
# Regulation of Biosurfactant Production by Quorum Sensing in *Pseudomonas fluorescens* 5064, the Cause of Broccoli Head Rot Disease

**by**

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# Declaration

I hereby declare that all of the results presented in this thesis are generated by myself, and have not been submitted previously for another degree. Work by others has been specifically acknowledged.

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## Regulation of biosurfactant production by quorum sensing in *Pseudomonas fluorescens* 5064, the cause of broccoli head rot disease

Broccoli head rot is a destructive disease found in most broccoli production areas. The main pathogen is the bacterium *Pseudomonas fluorescens*. *P. fluorescens* 5064, which was first isolated from an infected broccoli head in SE Scotland, produces biosurfactants that are important for bacterial establishment on the plant surface prior to causing disease in broccoli.

Preliminary experiments performed in this study showed that biosurfactant production in *P. fluorescens* 5064 was cell density dependent, which is a typical characteristic of the quorum sensing mechanism. Quorum sensing is a bacterial communication mechanism, which controls a number of key processes in growth, reproduction and virulence via signalling molecules (quorum sensing signal) in many gram-negative bacteria.

One aim of this study was to determine if biosurfactant production in *P. fluorescens* 5064 is controlled via quorum sensing. To do this, 35 surfactant-minus Tn5 mutants of *P. fluorescens* 5064 were screened for their abilities to produce a quorum sensing signal. Six of these biosurfactant-deficient mutants showed a large reduction in quorum sensing signal production. In one mutant 6423, which contains a single Tn5 insertion, the production of the quorum sensing signal was almost eliminated. Addition of quorum sensing signal, either synthetic or extracted from wild type *P. fluorescens* 5064, was able to restore biosurfactant production in mutant 6423. This strongly suggests that quorum sensing regulates biosurfactant production in *P. fluorescens* 5064.

Attempts were made to clone and sequence the Tn5 disrupted gene in mutant 6423, but the identity of the gene remains inconclusive.

The quorum sensing signal in wild type *P. fluorescens* 5064 was identified in this study by High Pressure Liquid Chromatography and Mass Spectrometry as N-3-

hydroxyoctanoyl-homoserine lactone, which has been shown by other researchers to be present in *P. fluorescens* strain 2-79, but not in the strains F113, 7-14 and NCIMB 10586.

The discovery that biosurfactant production in *P. fluorescens* 5064 is regulated by quorum sensing opens up a possibility for novel control of broccoli head rot. Although only the control of biosurfactant production by quorum sensing was examined in this study, it is possible that other virulence factors, such as pectic enzyme production, are also controlled by quorum sensing as in other pathogenic bacteria. By blocking the quorum sensing system, the pathogenic *P. fluorescens* that use this mechanism to control virulence could potentially be rendered avirulent. In greenhouse pathogenicity tests, a quorum sensing signal-degrading bacterium, *Bacillus* sp. A24, was evaluated for biocontrol of head rot disease caused by *P. fluorescens* 5064 on broccoli. However, the *Bacillus* sp. A24 showed only limited control effects, despite its strong quorum sensing signal-degrading ability towards the pathogen *in vitro*. A subsequent test proved that *Bacillus* sp. A24 is a surfactant producer itself and this could explain its ineffectiveness in disease control.

When screening the quorum sensing signals of the 35 biosurfactant mutants, mutant 6418 was found to produce a potent antibiotic-like compound. This was identified by thin-layer chromatography as pyrrolnitrin. Unlike wild-type *P. fluorescens* 5064, mutant 6418 has lost its ability to produce virulence factors and is thus non-pathogenic. It was therefore of interest to determine if mutant 6418 could be used as a biocontrol agent to control broccoli head rot disease. In greenhouse pathogenicity tests, mutant 6418 significantly reduced disease by 41 %.

The practical application of this research to bacterial disease control – via the manipulation of quorum sensing to inhibit virulence gene expression – is discussed.

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**LIST OF ABBREVIATIONS**

ACP – Acyl Carrier Protein

AdoMet - S-adenosyl-l-methionIne

ADSA – Axisymmetric Drop Shape Analysis

AHL – Acyl-Homoserine Lactone

Ais – Autoinducer molecules

CFU – Colony Forming Units

CMC – Critical Micelle Concentration

CV026 – Biosensor based on *Chromobacterium violaceum* mutant

DKP – Diketopiperazine (cyclic dipeptides)

DNA - Deoxyribonucleic Acid

EDTA – Ethylenediamine tetra-acetic acid

EPS – Extracellular Polysaccharide

HHL – N-Hexanoyl-Homoserine Lactone (C6-HSL)

HLB – Hydrophilic-lipophilic Balance

HPLC – High Pressure Liquid Chromatography

HSL – Homoserine Lactone

KB - King's B broth

KBA - King's B agar

LB – Luria-Bertani Medium

LSD – Least Significiant Difference

MS – Mass Spectrometry

NMR – Nuclear Magnetic Resonance

OD – Optical density

OHHL – (3-oxohexanoyl)-Homoserine Lactone

PCN - Phenazine-1-carboxamIde

PCR – Polymerase Chain Reaction

PDA - Potato Dextrose Agar

PQS – Pseudomonas quinolone signal, 2-heptyl-3-hydroxy-4-quinolone

QSC – Quorum Sensing Controlled

QscR – Quorum Sensing Controlled Repressor

QSR – Quorum Sensing Related

$R_f$ – Retardation factor

RLU – Relative Luminescence Unit

rpm – rotations per minute

SAC – Scottish Agricultural College

SDW – Sterile Deionized Water

SEM – Standard Error of the Mean

TAE – Tris-acetate-EDTA

TBE – Tris-borate-EDTA

TDP – Thymidine Diphosphate

TLC – Thin-layer chromatography

UV – Ultra Violet

VAI – *Vibrio fischeri* autoinducer and also known as OHHL (3-oxohexanoyl)-Homoserine Lactone)

# Chapter 1. Introduction and Literature Review

## 1.1. Broccoli head rot

### 1.1.1. Introduction

Broccoli (*Brassica oleracea* L *var italica* Plenck) is an annual plant consisting of a stem holding a primary head of green flower buds (Anon, 1984). Its edible primary head (broccoli spear) is a popular vegetable and the popularity continues to increase due in part to its increasingly well documented antioxidant health properties (Figure 1.1).

Broccoli heads are programmed to mature from June through to December. If the head maturity coincides with periods of persistent wet weather, then a head rot, which is a destructive disease of broccoli, may occur (Canaday *et al*., 1987). Broccoli head rot was first described in Britain by Dowson and Dillon-Weston (1937). The first symptoms of this bacterial disease appear in the water-soaked area of the broccoli head. In the right environmental conditions this head deteriorates rapidly into a brown or black coloured soft pulp (Figure 1.1). It might ultimately result in the downgrading of the head or render it completely unmarketable (Wimalajeewa *et al*., 1987).

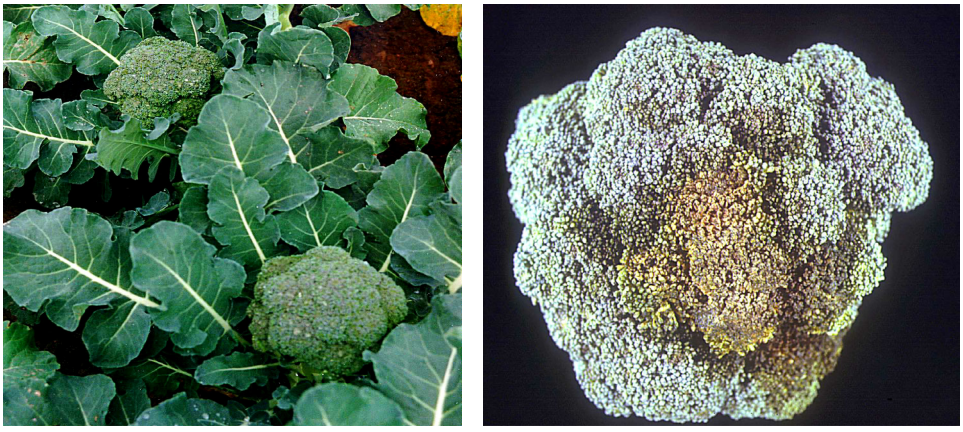


**Figure 1. 1: Healthy broccoli (left) and broccoli with head rot caused by the bacterium *Pseudomonas fluorescens* (right)**

Head rot is a serious problem in the broccoli production areas of Australia, the US, Canada, Ireland and the United Kingdom (Wimalajeewa *et al*.,1987; Canaday *et al*., 1987; Hildebrand, 1986; Ludy *et al*., 1989). Disease losses may run up from 30% to 100% in some fields in USA (Hildebrand 1989) while losses of about 35% are not uncommon in Australia (Wimalajeewa *et al*., 1987). Additional losses of the partially damaged, softened crop might occur in cold storage and transit (Brocklehurst *et al*., 1981; Ceponis *et al*., 1987; Liao *et al*., 1987).

1.1.2. Broccoli head rot pathogens

Head rot is an opportunistic bacterial disease caused by *Pseudomonas* and *Erwinia* species. Both groups of pathogen coexist on broccoli heads and are capable of causing disease. The representative head rot bacteria are *Pseudomonas fluorescens, P. viridiflava, P. marginalis* and *Erwinia carotovora* subsp. *carotovora* ( Wimalajeewa *et al*., 1987; Hildebrand, 1986; 1989; Canaday *et al*., 1987; 1991; Brokenshire & Robertson, 1986; Robertson *et al*., 1993; Liao & Sapers, 1999).

Though *Erwinia carotovora* can cause disease in the field, both Wimalajeewa (1985) and Hildebrand (1986) concluded that it is not the primary pathogen because it can only induce disease on wounded heads. Hildebrand (1989) subsequently demonstrated that the possible reason why *E. carotovora* does not cause disease on unwounded heads is due to its lacking of a surfactant-like substance. However, in Scotland, *Erwinia carotovora* ssp. *carotovora* and *E. carotovora* spp. *atroseptica* have been isolated from diseased heads and shown to be pathogenic on non-wounded heads (Robertson *et al*., 1993). The most likely explanation for this is that residual bacteria on the heads, although precautions are taken to reduce the unwanted contamination on the head, can possibly supply *E. carotovora* with the missing surfactant-like substance (Harling *et al*., 1994).

The fluorescent pseudomonads are cited as the primary causal organisms in broccoli head rot in some reports (Hildebrand, 1986; Wimalajeewa *et al*., 1987).

### 1.1.3. Bacterial pathogenicity factors

Pathogenicities of the head rot pathogens are mostly correlated with their abilities to produce surfactant and pectolytic enzymes. Hildebrand (1989) reported that the individual isolates lacking either pectolytic enzymes or biosurfactants (surfactant produced by microorganisms) could not attack the waxy plant surface and thus could not initiate disease development on unwounded tissue. The majority of pathogenic Pseudomonads on broccoli are capable of producing both pectolytic enzymes and biosurfactants while *Erwinia carotovora* cannot produce biosurfactants (Hildebrand, 1989; Robertson *et al*., 1993; Harling *et al*., 1994; Liao *et al*., 1992).

Strong pectolytic activity has been suggested to be a key factor in causing head rot disease (Wimalajeewa *et al*., 1987). Pectolytic enzymes macerate plant tissue by degrading the pectic substances within the middle lamella of plant cell walls (Lund, 1982). Production of pectolytic enzymes in most strains of soft-rotting Pseudomonads appears to be induced by pectic substrates (Fuchs, 1965; Hagar and McIntyre, 1972; Nasuno and Starr, 1966) or by plant tissues (Zucker and Hankin, 1970; Zucker *et al*., 1972). In some strains the production of pectolytic enzymes is not affected by the type of carbon source included in the medium (Nasuno and Starr, 1966; Zucker *et al*., 1972), but is induced by $Ca^{2+}$ and the $Ca^{2+}$ induction cannot be replaced by the other divalent cations (Liao *et al*., 1993).

In addition to the production of pectolytic enzymes, the highly virulent *P. fluorescens* produces a surfactant-like substance termed viscosin that apparently plays a major role in successful pathogenesis (Hildebrand, 1989). Viscosin is a potent lipopeptide biosurfactant that enables the bacteria to come into intimate contact with the difficult-to-wet waxy heads of broccoli. *P. fluorescens* Tn*5* mutants that lack biosurfactant production ($vis^-$) but retain pectolytic capability are still able to decay potato slices. On broccoli, however, these $vis^-$ mutants can cause decay of wounded florets but the decay fails to spread to adjacent nonwounded florets as occurs with the wild-type *P.*

*fluorescens* (Braun *et al*, 2001). This suggests that viscosin is an important virulence factor in the head rot of broccoli. The crucial role of biosurfactant in head rot disease incidence is also enforced by the fact that pectolytic-positive, biosurfactant-lacking strains of *Erwinia carotovora* var. *carotovora* and *E. carotovora*. var. *atroseptica,* which are incapable of causing decay on unwounded broccoli heads, can cause extensive decay when co-inoculated with the other biosurfactant-producing but non-pathogenic strains.

Biosurfactants do not alter the plant surface wax structure, but act as wetting agents by reducing the surface tension of water, leading to water soaking, thereby allowing bacterial enzymes to attack the plant surface and disrupt cell membranes (Laycock *et al*., 1991). Biosurfactant production may also play a significant role in the physical spread of bacteria during the early colonization of broccoli florets and after decay has been initiated. The crucial role of biosurfactants in the incidence of broccoli head rot will be addressed later.

It is not known whether other bacterial pathogenicity factors, such as cellulases, lyases and toxins, are involved in the disease incidence of broccoli head rot.

### 1.1.4. Environmental factors that affect the incidence of head rot

The incidence of broccoli head rot and its severity increase with the presence of wet weather and high humidity (Canaday and Wyatt, 1992; Canaday *et al*., 1991). Higher disease levels are typically found in autumn due to high daytime temperatures followed by low night-temperatures, which create a longer period of dew formation and hence an increased amount of free water available on the heads (Campbell *et al*., 1995). Ludy *et al*. (1997) demonstrated the importance of free water for disease initiation when they reported that decreasing the frequency of overhead irrigation, from every two to every eight days, reduced the incidence of head rot.

The effect of temperature on disease occurrence is conflicting and uncertain. Canaday *et al* (1991) reported that warm wet weather promoted disease, whilst both Wimalajeewa *et al*. (1987) and Robertson *et al*. (1993) found that cool wet weather was most suitable to disease development. Wimalajeewa *et al*. (1987) concluded that the disease could also occur under warm and wet conditions, but that it was less severe when compared with its development during cool and wet weather.

In addition to the effect of free water availability and temperature, Canaday and Wyatt (1992) reported that increased nitrogen (in the form of ammonium nitrate) could increase disease incidence and the severity of head rot in a susceptible broccoli cultivar. Everaarts's (1994) observations were in agreement with the above points, and he also demonstrated that a higher percentage of dry matter in the head was associated with low nitrogen treatments. Smaller amounts of nitrogen led to a tougher tissue, which was the main reason why the heads were less susceptible to bacterial attack.

## 1.2. Bacterial biosurfactant production

### 1.2.1. Introduction

Biosurfactants are a structurally diverse group and unlike chemically synthesized surfactants, which are classified according to the nature of their polar grouping, they are categorized mainly by their chemical composition and their microbial origin. In general, their structure includes a hydrophilic moiety consisting of amino acids, peptides anions or cations, mono-, di, or polysaccharides, and a hydrophobic moiety consisting of unsaturated or saturated fatty acids. Biosurfactants can have either a positive, negative or a neutral charge and this is determined by their hydrophilic moieties.

Due to their amphipathic properties, surface-active molecules concentrate at the interface of a solution with their hydrophobic regions aligned away from the liquid and the hydrophilic ones immersed (Fiechter, 1992). This then alters the physical forces governing the arrangement of liquid molecules, by influencing the formation of hydrogen-bonds, and results in a reduction in surface tension - the force required to keep a water droplet spherical (Denny, 1993).

The major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants and particulate surfactants.

**(a):Glycolipids**. Most known biosurfactants are glycolipids. They are carbohydrates combined with long-chain aliphatic acids or hydroxyaliphatic acids. The best known glycolipids are rhamnolipids, trehalolipids and sophorolipids. Rhamnolipids, in which one or two rhamnose subunits are linked to one or two β-hydroxydecanoic acids in a side chain, are the ones that have been well studied. Production of rhamnose-containing glycolipids was first described in *P. aeruginosa* by Jarvis and Johnson (1949). Several

different structural types of microbial trehalolipid biosurfactants have been reported (Lang and Wagner, 1987; Li *et al.,* 1984). Trehalolipids from different organisms differ in the size and stucture of mycolic acid, the number of carbon atoms, and the degree of unsaturation (Asselineau and Asselineau, 1978; Cooper *et al.*, 1989; Lang and Wagner, 1987). Trehalose dimycolate produced by *Phodococcus erythropolis* has been extensively studied (Kretschmer *et al.*, 1982; Rapp *et al.*, 1979). *Sophorolipids*, which are produced mainly by yeasts, consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxy fatty acid. These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides.

**(b) Lipopeptides/lipoproteins.** A large number of cyclic lipopetides including decapeptide antibiotics and lipopeptide antibiotics possess remarkable surface-active properties. The cyclic lipopeptide surfactin, produced by *Bacillus subtilis*, is one of the most powerful biosurfactants. It lowers the surface tension from 72 to 27.9 mN/m at concentrations as low as 0.5μg/mL (Arima *et al.*, 1968). Viscosin, produced by the broccoli head rot pathogen *P. fluorescens*, is also a potent lipopeptide biosurfactant. It can reduce the water surface tension from 72 to 27 mN/m at a concentration of 4μg/mL (Laycock *et al.*, 1991).

**(c) Phospholipids and fatty acids.** Several bacteria and yeasts produce large quantities of fatty acid and phospholipid surfactants during growth on n-alkanes (Asselineau and Asselineau, 1978; Cirigliano and Carman, 1985; Cooper *et al.*, 1978). Phosphatidylethanolamine produced by *Rhodococcus erythropolis* grown on n-alkane causes a lowering of interfacial tension between water and hexadecane to less than 1mN/m and a CMC (critical micelle concentration) of 30mg/L (Kretschmer *et al.*, 1982).

**(d) Polymeric biosurfactants.** Polymeric biosurfactants usually have a high molecular weight. The best studied polymeric biosurfactants are emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes. Emulsan, produced by *Acinetobacter calcoaceticus*, is a very effective emulsifying agent for hydrocarbons in water even at a

concentration as low as 0.001% to 0.01%. It is one of the most powerful emulsion stabilizers known today and resists inversion even at a water-to-oil ratio of 1:4 (Gutnick and Shabtai, 1987; Belsky *et al.*, 1979; Zosim *et al.*, 1982). Desai *et al.* (1988) demonstrated the production of bio-emulsifier by *P. fluorescens* during growth on gasoline. This bio-emulsifier is composed of 50% carbohydrate, 19.6% protein and 10% lipid.

### 1.2.2. Biosufactants in the incidence of head rot

Most broccoli head rot pathogens produce biosurfactants. Hildebrand (1986) suggested that *Pseudomonas fluorescens* was the predominant microbe in causing broccoli head rot. He reasoned that these highly virulent strains could produce not only pectolytic enzymes, but also a surfactant-like substance (biosurfactant). By reducing the surface tension of rain water or dew droplets present on the broccoli head, biosurfactants allow water to spread over the waxy tissue leading to the development of water soaking, enabling pectolytic enzymes to attack the otherwise hydrophobic waxy tissues of broccoli. Biosurfactants thereby enhance bacterial colonization and aid disease progression (Hildebrand, 1989). In addition, the biosurfactants can alter membrane permeability and thus result in nutrient leakage from the epidermis (Hildebrand *et al*., 1990). Biosurfactant production may also play a significant role in the physical spread of bacteria during the early colonization of broccoli florets and after decay has been initiated (Hildebrand, 1989). Further proof was obtained by Gladstone (1997) who inoculated broccoli with either a biosurfactant-producing strain of *P. fluorescens* or with purified viscosin (biosurfactant from *P. fluorescens*) and found that black lesions were induced on both trials. Support also came from Bussotti *et al.* (1997) who claimed that surfactant molecules were absorbed through the stomata of pine needles causing deterioration in the epistomatal wax structures.

Despite Hildebrand's claim that biosurfactants were a crucial virulent factor in bacteria head rot incidence, conflicting conclusions were drawn by Robertson *et al*. (1993), who

found that two biosurfactant negative *Erwinia* isolates were also pathogenic, and by Darling (1998), whose research indicated that Tn*5* biosurfactant-minus mutants of pathogenic *Pseudomonas fluorescens* could still cause head rot disease.

### 1.2.3. Biosurfactant production in *Pseudomonas fluorescens*

*Pseudomonas fluorescens*, the primary virulent strain of broccoli head rot, is known as a multi-type biosurfactant producer (Persson *et al.*, 1988; Healy *et al.*, 1996). Its principal extracellular surfactant component is identified as viscosin (Laycock *et al.*, 1991). Viscosin is a lipopeptide composed of a hydroxydecanoic acid moiety and was first isolated from *Pseudomonas viscosa* (Kochi *et al.*, 1951). It has strong antibiotic activity (Groupe *et al.*, 1951; Ohno *et al.*, 1953). The original structure of viscosin was published in 1953 (Ohno *et al.*, 1953) and revised in 1970 (Hiramoto *et al.*, 1970). Burke *et al.* (1989) described its synthesis. Viscosin has a critical micelle concentration (CMC) of 4µg/mL and can reduce the surface tension of water from 72 to 27 mN/m (Laycock *et al.*, 1991). Viscosin does not affect membranes below the CMC but lyses erythrocytes by a general detergent action at concentrations above the CMC, so it is not considered to have membrane-specific toxicity (Hildebrand *et al.*, 1998). On broccoli, a viscosin concentration of 10µg/mL causes wetting of the waxy florets and electrolyte leakage. The presence of viscosin restores the ability of viscosin deficient mutants of *P. fluorescens* to penetrate stomata, colonize, and decay broccoli tissues. Viscosin is similar in size, structure and composition to other lipopeptides, such as tolaasin produced by *P. tolaasii*, surfactin produced by *Bacillus subtilis*, syringomycin and syringopeptin produced by *P. syringae* pv. s*yringae* (Rainey *et al.*, 1991; Hutchison and Gross, 1997; Bernheimer and Avigad, 1970). These four lipopeptides have hydrocarbon chains ranging from $C_8$ to $C_{15}$ and chains of 7-25 amino acids of which 5-14 form a lactone ring. Unlike viscosin, these lipopeptides have considerably higher CMCs ranging from 0.8 to 1.25mg/mL and reduce the surface tension of water to between 41 and 27 mN/m. Viscosin production can be unstable. It is found to be declining in liquid culture and only low levels of biosurfactant remain after about 48 hours (Darling 1998).

Other compounds with surfactant activities similar to that of viscosin have been isolated from various isolates of *P. fluorescens*. Harling and Dubickas (unpublished data, 1998) found that there was at least one other biosurfactant type besides viscosin in their *P. fluorescens* isolates. Healy *et al*. (1996) found that *P. fluorescens* NCIMB11712 produced a non-haemolytic biosurfactant and a rhamnolipid biosurfactant. Nielsen *et al*. (1999) identified a new bacterial cyclic lipdepsipeptide from *P. fluorescens* DR54. It has biosurfactant properties and is termed viscosinamide. However, it is different from viscosin in that it contains glutamine rather than glutamate at the amino acid position 2. Unlike viscosin, viscosinamide production is not affected by C, N or P nutrient levels, starvation or different growth phases. Recently more lipopeptide biosurfactants from *P. fluorescens* have been identified. Henriksen *et al*. (2000) described a new lipopeptide biosurfactant termed tensin in *P. fluorescens* strain 96.578. A cyclic lipopeptide biosurfactant produced by *P. fluorescens* DSS73 was purified and named amphisin (Sorensen *et al*., 2001). So far, all the cyclic lipopeptide biosurfactants identified from *P. fluorescens* strains contain either 9 or 11 amino acids in the peptide ring and a $C_{10}$ fatty acid moiety at one of the amino acids (Nielsen *et al*., 2002; Nielsen and Sorensen, 2003). Viscosinamide has a strong cell-binding affinity to the producing cells of strain DR54 (Nielsen *et al*., 1999), while both tensin and amphisin are largely released into the surrounding medium (Nielsen *et al*., 2000). The accumulation of those cyclic lipopeptides will only take place in specific habitats, such as the rhizosphere of germinating sugar beet, and decline rapidly in the bulk soil. This suggests that indigenous soil microorganisms that are capable of degrading the biosurfactants exist (Nielsen and Sorensen, 2003). It is likely that more than one of the biosurfactants produced by *P. fluorescens* are involved in broccoli head rot.

A number of investigators have demonstrated that there is an overproduction of biosurfactants by *Pseudomonas spp*. once the cultures reach the stationary phase of growth (Guerra-Santos *et al.*,1986; Mulligan and Gibbs, 1989; Ramana and Karanth, 1989). In *P. fluorescens*, biosurfactant production generally requires low oxygen and nitrogen concentration in liquid culture (Persson *et al*., 1988). The limitation of

multivalent cations also causes an overproduction of biosurfactant. Iron limitation stimulates biosurfactant production in *P. fluorescens* (Persson *et al*., 1990a, 1990b). Carbon source plays an important role in yield and structure of microbial surfactants. Viscosin production is optimum *in vitro* on a medium containing glycerol as the carbon source for its synthesis (Georgiou *et al*., 1992).

Different types of biosurfactants may be produced by a bacterial species depending on the carbon source and the other nutrients available to it (Desai *et al*., 1988). *P. fluorescens* is found to produce a rhamnolipid instead of viscosin when grown on virgin olive oil (Healy *et al*., 1996). Laycock *et al.* (1991) noted that two additional HPLC peaks were obtained when biosurfactants were extracted from broccoli tissue, as opposed to agar plates. This indicates that the various bacterial environments may induce or suppress the production of certain biosurfactants.

### 1.2.4. Detection of biosurfactants

In order to study microbial surfactant production, an accurate, rapid and sensitive detection method is required. A variety of different test methods have been described for the screening of potential biosurfactant-producing micro-organisms.

(1) Van der Vege *et al.* (1991) developed an axisymmetric drop shape analysis (ADSA) method for the assessment of potential biosurfactant-producing bacteria. In this technique, drops of culture broth are placed on a fluoroethylene-propylene surface and the profile of the droplet is determined with a contour monitor. Surface tension is calculated from the droplet profiles by ADSA. Only biosurfactant-producing bacterial suspensions show reduction in surface tensions.

(2) Siegmund and Wagner (1991) described a colorimetric estimation of biosurfactants based on the fact that anionic surfactants are capable of reacting with the cationic-indicator and forming a coloured complex. This method is semi-quantitative as the diameter of the coloured halo increases with surfactant concentration. It has been further developed by Shulga *et al.* (1993) and Hansen *et al.* (1993) for screening of rhamnolipid-producing and hydrocarbon-degrading bacteria.

(3) A rapid drop-collapsing test, in which a water droplet will collapse while biosurfactant containing bacterial colonies (or bacterial suspensions) are added, was developed by Hildebrand (1989). Jain *et al.* (1991) further developed this method by placing a drop of cell suspension on an oil-coated surface. In this method, drops containing biosurfactants collapse whereas non-surfactant-containing drops remain stable.

(4) A direct thin-layer chromatographic (TLC) technique for rapid characterization of biosurfactant-producing bacterial colonies was described by Matsuyama *et al.* (1987). They identified *Serratia macrescens* biosurfactants by applying a single bacterial colony directly to the TLC plate without any sample preparation procedure.

(5) Testing a solution's surface tension with a surface tension meter leads to an accurate quantification of the presence of a biosurfactant. Biosurfactant activities can be determined by measuring the changes in surface and interfacial tensions, stabilization or destabilization of emulsions, and hydrophilic-lipophilic balance (HLB). Surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer. The surface tension of distilled water is 72 mN/m. When a surfactant is added to air/water or oil/water systems at increasing concentrations, a reduction of surface tension is observed up to a critical level, above which amphiphilic molecules associate readily to form supra molecular structures like micelles, bilayers, and vesicle. This value is known as the Critical Micelle Concentration (CMC). CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant.

(6) Bunster *et al.* (1989) studied the surface activity of bacteria by measuring the contact angles obtained after adding the bacteria to a water drop. The contact angle is located at the interface between the droplet and the solid surface. In the absence of surfactant, water molecules adhere strongly to each other and so the water droplet retains a round appearance with a contact angle of more than 90°, while in the presence of biosurfactants, the adherance forces are reduced causing the droplet to spread out flat creating a contact angle of less than 90°. Bunster *et al.* (1989) used a 5µl aliquot from a broth suspension to form a droplet and measured the contact angle obtained. This

method has the advantage of being quantitative and will detect all type of biosurfactant produced, although it may lack sensitivity towards low biosurfactant levels.
(7) Fungal growth inhibition is used to detect biosurfactants with antibiotic activity (Itoh *et al.*, 1971).

Darling (1998) discussed some of the above detection methods and the Water Droplet Test was proved to be the most sensitive and rapid assay for detecting *P. fluorescens* biosurfactant.

### 1.2.5. Biosurfactant synthesis and genetic characterization

In the biosurfactant amphiphathic structure, the hydrophobic moiety is either a long-chain fatty acid, a hydroxy fatty acid or a α-alkyl-β-hydroxy fatty acid, and the hydrophilic moiety may be a carbohydrate, carboxylic acid, phosphate, amino acid, cyclic peptide, or an alcohol. According to Syldatk and Wagner (1987), there are four possible ways how the different moieties of biosurfactants and their linkage are synthesised: (i) the hydrophilic and hydrophobic moieties are synthesized *de novo* by two independent pathways; (ii) the hydrophilic moiety is synthesized *de novo* while the synthesis of the hydrophobic moiety utilises a pre-existing substrate; (iii) the hydrophobic moiety is synthesized *de novo*, while the synthesis of the hydrophilic moiety is substrate dependent; and (iv) the synthesis of both the hydrophobic and hydrophilic moieties are formed from pre-existing substrates.

Nitrogen- or multivalent ion-dependent regulation also plays a prominent role in the synthesis of biosurfactants. The synthesis of rhamnolipids in *P. aeruginosa* upon exhaustion of nitrogen and commencement of the stationary phase of growth has been observed by several investigators (Guerra-Santos *et al.*, 1984; Ramana and Karanth, 1989). Moreover, the addition of a nitrogen source caused inhibition of rhamnolipid synthesis in resting cells of *Pseudomonas* sp. strain DSM-2874 (Syldatk *et al.*, 1985). The limitation of multivalent cations also causes overproduction of biosurfactants.

Guerra-Santos *et al.* (1986) demonstrated that by limiting the concentrations of salts of magnesium, calcium, potassium, sodium and trace elements, a higher yield of rhamnolipid can be achieved in *P. aeruginosa* DSM 2695. Iron limitation stimulates biosurfactant production in *P. fluorescens* (Persson *et al.*, 1990a; 1990b) and *P. aeruginosa* (Guerra-Santos *et al.*, 1984; 1986), whereas addition of iron and manganese salts stimulates biosurfactant production in both *B. subtilis* (Cooper *et al.*, 1981) and *Rhodococcus* sp. (Abu-Ruwaida *et al.*, 1991).

A clear understanding of the genetic characterization of biosurfactants has emerged only through studies of the genes involved in rhamnolipid synthesis in *P. aeruginosa* and surfactin synthesis in *Bacillus subtilis.*

In *P. aeruginosa*, several genes have been found to be involved in rhamnolipid biosynthesis. Ochsner *et al*. (1994a) discovered a 2-kb fragment capable of restoring rhamnolipid biosynthesis in a rhamnolipid deficient mutant strain of *P. aeruginosa*. In this fragment, a single open reading frame (*rhlR*) of 723 bp specifying a putative 28-kDa protein (RhlR) was identified. Disruption of the *P. aeruginosa* wild-type rhlR locus led to rhamnolipid-deficiency, thus confirming directly that this gene is necessary for rhamnolipid biosynthesis. Genes (termed *rhlAB*) encoding a rhamnosyltransferase, RhlAB, which catalyzes the transfer of rhamnose from TDP-rhamnose to β-hydroxydecanoyl-β-hydroxydecanoate, have recently been isolated and further sequenced (Ochsner *et al*., 1994b). The transcriptional activation of *rhlAB* appears to depend on a functional RhlR regulatory protein. The sequence upstream of the *rhlA* promoter contains two inverted repeats that define putative binding sites for the RhlR regulator. Another gene, *rhlI*, which is also required for rhamnolipid synthesis, has been identified downstream of the rhlABR gene culster. The *rhlI* gene production, RhlI, has been proved to be a bacterial autoinducer (usually belongs to homoserine lactone family) synthase. The *P. aeruginosa rhlA* promoter is actived only when both the *rhlR* and *rhlI* genes are present or when the *rhlR* gene alone is supplied together with synthetic autoinducers (Ochsner and Reiser, 1995). The RhlR-RhlI regulatory

mechanism is known as quorum sensing. The bacterial quorum sensing system will be further addressed later.

A study of peptide antibiotic biosynthesis in *Bacillus subtilis* led to the isolation of genes required for the production of the cyclic lipopeptide surfactin (biosurfactant). Three genes, *sfp, srfA* and *comA* were identified (Nakano *et al*., 1988; 1991; 1992; Nakano and Zuber, 1989,1990,1991). *Sfp* was found in the surfactin producing strain of *Bacillus subtilis*. When transferred by genetic transformation, *sfp* was necessary and sufficient to render cells of a non-producing strain surfactin positive through an unknown mechanism (Nakano *et al*., 1988). ComA mutations, in addition to blocking competence development at an early stage, also rendered sfp-bearing cells surfactin negative. The ComA product is homologous to the two-component regulatory proteins and is likely to be a DNA-binding protein (Weinrauch *et al*., 1989). The *srfA* operon, which encodes the multifunctional enzymes, is required for surfactin production in *Bacillus subtilis* (Nakano *et al.*, 1991; Nakano and Zuber, 1993). ComA is necessary for the expression of *srfA*. A subsequent study showed that ComA acts as a phosphorylated response regulator protein. It binds to the promoter region of *srfA*, thereby controlling surfactin production (Roggiani and Dubnau, 1993).

There is currently very little information regarding viscosin gene expression and regulation in *P. fluorescens*. Recently, Braun *et al.* (2001) have identified a 25kb chromosomal DNA region that affects the production of three high molecular mass proteins required for viscosin synthesis. They assume that these proteins, approximately 218, 215 and 137 kDa in size, compose a synthetase complex that assembles the nine amino acid peptide of viscosin and subsequently attaches this to the hydrophobic fatty acid component of the molecule. Further sequence analysis of portions of the 25kb gene cluster may shed some light on the viscosin gene regulation. Quorum sensing has been reported to control biosurfactant production in a variety of bacteria (Huber *et al.*, 2001). But so far it remains to be proved that whether the biosurfactant production in *P. fluorescens* is controlled by the quorum sensing system.

## 1.3. Quorum sensing — wide spread bacterial communication system

### 1.3.1. Introduction

Recent advances in the study of bacterial gene expression have discovered that many bacteria employ a dedicated intra-cellular communication system. This bacterial decision-making system enables a given species to sense, integrate and process information from its surroundings, communicate with each other, and monitor its own population density and, as a response, activate or repress specific gene expression. This bacterial cell-density-dependent communication system is known as quorum sensing (Fuqua *et al*., 1994).

To sense the surrounding bacterial population density, the bacterial quorum sensing system relies on one or more small signal molecules (also called "autoinducers"), which are produced and released by bacteria. In gram-negative bacteria, the most commonly utilized and intensively investigated autoinducers are N-acyl-homoserine lactones (AHLs) (Figure 1.2). The acyl side-chain length and the substitutions on the side chain provide signal specificity (Fuqua *et al*., 1996; Eberhard *et al*., 1981).

The regulation of bioluminescence in *Vibrio fischeri* was the first quorum sensing phenomenon to be studied and *V. fischeri* has subsequently become a model for studies of the mechanism of quorum sensing (Eberhard, 1972; Nealson *et al.* 1970). In this section, the *V. fischeri* quorum sensing system is reviewed in order to shed light on how the quorum sensing system functions in correlating gene expression with bacterial population density.

In the early 1970's, it was discovered that luminescence in *Vibrio fischeri* is subject to cell density-dependent auto-regulation (Eberhard, 1972; Nealson *et al.* 1970). When the bacteria are cultured in broth, they exhibit a lag in their luminescence gene (lux) expression during the early and mid-exponential growth phases, followed by a rapid

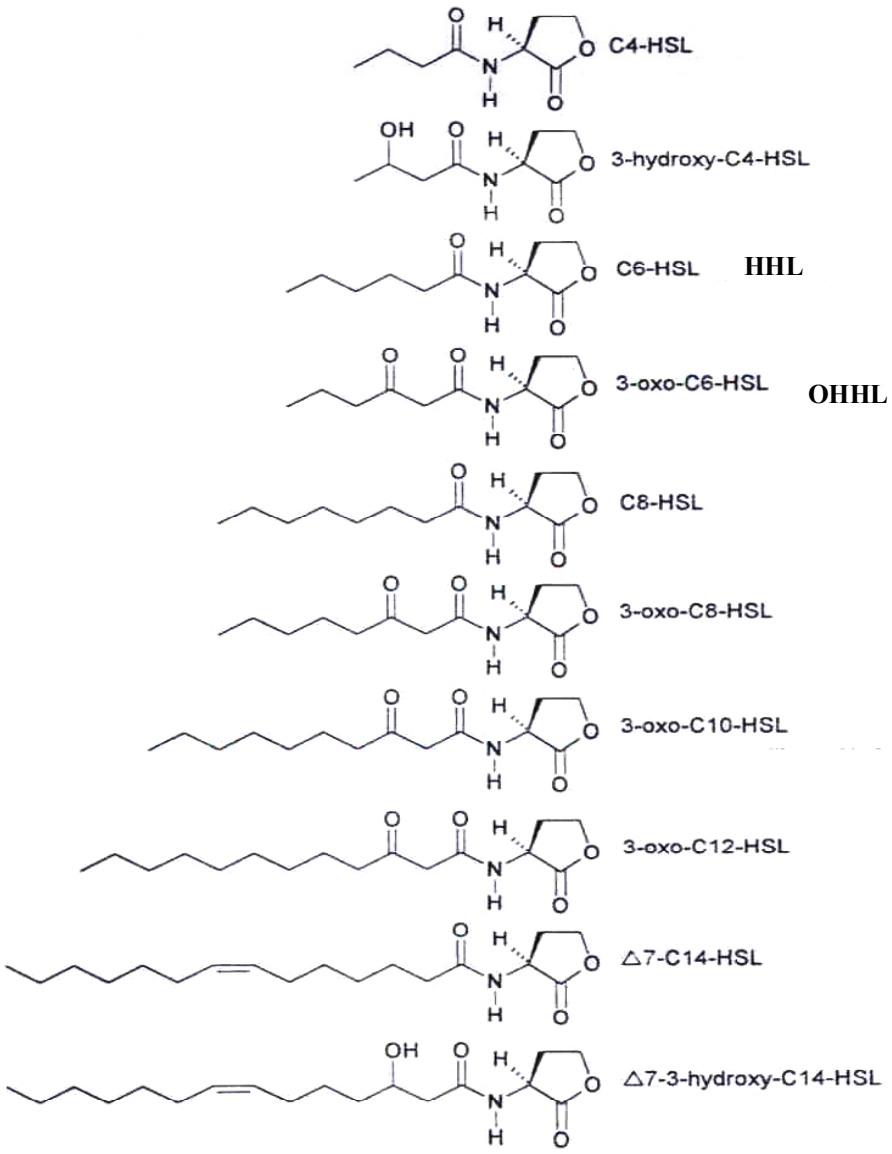



**Figure 1.2: Comparison of acyl-HSL structures.**
**(adapted from Fuqua and Eberhard, 1999)**

increase in expression during the late exponential and early stationary growth phases. Luminescence in early-log-phase cultures is induced by the addition of cell free fluid extracts from stationary-phase cultures (Eberhard, 1972; Nealson *et al*., 1970; Nealson, 1977). Kaplan and Greenberg (1985) found that *V. fischeri* produces a diffusible compound termed "autoinducer" (AI) that accumulates in the surrounding environment during growth. At low cell densities, the autoinducer is at low concentrations, and at high cell densities the autoinducer can accumulate to a concentration sufficient for activation of the luminescence genes. The autoinducer in *V. fischeri* is identified as N-(3-oxohexanoyl)-homoserine lactone (OHHL, Figure 1.2) (Eberhard *et al*., 1981). The cell-density-sensing mechanism mediated by an autoinducer molecule is known as quorum sensing (Fuqua *et al*., 1994).

Since the original discovery and descriptions of autoinduction, the mechanism of quorum sensing in *V. fischeri* has been studied in numerous laboratories. Quorum sensing depends on two proteins. The LuxR, which is a transcriptional factor (also known as autoinducer receptor protein) and the LuxI, which is the AHL synthase (Engebrecht *et al*., 1983; Engebrecht and Silverman, 1984). The LuxI and LuxR proteins as well as a proposed LuxR binding site lux box (a 20 bp inverted repeat situated within the *lux* regulon) are necessary for the activation of quorum sensing related gene expression (Figure 1.3). Once a critical concentration of OHHL has been reached, OHHL is thought to bind to LuxR. Binding to an autoinducer molecule (OHHL) enables the LuxR protein to bind with the lux box and act as a transcriptional activator, thus enhancing the expression of the rightward transcript. Since luxI is located within this operon, more OHHL is produced, more OHHL: LuxR binding occurs and increases transcriptional activation of the luxI gene, thereby generating a positive feedback regulatory loop in which OHHL controls its own synthesis (Figure1.3).

Although in *V. fischeri* luxR is transcribed divergently from luxI, there is no theoretical requirement for the genes encoding autoinducer molecules to be linked to the corresponding regulatory genes. Assuming that the AHL is freely diffusible and can

bind to its LuxR homologue and that the resultant complex can then find its appropriate binding site on the DNA, any form of genetic arrangement is possible (Salmond *et al*., 1995). Several homologous pairs of quorum sensing regulatory genes have proved to transcribe convergently and are not linked to the genes they regulate (Beck Von Bodman and Farrand, 1995; Pirhonen *et al*., 1993; Throup *et al*., 1995a; Wood and Pierson, 1996).

