QUORUM SENSING AND QUORUM QUENCHING STUDIES IN CLINICAL *Acinetobacter* ISOLATES FROM UMMC

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FACULTY OF MEDICINE
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ABSTRACT

Quorum sensing is a term that describes an environmental sensing system that allows bacteria to monitor their own population density. Many gram negative bacteria use *N*-acyl-homoserine lactones (AHLs) as autoinducing quorum sensing signal molecules. This cell-density dependent regulation of gene expression contributes significantly to the size and development of the biofilm. *Acinetobacter* spp. are now emerging as important nosocomoial pathogens and it has been shown that they form biofilms with enhanced antibiotic resistance. In this study, we sought to find out if the biofilm formation among clinical isolates of *Acinetobacter* spp. is under the control of autoinducing quorum sensing molecules. We have also evaluated various quorum quenching strategies which can be used to attenuate the pathogenicity of these organisms.

Biofilm formation among clinical isolates of *Acinetobacter* spp. was assessed and the production of signal molecules were detected with *Chromobacterium violaceum* CV026 biosensor system. Characterisation of autoinducers was carried out by thin layer chromatography bioassay followed by mass spectrometric analysis. An autoinducer synthase gene, abaI was identified among the isolates that produce quorum sensing signal molecules. The *abaI* gene was cloned, a mutant of *abaI* gene was created, and the biofilm forming capability of the mutant *abaI* gene was analysed. The quorum quenching property of the extracts from two soil isolates with a strong AHL-inactivating enzyme activity and the plant extracts of *Phyllanthus* spp., garlic bulb and lemon on clinical *Acinetobacter* spp. isolates was elucidated.

Using a microtitre-plate assay it was shown that 60% of the 50 *Acinetobacter* spp. isolates significantly formed biofilms. *Chromobacterium violaceum* CV026 biosensor system detected the production of long chain AHLs among seven of these biofilm

forming isolates. Thin layer chromatography bioassay and mass spectrometric analysis revealed that five of these isolates produced *N*-decanoyl homoserine lactone and two isolates produced acyl-homoserine lactone with a chain length equal to C_{12}. The abaI gene was identified and it was shown that there was considerable inhibition in biofilm formation in the *abaI*::Tc mutant. Further, two soil isolates of the genus *Bacillus* with a strong AHL-inactivating enzyme activity were identified and the extracts from these soil bacilli was used to inhibit the biofilm forming capabilities of quorum sensing signal molecule producing clinical isolates of *Acinetobacter* spp. The quorum quenching properties of *Phyllanthus* spp. extracts, garlic bulb and lemon on the clinical *Acinetobacter* spp. isolates have also been reported.

These data are of great significance as the signal molecules aid in biofilm formation which in turn confer various properties of pathogenicity to the clinical isolates including drug resistance. The use of quorum sensing signal blockers to attenuate bacterial pathogenicity is therefore highly attractive, particularly with respect to the emergence of multi antibiotic resistant bacteria. The results also showed that the extracts were capable of inhibiting the biofilm formation and also degrade the AHLs produced by these clinical isolates of *Acinetobacter* spp. Our results indicate that the AHLs -inactivation approach represents a promising strategy for prevention of diseases in which virulence is regulated by quorum sensing.

ABSTRAK

"Quorum sensing" adalah istilah yang menggambarkan sistem "sensing" (perangsangan) bakteria terhadap alam sekitar yang membolehkan pemantauan kepadatan populasinya. Kebanyakan bakteria gram negatif menggunakan "*N*-acyl-homoserine lactones (AHLs)" sebagai isyarat molekul untuk "autoinducing quorum sensing". Pengaturan ekspresi gen ini yang bergantung terhadap kepadatan sel menyumbang kepada saiz dan pembentukan "biofilm". *Acinetobacter* spp. kini muncul sebagai patogen nosokomial yang penting dalam pembentukan "biofilm" dengan rintangan antibiotik. Kajian ini bertujuan untuk mengetahui sama ada pembinaan "biofilm" oleh *Acinetobacter* spp. pencilan klinikal adalah di bawah kawalan molekul "autoinducing quorum sensing". Pelbagai strategi "quorum quenching" yang dapat digunakan untuk melemahkan kepatogenan organisma ini juga dinilai.

Dalam kajian ini, pembentukan "biofilm" oleh *Acinetobacter* spp. pencilan klinikal dinilai dan produksi isyarat molekul dikesan dengan menggunakan sistem biosensor *Chromobacterium violaceum* CV026. Pencirian "autoinducers" dijalankan dengan menggunakan bioassai kromatografi lapis tipis dan ini diikuti dengan analisis spektrometrik jisim. Suatu gen, *abaI*, yang merupakan gen "autoinducer synthase" telah dikenalpasti di dalam pencilan tersebut sebagai pembentuk isyarat molekul "quorum sensing". Gen *abaI* tersebut telah diklon untuk menghasilkan gen mutan *abaI* dan kebolehan gen mutan ini untuk menghasilkan "biofilm" dianalisa. Selain itu, ciri-ciri "quorum quenching" yang dimiliki oleh dua jenis ekstrak tanah dan juga ekstrak tumbuhan *Phyllanthus* spp., bawang putih dan lemon telah diuji.

Dengan menggunakan asai "microtitre-plate", sebanyak enam puluh peratus daripada lima puluh pencilan *Acinetobacter* spp. telah menunjukkan pembentukan

"biofilm" yang signifikan. Tujuh daripada pencilan pembentuk "biofilm" ini menunjukkan produksi rantai panjang AHL yang telah dikesan oleh sistem biosensor *Chromobacterium violaceum* CV026. Dalam analisis spektrometrik jisim dan kromatografi lapis tipis pula, lima daripada pencilan ini menunjukkan produksi "*N*-decanoyl homoserine lactone" dan dua lagi menunjukkan produksi "acyl-homoserine lactone" dengan kepanjangan rantai bersamaan dengan C_{12}. Gen *abaI* telah dikenalpasti dan ditunjukkan bahawa terdapat perencatan dalam pembentukan "biofilm" dalam mutan *abaI*::Tc. Kajian lanjutan juga telah dapat mengenalpasti dua pencilan tanah dari genus *Bacillus* dengan aktiviti enzim pentakaktifan AHL yang kuat. Ekstrak daripada *bacilli* tanah ini telah digunakan untuk merencatkan kebolehan pembentukan "biofilm" dalam isyarat molekul "quorum sensing" *Acinetobacter* spp. pencilan klinikal. Selain itu, pencirian "quorum quenching" ekstrak *Phyllanthus* spp., bawang putih dan lemon pada *Acinetobacter* spp. pencilan klinikal dapat dilaporkan.

Penemuan kajian ini adalah sangat signifikan kerana isyarat molekul yang terlibat dalam pembentukan "biofilm" dapat mencirikan kepatogenan *Acinetobacter* spp. pencilan klinikal yang salah satunya termasuk kerintangan terhadap pelbagai jenis ubat. Oleh yang demikian, penggunaan "quorum sensing signal blockers" untuk melemahkan kepatogenan bakteria memainkan peranan yang amat penting terutamanya dengan kemunculan bakteria yang rintang terhadap pelbagai antibiotik. Selain itu, hasil kajian ini juga menunjukkan ekstrak-ekstrak yang diuji dapat merencatkan pembentukan "biofilm" dan juga menurunkan penghasilan "AHLs" oleh *Acinetobacter* spp pencilan klinikal. Penemuan kajian ini juga membuktikan bahawa pendekatan pentakaktifan AHLs merupakan strategi yang menjanjikan dalam pencegahan penyakit di mana "virulence" (kemudaratan) dikawal oleh "quorum sensing".

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µg	microgram
µl	microliter
µM	micromolar
µm	micromole
bp	base pair
g	gram
L/ min	Litre per minute
M	molar
m/ z	mass- to- charge ratio
ml	milliliter
mM	milimolar
mm	millimeter
nM	nanomolar
nm	nanometer
ºC	degree Celcius
U	unit
V	volt
v/ v	volume/ voloume
vs.	versus
ABC	*Acinetobacter baumannii- calcoaceticus* complex
ACN	acetonitrile
AHLs	acyl homoserine lactones
ARDRA	amplified ribosomal DNA restriction analysis
BLAST	basis local alignment search tool
CFU	colony forming units
CIP	calf intestinal phosphatase
CviR	*Chromobacterium violaceum* repressor
dATP	2'- deoxyadenosine triphosphate
DHL	*N*- Decanoyl homoserine lactone
DMRM	Dynamic multiple reaction monitor

DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylene diamine tetraacetic acid
FA	formic acid
HHL	*N*- Hexanoyl homoserine lactone
HPLC	High- performance liquid chromatography
ICUs	Intensive care units
IPTG	isopropyl-beta-D-thiogalactopyranoside
LB	Luria-Bertani
LPS	lipopolysaccharide
MS	mass spectrometry
NA	nutrient agar
NCBI	National Centre for Biotechnology Information
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Rf	retention factor
RNA	ribonucleic acid
Rpm	rotation per minute
TAE	tris-acetic EDTA
TLC	thin layer chromatography
UMMC	University Malaya Medical Centre
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Chapter 1

Introduction

1.1 Taxonomy and epidemiology of *Acinetobacter* spp.

Acinetobacter spp. are gram negative aerobic coccobacilli. The members placed under this genus have a long history of taxonomic changes. Taxonomic changes occurred frequently with increasing knowledge of the characteristics of the microorganisms and with the emergence of new genetic analysis tools. Thus, the taxonomic place of some species among a group was often changing and these were published in the subsequent editions of *Bergey's Manual of Determinative Bacteriology*, the reference book for bacteriology.

A series of Gram-negative bacilli within a similar morphology exhibited divergent biochemical tests, conditions of growth, epidemiology, and pathogenic roles which varied with different potential sites in human or in different environmental sites. Technical uncertainties made the identification and classification of these organisms difficult. Thus, these Gram-negative bacteria remained unclassified for a long time. Some of these bacteria were designated as *Bacterium anitratum* (Schaub *et al*, 1948); *Herellea vaginicola* and *Mima polymorpha* (DeBord, 1939); or *Achromobacter, Alcaligenes, Neisseria, Micrococcus calcoaceticus, Diplococcus, B5W, and Cytophaga* (Juni, 1972). The common characteristics of these organisms were that they were all Gram negative and all of them were strict aerobes. With some of these organisms a confusing morphology in Gram stained preparations has contributed towards the difficulty in identifying and classifying these bacteria. They appear as coccoid or coccobacilli cells with a trend toward diplococcic arrangement which make them look like *Neisseria* spp. That's why these bacteria were designated as *Micrococcus* and *Diplococcus* earlier. In the same time two other species, *Moraxella glucidolytica* and *Moraxella lwoffi* were recognized. This was the beginning of the classification of these aerobic Gram-negative bacilli. The final designation *Acinetobacter* was proposed by

Brisou in 1957 and confirmed by the Subcommittee of the Taxonomy of *Moraxella* and allied bacteria. The genus *Acinetobacter* was definitively accepted in the *Bergey's Manual of Determinative Bacteriology* only in June 1984.

The genus *Acinetobacter*, as currently defined, comprises gram-negative, strictly aerobic, nonfermenting, nonfastidious, nonmotile, catalase-positive, oxidase-negative bacteria with a DNA G-C content of 39% to 47%. Based on more recent taxonomic data, it was proposed that member of the genus *Acinetobacter* should be classified in the new family *Moraxellaceae* within the order *Gammaproteobacteria*, which includes the genera *Moraxella*, *Acinetobacter*, *Psychrobacter*, and related organisms (Rossau *et al*., 1991). There was a major breakthrough in the history of the genus *Acinetobacter* was achieved in 1986 by Bouvet and Grimont, who, based on DNA-DNA hybridization studies, distinguished 12 DNA (hybridization) groups or genospecies. Some of these genospecies were given formal species names, including *A. baumannii*, *A. calcoaceticus*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, and *A. lwoffii* (Bouvet and Grimont, 1986). Work done by some of the researchers (Bouvet and Jeanjean, 1989; Tjernberg and Ursing, 1989; Nishimura *et al*, 1987) resulted in the description of further *Acinetobacter* genomic species, including the named species *A. radioresistens*, which corresponds to *Acinetobacter* genomic species 12 described previously (Bouvet and Grimont, 1986). Some of the independently described (genomic) species turned out to be synonyms, for example, *A. lwoffii* and *Acinetobacter* genomic species 9 or *Acinetobacter* genomic species 14, described by Bouvet and Jeanjean (14BJ), and *Acinetobacter* genomic species 13, described by Tjernberg and Ursing (13TU). More recently, 10 additional *Acinetobacter* species were described, including 3 species of human origin, *A. parvus*, *A. schindleri*, and *A. ursingii* (Nemec *et al*., 2003; Nemec *et al*., 2001), and 7 species isolated from activated sludge, *A. baylyi*, *A. bouvetii*, *A. grimontii*, *A. tjernbergiae*, *A. towneri*, *A. tandoii*, and *A. gerneri* (Carr *et al*., 2003;

Anton *et al*., 2008), increasing the actual number of validly described (genomic) species to 31, of which 17 have been given valid species names (Table 1.1). Four of the above listed species, *A. calcoaceticus*, *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU, are very closely related and difficult to distinguish from each other by phenotypic properties. It has therefore been proposed to refer to these species as the *A. calcoaceticus-A. baumannii* complex (Gerner-Smidt, 1992; Gerner-Smidt *et al*., 1991). However, this group of organisms comprises not only the three most clinically relevant species, *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU, but also an environmental species, *A. calcoaceticus*, that has frequently been recovered from soil and never been implicated in serious clinical disease.

Table 1.1: Delineation of *Acinetobacter* genomic species

Species name	Genomic species number	Type strain
A. baumannii	2	ATCC 19606
A. baylyi		DSM 14961
A. bouvetii		DSM 14964
A. calcoaceticus	1	ATCC 23055
A. gerneri		DSM 14967
A. grimontii		DSM 14968
A. haemolyticus	4	ATCC 17906
A. johnsonii	7	ATCC 17909
A. junii	5	ATCC 17908
A. lwoffii	8/9	ACTC 15309; ATCC 9957
A. parvus		NIPH384
A. radioresistens	12	IAM 13186
A. schindleri		NIPH1034
A. tandoii		DSM 14970
A. tjernbergiae		DSM 14971
A. towneri		DSM 14962
A. ursingii		NIPH137
A. venetianus		ATCC 31012
	3	ATCC 19004
	6	ATCC 17979
	10	ATCC 17924

Table 1.1 Continued

	11	ATCC 11171
	13TU	ATCC 17903
	13BJ, 14TU	ATCC 17905
	14BJ	CCUG 14816
	15BJ	SEIP 23.78
	15TU	M 151
	16	ATCC 17988
	17	SEIP Ac87.314
	Between 1 and 3	10095
	Close to 13TU	10090

(Adapted from Anton *et al*., 2008)

Members of the genus *Acinetobacter* are considered ubiquitous organisms. This is true as acinetobacters can be recovered after enrichment culture from virtually all samples obtained from soil or surface water (Baumann, 1968a). These earlier findings have contributed to the common misconception that *A. baumannii* is also ubiquitous in nature (Fournier and Richet, 2006). In fact, not all species of the genus *Acinetobacter* have their natural habitat in the environment. However, a systematic study to investigate the natural occurrence of the various *Acinetobacter* species in the environment has never been performed. Most *Acinetobacter* species that have been recovered from human clinical specimens have at least some significance as human pathogens (Seifert *et al*., 1993; Seifert *et al*., 1994). *Acinetobacters* are part of the human skin flora. In an epidemiological survey performed to investigate the colonization of human skin and mucous membranes with *Acinetobacter* species, up to 43% of nonhospitalized individuals were found to be colonized with these organisms (Seifert *et al*., 1997). The most frequently isolated species were *A. lwoffii* (58%), *A. johnsonii* (20%), *A. junii* (10%), and *Acinetobacter* genomic species 3 (6%). In a similar study, a carrier rate of 44% was found for healthy volunteers, with *A. lwoffii* (61%), *Acinetobacter* genomic species 15BJ (12%), *A. radioresistens* (8%), and *Acinetobacter* genomic species 3 (5%) being the most prevalent species (Berlau *et al*., 1999b). In patients hospitalized on a

regular ward, the carriage rate of *Acinetobacter* species was even higher, at 75% (Seifert *et al*., 1997). The fecal carriage of *Acinetobacter* was studied and found a carrier rate of 25% among healthy individuals, with *A. johnsonii* and *Acinetobacter* genomic species 11 predominating (Dijkshoorn *et al*., 2005). In contrast, *A. baumannii*, the most important nosocomial *Acinetobacter* species, was found only in 0.5% and 3% on human skin (Berlau *et al*., 1999b; Seifert *et al*., 1997) and in human feces (0.8%) (Dijkshoorn *et al*., 2005), and *Acinetobacter* genomic species 13TU was not found at all (Berlau *et al*., 1999b; Dijkshoorn *et al*., 2005; Seifert *et al*., 1997). More recently, the nares of healthy U.S. soldiers were investigated and acinetobacters were not found, but they did not use enrichment culture to increase the recovery rate (Griffith *et al*., 2006). In a subsequent study, Griffith *et al*. did not detect skin carriage of the *A. calcoaceticus-A. baumannii* complex among a representative sample of 102 U.S. Army soldiers deployed in Iraq, but again, they performed cultures without enrichment and with an extremely long transport time that may have contributed to this finding (Griffith *et al*., 2007). Notably, in tropical climates, the situation may be different. In Hong Kong, it was found that 53% of medical students and new nurses were colonized with acinetobacters in summer versus 32% in winter (Chu *et al*., 1999). Such a seasonal variability in skin colonization may contribute to the seasonal variation seen in the prevalence of *A. baumannii* in clinical samples (McDonald *et al*., 1999). *Acinetobacter* genomic species 3 (36%), *Acinetobacter* genomic species 13TU (15%), *Acinetobacter* genomic species 15TU (6%), and *A. baumannii* (4%) were the most frequently recovered species, while *A. lwoffii*, *A. johnsonii*, and *A. junii* were only rarely found. Although various *Acinetobacter* species have been isolated from animals and *A. baumannii* was occasionally found as an etiologic agent in infected animals (Francey *et al*., 2000; Vaneechoutte *et al*., 2000), the normal flora of animals has never been studied systematically for the presence of acinetobacters. *A. baumannii* was recovered from

22% of body lice sampled from homeless people (La Scola *et al*., 2004). The inanimate environment has also been studied for the presence of acinetobacters. Berlau *et al*. investigated vegetables in the United Kingdom and found that 30 of 177 vegetables (17%) were culture positive for *Acinetobacter* (Berlau *et al*., 1999a). Interestingly, *A. baumannii* and *Acinetobacter* genomic species 11 (each at 27%) were the predominant species, followed by *A. calcoaceticus* and *Acinetobacter* genomic species 3 (each at 13%), while *Acinetobacter* genomic species 13 was found only once. In Hong Kong, 51% of local vegetables were culture positive for *Acinetobacter* species, the majority of which were *Acinetobacter* genomic species 3 (75%), but one sample grew *A. baumannii* (Houang *et al*., 2001). They also found acinetobacters in 22 of 60 soil samples in Hong Kong, and the most frequent species were *Acinetobacter* genomic species 3 (27%) and *A. baumannii* (23%), with only one sample yielding *A. calcoaceticus* (Houang *et al*., 2001). Some recently described *Acinetobacter* species are *A. baylyi*, *A. bouvetii*, *A. grimontii*, *A. tjernbergiae*, *A. towneri*, and *A. tandoii*. These were isolated from activated sludge and are environmental species and have never been found in humans (Carr *et al*., 2003). In contrast, two other recently described species, *A. schindleri* and *A. ursingii*, have been recovered only from human specimens, while *A. parvus* was found in humans and was also cultured from a dog (Dortet *et al*., 2006; Nemec *et al*., 2001; Nemec *et al*., 2003). In conclusion, although available data derive from only a few studies, some *Acinetobacter* species indeed seem to be distributed widely in nature. *A. calcoaceticus* is found in water and soil and on vegetables; *Acinetobacter* genomic species 3 is found in water and soil, on vegetables, and on human skin; *A. johnsonii* is found in water and soil, on human skin, and in human feces; *A. lwoffii* and *A. radioresistens* are found on human skin; and *Acinetobacter* genomic species 11 is found in water and soil, on vegetables, and in the human intestinal tract. The natural habitats

of both *A. baumannii* and *Acinetobacter* genomic species 13TU still remain to be defined.

As it is a multi-drug resistant organism, infections are difficult to treat (Falagas and Karveli, 2007), resulting in mortalities of 23% for hospitalised patients and 43% for patients under intensive care (Falagas *et al*., 2006). The Antimicrobial Availability Task Force of the infectious Diseases Society of America recently identified *A.baumannii*, along with *Aspergillus* spp., extended–spectrum β-lactamase producing *Enterobacteriaceae*, Vancomycin-resistant *Enterococcus faecium*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*, as 'particularly problematic pathogens' for which there is a desperate need for new drug development (Talbot, 2006). Similarly, the SENTRY Antimicrobial Surveillance Program lists *Acinetobacter* spp. as the causative agent in 2.3 to 3.0% of health care-associated pneumonia and as the eighth most common pathogen (4.0%) isolated from ICU patients worldwide (Jones, 2003). Thus, *Acinetobacter* spp. is emerging as an increasingly important multidrug resistant pathogen, spreading in hospitals, and causing severe adverse outcomes. Besides that, *Acinetobacter* spp. seems to be spreading from hospital to hospital, and it has caused endemic infections in various geographical areas through multiple hospital outbreaks. It has become a leading nosocomial pathogen in many hospitals as compared to other non-fermenting Gram-negative bacilli.

1.2 Phenotypic and genotypic characteristics of *Acinetobacter* spp.

The genus *Acinetobacter* originally (Brisou, 1957; Brisou and Pre´vot, 1954; Pie´chaud *et al*., 1956) included a heterogeneous collection of nonmotile, gram-negative, oxidase-positive, and oxidase-negative saprophytes that could be distinguished from other bacteria by their lack of pigmentation (Ingram and Shewan, 1960). Extensive nutritional studies (Baumann *et al*.,1968b) showed clearly that the

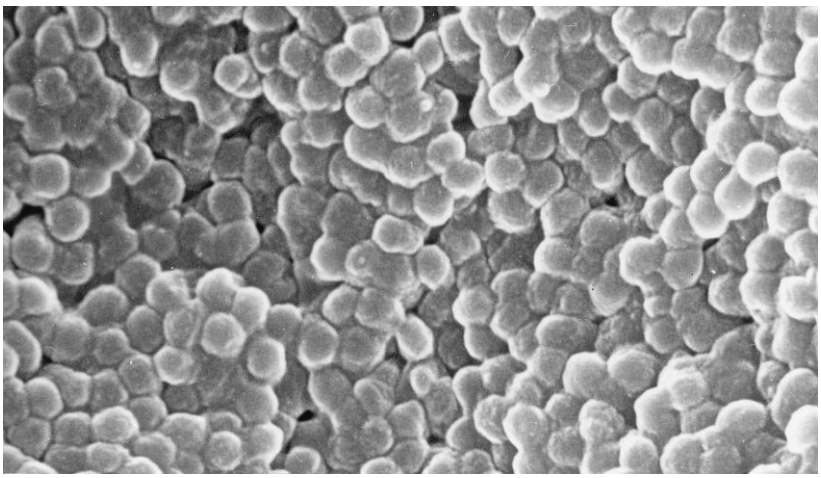
oxidase-negative strains differed from the oxidase- positive strains, and in 1971, the Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria recommended that the genus *Acinetobacter* comprise only oxidase-negative strains (Lessel, 1971). This division has been supported by the use of transformation tests (Juni, 1972), which have now been used for over two decades as the basis for inclusion of individual isolates within the genus.

Acinetobacters are short, plump, gram-negative rods, typically 1.0 to 1.5 by 1.5 to 2.5 mm in the logarithmic phase of growth but often becoming more coccoid in the stationary phase (Figure 1.1). They are often seen as pairs or clusters of cells. Variations in Gram staining (sometimes does not destain), variations in cell size and arrangement, can often be observed within a single pure culture (Baumann *et al*., 1968b). *Acinetobacter* spp. normally form smooth, sometimes mucoid, pale yellow to greyish-white colonies on solid media, although some environmental strains that produce a diffusible brown pigment have been described (Pagel and Seyfried, 1976). The colonies are comparable in size to those of enterobacteria. All members of the genus are strict aerobes, oxidase negative, catalase positive, and nonfermentative. It is the negative oxidase test that serves as a rapid presumptive test to distinguish *Acinetobacter* spp. from otherwise similar nonfermentative bacteria. Most strains are unable to reduce nitrate to nitrite in the conventional nitrate reduction assay. Some clinical isolates, particularly those belonging to genomic species 4 *(A. haemolyticus*), may show hemolysis on sheep blood agar plates.

Most of the *Acinetobacter* strains can grow in a simple mineral medium containing a single carbon and energy source. A wide variety of organic compounds can be used as carbon sources, although relatively few strains can use glucose (Baumann *et al*., 1968b), but no single metabolic test enables unambiguous differentiation of this genus from other similar bacteria. Clinical isolates can grow at 37°C, but some

environmental isolates prefer incubation temperatures of between 20 to 30°C. However, only *A. baumannnii* are able to grow at a higher temperature of 44°C which is the basis of differentiation between *A. baumannii* and *A. calcoaceticus* (Bouvet and Grimont, 1987).

Figure 1.1: Scanning electron micrograph of *A. baumannii* type strain ATCC 19606. (Final magnification, 318,000).



(Adapted from Bergogne and Towner, 1996).

A scheme of 28 phenotypic tests that claimed to discriminate between 11 of the initial 12 genomic species described was originally proposed (Bouvet and Grimont, 1986). Following this, a simplified scheme of 16 tests which, except for tests for glucose acidification and detection of hemolysis on sheep blood agar, comprised growth temperature and carbon source utilization was described (Bouvet and Grimont, 1987). However, these tests failed to differentiate between closely related genomic species. A more detailed and successful phenotypic identification scheme has been described (Ka¨mpfer *et al.*, 1993), but it seems that single or even a few tests cannot be used for unambiguous phenotypic identification of the different genomic species of *Acinetobacter*.

As far as commercial identification systems are concerned, the widely used API 20NE system, based largely on carbon source assimilation tests, contained only *A.*

baumannii, A. haemolyticus, and *A. lwoffii* in the 1993 database release, together with *A. junii* and *A. johnsonii* as a combination, whereas the type species *A. calcoaceticus* and the other genomic species were not included at all. This system sometimes has problems with sensitivity and reproducibility (Kropec *et al*., 1993). Indeed, two studies comparing the API 20NE system with species identification by DNA-DNA hybridization have demonstrated a poor correlation (Horrevorts *et al*., 1995; Weernink *et al*., 1995). Another commercial system, Biolog, differentiates bacteria on the basis of their oxidation of 95 different carbon sources and this system has been shown to give promising results for those strains that have been previously identified by DNA-DNA hybridization (Bernards *et al*., 1995).

Among the few methods that have been validated for identification of *Acinetobacter* species, DNA-DNA hybridization remains the standard method (Bouvet and Grimont, 1986). The phenotypic identification scheme proposed in 1986 is based on 28 phenotypic tests (Bouvet and Grimont, 1986). This identification scheme was refined in 1987 by the same authors and includes growth at 37°C, 41°C, and 44°C; production of acid from glucose; gelatine hydrolysis; and assimilation of 14 different carbon sources (Bouvet and Grimont, 1987). This method does not permit identification of the more recently described genomic species. In particular, the closely related and clinically most relevant species *A. baumannii* and *Acinetobacter* genomic species 13TU cannot be distinguished, while *A. calcoaceticus* and *Acinetobacter* genomic species 3 can only be separated by their growth properties at different temperatures (Gerner-Smidt *et al*., 1991). Unfortunately, simple phenotypic tests that are commonly used in routine diagnostic laboratories for identification of other bacterial genera to the species level are unsuitable for unambiguous identification of even the most common *Acinetobacter* species. Both DNA-DNA hybridization and the phenotypic identification system of Bouvet and Grimont are laborious and far from being suitable for routine microbiology

laboratories. Molecular methods that have been developed and validated for identification of acinetobacters include amplified *16S rRNA* gene restriction analysis (ARDRA) (Vaneechoutte *et al*., 1995; Dijkshoorn *et al*., 1998; Wong *et al*., 2010), high-resolution fingerprint analysis by amplified fragment length polymorphism (AFLP) (Nemec *et al*., 2001; Janssen & Dijkshoorn, 1996), ribotyping (Gerner-Smidt**,** 1992), tRNA spacer fingerprinting (Ehrenstein *et al*., 1996), restriction analysis of the 16S-23S rRNA intergenic spacer sequences (Dolzani *et al*., 1995), sequence analysis of the *16S-23S rRNA* gene spacer region (Chang *et al*., 2005), and sequencing of the *rpoB* (RNA polymerase β-subunit) gene and its flanking spacers (La Scola *et al*., 2006). ARDRA and AFLP analysis are currently the most widely accepted and validated reference methods for species identification of acinetobacters. Both ribotyping and sequence analysis of the *16S-23S rRNA* gene spacer region were found to discriminate between species of the *A. calcoaceticus- A. baumannii* complex but have not been applied to other *Acinetobacter* species. All of these methods have contributed to a better understanding of the epidemiology and clinical significance of *Acinetobacter* species during recent years, but they are too laborious to be applied in routine diagnostic microbiology, and their use for the time being is also confined mainly to reference laboratories. More recent developments include the identification of *A. baumannii* by detection of the *bla*OXA-51-like carbapenemase gene intrinsic to this species (Turton *et al*., 2006), PCR-electrospray ionization mass spectrometry (PCR–ESI-MS) (Ecker *et al*., 2006), and a simple PCR-based method (Higgins *et al.*, 2007) that exploits differences in their respective *gyrB* genes to rapidly differentiate between *A. baumannii* and *Acinetobacter* genomic species 13TU. Promising results with matrix-assisted laser desorption ionization–time-of-flight MS have been obtained for species identification of 552 well characterized *Acinetobacter* strains representing 15 different species (Seifert *et al*., 2007). Matrix-assisted laser desorption ionization–time-of-flight MS allows for

species identification in less than 1 hour, but it requires expensive equipment and needs further evaluation. Species identification with manual and semiautomated commercial identification systems that are currently used in diagnostic microbiology, such as the API 20NE, Vitek 2, Phoenix, and MicroScan WalkAway systems, remains problematic (Bernards *et al*., 1995; Bernards *et al*., 1996; Horrevorts *et al*., 1995). This can be explained in part by their limited database content but also because the substrates used for bacterial species identification have not been tailored specifically to identify acinetobacters. In particular, the three clinically relevant members of the *A. calcoaceticus-A. baumannii* complex cannot be separated by currently available commercial identification systems. *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13TU share important clinical and epidemiological characteristics (Dijkshoorn *et al*., 1993; Lim *et al*., 2007; Seifert and Gerner-Smidt. 1995). From a clinical and infection control point of view, it is necessary to distinguish between the *A. baumannii* group and acinetobacters outside the *A. baumannii* group since the latter organisms rarely have infection control implications. In addition, these organisms are usually susceptible to a range of antimicrobials, and infections caused by these organisms are most often benign. In research it is necessary that proper methods for species identification of acinetobacters, including those within the *A. baumannii* group, are mandatory to increase our knowledge of the epidemiology, pathogenicity, and clinical impact of the various species of this genus.

1.3 Emergence of nosocomial infections caused by *Acinetobacter* spp.

The majority of *A. baumannii* isolates are from the respiratory tracts of hospitalized patients. In many circumstances, it is very difficult to distinguish upper airway colonization from true pneumonia. There is no doubt, however, that true ventilator-associated pneumonia (VAP) due to *A. baumannii* is reported. In large

surveillance studies from the United States, between 5 and 10% of cases of ICU-acquired pneumonia were due to *A. baumannii* (Gaynes and Edwards, 2005). Patients with *A. baumannii* infections have had prolonged ICU stays (Garnacho *et al*., 2005). Community-acquired pneumonia due to *A. baumannii* has been described for tropical regions of Australia and Asia (Anstey *et al*., 2002; Anstey etal., 1992; Bick and Semel, 1993; Gottlieb and Barnes, 1989; Leung *et al*., 2006). The disease most typically occurs during the rainy season among people with a history of alcohol abuse and may sometimes require admission to an ICU (Anstey *et al*., 2002). It is characterized by a fulminant clinical course, secondary bloodstream infection, and mortality rate of 40 to 60% (Leung *et al*., 2006). The source of infection may be throat carriage, which occurs in up to 10% of community residents with excessive alcohol consumption (Anstey *et al*., 2002).

A. baumannii was the 10th most common etiologic agent of bloodstream infection in a large study in the United States (1995–2002). They were responsible for 1.3% of all monomicrobial nosocomial bloodstream infections (Wisplinghoff *et al*., 2004). *A. baumannii* was a more common cause of ICU-acquired bloodstream infection than of non-ICU-ward infection. Crude mortality overall from *A. baumannii* bloodstream infection was 34.0% to 43.4% in the ICU and 16.3% outside the ICU. *A. baumannii* bloodstream infection had the third highest crude mortality rate in the ICU, exceeded only by *P. aeruginosa* and *Candida* sp. infections. *A. baumannii* infections were the latest of all bloodstream infections to occur during hospitalization (Wisplinghoff *et al*., 2004). It is notable that 102 patients had bloodstream infections at sites treating U.S. military members injured in Iraq or Afghanistan from 1 January 2002 and 31 August 2004 (CDC, 2004). The sites of origin of these infections were not described in this report.

