Role of quorum sensing in the virulence of *Pantoea ananatis*

by

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: …….. Date: ……..

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God bless you all !!!!!!!!

SUMMARY

Role of quorum sensing in the virulence of *Pantoea ananatis*

by

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Pantoea ananatis, a plant pathogenic bacterium, inflicts significant economic losses to the agricultural and forestry industries. It is ubiquitous and capable of surviving in a diverse range of environmental conditions. The mechanism underlying host infection and colonization by this pathogen is poorly understood. The genome sequence of *P. ananatis* led to the discovery of putative pathogenicity determinants such as quorum sensing. In this study, a PCR-mediated protocol that makes use of the lambda (λ) red genes was used to knockout the genes for the three quorum sensing systems in *P. ananatis* LMG 2665^{T}. The mutant strain was named EanΔI/R,RhlΔI/R,ΔLuxS. Growth assays conducted using this mutant and the wild-type strain showed that the mutations did not affect its growth in liquid broth. This mutant was used to determine the role of quorum sensing in the virulence of *P. ananatis*. Virulence assays conducted showed that quorum sensing is required for virulence in *P. ananatis*.

To elucidate the role of individual quorum sensing systems in the virulence of *P. ananatis,* mutants lacking one system were constructed following the λ Red-mediated PCR protocol. The mutant strains were complemented by cloning the wild-type genes for the respective quorum sensing systems into the broad-host-range plasmid pBR1MCS-5. The mutant strains were named EanΔI/R, RhlΔI/R and ΔLuxS based on

the quorum sensing genes that were mutated. The complemented strains were named EanΔI/R::EanI/R, RhlΔI/R::RhlI/R and ΔLuxS::LuxS, respectively. *In vitro* growth studies showed that the genetically modified *P. ananatis* strains were not impaired in their growth.

The *P. ananatis* quorum sensing mutant strains and complemented mutant strains were used to determine the functional role of each quorum sensing system in *P. ananatis*. Characterization of the quorum sensing mutant strains revealed that the three quorum sensing systems are required for virulence of *P. ananatis* in onion seedlings. The virulence assays conducted showed that the LuxS quorum sensing system is the most crucial system for virulence in *P. ananatis.* Furthermore, *in vitro* studies of quorum sensing regulation of specific phenotypes of *P. ananatis* showed that quorum sensing governs biofilm formation and exopolysaccharide production. The phenotypes that were impaired in the quorum sensing mutant strains were restored to wild-type levels by genetic complementation. This study also showed that swarming and twitching motility, as well as rhamnolipid production are not influenced by quorum sensing in *P. ananatis*. The dependence of specific phenotypes on quorum sensing indicates the significance of the functional role of quorum sensing genes in the virulence of *P. ananatis*.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
µg	microgram
µl	microlitre
µM	micromolar
AHL	acyl homoserine lactone
AI	autoinducer
Amp^r	ampicillin resistance
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCC	Bacterial Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	carboxy
CFU	colony forming unit
Chl^r	chloramphenicol resistance
cm	centimetre
CWDE	cell-wall-degrading enzyme
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPD	4,5-dihydroxy-2,3-pentanedione
dpi	days post-inoculation
DSF	diffusible signal factor
dUTP	deoxyuridine triphosphate
eDNA	extracellular DNA
EPS	exopolysaccharide
Fig.	figure
FLP	flippase
FMN	flavin mononucleotide
$FMNH_2$	flavin mononucleotide, reduced
FRT	flippase recognition target

g	gram
gDNA	genomic DNA
GFP	green fluorescent protein
Gm^r	gentamycin resistance
h	hour
H_2O	water
IPTG	isopropyl β-D-1-thiogalactopyranoside
kan	kanamycin resistance gene
Kan^r	kanamycin resistance
kb	kilobase pair
kV	kilovolt
l	litre
LB	Luria-Bertani
LDP	lipodepsipeptides
LSD	least significant difference
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
ms	millisecond
N	amino
NaOAc	sodium acetate
NCBI	National Centre for Biotechnology Institute
ng	nanogram
nm	nanometre
OD	optical density
Pcc	*Pectobacterium carotovorum* subsp. *carotovorum*
PCR	polymerase chain reaction
pmol	picomol
pv.	patovar
PVC	polyvinyl chloride
rpm	revolutions per minute

rRNA	ribosomal ribonucleic acid
s	second
SAH	*S*-adenosyl-L-homocysteine
SAM	*S*-adenosylmethionine
SOC	Super Optimal Catabolite Repression
SRH	*S*-ribosyl-L-homocysteine
subsp.	subspecies
U	unit
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
α	alpha
β	beta
λ	lambda
Ω	ohm

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PREFACE

Pantoea ananatis causes disease symptoms in a wide variety of plants including, for example, blight disease in *Eucalyptus* and centre rot of onions. Most research on *P. ananatis* has focused on developing methods to reliably identify isolates to species level. The complete genome sequence of a number of strains of *P. ananatis* is currently available. These genome sequences revealed a number of putative pathogenicity determinants including genes that encode for different quorum sensing systems and secretion systems, as well genes that encode for cell-surface appendages implicated in motility and genes that encode for the production of exopolysaccharides.

Whilst several studies on the virulence determinants of other plant pathogenic bacteria have been conducted, there is still limited knowledge on the virulence factors used by *P. ananatis* to infect their hosts. The first goal of this study was to determine the role played by quorum sensing in the virulence of *P. ananatis*. The second goal was to conduct functional analyses of individual quorum sensing systems in *P. ananatis*.

In the first chapter, the literature on quorum sensing and quorum sensing systems in Gram-negative bacterial species was reviewed. The Literature Review also focused on the virulence factors under quorum sensing gene regulation in various bacteria.

In the second chapter, three quorum sensing systems identified in *P. ananatis* LMG2665^{T} were targeted for mutagenesis. The quorum sensing systems were EanI/R, RhII/R and LuxS. A quorum sensing mutant strain lacking all three quorum sensing systems was generated and used to determine whether quorum sensing plays a role in the virulence of *P. ananatis*. Strains that lacked each of the quorum sensing systems were also constructed. These *P. ananatis* mutant strains were generated for use in further studies to identify the quorum sensing system that is crucial for virulence, and also to unveil the biological significance of quorum sensing in this pathogen. The mutant strains were also complemented with plasmid-borne copies of the wild-type alleles in order to verify the results of successive studies conducted on the mutant strains.

In the third chapter, the role of quorum sensing in the virulence of *P. ananatis* is determined. An analysis of the role of individual quorum sensing systems in *P. ananatis* in the regulation of specific phenotypes was conducted. The phenotypes that were investigated included virulence assays in onion seedlings, biofilm formation, swarming and twitching motility, exopolysaccharide production and rhamnolipid synthesis.

CHAPTER ONE

OVERVIEW OF QUORUM SENSING AND VIRULENCE IN GRAM-NEGATIVE BACTERIA

1.1 INTRODUCTION

Quorum sensing refers to a cell density-dependent communication between bacterial cells and coordination of activities through signal molecules called autoinducers or pheromones (Winzer *et al.*, 2003). The main significance of quorum sensing in bacteria is the global regulation of expression of virulence traits. Quorum sensing also helps bacteria adapt to a changing environment by regulating the expression of specific genes essential for adaptation and survival (Gospodarek *et al.*, 2009).

Research focusing on quorum sensing started in the late 1960s with studies on *Vibrio fischeri*. This Gram-negative bacterium is capable of living planktonically, as well as in a biofilm in the light organs of fishes and squids. There were suggestions that pure cultures of *V. fischeri* cells were inhibited in producing bioluminescence by an unknown inhibitor present in culture media (Kempner and Hanson, 1968). Further suggestions were that bacteria at high cell density were able to eliminate the inhibitor from the media, resulting in bioluminescence. However, attempts to identify the inhibitor and mechanisms of its removal were unsuccessful.

In 1970 quorum sensing gene regulation was discovered in *V. fischeri* bacterial cells (Nealson *et al.*,1970). It was shown that bioluminescence in *V. fischeri* was a result of the accumulation of small diffusible signal molecules called autoinducers (Nealson *et al.*, 1970). The emission of light benefits both the bacteria and its marine host/s in a symbiotic relationship where light emitted provides the host/s with counter-illumination and protection against predators. The bacteria benefit from the relationship through acquisition of nutrients, amino acids and nourishment from the nutrient-rich light organs (Dunlap and Kita-Tsukamoto, 2006).

It was initially believed that quorum sensing was confined only to marine bacteria. However, research has shown the existence of different quorum sensing systems in a variety of non-marine Gram-positive and Gram-negative bacteria. This has led to an increased research focus on the mechanism of quorum sensing in a wide variety of bacterial species. There are basically four common types of quorum sensing systems,

the autoinducer 1 (AI-1)-dependent, the AI-2-dependent, the AI-3-dependent and the polypeptide-dependent system that is mainly found in Gram-positive bacteria (Gospodarek *et al.*, 2009). This literature review will focus mainly on AI-1 and AI-2 cell-to-cell communication found in Gram-negative bacteria, as well as discuss the quorum sensing systems that have been found and identified in *Pantoea ananatis*.

Pantoea ananatis is a member of the Gamma-proteo *Enterobacteriaceae,* and causes diseases in both plants and humans. The plants that are affected by *P*. *ananatis* include staple food crops such as maize, rice, sorghum (Cota *et al.*, 2010), honeydew melons, pineapples, onions, as well as *Eucalyptus* (Coutinho *et al.*, 2002; Coutinho and Venter, 2009). Typical symptoms of diseases caused by *P*. *ananatis* include leaf blight, seed stalk rot and bulb decay in onions, severe grain discolouration in rice (Yan *et al.*, 2010), brown spots in honeydew melons and brown rot in pineapples. In *Eucalyptus*, this bacterium causes bacterial blight disease that is characterized by tip dieback and spots on young leaves (Coutinho *et al.*, 2002). *P. ananatis* is present on all continents and is recognized internationally as an “emerging pathogen”. Since 2001, the incidence of outbreaks, their geographical location, and the range of hosts infected by *P. ananatis* have all increased. This is best illustrated by the appearance of white leaf spot on different grass species in South America (Cota *et al*., 2010, Alippi and López, 2010).

The effective control of *P. ananatis* has not yet been achieved and there is limited knowledge on the pathogenicity and virulence determinants of this bacterium. According to Kido *et al.* (2010), effective control of *P. ananatis* can be achieved through identification of the source of inocula. The only obstacle with the identification of the source of inoculum is the availability of effective techniques for distinguishing plant pathogenic strains from saprophytes and epiphytes (Kido *et al.*, 2010). Also, resistance breeding has not been a success to date. Studies on the pathogenicity determinants such as significance of quorum sensing genes in *P. ananatis* could help contribute to our understanding of the virulence of this pathogen and thus pave the way to development of an effective control strategy.

Studies focusing on phenotypes of densely populated bacteria have shown that quorum sensing gene regulation governs common traits in most bacteria (Greenberg, 2003). Bacteria use quorum sensing for collective regulation of gene expression and expression of specific phenotypes such as virulence. Virulence phenotypes include the production of exopolysaccharides, extracellular enzymes, surfactants such as rhamnolipids, antibiotics (Derzelle *et al.*, 2002), biofilm formation (Morohoshi *et al.*, 2007), swarming, twitching and swimming motility, as well as secretion of host response and pathogenicity (Hrp) proteins (Bassler and Losick, 2006).

1.2 SIGNIFICANCE OF QUORUM SENSING RESEARCH

Manipulation of quorum sensing systems is an emerging and promising approach towards biological control of bacterial infections and diseases (Dong *et al.*, 2000; Mae *et al.*, 2001; Dong *et al.*, 2001). Research and thus elucidation and a better understanding of quorum sensing systems in bacteria is crucial before quorum sensing manipulation can be employed as a biological tool against bacterial pathogens. Control of bacterial diseases through disruption of quorum sensing has been proved a feasible strategy (Fray, 2002) and can be done enzymatically through degradation of signals or through inactivation of quorum sensing signal molecules in a process termed quorum quenching or quorum inhibition (Denfoirdt *et al.*, 2008).

To date, the quorum quenching enzymes that have been identified and characterized using acylated homoserine lactone (AHL) substrates are AHL lactonase and acylase (Dong and Zhang, 2005). Quorum quenching enzyme activity in nature was identified in seedlings of *Lotus corniculatus*, a leguminous plant (Delalande *et al.*, 2005), and also in seedlings of various plants that include garlic, pea (Teplitski *et al.*, 2000), crown vetch (Zhang and Dong, 2004), rice, tomato, soybean and *Medicago* species (Gao *et al.*, 2003) such as alfalfa plants. Some other plants with quorum quenching activity include *Allium satiyum*, *Lycopersicum esculentum* and *Oryza sativa* (Sanchez-Contreras *et al.*, 2007). Other natural quorum quenchers include penillic acid produced by *Penicillium*

spp., patulin produced by fungi and L-canavanine from alfalfa and legumes (Keshavan *et al.*, 2005)

Bacteria that encode novel AHL-hydrolyzing enzymes have been isolated from soil (Riaz *et al.*, 2008), leaf samples of *Solanum tuberosum* (Morohoshi *et al.*, 2009), the rhizosphere (Uroz *et al.*, 2003) and fish guts (Morohoshi *et al.*, 2005). Such AHL-degrading bacteria can be used as antagonist/s to quorum sensing in bacterial pathogens and thus as biological control measures for effective control of bacterial infections and diseases. One such example is that of where potato root surface-associated *Chryseobacterium* spp. has been shown to degrade AHLs (Rashid *et al.*, 2011). A study by Uroz *et al.* (2003) showed that co-inoculation of *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) and *Rhodococcus erythropolis* resulted in inhibition of Pcc in potato tubers. The main advantage of quorum quenching as a biocontrol measure is that it leads to the disruption of quorum sensing in bacteria, but does not affect growth of quorum quenched bacteria or other non-targeted organisms. This was demonstrated by Uroz *et al.* (2003), where *R. erythropolis* was used to quorum quench *Chromobacter violaceaum, Agrobacterium tumefaciens* and Pcc. Growth assays conducted in the presence and in the absence of the quencher showed similar patterns (Uroz *et al.*, 2003), thus revealing that quorum quenching does not affect growth of quenched bacteria.

1.3 QUORUM SENSING AUTOINDUCERS

An autoinducer is defined as a small signal molecule that can move freely across a cell membrane. The basic features of an autoinducer include the ability to accumulate in an extracellular space at specific bacterial growth stage/s or in response to environmental changes, the capability for recognition by a specific receptor protein and the ability to trigger a cellular response (Winzer *et al.*, 2002). To date, the quorum sensing signal molecules that have been identified and studied extensively in Gram-negative bacteria are the acylated homoserine lactones (AHLs). The acylated homoserine molecules are unique from each other and the number of carbon atoms in their acyl side chain ensures

specificity in bacterial species (Xavier and Bassler, 2003; Choudhary and Schmidt-Dannert, 2010). Classification of the AHLs is based on the length of the acyl chain and substitution at carbon 3 of the acyl chain by either an oxo or hydroxyl group. Most studies on AHL signalling were done extensively on bacteria that include *Vibrio (*Milton, 2006), *Pseudomonas (*Williams and Cámara, 2009), *Agrobacterium* (White and Winans, 2007), and *Yersinia* (Atkinson *et al.*, 2006).

Another common type of signal molecule in Gram-negative bacteria is called autoinducer 2 (AI-2). The specificity of autoinducer 2 signals to bacterial species is owed to the difference in the stereochemistry at carbon 1 or the chelation of the boron molecule (Tsuchikama *et al.*, 2011). Detailed studies of AI-2-based signaling have been done in *V. harveyi* and *V. cholerae* (Lenz *et al.*, 2004; Neiditch *et al.*, 2005). In the Vibrios, AI-2 regulates the expression of virulence factors such as biofilm formation (Milton, 2006).

Other types of signal molecules in Gram-negative pathogenic bacteria include diffusible signal factor (DSF) (González and Keshavan, 2006) and cyclic dipeptides that were first identified in *Pseudomonas* spp*.* and later in other bacteria such as *Proteus mirabilis, Citrobacter freundii* and *Enterobacter agglomerans* (González and Keshavan, 2006*).* A study by Degrassi *et al.* (2002) revealed that cyclic dipeptides are capable of cross-strain induction of genes. However, Holden *et al.* (1999) reported that cyclic dipeptides activate genes at concentrations 30 000-times higher than that of acylated homoserine lactones, and thus postulated that cyclic dipeptides might not be important quorum sensing inducers under natural environmental conditions. This suggestion can only be proved through further investigations and determination of whether such high concentrations of cyclic dipeptides are reached in nature. Thus, more research on quorum sensing is needed before conclusions on the role and mechanisms of various quorum sensing systems can be drawn.

The diffusible signal factor (DSF) was first identified in *Xanthomonas campestris* and then later in *Stenotrophomonas maltophilia* (Fouhy *et al.*, 2007), *Burkholderia*

cenocepacia (Boon *et al.*, 2008) and *Xylella fastidiosa* (Colnaghi *et al.*, 2007). Chemical characterization of DSF identified it as cis-11-methyl-2-dodecenoic acid (Wang *et al.*, 2004). The DSF has been shown to regulate phenotypes that are normally influenced by quorum sensing (Barber *et al.*, 1997), for example, production of exopolysaccharide, exoenzymes, xantham and virulence-related phenotypes such as swimming motility, as well as antibiotics resistance (Slater *et al.*, 2000).

Recently, it was reported that *Xanthomonas oryzae* pv. *oryzae* has another quorum sensing system that is regulated by small protein Ax21 (Ronald, 2011). The Ax21 protein was shown to exist in all sequenced species of *Xanthomonas* with 98% to 99% amino acid sequence identity. The newly discovered quorum sensing system was found to regulate biofilm formation and virulence in *Xanthomonas* spp. Nonetheless, the majority of studies on quorum sensing have been undertaken with the AHL- and AI-2-based quorum sensing regulation mechanisms (Jones *et al.*, 1993; Beck von Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Freeman and Bassler, 1999; Mok *et al.*, 2003; Henke and Bassler 2004; Molina *et al.*, 2005; Morohoshi *et al.*, 2007; Licciardello *et al.*, 2011; Morohoshi *et al.*, 2011).

1.4 QUORUM SENSING SYSTEMS IN GRAM-NEGATIVE BACTERIA

1.4.1 Lux I/R or AI-1-dependent systems

The first study on quorum sensing genes was conducted by Engebrecht *et al.* in 1983 when the basics of quorum sensing systems found in most Gram-negative bacteria were modelled. In *V. fischeri* cells the enzyme that produces the signal molecule, the autoinducer synthase, is called LuxI, and the autoinducer receptor protein or transcriptional activator is called LuxR. The nomenclature of these proteins is based on that this quorum sensing system regulates the luciferase operon that codes for light emission. The autoinducer molecule that regulates quorum sensing in *V. fischeri* is an acylated homoserine lactone called 3-oxo-C_6-homoserine lactone. The genes that code for emission of bioluminescence in *V. fischeri* are *luxICDABE,* also called the Luciferase (*lux*) operon.

Each gene in the *lux* operon plays a role in bioluminescence emission. The *luxA* and *luxB* genes code for the β and α subunits of the enzyme luciferase. The *luxC, luxD* and *luxE* genes code for components of the reductase that catalyses the production of the substrate for the luciferase (Fuqua and Greenberg, 2002). The substrate for the enzyme luciferase is tetradecanal, a fatty aldehyde produced by reduction of a long fatty acid tetradecanoate. Luciferase catalyses the oxidation of fatty aldehyde and flavin mononucleotide ($FMNH_2$), resulting in the emission of light. The chemical equation for light emission in bacterial cells is $FMNH_2 + O_2$ + fatty aldehyde → light + H_2O + fatty acid + FMN (Abu-Soud *et al.*, 1992).

Quorum sensing genes are transcribed at low levels when the bacterial population density is low. Also, at low cell densities, the autoinducer molecules are not accumulated but are rather lost into the surrounding environment by either inactivation or diffusion out of cells (Kaplan and Greenberg, 1985). Production of quorum sensing autoinducer molecules and protein receptor molecules occurs at one site in bacterial cells, in the cytoplasm. The loss of signal molecules at low cell densities, as well as a reduced half-life of protein receptor molecules helps prevent autoinduction when bacterial cell densities are below threshold levels. This instability of the unbound signal molecule was demonstrated in *Agrobacterium tumefaciens* where it was shown that the receptor TraR degrades faster when free and unbound to quorum sensing signal molecules (Zhu and Winans, 2001).

As the bacterial cell density increases to threshold levels, for example in *V. fischeri* at cell concentrations of approximately 10^{10} cells/ml, quorum sensing is induced. The increase in cell density leads to accumulation of autoinducers in the bacterial surrounding environment, as well as in the intracellular space. Once the autoinducers are accumulated to a threshold level they bind to the receptor proteins (LuxR). The resulting complex of LuxR and autoinducer molecule binds to the *lux* box, which constitute the promoter sequence of the luciferase operon. This results in a further increase in the production of the autoinducer, transcription of the *luxICDABE* genes and expression of bioluminescence, as well as other genes and phenotypes governed by

this system. Figure 1.1 shows a schematic representation of an acylated homoserine lactone (AHL)-dependent quorum sensing system, also termed the LuxI/R system (Choudhary and Schmidt-Dannert, 2010).

The LuxI/R system has been found in a variety of Gram-negative bacteria, including the plant pathogenic bacterium *Pantoea ananatis* discussed above. The *luxI*/*R* quorum sensing genes are made up of two distinct families that are unique. One family is restricted to the gamma proteobacteria, whilst the other is found in gamma, beta and alpha proteobacteria (Lerat and Moran, 2004). The LuxI/R quorum sensing systems exhibit extreme specificity to bacterial species, owing to the specificity of the acyl binding pocket of the LuxR proteins that recognize the corresponding acyl chain of the autoinducer molecules (Zhang *et al.*, 2002). LuxR proteins are basically made up of the N-terminal residue that interacts with autoinducers, a C-terminal with a helix-turn-helix motif for binding to DNA and a conserved amino acid region (Nasser and Reverchon, 2006). In mixed populations, each species detects and responds to the presence of its own autoinducer molecule (Waters and Bassler, 2005).

Bacteria use one or more quorum sensing systems to effectively express specific genes and phenotypes. A quorum sensing system is made up of a quorum sensing autoinducer synthase, the autoinducer and the autoinducer receptor protein, the signal response regulator and the genes that are influenced by the system (Williams, 2007; Swift *et al.*, 2008). Different quorum sensing systems can also be used in conjuction with each other in the establishment of effective expression of various phenotypes. Some quorum sensing systems regulate expression of other quorum sensing systems, for example, the LasI/R system regulates expression of the RhlI/R system in *Pseudomonas aeruginosa* (Waters and Bassler, 2005). In a bacterium with multiple quorum sensing systems, three classes of genes are portrayed. These constitute the genes that are regulated by only one system, genes that require two or more systems to be expressed and genes that are dependent on either of the present quorum sensing systems (Henter *et al.*, 2003; Wagner *et al.*, 2004).

The RhlI/R is one other unique classical example of an AHL-dependent quorum sensing system. The RhlI/R quorum sensing system was first identified in *P. aeruginosa*, a bacterium with a wide host range that causes diseases in both humans and plants such as tobacco (Elrod and Braun, 1942; Rahme *et al.*, 1997), as well as *Arabidopsis* and sweet basils roots where it forms biofilms causing infections and death of plants (Walker *et al.*, 2004). *P. aeruginosa* possesses two LuxI/R quorum sensing systems, the LasI/R system that influences elastase production and the RhlI/R quorum sensing system that regulates rhamnolipid production (Jones *et al*., 1993; Reis *et al*., 2011).

In *P. aeruginosa* the LasI/R and RhlI/R quorum sensing systems are dependent on the signals *N*-(3-oxo-dodecanoyl)-l-homoserine lactone (3-oxo-C_{12}-AHL) and *N*-(butanoyl)-l-homoserine lactone (C_4-AHL), respectively (Venturi, 2006). Like all other LuxI/R systems, the RhlI/R system is comprised of the autoinducer synthase gene (*rhlI*) and the protein receptor synthase gene (*rhlR*). The autoinducer/protein receptor complex binds to the promoter of the operon that code for rhamnosyltransferase, an enzyme that catalyzes the synthesis of rhamnolipids. The RhlI/R system produces its own autoinducer and regulates its own sets of target genes independent of the LasI/R system (Waters and Bassler, 2005). Thus, the existence of the two systems in a bacterium results in an effective regulation of genes and phenotypes.

In *P. ananatis* (Fig. 1.2), two distinct quorum sensing systems, namely PANA_1416-1417 (RhlI/RhlR) and PANA_1955-1956 (EanlI/EanR), have been identified in the complete genome sequence (De Maayer *et al.*, 2010). PANA_1416-1417 (RhlI/RhlR) is made up of two genes, the *rhlI* responsible for signal molecule synthesis and the *rhlR* which is a transcriptional activator. The RhlI/R quorum sensing system is responsible for production of a biosurfactant termed rhamnolipid that is an essential pathogenicity determinant in *P. aeruginosa* (Oschner *et al.,* 1995). Rhamnolipids contribute to *P. aeruginosa* pathogenicity in the amoeba, *Dictyostelium discoideum,* by causing cells to rupture (Cosson *et al.*, 2007). The *rhlI/rhlR* genes in *P. aeruginosa* are clustered with rhamnolipid genes, whereas in *P. ananatis* they occur separately (De Maayer *et al.*, 2010).

The PANA_1955-1956 (EanI/EanR) quorum sensing system is also made up of two genes, the *eanI* responsible for signal molecule synthesis and the *eanR* which is a transcriptional activator. This quorum sensing system regulates the production hexanoyl-homoserine lactone (Morohoshi *et al.*, 2007). The EanI/R quorum sensing system typically resembles the LasI/R quorum sensing system in *P. aeruginosa.* Studies on the LasI/R system in *P. aeruginosa* showed that this quorum sensing system regulates expression of virulence traits such as biofilm formation, swarming and twitching motility (Venturi, 2006).

1.4.2 AI-1 or AHL biosynthetic pathway

The expression of LuxI protein is required for the production of AHLs in most bacteria that produce AHLs (Fuqua *et al.*, 2001). A fatty acyl side chain and a source of the homoserine lactone ring are required for the catalytic formation of the amide bond by AHL synthases (Atkinson and Williams, 2009). There are essentially two classes of AHL synthases that have been identified and characterized, the LuxI and the LuxM (Milton, 2006) identified in *V. harveyi* and shares no identity to LuxI. More than a hundred AHL synthases are available on the bacterial genome databases (Cha *et al.*, 1998).

The initial substrates for AHL biosynthesis are the same for both LuxI and LuxM proteins. These substrates constitute *S*-adenosylmethionine (SAM) and 3-oxo-hexanoyl-CoA (More *et al.*, 1996; Parsek and Greenberg, 1999) or aroyl-CoA (Schaefer *et al.*, 2008). In general, AHL synthases utilize a variety of acyl-carrier proteins (ACPs), resulting in the production of various AHL molecules in bacteria (Ortori *et al.*, 2007). The nature of the AHL molecules synthesized is governed by available subtrates, as well as the specificity and availability of AHL synthases.

1.4.3 Autoinducer 2 (AI-2)-dependent quorum sensing

A second type of quorum sensing system was discovered in the marine bacterium, *Vibrio harveyi.* The mechanism of quorum sensing gene regulation in *V. harveyi* provided the first evidence that bacteria are capable of quorum sensing using multiple

autoinducers. Regulation of bioluminescence in *V. harveyi* is influenced by two signal molecules, the 3-hydroxy-C_4-homoserine lactone and a second quorum sensing autoinducer molecule called autoinducer 2 (AI-2). Regulation of quorum sensing in this bacterium is based on a two-component system where phosphorylation and dephosphorylation reactions result in autoinduction and regulation of bioluminescence emission (Mok *et al.*, 2003). Figure 1.3 shows a schematic representation of the two-component quorum sensing system in *V. harveyi.*

At low bacterial cell population density, the protein kinases LuxQ and LuxN transfer the phosphate molecule via LuxU to LuxO, a DNA-binding response regulator molecule (Coulthurst *et al.*, 2002). The phosphorylated LuxO activates the transcription of a protein repressor molecule that represses the production of the protein receptor molecule, LuxR. The LuxR protein is required for expression of the *luxCDABE* genes that code for expression of bioluminescence (Fuqua and Greenberg, 2002).