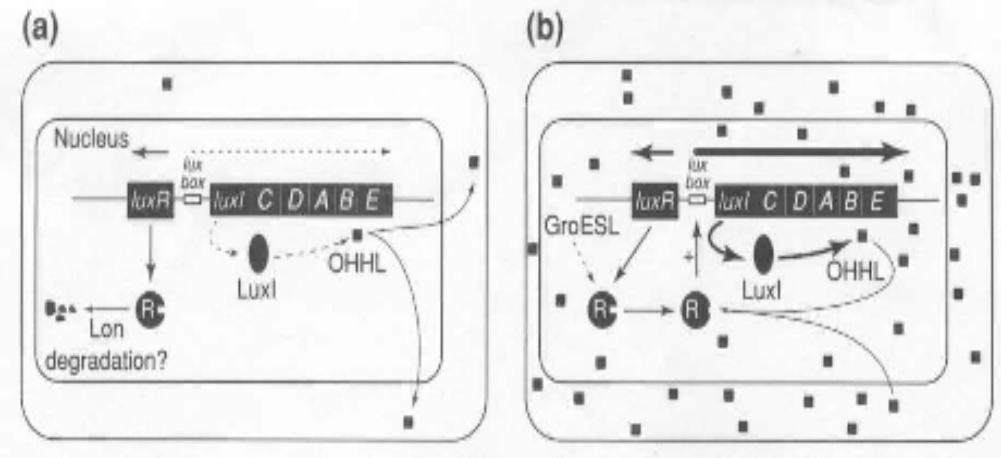



**Figure1. 3: Luminescence (*lux*) gene regulation in Vibrio fischeri (Swift *et al*., 1996)**

(a) At low population density, *luxI* and *luxR* are transcribed at a low level and there is insufficient accumulation of the signal OHHL to elicit LuxR dependent transcription of the *luxICDABE* operon for visible bioluminescence. As the population grows, however, transcription of the *luxICDABE* operon continues at a basal level sufficient to enable the concentration of OHHL to gradually increase. (b) At a critical level of OHHL, which reflects a critical population density, an OHHL-LuxR complex is thought to activate transcription of the *luxICDABE* operon, resulting in increased levels of OHHL and bioluminescence.

In different bacterial quorum sensing systems, the AHL synthase (LuxI homologues) and the transcriptional factors (LuxR homologues) are highly conserved in their gene sequences and the AHL-mediated quorum sensing is widespread among gram-negative bacteria (Swift *et al*., 1993; Bainton *et al*., 1992a, 1992b).

In bacteria, quorum sensing usually depends on a quorum sensing signal and a signal receptor protein, but not necessarily. Recently, it was discovered that some

microorganisms produce AI receptor proteins but lack the ability to produce quorum sensing signals. For example, SdiA in the human pathogen *Salmonella enterica* "recognises" quorum sensing signals that are produced by other microorganisms (Michael *et al.*, 2001). This may enable non-quorum sensing signal producers to "eavesdrop" on communications among other microbes sharing the same niche and regulate the expression of specific genes by the density of signals produced by the microbial community, perhaps without having to produce signals themselves. Thus, our perception of what constitutes a "quorum" on a plant surface may need to be redefined to include the diversity of microbes capable of producing signals and those capable of monitoring signals in a shared niche (Loh *et al.*, 2002).

### 1.3.2. Quorum sensing signals, LuxI and LuxR homologues

#### 1.3.2.1. AHL signal structure and synthesis

All AHLs thus far reported have the general structure of an acyl chain ligated to the amino nitrogen of the homoserine lactone moiety via an amide bond. The acyl side chain length and the substitutions provide signal specificity. A primary site of variation is the third position on the acyl chain, it can be a carbonyl group, a hydroxyl, or fully reduced in different bacteria (Figure 1.2). Acyl side chains of these AHL signals can be fully saturated, and can have lengths with an even number of carbons ranging from 4 to 16 (Fuqua *et al*., 1996). To date, only two compounds with acyl chains containing double bonds have been identified. These are 7,8-cis-N-(3-oxohexanoyl) homoserine lactone and 7,8-cis-N-(tetradecenoyl) homoserine lactone produced by *Rhizobium leguminosarum* (Schripsema *et al*., 1996; Gray *et al*., 1996) and *Rhodobacter sphaeroides* (Puskas *et al*., 1997), respectively.

Acyl-HSLs are chemically stable at neutral or acidic pH in aqueous solutions (Schaefer *et al.*, 2000). However, the HSL ring is subject to alkaline hydrolysis (Voelkert and Grant, 1970). The fact that a signal, N-(3-oxohexanoyl) homoserine lactone, freely diffuses through *V. fischeri* and *E. coli*. cells has led to the assumption that all AHL

autoinducers are freely diffusible (Kaplan and Greenberg, 1985 ; Pearson *et al*., 1997). However, a subsequent study focused on *P. aeruginosa* AHL proves that short chain signals, such as $C_4$-HSL, diffuse freely into or out of the cell membrane (Pearson *et al*., 1999) and that $C_{12}$-HSL can only diffuse slowly through the membrane. In addition to its slow diffusion, $C_{12}$-HSL is actively pumped from cells by the MexAB-OprM, and perhaps other, efflux pumps (Pearson *et al*., 1999; Evans *et al*., 1998). Presumably, the difference in the length and /or the degree of substitution of the acyl side chains determines whether an AHL signal is freely diffusible or is subject to active efflux. Regardless of this, the cellular concentration of an AHL is defined by its environmental concentration, and environmental concentrations can rise only when there is a sufficient population of the signal producing bacterium.

The structure of the *V. fischeri* autoinducer (3-oxo-$C_6$-HSL) suggests that this signal molecule is synthesised from fatty acid and amino acid precursors (Eberhard *et al*., 1981). When the intracellular pools of AdoMet (S-adenosyl-L-methionine) in *E. coli* were depleted by employing the AdoMet hydrolase from bacteriophage T7, the synthesis of 3-oxo-$C_8$-HSL by the TraI (AHL synthase of *Agrobacterium tumefaciens*) protein is severely reduced in proportion to the observed reduction in AdoMet pools. This study provides indirect evidence for the role of AdoMet as a substrate in acyl-HSL synthesis (Val and Cronan, 1998).

In addition to AdoMet, acylated ACP, which is an intermediate in fatty acid biosynthesis, was also proved to be indispensable for AHL synthesis (Schaefer *et al*., 1996a; Kuo *et al*., 1994). An acyl-HSL synthase RhlI protein has been purified successfully under denaturing conditions from *E. coli* by using a low-copy number expression vector. Further analysis of its enzymatic activity indicate that only ACP and AdoMet are required for synthesis of acyl-homoserine lactone; CoA and NADPH are not substrates for RhlI (AHL synthase of *P. aeruginosa*) when present at physiological concentrations (Hoang *et al*, 1999). The biochemical mechanism for conversion of SAM and specific acyl-ACPs to HSL autoinducers has now been demonstrated for several LuxI-like proteins from different bacteria. These enzymes include LuxI from *V.*

*fischeri* (Hanzelka and Greenberg, 1996), TraI from *A. tumefaciens* (More *et al*, 1996), and RhlI from *Pseudomonas aeruginosa* (Parsek *et al*, 1999).

The level of conservation within the AHL synthase family ranges from 28 to 34%. While conserved residues are dispersed throughout the sequence of *luxI* homologs, there are 10 invariant residues that are clustered in the amino-terminal half of the proteins. The carboxy-terminal half of acyl-HSL synthase, while lacking any absolutely conserved amino acid residues, does contain residues that are highly conserved or stucturally conserved. Using both AdoMet and acyl-ACP to synthesize acyl-HSLs suggests that the active site of this *luxI* type synthase must be able to accommodate two very different substrates. The mechanism of the reaction ligating the amino acid portion of AdoMet with the acyl chain is likely to be conserved between different acyl-HSL synthases, and therefore the amino acid residues that direct this reaction are likely to be among those highly conserved between all acyl-HSL synthases (Fuqua and Eberhard, 1999). Recent studies of *V. fischeri* and *V. harveyi* have identified proteins that are likely acyl-HSL synthases but bear no sequence similarity to *luxI*. In *V. harveyi*, synthesis of 3-hydroxy-$C_4$-HSL requires the luxM gene (Bassler *et al*, 1993). Likewise, production of $C_8$-HSL in *V. fischeri* is mediated by a protein called AinS (Gilson *et al*, 1995). The LuxM and AinS proteins share a region of strong conservation with each other but are not homologous to LuxI. The relationship between *luxI*-type and AinS-type acyl-HSL synthases remains unknown. LuxM and AinS are thus known as a second class of Acyl-HSL synthases.

Identification of AdoMet and acyl-ACP as substrates for acyl-HSL synthesis suggests a plausible enzymatic mechanism (Eberhard *et al*., 1991). The amino group of AdoMet provides a strong nucleophile for attacking the carbonyl group of the fatty acid moiety of the acyl-ACP. The reaction ligates the acyl chain to AdoMet via an amide bond. Lactonization of the acyl-AdoMet intermediate will generate the free acyl-HSL and 5'-methylthioadenosine (Hanzelka and Greenberg, 1996; More *et al*., 1996; Schaefer *et al*., 1996a) (Figure 1.4).

Each AHL autoinducer synthase manufactures a specific signal. The newly reported structure of the EsaI (homologue of RhlI, autoinducer synthase of *E. carotovora*) reveals clues as to how a series of AHL molecules can be generated that differ only in side chain length. The EsaI structure predicts the existence of a hydrophobic cavity into which the acyl side chain (3-oxo-hexanoyl) neatly packs (Watson *et al*., 2002). Modelling suggests that the corresponding cavity in LasI (AHL synthase of *P. aeruginosa*) is larger, and this is consistent with LasI synthesising the longer autoinducer, 3-oxo-dodecanoyl AHL.

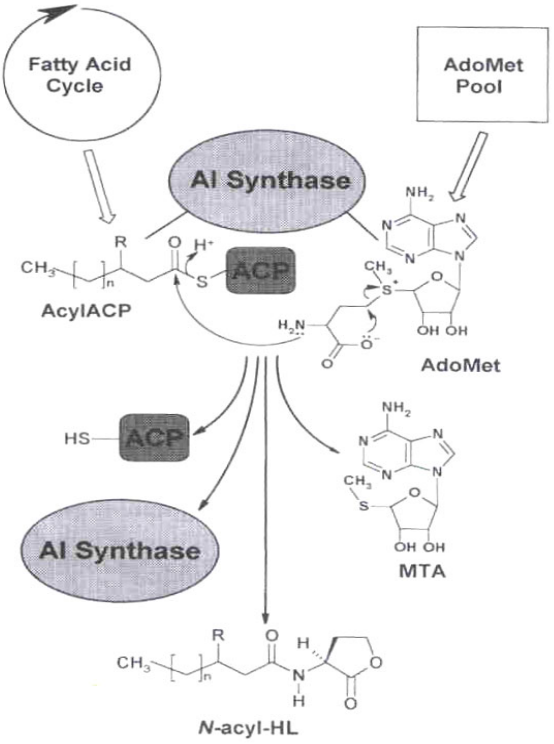



**Figure 1.4 : Acyl-HSL catalytic mechanism**

Putative enzymatic mechanism for acyl-HSL synthesis by LuxI type proteins indicating precursors and probable products (Fuqua *et al*., 1996).

1.3.2.2. Extraction of AHL

Crude AHL is readily soluble in ethanol, methanol, acetone, ethylacetate, acetonitrile and dichloromethane, but not in hexane. It is best extracted from aqueous media with

ethyl acetate. Dichloromethane is almost as effective while diethyl ether is much less so (Eberhard *et al.*, 1981).

AHLs are chemically stable. Crude AHL preparations lose only some activity upon treatment for one hour at room temperature with either 3% $H_2O_2$ or $H_2$/Pt or upon heating at 140 °C for 10 minutes as a dry thin film in air. These films of crude AHL lose no activity upon standing at room temperature for several days either in air or *in vacuo* (Eberhard *et al.*, 1981).

The chemical properties of AHL suggest that it should be stable enough to be isolated. Currently the most popular procedure to isolate AHL is as follows: AHLs are concentrated from spent culture filtrate by extracting into an organic solvent (e.g. dichloromethane, ethyl acetate) (Williams and Fleming, 1987; Kemp, 1991; Camara *et al.*, 1998) and purified to homogeneity by using semi-preparative high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) (Bainton *et al.*, 1992a; Camara *et al.*, 1998; Shaw *et al.*, 1997; Swift *et al.*, 1997). The purified AHL can then be identified by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Eberl *et al.*, 1996).

1.3.2.3. Other quorum sensing signal molecules

The acyl-homoserine lactone (AHL) family is the most common quorum sensing signal, but several other chemically distinct signal families have also been identified.

Two sets of quorum sensing systems employing the LuxRI homologues (RhlRI and LasRI) have been identified from *P. aeruginosa* (Passador *et al.*, 1993; Pearson *et al.*, 1994; 1995; Winson *et al.*, 1995). Except for the AHL-type quorum sensing signals ($C_4$- and $C_{12}$-homoserine lactone), two new groups of diffusible signal molecules have been identified in *P. aeruginosa* spent culture supernatants. By studying the expression of *lasB* in a *lasR*-negative mutant, Pesci *et al.* (1999) discovered another quorum sensing signal. The signal, which is chemically characterised as 2-heptyl-3-hydroxy-4-

quinolone (termed PQS), is unrelated to any previously known signal. PQS production is LasR dependent, indicating that the genes responsible for PQS synthesis are part of the LasR regulon. They also demonstrate that exogenous PQS exhibits bioactivity in the *lasR* mutant but not in the *lasR-rhlR* double mutant strain. These results suggest that LasR is required for PQS production while RhlR is crucial for PQS bioactivity (Pesci *et al*., 1999). A later work indicates that PQS is not produced maximally until the culture reaches the late stationary phase of growth (McKnight *et al.*, 2000). This implies that PQS acts as a link between the two (*las* and *rhl*) quorum sensing systems and that this signal is not involved in sensing cell density.

The discovery of PQS has added an exciting new dimension to the quorum sensing cascade of *P. aeruginosa*. Apart from AHL and PQS, *P. aeruginosa* has recently been shown to produce another family of small diffusible signal molecules, the diketopiperazines (DKPs) (Holden *et al*., 1999). These cyclic dipeptides, at high concentrations, are able to cross-activate AHL-dependent biosensors. In higher plants and animals, DKPs exhibit a remarkable spectrum of biological and pharmacological activity. Therefore, it is quite feasible that the DKPs do not function as bacteria-to-bacteria signalling molecules *per se*, but they might have a role in modulating prokaryotic-eukaryotic interactions (Holden *et al*., 2000). In bacteria, the production of DKPs is not limited to *P. aeruginosa*, as other gram-negative bacteria, including *Proteus mirabilis, Citrobacter freundii, Enterobacter agglomerans, Pseudomonas fluorescens,* and *Pseudomonas alcaligenes*, have been found to produce the same or related molecules (Holden *et al*., 1999).

Although there is, as yet, no chemically characterised example of quorum sensing signal molecules common to both gram-negative and gram-positive bacteria, the latter prefer peptides as their quorum sensing signals. Surette *et al.* (1999) identified a gene termed LuxS, which has homologues in both bacterial types. The LuxS protein appears to be responsible for the production of a novel class of quorum sensing signal molecules which is known as AI-2 (autoinducer-2) and in this case, the AHL-type quorum sensing signals are known as AI-1. AI-2 activity is produced maximally in mid-exponential

phase in *Escherichia coli* and *Salmonella typhimurium*, but are degraded when the bacteria enter stationary phase (Surette and Bassler, 1998).

The AI-2 synthase gene luxS, which has been identified from *E. coli, S. typhimurium, V. harveyi*, is now shown to be present in at least 25 discrete species, including both gram-negative and gram-positive bacteria. Sequencing analysis shows that LuxS proteins are highly conserved and some of the proteins can even function across species (Surette *et al.*, 1999).

Schauder *et al.* (2001) provided proof showing that AI-2 is produced from AdoMet in three enzymatic steps. The substratum for LuxS is S-ribosylhomocysteine, which is cleaved to form two products, one of which is homocysteine, and the other is AI-2. The authors also provided evidence that the biosynthetic pathway and biochemical intermediates in AI-2 biosynthesis are identical in *E. coli, S. typhimurium, V. harveyi, V. cholerae* and *Enterococcus faecalis*. This result suggests that, unlike homoserine lactone-type autoinducers, AI-2 is a 'universal' signal that could be used by a variety of bacteria for communication among and between species (Schauder *et al*., 2001).

The chemical structure of AI-2 could not be identified for a long time as the AI-2 signals could not be purified by conventional techniques used for the isolation of AHL autoinducers (Surette and Bassler, 1998; 1999). Finally, Chen *et al*. (2002) determined the structure of the *V. harveyi* AI-2. It consists of two fused 5 membered rings with a boron atom bridging the diester (Figure 1.5).

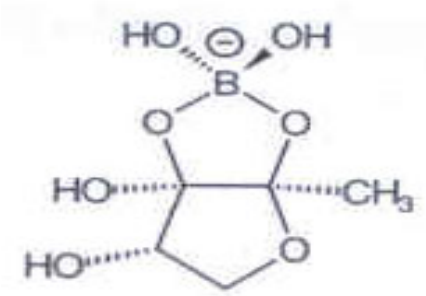



**Figure 1.5: The structure of AI-2 (Chen *et al*., 2002)**

1.3.2.4. Quorum sensing signal turnover

Given the control role played by the quorum sensing signal in the bacterial gene expression regulatory system, we would expect that its concentration is tightly regulated and a signal turnover system might exist. However, little is known about how bacterial cells exit the quorum sensing phase, once they have entered it. In the study of plasmid transfer in *Agrobacterium tumefaciens*, Zhang *et al*. (2002a) identified a gene, designated as *attM*. The enzyme encoded by *attM* inactivates the *A. tumefaciens* quorum sensing signal by hydrolysis of its homoserine lactone ring. Furthermore, they present evidence that *attM* is negatively controlled by a transcription factor AttJ, the expression of which is enhanced substantially when *A. tumefaciens* bacterial cells enter the stationary phase. The enzyme is capable of degrading 3-O-$C_8$-HSL and terminating the conjugation-related quorum sensing.

However, Byers *et al*. (2002) provide a different view in the *Erwinia carotovora* quorum sensing signal turnover mechanism. They demonstrated that the concentration of OHHL in *E. carotovora* spp. c*arotovora* culture supernatants rapidly decreases in the stationary phase of the growth curve and the stationary-phase-decrease is due to non-enzymatic turnover of the signal. The non-enzymatic degradation of OHHL is shown to involve alkalization of growth medium. They discovered that the pH of the supernatant is increased as the growth phase progressed in cultures of LB medium from pH 7 to ~ 8.5. OHHL becomes unstable over a narrow pH range (pH 7 - 8).

1.3.2.5. The quorum sensing signal receptor protein (LuxR homologues)

Many of the molecular genetic analyses of LuxR have been performed in an *E. coli* background. Such studies on the structure and function of the protein have offered an insight into the mechanisms involved in the activation of quorum sensing system by LuxR and its homologues. LuxR is a 250-amino acid polypeptide that requires the presence of the GroESL molecular chaperones to enable folding into an active form (Devine *et al*, 1988; Adar *et al.*, 1992; Adar and Ulitzur, 1993; Dolan and Greenberg,

1992). Indeed, it has been proposed that, in the absence of these protein chaperones, LuxR may become inactive due to its instability (Adar and Ulitzur, 1993). Amino acid sequence alignments of LuxR with its homologues reveal them to be surprisingly disparate in terms of sequence identity (18-25%). Only five residues are completely conserved in all of the LuxR homologues.

Evidence suggests that LuxR is associated with the inner layer of the cytoplasmic membrane through its N-terminal domain (Kolibachuk and Greenberg, 1993). LuxR proteins can activate the target gene expression while binding with their cognate AHL molecules. Based on an extensive analysis of the LuxR family, it has been proposed that LuxR consist of 2 domains, a C-terminal DNA binding domain, and a N-terminal acyl-HSL-binding domain (Stevens and Greenberg, 1998). Within the C-terminal domain there is a helix-turn-helix motif. For LuxR this motif has been shown to make contact with the regulatory DNA in the promoter of the luminescence genes (Stevens and Greenberg, 1997). The DNA-binding domains of LuxR-type proteins share sequence similarity with a much larger group of transcription factors (Henikoff *et al*., 1990; Kahn and Ditta, 1991). The specificity of gene activation will be determined by the DNA-binding domain. A widely accepted model by which the LuxR-type proteins are thought to activate gene expression is that the AHL signals binding to the N-terminal domain of the protein cause a conformational change, which leads to exposure of the DNA-binding domain at the C-terminus of the protein (DaRe *et al*., 1994). A recent variation on this mechanism is that the binding of AHL causes the protein to become capable of binding DNA (Choi and Greenberg, 1992; Zhu and Winans, 1998; 1999). While studying the TraR protein (LuxR homologue), Qin *et al*. (2000) concluded that binding to AHL signals drives dimerisation of TraR and prompts its release from membranes into the cytoplasm. Interestingly, a truncated LuxR molecule, lacking its amino-terminal region (and therefore designated LuxRΔN), has been shown to activate lux genes in an OHHL-independent manner. Although results from work using LuxRΔN should be assessed in the knowledge that this protein may not behave in the same way as its full-sized progenitor protein, this finding would suggest that the DNA binding domain of LuxR is contained within LuxRΔN (Choi and Greenberg,

1991). It also indicates that the amino-terminal region of LuxR plays a role in retaining LuxR in an inactive conformation in the absence of OHHL. This is probably achieved via structural occlusion of the multimerisation and/or DNA binding domains of this protein (Choi and Greenberg, 1991; 1992). It is therefore likely that the interaction between OHHL and LuxR serves to free the amino-terminal region from performing such an inhibitory function and effectively activate the protein. To this end, point mutations capable of activating LuxR in the absence of OHHL have been mapped to locations throughout the whole protein (Poellinger *et al.*, 1995; Sitnikov *et al.*, 1996). This suggests that important amino acid interactions exist within the individual proteins that are responsible for the maintenance of LuxR and its homologues in an inactive form. Studies on TraR and CarR have both offered evidence that, when these LuxR homologues bind their appropriate ligand, a conformational change in the proteins ensues (Qin *et al.*, 2000; Welch *et al.*, 2000).

The X-ray structure of TraR (from the plant pathogen *Agrobacterium tumefaciens*) bound to the cognate AHL has recently been solved. Surprisingly, the autoinducer is fully embedded within the protein with virtually no solvent contact (Zhang *et al.*, 2002b). This structure is consistent with earlier findings that folding of TraR requires autoinducer binding (Zhu and Winans, 2001).

From the evidence described above, a widely accepted picture of the processes involved in the activation of TraR and LuxR emerges. The respective protein (TraR or LuxR) firstly interacts with its cognate acyl HSL and this promotes a conformational change that allows the protein to dimerise. Such dimers are then free to bind to the relevant DNA promoter sequences and enact their transcriptional regulatory processes in conjunction with RNA polymerase. However, studies performed on other LuxR homologues indicate that this pathway is not conserved in all members of this group of proteins. Indeed, CarR and ExpR (a LuxR homologue in *Erwinia chrysanthemi*) are both capable of binding to promoter sequences of target genes in the absence of acyl HSL (Welch *et al.*, 2000; Nasser *et al.*, 1998; Reverchon *et al.*, 1998). Thus, as first proposed by Qin *et al.*, there may be at least two subgroups within the LuxR family of

proteins — those that bind DNA in the absence of acyl-HSLs and those that require the presence of these ligands to enable their DNA binding activity (Qin *et al*., 2000).

1.3.2.6. Lux box - LuxR binding region

The DNA regions to which LuxR binds are known as lux boxes. In *V. fischeri*, the *lux* box is a 20-bp DNA element of dyad symmetry and located around 40 bp upstream of LuxR-regulated genes. Lux-box-type sequences have been identified upstream of quorum sensing regulated genes in many other organisms, although it is not the case that all quorum sensing regulated genes are preceded by lux box-type sequences. DNA band shift assays and DNase I footprinting experiments showed that, *in vitro*, ligand-activated TraR dimers bind specifically to a *lux* box-type sequence, known as a *tra* box (Zhu and Winans, 1999; Qin *et al*, 2000; Zhu and Winans; 2001). Luo and Farrand (1999) created a lacZ reporter element and demonstrated that, as was shown more recently with LuxR, TraR could function as a repressor of β-galactosidase activity. Such activity was dependent upon the helix-turn-helix domain at the C-terminus of TraR. Together, these studies show that disruption of the tra box sequence abolishes TraR binding activity. Similarly, they also show that DNA binding activity of TraR is dependent upon the prior activation of the protein by its cognate acyl HSL (Zhu and Winans, 1999; Qin *et al*., 2000; Zhu and Winans, 2001; Luo and Farrand, 1999). While a minimal *lux* box has not been defined, a consensus *lux*-box-like sequence has been determined from an alignment of the *lux* box with sequences upstream of promoters regulated by LuxR homologs (Fuqua *et al*., 1994; Gray *et al*., 1994) (Figure 1.6).

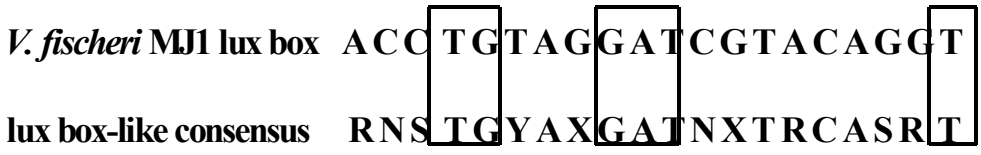



**Figure 1.6: A consensus *lux*-box-like sequence**

The nucleotide sequence of the *V. fischeri* MJ1 lux box and a consensus sequence for *lux*-box-like elements found in promoter regions of genes regulated by LuxR homologs. Consensus sequence abbreviations: N=A, T, C or G; S=C or G; Y=T or A; X=N or a gap in the sequence (Greenberg, 1997)

### 1.3.3. Detection of AHL

#### 1.3.3.1. Biological detection

The following two facts prompted Bainton *et al.* (1992a) to construct a lux-based AHL biosensor (termed pSB237) to screen a range of bacteria for their abilities to produce AHL. Firstly, many Gram-negative bacteria regulate gene expression in response to their population size by sensing the level of the autoinducer molecules (AIs). In most known cases these quorum sensing AIs are acyl-homoserine lactones. Secondly, although a given *luxR* homology protein is activated most efficiently by its cognate autoinducer, analogs differing in chain length and the nature of the substitution at carbon 3 can show activity, albeit at much higher concentrations (Eberhard *et al*., 1986; Schaefer *et al*., 1996b; Passador *et al*., 1996). This biosensor contains *luxR* and the *lux* promoter regions from the *V. fischeri* regulon, fused to the *luxAB* genes from *V. harveyi* but without *luxI* activity. When this recombinant plasmid is introduced into *E.coli*, no light is emitted unless the organism is supplied with exogenous OHHL or a structurally related analogue. By exposing the biosensor to cell-free stationary phase culture supernatants, high levels of light output are observed with many different Gram-negative bacteria that produce AHL (Bainton *et al*., 1992a; Williams *et al*., 1992).

A number of AHL biosensors, dependent on either lux or lacZ reporter fusions have been constructed in several laboratories. These plasmid-based biosensors carry:
*luxRI*⁻:: *luxAB* (Swift *et al*., 1993);
*luxRI*⁻:: *luxCDABE* (Eberl *et al*, 1996; Swift *et al*. 1997; Winson *et al*. 1998a);
*rhlRI*⁻:: *luxCDABE* (Winson *et al*, 1995;1998b);
*ahyRI*⁻:: *lux CDABE* (Swift *et al*., 1997);
*lasRI*⁻:: *luxCDABE* (Winston *et al*., 1998a);
*luxRI*⁻:: *lacZ* (Dunlap and Kuo, 1992)
*traG*⁻:: lacZ/traR (Piper *et al.,* 1993);
*rhlA*⁻::*lacZ* (Pesci *et al.* , 1997);
*lasB*⁻:: *lacZ* (Gray *et al*., 1994).

Strains harbouring these reporter plasmids will not produce their own N-acyl-L-homoserine lactone signal molecules. However, the transcription of the reporter fusion will be activated in response to an exogenous source of active autoinducer.

A *Chromobacterium violaceum* based biosensor was described by McClean *et al*. (1997). *C. violaceum* characteristically produces violacein, a water insoluble purple pigment. When subjected to transposon mutagenesis, a white mutant is obtained which no longer produces the HHL (Figure1.2) (Throup *et al*., 1995b; McClean *et al*., 1997). When exposed to exogenous AHL, this white *C. violaceum* mutant turns purple and has proved to be a very versatile, simple-to-use AHL biosensor (Latifi *et al*., 1995; McClean *et al*., 1997; Milton *et al*., 1997; Swift *et al*., 1997). It is extremely sensitive to the short- and medium-chain length acyl-HSL, but lacks sensitivity to acyl-HSL with longer chains and most of the 3-oxo-derivatives. The strain cannot detect any of the 3-hydroxy-derivatives (Cha *et al*., 1998).

Cha *et al*. (1998) evaluated four AHL-responsive biosensors that were based on tra of *A. tumefaciens*, lux of *V. fischeri*, las of *P. aeruginosa* and pigment production of *C. violaceum*, and examined their abilities to detect sets of acyl-HSLs with chain lengths ranging from $C_4$ to $C_{12}$. The *traG*:: *lacZ* fusion reporter from the *A. tumefaciens* Ti plasmid was found to be the most sensitive and versatile detector of the four. It could detect 3-oxo-substituted HSL derivatives with acyl chain lengths from 4-12 carbons as well as 3-hydroxy derivatives with acyl chain lengths of 6, 8 and 10 carbons. This biosensor could also detect signal molecules with acyl chain lengths longer than 12. However, the *A. tumefaciens* reporter responded less well to most of the 3-unsubstituted acyl-HSLs, and it did not respond at all to N-butanoyl-L-HSL.

Recently, a few more AHL biosensors based on different reporters have been reported. Thomson *et al*. (2000) described a *Serratia* sp. ATCC39006 mutant that could not produce prodigiosin, a red pigment, and restored the prodigiosin production by applying exogenous AHL. This prodigiosin-based bioassay can thus be used for detection of some N-acyl-homoserine lactones.

In order to perform single-cell analysis and online studies of N-acyl homoserine lactone mediated communication among bacteria, Andersen *et al.* (2001) constructed a new biosensor by fusing components of the *V. fischeri* quorum sensor with the modified versions of *gfpmut*3 genes encoding unstable green fluorescent proteins. Bacterial strains harbouring this green fluorescent sensor can detect a broad spectrum of AHL molecules and are capable of sensing the presence of 5nM N-3-oxohexanoyl-L-homoserine lactone in the surroundings. In combination with epi-fluorescent microscopy, the sensitivity of the sensor enables AHL detection at the single cell level and allows for real-time measurements of fluctuations in AHL concentrations (Andersen *et al*., 2001).

All these biosensors have greatly facilitated the screening of microorganisms for AHL and accelerated the identification of the *luxI* family of AHL synthases (Swift *et al*., 1993; Pearson *et al*., 1994; 1995; Hwang *et al*., 1994; Shaw *et al*., 1997).

Because of the specific requirements of the *luxR* homologous protein, most of the detection systems are limited in the range of the acyl-HSLs to which they respond. Thus the assay must be used with caution. First, detection is limited to those acyl-HSLs to which the biosensor will respond. In this regard, a thorough screening for production of these compounds can require the use of several reporter systems, each responding to signal molecules with different structural features. Second, the signal must be present at levels detectable by the reporter. The R proteins are highly specific for their cognate acyl-HSL signals, but will only respond to analogs if present at sufficiently high concentration. Third, it cannot be concluded from the absence of a signal that the tested bacterium does not produce one or more acyl-HSLs. Such organisms may produce signals that are not detectable by the reporter, or they may produce AHL molecules at levels below the threshold of the reporter.

1.3.3.2. AHL detection by chemical method

The traditional bioassay methods of employing heterologous reporter constructs have certain limitations. They are labour intensive, require construction of an appropriate indicator strain, and are biased towards detection of acyl-HSLs close enough in structure to the natural signal to be recognised by specific LuxR homologs. Schaefer *et al.* (2001) developed a radio tracer assay method to detect AHLs and overcame these limitations. The radio tracer assay depends upon incorporation of radio label from carboxyl-[$_{14}$C]-methionine into acyl-HSLs. The labeled AHLs are separated and quantified by HPLC and the radioactivities are monitored with a liquid scintillation at the same time.

### 1.3.4. Quorum sensing is widespread among gram-negative bacteria

In recent years it has become clear that the AHL mediated cell-density-dependant quorum sensing system is widespread in Gram-negative bacteria. As indicated earlier, the use of a biosensor to screen spent culture supernatants has led to the discovery that AHLs are produced by a plethora of unrelated bacteria. These AHL autoinducers control a variety of physiological processes, including bioluminescence, swarming, swimming and twitching motility, antibiotic biosynthesis, biofilm differentiation, sporulation, alternative sigma factor synthesis, plasmid conjugal transfer and the production of virulence determinants (Table1.1).

Interestingly, no gram-positive bacteria have so far been shown to produce AHL. Pathogenic gram-negative bacteria, which so far have been reported as AHL-negative, include *Actinobacillus pleuropneumonial, Haemophilus influenzae, Klebsiella pheumonial, Neiseria meningitidis, Salmonella spp., Vibrio cholerae, Campylobacter jejuni* and *Helicobacter pylori* (Swift *et al*., 1999c).

**Table 1.1. Summary of AHL based quorum sensing in gram-negative bacteria**

| Organism | Major signal molecule | Regulatory Proteins | Phenotype Control | Reference |
|---|---|---|---|---|
| *Aeromonas salmonicida* | C4-HSL | AsaI/AsaR | Extracellular protease | Swift et al., 1997 |
| *Aeromonas hydrophila* | C4-HSL | AhyI/AhyR | Exoprotease production | Swift et al., 1997 |
| *Agrobacterium tumefaciens* | 3-oxo-C8-HSL | TraI/TraR | Ti plasmid conjugation | Zhang et al., 1993; Hwang et al., 1995; Piper et al., 1993 |
| *Burkholderia cepacia* | C8-HSL | CepI/CepR | Protease, siderophores | Lewenza et al., 1999. Aguilar et al., 2003 |
| *Burkholderia vietnamiensis* | C10-HSL | BviI/BviR | ? | Conway and Greenberg, 2002. |
| *Chromobacterium violaceum* | C6-HSL | CviI/CviR | Exoenxymes, antibiotics, cyanide, violacein | McClean et al., 1997 |
| *Enterobacter agglomerans* | 3-oxo-C6-HSL | EagI/EagR | ? | Swift et al., 1993 |
| *Erwinia carotovora subsp. carotovora* | 3-oxo-C6-HSL | ExpI/ExpR<br>CarI/CarR | Exoenzymes<br>Carbapenem antibiotics | Bainton et al., 1992; Jones et al., 1993; McGowan et al., 1995; Pirhonen et al., 1993 |
| *Erwinia chrysanthemi* | 3-oxo-C6-HSL<br>C6-HSL | ExpI/ExpR | Pectate lyases | Nasser et al., 1998; Reverchon et al., 1998. |
| *Erwinia stewartii* | 3-oxo-C6-HSL | EsaI/EsaR | Exopolysaccharide, virulence factors | Beck von Bodman & Farrand, 1995 |
| *Escherichia coli* | ? | ?/SdiA | Cell division, attachment and effacing lesion formation | Sharma et al., 1986; Sperandio et al., 1999; Withers & Nordstrom, 1998 |
| *Nitrosomonas europea* | 3-oxo-C6-HSL | ? | Emergence from lag phase | Cooper et al., 1995 |
| *Paracoccus denitrificans* | C16 - HSL | ? | ? | Schaefer et al., 2002 |
| *Pseudomonas aeruginosa* | 3-oxo-C12-HSL | LasI/LasR | Multiple extracellular enzymes, RhlR, Xcp, biofilm formation | Davies et al.,1998; Gambello et al., 1993; Pearson et al., 1994,1995 |
| | C4-HSL | RhlI/RhlR | RpoS, Multiple extracellular enzymes, rhamnolipid, secondary metabolites | Latifi et al., 1995; Ochsner et al., 94, 95; Winson et al., 1995 |
| *Pseudomonas aureofaciens* | C6-HSL | PhzI/PhzRCsaI/CsaR | Phenazine antibiotics<br>Cell surface components | Pierson et al., 1994; Zhang & Pierson, 2001; Pierson & Thomashow et al., 1992. |

| | | | | |
|---|---|---|---|---|
| *Pseudomonas fluorescens* | C6,C10,$C_{14:1}$-HSL | MupI/MupR | Mupirocin antibiotic | EI-Sayed et al., 2001 |
| | | HdtS/? | ? | Laue et al., 2000 |
| | | PhzI/PhzR | Phenazine antibiotic | Gene Bank accession : L48616 |
| *Pseudomonas putida* | OHHL | PpuI/PpuR | Influences biofilm structural development | Elasri et al.,2001 |
| | OOHL, ODHL | | | Steidle et al., 2002 |
| *Pseudomonas syringae* | HHL OHHL | PsyI/PsyR | ? | Elasri et al., 2001 |
| *Ralstonia solanacearum* | C8-HSL | SolI/SolR | ? | Flavier et al., 1997 |
| *Rhizobium etli* | ? | RaiI/RaiR | Restriction of number of nitrogen-fixing nodules | Rosemeyeer et al., 1998. |
| *Rhizobium leguminosarum* | C6-HSL; C8-HSL | RhiI/RhiR | RhiABC rhizosphere-expressed genes, nodulation | Cubo et al., 1992; Gray et al., 1996; Rodelas et al., 1999. |
| | 3-hydroxy-7-cis-C14-HSL | CinI/CinR | Part of quorum sensing network, control rhiI/rhiR expression. | Wisniewski-Dye et al., 2002. |
| | 3- O-C 8 – HSL | Rai I/Rai R | Part of quorum sensing network, | Rodelas et al., 1999 |
| | 3 –OH-C8-HSL | | function unknown | Lithgow et al., 2000 |
| *Rhodobacter capsulatus* | C14 – HSL C16 - HSL | GtaI/? | Regulate gene transfer agent production | Schaefer et al., 2002 |
| *Rhodobacter sphaeroides* | 7-cis-C14-HSL | CerI/CerR | Dispersal from bacterial aggregates | Puskas et al., 1997 |
| *Salmonella typhimurium* | ? | ?/ Sdi A | Rck (resistence to competence killing) | Ahmer et al., 1998 |
| *Serratia liquifaciens* | C4-HSL C6-HSL | SwrI/SwrR | Extracellular protease, swarming. lipase | Eberl et al., 1996; Givskov et al., 1998; Riedel et al., 2001 |
| *Sinorhizobium meliloti* | Different strain produce different AHLs | TraR/TraM SinI/SinR | Plasmid transfer EPS synthesis | Marketon and Gonzalez, 2002 Marketon et al., 2003 |
| *Vibrio anguillarum* | 3-oxo-C10-HSL | VanI/VanR | ? | Milton et al., 1997 |
| *Vibrio fischeri* | 3-oxo-C6-HSL | LuxI/LuxR | Bioluminescence | Eberhard et al.,1981 |
| | C8-HSL | AinS/AinR[c] | Bioluminescence | Gilson et al.,1995; Kuo et al.,1996 |
| *Vibrio harveyi* | 3-hydroxy-C4-HSL | LuxLM/LuxN | Bioluminescence | Cao & Meighen 1989; Showalter et al., 1990 |
| | ? | Lux?/LuxPQ | | |
| *Xenorhabdus nematophilus* | HBHL | ? | Virulence factor | Dunphy et al., 1997 |
| *Yersinia enterocholitica* | C6-HSL | YenI/YenR | ? | Throup et al., 1995a; |
| *Yersinia pseudotuberculosis* | C8-HSL OHHL | YesI/YesR YtbI/YtbR | ?; Regulate bacterial aggregation and motility. | Atkinson et al., 1999 |

Table 1 (continued) : Summary of AHL based quorum sensing in gram-negative bacteria

### 1.3.5. Of what advantage to pathogenic bacteria is quorum sensing?

Quorum sensing is a result of co-evolution between plants and bacteria. For the pathogenic bacteria, the ability to successfully invade specific hosts depends on the evolution of unique gene systems – virulence or pathogenicity genes. These genes are frequently activated in the presence of the host, and the products of these activated genes alter the plant host in some way to produce disease (Dunny and Winans, 1997). It is rapidly becoming clear that AHL-mediated quorum sensing plays an important role in the bacterial virulence gene expression (Beck von Bodman and Farrand, 1995; Tang *et al*., 1996). A large number of pathogenic bacteria control the expression of many of their extracellular virulence factors by quorum sensing systems. Examples of these are *E. carotovora,* which regulates its pectate lyase, pectin lyase, polygalacturonase, cellulase and protease production through quorum sensing (Jones *et al*., 1993; Kohler *et al*., 1997); *P. stewartii* subsp. *stewartii*, which regulates its expression of major virulence factor polysaccharide capsule through quorum sensing (Beck Von Bodman and Farrand, 1995); *Ralstonia solanacearum*, which regulates its exopolysaccharide fraction production through quorum sensing (Flavier *et al*., 1997); *P. aeruginosa*, which regulates its elastase, alkaline protease, exotoxin A through quorum sensing; *Agrobacterium tumefaciens*, which regulate the conjugal transfer of the Ti plasmid through quorum sensing (Piper *et al*., 1993; Zhang and Kerr, 1991); *Shigella flexneri*, which regulates its expression of ipa, mxi, and spa invasion operons by quorum sensing (Bahrani *et al*., 1997; William *et al*., 2001).

What are the particular advantages of possessing a population dependent regulatory system for pathogenicity and virulence genes in bacterial pathogens? A major objective of an invading bacterium is to overwhelm its host's defenses. Therefore, the premature expression of bacterial virulence factors, which could possibly alert the plant host and elicit a subsequent defensive response, would not be a sensible strategy for such pathogens. Quorum sensing provides an effective timing-control mechanism in virulence factor expression and allows the pathogens to amass, without being sensed by the host, until a big enough population is achieved to overwhelm the plant host and

make the aggressive phenotype effective. In some bacteria, as well as in *V. fischeri*, the quorum sensing system forms a positive feedback circuit, where the AHL synthase is part of the quorum sensing induced regulon. This will allow for rapid signal amplification and thus an accelerated induction of the aggressive phenotype to full potential (Engebrecht *et al*., 1983; Hwang *et al*., 1994; Chan *et al*., 1995; Seed *et al*., 1995; Winson *et al*., 1995; Swift *et al*., 1997; Cui *et al*., 2004).