A. baumannii may occasionally cause skin/soft tissue infections outside of the military population. The organism caused 2.1% of ICU-acquired skin/soft tissue infections in one assessment (Gaynes and Edwards, 2005). It is a well-known pathogen in burn units and may be difficult to eradicate from such patients (Trottier *et al*., 2007). However, its contribution to poor outcome in burn patients is debated (Albrecht *et al*., 2006, Wisplinghoff *et al*., 1999). *A. baumannii* is commonly isolated from wounds of combat casualties from Iraq or Afghanistan (Johnson *et al*., 2007; Murray *et al*., 2006; Petersen *et al*., 2007; Scott *et al*., 2007; Whitman, 2007; Yun *et al*., 2006). It was the most commonly isolated organism (32.5% of cases) in one assessment of combat victims with open tibial fractures (Johnson *et al*., 2007).

A. baumannii is an occasional cause of UTI, being responsible for just 1.6% of ICU-acquired UTIs in one study (Gaynes and Edwards, 2005). Typically, the organism is associated with catheter associated infection or colonization. It is not usual for this organism to cause uncomplicated UTI in healthy outpatients.

Nosocomial, postneurosurgical *A. baumannii* meningitis is an increasingly important entity. The microbial epidemiology of nosocomial meningitis is evolving to include more gram negative pathogens (Briggs *et al*., 2004; Durand *et al*., 1993; Palabiyikoglu *et al*., 2006; Siegman *et al*., 1993) and thus multidrug-resistant *A. baumannii* is included among the pathogens implicated (Metan *et al*., 2007; Nguyen *et al*., 1994; Nunez *et al*., 1998; O'Neill *et al*., 2006). Typical patients have undergone neurosurgery and have an external ventricular drain (Metan *et al*., 2007). Mortality may be as high as 70%. There is a report of neurosurgically related nosocomial *Acinetobacter baumannii* meningitis (Krol *et al*., 2009).

A small number of case reports of *Acinetobacter* endocarditis exist (Menon *et al*., 2006; Olut and Erkek, 2005; Rizos *et al*., 2007; Starakis *et al*., 2006; Valero *et al*., 1999). Most of the cases have involved prosthetic valves. *Acinetobacter* spp. may cause

endophthalmitis or keratitis, sometimes related to contact lens use or following eye surgery (Corrigan *et al*., 2001; Kau *et al*., 2002; Levy *et al*., 2005; Lindbohm *et al*., 2005). A single case report exists of a Shiga toxin-producing *A. haemolyticus* strain, which was associated with bloody diarrhea in a 3-month-old infant (Grotiuz *et al*., 2006).

1.4 Treatment of *Acinetobacter* spp. infections

There are many reports that have documented the high rates of antibiotic resistance in *Acinetobacter* spp. (Bergogne-Be´re´zin and Joly-Guillou, 1985; Buisson *et al*., 1990; French *et al*., 1980; Joly-Guillou *et al*., 1992; Larson, 1984; Belkum, 1993; Bou *et al*., 2000; Quale *et al*., 2003; Wong *et al*., 2010; Wong *et al*., 2009; Neonakis *et al*., 2011).

The emergence of frequent multiple antibiotic resistance exhibited by nosocomial *Acinetobacter* spp. and the resulting therapeutic problems involved in treating patients with nosocomial infections in ICUs is of particular concern. Thus far, carbapenems have been thought of as the agents of choice for serious *A. baumannii* infections. However, although these drugs are still active against the vast majority of *A. baumannii* strains worldwide, the clinical utility of this class of antimicrobial is increasingly being jeopardized by the emergence of both enzymatic and membrane-based mechanisms of resistance (Bou *et al*., 2000; Quale *et al*., 2003). Sulbactam is one of three commercially available β-lactamase inhibitors. Unlike clavulanic acid and tazobactam, it has clinically relevant intrinsic antimicrobial activity against certain organisms, specifically *Acinetobacter* (Brauers *et al*., 2005; Corbella *et al*., 1998; Higgins *et al*., 2004; Levin *et al*., 2002; Levin *et al*., 2003; Obana *et al*., 1990; Rodriguez *et al*., 2001) and *Bacteroides* spp. (Williams, 1997) which is mediated by its binding to penicillin-binding protein 2 (Noguchi and Gill, 1988). Sulbactam is commercially available in a

combined formulation with either ampicillin or cefoperazone and also as a single agent in France, Germany, and Spain (Brauers *et al*., 2005; Levin, 2002). Studies assessing the activity of sulbactam alone compared to its combination with a β-lactam clearly demonstrate the intrinsic activity of the agent rather than its ability to inhibit β-lactamase (Brauers *et al*., 2005; Corbella *et al*., 1998; Higgins *et al*., 2004). Efficacy of sulbactam to that of imipenem has also been shown for treatment of *A. baumannii* bloodstream infection (Choi *et al*., 2006; Cisneros *et al*., 1996; Jellison *et al*., 2001), with one study demonstrating a reduction in pharmaceutical costs (Jellison *et al*., 2001). In a further study, treatment of highly drug-resistant *A. baumannii* bacteremia (susceptible only to sulbactam and polymyxin E) with ampicillin- sulbactam was comparable to treatment of more susceptible strains with other therapies, including imipenem, aminoglycosides, and quinolones (Smolyakov *et al*., 2003; Betrosian and Douzinas, 2009). These data indicate that when *A. baumannii* is susceptible to sulbactam, this antimicrobial is efficient. There are in vitro studies which have shown enhanced activity when sulbactam is combined with cefepime (Sader and Jones, 2005; Tong *et al*., 2006), imipenem (Choi *et al*., 2004; Song *et al*., 2007), meropenem (Kiffer *et al*., 2005; Ko *et al*., 2004), amikacin (Savov *et al*., 2002), rifampin (Appleman *et al*., 2000), and ticarcillin-clavulanate (Joly-Guillou *et al*., 1995).

The emergence of *A. baumannii* strains resistant to all routinely tested antimicrobials has led to the necessary revival of the polypeptide antibiotics known as the polymyxins (colistin or polymyxin E and polymyxin B). These positively charged antimicrobial peptides were discovered in 1947, originating from *Bacillus polymyxa*. They target the anionic LPS molecules in the outer cell membranes of gram-negative bacteria, leading to interactions between the inner and outer cell membranes, with associated lipid exchange, membrane disturbance, osmotic instability, and eventual cell death (Clausell *et al*., 2007; Falagas and Kasiakou, 2005). There are two commercially

available forms of colistin, namely, colistin sulfate for oral and topical use, and CMS, also known as sodium colistin methanesulfonate or colistin sulfomethate sodium, for parenteral use (Li *et al*., 2006a). Both forms are available for nebulization. Our understanding of the critical pharmacological parameters that govern dosing for maximal efficacy and minimal toxicity is poor. As a consequence, confusion exists among clinicians and in the literature regarding formulations, nomenclature, and dosing (Falagas and Kasiakou, 2006; Li *et al*., 2006a; Li *et al*., 2006b). In vitro, colistin demonstrates concentration-dependent bactericidal activity against *A. baumannii* strains with various susceptibility profiles, as determined by time-kill analyses (Montero *et al*., 2002; Owen *et al*., 2007; Rodriguez *et al*., 2004). Colistin in combination with a carbapenem and/or rifampin appears most promising (Giamarellos *et al*., 2001; Hogg *et al*., 1998; Manikal *et al*., 2000; Montero *et al*., 2004; Pantopoulou *et al*., 2007; Song *et al*., 2007; Tascini *et al*., 1998; Timurkaynak *et al*., 2006; Yoon *et al*., 2004). Understandably, in the presence of significant carbapenemase activity, as opposed to membrane-based changes (porins and efflux pumps) that are likely disrupted by the polymyxins, synergy with carbapenems may be lost (Wareham and Bean, 2006).

A concerning void of new therapeutic options exists for *A. baumannii* infections. Of the recently licensed antimicrobials, tigecycline, a 9-*t*-butylglycylamido semisynthetic derivative of minocycline, has provided some hope, but clinical data are still limited. As with other tetracycline derivatives, tigecycline inhibits the 30S ribosomal subunit (Fluit *et al*., 2005; Petersen *et al*., 1999). Thus far, the in vitro activity of tigecycline against *A. baumannii* has been assessed largely by MIC testing. More recently, combination therapy with tigecycline has been studied using time-kill and Etest synergy methodology (Sands *et al*., 2007; Scheetz *et al*., 2007). When it was tested against a noncarbapenem- susceptible *A. baumannii* strain, tigecycline alone allowed maximal killing at concentrations near the MIC, which was 1 g/ml, with no

benefit of using higher concentrations (Scheetz *et al*., 2007). There are reports for effective control of *A. baumannii* outbreak (Jamal *et al*., 2009). However, the pharmacokinetic considerations exist for the tigecycline treatment (Cunha, 2009). Recently fosfomycin for the treatment of infections caused by multidrug-resistant non-fermenting Gram-negative bacilli has been reported (Falagas *et al*., 2009).

The use of combination therapy to treat multidrug- or pandrug- resistant gram-negative organisms has become an area of great interest (Rahal, 2006). This strategy aims to create an active combination out of two agents to which the organism tests nonsusceptible in the laboratory. Apart from trying to improve efficacy, combination therapy may also help to prevent the emergence of resistance when at least one agent is active in vitro (Chait *et al*., 2007; Pachon *et al*., 2006). The studies involving combination therapy with either sulbactam or the polymyxins are the most promising group. Other combinations have also been studied using in vitro techniques and animal models, including various combinations of quinolones, β-lactams, and/or amikacin (Bonapace *et al*., 2000; Chang *et al*., 1995; Drago *et al*., 2004; Jung *et al*., 2004; Roussel *et al*., 1996). The results for quinolone combination therapy are varied, with reduced efficacy being described when ciprofloxacin was used for ciprofloxacin-resistant *A. baumannii* (Ermertcan *et al*., 2001), as well as a lack of enhanced activity with levofloxacin combined with imipenem or amikacin in a mouse pneumonia model (Joly *et al*., 2000). Interestingly, enhanced activity was seen when aztreonam was tested in combination with other β-lactams against a select group of MBL-producing *A. baumannii* strains (Sader *et al*., 2005).

The increasing incidence of multidrug-resistant *A. baumannii* in addition to a lack of new antimicrobial agents has reawakened interest in the utilization of colistin due to its good activity against this organism (Katragkou and Roilides, 2005). It has been

recently shown that intrathecal colistin is a safe and curative treatment drug for multidrug-resistant *Acinetobacter* spp meningitis (Sahin *et al*., 2008). Optimal therapy for the multi-drug resistant *Acinetobacter baumannii* has been suggested (Cunha, 2010; Fishbain *et al*., 2010; Garnacho and Amaya, 2010). Also the length of the course of treatment has to be minimized for prevention of the development of drug resistance (Giamarellou, 2010).

In general, the recommended drugs in most recent studies have been extended-spectrum penicillins, broad-spectrum cephalosporins, or imipenem, combined with an aminoglycoside.

1.5 Resistance mechanisms in *Acinetobacter* spp.

Most *Acinetobacter* spp. research to date has focused on cataloguing and understanding the variety of antimicrobial resistance genes and mechanisms found within the species. Since it is largely a nosocomial pathogen, *Acinetobacter* spp. is bombarded by the selective pressure of a broad variety of antibiotics. An overview of the intrinsic and acquired mechanisms of antibiotic resistance reported for this organism is provided in Table 1.2 and Table 1.3, respectively. In addition, *A. baumannii* has a number of intrinsic mechanisms, including ß-lactamases and efflux pumps, which contribute to resistance.

Clinical isolates of *A. baumannii* encodes an array of ß-lactamases. Some of the genes encoding these are intrinsic to essentially all isolates of this species and are encoded on the chromosome, while the majority of the genes are acquired from other species or other *Acinetobacter* isolates, with the determinants encoded on mobile genetic elements. As illustrated in Table 1.2, all isolates of *A. baumanii* have a chromosomally encoded *ampC* cephalosporinase gene analogous to the species-specific *ampC* gene found in *Escherichia coli.* Consensus reached from many studies indicates

that this *ampC* gene is not inducible with ß-lactams and is normally expressed at low levels (Poirel *et al.*, 2006; Lopez *et al.*, 2001; Perilli *et al*., 1996).

More recent DNA sequence analysis and enzymatic characterization of *ampC* cephalosporinases from various *Acinetobacter* isolates have led to a more uniform designation of these enzymes, defining variants including ADC (for *Acinetobacter*-derived cephalosporinases)-1 to -7 (Hujer *et al*., 2006). Another variant, recently characterized from *A. baylyi*, ADC-8, shares less than 50% amino acid identity with the other ADC variants, yet it appears to confer a similar cephalosporin resistance phenotype in this species (Beceiro *et al.*, 2007).

Table 1.2 Intrinsic Mechanisms of Antibiotic Resistance in *Acinetobacter baumannii*

Antibiotic class	Resistance gene(s)	Antibiotics affected	Reference
		ß-lactams	
Penicillins	Chromosomally encoded *AmpC* (basal)	Penicillin and ampicillin	Poirel & Nordmann, 2006; Joly *et al*., 1988; Lopez *et al*., 2001; Blechschmidt *et al*., 1992; Perilli *et al*., 1996; Beceiro *et al*., 2004
Cephalosporins	*ampC* overexpression via insertion sequence promoters	Cephaloridine, cefotaxime and ceftazidime	Beceiro *et al*., 2007; Segal *et al*., 2004; Heritier *et al*., 2006
	Chromosomally-encoded *bla* $_{\text{OXA-51}}$ Low carbapenemase activity	Low-level cephalosporin resistance	Poirel and Nordmann, 2006
	Changes in penicillin-binding proteins	Penicillins and cephalosporins	Fernandez *et al*., 2003
	Loss of porins (e.g., CarO)	Cephalosporin resistance, particularly with overexpression of *ampC*	Vila *et al*., 2007; Siroy *et al*., 2005; Mussi *et al*., 2005
	Overexpression of efflux pump AdeABC	High-level cephalosporin resistance	Marchand *et al*., 2004; Ruzin *et al*., 2007
Fluoroquinolones	Point mutations in *gyrA* alone or combined with mutations in *parC*	Sequential, multiple mutations elevate MICs to all available fluoroquinolones	Bonomo & Szabo, 2006; Wisplinghoff *et al*., 2003
	Porin loss and efflux AdeABC overexpression with *gyrA* and *parC* mutations	High-level resistance to all available agents	Van *et al*., 2004
Tigecycline	Overexpression of AdeABC efflux pump	MICs to tigecycline elevated to 4-32 µg/ml	Peleg *et al*., 2007; Navon *et al*., 2007
Polymyxin B	Mutation in genes leading to membrane alterations		Van *et al*., 2004; Hawley *et al*., 2007
Colistin	Mutations in genes leading to membrane changes		Van *et al*., 2004; Antoniadou *et al*., 2007; Hawley *et al*., 2007

(Adapted from Thomas *et al*., 2008)

Table 1.3 Acquired Resistance Mechanisms to Antibiotics in *Acinetobacter baumannii*

Antibiotic class	Resistance gene(s)	Antibiotics affected	Reference
ß-lactams	Class A, extended-spectrum ß-lactamase genes; *TEM-92, VEBv1* and *PER*	Penicillins and cephalosporins	Bonomo & Szabo, 2006; Endimiani *et al*., 2007; Naas *et al*., 2006; Arduino *et al*., 2002
Class D, OXA carbapenemases *bla* OXA-23, -24, -25, -26, -27, -40 and -58	Penicillins, cephalosporins and carbapenems		Poirel and Nordmann, 2006; Brown and Amyes, 2006; Brown and Amyes, 2005; Heritier *et al*., 2005
Class B metallo-ß-lactamases *VIM, IMP* and *SIM* variants	Primarily carbapenems and some cephalosporins		Poirel and Nordmann, 2006; Lee *et al*., 2005; Ellington *et al*., 2007; Yong *et al*., 2006
Tetracyclines	Tet(A) efflux pump	Resistance to tetracycline	Van *et al*., 2004; Vila *et al*., 2007; Ribera *et al*., 2003
Tet(B) efflux pump	Resistance to tetracycline and minocycline		Van *et al*., 2004; Vila *et al*., 2007; Ribera *et al*., 2003
Aminoglycosides	AbeM efflux pump	Fluoroquinolones and aminoglycosides	Vila *et al*., 2007
Acquired genes encoding acetyltranserases, phosphotransferases and nucleotidyltransferases 16S rRNA methylation	All available aminoglycosides depending on the modifying enzyme and its level of expression		Bonomo & Szabo, 2006; Noppe *et al*., 1999; Huys *et al*., 2005
Chloramphenicol	Acquisition of gene encoding chlorapmphenicol acetyl transferase	Chloramphenicol	Van *et al*., 2004

(Adapted from Thomas *et al*., 2008)

In one study, 42 *A. baumannii* strains, including strains susceptible (MIC ≤ 8 μg/ml), intermediate (MIC 32 μg/ml) or highly resistant (MIC ≥ 256 μg/ml) to ceftazidime, were examined at the *ampC*-gene sequence, including regions adjacent to its 5'-end. An 1180-bp IS was detected in all the non-susceptible isolates (Corvec *et al*., 2003). Since the initial studies describing the acquisition of this IS element fused to the ADC gene, additional observations have demonstrated that this arrangement leads to the overproduction of cephalosporinase. Cloning and sequencing of this region from the *A. baumannii* strain, RAN, identified several putative promoters in the IS that could increase *ampC* expression (Segal *et al*., 2004). The IS, now identified as 'IS Aba1-like' once inserted ahead of the structural gene, resulted in a single nucleotide change in the ribosome binding site (TGAG to GGAG) in the *bla* $_{\text{AMPC}}$ gene (Heritier *et al*., 2006). Apparently, *A. baumannii* applies this elegant mechanism of ISAba1 for upregulating other resistance genes, such as the *bla* $_{\text{OXA-23}}$ and *bla* $_{\text{OXA-27}}$ carbapenemases and the *sulII* gene (Segal *et al*., 2005).

In addition to the presence of the ADC and OXA-51 intrinsic ß-lactamases, similar to other Gram-negatives, *A. baumannii* has a number of acquired *bla* genes, including several Ambler class A serine ß-lactamases (Table 1.3). Many variants of the TEM, SHV, VEB, PER and CTX-M enzymes are found occasionally in this organism, including many that are extended-spectrum ß-lactamases (ESBLs) with potent activity against third-generation cephalosporins (Bonomo and Szabo, 2006; Endimiani *et al*., 2007; Sechi *et al*., 2004; Naas *et al*., 2006; Walther and Hoiby, 2004; Naas *et al*., 2007; Carbonne *et al*., 2005; Paterson and Bonomo, 2005). The Ambler class D oxacillinases appear to be more widely distributed in clinical strains of *A. baumannii* (Poirel and Nordmann, 2006). A study showed that, of the 39 carbapenem resistant isolates of *Acinetobacter* spp., only 2 harbored a $bla_{\text{IMP-4}}$ carbapenemase and the remaining 37 isolates were positive for bla_{OXA} (Wong *et al*., 2010). The OXA carbapenemases found

in *Acinetobacter* have been organized into either three or four subgroups (OXA-23-like, OXA-24-like, OXA-51-like and OXA-58) based on their sequence divergence (Brown and Amyes, 2005). Outbreaks of MDR acinetobacters where these *bla* and *oxa* genes are implicated have occurred (Hujer *et al*., 2006; Bogaerts *et al*., 2006; Pournaras *et al*., 2006; Bertini *et al*., 2007; Giordano *et al*., 2007; Navia *et al*., 2002; Brown *et al*., 2005; Valenzuela *et al*., 2007; Koh *et al*., 2007; Zhou *et al*., 2007; Depardieu *et al*., 2007).

In addition to the Ambler class D carbapenemases, clear evidence exists that the Ambler class B metallo-ß-lactamases VIM, IMP and SIM are present in acinetobacters (Lee *et al*., 2005). ß-lactamases have spread widely in populations of *A. baumanii*, including those that can encode carbapenem resistance, leaving few choices for effective therapy. The available evidence strongly suggests that additional mechanisms of resistance can be found in the same MDR strain, such as overexpressed efflux and outer membrane alterations that can augment the level of resistance to important antimicrobials.

Aminoglycoside resistance is common in clinical isolates of acinetobacters. As with other Gram-negative bacilli, the major mechanism of resistance to these agents is due to the dissemination of genes encoding aminoglycoside-modifying enzymes (Bonomo and Szabo, 2006).

Porins are outer membrane proteins that form water-filled pores, allowing the access of nutrients and antibiotics into the cell. Acinetobacters can easily downregulate porin expression in the presence of antibiotic selective pressure without acquiring new genetic material. As discussed in a recent review (Vila *et al*., 2007), the relatively low permeability of *Acinetobacter* to antibiotics may be due to either a reduced number of porins in the outer membrane or their poor diffusion characteristics for small molecules. There have been at least five putative porins detected in acinetobacters. The best

characterized is the 35,636-Da heat-modifiable protein (HMP-AB). The second most characterized putative porin in acinetobacters is the 29-kDa CarO protein (Vila *et al*., 2007). Loss of other putative porins of *A. baumannii* have been implicated in resistance to carbapenems, either alone or in combination with efflux systems or carbapenemase genes, such as *bla* $_{OXA}$ (Bou *et al*., 2000; Limansky *et al*., 2002; Clark, 1996). A 29-kDa OMP has been identified to be the major outer membrane protein in *A. baumannii* / *calcoaceticus* and loss of this porin and overexpression of IROMPs have been shown to contribute to carbapenem resistance (Wong *et al*., 2010).

Efflux of structurally unrelated classes of antibiotics from MDR, Gram-negative bacilli is now established as an important mechanism of resistance. The most extensively studied efflux system is the *AdeABC* efflux operon that is under regulation by a two-component signal transduction system (Vila *et al*., 2007; Magnet *et al*., 2001; Marchand *et al*., 2004; Piddock, 2006). Disruption of the *ade* gene resulted in lower MIC values than the parent strains for ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin, amoxicillin and meropenem. Knocking the gene out showed that the decrease in the MICs was greater in the mutants lacking *adeB* gene, *adeR* gene and *adeS* gene, while all the mutants showed no differences in the MIC values for imipenem as compared to the wild-type strains. This shows that the efflux pump has an effect on the resistance to meropenem compared to imipenem, and the resistance may be due to meropenem upregulating the efflux pump (Wong *et al*., 2009). The AdeABC efflux pump has been implicated in the enhanced resistance to aminoglycosides, ß-lactams, chloramphenicol, tetracyclines and fluoroquinolones (Vila *et al*., 2007). Recent studies indicate that the AdeABC efflux pump is implicated in decreased susceptibility to the new antimicrobial, tigecycline.

The Tet(A), Tet(B) and AbeM efflux pumps (Table 1.2) have also been detected in *A. baumannii* (Vila *et al*., 2007). The Tet(A) pump confers resistance to tetracycline,

while Tet(B) confers resistance to tetracycline and minocycline, the AbeM pump belongs to the MATE family of transporters and its presence has been implicated in raising MICs to norfloxacin, ofloxacin, ciprofloxacin and gentamicin. Resistance to fluoroquinolones in acinetobacters most commonly results from sequencial accumulation of point mutations in the topoisomerase *gyrA* and *parC* genes (Bonomo and Szabo *et al*., 2006).

Apart from being highly resistant to antibiotics, the members of the *Acinetobacter* spp. are highly resistant to desiccation. These organisms can persist for a long time in inanimate surfaces. *A. baumannii* is one of the most common causes of device-related nosocomial infections, possibly because it is able to resist physical and chemical disinfection. Biofilm formation is a possible explanation for the resistance to desiccation and disinfection. Further, it could also be responsible for an important number of the device-related nosocomial infections (Donlan, 2002; Tomaras *et al*., 2003).

1.6 Biofilms

1.6.1 Biofilm formation

Biofilms are microbially derived sessile communities characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription. A biofilm is composed of a congregation of bacterial cells to a solid surface and enclosed within a polysaccharide matrix. The basic building block of the biofilm is the microcolony, and an elucidation of basic biofilm processes, such as quorum sensing, antimicrobial resistance, and detachment and may hinge on an understanding of the physiological interactions of microcolonies within a developed biofilm. Confocal laser scanning

microscopy (CLSM) observations of living biofilms have shown that this basic community structure is universal.

1.6.2 Resistance to antimicrobial agents

The nature of biofilm structure and the physiological attributes of biofilm organisms confer an inherent resistance to antimicrobial agents, whether these antimicrobial agents are antibiotics, disinfectants, or germicides. Mechanisms responsible for resistance may be one or more of the following: (i) delayed penetration of the antimicrobial agent through the biofilm matrix, (ii) altered growth rate of biofilm organisms, and (iii) other physiological changes due to the biofilm mode of growth.

The encased cells in the biofilm matrix can be inactivated by the use of antimicrobial molecules only if the antimicrobials diffuse through the matrix. The extracellular polymeric substances constituting this matrix present a diffusional barrier for these molecules by influencing either the rate of transport of the molecule to the biofilm interior or the reaction of the antimicrobial material with the matrix material. Suci *et al*., (1994) demonstrated a delayed penetration of ciprofloxacin into *Pseudomonas aeruginosa* biofilms; what normally required 40 s for a sterile surface required 21 min for a biofilm-containing surface. Hoyle *et al*., (1992) found that dispersed bacterial cells were 15 times more susceptible to tobramycin than were cells in intact biofilms. DuGuid *et al*., (1992) examined *Staphylococcus epidermidis* susceptibility to tobramycin and concluded that the organization of cells within biofilms could in part explain the resistance of this organism to this antimicrobial agent.

Another proposed mechanism for biofilm resistance to antimicrobial agents is that biofilm-associated cells grow significantly more slowly than planktonic cells and, as a result, take up antimicrobial agents more slowly. Using a method of cell culture designed to determine the effect of growth rate apart from other biofilm processes, Evans *et al*., (1990) found that the slowest growing *Escherichia coli* cells in biofilms

were the most resistant to cetrimide. At growth rates higher than 0.3 per h, biofilm and planktonic cells were equally susceptible.

Nutrient limitation and increases in toxic metabolite concentrations might be particularly acute within the depths of established biofilms. Tresse *et al*., (1995) found that agar-entrapped *E. coli* cells were more resistant to an aminoglycoside as oxygen tensions were decreased. They suggested that the persitence was due to lowered uptake of the antibiotic by the oxygen-starved cells. Dagostino *et al*. (1991) proposed that initial bacterial association with a surface may result in the repression or induction of genes, which in turn results in a number of physiological responses.

1.6.3 Biofilms on medical devices and its relationship with the disease

There are varied environments that are suitable for microorganisms to colonize and establish biofilms. Costerton *et al*., (1999) provided a partial listing of medical devices that have been shown to become colonized by biofilms. Biofilms of various medical devices have been studied extensively over the last 20 years, through techniques such as viable culture techniques and scanning electron microscopy, to characterize the microbial diversity and visualize the biofilms.

Prosthetic valve endocarditis following the valve replacement is due to the infection caused by organisms such as streptococci, enterococci, *S. aureus*, gram-negative coccobacilli, or fungi which form biofilm on the valve (Karchmer and Gibbons, 1994). Central venous catheters pose a greater risk of device-related infection than does any other indwelling medical device, with infection rates of 3 to 5%. Catheters may be inserted for administration of fluids, blood products, medications, nutritional solutions, and hemodynamic monitoring (Flowers *et al*., 1989). Biofilms have been shown by scanning electron microscopy and transmission electron microscopy to be universally present on central venous catheters and may be associated with either the outside of the catheter or the inner lumen (Raad *et al*., 1993). Organisms

like *S. aureus* and *S. epidermidis* that colonize originate either from the skin insertion site, migrating along the external surface of the device, or from the hub, due to manipulation by health care workers, migrating along the inner lumen (Elliott *et al*., 1997; Raad, 1998). Urinary catheters are tubular, latex, or silicone devices that are inserted through the urethra into the bladder to measure urine output, collect urine during surgery, prevent urinary retention, or control urinary incontinence (Kaye and Hessen, 1994). Stickler (1996) noted one study that measured *P. aeruginosa* cells at catheter surface. Catheters are colonized by single species, such as *S. epidermidis*, *Enterococcus faecalis*, *E. coli*, or *Proteus mirabilis.* As the catheter remains in place, the number and diversity of organisms increase. Mixed communities develop, containing such organisms as *Providencia stuartii*, *P. aeruginosa*, *Proteus mirabilis*, and *Klebsiella pneumoniae* (Stickler, 1996). Other organisms isolated from urinary catheter biofilms include *M. morganii*, *Acinetobacter calcoaceticus* (Stickler *et al*., 1993a), and *Enterobacter aerogenes* (Stickler *et al*., 1993b). Hard contact lenses are constructed of polymethylmethacrylate and move with each blink, allowing oxygen-containing tears to flow underneath the lens (Dart, 1996). Bacteria adhere readily to both types of lenses (Dart, 1996; Miller and Ahearn, 1987; Stapleton and Dart, 1995; Stapleton *et al*., 1993). Organisms that have been shown to adhere to contact lenses include *P. aeruginosa, S. aureus*, *S. epidermidis*, *Serratia* spp., *E. coli*, *Proteus* spp., and *Candida* spp. (Dart, 1996). Intrauterine devices use has been shown to result in pelvic inflammatory disease (Chesney, 1994; Lewis, 1998; Wolf and Kreiger, 1986). Dental units are equipped with small-bore flexible plastic tubing that supplies water to different hand pieces, such as the air-water syringe, the ultrasonic scaler, and the high-speed hand piece. Organisms generally isolated from these units include *Pseudomonas* spp., *Flavobacterium* spp., *Acinetobacter* spp., *Moraxella* spp., *Achromobacter* spp. (Tall *et al*., 1995), *Methylobacterium* spp. (Whitehouse, 1991), *Rhodotorula* spp.,

hyphomycetes (*Cladosporium* spp., *Aspergillus* spp., and *Penicillium* spp.), *Bacillus* spp., *Streptococcus* spp., CoNS, *Micrococcus* spp., and *Corynebacterium* spp. (Mills *et al*., 1986). *Legionella pneumophila* has also been isolated from these systems (Atlas *et al*., 1995; Callacombe and Fernandes, 1995; Pankhurst *et al*., 1990).

It is clear from epidemiologic evidence that biofilms have a role in infectious diseases, both for specific conditions such as cystic fibrosis and periodontitis and in bloodstream and urinary tract infections as a result of indwelling medical devices. The process may be particularly relevant for immunocompromised patients, who lack the ability to combat invading organisms.

1.6.4 Regulation of biofilms

Antibiotic susceptibility of planktonic bacteria and resistance of corresponding biofilm cells is a well-established phenomenon (Tenke *et al*., 2006). In most cases, treatment with antibiotics slows down biofilm progression by eliminating planktonic cells and interfering with biofilm metabolism (Tenke *et al*., 2006). However, neither the biofilm nor the infection are eliminated effectively, and there is growing concern across all industry sectors that deal with biofilms in relation to cross-resistance exhibited by antibiotic- resistant strains to other antimicrobial agents, such as disinfectants (Langsrud *et al*., 2003; Lunde´n *et al*., 2003).

One of the biofilm control strategy is to prevent initial attachment of the biofilm. Some examples include materials coated with silver ions or within which silver ions are incorporated, materials containing antibiotics which are released slowly, materials in which intrinsic properties like surface hydrophobicity have been altered, and the use of anti-adhesive surfaces such as heparin coatings (Donlan and Costerton, 2002; Tenke *et al*., 2006; Morris and Stickler, 1998; Chilukuri and Shah, 2005).

The other strategy to intervene biofilm is to minimize biofilm formation. Cell attachment to surfaces has been shown to occur within a few minutes to hours, making frequent cleaning unfeasible as a means to prevent cell attachment (Meyer, 2003).

More recent novel approaches include disruption of quorum-signalling molecules, which may, in turn, disrupt the biofilm structure allowing for better inactivation and removal (Donlan and Costerton, 2002). Davies *et al*., (1998) showed that Quorum Sensing (QS) signaling molecules, acylhomoserine lactones (AHLs) were involved in biofilm architecture and detachment, and it has been suggested that novel treatments might be based on disruption of these quorum-sensing systems (Davies *et al*., 1998; Hartman and Wise, 1998; Stickler *et al*., 1998). A number of laboratories are currently attempting to elucidate the genes that are activated or repressed during initial biofilm formation. In the future, treatments that inhibit the transcription of these genes might be able to completely inhibit biofilms.

1.6.5 Biofilm formation in *Acinetobacter* spp.

As well as being highly resistant to the antibiotics, the members of the *Acinetobacter* spp., especially *A. baumannii*, are highly resistant to desiccation. These organisms can persist for long periods of time on inanimate surfaces. In addition *A. baumannii* is one of the most common causes of device-related nosocomial infection because it is able to resist chemical and physical disinfectants. Biofilm formation is one of the possible explanations for the resistance to desiccation and disinfection. It could also be responsible for a number of device-related nosocomial infections (Donlan, 2002; Tomaras *et al*., 2003).