At high bacterial cell population density, the quorum sensing signal molecules, AI-2 and 3-hydroxy-C_4-homoserine lactone, are accumulated. Once a threshold concentration of autoinducers is reached, the quorum sensing signals interact with the protein sensors LuxP and LuxN and thus result in the removal of the phosphate group from LuxO through LuxU (Freeman and Bassler, 1999). Desphosphorylation of LuxO causes deactivation of expression of the repressor proteins, translation of the protein receptor molecule, LuxR, and activation of the *luxCDABE* genes, and thus expression of bioluminescence. This two-component quorum sensing system in *V. harveyi* regulates a variety of genes and phenotypes (Mok *et al.*, 2003).

This type of AI-2-dependent quorum sensing system has also been identified in *P. ananatis* (De Maayer *et al.*, 2010). In this bacterium the LuxS quorum sensing system is made up of a single gene PANA_3027 and codes for the LuxS protein. Figure 1.1 depicts the arrangement of quorum sensing genes in the genome of *P. ananatis* (De Maayer *et al.*, 2010).

1.4.4 AI-2 biosynthetic pathway

The activated methyl cycle synthesizes methyl molecules that are used in successive methylation reactions, resulting in the production of AI-2 molecules (Miller *et al.*, 2004). Research has shown that the biosynthetic pathways and intermediates in the production of AI-2 are identical (Winzer *et al.*, 2002) in all the studied bacterial species that produces this autoinducer. AI-2 is synthesized from *S*-adenosylmethionine (SAM), a product of the catalytic conversion of methionine and ATP by SAM synthetase (Miller *et al.*, 2004).

The production of AI-2 is basically made up of three enzymatic steps, the first reaction is the catalytic formation of *S*-adenosyl-L-homocysteine (SAH) from SAM by the enzyme methyltransferase (Miller *et al.*, 2004). The second reaction is the hydrolysis of the glycosidic bond of SAH by the enzyme *S*-adenosylhomocysteine nucleosidase, resulting in the production of adenine and *S*-ribosyl-L-homocysteine (SRH) (Miller *et al.*, 2004). In the last and final catalytic reaction the metalloenzyme LuxS (Gopishetty *et al.*, 2009) converts SRH to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is highly unstable and forms furanone derivatives via cyclysation and dehydration reactions. The furanones then react with borate to form the AI-2. X-ray crystallography revealed the structure of AI-2 as a furanosyl diester. The biosynthesis of AI-2 is summarized in Fig. 1.4. AI-2 is thus a furanosyl-borate diester, which acts as a quorum sensing signal molecule. Research has shown that the LuxS quorum sensing system regulates virulence in several bacterial pathogens by facilitating transition from non-virulent to virulent bacteria through autoinduction by the signal molecule AI-2 (Gao *et al.*, 2009; Li *et al.*, 2011).

1.4.5 Other roles of AI-2

Autoinducer 2 is produced by more than 50 different Gram-positive and Gram-negative bacterial species (Vendeville *et al.*, 2005; Milton 2006; Atkinson and Williams 2009). The widespread production of AI-2, as well as the similarity of the biosynthetic pathways and intermediates in the production of AI-2 in all studied bacterial species has led to

conclusions that AI-2 is used for interspecies communication (Bassler *et al.*, 1997; Miller and Bassler 2001; Bassler, 2002). The role of AI-2 in interspecies communication still stands to be proven. AI-2 has also been shown to play a role in the detection of the environmental growth conditions of bacterial cells (Sperandio *et al.*, 2003). This is crucial for adaptation to a changing environment such as preparation for nutrient-limiting conditions (Withers and Nordström, 1998).

LuxS is believed to play an essential role in the recycling of nutrients in the activated methyl cycle where it helps to detoxify *S*-ribosylhomocysteine (SAH) and recycles homocysteine from *S*-adenosylmethionine, thus resulting in the production of methionine (Miller *et al.*, 2004). Based on this crucial role of LuxS in cell metabolism, mutants deficient in the *luxS* gene are expected to exhibit growth defects. However, some *luxS* mutants have exhibited growth patterns similar to wild-type strains. Thus, based on such findings, the result of *luxS* disruption can influence either quorum sensing or metabolism, depending on the bacterial strain in question (Atkison and Williams, 2009).

It has also been suggested that in some bacteria AI-2 is produced as a secondary metabolite (Rezzonico and Duffy, 2008) that interferes with signaling in other species (Winzer *et al.*, 2002). It is also believed that AI-2 acts as a transporter of boron (Coulthurst *et al.*, 2002) under certain growth conditions. The role of boron in bacteria has not been explored in detail and thus this suggestion still needs further clarification.

1.5 DETECTION OF AUTOINDUCERS

A variety of techniques have been employed for detecting the presence of autoinducers in bacterial cultures, for example, gas chromatography with mass spectra, thin-layer chromatography and biosensors (Annous *et al.*, 2009). The most common and most widely used method makes use of bacterial biosensors. These have been used as a tool for identification of quorum sensing systems in bacteria (Steindler and Venturi, 2007). Bacterial biosensors are composed of mutants that are incapable of producing

autoinducers but express the receptor protein and contain a promoter that is activated by the presence of autoinducer molecules, fused to a reporter gene. The presence of autoinducers is thus detected through expression of reporter genes in biosensors (Steindler and Venturi, 2007).

Typical examples of biosensors include several *Agrobacterium tumefaciens* biosensors with a broad range and high autoinducer detection capacity (Steindler and Venturi, 2007). The quorum sensing genes *traI/R* of *A. tumefaciens* are located in the conjugal Ti plasmid. The AHL-dependent expression of a blue-white phenotype of the β-galactosidase gene (Kawaguchi *et al.*, 2008) is used to detect the presence of AHLs by mutant (*lacZ*) strains of *A. tumefaciens*. The biosensor is constructed by mutating the *traI* gene or by curing the *A. tumefaciens* bacterial strain of the Ti plasmid and thus rendering it incapable of producing the AHL molecules. Plasmids containing the reporter fusion genes, for example, the *traG-lacZ* are then used as biosensors in respective mutant strains. One typical *A. tumefaciens* biosensor comprises of three plasmids, i.e. pJZ384 that contains the protein receptor gene *traR* regulated by the phage T7 promoter, pJZ410 that contains the T7 RNA polymerase gene and pJ372 that contains the reporter gene *traI-lacZ* (Steindler and Venturi, 2007).

Another example of a simple and extensively used biosensor is *Chromobacterium violaceum* CV026 (McClean *et al.*, 1997). The production of a purple pigment called violacein in wild-type strains of C*. violaceum* is regulated by quorum sensing. The biosensor CV026 was constructed by transposon mutagenesis in the *luxI* gene homologue. In the absence of AHLs the mutant portrays a white phenotype and a purple phenotype is portrayed in the presence of AHLs. CV026 is used to detect both short-chain and long-chain AHLs (McClean *et al.*, 1997). It has also been used in the detection and identification of the LuxI/R quorum sensing system in *P. ananatis* (Morohoshi *et al.*, 2007).

Reporter strains have also been employed in the detection of AI-2 (Bassler *et al.*, 1997). The *V. harveyi* mutant strain is rendered incapable of responding to AI-1 by mutating

the *luxN* gene (Bassler *et al.*, 1994). The presence of AI-2 results in premature emission of bioluminescence and thus detection of the signal molecules. This assay has also been employed in the quantification of AI-2 through comparison of light emitted to standards or controls (Winzer *et al.*, 2002). Other biosensors include those that show phenotypes such as the green fluorescent protein (GFP) (Steidle *et al.*, 2001), bioluminescence (Engebrecht *et al.*, 1983) and synthesis of orange-coloured phenazine (Wood *et al.*, 1997).

1.6 ENVIRONMENTAL FACTORS IMPACTING QUORUM SENSING

Quorum sensing is modulated by environmental factors that include nutrition, pH, temperature and rate of diffusion of signal molecules. High temperatures above 37ºC and alkaline pHs significantly affect the half-life of AHLs. Alkalization is used by plants as a defense mechanism against bacterial infections, for example, Pcc causes alkalization of the zone of infection resulting in inactivation of AHLs and thus interfering with quorum sensing regulation of virulence traits (Boyer and Wisniewski-Dyé, 2009). However, the sensitivity of autoinducers to pH and elevated temperatures is dependent on the length of the acyl chain where long-chain AHLs of more than 8 carbons are less sensitive to elevated temperatures and alkaline pHs compared to their short-chain counterparts (Yates *et al*. 2002; Decho et al. 2009).

Nutritional growth conditions also impact on the expression of quorum sensing systems in bacteria (Wagner *et al.*, 2003; Duan and Surette, 2007). A study conducted in Pcc indicated that various carbon sources affected expression of different quorum sensing systems in different ways. McGowan *et al.* (2005) showed that the levels of AHLs produced by Pcc differed significantly in different media containing sucrose, glucose and glycerol, respectively, as carbon sources. The nutrient composition also affected the amounts and ratios of autoinducers produced by *Rhizobium leguminosarum* (Lithgow *et al.*, 2001). Despite the intriguing effect of carbon sources on quorum sensing systems in bacteria, the mechanism underlying the link of carbon sources to quorum sensing still stands to be better understood.

Iron availability is another limiting factor that affects quorum sensing systems in bacteria. The lack of iron causes increased formation of virulence factors (Kim *et al.*, 2003) in some bacteria and also increases expression of quorum sensing systems (Bollinger *et al.*, 2001; Duan and Surette, 2007). This could lead to early detection by host/s immune defense systems and thus could be detrimental to the pathogen. Oxygen is also a limiting factor affecting expression of quorum sensing systems in bacteria. In *P. aeruginosa,* LasI is expressed at a higher level compared to RhlI when bacteria are grown under anaerobic conditions (Alvarez-Ortega and Harwood, 2007).

The accumulation of autoinducers in a particular niche is influenced by the autoinducer diffusion gradient. The rate of diffusion is dependent on the nature of the autoinducer molecule, the hydrophobicity of the culture medium, as well as the bacterial species in question as some bacteria produce exopolysaccharides (EPS) that regulate diffusion into and out of cells. A study conducted by Charlton *et al.* (2000) showed that the movement of autoinducers is influenced by EPS in biofilms of *P. aeruginosa* where autoinducers are retained within a bioflm and made unavailable at the surface-fluid interface. This factor is mainly important for the highly hydrophobic 3-oxo-C_{12}-homoserine lactone and less important for the C_4-homoserine lactone, which thus does not interact much with the biofilm matrix.

AHLs are prone to being washed away by circulating fluids and thus hindered from accumulating to threshold levels that are essential for a quorum to be reached. In *Pseudomonas syringae* the accumulation of autoinducers is favoured by dry environments, suggesting that humidity or moisture content is a prime factor influencing diffusion of autoinducers to the external environment (Dulla and Lindow, 2008). Thus, it was postulated that rain water flow disrupts quorum sensing (Boyer and Winsniewski-Dyé, 2009). The speed of circulating fluid affects biofilm maturation in *P. aeruginosa.* In such environments autoinducers accumulate more within the biofilm than on the surface, resulting in premature autoinduction of cells within the biofilm (de Kievit *et al.*, 2001).

1.7 ASSOCIATION OF PLANTS WITH AUTOINDUCERS

Plants have been found to associate with autoinducer molecules that are produced by bacterial cells. A typical example is the zoospores produced by the sea weed *Enteromorpha*. These zoospores depend on autoinducers in the selection of the appropriate bacterial biofilms for settlement. A study by Joint *et al.* (2002) showed that this selection of biofilms is dependent on 3-oxo-C_{12}-homoserine lactone and 3-oxo-C_{10}-homoserine lactone. Their study showed that quorum sensing autoinducers influence the ecology of *Enteromorpha*. The report by Joint *et al.* (2002) was further supported by a study by Tait *et al.* (2005) that showed that AHL-deficient mutant strains of marine bacteria were not able to attract zoospores of the sea weed. However, the molecular mechanism underlying detection and response to AHLs by sea weed zoospores is still unknown.

A proteomic study conducted on *Medicago truncatula* showed that the presence of bacterial autoinducer molecules triggers expression of various plant defense proteins (Mathesius *et al*., 2003). Another example of plant-autoinducer interaction is in tomato plants where the presence of autoinducer molecules from *Serratia marcescens* triggered the immune defence of plants and conferred increased resistance to a leaf pathogen, *Alternaria alternata* (Schuhegger *et al.,* 2006). Such studies showed the significance of autoinducers in influencing the result of pathogen-host interaction. The mechanism of plant/s response to autoinducers is still not yet fully understood. Mathesius *et al.* (2003) postulated that plant responses to AHLs could have evolved as a means of detecting the presence of bacteria in the environment. Such postulations could only be proved through further investigations.

Some autoinducer-plant associations include species such as *Lotus corniculatus*, a leguminous plant (Delalande *et al.*, 2005), garlic, pea (Teplitski *et al.*, 2000), crown vetch (Zhang and Dong, 2004), rice, tomato, soybean, *Allium satiyum*, and (Sanchez-Contreras *et al.*, 2007) and *Medicago* species (Gao *et al.*, 2003) such as alfalfa that are capable of degrading autoinducer molecules. The subject of quorum sensing is very complex and complicated, thus much more research is required for it to be better

understood and possibly used for the benefit all living organisms such as in biological control.

1.8 VIRULENCE FACTORS

The increased development of bacterial strains resistant to antibiotic treatment has led to increased research focusing on alternative control strategies. Virulence factors are expressed by bacteria during pathogenesis and promote survival of pathogens in host tissues (Gospodarek *et al.*, 2009). Virulence refers to the quantitative and qualitative features of a microorganism that contribute to its ability to cause disease and infection/s (Casadevall and Pirofski, 1999). Virulence factors are molecules produced and phenotypes expressed by bacteria that enable them to successfully infect, colonize and cause disease in their hosts. Expression of virulence traits is influenced by quorum sensing in most bacteria. The availability of complete genome sequences and advanced technologies for bacterial genetic manipulation has led to improved research on bacterial virulence factors. Such knowledge on virulence traits is crucial in the development of control strategies for bacterial infections and diseases (Casadevall and Pirofski, 1999). This literature discusses virulence traits that are affected by quorum sensing, and that have been identified and studied in pathogenic bacteria.

Quorum sensing is used in the regulation of host-pathogen interactions where, in most cases, it results in the induction of virulence factors in numerous bacterial species (Antunes *et al.*, 2010). Research based on quorum sensing mutant strains of different bacterial strains has indicated intriguing roles of quorum sensing systems with regards to virulence. It has been reported that the *esaI*$^{-}$/*esaR*$^{-}$ mutant strain of *Pantoea stewartii* subsp. *stewartii* is less virulent compared to the wild-type strain (Beck von Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Koutsoudis *et al.*, 2006). Conversely to that, the *Pseudomonas syringae* quorum sensing double mutant strain *ahlI*$^{-}$*ahlR*$^{-}$ showed a higher degree of virulence on bean and tobacco plants compared to the wild-type strain (Quinones *et al.*, 2005). Balestrino *et al.* (2005) showed that the LuxS quorum sensing system plays a role in biofilm formation in *Klebsiella pneumoniae*. The

above cited literature on quorum sensing shows that different quorum sensing systems influence virulence in various bacteria in different ways (Beck von Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Balestrino *et al.*, 2005; Quinones *et al.*, 2005; Koutsoudis *et al.*, 2006).

A study by Zhao *et al.* (2005) showed that the *luxS* gene is not an important virulence factor in a phytopathogen, *Erwinia amylovora*. However, their study revealed that *luxS* downregulates two virulence genes, *hrpL* and *dspA*. These two genes are essential for bacterial growth and detection of change in the environment (Wei *et al.,* 2000). Thus, it can be postulated that the *luxS* gene plays a role in the virulence of *E. amylovora* to a lesser extent. LuxS has also been shown to play a role in biofilm formation and iron metabolism in *Actinobacillus pleuropneumoniae* (Li *et al.*, 2011) as well as swimming motility in *Escherichia coli* (Ling *et al.*, 2010), but to play no role in the virulence of *Salmonella typhimurium* (Charlotte *et al.*, 2009). The controversy with quorum sensing gene regulation is inevitable. The LuxS quorum sensing system in *E. amylovora* plays no role in regulation of virulence and virulence-associated phenotypes such as motility in apple blossoms, apple trees and pear fruits (Rezzonico and Duffy, 2007). However, in contrast to that, Gao *et al.* (2009) reports involvement of the LuxS quorum sensing system in virulence, EPS production and motility of *E. amylovora*. Research has confirmed that quorum sensing regulation is bacterial strain-specific and dependent on the physiological conditions of the host/s.

Whilst research on quorum sensing has been conducted on other bacterial plant pathogens (such as those mentioned above), not much research has been done on quorum sensing in *P. ananatis.* However, there are reports on the effects of the *eanI* and *rhlI* genes in cell aggregation and virulence in *P. ananatis* (Morohoshi *et al.*, 2007; Morohoshi *et al.*, 2011). Morohoshi *et al.* (2007) showed that the *eanI*$^-$ *rhl*$^-$ mutant strain of *P. ananatis* is defective in biofilm formation and less aggressive on onion seedlings compared to the wild-type strain. Their study led to the identification and characterization of two different AHLs produced by *P. ananatis,* the N-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C_6-HSL) and N-hexanoyl-L-homoserine lactone (C_6-HSL).

Despite the work on *P. ananatis* discussed above, there is still limited knowledge pertaining to the significance of quorum sensing systems in this plant pathogen.

1.8.1 Biofilm formation

The theory of biofilms was first outlined by Costerton *et al.* in 1978. Despite the fact that biofilms in nature exist as a mixture of different bacterial strains and species, most *in vitro* studies have employed single species. Biofilms of single species do exist in nature and have been identified mostly in clinical situations (Davey and O'Toole, 2000). A biofilm is defined as a community of microorganisms permanently attached to either a surface or to each other, enclosed in an exopolysaccharide matrix (produced by bacteria) and expressing an altered phenotype with regards to gene expression and growth rate (Donlan and Costerton, 2002). Research has shown that biofilm formation is influenced by quorum sensing in most bacterial species (Swift *et al.*, 2008).

For quorum sensing to occur, cells should be in close proximity to each other so as to facilitate accumulation of quorum sensing signals (von Bodman *et al.*, 2003). Bacteria achieve this through biofilm formation, thus it is a crucial virulence factor. Biofilms result in concentrating a high mass of cells in one site for a time frame sufficient for bacteria to initiate successful infection of the host/s. The cell population density in biofilms allows bacteria to act as a multicellular organism and perform activities that can not be performed by their planktonic counterparts. One mechanism of plant defence against bacterial plant pathogens is inhibition of biofilm formation. A typical example is the production of salicyclic acid that inhibits attachment and cell aggregation in *P. aeruginosa* on *A. thaliana* roots (Prithiviraj *et al.*, 2005). Biofilms help protect bacteria from host defence systems. For example, an antimicrobial molecule called rosmarinic acid produced by sweet basil is effective against planktonic *P. aeruginosa* cells and has no effect on cells in a biofilm (Walker *et al.*, 2004). Bacterial biofilms are a crucial bacterial phenotype since they confer bacteria with other competitive advantages, for example, they provide a site for horizontal gene transfer where bacteria acquire genes that could be beneficial for adaptation and survival in a changing environment (Jefferson, 2004).

In many bacterial species, biofilm formation is regulated by quorum sensing and consists of five distinct stages depicted in Fig. 1.5. The first stage is the initial attachment of bacterial cells to a surface; during this stage the genes that are essential for biofilm formation are transcribed (Annous *et al.*, 2009). The second stage is the production of an exopolysaccharide that leads to a firm irreversible adherence, the third and fourth stages entail initial and final stages of development of the biofilm architecture, respectively (Stoodley *et al.*, 2002). The development of microcolonies with water channels occurs during the third and fourth stages. The cells also alter their metabolic processes and adapt to that particular niche. The fifth and last stage is the release of bacterial cells from the biofilm. This not only results in biofilm formation at a different site, but it also facilitates the spread and distribution of the pathogen (Annous *et al.*, 2009).

Microbial cells in a biofilm exist as single cells or microcolonies (Costerton *et al.*, 1987). The water channels that occur between cell clusters help deliver nutrients and oxygen deep into the inside of the biofilm and also help in the removal of metabolic waste (Soncransky and Haffajee, 2002). These water channels are maintained open by surfactants (Davey *et al.*, 2003), for example, rhamnolipids in *P. aeruginosa*. The hydrophobic and hydrophilic nature of biosurfactants helps reduce the interfacial forces between lipids and water, and result in cell-to-surface association rather than cell-to-cell or cell-to-matrix association (Boles *et al.*, 2005).

The biofilm structure is influenced by a number of factors that include quorum sensing, exopolysaccharide production, rate of cell proliferation, motility, surfactants (Davey *et al.*, 2003) and growth conditions (Stoodley and Stoodley, 2002). Three modes of biofilm formation include motility and attachment of cells to a surface, cell proliferation and recruitment of cells from the bulk fluid to the developing biofilm (Stoodley and Stoodley, 2002). The mode of biofilm formation is dependent on the bacteria in question, the attachment surface and other environmental conditions such as availability of nutrients (Stoodley and Stoodley, 2002).

Studies have shown that the attachment of cells to surfaces triggers expression of genes that play a role in the formation of biofilms. One typical example is the gene *algC* that controls the production of an exopolysaccharide, alginate, which is synthesized during the initial attachment of *P. aeruginosa* cells to a surface (Davies and Geesey, 1995). Other genes that are involved in biofilm formation include motility genes, which are activated and deactivated at various stages of biofilm formation (Klausen *et al.,* 2003).

Bacterial biofilms are found in most natural ecosystems. According to Donlan and Costerton (2002), more than 99.9% of bacteria are capable of forming biofilms on various surfaces. Research has shown that biofilms are responsible for persistant infections and virulence of bacteria (Li and Tian, 2012; Wang *et al.*, 2012; Rendueles and Ghigo, 2012; Djeribi *et al.*, 2012). The nature and structure of biofilms contribute to increased resistance of biofilms to antimicrobial agents, for example, the extracellular matrix influences the rate of diffusion of substances such as antibiotics into a biofilm. A study conducted by Hoyle *et al.* (1992) showed that bacteria in a biofilm are more than 15-times more resistant to an antibiotic, tobramycin, compared to their planktonic counterparts. An exopolysaccharide, alginate, was found to prevent diffusion of gentamycin and tobramycin into cells in a biofilm (Hatch and Schiller, 1998).

1.8.2 Motility

Motility has been implicated in the virulence of most motile bacteria where it helps in the movement of bacterial cells to favoured niches, penetration of host tissues and translocation into the intracellular spaces of host cells (Quinones *et al.*, 2005; Syed *et al.*, 2009; Zhao *et al.*, 2009; Lertsethtakarn *et al.*, 2011). The mechanisms of bacterial movement include flagella-mediated swimming and swarming motility (Rashid and Kornberg, 2000), gliding motility and type IV pili-mediated twitching motility. Swimming motility is defined as movement on an aqueous thick surface and swarming motility is movement on a relatively thin aqueous surface. Twitching motility is a type IV pili-mediated form of movement on a solid surface, resulting in an unorganized macro-morphological pattern (Rashid and Kornberg, 2000).

Swimming motility has been shown to be crucial in the initial stages of infection of plants by *R. solanacearum* (Tans-Kersten *et al.*, 2001). Quorum sensing-deficient strains of *Pantoea stewartii* subsp. *stewartii* lacking motilities formed thin disperse poor biofilms (Koutsoudis *et al.*, 2006) and conferred reduced virulence on maize plants. The LuxS quorum sensing system in Pcc has been shown to play a role in motility and virulence (Coulthurst *et al.*, 2006). Quorum sensing-regulated motility has also been identified in the bean plant pathogen *P. syringae*. In *P. syringae,* inactivation of the *luxS* gene resulted in reduced virulence and swimming motility (Quinones *et al.*, 2005). Based on the above literature, motility is thus a crucial virulence trait governed by quorum sensing.

1.8.3 Exopolysaccharides

Exopolysaccharides (EPS) are an important virulence factor in most bacterial-plant associations (Geider, 2000; Skurnik and Bengoechea, 2003). Exopolysaccharides are made up of polysaccharides, glycoproteins, glycolipids and, in some cases, extracelluar DNA (eDNA) (Vilain *et al.*, 2009) that form a matrix and help retain water and keep bacterial cells in close proximity. Extracellular DNA in exopolysaccharides contributes to the stability of biofilms during the initial stages of biofilm formation in Gram-negative bacteria such as *P. aeruginosa (*Whitchurch *et al.*, 2002). The extracellular DNA in EPS is produced by a quorum sensing-regulated bacterial cell lysis at suboptimal concentrations.

Pantoea stewartii causes wilting in maize. EPS production in *P. sterwartii* subsp. *sterwartti* is under quorum sensing influence. The EPS produced by this bacterium, sterwartan, causes vascular occlusion, collapse and wilting in maize (Geider, 2000). Quorum sensing has also been shown to play a role in the virulence of *E. amylovora* where it regulates the production of amylovoran, an exopolysaccharide that causes progression of symptoms of infection and development of disease symptoms in plants belonging to the Rosaceae family (Geider, 2000) such as pears, apples and raspberries.

Quorum sensing autoinduction has also been described in several plant pathogenic bacteria, including *R. solanacearum* (Flavier *et al.*, 1997). The virulence of *R. solanacearum* is dependent on secretion of an EPS called EPS I (Schell, 1996). In *P. syringae,* quorum sensing regulates production of an EPS (Quinones *et al.*, 2005) that is crucial for virulence of this bacterium on bean leaves and other leaf-associated plants. An EPS, xantham from *X. campestris,* the black rot pathogen of cruciferous plants, is essential for cell aggregation and biofilm formation (Dow *et al.*, 2003). In *A. tumefaciens,* cellulose production results in increased bacteria-to-plant attachment and biofilm formation (Rodríguez-Navarro *et al.*, 2007). Structural and chemical differences of EPSs from different plant pathogens suggest that these molecules are specific and have different roles in plant-pathogen interactions.

In summary, exopolysaccharides play essential roles in plant-pathogen interactions where they are believed to act as signals (de Pinto *et al.*, 2003) in plant-pathogen interactions and aid in attachment of pathogens to hosts. EPS act as a microenvironment for bacteria in a biofilm and help protect bacteria from host defense mechanisms, predators (Sutherland, 2001) and environmental stress such as osmotic shock, ultraviolet radiation and desiccation (Flemming, 1993). The exopolysaccharides also aid in sequestering nutrients by binding to nutrients necessary for growth (Costerton *et al.*, 1994) from the environment. Exopolysaccharides act as an ion exchanger in biofilms by restricting diffusion of compounds into the biofilm (Gilbert *et al.*, 1997) and thus protect bacteria from the action of antimicrobials (Mah and O'Toole, 2001). Thus, it can be concluded that exopolysaccharides influence the survival of bacteria in adverse environments and hence can be a direct or indirect virulence factor, depending on the bacterial strain in question.

1.8.4 Cell-wall-degrading enzymes (CWDEs)

Some bacteria produce plant cell-wall-degrading enzymes that aid in host invasion and colonization. A typical example is Pcc (Molina *et al.*, 2005) that secretes a battery of cell-wall-degrading enzymes. The successful colonization and invasion of host tissues by this bacterium (Põllumaa *et al.*, 2012) is achieved by quorum sensing regulation of

secretion of the CWDEs (Jones *et al.*, 1993; Barnard *et al.*, 2007) such as proteases, polygalacturonases and cellulases.

A soft rot and wilt causing bacterium, *Dickeya chrysanthemi,* secretes CWDEs such as pectate lyase, which is crucial for its virulence (Grenier *et al.*, 2006). *X. campestris* is another CWDE-secreting bacterial pathogen that secretes enzymes such as endo-β-(1,4)-mannanase that aid in successful plant infections (Dow *et al.*, 2003). Plant CWDEs have also been implicated in the virulence of *R. solanacearum*, a bacterium that causes lethal wilt in various plants (Liu *et al.*, 2005).