Another potential advantage for employing such a density-sensing system is to ensure the bacteria an economic life style. Usually, a mass of cells is required to produce sufficient quantities of extracellular factors to influence the surrounding environment. It would be a waste of resources if bacteria start to produce these factors before a critical population has been reached (Greenberg, 2000).

In addition to intercellular signalling to ensure the presence of an effective number of cells, it is not clear what range of other regulatory mechanisms interfere with quorum sensing to ensure that a suitable niche is occupied before the individual bacterium activates its virulence determinants. Recently, a quorum sensing control repressor (QscR) has been identified in *P. aeruginosa*. QscR, a homolog of LasR and RhlR, is found to function as a negative regulator of quorum-sensing-controlled (qsc) genes and thus ensure that qsc genes are not prematurely activated in an unsuitable environment (Chugani *et al*, 2001).

In plant pathogens such as *Erwinia carotovora*, the direct relationship between virulence and AHL production underlines the crucial role of quorum sensing in bacterial pathogenicity (Jones *et al*., 1993; Pirhonen *et al*., 1993). AHL deficient mutants of *E. carotovora* are avirulent. When the mutant bacteria are pre-incubated with their species-specific AHL and inoculated onto plant leaves, the virulence is at least partially restored. Further tests show that AHL is required not only for *E. carotovora* to initiate the infection, but also for the infection to persist (Pirhonen *et al*., 1993). Using AHL to mediate virulence factor production makes considerable sense for this organism in its natural environment during plant infection. Small amounts of *E. carotovora* cells could

never produce sufficient tissue-degrading enzymes to overwhelm the plant host. Once invasion has been successful, the suddenly released nutrients from the plant could result in competition from other soil and plant-borne bacteria. To minimize this potential disadvantage, *E. carotovora* has adopted a strategy of coordinating the release of a broad-spectrum antibiotic (carbapenem) along with plant-degradating enzymes (Swift *et al*., 1999a). The package is apparently evolutionarily successful as the formula appears to be repeated by *P. aeruginosa* and *C. violaceum*, both of which coordinate antibiotic and cyanide production via quorum sensing (Flaishman *et al*., 1996; Schippers *et al*., 1990; Winson *et al*., 1994; Latifi *et al*., 1995; Taylor, 1997).

### 1.3.6. Quorum sensing - a novel target for anti-microbial therapy

The discovery that a wide spectrum of bacteria use quorum sensing to control virulence factor production makes it an attractive target for antimicrobial therapy (Finch *et al*., 1998). Mechanisms by which quorum sensing may be blocked – and disease control achieved – are discussed below.

#### 1.3.6.1.Using AHL analogs as antagonists

In most of bacterial quorum sensing systems, the AHLs and their cognate R receptor proteins (LuxR homologues) have a high affinity for one another. Noncognate signals-R protein complexes might have the ability to activate the quorum sensing-related genes weakly. However, more often, noncognate AHLs act as antagonists, bind to but do not activate R proteins (Pearson *et al*., 1995; Eberhard *et al*., 1986; Hanzelka and Greenberg, 1995; Zhu *et al*., 1998). The potential possibilities of using AHL analogs to attenuate quorum sensing regulation systems have been explored in *Vibrio fischeri, Aeromonas hydrophila, Aeromonas salmonicida, A. tumefaciens, E. carotovora* and *Chromobacterium violaceum* (Schaefer *et al*., 1996b; Eberhard *et al*., 1986; Hanzelka and Greenberg, 1995; Zhu *et al*., 1998; Chhabra *et al*., 1993; Swift *et al*., 1997; 1999b). For example, in *A. hydrophila,* the $C_4$-HSL-dependent exoprotease production can be

virtually abolished by the addition of AHL with acyl side chains of 10, 12 or 14 carbons (Swift *et al.,* 1999b).

Recently, Teplitski *et al* (2000) tested pea and other plant species for the production of potential AHL mimicking substances using several bacterial reporter strains carefully developed to detect exogenous AHLs. When a lawn of reporter *Chromobacterium violaceum* CV026 cells is exposed to aseptically grown pea seedlings, the plants do not stimulate violacein synthesis. However, when the lawn of CV026 cells is induced to synthesize violacein by the addition of $C_4$-HSL to the agar, the pea seedlings secrete substances which substantially inhibit violacein synthesis. In subsequent tests to confirm this inhibitory activity, the methanol-soluble compounds extracted from lyophilized pea seedling exudates were found to have parallel, dose-dependent inhibitory effects on AHL-regulated synthesis of exoprotease, exochitinase and violacein (Teplitski *et al*., 2000). In contrast to the *Chromobacterium* CV026 reporter, pea seedlings and exudates stimulated AHL-dependent surface swarming of the *Serratia liquefaciens* AHL deficient mutant MG44. These results suggest that pea secretes one or more AHL analogs that are capable of interfering with bacterial quorum sensing (Teplitski *et al*. 2000). Teplitski *et al.* (2000) have also tested various plant species for their AHL-mimic production and most of the plants tested, including rice, soybean, tomato, pea and crown vetch, stimulate lux-based AHL reporters to produce luminescence.

1.3.6.2. Furanone compounds

Halogenated furanone compounds (Figure 1.7) produced by the Australian marine macro-alga *Delisea pulchra* have been found to be capable of inhibiting AHL-regulated processes, including swarming in *Serratia liquefaciens,* virulence of *Vibrio harveyi*, bioluminescence in *V. fischeri* and antibiotic and exoenzyme production in *E. carotovora* (Givskov *et al*., 1996; Kjelleberg *et al*., 1997; Srinivasan *et al*., 1998; Rasmussen *et al*., 2000; Manefield *et al*, 2000; 2001). The alga uses quorum sensing inhibition to prevent its bacterial over-colonisation. As the inhibitory effect can be

overcome by the addition of an excess of the cognate AHL, the furanones appear to be acting as competitive antagonists. But recently, work by Manefield and colleagues' (Manefield *et al.*, 2002) reveals that halogenated furanones modulate LuxR activity through accelerating LuxR turnover, rather than protecting the AHL-dependent transcriptional activator. They suggest that the reduction in LuxR concentration is the mechanism by which furanones control expression of AHL-dependent phenotypes.

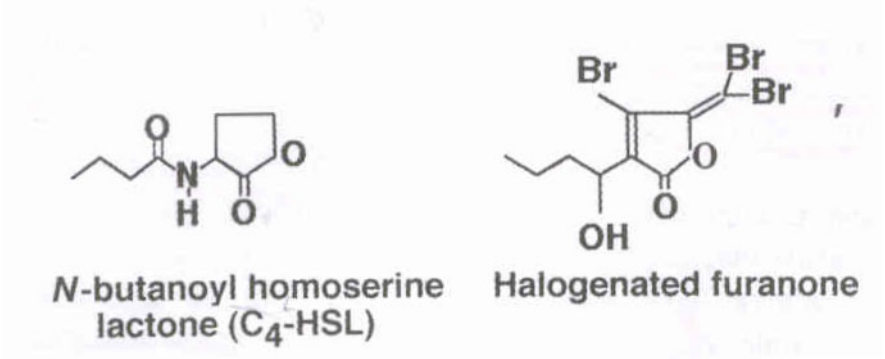



**Figure 1.7: Structural comparison of AHL and halogenated furanone (adapted from Wolfgang and Max, 2001)**

1.3.6.3. Quorum sensing signal degradation

Many acyl-HSLs are quite stable in mildly acidic or neutral pH environments. However, there is no evidence that they accumulate in such environments. If they accumulated over long periods of time, their function as quorum sensing signals would be disarmed and signal concentration would not reflect cell number after fluctuations in population density. What is more, as AHL-producing bacteria are ubiquitously present in natural environments, it can be expected that other organisms have evolved means to interfere with this type of communication. The above facts imply that a possible natural acyl-HSL signal degradation pathway exists.

Dong *et al*. (2000) discovered an enzyme from *Bacillus* 240 B1 that is capable of degrading AHL. This enzyme is encoded by the *aii* gene and contains two domains. The enzyme inactivates AHL activity by hydrolysing the lactone bond of AHL and is

named AHL lactonase (Dong *et al*., 2001). Later studies discovered that the AHL-degrading enzyme encoding genes are widespread in many subspecies of *Bacillus.* Lee *et al*. (2002) studied the *aiiA* homologue genes in 16 subspecies of *B. thuringiensis* genome sequences. When compared with the *Bacillus* 240 B1 *aiiA* gene (Dong *et al*., 2000), these *aiiA* showed high homologies of 89% to 95% in the nucleotide sequences and 90% to 96% in the deduced amino acid sequences, respectively. These results indicated that insecticidal *B. thuringiensis* strains might have the potential to compete with gram-negative bacteria in natural ecosystems by autoinducer-degrading activity. Apart from these *B. thuringiensis* species, two closely related species *B. cereus* and *B. mycoides* were shown to produce AHL-inactivating enzymes, while *B. fusiformis* and *B. sphaericus* strains do not (Dong *et al*., 2002). Expression of *aiiA* in the transformed *E. carotovora* strain SCG, a pathogen that causes soft rot disease in many plants, significantly reduces release of AHL, decreases extracellular pectolytic enzyme activity, and attenuates pathogenicity in potato, egg-plant, Chinese cabbage, carrot, celery, cauliflower and tobacco (Dong *et al*., 2000). In a recent study, Dong *et al*. (2004) discovered that *B. thuringiensis*, when co-incubated with *E. carotovora*, abolished its accumulation of AHL signal and significantly decreased the symptom development of potato soft rot caused by this pathogen. Transgenic plants expressing AHL lactonase exhibit significantly enhanced resistance to *E. carotovora* infection and delay development of soft rot symptoms (Dong *et al*., 2001).

A motile, rod-shaped soil bacterium, *Variovorax paradoxus* has been shown to degrade and utilize N-(3-oxohexanoyl)-L-homoserine lactone as the sole source of energy and nitrogen (Leadbetter and Greenberg, 2000). Further tests showed that the *V. paradoxus* isolate is capable of growth on all of the acyl-HSLs tested. From the same enrichment culture that yielded *V. paradoxus*, Flagan *et al*. (2003) discovered that an *Arthrobacter* strain VAI-A, though not capable of degradation of AHLs, could utilize the nitrogenous degradation products of AHLs as sources of energy and nitrogen. They therefore hypothesized that the original co-enrichment of these two organisms from the same soil sample was not coincidental and that the consortia might play a role in quorum sensing turnover (Flagan *et al*., 2003).

Recently, Lin *et al*. (2003) cloned a gene, named *aiiD*, that is capable of encoding a novel and potent AHL acylase in a *Ralstonia* isolate. The gene product (AiiD) is a polypeptide of 794 amino acids. Sequence homology searches indicated that AHL acylase is conserved in many different bacterial species. HPLC and MS analysis demonstrated that AiiD hydrolyses the AHL amide, releasing homoserine lactone and the corresponding fatty acid (Lin *et al*., 2003). AiiD has been proved to be a potent enzyme, it abolishes the AHL-based cell-cell communication in *P. aeruginosa* and virulence in *Caenorhabditis elegans* (Lin *et al*., 2003).

Uroz *et al*. (2003) report the isolation of several bacteria that are capable of degrading AHLs, all with different specificity and kinetics. One of these isolates, *Rhodococcus erythropolis* W2, can interfere with the violacein production of *Chromobacterium violaceum* and the pathogenicity of *Agrobacterium tumefaciens* when co-cultured together. In plant, *R. erythropolis* significantly reduces the pathogenicity of *Pectobacterium carotovorum* subsp. c*arotovorum* (also known as *E. carotovora* subsp. *carotovora*) in potato tubers (Uroz *et al*., 2003).

1.3.6.4. Interrupting the quorum sensing signal biosynthetic pathway and global repressor genes

Two biosynthetic pathways related to AHL synthesis have been elucidated. One is the fatty acid biosynthesis pathway, by which the acyl side chain is synthesized. Another is the synthesis of homoserine lactone from S-adenosylmethionine (Figure 1.4) (Schaefer *et al*., 1996a; Hanzelka and Greenberg, 1996; Jiang *et al*., 1998; More *et al*., 1996). Interrupting the AHL biosynthetic pathway and shutting down AHL synthesis, perhaps through the use of analogs of AHL precursors or inhibition of the enzymes that are necessary for the synthesis, would be a highly effective means of blocking the quorum sensing cascade (de Kievit and Iglewski, 2000).

Enoyl-ACP reductase is an important enzyme in AHL biosynthesis. An enoyl-ACP reductase inhibitor, triclosan, has been reported that is capable of reducing AHL production *in vitro*. (Hoang and Schweizer, 1999).

The recent significant advances in defining the enzymatic activities and substrate requirements of *luxI* homologues emphasise the potential of using the AHL synthase as an antimicrobial target (More *et al*., 1996; Schaefer *et al*., 1996a; Jiang *et al*., 1998; Parsek *et al*., 1999). So far, several global repressor genes have been found to reduce the levels of transcripts of *luxI* homologues. Branny *et al.* (2001) have isolated a multicopy suppressor gene *dksA* from *P. aeruginosa*, which is a homologue to the *E. coli dnaK*. Over-production of this *P. aeruginosa dksA* gene inhibits quorum sensing dependent virulence factor production by down-regulating the transcription of the autoinducer synthase gene *rhlI*. Another global repressor gene *qscR* has recently been described as a modulator of quorum sensing signal synthesis and virulence in *P. aeruginosa* (Chugani *et al*., 2001). The *qscR* gene product governs the timing of quorum sensing controlled gene expression. Its primary role is to repress the *lasI* function. The repression of *lasI* by QscR could serve to ensure that quorum-sensing-controlled genes are not activated in environments where they are not useful. A *qscR* mutant produces the *lasI*-generated signal prematurely, and this results in premature transcription of a number of quorum sensing-regulated genes.

In additional to *qscR* and *dksA*, other global repressor genes such as *rsaL*, which is located downstream from *lasR* in *P. aeruginosa* (de Kievit *et al*., 1999) and rsmA, which is identified in *E. carotovora* subsp. *carotovora* $T_1$ mutant (Cui *et al*., 1995), have also been reported. Overproduction of these AHL synthase repressor genes in plant pathogenic bacteria could be an attractive strategy to quench AHL production as a means of disease control.

1.3.6.5. Interference with the bacterial membrane multi-drug efflux pump

Originally described in bacteria, efflux pumps (drug transporters) are now recognised as common membrane components in all cell types, from prokaroytes to superior eukaryotes (van Bambeke *et al*., 2003). It confers bacteria a common and basic mechanism of resistance by extruding antibiotic or other toxic molecules from the cell (McMurry *et al*., 1980). In an investigation of whether AHL can diffuse freely in and out of *P. aeruginosa* cells, it was discovered that, in addition to its slow diffusion, 3-oxo-$C_{12}$-homoserine lactone is actively pumped from cells by the MexAB-OprM pump (Pearson *et al*., 1999). This finding is intriguing because it suggests that antimicrobial therapy designed to interfere with bacterial membrane pump and increase the antibiotic susceptibility of pathogenic bacteria will also affect this bacterial quorum sensing-controlled gene expression and thus become more effective (de Kievit and Iglewski, 2000).

1.3.6.6. Macrolides inhibit quorum sensing

Macrolides, such as azithromycin, are a family of antibiotics used to treat a wide range of bacterial infections. The mechanism by which macrolides affect bacterial virulence factor production has been demonstrated by Tateda *et al.* (2001). They reported that azithromycin (2µg/ml) inhibits the quorum sensing circuitry of *P. aeruginosa* strain PAO1. Addition of synthetic autoinducer partially restored the expression of the transcriptional activator-encoding gene lasR and rhlR, but not that of the AHL synthase-encoding gene *lasI*. They thus proposed that azithromycin interferes with the synthesis of quorum sensing signals leading to a reduction of virulence factor production. The production of rhamnolipids, which is controlled by *rhlR*/*rhlI* quorum sensing system, progressively decreased with increasing azithromycin concentrations without a parallel drop in growth (Tateda *et al*., 2001). For inhibition of virulence factor production to occur, prolonged exposure to azithromycin is required (Tateda *et al*., 2001).

1.3.6.7. Manipulating plants to interfere with pathogenic bacterial quorum sensing

The fact that AHL signals isolated and purified from one bacterium can alter gene expression in a second bacterium *in vitro* (Greenberg *et al.,* 1979; McKenney *et al*., 1995) has raised the question whether a plant can be engineered to produce AHLs and whether these AHLs can alter bacterial gene expression. Recently, Fray and colleagues (1999) have taken a first step in engineering plants to interact with bacteria in this way. They constructed a vector that fused the *Yersinia enterocolitica* AHL synthase gene *yenI* to the *Petunia rbcS* ribulose biphosphate chloroplast-targeting sequence. This translational fusion was placed under the control of the 35S CaMV promoter and transformed into tobacco. AHL was isolated from homogenised transgenic plant tissue and the quantity produced was sufficient to induce target gene expression in several recombinant bacterial biosensors and to restore the biocontrol activity of an AHL-deficient *P. aureofaciens* strain. Because tobacco is not a normal host for *E. carotovora*, they created transgenic *yenI*-expressing potato lines in order to test whether small inocula of wild type *E. carotovora* could be induced to attempt a pathogenic attack prematurely on AHL-producing potato plants, and whether such an infection would result in increased resistance. Surprisingly, the plants proved to be more susceptible than the untransformed control plants (refer to Fray, 2002 for possible explanations). Mae *et al*. (2001) observed that the transgenic tobacco carrying the *expI* gene could, on the one hand, partially complement the avirulent phenotype of a *expI*-negative *E. carotovora* mutant and, on the other hand, exhibit enhanced resistance to infection caused by wild type *E. carotovora*. These contradictory results imply that the quorum sensing mechanisms in different phytopathogens might vary and the bacteria might have adapted different threshold settings for quorum sensing-related gene activation. Extra care should be taken into account for specific host-pathogen combinations when attempting to attenuate bacterial virulence by this approach (Zhang, 2003). Fray (2002) suggests that supplying transgenic plants with the ability to block or degrade AHL signals may provide an alternative, more direct approach for engineering resistance to *E. carotovora* rather than giving plants the ability to produce AHL themselves (and thus induce premature virulence expression). As described earlier,

Dong *et al*. (2001) transformed a copy of *aiiA* (AHL degrading) gene into tobacco and potato. The transgenic plants showed significantly enhanced resistance to *E. carotovora*.

### 1.3.7. Quorum sensing in *Pseudomonas fluorescens*

A quorum sensing system has been found very recently in *P. fluorescens* strain NCIMB 10586 (EI-Sayed *et al*., 2001). Two genes, *mupR* and *mupI*, have been cloned and sequenced and the gene products showed significant identity to LasR/LuxR and LasI/LuxI respectively. *mupR* encodes a predicted protein of 234 amino acids and molecular mass 26kDa, while mupI produces a 191 amino acid protein with a molecular mass of 21kDa. Both LasR and LuxR C-terminal domains contain a putative helix-turn-helix motif, which is thought to bind with the lux/las promoter target and activate the *lux/las* operon. This motif is also found in MupR. MupI produces a diffusible inducer and the MupI$^-$ phenotype can be overcome by the diffusible product from the wild-type strain. A MupR$^-$ mutant cannot produce the diffusible activator even if it contains a complete copy of *mupI*. Therefore, the *mupR* region is needed to allow *mupI* to generate the diffusible product. Further work has found that this diffusible product can light up the AHL biosensor pSB1075 (Winson *et al*., 1998a) but not CV026 (McClean *et al*., 1997). The *mupR/mupI* quorum sensing has been found to regulate the mupirocin biosynthetic gene cluster in *P. fluorescens* NCIMB 10586. Mupirocin is a polyketide antibiotic and usually used as topical treatment for staphylococcal infections (Rode *et al*., 1991).

Although the autoinducer molecule produced by *P. fluorescens* NCIMB 10586 has not been identified so far, several quorum sensing signals have been reported to be present in different *P. fluorescens* strains. Laue *et al*. (2000) found that *P. fluorescens* F113 produces at least three different AHLs. These are (1). N-(3-hydroxy-7-cis-tetradecenoyl) homoserine lactone (3 OH, $C_{14:1}$-HSL), a molecule previously known as the *Rhizobium leguminosarum* small bacteriocin that is exclusively associated with growth inhibition in *R. leguminosarum*; (2). N-decanoyl homoserine lactone ($C_{10}$-HSL)

and (3). N-hexanoyl homoserine lactone ($C_6$-HSL). $C_{10}$-HSL has not been previously found as a naturally occurring AHL. Furthermore, Laue *et al*. (2000) identified a gene in *P. fluorescens* F113, termed *hdtS*, capable of directing synthesis of all three AHLs. The HdtS, a 33 KDa protein, does not belong to either of the known AHL synthase families (LuxI or LuxM homologues) and is related to the lysophosphatidic acid acyltransferase family. They therefore concluded that HdtS is from a third protein family capable of AHL biosynthesis.

Interestingly, none of the three AHLs identified in *P. fluorescens* F113 have yet been identified in other *P. fluorescens* strains. Shaw *et al*. (1997) have reported up to 5 different AHL molecules in *P. fluorescens* 2-79 by TLC and MS analysis. Three of which were identified as N-(3-hydroxyhexanoyl)-L-HSL, N-(3-hydroxyoctanoyl)-L-HSL and N-(3-hydroxydecanoyl)-L-HSL, one could be N-octanoyl-L-HSL, another one was identified as being N-hexanoyl-HSL later by Cha *et al.* (1998). However, no AHL molecules are detected in the culture supernatants of either *P. fluorescens* 1855.344 (Cha *et al.*, 1998) or *P. fluorescens* pf 7-14 (Dumenyo *et al.,* 1998).

In *P. fluorescens* NCIMB 10586, instead of AHL molecules, Holden *et al*. (1999) identified a compound that could light up an N-acyl homoserine lactone biosensor. This compound is chemically characterized as cyclo(L-Phe-L-Pro), a diketopiperazine (DKP). Their studies imply the complexity of *P. fluorescens* quorum sensing systems and the existence of cross-talk among bacterial signalling systems in *P. fluorescens*.

Given the differences in AHL profiles of the *P. fluorescens* strains examined to date, it will clearly be interesting to determine whether there is any correlation between AHL profile and strain habitat.

Elasri *et al*. (2001) tested a total of 137 soil-borne and plant-associated *Pseudomonas* species for their abilities to synthesize AHL molecules. They found that AHL production appears to be more common among plant-associated than among soil-borne *Pseudomonas spp*.

### 1.3.8. Quorum sensing controlled genes

Quorum sensing represents a global gene regulation system in gram-negative bacteria. This raises the question about which genes in a bacterium of interest are regulated by AHL-mediated quorum sensing.

To begin a systematic investigation of global gene regulation by quorum sensing in *P. aeruginosa*, Whiteley *et al.* (1999) constructed a library of AHL-regulated genes by using saturation Tn*5* mutagenesis, with which random *lacZ* transcription fusions in the chromosome of a *lasI-rhlI* double mutant have been generated. Insertions in quorum sensing-regulated genes were identified by monitoring β-galactosidase expression in the presence and absence of 3-O-$C_{12}$-HSL and $C_4$-HSL. This is a powerful and quite general approach to the identification of genes affected by AHLs. They identified 39 quorum sensing controlled genes. However, their Tn*5* mutagenesis failed to detect a number of genes previously known to be regulated by AHLs. Among those quorum sensing controlled genes identified by Whiteley and colleagues (1999), some were regulated by additional factors other than AHL. Although they required acyl-HSL signals for activation, they were repressed until stationary phase even in the presence of acyl-HSL. These genes were termed late quorum sensing controlled genes (late qsc). Other genes, early qsc genes, showed activation upon addition of acyl-HSL signals even in early logarithmic phase. The promoters of several *P. aeruginosa* early qsc genes were analysed (Whiteley & Greenberg 2001). These promoters all possessed lux-box-like elements indicating that they are involved in qsc transcription. It seems possible that future screens using specific late-response qsc genes may lead to the identification of additional novel regulators of quorum sensing (Pearson 2002).

A second general approach in identifying AHLs and mimic-responsive genes in bacteria is 2-dimensional gel analysis of the proteins produced in the presence and absence of added AHLs. Givskov *et al*. (1998) used this method to identify 28 polypeptides that

increase or decrease in amount within 20 min of exposing *S. liquefaciens* MG44 to 2μM $C_4$-HSL.

Recently, the sequence of the genome of *P. aeruginosa* strain PAO1 was published (Stover *et al*., 2000), and one year later a commercially produced DNA microarray chip (Affymetrix Genechip) was made available to investigators. The DNA microarray chip, which represents an entire genome, is a very powerful experimental tool in quorum sensing analysis. Both Schuster *et al*. (2003) and Wagner *et al*. (2003) provided impressive assemblages of data from microarray experiments in which they examined the transcriptional responses of *P. aeruginosa* genes to the presence of AHL signalling components. Schuster *et al*. (2003) performed a transcriptional analysis to identify quorum sensing controlled genes in *P. aeruginosa*. They compared gene expression in a LasI-RhlI signal mutant grown with added signals to gene expression without added signals, and they compared a LasR-RhlR signal receptor mutant to its parent. In all, they identified 315 quorum-sensing-induced and 38 quorum-sensing-repressed genes, representing about 6% of the *P. aeruginosa* genome. Wagner *et al.* (2003) identified even more quorum-sensing-related genes in *P. aeruginosa* by using high-density oligonucleotide microarrays conservatively. 616 genes showed statistically significant differential expression ($p \leq 0.05$) in response to the exogenous autoinducers and were classified as quorum-sensing-regulated. A total of 244 genes were identified as being quorum-sensing-regulated at the mid-logarithmic phase, and 450 genes were identified as being quorum-sensing-regulated at the early stationary phase. 222 genes were identified as being quorum-sensing-repressed, thus representing a remarkably high level of discordance with the results from Schuster *et al*. (2003).

# Chapter 2. Quorum Sensing Control of Biosurfactant Production in *Pseudomonas fluorescens* 5064

## 2.1. Introduction

Since the term "quorum sensing" was introduced a decade ago (Fuqua *et al*., 1994), this fascinating area has attracted considerable attention and diverse cell-to-cell communication systems have been identified in numerous bacteria. The quorum sensing system of various bacteria has been proved to control a variety of physiological processes including bioluminescence, swarming, swimming, twitching motility, antibiotic synthesis, biofilm differentiation, sporulation, plasmid conjugal transfer and the production of virulence determinants (Table 1.1).

As addressed in the literature review, bacteria benefit greatly from employing quorum sensing to regulate their virulence gene expression. The primary objective of an invading bacterium is the evasion of its host defenses. Therefore, the premature expression of bacterial virulence factors could alert the host and elicit a defensive response and thus would be a poor strategy for such pathogens. Quorum sensing can be used, in this respect, for a concerted activation of a regulon coding for the components of an aggressive phenotype only when sufficient bacteria exist to make the phenotype effective (Greenberg, 2000; de Kievit and Iglewski, 2000; Swift *et al*., 1999c). A large number of pathogenic bacteria control the expression of many of their extracellular virulence factors by quorum sensing. Examples of these are *Erwinia carotovora,* which regulate its pectate lyase, pectin lyase, polygalacturonase, cellulase and proteases production through quorum sensing (Jones *et al*., 1993; Kohler *et al*., 1997); *Erwinia stewartii* subsp. *stewartii*, which regulates its major virulence factor, polysaccharide capsule expression, through quorum sensing (Beck Von Bodman and Farrand, 1995); *Ralstonia solanacearum*, which regulates its exopolysaccharide fraction production through quorum sensing (Flavier *et al*., 1997); *Pseudomonas aeruginosa*, which regulates its elastase, alkaline protease and exotoxin A through quorum sensing;

*Agrobacterium tumefaciens*, which regulates the conjugal transfer of the Ti plasmid through quorum sensing (Piper *et al*., 1993; Zhang and Kerr, 1991); *Shigella flexneri*, which regulates its expression of *ipa*, *mxi*, and *spa* invasion operons by quorum sensing (Bahrani *et al*., 1997; William *et al*., 2001).

The bacterium *Pseudomonas fluorescens* 5064 is an opportunistic soft rot pathogen of broccoli (*Brassica oleracea* var *italica*). It produces an extracellular lipopeptide biosurfactant, which has been suggested to play a role in disease incidence (Hildebrand, 1989). The synthesis of biosurfactants in *P. fluorescens* upon exhaustion of nitrogen and commencement of the stationary phase of growth has been observed by several investigators (Guerra-Santos *et al.*, 1986; Mulligan and Gibbs, 1989; Ramana and Karanth, 1989; Persson *et al.*, 1988). A previous study in our laboratory has also shown that *P. fluorescens* 5064, isolated from infected broccoli, will only commence biosurfactant production after a 30 h incubation period, once the culture has reached a late exponential growth phase (Darling, 1998). As quorum sensing-dependent-gene expression is always associated with high cell densities in the late exponential or stationary phase, we hypothesised that biosurfactant production in *P. fluorescens* 5064 might be controlled by quorum sensing. This chapter provides evidence to support this hypothesis.

## 2.2. Is biosurfactant production of *P. fluorescens* 5064 coupled with cell population density?

### 2.2.1.Aim

In a number of different *P. fluorescens* strains, biosurfactant production increases sharply when a big population density is reached (Guerra-Santos *et al*., 1986; Mulligan and Gibbs, 1989; Ramana and Karanth, 1989). Previous work in our laboratory (Darling, 1998) concluded that the biosurfactant expression in *P. fluorescens* 5064 starts at a late growth phase. However, Henriksen *et al*. (2000) found that, in *P. fluorescens* strain 96.578, biosurfactant production (viscosinamide) was not coupled with its growth phases. In this study, an experiment was designed to re-confirm that the biosurfactant production in *P. fluorescens* 5064 strain is induced only when the cultures have reached a late phase of growth.

### 2.2.2. Materials and methods

2.2.2.1. Medium and bacterial strain

KB medium: (see appendix A).
We chose to use KB medium in biosurfactant detection, because optimum viscosin production *in vitro* occurs on a medium containing glycerol as the carbon source for its synthesis (Georgiou *et al*., 1992).

Bacterial strain: The *P. fluorescens* 5064 strain used in this study was isolated in the Scottish Agricultural College from an infected broccoli collected in SE Scotland. It was grown at 26°C in KB medium with or without Ampicillin (50µg/ml).

2.2.2.2. Biosurfactant assay method — Water Droplet Test

A biosurfactant assay was carried out by using the Water Droplet Test (Hildebrand, 1989). After incubation for 30h, a loopful of bacteria from KB agar was transferred, using a sterile toothpick, to a 50 µl water droplet situated on a plastic petri dish. If biosurfactants were present, the surface tension of the water droplet would be immediately reduced giving a corresponding collapse or spreading of the water droplet.

2.2.2.3. Experimental procedure

*P. fluorescens* 5064 was grown on KB agar plates at 26°C. During the incubation, samples were taken every 3h (or as specified in table 2.1) to carry out the biosurfactant bioassay using the Water Droplet Test method. Three replicates were carried out for each test.

### 2.2.3. Results and discussion

For all the tested replicates (without exception), there were no detectable biosurfactant activities even after 27h incubation. However, biosurfactant activities appeared for the tests carried out upon 30h incubation (Table 2.1). The biosurfactant activity present was so strong that the testing water droplets collapsed immediately. A later experiment showed that the stationary phase of *P. fluorescens* 5064 commenced after about 30h incubation (Figure 2.4). The results provide sufficient evidence to prove that *P. fluorescens* 5064 will only start producing biosurfactant at a late phase of growth.

**Table 2.1 The biosurfactant production during different growth phases**

| Incubation time (h) | 5 h | 8 h | 11 h | 15 h | 18 h | 21 h | 24 h | 27 h | 30 h |
|---|---|---|---|---|---|---|---|---|---|
| Biosurfactant formation | No | No | No | No | No | No | No | No | Yes |

This experiment confirmed Darling's (1998) result that there was an over-production of biosurfactant in *P. fluorescens* 5064 at a late growth phase and predicted that the biosurfactant production in *P. fluorescens* was regulated by a quorum sensing mechanism.

## 2.3. Does *P. fluorescens* 5064 produce a quorum sensing signal (QSS)?

### 2.3.1. Aim

Several types of QSS have been reported from different *P. fluorescens* strains. Laue *et al.* (2000) found that *P. fluorescens* F113 produces at least three different AHLs. Shaw *et al.* (1997) reported up to 5 different AHLs in *P. fluorescens* 2-79 by Thin Layer Chromatography (TLC) and Mass Spectrometry (MS) analysis. However, no AHL molecules were detected in *P. fluorescens* 1855.344 (Cha *et al.*, 1998) and *P. fluorescens* pf 7-14 (Dumenyo *et al.*, 1998). To prove the existence of a quorum sensing system in *P. fluorescens* 5064, we need first to confirm that there is a QSS present in this strain.

The use of biosensor strains, which have been developed in several laboratories, made the AHL detection easier. Such biosensor strains do not express an AHL synthase, but do express an AHL-responsive transcriptional activator (LuxR homologue) and contain an AHL-activated promoter fused to a reporter gene(s) such as *lacZ* or *luxCDABE*. Since such a strain does not produce any AHL, expression of the reporter requires an exogenous source of AHL (McClean *et al.,* 1997; Swift *et al*., 1999c).

### 2.3.2.Materials and methods

2.3.2.1. Media:

**Luria-Bertani (LB) broth**: (see Appendix B).
**LB agar**: Agar (technical grade No.3, Oxoid Ltd., England) was added to LB broth before autoclaving to a concentration of 1.2 % (w/v).

**Semi-solid LB agar**: Agar was added to LB broth before autoclaving to a concentration of 0.5 % (w/v).

2.3.2.2. AHL-dependent biosensors

- Biosensor ***E. coli* (pSB401)**: *E. coli* JM109 carrying plasmid pSB401. pSB401 contains *luxR* from *Vibrio fischeri* and *luxCDABE* fused to *luxI* promoter (*luxR luxI*$^{p}$:: *lux CDABE*), but does not have *luxI* gene activity. It will only produce light in the presence of exogenous AHL. Plasmid pSB401 carries tetracycline resistance and is preferentially activated by AHL with 6-8 carbon side chains (Winson *et al*., 1995).
- Biosensor ***E. coli* (pSB1075)**: *E. coli* JM109 carrying plasmid pSB1075. pSB1075 contains *lasR* from *Pseudomonas aeruginosa* and *luxCDABE* genes fused to *lasI* promoter (*lasR lasI*$^{p}$:: *luxCDABE*), but does not have *lasI* gene activity. It is based on plasmid pUC18 and therefore contains ampicillin resistance. *E. coli* (pSB1075) responds most sensitively to AHL with long (10-14 carbon) side chains by emitting light (Winson *et al*., 1995).
- Biosensor ***Chromobacterium violaceum* CV026**: Biosensor CV026 is violacein-negative, double mini-Tn*5* mutant of *Chromobacterium violaceum* ATCC31532. It can be grown without antibiotics but is also kanamycin resistant. *C. violaceum* CV026 responds most sensitively to AHL with 4-6 carbon acyl side chains, irrespective of the substituent at the C-3 position of the acyl chain, by producing the purple pigment violacein (Latifi *et al*., 1995).

All the three biosensors were kindly provided by Prof. Paul Williams, University of Nottingham.

2.3.2.3. Acyl-HSL standards

- **HHL**: N-hexanoyl-homoserine lactone (Figure 1.2), the signal molecule that was first identified in a *P. fluorescens* strain (Cha *et al*., 1998). Commercially available HHL (Sigma Chem. Co.) was dissolved in dichloromethane (Fluka Chem. Co. HPLC grade) reaching a working concentration of 0.1 mM and stored at –20°C as a standard solution.
- **VAI**: VAI is N-(3-oxohexanoyl) homoserine lactone (commercial name is N-ketocaproyl-homoserine lactone, from Fluka Chem. Co.) and also commonly known as OHHL (Figure 1.2). It was first isolated from *V. fischeri*. VAI was suggested to be widely involved in bacterial signalling. VAI was dissolved in dd$H_2O$ reaching a working concentration of 0.1 mM and stored at –20°C as a standard solution.

2.3.2.4. Crude AHL extraction

Bacteria, *P. fluorescens* 5064 or its Tn*5* surf$^-$ mutants as described later, were grown in LB broth (1-3 litres as specified in different experiments) at 30°C with shaking to ensure adequate aeration. The bacteria were grown for about 50 – 55h (optical density of 2.1 at 500nm) to the late exponential or early stationary phase and then subjected to the following extraction.

- 1 litre of the *P. fluorescens* 5064 late-exponential-phase LB broth was centrifuged at 5000 rpm for 20 minutes to precipitate the cells.
- The supernatant was extracted twice with dichloromethane (1000:700/supernatant: solvent) and the organic and aqueous phases were mixed gently. The emulsified mixtures were extracted for more than 2h until the complete separation of the organic and aqueous phase.
- The combined dichloromethane organic phases were dried over approximately 5g of anhydrous magnesium sulphate in order to remove any aqueous residue.

- The solvent was filtered (Whatman filter paper, 180mm, Whatman Ltd. England) to discard the magnesium sulphate before being rotary evaporated to dryness.
- The organic solvent was evaporated by rotary vacuum evaporation at 37°C (Grant 349/2, Grant Instruments Cambridge Ltd).
- The residue was re-suspended in 1.5 ml of 95% ethanol, transferred to a vial and centrifuged at 10000 rpm for 10 min, the supernatant was removed to a clean vial and air dried in a clean cabinet.
- The residue was re-dissolved in 95% ethanol (100 µl 95% ethanol per litre of original supernatant or as specified in different experiments) and sterilised with a 0.22 µl filter (Millex® - GS, Millipore Co. U.S.A).

2.3.2.5. Biosensor culture preparation

The biosensor *E. coli*. (pSB401) [or *E. coli* (pSB1075)] was inoculated in 5 ml of LB broth containing the appropriate antibiotic [for *E. coli* (pSB401), the tetracycline concentration was 50 µg/ml and for *E.coli* (pSB1075), the ampicillin concentration was 60 µg/ml]. The biosensor was then incubated with shaking at 37°C for 4 h before the AHL bioassay.

2.3.2.6. Luminescence measurement

Bioluminescence produced by the biosensors was measured with a ID-20/20 Luminometer (DOP Solutions LTD, England). The ID-20/20 Luminometer is very sensitive and its sample adapter can accommodate 35mm petri dishes. The data were recorded as Relative Luminescence Units (RLU).

The images of the bioassay plates showing induced luminescence were taken by a ChemiImager™ 4400 camera (Alpha Innotech Corporation, USA).

2.3.2.7. AHL bioassay using the AHL-dependent biosensors

QSSs extracted from *P. fluorescens* 5064 and its mutants were detected by different bacterial biosensors. Positive assays were judged as induction of purple pigment in *Chromobacterium violaceum* CV026 or induction of bioluminescence in *E. coli* (pSB401) and *E. coli* (pSB1075) detected by ID-20/20 Luminometer or photographed by the ChemiImager $^{TM}$ 4400 camera. The AHL bioassay method was based on McClean *et al.* (1997) with some modifications: 5 ml of pre-warmed semi-LB agar [0.5% (w/v), heated up in a microwave until melted and then cooled down to about 50°C] was seeded with 50µl of the appropriate bacterial biosensor prepared as described before (2.3.2.5). In addition, 30µl of QSS extract (substance to be detected) was also added. The mixture was mixed well and immediately overlaid on the surface of 35 mm petri dishes containing pre-warmed LB agar. The plates were left on the bench for about 20min until the overlaid semi-LB agar solidified and were then subjected to the first (zero time) bioluminescence detection (except for the CV026 plates). The data were recorded as background luminescence. The plates were incubated at 37°C (30°C for CV026) in an upright position for 4 h and were then subjected to the second luminescence detection, or examined for the stimulation of purple pigment (for CV026 treatments). Instead of the QSS extracts, 20 µl of synthetic AHL signal solution [either 3-oxohexanoyl-homoserine lactone (OHHL, Sigma Chem Co.) or N-hexanoyl-homoserine lactone (Fluka Chem. Co.) was dissolved in 95% ethanol to a concentration of 0.1mM] was added to the semi-LB agar as positive control, and 20 µl of solvent (95% ethanol) was added as a negative control.

### 2.3.3. Results and discussion

QSS production extracted from *P. fluorescens* 5064 supernatant was assayed by the AHL-dependent biosensors *E.coli* (pSB401), *E. coli* (pSB1075) and *C. violaceum* CV026. CV026 is a violacein-negative strain that responds to exogenous AHLs by

producing the purple pigment violacein. The remaining two biosensors are *E. coli* strains carrying bioluminescence reporter plasmids that will respond to exogenous AHLs by emitting light. QSS extracted from *P. fluorescens* 5064 was found to induce light emission in both pSB401 (figures 2.1, 2.2) and pSB1075 (figure 2.1). The light output induced in pSB401 was much stronger than that induced in pSB1075 under the same incubation conditions (figure 2.1). This indicated that pSB401 was more sensitive than pSB1075 in responding to *P. fluorescens* 5064 QSS production. The *P. fluorescens* 5064 QSS could not induce any purple pigment formation in CV026 even after 4 days' incubation. Failure to induce pigment production in CV026 was also observed by Laue *et al.* (2000) in *P. fluorescens* F113 and EI-Sayed *et al.* (2001) in *P. fluorescens* NCIMB 10586.