In *A. baumannii* ATCC 19606, in vitro biofilm formation occurs mainly at the liquid-air interface and produces a biofilm-ring structure just above the surface of the medium. This structure grows further upwards from the liquid-air interface onto the walls of the tube. Within this biofilm, bacterial cells are attached to each other by pili-

like structures and there are channels to provide nutrients to the individual bacteria and remove the waste products. A polycistronic operon involved in pili assembly (*csu* genes) has been described in this strain as a requirement for pili formation and therefore initial bacterial attachment to the surface (Tomaras *et al*., 2003).

In addition, a biofilm-associated protein (Bap) has been described in *A. baumannii* which may have a function in supporting the development of the mature biofilm structure. This protein is a surface-expressed protein that is structurally similar to bacterial adhesions and its disruption produces a reduction in the volume and thickness of this biofilm (Loehfelm *et al*., 2008). The expression of an operon, *pga*ABCD, encoding a poly *N*-acetyl glucosamine (PGA) extracellular matrix has been shown to have a role in biofilm formation (Amini *et al*., 2009; Bosse *et al*., 2010).

1.7 Quorum Sensing (QS)

1.7.1 Bacterial cell-cell communication

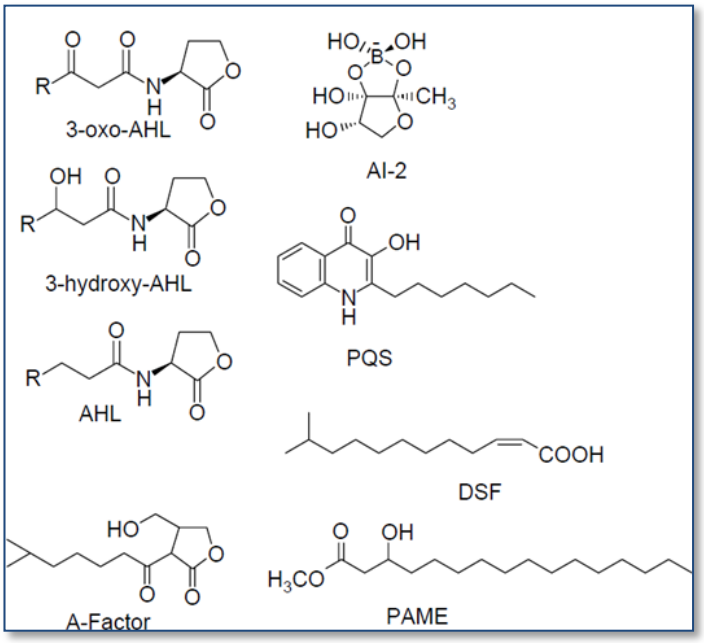
Bacteria display complex social behaviors and form communities of cells coordinating their activities through chemical communication. Because such cooperative behaviour is often dependent on cell population density, it is usually referred to as ‘‘quorum sensing’’ (QS). QS involves the activation of a receptor by a diffusible signal molecule. The concentration of QS signal reflects the number of bacterial cells and the sensing of a threshold level of signal indicates that there is required population of bacteria (Fuqua and Greenberg, 2002; Lazdunski *et al*., 2004; Williams *et al*., 2007).

Although QS was originally used to describe *N*-acylhomoserine lactone (AHL)-dependent cell-to-cell communication in Gram-negative bacteria, QS signal molecules exhibit significant chemical diversity. Structures of some of the representative QS molecules are given in figure 1.2. These molecules are autoinducers and bacteria may employ more than one QS signal molecule from the same or a different chemical class

and their activities may be coordinated by interacting QS systems, each of which incorporates a signal molecule synthase and a sensor/receptor (Fuqua and Greenberg, 2002; Lazdunski *et al*., 2004; Williams *et al*., 2007).

Because bacteria co-exist in ecosystems with many other organisms, it is perhaps not surprising to discover that QS signals can modulate the behaviour of both bacteria other than the QS signal producer itself and of higher organisms, in ways advantageous for bacterial survival (Williams *et al*., 2007). Higher organisms can sometime manipulate QS by producing signal mimics or by modulating QS pathways through the action of cytokines or by blocking QS through the deployment of inhibitors or through the enzymatic inactivation of QS signals.

Figure 1.2: Structures of some representative quorum sensing signalling molecules



(Adapted from Williams *et al*., 2007)

3-oxo-AHL, *N*-(3-oxoacyl) homoserine lactone; 3-hydroxy-AHL, *N*-(3-hydoxyacyl) homoserine lactone and AHL, *N*-acylhomoserine lactone where R ranges from C1 to C15. The acyl side chains may also contain one or more double bonds: A-factor, 2-isocapryloyl-3-hydroxymethyl-g-butyrolactone; AI-2, autoinducer-2, furanosyl borate ester form; PQS, Pseudomonas quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; DSF, 'diffusible factor', methyl dodecenoic acid; PAME, hydroxyl-palmitic acid methyl ester.

1.7.2 Types of quorum sensing signal molecules

The term ‘quorum sensing’ was coined to describe AHL mediated signalling (Dunny and Winans, 1999). AHL-mediated QS was found initially in few marine *vibrios*, but now it has been demonstrated in diverse Gram-negative genera including *Agrobacterium, Aeromonas, Burkholderia, Chromobacterium, Citrobacter, Enterobacter, Erwinia, Hafnia, Nitrosomonas, Obesumbacterium, Pantoea, Pseudomonas, Rahnella, Ralstonia, Rhodobacter, Rhizobium, Acinetobacter, Serratia* and *Yersinia*. The key protein components are the LuxI family of AHL synthases and the LuxR family of transcriptional activators. The LuxI proteins catalyse the formation of AHL signal molecules, and *in vitro* and *in vivo* studies have revealed that the homoserine lactone moeity is derived from *S*-adenosyl methionine (Moré *et al*., 1996; Jiang *et al*., 1998). The *N*-acyl chains, which range in length from 4–14 carbons that may be saturated or unsaturated and may or may not contain a C3 hydroxy or oxo group, are provided through the appropriately charged acyl–acyl carrier protein or acyl–coenzyme A (Moré *et al*., 1996; Jiang *et al*., 1998). Although fatty acids and polyketides with an odd number of acyl chain carbons have been described, AHLs with odd-numbered *N*-linked acyl chains have been identified only recently.

A second family of AHL synthases, LuxM, has been identified in *Vibrio* spp. (Bassler *et al*., 1993; Hanzelka *et al*., 1999) and these direct the synthesis of *N*-(3-hydroxybutanoyl)-homoserine lactone (3-hydroxy-C4-HSL and *N*-octanoyl-homoserine lactone (C8-HSL). Despite the lack of homology with the LuxI family, they appear to catalyse AHL formation from the same substrates as the LuxI proteins (Hanzelka *et al*., 1999).

Most recently, evidence for a third AHL synthase, HdtS, has been obtained from studies of *Pseudomonas fluorescens* F113 (Laue *et al*., 2000). When expressed in *Escherichia coli*, *hdtS* directs the production of three AHLs, namely *N*-(3-hydroxy-7-*cis*-tetradecenoyl) homoserine lactone, *N*-decanoyl-homoserine lactone (C10-HSL) and

N-hexanoyl-homoserine lactone (C6-HSL) (Laue *et al*., 2000). The HdtS protein sequence bears no homology to either the LuxI or LuxM families. Database searches reveal that HdtS is most closely related to the lysophosphatidic acid (LPA) acyl transferase family, which includes the PlsC protein of *E. coli*, responsible for the transfer of an acyl chain from either acyl–ACP or acyl–CoA to LPA to produce phosphatidic acid. It is therefore possible that HdtS transfers acyl chains from acyl–ACP or acyl–CoA to a substrate such as S-adenosyl methionine to generate AHLs. New research shows that bacteria have elaborate chemical signaling systems that enable them to communicate within and between species. One signal called AI-2 is universal and facilitates interspecies communication. Many processes, including virulence factor production, biofilm formation, and motility, are controlled by AI-2 (Federle and Bassler, 2003). Strategies that interfere with communication in bacteria are being explored in the biotechnology industry with the aim of developing novel antimicrobials.

Gram-negative bacteria also utilise QS signal molecules unrelated to the AHLs. The study of AHL-signalling in *Pseudomonas aeruginosa* identified two LuxRI QS systems—LasRI/*N*-(3-oxododecanoyl)homoserine lactone (3-oxo-C12-HSL) and RhlRI/*N*-butanoyl homoserine lactone (C4-HSL) — that form a hierarchical cascade for the regulation of multiple structural and regulatory genes (Latifi *et al*., 1996). Recently, a new QS molecule, termed the *Pseudomonas* quinolone signal (PQS), whose chemical structure is 2-heptyl-3-hydroxy-4-quinolone, was identified as a further component of QS hierarchy (Pesci *et al*., 1999). As well as AHLs and PQS, *P. aeruginosa* has recently been shown to produce another family of putative QS signal molecules (Holden *et al*., 1999). These are the cyclic dipeptides, which are capable of activating or antagonising LuxR-mediated QS systems possibly by competing for the AHL binding site on LuxR-type proteins.

While Gram-negative bacteria employ hydrophobic low molecular weight signal molecules, posttranslationally modified peptides are engaged by Gram-positive bacteria as quorum sensing signal molecules.

1.7.3 Quorum sensing regulation through networking

The information about the cell population density provided by QS forms a part of network of sensory input reflecting the physical and chemical status of the prevailing environment. The outline of this network is shown in figure 1.3. Perhaps the simplest case is the integration of different AHL signals.

In *V. harveyi*, two distinct QS signals are integrated in concert with cell density to control bioluminescence, colony morphology and siderophore production (Freeman and Bassler, 1999; Lilley and Bassler, 2000). *V. harveyi* appears capable of making decisions on the basis of multiple sensory inputs and, given the two distinct QS systems, can use the LuxS derived signal to determine not only its own population density, but also its numerical strength within a mixed bacterial community. The mass action of a high density population of *V. harveyi* cells may therefore be required to induce a significant bioluminescent signal. The presence of other bacteria, may be a key factor in verifying the location of the *V. harveyi* population before committing it to such an energy-expensive phenotype.

QS input is also integrated with information regarding aspects of the bacterial environment other than cell density. In many plant-associated *pseudomonads*, a two-component signal transduction system termed GacA/GacS for global antibiotic and cyanide control respectively, regulates the expression of many different phenotypes. In *P. aeruginosa* (Reimmann, 1997) and *Pseudomonas aureofaciens*, the GacA/GacS system also modulates quorum sensing by influencing C4-HSL production in the former, and regulating expression of the *luxI* homologue *phzI* in the latter (Chancey *et*

al., 1999). In *P. aeruginosa*, cyanide production is produced maximally at low oxygen tension and high cell densities.

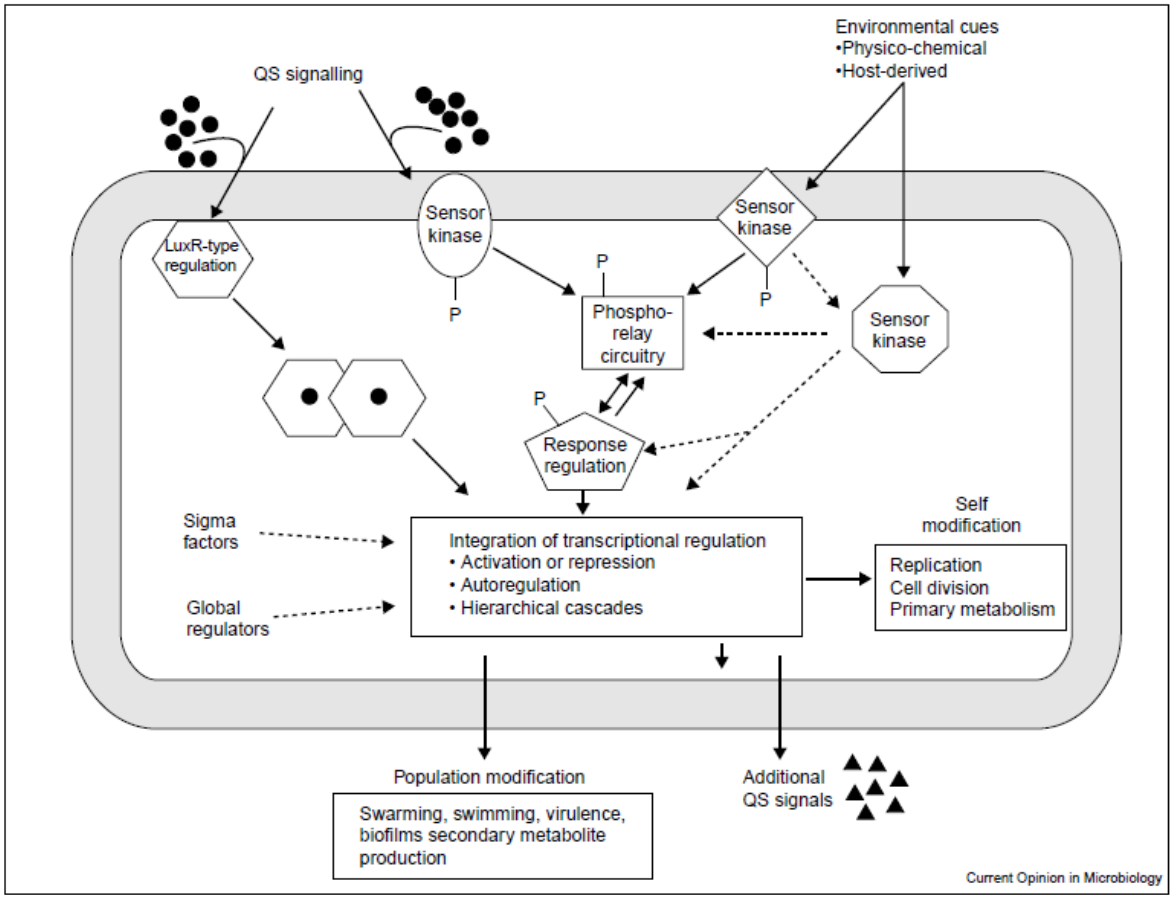
Another particularly important factor for a pathogen is the presence of the host. In *Agrobacterium tumefaciens*, plants opine hormones control the QS-dependent conjugal transfer of Ti plasmids (Oger *et al*., 1998). In *E. carotovora*, QS is necessary to activate both a battery of plant cell wall degrading exoenzymes and, in some strains, a carbapenem antibiotic (Jones *et al*., 1993). In *Yersinia* and *Salmonella*, SlyA homologues, which are responsible for the activation of genes required for virulence (Revell and Miller, 2000), are induced upon interaction with host eukaryotic cells.

The QS systems thus constitute mechanisms by which bacterial cells act in concert for the wellbeing of the population as a whole. A second, interrelated QS regulation involves phenotypic changes that increase the ability of the individual cell to survive. Examples include regulation of catabolism of specific amino acids during entry into stationary phase (Baca-Lancey *et al*., 1999), cell division (Garcia-Lara *et al*., 1996) and DNA replication (Withers and Nordström, 1998) in *E. coli*, and the transcriptional regulation of *rpoS* in *P. aeruginosa* (Latifi *et al*., 1996). All these processes are important for cell survival under conditions such as nutrient limitation and stress, most of which occur at high cell density and upon entry into stationary phase. It is also likely that these are the conditions most bacterial cells encounter outside the laboratory, and therefore the co-ordination of these processes would seem desirable.

A single bacterial cell contains a diverse array of signalling modules that may act in parallel or may be organised into a hierarchical cascade to give rise to the concept of 'neural' regulatory networks in bacteria. QS, as the determinant of cell population density, is therefore just one parameter amongst many that the cell must integrate in order to determine subsequent behaviour. QS signal molecules, in common with other environmental signals, may be internalised or detected at the cell envelope such that

signal transduction leads to self modulation or population modification (Withers *et al*., 2001).

Figure 1.3: QS regulation through networking



(Adapted from Withers *et al*., 2001)

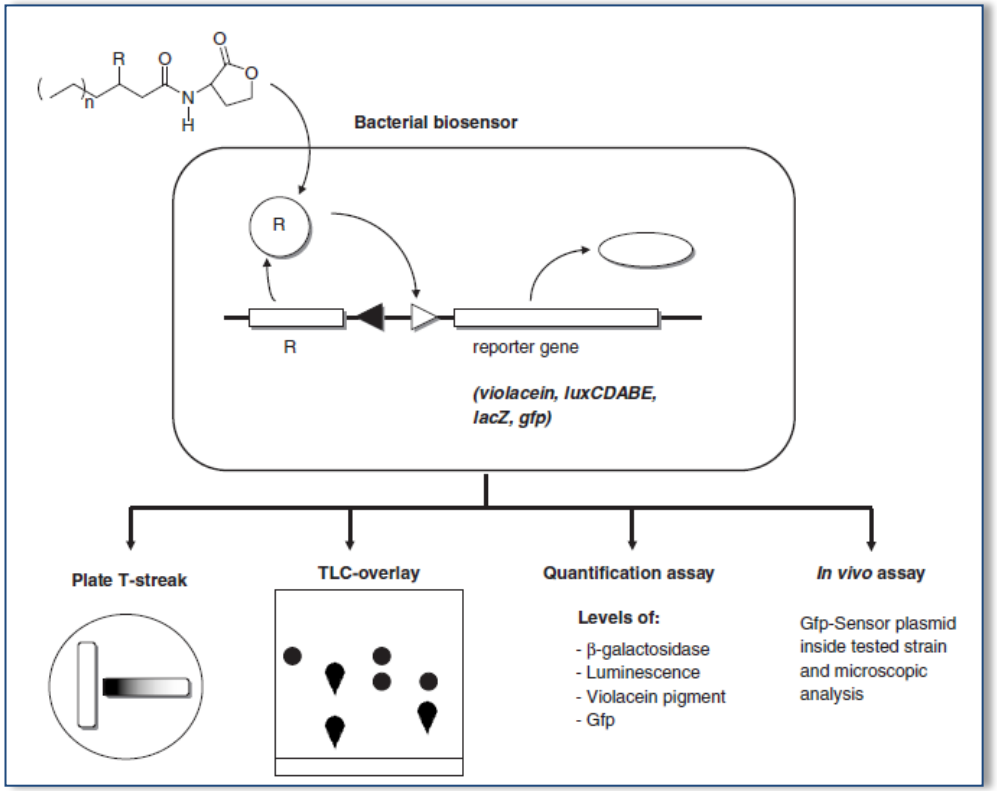
1.7.4 Bacterial AHL biosensor reporter strains

The very large number of AHL QS systems identified has been possible mainly by the use of bacterial biosensors that are able to detect the presence of AHLs. These biosensors do not produce AHLs and contain a functional LuxR-family protein cloned together with a cognate target promoter, which positively regulates the transcription of a reporter gene (Figure. 1.4).

AHLs can also be extracted from spent supernatants of late exponential phase cultures (Shaw *et al*., 1997; Schaefer *et al*., 2000), and partial characterization can be carried out by Thin Layer Chromatography (TLC) on C18 reversed phase plates. This

organic extraction increases many fold the sensitivity of biosensors. The TLC plates are loaded with the sample extracts and with different standards and, after chromatography, overlaid with a soft-agar suspension of the AHL biosensor strain (McClean *et al*., 1997; Shaw *et al*., 1997; Schaefer *et al*., 2000). Each AHL migrates with a characteristic mobility and results in a spot shape depending on the reporter of the biosensor strain. The 3-oxo-AHLs produce tear-shaped spots, whereas alkanoyl- AHLs and 3-OH-AHLs migrate and form well-defined circles. It is sometimes advantageous that before TLC, the sample extracts are further purified by C18-reverse phase HPLC and the resulting fractions tested for activity against AHL-biosensors either directly or via TLC (Schaefer *et al*., 2000). Separation by TLC coupled with detection by AHL biosensors gives a rapid and direct visual index of the AHL(s) produced by the tester bacteria. AHLs cannot be unambiguously identified using TLC. However, their chromatographic properties can be used to assign tentative structures, as retention factors (Rfs) calculated for the samples can be compared with Rfs of AHL standards. AHL structures are unequivocally determined on the basis of spectroscopic properties (Schaefer *et al*., 2000) including Mass Spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). Several of the AHL biosensors can also be used for quantifying AHLs by measuring the activity of the reporter system present in the biosensor bacterial strain. This is useful for studying regulation of AHL synthesis and for identifying strain-level differences in AHL production. In order to quantify accurately one must determine, using the synthetic AHL, the minimal amount of AHL required for a response as well as the amount necessary for a saturated response in order to plot the linear dose response.

Figure 1.4: Construction and use of a bacterial AHL biosensor



(Adapted from Steindler and Venturi, 2007)

'R' refers to the moiety at position C3, which can be either unmodified or carries an oxo- or hydroxyl group.

'n' refers to the length of the acyl chain, which is most commonly from 4 to 12 and in some cases bacteria can produce AHLs having chains of 14 to 18 carbons.

Some AHL biosensors have been constructed based on several LuxR-family proteins that are able to detect AHLs having acyl chains of C4 to C8 in length. A commonly used biosensor is based on *Chromobacterium violaceum*, a Gram negative water and soil bacterium that produces the antibacterial purple pigment violacein. *Chromobacterium violaceum* regulates violacein production via the CviI/R AHL QS system, which produces and responds to C6-AHL (McClean *et al*., 1997). McClean *et al*. (1997) constructed *C. violaceum* CV026, a violacein and AHL-negative double miniTn5 mutant. One transposon is inserted into the cviI AHL synthase gene, and the other is inserted into a putative violacein repressor locus. Exposure of strain CV026 to

exogenous AHLs, which are able to interact with CviR, results in rapid production of a visually clear purple pigmentation. Unsurprisingly, the most active agonist AHL for CV026 is C6-AHL, the natural *C. violaceum* AHL. Other AHLs that induce reasonably well include C6-3-oxo-AHL and C8-AHL, C8-3- oxo-AHL and C4-AHL. AHLs with acyl chains of C10 and longer chain lengths, with or without oxygen at the third position, could be detected by inhibition of HHL mediated activation of violacein production. This strain is well suited for detection on solid media via a 'T' streak analysis as well as the TLC soft-agar overlay technique.

Several other biosensors rely on a plasmid construct harboring the luxCDABE operon of Photorhabdus luminescens resulting in bioluminescence as a reporter system (Winson *et al*., 1998b). These plasmids are usually harboured in *Escherichia coli*, which do not produce AHLs. Plasmid pSB401 (Winson *et al*., 1998a) and pHV200I (Pearson *et al*., 1994) are both based on LuxR of *V. fischeri* and cognate luxI promoter controlling luxCDABE expression. They are most sensitive to cognate C6-3-oxo-AHL and display good sensitivity towards C6-AHL, C8-3-oxo-AHL and C8-AHL. Little agonist activity was observed by C4- AHLs, C10- and longer acyl chain AHLs. The presence of AHLs therefore induces bioluminescence, which, in a TLC analysis, can be conveniently detected by exposing the TLC overlaid with the biosensor to autoradiographic paper. These biosensors can also be used for 'T' streak analysis. They are not used as frequently as CV026 as they require a photon camera.

AHL biosensors for specific detection of C10-AHL, C12- AHL and their 3-oxo derivatives are based on the LasI/R system of *P. aeruginosa*, which produces and responds to C12-3-oxo-AHL. Plasmid sensor pSB1075 contains the lasR gene and cognate lasI gene promoter controlling luxCDABE expression (Winson *et al*., 1998a). This plasmid can only be harbored in *E. coli* and can be conveniently used in TLC analysis responding well to C12-3-oxo-AHL, C10-3-oxo- AHL and C12-AHL.

The *Chromobacterium violaceum* CV026 biosensor can detect the presence of long acyl-chains by the inhibition of AHL-induced violacein synthesis (McClean et al., 1997). Thus CV026 can detect the presence of a broad range of AHLs. Quorum sensing in *A. tumefaciens* is involved in the regulation of conjugal transfer of many plasmids (Farrand *et al*., 2002; Von Bodman *et al*., 2003). AHL biosensor *A. tumefaciens* NT1 (pZLR4) consists of strain NT1 cured of the Ti plasmid and thus unable to produce AHLs, and plasmid pZLR4. The plasmid contains the traR gene and one of the tra operons, responsible for Ti plasmid conjugal transfer, containing a traG::lacZ reporter fusion, the transcription of which is known to be regulated by the TraI/R AHL QS system (Cha *et al*., 1998; Farrand *et al*., 2002). *A. tumefaciens* displays the broadest sensitivity to AHLs at the lowest concentrations. This β-galactosidase-based biosensor is particularly well suited for TLC analysis. It is so sensitive to many AHLs that it requires only small volumes of AHL extracts from spent supernatants (Farrand *et al*., 2002). This sensor can also be used by spotting colonies, culture supernatants or sample extracts onto an overlay of the sensor grown in a suitable medium containing X-Gal. After overnight incubation, the presence of AHLs will result in a blue zone around the site of application (Farrand *et al*., 2002). It is also particularly suited to the study of AHL production profiles of a large number of bacterial strains. This biosensor detects 3-oxo-substituted AHL-derivatives with acyl chain lengths from 4 to 12 carbons and also 3-unsubstituted AHLs, with the exception of C4-AHL.

1.7.5 QS in *Acinetobacter* spp.

There are reports suggesting that quorum sensing signal molecules play an important role in biofilm formation (Camilli and Bassler, 2006; Domka *et al*., 2007). Studies on two fundamental bacterial small-molecule signaling pathways: extracellular quorum-sensing signaling and intracellular cyclic dinucleotide signalling suggested how

these two pathways may converge to control complex processes including multicellularity, biofilm formation, and virulence (Camilli and Bassler, 2006).

Previous studies showed AHL production by *Acinetobacter* spp. In *A. calcoaceticus* BD413 supernatants four compounds were detected in a time-dependent manner, and maximal activity was reached at stationary phase (Gonzalez *et al*., 2001). In a variety of gram-negative bacteria, it has also been demonstrated that biofilm development can be dependent on AHL signaling (Davies *et al*., 1998; Huber *et al*., 2001; Lynch *et al*.,2002). In *A. baumannii*, very little is known regarding factors required for biofilm formation (Loehfelm *et al*., 2008; Tomaras *et al*., 2003; Vidal *et al*., 1996). A putative chaperone that is required for this process has been identified (Tomaras *et al*., 2003). In addition, a homolog of a staphylococcal biofilm-associated protein (Bap) has been characterized in *A. baumannii*, where it appears to act as an extracellular adhesin (Loehfelm *et al*., 2008).There is also a report on the identification and characterization of an autoinducer synthase from *A. baumannii* that was designated AbaI. Mass spectrometry was used to identify AHL signals that were directed by AbaI. The *abaI* gene was activated in a positive-feedback loop by an AbaI-dependent AHL signal(s). An *abaI* mutant was impaired in the later stages of biofilm development, and this phenotype was rescued by ethyl acetate extracts of cell supernatants from a wild-type strain, thus proving the QS molecules aid biofilm formation in *Acinetobacter baumannii* (Niu *et al.*, 2008)

The identification of *abaI gene* and the corresponding AHL signals will now allow the identification of signal antagonists that inhibit biofilm development. These antagonists may also reduce the ability of *A. baumannii* to survive on environmental surfaces for extended periods, a key component of its ability to persist in intensive care wards.

1.8 Quorum Quenching

From the discovery of QS systems and their role in pathogenesis, elucidation of methods to interfere with QS, quorum quenching, as an effective infection control strategy has been developed. Numerous studies have established the presence of a globally controlled QS regulon in *P. aeruginosa*, of which roughly one third of the genes encode virulence factors. These are mainly secreted components including elastase, alkaline protease, rhamnolipids, phenazines, cyanide, lectins, chitinases and numerous proteins with unknown functions (Hentzer *et al*., 2003; Rasmussen *et al*., 2005b; Schuster *et al*., 2003; Vasil, 2003; Wagner *et al*., 2003). Several animal infection models highlight the involvement of these QS-regulated virulence factors in pathogenicity of *P. aeruginosa*. The simplest infection model has been established in the nematode *Caenorhabditis elegans*. This small worm can feed on bacteria but in the case of an opportunistic pathogen such as *P. aeruginosa*, the worm is often killed within a short period of time after ingesting the bacteria. When feeding on *P. aeruginosa*, the worms are killed by cyanide and phenazines secreted by the bacteria. On the contrary, with mutations in QS regulatory systems killing is abolished and they can be maintained on bacteria (Darby *et al*., 1999; Mahajan–Miklos *et al*., 1999; Rasmussen *et al*., 2005a). The RhlR based QS system has been found to be important for virulence of *P. aeruginosa* towards the amoeba *Dictyostelium discoideum* (Cosson *et al*., 2002). Functional QS systems have been found to be important for establishment and reduced clearance of *P. aeruginosa*. If the animals are infected with QS mutants, the immune response is faster, the polymorphonuclear leucocytes respond by development of stronger oxidative bursts and antibodies accumulate faster in the infected lung (Bjarnsholt *et al*., 2005; Smith *et al*., 2002; Wu *et al*., 2001). From the above studies it is clear that QS is indeed involved in virulence and pathogenesis of *P. aeruginosa*. A similar relationship is found in many other predominantly opportunistic pathogens

including *Serratia liquefaciens* and *Chromobacterium violaceum* both of which are human pathogens, and *Agrobacterium tumefaciens* and *Erwinia carotovora* both of which are plant pathogens (Brito *et al*., 2004; Grimont and Grimont, 1978; Sheng and Citovsky, 1996; Whitehead *et al*., 2002).

1.8.1 Biological screening for quorum sensing inhibitors

The discovery of quorum sensing inhibitor (QSI) compounds requires live screening systems which can identify the presence of QSI activities of pure as well as mixtures of compounds like plant, fungal, algal or microbial extracts. Such systems used for screening the QSI compound, make use of a QS-controlled promoter fused to a reporter gene/operon such as the violacin operon from *C. violaceum*, the lux gene cluster from *V. fischeri*, lacZ from *Escherichia coli* or gfp from *Aequorea victoria*. In presence of exogenously added AHL signal molecules, these reporters are activated to express their corresponding reporters. In the presence of exogenous QSI activity expression of the reporters is reduced or abolished and a positive result of the screen is manifested by a reduced signal. Several genetically modified bacteria, termed QSI selectors (QSIS), based on both the lux and the rhl/ las systems have been developed. These bacteria are killed in the presence of AHL signal molecules but rescued in the presence of both AHL and a QSI candidate (Rasmussen *et al*., 2005a).

1.8.2 Quorum sensing inhibitors

In *A. tumefaciens*, the activity of the quorum-sensing regulator protein TraR is antagonized by many quorum-sensing antiactivator proteins. TraM is one among these proteins which is encoded by the Ti plasmid in *A. tumefaciens* and also found in other members of the family *Rhizobiaceae* (Luo *et al*., 2000; Swiderska *et al*., 2001). Therefore, the main role of TraM is to regulate the activity of the AHL-TraR complex such that it functions effectively only when a particular quorum is reached (Hwang *et al*., 1999, Piper and Farrand, 2000).

TrlR (*T*raR-*l*ike *r*egulator) is another protein which is responsible for antagonizing the activity of TraR in *A. tumefaciens*. TrlR binds to one molecule of AHL per monomer and thus it can potentially titrate out the amount of AHL available to activate TraR, especially at low population densities when AHL production is at basal levels (Chai *et al*., 2001).

The QscR (quorum-sensing control repressor) protein is a homolog of LasR and RhlR, which are the transcriptional regulators of *Pseudomonas aeruginosa* quorum-sensing systems (Chugani *et al*., 2001; Ledgham *et al*., 2003).

The AHL that is made by the Tra quorum sensing system of *A. tumefaciens* is hydrolysed by the AHL-degrading enzyme coded by *attM* gene (Zhang *et al*., 2002). It was recently shown by Zhang *et al*., (2004) that this degradation system is highly regulated by the stress alarmone signal (Zhang *et al*., 2004). A second enzyme, AiiB, which is homologous to AttM, was identified in *A. tumefaciens* and this was shown to degrade AHLs (Carlier *et al*., 2003).

Recently, AHL acylase and lactonase enzyme activities were identified in soil pseudomonads and *P. aeruginosa* PAO1 strains (Huang *et al*., 2003). The AHL-degrading activity was specific towards the long-chain AHLs.

Recent work on the regulation of the quorum sensing systems in *V. harveyi* and *Vibrio cholerae* shows that small, regulatory RNAs (sRNAs) are involved in specifically repressing quorum sensing (Lenz *et al*., 2004; Cui *et al*., 1995).

A. tumefaciens causes crown gall disease in a variety of plants, causing large tumors in the crown of the plant. The tumor-inducing Ti plasmid of *A. tumefaciens* contains genes responsible for virulence and harbors the ability to transfer a fragment of its DNA (T-DNA) into the nuclear genome of the host plant. This bacterial DNA then directs the plant cells to synthesize specific carbohydrates called opines which are readily metabolized by *Agrobacterium* around the crown gall tumors (Zhu *et al*., 2000).