1.8.5 Antibiotics

Antibiotics have not been implicated in host infection and colonization, but give bacterial plant pathogens a competitive advantage during plant-pathogen interaction. One exceptional antibiotic, phenazine, produced by *Pseudomonas chloraphis* has been shown to play a significant role in biofilm formation and virulence of this bacterium on the seeds and roots of wheat. The production of phenazine is under quorum sensing regulation (Maddula *et al.*, 2006). Phenazine produced by Pseudomonads influences swarming motility, attachment and biofilm formation (Ramos *et al.*, 2010). Wang *et al.* (2011) showed that phenazine causes an increase in iron acquisition and thus plays a role in cell nutrition, growth and biofilm formation.

1.8.6 Toxins

Quorum sensing has also been shown to regulate the production of phytotoxic lipodepsipeptides (LDPs), which are believed to play a role in the virulence of plant pathogenic *Pseudomonas corrugata* and *Pseudomonas mediterranea* in tomato plants. Pathogenicity tests of the mentioned *Pseudomonas* spp. and their respective quorum sensing mutants showed that mutants produced weak symptoms of tomato pith necrosis disease compared to wild-type strains (Licciardello *et al.*, 2011). Toxin production by the bacterium *Clostridium perfringes* the causal pathogen for clostridial myonecrosis, food poisoning and enteritis necroticans in humans is regulated by the

LuxS quorum sensing system where precise timing for toxin production plays a crucial role as a pathogenicity factor (Ohtani *et al.*, 2002).

1.8.7 Biosurfactants

Research has shown that biosurfactants such as rhamnolipids are important for virulence in bacteria (Rahme *et al.*, 2000; Zulianello *et al.*, 2006; Murray *et al.*, 2007). Various classes of biosurfactants produced by bacteria have been identified, including rhamnolipids and cyclic lipopeptides. Biosurfactant production is a quorum sensing-regulated process, for example, the Rhll/R quorum sensing system has been shown to influence production of rhamnolipids in *Pseudomonas* spp. (Oschner *et al.*, 1995; Fuqua and Greenberg, 2002; Girard and Bloemberg, 2008). Biosurfactants have anti-microbial properties and thus confer competitive advantages to bacteria that produce them. Despite the significance of biosurfactants in biofouling, they have been found to be essential virulence factors of bacteria, where they serve to reduce the surface tension by acting as wetting agents and thus facilitate motility (Tremblay *et al.*, 2007), and biofilm formation (Davey *et al.*, 2003; Stanley and Lazazzera, 2004).

P. aeruginosa is capable of adaptation, growth and survival in various niches. Its ability to produce rhamnolipids has been shown to contribute to its ability to cope in changing environments through facilitation of cell motility and maintenance of biofilm water channels. Davey *et al.* (2003) showed that in this bacterium the absence of rhamnolipids or presence of too much rhamnolipids cause defects in the biofilm structure. Their study signified the importance of quorum sensing gene regulation in the production of biosurfactants by bacteria.

1.8.8 Pigmentation

Microbial cell pigmentation plays a role in the virulence of the pigmented bacterial species through interference with the host immune system (Liu and Nizet, 2009). Pigments also confer microorganisms with an ability to survive stressful environmental conditions such as ultraviolet radiation, oxidants, extreme temperatures and presence of

antimicrobials (Nair *et al.*, 1992; Gao *et al.*, 2003). The pigment produced by *P. aeruginosa,* pyocianin, has been implicated in the virulence of this pathogen. The production of pyocianin is influenced by quorum sensing (Rahme *et al.*, 2000) and pyocianin results in the generation of reactive oxygen compounds that causes toxicity to human cells (Becerra *et al.*, 2001). Though the study of involvement of pigments in bacterial virulence was conducted on mammalian hosts, it can not be ruled out as a important phenotype for virulence in plants since bacteria with broad host range have shown conserved mechanisms of virulence (Rahme *et al.*, 2000).

1.8.9 Secretion systems

Bacterial secretion systems help transport microbial proteins that are important for pathogenicity (Waksman 2012; Beeckman and Vanrompay, 2012). The secretion systems that have been shown to impact on the virulence of bacteria are type I, II, III and IV. The type I secretion system helps transport toxins to the extracellular space (Remaut and Waksman, 2004), whereas the type II secretion system transports enzymes and toxins (Sandkvist, 2001). The type III secretion system transports proteins that are detrimental to host tissues directly into the host's cells. The type IV secretion system (Mougous *et al.*, 2006) transports molecules such as DNA and proteins that influence the host's metabolic pathways (Nagai and Roy, 2003), for example, the Ti plasmid of *A. tumefaciens* (Aguilar *et al.*, 2011). Quorum sensing regulation of secretion systems has been reported in bacteria such as in *Vibrio parahaemolyticus* and *V. harveyi* where quorum sensing influences the type III secretion system (Henke and Bassler, 2004). The secretion of microbial proteins that are important for bacterial virulence traits is crucial and thus regulation by quorum sensing helps to establish successful infections.

1.9 CONCLUSIONS

Plant pathogenic bacteria cause significant economic losses to the agricultural and forestry industries. Different quorum sensing systems exist in plant pathogenic bacteria where they are crucial for regulation of host-pathogen interactions. Quorum sensing

influences expression of virulence in bacterial biofilms that form on and around plant tissues, resulting in effective host/s infections and colonization. A better understanding of how different plant pathogenic bacteria utilize quorum sensing systems to achieve successful host infections and establishment of virulence phenotypes is essential. Such knowledge could help uncover crucial role/s of quorum sensing systems in plant-pathogen associations and thus open avenues for control of bacterial plant diseases.

The literature discussed above indicates that quorum sensing is an essential mechanism for bacterial survival, virulence and adaptation to various niches. The study of quorum sensing-deficient strains is a useful tool for better clarification and understanding the importance of quorum sensing in various plant-pathogen interactions. Research focusing on the association of quorum sensing and virulence in bacteria is thus a requirement. The knowledge thus acquired can be used in the formulation of new biological control measures so as to alleviate the current challenge of development of antibiotic-resistant strains (Antunes *et al.*, 2010).

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Figures

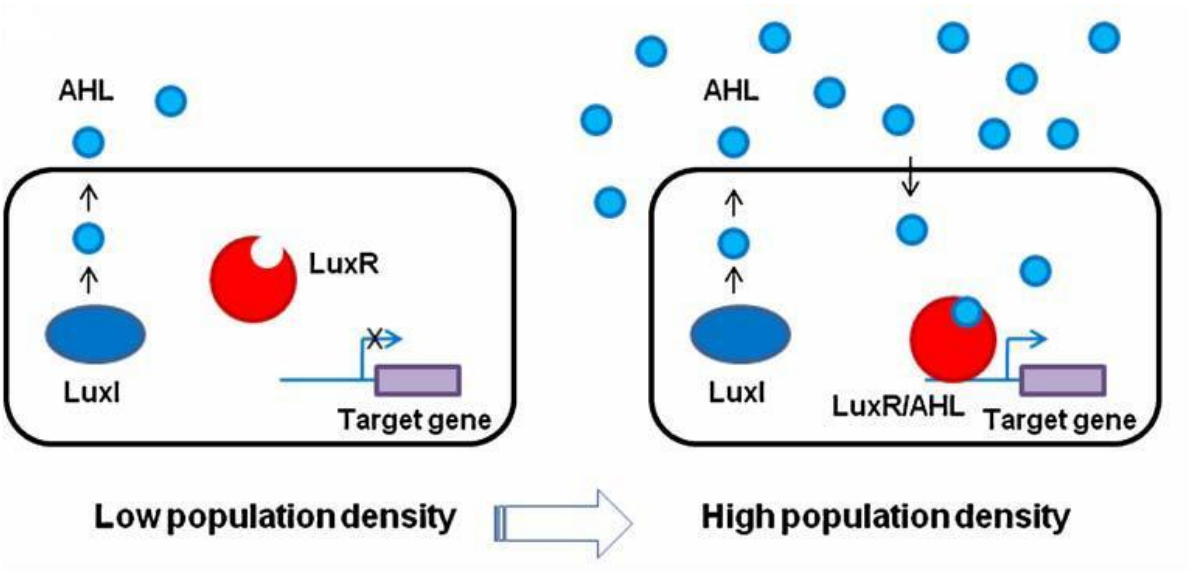
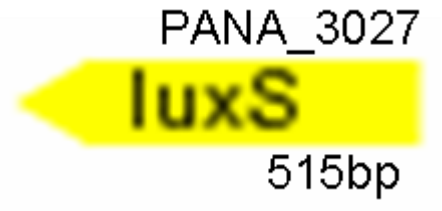


Fig. 1.1 Schematic representation of the model of an AHL-based quorum sensing system in Gram-negative bacteria. The diagram shows that at low cell densities the receptor proteins are free and unbound to AHLs and thus no quorum sensing gene regulation occurs. In contrast, at high cell densities the receptor proteins bind to AHLs and trigger expression of quorum sensing-regulated genes (modified from Choudhary and Schmidt-Dannert, 2010).

(a)



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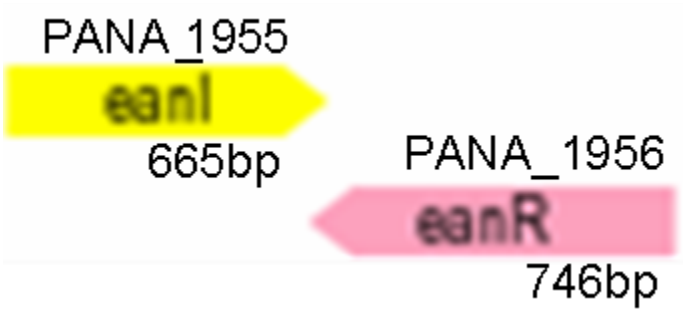

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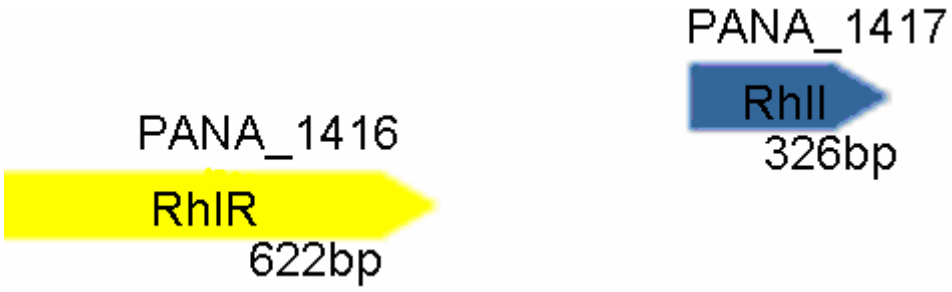

Fig. 1.2 Schematic presentation of quorum sensing genes of *P. ananatis* showing specific gene sizes. **(a)** shows the *luxS* gene, a single copy made up of PANA_3027, **(b)** shows the two genes (PANA_1955 and PANA_1956) coding for the EanI/R quorum sensing system, and **(c)** shows the two genes (PANA_1416 and PANA_1417) coding for the RhlI/R quorum sensing system (De Maayer *et al.*, 2010).

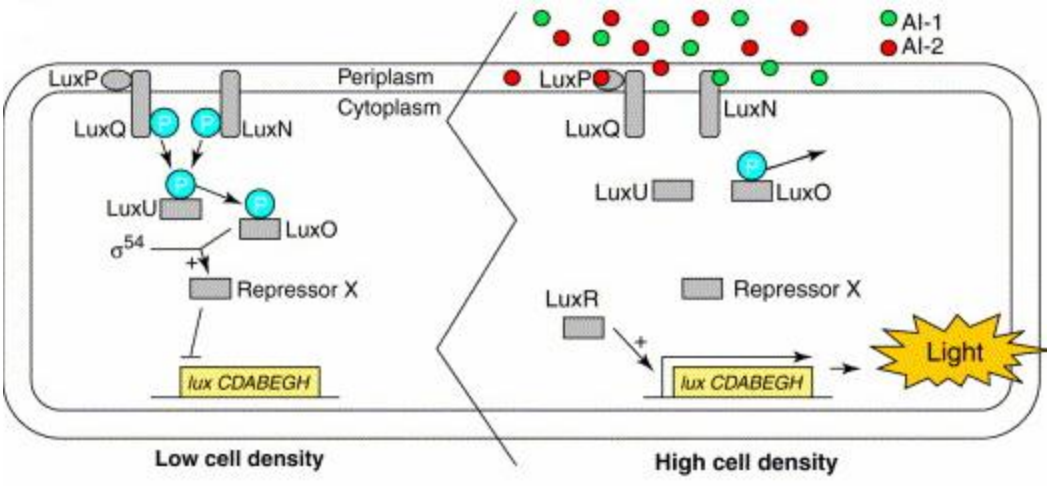

Fig. 1.3 Schematic representation of the two-component quorum sensing system in *V. harveyi*. At low population density the repressor protein is phosphorylated and inhibits transcription of the quorum sensing-governed light emission, whereas at high cell densities the repressor protein is dephosphorylated resulting in deactivated repressor protein and light emission (modified from Coulthurst *et al.*, 2002).

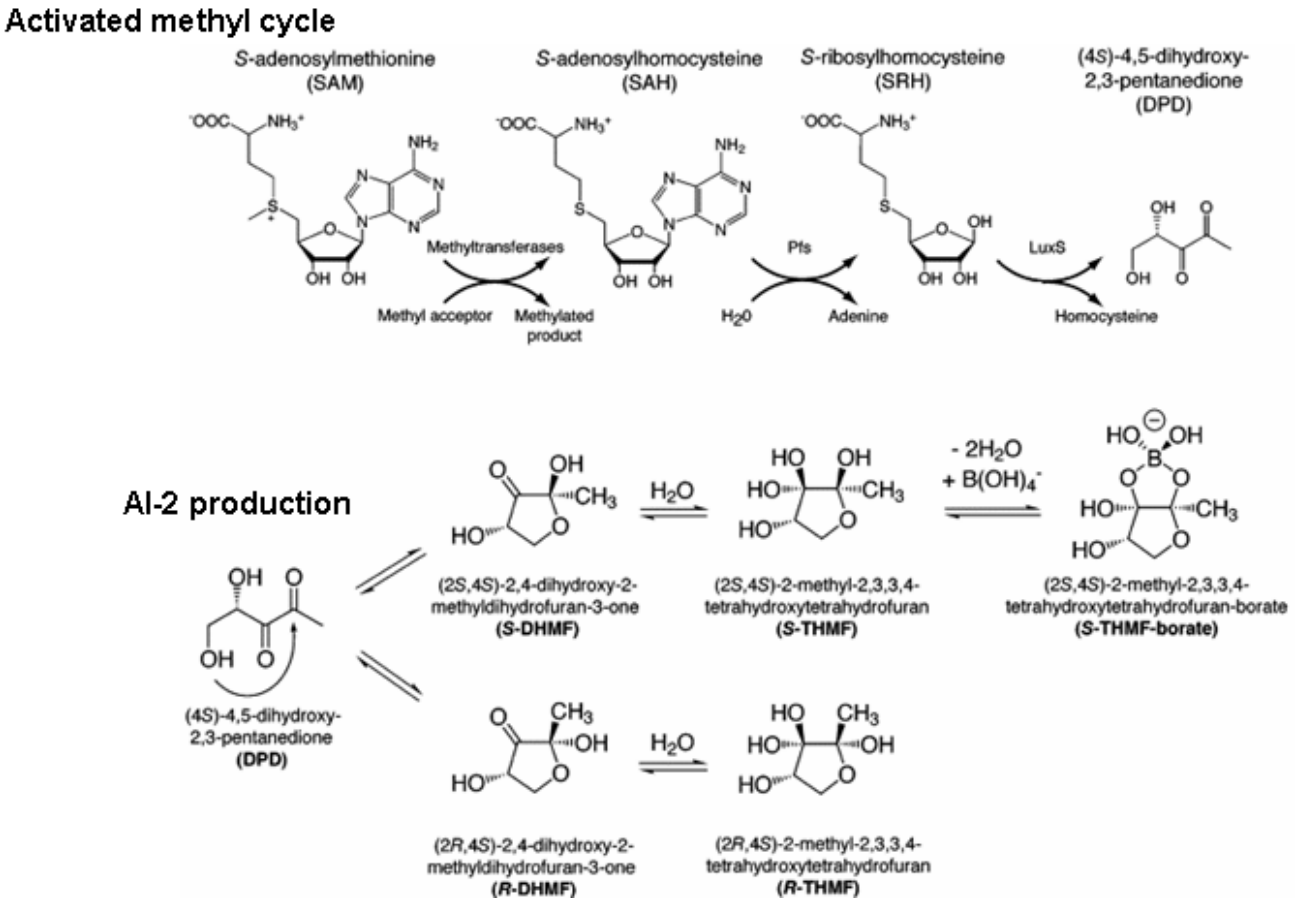

Fig. 1.4 Summary of chemical reactions in the activated methyl cycle resulting in the production of the quorum sensing signal AI-2. Many methyltransferases act on SAM and transfer a methyl group to various substrates. These reactions also produce *S*-adenosylhomocysteine (SAH). Pfs hydrolyzes adenine from SAH to form *S*-ribosylhomocysteine (SRH). LuxS acts on SRH to produce 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine. As shown in the bottom diagram, DPD is able to undergo spontaneous cyclization to either of the two forms (*R* or *S*) of 2,4-dihydroxy-2-methylhydro-3-furanone (DHMF). Hydration of *S*-DHMF yields *S*-TMHF that subsequently forms a diester with boric acid to generate the active form of AI-2 in *V. harveyi*. On the other hand, *R*-DHMF hydrates to form *R*-THMF, the active form of enteric AI-2 (Miller *et al.*, 2004).

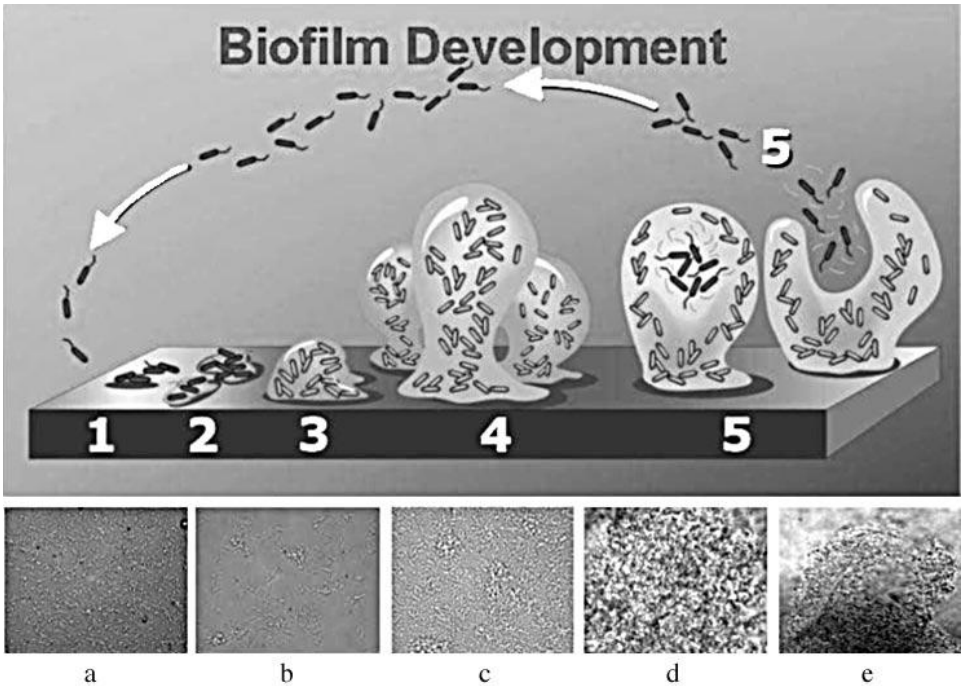

Fig. 1.5 Schematic representation of the five stages of biofilm formation in bacteria. Stage 1 - initial attachment to surface; stage 2 - EPS production; stage 3 - initial development of biofilm architecture; stage 4 - final development of biofilm architecture, stage 5 - release of cells from biofilm. The picture frames a-e shows each of the five stages when viewed under the microscope (Stoodley *et al.*, 2002).

CHAPTER TWO

CONSTRUCTION AND COMPLEMENTATION OF *Pantoea ananatis* QUORUM SENSING MUTANT STRAINS: AN ADAPTATION OF THE λ RED-RECOMBINEERING PROTOCOL

ABSTRACT

The genes for three quorum sensing systems of a plant pathogenic strain of *Pantoea ananatis* (LMG2665^T) were identified from its genome sequence. The three systems are EanI/EanR, RhlI/RhlR and LuxS. A PCR-based lambda (λ) Red-mediated gene knockout technique was used to generate the three mutant strains, EanΔI/R, RhlΔI/R and ΔLuxS. In addition, a mutant strain lacking all three quorum systems was also engineered. The mutant strains were complemented by cloning the respective quorum sensing genes into the multiple cloning site of the broad-host-range cloning vector pBR1MCS-5, and then introducing these recombinant plasmids into the corresponding mutant strain. The engineered mutant strains can be used towards determining the importance of the quorum sensing systems in the biology of *P. ananatis*.

2.1 INTRODUCTION

The advent of whole-genome sequencing has provided a key to gene identification and thus paved the way to determine gene function/s. The most effective approach for functional analysis of specific genes is studying a strain that lacks that particular gene or has an altered version of that specific gene (Alberts *et al.*, 2002). Indeed, the characterization of mutant strains of various organisms has helped to unveil the biological role/s of numerous genes and the proteins they encode (Beck von Bodman *et al.*, 1998; Quinones *et al.*, 2005; Koutsoudis *et al.*, 2006; Charlotte *et al.*, 2009; Gao *et al.*, 2009; Ling *et al.*, 2010; Li *et al.*, 2011; Morohoshi *et al.*, 2011). The initial step in these types of studies, however, is to engineer specific strains that lack the gene or contain a modified version of the gene of interest.

Conventional gene replacement protocols typically involve construction of gene disruption cassettes on a suitable plasmid before recombining it into the chromosome of the organism. This approach entails sub-cloning of the target gene into a suitable plasmid vector and the use of restriction enzymes to digest the cloned target gene, followed by cloning of a selectable marker gene into the deletion site (Kuwayama *et al.*, 2002). The protocol is laborious and the efficiency by which a mutant strain is generated is generally low. Alternative technology makes use of a PCR-based protocol to construct a gene disruption cassette that is then recombined into the host genome in order to inactivate or replace the targeted gene (Datsenko and Wanner, 2000).

The PCR-based gene disruption method has been employed for gene disruption in yeasts and fungi such as *Saccharomyces cerevisiae* (Baudin *et al.,* 1993), *Candida albicans* (Wilson *et al.*, 1999) and *Aspergillus nidulans* (Chaveroche *et al.*, 2000). The method has also been used in making mutants of the amoeba *Dictyostelium discoideum* (Kuwayama *et al.*, 2002; Betapudi *et al.*, 2004). This PCR-mediated protocol has been expanded for use in bacterial species, and was used successfully to make targeted mutations in, for instance, *Escherichia coli* (Datsenko and Wanner, 2000), *Pseudomonas aeruginosa* (Lesic and Rahme, 2008), *Yersinia* spp. (Derbise *et al.*, 2003; Lesic *et al.*, 2004), *Salmonella enterica* Serovar Typhimurium (Husseiny and

Hensel 2005), *Shigella flexneri* (Beloin *et al.*, 2003), *Serratia marcescens* (Rossi *et al.*, 2003) and *Erwinia amylovora* (Zhao *et al.*, 2009). In this study, we describe the inactivation of chromosomal genes encoding three putative quorum sensing systems of the plant pathogen *Pantoea ananatis* LMG2665^T by making use of PCR-generated gene disruption cassettes and a bacteriophage lambda (λ) Red recombinase system.

The availability of the complete genome sequence of *P. ananatis* LMG20103 (De Maayer *et al.*, 2010) has facilitated the feasibility of generating quorum sensing mutants of *P. ananatis* LMG2665^T using the λ Red-recombineering method. The genome data of strain LMG20103 was used to identify homologous genes in the *P. ananatis* LMG2665^T genome, thus enabling the design of appropriate primers so as to make gene disruption cassettes. Construction of a PCR-generated gene disruption cassette relies on a three-step PCR protocol to obtain a DNA fragment that carries an antibiotic resistance cassette flanked by a region homologous to the target locus (Fig. 2.1). To enable inactivation of the chromosomal gene, the gene disruption cassette is subsequently introduced into a strain that expresses the bacteriophage λ Red recombinase system (Katashkina *et al.*, 2009).

As indicated above, the λ Red-recombineering method makes use of a homologous recombination system of bacteriophage λ, designated the λ Red system. The λ Red operon comprises three genes that code for the Exo, Beta and Gam proteins, respectively. Each of the three proteins has a significant role in recombination. The Gam protein serves to inhibit recognition of foreign DNA by the host cell's nucleases, thereby protecting the disruption cassette from degradation; the Exo protein degrades linear double-stranded DNA from each end and in a 5' to 3' direction, resulting in double-stranded DNA with single-stranded DNA overhangs at the 3' ends; and the Beta protein facilitates the binding and pairing of homologous DNA strands to allow for homologous recombination to occur (Datsenko and Wanner, 2000). In this study, the easily curable, low-copy-number plasmid pRSFRedTER (Katashkina *et al.*, 2009), which enables inducible expression of the Exo, Beta and Gam proteins, was used to facilitate genetic engineering of *P. ananatis* LMG2665^T.

In addition to the engineering of mutant strains, it is also important to complement the mutations present in the mutant strains in order to verify the phenotypes observed. In this study, the pBR1MCS-5 plasmid was chosen for the construction of recombinant plasmids whereby *P. ananatis* LMG2665^T mutant strains could be complemented with the wild-type allele. This plasmid has a relatively small size (about 4.8 kb), it allows direct selection of transformants using the gentamycin resistance gene (Kovach *et al.*, 1995) and it is also stably maintained in host cells in the absence of antibiotic selection pressure (Lagendijk *et al.*, 2010). The stability of the pBR1MCS-5 plasmid was verified by confirming the presence of the plasmid in bacterial cells after sub-culturing for 30 generations in gentamycin-free media (Lagendijk *et al.*, 2010).

Towards determining the importance of quorum sensing systems identified in the genome of *P. ananatis* LMG2665^T, this study reports on the construction of isogenic mutants strains by directed mutagenesis using the λ Red recombineering approach and the verification of the presence of the mutant alleles in the derived *P. ananatis* mutants. Furthermore, the construction and characterization of complementation plasmids whereby the mutations could be complemented in mutant strains is also described.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains, plasmids and culturing conditions

The bacterial strains and plasmids used in this study are listed in Table 2.1. The bacterial strains were cultured routinely in Luria-Bertani broth (LB: 0.5% [w/v] yeast extract; 1% [w/v] tryptone; 1% [w/v] NaCl, pH 7) at 30°C (*P. ananatis*) or 37°C (*E. coli*) with shaking at 150 rpm, and maintained at 4°C on LB agar or at -80°C as glycerol cultures. For plasmid DNA selection and maintenance, the growth medium was supplemented with kanamycin (40 µg/ml), gentamycin (10 µg/ml), chloramphenicol (50 µg/ml) or ampicillin (50 µg/ml). All antibiotics were purchased from Sigma-Aldrich.

2.2.2 PCR-based construction of gene disruption cassettes

2.2.2.1 Primers

Nucleotide sequences of *P. ananatis* LMG2665^T potentially coding for proteins similar to the *eanI/eanR*, *rhlI/rhlR* and *luxS* gene products of *P. ananatis* LMG20103 (De Maayer *et al.*, 2010) were identified using the BLAST-N alignment program (Altschul *et al.*, 1997). Primers whereby upstream and downstream regions flanking the genes for the above quorum sensing systems of *P. ananatis* LMG2665^T could be amplified were subsequently designed from the relevant genome sequences using Oligo Analyzer (available at: http://eu.idtdna.com). The primer sequences were then compared to the complete genome sequence of *P. ananatis* LMG2665^T by BLAST-N analysis to ensure that they did not anneal to non-targeted regions on the genome. The primers, indicated in Table 2.2, were synthesized by Inqaba Biotechnical Industries.