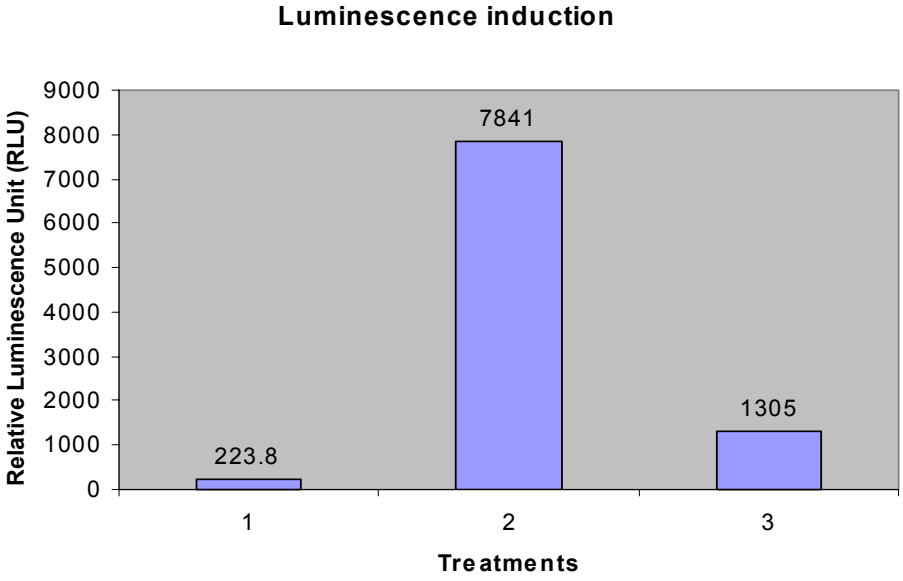
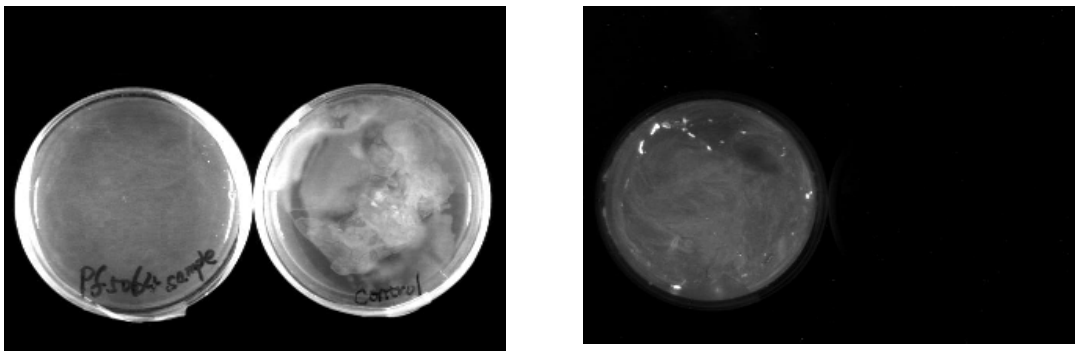



**Figure 2.1: Luminescence induced by *P. fluorescens* 5064 QSS extract detected by biosensors *E. coli* (pSB 401) and *E. coli* (pSB1075)**

1. Negative control (biosensor pSB1075 was exposed to same amount of ethanol) showing background luminescence
2. *E. coli* (pSB401) biosensor strain was exposed to the quorum sensing signal extracted from *P. fluorescens* 5064.
3. *E. coli* (pSB1075) biosensor strain was exposed to the quorum sensing signal extracted from *P. fluorescens* 5064.

Since CV026 responds most sensitively to AHL with 4-6 carbon acyl side chains, pSB401 prefers 6-8 carbon side chains and pSB1075 detects compounds with 10-14 carbon side chains (Winson *et al*., 1998), it was hypothesized that the QSS produced by *P. fluorescens* 5064 is a 8-10 carbon in side chain length. Further purification and chemical identification of AHL produced by *P. fluorescens* 5064 were performed and this will be addressed in Chapter 3.




**a** **b**

**Figure 2.2: Luminescence induced by the QSS extracted from *P. fluorescens* 5064 and detected by biosensor *E. coli* (pSB401)**

**a**: Photographed in white light
Left: pSB401 biosensor strain was exposed to quorum sensing signal extracted from *P. fluorescens* 5064.
Right: Biosensor strain only (as negative control).

**b**: Photographed in darkness
light emitted by the biosensor strain confirms production of quorum sensing signal by *P. fluorescens* 5064.

The experiments suggest that *P. fluorescens* 5064 produces at least one detectable AHL signal and imply that a quorum sensing system exists in this bacterial strain.

## 2.4. *P. fluorescens* 5064 quorum sensing signal synthesis dynamics

### 2.4.1. Introduction

Quorum sensing-dependent gene expression is cell-density-dependent. The maximum gene production occurs during the late logarithmic and early stationary phase of growth (Whooley and McLoughlin, 1983). A bacterium gauges its population density by sensing the concentration of QSS. As was mentioned in the literature review, in the *V. fischeri* quorum sensing system, the OHHL, having reached a critical concentration, will bind to LuxR. Binding to an OHHL enables the LuxR protein to combine with the lux box and act as a transcriptional activator, thus enhancing the expression of the rightward transcript of the operon. Since the *luxI* is located within this operon, more OHHL is produced, leading to the allowance of more OHHL:LuxR binding and increasing the transcriptional activation of the *luxI* gene, thereby generating a positive feedback regulatory loop, in which OHHL controls its own synthesis and shows a sudden logarithmic increase once the quorum sensing has been triggered (Figure 1.3).

Although in *V. fischeri*, *luxI* is located within the quorum sensing regulated gene cluster, there is no theoretical requirement for the genes encoding QSS to be linked to the corresponding regulatory genes. Assuming that the AHL is freely diffusible and can bind to its LuxR homologue and that the resultant complex can then find its appropriate binding site on the DNA, any form of genetic arrangement is possible (Salmond *et al*., 1995). Several homologous pairs of quorum sensing regulatory genes have proved to transcribe convergently and are not linked to the genes they regulate (Beck Von Bodman and Farrand, 1995; Pirhonen *et al*., 1993; Throup *et al*., 1995a; Wood and Pierson, 1996).

Assessing the QSS producing dynamics could provide evidence in determining whether or not the luxI homologue in *P. fluorescens* 5064 is linked to the genes it regulates.

### 2.4.2. Materials and methods

To determine the QSS production at different growth phases of *P. fluorescens* 5064, QSS extracts at different incubation times were assessed for luminescence induction by the biosensor *E. coli* (pSB401). *P. fluorescens* 5064 was grown in 5 flasks (each containing 300 ml LB broth) with shaking at 26°C. After 5, 11, 19, 25 and 30h incubation, samples were taken to detect their QSS production and OD value (500 nm), respectively. The QSS extraction and QSS bioassay methods were the same as described in 2.3.2. In this experiment, we chose to use pSB401 as the biosensor, because it was more sensitive than the pSB1075 in detecting the *P. fluorescens* 5064 QSS.

### 2.4.3. Results and discussion

*P. fluorescens* 5064 QSS extracts at different incubation times were assessed for their luminescence induction with the biosensor *E.coli* (pSB401). The results showed that *P. fluorescens* 5064 exhibited a lag period in AHL production during early- and mid-exponential growth phases, followed by a rapid increase during the late exponential phase and reached maximum production at early stationary growth phases (Figure 2.3, 2.4). This is indicative of the quorum sensing mechanism for controlling gene expression in response to an expanding bacterial population and implies that the quorum sensing signal synthase gene (*luxI* homologue) may be linked to the quorum-sensing-regulated genes.

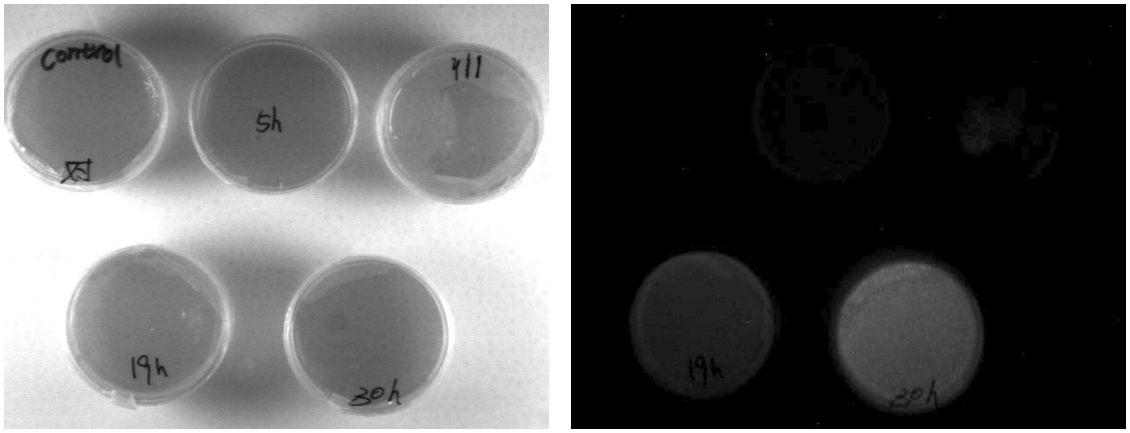



**Figure 2.3: *P. fluorescens* 5064 QSS formation at different incubation times**

AHL extracts of *P.fluorescens* 5064 collected at different incubation times (5h, 11h, 19h, 30h) were assessed for their ability to induce luminescence in the biosensor *E.coli* (pSB401). A sudden appearance of luminescence after 19h incubation indicating a rapid increase of QSS.

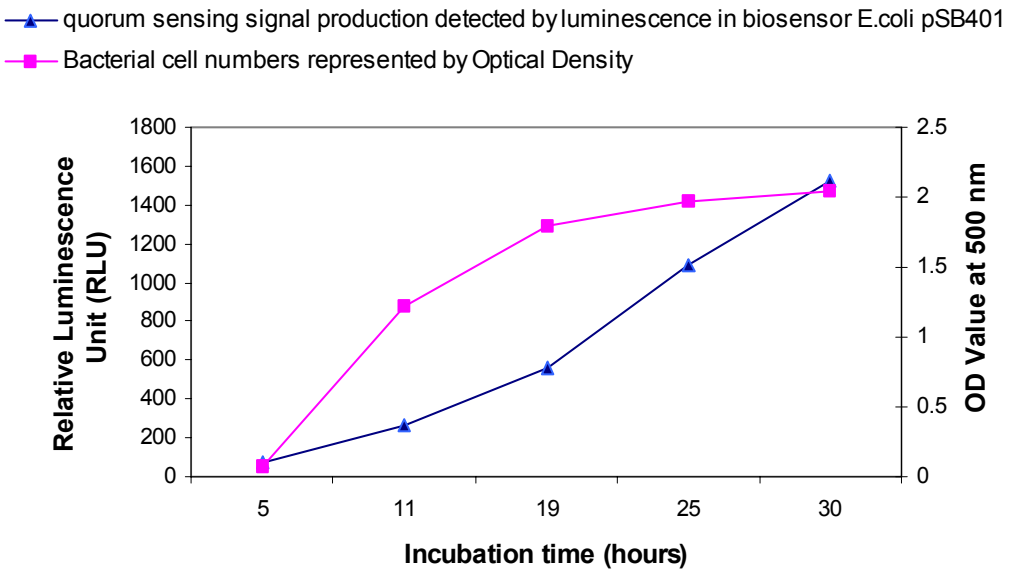



**Figure 2.4. Dynamics of quorum sensing signal production in *P. fluorescens* 5064**

Quorum sensing signal production showed a lag period during early- and mid- exponential growth phases, followed by a rapid increase during the late exponential and early stationary phases.

## 2.5. Screening the surf⁻ mutants for their QSS production

### 2.5.1. Introduction

In previous work in our laboratory, a library consisting of 35 mutants that no longer produce biosurfactants was generated by Tn*5* mutagenesis of *P. fluorescens* 5064. These 35 biosurfactant-minus mutants (surf⁻) were labelled 6401 through to 6435 (m6401 to m6435). The pathogenicity and phenotypic characterization of the 35 surf⁻ mutants are shown in Table 2.2 (adapted from Darling 1998). Southern blot has been carried out to determine the number of the Tn*5* transposon present in the mutants (Darling 1998). Restriction enzyme *Eco*RI does not cut within Tn*5*, while *Sal*I cut Tn*5* centrally (one cutting site) (see appendix N for Tn*5* map). The DNA of the mutants was digested by *Eco*RI and *Sal*I and the digested DNA was hybridised by Tn*5* probe. If only one copy of Tn*5* exists in the mutant DNA, there should be one hybridised band present in the *Eco*RI digested DNA profile and two bands in the *Sal*I profile. Additional Tn*5* insertion might have occurred if there are more bands present (see appendix M for the hybridisation results of the 35 mutants). All the surf⁻ mutants have kanamycin resistance.

This experiment aimed to assess AHL production among these 35 biosurfactant mutants and to find a possible relationship between biosurfactant production and quorum sensing signal production in *P. fluorescens* 5064.

### 2.5.2. Materials and method

To determine the 35 surf⁻ mutants QSS producing profile, the AHLs were extracted from the mutants using the same method described in 2.3.2. and were subjected to the biosensor assay (2.3.2.).

## Table 2.2: Pathogenicity and phenotypic characterization of *P. fluorescens* 5064 surf mutants (Darling, 1998)

| Isolation Number | % wild-type disease on broccoli | Doubling time(minute) | Fluorescent pigment | Protease | Pectinase | Number of hybridising bands following EcoRI Digestion | Number of hybridising bands following SalI Digestion | Additional mutation |
|---|---|---|---|---|---|---|---|---|
| 6416 | 18.8 | 73.3 | - | - | - | 2 | 3 | Yes |
| 6417 | 25.0 | 80.0 | - | - | - | 2 | 3 | Yes |
| 6418 | 25.0 | 73.3 | - | - | - | 2 | 3 | Yes |
| 6419 | 25.0 | 73.3 | - | - | - | 2 | 3 | Yes |
| 6427 | 25.0 | 86.6 | + | + | + | 2 | 3 | Yes |
| 6407 | 31.2 | 86.6 | - | - | - | 2 | 3 | Yes |
| 6415 | 31.2 | 73.3 | + | + | + | 2 | 3 | Yes |
| 6428 | 31.2 | 66.6 | + | + | + | 2 | 3 | Yes |
| 6414 | 43.8 | 80.0 | + | + | + | 2 | 3 | Yes |
| 6422 | 43.8 | 66.6 | + | + | + | 1 | 2 | No |
| 6425 | 43.8 | 73.3 | + | + | + | 1 | 2 | No |
| 6434 | 43.8 | 73.3 | + | + | + | 2 | 3 | Yes |
| 6426 | 50.0 | 66.6 | + | + | + | 1 | 2 | No |
| 6429 | 50.0 | 66.6 | + | + | + | 1 | 2 | No |
| 6403 | 56.2 | 66.6 | + | + | + | 1 | 2 | No |
| 6404 | 62.5 | 73.3 | + | + | + | 1 | 2 | No |
| 6420 | 62.5 | 66.6 | + | + | + | 1 | 2 | No |
| 6424 | 68.8 | 66.6 | + | + | + | 1 | 2 | No |
| 6431 | 68.8 | 73.3 | + | + | + | 2 | 5 | Yes |
| 6432 | 75.0 | 66.6 | + | + | + | 1 | 2 | No |
| 6406 | 81.2 | 66.0 | + | + | + | 2 | 3 | Yes |
| 6409 | 81.2 | 80.0 | + | + | + | 2 | 3 | Yes |
| 6410 | 81.2 | 80.0 | + | + | + | 1 | 2 | No |
| 6421 | 81.2 | 86.6 | + | + | + | 1 | 2 | No |
| 6433 | 81.2 | 73.3 | + | + | + | 1 | 2 | No |
| 6401 | 87.5 | 66.6 | + | + | + | 1 | 2 | No |
| 6408 | 87.5 | 73.3 | + | + | + | 1 | 2 | No |
| 6412 | 87.5 | 73.3 | + | + | + | 2 | 3 | Yes |
| 6413 | 87.5 | 66.6 | + | + | + | 2 | 3 | Yes |
| 6405 | 93.8 | 80.0 | + | + | + | 1 | 2 | No |
| 6435 | 93.8 | 60.0 | + | + | + | 2 | 3 | Yes |
| 6402 | 100.0 | 66.6 | + | + | + | 1 | 2 | No |
| 6423 | 100.0 | 73.3 | + | + | + | 1 | 2 | No |
| 6411 | 112.5 | 66.6 | + | + | + | 2 | 3 | Yes |
| 6430 | 112.5 | 66.6 | + | + | + | 2 | 6 | Yes |

### 2.5.3. Results and discussion

In order to determine the potential relationship between biosurfactant production and quorum sensing signal production in *P. fluorescens* 5064, 35 *P. fluorescens* 5064 biosurfactant-deficient mutants, generated by Tn*5* mutagenesis (Darling, 1998), were screened for their abilities to produce QSS. Culture filtrates from each mutant were assayed for QSS production using the *E. coli* (pSB401) biosensor. According to the screening results, the 35 mutants were divided into 3 groups based on their QSS producing abilities (Table 2.3). Twenty-four mutants (group 1) showed strong luminescence induction, i.e. were similar to wild type in production of QSS. Six mutants (group 2) showed greatly reduced luminescence induction. The luminescence induction of mutant 6423 (m6423) was almost 100 times lower than the wild-type *P. fluorescens* 5064 (Figure 2.5). No luminescence could be detected in 5 of the mutants in group 3 (Table 2.3). Among the mutants in group 2 that showed reduced QSS production, mutants 6423, 6421, and 6429 are more interesting due to their lack of additional transposon insertions (Table 2.2. and appendix M). A single Tn*5* insertion into genomic DNA makes further gene analysis easier. Mutant 6423, in particular, warrants further genetic analysis to determine the nature of the mutation because its QSS production was almost eliminated.

Interestingly, AHL extracts from five mutants in group 3 showed very strong antibiotic activity: they killed not only the *E. coli* biosensors but also the wild-type *P. fluorescens* 5064 in a subsequent experiment. A possible reason for this might be due to the Tn*5* insertion blocking a transcriptional repressor gene function, thereby restoring antibiotic production that is normally repressed by this particular gene. Recently, Delany *et al.* (2000) identified a putative regulatory gene (*phlF*) upstream and divergently transcribed from the phloroglucinol (antibiotic) biosynthetic genes in *P. fluorescens* F113. Introduction of *phlF* into *P. fluorescens* F113 in multiple copies resulted in repression of phloroglucinol production and inactivation of *phlF* resulted in derepression of the antibiotic production in this strain. These facts indicate that *phlF* functions as a gene repressor. Whether the restored antibiotic production in mutants in group 3 is due to a

mutation of a repressor gene needs further investigation. Because these mutants killed the biosensors, it is not possible to draw any conclusion on their AHL producing abilities by the bioassay method described in 2.3.2. In addition, because the mutants in group 3 exhibited antibiotic activity, they could be used as a potential biocontrol agent. This will be further addressed in Chapter 5.

A greatly reduced AHL production was observed within group 2 mutants, especially m6423, which suggests that the Tn*5* insertions might have disrupted a gene in the AHL synthesis pathway. But none of these 35 surf$^-$ mutants showed a complete blockage in AHL expression. The lower level of luminescence in *E. coli* (pSB401) induced by group 2 mutants suggested that the AHL synthesis mechanism in *P. fluorescens* 5064 might be very complicated. Two quorum sensing systems have been identified in *Pseudomonas aeruginosa* including the LasR/LasI system, which produces QSS 3-oxo-$C_{12}$-HSL; and the RhlR/RhlI system, which produces C4-HSL as its cognate QSS. It is now apparent that both of these quorum sensing systems regulate the production of rhamnolipid (a biosurfactant produced by *P. aeruginosa*), though the LasR/LasI system also plays a role in expression of many other virulence genes. In this study, surf$^-$ mutant 6423 produced wild-type levels of protease, pectinase and siderophores (Darling, 1998). The production of these compounds is regulated in a cell-density-dependent manner in *P. aeruginosa* and also in a number of other bacteria including *P. fluorescens*. This implies that there may be more than one quorum sensing system in *P. fluorescens* 5064 and that these quorum sensing systems may interact with each other in controlling the expression of biosurfactant. This can explain why the AHL production of m6423 was reduced greatly but not eliminated. In fact, a MupR/MupI quorum sensing system that regulates the mupirocin biosynthetic gene cluster has very recently been identified in *P. fluorescens* NCIMB 10586 (EI-sayed *et al*., 2001). Both MupR and MupI genes have been cloned. It would be interesting to know if the MupR/MupI quorum sensing system affects biosurfactant synthesis in *P. fluorescens* 5064.

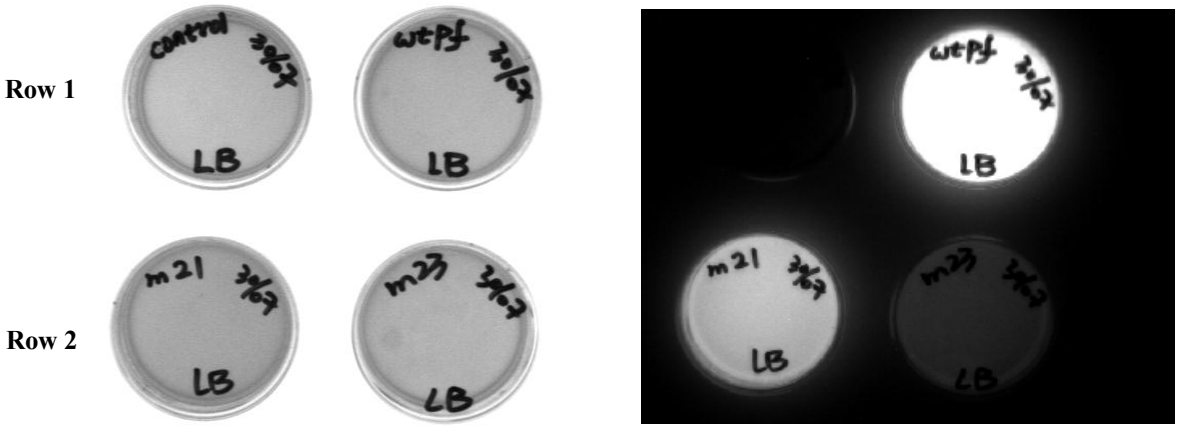



**Figure 2.5. Quorum sensing signal production in biosurfactant-negative mutants using the *E.coli* (pSB401) biosensor assay**

A: Plates photographed under white light
B: Plates photographed in darkness
Row 1 left: Water as negative control (Relative Luminescence Unit (RLU) = 11)
Row 1 right: *P. fluorescens* 5064 wild type as positive control (RLU = 5600)
Row 2 left: mutant 6421 (RLU = 618)
Row 2 right: mutant 6423 (RLU = 62)
Quorum sensing signal produced in mutant 6421 was less than in wild type, and almost eliminated in mutant 6423.

**Table 2.3: The quorum sensing signal production in 35 biosurfactant-negative mutants of *P. fluorescens* 5064**

| Phenotypic Group | Quorum sensing signal production detected by biosensor *E.coli* pSB401 | *P. fluorescens* 5064 mutants | Other phenotypic characteristics that are different from the wild type * |
|---|---|---|---|
| 1 | Same as wild type *P. fluorescens* 5064. | 6401, 6402, 6403, 6404, 6405, 6406, 6408, 6409, 6410, 6411, 6413, 6414, 6420, 6422, 6424, 6425, 6426, 6428, 6430, 6431, 6432, 6433, 6434, 6435. | None |
| 2 | Quorum sensing signal production was greatly reduced compared to wild type *P. fluorescens* 5064. | 6412, 6415, 6421, 6423[1], 6427, 6429. | None |
| 3 | No quorum sensing signal production could be detected – because culture filtrates from these mutants killed the biosensor strains. | 6407, 6416, 6417, 6418[2], 6419 | No extracellular product expression (i.e. protease, pectinase, siderophore production). Powerful unknown antibiotic compound produced. |

* Darling, 1998

[1].m6423 has one copy of Tn5 insertion (appendix M).

[2].m6418 has additional Tn5 insertion (appendix M).

## 2.6. Can exogenous quorum sensing signal restore biosurfactant activity in surf $^{-}$ mutants?

### 2.6.1. Materials and methods

2.6.1.1. Acyl-HSL standard solutions used in the biosurfactant restoration tests

**HHL**: the same as described in 2.3.2.3.

**VAI:** the same as described in 2.3.2.3.

**QSS extracted from wild type *P. fluorescens* 5064**: the QSS was extracted from original one litre of *P. fluorescens* 5064 LB broth and the extraction method was the same as described in 2.3.2..

2.6.1.2. Bacterial strains:

Mutant 6423 (m6423) was chosen to carry out the biosurfactant restoration test due to its greatly reduced AHL production and lack of additional transposon insertions (Table 2.2). A single Tn*5* insertion into genomic DNA makes further gene analysis easier. m6423 was grown in KB broth with kanamycin (50μg/ml) and incubated at 30°C.

2.6.1.3. Biosurfactant extraction:

The biosurfactant extracting method was based on Laycock (1991). m6423 was grown in 200ml KB broth for about 30h, or as indicated for specific experiments and then subjected to the following procedures:

1. The bacterial cell suspensions obtained from the above KB broth were centrifuged at 20000 *g* for 30 min to precipitate the cells.
2. The cell free supernatant was acidified to pH 2 with 2N HCl and allowed to stand at 2°C for at least 1 h.

3. The white precipitate that formed upon acidification was centrifuged at 20000 g for 15 min, the supernatant was decanted and the pellet was extracted with 60 ml of 95% ethanol.
4. The undissolved material was filtered off and re-extracted with 95% ethanol.
5. The residue was discarded and the two ethanolic extracts were combined and concentrated on a rotary evaporator to 3~5 ml.
6. A small amount of activated charcoal was added to remove any colour, and the solution was then filtered through Celite.
7. The final solution was kept for the detection of biosurfactant production.

2.6.1.4. Biosurfactant restoration test:

Two biosurfactant restoration tests, both based on the Water Drop Test (see 2.2.2.2), were assayed in this experiment.

Mutant 6423 was streaked in the middle of KB agar plates containing kanamycin and grown at 30°C for 6h before adding 20 µl of each of the three **AHL standard solutions** (2.6.1.1), respectively, around the bacterial colonies. The bacteria and AHLs mixtures were allowed to grow for another 4h, 8h and 24h before the samples (colonies) were taken for the biosurfactant assay (using Water Drop Test). The bacterial colonies were transferred using a sterile toothpick to a 50 µl water droplet situated on a plastic petri dish. If biosurfactant production is restored, the surface tension of the water droplet will be reduced and the water droplet will collapse. Six replicates were carried out and mutant 6423 growing without exogenous AHL was used as negative control.

Mutant 6423 was grown in 200 ml KB broth (without antibiotic) shaking at 30°C for 6 hours before adding 25 µl of each of the three **AHL standard solutions** (2.6.1.1.), respectively, and reaching a final concentration at about 12.5mM. The bacteria and AHLs mixtures were allowed to grow. After a further 4h, 8h and 24 h incubation, samples were taken for biosurfactant extraction using the method described in 2.6.1.3. The extracts were then used to carry out the biosurfactant bioassay using the Water Drop Test method (2.2.2.2). For the biosurfactant bioassay, 5 µl of the biosurfactant

extract was pipetted on to a 50 µl water droplet situated on a plastic petri dish. The reduction of the surface tension of the water droplets indicates the presence of biosurfactant. Three replicates were carried out and mutant 6423 growing without exogenous AHL was used as negative control.

### 2.6.2. Results and discussion

For the biosurfactant restoration test that used KB agar plates to grow mutant 6423, we could not draw an accurate conclusion, because the biosurfactant formation in mutant 6423 varied with different replicates. The results showed that the exogenous AHL affected the biosurfactant restoration in mutant 6423 randomly. The most likely reason could be that the exogenous AHL could not diffuse evenly within the agar plates and therefore generated an uncertain result.

For the second test method that used KB broth to grow mutant 6423, biosurfactant restorations were observed when either synthetic OHHL or QSS extracts from wild type *P. fluorescens* 5064 were added to mutant 6423 following a further 24h incubation (Figure 2.6). This experiment provides strong evidence for the regulation of biosurfactant production by the quorum sensing system in *P. fluorescens* 5064.

In both test methods, no biosurfactant restorations were observed when mutant 6423 was grown for 4 h and 8 h after inoculating with exogenous AHLs (either synthetic OHHL or QSS from wild type). As 10 h (6 + 4 h) and 14 h (6 + 8 h) represent an early and a mid-logarithmic growth phase, respectively, of mutant 6423, this experiment provided evidence, once again, that biosurfactant gene expression in *P. fluorescens* 5064 is triggered during the late logarithmic growth phase.

Surprisingly, HHL, which was first identified in a *P. fluorescens* strain (Cha *et al*., 1998), did not induce biosurfactant restoration in mutant 6423. It might be due to the significant structural difference between HHL and the AHL produced by *P. fluorescens* 5064. Later work undertaken in this study has proved this hypothesis (chapter 3).

The second test method was more sensitive, which was predicted. Firstly, it used extracted biosurfactant, which had a much higher concentration than the biosurfactant present in the colonies (first method). Secondly, in the second test method, the added exogenous AHLs could be mixed better and more evenly in a liquid culture with shaking than on a solid agar plate, which had been used in the first method.

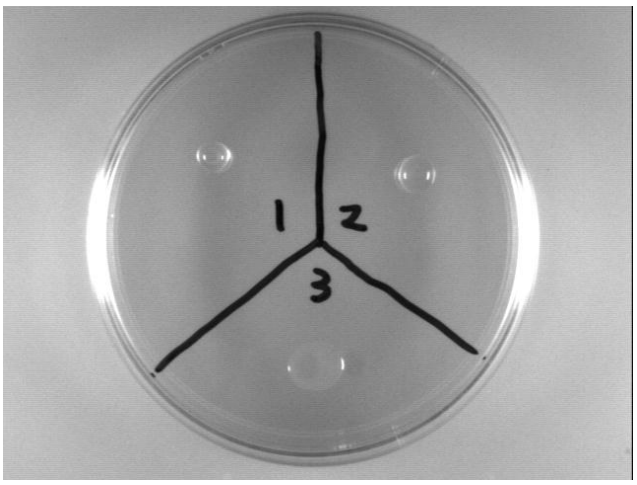



**Figure 2.6. Restoration of biosurfactant production in *P. fluorescens* mutant 6423 in response to exogenous quorum sensing signal using the Water Drop Test.**

**1**: Water droplet alone on plastic petri dish.
**2**: Water droplet containing extracted biosurfactant from mutant 6423 grown without exogenous QSS
**3**: Water droplet containing extracted biosurfactant from mutant 6423 grown in presence of exogenous QSS

Loss of surface tension in droplet in (3) shows that biosurfactant production was restored in mutant 6423 by addition of QSS (either extracted from wild type *P. fluorescens* 5064, or synthetic 3-oxohexanoyl-homoserine lactone (VAI)).

## 2.7. General discussion and conclusion

Quorum sensing controls a number of key processes in growth, reproduction and virulence in many gram-negative bacteria (Gambello *et al*., 1993; Pierson *et al*., 1994; Zhang *et al*., 1993).

The bacterium *P. fluorescens* 5064 is an opportunistic soft rot pathogen of broccoli (*Brassica oleracea* var *italica*). It produces an extracellular lipopeptide biosurfactant which has been suggested to play a role in disease incidence (Hildebrand, 1989). In this study, we provided evidence for the regulation of biosurfactant production via quorum sensing in *P. fluorescens* 5064. *P. fluorescens* 5064 biosurfactant production has always been observed to be coupled with its cell population density (Darling, 1998 and this study), which is a characteristic phenomenon of quorum sensing. A valuable *P. fluorescens* 5064 mutant that lacks both biosurfactant and QSS production has been identified. Supplying exogenous QSS to this mutant, mutant 6423, leads to a restoration of its biosurfactant production. This result strongly indicates that biosurfactant production in *P. fluorescens* 5064 is regulated by quorum sensing.

Whiteley *et al*.(1999) studied the number and type of genes controlled by quorum sensing in *P. aeruginosa* systematically. They found that some *P. aeruginosa* quorum sensing controlled (qsc) genes were regulated by additional factors. Although they required acyl-HSL signals for activation, they were repressed until the stationary phase was reached, even in the presence of acyl-HSLs. These qsc genes are termed late qsc genes. Other genes, early qsc genes, showed activation upon addition of acyl-HSL signals even in early logarithmic phase (Whiteley and Greenberg, 2001). In this study, no biosurfactant restoration was observed in the samples that were taken either 4h or 8h after exogenous QSS addition. This suggests that the biosurfactant-regulation-gene that is controlled by quorum sensing in *P. fluorescens* 5064 is a late qsc gene.

When screening the 35 surf⁻ mutants for QSS production, five mutants showed very strong antibiotic activity. They killed not only the *E. coli* biosensors but also the wild type *P. fluorescens* 5064 in a subsequent experiment (data shown in Chapter 5). A possible reason might be that Tn*5* insertion had blocked a transcriptional repressor gene function, thereby restoring antibiotic production that is normally repressed by this particular gene. These *P. fluorescens* 5064 mutants are potentially important because of their possible application as biocontrol agents. *P. fluorescens* strains have been widely used as biocontrol agents since they are widely occurring, usually non-pathogenic, and highly effective at eliminating competing microbes (Burr *et al.*, 1978; Schroth and Hancock, 1983; Weller and Cook, 1983). The possibility of using one of these mutants, mutant 6418, to control broccoli head rot disease is evaluated in Chapter 5.

A large number of pathogenic bacteria control the expression of many of their extracellular virulence factors by a quorum sensing system and quorum sensing plays an important role in bacterial disease development and spread (Beck Von Bodman and Farrand, 1995; Flavier *et al.*, 1997). A major objective of an invading bacterium is the evasion of its host defense. The advantage of employing such a quorum sensing mechanism is to avoid premature gene expression, which could alert the host and elicit a defensive response, and to make sure that the invading bacteria will only commence virulence gene expression when they have proliferated to a level where the coordinated production of virulence determinants may overwhelm the host (Pierson *et al.*, 1999). By artificially blocking the cell-to-cell signalling mechanism, pathogenic organisms that use quorum sensing to control virulence could potentially be rendered avirulent (Beck Von Bodman and Farrand, 1995). The finding that *P. fluorescens* 5064 regulates biosurfactant production and, we speculate, other virulence factors by quorum sensing opens the possibility for novel disease control methods.

It is worth mentioning that, although *P. fluorescens* has been reported to produce many different biosurfactants (Harling and Dubickas, unpublished data, 1998; Healy *et al.*, 1996; Henriksen *et al.*, 2000), not all of them are regulated by quorum sensing. Nielsen *et al.* (1999) reported a bacterial cyclic lipodepsipeptide with biosurfactant properties

from *P. fluorescens* DR54, termed Viscosinamide. Unlike Viscosin (the possible biosurfactant production in *P. fluorescens* 5064), Viscosinamide production can not be affected by C, N, P nutrient levels, starvation or different growth phases, which implies its non-quorum-sensing dependent nature.

# Chapter 3. Chemical Identification of the QSS Produced by *P. fluorescens* 5064

## 3.1. Introduction

Quorum sensing has been proved to exist in *P. fluorescens* 5064 and to regulate its biosurfactant production. It would be of interest to identify the QSS that is present in this strain. Previous work in this study (chapter 2) implied that the *P. fluorescens* 5064 QSS might belong to the AHL family. Firstly, because it could promote light emission in the AHL-dependent biosensors. Secondly, because exogenously applied AHL molecules (OHHL) were able to restore the biosurfactant production in a *P. fluorescens* 5064 surf$^-$ mutant.

Thus far, several QSS have been reported to be present in different *P. fluorescens* strains. Laue *et al*. (2000) found that *P. fluorescens* F113 produces at least three different AHLs. These are (1). N-(3-hydroxy-7-cis-tetradecenoyl) homoserine lactone (3-OH, $C_{14:1}$-HSL). (2). N-decanoyl HSL ($C_{10}$-HSL) and (3). N-hexanoyl HSL ($C_6$-HSL). Interestingly, none of these three AHLs have yet been identified in other *P. fluorescens* strains. Shaw *et al*. (1997) have reported up to 5 different AHL molecules in *P. fluorescens* 2-79 by Thin Layer Chromatography (TLC) and Mass Spectrometry (MS) analysis. Three of which were identified as N-(3-hydroxyhexanoyl)-HSL, N-(3-hydroxyoctanoyl)-HSL, and N-(3-hydroxydecanoyl)-HSL. Later on, N-octanoyl-HSL and N-hexanoyl-HSL were identified in this strain by Cha *et al*. (1998). However, no AHL molecules were detected in *P. fluorescens* 1855.344 (Cha *et al*.,1998), *P. fluorescens* 7-14 (Dumenyo *et al*., 1998) or *P. fluorescens* NCIMB 10586 (Holden *et al*., 1999). Given the differences in AHL profiles among the *P. fluorescens* strains examined to date, it would be of future interest to determine whether there is any association between AHL profile and strain habitat. It would be interesting to discover what kind of QSS is present in *P. fluorescens* 5064.

The most commonly used procedure for characterizing AHL molecules was developed by Eberhard *et al*. (1981). The procedure makes use of high performance liquid chromatography (HPLC) and Mass Spectrometry (MS). HPLC coupled to MS has become an important analytical methodology during the last 10 years. Through the combination of HPLC and MS, both the separation of different substances (through HPLC) and the structural identification of those compounds (through MS) can be achieved.

In this experiment, a HPLC system with a reverse $C_{18}$ column in combination with MS have been employed in order to identify the QSS production in *P. fluorescens* 5064.

## 3.2. Materials and methods

### 3.2.1. HPLC analysis of QSS extract from *P. fluorescens* 5064

3.2.1.1. Crude QSS preparation

QSS was extracted using the method described in 2.3.2 to which some changes were made. *P. fluorescens* 5064 was grown in 3 L of LB broth for 50-55 h at 26°C with shaking (optical density at 500nm of 2.2). The culture was then centrifuged to precipitate the cells and the supernatant was extracted twice with equal volumes of dichloromethane (HPLC grade, Fisher Chemicals). The dichloromethane organic phases were combined (the trace aqueous residue was removed by anhydrous magnesium sulphate) and dried by rotary vacuum evaporation (Grant 349/2, Grant Instruments Cambridge Ltd) at 37°C. The resulting residue was re-dissolved in 500µl of dichloromethane for further HPLC analysis.

3.2.1.2. HPLC system and columns

The HPLC was performed on a Hewlett Packard 1100 Series LC System, employing a $C_{18}$ reverse-phase column [Lichrosorb, 5RP18 (250 x 8mm), particle size 5µm, Technical Ltd, Congleton, UK] for the preparative HPLC and a PLRP-S reverse-phase column (250 x 4.6mm, 100/, 5µm, Polymer Labs) for the analytical HPLC.

3.2.1.3. Chromatography procedure

(1). For preparative HPLC, 100µl of the sample was applied to a $C_{18}$ reverse-phase column eluted with a mobile phase of acetonitrile ($CH_3CN$, HPLC grade, Fisher Chemicals) in water (purified using a Millipore Elix 5 system). The mobile phase flow rate was 2ml/min and the system was monitored at 210nm. The gradient of acetonitrile was as follows: 15% from 0 to 8 min; 35% from 10 to 20 min; 60% from 22 to 30 min.
(2). After preparative HPLC, the fractions showing luminescence induction in the *E. coli* (pSB401) biosensor were pooled and re-chromatographied using an analytical PLRP-S reverse-phase column. 10µl of the sample was applied to the column at each cycle, eluted with a mobile phase of acetonitrile in water (using a linear gradient of 0 to 35% over the course of 20 min and remaining on 35% for another 2 min) at a flow rate of 1ml/min and monitored at 210nm.

3.2.1.4. Collection of fractions and bioassay

For both preparative and analytical HPLC, the eluted fractions were collected and freeze-dried by using a lyophilizer (Edwards, Super Modulyo, USA).
The fraction collecting and detecting methods were as follows:
(1). After each cycle of HPLC, the fractions were collected and pooled separately.
(2). All the fraction samples were kept in a – 70°C freezer for approximately half an hour before being applied to the lyophilizer machine.

(3). Once the lyophilization finished (usually 50 hours, depending on the amount of sample loaded), these samples were re-dissolved in 200µl of 95% ethanol.
(4). The samples were assayed for their biological activities using the AHL dependent biosensor *E. coli* (pSB401) as described in 2.3.2.

### 3.2.2. Mass spectrometry

Mass Spectrometry was performed on a Micromass Platform II quadrupole mass spectrometer equipped with an electrospray ion source. The collision energy was set to 25eV and range of m/z 0-300 were recorded. The average molecular mass was determined using the MaxEnt algorithm of MassLynx software.

## 3.3. Results

### 3.3.1. HPLC analysis of QSS extracts from *P. fluorescens* 5064

3.3.1.1. Preparative HPLC and fraction analysis

A series of peaks were observed after preparative HPLC. These peaks were divided into 4 groups and collected separately (as shown in Figure 3.1.). These 4 samples, designated P1, P2, P3 and P4, were collected, pooled and freeze-dried using a lyophilizer and re-dissolved in 200µl of 95% ethanol after lyophilization (3.2.1.4.). Further bioassays were carried out among these 4 samples in order to determine their abilities of inducing light emission in the AHL dependent biosensor *E. coli* (pSB401). Sample P4 was selected and subjected to further analytical HPLC due to its strong activity in stimulating bioluminescence in *E.coli* (pSB401) (Figure 3.2). Two more cycles of the QSS extraction and HPLC separation experiments had to be repeated in order to obtain enough quantity of sample P4 for the analytical HPLC.