TraR is expressed only in the presence of plant-produced opines and so conjugal transfer of the Ti plasmid occurs only in the vicinity of host tumors (Oger and Farrand, 2001, 2002; Piper *et al*., 1999).

Halogenated furanones are naturally produced by the Australian red alga Delisea pulchra and these are known to have strong inhibitory activity against fouling organisms and herbivores. Interestingly, the furanones have structural similarity to AHLs. Previous research has shown that furanones are capable of interfering with the quorum-sensing behavior of several bacterial strains. The Swr quorum-sensing system of *S. liquefaciens* comprises the response regulator SwrR and the synthase SwrI, which are responsible for the production of C4-HSL and C6-HSL (Daniels *et al*., 2004; Lindum *et al*., 1998). Binding of furanone to LuxR results in the rapid disruption of the quorum-sensing-mediated gene regulation (Manefield *et al*., 1999; Manefield *et al*., 2002).

L-Canavanine is an arginine analog found exclusively in the seeds of legumes. It has been reported to be as abundant as up to 5% (dry weight) of some leguminous seeds (Weaks, 1977). L-canavanine has been identified as a QSI compound and showed that the QSI activity is independent of its effect on bacterial growth (Keshavan *et al*., 2005).

A recent study on enterohemorrhagic *Escherichia coli* (EHEC) showed that human hormones cross communicated with the bacterial quorum-sensing system (Sperandio *et al*., 2003). The LuxS dependent quorum-sensing system in EHEC controls several phenotypes, including pathogenicity and type III secretion, which eventually results in virulence on HeLa epithelial cells (Sircili *et al*., 2004).

Several AHL-degrading enzymes identified in various bacteria have the potential to be used as quorum quenchers. Dong *et al*., (2000) initially identified AiiA from *Bacillus* species and showed that this enzyme inactivates the AHL signal and attenuates virulence when expressed in *Erwinia carotovora*. This enzyme is an AHL lactonase, known to act by hydrolyzing the lactone bond in the AHL (Dong *et al*., 2001). Another

study showed that a soil isolate of *Variovorax paradoxus* not only breaks down AHLs but is capable of utilizing them as the sole source of carbon and nitrogen (Leadbetter and Greenberg, 2000). Other bacteria producing the AHL degrading enzymes belonged to the genera *Pseudomonas, Comamonas, Variovorax,* and *Rhodococcus.*

A second class of quorum-quenching enzymes was identified in *Ralstonia* strain XJ12B. The acylase AiiD isolated from this strain is a 794-amino-acid polypeptide that is capable of hydrolyzing the AHL amide (Huang *et al*., 2003).

Quorum sensing appears to be crucial for plant-bacterial interactions such as pathogenesis or symbiosis (Gonza´lez and Marketon, 2003; Loh *et al*., 2002; Wisniewski-Dye and Downie. 2002; Ma¨e *et al*., 2001; Zhang, 2003). This idea has been exploited to create transgenic plants encoding bacterial AHL synthases, such that the plants are now capable of producing AHL signal molecules. Fray *et al*., (1999) cloned the *yenI* AHL synthase from *Yersinia enterocolitica* and targeted it to the chloroplasts of tobacco plants to create transgenics that produced 3-oxo-C6-HSL and C6-HSL. The AHLs produced by the transgenic tobacco plants activated quorum-sensing-related phenotypes in AHL synthase mutants of *Pseudomonas aureofaciens* and *E. carotovora* strains (Fray *et al*., 1999).

Many of the structural analogs for different AHL molecules have been studied for their effects on the quorum-sensing system of the related bacterial strain. A study on the analogs of the 3-oxo-C8-HSL revealed that the nature of the antagonistic activity strongly depends on the expression of the TraR protein (Zhu *et al*., 1998; Reverchon *et al*., 2002). Several furanone-based structural analogs have also been synthesized and analyzed for their QSI activity. A study reported the isolation of two natural products from a marine sponge and a *Pseudomonas* sp. that were structurally similar to furanones. These compounds, isocladospolide and acaterin, were used as templates for further modifications, and the resulting compounds were tested against LuxR-based *E.*

coli biosensor strains. The 5H-furan-2-ones substituted with short alkyl chains were in general more antagonistic than the longeralkyl- chain counterparts (Hjelmgaard *et al*., 2003). Another interesting study using synthetic furanones tested on mouse lungs infected with different bacterial strains indicated that the QSI effect of furanones was functional in vivo and helped to enhance clearance of *P. aeruginosa* from infected lungs (Wu *et al*., 2004).

1.9 Justification and objectives of the study

Acinetobacter spp. are reported to be persistent in the hospital environment causing a variety of opportunistic nosocomial infections. *Acinetobacter* spp. have been isolated from various types of opportunistic infections, including septicemia, pneumonia, endocarditis, meningitis, skin and wound infection, and urinary tract infection. They have now emerged as important pathogens in the ICU setting, and this is probably due to the increasingly invasive diagnostic and therapeutic procedures used in hospital ICUs. The development of multidrug resistance in this organism in recent times had made the treatment of the infections difficult. Thus, *Acinetobacter* spp. is emerging as an increasingly important multidrug resistant pathogen, spreading in hospitals, and causing severe adverse outcomes. Besides that, *Acinetobacter* spp. seems to be spreading from hospital to hospital, and it has caused endemic infections in various geographical areas through multiple hospital outbreaks. It has become a leading nosocomial pathogen in many hospitals as compared to other non-fermenting Gram-negative bacilli. Therefore, a new strategy in the successful treatment of *Acinetobacter* infections is an absolute necessity.

Research findings have shown that *A.baumannii* forms biofilm with enhanced antibiotic resistance. The current view of biofilm infections leads to the realization that their effective control will require a concerted effort to develop therapeutic agents that target the biofilm phenotype and community signaling–based agents that prevent the

formation, or promote the detachment, of biofilms. Although such a model fits the concept of density-driven cell–cell communication and appears to describe biofilm development in several bacterial species and conditions, biofilm formation is multifactorial and complex. These include QS signal molecules, oligopeptides, amino acids such as glutamate and aspartate, and fatty methyl esters. Genetic analysis of biofilm formation has led to the proposal that extracellular signals and quorum sensing regulatory systems are essential for differentiated biofilms.

Disruption of the quorum sensing signals would therefore attenuate the pathogenicity of the organism by inhibiting the biofilm formation. There are a variety of quorum quenchers available which can effectively disrupt quorum sensing.

We through this study, proposed to elucidate the quorum sensing properties of clinical isolates of *Acinetobacter* spp. and interrupt these QS molecules by some of the quorum quenching strategies. Thus, the study was designed with the following objectives:

1. To determine the QS properties of the *Acinetobacter* spp. isolates.
2. To perform various assays to identify, quantify and characterise the QS molecules (*N*-acyl homoserine lactones (AHL) in gram negative organisms).
3. To investigate the gene involved in quorum sensing and creating mutant in the later stages of biofilm development which can be used for quorum quenching.
4. To record the effects of natural sources as potential quorum quenchers.

Chapter 2
Materials & Methods

2.1 Bacterial strains

2.1.1 Sample strains

Fifty strains that were identified as *Acinetobacter* spp. were obtained from the Diagnostic Bacteriology Laboratory, University Malaya Medical Centre (UMMC) between August 2003 to March 2004. The strains were obtained from invasive and non-invasive sites which include blood, tracheal secretion, sputum, throat swab, peritoneal fluid, wound, bronchial lavage, and urine. The identity of the isolates was confirmed using standard laboratory methods which include Gram-stain, colony morphology, lactose fermention, and the oxidase test. The isolates were further confirmed to belong to the *Acinetobacter baumannii/calcoaceticus* complex using API20NE kit (bioMerieux, France).

Further species level identification was done using the molecular method of ARDRA (Amplified ribosomal DNA restriction analysis) (Koeleman *et al*., 1998). All the strains were shown to belong to the *Acinetobacter baumannii- calcoaceticus* complex (ABC). This has been reported in our previous study with the same isolates (Wong *et al*., 2010). The test isolates were grown at 37ºC on Luria Bertani broth (LB, Difco) (1% peptone, 0.5% yeast extract, 0.5% NaCl; PH 7.00) (Bertani, 1951).

2.1.2 Biosensor strains

The biosensor strains detect AHL signals by the activation of a reporter gene such as lacZ or lux or by the production of or inhibition of a purple pigment in *Chromobacterium violaceum*. *Chromobacterium violaceum* strain CV026 was the monitor strain used to screen for AHL-producing *Acinetobacter* strains. The CviR (*Chromobacterium violaceum* Repressor) of *Chromobacterium violaceum* strain CV026 (Throup *et al*., 1995) regulates the production of a purple pigment when induced by AHL. Certain long-chained AHLs reversibly inhibit the induced CviR resulting in a lack of pigment when compared to a control. The CV026 strain was routinely grown in a

shaker incubator in LB solidified with 1.25% agar when required and supplemented with 20 μg/μl of kanamycin. For screening AHLs, TY medium containing (per liter) 8 g of Bacto Tryptone (Difco Laboratories), 5 g of yeast extract, and 5 g of NaCl (pH 7) was used.

2.1.3 Characterization of the bacterial isolates

Pure cultures of the isolates were obtained and all the standard routine methods of identification and characterization were carried out. These include Gram staining reaction, motility test, and biochemical tests including Methyl Red-Voges- proskauer (MR-VP) test, Triple sugar iron agar test, Simmon's citrate, Oxidase test. All these tests were performed using the standard protocols available.

For further characterization, API20NE was used. This is a standardized system for the identification of non-fastidious, non-enteric Gram-negative rods, combining 8 conventional tests which include potassium nitrate (NO_3), L-tryptophane (TRP), D-glucose (GLU), L-arginine (ADH), urea (URE), esculin ferric citrate (ESC), gelatin (GEL), and 4-nitrophenyl-β-D-galactopyranoside (PNPG), and also the 12 assimilation tests which include D-glucose (GLU), L-arabinose (ARA), D-mannose (MNE), D-mannitol (MAN), *N*-acetyl-glucosamine (NAG), D-maltose (MAL), potassium gluconate (GNT), capric acid (CAP), adipic acid (ADI), malic acid (MLT), trisodium citrate (CIT), and phenylacetic acid (PAC). Following the tests, results were analyzed using the database incorporated in the APILAB software (bioMerieux, France). These tests were performed according to the manufacturer's instructions.

2.2 Quantification of biofilm formation

2.2.1 Microtiter plate method

Biofilm formation was determined by the ability of cells to produce extracellular polymeric substances and adhere to the base of 96-well polystyrene plate using the method of Boddey *et al*. (2006) with modification. Briefly, 100 µl of LB broth was added into each well of sterile 96-well polystyrene plate followed by the addition of 1 µl of bacterial culture that was grown at 37°C overnight with shaking at 150 rpm. The plate was incubated without shaking at 37°C for 18 hours. Thereafter, 1 µl from each well was transferred into triplicate wells of a fresh 96-well plate containing 100 µl of fresh LB and the plates were incubated without shaking for 24 hours at 37°C. Following incubation the supernatant was discarded carefully and the wells were stained with 150 µl of 1% crystal violet at room temperature for 30 minutes. The stain was then removed and the wells were washed twice with 175 µl sterile deionized water, before the addition of 175 µl of dimethyl sulfoxide (DMSO) to solubilize the crystal violet. The plate was then read spectrophotometrically at an absorbance wavelength of 570 nm. For each strain the assay was run in triplicate wells and in three independent experiments. Wells containing only the medium was used as control. The same protocol was followed to quantify the biofilm after prolonged incubation at 37°C for 48 hours and then they were compared to the biofilms formed after incubation for 24 hours.

2.2.2 Statistical Analysis

The significance of the biofilms formed by the 50 isolates were statistically analysed by using the independent sample two-tailed t test. All the t tests were performed by SPSS software.

2.3 Screening for Acyl Homoserine Lactones (AHLs)

2.3.1 *Chromobacterium violaceum* CV026 induction assay

Screening for AHL production among the 50 isolates was done as follows: A fresh plate of *C.violaceum* CV026 was prepared on LB agar plates containing 20 μg/ml kanamycin. This plate was incubated overnight at 37°C. A loopful of cells was taken and resuspended in 10 μl of sterile water and adjusted to an $OD_{600\ nm}$ of approximately 0.1. Then 5 ml of this culture was added to 200 ml of cooled TY agar and poured as a thin plate. The test strains were grown separately in 5 ml of TY broth for 24 hours and then diluted to an $OD_{600\ nm}$ of 1.0. About 5 μl drops of the test strains were then added to the previously prepared chromoplate and allowed to dry. The tests are done in duplicate and incubated for 2-3 days at 28°C. This test can detect the presence of short chain AHL producing test strains. Monitor strains themselves served as negative control and monitor strains in plate containing 75nM HHL served as positive control. The basic concept of this method was adopted from McClean *et al*. (1997). This method was previously not used for directly screening AHL producing bacteria.

2.3.2 *Chromobacterium violaceum* CV026 inhibition assay

For detecting the long chain AHLs producing test strains a variant of *Chromobacterium violaceum* CV026 induction assay was carried out in which the chromoplate was prepared along with 75 nM concentration of *N*-Hexanoyl Homoserine lactone (HHL) incorporated in the medium. The inhibition of the induced violacein production reveals a positive result.

2.4 Quantification of AHLs

2.4.1 Well-diffusion assays (Induction and Inhibition)

Quantifications of the AHLs produced by the test strains can be done semi-quantitatively by well-diffusion assays. This was done as described by Ravn *et al*. (2001) with some modifications. The agar plates were prepared as follows: a preculture of *Chromobacterium violaceum* CV026 was grown in LB for 24 hours at 25°C with aeration and 2 ml of the preculture was used to inoculate 100 µl of LB broth. The culture was grown for 24 hours at 25°C with aeration and was poured into 200 ml of LB agar (1.2 % agar) containing 20 µg/µl kanamycin maintained at 46°C. The agar culture solution was immediately poured as 20 ml portions in petri dishes. Fifty µl volumes of AHL containing solutions (different concentrations of the standards) were pipetted into wells (6mm) punched in the solidified agar. The plates were incubated at 25°C for 48 hours before the diameter of the AHL induced purple pigmented zones surrounding the wells were measured. For the inhibition of the induced CV026, the LB-agar was supplemented with 75 nM *N*-hexanoyl L-homoserine lactone (HHL, Sigma- Aldrich). Here the inhibited colourless zones were measured.

2.4.2 Standard curve derivation

Standard curve showing the relationship between concentration of acylated homoserine lactone (*N*-hexanoyl homoserine lactone (HHL) & *N*-Decanoyl homoserine lactone (DHL)) and resulting diameters of induced / inhibited zones in *Chromobacterium violaceum* CV026 monitor system was derived. This curve was used to test the extraction efficiencies of the isolates that tested positive for the production of the AHL signal molecules.

2.4.3 Well-diffusion assay for the positive isolates

For detecting the quantity of AHLs present in each of the test isolates which tested positive for the presence of AHL in the preliminary screening tests, their zones of induction or inhibition were compared with that of the standards. This was done as described by Ravn *et al*. (2001) with some modifications. The agar plates were prepared as follows: a preculture of *Chromobacterium violaceum* CV026 was grown in LB for 24 hours at 25°C with aeration and 2 ml of the preculture was used to inoculate 100 µl of LB broth. The culture was grown for 24 hours at 25°C with aeration and was poured into 200 ml of LB agar (1.2 % agar) containing 20 µg/µl kanamycin maintained at 46°C. The agar culture solution was immediately poured as 20 ml portions in petri dishes. Fifty µl volumes of AHL containing solutions (the extraction from culture supernatants)) were pipetted into wells (6 mm) punched in the solidified agar. The plates were incubated at 25°C for 48 hours before the diameter of the AHL induced zones surrounding the wells was measured. For the inhibition of the induced CV026 (detection of long chain AHL molecules), the LB-agar was supplemented with 75 nM *N*-hexanoyl L-homoserine lactone (HHL, Sigma- Aldrich). Quantifications were done based on the comparison of the induction and inhibition zone diameters of the test isolates with the standard curves derived.

2.5 Identification of AHLs

2.5.1 Extraction of AHLs from the culture Supernatants

Extractions for Thin Layer Chromatography (TLC) were prepared as described by Shaw *et al*. (1997) with minor modifications. Ten-milliliter volumes of the bacterial cultures were centrifuged and the supernatants were extracted with an equivalent volume of ethyl acetate acidified by supplementing with 0.5% formic acid. The mixture was shaken vigorously for 30 seconds and the two phases were allowed to separate. The shaking was repeated three times before the ethyl acetate containing fraction was removed and another 10 ml fraction was added. The whole extraction process was repeated three times. The combined extracts were dried filtered and evaporated to dryness. Residues were dissolved in 50-100 µl of HPLC–grade ethyl acetate. Comparisons were made of extraction efficiencies using both acidified and non-acidified ethyl acetate exracts.

2.5.2 Thin Layer Chromatography Bioassay

This assay is used for semi-qualitatively identifying the AHLs produced by the isolates. Synthetic AHLs or test sample extracts dissolved in ethyl acetate in volumes of 10-20 µl were spotted onto C^{18} reversed phase TLC plates (aluminum sheets 20x20 cm; RP – 18 F_{254S}, 1.05559, Merck 64271, Darmstadt, Germany) and the chromatogram was developed using a solvent system of Methanol/water (60:40v/v) as described by Shaw *et al*, (1997). After development, the solvent was evaporated and the dried plates were overlaid with a culture of the CV026 monitor strain (induction or inhibition). A 30 ml overnight culture of CV026 was used to inoculate 150 ml of LB medium, and the culture was spread over the surface of the developed plates. After the agar had solidified, the plates were incubated overnight at 30°C in a sterilized closed plastic container. For CV026 inhibition assay the medium was incorporated with 75nM *N*-

hexanoyl L-homoserine lactone obtained commercially. Synthetic Decanoyl Homoserine Lactone (DHL) and Dodecanoyl Homoserine Lactone (dDHL) were used as standards in this assay.

2.5.3 Mass Spectrometry (MS)

2.5.3.1 Preparation of extracts for MS

Extracts used for TLC were also subjected to Mass Spectrometric analysis. Sample pellets were re-suspended in a mixture of 50% acetonitrile:distilled water 0.1% formic acid (200 µl) initially using a pipette, then sonicated for several minutes. The samples were centrifuged for 5 minutes at maximum rpm and then the supernatant was carefully removed and transferred to a new vial. 10 µl of each sample was transferred to a sample vial with insert and diluted 1 in 5 by addition of 50% acetonitrile, 0.1 % formic acid (40 µl).

2.5.3.2 Preparation of Standards

The standards of beta-ketocaproyl, hexanoyl, heptanoyl, octanoyl and decanoyl were diluted using 100% acetonitrile. The diluted standards were mixed to provide a stock solution containing all standards with the following concentrations: 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000, 5000 and 10,000 micromolar (µM). Standard curves were generated for the initial quantification experiment using all the above mentioned standards. The AHLs present in the isolates culture extracts were quantified by using these standard curves.

2.5.3.3 Instrumentation

An Agilent 6420 LC-ESI-QQQ using a dynamic multiple reaction monitor (DMRM) was used for quantification. Instrument conditions were: Gas Temperature: 300°C, Gas Flow: 10.5 L/min, Nebulizer: 45 psi, Capillary (positive): 4000V. Samples were injected (2µl) and separated by reverse-phase chromatography on an Agilent

Poroshell 120 EC-C18 (2.1 x 100 mm, 2.7 µm, 600 Bar) column, using the following buffers (100% water/0.1% FA) and (100% ACN/0.1% FA), with temperature maintained at 35°C.

The samples were analysed using an Agilent 6520 LC-ESI-QTOF in full scanning positive ion mode or auto-MS/MS mode in a range of 50-1600 *m/z* was. Instrument conditions were: Gas Temperature: 300°C, Gas Flow: 10.5 L/min, Nebulizer: 45 psi, Capillary (positive): 4000V, Fragmentor: 150V, Skimmer 65V. Samples were injected in 1 µl volumes and separated by reverse-phase chromatography on an Agilent Poroshell 120 EC-C18 (2.1 x 100 mm, 2.7 µm, 600 Bar) column, using the following buffers (100% water/0.1% FA) and (100% ACN/0.1% FA), with temperature maintained at 35°C.

2.5.3.4 MS-MS

MS/MS Spectra were collected using the following parameters: MS (Full scan) range: 100-1600 *m/z* collected at a rate of 1 spectra/s. Auto MS/MS range: 50-1600 *m/z* collected at rate of 3 spectra/s with a maximum of 5 precursors selected and fragmented using collision energy of 25V. Precursors were excluded after 1 spectra for a period of 30 seconds.

Table 2.1: Chromatographic conditions.

Time (minutes)	% Solvent B	Flow Rate (µl.min^{-1})
0.00	15	400
1.00	15	400
6.00	100	400
7.00	100	400
7.10	15	400
10.00	15	400

Table 2.2: DMRM List used for quantification.

Compound Name	Pre-cursor Ion	MS1 Res	Product Ion	MS2 Res	Frag-mentor	Collision Energy	Ret Time (min)	Delta Ret Time
β-Ketocaproyl	214.1	Unit	113	Unit	75	9	1.8	1
β-Ketocaproyl	214.1	Unit	102.1	Unit	75	9	1.8	1
β-Ketocaproyl	214.1	Unit	74.1	Unit	75	21	1.8	1
Hexanoyl	200.1	Unit	102	Unit	75	5	3.6	0.75
Hexanoyl	200.1	Unit	74.1	Unit	75	13	3.6	0.75
Hexanoyl	200.1	Unit	71.1	Unit	75	9	3.6	0.75
Heptanoyl	214.1	Unit	113.1	Unit	80	5	4.3	0.75
Heptanoyl	214.1	Unit	102	Unit	80	9	4.3	0.75
Heptanoyl	214.1	Unit	85.1	Unit	80	9	4.3	0.75
Octanoyl	228.2	Unit	102	Unit	80	9	4.8	0.75
Octanoyl	228.2	Unit	74.1	Unit	80	13	4.8	0.75
Octanoyl	228.2	Unit	57.1	Unit	80	17	4.8	0.75
Decanoyl	256.2	Unit	102.1	Unit	70	9	5.75	0.75
Decanoyl	256.2	Unit	71.1	Unit	70	13	5.75	0.75
Decanoyl	256.2	Unit	57.1	Unit	70	17	5.75	0.75

2.6 Identification of the QS gene in *Acinetobacter* spp. and construction of its mutant

The autoinducer synthase coding gene *abaI* was identified among the clinical isolates and the PCR purified product of this gene was cloned and transformed into competent cells. The recombinant plasmid carrying the *abaI* gene was further isolated and a mutant of this gene was created by insertional inactivation of the coding region of the gene. The biofilm forming capabilities of the wild-type and the mutant genes were evaluated.

2.6.1 Identification of *abaI* gene in *Acinetobacter* spp. producing the QS signal molecules by Polymerase Chain Reaction (PCR)

The presence of *abaI* gene encoding an autoinducer synthase was identified by polymerase chain reaction (PCR) using the primers published by Niu *et al*, (2008). The primers that were specific to the *abaI* gene, F-5'-GTACAGTCGACGTATTTGTTGAATATTTGGG-3' and R-5'-CGTACGTCTAGAGTAATGAGTTGTTTTGCGCC-3' were used. The template DNA for PCR was prepared using the epicentre masterpure DNA isolation kit following the manufacturer's instructions. The final PCR amplification reaction mixture contained 5.0 mM PCR buffer, 8.3 mM $MgCl_2$, 200 nM dNTP (MBI, Fermentas, Vilnius, Lithuania), 0.3 µM of each primer pair, 2 units of Taq DNA polymerase (MBI, Fermentas) and 3 µl of DNA template in a final volume of 25 µl. The PCR amplification was performed with mycyler thermocycler (BioRad laboratories, Hercules, CA). The parameters for amplification of *abaI* gene are shown in Table 2.3.

The PCR products were then electrophoresed on a 2% agarose gel (Pronadisa, Hispanlab, Spain) with ethidium bromide (0.5 µg/ml) incorporated into the gel, at 70 to 90 V for one and half hours. A 100 bp DNA ladder (Promega, Wisconsin, USA) was

used to estimate the size of the PCR products. The gel was then documented using the gel documentation system. The resulting amplicons of all the isolates that were positive for the AHL production were subjected to sequence analysis.

Table 2.3: Parameters for amplification of *abaI* gene.

Step and Temperature	Time
Initial denaturation, 94°C	10 minutes
Denaturation, 94°C	30 seconds
Annealing, 66.5°C	30 seconds
DNA extension, 72°C	60 seconds
Final extension, 72°C	5 minutes
Cycles	30

2.6.2 Sequencing and identity of the *abaI* gene PCR product

PCR fragments from the representative isolates for the *abaI* gene were sequenced to confirm the identity of the genes. Template DNA to be sequenced had to be sufficient in quantity and free of proteins, RNA, genomic DNA, EDTA and other salts to ensure good results. The quality and quantity of the purified DNA were measured by scanning the samples in a nanospectrophotometer. This was used to determine the ratio of A260/A280 and the concentration of the DNA sample. An optimum ratio (A260/A280) of > 1.8 and a concentration of between five to 20 ng/µl for a nucleotide length of 200 to 1,000 bp were deemed to be suitable for sequencing.

The purified DNA samples were sequenced at the First-Base Laboratory (Malaysia) using the Applied Biosystems 3730 DNA Analyser (Life Technologies, USA). The sequences were then downloaded and analysed using the BLAST tool available at the GenBank (http://www.ncbi.nlm.nih.gov), which compared the sequence with the published sequences in the GenBank by giving the percentage of homology.

2.6.3 Sequence analysis and Bioinformatics tools

All sequencing outputs for the respective genes (forward and reverse compliment sequences) were aligned and combined to produce a single targeted nucleotide length using ClustalW program (Table 2.4). The finalized longer partial sequence of the respective genes were analysed for their identity, amino acid sequence homology and protein structure using the bioinformatics tools available online at their respective websites. Table 2.4 lists all the tools with their respective functions.

Table 2.4. Bioinformatics tools, websites and their functions.

Tools [a]	Website addresses and functions
BioEdit	http://www.mbio.ncsu.edu/bioedit/bioedit.html (To do simple sequence and alignment editing and manipulations)
Sequencher	http://www.genecodes.com/ (To perform DNA sequence assembly and analysis)
Reverse Compliment	http://www.bioinformatics.org/sms/rev_comp.html (To return the reverse compliment of the antisense DNA strand)
ClustalW	http://www. Ebi.ac.uk/Tools/clustalW/index.html (To align and compare multiple sequences)
DNA-Protein	http://www.expasy.org/tools/ (To translate nucleotide sequences to amino acid sequences)
BLAST	http://www.ncbi.nlm.nlh.gov (To search for homologous nucleotide or amino acid sequences in the GenBank and predict the sequence identity)

[a]Detailed instructions for using the tools are available at the respective websites.

2.6.4 Cloning of the *abaI* gene

2.6.4.1 Purification of the *abaI* PCR product

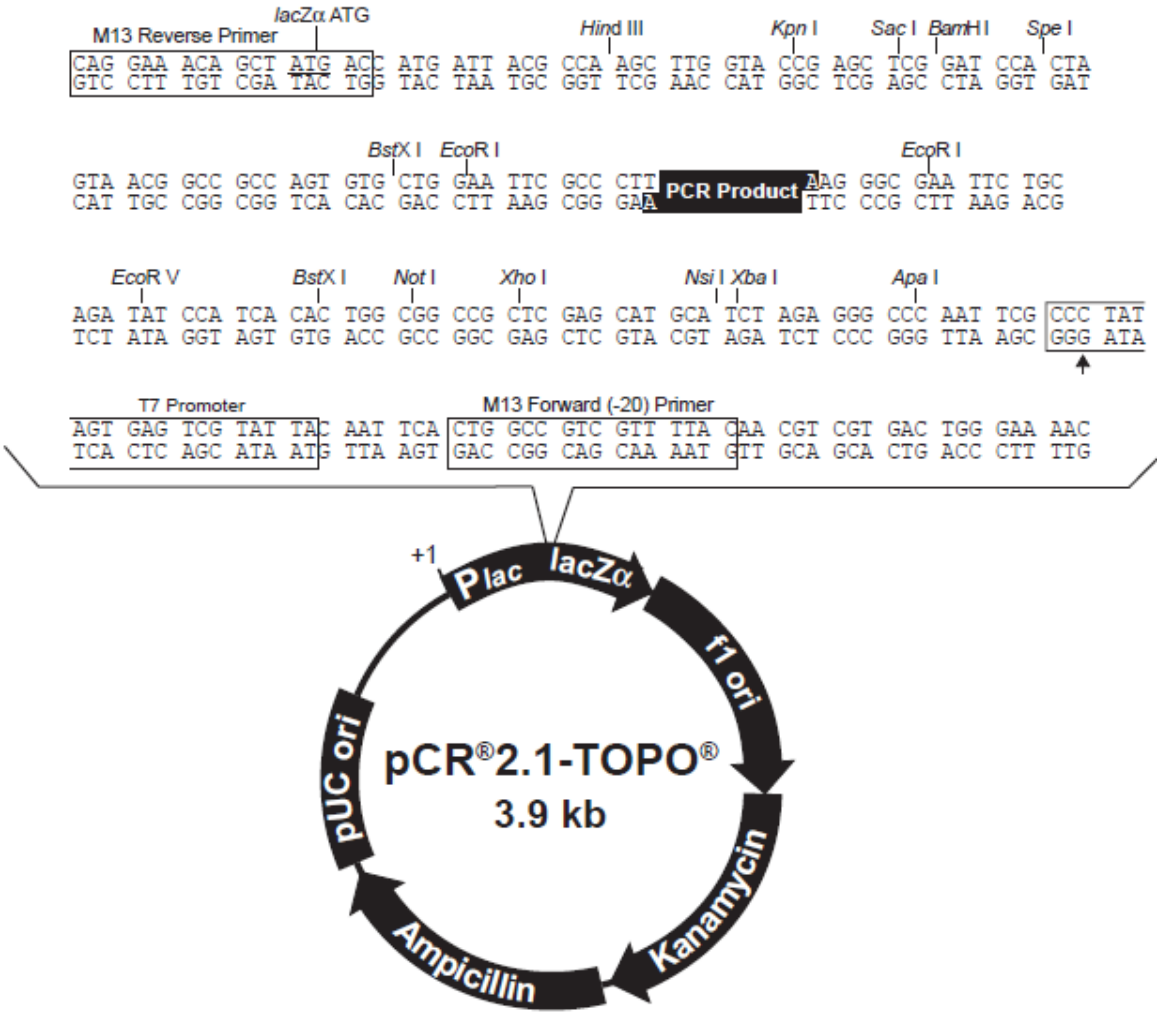
The PCR product to be cloned was purified using the Expin PCR purification kit (GeneAll Biotechnology, Korea) following the manufacturer's instructions. Briefly, about 5 volumes of buffer PB was added to 1 volume of the PCR product to be purified and mixed thoroughly and then the mixture was transferred to a SV column. This was

then centrifuged at 13,000 rpm for 30 seconds. The pass-through was discarded and the column was inserted back into the same tube. About 700 µl of buffer NW was added and centrifuged at 13,000 rpm for 30 seconds. The pass-through was again discarded and the column was inserted back into the collection tube. An additional centrifugation for 1 minute was done to remove any residual wash buffer and the SV column was transferred to a new 1.5 ml eppendorf tube. About 50 µl of buffer EB was then added to the centre of the membrane in the SV column and this was allowed to stand for 60 seconds and then centrifuged at 13,000 rpm for 60 seconds. The purified PCR DNA was thus obtained. This purified DNA was further used in the cloning experiment.

2.6.4.2 Cloning of the *abaI* gene

Cloning of the *abaI* gene was done using the TOPO TA cloning kit (Invitrogen Corporation, California) following the manufacturer's instructions. The purified PCR product was first treated with Taq polymerase buffer with dATP and 0.5 U Taq polymerase. This mixture was then incubated for 10-15 minutes at 72ºC and used immediately in the cloning reaction. The purified PCR product was cloned using the vector pCR 2.1-TOPO (Figure 2.1). Table 2.5 describes how the cloning reaction was set up. The total volume of the cloning reaction was 6 µl. This was eventually used to transform chemically competent *E.coli*. All the components were added in the same order as in table 2.5, mixed gently and incubated for 5 minutes at room temperature. This reaction mix was then placed on ice and was be stored at -20ºC overnight before being transformed into competent *E.coli*.

Figure 2.1: Map of pCR TOPO 2.1



pCR 2.1-TOPO (3931 nucleotides):

*Lac*Za fragment: bases 1-547

M13 reverse priming site: bases 205-221

Multiple cloning site: bases 234-357

T7 promoter/priming site: bases 364-383

M13 Forward (-20) priming site: bases 391-406

f1 origin: bases 548-985

Kanamycin resistance ORF: bases 1319-2113

Ampicillin resistance ORF: bases 2131-2991

pUC origin: bases 3136-3809

Table 2.5: Components of the cloning reaction.