2.2.2.2 PCR

The gene disruption cassettes were constructed according to a three-step PCR protocol described by Datsenko and Wanner (2000). Genomic DNA of *P. ananatis* LMG2665^T was extracted with the Zymoclean™ gDNA isolation kit (Zymo Research Corp.) according to the instructions from the manufacturer, and used as template DNA in the PCR reactions. The upstream and downstream regions flanking the targeted genes were PCR amplified with the appropriate F_{up}/R_{up}-kan and F_{down}-kan/R_{down} primers (Table 2.2; Figs. 2.2a-2.4a). Each of the R_{up}-kan and F_{down}-kan primers contain, respectively, at their 5' ends 20 and 22 nucleotides that are homologous to the 5' and 3' termini of a kanamycin resistance gene. The plasmid pkD13 was used as template to amplify the kanamycin resistance gene with long flanking regions homologous to the target gene using the appropriate Kan-F and Kan-R primers (Table 2.2). Each PCR reaction mixture (50 µl) contained 50 ng of the DNA template, 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP), 100 pmol of each of the forward and reverse primer and 1 U of Phusion® High-Fidelity DNA polymerase (Fermentas). The PCR tubes were placed in a Perkin-Elmer Model 2720 thermocycler. Following initial denaturation at 94°C for 3 min, the reactions were subjected to 30 cycles of

denaturation at 94°C for 30 s, annealing at 60°C for 20 s, and extension at 72°C for 2 min. After the last cycle, the reactions were kept at 72°C for 7 min.

In a second PCR, 10 ng of each of the purified amplified DNA fragments (*i.e.* kanamycin resistance gene, as well as the upstream and downstream regions flanking the target sequence) were mixed with the appropriate F_{up}/R_{down} primers. The overlap PCR reactions were performed as described above, except that the thermocycling parameters were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 3 min, and a final extension at 72°C for 7 min. A third PCR was finally performed using again the F_{up}/R_{down} primers and the product of the second PCR as the template in order increase the yield of the gene disruption cassette.

2.2.3 Agarose gel electrophoresis and purification of DNA fragments

The PCR products were analyzed by electrophoresis in a 1% (w/v) agarose gel at 85 V for 50 min, in the presence of an appropriate DNA molecular weight marker, to determine their size. The DNA fragments of interest were excised from the gel and purified with the Zymoclean™ Gel DNA Recovery kit according to the instructions from the manufacturer (Zymo Research Corp.). Following purification, the PCR-generated gene disruption cassettes were concentrated by ethanol precipitation. For this purpose, 0.1 volume of 3 M NaOAc (pH 4.6) and two volumes of absolute ethanol was added to each sample. Following incubation at -20°C for 20 min, the DNA was collected by centrifugation at 15 000 rpm for 5 min. The DNA pellet was rinsed twice with 70% ethanol, vacuum-dried and suspended in 15 µl of nuclease-free water. The concentration of the purified DNA was determined with a NanoDrop ND-1000 spectrophotometer.

2.2.4 Transformation of *P. ananatis* LMG2665^T with the λ RED plasmid, pRSFRedTER

2.2.4.1 Preparation of competent cells

Competent cells of *P. ananatis* LMG2665^T were prepared according to the protocol described by Sambrook and Russel (2001). A single colony was inoculated into 100 ml of SOC medium (2% [w/v] tryptone; 0.5% [w/v] yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM glucose) and cultured overnight at 30°C with shaking. Subsequently, 1 ml of the overnight culture was inoculated into 50 ml of SOC medium and grown with shaking to an OD_{600} of approximately 0.4. Following incubation on ice for 20 min, the cells were collected by centrifugation at 2500 rpm for 10 min at 4°C and suspended in 30 ml of ice-cold 0.1 M $CaCl_2$. The cells were incubated on ice for 30 min, collected as above, and then suspended in 8 ml of ice-cold 0.1 M $CaCl_2$ containing 15% (v/v) glycerol. The cells were aliquoted into 1.5-ml Eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80°C until required.

2.2.4.2 Transformation

After allowing the competent cells to thaw on ice, the cells were transformed with the heat shock-method (Micklos and Freyer, 1990). An aliquot of the cells (50 µl) was mixed with 2 µl (10 ng) of pRSFRedTER plasmid DNA. Following incubation on ice for 30 min, the transformation mixture was incubated at 42°C for 90 s and then incubated on ice for 2 min. This was followed by addition of 800 µl of SOC medium and incubation at 30°C for 3 to 4 h with shaking. As a negative control, transformation was also conducted in the absence of the plasmid DNA. Transformants were selected by plating the culture onto LB agar containing 50 µg/ml of chloramphenicol, and the agar plates were incubated overnight at 30°C.

2.2.5 Construction of *P. ananatis* LMG2665^T mutant strains

2.2.5.1 Induction of λ Red gene expression in *P. ananatis*(pRSFRedTER)

A single colony of *P. ananatis* LMG2665^T harbouring the pRSFRedTER plasmid was inoculated into 50 ml of LB broth containing 50 µg/ml of chloramphenicol and incubated

overnight at 30°C with shaking. To induce expression of the λ Red genes, 2 ml of the overnight culture was transferred into 100 ml of SOC broth containing chloramphenicol and supplemented with 2 mM IPTG, followed by incubation at 30°C for 4 to 5 h with shaking. Following incubation, the culture was used immediately for the preparation of electro-competent cells.

2.2.5.2 Preparation of electro-competent *P. ananatis*(pRSFRedTER) cells

Electro-competent cells were prepared according to the protocol of Sambrook and Russel (2001). Equal volumes (50 ml) of the IPTG-induced culture of *P. ananatis*(pRSFRedTER) were transferred to two 100-ml sterile Falcon tubes (BD Biosciences) and chilled on ice for 15 min. The cells were collected by centrifugation at 5000 rpm for 10 min at 4°C, washed twice with 30 ml of ice-cold sterile water and again twice with 15 ml of 10% (v/v) glycerol. The cells were finally suspended in 10 ml of 10% (v/v) glycerol.

2.2.5.3 Electroporation

The PCR-generated gene disruption cassettes were introduced into the electro-competent *P. ananatis*(pRSFRedTER) cells by electroporation with an Eppendorf Multiporator. For this purpose, 10 µl (70 ng) of the gene disruption cassette DNA was mixed with 50 µl of the electro-competent cells in a 0.1-cm electrode gap electroporation cuvette (EquiBio). Following electroporation at 2.5 kV and 200 Ω for 5 ms, 800 µl of SOC medium was added immediately and the transformation mixtures were incubated at 30°C for 4 h. As a negative control, electroporation of cells was also done in the absence of the gene disruption cassette DNA. Transformed cells were selected by plating onto LB agar containing 40 µg/ml of kanamycin, and the agar plates were incubated overnight at 30°C.

2.2.5.4 Screening for mutant strains

A colony-PCR was performed to screen for putative *P. ananatis* LMG2665^T mutant strains. Selected colonies from replica plates were transferred into Eppendorf tubes

containing 500 µl of nuclease-free water, vortexed and boiled at 95°C for 5 min. The cellular debris were pelleted by centrifugation at 14 000 rpm for 2 min and 2 µl of the supernatant, containing the DNA, was used as template in PCR reactions. The PCR reactions were performed, as described previously (Section 2.2.2.2), using the appropriate F_{up}/R_{down} primers to amplify the new mutant-specific fragment.

2.2.6 Verification of *P. ananatis* LMG2665^T mutant strains

The replacement of the wild-type genes with the mutant alleles in the selected *P. ananatis* LMG2665^T strains was verified by Southern blot analysis (Southern, 1975) and PCR analyses.

2.2.6.1 Southern blotting

- **Preparation of labeled probe**

For Southern blot analysis, the kanamycin resistance gene was used as probe and labeled with digoxigenin-dUTP (DIG-dUTP) during the PCR reaction, using the DIG PCR Probe Synthesis kit (Roche Diagnostics) according to the manufacturer's instructions. The kanamycin resistance gene was PCR-amplified using plasmid pKD13 as template and primers km2 and KanF (Table 2.2), as described previously (Section 2.2.2.2), except that primer annealing was performed at 58°C for 30 s.

- **Nucleic acid hybridization**

Aliquots of extracted genomic DNA of the *P. ananatis* LMG2665^T wild-type and mutant strains were digested with *Eco*RI, *Cla*I and *Xho*I at 37°C overnight (5 U of each enzyme per 5 µg of genomic DNA). The digested genomic DNA samples were electrophoresed on a 0.7% (w/v) agarose gel, along with an unlabeled PCR-amplified kanamycin resistance gene as a positive hybridization control. The DNA was subsequently transferred from the agarose gel to a nylon membrane (Hybond™-N; Amersham Biosciences) using a BioRad vacuum blotter and then fixed to the membrane by UV irradiation for 5 min. The probe DNA was denatured by heating in a boiling water bath for 5 min and placed immediately on ice prior to being added to the membrane.

Hybridization was allowed to proceed overnight at 42°C. The hybridized DNA was detected immunologically with alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche Diagnostics) according to the manufacturer's instructions. Once the bands became visible, the colour reaction was stopped by adding TE buffer to the membrane.

2.2.6.2 PCR analyses

The mutant strains were analyzed for replacement of the targeted genes with the kanamycin resistance gene by PCR analyses. For this purpose, three PCRs were performed for each mutant strain. A PCR reaction was carried out with the appropriate F_{up}-out and R_{down}-out flanking locus-specific primers (Table 2.2; Figs. 2.6a-2.8a) to verify simultaneous loss of the wild-type (non-mutant) fragment and gain of the new mutant-specific fragment. A further two PCR reactions were performed by using the flanking locus-specific primers together with the appropriate kanamycin gene-specific test primer (F_{up}-out/km2 or R_{down}-out/km1; Table 2.2) to test for both new junction fragments. The PCR reactions were performed, as described previously (Section 2.2.2.2), except that primer annealing was performed at 55°C for 30 s and extension at 72°C for 4 min for all primer pairs. For all of the analyses, nuclease-free water served as a negative control, while chromosomal DNA extracted from the wild-type and mutant strains provided sample template DNA. Following PCR amplification, aliquots of the respective reaction mixtures were analyzed by electrophoresis on 1% (w/v) agarose gels in the presence of an appropriate DNA molecular weight marker.

2.2.7 Curing of plasmid pRSFRedTER from *P. ananatis* LMG2665^T mutant strains

The pRSFRedTER plasmid was cured from the *P. ananatis* LMG2665^T mutant strains by streaking the strains onto LB agar containing 10% (w/v) sucrose and 40 µg/ml of kanamycin. The agar plates were incubated at 30°C for 48 h. Single colonies were then selected randomly and replica-plated onto LB agar containing kanamycin and LB agar containing 50 µg/ml of chloramphenicol. Following incubation overnight at 30°C, strains

that were kanamycin-resistant but chloramphenicol-sensitive, thus indicating loss of the pRSFRedTER plasmid DNA, were selected for further use.

2.2.8 Complementation of quorum sensing mutant strains

To allow for complementation of the *P. ananatis* mutant strains, the respective quorum sensing genes were cloned into the pBR1MCS-5 plasmid (Kovach *et al.*, 1995). To overcome problems associated with the cloning PCR products, two cloning steps were conducted where the cloning vector pJET1.2/blunt (Fermentas) was used as an intermediate vector before cloning into pBR1MCS-5.

2.2.8.1 Primers

Primers used to amplify the *rhlI*/*rhlR*, *eanI*/*eanR* and *luxS* genes, inclusive of the native promoter regions, were designed based on the complete genome sequence of *P. ananatis* LMG2665^T using Oligo Analyzer (available at: http://eu.idtdna.com). Thus, the RhlI/R-CompF primer was designed to anneal at a position 913 bp upstream of the *rhlR* gene, whereas the LuxS-CompF primer annealed at a position 111 bp upstream of the *luxS* gene. In the case of the *eanI/R* allele, the EanI/R-CompF primer was designed to anneal at a position 357 bp upstream of *eanI* and the EanI/R-CompR primer at a position 597 bp upstream of *eanR*. To facilitate subsequent cloning procedures involving the PCR-amplified products, unique restriction endonuclease recognition sites (*Apa*I) were included at the 5'-terminus of the different primers. The primers, indicated in Table 2.2, were synthesized by Inqaba Biotechnical Industries.

2.2.8.2 PCR amplification of quorum sensing genes

The wild-type alleles of *rhlI*/*R*, *eanI*/*R* and *luxS* were amplified by PCR using genomic DNA of *P. ananatis* LMG2665^T as a template and the appropriate CompF and CompR primers (Table 2.2). The PCR reactions were performed as described previously (Section 2.2.2.2). For control purposes, identical reaction mixtures were also prepared, except that template DNA was omitted. The PCR reaction mixtures were subsequently analyzed by electrophoresis on 1% (w/v) agarose gels in the presence of an appropriate

DNA molecular weight marker, and the PCR products were purified from the agarose gels with the Zymoclean™ Gel DNA Recovery kit (Zymo Research Corp.).

2.2.8.3 Construction of recombinant pJET1.2/blunt plasmids

To enable cloning of the PCR products, which contain 3'-dA overhangs generated by the *Taq* DNA polymerase, it was first necessary to generate blunt-end DNA fragments. For this purpose, 150 ng of purified PCR product was incubated with 10 µl of 2 × Reaction buffer and 1 µl of DNA Blunting Enzyme, a proprietary thermostable DNA polymerase with proofreading activity, in a final volume of 18 µl (CloneJet Cloning kit; Fermentas). Following incubation at 70°C for 5 min, the reaction mixture was chilled on ice and then added to 50 ng of pJET1.2/blunt cloning vector and 1 U of T4 DNA ligase. The ligation reaction mixture was incubated at room temperature (25°C) for 1 h and subsequently introduced into competent *E. coli* DH5α cells, which had been prepared and transformed with the heat shock-method as described previously (Section 2.2.4). The transformation mixtures were plated onto LB agar containing 50 µg/ml of ampicillin, and the agar plates were incubated overnight at 37°C. Since the cloning vector does not allow for blue/white selection, recombinant transformants were identified by performing colony-PCR on randomly selected colonies. The colonies from replica agar plates were each suspended in sterile distilled water, lysed by boiling (95°C for 5 min) and then 2 µl of the supernatant, collected after centrifugation (14 000 rpm for 2 min), was used as a template in the PCR reactions. The primers used in the colony-PCR annealed to regions flanking the multiple cloning site of the vector and were designated pJET-F and pJET-R (Table 2.2). The PCR reactions were performed as described in Section 2.2.2.2.

2.2.8.4 Nucleotide sequencing and sequence analysis

The nucleotide sequence of cloned insert DNA was determined by automated sequencing with the ABI PRISM® BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems) according to the manufacturer's instructions. In addition to the pJET-F and pJET-R vector-specific primers, gene-specific internal

primers were also used in the sequencing reactions (Table 2.2). The reaction mixtures contained 100 ng of purified recombinant plasmid DNA, 0.5 µl of BigDye™ Termination Mix, 2.5 µl of sequencing buffer, 12.5 pmol of the appropriate sequencing primer and nuclease-free water to a final volume of 12 µl. Cycle sequencing reactions were performed in a Perkin-Elmer Model 2720 thermal cycler with 25 of the following cycles: denaturation at 96°C for 10 s, annealing at 55°C for 5 s, and elongation at 60°C for 4 min. The extension products were precipitated by addition of 2 µl of 3 M NaOAc (pH 4.6) and 16 µl of absolute ethanol. The tubes were incubated for 20 min at room temperature in the dark, centrifuged at 15 000 rpm for 30 min and the supernatant carefully aspirated. The extension products were resolved on an ABI PRISM® Model 3120 automated sequencer (Applied Biosystems). The nucleotide sequences obtained were compared against the entries in the GenBank database with the BLAST-N programme, available on the National Centre for Biotechnology Information web page (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.8.5 Construction of recombinant pBR1MCS-5 complementation plasmids

To construct the desired complementation plasmids, the insert DNA was recovered from recombinant pJET1.2/blunt plasmid DNA by digestion with *Apa*I. Restriction endonuclease digestions were performed in Eppendorf tubes in a final volume of 20 µl and contained the appropriate concentration of salt (using the 10× buffer supplied by the manufacturer) for the enzyme and 1 U of enzyme per 1 µg of plasmid DNA. The reaction mixtures were incubated at 37°C for 1 h. Following purification of the insert DNA fragments from an agarose gel, they were cloned into the *Apa*I site of pBR1MCS-5. For this purpose, ligation was conducted at 25°C for 1 h in a final volume of 10 µl, which contained 1 µl of T4 DNA ligase (5 U/µl) and 2 µl of a 5 × Rapid Ligation Buffer (Rapid DNA Ligation Kit; Fermentas). The ratio of vector to insert DNA was 1:3. Following transformation of competent *E. coli* DH5α, the cells were plated onto LB agar containing 10 µg/ml of gentamycin. Using a strategy similar to that described above, recombinant transformants were identified by colony-PCR using primers pBR1MCS-5F and pBR1MCS-5R that flank the multiple cloning site of the pBR1MCS-5 vector (Table

2.2), and successful cloning of the insert DNA was confirmed by restriction enzyme digestion of the recombinant plasmid DNA with *Apa*I.

2.2.9 Complementation of *P. ananatis* LMG2665^T mutant strains

The recombinant pBR1MCS-5 complementation plasmid DNA was introduced into the corresponding *P. ananatis* LMG2665^T mutant strain, which had been made competent and transformed according to the procedures described in Section 2.2.4. The transformation mixtures were plated onto LB agar containing 10 µg/ml of gentamycin and 40 µg/ml of kanamycin. The agar plates were incubated overnight at 30°C. Strains that displayed resistance to both gentamycin and kanamycin were selected and used in all subsequent investigations.

2.2.10 Construction of a *P. ananatis* mutant lacking quorum sensing systems

The EanΔI/R mutant strain served as the source for the construction of a *P. ananatis* LMG2265^T strain lacking the RhlI/R, EanI/R and LuxS quorum sensing systems. All procedures used during the construction of the triple-knockout strain were performed in accordance with the methods described in the preceding sections. To eliminate the kanamycin resistance gene from the EanΔI/R mutant strain, the mutant strain was transformed by the heat-shock method with plasmid pCP20, which is an ampicillin- and chloramphenicol-resistant plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis (Datsenko and Wanner, 2000). Transformants were selected at 30°C on LB agar containing 40 µg/ml of kanamycin and 50 µg/ml of chloramphenicol. Single colonies were then picked and streaked onto non-selective LB agar, followed by incubation at an elevated temperature of 43°C. Colonies that grew at this temperature were re-tested for growth in selective media containing kanamycin and chloramphenicol. A colony that was unable to grow on the selective media, thus indicating loss of the FRT-flanked kanamycin resistance gene and the FLP helper plasmid, was selected in further experiments. Competent cells prepared of the selected strain were transformed with the pRSFRedTER plasmid, after which expression of the λ Red genes was induced with IPTG and electro-competent cells prepared. The cells

were electroporated with a PCR-generated *luxS* gene disruption cassette, transformants were selected on LB agar containing kanamycin and the transformed cells were then cured of the pRSFRedTER plasmid using sucrose. The elimination of the kanamycin gene from the genome of the double-knockout mutant strain was then repeated as described above. The procedure was again repeated by making use of the cells that had lost the kanamycin resistance gene to replace the *rhlI/R* locus with a PCR-generated gene disruption cassette, except that the kanamycin gene was not excised following curing of the pRSFRedTER plasmid from the mutant strain. Successful engineering of the EanΔI/R,RhlΔI/R,ΔLuxS quorum sensing mutant strain was verified by Southern blot hybridization and PCR analyses. The Southern blot was conducted as described previously (Section 2.2.6.1), whereas the PCR assays were conducted by making use of the respective CompF and CompR primers (Table 2.2), and the reaction conditions were the same as that described in Section 2.2.2.2.

2.3 RESULTS

2.3.1 Generation of gene disruption cassettes

Since homologous recombination events that incorporate a mutant allele into the chromosome of an organism are rare, it is tedious and time-consuming to screen for such events if the cloned gene cannot be directly selected. However, by inactivating the cloned gene with a readily selectable marker, such as an appropriate antibiotic resistance gene, it is possible to directly screen for potential mutants based on their newly acquired resistance to the antibiotic. In this study, the kanamycin resistance gene was used to replace the putative *rhlI/rhlR* and *eanI/eanR* loci, as well as the putative *luxS* gene of *P. ananatis* LMG2665^{T}. By making use of the kanamycin resistance gene, it would thus be possible to rapidly and directly screen for potential *P. ananatis* mutant strains based on their newly acquired resistance to kanamycin. Consequently, a three-step PCR procedure was used to generate gene disruption cassettes containing the kanamycin resistance gene flanked by regions surrounding the target genes.

- **Construction of a *rhlI/rhlR* disruption cassette**

The three-step PCR procedure that was used to construct a disruption cassette for the *rhlI* (PANA_1417) / *rhlR* (PANA_1416) locus yielded the following results. In the first PCR step, *P. ananatis* LMG2665^T genomic DNA was used together with primers RhlI/R-F_{up} and RhlI/R-R_{up}-kan to PCR amplify a 916-bp region upstream of the *rhlR* gene, as well as with primers RhlI/R-F_{down}-kan and RhlI/R-R_{down} to PCR amplify a 1.073-kb region downstream of the *rhlI* gene (Fig. 2.2a). Simultaneously, plasmid pkD13 was used with primers RhlI/R-F-kan and RhlI/R-R-kan to amplify the 1.310-kb kanamycin resistance gene. In the second PCR step, the purified PCR products were mixed with primers RhlI/R-F_{up} and RhlI/R-R_{down}, and a 3.299-kb product was obtained that comprised the kanamycin resistance gene flanked by the locus-specific upstream and downstream regions. A third PCR was subsequently performed with the same two primers and the product of the second PCR as the template in order to obtain a large quantity of the gene disruption cassette (Fig. 2.2b).

- **Construction of a *eanI/eanR* disruption cassette**

The same three-step PCR procedure was used to construct a disruption cassette for the *eanI* (PANA_1955) / *eanR* (PANA_1956) locus, except that the *P. ananatis* LMG2665^T genomic DNA was used with primers EanI/R-F_{up} / EanI/R-R_{up}-kan and EanI/R-F_{down}-kan / EanI/R-R_{down}, respectively, to amplify regions of 1.263-kb and 1.198-kb flanking the locus (Fig. 2.3a). These DNA fragments and the PCR-amplified 1.310-kb kanamycin resistance gene were mixed with primers EanI/R-F_{up} and EanI/R-R_{down} to generate a 3.771-kb PCR product. A large quantity of the gene disruption cassette was obtained in a final PCR step performed with primers EanI/R-F_{up} and EanI/R-R_{down} (Fig. 2.3b).

- **Construction of a *luxS* gene disruption cassette**

To construct a gene disruption cassette for the *luxS* gene (PANA_3027) of *P. ananatis* LMG2665^T, the genomic DNA was used as template in a PCR with primers LuxS-F_{up} and LuxS-R_{up}-kan to amplify a 1.006-kb region downstream of the *luxS* gene, and with primers LuxS-F_{down}-kan and LuxS-R_{down} to amplify a 948-bp region upstream of the *luxS*

gene (Fig. 2.4a). The two PCR products and the 1.310-kb kanamycin resistance gene were mixed with primers LuxS-F_{up} and LuxS-R_{down}, and the overlap PCR reaction resulted in the amplification of a 3.264-kb PCR product. This PCR product was re-amplified with the same primers to increase the yield of the gene disruption cassette (Fig. 2.4b).

2.3.2 Construction of mutant strains of *P. ananatis* LMG2665^T

Mutagenesis was subsequently carried out by electroporating the above PCR products, containing a kanamycin resistance gene flanked by sequences homologous to the targeted DNA, into a *P. ananatis* LMG2665^T strain expressing the λ Red operon. The presence of the specific PCR product promotes the deletion of the chromosomally located targeted region via homologous recombination between the genomic region and the flanking sequences of the PCR product. The λ Red proteins are required to mediate this homologous recombination event. The Gam protein inhibits the host RecBCD exonuclease V so that the Beta and Exo proteins can gain access to DNA ends to promote homologous recombination (Datsenko and Wanner, 2000; Katashkina *et al.*, 2009).

To generate mutants of *P. ananatis* LMG2665^T, the wild-type strain was thus first transformed with the λ Red plasmid, pRSFRedTER, and a chloramphenicol-resistant strain was then cultured in the presence of IPTG to induce expression of the λ Red proteins. Subsequently, mutant strains were generated by electroporating the PCR-generated gene disruption cassettes into the LMG2665^T(pRSFRedTER) strain, and selecting for subsequent homologous recombination events between the *P. ananatis* DNA flanking the kanamycin resistance cassette and the wild-type locus on the genome. Recipient *P. ananatis* strains harbouring an integrated copy of the respective mutant alleles (*rhlI/rhlR*::*kan*^{r}, *eanI/eanR*::*kan*^{r} or *luxS*::*kan*^{r}) were thus selected by plating onto LB agar supplemented with kanamycin. The kanamycin-resistant *P. ananatis* mutant strains were designated EanΔI/R, RhlΔI/R and ΔLuxS, respectively. The presence of the null alleles in the respective mutant strains was subsequently verified by two methods, Southern blot hybridization and PCR analyses.

2.3.3 Verification of the putative *P. ananatis* LMG2665^T mutant strains

2.3.3.1 Southern blot analysis

To determine whether the kanamycin resistance gene was present in the genome of the mutant EanΔI/R, RhlΔI/R and ΔLuxS strains, Southern blot analysis was performed. The genomic DNA of these mutant strains was isolated, digested with EcoRI, ClaI and XhoI restriction enzymes and separated by agarose gel electrophoresis. The DNA fragments were transferred onto a nylon membrane by vacuum blotting and the membrane was then hybridized with DIG-dUTP labeled DNA probe specific for the kanamycin resistance gene. In this analysis, the unlabeled PCR-amplified kanamycin resistance gene was included as a positive hybridization control, while restriction enzyme-digested genomic DNA of the wild-type *P. ananatis* LMG2665^T strain was included as a negative hybridization control.

The result (Fig. 2.5) indicated that the probe specific for the kanamycin resistance gene hybridized with the PCR-amplified kanamycin resistance cassette, as well as with a DNA restriction fragment from each the EanΔI/R, RhlIΔ/R and ΔLuxS genomic DNA. The probe, however, did not hybridize with the digested genomic DNA of the wild-type *P. ananatis* LMG2665^T strain. From these results it was thus concluded that a single copy of the mutant allele was integrated into the genome of the respective mutant strains.

2.3.3.2 PCR analyses of *P. ananatis* LMG2665^T mutant strains

To confirm the location of the integrated mutant allele in each of the *P. ananatis* LMG2665^T mutant strains, PCR analyses were performed using different pairs of primers that amplified the new junction regions and the genome sequence spanning the region in which the mutant allele was integrated.

- **Analysis of the RhlΔI/R mutant strain**

Primers RhlI/R-F_{up}-out and RhlI/R-R_{down}-out, which anneal to gene sequences flanking the *rhlI*/*rhlR* locus, were used to amplify either a 3.806-kb product in the absence of the kanamycin resistance gene, or a 3.982-kb product in the presence of the gene. Although PCR products of the expected sizes could be observed on an agarose gel, the similarity in size of the respective DNA fragments did not allow for clear conclusions to be drawn (Fig. 2.6). Consequently, primers RhlI/R-F_{up}-out / km2 and RhlI/R-R_{down}-out / km1 were used to respectively amplify hybrid products of 2.320 kb and 1.598 kb only if the kanamycin resistance gene was located within the deleted *rhlI*/*rhlR* locus. The respective products were produced when RhlΔI/R genomic DNA was used as template, but they were absent when wild-type *P. ananatis* LMG2665^T genomic DNA was used as template in the respective PCR reactions (Fig. 2.6).