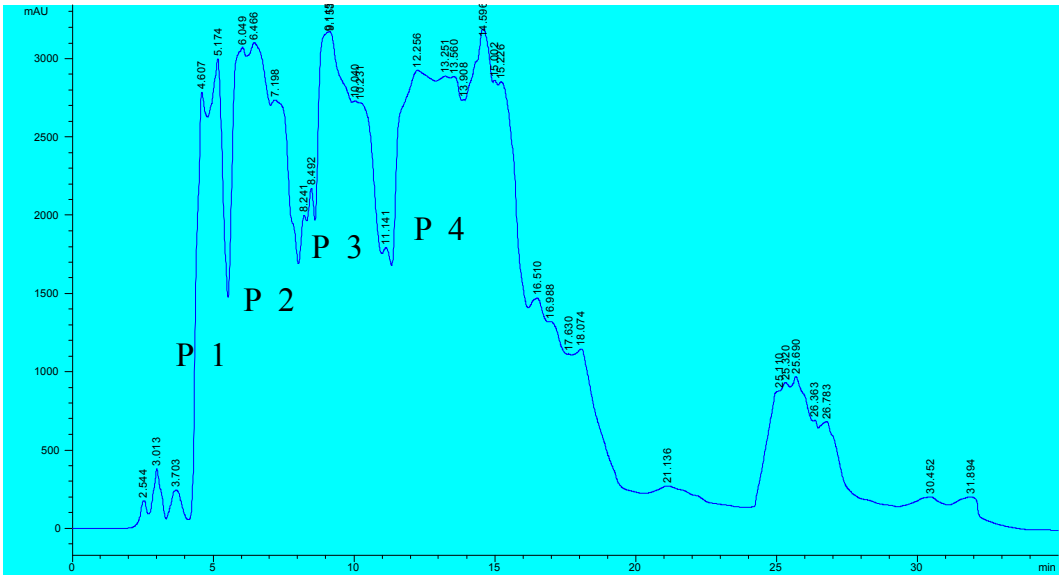



**Figure 3.1. Preparative HPLC Chromatograph**
The eluted peaks were divided into 4 groups and collected separately.

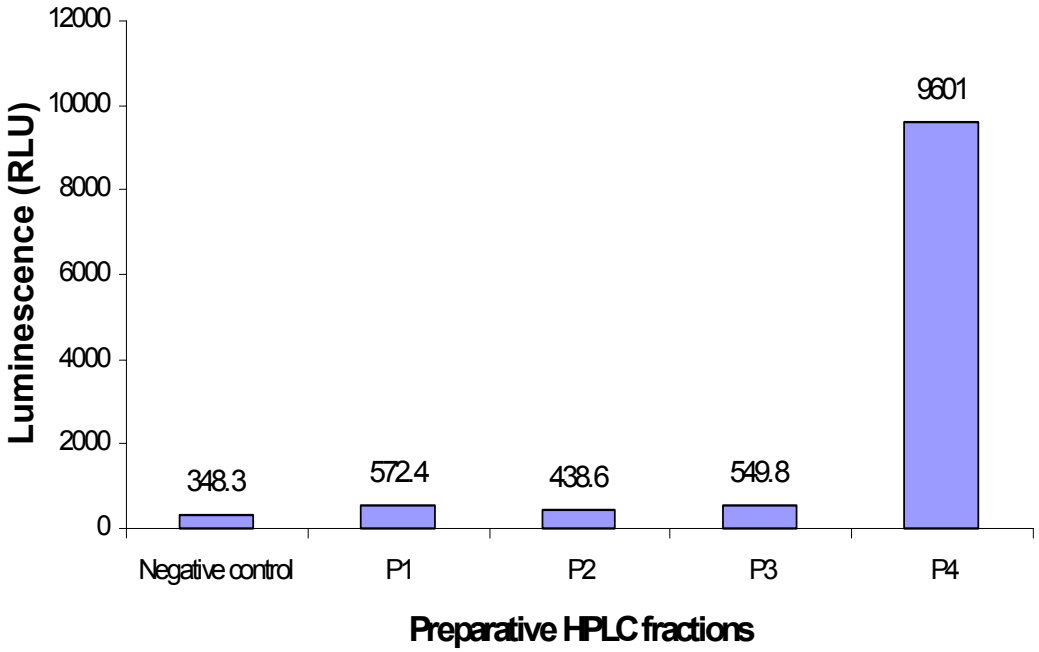



Figure 3.2: Luminescence induced by the preparative HPLC fractions

3.3.1.2. Analytical HPLC and fraction analysis

Sample P4 was applied to a PLRP-S reverse-phase analytical column. The column was eluted with a mobile phase of acetonitrile in water (0-35% in 20 min and remaining on 35% for another 2 min) and monitored at 210nm. Three peaks were observed and collected, respectively (Figure 3.3). The three samples, named A1, A2, A3, were then subjected to the QSS bioassay using the *E. coli* (pSB401) reporter. Sample A1 showed great activity and induced strong luminescence in *E. coli* (pSB401) (Figure 3.4). Because Sample A1 was collected from a very sharp peak, we assumed it would be pure enough for further Mass Spectrometry analysis. Again, the QSS extraction, the preparative and analytical HPLC had to be repeated three times to ensure that enough samples were collected for the MS analysis.

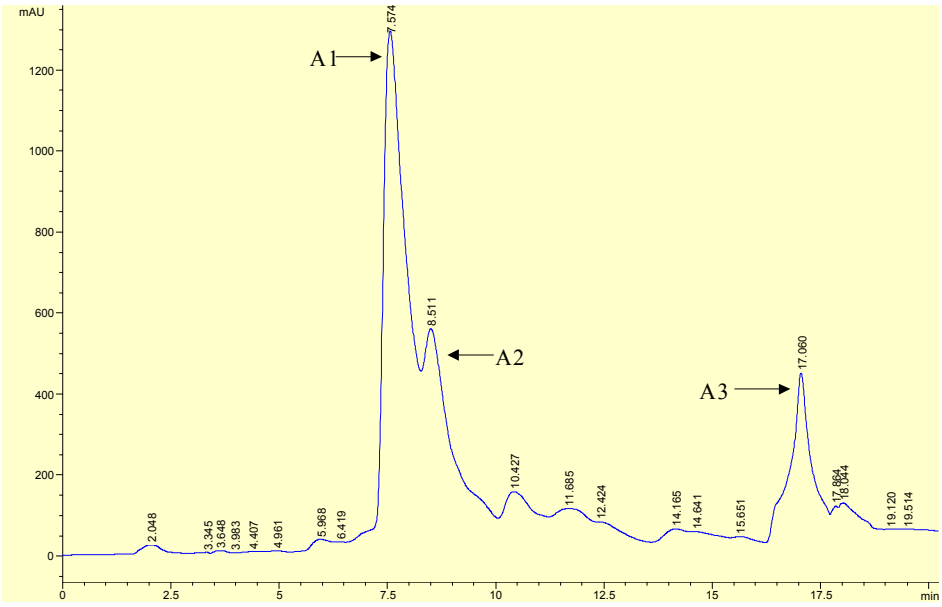



**Figure 3.3: Analytical HPLC profile**

The eluted peaks were divided into 3 groups and collected separately

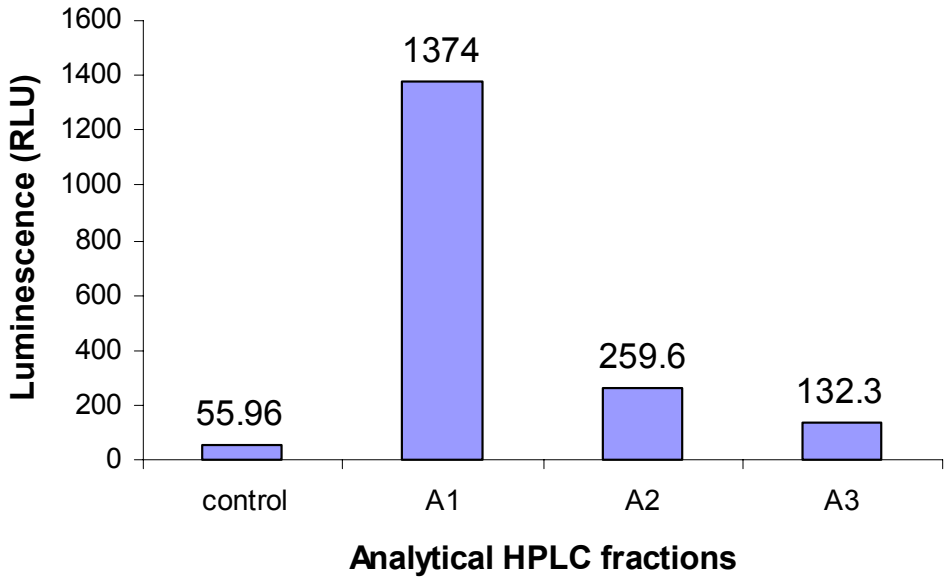



**Figure 3.4: Luminescence induced by analytical HPLC fractions**

### 3.3.2. Mass Spectrometry

MS analysis (Figure 3.5) of the A1 fraction yielded a parent peak of M+H ions 244. This matched the formula N-(3-hydroxyoctanoyl)-HSL and an ion at m/z 102 indicating the existence of a homoserine lactone moiety. The other peaks that displayed on the MS graph resulted from the loss of $C_2H_4$ (m/z 29), $C_3H_7$ (m/z 41-43), $C_4H_6O$ (m/z 70) and $C_6H_9O_2$ (m/z 113). The presence of the parent peak at m/z 244 and the classic m/z 102 peak corresponding to the homoserine lactone moiety, along with the intense fragments at m/z 144 indicating the 3-hydroxyoctanoyl moiety, leave little doubt that the quorum sensing signal isolated from *P. fluorescens* 5064 has the structure of N-3-hydroxyoctanoyl-homoserine lactone (Figure 3.5). The m/z 125 indicates a loss of water from the $C_8$-HSL side chain. This characteristically occurs during MS analysis of compounds with hydroxyl groups (Shaw *et al*. 1997).

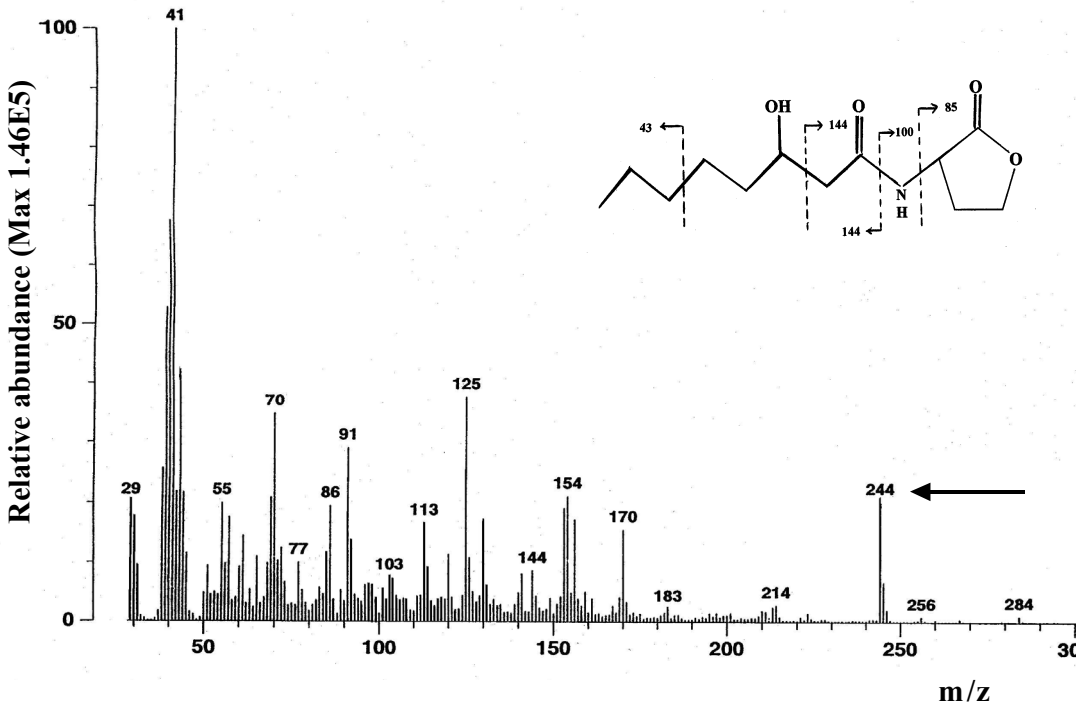



**Figure 3.5 Chemical identification of the quorum sensing signal in *P. fluorescens* 5064 by Mass Spectrometry**

The arrow shows the parent peak with m/z of 244.

## 3.4. Conclusion and discussion

The detection of AHL molecules has been greatly facilitated by the construction of *lux* or *lacZ*-based reporter fusions driven by specific LuxR homologues (Bainton *et al*., 1992a; Swift *et al*., 1993; Hwang *et al*., 1995; Shaw *et al*., 1997; Winson *et al*., 1998a,b). In a previous chapter (chapter 2), we discussed how both biosensor *E.coli* (pSB401) and *E.coli* (pSB1075) are able to detect the QSSs produced by *P. fluorescens* 5064, and how pSB 401 is much more sensitive than pSB1075 in this detection. We also found that the biosensor *Chromobacterium violaceum* CV026 does not show any response when supplied with QSS extract from *P. fluorescens* 5064. Since CV026 responds most sensitively to AHLs with 4-6 carbon acyl side chains, pSB401 prefers 6-8 carbon side chains, and pSB1075 detects compounds with 10-14 carbon side chains, the QSS produced by *P. fluorescens* 5064 should have 8-10 carbons in side chain length. By using HPLC and MS analysis, the QSS produced by *P. fluorescens* 5064 has been identified as acyl-hydroxyoctanoyl-homoserine lactone (Figure 3.5). Cha *et al.*(1998) pointed out that the CV026 biosensor could not detect any of the 3-hydroxy-derivatives of AHL. This explained why *P. fluorescens* 5064 AHL (which is a 3-hydroxy-derivative) did not induce any purple pigment production in CV026.

The acyl-hydroxyoctanoyl-homoserine lactone has been reported to be present in *P. fluorescens* 2-79 (biocontrol agent isolated from wheat roots) (Shaw *et al.*, 1997), but not in the strains F113, 7-14 and NCIMB 10586 (Dumenyo *et al*., 1998; Laue *et al*., 2000; Holden *et al*., 1999). It is possible that more than one QSS exists in *P. fluorescens* 5064, although we only identified one AHL so far. The other putative QSSs may not be in sufficient quantities to be detected by the biosensors we used in this study.

# Chapter 4. Cloning of the Tn*5* Disrupted Gene in m6423

## 4.1 The Vectorette Gene Cloning System

Since the biosurfactant deficient mutant 6423 (m6423) does not produce as much quorum sensing signal as the wild type and both synthetic AHL and AHL extracted from *P. fluorescens* 5064 can restore m6423 biosurfactant production, it can be argued that the Tn*5* disrupted gene in m6423 might play a role in the quorum sensing regulation system in *P. fluorescens* 5064.

In this chapter, we describe the attempts made to clone the Tn*5* flanking DNA in m6423 by employing vectorette genomic walking technique (Sigma-Genosys Ltd.). m6423 had only one copy of the Tn*5* insertion (chapter 2 and appendix M), which could make the gene cloning much easier to achieve.

Polymerase chain reaction (PCR) has revolutionised molecular biology since its original development in 1985 (Saiki *et al*., 1985). The ability to amplify specific fragments of DNA has found a wide range of applications in all aspects of life science research. Typically, PCR requires prior knowledge of the DNA sequence of the 5' and 3' ends of the fragment to be amplified. However, for a large number of applications, it is useful to be able to amplify DNA fragments where the sequence of only one end is known. Vectorette PCR is one of the methods capable of performing this process, as it allows amplification of an uncharacterized sequence adjacent to a known region.

The Vectorette system consists of three key steps (Figure: 4.1):

1. Digestion of a DNA sample with a restriction enzyme.
2. Ligation of the appropriate Vectorette adaptors to the restriction digested DNA fragments, generating a Vectorette library.

3. Performance of PCR using one primer (vectorette primer) that anneals to the vectorette adaptor and another primer (custom primer) that targets at the known DNA sequence. This allows for the *in vitro* amplification of a fragment of DNA located between the known sequence and the restriction site used to cut the target DNA.

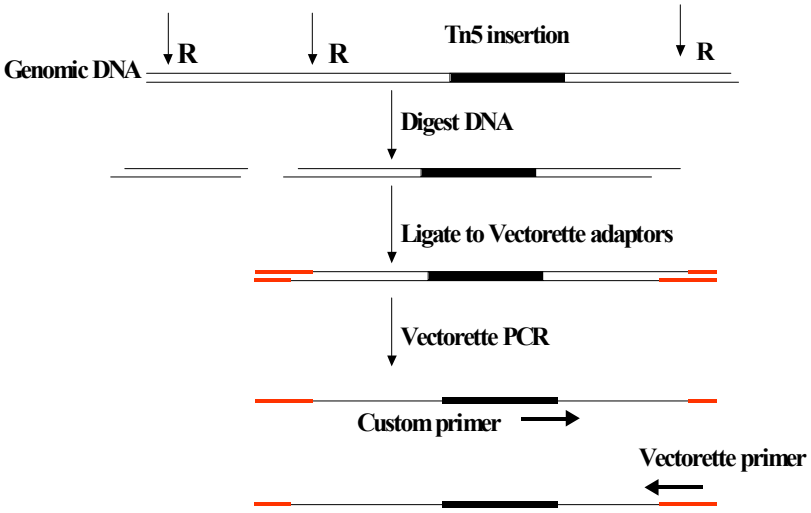



**Figure 4.1: Amplification of Tn*5* flanking DNA using the Vectorette genome walking technique**

In most of the gene walking experiments, the distance between the known sequence and the restriction site is not known. Hence it is necessary to make several libraries with different restriction enzymes to ensure amplification of an optimum sized PCR fragment, as well as differing sizes of PCR products containing the region of interest. The longer the PCR product, the farther away the custom primer sequence is from the restriction site. Sequences from the restriction site end of the longest PCR fragment can be used to design another primer for the next cycle of gene walking.

One feature of the sequence at the end of the vectorette adaptor is that, although it is able to ligate to a particular sticky/blunt end, the original restriction site is not re-formed

in the target-vectorette construct. This means that during vectorette library construction, there is no need to heat-inactivate the restriction enzyme before the ligation step. The self-annealed molecules will be cut by the restriction enzyme, which helps to increase the overall yield of desirable molecules (target-vectorette constructs).

## 4.2. Materials and Methods

### 4.2.1.Vectorette PCR to amplify the target DNA fragment

The Vectorette genome walking technique was employed in order to characterise the Tn*5* disrupted gene in m6423. The m6423 genome DNA was extracted and digested by restriction enzymes. The digested mutant 6423 DNA was then ligated with the cognate vectorette units to generate Vectorette libraries. The vectorette PCR was carried out by using the above Vectorette libraries as templates. Sometimes, to obtain a sharper PCR product band, a nested PCR is necessary. A nested PCR is a second round of PCR using primers internal to the primers used in the first round of PCR. It allows increased specificity and may produce cleaner bands.

4.2.1.1. Genome DNA extraction from mutant 6423

The mutant 6423 genome DNA was extracted and purified using the DNA purification kit by PUREGENE® (Gentra, Cat. No. D-6001) and the protocol is described in appendix F.

4.2.1.2. DNA detection by electrophoresis.

Gel, sample preparation and electrophoresis procedures were carried out according to the standard protocols (Sambrook *et al*., 1989) described in appendix G. For photo-documentation of the electrophoresis gel, the gels were placed onto a UV

transilluminator connected to a camera (Kaiser RS 1 Germany) and a gel documentation system ChemiImager 4400 v 5.5 (Alpha Innotech Corporation). Images were captured digitally, saved and printed on thermal paper.

Two methods were used for the quantification of nucleic acid samples:

a. Spectrophotometric method

The readings were performed using a spectrophotometer (Biophotometer, Eppendorf, Germany). This was used to derive not only information about the concentration of the sample, but also about its purity. Quantitation of nucleic acid samples by spectrophotometer relies on the fact that nucleic acids absorb light maximally at 260nm. This measurement permits the direct calculation of the concentration of nucleic acid in a sample:

[DNA] µg/ml=$A_{260}$ x 50 x dilution

[RNA] µg/ml=$A_{260}$ x 44.19 x dilution

Where: $A_{260}$ = absorbance at 260nm

50 = extinction coefficient of DNA

44.19 = extinction coefficient of RNA

Dilution = dilution factor of the sample

Calculation of the $A_{260}$: $A_{280}$ ratio provided a reasonable estimate of the purity of the preparation. A pure sample of DNA should have an $A_{260:}$ $A_{280}$ ratio of 1.8 ± 0.05; a pure RNA sample should have an $A_{260:}$ $A_{280}$ ratio of 2 ± 0.05.

b. Non-spectrophotometric method

This method involves the DNA quantification by gel analysis with the aid of an image analysis system. A mass ladder (High DNA Mass™ Ladder, Invitrogen, 4µl/appl, 520ng/4µl) is used for estimating the concentration of unknown DNA samples. This ladder consists of an equimolar mixture of six blunt-ended DNA fragments of 10, 6, 4,

3, 2 and 1kb. Electrophoresis of 4µl of the High DNA Mass Ladder results in bands containing 200, 120, 80, 60, 40 and 20 ng (520 ng total) of DNA, respectively. The amount of DNA or RNA sample is estimated by comparing the strength of fluorescence it produces with the fluorescence produced by the similar size of standard in the mass ladder (both run on the same gel).

4.2.1.3. Restriction enzyme digestion

The protocol for restriction enzyme digestion was as follows:
The following were combined in a sterile eppendorf tube.
a. 100 ng sample DNA in water or 1 x TE buffer,
b. 5µl of 10 x restriction buffer,
c. 0.1µl of *Eco*RI (GibcolBRL, 10 U/µl),
d. Sterile deionized water (SDW) was added to make up the reaction volume to 50 µl.
The sample was incubated at 37°C for at least 2 hours.
Digested fragments were analysed by agarose gel electrophoresis.

4.2.1.4.Ligation of the digested target DNA to the Vectorette units

5 µl of the corresponding Vectorette units was ligated to the digested ends of the DNA sample using 1 unit of $T_4$ DNA ligase (GibcoBRL®). ATP and DTT were added to a final concentration of approximately 2mM. This reaction was carried out in the same tube of the restriction enzyme digest since most restriction buffers are compatible with that for $T_4$ DNA ligase.

The ligation protocol was as follows:
To the tube containing the restriction digest, the following reagents were added:
a. 5µl Vectorette units (3 pmol)
b. 1µl 100mM ATP

c. 1µl 100mM DTT

d. 1 unit T4 DNA ligase (GibcoBRL, 1U/µl),

The eppendorf tube was incubated at 20°C for 60 min followed incubation at 37°C for 30 min and this procedure was repeated three times. A thermo-block (Biometra) was used to perform this incubation procedure. The resulting Vectorette libraries were then stored at -20°C.

4.2.1.5. Vectorette PCR

(1). The following were combined in a sterile eppendorf tube:

1µl of the Vectorette library as template

10 µl of 10 x PCR buffer [Taq polymerase buffer with $MgCl_2$ (25Mm), Q. BIO gene]

50µM (final concentration) of each dNTPs (GibcoBRL)

100 pmol of universal Vectorette Primer

100 pmol of custom primer

1 µl of Taq DNA polymerase (Q.BIO gene, 5U/µl)

Reaction was made up to 100 µl with SDW

Appropriate negative controls for this experiment included a reaction with no template, a reaction with only the vectorette PCR primer and a reaction with only the gene specific custom primer.

(2). Custom PCR primers design

Since the target gene in mutant 6423 is disrupted by Tn*5*, the first custom primer (Hozbor) and nested primer were designed according to the Tn*5* IS50R transposase gene sequence (Hozbor *et al*.,1998)(Figure 4.2).

(3). PCR conditions were as follows:

a. 94°C for 4 min

b. 94°C 1 min→ 55°C for 1 min→ 72°C for 1 min (35 cycles).

c. 72°C for 10 min

(4). PCR products were analysed on a 1.5% agarose gel


**Figure 4.2: Custom primers designed for the vectorette PCR**

4.2.1.6. Nested PCR

It was often observed that there were faint bands or no bands at all after the initial vectorette PCR. Therefore, nested PCR was performed in order to obtain the desirable band at a higher concentration. The nested primer is internal to the Hozbor custom primer (Figure 4.2).

The nested PCR was prepared as follows:

1 µl (of a 1x, 10x and 100x dilution) of the primary PCR product

10 µl of 10 x PCR buffer [Q.BIO gene, with $MgCl_2$(25mM)]

50µM (final concentration) of each dNTPs (GibcoBRL)

100 pmol of nested Vectorette Primer (included in the kit)

100 pmol of nested custom primer

Similar PCR conditions as described for the initial vectorette PCR were used and the same control experiments were set up. The nested PCR product was analysed by electrophoresis.

### 4.2.2. Cloning of the vectorette PCR product

4.2.2.1. Gel Purification

In this experiment, a QIAquick gel extraction kit (QIAGEN, Germany) was used to purify the vectorette PCR product from the gel. This kit is designed to extract and purify DNA 70bp to 10kb in size from standard or low-melt agarose gels in TAE or TBE buffer. Detailed procedures are described in appendix H.

4.2.2.2.Cloning the PCR product into pCR®4-TOPO® Vector

A TOPO TA cloning kit (Invitrogen Co.) was used to clone the vectorette PCR product. The Taq polymerase used in Vectorette PCR has a non-template dependent terminal transferase activity, which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector (pCR®4-TOPO®) (Figure 4.3) supplied in this TA cloning kit has single, overhanging 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently to the vector.

The gel purified vectorette PCR product was ligated into pCR®4-TOPO®.
The ligation reaction (6µl) was set up as follows:

| Samples | Amount |
|---|---|
| Fresh PCR product | 0.5 to 4 µl |
| Salt solution (provided in kit) | 1µl |
| pCR®4-TOPO® Vector | 1µl |

The reaction was mixed gently and incubated for 15 min at room temperature, then stored on ice for 30 min before the transformation procedure.

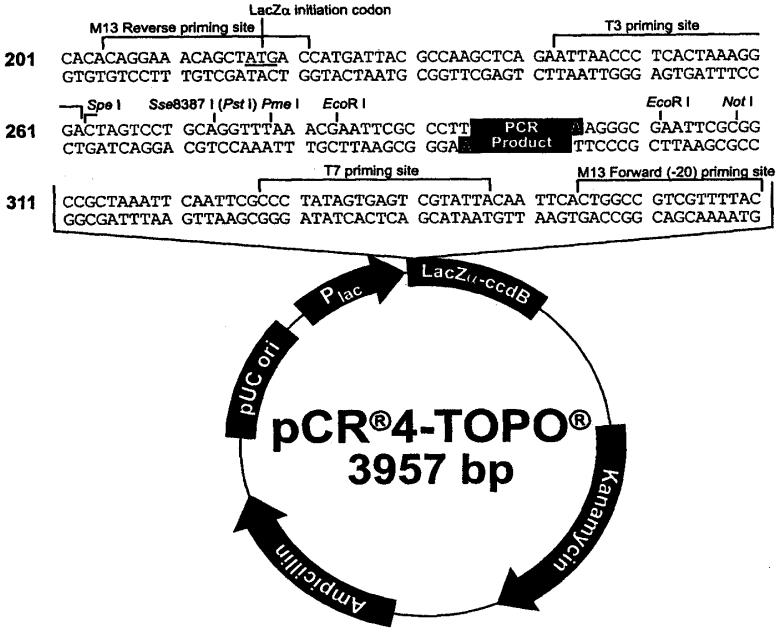



**Figure 4.3: Map of the linearized vector pCR®4-TOPO®**

The map shows the features of pCR®4-TOPO®and the sequence surrounding the TOPO®cloning site. Restriction sites are labeled to indicate the actual cleavage site.

4.2.2.3. Transformation

Transformation was carried out using the standard protocol (Sambrook *et al*., 1989) and the procedures are described in Appendix I. TOP10 OneShot® chemically competent *E. coli* (which were included in the TOPO TA cloning kit) were used and plated on selective LB plates containing 100µg/ml ampicillin or 50µg/ml kanamycin.

4.2.2.4. Analysis of positive colonies by PCR

pCR®4-TOPO vector contains a M13 reverse priming site, a T3 priming site, a M13 forward priming site and a T7 priming site (Figure 4.3), so it is possible to use one pair of these primers to analyze and select the positive transformation colonies.

The PCR detection procedure was as follows:

(1).For each PCR tube, these reagents were added:

1.2 units of Taq polymerase (Promega, Co.)

5µl PCR buffer (Promega, Co.)

5µl dNTPs (2mM)

0.5µl M13 forward primer (1µg/µl)

0.5µl M13 reverse primer (1µg/µl)

and SDW was used to make up total volume to 50µl.

(2). 10 colonies were picked from the transformation plates and resuspended individually into each PCR reaction tube.

(3). The reactions were incubated for 10 min at 94°C to lyse the cells and inactivate nucleases.

(4). Reactions were amplified for 20 to 30 cycles (94°C for 1min; 55 °C for 1min and 72 °C for 1min).

(5). For the final extension, reactions were incubated at 72°C for 10min and then held at 4°C.

(6). PCR products were analyzed by agarose gel electrophoresis.

### 4.2.3. Sequencing of vectorette products

4.2.3.1 Plasmid purification

Plasmid purification was carried out from liquid cultures of the positive colonies obtained from the transformation described above. Plasmids containing amplified vectorette products were purified by a High Pure Plasmid Isolation Kit (Roche Diaghostics Co., Germany). A detailed protocol is attached in Appendix J.

4.2.3.2. Restriction enzyme digestion of the plasmids

Before sequencing the insert, it was necessary to check that the pCR®4-TOPO® Vector contained the correct DNA fragment. Since pCR®4-TOPO® Vector has *Eco*RI restriction sites on both sides of the insertion (Figure 4.3), we digested the plasmids with *Eco*RI. The *Eco*RI digestion should release the target DNA fragment from the vector and make it visible on an agarose gel after electrophoresis.

The restriction enzyme digestion was carried out as follows:
The following reagents were added to a microcentrifuge tube:
a. 2-5 µl of plasmid DNA (depending on the concentration)
b. 2.5 µl of 10 x *Eco*RI enzyme buffer
c. 1 µl of restriction enzyme *Eco*RI (GibcoBRL, 10u/µl)
SDW was used to make up total volume to 25 µl
The reaction was incubated at 37 °C for 4-5 hours and visualized under UV light after agarose gel electrophoresis and ethdium bromide staining.

4.2.3.3. Sequencing

Plasmids of interest were sent to the MBSU DNA sequencing service, University of Glasgow for sequencing of the DNA insert.

## 4.3. Results

### 4.3.1. Vectorette PCR

4.3.1.1. Genomic DNA extraction of mutant 6423

For the extraction of genomic DNA of mutant 6423, DNA at a concentration of 713 ng/µl (detected by spectrophotometer, Biophotometer, Germany) was obtained and visualized on an agarose gel (0.8%) (Figure 4.4).

4.3.1.2. Restriction enzyme digest of m 6423 genome DNA

To obtain different Vectorette libraries, genomic DNA from mutant 6423 was digested with *Eco*RI, *Bam*HI and *Hin*dIII respectively. The digestion results were visible on an agarose gel (0.8%)(Figure 4.5).

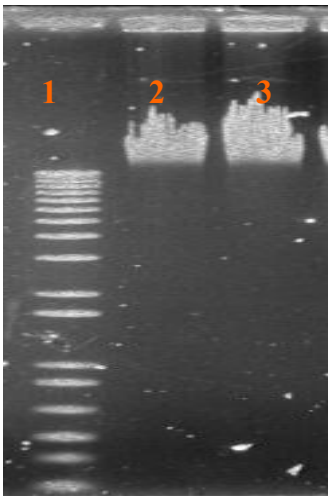



**Figure 4.4: Mutant 6423 genomic DNA**

**Lane 1: 1kb plus ladder (GibcoBRL, 1µl loaded at 1µg/µl)**
**Lane 2: Mutant 6423 genome DNA (1µl)**
**Lane 3: Mutant 6423 genome DNA (2µl)**

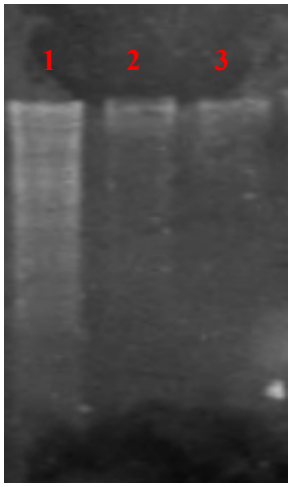



**Figure 4.5: Restriction enzyme digestion of m 6423 genomic DNA**

**Lane 1: 6423 DNA digested with *Eco*RI**
**Lane 2: 6423 DNA digested with *Bam*HI**
**Lane 3: 6423 DNA digested with *Hin*dIII**

4.3.1.3. Vectorette PCR

Vectorette PCR was carried out using the Vectorette Libraries as templates. Agarose gel electrophoresis (1.5% agarose gel, TBE buffer, 100 V) showed that a 300bp PCR fragment was present when using *Bam*HI library as template, whilst the same band could not be observed in the negative controls (Figure 4.6).

4.3.1.4. Nested vectorette PCR

Since the positive band (300bp) obtained by the first PCR was very faint, a nested PCR was carried out in order to obtain a larger quantity of sample. A sharper PCR band was obtained after nested PCR was performed (Figure 4.7).

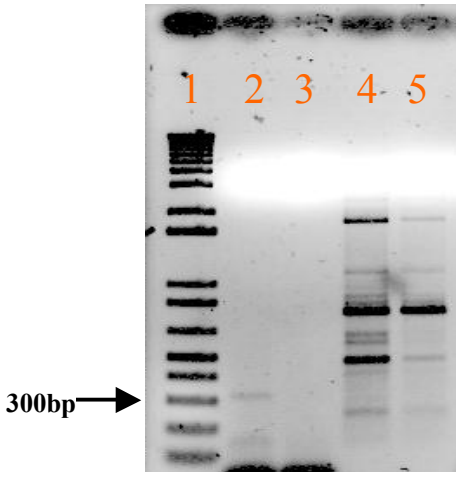



**Figure 4.6: Vectorette PCR**

**Lane 1: 1Kb Plus Ladder**
**Lane 2: Vectorette PCR showed a positive band (300 bp)**
**Lane 3: PCR control with no template**
**Lane 4: PCR control with no custom primer**
**Lane 5: PCR control with no Vectorette primer**

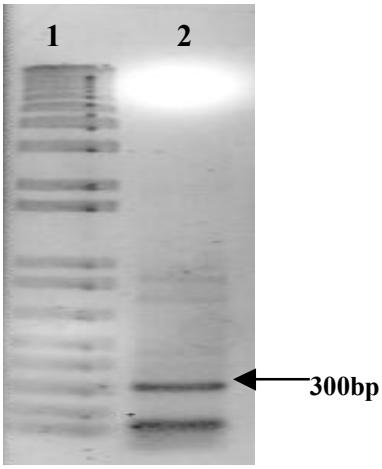



**Figure 4.7: Nested PCR**

**Lane 1: 1Kb plus ladder**
**Lane 2: The nested PCR band (300 bp) of interest is shown by the arrow**

### 4.3.2. Sub-cloning of Vectorette PCR product

The 300bp fragment was purified from the gel and ligated into the pCR®4-TOPO®vector. The vector was used for transformation of TOP10 *E. coli* competent cells. PCR detection, using M13 forward and M13 reverse primers, was carried out in order to select positive colonies. Two colonies, colony 3 and colony 5, showed bands of the expected size (Figure 4.8).

### 4.3.3. Sequencing of Vectorette PCR product

Plasmid purification was performed from liquid cultures of colony 3 and 5. To confirm that the resulting plasmids contained the 300bp target band, we digested the plasmids with *Eco*RI restriction enzyme. The 300bp bands were released from the vectors (Figure 4.9). Sequencing analysis of the plasmid from colony 3 revealed a 323bp sequence of the "Tn*5*-disrupted DNA fragment" from mutant 6423. This DNA fragment was named PF6423-3 (Figure 4.10).

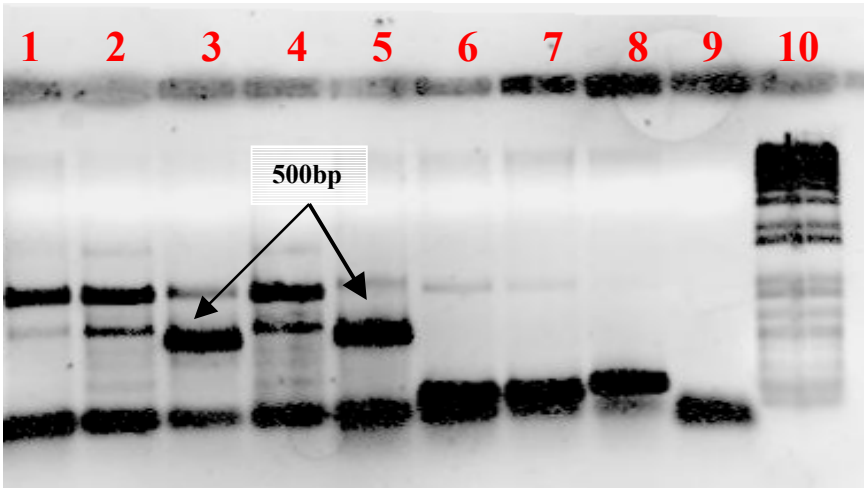



**Figure 4.8: PCR Detection of Colonies**

Lane 3 and lane 5 showed the positive 500bp bands, which correspond to the 300 bp insert plus vector sequence between the two M13 primers.
Lane 9: Negative control (no template PCR)
Lane 10: 1Kb plus ladder

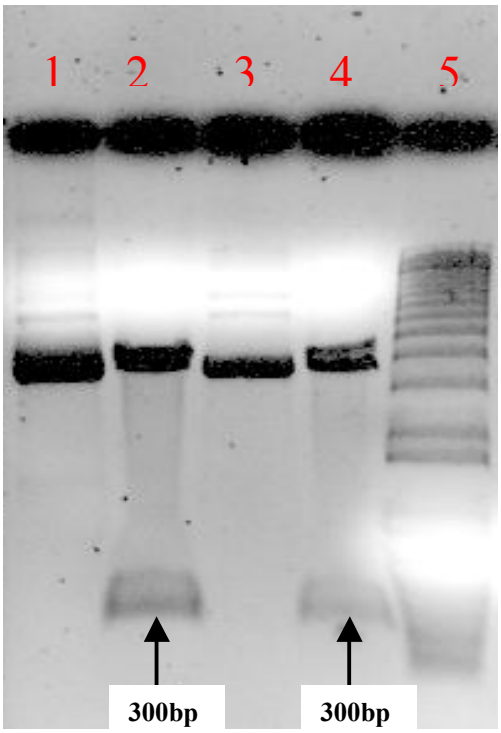



**Figure 4.9: Plasmid and Plasmid *Eco*RI Digestion**

Lane 1: Purified plasmid 3 ( from colony 3)
Lane 2: *Eco*RI digest of plasmid 3
Lane 3: Purified plasmid 5 (from colony 5)
Lane 4: *Eco*RI digest of plasmid 5
Lane 5: 1 Kb plus ladder

**5'CACATGGAAGATCAGATCCTGGAACAACCGCTCGGCCCTACGAATC**
**ACCGCGTCCTCGGCCTCGTAGGACAAGTCGGTGTCGAAGTAGAAAAC**
**GTCGTAGTCCTATACGCCCTGCGCGGCAGGAAGATTGCACTGGTGAT**
**TCCACACCGCCTGGAACAGACACCCCGCCGTGAGCATGCACTGCTCC**
**ACACCGAGGTCGGGCAGGCGCTTGGTCATTTCAGCATTGATGGGGTT**
**GGCCATGGCANGGTGGAGCAAGGTGTCGACGGTCAAGGTCATGGGG**
**ATCGCAGGACAACCCCATGAAGCTTGAATTCGGATCCACGTTG 3'**

**Figure 4.10: The DNA sequence of the cloned 323bp gene fragment (PF6423-3)**

The Custom primer sequence is shown in red colour (24bp)
The Vectorette primer DNA sequence is shown in blue colour (24bp)
The sequence of the cloned gene fragment is shown in black colour (275bp)

### 4.3.4. Second run of Vectorette PCR

The result of the DNA data analysis of the 323 bp Tn*5* disrupted gene fragment was insufficient to determine the gene function. Further gene walking was therefore needed.

We designed a new custom primer (PF6423VEC) annealing to the 3' end of the 323 bp sequence obtained in the first run vectorette PCR using the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer) (Figure 4.11).

**5'CAACCGCTCGGCCCTACGAATCACCGCGTCCTCGGCCTCGTAGGAC**
**AAGTCGGTGTCGAAGTAGAAAACGTCGTAGTCCTATACGCCCTGCGC**
**GGCAGGAAGATTGCACTGGTGATTCCACACCGCCTGGAACAGACACC**
**CCGCCGTGAGCATGCACTGCTCCACACCGAGGTCGGGCAGGCGCTTG**
**GTCATTTCAGCATTGATGGGGTTGGCCATGGCANGGTGGAGCAAGGT**
**>>**
**GTCGACGGTCAAGGTCATGGGGATCGCAGGACAACCCCATG 3'**
**>>>>>>>>>>>>>>>>>>>>>>>>**

**Figure 4.11: Custom primer (PF6423VEC) for the second run of vectorette PCR**

The sequence in black colour shows the 323bp gene fragment we identified in the first run of vectorette PCR. The 20nt custom primer that was designed for the second run of vectorette PCR is shown in red colour.

All the methods for the second run of vectorette PCR were the same as described before except for the vectorette PCR template. Since *Bam*HI restriction enzyme Vectorette Library was used for the first run of vectorette PCR, a different restriction enzyme Vectorette Library (the *Eco*RI library) was chosen to perform the second run of vectorette PCR.

4.3.4.1. Vectorette PCR using *Eco*RI Vectorette library

A positive band about 550 bp was obtained in the vectorette PCR reaction, but not in the negative control reactions (Figure 4.12). This 550bp band was purified from a 1.5% agarose gel for further transformation (Figure 4.13).

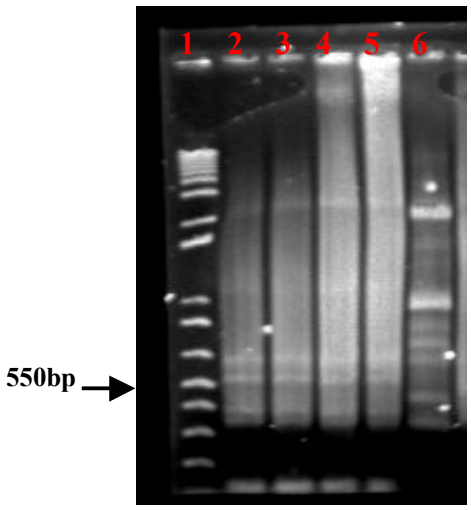



**Figure 4.12: Vectorette PCR**

Lane 1: 1Kb plus ladder
Lane 2-5: Vectorette PCR reactions
Lane 6: Control reaction (without custom primer)
Lane 7: Control reaction (without Vectorette primer)

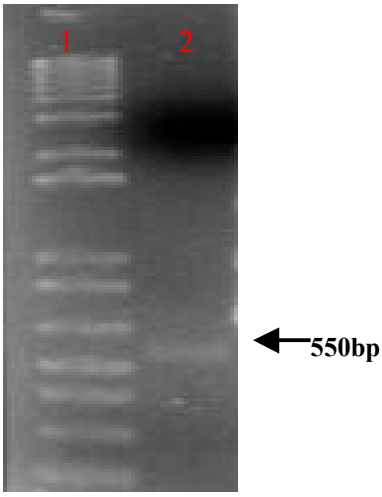



**Figure 4. 13: Gel purification**

Lane 1: 1Kb plus ladder
Lane 2: The 550bp band was purified from the total Vectorette PCR products and showed by arrow.