Reagent	Volume of the component reagents
Fresh purified PCR product	2 µl
Salt solution	1 µl
Water	2 µl
TOPO Vector	1 µl
Final Volume	6 µl

2.6.5 Transformation of One Shot TOP10 *E.coli* competent cells

The transformation was done following the instructions of the manufacturer. Before starting the transformation reaction selective LB plates containing 50 µg/ml ampicillin was prepared. For each transformation reaction one vial of competent cells was used. A water bath was set at 42ºC and S.O.C. medium was warmed to room temperature. The selective plates were also warmed at 37ºC for 30 minutes. 40 µl each of 40 mg/ml X-gal and 100 mM IPTG were spread plated on the LB selective plates and incubated at 37ºC until it was used. The vial containing the competent cells was thawed on ice.

The chemically competent cells of *E.coli* were treated with 2 µl of cloning reaction mixture from the previous experiment and were mixed gently. The mixture was incubated on ice for 30 minutes. The cells are now heat-shocked at 42ºC for 30 seconds without shaking and the tubes were immediately transferred to ice. Then to this tube 250 µl of S.O.C. medium was added and capped. The tube was incubated at 37ºC for one hour with shaking at 200 rpm. About 20 µl of the transformation mixture was mixed with equal volume of S.O.C. medium and spread plated onto prewarmed selective LB plate and incubated at 37ºC overnight. Approximately 10 white or light blue colonies were picked as positive transformants for further analysis. The dark blue colonies were negative for transformation and so they were not carried forward for further analysis.

2.6.5.1 Scale-up of the Competent *E.coli* cells

The competent cells provided were grown and scaled-up for further transformation experiments. A loopful of the competent bacteria provided was inoculated into 5 ml of LB broth containing 50 mg/ml ampicillin and incubated at 37ºC overnight at 150 rpm. About 0.5 ml of the overnight culture was pipette into 50 ml of fresh LB broth and allowed to grow until it reaches an OD of 0.5 at A_{600nm}. Then the culture was incubated on ice for one to two hours. The culture was then centrifuged at 5000 rpm at 4ºC for 10 minutes. The supernatant was discarded. The pellet was resuspended in 1.7 ml of 0.1 M $CaCl_2$ and 0.3 ml of 50-80% glycerol and incubated for one hour. About 100 µl of this mixture was aliquotted into separate 1.5 ml eppendorf tubes. The tubes were snap freezed in liquid nitrogen and then was stored at -80ºC until used for transformation experiment.

2.6.6 Confirmation of the clone by colony PCR

The positive transformants were analysed directly by using PCR. The primers specific for the *abaI* gene F-5'-GTACAGTCGACGTATTTGTTGAATATTTGGG-3' and R-5'-CGTACGTCTAGAGTAATGAGTTGTTTTGCGCC-3' were used (Niu *et al*, 2008). For PCR reaction the Taq Mastermix (Geneall Biotechnology, Korea) and 20 µM each of the forward and the reverse primers were used. About 48 µl of the mastermix was added into the microcentrifuge tube and 1 µl each of the forward and reverse primers were added to the mastermix. Around 10 colonies from the suspected positive transformants were picked and individually resuspended into the 50 µl of PCR cocktail from the above step. The colonies were preserved for further analysis. The reaction was incubated for 10 minutes at 94ºC to lyse cells and inactivate nucleases. The PCR amplification was performed with mycyler thermocycler (BioRad laboratories, Hercules, CA) for around 30 cycles. The parameters for amplification are shown in Table 2.3.

The PCR products were then electrophoresed on a 2% agarose gel (Pronadisa, Hispanlab, Spain) with ethidium bromide (0.5 µg/ml) incorporated into the gel, at 70 to 90 Vfor one and half hours. A 100 bp DNA ladder (Promega, Wisconsin, USA) was used to estimate the size of the PCR products. The gel was then documented using the gel documentation system. The resulting amplicons of all the isolates that were positive for the presence of the *abaI* gene were subjected to further analysis.

2.6.7 Confirmation of the clone by restriction digestion

The transformants were also analysed for the presence of the *abaI* gene by restriction analysis. About 2-6 white or light blue colonies from the selective plate were cultured overnight in LB medium containing 50 µg/ml ampicillin. The plasmid DNA from this culture was isolated using the Exprep Plasmid isolation kit (GeneAll Biotechnology, Korea), following the manufacturer's instructions. Briefly, a single colony of the recombinant *E.coli* carrying the plasmid with *abaI* gene was inoculated into LB broth containing ampicillin and incubated at 37ºC for 24 hrs. About 1.5 ml of the overnight culture was aliquoted into 1.5 ml eppendorf tube and centrifuged at 13,000 rpm at room temperature for one minute. The supernatant was discarded and the pellet was stored at -20 C until use. The pellet was then resuspended thoroughly in 170 µl of buffer S1 and 170 µl of buffer S2. The tubes were then mixed by inverting three to four times. This mixture is incubated for less than 5 minutes until the cell suspension becomes clear. Then the lysate is treated with 250 µl of buffer G3 and immediately mixed by inverting the tube 3 to 4 times. The lysate was then transferred carefully to the EzClear column stack by decanting or pipetting. The column was then centrifuged for 30-60 seconds, the upper filter column unit discarded and the SV column was removed. The pass-through was discarded and the SV column was re-inserted into the collection tube. After this 500 µl of buffer AW was added and centrifuged for 30 seconds. The SV column was then removed and the pass-through was discarded and the column re-

inserted into the collection tube. Then 700 µl of buffer PW was added and centrifuged for 30 seconds, SV column removed and the pass-through discarded and the column re-inserted into the collection tube. Then an additional step of centrifugation for 1 minute was done to remove any residual wash buffer. The SV column was transferred to a new 1.5 ml eppendorf tube. About 50 µl of buffer EB was added and centrifuged for 1 minute. The tube contains the purified plasmid DNA.

The plasmid DNA isolated was then subjected to restriction digestion using the EcoRI (FastDigest EcoRI, Fermentas, Lithuania) following the manufacturer's instruction. Briefly, the reaction components listed in table 2.6 were mixed at room temperature in the same order as in the table. The mixture was then gently mixed and spinned down. The mixture was then incubated at 37ºC in a heat block for 5 minutes and the enzyme was inactivated by heating for 5 minutes at 80ºC.

The digested plasmids along with the uncut plasmid DNA was then electrophoresed on a 0.7% agarose gel (Pronadisa, Hispanlab, Spain) with ethidium bromide (0.5 µg/ml) incorporated into the gel, at 70 to 90 V for one and half hours. A 1000 bp DNA ladder (Promega, Wisconsin, USA) was used to estimate the size of the plasmid DNA. The gel was then documented using the gel documentation system. The resulting cut fragments of the digested plasmid DNA was analysed for the presence of the *abaI* gene.

Table 2.6: Reaction components used for the restriction digestion of the recombinant plasmid.

Components	Volume of the components
Water (nuclease-free)	15 µl
10X Fast Digest buffer	2 µl
Pasmid DNA	2 µl
FastDigest enzyme	1 µl
Total volume	20 µl

2.6.8 Confirmation by sequencing

The positive clones were further confirmed for the presence and also for proper orientation of the cloned gene *abaI* by sequencing. The M13 forward and M13 reverse primers were used for the sequencing purpose as the cloned gene is flanked by these primer sequences. The map of the pCR 2.1-TOPO is given in Figure 2.1.

The purified PCR DNA products were sequenced at the First-Base Laboratory (Malaysia) using the Applied Biosystems 3730 DNA Analyser (Life Technologies, USA). The sequences were then downloaded and analysed using the BLAST tool available at the GenBank (http://www.ncbi.nlm.nih.gov), which compared the sequence with the published sequences in the GenBank by giving the percentage of homology and the presence of *abaI* gene.

2.6.9 Construction of the mutant of *abaI* gene

The recombinant plasmid containing the *abaI* gene from the previous experiment was isolated using Exprep Plasmid isolation kit (GeneAll Biotechnology, Korea), following the manufacturer's instructions as described in section 2.7.7. The plasmid DNA was then checked for purity and quantified using the nanospectrophotometer and was run on 0.8% agarose gel prepared in TAE buffer with ethidium bromide incorporated within the gel in order to view the plasmids. A 1 kb DNA ladder (Promega, Wisconsin, USA) was run along as a marker. This plasmid was digested in the middle of the coding region of the *abaI* gene and an insertion was done to inactivate the *abaI* gene.

2.6.9.1 Selection of the restriction enzyme for the blunt end digestion

The coding region of the *abaI* gene within the plasmid was cut by a blunt-end cutter SspI. This restriction enzyme was selected from the list generated by using the software NEBcutter V2.0. The data generated is shown in the table 2.7.

Table 2.7: NEBcutter V2.0 analysis of the *abaI* gene for the selection of the restriction enzyme for blunt end ligation.

#	Enzyme	Specificity	Sites & flanks	Cut positions (blunt - 5' ext. - 3' ext.)
1	AccI	GT^MK_AC	list	*18/20
2	AciI	C^CG_C	list	*203/205
3	ApeKI	G^CWG_C	list	*66/69
4	ApoI	R^AATT_Y	list	159/163
5	AseI	AT^TA_AT	list	125/127
6	BbvI	GCAGC(N)$_8$^NNNN_	list	53/57
7	BccI	CCATCNNNN^N_	list	95/96
8	BceAI	ACGGC(N)$_{12}$^NN_	list	*170/172
9	BfuCI	^GATC_	list	1/5
10	BpuEI	CTTGAG(N)$_{14}$_NN^	list	24/22
11	BsmI	GAATG_CN^	list	141/139
12	BstUI	CG^CG	list	*71
13	BstXI	CCAN_NNNN^NTGG	list	162/158
14	BtsI	GCAGTG_NN^	list	231/229
15	DpnI	GA^TC	list	3
16	DpnII	^GATC_	list	#1/5
17	FauI	CCCGCNNNN^NN_	list	*210/212
18	Fnu4HI	GC^N_GC	list	*67/68
19	HhaI	G_CG^C	list	*71/69
20	HinP1I	G^CG_C	list	*69/71
21	HincII	GTY^RAC	list	*19
22	HinfI	G^ANT_C	list	50/53
23	Hpy166II	GTN^NAC	list	19
24	HpyCH4V	TG^CA	list	256
25	MboI	^GATC_	list	#1/5
26	MseI	T^TA_A	list	125/127
27	MwoI	GCNN_NNN^NNGC	list	221/218
28	SalI	G^TCGA_C	list	*17/21
29	Sau3AI	^GATC_	list	1/5
30	SmlI	C^TYRA_G	list	39/43
31	SspI	AAT^ATT	list	114
32	TfiI	G^AWT_C	list	50/53
33	TseI	G^CWG_C	list	66/69
34	TspRI	_NNCASTGNN^	list	231/222

2.6.9.2 Restriction digestion of the recombinant plasmid with *abaI* gene

The recombinant plasmid DNA with *abaI* gene was isolated and then subjected to restriction digestion using the SspI (FastDigest SspI, Fermentas, Lithuania) following the manufacturer's instruction. Briefly, the reaction components listed in table 2.8 were mixed at room temperature in the same order as in the table. The mixture was then

gently mixed and spinned down and incubated at 37ºC in a heat block for 5 minutes and the enzyme was inactivated by heating for 5 minutes at 80ºC. The resulting cut fragments of the digested plasmid DNA was used for ligation reaction with the tetracycline gene cassette.

Table 2.8: Reaction components used for the restriction digestion of the recombinant plasmid.

Components	Volume of the components
Water (nuclease-free)	15 µl
10X Fast Digest buffer	2 µl
Pasmid DNA	2 µl
FastDigest enzyme	1 µl
Total volume	20 µl

2.6.9.3 Blunt end ligation of the tetracycline gene and the recombinant plasmid

The linearized vector from section 2.6.9.1 was dephosphorylated with calf intestinal phosphatase (CIP) purchased from New England Biolabs following the manufacturer's instructions. Briefly, the reaction components listed in table 2.9 were mixed in the same order as in the table. The mixture was then gently mixed and incubated at 37ºC in a heat block for 60 minutes. The resulting dephosphorylated fragment was treated with 20 µl of 200mM ethylene diamine tetraacetic acid (EDTA) and incubated at 65ºC for 10 minutes. The digested plasmid DNA was then used for ligation reaction with the tetracycline gene cassette. For ligation reaction Fast DNA ligation kit (Fermentas, Lithuania) was used following the manufacturer's instructions. Briefly, the reaction components listed in table 2.10 were mixed at room temperature in the same order as in the table. The mixture was then gently vortexed and spinned down and incubated at 22ºC for 5 minutes. About 2 µl of the ligation mixture was used for transformation.

Table 2.9: Reaction components used for the dephosphorylation of the restriction digested recombinant plasmid.

Components	Volume of the components
10X CIP buffer	20 µl
CIP (1unit/ml)	10 µl
Vector DNA	20 µl
Water (nuclease-free)	150 µl
Total volume	200 µl

Table 2.10: Reaction components used for the ligation of restriction digested *abaI* gene and tetracycline gene cassette.

Components	Volume of the components
Water (nuclease-free)	1 µl
5X Ligation buffer	4 µl
Linearized vector DNA	12 µl
Insert (Tetracycline gene cassette)	2 µl
T4 DNA ligase (5units/ml)	1 µl
Total volume	20 µl

2.6.10 Transformation of the *abaI*:Tc mutant plasmid

The transformation was done as described in section 2.6.5. After the completion of transformation the positive transformants containing the *abaI*::Tetracycline (*abaI*::Tc) mutant plasmid were screened by plating on the selective plate containing 50 µg/ml ampicillin and 20 µg/ml tetracycline. Approximately 10 colonies were picked as positive transformants for further analysis.

2.6.11 Inactivation of the *abaI* gene in the chromosome of the *Acinetobacter* spp. S117

To inactivate the *abaI* gene in the chromosome of the *Acinetobacter* spp. S117, the insertion that disrupted the *abaI* gene in the middle along with the flanking chromosomal DNA was cloned into pUC18- based suicide delivery vector. This

plasmid pUC18.*abaI*::Tc was introduced into *Acinetobacter* spp. S117 by conjugation. *E.coli* K12 carrying the pUC18.*abaI*::Tc plasmid was the donor. Matings were performed on filter membrane. Following conjugation, the suicide vector pUC18.*abaI*::Tc would be integrated at the wildtype *abaI* gene, that results in the wildtype copy of the *abaI* gene and second copy with the *abaI*::Tc disruption. These exconjugants with both the wildtype and the mutant copy of the *abaI* gene (single crossover event) were selected based on their tetracycline and ampicillin resistance (tetracycline marker was present on the disrupted *abaI* gene and ampicillin marker was on the pUC18 vector). Following this, the exconjugants were grown without the antibiotic pressure. This removes the pressure to maintain the plasmid integration. Then the exconjugants that carry only the mutant *abaI* gene (double crossover event) were selected by eliminating any bacteria that have maintained the plasmid integration and selecting the bacteria that does not grow in the presence of the vector marker (ampicillin).

2.6.12 Confirmation of the exconjugant clone for the presence of tetracycline gene by colony PCR

The positive transformants were analysed directly by using PCR. The primers specific for the tetracycline gene F-5'-CGGCTTAGATCTAGGTCGAGGTGGCC-3' and R-5'-TCCAACAGATCTATTTGCCGACTACCTTGGTG-3' were used. The PCR amplification reaction mixture consisted of 10X ipfu buffer, 5 % DMSO, 200 nM dNTP (MBI Fermentas, Vilnius, Lithuania), 0.3µM of each primer pair, 2 units of ipfu DNA polymerase (MBI Fermentas) to a final volume of 25 µl. The PCR amplification was performed with Mycycler Thermocycler (BioRad Laboratories, Hercules, California). Around 10 colonies from the suspected positive transformants were picked and individually resuspended into the 25 µl of PCR cocktail from the above step. The colonies were preserved for further analysis. The reaction was incubated for 10 minutes

at 95ºC to lyse cells and inactivate nucleases. The PCR amplification was performed with mycyler thermocycler (BioRad laboratories, Hercules, CA) for around 35 cycles. The parameters for amplification are shown in Table 2.11.

The PCR products were then electrophoresed on a 2% agarose gel (Pronadisa, Hispanlab, Spain) with ethidium bromide (0.5 µg/ml) incorporated into the gel, at 70 to 90 Vfor one and half hours. A 1 Kp DNA ladder (Promega, Wisconsin, USA) was used to estimate the size of the PCR products. The gel was then documented using the gel documentation system. The resulting amplicons of all the isolates that were positive for the presence of the tetracycline gene were considered to be the *abaI*::Tc mutants.

Table 2.11: Parameters for amplification of tetracycline gene.

Step and Temperature	Time
Initial denaturation, 95°C	5 minutes
Denaturation, 95°C	30 seconds
Annealing, 60°C	60 seconds
DNA extension, 72°C	90 seconds
Final extension, 72°C	10 minutes
Cycles	35

2.6.13 AHL production in wildtype and mutant *abaI* gene

The production of AHLs in *Acinetobacter* spp. S117 with the wildtype and mutant *abaI* gene was carried out as described in section 2.3. Both CV026 induction and inhibition assays were carried out and the results were recorded.

2.6.14 Biofilm formation in wildtype and mutant *abaI* gene

Biofilm formation was determined by the ability of the cells to produce extracellular polymeric substances and adhere to the base of 96-well polystyrene plate using the method of Boddey *et al*. (2006) with modification as previously described in section 2.2. Both the wild type and mutant *abaI* were studied for their ability to form biofilm after 24 and 48 hours of incubation. The experiment was run in triplicate wells and in three independent experiments. Wells containing only the medium was used as

control. The difference in biofilm formation in *Acinetobacter* spp. S117 with the wildtype and mutant *abaI* genes were compared.

To demonstrate that the biofilm inhibition in the mutant was due to the loss of the AHL signal, mutation complementation experiment with the exogenously added AHLs as the ethyl-acetate extracts of the culture supernatants from the wildtype and the mutants was performed. Only ethyl-acetate was added in the negative control samples.

2.7 Natural sources as potential quorum quenchers

2.7.1 AHL inactivation activity of environmental bacterial isolates

2.7.1.1 Isolation of soil *Bacilli*

Soil samples were collected and sampling was done at the surface and subsurface using a clean spatula. The soil samples were stored in a clean and sterile container. The sample was processed by removing all large particles and plant materials such as leaves. Each of the soil samples were suspended in sterile distilled water. The soil samples were vortexed and the soil bacilli were isolated by plating a serially diluted soil suspension (10 g/10 ml of sterile distilled water), which was previously heat treated at 100ºC for 10 minutes to kill any vegetative forms of bacteria in the soil. The plates were incubated at 28ºC overnight. Pure colonies were picked for further analysis.

2.7.1.2 DNA extractions & PCR amplifications of *16S rDNA* gene

The DNA from pure cultures of the bacterial soil isolates was extracted using the Masterpure complete DNA purification kit (Epicenter, USA) following the manufacturer's instructions except that the cells were initially treated with 200 µM of lysozyme since they are suspected to be gram positive bacteria.

Soil bacteria were first subjected to PCR amplification to detect the *16S rDNA* gene sequence specific for *Bacillus* spp. The PCR amplification reaction mixture consisted of 5.0 mM PCR buffer, 8.3 mM $MgCl_2$, 200 nM dNTP (MBI Fermentas, Vilnius, Lithuania), 0.3 µM of each primer pair, 2 units of TaqDNA polymerase (MBI Fermentas) and 3 µl of template DNA in a final volume of 25 µl. The PCR amplification was performed with Mycycler Thermocycler (BioRad Laboratories, Hercules, California). The parameters for amplification of *16S rDNA* gene are shown in Table 2.12. The forward and reverse primers F-5'-AGAGTTTGATCCTGGCTCAG-3'& R-5'-AAGGAGGTGATCCAGCC-3' published by Dong *et al*., (2002) were used

to detect the presence of *16S rDNA* gene of the *Bacillus* spp. The expected amplicon size was approximately equal to 1500 bp.

The PCR products were then electrophoresed on a 0.7% agarose gel (Pronadisa, Hispanlab, Spain) with ethidium bromide (0.5 µg/ml) incorporated into the gel, at 70 to 90 V for one and half hours. A 1000 bp DNA ladder (Promega, Wisconsin, USA) was used to estimate the size of the PCR products. The gel was then documented using the gel documentation system.

Table 2.12.: Parameters for amplification of *16S rDNA* gene of *Bacillus* spp.

Step and Temperature	Time
Initial denaturation, 94°C	5 minutes
Denaturation, 94°C	30 seconds
Annealing, 50.75°C	30 seconds
DNA extension, 72°C	60 seconds
Final extension, 72°C	5 minutes
Cycles	30

2.7.1.3 *16S rDNA* gene sequence analysis

The sequences of the *16S rDNA* products were determined using the Applied Biosystems 3730 DNA Analyser (Life Technologies, USA). All sequencing outputs for the respective genes (forward and reverse compliment sequences) were aligned and combined to produce a single targeted nucleotide length (http://www.genecodes.com/, http://www.mbio.ncsu.edu/bioedit/bioedit.html,). The finalized longer partial sequences of the *16S rDNA* genes were analysed for their identity, amino acid sequence homology and protein structure with the sequences already available in the GenBank (www.ncbi.nlm.nih.gov) databases using the standard nucleotide–nucleotide BLAST program (www.ncbi.nlm.nih.gov). This would reveal the closest relatives to the determined sequences.

2.7.1.4 Detection of *aiiA* homologue gene

Bacteria which were positive for the *Bacillus* spp. identification were screened for the presence of *aiiA* homologue gene sequence using PCR. The presence of *aiiA* gene among the *Bacillus* sp. was detected by running a PCR for this gene using the forward primer of F-5'-ATG GGA TCC ATG ACA GTA AAG AAG CTT TAT-3' & a reverse primer of R-5'-GTC GAA TTC CTC AAC AAG ATA CTC CTA ATG-3' as reported by Dong *et al*., (2002). The expected amplicon size was approximately equal to 800 bp. The parameters for amplification of *aiiA* gene are shown in Table 2.13

The PCR products were then electrophoresed on a 0.7% agarose gel (Pronadisa, Hispanlab, Spain) with ethidium bromide (0.5 µg/ml) incorporated into the gel, at 70 to 90 V for one and half hours. A 1000 bp DNA ladder (Promega, Wisconsin, USA) was used to estimate the size of the PCR products. The gel was then documented using the gel documentation system.

The protocol used was an initial denaturation at 94°C for 10 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds; primer annealing at 58.35°C for 30 seconds, primer extension at 72°C for 1 minute and the final extension at 72°C for 5 minutes.

Table 2.13.: Parameters for amplification of *aiiA* gene of *Bacillus* spp.

Step and Temperature	Time
Initial denaturation, 94°C	10 minutes
Denaturation, 94°C	30 seconds
Annealing, 58.35°C	30 seconds
DNA extension, 72°C	60 seconds
Final extension, 72°C	5 minutes
Cycles	35

2.7.1.5 AHL inactivation assay for the soil isolate extracts

To determine AHL-inactivating activity, method described by Dong *et al*., (2002) was followed with slight modification. Briefly, *N*-hexanoyl homoserine lactone (HHL) was added at a final concentration af 20 µM to an overnight soil bacterial culture which was diluted to an optical density of 1.1 at 600 nm. The reaction mixture was incubated at 28°C. At 0, 3, 6, 9, 12 hours after incubation, cultures were withdrawn and the supernatant was UV irradiated. Then amount of HHL remaining in the supernatant was determined. The heat inactivated suspension (10 µl) was inoculated onto the LB agar seeded with the bioreporter *Chromobacterium violaceum* strain CV026 and incubated at 28 ºC. *Escherichia coli* DH5α strains served as negative controls. Degradation of HHL was visualized as loss of purple pigmentation shown by CV026 and the results were recorded.

2.7.2 Inhibition of biofilm and inactivation of AHLs among the clinical *Acinetobacter* spp. isolates

Acinetobacter spp. isolates were cultured in 1.5-ml eppendorf tubes. After incubation for 16 h, the bacterial culture was reinoculated into 1.5 ml of fresh medium and mixed with an equal volume of culture supernatant from the O/N culture of the soil *Bacillus* sp. which was previously uv-irradiated for 60 min. The mixtures were incubated at 28°C for 9 hours for soil isolate B1 and 3 hours for isolate B2, followed by the assays to detect the inactivation of AHL and inhibition of biofilm formation.

Screening for AHLs among the 7 clinical isolates was done as follows: A fresh plate of *C.violaceum* CV026 was prepared an LB agar plates containing 20 µg/ml kanamycin. This plate was incubated overnight at 37°C. A loopful of cells was taken and resuspended in 10 µl of sterile water and adjusted to an $OD_{600\ nm}$ of approximately

0.4. Then 5 ml of this culture was added to 200 ml of cooled LB agar and poured as a thin plate along with 75 nM concentration of *N*-Hexanoyl Homoserine lactone (HHL) incorporated in the medium. About 5 µl drops of the test strains (*Acinetobacter* spp. isolates treated with the soil extracts) were then added to the previously prepared chromoplate and allowed to dry. The tests are done in replicate and incubated for 2-3 days at 28°C. Monitor strains themselves served as negative control and monitor strains in plate containing 75nM HHL served as positive control. The basic concept of this method was adopted from McClean *et al*, (1997).

The extracts from *Acinetobacter* spp. treated with the soil bacilli were also subjected to thin layer chromatography (TLC) bioassay by the method described by Shaw *et al*. (1997) to detect the presence of any AHLs.

About 1µl of the treated sample was further analysed for biofilm formation. Biofilm formation was determined by the ability of cells to produce extracellular polymeric substances and adhere to the base of 96-well polystyrene plate using the method of Boddey *et al*. (2006) with modification. The biofilm forming capabilities of both the soil extract treated and untreated clinical isolates were analysed in this study.

2.7.3 AHL inactivation activity of plant extracts

2.7.3.1 Preparation of the plant extracts

Methanolic extracts of four *Phyllanthus* species (*P.amarus, P.niruri, P.urinaria, and P.watsonii*), garlic bulb and lemon were analysed for their quorum sensing inhibition properties. Freshly harvested plant samples were washed, dried in room temperature and then freeze dried. For preparing the methanol extract, dried plant sample were soaked with absolute methanol. The samples were then homogenized with extraction buffer and the supernatant collected after three rounds of extraction.

For garlic and lemon, they were cut into small pieces and dried for 24 hours. Dried materials were then ground and 95% ethanol (100 g/1 litre) was added and allowed to stand for 24 hours. This was then filtered through whatman no.1 filter paper and the filtrate evaporated to dryness under vacuum using a rotary evaporator. The resultant residues were stored at -20 ºC until used. Before being used for AHL inactivation assay it was reconstituted in 95% ethanol.

2.7.3.2 AHL inactivation assay for the plant extracts

The *Chromobacterium violaceum* quorum sensing system was used for this assay. Cultures of *Chromobacterium violaceum* CV026 were prepared by growing bacteria in Luria Bertani broth (Merck, Germany) and incubated for 16-18 h in an orbital incubator running at 30 °C and 150 rpm. Cultures were then adjusted to 0.5 McFarland standard (Ca. 10_8 CFU/mL). CV026 was spread plated on chromoplate containing 75 nM concentration of *N*-Hexanoyl Homoserine lactone (HHL) incorporated in the medium. Wells (6 mm diameter) were bored on the chromoplate and ethanol extracts of plants dissolved in sterile DMSO were dispensed into the wells. Luria Bertani plates were then incubated at 30 °C for 24 h after which results were recorded. To ensure the sterility of the sample and to minimize any introduction of exogenous anti QS compounds, extracts were membrane (0.2 μm) sterilized and were tested for microbial contamination before the anti QS assay by streaking onto Luria Bertani agar (LB) plates and incubation at 37 °C for overnight. Control plate with DMSO that was used to dissolve the extracts was also included to exclude the potential effect of DMSO on the quorum quenching.

2.7.4 Inhibition of biofilm formation and inactivation of AHLs among the clinical *Acinetobacter* spp. isolates after treatment with plant extracts

Acinetobacter spp. isolates were cultured in 1.5-ml Eppendorf tubes. After incubation for 16 h, the bacterial culture was reinoculated into 1.5 ml of fresh medium

and mixed with an equal volume of extracts from all the four *Phyllanthus* species, garlic and lemon as used for the above mentioned AHL inactivation assay. The treated clinical isolate mixtures were incubated at 28°C for 12 hours after the addition of the extracts, followed by the assays to detect the inactivation of AHL and inhibition of biofilm formation.

Screening for AHLs among the 7 clinical isolates was done as follows: A fresh plate of *C.violaceum* CV026 was prepared on LB agar plates containing 20 μg/ml kanamycin. This plate was incubated overnight at 37°C. A loopful of cells was taken and resuspended in 10 μl of sterile water and adjusted to an $OD_{600\ nm}$ of approximately 0.4. Then 5 ml of this culture was added to 200 ml of cooled LB agar and poured as a thin plate along with 75 nM concentration of *N*-Hexanoyl Homoserine lactone (HHL) incorporated in the medium. About 5 μl drops of the test strains (*Acinetobacter* spp. isolates treated with the soil extracts) were then added to the previously prepared chromoplate and allowed to dry. The tests are done in replicate and incubated for 2-3 days at 28°C. Monitor strains themselves served as negative control and monitor strains in plate containing 75 nM HHL served as positive control. The basic concept of this method was adopted from McClean *et al*, (1997). This method was previously not used for directly screening AHL producing bacteria.

The extracts from *Acinetobacter* spp. treated with the plant extracts, garlic and lemon were also subjected to thin layer chromatography (TLC) bioassay by the method described by Shaw *et al*., (1997) to detect the presence of any AHLs. About 1μl of the treated sample from the above experiment was further analysed for biofilm formation. Biofilm formation was determined by the ability of cells to produce extracellular polymeric substances and adhere to the base of 96-well polystyrene plate using the method of Boddey *et al*. (2006) with modification. The biofilm forming capabilities of both the treated and untreated clinical isolates were analysed in this study.

Chapter 3
Results

3.1 Bacterial strains

A total of 50 strains were obtained from the Diagnostic Bacteriology Laboratory, University Malaya Medical Centre (UMMC) between August 2003 and March 2004. The strains were isolated from various body sites of infections from patients who were hospitalized mainly in the intensive care unit (ICU), surgical and general wards as listed in Table 3.1. All the *Acinetobacter* spp. isolates were sporadic cases of bacterial infections and could have been community-acquired. Majority of the isolates were from tracheal secretion (45%), wound swab (12%), bronchoalveolar lavage (11%), sputum (8%), blood (6%), peritoneal fluid (4%), followed by urine (2%), and other body sites such as epidermal catheter, abrasion over back, suture line swab, foot pressure sore swab, pus swab, and double lumen tips (12%). 89.8% of the strains were from non-invasive sites while 10.2% were from invasive sites. However, for simplicity of analysis, the sites of isolation were re-assigned as upper respiratory tract (tracheal secretion, nasopharyngeal secretion) (46%), lower respiratory tract (sputum, bronchoalveolar lavage) (18%), peritoneal fluid (4%), blood (6%), wound (12%), urine (2%), and others (12%).

Table 3.1: Strain number, patient number, ward, source and date of isolation of 50 *Acinetobacter* spp. isolates.