- **Analysis of the EanΔI/R mutant strain**

To confirm replacement of the *eanI*/*eanR* locus with the kanamycin resistance gene, primers EanI/R-F_{up}-out and EanI/R-R_{down}-out were used to amplify either a 3.922-kb product in the absence of the kanamycin resistance gene, or a 4.311-kb product in the presence of the kanamycin resistance gene. Although PCR products of the expected sizes could be observed on an agarose gel, the similarity in size of the respective DNA fragments did not allow for clear conclusions to be drawn (Fig. 2.7). Confirmation for the replacement of the *eanI*/*R* locus with the kanamycin resistance gene was obtained from the results obtained by PCR amplification of the junction fragments. Primers EanI/R-F_{up}-out and km2, as well as primers EanI/R-R_{down}-out and km1 were used to amplify a 2.279-kb and 1.942-kb hybrid product, respectively, only if the kanamycin resistance gene was located within the deleted *eanI*/*R* locus. In contrast to the wild-type strain, these products were produced when genomic DNA of the EanΔI/R mutant strain was used as a template in the PCR (Fig. 2.7).

- **Analysis of the ΔLuxS mutant strain**

Primers LuxS-F_{up}-out and LuxS-R_{down}-out, which anneal to gene sequences located upstream and downstream of the *luxS* gene, respectively, were used to amplify either a

2.866-kb product in the absence of the kanamycin resistance gene, or a 3.512-kb product in the presence of the kanamycin resistance gene. As expected, a 3.512-kb product was produced when strain ΔLuxS genomic DNA was used as template, but template DNA of wild-type *P. ananatis* LMG2665^T generated the 2.866-kb product (Fig. 2.8). Moreover, primers LuxS-F_{up}-out and km2, as well as LuxS-R_{down}-out and km1 were used to amplify hybrid products of 2.320 kb and 1.572 kb, respectively, if the kanamycin resistance gene was located within the deleted *luxS* gene. These products were produced when ΔLuxS genomic DNA was used as template. No products were amplified when wild-type *P. ananatis* LMG2665^T genomic DNA was used as template in the PCR reactions (Fig. 2.8).

Cumulatively, these results confirmed that a single copy of the mutant allele was integrated into the genome of the engineered *P. ananatis* LMG2665^T mutant strains and that the integration occurred at the targeted loci. Since expression of the λ Red plasmid-encoded Red genes can lead to toxic effects in bacterial cells (Sergueev *et al.*, 2001; Katashkina *et al.*, 2009), is was necessary to cure these mutant strains from the plasmid DNA. The λ Red plasmid contains the levansucrase (*sacB*) gene from *Bacillus subtilis* that allows for rapid and efficient eviction from the cells in medium containing sucrose (Katashikina *et al.*, 2009). Thus, the mutant strains were cured from the λ Red plasmid by streaking onto LB agar containing sucrose and loss of the plasmid was confirmed by streaking the strains onto medium containing chloramphenicol. Mutant strains that were chloramphenicol-sensitive, but kanamycin-resistant were selected for all further assays.

2.3.4 Construction of recombinant complementation plasmids

To investigate whether any altered phenotypes that may be displayed by the respective *P. ananatis* LMG2665^T mutant strains were due to mutagenesis of the wild-type genes, complementation plasmids, containing a wild-type copy of the genes under transcriptional control of the native promoters, were constructed whereby the mutant strains could be complemented. Since complementation studies would require that the plasmid DNA is capable of replicating in *P. ananatis*, the broad-host-range plasmid pBR1MCS-5 was selected for construction of the complementation plasmids. This

plasmid vector permits replication in a wide variety of Gram-negative bacteria, and it also harbours a gentamycin resistance marker to allow for plasmid selection and maintenance in the bacterial hosts (Lagendijk *et al.*, 2010). To facilitate cloning, PCR amplicons were first cloned into pJET1.2 vector DNA and then recloned into pBR1MCS-5. The pJET1.2 vector DNA contains an ampicillin resistance gene as a selective marker that was not suitable for use in *P. ananatis* due to its high natural resistance to ampicillin (De Maayer *et al*., 2010).

Using genomic DNA of *P. ananatis* LMG2665^T as template, PCR amplifications were performed using the appropriate CompF and CompR primers for either the *rhlI*/*R* locus, *eanI*/*R* locus or *luxS* gene, inclusive of upstream regions containing putative promoter sequences, as described under Materials and Methods (Section 2.2.8.2). An aliquot of the reaction mixtures were analyzed by agarose gel electrophoresis and a single DNA fragment of the expected size were observed in each case, namely 2.375 kb for the *rhlI*/*R* locus, 2.306 kb for the *eanI*/*R* locus and 646 bp for the *luxS* gene. In contrast, no amplification products were observed in the negative control reaction mixture in which template DNA was omitted (Fig. 2.9a).

The PCR products were purified from the agarose gel, blunt ended and then ligated into pJET1.2/blunt vector DNA. Following transformation of competent *E. coli* DH5α cells, recombinant transformants were identified by colony-PCR using primers that flank the multiple cloning site of the cloning vector (results not shown). To confirm successful cloning of the PCR products, the extracted plasmid DNA was also digested with *Apa*I of which the recognition sequence had been incorporated during the design of the primers. Digestion of the recombinant plasmids with *Apa*I resulted in the excision of DNA fragments of the expected size, indicating that the amplicons were successfully cloned into the pJET1.2/blunt vector DNA (Fig. 2.9b). As a final confirmation regarding the integrity of the cloned insert DNA, the nucleotide sequence of the cloned insert DNA was determined. No nucleotide differences were observed between the sequences of these genes and those reported previously (De Maayer, unpublished). The complete

nucleotide sequences of the cloned genes are provided in the Appendix to this dissertation.

To complete construction of the desired complementation plasmids, the insert DNA was recovered from the recombinant pJET1.2/blunt plasmid DNA by digestion with *Apa*I and ligated into pBR1MCS-5 that had been prepared identically. The ligation reaction mixtures were transformed into competent *E. coli* DH5α cells and the cells were plated onto LB agar medium containing gentamycin. Recombinant transformants were first identified by colony-PCR using primers flanking the multiple cloning site of the pBR1MCS-5 vector (results not shown), and the extracted recombinant plasmid DNA was then characterized by restriction enzyme digestion and agarose gel electrophoresis. Digestion of the recombinant plasmid DNA with *Apa*I yielded two DNA fragments each, which corresponded in size with the pBR1MCS-5 vector DNA (4.768 kb) and either the *rhlI*/*R* genes (2.375 kb), the *eanI*/*R* genes (2.306 kb) or the *luxS* gene (646 bp) (Fig. 2.9c). The derived complementation plasmids were designated pBR1MCS-5-EanI/R, pBR1MCS-5-RhlI/R and pBR1MCS-5-LuxS, respectively.

The complementation plasmids were subsequently introduced into the corresponding *P. ananatis* LMG2665^T mutant strains, and strains that displayed resistance to both gentamycin and kanamycin on LB agar were selected. These complemented strains were designated EanΔI/R::EanI/R, RhlΔI/R::RhlI/R and ΔLuxS::LuxS, respectively, and used in all subsequent assays described in this study.

2.3.5 Construction and verification of an EanΔI/R, RhlΔI/R, ΔLuxS triple-knockout *P. ananatis* LMG2665^T mutant strain

Having constructed three *P. ananatis* LMG2665^T mutant strains in which one each of the respective quorum sensing systems had been inactivated, it was next attempted to engineer a quorum sensing null mutant strain. This was accomplished by λ Red-mediated recombination, followed by FLP-mediated excision of the kanamycin resistance marker gene which is flanked by FRT (FLP recognition target) sites. Thus, the kanamycin resistance gene could be used in the next step of chromosomal

modification of the strain, thereby enabling the step-by-step modification of the *P. ananatis* genome. To enable elimination of the kanamycin resistance gene, the mutant strains were transformed with plasmid pCP20 (Cherepanov and Wackernagel, 1995; Datsenko and Wanner, 2000). This plasmid has a thermo-sensitive replicon and therefore provided thermo-inducible expression of the FLP recombinase under the control of the λ P_R promoter regulated by the temperature-sensitive λ CI857 repressor. By making use of the strategy, as detailed under Materials and Methods (Section 2.2.10), a strain lacking the *eanI/R* and *luxS* genes and in which the *rhI/R* locus was replaced with the kanamycin resistance gene was obtained.

To verify successful construction of the EanΔI/R,RhlΔI/R,ΔLuxS mutant strain, restriction enzyme-digested genomic DNA was subjected to Southern blot analysis with a DIG-dUTP labeled DNA probe specific for the kanamycin resistance gene. As expected, the probe hybridized with a single restriction DNA fragment from the mutant strain but not with digested DNA from the *P. ananatis* LMG2665^T wild-type strain (Fig. 2.10a). To furthermore confirm that the respective target genes were mutated, a PCR-based strategy was used. The approach that was used to construct the triple-knockout mutant strain relied on excision of the kanamycin resistance marker gene following mutagenesis of the *eanI/R* and *luxS* genes. PCR amplification of these target loci with primers EanI/R-CompF / EanI/R-CompR or LuxS-CompF / LuxS-CompR yielded expected products of 890 bp and 130 bp, respectively, thus indicating that the marker gene had indeed been eliminated from these genome regions of the EanΔI/R, RhlΔI/R, ΔLuxS mutant strain. As expected, no PCR product was obtained when genomic DNA of the mutant strain was used together with primers RhlI/R-CompF and RhlI/R-CompR. This was due to deletion of these genes and thus the absence of primer annealing sites for these primers (Fig. 2.10b). When genomic DNA of the wild-type strain was used as template together with the above-mentioned primers, PCR products of the expected sizes were obtained, *i.e.* 2.306 kb for the *eanI/R* locus, 2.375 kb for the *rhII/R* locus and 646 bp for the *luxS* gene (Fig. 2.10b).

Based on the above results, it could therefore be concluded that the engineered EanΔI/R,RhlΔI/R,ΔLuxS mutant strain lacks the *eanI/R* and *luxS* genes, whereas the *rhlI/R* locus was replaced with a kanamycin resistance marker gene. This mutant strain was included in all subsequent studies.

2.4 DISCUSSION

The construction of isogenic mutant strains from which specific functions have been eliminated is central to the analysis of various questions in microbiology. This approach has been used successfully to determine aspects relating to bacterial pathogenesis (Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Quinones *et al.*, 2005; Balestrino *et al.*, 2005; Koustoudis *et al.*, 2006; Beck von Morohoshi *et al*., 2007; Charlotte *et al.*, 2009; Gao *et al.*, 2009; Zhao *et al*., 2009; Ling *et al.*, 2010; Li *et al.*, 2011) and to determine the precise function of undefined open reading frames (Dolph *et al*., 1988; Bijlsma *et al.*, 1999; Camacho *et al*., 1999; Vidal *et al*., 2009; Rhodes *et al*., 2011). The approach has also helped unveil the mechanism of bacterial survival and adaptation to various niches such as in studies focusing on motility and chemotaxis (Weller, 2010; Olsen *et al*., 2012). To generate such complete loss-of-function mutations, both random transposon insertion mutagenesis and allelic exchange methods have been useful.

Transposons, being mobile genetic elements, have the capability of inserting themselves into genes on a bacterial chromosome or plasmid, thereby disrupting the gene itself and sometimes additional genes that are encoded downstream of the mutated gene. Nevertheless, this represents a powerful approach towards identifying genes involved in a specific function provided that an appropriate high-throughput screening method is available. Using such an approach, mutagenesis and functional analysis of specific genes has been addressed (Hamer *et al*., 2001; Hayes, 2003; Holeva *et al*., 2004; De Maayer, 2010; Weller, 2010; Olsen *et al*., 2012). With specific reference to *P. ananatis*, mutagenesis with a Tn5 transposon has been used to demonstrate the significance of exopolysaccharides in the pathogenisis of *P. ananatis*

(De Maayer, 2010), as well as the role of pili and fimbriae in attachment of *P. ananatis* to host tissue (Weller, 2010).

In this study, genes encoding predicted products that are significantly similar to those of the *P. ananatis* LMG20103 *rhlI*/*rhlR*, *eanI*/*eanR* and *luxS* genes were identified in the genome of *P. ananatis* LMG2665^T. These genes encode for three different quorum sensing systems, which are proposed to a play role in the pathogenesis and expression of virulence traits of this pathogen. In order to determe the importance of these genes in the biology and virulence of *P. ananatis* LMG2665^T, allelic exchange was used in this study as an alternative to transposon mutagenesis for constructing mutant strains.

One approach whereby alleles can be exchanged relies on the use of plasmids that are conditional for their replication ("suicide plasmids") as a means of introducing defined mutations within a target genome (Snyder and Champness, 2003; Horzempa *et al*., 2010; Rhodes *et al*., 2011; van Aartsen and Rajakumar, 2011). In such instances, a copy of a chromosomal gene, which has been disrupted through the insertion of an antibiotic resistance gene, is cloned into a plasmid and then introduced into a recipient strain where the plasmid cannot replicate. Since the plasmid cannot replicate, selection for some property of the plasmid, such as the newly introduced antibiotic resistance marker, results in isolates that have integrated the cloned disrupted DNA fragment into the host chromosome via homology between the DNA fragment and the corresponding region of the recipient chromosome. The construction of such mutations therefore represents a powerful approach to the definition of structure-function relationships and the identification of gene function (Beck von Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Balestrino *et al.*, 2005; Quinones *et al.*, 2005; Koustoudis *et al.*, 2006; Morohoshi *et al.*, 2007; Vidal *et al*., 2009). Mutations made by allelic exchange are also targeted, therefore making it a more attractive method of mutagenesis than random transposon insertion mutagenesis.

An alternative approach to mutagenesis relies on the use of PCR to generate appropriate gene disruption cassettes. In this targeted mutagenesis, a gene disruption

cassette made up of sequences that flank the targeted gene and an antibiotic resistance marker in the middle is generated by PCR. The host cells should overexpress the λ Red genes prior to insertion of the gene disruption cassette into the genome. The proteins encoded by the λ Red genes help to facilitate the gene knockout by inhibiting the recognition of foreign DNA (integrative cassette) by the host's exonucleases and facilitate the overall homologous recombination of the cassette, thus resulting in a mutated strain. The mutant strains are then selected based on the selective marker genes on the disruption cassette. The protocol has proved to be rapid and reliable for gene knockout in various microorganisms (Wilson *et al.*, 1999; Chaveroche *et al.*, 2000; Datsenko and Wanner, 2000; Kuwayama *et al.*, 2002; Beloin *et al.*, 2003; Rossi *et al.*, 2003; Derbise *et al.*, 2003; Betapudi *et al.*, 2004; Lesic *et al.*, 2004; Husseiny and Hensel 2005; Clements *et al*., 2007; Lesic and Rahme, 2008; Struve *et al*., 2009; Zhao *et al.*, 2009; Toba *et al*., 2011).

In this study, the *rhlI*/*rhlR*, *eanI*/*eanR* and *luxS* genes were targeted for deletion. To enable the generation of the desired mutant strains, gene disruption cassettes were generated by PCR in which a kanamycin resistance gene was flanked by upstream and downstream regions of the respective genes. The gene disruption cassettes were subsequently introduced into *P. ananatis* LMG2665^{T} by electroporation and presumptive mutant strains were selected following culturing on a selective medium, and characterized by Southern blot hybridization and by PCR analyses. In addition, the genes for the three distinct quorum sensing systems were also knocked out following a PCR λ Red-mediated protocol (Katashkina *et al*., 2009). To facilitate the successive integration of three integrative cassettes and mutagenesis, the kanamycin resistance marker had to be deleted from the mutants prior to introduction of the next disruption cassette so as to enable selection of mutants on selective media. The protocol makes use of the pCP20 plasmid and an elevated temperature. This plasmid encodes a FLP recombinase enzyme that aids deletion of the kanamycin resistance gene and the subsequent FLP recombination of sequences once the kanamycin resistance gene has been eliminated (Cherepanov and Wackernagel, 1995).

Not only is the construction of mutant strains required for investigating the functional importance of the quorum sensing encoding genes, but also an important step in such investigations would be to complement the mutations in the constructed *P. ananatis* LMG2665^T mutant strains by providing the wild-type genes *in trans* on a recombinant plasmid. Should the mutant phenotypes be complemented to wild-type levels, it would provide strong evidence that the altered phenotype is due to disruption of the specific wild-type gene under investigation. Consequently, complementation plasmids were constructed by cloning intact copies of the wild-type *rhlI*/*rhlR*, *eanI*/*eanR* and *luxS* genes, under transcriptional control of the native promoters, into plasmid pBR1MCS-5, a broad-host-range plasmid that would permit extrachromosomal replication in *P. ananatis* LMG2665^T.

The use of native promoters is favoured in situations where the expression of genes occurs at a specific point during bacterial growth such as in quorum sensing where it only occurs once a specific cell density has been achieved. In this study, we chose to make use of native promoters as opposed to non-native promoters because the strong constitutive promoters would have limited our comparison of the complemented strains to the wild-type control, as well as mutant strains. Inducible promoters were also not going to be ideal for our investigation, as they are controlled by specific external factors. Some inducible promoters require high temperatures to be active and since quorum sensing is affected by environmental factors, including change in temperature, such a promoter would thus not have been ideal. Gene expression based on inducible promoters has been found to be often ten-fold lower compared to other promoters (Primrose *et al*., 2001). Inducers are also bound to trigger expression of other non-targeted genes or result in cell toxicity if exposure is too long and thus with studies based on gene regulation this could be a delimitation.

The construction of the *P. ananatis* mutant strains and the complementation of these strains with the appropriate complementation plasmid, as detailed in this Chapter, provided the tools necessary to undertake further studies regarding the functional importance of the *P. ananatis* quorum sensing systems. The details of these

investigations and the results that were obtained during the course of these investigations are given in the following chapter.

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Tables and Figures

Table 2.1 Bacterial strains and plasmids used

Strain/plasmid	Relevant characteristics[a]	Reference or source
Strains		
E. coli DH5α	F⁻ *recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ (*lacZYA-argF*)U169 λ⁻ [Φ80d*lacZ*ΔM15]	BCC[b]
P. ananatis LMG2665^T	Virulent, natural isolate from pineapple	BCC[b]
P.ananatis(pRSFRedTER)	Derivative of *P. ananatis* LMG2665^T, transformed with pRSFRedTER	This study
EanΔI/R	*P. ananatis* LMG2665^T Δ(EanI, EanR), Kan^r	This study
EanΔI/R::EanI/R	EanΔI/R complemented with pBR1MCS-5-EanI/R, Gm^r, Kan^r	This study
RhlΔI/R	*P. ananatis* LMG2665^T Δ(RhlI, RhlR), Kan^r	This study
RhlΔI/R::RhlI/R	RhlΔI/R complemented with pBR1MCS-5-RhlI/R, Gm^r, Kan^r	This study
ΔLuxS	*P. ananatis* LMG2665^T ΔLuxS, Kan^r	This study
ΔLuxS::LuxS	ΔLuxS complemented with pBR1MCS-5-LuxS, Gm^r, Kan^r	This study
EanΔI/R,RhlΔI/R,ΔLuxS	*P. ananatis* 2665^T Δ((EanI, EanR), (RhlI, RhlR), (LuxS)), Kan^r	This study
Plasmids		
pCP20	FLP$^+$, λ cI857$^+$, λ P_R Rep^{ts}, Amp^r, Cm^r	Cherepanovad and Wackernagel, 1995
pRSFRedTER	Broad-host-range plasmid, λ *gam*, *bet* and *exo* genes under control of P_{lacUV5}, *sacB* gene, Chl^r	Katashkina *et al*., 2009
pkD13	Plasmid containing Kan^r cassette flanked by FRT sites	Datsenko and Wanner, 2000
pJet1.2/blunt	Cloning vector, Amp^r	Fermentas
pJet1.2-RhlI/R	Derivative of pJet1.2 vector containing wild-type *rhlI/R* allele, inclusive of native promoter region	This study
pJet1.2-EanI/R	Derivative of pJet1.2 vector containing the wild-type *eanI/R* allele, inclusive of native promoter regions	This study
pJet1.2-LuxS	Derivative of pJet1.2 plasmid containing wild-type *luxS* allele, inclusive of native promoter region	This study
pBR1MCS-5	Broad-host-range cloning plasmid, rep, mob, Gm^r	Kovach *et al.*, 1995
pBR1MCS-5-EanI/R	pBR1MCS-5 harbouring wild-type *eanI/R* allele, inclusive of native promoter regions, cloned into *Apa*I site, Gm^r	This study
pBR1MCS-5-RhlI/R	pBR1MCS-5 harbouring wild-type *rhlI/R* allele, inclusive of native promoter region, cloned into *Apa*I site, Gm^r	This study
pBR1MCS-5-LuxS	pBR1MCS-5 harbouring wild-type *luxS* allele, inclusive of native promoter region, cloned into *Apa*I site, Gm^r	This study

[a] Amp^r, Gm^r, Kan^r, Chl^r: resistant to ampicillin, gentamycin, kanamycin or chloramphenicol, respectively

[b] BCC: Bacterial Culture Collection, located in the Department of Microbiology and Plant Pathology, University of Pretoria

Table 2.2 Primers used

Primer	Sequence (5' to 3')[a]	Use
***rhlI/R* Primers**		
RhlI/R-F_{up}	GCAGATAGTAACAGGACAGCGCTG	Gene disruption cassette
RhlI/R-R_{up}-kan	AGCTCCAGCCTACACAATCGCCTTCACTTGTCATATTGCCTC	Gene disruption cassette
RhlI/R-F_{down}-kan	GGTCGACGGATCCCCGGAATATCTTAGCGTTACTGACGATAAGC	Gene disruption cassette
RhlI/R-R_{down}	CTTACTGAGATAACCTGCGGCACC	Gene disruption cassette
RhlI/R-F-kan	GAGGCAATATGACAAGTGAAGGCGATTGTGTAGGCTGGAGCT	Kanamycin resistance gene
RhlI/R-R-kan	GCTTATCGTCAGTAACGCTAAGATATTCCGGGGATCCGTCGACC	Kanamycin resistance gene
RhlI/R-F_{up}-out	TGGGCATTGAGCATGTTGGCAG	Verification of mutation
RhlI/R-R_{down}-out	AACAGCTGAAGGGAGATTCGGC	Verification of mutation
RhlI/R-CompF	AAA**gggccc**GGACAGCGCTGAGGGTAATTACG	Wild-type allele amplification
RhlI/R-CompR	AAA**gggccc**TAAACTCAACCGTGCGGGTGC	Wild-type allele amplification
***eanI/R* Primers**		
EanI/R-F_{up}	AAAGGGCCCATGGTGGAGGAGCCACGGGTTACCACT	Gene disruption cassette
EanI/R-R_{up}-kan	AGCTCCAGCCTACACAATCGCCTCTAAAAGTTACTGCAGCCCTCAC	Gene disruption cassette
EanI/R-F_{down}-kan	GGTCGACGGATCCCCGGAATCGTATCCGTTATTGTTTGATTTTC	Gene disruption cassette
EanI/R-R_{down}	AAAAGGGCCCGAAGAGGTGTTAATGATACAG	Gene disruption cassette
EanI/R-F-kan	GTGAGGGGCTGCAGTAACTTTTAGAGGCGATTGTGTAGGCTGGAGCT	Kanamycin resistance gene
EanI/R-R-kan	GAAAATCAAACAATAACGGATACGATTCCGGGGATCCGTCGACC	Kanamycin resistance gene
EanI/R-F_{up}-out	ACCACATCCGAGAACATGTTCC	Verification of mutation
EanI/R-R_{down}-out	TCAGAGCACCATACCTGCCC	Verification of mutation
EanI/R-CompR	AAAA**gggccc**TCCTTGAACAGGGTGGTCAGAGAG	Wild-type allele amplification
EanI/R-CompF	AAAA**gggccc**GCAGTGCATATTGATAACTTATCG	Wild-type allele amplification
***luxS* Primers**		
LuxS-F_{up}	GATGGTGCGATCAGTTCAGCACTCAG	Gene disruption cassette
LuxS-R_{up}-kan	AGCTCCAGCCTACACAATCGCCTGAAAGAGCTGCACATCTAGTC	Gene disruption cassette
LuxS-F_{down}-kan	GGTCGACGGATCCCCGGAATTACGTCACCTCTTGGTCTGA	Gene disruption cassette
LuxS-R_{down}	CCAATAAGTCGCAGAGCTCGCT	Gene disruption cassette
LuxS-F-kan*	GACTAGATGTGCAGCTCTTTCAGGCGATTGTAGGCTGGAGCT	Kanamycin resistance gene
LuxS-R-kan	TCAGACCAAGAGGTGACGTAATTCCGGGGATCCGTCGACC	Kanamycin resistance gene
LuxS-F_{up}-out	ACCGCTATGGTGCGTTGATG	Verification of mutation
LuxS-R_{down}-out	CTGGTGTGGTTGCTTAACACC	Verification of mutation
LuxS-CompF	AAA**gggccc**CTGTCCGGACTAACCTAACTGA	Wild-type allele amplification
LuxS-CompR	AAA**gggccc**TCTCTGTTGTCAGGGATGGATG	Wild-type allele amplification
Other Primers		
km1	CAGTCATAGCCGAATAGCCT	Verification of mutation
km2	CGGTGCCCTGAATGAACTGC	Verification of mutation
KanF	GCGATTGTAGGCTGGAGCT	Kanamycin resistance gene
pJET-F	CGACTCACTATAGGGAGAGCGGC	Colony-PCR/Sequencing
pJET-R	AAGAACATCGATTTCCATGGCAG	Colony-PCR/Sequencing
pBR1MCS-5F	CCTCTTCGCTATTACGCCAGCTG	Colony-PCR/Sequencing
pBR1MCS-5R	GCAGTGAGCGCAACGCAATTAATG	Colony-PCR/Sequencing
pEanI/RR	GAAAATCAAACAATAACGGATACG	Sequencing
pEanI/RF	GCAGTGCATATTGATAACTTATCGATCAA	Sequencing
pRhlI/R	GCTTATCGTCAGTAACGCTAAGATAT	Sequencing
pRhlI/F	CTT CAC TTG TCA TAT TGC CTC	Sequencing
pLuxSF	GACTAGATGTGCAGCTCTTTCAG	Sequencing
pLuxSR	TCAGACCAAGAGGTGACGTAAT	Sequencing

[a] In primer sequences the *Apa*I restriction endonuclease recognition sequence is indicated in bold lower case nucleotides

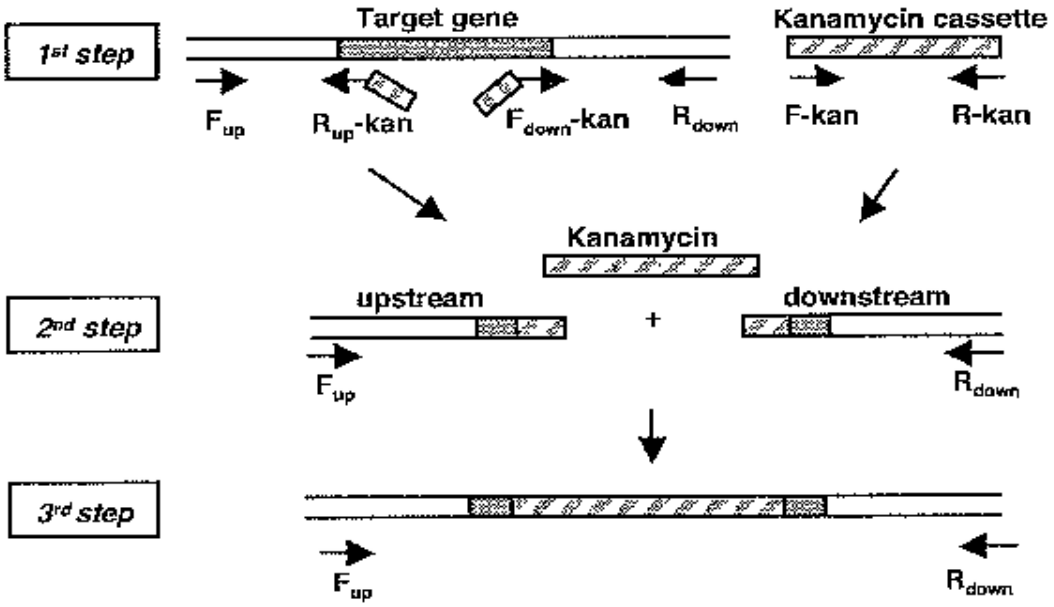
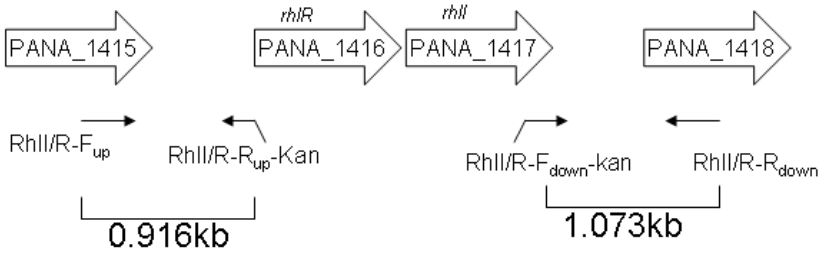


Fig. 2.1 Summary of the PCR reactions required for construction of a gene disruption cassette. The first step is to amplify the upstream and downstream sequences flanking the targeted gene, and the kanamycin resistance gene that is used as a selectable marker. The PCR products are then fused in an overlap PCR in step 2, whereas the PCR product is then re-amplified in a third PCR step to augment the yield of the product (taken from Lesic and Rahme, 2008).