4.3.4.2. Sub-cloning and transformation

The 500 bp band was cloned into the pCR®4-TOPO® vector and then used to transform TOP10 *E.coli* competent cells. 25 colonies were screened by PCR using either M13 forward-M13 reverse or PF6423VEC-vectorette universal primers. Two colonies, colony 2 and colony 13, exhibited a distinct band with both primer pairs (Figure 4.14).

4.3.4.3. Plasmids purification and *Eco*RI digestion

The two positive colonies were sub-cultured into selective LB broth (containing Ampicillin 100µg/ml). Bacterial cells harvested from these cultures were used for plasmid purification. The purified plasmids, named plasmid 2 and plasmid 13, were digested by *Eco*RI. A 550 bp insert was released from both plasmids after *Eco*RI digestion (Figure 4.15).

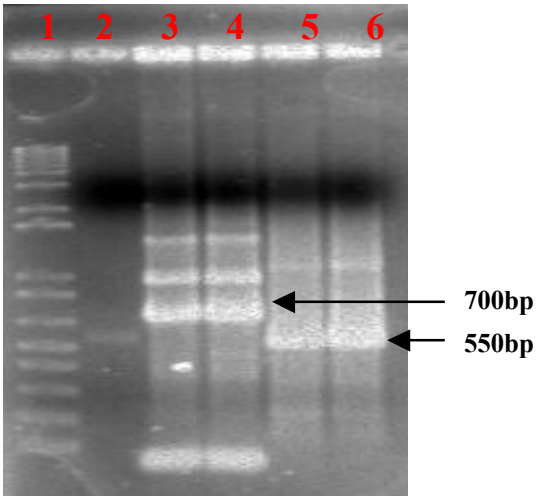



**Figure 4.14: PCR detection of the colonies**

Lane 1: 1Kb plus ladder
Lane 2: the original 550 bp fragment
Lane 3: PCR of colony 2 by M13 forward and reverse primers
Lane 4: PCR of colony 13 by M13 forward and reverse primers
Lane 5: PCR of colony 2 by PF6423VEC and vectorette primers
Lane 6: PCR of colony 13 by PF6423VEC and vectorette primers

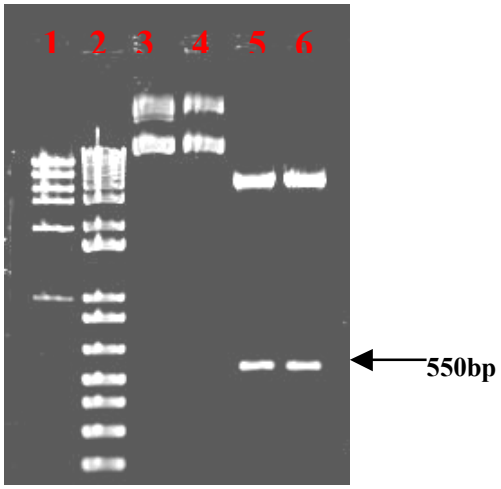



**Figure 4.15. Plasmid purification and *Eco*RI digestion**

Lane 1. Mass ladder (520 ng/4µl)
Lane 2. 1 kb plus ladder
Lane 3. Plasmid 2
Lane 4. Plasmid 13
Lane 5. Plasmid 2 *Eco* RI digestion
Lane 6. Plasmid 13 *Eco*RI digestion

4.3.4.4. Sequencing

Plasmid 2, carrying the 550 bp Vectorette PCR product, was sequenced. Both PF6423VEC and the vectorette primer were contained in the new sequence. However, the new sequence did not have any identity to the sequence from which we designed the custom primer. The 323 bp obtained from the first run of vectorette PCR did not have the remaining Tn*5* sequence either (Figure 4.16). These results indicated that the gene fragments cloned were unreliable and an alternative method should be performed.

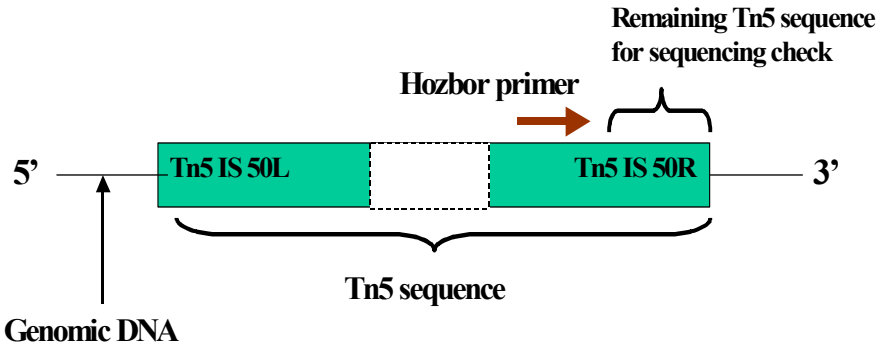



**Figure 4.16. Figure shows the remaining sequence for sequencing check of the cloned gene (not drawn to scale)**

## 4.4 Discussion

The primers used for vectorette PCR were designed to anneal 20-30bp downstream of the 3' end of either the Tn*5* transposase sequence or the PF6423-3 sequences. The 20-30bp ('remaining sequence')(Figure 4.16), which were designed to be used for sequencing check, should be contained in the cloned gene sequences. Although nearly 900bp in length of the target gene has been cloned, we could not identify the 'remaining sequence'. We therefore could not confirm that the DNA sequence obtained by Vectorette PCR belonged to the gene disrupted by Tn*5* in m6423. The PF6423-3 fragment was cloned by a technician and the second run vectorette PCR was carried out by myself. Both the sequences obtained by vectorette PCR encountered the same problem.

## 4.5. Complementary data added to Chapter 4

Before performing the second run of vectorette PCR, a short cut attempting to clone the Tn*5* disrupted gene in m6423 was tried. In collaboration work with Bogor Agricultural University, Indonesia, a partial DNA library of *P. fluorescens* 5067 had been constructed containing 50 recombinants that could be hybridized by the Tn*5* flanking DNA (300bp) cloned in mutant 6426 using the vectorette gene cloning kit. Because m6426 and m6423 have very similar restriction patterns (both *Eco*RI and *Sal*I) (Darling, 1998), we thought it might worth trying to see if the Tn*5* flanking sequence in mutant 6423 (PF6423-3), when used as a probe, could hybridize with any of the colony in the partial DNA library of *P. fluorescens* 5067.

(1) Plasmid purification and *Eco*RI digestion

The colonies 19 and 20 were chosen from the partial DNA library and used for plasmid purification (see appendix J). The plasmids were digested by *Eco*RI to release the insert (4.8kb) (Figure 4.17).

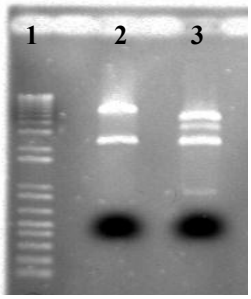



**Figure 4.17: *Eco*RI digestion of plasmid 19 and 20**

Lane 1: 1 kb plus ladder
Lane 2: Plasmid 19 digestion showed 4.8kb insertion
Lane 3: Plasmid 20 digestion

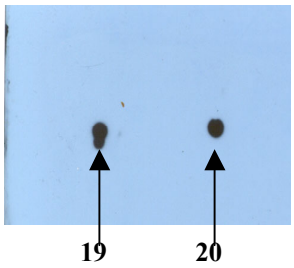



**Figure 4.18: High stringency hybridisation of colony 19 and 20 using PF6423-3 as probe**

(2) Probe labeling by PCR

PCR (using *dig-dNTPs) was performed in order to label the PF6423-3 sequence using NEBlot® Kit (New England Biolab, UK). The labeled PF6423-3 was used as a probe to hybridise clones 19 and 20.

(3). Hybridisation

The detailed hybridisation method (high stringency) is described in Appendix K.

(4). Detection method

The detailed detection method is described in Appendix L. The dot blot results are shown in Figure 4.18. Both colonies were positive.

(5). Sequencing

The 4.8kb fragment cloned in colony 19 was sequenced. The DNA data analysis showed that the longest ORF is an AMP-binding protein. Surprisingly, we could not locate the original PF6423-3 fragment (the probe) from the 4.8kb gene sequence, indicating that the cloning strategy was unsuccessful. We therefore could not draw any conclusion as to whether the AMP-binding protein is involved in either biosurfactant production or quorum sensing pathways. The possible reason for having such a false positive result in the dot blot hybridisation might be due to the unreliability of the CDP-star detection system (Roche, German). Controls (both positive and negative) should have been used to eliminate the false results.

Inverse PCR, which is often used to clone an unknown DNA sequence that flanks a core region of known sequence, was also tried in order to clone the Tn*5* disrupted gene in m6423. DNA containing the core region is digested with appropriate restriction enzymes to produce a fragment of suitable size for PCR amplification. The ends of the fragment are then ligated to form a circular molecule. Primers for PCR are homologous to the ends of the core region included within the circle, but oriented such that chain elongation proceeds across the uncharacterized region of the circle rather than across the core region separating the primers (Ochman *et al*., 1988; Triglia *et al*., 1988; Silver and Keerikatte, 1989). Unfortunately, the Inverse PCR also failed to clone the target gene and the m6423 Tn*5* flanking DNA remains unknown. Both Vectorette PCR and Inverse PCR are generally effective in cloning unknown genes but failed to clone the Tn*5* flanking DNA in *P. fluorescens* 5064 mutant 6423. A possible reason could be that the unknown gene product is harmful to the *E. coli* host, which has been used as cloning vector. Therefore, changing gene cloning vector or using a low copy vector might be an alternative solution.

# Chapter 5. Biocontrol of Broccoli Head Rot Disease

## 5.1. General introduction – fluorescent *Pseudomonas* species in biological control

Biological control, based on using a beneficial microorganism to control the activity of undesirable invasive species, is a powerful management tool that has proven effective at controlling numerous invasive species (Burr and Caesar, 1984). Biological control agents for plant disease are currently being examined as alternatives to synthetic pesticides due to their increased level of safety and minimal environmental impact. Three major mechanisms of biological control of phytobacterial disease include (1) induced resistance in the host; (2) competition or exclusion of the pathogen from the infection site and (3) biological control via antibiotics, bacteriocins and bacteriophages (Moore, 1981).

Bacterial strains from the *Pseudomonas fluorescens-putida* group have drawn considerable attention as biological control agents since they are widely occurring, usually non-pathogenic, and highly effective at eliminating competing microbes (Burr *et al*., 1978; Schroth and Hancock, 1983; Weller and Cook, 1983).

*Pseudomonas* spp have been reported to reduce both damping-off caused by *Pythium*, *Rhizoctonia solani* and *Sclerotium rolfsii*, and root rot caused by *Fusarium oxysporum*, *Sclerotium rolfsii* and *Thielaviopsis basicola* in several vegetable crops when applied as a seed treatment or a soil drench (Punja, 1997). Biopriming pearl millet seeds with *P. fluorescens* resulted in improved growth of the plants and also induction of resistance against downy mildew disease caused by the fungus *Sclerospora graminicola* (Raj *et al*., 2004). *P. fluorescens* 2-79 RN is capable of controlling wheat take-all disease caused by the ascomycetous fungus *Gaeumannomyces graminis* var. *tritici* (Weller and Cook, 1983). Elvira-Recuenco and van Vuurde (2003) described the potential use of *P.*

*syringae* pv. *pisi* as a biocontrol agent to control pea bacterial blight. In greenhouse experiments, *P. fluorescens* J3 was proved to be able to protect tomato against *Ralstonia solanacearum* and reduced bacterial wilt (Guo *et al*., 2004). A recent study showed that *P. fluorescens* can significantly reduce the plant root-knot disease caused by the nematode *Meloidogyne javanica* (Siddiqui and Shaukat, 2003).

Among the mechanisms which have been suggested for the microbial control of plant pathogens by *Pseudomonas* spp. are antibiotics, enzymic lysis, competition for space and nutrients and the production of siderophores (Campbell and Faull, 1979; Hornby 1979; Howell and Stipanovic, 1979; Kloepper *et al*., 1980). Iron-chelating compounds (siderophores) have both fungistatic and bacteriostatic effects in the laboratory under low-iron conditions. Under field conditions, these iron chelators are thought to contribute directly to the suppression of plant disease by the biocontrol agent. Indirectly, siderophores may play an important role in the general persistence and survival of biocontrol *Pseudomonas* strains by facilitating iron uptake to support metabolism in iron-limiting environments (Dowling and O'Gara, 1994). Siderophores are produced by many microorganisms for iron uptake since iron is not readily available due to the poor solubility of iron oxides and hydroxides formed in the environment. Apart from siderophores, pseudomonads produce a range of iron-chelating compounds, including salicylic acid, pyochelins, fluorescent pseudobactins and pyoverdines. Buysens *et al*. (1996) stated that the production of either pyoverdine or pyochelin was necessary for the wild-type level of protection against *Pythium*-induced post-emergence damping-off.

In addition to producing iron-chelating compounds, *Pseudomonas* spp. produce an array of low-molecular-weight metabolites, some of which are potent antimicrobial agents. Important metabolites include phenazines, pyoluteorin, pyrrolnitrin and the diacetylphloroglucinols as well as the complex macrocyclic lactone, 2,3-de-epoxy-2,3-didehydra-rhizoxin (Keel *et al*., 1992; Shanahan *et al*., 1992; Thomashow and Weller, 1992; Ligon *et al*., 2000). Direct killing or inhibition of the pathogen by the metabolite

has been observed in laboratory experiments, and there are a number of studies indicating a direct effect of antimicrobial metabolites on pathogen number in soil (Kloepper *et al*., 1981; Bull *et al*., 1991). 2,4-diacetylphloroglucinol (Phl) has been shown to be largely responsible for the prevention of damping-off caused by *Pythium ultimum*) of sugar beet (Shanahan *et al*., 1992) and responsible for the control of black root rot of tobacco (caused by *Thielabiopsis basicola*), although in the latter case hydrogen cyanide (HCN) also contributes to biocontrol (Voisard *et al*., 1989; Keel *et al.*, 1992). The antimicrobial metabolites 2,4-diacetylphloroglucinol and pyoluteorin of *P. fluorescens* CHA0 have been reported to contribute to plant root-knot disease control caused by *Meloidogyne javanica* (Siddiqui and Shaukat, 2003). *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 are capable of controlling foot and root rot of tomato caused by the fungus *Fusarium oxysporum*. The antifungal metabolite phenazine-1-carboxamide (PCN) is believed to be essential in the biocontrol mechanism (Bolwerk *et al*., 2003). Pyrrolnitrin is active against *Rhizoctonia spp*, *Fusarium spp*, and other plant pathogenic fungi and it has been used as a lead structure in the development of a new phenylpyrrole agricultural fungicide. In addition, pyrrolnitrin has been used for years as a model for the study of the mechanisms involved in the chlorination of organic molecules (Ligon *et al*., 2000).

Biological control agents may be genetically engineered as well as naturally occurring strains. Delany *et al.* (2001) evaluated the biocontrol efficacy of a genetically modified *P. fluorescens* F113 strain with enhanced 2,4-diacetylphloroglucinol production. The GM strain proved to be as effective at controlling damping-off disease as the appropriate fungicide treatment. Genetically modified *P. fluorescens* variants producing phenazine-1-carboxylic acid significantly reduced damping-off disease of pea seedlings caused by *Pythium ultimum*, even under conditions of heavy soil infestation (Timms-Wilson *et al.,* 2000). It is important that the large-scale introduction of antifungal metabolite-producing strains into the environment, for agricultural purposes, affects only the target pathogen and presents little risk to the beneficial microflora. There is evidence that the production of the antifungal compound

phenazine by *P. aureofaciens* contributes to the ecological fitness of the producing strain in wheat rhizosphere microcosm experiments (Mazzola *et al*., 1992). By contrast, in experiments with near-isogenic strains of *P. fluorescens* F113, where Phl producers were compared with non-producers following co-inoculation in sugar beet rhizosphere microcosm, it was found that even after 10 simulated cropping and resowing cycles over a total of 270 days, there was no significant advantage to the Phl producer under the experimental conditions (Carroll *et al*., 1995). These data suggest that the long-term impact on the target ecosystem following introduction of Phl producers is likely to be minimal. It is important to ensure that a particular agent is not ice nucleation active or itself phytopathogenic. The use of genetically engineered antagonists has implications for the introduction and spread of new genes in the general microflora, with unknown and possibly unpredictable results. However, in spite of these limitations, biological control is generally considered a more ecologically acceptable approach when compared to chemical control and it is more suited to modern concepts of integrated pest management (IPM) (Sigee, 1993).

Furthermore, the application of a bacterial antagonist has even been reported to increase plant growth yield of mature celery, cucumber, onion and tomato under both glasshouse and field conditions. For example, McCullagh *et al*. (1996) reported that applying *P. fluorescens* increased the growth of cucumber plants and their fruit, and provided a yield increase of 18% compared with untreated plants infected with *Pythium aphanidermatum*. Whilst in field trials, wheat yields have been increased by 27% by inoculation of seeds with *P. fluorescens*, and similar treatment of potato seed tubers has been found to increase tuber yields by as much as 70% (Lindsey and Jones, 1989).

Although Pseudomonads have been proved to have strong biocontrol activities against soil-borne disease, there are limited studies of biocontrol in aerial disease and most of them have been demonstrated under greenhouse and laboratory conditions. Sleesman and Leben (1976) showed that *P. cepacia* inhibited germination of *Bipolaris maydis* and controlled leaf blight in greenhouse tests when applied on corn seedlings (sprayed with

an aqueous suspension of the biocontrol bacteria). In a substance test, *P. cepacia* was found to inhibit germination of pathogenic *Alternaria alternata* at $10^7$ antagonist cells/ml (Spurr, 1978). Biological control of foliar disease is a highly desirable tactic to add to currently used disease control methods. Most antagonists studied so far have been isolated from the phyllosphere and the techniques used to select antagonists are not fully defined, and potentially effective candidates may be missed (Spurr, 1981).

## 5.2. Identification of the antibiotic metabolite overproduced in m6418

### 5.2.1. Introduction

*P. fluorescens* has been reported to produce a diverse array of antibiotics, which are largely responsible for the biocontrol effectiveness of this bacterium. The major and most important antimicrobial metabolites are phenazines (Thomashow and Weller, 1992), pyrrolnitrin (Howell and Stipanovic, 1979; Pfender *et al*., 1993), pyoluteorin (Howell and Stipanovic, 1980), 2,4-diacetylphloroglucinol (Nowak-Thompson *et al*., 1994; Shanahan *et al*., 1992). The structures of these antibiotics are shown in Figure 5.1.

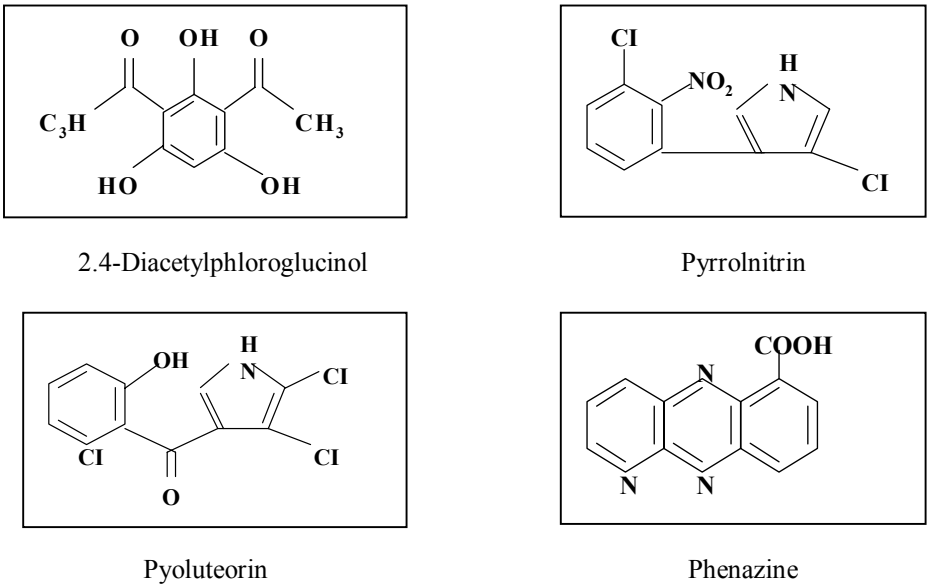



**Figure 5.1: Structure of antibiotics naturally produced by *Pseudomonas spp*.**

**2,4-diacetylphloroglucinol (Phl):**

Phl is a broad-spectrum antibiotic that exhibits antifungal, antibacterial, antihelmenthic and phytotoxic activities (Vincet *et al*., 1991; Bangera and Thomashaw, 1996; Abbas *et al*., 2002; Keel *et al*., 1992; Nowak-Thompsan *et al*., 1994; Reddi *et al*, 1969; Levy *et al*., 1992; Harrison *et al*., 1993). According to a few investigators, Phl is a major

determinant in the biocontrol activity of *P. fluorescens* CHA0 against black root rot of tobacco caused by *Thielaviopsis basicola*, take-all of wheat caused by *Gaeumannomyces graminis* var *tritici* and *P. fluorescens* F113 against damping-off of sugar beet caused by *Pythium ultimum* (Raaijmakers and Weller, 1998; Defago and Haas, 1990).

**Pyrrolnitrin (Prn):**

Pyrrolnitrin is a broad-spectrum antifungal metabolite produced by many strains of the genus *Pseudomonas* (Arima *et al*., 1964; Hamill *et al*., 1967; Elander *et al*., 1968; Howell and Stipanovic, 1979), a *Myxococcus* strain (Gerth *et al*., 1982) and *Enterobacter agglomerans* (Chernin *et al*., 1996). It was first described by Arima *et al*. (1964). A phenyl pyrrol derivative of Prn has been developed as an agricultural fungicide (Elander *et al*., 1968). Pyrrolnitrin persists actively in the soil for at least 30 days. Prn has been reported to be an effective antagonist of damping-off of cotton caused by *Rhizoctonia solani* (Hill *et al*., 1994), tan spot of wheat caused by *Pyrenophora tritici repentis* (Pfender *et al*., 1993), potato dry rot caused by *Fusarium sambucinum* (Burkhead *et al*., 1994) and other fungal pathogens such as *Botrytis cinerea, Sclerotinia sclerotiorum, Bipolaris maydis, Sclerotina homeocarpa* and *Drechslera poae* (Stevans *et al*., 1994; Rodriguez and Pfender, 1997; Mcloughlin *et al*., 1992; Hammer *et al*., 1993)

**Phenazines (Phz):**

Phenazines are N-containing heterocyclic pigments and currently over 50 naturally occurring Phz compounds have been described (Turner and Messenger, 1986; Mavrodi *et al*., 1998; Smirnov and Kiprianova, 1990). Almost all Phz exhibit broad-spectrum activity against bacteria and fungi (Smirnov and Kiprianova, 1990). Growth conditions determine the number and type of Phz synthesized by an individual bacterial strain. For example, *P. fluorescens* 2-79 produces mainly PCA (phenazine 1-carboxylic acid), whereas *P. aureofaciens* 30-84 not only produces PCA but also lesser amounts of 2-OH-phenazines (Winenberg, 1970). It has been shown that bacterization of wheat seeds

with *P. fluorescens* strains 30-84 and 2-79 provides primary protection against *G. graminis tritici* due to release of Phz (Mazzola *et al*., 1992).

**Pyoluteorin (Plt):**

Plt is an aromatic polyketide antibiotic consisting of a resorcinol ring. Plt is produced by several *Pseudomonas sp*. (Kraus and Loper, 1995; Maurhofer *et al*., 1992; 1994) and when applied to seeds, plt-producing biocontrol strains decrease the severity of *Pythium* damping-off (Nowak-Thompsan *et al*., 1999).

On screening the biosurfactant deficient library for QSS production, we found that 5 mutants, including m6418, overproduced an unknown antibiotic-like substance. An i*n vitro* test indicated that this antibiotic-like substance was capable of inhibiting bacteria such as *E. coli* and wild type *P. fluorescens* 5064 (Chapter 2).

The purpose of this experiment was to determine whether the antibiotic produced by m6418 belongs to one of the above antimicrobial metabolite groups.

### 5.2.2. Materials and Methods

5.2.2.1. Microorganisms and culture conditions

*P. fluorescens* 5064 wild type was cultivated at 28°C in Luria-Bertani (LB) broth or agar (appendix B, Sambrook *et al.,* 1989) with ampicillin (wild type *P. fluorescens* 5064 is naturally resistant to 100mg/L of ampicillin). The media used for mutant 6418 growth were LB broth or agar with both ampicillin and kanamycin (50mg/L). The pathogen *Pythium ultimum* was maintained, and the antifungal assay was carried out, on potato-dextrose agar (PDA, appendix C) plates at 28°C.

5.2.2.2. Antibiotic extraction

Since the antibiotic activity of mutant 6418 (m6418) was first discovered in its AHL crude extract, the method used to extract this unknown antibiotic substance was the same as the method described in 2.3.2.4 with minor changes.

The *P. fluorescens* 5064 wild type (as negative control in this experiment) and mutant 6418 were grown overnight on LB agar plates at 28°C with appropriate antibiotic before being sub-cultured into 1 L of LB broth (without antibiotic). The bacteria were grown for about 50-55 h with shaking to ensure adequate aeration until the late exponential or early stationary phase (optical density of 2.1 at 500nm) and subjected to the following extracting procedure:

- The bacterial suspensions were centrifuged at 5000 rpm for 20 minutes in order to precipitate the cells.
- The supernatant was extracted twice with dichloromethane (1000:700 /supernatant: solvent) and the organic and aqueous phases were mixed gently. The emulsified mixtures were extracted for more than 2h until the complete separation of organic and aqueous phase.
- The combined dichloromethane organic phases were dried over approximately 5g of anhydrous magnesium sulphate to remove any remaining aqueous residue.
- The solvent was filtered (Whatman filter paper, 180mm, Whatman Ltd. England) to discard the magnesium sulphate before being rotary evaporated to dryness.
- The organic solvent was evaporated by rotary vacuum evaporation at 37°C (Grant 349/2, Grant Instruments Cambridge Ltd).
- The residue was resuspended in 1.5 ml of methanol, transferred to a vial and centrifuged at 10000 rpm for 10 min, the supernatant was removed to a clean vial and air dried in a clean cabinet.

- The residue was redissolved in 1 ml of methanol (at a concentration of 1000× higher than that in the original culture) and sterilised with a 0.22µl filter (Millex® - GS, Millipore Co. U.S.A).

5.2.2.3. Thin-layer Chromatography (TLC)

TLC is a simple, quick and inexpensive technique that is useful for separating organic compounds. TLC is also used to support the identity of a compound in a mixture when the $R_f$ of an unknown compound is compared with the $R_f$ of a known compound (preferably both run on the same TLC plate). A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or aluminium). A small amount of the mixture to be analysed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action. As the solvent moves past the spot that was applied, equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid, and the molecules which are in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried further up the plate than others. When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualised. If the compounds are coloured, visualisation is straightforward. Usually the compounds are not coloured, so a UV lamp or different colour reactions will be used to visualise the plates.

**Retardation factor ($R_f$ ):**

The furthest point reached by the advancing solvent on the plate is termed the solvent front. This can be used as reference point for describing the relative distance of travel, which is also known as retardation factor ($R_f$ ). $R_f$ is the ratio of the distance travelled

by the centre of the sample spot to the distance simultaneously travelled by the solvent front from the origin.

$R_f = a/b$

a: the distance travelled by the centre of the sample spot

b: the distance travelled by the solvent front

By definition, $R_f$ values are always less than unity. They are usually expressed in up to two decimal places. For convenience, it can be expressed as a percentage by multiplying by 100. The $R_f$ is a useful value as it is constant when all conditions are exactly reproduced. Some of the factors which affect $R_f$ values are the type of plate and the temperature at which chromatography is run.

**Procedure for TLC**:

a. Preparing for the developing chamber

100 ml of developing solvent was poured into the chamber to a depth of just less than 0.5cm. The inside of the chamber was lined with filter paper (Whatman, Whatman International Ltd., England) to aid the saturation of the TLC chamber with solvent vapors. The chamber was covered with a watch glass and allowed to saturate for at least 40min while preparing the TLC plates.

b. Preparing for the TLC plates

The TLC plates used in this experiment were Silica Gel 60 $F_{254}$ (E. Merk, AG, Darmstadt, Germany). A line was drawn lightly across the plate at 2 cm from the bottom of the plate using a pencil. This is the origin, the line on which to "spot" the sample. The name of the samples to be analyzed were marked lightly just under this line (make sure to leave enough space between the samples so that they do not run together). Another line, the finishing line, was drawn across the plates at 10 cm above the origin line.

c. Spotting the TLC plates

10 µl of the antibiotic extract from m6418 and *P.fluorescens* 5064 were added to the plate in a process called 'spotting'. The samples were added slowly using a Gilson pipette on the origin line that had been marked earlier. The sample was applied to the plate as a spot not larger than 5 mm diameter. To do this, it might be necessary to apply the 10 µl sample in several stages. The spot was allowed to dry each time before applying another. Extra care was taken not to disturb the coating of the adsorbent. The whole spotting procedure was performed in a fume cabinet (Astecair 3000, Astec Environmental Systems Ltd., England).

d. Developing the plates

The pre-prepared plates were placed in the developing chamber without touching the 'side' of the chamber. The chamber was covered with a watch glass and the TLC was allowed to run undisturbed. When the solvent front touched the 'finishing' line, the plates were removed from the chamber to end the development. For one-way TLC (used for colour reactions and $R_f$ determination), chloroform: acetone (9:1; v/v) was used as developing solvent. For the two-dimensional TLC (used for the antibiotic spot purification), the sample was spotted on the left hand corner, but 2 cm away from either edge of the plate. The plates were developed first in chloroform: acetone (9:1; v/v), removed from the chamber and allowed to dry, then turned 90 degrees and placed into the second solvent (toluene: acetone, 4:1 (v/v)). When the solvent front reached the finishing line, the plates were removed from the chamber and allowed to dry.

**Visualising the TLC plates**:

a. UV detection of TLC plates: The developed chromatograms were observed under long-wave (365nm) and short-wave (254nm) UV lights for fluorescing and absorbing spots (Ultra-violet Products, Inc. California, USA).

b. Ehrlich's reagent: Ehrlich's reagent is the name of a mixture (see appendix D) which is used to detect indole- and pyrrol-form compounds that are separated on a TLC plate (Scott *et al*., 1981). After spraying with Ehrlich's solution on the plate, a coloured spot will form where such a pyrro-like compound lies.

c. Pauly's reagent: The developed chromatograms were (after viewing under UV lights) sprayed with Pauly's reagent (see appendix E) to detect compounds with phenol forms. The spot where such a phenolic compound lies will turn into a burnt-orange or a red colour.

**Purification of the antibiotic spot from the TLC plates:**

The developed plates were observed under UV light to visualise fluorescing and adsorbing bands, and marked with a pencil. The solid adsorbent coat (Sillica Gel) of the bands and blank areas were scraped separately from the plates and collected carefully into 15 ml centrifuge tubes. The collected sample was extracted twice with dichloromethane. The silica gel was discarded and the organic phases were combined and brought to dryness by a centrifugal evaporator (RC 10-22, Jouan S.A. France). The residue was redissolved in 20 µl of chloroform and kept at 4°C for further antifungal bioassay.

5.2.2.4. Antifungal bioassay

A hole was punched using a sterile pipette tip in the centre of a PDA plate and filled with a pre-prepared sterile-water suspension of spores of *P. ultimum*. A small piece of filter paper (Whatman International Ltd., England), which had been dipped into the eluted antibiotic solution, was placed on the plate about 2 or 3 cm away from the middle hole. Another piece of filter paper, which had been dipped in the solvent (chloroform), was placed on the other side of the hole and served as control. The plate was incubated upright at 28°C for 2 days before any inhibitory effects could be observed.

### 5.2.3. Results

5.2.3.1. Identification of the antibiotic production in m6418 by TLC

The antibiotic extracts from m6418 and wild type *P. fluorescens* 5064 were separated by one-way TLC in chloroform: acetone (9:1; v/v). With sample m6418, a dark spot, corresponding to an $R_f$ of 0.48 was observed under short-wave UV light. This spot, which could not be visualized under long-wave UV light, was impressively large compared to the other spots on the plate, reflecting a large amount of the compound. The corresponding spot was not present in the wild-type sample. As pyrrolnitrin typically shows a dark spot under short-wave UV light and is reported to have an $R_f$ value of 0.48 under the same chromatographic conditions used in this study (Burkhead *et al*., 1994), we tentatively identified the antibiotic produced by m6418 as pyrrolnitrin. The colour reaction results were further consistent with this conclusion. The antibiotic spot turned orange with Pauly's reagent and red with Erhlich's reagent (Figure 5.2), indicating the presence of phenolic and pyrrolic groups (Scott *et al*., 1981; Arima *et al*., 1965; Mahoney and Roitman, 1990).

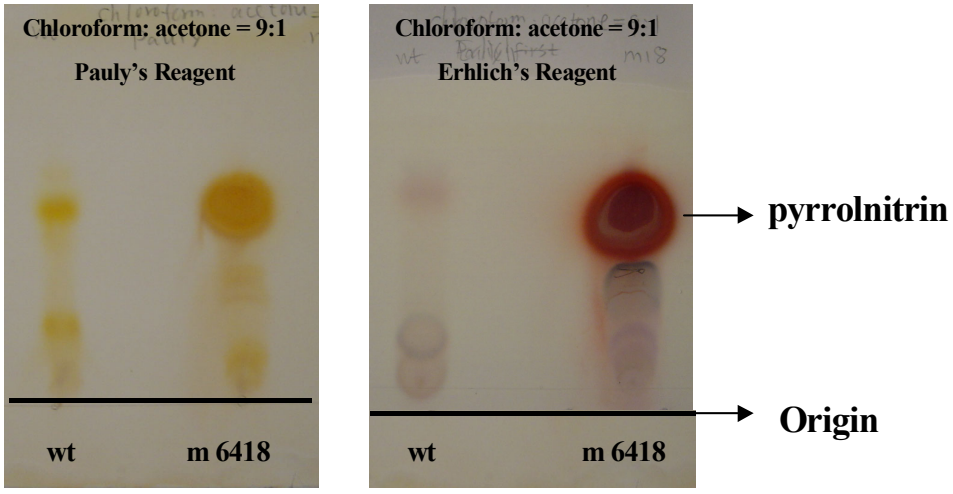



**Figure 5.2: Colour reactions of pyrrolnitrin on TLC plates**

Wt = *P. fluorescens* 5064, pathogenic to broccoli
M6418 = *P. fluorescens* 5064 mutant 6418, non-pathogenic to broccoli

Pyrrolnitrin showed an $R_f$ value of 0.53 in the developing solvent toluene-acetone (4:1; v/v).

5.2.3.2. Antifungal activity of Pyrrolnitrin

An antifungal bioassay using *Pythium ultimum* was performed to identify the antibiotic activities of the collected samples described in 'Materials and methods'. A spot responding to the $R_f$ 0.48 exhibited strong antifungal activity as determined by PDA plate assay. *P. ultimum* is a fast-growing fungus and after 2 days, fungal hyphal growth was inhibited on the side of the agar plates containing the pyrrolnitrin filter paper disc, whilst the control paper disk did not affect the fungal growth (Figure 5.3).

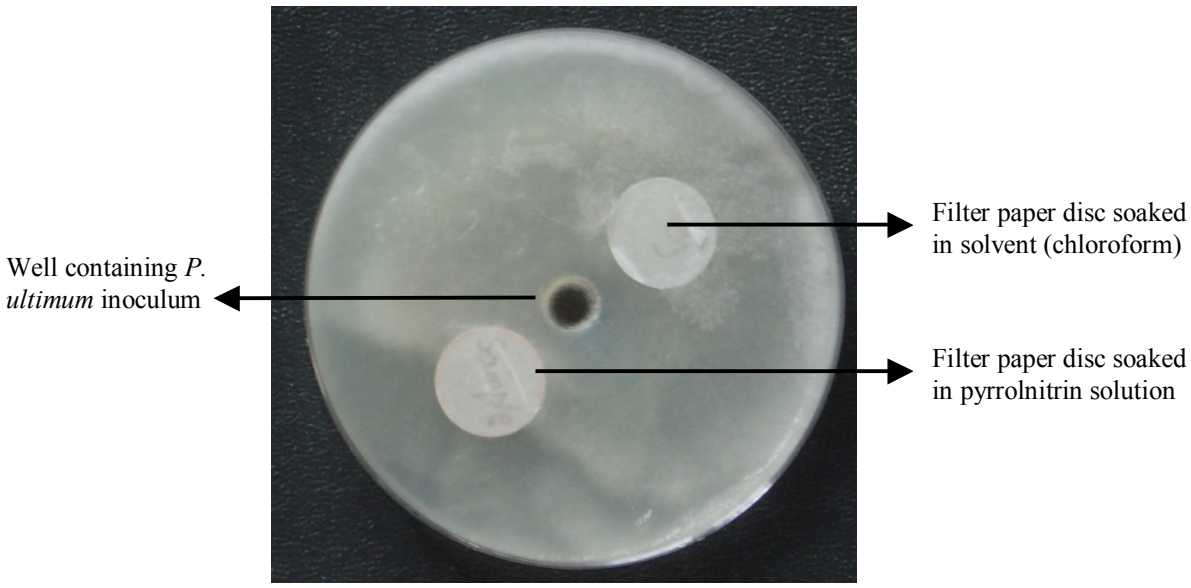



**Figure 5.3. Agar plate bioassay of pyrrolnitrin, produced by *P. fluorescens* 5064 mutant 6418, against *Pythium ultimum***

The TLC profile also provided evidence to assess the existence of other antibiotics, such as Phl, Plt and Phz, in m6418 and wild type *P. fluorescens* 5064 (Table 5.1). In addition to the major antibiotic Prn, m6418 also produced Phl, which was visible under long-wave UV light (blue fluorescence) with an Rf value of 0.41 (toluene:acetone/4:1) (Keel *et al*., 1992). In contrast to mutant 6418, wild type *P. fluorescens* 5064 did not produce any detectable antibiotics listed above. Although a colour spot also showed at $R_f$ 0.48 in the wild type *P. fluorescens* 5064 chromatograms (Figure 5. 2), this spot, under short-wave UV light, showed blue fluorescence instead of the dark non-fluorescent dot corresponding to pyrrolnitrin in m 6418 chromatogram, therefore we concluded this was not Prn.

**Table 5.1: Antibiotic production by wild type *Pseudomonas fluorescens* 5064 and mutant 6418 as detected by thin layer chromatography (TLC)**

| Antibiotics | Identity on TLC | Presence in m6418 | Presence in wild type |
|---|---|---|---|
| 2,4-diacetyl-phloroglucinol (Phl) | Rf = 0.41 (toluene-acetone = 4:1)<br>Fluorescence under longwave UV light | Yes | No |
| Pyoluteorin (Plt) | Rf = 0.27 (chloroform-acetone = 9:1)<br>Rf = 0.31 (toluene-acetone = 4:1)<br>Fluorescence under shortwave UV light | No | No |
| Pyrrolnitrin (Prn) | Rf = 0.48 (chloroform-acetone = 9:1)<br>Rf = 0.53 (toluene-acetone = 4:1)<br>Dark dot under shortwave UV light<br>Pauly's Reagent (red or orange colour)<br>Ehrlich's Reagent (Red colour) | Yes | No |
| Phenazine (Phz) | Colony pigmentation (dark zone surrounding colonies on agar) | No | No |

## 5.3. Evaluation of m6418 for biological control of broccoli head rot

### 5.3.1. Introduction

In a study by Darling (1998) a mutant of *P. fluorescens* (m6418) was produced which had lost most the ability to produce certain extracellular products, such as protease and pectinase, and had become non-pathogenic. The loss of virulence and the overproduction of pyrrolnitrin made m6418 a possible biocontrol agent for broccoli head rot disease. According to a previous study, the growth rate of m6418 was slightly slower than that of the wild type *P. fluorescens* (Darling, 1998). This generated a question as to whether m 6418 would show adequate fitness *in planta* to antagonise *P. fluorescens* 5064.

In this study, experiments were designed to monitor the changes in *P. fluorescens* 5064 growth when co-inoculated with m6418 both in culture and on broccoli leaves. A pathogenicity test on excised broccoli head was also carried out to determine m6418's biocontrol effectiveness.

### 5.3.2. Effects of m6418 to *P. fluorescens* 5064 in culture

5.3.2.1. Aim

As described earlier, the pyrrolnitrin extracted from m6418 was capable of inhibiting *P. fluorescens* 5064 growth (Chapter 2). In this section, an experiment was performed to determine whether m6418, when co-inoculated with *P. fluorescens* 5064 in liquid culture, could inhibit *P. fluorescens* 5064 without further extraction of the antibiotic.

5.3.2.2. Materials and methods

Antibiotic bioassay procedure:
(1). One single clone of m6418, from an overnight LB plate (kanamycin, 50mg/L), was inoculated into 200ml of LB broth and grown at 30°C for 2 days to ensure that enough pyrrolnitrin was produced.
(2). The above m6418 bacterial suspension was centrifuged at 5000 rpm (Sorvall RC5C Plus, USA) for 30min to precipitate the cells.
(3). The supernatant was sterilised using a 0.20µl filter (Millex® - GS, Millipore Co. U.S.A).
(4). *P. fluorescens* 5064 was inoculated into the sterilised supernatant and the mixture was incubated at 30°C for another 2 days.

5.3.2.3. Results and discussion

After 2 days' incubation, the m6418 culture filtrate, although inoculated with *P. fluorescens* 5064, remained clear. This indicated that the pyrrolnitrin produced by m6418 has a strong inhibitory effect against *P. fluorescens* 5064 (even without further concentration).

### 5.3.3. The introduction of a rifampicin-resistant marker to *P. fluorescens* 5064

5.3.3.1. Aim

*P. fluorescens* 5064 wild type is resistant to ampicillin and m6418 has both ampicillin and kanamycin resistance. A spontaneous rifampicin-resistant ($Rif^r$) derivative of *P. fluorescens* 5064 was selected in order to facilitate the monitoring of the cell numbers of co-inoculated bacteria in the pathogenicity tests.