Strain No.	Patient No.	Date Collected	Ward	Source
11	1147833	03/12/2003	8U	Tracheal secretion
124	1184548	25/02/2004	13U	Tracheal aspiration
1	1106728	29/08/2003	ICU	Tracheal aspiration
62	1161661	04/01/2004	13U	Tracheal secretion
80	1171825	27/01/2004	13U	Tracheal secretion
9	11464697	01/12/2003	6TE	Tracheal secretion
6	1147974	04/12/2003	6U	Tracheal secretion
35	1157917	26/12/2003	6U	Tracheal secretion
57	1164572	09/01/2004	6U	Tracheal secretion
48	1163171	07/01/2004	7U	Tracheal secretion
69	1167282	15/01/2004	7U	Tracheal secretion
12	1149607	08/12/2003	CICU	Tracheal secretion
21	1152147	12/12/2003	ICU	Tracheal secretion
93	1170717	25/01/2004	ICU	Tracheal secretion
14	1149626	08/12/2003	ICU	Tracheal secretion
15	1150413	09/12/2003	ICU	Tracheal secretion
30	1156504	22/12/2003	ICU	Tracheal Secretion
46	1160751	01/01/2004	ICU	Tracheal secretion
61	1162426	06/01/2004	ICU	Tracheal secretion
90	1171487	26/01/2004	ICU	Tracheal secretion
125	1185678	26/02/2004	ICU	Tracheal secretion
113	1186526	01/03/2004	8D	Throat swab
70	1166501	14/01/2004	P5	Throat swab
114	1186122	28/02/2004	12U	Sputum
49	1163189	07/01/2004	13U	Sputum
99	1176996	09/02/2004	13U	Sputum
52	1162203	05/01/2004	7U	Sputum
33	1157503	24/12/2003	7U	BAL
117	1188198	04/03/2004	ICU	BAL
59	1165166	11/01/2004	7U	Bronchial lavage
63	1162120	05/01/2004	P1	Bronchial lavage
73	1168513	18/01/2004	7U	Bronchial 3 plugged & mucous
77	1167614	15/01/2004	ICU	Peritoneal fluid
26	1155939	21/12/2003	P1	Peritoneal fluid
8	1146957	02/12/2003	P2	Blood
4	1137968	07/11/2003	6TD	Blood C+S
3	1133309	28/10/2003	6TD	Blood-PICC
107	1180637	16/02/2004	12U	Wound swab
55	1161540	03/01/2004	6U	Wound swab
96	1176799	09/02/2004	7D	Wound Swab
102	1179797	14/02/2004	7U	Wound swab
13	1149580	08/12/2003	8D	Wound swab
22	1151037	10/12/2003	CICU	Wound swab
54	1164238	09/01/2004	P5	Urine
45	1159599	30/12/2003	6U	Abrasion over back
104	1177833	10/02/2004	ICU	Double lumen tips
94	1176566	08/02/2004	6U	Pus swab
53	1161971	05/01/2004	8U	Suture line swab
81	1171359	26/01/2004	13U	Swab & foot pressure sore
29	1157633	24/12/2003	ICU	Tip for epidermal catheter

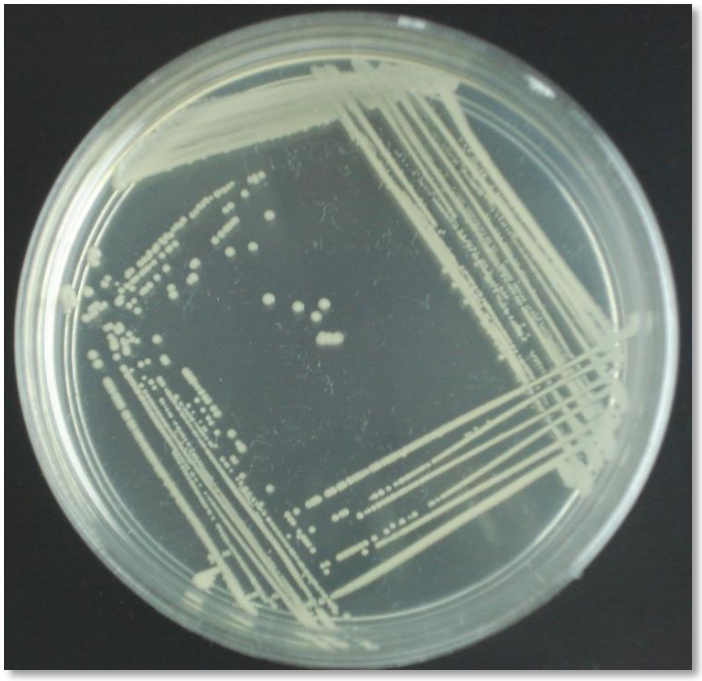
3.1.1 Identification using standard laboratory methods.

The identification of the organisms was performed using standard laboratory methods which include Gram staining and other routine sugar fermentation tests. The members of the genus *Acinetobacters* are Gram-negative coccobacilli that are strictly aerobic, non-fermentative, oxidase-negative, catalase-positive, and non-motile bacteria. They appear as white mucoid colonies on Mueller Hinton (MH) agar as shown in Figure 3.1. The Gram stained slide of *Acinetobacter* spp. under light microscope with 100X magnification showed that they appear as pink, plump and short rods as shown in Figure 3.2. They utilize carbon and ammonium as its only source of energy and the results of the sugar tests carried out to confirm this are shown in Figure 3.3. The results of standard identification tests of *Acinetobacter* spp. in comparison to other family members of *Enterobacteriaceae* and *Non-Enterobacteriaceae* are shown in Table 3.2.

In addition, API20NE test was carried out as confirmatory test for the identification of this organism. This test is also to differentiate between *Acinetobacter* spp. with other *Non-Enterobacteriaceae*. The API20NE test showed that all the 50 strains were identified as *Acinetobacter baumannii/calcoaceticus* complex as shown in Figure 3.4.

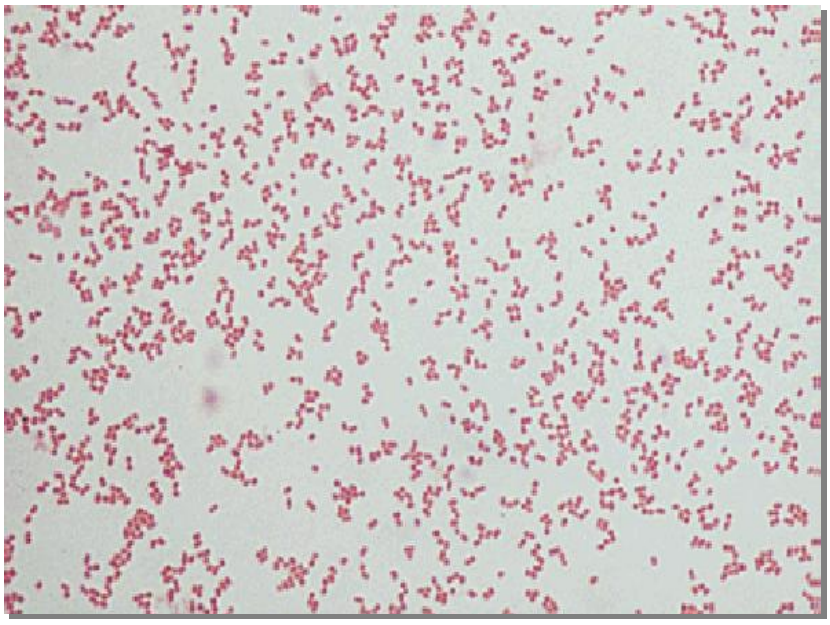
Further species level identification was done using molecular method ARDRA (Amplified ribosomal DNA restriction analysis). All the strains were shown to belong to the *Acinetobacter baumannii- calcoaceticus* complex. This has been reported in our previous study with the same isolates (Wong *et al*., 2010).

Figure 3.1: Morphology of *Acinetobacter* spp.



Acinetobacter spp. isolates appear as white mucoid colonies.

Figure 3.2: Gram stain of *Acinetobacter* spp.



Gram stain shows that they are Gram-negative coccobacilli or rod shaped.

Figure 3.3: Biochemical tests used for the identification of *Acinetobacter* spp.

Indole production test of *Acinetobacter* spp.	**MR test of *Acinetobacter* spp.**	**VP test of *Acinetobacter* spp.**	**Citrate utilization test of *Acinetobacter* spp.**	**TSI test of *Acinetobacter* spp.**
				
Negative test: The organism does not produce tryptophanase, an enzyme that cleaves tryptophan producing indole and other products.	**Negative test:** The organism does not ferment glucose and so no acid end products.	**Negative test:** No Fermentation of glucose.	**Positive test:** The organism produces Citrate permease and Citrate is utilized as its only source of carbon; and ammonium as its only source of nitrogen.	**Negative test:** No Fermentation of glucose, lactose or sucrose; peptone utilized.

Table 3.2: Comparison of biochemical test among family members of *Enterobacteriaceae* and *Non-Enterobacteriaceae.*

Species	A	B	C	D	E	F			G	H	I
						Slant	Butt	H_2S			
***Acinetobacter* spp.**	-	-	-	-	+	Alkaline	Alkaline	-	-	+	-
P. aeruginosa	+	-	-	-	+	Alkaline	Alkaline	-	+	+	-
E. coli	+	+	+	-	-	Acid	Acid	-	-	-	+
K. pneumoniae	-	-	-	+	+	Acid	Acid	-	-	-	+
C. freundii	+	-	+	-	+	-	-	+	-	-	+
***Enterobacter* spp.**	+	-	-	+	+	Acid	Acid	-	-	-	+
M. morganii	+	+	+	-	-	Acid	Alkaline	-	-	-	-

Abbreviation:

A **Motility test**
B **Indole production**
C **Methyl Red**
D **Voges-Proskauer**
E **Citrate utilization**
F **Triple Sugar Iron Agar test**
G **Oxidase test**
H **Catalase test**
I **Lactose fermentation**

Figure 3.4: Confirmation *Acinetobacter* spp. identification using API20NE.

***A.* spp**

E. coli

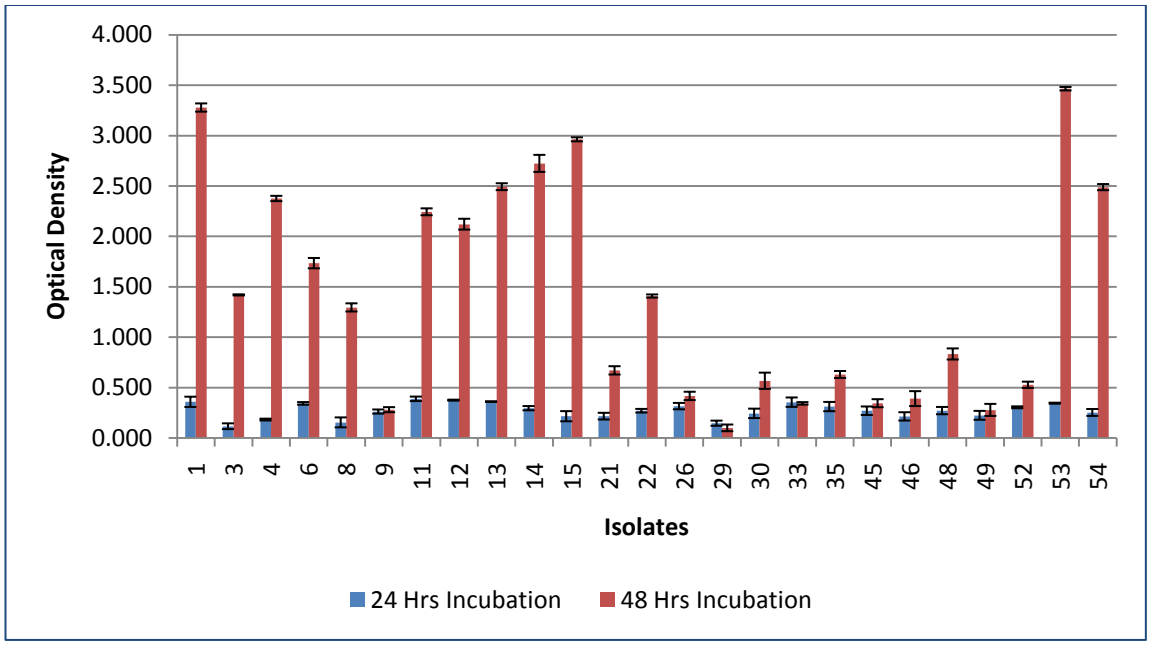
All 50 *Acinetobacter* spp. isolates were identified as *A. baumannnii/calcoaceticus* complex.

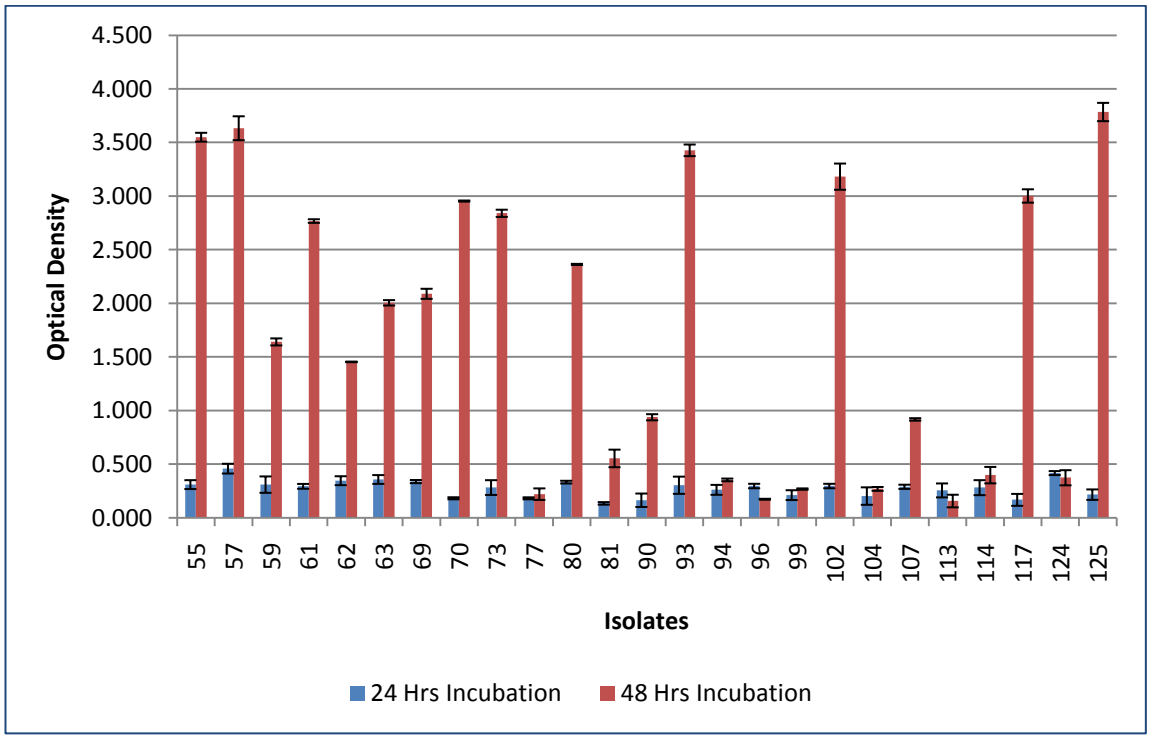
3.2 Quantification of biofilm formation using microtiter plate method

Quantification of biofilm produced by the 50 clinical isolates of *Acinetobacter* spp. was carried out using the microtiter plate method. The isolates which were capable of producing exopolysaccharide and bind to the surface of the polyvinyl plates were the ones that form efficient biofilms. The biofilms were stained with crystal violet and the stain was further extracted over by dimethyl sulfoxide and read spectrophotometrically at an absorbance wavelength of 570 nm. All the isolates were in the same logarithmic phase at the initial inoculation and the quantification of the biofilm was based on the biofilm absorbance at a wavelength of 570 nm.

The significance of the biolfilms formed by the 50 isolates were statistically analysed by using the independent sample two-tailed t test. All the t tests were performed by SPSS software. The analysis revealed that 30(60%) isolates significantly formed biofilms under 48 hrs of incubation (p value <0.05). Figure 3.5 shows the graphical representation of the biofilm formation among all the 50 isolates. Quantification of biofilm formation after prolonged incubation periods showed that in all the 50 isolates 48 hrs incubation showed higher biofilm formation compared to 24 hrs of incubation. The microtitre plate assay was done in triplicates and the data were pooled from these experiments.

Figure 3.5: Biofilm formation among the clinical isolates of Acinetobacter spp. in microtiter plates





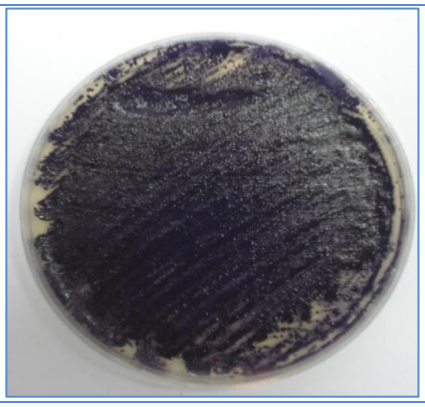
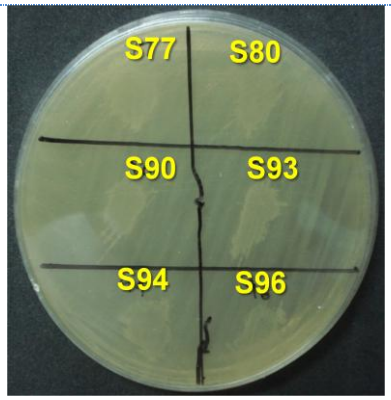
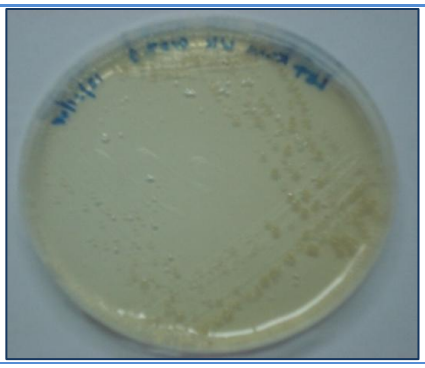
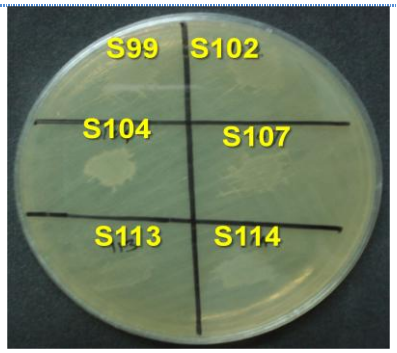
3.3 Screening for Acyl Homoserine Lactones (AHLs)

3.3.1 *Chromobacterium violaceum* CV026 induction assay

The preliminary screening for the production of AHLs by all the 50 clinical isolates of *Acinetobacter* spp. was done using the biosensor reporter strain *Chromobacterium violaceum* CV026. *Chromobacterium violaceum* produces the characteristic purple pigment violacein. Previously, a violacein-negative, mini-Tn5 mutant of *C. violaceum* (CV026) was described in which pigment production can be restored by incubation with supernatants from the wild-type strain. This mutant was developed to be used as a general biosensor for AHLs (Throup *et al*., 1995). In CV026, violacein is inducible by all the AHL compounds evaluated with *N*-acyl side chains from C_4 to C_8 in length, with varying degrees of sensitivity. Although AHL compounds with *N*-acyl side chains from C_{10} to C_{14} are unable to induce violacein production, if an activating AHL is incorporated into the agar, these long-chain AHLs can be detected by their ability to inhibit violacein production.

When CV026 induction test was carried out for the detection of short chain AHLs, all the 50 isolates produced colourless colonies. This showed that none of these *Acinetobacter* spp. clinical isolates produced short chain AHL molecules (Figure 3.6). The CV026 induction test was done on the chromoplate and the positive colonies producing the AHLs are expected to produce purple colonies. The isolates which are negative for the production of the AHL molecules produced colourless colonies. None of the tested isolates were positive for the induction test.

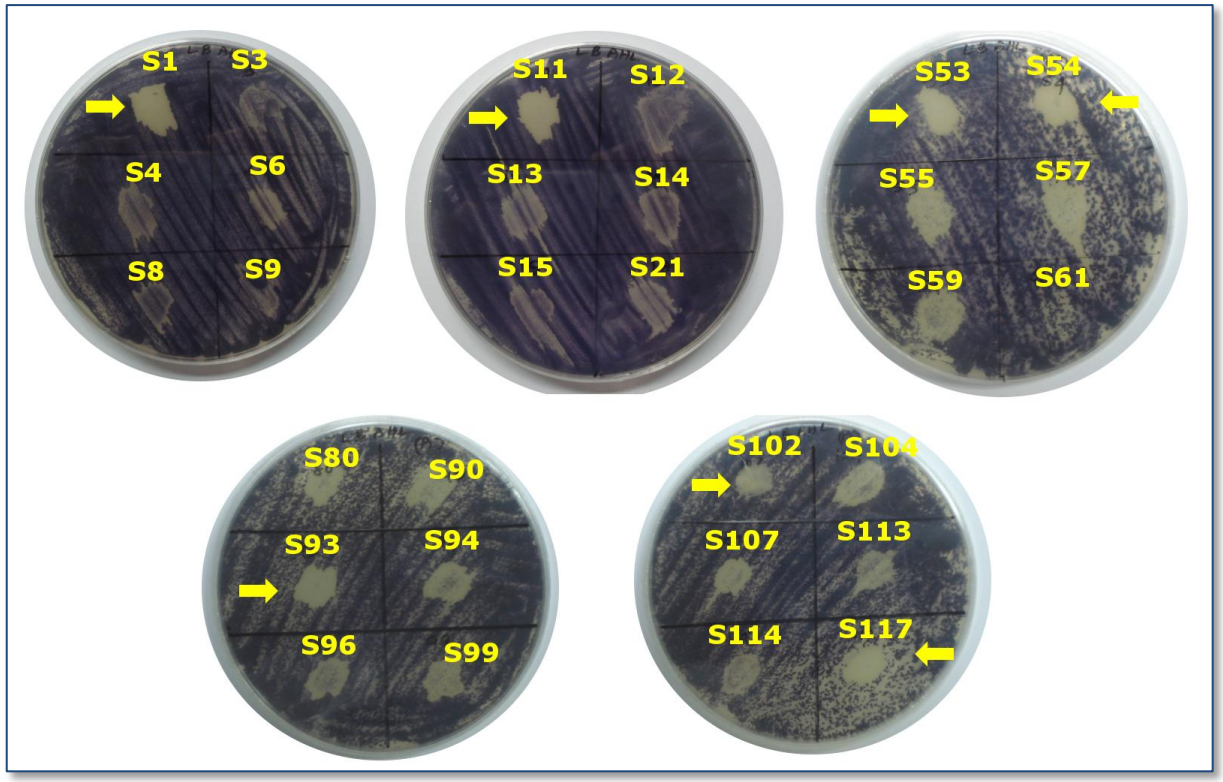
Figure 3.6: *Chromobacterium violaceum* CV026 induction assay

Controls	CV026 Induction Assay (for the detection of short chain AHLs)
	
Positive	Representative Negative Results
	
Negative	Representative Negative Results

3.3.2 *Chromobacterium violaceum* CV026 inhibition assay

In CV026 inhibition test, 7 of the isolates marked as S1, S11, S53, S54, S93, S102, S117 tested positive for long- chain AHL production. They produced colourless colonies in the induced chromoplate, while the negative isolates produced purple colonies (Figure 3.7). CV026 inhibition test was carried in a chromoplate to which the synthetic AHL was previously added. Here long-chain AHLs are expected to inhibit the induced CV026 and thus produce colourless colonies in the purple background. Seven of the *Acinetobacter* spp. clinical isolates tested positive for long-chain AHLs. These positive isolates producing AHLs were further quantified and characterized (Section 3.4).

Figure 3.7: *Chromobacterium violaceum* CV026 inhibition assay

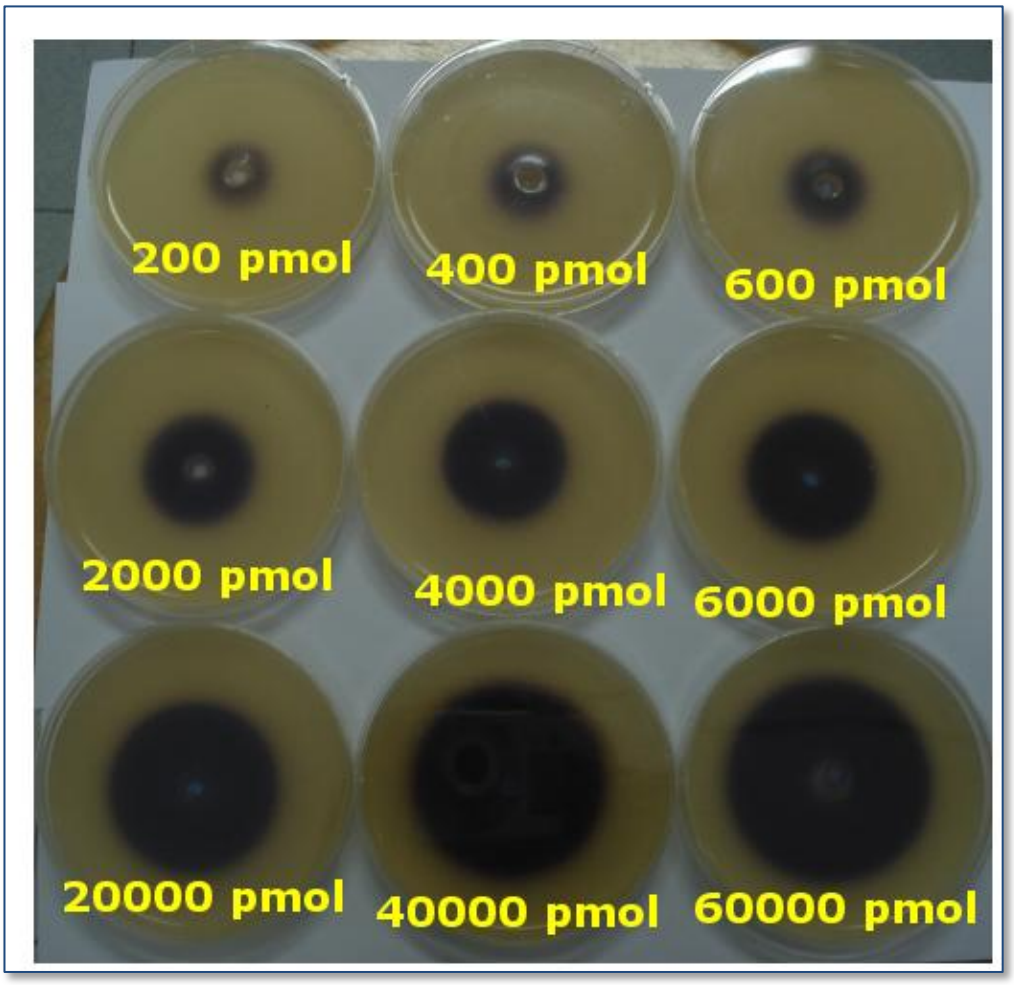


Arrows show isolates positive for AHL production

3.4 Quantification of the AHLs by well-diffusion assay

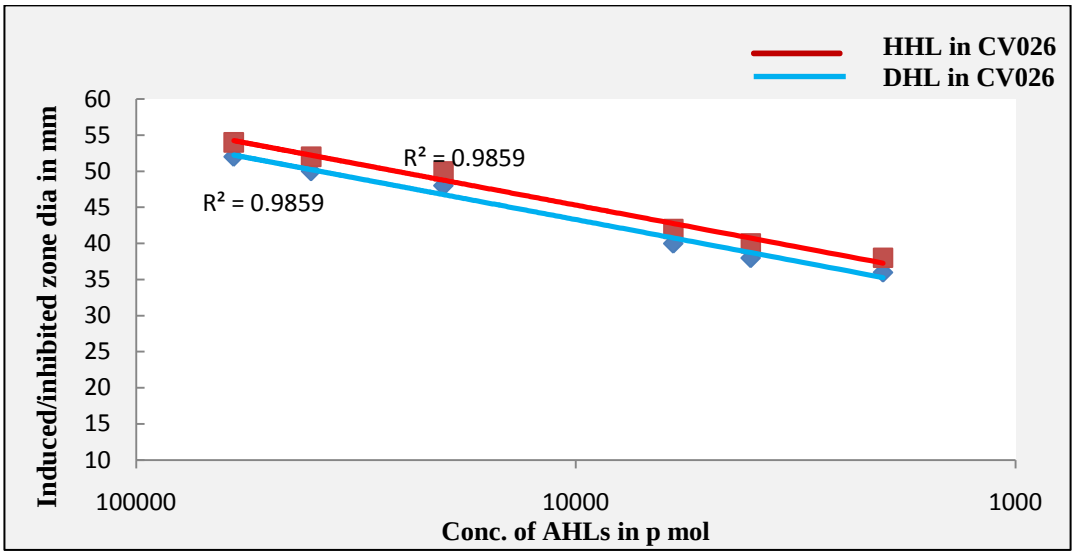
AHLs were extracted and quantified by the well diffusion assay. Increasing amounts of AHL added to the wells in the well-diffusion assay caused increase in the size of induced and inhibited zones surrounding the wells in *Chromobacterium violaceum* CV026 (Figure 3.8). Standard curves were created by comparing log (amount of AHL) to surface area of the zones (Figure 3.9). Based on the standard curves, extraction efficiencies were calculated for DHL (Decanyl Homoserine Lactone) and HHL (3-*N*-Hexanoyl homoserine lactone) using both acidified and non-acidified ethyl acetate. Approximately twice as much AHL was extracted when using acidified ethyl acetate as compared to non-acidified. Extraction efficiencies were tested on supernatants from *Acinetobacter* strains and it was observed that acidified extractions gave better yields as compared to the non-acidified extractions. Yields obtained were in the range of 1×10^{-9} moles to 6×10^{-9} moles of AHLs (Figure 3.10). The extraction efficiencies of the seven isolates that tested positive for the AHLs production are shown in the table 3.3.

Figure 3.8: *Chromobacterium violaceum* CV026 well-diffusion assay in LB agar.



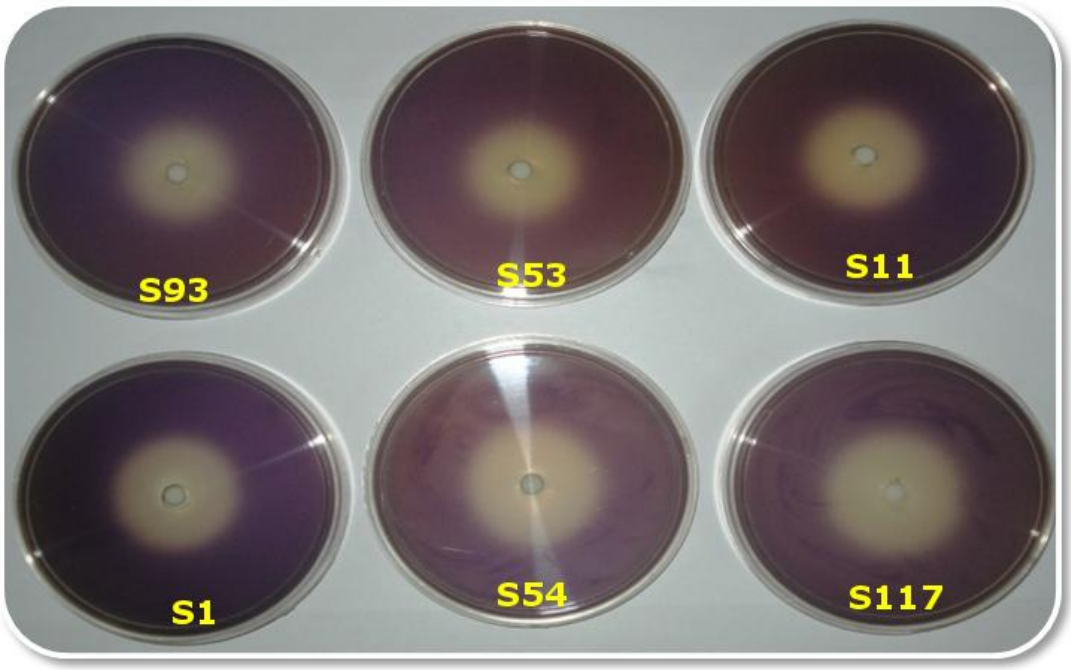
N-hexanoyl homoserine lactone (HHL) was added to wells in agar containing *Chromobacterium violaceum* CV026. Zones of HHL-induced violacin production is seen surrounding the wells. Increasing concentration of AHL shows the increase in zone diameters.

Figure 3.9: Std. curve for Extraction Efficiency of the Standard AHLs



Standard curve showing the relationship between concentration of acylated homoserine lactone (*N*-hexanoyl homoserine lactone (HHL) & *N*-Decanoyl homoserine lactone (DHL)) and resulting diameters of induced / inhibited zones in *Chromobacterium violaceum* CV026 monitor system.

Figure 3.10: Well-diffusion assay for the tested isolates.



Extraction efficiencies were tested on supernatants from *Acinetobacter* strains. Yields obtained were in the range of 1 x 10^{-9} moles to 6 x 10^{-9} moles of AHLs.

Table 3.3: Table showing the extraction efficiencies of the test isolates.