(a)



(b)

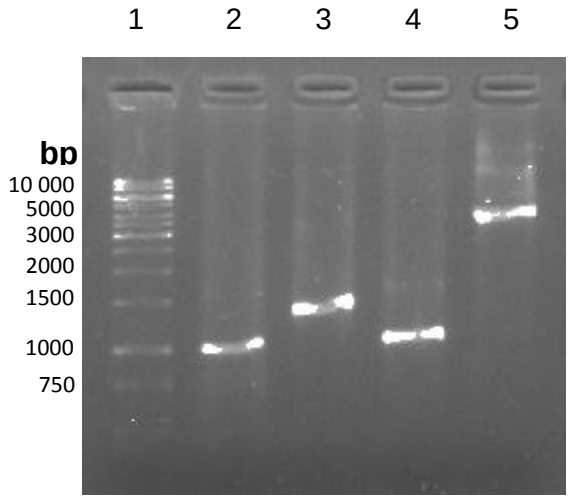
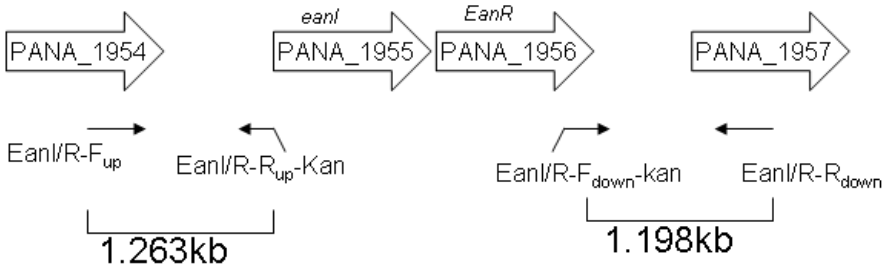


Fig. 2.2 Construction and verification of an *rhlI/R* disruption cassette. **(a)** Schematic presentation of the primers and their annealing positions that were used to PCR amplify upstream and downstream regions required for generating a disruption cassette for the *rhlI/rhlR* locus. The expected sizes of the PCR products are indicated. **(b)** Agarose gel electrophoretic analysis of the products obtained following PCR amplification using genomic DNA of *P. ananatis* LMG2665^{T} as template together with primers RhlI/R-F$_{up}$ and RhlI/R-R$_{up}$-kan (lane 2) or primers RhlI/R-F$_{down}$-kan and RhlI/R-R$_{down}$ (lane 4). The kanamycin resistance gene was PCR-amplified from plasmid pKD13 using primers RhlI/R-F-kan and RhlI/R-R-kan (lane 3). The DNA fragments were mixed and PCR-amplified with primers RhlI/R-F$_{up}$ and RhlI/R-R$_{down}$ to generate the desired *rhlI/R* disruption cassette (lane 5). The sizes of the DNA molecular weight marker (lane 1), O'GeneRuler™ 1-kb DNA Ladder (Fermentas), are indicated in base pairs to the left of the figure.

(a)



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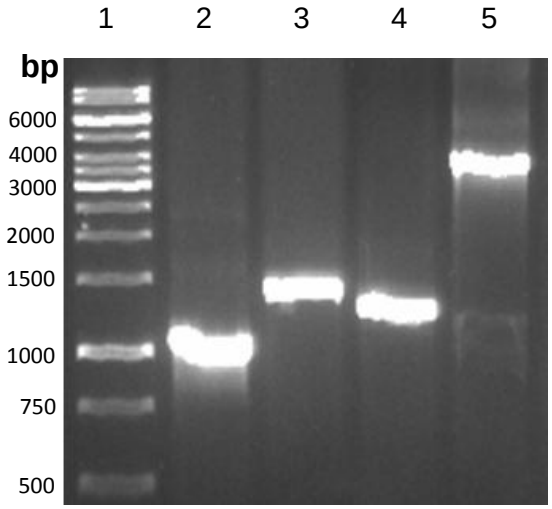
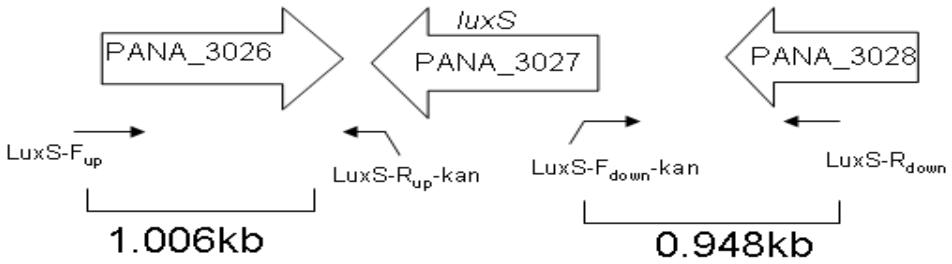


Fig. 2.3 Construction and verification of an *eanI/R* disruption cassette. **(a)** Schematic presentation of the primers and their annealing positions that were used to PCR amplify upstream and downstream regions required for generating a disruption cassette for the *eanI*/*eanR* locus. The expected sizes of the PCR products are indicated. **(b)** Agarose gel electrophoretic analysis of the products obtained following PCR amplification using genomic DNA of *P. ananatis* LMG2665^{T} as template together with primers EanI/R-F$_{up}$ and EanI/R-R$_{up}$-kan (lane 2) or primers EanI/R-F$_{down}$-kan and EanI/R-R$_{down}$ (lane 4). The kanamycin resistance gene was PCR-amplified from plasmid pKD13 using primers EanI/R-F-kan and EanI/R-R-kan (lane 3). The DNA fragments were mixed and PCR-amplified with primers EanI/R-F$_{up}$ and EanI/R-R$_{down}$ to generate the desired *eanI/R* disruption cassette (lane 5). The sizes of the DNA molecular weight marker (lane 1), O'GeneRuler™ 1-kb DNA Ladder (Fermentas), are indicated in base pairs to the left of the figure.

(a)



(b)

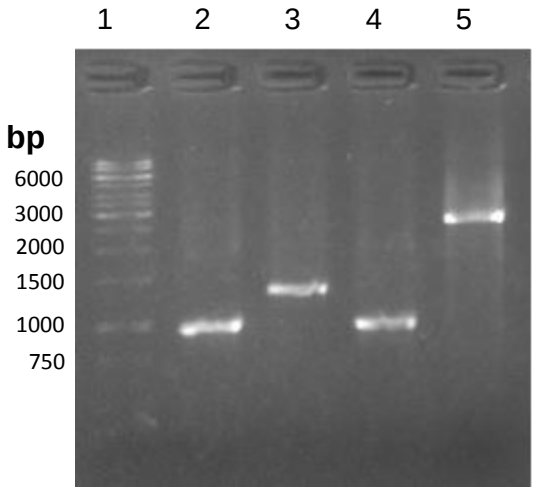
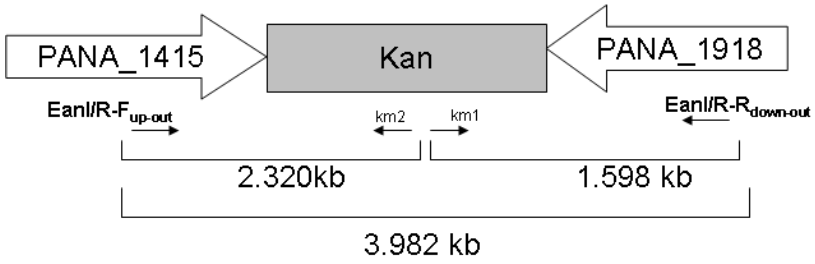


Fig. 2.4 Construction and verification of a *luxS* gene disruption cassette. **(a)** Schematic presentation of the primers and their annealing positions that were used to PCR amplify upstream and downstream regions required for generating a gene disruption cassette for the *luxS* gene. The expected sizes of the PCR products are indicated. **(b)** Agarose gel electrophoretic analysis of the products obtained following PCR amplification using genomic DNA of *P. ananatis* LMG2665^T as template together with primers LuxS-F$_{up}$ and LuxS-R$_{up}$-kan (lane 2) or primers LuxS-F$_{down}$-kan and LuxS-R$_{down}$ (lane 4). The kanamycin resistance gene was PCR-amplified from plasmid pKD13 using primers LuxS-F-kan and LuxS-R-kan (lane 3). The DNA fragments were mixed and PCR-amplified with primers LuxS-F$_{up}$ and LuxS-R$_{down}$ to generate the desired *luxS* gene disruption cassette (lane 5). The sizes of the DNA molecular weight marker in base pairs, O'GeneRuler™ 1-kb DNA Ladder (Fermentas), are indicated to the left of the figure.

Fig. 2.5 Southern blot analysis of genomic DNA extracted from the *P. ananatis* LMG2665^T mutant strains EanΔI/R, RhlΔI/R and ΔLuxS. Genomic DNA extracted from the wild-type *P. ananatis* LMG2665^T (lane 4) and mutant strains EanΔI/R (lane 1), RhlΔI/R (lane 2) and ΔLuxS (lane 3) were digested with *Eco*RI, *Cla*I and *Xho*I, resolved by agarose electrophoresis and transferred to a nylon membrane. A PCR-amplified kanamycin gene (lane 5) was included as a positive control. The membrane was probed with a DIG-labeled kanamycin resistance gene.

(a)



(b)

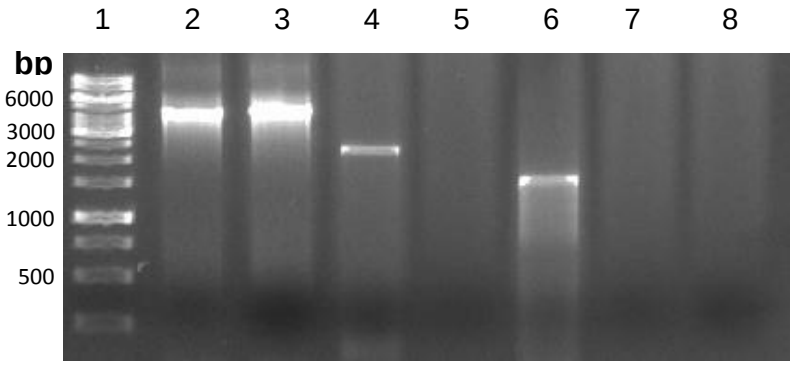
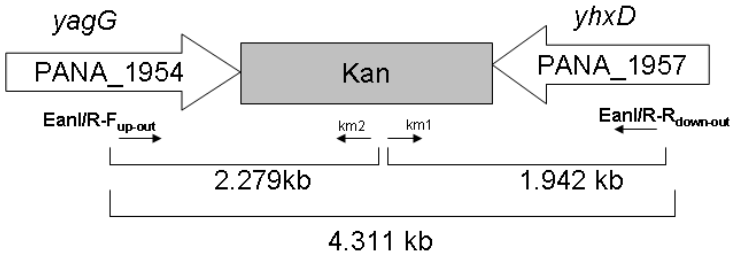


Fig. 2.6 Verification of the RhlΔI/R mutant strain by PCR analyses. **(a)** Schematic presentation of the primers and their annealing positions that were used to characterize the RhlΔI/R mutant strain, in which the *rhlI/rhlR* locus was replaced with a kanamycin resistance gene. The expected sizes of the PCR products are indicated by brackets. **(b)** Agarose gel electrophoretic analysis of the amplification products obtained following PCR analysis of the RhlΔI/R mutant strain and *P. ananatis* LMG2665^T using primers RhlI/R-F_{up}-out and RhlI/R-R_{down}-out (lanes 2 and 3), RhlI/R-F_{up}-out and km2 (lanes 4 and 5), and RhlI/R-R_{down}-out and km1 (lanes 6 and 7). Lanes 2, 4 and 6 represent genomic DNA from the RhlΔI/R mutant strain, whereas lanes 3, 5 and 7 represent genomic DNA from wild-type *P. ananatis* LMG2665^T. A control PCR reaction lacking template DNA was included (lane 8). The sizes of the DNA molecular weight marker in base pairs, O'GeneRuler™ 1-kb DNA Ladder (Fermentas), are indicated to the left of the figure.

(a)



(b)

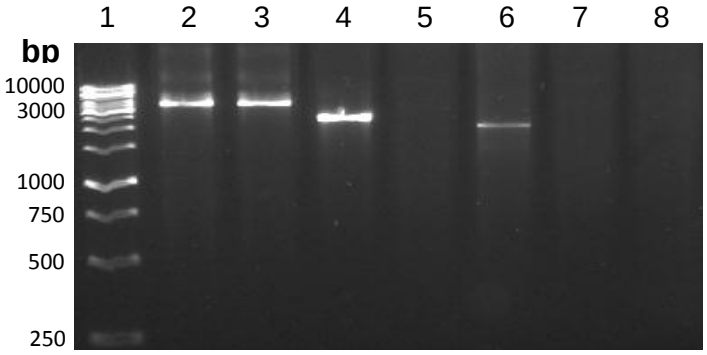
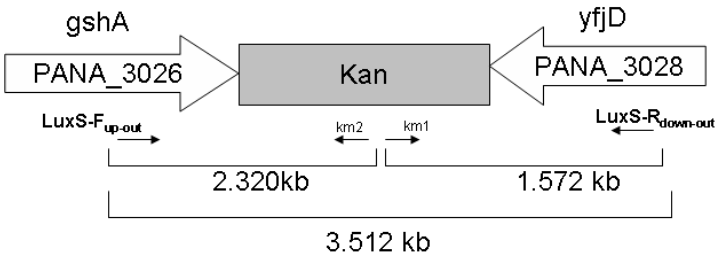


Fig. 2.7 Verification of the EanΔI/R mutant strain by PCR analyses. (a) Schematic presentation of the primers and their annealing positions that were used to characterize the EanΔI/R mutant strain, in which the *eanI/eanR* locus was replaced with a kanamycin resistance gene. The expected sizes of the PCR products are indicated. **(b)** Agarose gel electrophoretic analysis of the amplification products obtained following PCR analysis of the EanΔI/R mutant strain and *P. ananatis* LMG2665^T using primers EanI/R-F_{up}-out and EanI/R-R_{down}-out (lanes 2 and 3), EanI/R-F_{up}-out and km2 (lanes 4 and 5), and EanI/R-R_{down}-out and km1 (lanes 6 and 7). Lanes 2, 4 and 6 represent genomic DNA from the EanΔI/R mutant strain, whereas lanes 3, 5 and 7 represent genomic DNA from wild-type *P. ananatis* LMG2665^T. A control PCR reaction lacking template DNA was included (lane 8). The sizes of the DNA molecular weight marker in base pairs, O'GeneRuler™ 1-kb DNA Ladder (Fermentas), are indicated to the left of the figure.

(a)



(b)

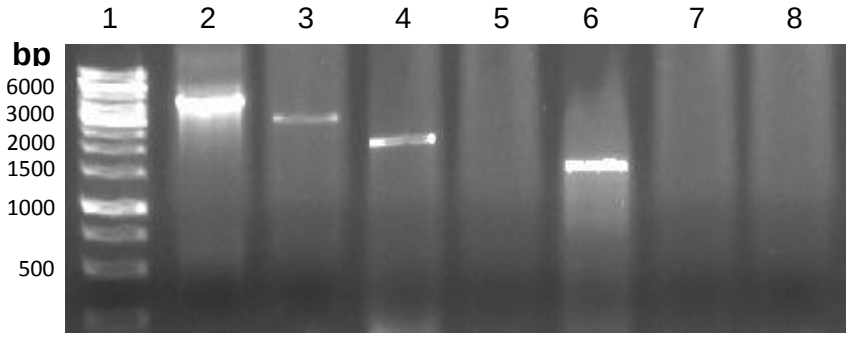


Fig. 2.8 Verification of the ΔLuxS mutant strain by PCR analyses. (a) Schematic presentation of the primers and their annealing positions that were used to characterize the ΔLuxS mutant strain, in which the *luxS* gene was replaced with a kanamycin resistance gene. The expected sizes of the PCR products are indicated. **(b)** Agarose gel electrophoretic analysis of the amplification products obtained following PCR analysis of the ΔLuxS mutant strain and *P. ananatis* LMG2665^{T} using primers LuxS-F_{up}-out and LuxS-R_{down}-out (lanes 2 and 3), LuxS-F_{up}-out and km2 (lanes 4 and 5), and LuxS-R_{down}-out and km1 (lanes 6 and 7). Lanes 2, 4 and 6 represent genomic DNA from the ΔLuxS mutant strain, whereas lanes 3, 5 and 7 represent genomic DNA from wild-type *P. ananatis* LMG2665^{T}. A control PCR reaction lacking template DNA was included (lane 8). The sizes of the DNA molecular weight marker in base pairs, O'GeneRuler™ 1-kb DNA Ladder (Fermentas), are indicated to the left of the figure.

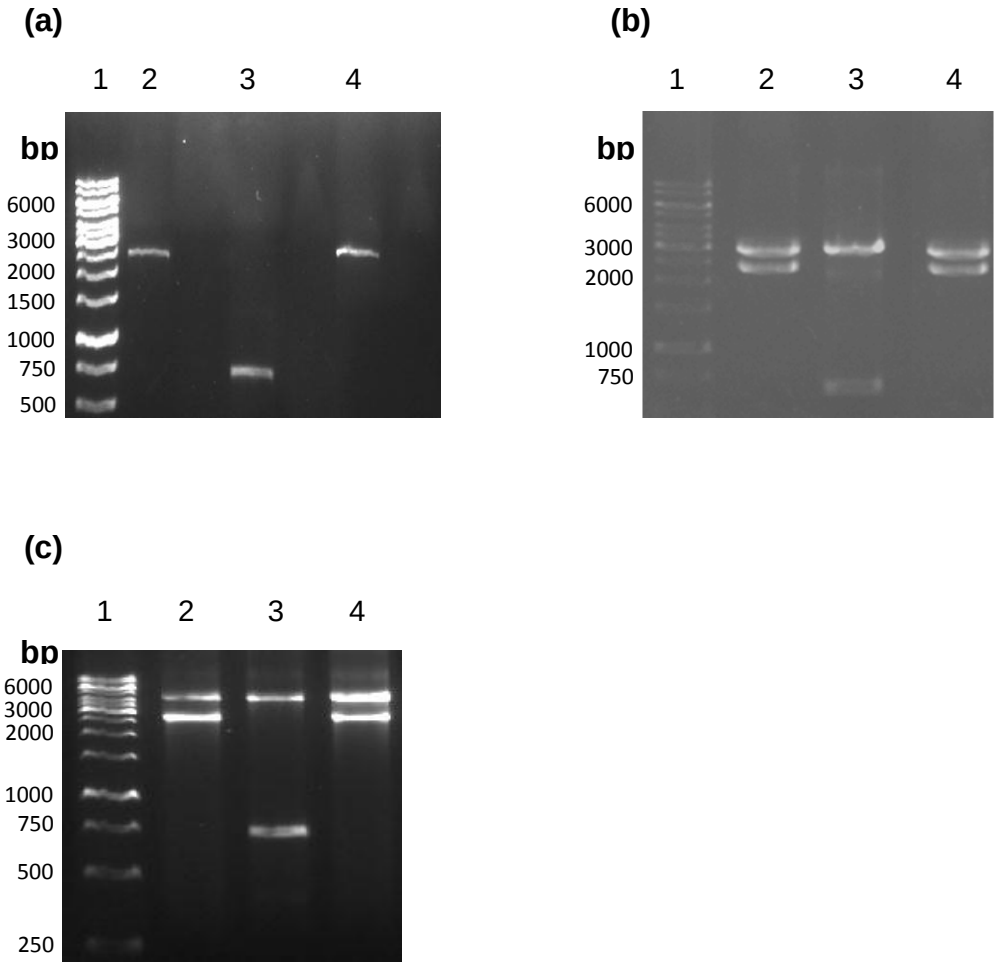
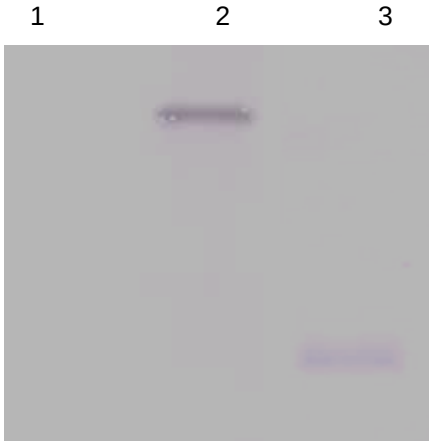


Fig. 2.9 Construction and verification of recombinant complementation plasmids. (a) Agarose gel electrophoretic analysis of PCR products obtained using *P. ananatis* LMG2665^T genomic DNA and primers Ean/IR-compF and EanI/R-compR to amplify the *eanI/R* genes (lane 2), primers LuxS-compF and LuxS-compR to amplify the *luxS* gene (lane 3) or primers RhlI/R-compF and RhlI/R-compR to amplify the *rhlI/R* genes (lane 4). **(b)** The PCR products were cloned into pJET1.2/blunt plasmid DNA to generate recombinant plasmids pJet1.2-EanI/R (lane 2), pJet1.2-LuxS (lane 3) and pJet1.2 RhlI/R (lane 4) that had been digested with *Apa*I to confirm successful cloning of the respective wild-type alleles. **(c)** The insert DNA was purified from the agarose gel and cloned into the *Apa*I site of the pBR1MCS-5 plasmid to generate the recombinant complementation plasmids pBR1MCS-5-EanI/R (lane 2), pBRMCS-5-LuxS (lane 3) and pBR1MCS-5-RhlI/R (lane 4), which were characterized by digestion with *Apa*I followed by agarose gel electrophoresis. In each of the figures, the sizes of the DNA molecular weight marker in base pairs, O'GeneRuler™ 1-kb DNA Ladder (Fermentas), are indicated to the left of the figure.

(a)



(b)

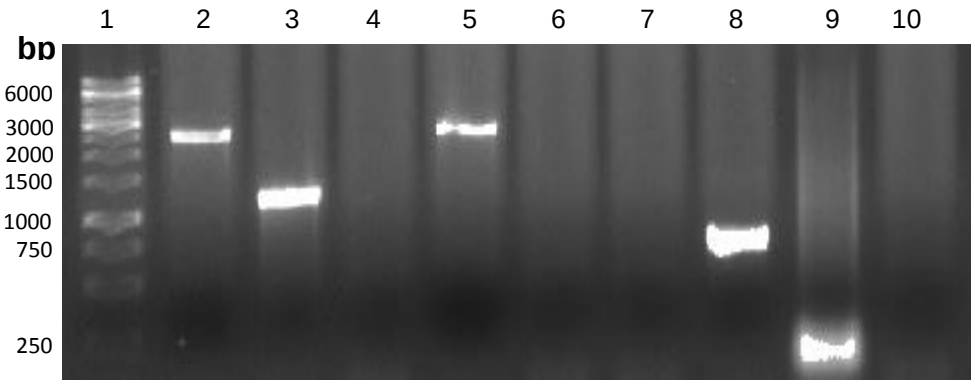


Fig. 2.10 Verification of an EanΔI/R,RhlΔI/R,ΔLuxS triple-knockout mutant strain of *P. ananatis* LMG2665^T. (a) Southern blot analysis of the mutant strain. Genomic DNA extracted from the wild-type *P. ananatis* LMG2665^T strain (lane 1) and mutant strain (lane 2) was digested with restriction enzymes, resolved by agarose electrophoresis and then transferred to a nylon membrane. A PCR-amplified kanamycin resistance gene (lane 3) was included as a positive control. The membrane was probed with a DIG-labeled kanamycin resistance gene. **(b)** PCR analyses of the mutant strain using different primer pairs, namely EanI/R-CompF and EanI/R-CompR (lanes 2 and 3), RhlI/R-CompF and RhlI/R-CompR (lanes 5 and 6) and LuxS-CompF and LuxS-CompR (lanes 8 and 9). Lanes 2, 5 and 8 represent genomic DNA from wild-type *P. ananatis* LMG2665^T, whereas lanes 3, 6 and 9 represent genomic DNA from the mutant strain. Control PCR reactions lacking template DNA were included in the assays (lanes 4, 7 and 10). The sizes of the DNA molecular weight marker in base pairs, O'GeneRuler™ 1-kb DNA Ladder (Fermentas), are indicated to the left of the figure.

CHAPTER THREE

FUNCTIONAL ROLES OF DIFFERENT QUORUM SENSING SYSTEMS IN *Pantoea ananatis*

ABSTRACT

The plant pathogen, *Pantoea ananatis*, is believed to regulate virulence in a cell density-dependent manner. Based on an analysis of the genome sequence of this pathogen, three quorum sensing systems were identified, namely, EanI/EanR, RhlI/RhlR and LuxS. In this study, specific virulence phenotypes governed by these quorum sensing systems were investigated in *P. ananatis*. The phenotypes examined included virulence assays on onion seedlings, biofilm formation, exopolysaccharide production, motility and rhamnolipid synthesis. *P. ananatis* mutants, defective in all or one of the three quorum sensing systems, were used. Firstly, we showed that quorum sensing regulates virulence of *P. ananatis* LMG2665^{T} on onion seedlings. Secondly, that the quorum sensing-deficient *P. ananatis* strains EanΔI/R,RhlΔI/R,ΔLuxS, EanΔI/R and RhlΔI/R are deficient in biofilm formation and strain EanΔI/R is impaired in exopolysaccharide synthesis. Motility and rhamnolipid synthesis is not regulated by quorum sensing in *P. ananatis*. The complemented mutant strains EanΔI/R::EanI/R, RhlΔI/R::RhlI/R and ΔLuxS::LuxS restored the phenotypes that were impaired in the mutant strains. The knowledge acquired in this study provides valuable insights into cell density-dependent gene regulation in *P. ananatis*.

3.1 INTRODUCTION

Quorum sensing is defined as a density-dependent cell-to-cell communication in bacteria that occurs as a result of the accumulation of small diffusible signal molecules, referred to as pheromones or autoinducers. Research has shown that several bacterial species depend on quorum sensing for the regulation of specific phenotypes that are crucial for bacterial virulence (Beck von Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Balestrino *et al.*, 2005; Quinones *et al.*, 2005; Koustoudis *et al.*, 2006; Gao *et al.*, 2009; Ling *et al.*, 2010; Li *et al.*, 2011). Bacteria use various quorum sensing systems for precise timing of the expression of specific regulons (Swift *et al*., 2008). Quorum sensing gene regulation is complex with some bacteria depending on one system, while others depend on more than a single quorum sensing system for effective regulation of specific phenotypes (Henter *et al.*, 2003; Wagner *et al.*, 2004). The typical components of a quorum sensing system include a quorum sensing autoinducer synthase, the autoinducer and the autoinducer receptor protein, the signal response regulator and the genes that are influenced by the system (Williams, 2007; Swift *et al.*, 2008).

The two main quorum sensing systems that have been identified in several bacterial species and studied extensively are the LuxI/R and the LuxS systems. The LuxI/R systems are dependent on acylated homoserine lactone (AHL) as a signal molecule and LuxS makes use of autoinducer 2 (AI-2) for signalling. The LuxI/R system has been found in a variety of Gram-negative bacteria, including the plant pathogenic bacterium *Pantoea ananatis* (Morohoshi *et al*., 2007; De Maayer, 2010). The LuxS system is unique in that it has been found in both Gram-negative and Gram-positive bacteria (Vendeville *et al.*, 2005; Milton, 2006; Atkinson and Williams, 2009). The existence of the LuxS system in *P. ananatis* has also been reported (De Maayer, 2010).