5.3.3.2. Materials and methods

For the wild type *P. fluorescens* 5064, Rif $^{r}$ was introduced by selecting spontaneous mutants on rifampicin-rich media. The whole selection procedure is shown in Figure 5.4. When the bacteria is resistant to 50µg/ml rifampicin in the medium, it is considered to be a rifampicin-resistant strain.

5.3.3.3. Results

After two generations' selection, a *P. fluorescens* 5064 Rif $^{r}$ derivative carrying stable rifampicin resistance was obtained. It was shown to be identical in terms of growth rate to wild type *P. fluorescens* 5064. The selected bacterium was kept in a -80°C freezer.

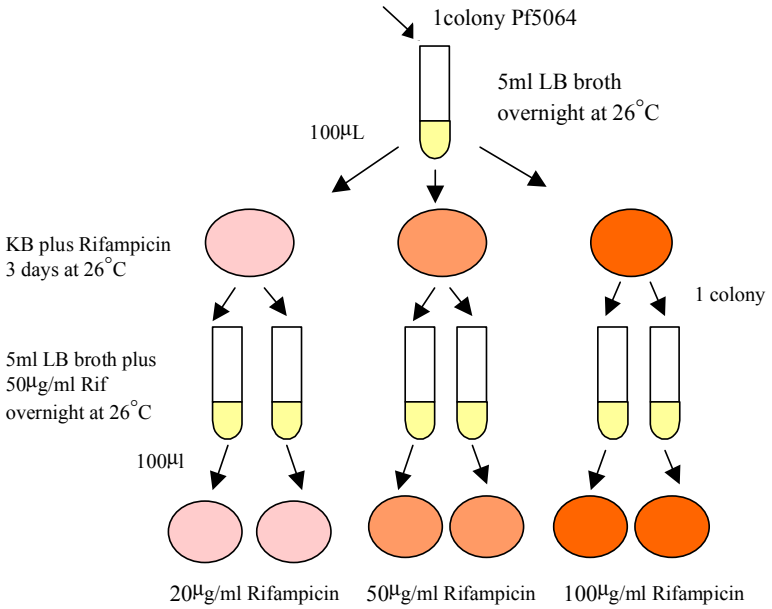



**Figure 5.4: Selection of a spontaneous rifampicin resistant derivative of *P. fluorescens* 5064 (Rif $^{r}$)**

### 5.3.4. The establishment of m6418 and *P.fluorescens* 5064 calibration curves

5.3.4.1. Aim

A very useful method for estimating bacterial cell numbers in liquid culture is to measure the turbidity of a suspension of the microbe using a spectrophotometer, which measures light lost from the culture due to scattering, this is called absorbance or optical density (OD). The amount of light scattered by the suspension is proportional to the number of cells present, but is also influenced by the size shape and nature of the cells. A standard curve relating OD values to cell numbers is therefore required for each individual species/strain under specific growth conditions for estimation of cell numbers.

The standard growth calibration curves of m6418 and *P. fluorescens* 5064, with colony forming units (cfu/ml) corresponding to the relevant $OD_{500nm}$ values, were established in order to allow a rapid estimation of concentrations of bacterial suspensions, once the $OD_{500nm}$ values of a culture are measured.

5.3.4.2. Materials and methods

m 6418 and *P. fluorescens* 5064 were sub-cultured from 24 h LB plates into 100ml LB broth. The sub-cultured bacteria were incubated at 30°C with gentle shaking. Immediately upon start of incubation and then every few hours after that (as specified in Figure 5.5 and 5.6), samples were collected and the $OD_{500nm}$ values were recorded as an indication of bacterial growth. To each recorded $OD_{500nm}$ value, the corresponding viable bacterial cell numbers were counted using the method described below.

Quantification of viable bacterial cell number: 2µl of each sample to be quantified were diluted in a series of 10 fold dilutions (1 ml of culture plus 9 ml of sterile distilled water). 100µl of each dilution were spread on LB plates with the appropriate antibiotic.

Plates were then incubated at 30°C for 2 days to allow colonies to develop, which were then counted at suitable dilutions. As the number of microbes in the original culture may be as high as $10^{10}$ and the ideal number of countable colonies on a plate is between 30 and 200, it is therefore necessary to dilute the culture in a series of 10-fold dilutions. Three replicates were carried out for each sample to ensure consistency of the results. The machine used to detect the OD value was a Beckman, DU-65 Spectrophotometer.

5.3.4.3. Results

Figure 5.5 and 5.6 present the standard growth curves of *P. fluorescens* 5064 and m6418 – the number of viable bacterial cells (cfu) obtained over time in response to their $OD_{500nm}$ values. These curves are useful in the broccoli seedling and excised head pathogenicity tests for estimating bacterial cell numbers promptly. For example, the $OD_{500nm}$ value of 1.5 for m6418 represents the concentration of $1 \times 10^8$ cfu/ml, one of the concentrations that was used in the pathogenicity tests.

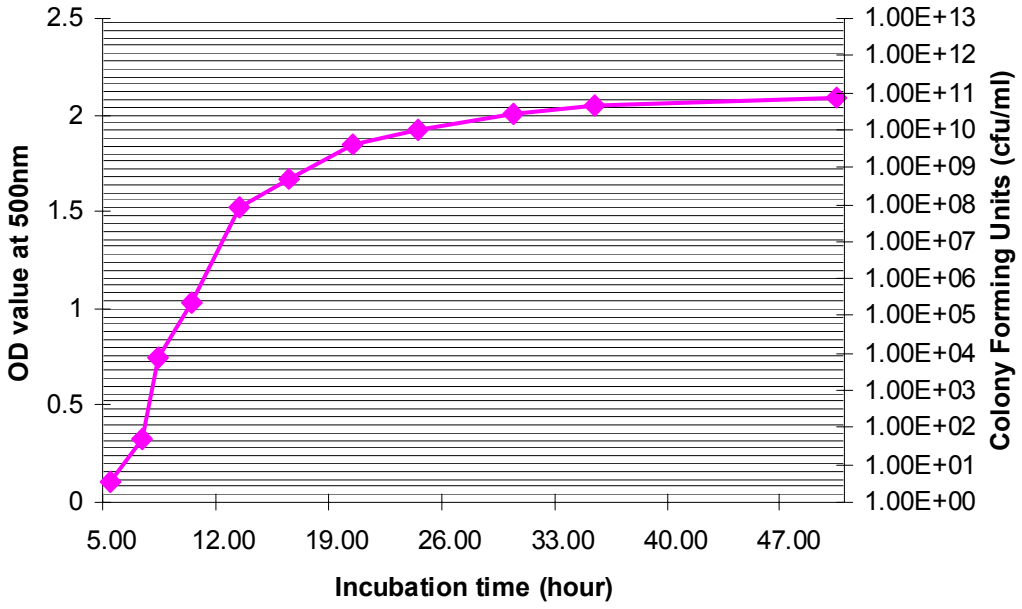



**Figure 5.5: m6418 standard growth curve**
This graph shows the mean number of viable bacterial cells corresponding to each $OD_{500nm}$ value.

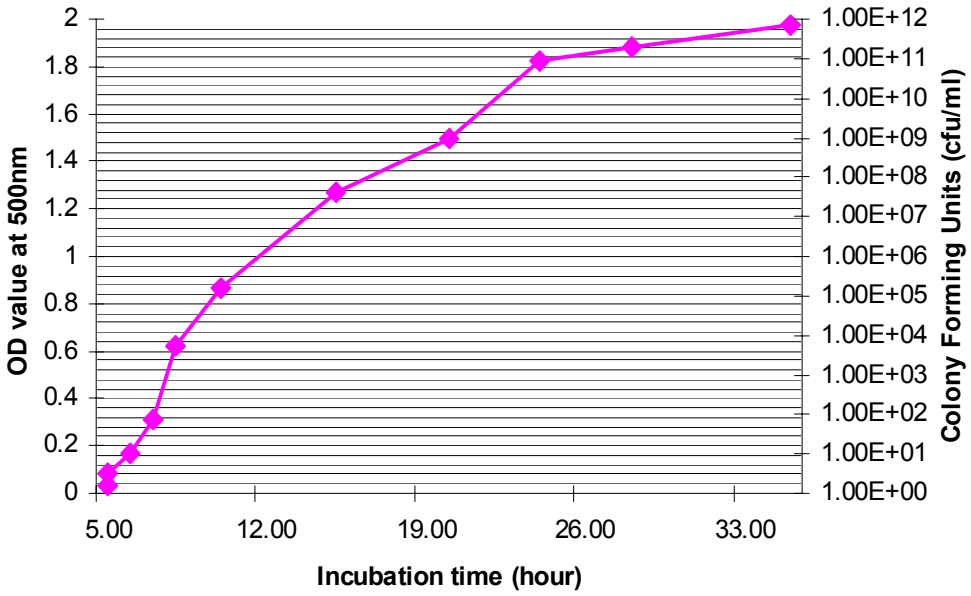



**Figure 5.6: *P. fluorescens* 5064 standard growth curve**
This graph shows the mean number of viable bacterial cells corresponding to each $OD_{500nm}$ value.

### 5.3.5. The effects on *P. fluorescens* 5064 growth when co-inoculated with m6418 in liquid culture

5.3.5.1. Materials and methods

Five co-inoculation tests were set up as follows: (1). m6418 was sub-cultured from a 24 h LB plate (kanamycin, 50µg/ml) into 100ml of LB broth. The sub-cultured bacterium was then incubated at 30°C with gentle shaking. Six hours after the incubation and every few hours after that (as specified in Figure 5.7 and 5.8), samples were taken for bacterial cell quantification (see below).

(2). m 6418 was sub-cultured from a 24 h LB plate (kanamycin, 50µg/ml) into 100ml LB broth. The sub-cultured bacterium was then incubated at 30°C for 6 h before inoculation with *P. fluorescens* 5064 ($Rif^r$), which was grown on a 24 h LB plate

containing rifampicin (50µg/ml). The bacterial mixture was allowed to continue to grow in a 30°C incubator. Four hours later and every few hours after that (as specified in the results), samples were taken for bacterial cell quantification.

(3). m6418, which was growing on a 24 h LB plate (kanamycin, 50µg/ml) and *P. fluorescens* 5064 Rif $^{r}$, which was growing on a 24 h LB plate (rifampicin, 50µg/ml) were co-cultured into 100ml of LB broth. The bacterial mixture was then incubated at 30°C with gentle shaking, 6 h after the incubation and every few hours after that, samples were taken for bacterial cell quantification**.**

(4). *P. fluorescens* 5064 Rif $^{r}$ was sub-cultured from a 24 h LB plate (rifampicin, 50µg/ml) into 100ml LB broth. The bacterium was incubated at 30°C with gentle shaking. Six hours after the incubation and every few hours after that, samples were taken for bacterial cell quantification.

(5). *P. fluorescens* 5064 Rif $^{r}$ was sub-cultured from a 24 h LB plate (rifampicin, 50µg/ml) into 100ml LB broth. The bacterium was then incubated at 30°C with gentle shaking for 6 h before addition of m6418. The bacterial mixture was allowed to continue to grow at 30°C. Six hours later, and every few hours after that, samples were taken for bacterial cell quantification.

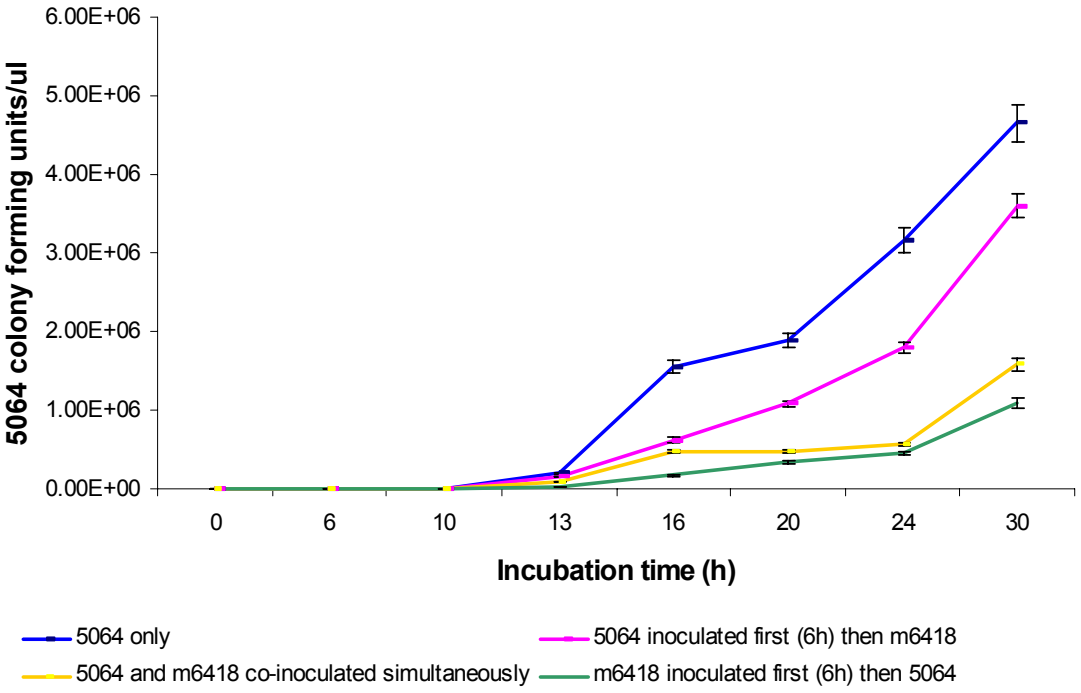
**The viable bacterial cell quantification:**

The bacterial cell quantification method was the same as described in 5.3.4.2, 100µl of each sample was serially diluted and plated onto LB plates. LB plates with kanamycin (50µg/ml) were used to quantify the m6418 cell numbers in a (mixed) sample and LB plates with rifampicin (50µg/ml) were used to quantify the *P. fluorescens* 5064 Rif $^{r}$ cell numbers in a (mixed) sample. Three replicates were carried out for each treatment.

5.3.5.2. Results and discussion

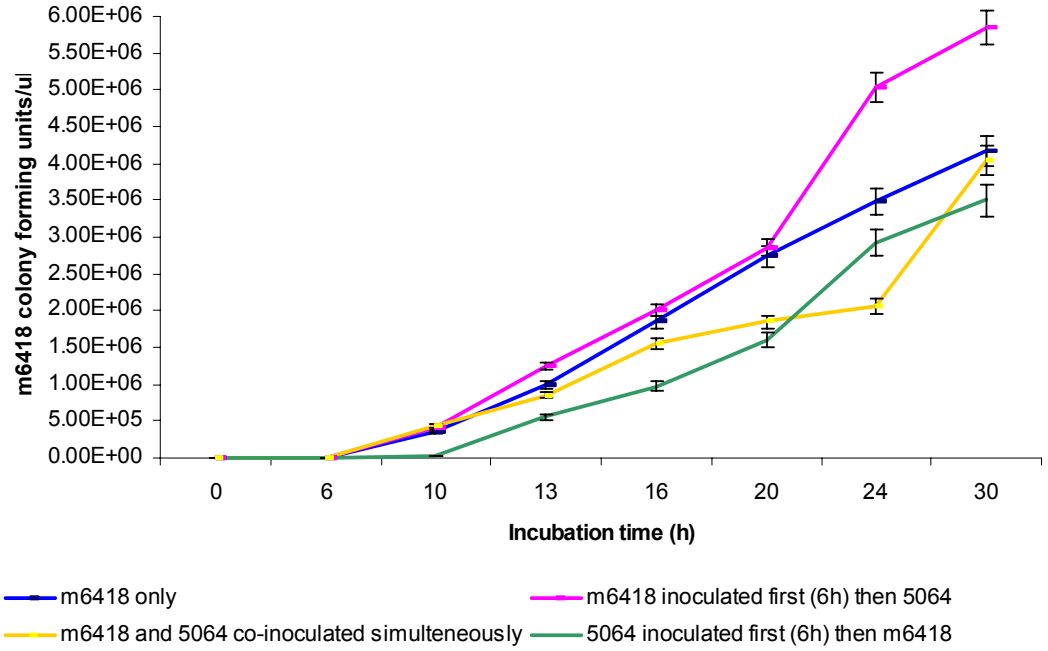
The growth curves of *P. fluorescens* 5064 and m6418 upon co-inoculation are shown in Figure 5.7 and Figure 5.8.

m6418 significantly inhibited the growth of *P. fluorescens* 5064 in liquid culture. The greatest inhibitory effect resulted from m6418 when inoculated 6h before 5064, when the *P. fluorescens* 5064 cell number was 5 times less than that of the control. The m6418 population initially remained low when inoculated 6h later than 5064, but it increased quickly after 20 h co-incubation (Figure 5.8). The 5064 population remained at a low level throughout the experiment when inoculated 6h later than m6418 (Figure 5.7).




Bars = standard error of the mean (SEM)

**Figure 5.7: Effects of *P. fluorescens* m6418 on *P. fluorescens* 5064 growth in liquid culture**




Bars = standard error of the mean (SEM)

**Figure 5.8: Effects of *P. fluorescens* 5064 on *P. fluorescens* m6418 growth in liquid culture**

### 5.3.6. Population dynamics of m6418 and *P. fluorescens* 5064 when co-inoculated onto broccoli leaves.

#### 5.3.6.1. Materials and methods

Initially, broccoli seeds (CV Skiff FI) were surface sterilized in 1% chlorine solution for 30min, rinsed in distilled water three times, then sown into 5 pots (10cm × 18cm) to germinate. After 4 weeks when there were sufficient true leaves present, 25 true leaves from each pot were selected and marked to distinguish them from leaves which appeared later in plant growth (Figure 5.9). The marked leaves were sprayed evenly with bacterial suspension according to the different treatments. Five treatments, which are listed in Table 5.2, were carried out using the marked leaves in the 5 pots (i.e. one pot for each treatment).

**Table 5.2: Different co-inoculating treatments on broccoli leaves**

| Pot No. | Different co-inoculum treatment |
|---|---|
| 1 | *P. fluorescens* 5064 Rif $^{r}$ only |
| 2 | *P. fluorescens* 5064 Rif $^{r}$ and m6418 (kan$^{r}$) were inoculated simultenously |
| 3 | *P. fluorescens* 5064 Rif $^{r}$ was inoculated one day earlier than m6418 (kan$^{r}$) |
| 4 | m6418(kan$^{r}$) was inoculated one day earlier than *P. fluorescens* 5064 Rif $^{r}$ |
| 5 | m6418 (kan$^{r}$)only |

**Figure 5.9: The tested broccoli seedlings with marked leaves**

Preparation of the bacterial spray suspension for each treatment:

m6418 or *P. fluorescens* 5064 Rif $^{r}$ was inoculated into 100ml of LB broth (without antibiotic) and grown at 30°C for 12-16 hours. The $OD_{500nm}$ values of both bacteria were measured. The concentrations of the bacterial suspension were then determined using the m6418 or *P. fluorescens* 5064 growth calibration curve established earlier (see 5.2.4). The bacterial suspensions were diluted with LB broth to a final concentration of $1 \times 10^{8}$ cfu/ml and placed into aerosol sprays. One drop of Tween 20 was added to each aerosol spray. After spraying, the leaves were placed in a Fisons environmental cabinet

with a 16 h photoperiod and high humidity (100%) at 20°C (day)/10°C (night)(Figure 5.10).

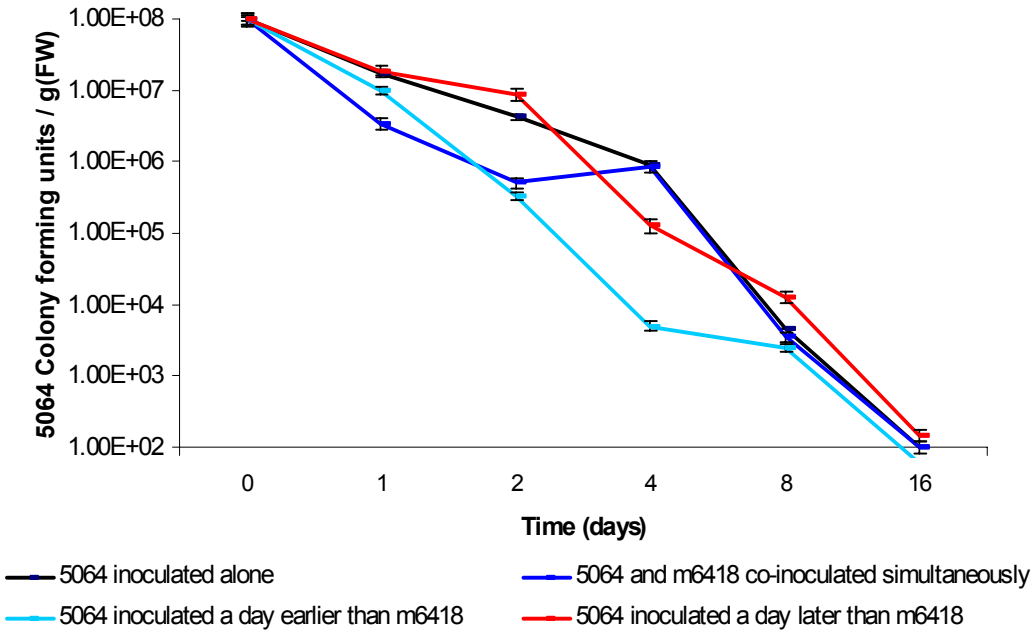
**Figure 5.10: Fisons environmental cabinet**

Quantifying the cell numbers on leaves:

The marked leaves were harvested on the first, the second, the fourth, the eighth and the sixteenth day after inoculation, weighed and placed into sterile tubes containing 10ml of sterile distilled water. The tubes were sonicated at 40 KHz in a water bath (Decon FS200, Decon Ultrasonics Ltd., England) for 7 minutes to remove the bacteria from the leaf surfaces and to distribute the cells evenly in the suspensions. The bacterial suspensions were then serially diluted in sterile distilled water. A 100 µl aliquot of each dilution was spread onto the appropriate LB plates (*P. fluorescens* 5064 onto LB plates with rifampicin, m6418 on to LB plates with kanamycin, whilst co-inoculated samples were plated onto both type of plates). All the plates were incubated at 30°C for 48 h to allow the total number of colonies to develop and to be counted. The number of bacterial cells was related to the fresh weight of the leaf where they were originally from. Five replicates were carried out for each treatment on each day.

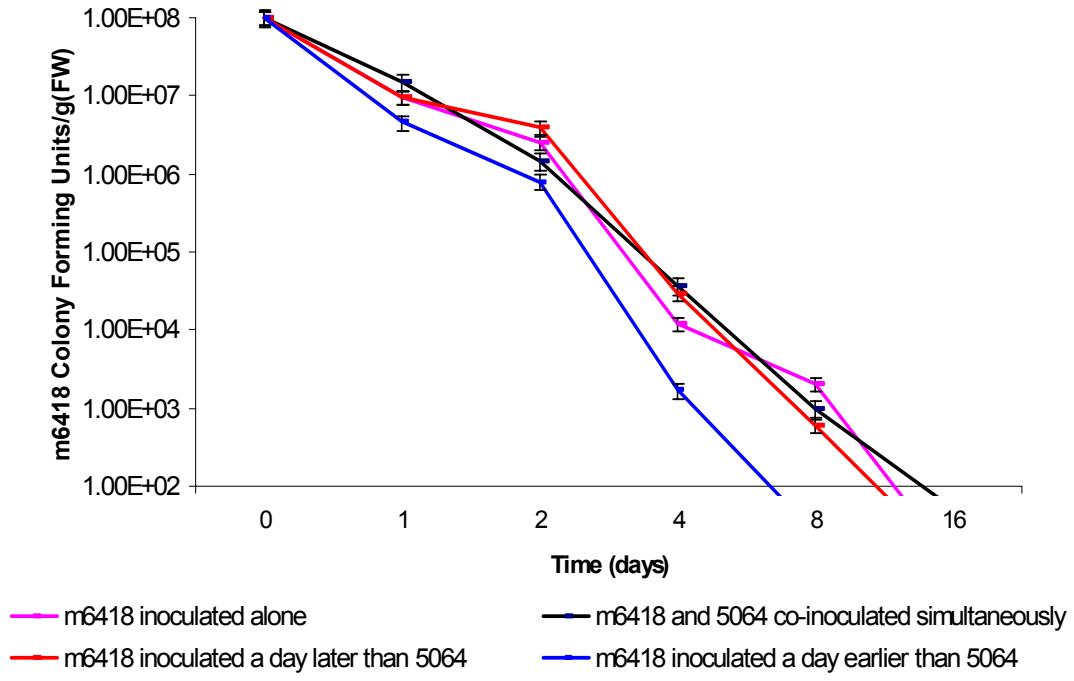
5.3.6.2. Results and discussion

M6418 showed similar growth curves in most of the co-incubation treatments, which indicated that *P. fluorescens* 5064 did not significantly interfere with m6418 growth (Figure 5.12). When inoculated one day later than the wild type, m6418 reduced the growth rate of the wild type, but not when inoculated one day before it. When 5064 and m6418 were co-inoculated simultaneously, m6418 inhibited 5064 growth on the first and second day, however the inhibitory effects were lost thereafter (Figure 5.12). These results might be due to m6418 exhibiting less fitness on the leaf surfaces when compared to 5064, which is reflected by the faster decline in its population (Figure 5.11 and Figure 5.12).




Bars = standard error of the mean (SEM)

**Figure 5.11: Effects of *P.fluorescens* m6418 on *P.fluorescens* 5064 growth on broccoli leaves**




Bars = standard error of the mean (SEM)

**Figure 5.12: Effects of *P.fluorescens* 5064 on *P.fluorescens* m6418 growth on broccoli leaves**

### 5.3.7. Effects of m6418, as a biocontrol agent, on broccoli head rot disease symptom development in the excised head pathogenicity test

5.3.7.1. Aim

Pathogenicity tests involve inoculating plant tissue with a microorganism of interest to determine whether it can induce disease. A good pathogenicity test should be sensitive to low inoculum concentration, accurate enough to distinguish pathogenic from non-pathogenic strains, and reliable enough to produce the same results upon repetition. Although we are ultimately interested in the disease process as it occurs in the field, field-based experimentation is expensive to conduct, time consuming and labour intensive. Laboratory/greenhouse based pathogenicity test methods, on the other hand,

are more cost effective on labour, materials, space, time and the conditions can be closely controlled. If designed carefully, the laboratory-based tests should enable the main disease characteristics to be reflected and provide a useful research tool. In this study, a laboratory based excised broccoli head pathogenicity test (Darling *et al*., 2000) was used to study the effects of m6418, as a biocontrol agent, on disease incidence when co-inoculated with the pathogen *P.fluorescens* 5064.

5.3.7.2. Materials and methods

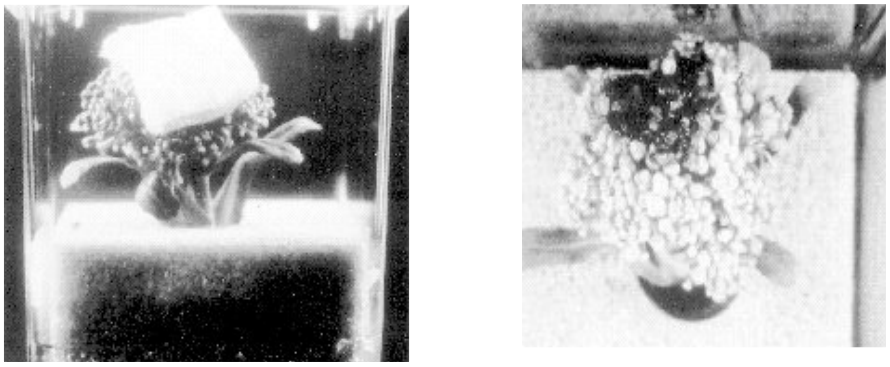
Pathogenicity test procedures:

Mature broccoli heads (purchased from a supermarket) were inoculated by placing two stacked 2 × 2 cm pieces of cotton lint, soaked in bacterial suspension, onto the broccoli head. Heads were kept upright with sponge collars in closed "Magenta GA7" vessels (Sigma Chemicals) with 20 ml of sterile distilled water to maintain humidity (Figure 5.13). Heads were incubated for five days at 20°C (day)/10°C (night) with a 16 h photoperiod in a Fisons environmental cabinet (Figure 5.10). The split temperature regime of 20°C/10°C was established to encourage dew formation. It has been suggested that dew droplets enhance bacterial multiplication by providing free water (Campbell *et al*., 1995).

Four treatments were carried out:

a. the broccoli heads were inoculated with "*P. fluorescens* 5064 only" (as positive control),
b. The broccoli heads were inoculated with sterile water (as negative control),
c. The broccoli heads were inoculated with m6418 only,
d. The broccoli heads were inoculated with a m6418 and *P. fluorescens* 5064 mixture. The co-inoculum ratio of m6418 to *P. fluorescens* 5064 was 1: 1.

The concentration of the bacterial suspensions were $1\times 10^8$ cfu/ml for both bacteria and ten replicates were carried out for each treatment.



**a** **b**

**Figure 5.13: Excised broccoli head pathogenicity test**

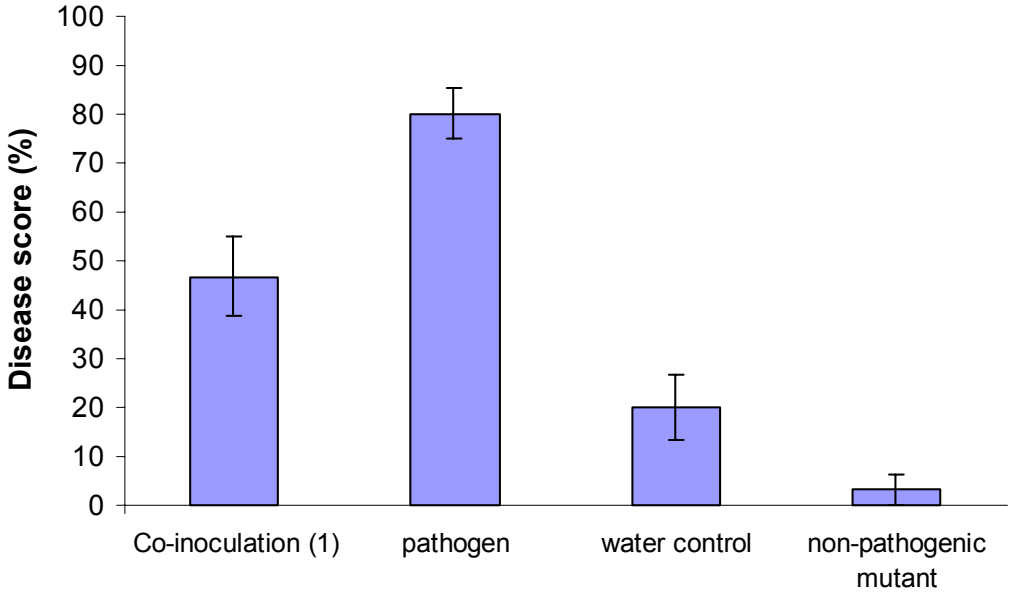
**a**, **Portion of broccoli head inoculated with cotton lint, which had been soaked in a bacterial suspension**
**b, Top view of broccoli head, lint having been removed after a few days inoculation, showing head rot.**

A five point scoring scale was used to assess the severity of disease symptoms (Darling *et al*., 2000): 0 = no symptoms, 1 = water soaking (loss of waxy bloom from florets), 2 = water soaking and tissue browning but no rot, 3 = tissue browning and softening (with rot), 4 = extensive black soft rot. Data were subjected to percentile angular transformation using arc sine (Schefler, 1969), thus the above categories 0-4 became 0% (0), 33% (1), 50% (2), 67% (3) and 100% (4), respectively.

5.3.7.3. Results and discussion

Inoculation of broccoli head with pathogenic *P. fluorescens* 5064 resulted in 80% disease after 5 days with extensive black soft rot (Figure 5.14). Simultaneous co-inoculation of the pathogen with the non-pathogenic mutant m6418 reduced disease to 47% (41% reduction), this score means that tissue is brown but not soft-rotted. Heads inoculated with water alone had a disease score of 20% (heads were slightly darkened and showed a loss of the waxy bloom), which is normal for negative controls due to the

presence of residual non-pathogenic bacterial activity beneath the water-soaked cotton lints. Heads inoculated with mutant m6418 showed virtually no symptoms at all (3%), which suggests that m6418 has very strong biocontrol activity and is capable of inhibiting even the residual bacteria.




0% = No disease symptoms; 100% = Severe head rot
(Data arc sine transformed)
Bars = standard error of the mean (SEM)
(1) Co-inoculation: Pathogen (*P. fluorescens* 5064) and non-pathogenic mutant (*P. fluorescens* m6418) simultaneously co-inoculated on broccoli heads in a ratio 1:1 (each at 1 x $10^8$ cfu/ml).

**Figure 5.14: Biocontrol of head rot on excised broccoli heads using non-pathogenic *P. fluorescens* mutant m6418**

## 5.4. The effects of the AHL-degrading strain *Bacillus* sp. A24 on broccoli head rot disease control

### 5.4.1. Introduction

As has been proved earlier in this study, biosurfactant production is regulated by quorum sensing in the head rot pathogen *Pseudomonas fluorescens* 5064 (refer to chapter 2). Interference with the quorum sensing regulation pathway may therefore be a new strategy for controlling this disease. Several potential target sites in the quorum sensing regulatory network can be envisaged (refer to literature review). In this study, we chose enzymatic signal destruction as an approach to block the *P.fluorescens* 5064 quorum sensing pathway and in an attempt to reduce disease severity.

In an exciting recent report, an enzyme (AHL lactonase) from an isolate of *Bacillus* capable of degrading AHL was discovered (Dong *et al*., 2000). Later work proved that the gene encoding the AHL-degrading enzyme (*aiiA* gene) is widespread in many species of *Bacillus* (Reimmann *et al*., 2002; Lee *et al*., 2002; Dong *et al*., 2002). Expression of the *aii*A gene in transformed *E. carotovora* strain SCG1, a pathogen that causes soft rot disease in many plants, significantly reduces release of AHL, decreases extracellular pectolytic enzyme activities and attenuates its pathogenicity for potato, egg-plant, Chinese cabbage, carrot, celery, cauliflower and tobacco (Dong *et al*., 2000). It would be interesting to find out whether the AHL degrading strain, *Bacillus sp*., can be used as a biocontrol agent by degrading the *P. fluorescens* 5064 quorum sensing signal and therefore reduce its pathogenicity.

*Bacillus* spp strains have been used as biocontrol agents to control a wide variety of pathogens (Krause *et al*., 2003; Wulff *et al*., 2003; Kim *et al*., 2003). Applications of *Bacillus* AS 43.4 isolated from wheat anthers decreased disease severity of *Fusarium* head blight under glasshouse conditions by 67-95% (Khan *et al*., 1999) and 48-95% in

another glasshouse investigation (Schisler *et al*., 2002). *Bacillus subtilis* HS93 and *B. licheniformis* LS674 have been shown to antagonise *Phytophthora capsici* and *Rhizoctonia solani* and to limit root rot disease in pepper plants under greenhouse conditions (Ahmed *et al*., 2003). Abdelzaher (2003) demonstrated that control of cauliflower root rot disease caused by *Pythium ultimum* can be obtained by applying *B. subtilis* to the soil as a biological control agent. Optimal control was obtained by mixing the bacteria with the soil rather than by dipping the cauliflower roots in the bacterial suspension immediately before planting. *B. subtilis* has been shown to effectively reduce foliage disease (bean leaf rust) caused by *Uromyces phaseoli*, and give 95-98% reduction in the number of rust pustules when *Bacillus* suspensions are sprayed onto plants 2 hours – 5 days before inoculation with *U. phaseoli* (Baker *et al*., 1983).

### 5.4.2. Degradation of *P. fluorescens* 5064 AHL by *Bacillus* sp. A24 *in vitro*

#### 5.4.2.1. Aim

Although the *Bacillus* sp. A24 has been reported as capable of degrading AHL quorum sensing signals of many different bacteria (Reimmann *et al*., 2002; Molina *et al*., 2003), it is necessary to test if it can degrade the 3-OH-$C_8$-homoserine lactone produced by *P. fluorescens* 5064.

#### 5.4.2.2. Materials and methods

Bacterial strains:

The plant pathogen wild type *P. fluorescens* 5064 and the AHL-degrading strain *Bacillus* sp. A24 were used in this study. *Bacillus* sp A24, which was kindly provided by Professor Genevieve Defago (ETH, Zurich, Switzerland), was isolated from soil in Switzerland. This bacterium was grown in LB medium at 30°C with no antibiotic presence. Wild type *P. fluorescens* 5064 was grown in LB broth at 30°C.

Crude AHL extract preparation:

The crude AHL extract was prepared using the method described in chapter 2. The resulting 100µl of $C_8$-OH-AHL extract (in 95% ethanol) was used for the following AHL-degradation assays.

AHL degradation assays:

*Bacillus* sp A24 was incubated at 30°C in 300 ml LB broth containing half (50 µl) of the above $C_8$-OH-AHL extract (the other half of the AHL extract was kept at 4°C and used for luminescence induction as a positive control, plate 2 in table 5.3). The bacterial culture was grown for 24 h to allow enough time for degradation of AHL before proceeding to AHL extraction (method as described in chapter 2). The resulting AHL extracts were re-dissolved in 50 µl of 95% ethanol and used for luminescence induction (AHL bioassay) (plate 3 in table 5.3). At the same time, AHL extract from 300ml of *Bacillus* sp A24 LB broth (*Bacillus* sp A24 grown alone without exogenous $C_8$-OH-AHL) was also prepared and the resulting 50 µl of sample was used in the luminescence induction assay (plate 4 in table 5.3).

AHL bioassays (luminescence induction):

The AHL bioassays were carried out using *E. coli* (pSB401) as biosensor. The assay method was the same as described previously (Chapter 2). The bioassay samples were prepared as shown in table 5.3. The 4 LB plates were placed on the bench for about 20 min until the semi-LB broth on the top layer solidified and then bioluminescence was detected. The data were recorded (referred to as background luminescence) and the plates were incubated at 37°C for 4 h before taking the second luminescence readings. The luminescence induced by each sample was recorded (Relative Luminescence Units, RLU), taking into account the background reading.

**Table 5.3: AHL degradation assays**

| Pre-warmed LB plates | Sample description* |
|---|---|
| Plate 1 | *E.coli* pSB(401) biosensor only as negative control |
| Plate 2 | Biosensor and AHL extract from *P. fluorescens* 5064 as positive control |
| Plate 3 | Biosensor and AHL extract from *Bacillus* sp. A24 growing with exogenous AHL (same amount as that in the positive control). |
| Plate 4 | Biosensor and AHL extract from *Bacillus* sp. A24 growing without any exogenous AHL |

* Each sample, as described in the table, was mixed well with 5ml semi-LB broth (seeded with the biosensor strain) and poured evenly on top of the pre-warmed LB plates.

5.4.2.3. Results and discussion

The luminescence induced in plate 3 was greatly reduced compared to the positive control plate 2 and was similar to the negative control in plate 4. This indicated that, since AHL extracts were added to plate 2 and plate 3 in the same amounts, *Bacillus* sp. A24 had degraded most of the $C_8$-OH-HSL produced by *P. fluorescens* 5064 (Figure 5.15).

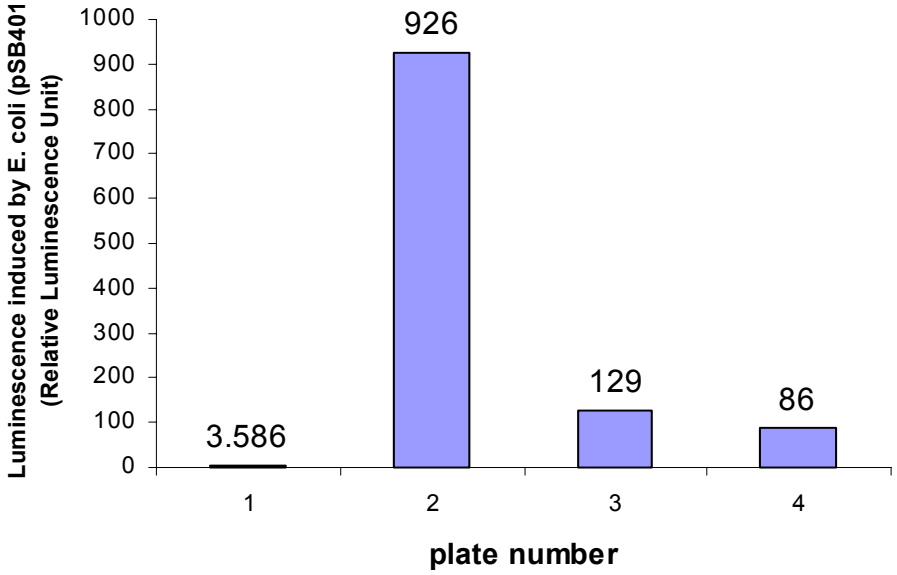



**Figure 5.15: Effect of *Bacillus sp.* A24 on AHL production as measured by luminescence induction (refer to table 5.3)**

### 5.4.3. *Bacillus* sp. A24 and *P. fluorescens* 5064 co-incubation in liquid culture

#### 5.4.3.1. Aim

This experiment was performed to assess the AHL degrading ability of *Bacillus* sp. A24 and to determine its growth rate when co-inoculated with *P. fluorescens* 5064 in a liquid culture.

#### 5.4.3.2. Materials and methods

*Bacillus* sp. A24 was co-inoculated with *P. fluorescens* 5064 in 300ml LB broth at the same time, 6h earlier or 6h later. The bacterial mixtures were grown for 48 h before proceeding to QSS extraction (as described in chapter 2).

Quantification of bacterial cell number

For each co-inoculation test described above, samples were also taken to determine the viable bacterial cell number besides QSS extraction. The quantification procedure was as follows:

a. 2 µl of each sample was pipetted into 50ml sterile distilled water and mixed well.
b. 2 µl of the diluted bacterial solution was pipetted onto a LB plate without antibiotic and 2 µl to a LB plate containing 50µg/ml ampicillin. Since *P. fluorescens* 5064 naturally carries an ampicillin resistance gene, and *Bacillus* is non-ampicillin resistant, the different bacterial colonies can be distinguished by plating the bacterial mixture, simultaneously, on to different LB plates.
c. The samples were spread evenly on the plates.
d. All the plates were then incubated at 30°C for 2 days to allow the colonies presented on the plates to develop and the viable bacterial cell numbers were calculated (taking dilution factor into account).