Sample No	Extraction Efficiencies (p mol/50 µl)
1	≈ 4000
11	≈ 4000
53	> 2000
54	> 4000
93	= 2000
102	< 2000
117	6000

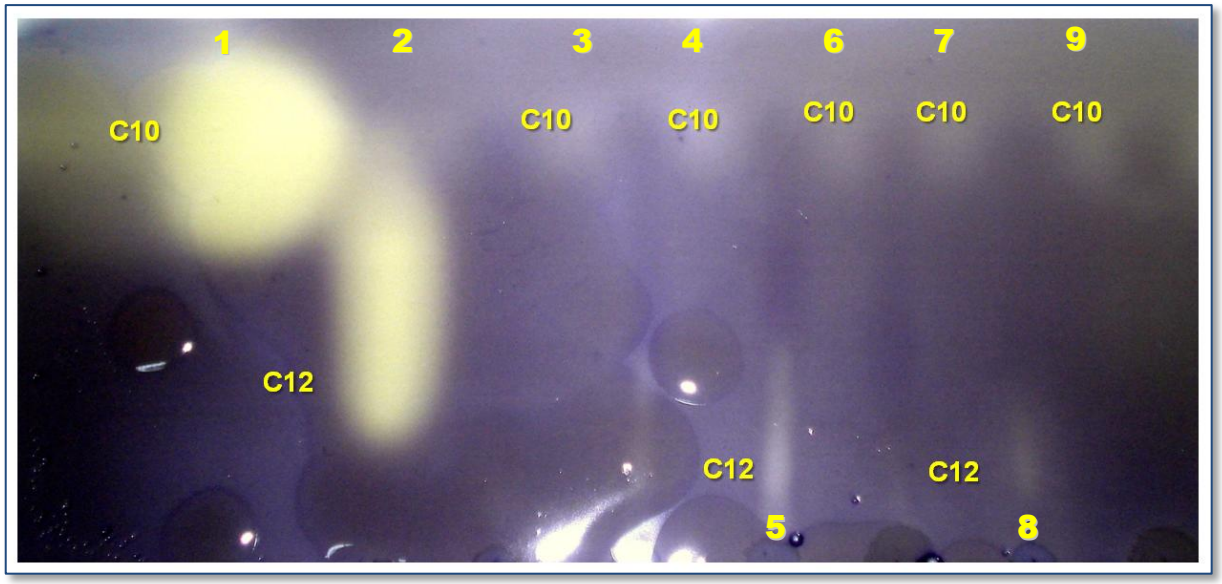
3.5 Identification of AHLs

3.5.1 Thin Layer chromatography and Bioassay

As the screening for AHLs showed that none of the clinical isolates of *Acinetobacter* spp. produced short-chain AHL molecules, TLC induction bioassay was carried out to confirm this. However, the TLC inhibition bioassay carried out for the 7 isolates which tested positive for long-chain AHL molecules showed varying inhibition zones (Figure 3.11). After the extracts were chromatographed on the C18 reversed phase TLC plates, the plates were dried and CV026 along with synthetic AHL was poured on the TLC plate as an overlay and the positive result was visualized as the zone of inhibition in a purple background. On C18 reversed-phase plates, of the solvent systems evaluated, methanol/water (60:40, vol/vol) separated the two standard compounds tested (Figure 3.11). The CV026 reporter detected the two 3-oxo-acyl-HSL standards with side chains C_{10} and C_{12} (Lane 1 and 2 respectively). The reporter also detected the AHLs produced by the isolates. As shown in the figure, the zone of inhibition of the 5 isolates (lanes 3, 4, 6, 7 and 9) corresponded to the synthetic standard DHL (lane 1) (*Rf* value of 0.18) and so all these isolates produced AHLs with C_{10}. These isolates were designated as S1, S11, S54, S93 and S117. For isolates S53 and S102 (lane 5, 8) the zone of inhibition tentatively corresponded to C_{12}. Here, the standard used for C_{12} is *N*-3-Oxo-Dodecanoyl-L-Homoserine Lactone. But the signal C_{12} produced by the isolates S53 and S102 presented spots in a different position. This is because, each of the 3-oxo and 3-unsubstituted derivatives migrated with a characteristic *Rf* values. The 3-Oxo-C12 (Standard used in lane 2) has a *Rf* value of 0.07, whereas, the 3-Unsubstituted- C12 has a *Rf* value of 0.02 (S53 and S102 in lane 5 and 8 respectively). Thus, the isolates S53 and S102 would have produced 3-

Unsubstituted- C12 signals. All the comparisons were done based on the inhibition zone produced by standard DHL and dDHL.

Figure 3.11: Thin layer chromatography bioassay.



Acylated homoserine lactones (AHLs) chromatographed on a C_{18} reversed phase thin layer plate developed with methanol / water (60:40). The plate after development was visualised using induced *Chromobacterium violaceum* CV026.

Lane 1	:	Standard Decanoyl Homoserine lactone (3-Oxo-C10)
Lane 2	:	Standard Dodecanoyl Homoserine Lactone (3-Oxo-C12)
Lane 3 and 4	:	Extracts from strain 1and 11 showing zone corresponding to C_{10} (3-Oxo-C10)
Lane 5	:	Extract from strain 53 showing zone of inhibition corresponding to C_{12} (3-Unsubstituted-C12)
Lane 6 and 7	:	Extracts from strains 54 and 93 showing zones corresponding to decanoyl homoserine lactone (3-Oxo-C10)
Lane 8	:	Extract from strain 102 showing zone of inhibition corresponding to C_{12} (3-Unsubstituted-C12)
Lane 9	:	Extracts from strain 117 shows zone corresponding to C_{10} (3-Oxo-C10)

3.5.2 Mass Spectrometry (MS)

3.5.2.1 Quantitation

Agilent MassHunter Quantitation Version B.04.00 was used to generate standard curves and calculate concentrations of AHLs present. Linear curves were fit to the linear portion of the curve with an acceptable R^2 = 0.98. In the case of high concentration standards significant signal suppression was observed, in these cases high concentration points were removed from the standard curve.

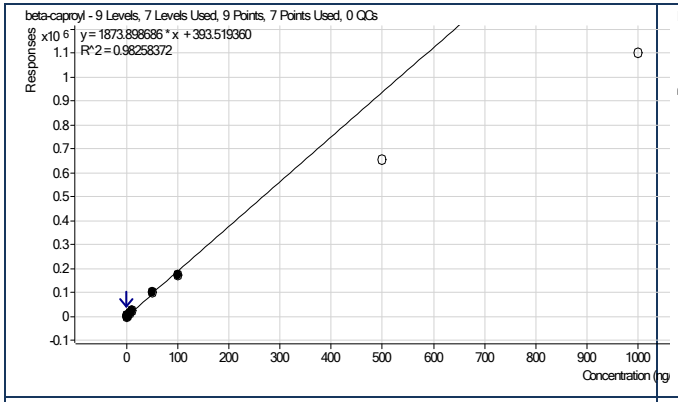
3.5.2.2 Standard Curves

A standard curve was run between the concentrations of 0.1 μM – 1000 μM (Figure 3.12). The concentrations of 500 and 1000 μM displayed significant signal suppression indicating that the standard curve had reached the limits of the dynamic range of the instrument. In each case a curve was fit over the appropriate range, with a linear curve attempted in all cases, except where high concentration points were required. For the experiment where concentrated samples were tested a limited concentration range 0.1 μM – 100 μM was used and this significantly reduced signal suppression and turnover of the standard curve.

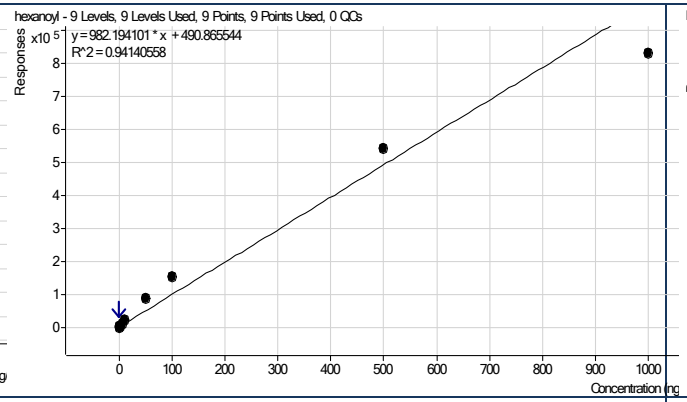
From the standard curves generated, the concentrations of the AHLs produced by the isolates were quantified. Care was taken when interpreting results below concentrations of 0.5 μM as there is significant variation and error found within the lowest concentrations measured (0.1 μM). Results above 0.5 μM indicated a positive result and the presence of the analyte. Initial results indicated samples from isolates S1, S11, S117, S53, S102, S93 and S54 contained measureable levels of longer chain AHL's (Table 3.4). Samples 1, 11, 117, 93 and 54 contained high levels of decanoyl homoserine lactone with concentrations ranging from 0.8-2.6 mM. Concentrations of octanoyl were

also observed in significant amounts. Isolates S53 and S102 did not show considerable concentrations of the AHLs when compared with the standards used, as C12 standard was not included in the standard curve derivation. But, further mass spectrometric analysis showed the presence of C12 AHLs in S53 and S102. This result confirms the detection of AHLs by TLC (Section 3.5.1).

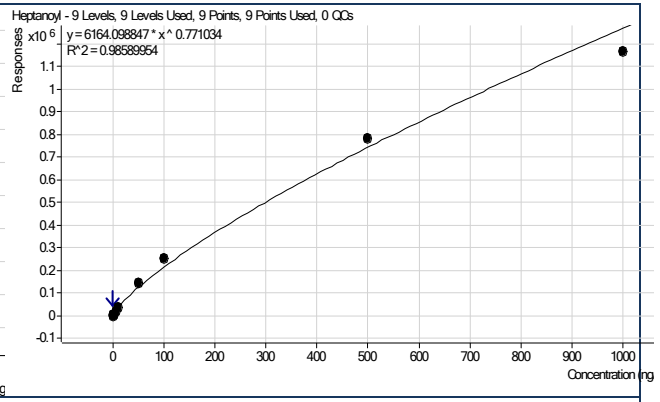
Figure 3.12: Standard curves generated for the initial quantification experiment measuring all samples.



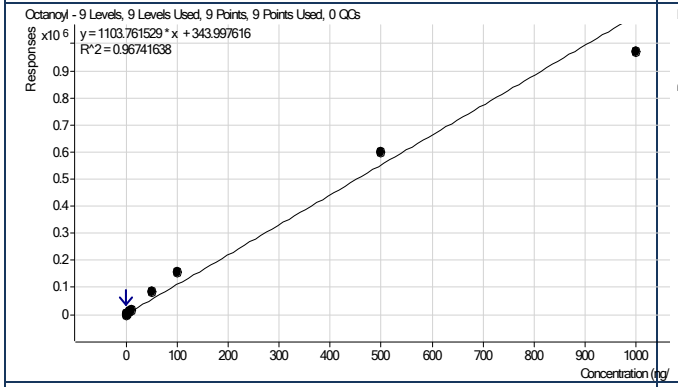
a. Standard curve for β-Ketocaproyl Homoserine Lactone, using a linear fit weighted (1/x) with standards 100 and 500 µM removed.



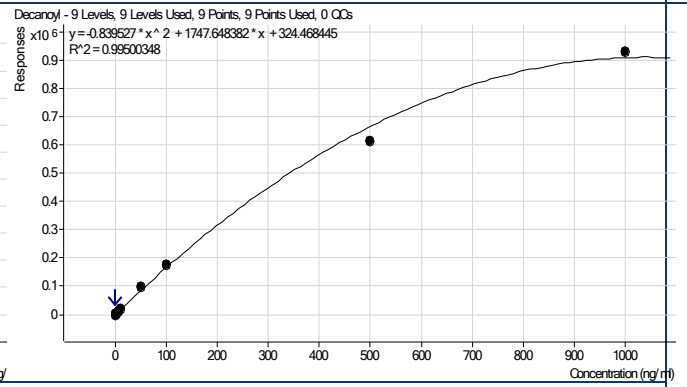
b. Standard curve for Hexanoyl Homoserine Lactone using a linear fit weighted (1/x);



c. Sstandard curve for Heptanoyl Homoserine Lactone using a power curve fit, weighted (1/x);



d. Standard curve for octanoyl using a linear fit, weighted (1/x);



e. Standard curve for Decanoyl Homoserine Lactone using a quadratic fit weighted (1/x).

Table 3.4: Initial quantification results expressed in micromolar (µM)

Sample			β-Ketocaproyl Results	Hexanoyl Results	Heptanoyl Results	Octanoyl Results	Decanoyl Results
Name	Type	Exp. Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.
Standard1	Cal	0.1	0.0000	0.0000	0.0287	0.0000	0.0326
Standard2	Cal	0.5	0.7174	0.5773	0.2210	0.5356	0.5351
Standard3	Cal	1	1.3390	1.5188	0.4767	1.1765	1.0372
Standard4	Cal	5	7.1298	10.3666	4.1723	8.4506	6.2339
Standard5	Cal	10	12.8769	19.4301	9.3205	16.0361	11.8248
Standard6	Cal	50	52.4186	86.5213	60.0758	76.8614	56.8389
Standard7	Cal	100	92.1640	154.7764	122.1592	141.5222	105.9861
Standard8	Cal	500	349.5496	551.5049	536.2855	541.2738	448.4509
Standard9	Cal	1000	589.2719	842.1959	894.7237	880.8895	
Sample_1	Sample		0.2938	0.6715	0.3076	16.5728	**1054.3743**
Sample_11	Sample		0.0000	0.0122	0.2605	12.4294	**808.3177**
Sample_117	Sample		0.0000	0.0000	0.2092	18.3667	**1531.9985**
Sample_53	Sample		0.0000	0.0000	0.2538	0.0000	6.1387
Sample_102	Sample		0.0000	0.0000	0.1671	0.0000	2.4168
Sample_93	Sample		0.0000	0.0000	0.1229	30.4455	**2684.0398**
Sample_54	Sample		0.0000	0.0000	0.0285	28.8595	**1675.7330**

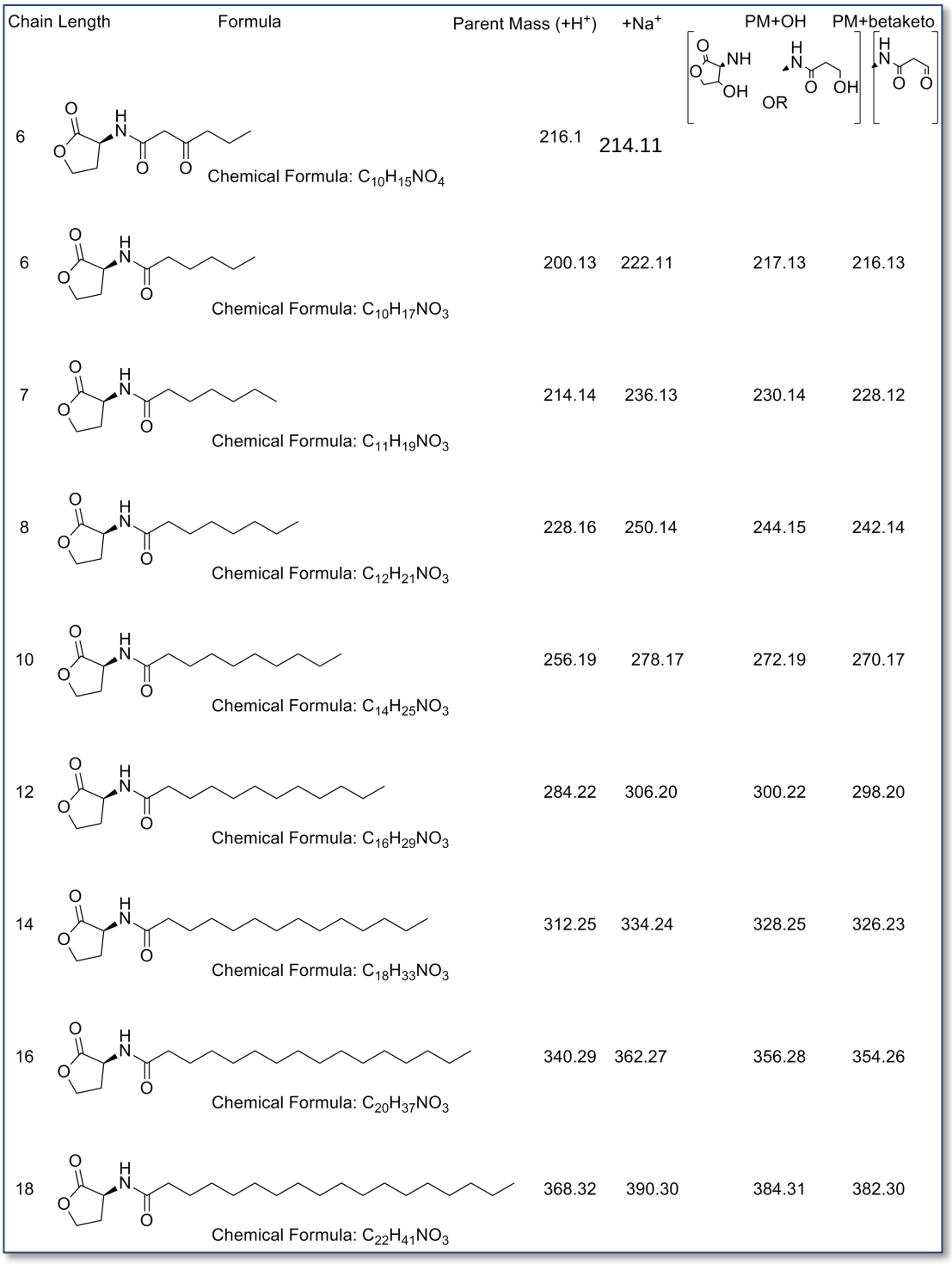
The concentrations of decanoyl homoserine lactone in samples 1, 11, 117, 93 and 54 ranging from 0.8-2.6 mM are highlighted in green.

3.5.2.3 Profiling of AHLs

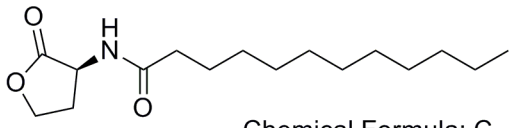
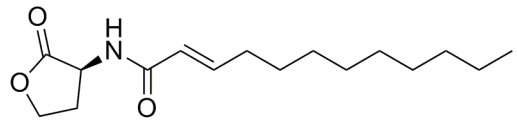
An experimental setup where a blank was included between every sample injection was used to limit the potential of cross-contamination and carry-over between samples. Agilent MassHunter Qualitative Analysis Software B.03.01 was used to mine data generated from precursor ion scanning experiments. An automated Find Compounds by Targetted MS/MS procedure was used to identify compounds present. Spectra were searched in the mass range 100-1000 *m/z* with a peak filter of 5% of largest peak. An ‘excellent’ hit provided all 3 product ions (71.1, 74.1 or 102.1) from the same parent peak within a 0.5 minute time window, a ‘good’ hit was identified by observing at least the presence of two separate product ions and a ‘poor’ hit only a single product ion. Summary of calculated potential AHLs are shown in figure 3.13. Here, the parent mass and the fragment breakdown for all the potential AHLs were detected using the standards and the possible chain lengths of the different AHLs were elucidated.

Figure 3.13: AHL Profiling - Summary of Calculated Potenial AHLs.

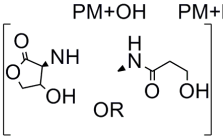
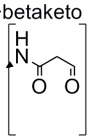
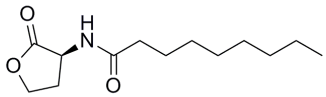
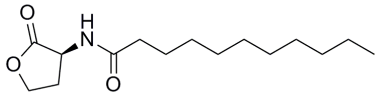
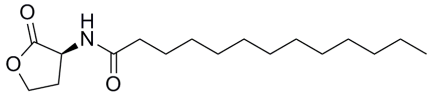
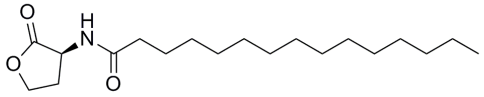
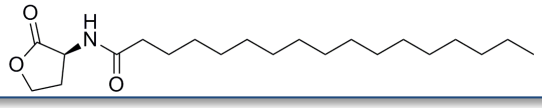
Potential even chain length AHLs and supplied standards:



Potential C12 AHLs:

Chain Length	Formula	Parent Mass ($+H^+$)
12	 Chemical Formula: $C_{16}H_{29}NO_3$	284.22
12	 Chemical Formula: $C_{16}H_{27}NO_3$	282.21

Potential odd chain length AHLs:

Chain Length	Formula	Parent Mass ($+H^+$)	$+Na^+$	PM+OH 	PM+betaketo 
9		242.18	264.16	258.17	256.19
11		270.21	292.19	286.20	284.19
13		298.24	320.22	314.23	312.22
15		326.27	348.25	342.26	340.25
17		354.30	376.28	370.30	368.28

3.5.2.4 Identification of the AHLs

ChemBioDraw Ultra Version 11 was used to generate AHL structures and calculate molecular masses of the protonated and sodiated forms of the unsubstituted straight chain AHL's (C6-C18). β-keto/β-hydroxy derivatives and a C12 with a single alkene were calculated as only the protonated forms.

The samples were re-suspended in 200 µL of solvent. All quantification results have been corrected and are expressed as a concentration (µM) relative to this volume. Concentrations for the standard curve are expressed in µM. To derive actual concentrations extracted from the initial cell culture the final concentrations will require correction to the initial volume and cell mass. A DMRM method was generated to quantify concentrations of beta-ketocaproyl, hexanoyl, heptanoyl, octanoyl and decanoyl AHLs. Good separation and signal was observed for each AHL with longer chain AHLs eluting later. The trace ion current (TIC) of a mixture of standards is shown in figure 3.14 and the TIC of the tested samples are shown in figures 3.15 to 3.21. The selected compounds with observed masses, retention times and observed fragment ions are shown in Appendix 2 (Tables A1.1 to A1.14).

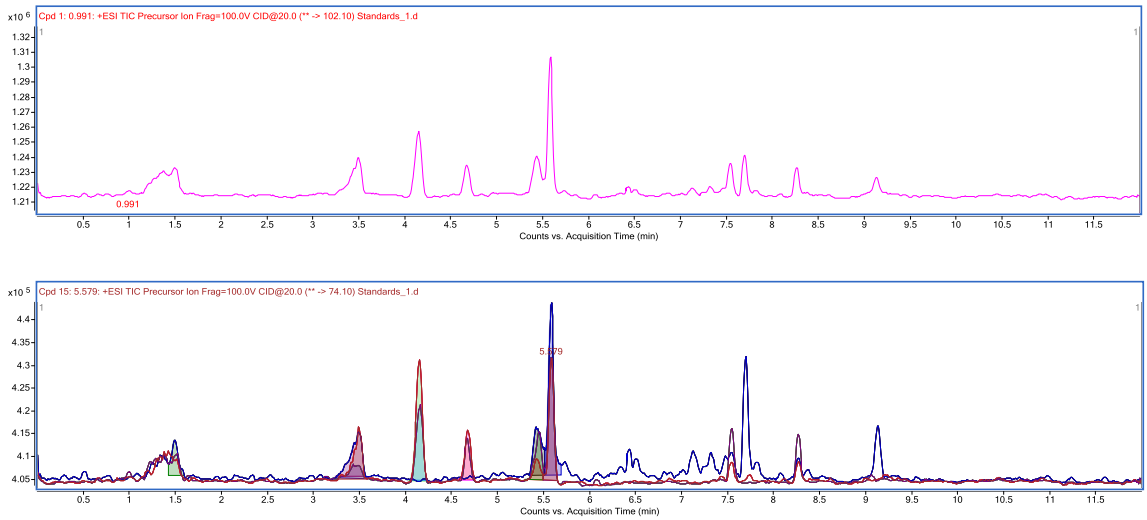
Extraction of AHLs was performed and concentrations of AHLs with beta-ketocaproyl, hexanoyl, heptanoyl, octanoyl and decanoyl side chains were determined. Further identification of AHLs present was conducted by performing MS/MS experiments searching for characteristic AHL fragments. Each sample was analysed using a Precursor Ion method monitoring for the following common fragments: 102.1, 74.1, 71.1. These fragments had been observed to be fairly common when performing optimization studies on the standards stated (β-ketocaproyl, hexanoyl, heptanoyl, octanoyl and decanoyl).

Mass spectrometric analysis confirmed the presence of C_{10} AHLs in the tested 5 isolates (S1, S11, S54, S93 and S117) and 2 isolates (S53, S102) produced AHLs with

chain lengths C_{12} (Figures 3.15 to 3.21). Of the masses that were reported the only ones that seem to make sense are 284 and 282 as an AHL with a 12 carbon acyl chain, with the lower mass (282) containing a single alkene (Tables A1.1-A1.14). An alkene could be anywhere along the chain length, however it can be rationalized that it could be a dehydration product from a beta-hydroxy derivative. The retention time at a later time than the decanoyl standard fits well. These masses were found in samples 53 and 102, which well confirms the TLC report. The TIC trace of each sample (1, 11, 53, 54, 93, 102, 117) with overlaid common precursor ions are shown in Figures 3.15 -3.21.

Results indicated the presence of decanoyl homoserine lactone in the isolates S1, S11, S54, S93 and S117. Other AHL's with higher masses (longer chain) than the provided standard were seen in S53 and S102 with C12 (Dodecanoyl homoserine lactone).

Figure 3.14: TIC of a mixture of standards



Individual standards are highlighted; later eluting peaks correspond to other contaminants.

Figure 3.15a: TIC trace of Sample 1

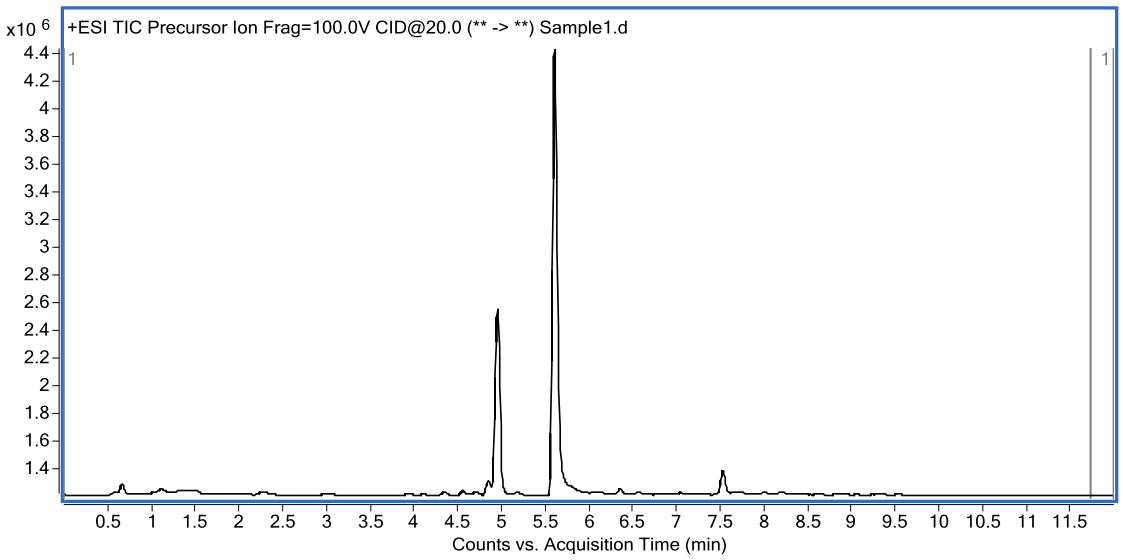


Figure 3.15b: Combined overlay of TIC and Extracted compounds of sample 1

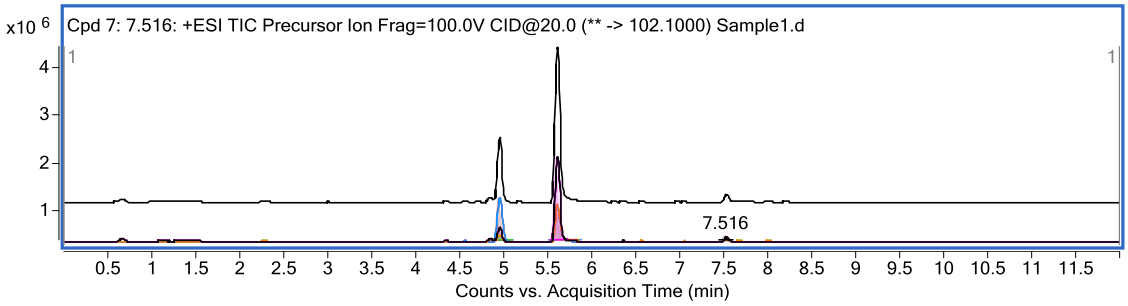


Figure 3.15c: Magnified view of extracted compounds in sample 1

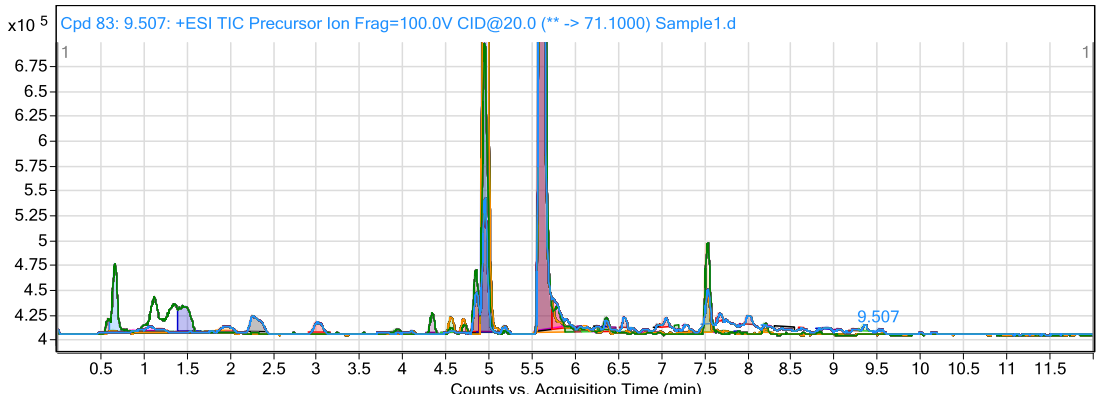


Figure 3.15d: Overlaid spectra of all common precursor Ions common in sample 1 providing fragment Ions (71.1, 74.1, and 102.1):

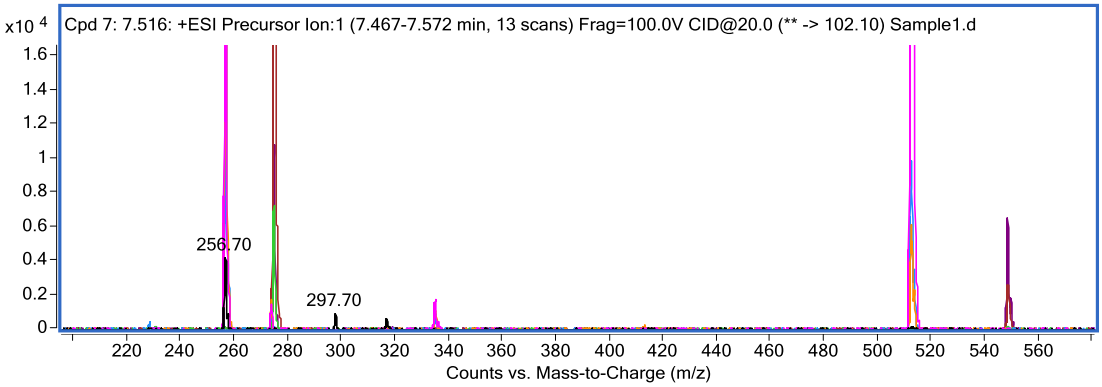


Figure 3.16a: TIC trace of Sample 11

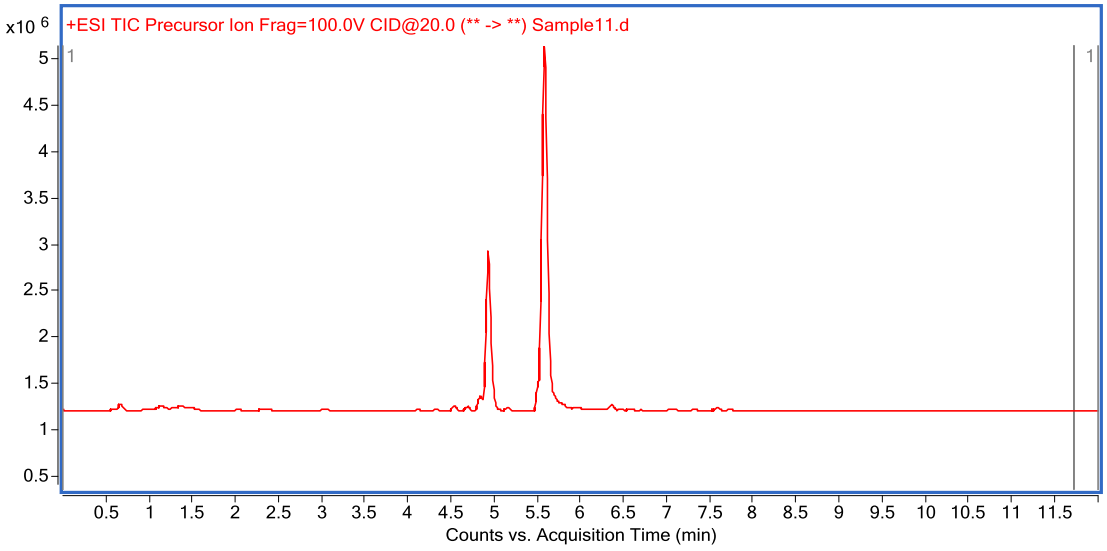


Figure 3.16b: Combined overlay of TIC and extracted compounds of sample 11

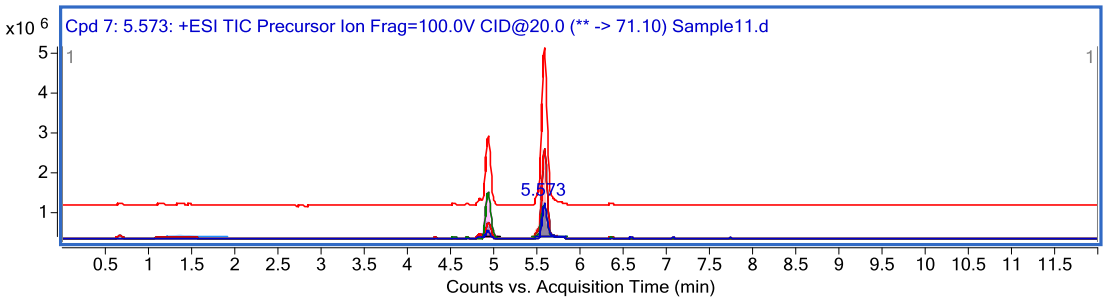


Figure 3.16c: Magnified view of extracted compounds in sample 11

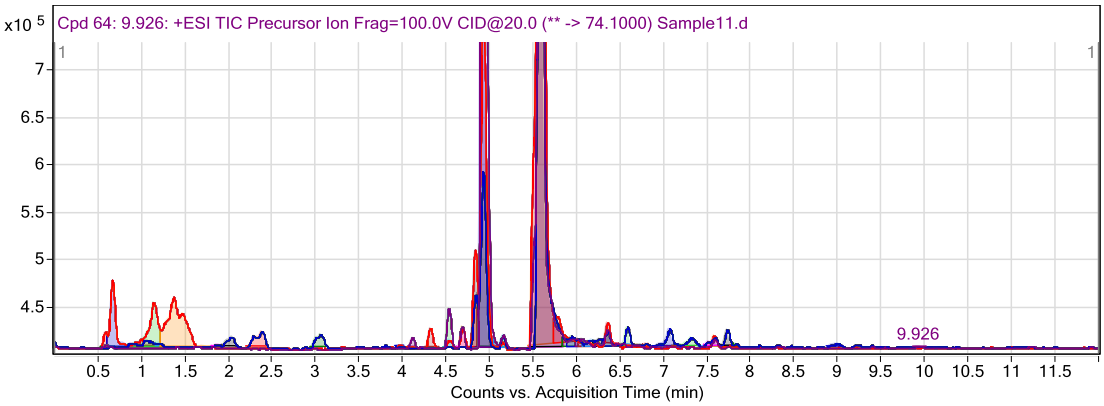


Figure 3.16d: Overlaid spectra of all common precursor Ions common in sample 11 providing fragment Ions (71.1, 74.1, and 102.1):