Studies have shown that quorum sensing is required for effective host colonization, expression of virulence and development of infection symptoms (Antunes *et al.*, 2010). Research based on quorum sensing mutant strains of different bacterial species has shown that quorum sensing regulation of phenotypes is bacterial species specific and

thus expression of specific phenotypes with regards to virulence is different depending on the bacterial species in question (Beck von Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Balestrino *et al.*, 2005; Quinones *et al.*, 2005; Koustoudis *et al.*, 2006). Quorum sensing has also been shown to be influenced by the physiological status of bacteria, as well as environmental conditions such as temperature, pH, rate of diffusion of autoinducers and nutrient availability (Charlton *et al.*, 2000; Bollinger *et al.*, 2001; de Kievit *et al.*, 2001; Lithgow *et al.*, 2001; Yates *et al*., 2002; Kim *et al.*, 2003; McGowan *et al.*, 2005; Alvarez-Ortega and Harwood, 2007; Duan and Surette, 2007; Dulla and Lindow, 2008; Boyer and Wisniewski-Dyé, 2009; Decho *et al.*, 2009).

Several studies have indicated that quorum sensing needs to be better understood. In some cases quorum sensing is required for virulence in bacteria, while in others it is not (Beck von Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Balestrino *et al.*, 2005; Quinones *et al.*, 2005; Zhao *et al*., 2005; Koustoudis *et al.*, 2006; Morohoshi *et al.*, 2007; Rezzonico and Duffy, 2007; Charlotte *et al.*, 2009; Gao *et al.*, 2009; Ling *et al.*, 2010; Li *et al.*, 2011). One example of a study that showed the significance of quorum sensing in bacterial virulence was an investigation that was conducted on the plant pathogenic *Pseudomonas syringae* (Quinones *et al.*, 2005), where the quorum sensing double mutant strain *ahlI*$^{-}$*ahlR*$^{-}$ was more aggressive on bean and tobacco plants compared to the wild-type strain (Quinones *et al.*, 2005).

Quorum sensing has also been studied in *Pantoea stewartii* subsp. *stewartti* and *Erwinia amylovora*, both of which are close relatives of *P. ananatis*. It was shown that the *esaI*$^{-}$/*esaR*$^{-}$ mutant strain of *Pantoea stewartii* subsp. *stewartti* has impaired virulence compared to the wild-type strain (Beck von Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Koustoudis *et al.*, 2006). In *Erwinia amylovora,* it was reported that the LuxS quorum sensing system regulates virulence, exopolysaccharide production and motility (Gao *et al.*, 2009). It was also shown that *luxS* down-regulates two virulence genes, *hrpL* and *dspA* (Zhao *et al*., 2005), which are required for detection of environmental change and bacterial growth (Wei *et al.*, 2000).

However, there have been studies that showed that quorum sensing is not essential for virulence, for example, in *E. amylovora*. Although *luxS* influenced the above-mentioned virulence genes, it was shown that the LuxS quorum sensing system plays no role in regulation of virulence and virulence-associated phenotypes such as motility in apple blossoms, apple trees and pear fruits (Zhao *et al.*, 2005; Rezzonico and Duffy, 2007). The significance of the *luxS* gene in the virulence of several other non-plant pathogenic bacteria has been shown, for example, in *Actinobacillus pleuropneumoniae* where *luxS* had been shown to be involved biofilm formation and iron metabolism (Li *et al.*, 2011). In *Klebsiella pneumoniae* the LuxS quorum sensing system also plays a role in biofilm formation (Balestrino *et al.*, 2005), whereas in *Escherichia coli* it controls swimming motility (Ling *et al.*, 2010). In *Salmonella typhimurium,* on the other hand, the LuxS quorum sensing system was reported to be unimportant and thus not required for virulence (Charlotte *et al.*, 2009). The literature cited above indicates that the functional role of quorum sensing in various bacterial species differs.

Pantoea ananatis is the causal agent of a bacterial disease that affects numerous economically important crops including, for example, maize, rice, honeydew melons, pineapples, onions and *Eucalyptus* (Coutinho and Venter, 2009). The symptoms caused by this pathogen are varied depending on the host and include leaf blight, stalk rot, grain discolouration and brown rot. An analysis of the completed genome sequence of *P. ananatis* (De Maayer *et al.*, 2010) revealed the presence of three distinct quorum sensing systems, namely, EanI/EanR, RhlI/RhlR and LuxS. An understanding of the significance of these quorum sensing systems, as well as virulence traits that are essential for successful infection and colonization of hosts could assist with the development of appropriate management strategies against this bacterial plant pathogen.

In this study, the role of quorum sensing in the virulence of *P. ananatis* LMG2665^{T} is investigated. The mutant strain EanΔI/R,RhlΔI/R,ΔLuxS that lacked all three the quorum sensing systems revealed that quorum sensing is required for virulence of *P. ananatis*. Functional characterization of the quorum sensing mutant strains of *P. ananatis*

LMG2665^T, namely, EanΔI/R, RhlΔI/R and ΔLuxS, helped to unveil the importance of each system in virulence. The dependence of specific phenotypes on quorum sensing gene regulation was also determined. The phenotypes that were examined included virulence in onion seedlings, exopolysaccharide synthesis, motility, biofilm formation and rhamnolipid production.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and culturing conditions

The bacterial strains used in this study are listed in Table 3.1. To prepare inoculums for use in assays all *P. ananatis* strains were cultured at 30°C in Luria-Bertani broth (LB: 0.5% [w/v] yeast extract; 1% [w/v] tryptone; 1% [w/v] NaCl, pH 7) or in M9 salts minimal medium (M9 salts: 1.28% [w/v] Na_2HPO_4-$7H_2O$; 0.3% [w/v] KH_2PO_4; 0.05% [w/v] NaCl; 0.1% [w/v] NH_4Cl; 1 M $MgSO_4$; 20% [w/v] glucose; 1 M $CaCl_2$). The growth medium of quorum sensing-deficient strains of *P. ananatis* was supplemented with kanamycin (40 µg/ml), whereas the growth medium of mutant strains complemented with the wild-type allele *in trans* on a plasmid was supplemented with both gentamycin (10 µg/ml) and kanamycin (40 µg/ml). All antibiotics were purchased from Sigma-Aldrich.

To prepare inoculum for use in assays described below, the *P. ananatis* cultures were grown overnight at 30°C in LB broth supplemented with the appropriate antibiotics. Following incubation, the cells from 30 ml of the culture were harvested by centrifugation at 10 000 rpm for 2 min and suspended in sterile water to an OD_{600} of 0.2 (approximately 1 x 10^8 CFU/ml as determined by dilution plating), unless indicated otherwise.

3.2.2 Bacterial growth assays

Growth curves were determined in LB medium and/or M9 salts minimal medium for the *P. ananatis* wild-type strain LMG2665^T, the quorum sensing mutant strains (EanΔI/R, RhlΔI/R, ΔLuxS and EanΔI/R,RhlΔI/R,ΔLuxS) and the complemented mutant strains

(EanΔI/R::EanI/R, RhlΔI/R::RhlI/R and ΔLuxS::LuxS). Bacterial cultures from glycerol stocks were inoculated into LB broth or M9 salts broth, supplemented with the appropriate antibiotics, and cultured overnight at 30°C with shaking at 150 rpm. An aliquot (1 ml) of the overnight cultures was diluted in sterile water to an OD_{600} of 0.8 and then used to inoculate 100 ml of the respective broths to an OD_{600} of 0.02. The flasks were incubated at 30°C with shaking and the OD_{600} reading of each culture was determined at 1-h intervals with a Spectronic®-20 Genesys™ spectrophotometer until the culture entered the stationary growth phase. For each bacterial strain, the assay was repeated three times independently with three replicates each time.

3.2.3 Virulence assay

To determine if quorum sensing plays a role in the virulence of *P. ananatis*, the mutant strain EanΔI/R,RhlΔI/R,ΔLuxS that lacks all three quorum sensing systems was used initially to conduct virulence assays. In these assays, the wild-type strain LMG2665^T was used as a positive control. Virulence tests of these *P. ananatis* wild-type and mutant strains were conducted on onion seedlings by stab inoculation using a sterile needle and adding drops (100 µl) of inoculum through a sterile syringe into the onion leaves, as described by Kido *et al*. (2010). As a negative control, onion seedlings were likewise inoculated with sterile water. The plants were kept in a greenhouse at 25°C for 3 days inside a Perspex container in order to maintain a high atmospheric humidity that favours disease development. The development of disease symptoms was monitored at 1, 2 and 3 days post-inoculation (dpi), and scored as follows: 0 = no symptoms, 1 = water soaking lesion (< 1 cm), 2 = necrotic lesion (> 1 cm), and 3 = collapse of tissue. Disease symptoms were photographed at different days post-inoculation with a Canon PowerShot A3100 IS digital camera. The virulence assay was furthermore repeated using an inoculum with an OD_{600} of 0.02 (approximately 1 x 10^7 CFU/ml), prepared as indicated in Section 3.2.1. For each bacterial strain, the test was repeated six times independently with two replicates each time.

3.2.4 Virulence assay of *P. ananatis* single knockout mutant strains

Based on the results obtained from the above virulence assays, the different *P. ananatis* mutant strains deficient in a single quorum sensing system each were characterized in order to determine which of these quorum sensing systems is crucial for virulence. Thus, the virulence assay described above was repeated using the *P. ananatis* wild-type (LMG2665^T), mutant (EanΔI/R, RhlΔI/R and ΔLuxS) and complemented mutant (EanΔI/R::EanI/R, RhlΔI/R::RhlI/R and ΔLuxS::LuxS) strains.

3.2.5 Swarming motility: Plate assay

Flagella-mediated swarming motility was assayed as described by Rashid and Kornberg (2000). Standardized cultures (OD_{600} = 0.2) of the *P. ananatis* wild-type, mutant and complemented mutant strains were stab inoculated into nutrient agar (0.5% [w/v] agar; 0.5% [w/v] glucose) with a sterile needle. In a single plate, a mutant strain, the corresponding complemented mutant strain and control wild-type (LMG2665^T) strain were inoculated. The agar plates were incubated overnight at 30°C and the swarming motility was then measured (in mm) as the radius of growth expansion. For each bacterial strain, the assay was repeated three times independently with three replicates each time.

3.2.6 Twitching motility: Slide culture assay

Type IV pili-mediated twitching motility was assayed using slide culture assays as described previously (Glessner *et al*., 1999; Rashid and Kornberg, 2000). For this purpose, standardized cultures (OD_{600} = 0.2) of the *P. ananatis* wild-type, mutant and complemented mutant strains were point inoculated onto the surface of a slab of LB agar, placed on a microscope slide, using a sterile needle. The inoculated LB agar was then covered with a coverslip and the slide cultures were incubated at 30°C for 5 h in a humid Petri dish. Humidity was maintained by placing the microscope slide on moist paper towels in the Petri dish. Following incubation, the microscope slides were examined for twitching motility under a Nikon Optiphot light microscope. The images

were acquired with a Nikon DXM 1200 digital camera. For each bacterial strain, the assay was repeated three times independently and representative results are shown.

3.2.7 Biofilm assay

Biofilm formation on an abiotic surface was determined using a quantitative plate assay (Seper *et al*., 2011; Waruiri *et al*., 2012). Wild-type *P. ananatis* LMG2665^T was used as positive control and sterile LB broth as negative control. A 96-well polyvinyl chloride (PVC) plate (Nunc) was inoculated with 150 µl of standardized cultures (OD_{600} = 0.02) of the *P. ananatis* wild-type, mutant and complemented mutant strains. The plates were incubated overnight at 30°C under static conditions. Following incubation, the cultures were aspirated and the wells rinsed six times with 200 µl of sterile water to remove unattached cells, followed by staining with 180 µl of a 1% (w/v) crystal violet solution (Sigma-Aldrich). Following incubation at room temperature for 15 min, the wells were rinsed four times with sterile water to remove excess stain and then filled with 250 µl of 95% ethanol prior to measuring the optical density at 600 nm. For each bacterial strain, the assay was repeated three times independently with six replicates each time.

3.2.8 Quantification of exopolysaccharides

The amount of exopolysaccharides (EPS) produced by the different *P. ananatis* strains in culture supernatants was measured according to the methodology described by Guo *et al*. (2010) with slight modifications. Single colonies of the *P. ananatis* wild-type, mutant and complemented mutant strains were inoculated into 50 ml of LB broth and cultured overnight at 30°C with shaking at 150 rpm. Subsequently, a 10-ml volume (5 x 10^8 CFU/ml) of the cultures was removed and the cells were removed by centrifugation at 5000 rpm for 20 min. To the recovered supernatants, 30 ml of 99% ethyl alcohol were added in order to precipitate the EPS (Cerning *et al.*, 1994; van Calsteren *et al.*, 2002; Harding *et al.*, 2003; Ruas-Madiedo and de los Reyes-Gavilán, 2005). The precipitated EPS was pelleted by centrifugation (10 000 rpm, 10 min), dried in a pre-heated oven at 100°C and then weighed. For each bacterial strain, the assay was repeated three times independently with two replicates each time.

3.2.9 Quantification of rhamnolipids

3.2.9.1 Preparation of reagents

The reagents required to perform the assay were prepared according to Jurado *et al*. (2006). A 50 mM Borax buffer was prepared by dissolving 9.5 g of sodium tetraborate ($Na_2B_4O_7.10H_2O$) in 425 ml of sterile distilled water and the pH was then adjusted to 11 with NaOH. The methylene blue reagent was prepared by dissolving 0.05 g of methylene blue (Merck) in 50 ml of a 10 mM Borax buffer. The pH was then adjusted to 8.6 with 50 mM Borax buffer.

3.2.9.2 Quantification of rhamnolipids

Quantification of rhamnolipids was conducted following the methylene blue complexation protocol (Meyer-Hoffert *et al*., 2011) with slight modification. *P. ananatis* wild-type, mutant and complemented mutant strains were cultured in 50 ml of LB broth to an OD_{600} of 1 (5 x 10^8 CFU/ml), followed by centrifugation of 10 ml of the culture at 10 000 rpm for 30 min at 4°C to pellet the cells. The recovered supernatants were filter-sterilized and acidified with 1 N HCl to pH 3. Rhamnolipids were extracted twice by addition of two volumes of ethylacetate. Subsequently, 4 ml of the rhamnolipid extract was mixed with the freshly prepared methylene blue solution containing 200 µl of the 0.05 g/50 ml methylene blue reagent, and 4.9 ml of sterile distilled water. Following rigorous mixing for 4 min, the samples were left to stand for 15 min at room temperature. The organic phase was then transferred into a cuvette and the optical density was measured at 600 nm, using ethylacetate as the blank. The amount of rhamnolipid was calculated from a standard plot, prepared as described below. For each bacterial strain, the assay was repeated three times independently with two replicates each time.

3.2.9.3 Preparation of a standard plot

Rhamnolipid solutions of different concentrations were prepared by dissolving the rhamnolipid sample, obtained from Sigma-Aldrich, in ethylacetate. The methylene blue complexation protocol, detailed above, was then repeated, followed by measuring the

optical density at 600 nm. A standard plot was prepared where the OD_{600} readings were plotted against increasing concentrations of rhamnolipid (1-5 mg/ml).

3.2.10 Statistical analyses

All data were analyzed using a one-way ANOVA with the programme SPSS version 15 (2006). Differences between isolates were tested using least significant difference (LSD) at the 5% level. The normality test was done using the Mann-Whitney's test.

3.3 RESULTS

3.3.1 Bacterial growth curves

Since several reports have noted that insertion mutagenesis may influence the growth properties of a particular mutant strain (Kadurugamwa *et al.*, 1993; Hoang *et al.*, 2000; Duval *et al.*, 2010), it is possible that the observed effect following mutagenesis may be due to growth impairment of the strain rather than inactivation of a specific gene. To investigate whether the introduced mutations influenced the growth properties of the mutant strains, the *P. ananatis* wild-type, mutant and complemented mutant strains were cultured in nutrient-rich LB broth and M9 salts minimal medium, and their growth was followed by taking optical density readings at 600 nm. The results indicated that the mutant strains (EanΔI/R, RhlΔI/R, ΔLuxS and EanΔI/R,RhlΔI/R,ΔLuxS) displayed a growth rate indistinguishable to that of the wild-type LMG2665^T strain in LB medium (Fig. 3.1a) and M9 salts minimal medium (Fig. 3.1b). Furthermore, the complemented mutant strains (EanΔI/R::EanI/R, RhlΔI/R::RhlI/R and ΔLuxS::LuxS) displayed a growth rate very similar to the wild-type LMG2665^T strain in LB broth (Fig. 3.1c). Although the ΔLuxS::LuxS strain appeared to grow at a faster rate, no statistically significant differences between this strain and the wild-type strain were detected at any of the time intervals ($p > 0.05$).

3.3.2 Quorum sensing regulates virulence in *P. ananatis*

To determine whether quorum sensing plays a role in the virulence of *P. ananatis*, virulence tests were conducted by stab inoculation of onion seedlings with the wild-type LMG2665^T strain and the mutant EanΔI/R,RhlΔI/R,ΔLuxS strain, which lacks all three quorum systems identified in *P. ananatis*. The wild-type strain LMG2665^T was aggressive, causing massive water-soaked lesions and collapse of onion tissue (Fig. 3.2), and had a disease score of 2 at two days post-inoculation and 3 at three days post-inoculation. In contrast, onion seedlings inoculated with the quorum sensing-deficient mutant strain did not show any symptoms of infection (Fig. 3.2), and had a disease score of 0 at three days post-inoculation. No difference in the results was observed when a 10-fold lower concentration of the inoculum (1 x 10^7 CFU/ml) was used, except that disease symptom expression was slower and symptoms first appeared at four days post-inoculation (results not shown). Based on these results, it could be concluded that quorum sensing is required for virulence in *P. ananatis.* These results prompted us to delineate the contribution of the individual quorum sensing systems in the virulence of *P. ananatis*.

3.3.3 Characterization of *P. ananatis* mutant strains lacking single quorum sensing systems

3.3.3.1 Quorum sensing systems are unique in regulating virulence in *P. ananatis*

To identify which of the three quorum sensing systems is more crucial in influencing virulence in *P. ananatis*, virulence assays were conducted with the mutant strains EanΔI/R, RhlΔI/R and ΔLuxS, as well as with the complemented mutant strains EanΔI/R::EanI/R, RhlΔI/R::RhlI/R and ΔLuxS::LuxS. The progression of disease development was slow in onion seedlings stab inoculated with the EanΔI/R and RhlΔI/R strains when compared to the wild-type LMG2665^T strain (Fig. 3.2). The disease score for both the EanΔI/R and RhlΔI/R strains was 1 at two days post-inoculation and 2 at three days post-inoculation. The virulence phenotype was restored in the complemented mutant strains EanΔI/R::EanI/R and RhlΔI/R::RhlI/R. The mutant strain ΔLuxS showed no disease symptoms (Fig. 3.2) and thus had a disease score of 0.

However, the complemented mutant strain, ΔLuxS::LuxS, was slower in causing disease symptoms compared to the wild-type LMG2665^T strain, and had a disease score of 2 at three days post-inoculation and 3 at three days post-inoculation. These results indicate that although the RhlI/R and EanI/R quorum sensing systems influence virulence, the LuxS system is more crucial for virulence in *P. ananatis* compared to the latter two quorum systems.

3.3.3.2 Motility is not influenced by quorum sensing in *P. ananatis*

To determine if quorum sensing influences motility in *P. ananatis*, both swarming motility mediated by flagella and twitching motility mediated by Type IV pili was assessed. To evaluate swarming motility, the wild-type, mutant and complemented mutant strains were stab inoculated into nutrient agar and examined after incubation overnight at 30°C. The radius of growth expansion indicated that there is no significant difference ($p > 0.05$) in the swarming motility of the respective bacterial strains (Fig. 3.3). To assess twitching motility, slide cultures of the bacterial strains were prepared, incubated under humid conditions for 5 h and then examined under a light microscope. Light microscopy revealed that the twitch zones of the wild-type, mutant and complemented mutant strains were comparable to each other (Fig. 3.4). Motile cell "rafts" were also observed at the edges of the respective colony peripheries, indicative of characteristic twitching motility in *P. ananatis* strains.

3.3.3.3 Quorum sensing is required for exopolysaccharide (EPS) production in *P. ananatis*

To determine if quorum sensing influences the production of EPS in *P. ananatis*, the wild-type, mutant and complemented mutant strains were grown overnight in LB broth. The EPS was quantified by precipitating the exopolysaccharides from the cell-free culture supernatants with ethyl alcohol and then measuring the dry weight. The results indicated that, with the exception of the EanΔI/R mutant strain, the respective *P. ananatis* strains produced comparable amounts of EPS (Fig. 3.5). Complementation of the EanΔI/R mutant strain with the wild-type *eanR/I* genes restored EPS production to

wild-type levels. These results therefore indicate that EPS production in *P. ananatis* is under control of the EanI/R quorum sensing system.

3.3.3.4 Biofilm formation in *P. ananatis* is dependent on quorum sensing

Many plant pathogenic bacteria form biofilms in contact with biotic or abiotic environments, and are suspected of playing a role in bacteria-host interaction (O'Toole *et al.*, 2000; Danhorn and Fuqua, 2007). To examine the significance of quorum sensing in biofilm formation by *P. ananatis*, a quantitative microtitre plate assay was used as described in Materials and Methods (Section 3.2.7). The results indicated that the ΔLuxS mutant strain was not impaired in biofilm formation, forming a similar amount of biofilm as the wild-type LMG2665^T strain. In contrast, the EanΔI/R,RhlΔI/R,ΔLuxS mutant strain, as well as the EanΔI/R and RhlΔI/R mutant strains were unable to form biofilm bands at the air-liquid interface (Fig. 3.6). However, strains EanΔI/R::EanI/R and RhlΔI/R::RhlI/R, in which the respective mutations were complemented *in trans*, produced a similar amount of biofilm compared to the wild-type LMG2665^T strain (Fig. 3.6). Based on these results, it could be concluded that the EanI/R and RhlI/R quorum sensing systems have a significant influence on the biofilm formation capability of *P. ananatis* on an abiotic surface under the tested conditions.

3.3.3.5 Rhamnolipid synthesis in *P. ananatis* does not require quorum sensing

To determine whether quorum sensing influences rhamnolipid production in *P. ananatis*, the wild-type, mutant and complemented mutant strains were cultured in LB broth and the amount of rhamnolipid was quantified from cell-free culture supernatants using a methylene complexation method (Meyer-Hoffert *et al*., 2011). The results indicated that the respective *P. ananatis* strains produced comparable amounts of rhamnolipid (0.28-0.30 mg/ml) (Fig. 3.7). Based on the results, it was concluded that quorum sensing does not affect rhamnolipid production in *P. ananatis*.

3.4 DISCUSSION

The results of this study indicate the biological significance of quorum sensing gene regulation in *P. ananatis*. Virulence assays conducted using the mutant strain EanΔI/R,RhlΔI/R,ΔLuxS showed that quorum sensing is required for virulence in *P. ananatis.* The EanΔI/R,RhlΔI/R,ΔLuxS strain did not cause disease in onions, possibly due to the lack of all three the quorum sensing systems. However, using the mutant strains lacking one quorum sensing system, we were able to identify which system is most essential for virulence in *P. ananatis*. Furthermore, the similarities and difference of the three quorum sensing systems in influencing different phenotypes in this plant pathogen were determined.

The phenotypic characterization of the quorum sensing mutant strains RhlΔI/R, EanΔI/R and ΔLuxS indicated defects in one or two phenotypes in *P. ananatis*. Our results show that the three quorum sensing systems in *P. ananatis* regulate infection and disease progression in onions. The virulence results exhibited by the RhlΔI/R and EanΔI/R quorum sensing mutants could be as a result of a defect in quorum sensing, thus resulting in a decreased rate of disease initiation and development. The significance of the LuxI/R quorum sensing systems in regulating virulence in different bacterial strains and various hosts have been shown (Beck von Bodman and Farrand, 1995; Beck von Bodman *et al.*, 1998; Quinones *et al*., 2005; Koustoudis *et al.*, 2006; Morohoshi *et al.*, 2007). Our results are in agreement with a previous study that showed that the *eanI* and *rhlI* genes in *P. ananatis* are required for virulence (Morohoshi *et al*., 2007). Based on our findings, we concluded that the two quorum sensing systems are thus required for effective virulence in *P. ananatis*.

The significance of the LuxS quorum sensing system in the virulence of *E. amylovora* (Gao *et al*., 2009), *Streptococcus suis* (Wang *et al*., 2011), virulence factor expression in *Bacillus anthracis* (Jones *et al*., 2010), and in the expression of the virulence genes *hrpL* and *dspA* in *E. amylovora* (Zhao *et al*., 2005) has been shown. Our study reports for the first time the involvement of the LuxS quorum sensing system in the

pathogenesis of *P. ananatis*. Our data showed that the LuxS quorum sensing system is the most significant quorum sensing system regulating virulence in *P. ananatis*, since a mutant strain lacking the *luxS* gene was unable to cause disease symptoms in onions. We thus concluded that the LuxS system could possibly control a phenotype that is crucial in the virulence of *P. ananatis*.

Motility has been shown to be a requirement for host infection and colonization in various bacteria (Allison *et al*., 1994; Young *et al*., 2000; Quinones *et al.*, 2005; Coulthurst *et al.*, 2006; Herrera *et al*., 2008; Syed *et al.*, 2009; Zhao *et al.*, 2009; Lertsethtakarn *et al.*, 2011). Quorum sensing has been shown to influence motility in *P. syringae* (Quinones *et al*., 2005), *P. stewartii* subsp. *stewartii (*Koutsoudis *et al.*, 2006), *Pseudomonas aeruginosa* (Glessner *et al*., 1999; Reimmann *et al*., 2002), *Yersinia enterolitica* (Atkison *et al*., 2006) and *Pectobacterium carotovorum* subsp. *carotovorum* (Coulthurst *et al.*, 2006). However, in our study, all the mutant strains, namely RhlΔI/R, EanΔI/R, ΔLuxS and EanΔI/R,RhlΔI/R,ΔLuxS, were not impaired in their ability to swarm or twitch. Based on our results, we concluded that the results for the virulence experiment were therefore not as a result of impairment of motility of the mutant strains.

The synthesis of exopolysaccharides by pathogenic bacteria is believed to be a crucial phenotype contributing to virulence (Schell, 1996; Geider, 2000; Skurnik and Bengoechea, 2003). Reports of the involvement of exopolysaccharides in host infection and colonization have been made in close relatives of *P*. *ananatis*, namely *P. stewartii* subsp*. stewartti* and *E. amylovora* (Geider, 2000). The involvement of the acetylated homoserine lactone (AHL)-dependent quorum sensing system in exopolysaccharide production has been reported in *P. syringae* (Quinones *et al*., 2005) and *P. stewartii* subsp. *stewartii* (Beck von Bodman and Farrand, 1995). In the current study, the EanI/R quorum sensing system was shown to regulate exopolysaccharide production in *P. ananatis* in a cell density-dependent manner, as was evidenced by the significant reduction in the amount of exopolysccharide produced by a mutant strain lacking the *eanI/R* genes. However, although exopolysaccharide production is a quorum sensing influenced phenotype, it has been reported not to be a direct virulence factor in *P.*

ananatis (Morohoshi *et al*., 2011). We thus concluded that in *P. ananatis*, though exopolysaccharides are possibly not a direct virulence determinant, their production is regulated by quorum sensing.

Our study showed that exopolysaccharide production is not influenced by the LuxS and the RhlI/R quorum sensing systems, although these two systems influence the virulence of *P. ananatis* in onions. In *P. stewartii* subsp. *stewartii* the amount of exopolysccharide produced, as well as the precise timing for its production is crucial for normal infection and disease progress (Koutsoudis *et al*., 2006). We thus reason that in *P. ananatis* the slowed progression of symptoms of infection that was exhibited by the EanΔI/R mutant strain may have been due to less exopolysaccharides produced, which then interfered with normal infection. The EanΔI/R,RhlΔI/R,ΔLuxS strain, however, was not impaired in exopolysaccharide production. This is possibly due to the three quorum sensing systems being required for precise timing for up-regulation or down-regulation of exopolysaccharide production during the infection process.