Three replicates were made for each bacterial count.

5.4.3.3. Results and discussion

Degradation of *P. fluorescens* 5064 QSS by *Bacillus* sp. A24

The biosensor *E.coli* (pSB401) produces luminescence in response to the presence of the AHL produced by *P. fluorescens* 5064 (as shown in chapter 2). No visible luminescence was observed when either *Bacillus* sp. A24 was inoculated in the medium before m6418, or when they were co-inoculated (Figure 5.16). Therefore, *Bacillus* sp. A24 has a strong ability to degrade the QSS produced by *P. fluorescens* 5064. When *P. fluorescens* 5064 was incubated 6h before the *Bacillus*, faint but visible luminescence occurred (Figure 5.16). This indicated that, although inoculated 6 h later than *P. fluorescens* 5064, *Bacillus* sp. A24 could still grow normally and destroy most, but not all, of the QSS produced by *P. fluorescens* 5064.

Effect of *Bacillus* sp A24 on growth of *P. fluorescens* 5064 in culture

When *P.fluorescens* 5064 and *Bacillus* sp. A24 were co-inoculated at the same time, the final cell number ratio of *P. fluorescens* to *Bacillus,* after 2 days incubation, was 1:25 (Table 5.4). Even when inoculated 6 h later than *P.fluorescens* 5064, *Bacillus* sp. A24 could still grow unhindered and showed a ratio of *P.fluorescens* to *Bacillus* of 1**:**1. These results demonstrated that *Bacillus* sp. A24 could out-compete *P. fluorescens* 5064, which is consistent with the conclusion we drew earlier in this study that *P. fluorescens* 5064 does not produce detectable antibiotics, at least not in culture. The faint luminescence induced by *P.fluorescens* 5064 when inoculated 6 h earlier than the *Bacillus* (Figure 5.16) implied that *Bacillus* sp. A24 needs a bigger population than *P. fluorescens* 5064 in order to abolish its QSS production.

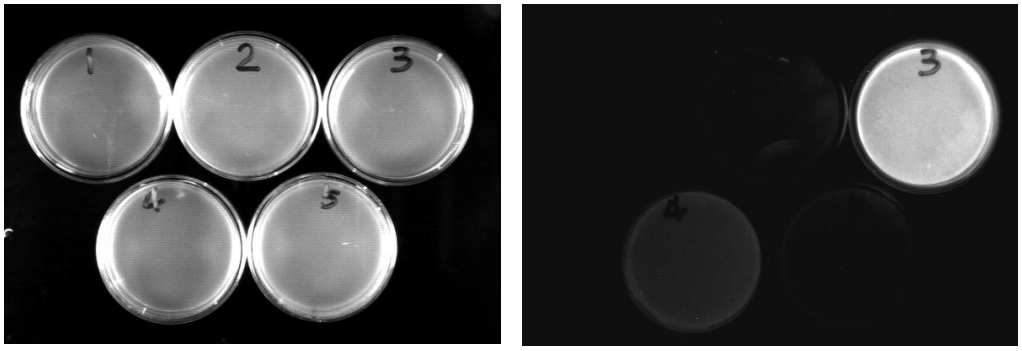



**Figure 5.16.: Quorum sensing signal degradation in *P. fluorescens* 5064 by *Bacillus* sp.A24**

Quorum sensing signal was extracted from culture filtrates of different bacterial co-inoculation mixtures after 48 h, added to semi-LB agar seeded with biosensor *E.coli* (pSB401), and overlaid on LB agar plates. The biosensor produces light in the presence of quorum sensing signal.

Left: Photographed in white light Right: Photographed in darkness

1.Negative control (biosensor only)
2.Co-incubation of *Bacillus* sp A24 and *P. fluorescens* 5064 at the same time
3.Positive control (*P. fluorescens* 5064 only)
4.*P. fluorescens* 5064 inoculated 6h earlier than *Bacillus* sp. A24
5.*Bacillus* sp. A24 inoculated 6h earlier than *P. fluorescens* 5064

**Table 5.4: Ratio of cell numbers of *P.fluorescens* 5064 to *Bacillus* sp. A 24, 2 days after co-inoculation**

| co-inoculation | Ratio of cell number of Pf* to Ba* |
|---|---|
| Co-inoculated at the same time | 1/25 |
| Pf was inoculated 6 h earlier | 1/1 |
| Ba was inoculated 6 h earlier | No Pf was detected |

*Pf: *P.fluorescens* 5064; Ba: *Bacillus* sp. A 24

### 5.4.4. Biocontrol efficacy of *Bacillus* sp. A24 in broccoli excised pathogenicity tests

5.4.4.1. Materials and methods

Establishment of a *Bacillus* sp. A 24 growth curve:
*Bacillus* sp. A24 growth calibration curve (colony forming units corresponding to $OD_{500}$ value) was established in order to estimate the concentration of bacterial suspension promptly once its corresponding OD value was detected. The experimental procedures were the same as described in 5.3.4.

Pathogenicity test procedure:
The broccoli excised head pathogenicity test procedures were the same as described in 5.3.7.2. Two bacterial inoculum concentrations, $1\times 10^8$ cfu/ml and $1\times 10^6$, were tested. six treatments with different inoculum ratio of *Bacillus* sp. A24 to *P. fluorescens* 5064 (Ba**:**Pf) were evaluated: (1). The cotton lint was soaked in sterile distilled water as a control; (2) The cotton lint was soaked in a mixed bacterial suspension of Ba**:**Pf = 1**:**3; (3) The cotton lint was soaked in a mixed bacterial suspension of Ba**:**Pf = 1**:**1; (4) The cotton lint was soaked in a mixed bacterial suspension of Ba**:**Pf = 3**:**1; (5) The cotton lint was soaked in *Bacillus* sp. A24 suspension only; (6) The cotton lint was soaked in *P. fluorescens* 5064 suspension only. Ten replicates were carried out for each treatment.

A five point scoring scale was used to assess the severity of disease symptoms (Darling *et al*., 2000): 0 = no symptoms, 1 = water soaking (loss of waxy bloom from florets), 2 = water soaking and tissue browning but no rot, 3 = tissue browning and softening (with rot), 4 = extensive black soft rot. Data were subjected to percentile angular transformation using arc sine (Schefler, 1969), thus the above categories 0-4 became 0% (0), 33% (1), 50% (2), 67% (3) and 100% (4) respectively.

5.4.4.3. Results and discussion

Figure 5.17 shows the *Bacillus* sp. A24 growth curve, where the number of CFU obtained over time are expressed in a manner which directly relates to the corresponding $OD_{500nm}$ values. This growth curve was used to estimate the *Bacillus* sp. A24 concentration of a sample once its OD value was determined.

To test the biocontrol activity of *Bacillus* sp. A24, we co-inoculated it with the pathogen at different ratios of *Bacillus* to *P. fluorescens* 5064 and at two different final inoculum concentrations (Figure 5.18). When the pathogen was inoculated at a rate of $1 \times 10^8$ cells/ml, the *Bacillus* was ineffective, but at a rate of $1 \times 10^6$, the *Bacillus* gave a disease reduction of 26% (78-58/78). The *Bacillus* itself, when inoculated alone, induced greater disease symptoms (tissue darkening) compared to the water control.

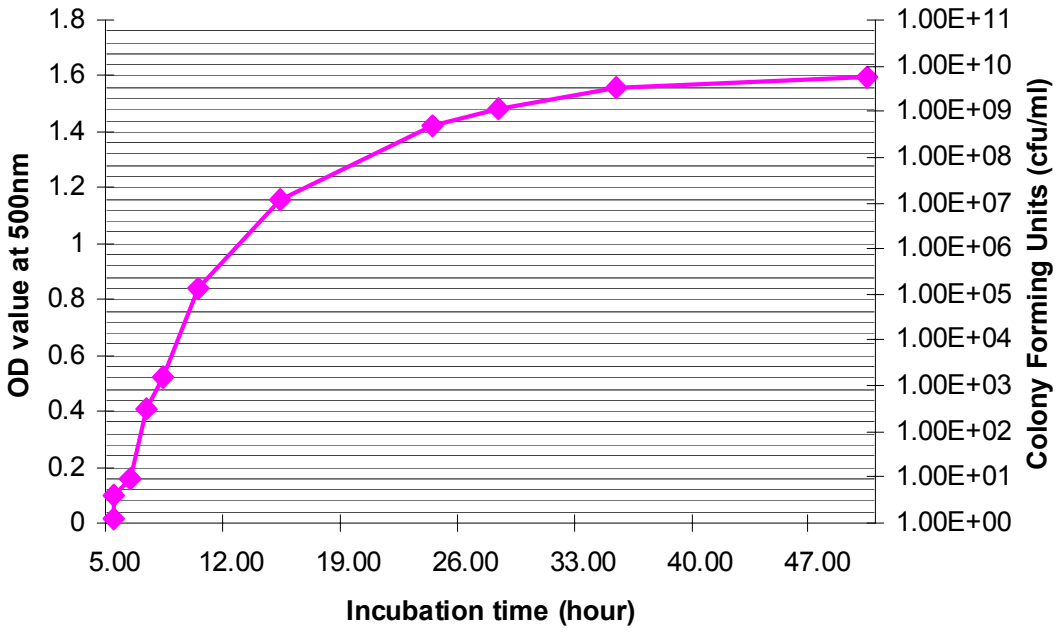
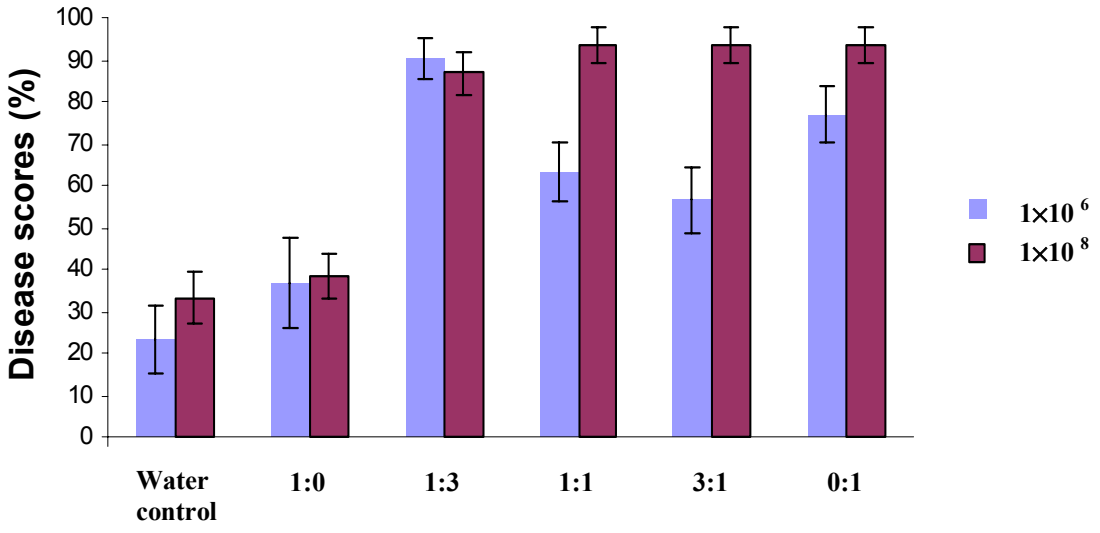



**Figure 5.17: *Bacillus* sp. A24 standard growth curve**

This graph shows the mean number of viable bacterial cells corresponding to each $OD_{500nm}$ value.




0% = No disease symptoms; 100% = Severe head rot
(Data arc sine transformed )
Bars = standard error of the mean (SEM)

**Figure 5.18: Biocontrol of head rot on excised broccoli heads using *Bacillus* sp. A24**

## 5.5. General discussion and conclusion

In this study, we have examined the potential for the biocontrol of broccoli head rot, caused by GpIV *P. fluorescens* strains, with two bacterial isolates: a Tn*5* mutant derived from the pathogen, *P. fluorescens* m6418, and *Bacillus* sp. A24. We have shown that the two biocontrol isolates are inhibitory towards the pathogen in two different ways: m6418 produces large amount of the antimicrobial substance pyrrolnitrin, and *Bacillus* sp. A24 is capable of degrading the 3-hydroxyoctanoly homoserine lactone produced by the pathogen. m6418 was able to reduce head rot in an excised head bioassay by 41%; the *Bacillus* sp. A24 was less effective at 26%.

TLC is a very useful technique of separation and identification of chemical substances. In this study, TLC performed on m6418 culture filtrates together with the results of antifungal assay revealed that pyrrolnitrin is the main antibiotic produced by mutant m6418. m6418 produces a large amount of pyrrolnitrin. This can be predicted not only by the size and colour intensity of the visualized pyrrolnitrin spot present on the TLC plates (Figure 5.2), but also by the strong antibiotic activity it exhibits against *P. ultimum* (Figure 5.3), the wild type *P. fluorescens* 5064 and *E. coli* (Chapter 2).

*P. ultimum* is a wide-spread and important oomycete plant pathogen causing seed decay, pre- and post-emergence damping-off, and root rot in many plant species (Hendrix *et al*., 1973). Biocontrol agents such as *P. fluorescens* and *Bacillus* species have been reported to effectively suppress *Pythium* disease through the biosynthesis of the antibiotics Phl and Plt (Elad and Baker, 1985; Trutmann and Nelson, 1992; vanDijk and Nelson, 1998). Although pyrrolnitrin (Prn) has been reported to effectively suppress a wide rage of fungal and bacterial pathogens (Arima *et al*., 1965; EI-Banna and Winkelmann, 1998), controversial conclusions were published regarding to its ability of inhibiting *P. ultimum*. EI-Banna and Winkelmann (1998) tested the antimicrobial effect of pyrrolnitrin on almost 40 bacterial and fungal strains. Their study showed that pyrrolnitrin was active against several filamentous fungi including *P.*

*ultimum*, yeasts and gram-positive bacteria (including some *Streptomyces* species, especially *S. antibioticus*). However, almost all tested gram-negative bacteria, including *E. coli* K12 and *P. fluorescens* ATCC 13525, were resistant to the doses tested except for *Proteus vulgaris* ATCC 13315. Levenfors and colleagues (2004) provided data that pyrrolnitrin showed an inhibitory effect against *P. ultimum* when applied at a high concentration (50mg/L). However, Howell and Stipanovic (1979) concluded that pyrrolnitrin was ineffective against *P. ultimum*. In this study, pyrrolnitrin produced by m6418 showed strong antibiotic activity against *P. ultimum*, as well as wild type *P. fluorescens* 5064 and *E. coli* JM109 (Chapter 2). The inhibitory effects shown by pyrrolnitrin towards *P. ultimum* in this study might partly be due to the high concentration of pyrrolnitrin produced by m 6418.

The research conducted in this study has provided evidence that the antibiotic producing strain m 6418 could be used as a biocontrol agent to control broccoli head rot disease. m6418 showed significant inhibitory effects against *P. fluorescens* 5064 in culture, on broccoli seedlings and excised broccoli heads. In culture, the inhibitory effect of m 6418 was greater when it was inoculated and allowed to establish before *P. fluorescens* 5064, although this was not the case on broccoli leaves, possibly because of its lower fitness on leaves, which was reflected by the faster decline in its population on the broccoli seedlings (Figure 5.11, 5.12).

With *Bacillus* sp. A24, no disease control effect was observed at the higher pathogen inoculum concentration of $1 \times 10^8$ cfu/ml. However, at a pathogen inoculum concentration of $1 \times 10^6$ cfu/ml, the *Bacillus* sp. A24 did show some control of head rot. The possible reasons for the lower effectiveness of *Bacillus* sp. A 24 as a biocontrol agent might be because: 1). A level of $1 \times 10^8$ cfu/ml for the pathogen is very high and represents a severe test for a biocontrol agent. In natural infections, disease symptoms are initiated at levels of $10^5 - 10^6$ cells of *P. fluorescens* on the head (data not shown); 2). As we mentioned before, when *P. fluorescens* 5064 reaches a threshold population, its quorum sensing system will be activated and its virulence factors start to be

expressed. It, therefore, will not make much difference for *Bacillus* sp. A24 to degrade the AHL of *P. fluorescens* after the quorum sensing system has already been triggered and the virulence factors have been expressed. *Bacillus* sp. A24 might have a better disease control effect in the natural environment, where *P. fluorescens* concentrations are less than we created in the laboratory tests; 3). The prospective disease control effect of *Bacillus* depends on its ability to degrade the quorum sensing signal and therefore block biosurfactant production, one of the pathogen's virulence factors (controlled by quorum sensing). However, subsequent assay showed that *Bacillus* sp. A24 itself is a biosurfactant producer. Given the role of biosurfactant in broccoli head rot (Hilderbrand 1989), it is possible that the biosurfactant produced by *Bacillus* sp. A24 enhanced disease – either by direct damage to the head surface and/or by encouraging damage by *P. fluorescens*. Although the biosurfactant produced by *Bacillus* sp A24 has not been identified, *Bacillus subtilis* has been reported to produce one of the most powerful cyclic lipopeptide biosurfactant – surfactin. It lowers the surface tension from 72 to 27.9 mN/m at concentrations as low as 0.005% (Arima *et al*., 1968). As the principal surfactant produced by *P. fluorescens* (viscosin) is a lipopeptide as well (Kochi *et al*., 1951), it is very likely that *Bacillus* sp. A24 provided *P. fluorescens* 5064 with the missing biosurfactant and therefore decreased its biocontrol ability; 4) A recent study demonstrated that bacteria employ a 'population control' circuit that automatically regulates population density. The cell density is broadcast and detected by the bacterial quorum sensing system. Accumulation of QSS will activate a 'killer gene', which in turn increases the death rate of the bacteria (You *et al*., 2004). According to their studies, degradation of *P. fluorescens* 5064 AHL will deactivate the 'population control' circuit and increase the pathogen cell number (You *et al*., 2004). This could possibly explain the enhanced disease development in the Ba:Pf = 1:3 treatment.

A negative control was not set up for the tests performed on broccoli seedlings. This was because in a previous study, a similar study had been done and the chance of

residual bacteria affecting test results was very low (Amandine Cochet, SAC exchange student report).

The broccoli used in the pathogenicity test were bought in a local supermarket. Unfortunately, head sterilisation is not possible with this technique, as soaking heads in surface sterilants was found to lead to the development of rotting (Simpson, 1993). To compensate for this, a negative control, where applied lint had been soaked in sterile distilled water only, was set up and encouragingly it showed a low disease score (less than 50%, no head rot). Therefore, results generated here are considered reliable.

Fluorescent Pseudomonads, such as *P. fluorescens*, are particularly important as biological control agents since they are widely occurring and highly effective at eliminating competing microbes. The *P. fluorescens* 5064 mutant m6418, a pyrrolnitrin overproducing strain which also has lost its ability to produce virulence factors, had significantly reduced the severity of broccoli head rot in an excised head pathogenicity test. This indicated that using m6418 as a biocontrol agent might be a satisfactory method in controlling the disease.

# Chapter 6. Summary of Results and Possible Future Work

Bacteria are amazing creatures in coping with extreme environments, finding gaps to thrive and maintaining a resource-economic life style. Quorum sensing is one of the sophisticated mechanisms bacteria use, it allows bacteria to communicate with each other and regulate gene expression in response to their population size. A large number of pathogenic bacteria control their virulence factor expression by quorum sensing. This is particularly practical as quorum sensing provides an effective timing-control mechanism and allows the pathogens to amass, without being sensed by the host, until the pathogen population size is large enough to overwhelm the plant host defenses and to make the virulent phenotype effective.

In this study, evidence has been provided to prove that the opportunistic broccoli soft rot pathogen *P. fluorescens* 5064 regulates at least one of its virulence factors – biosurfactant production by quorum sensing. Bacteria have been reported to produce many different types of quorum sensing signals, such as acyl-homoserine lactone (AHL), 2-heptyl-3-hydroxy-4-quinolone (PQS) (Pesci *et al*., 1999) and diketopiperazines (DKPs) (Holden *et al*., 1999). Among gram-negative bacteria, the most commonly used quorum sensing signals are the AHL homologues. In this study, the quorum sensing signal produced by *P. fluorescens* 5064 was identified as 3-OH-$C_8$-homoserine lactone using High Performance Liquid Chromatography and Mass Spectrometry.

The discovery that biosurfactant production in *P. fluorescens* 5064 is regulated by quorum sensing opens up the possibility for a novel control method of broccoli head rot. Although only the control of biosurfactant production by quorum sensing was examined in this study, it is possible that other virulence factors, such as pectic enzyme production, are also controlled by quorum sensing as in other plant pathogenic bacteria. By blocking the quorum sensing system, the pathogenic *P. fluorescens* strains that use

this mechanism to control virulence could potentially be rendered avirulent. Several possible ways of interrupting the quorum sensing circuitry have been discussed in the literature review. An AHL degrading strain *Bacillus* sp. A24 (kindly donated by Prof. Genevieve Defago) was assessed, as a biocontrol agent, for its efficacy in controlling the broccoli head rot disease. Although *Bacillus* sp. A24 was capable of abolishing *P. fluorescens* 5064 AHL production and growing competitively when co-inoculated with this pathogen, it did not show a disease control level as high as expected of an effective biological control agent. It reduced the disease score by 26% when inoculated with a low quantity of pathogen ($1 \times 10^6$), but was ineffective when inoculated with a higher rate of pathogen ($1 \times 10^8$). A subsequent test discovered that *Bacillus* A24 is a potent biosurfactant producer. It is very likely that *Bacillus* sp. A24 provided *P. fluorescens* 5064 with extra biosurfactant and therefore lost its disease control ability. Although *Bacillus* sp. A24 was less effective than expected, the original idea of employing quorum sensing as a target for controlling bacterial infections is still an attractive strategy. As plant-microbe interactions are complicated, comprehensive considerations are necessary in order to make this therapeutic strategy most effective.

During the screening of a biosurfactant-deficient *P. fluorescens* 5064 genomic library, mutant 6418 stood out due to its strong antibiotic producing ability. The antibiotic overproduced by mutant 6418 was capable of inhibiting *E. coli* and wild type *P. fluorescens* 5064 and was identified as pyrrolnitrin by thin layer chromatography combined with chemical colour reactions. Antifungal bioassays showed that the pyrrolnitrin produced by mutant 6418 strongly inhibited *Pythium ultimum*. *P. ultimum* is a widespread and important oomycete plant pathogen that causes seed decay, pre- and post-emergence damping off, and root rot in many plant species (Hendrix *et al*., 1973). Biocontrol agents such as *P. fluorescens* and *Bacillus* species have been reported to effectively suppress *Pythium* disease through the biosynthesis of antibiotics Phl and Plt (Elad and Baker, 1985; Trutmann and Nelson, 1992; vanDijk and Nelson, 1998). Although pyrrolnitrin has been reported to effectively inhibit filamentous fungi, yeasts and gram-positive bacteria (Arima *et al*., 1965; EI-Banna and Winkelmann, 1998), so

far there is no evidence showing that it antagonizes *P. ultimum* and gram-negative bacteria. In fact, Howell and Stipanovic (1979) concluded that pyrrolnitrin is totally ineffective against *P. ultimum*. EI-Banna and Winkelmann (1998), after testing the antimicrobial effect of pyrrolnitrin on almost 40 bacterial and fungal strains, concluded that all tested gram-negative bacteria were resistant to the pyrrolnitrin doses applied except for *Proteus vulgaris* ATCC 13315. The experiments conducted in this study have, for the first time, provided evidence that pyrrolnitrin is capable of inhibiting *P. ultimum* and gram-negative bacteria *E.coli* and *P. fluorescens*. The discrepancy of results might be partly due to the high concentration of pyrrolnitrin produced by mutant 6418.

Because Pyrrolnitrin produced by *P. fluorescens* plays an important role in the biocontrol of fungal plant pathogens, extensive work has been carried out to elucidate its gene expression and regulation. In addition to the identification of the prn synthesis gene operon (including 4 genes) (Hammer *et al*., 1997; Kirner *et al*., 1998), pyrrolnitrin has also been reported to be under global genetic control by a two-component regulatory system composed of the sensor protein ApdA (also called LemA) (Corbell and Loper, 1995) and the response regulator GacA (Gaffney *et al*., 1994; Laville *et al*., 1992). A mutation in *apdA* or *gacA* of *P. fluorescens* abolishes the production of Prn (Corbell and Loper, 1995; Laville *et al*., 1992). A sigma factor encoded by *ropS* is also reported to be necessary for Prn production (Sarniguet *et al*., 1995; Pfender *et al*., 1993). In this study, the over-expression of Prn in the Tn*5* mutant 6418 implies that the gene disrupted by Tn*5* is a repressor within the pyrrolnitrin biosynthesis or regulation pathway. Since a negative regulatory gene has not previously been described in the literature, the Tn*5* disrupted gene in mutant 6418 might be a novel regulatory gene involved in the Prn synthesis and further gene isolation would be of interest.

The research conducted in this study has provided evidence that the antibiotic producing strain mutant 6418 could be used as a biocontrol agent to control broccoli head rot disease. mutant 6418 showed significant control effects against *P. fluorescens* 5064 in

dual cultures, on greenhouse-grown seedling and excised broccoli heads. In the excised broccoli head rot pathogenicity test, *P. fluorescens* mutant 6418 was able to reduce head rot by 41%.

Although mutant 6418, as a biocontrol agent, was significantly effective in reducing the severity of broccoli head rot disease in the excised head pathogenicity tests, it showed weaker ecological fitness than that of the wild type *P. fluorescens* 5064. Biological control is generally considered a more ecologically acceptable approach when compared to chemical control, and the potential of developing a biocontrol method to reduce the incidence of head rot is very attractive. However, the discrepancy between the performance under environmentally controlled conditions, such as glasshouse, and field conditions could be a major obstacle to the development of commercial biocontrol products. Obviously, many physical and biological factors of the phyllosphere would affect the outcome or antagonist efficacy. Low humidity and ultraviolet irradiation are cited as major factors responsible for the rapid death of bacterial antagonists on foliage (Leben, 1974). Considerable research work in the fields is necessary to determine whether mutant 6418 is capable of controlling the disease in a field test. To make sure that the biocontrol is effective, sufficient numbers of the live antagonist might be needed at the appropriate time.

The gene cloning work revealed the existence of an AMP-binding protein in *P. fluorescens* 5064. Although AMP-binding protein has been reported to be necessary in the *Vibrio fischeri* quorum sensing regulatory circuit (Dunlap and Greenberg, 1988), insufficient evidence was found to prove that it was involved in the quorum sensing regulatory system in *P. fluorescens* 5064. The quorum sensing system in *P. fluorescens* 5064 remains unknown and future work would be of interest to identify these quorum sensing related genes.

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# Appendixes

**Appendix A:**

KB medium:

To 950 ml of deionized $H_2O$, add:

Neutralized bacteriological peptone 20 g,

| | |
|---|---|
| $K_2HPO_4 \cdot 3H_2O$ | 1.5 g |
| $MgSO_4 \cdot 7 H_2O$ | 1.5 g |
| Agar | 15 g |
| Glycerol | 15 ml |

Shake until the solutes have dissolved. Adjust the volume of the solution to 1 liter with deionized $H_2O$. Sterilize by autoclaving for 20 minutes at 15 Ib/sq. in.on liquid cycle.

**Appendix B:**

LB medium:

To 950 ml of deionized $H_2O$, add:

| | |
|---|---|
| Bacto-tryptone | 10 g |
| Bacto-yeast extract | 5 g |
| NaCI | 10 g |

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with deionized $H_2O$. Sterilize by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle.

**Appendix C:**

PDA medium:

Suspend 39g of potato dextrose (Oxoid Ltd. England) in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving for 15 minutes.

**Appendix D:**

Ehrlich's reagent: The reagent is made from 0.1% p-dimethylaminobenzaldehyde inconcentrated HCI, followed by 0.1% sodium nitrite in water.

**Appendix E**:

Pauly's reagent: It consists of three separate solutions: 0.5% $NaNO_2$, 0.5% sulfanilic acid in 2% HCI, and 5% NaOH in 50% ethanol. Equal volumes of $NaNO_2$ and sulfanilic acid solutions are mixed immediately prior to use, and plates are sprayed with this mixture and then with NaOH before being warmed with a heat gun to give burnt-orange and red colours for phenolic compounds.

**Appendix F**:

Genome DNA extraction protocol using PUREGENE® Kit

a. Preparation of overnight cultures:

①. Pour 5ml of LB broth with kanamycin (50μg/ml) into sterilised 50ml tube.

②. Using sterile techniques remove a single colony of *P. fluorescens* mutant 6423 and gently stir into the above medium.

③. Place the tube into an incubator shaking at 26°C overnight.

b. Cell lysis

①. Turn on the water bath and heat to 80°C.

②. Centrifuge the cell suspension (step1) at 1000 x g for 3 minutes to pellet cells. Carefully remove as much supernatant as possible using a pipet.

③. Add 5ml **Cell Lysis Solution** provided in the kit and gently pipet up and down until the cells are suspended.

④. Incubate samples at 80°C for 5 min to lyse cells.

c. Rnase treatment

①. Add 15μl **Rnase A Solution** to the cell lysate.

②. Mix the sample by inverting the tube 25 times and incubate at 37°C for 15-60 min.

d. Protein precipitation.

①. Cool sample to room temperature.

②. Add 1ml **Protein Precipitation Solution** to the cell lysate.

③. Vortex vigorously at high speed for 20 seconds to mix the **Protein Precipitation Solution** uniformly with the cell lysate. For species with a high mucopolysaccharide content, placing the sample on ice for 5 min before centrifugation may be required.

④. Centrifuge at 2000 x g for 10 min. For species with a high mucopolysaccharide content, centrifugation at 4°C may be required. The precipitated proteins should form a tight pellet.

e. DNA Precipitation

①. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 15ml centrifuge tube containing 3ml 100% Isopropanol (2-propanol) (Sigma Co.).

②. Mix the sample by inverting gently 50 times.

③ Centrifuge at 2000 x g for 3 min, the DNA should be visible as a small white pellet.

④. Pour off supernatant and drain tube on clean absorbent paper. Add 3 ml 70% Ethanol and invert the tube several times to wash the DNA.

⑤. Centrifuge at 2000 x g for 1min. Carefully pour off the ethanol.

⑥. Invert and drain the tube on clean absorbent paper and allow to air dry 10-15 min.

f. DNA Hydration

①. Add 500µl of DNA Hydration Solution.

②. Rehydrate DNA by incubating sample for 1 hour at 65°C and overnight at room temperature. Tap tube periodically to aid in dispersing the DNA.

③. Sample was centrifuged briefly and then transferred to a 1.5 ml eppendorf tube.

④. Store DNA at -20°C.

**Appendix G**

The gel and sample preparation and electrophoresis procedures

(1). Gel Preparation

The required amount (depending on the percentage of gel required, usually, a higher concentration of the gel is better to separate smaller sizes of DNA bands) of agarose was weighed and adjusted to the appropriate concentration by adding Tris-acetate-EDTA (TAE) buffer (see appendices). The mixture was microwaved until molten, mixed by shaking the bottle and micro waved again until boiling. The gel was allowed to cool down to about 60°C then cast into a gel tray to a height of 0.5-0.75 cm depending on the amount of sample that need to be loaded.

(2). Sample Preparation:

To the samples, loading buffer (LB, see appendices) was added (sample : LB = 1:1) and mixed prior to electrophoresis in order to increase its density. In addition, it contains a visible dye that allows the direction of the electrophoresis to be monitored.

(3). Electrophoresis

Gel electrophoresis involved the loading of a small sample of DNA and/or RNA into a preformed well in the gel. This was then subjected to an electric current in a horizontal tank, via an ionic buffer solution (TAE or TBE), for a set amount of time depending on the agarose concentration, gel size, voltage applied and type of nucleic acid sample. This technique effectively separates fragments of DNA and/or RNA on the basis of molecular size. As the electric field was applied across the gel, DNA and/or RNA, which are negatively charged at neutral Ph, migrated towards the anode.

The rate of migration is dependent on:

a. the molecular size of the nucleic acid – larger molecules migrate more slowly.
b. the agarose concentratio of the gel – there is a linear relationship between agarose concentration and efficient range of separation.
c. Voltage – at low voltage the rate of migration is proportional to the voltage applied, and to obtain the maximum resolution of DNA fragments greater than 2 kb in size, agarose gels should be run at no more than 5.0v/cm.
d. The composition of the electrophoretic buffer – in buffers of high ionic strength electric conductance is very efficient.

The running electrophoresis parameters included:

Setting Voltage: 120 V or 100 V

Time: 1h or 1.5h,

However, it depends on the electrophoresis system used.

(4). Staining Procedure:

A stock solution of 10.00mg/ml ethidium bromide was prepared and not more than 0.5µg/ml used for staining (ethidium bromide was diluted by distilled water). The gel was placed into the ethidium bromide solution and left in the dark for about 10 to 15 minutes and then washed in distilled water. Ethidium bromide is a very powerful mutagen used for visualization of nucleic acids in gels under UV light. Consequently contaminated buffers and gels were treated as toxic waste.

(5). Photodocumentation of Electrophoresis Gel

Photographs of the gels were obtained by using UV illumination, due to the fluorescent yield that ethidium bromide/DNA complexes emitted.

**Appendix H**

DNA purification from the gel (QIA quick gel extraction kit protocol, QIAGEN, Germany:

(1). Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.

(2). Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100mg ~ 100µl).

(3). Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.

(4). After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

(5). Add 1 gel volume of isopropanol to the sample and mix (This step increases the yield of DNA fragments less than 500bp and more than 4kb).

(6). Place a QIAquick spin column in a provided 2 ml collection tube.

(7). To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.

(8). Discard flow-through and place QIAquick column back in the same collection tube.

(9). To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

(10). Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at about 13000 rpm.

(11). Place QIAquick column into a clean 1.5-ml microfuge tube.

(12). To elute DNA, add 50 µl of Buffer EB (10mM Tris HCI, pH 8.5) to the center of the QIAquick membrane and let the column stand for 1 min, then centrifuge the column for 1 min at maximum speed.

**Appendix I**

Transformation of *E. coli*

(1). Preparation for Transformation:

a. Equilibrate a water bath to 42°C; Warm the vial of SOC medium to room temperature;
b. Pre-warm the selective plates at 37°C for 30 min;
c. Thaw on ice 1 vial of competent *E.coli* for each transformation.

(2). Performing the OneShot® Transformation

a. Add 2µl of the TOPO Cloning reaction into a vial of TOP10 OneShot® Chemically Competent *E. coli* and mixed gently.
b. Incubate on ice for 5 to 30 min.
c. Heat-shock the cells for 30 seconds at 42 °C without shaking.
d. Immediately transfer the tubes to ice.
e. Add 250 µl of room temperature SOC medium.
f. Cap the tube tightly and shake the tube horizontally (200rpm) at 37°C for 1 hour.
g. Spread 50 and 200 µl from each transformation on pre-warmed selective plates, respectively and incubate overnight at 37°C.

10 colonies are picked up for the further analysis.

**Appendix J**

The plasmid purification procedure (High Pure Plasmid Isolation Kit, Roche Diaghostics Co., Germany):

(1). Grew the white colonies in 5 ml LB broth containing 100µg/ml Ampicillin at 37°C overnight, with shaking.

(2). Placed binding buffer (the green cap) on ice.

(3). Centrifuged the bacteria cells from step (1) and discarded the supernatant.

(4). Resuspended the pellet in 250 µl suspension buffer (white cap and was kept in 4°C fridge) and mixed well (up and down).

(5). Added 250µl lysis buffer (red cap), mixed gently by inverting the tube 3 to 6 times and incubated for 5min at room temperature.

(6). Added 350µl chilled binding buffer (green cap), mixed gently by inverting the tubes 3 to 6 times, incubated on ice for 5 min. The solution became cloudy and flocky.

(7). Centrifuged for 10min at 13000 rpm in a standard table top centrifuge (Heraeus, Biofuge 13).

(8). Combined the High Pure Filter tube and the collection tube, pipetted the supernatant to the upper reservoir and centrifuged for 1 min at 13600 rpm.

(9). Discarded the flowthrough, added 700µl washing buffer II (blue cap) and centrifuge for 1min.

(10). Discarded the flowthrough, centrifuged for an additional 1 min to remove residual washing buffer.

(11). Discarded the collection tube and insert the filter tube in a clean 1.5ml eppendorf tube, added 100µl elution buffer (colorless cap) and centrifuged for 1min.

(12). Visualize by agarose gel electrophoresis.

**Appendix K**

Hybridisation procedure:

a. Place 10µl of plasmids 19 and 20 in a vial, respectively and denature at 100°C for 10 min in a heating block.

b. Place the denatured plasmids on ice immediately
c. Quickly spin the sample to collect the plasmids and then add them on a Hybond-$N^+$ nylon membrane carefully.
d. Crosslink the plasmids to the membrane using a UV crosslinker (Stratalinker ®2400, Stratagene, USA).
e. Add 10ml of 'easyhyb' liquid in a 50ml hybridisation tube. Carefully place the membrane in the tube, set up on the hybridisation oven and pre-hybrid for 3 hours at 42°C.
f. The labeled probe was denatured at 100°C for 10 min and then pipitted into the above hybridisation solution (make sure that the probe does not touch the membrane directly). Shake slightly to mix the probe and then place the vial back to the oven and hybrid for over night.

**Appendix L:**

The hybridisation detection method:

a. Wash the membrane twice, for 5 min in 2x wash solution at room temperature with gentle agitation (2x wash solution: 2 x SSC with 0.1% SDS).
b. Wash the membrane twice for 15 min in 0.5x wash solution at 65°C with gentle agitation.
c. Place the membrane in a petri-dish. Block the membrane by shaking gently with 5% blocking reagent in 100mM Maleic Acid pH 7.5, 150mM NaCI for an hour (total volume 10ml for a small membrane).
d. Centrifuge the antidigoxygenin for 5 min prior to use. Add a 1 in 10,000 dilution of antibody to the blocking solution (i.e. 1µl of the antibody to 10 ml of blocking solution). Incubate for an hour
e. Wash the membrane twice in Buffer 1 (100mM Maleic acid pH 7.5, 150 mM NaCI) for 15 min each time.
f. Wash the membrane twice in Washing buffer (Maleic acid buffer with Tween 20, 0.3% v/v) for 15 min each time.

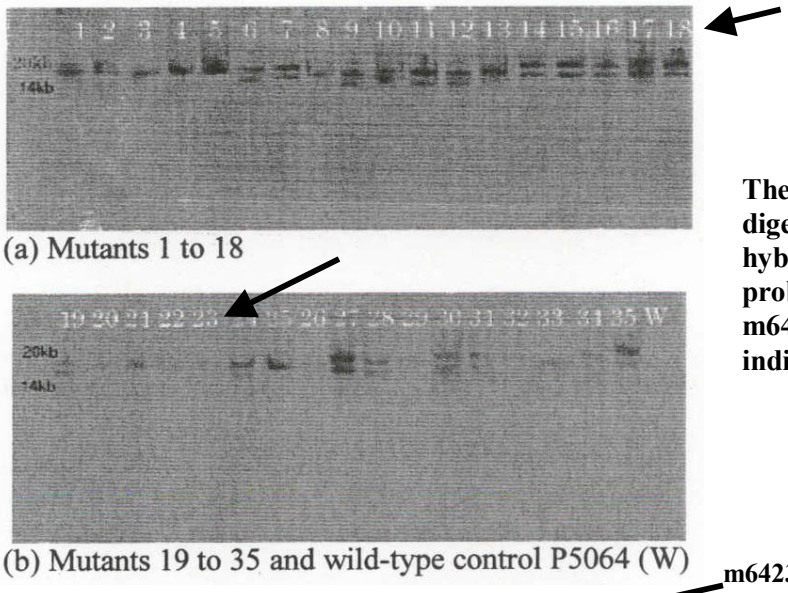
g. Equilibrate for 3 min in 20 ml buffer 3 (0.1M Tris HCI, 0.1M NaCI, pH 9.5).

h. Dilute CDP-STAR 1:100 in the above buffer 3 and incubate the membrane in CDP-STAR for 5 min.

i. Cover the damp membrane with clingfilm. Expose the membrane to film for a few min in dark room, then place the film in developer for 5 min, wash in water for 1 min, place in fixer for 5 min, wash in water for 5 min.

## Appendix M

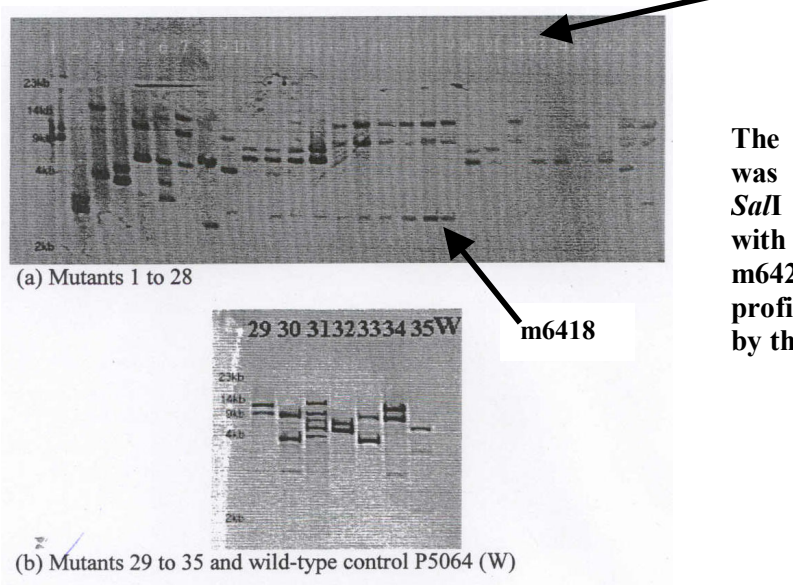
Southern blots profile of 35 *P. fluorescens* 5064 Tn*5* mutants (Darling, 1998).




(a) Mutants 1 to 18

(b) Mutants 19 to 35 and wild-type control P5064 (W)

**The mutant DNA was digested with *Eco*RI and hybridised with Tn*5* probe. m6423 (23) and m6418 (18) profiles are indicated by the arrows.**




(a) Mutants 1 to 28

(b) Mutants 29 to 35 and wild-type control P5064 (W)

**The mutant DNA was digested with *Sal*I and hybridised with Tn*5* probe. m6423 and m6418 profile are indicated by the arrows.**

## Appendix N

Tn*5* map (refer to Simon *et al*., 1983) showing the restriction enzyme sites.