Biofilm formation has been reported to contribute to bacterial survival (Hoyle *et al.*, 1992; Hatch and Schiller, 1998) and virulence (Li and Tian, 2012; Wang *et al.*, 2012; Rendueles and Ghigo, 2012; Djeribi *et al.*, 2012) in several bacterial pathogens. AHL-dependent biofilm formation has been reported in *P. ananatis* (Morohoshi *et al*., 2007) and other bacteria such as *Serratia plymuthica* (Liu *et al*., 2011) and *Burkholderia cenocepacia* (Tomlin *et al*., 2005). Cell aggregation has also been reported to be influenced by AHL-dependent quorum sensing in *P. ananatis* (Morohoshi *et al*., 2011). In this study, we showed that the RhlΔI/R, EanΔI/R and EanΔI/R,RhlΔI/R,ΔLuxS strains were significantly impaired in their ability to form biofilms *in vitro*, indicating that the AHL-dependent quorum sensing systems regulate this phenotype in *P. ananatis*. A different study has also reported the dependence of biofilm formation on quorum sensing (Morohoshi *et al*., 2007). The complemented mutant strains (EanΔI/R::EanI/R and RhlΔI/R::RhlI/R) were able to restore the biofilm formation phenotype to wild-type

level, thus confirming that biofilm formation is a cell density-dependent phenotype in *P. ananatis*.

The LuxS quorum sensing system has been shown to regulate biofilm formation in *Streptococcus suis* (Wang *et al*., 2011), *Shewanella oneidensis* (Bodor *et al*., 2011), *Actinobacillus pleuropneumoniae* (Li *et al.*, 2011) and *Klebsiella pneumoniae* (Balestrino *et al*., 2005). However, in our study, the ΔLuxS strain was not affected in its ability to form biofilms *in vitro.* We thus concluded that this quorum sensing system plays no role in biofilm formation in *P. ananatis*. The role of the *luxS* gene in bacteria is intriguing, as available literature shows that its involvement in virulence and virulence factor expression is not universal in all bacterial species (Balestrino *et al.*, 2005; Zhao *et al.*, 2005; Rezzonico and Duffy, 2007; Gao *et al.*, 2009).

Rhamnolipids are believed to act as wetting agents that reduce surface tension and thus facilitate motility in bacterial cells (Tremblay *et al.*, 2007). However, the importance of rhamnolipids in facilitating bacterial motility has been shown to be not solely responsible for defects on motility of some bacterial strains. An example is where it was demonstrated that the addition of exogenous rhamnolipids to media does not restore the impaired swarming phenotype of *Y. enterocolitica* quorum sensing mutants (Atkison *et al*., 2006).

The synthesis of rhamnolipids in all the studied *P. ananatis* strains is not influenced by quorum sensing. These results were unexpected for the RhlΔI/R, EanΔI/R and EanΔI/R,RhlΔI/R,ΔLuxS mutant strains that lacked the *rhlI/R* genes, since the RhlI/R quorum sensing system is well known for influencing the synthesis of rhamnolipids and thus motility in *P. aeruginosa* (Köhler *et al*., 2000; Chen *et al*., 2004; Caiazza *et al*., 2005; Tremblay *et al*., 2007). The unchanged rhamnolipid production could be one possible reason for the unchanged motility, where it was observed that there was no difference in both the swarming and twitching motility of the respective bacterial strains. We thus concluded that the motility of the mutant *P. ananatis* strains were comparable

to that of wild-type strain possibly due to the fact that rhamnolipid synthesis was not impaired.

In conclusion, our data provides evidence of the involvement of quorum sensing in regulating virulence in *P. ananatis*. We showed for the first time the involvement of the LuxS quorum sensing system in the virulence of a plant pathogenic strain of *P. ananatis*. This research also showed that quorum sensing gene regulation plays a role in biofilm formation, exopolysaccharide production and does regulate neither swarming and twitching motility nor rhamnolipid production in *P. ananatis*. Regulation of different phenotypes by the three quorum sensing systems indicates the importance of these systems for the establishment of infection of the host. The major challenge now is to determine which genes are under the control of the LuxS quorum sensing system.

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Tables and Figures

Table 3.1 Bacterial strains used

Strain	Relevant characteristics[a]	Source
P. ananatis LMG2665^T	Virulent, natural isolate from pineapple	BCC[b]
EanΔI/R	*P. ananatis* LMG2665^T Δ(EanI, EanR), Kan^r	This study
EanΔI/R::EanI/R	EanΔI/R complemented with pBR1MCS-5-EanI/R, Gm^r, Kan^r	This study
RhlΔI/R	*P. ananatis* LMG2665^T Δ(RhlI, RhlR), Kan^r	This study
RhlΔI/R::RhlI/R	RhlΔI/R complemented with pBR1MCS-5-RhlI/R, Gm^r, Kan^r	This study
ΔLuxS	*P. ananatis* LMG2665^T ΔLuxS, Kan^r	This study
ΔLuxS::LuxS	ΔLuxS complemented with pBR1MCS-5-LuxS, Gm^r, Kan^r	This study
EanΔI/R,RhlΔI/R,ΔLuxS	*P. ananatis* 2665^T Δ((EanI, EanR), (RhlI, RhlR), (LuxS)), Kan^r	This study

[a] Kan^r, Gm^r: resistant to kanamycin or gentamycin, respectively

[b] BCC: Bacterial Culture Collection, located in the Department of Microbiology and Plant Pathology, University of Pretoria

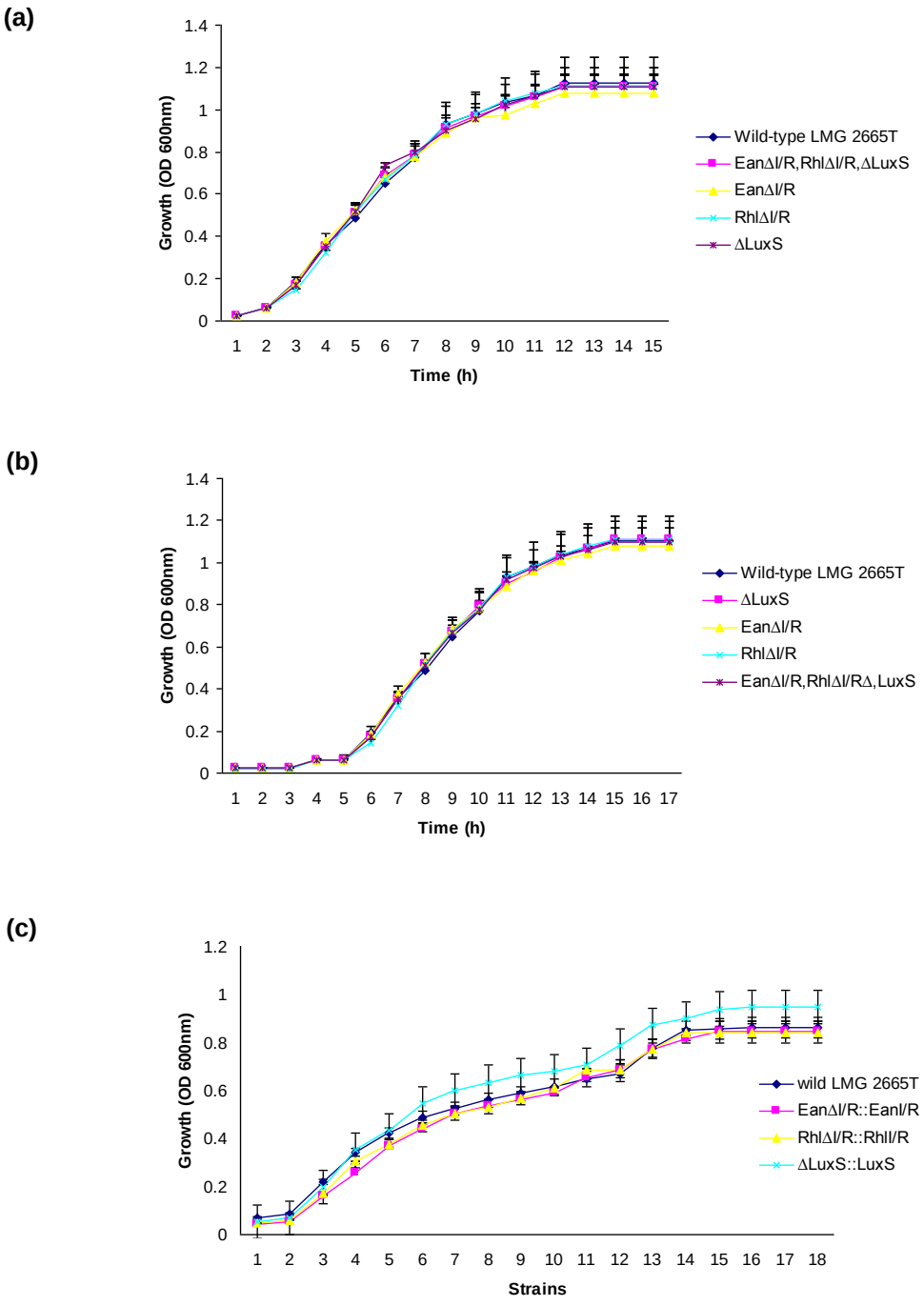

Fig. 3.1 Growth curves of *P. ananatis* strains. The wild-type LMG2665^T and mutant strains were cultured in LB broth **(a)** or M9 salts minimal medium **(b)**, whereas the complemented mutant strains were cultured in LB broth only **(c)**. The results are the means of three independent experiments and the error bars represent the standard error of the mean.

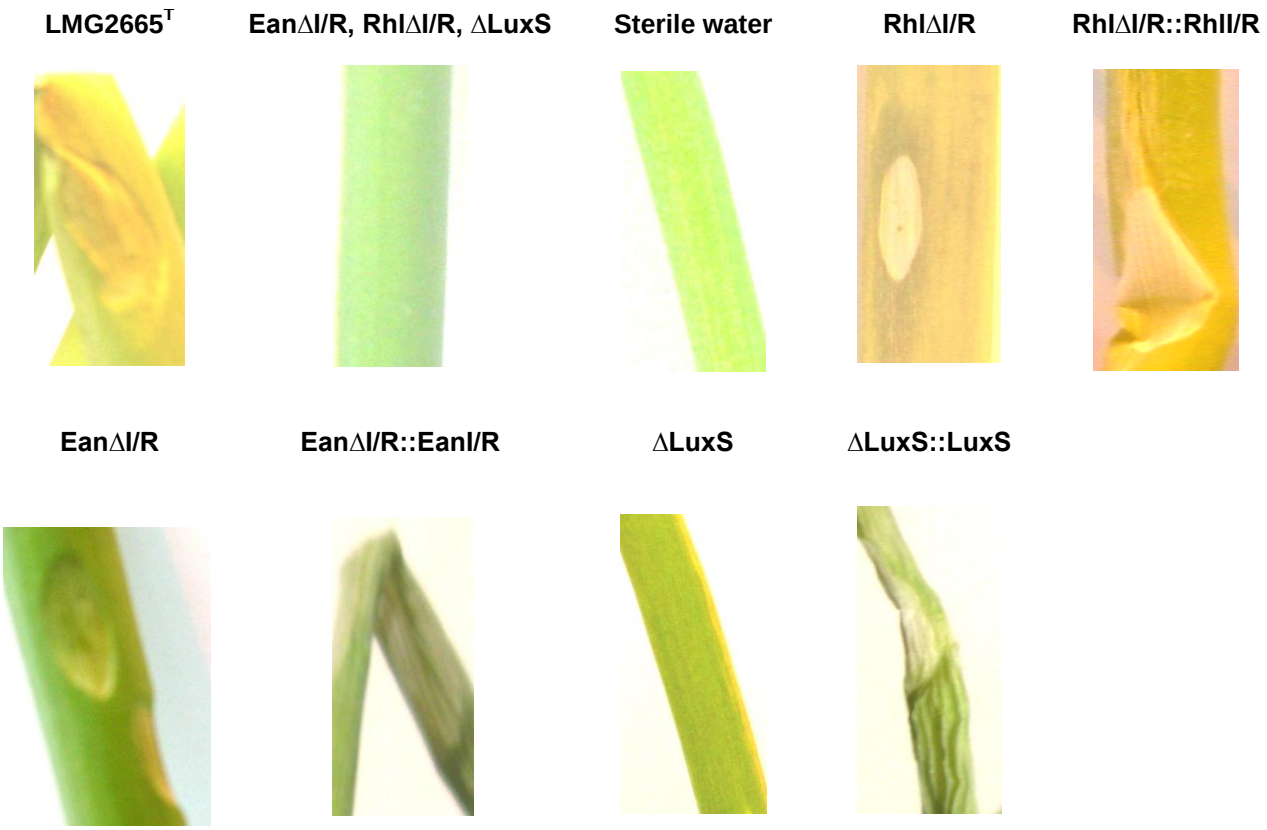

Fig. 3.2 Virulence assay of *P. ananatis* wild-type, mutant and complemented mutant strains. Bacterial strains (1 x 10^8 CFU/ml) were stab inoculated into onion seedlings. In these assays, onion seedlings inoculated with sterile water were included as a negative control. Inoculated onion seedlings were incubated in a greenhouse for 3 days and monitored for the development of disease symptoms. Photographs were taken at 2 days post-inoculation.

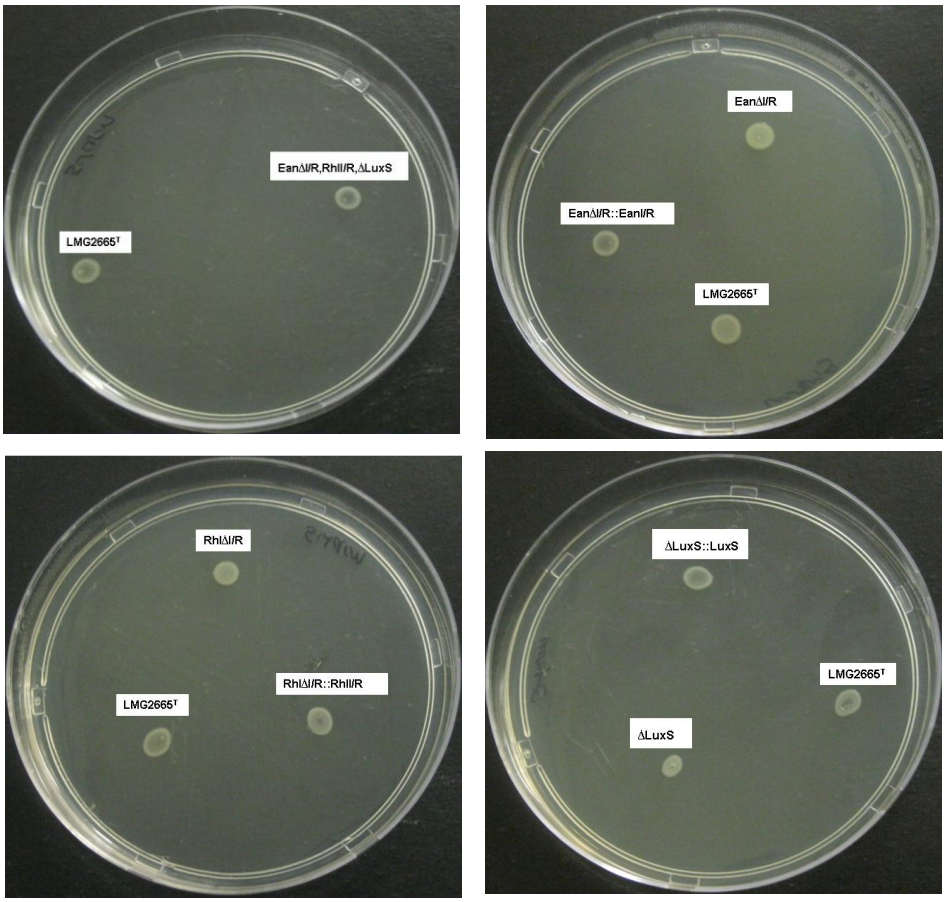

Fig. 3.3 Swarming motility assays of *P. ananatis* wild-type, mutant and complemented mutant strains. The bacterial strains, as indicated in the figure, were stab inoculated into nutrient agar supplemented with glucose and the zone of growth expansion was compared after incubation overnight at 30°C. The assay was repeated three independent times, and representative results are shown.

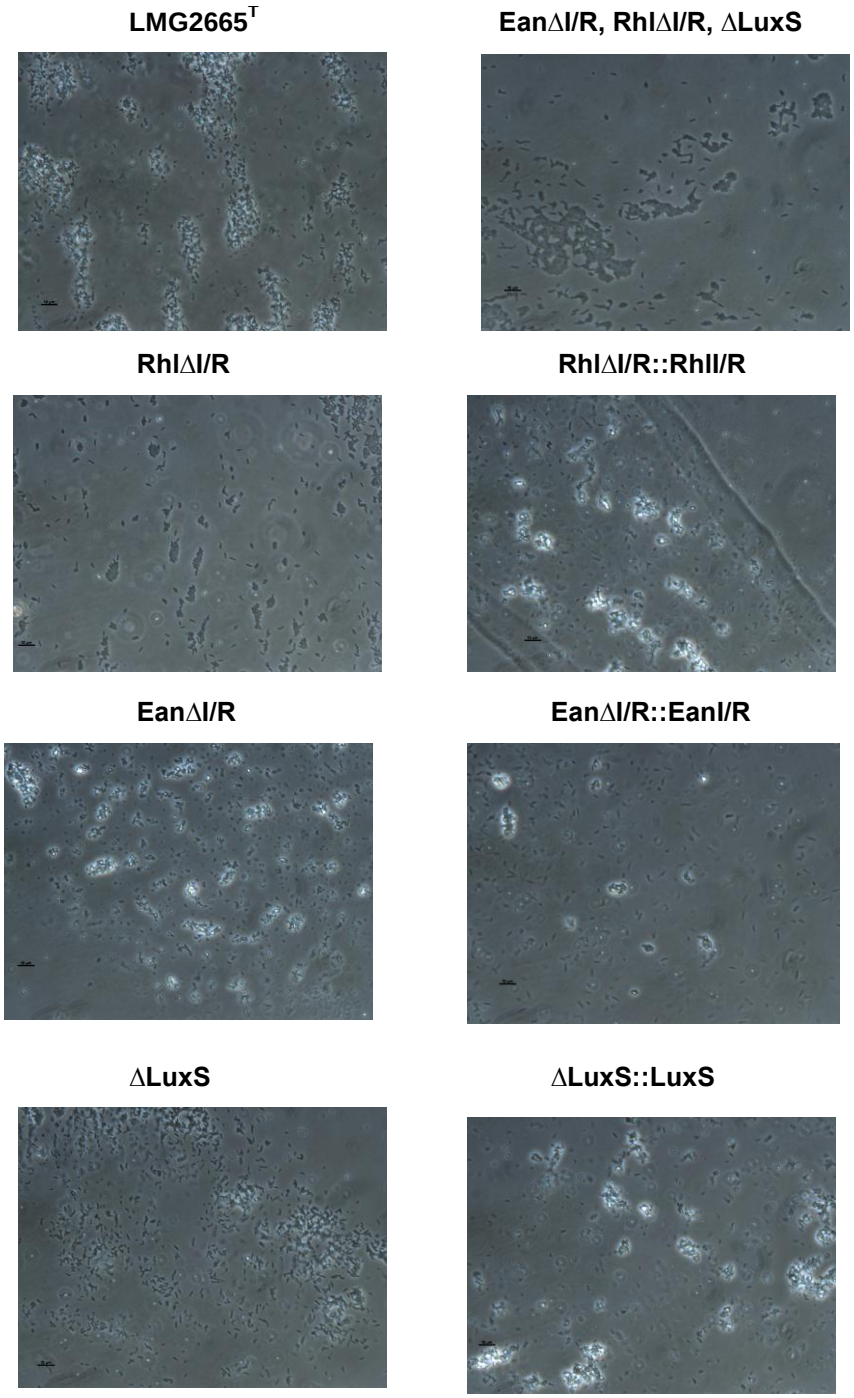

Fig. 3.4 Twitching motility assays of *P. ananatis* wild-type, mutant and complemented mutant strains. The bacterial strains, as indicated in the figure, were inoculated onto the surface of a slab of LB agar placed on microscope slides and incubated for 5 h at 30°C. The slide cultures were then examined under a light microscope. For the wild-type, mutant and the complemented mutant strains, motile rafts could be seen, indicative of twitching motility across the agar surface. Images are shown at a magnification of x1000.

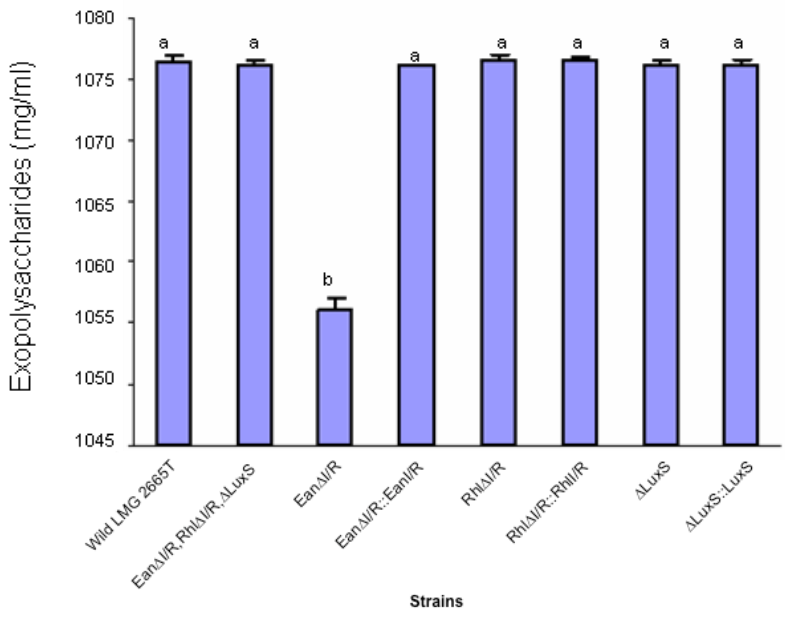

Fig. 3.5 Exopolysaccharide (EPS) production by *P. ananatis* wild-type, mutant and complemented mutant strains. The respective *P. ananatis* strains, as indicated in the figure, were cultured overnight in LB broth at 30°C. The exopolysaccharides were precipitated with ethyl alcohol from the cell-free culture supernatants, dried and then weighed. The results are the mean of three independent experiments and the error bars represent the standard error of the mean. Different letters indicate significant differences ($p > 0.05$) among the strains.

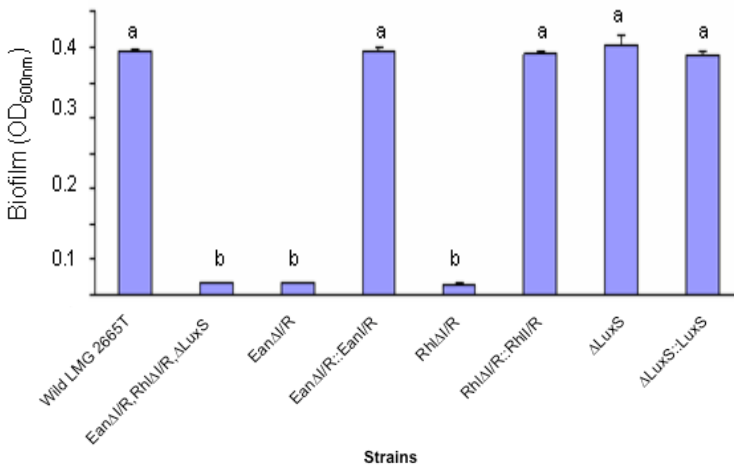

Fig. 3.6 Biofilm formation by *P. ananatis* wild-type, mutant and complemented mutant strains. Biofilm formation was quantified by measuring the OD_{600} of crystal violet-stained PVC microtitre wells. The results are the mean of three independent experiments with six replicates each and the error bars represent the standard error of the mean. Different letters indicate significant differences ($p > 0.05$) among the strains.

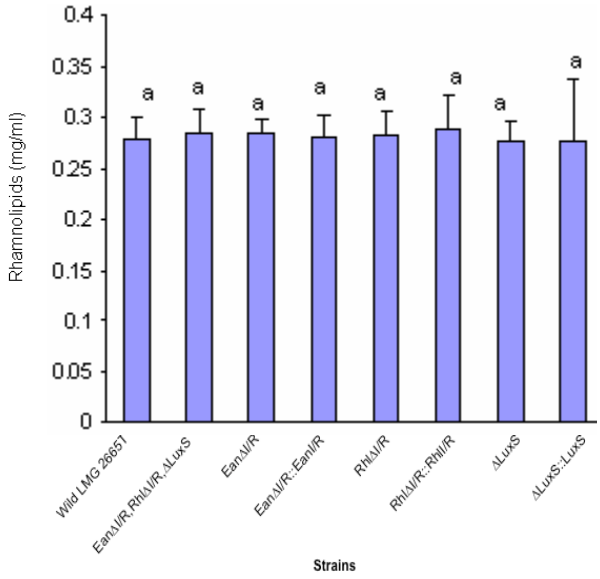

Fig. 3.7 Rhamnolipid production by *P. ananatis* wild-type, mutant and complemented mutant strains. The respective *P. ananatis* strains, as indicated in the figure, were cultured overnight in LB broth at 30°C. The rhamnolipids were extracted from culture supernatants with ethylacetate and quantified with the methylene blue complexation protocol of Meyer-Hoffert *et al*. (2011). The results are the mean of three independent experiments and the error bars represent the standard error of the mean. No significant differences ($p > 0.05$) were observed among the strains.

APPENDIX

Nucleotide sequence alignment of *rhlI/R*, *eanI/R* and *luxS* quorum sensing genes of *P. ananatis* LMG20103 and *P. ananatis* LMG 2665^{T}, inclusive of upstream sequences, that were used in complementation studies

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LuxS 20103    101 GTTGCGGGCAATTTGCTGAGCTTCATCCAGAGAATGCAGTTTATAGCTGCCACACTGATATTCGTTGAGCTCTGGAATCTGGTTC

LuxS 2665     201 AGCACGTCTTCCATCGCGCCTTTCCAGGCTTGTGCCACACGTTGCTCGTCTGGTGTACCAATCAGGCTCATGTAGAAGCCTGTGC
LuxS 20103    201 AGCACGTCTTCCATCGCGCCTTTCCAGGCTTGTGCCACACGTTGCTCGTCTGGTGTACCAATCAGGCTCATGTAGAAGCCTGTGC

LuxS 2665     301 AAATATCAACAATCTCTACGCCGTTACCGTTGAGATGGTTACGCATAAAGCCCGCAAACAAATGCTCAAGCGTGTGGATCCCACG
LuxS 20103    301 AAATATCAACAATCTCTACGCCGTTACCGTTGAGATGGTTACGCATAAAGCCCGCAAACAAATGCTCAAGCGTGTGGATCCCACG

LuxS 2665     401 GATATTGGGGCGACAAAAACGCAGGTCAAAAACAGTAATCGTATCGCCATGGGGAGTCTTCATGGTTTTTGCTACACGCACTGCG
LuxS 20103    401 GATATTGGGGCGACAAAAACGCAGGTCAAAAACAGTAATCGTATCGCCATGGGGAGTCTTCATGGTTTTTGCTACACGCACTGCG

LuxS 2665     501 GTGTGATCAACAGTAAAACTATCCAGTAATGGCATACGTCACCTCTTGGTCTGAAAAATTTTTTGAGAACGATGAAACTTTTCCG
LuxS 20103    501 GTGTGATCAACAGTAAAACTATCCAGTAATGGCATACGTCACCTCTTGGTCTGAAAAATTTTTTGAGAACGATGAAACTTTTCCG

LuxS 2665     601 AATATATGAAAGACGCGCATTTGTTATCATCCATCCCTGACAACAG
LuxS 20103    601 AATATATGAAAGACGCGCATTTGTTATCATCCATCCCTGACAACAG
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