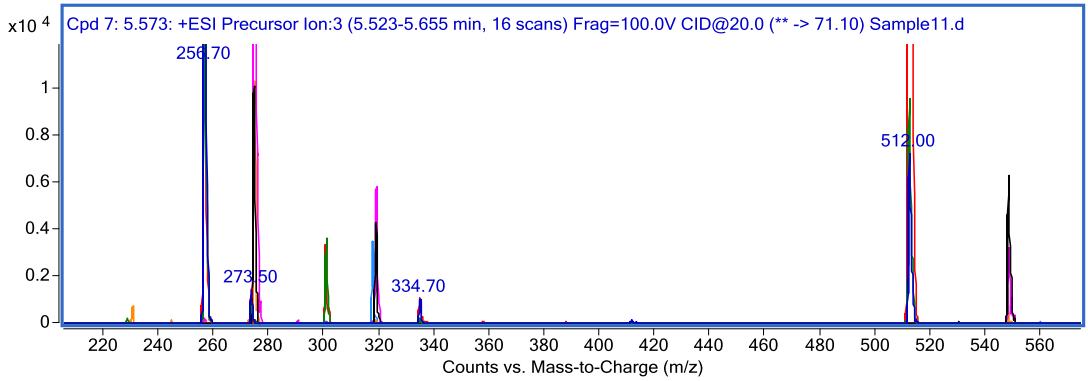


Figure 3.17a: TIC trace of Sample 53

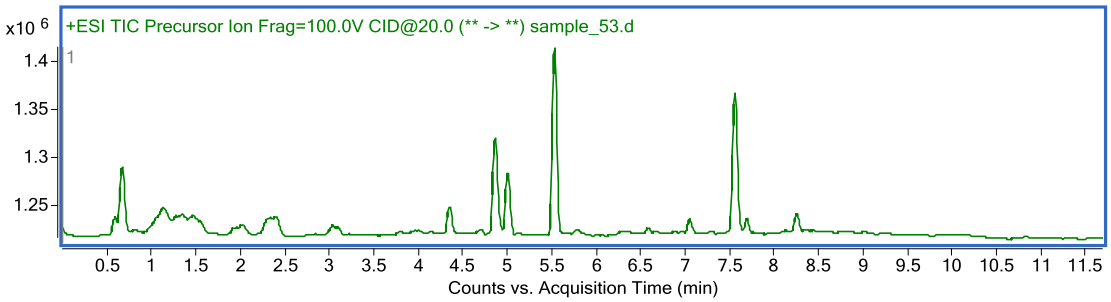


Figure 3.17b: Combined overlay of TIC and Extracted compounds of sample 53

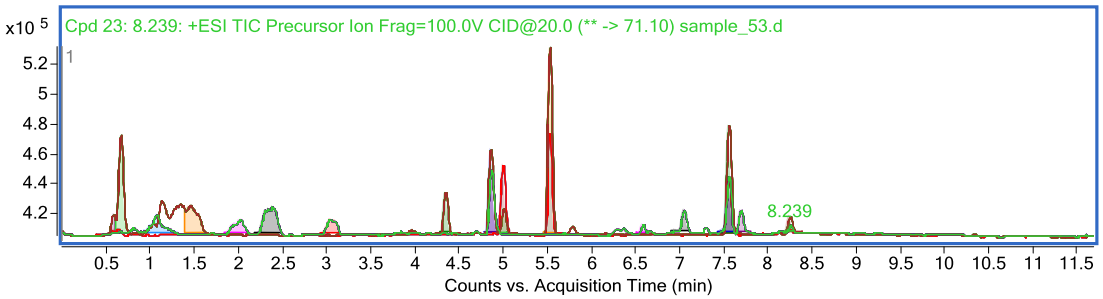


Figure 3.17c: Overlaid spectra of all common precursor Ions common in sample 53 providing fragment Ions (71.1, 74.1, and 102.1):

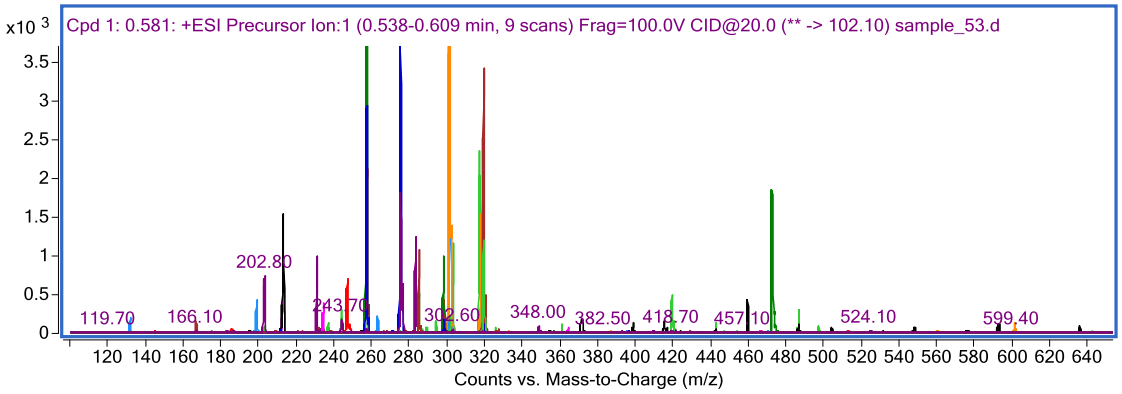


Figure 3.18a: TIC trace of Sample 54

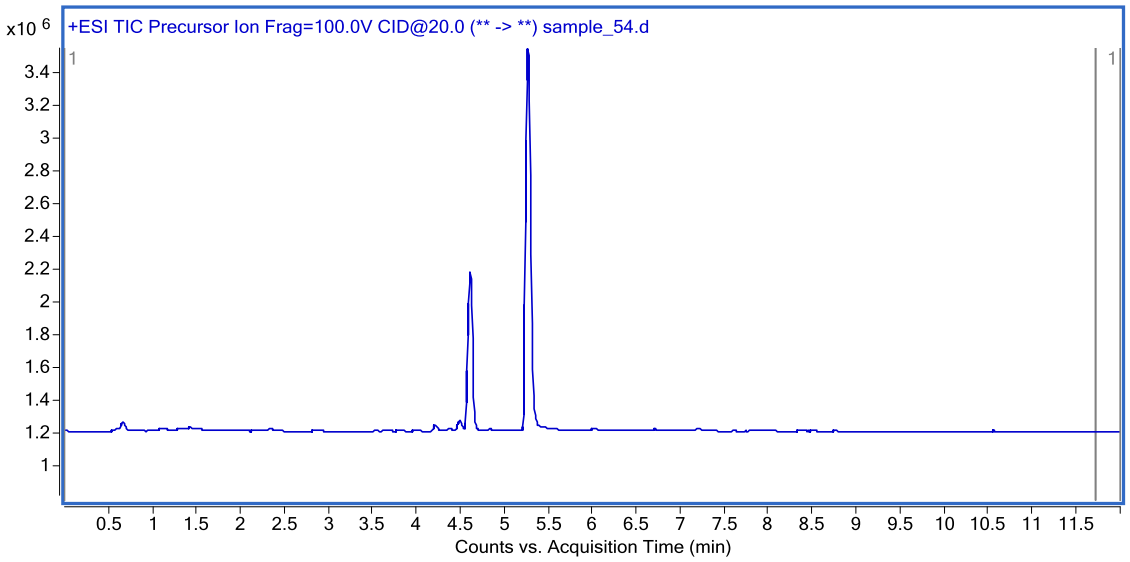


Figure 3.18b: Combined overlay of TIC and Extracted compounds of sample 54

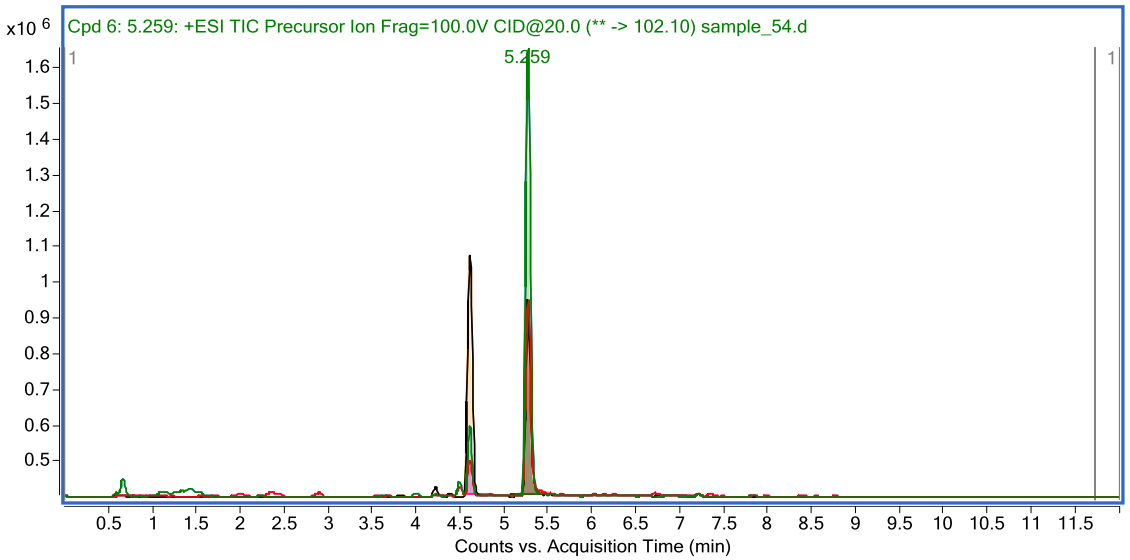


Figure3.18c: Magnified view of extracted compounds in sample 54.

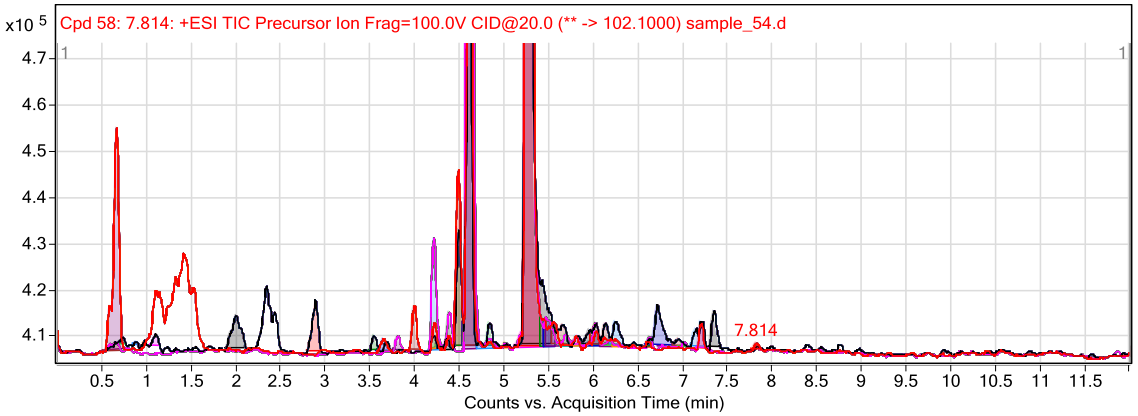


Figure 3.19a: TIC trace of Sample 93

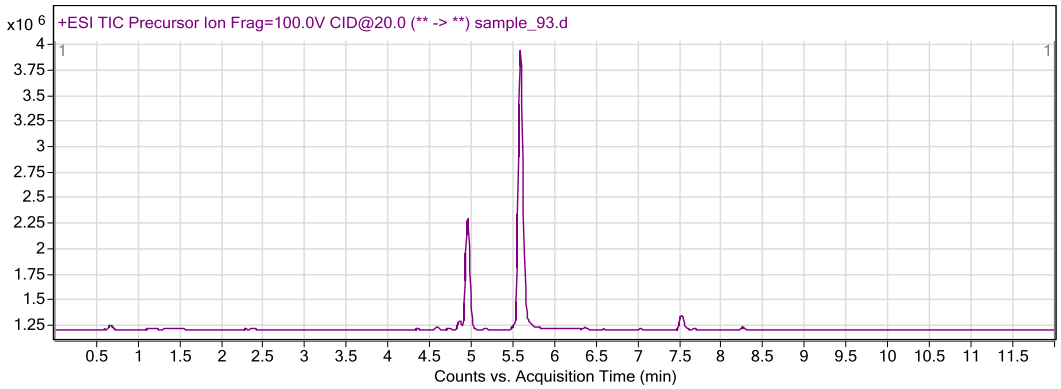


Figure 3.19b: Magnified view of extracted compounds in sample 93.

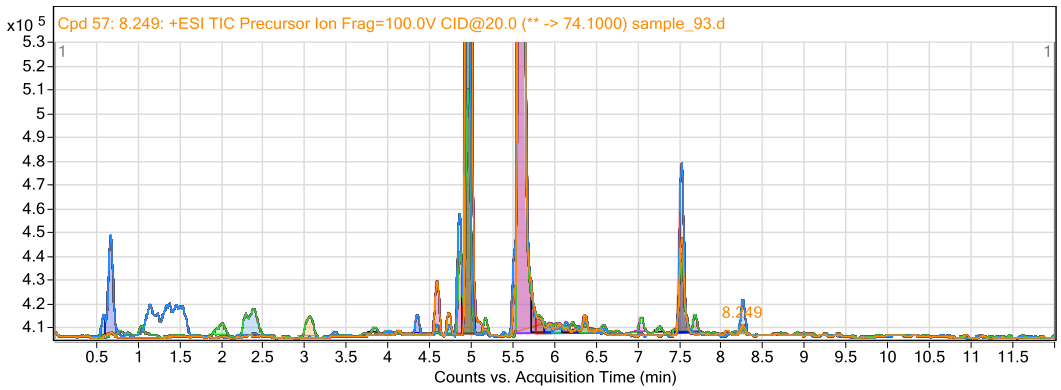


Figure 3.20a: TIC trace of Sample 102

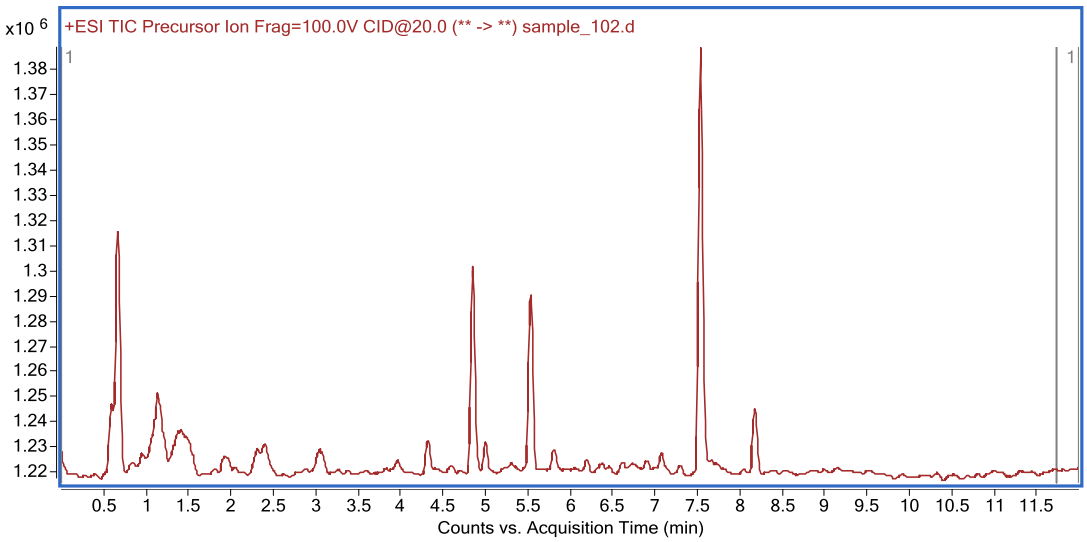


Figure 3.20b: Combined overlay of TIC and Extracted compounds of sample 102

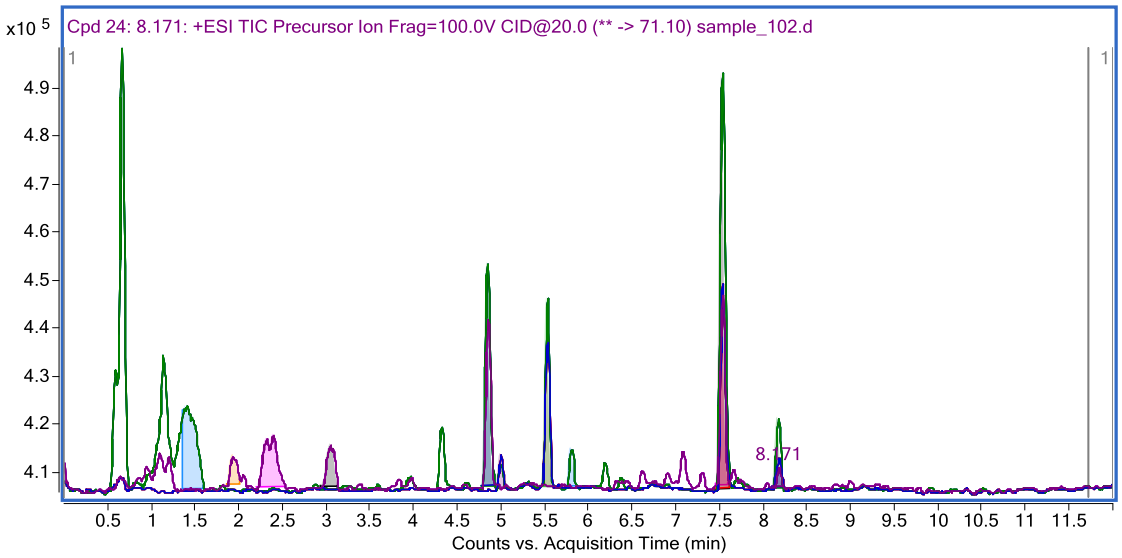


Figure 3.21a: TIC trace of Sample 117

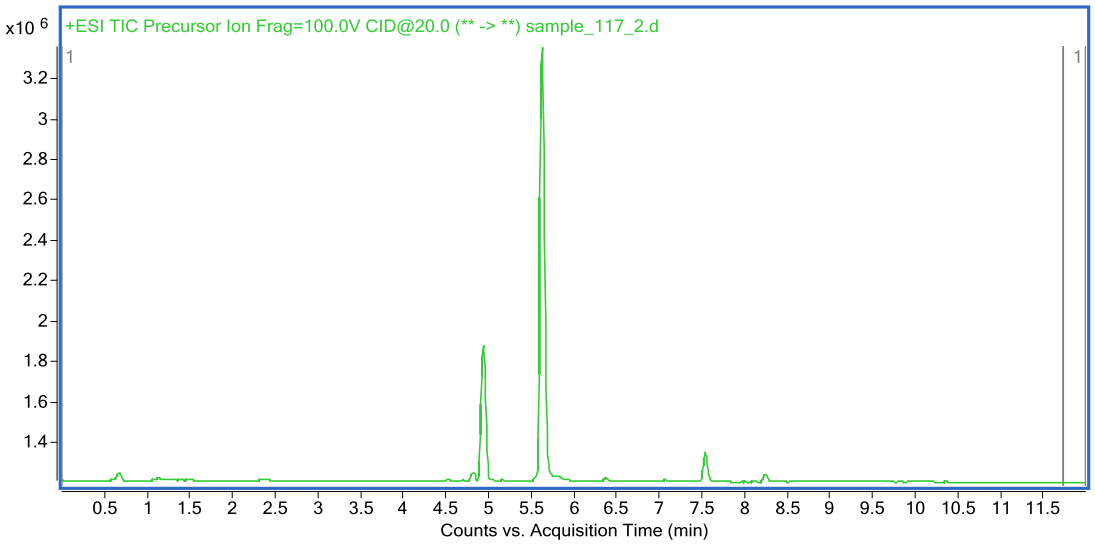


Figure 3.21b: Combined overlay of TIC and Extracted compounds of sample 117

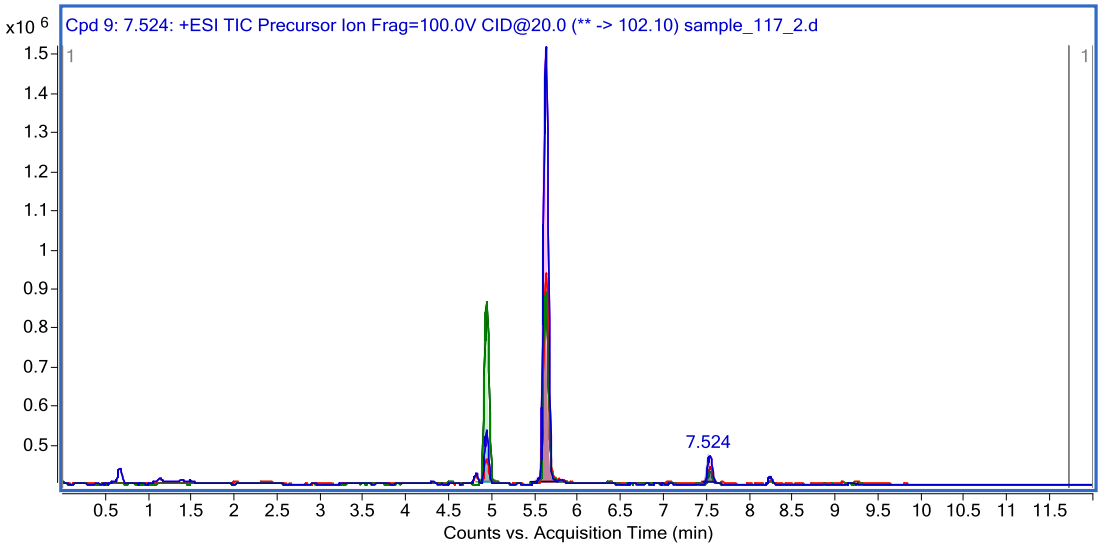
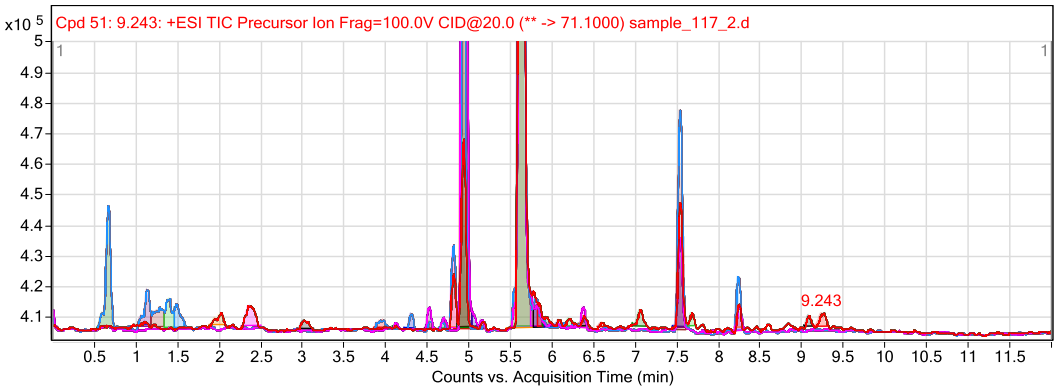


Figure 3.21c: Magnified view of extracted compounds in sample 117

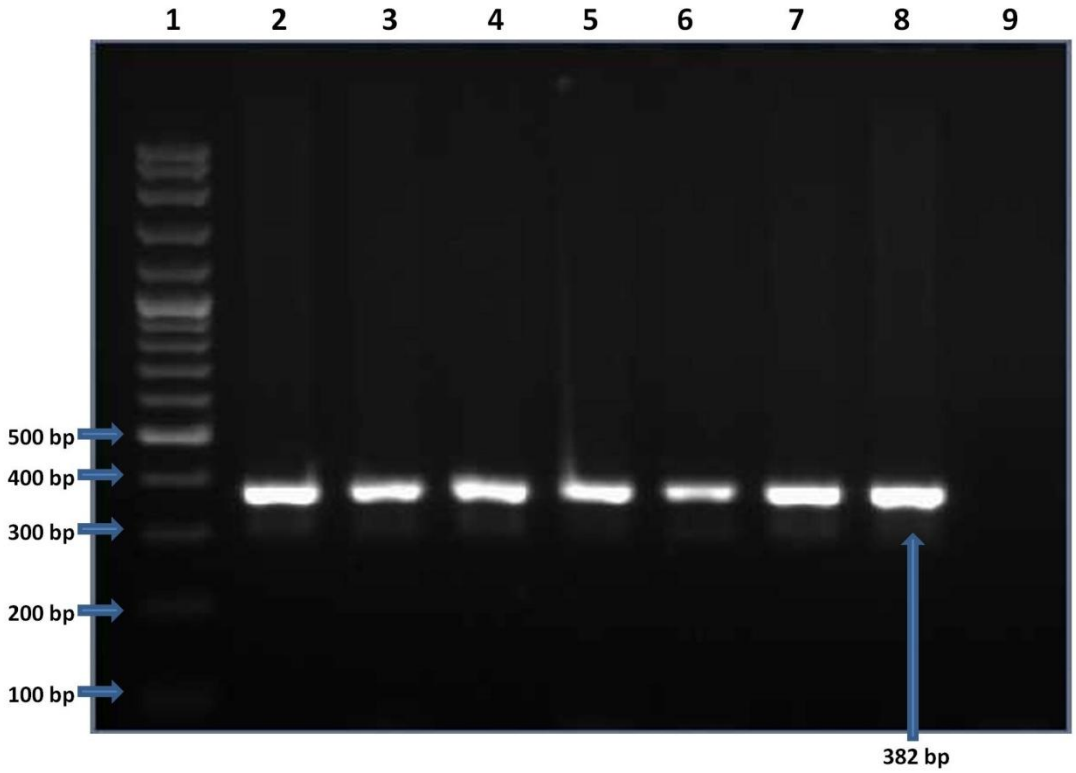


3.6 Identification of the QS gene in *Acinetobacter* spp. and construction of its mutant

3.6.1 Identification of *abaI* gene in *Acinetobacter* spp. producing the QS signal molecules

Polymerase Chain Reaction (PCR) for *abaI* gene, produced amplicons of 382 bp in all the 7 isolates (isolate no.S1, S11, S53, S54, S93, S102, S117) which produced the QS signal molecules. The electrophoretic banding patterns are shown in figure 3.22. PCR for *abaI* gene was carried out in all the 50 test isolates and the *abaI* gene product was also detected among 31 isolates which were negative for the AHL molecules. It is significant that all the seven isolates positive for the long chain AHL molecules showed the presence of the *abaI* gene. This *abaI* gene present in 31 of the other isolates that were negative for AHL molecules may not play a significant role in the AHL-mediated pathways.

Figure 3.22: The electrophoretic banding patterns of the PCR products using the primers for the *abaI* gene



Lane 1 – 100bp DNA ladder
Lane 2, 3, 4, 5, 6, 7, 8 – amplicons from isolates S1, S11, S53, S54, S93, S102 and S117 respectively
Lane 9 – Negative control.

3.6.2 Sequencing of the *abaI* gene of the test isolates

Sequencing of the detected *abaI* gene was done for all the seven isolates and softwares were used to compare and calculate the differences in the aminoacid sequences of the *abaI* gene. When compared to the sequence of the already reported AbaI protein (Niu *et al.,* 2008), eight amino acid changes have been reported in AbaI protein of S117. Among the seven isolates, isolate S117 thus showed significant differences when compared to the already reported *abaI* gene. The amplicon of the *abaI* gene sequence from isolate S117 was further analysed. The BLAST analysis program revealed that the *abaI* gene identified in *Acinetobacter* spp. isolate S117 is 92% identical to the corresponding protein reported in recently published *A. baumannii* ATCC 17978 genome. The AbaI protein shared 91% identity and 97% similarity with the already reported AbaI protein of *A. baumannii* M2 strain. Further, the BLAST analysis also revealed that the AbaI protein exhibited 61% identity and 79% similarity to the acyl homoserine lactone synthase of *Halothiobacillus neapolitanus* c2, 65% identity and 75% similarity to the autoinducer synthesis protein of *Acidithiobacillus ferrooxidans* ATCC 53993, 47% identity and 67% similarity with autoinducer synthatase family protein of *Burkholderia mallei* ATCC 23344 and 49% identity and 63% similarity with *N*-acyl homoserine lactone synthase from *Pseudomonas fluorescens* (Table 3.5). The sequence analysis results of the seven isolates are shown in Figure 3.23 to Figure 3.29. The *abaI* gene sequence of *Acinetobacter* spp. isolate S117 has been submitted to the NCBI Genbank (Accession number HQ013310). This is shown in figure 3.30.

Table 3.5: Homology percentages of the *abaI* region in *Acinetobacter* spp. (S117)

Identity	Similarity	Protein / Organism
92%		*A. baumannii* ATCC 17978 genome
91%	97%	AbaI protein of *A. baumannii* M2 strain
61%	79%	AHL synthase of *Halothiobacillus neapolitanus* c2
65%	75%	Autoinducer synthesis protein of *Acidithiobacillus ferrooxidans* ATCC 53993
47%	67%	Autoinducer synthatase family protein of *Burkholderia mallei* ATCC 23344
49%	63%	*N*-acyl homoserine lactone synthase from *Pseudomonas fluorescens*

Figure 3.23: Sequence analysis of *abaI* gene from isolate S1

Contig Sequence:

```
NNNGGNNGTTGN

CTGTCCAAACAATGAAGAACTAGATCAATTTGATAAAGTCGATACAGCTTATGTCGTGGCTCAAGACAGAG
AATCTAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACCTTATTTACTCGGGGAAATATTTCCC
CAACTTCTCAATGGAATGCCTATTCCCTGCTCACCAGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGA
TTTTTCAAATCCACCTTCCTCTAGCAGTCAGGCTGTGTCATCACCGGTCTCAATTGCAATTCTGCAAGAA

GCAATCAACTTTGCAAGAGAACAAGGCGCAAAACAACTCATTACTCTAGACGTACG
```

Blast Analysis (Nucleotide):

```
Score =  464 bits (514),Identities = 276/286 (96%), Gaps = 3/286 (1%)

Query  1    CTGTCCAAACAATGAAGAA-CTAGAT--CAATTTGATAAAGTCGATACAGCTTATGTCGT
57
            |||||||||||||||||| |||||   ||||||||||||||||||||||||||||||||
Sbjct  108  CTGTCCAAACAATGAAGAAACTAGAATCCAATTTGATAAAGTCGATACAGCTTATGTCGT
167

Query  58   GGCTCAAGACAGAGAATCTAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACC
117
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  168  GGCTCAAGACAGAGAATCCAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACC
227

Query  118  TTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAATGCCTATTCCCTGCTCACC
177
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  CTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAATGCCTATTCCCTGCTCACC
287
            |||||||||||||||||||||||||||||||||||||||||||| || ||||||||||||
Sbjct  288  AGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAAACCGCCTTCCTCTAG
347

Query  238  CAGTCAGGCTGTGTCATCACCGGTCTCAATTGCAATTCTGCAAGAA  283
            ||||||||||||||||||||  ||||||||||||||||||||||||
Sbjct  348  CAGTCAGGCTGTGTCATCACCTATCTCAATTGCAATTCTGCAAGAA  393
```

Protein Analysis:

```
Score =  157 bits (398),Identities = 90/95 (94%), Positives = 91/95 (95%),
Gaps = 1/95 (1%)

Query  1    CPNNEELD-QFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
59
            CPNNEE   QFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
Sbjct  37   CPNNEETRIQFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
96

Query  60   EIWELSRFSAVDFSNPPSSSSQAVSSPVSIAILQE  94
            EIWELSRFSAVDFS PPSSSSQAVSSP+SIAILQE
Sbjct  97   EIWELSRFSAVDFSKPPSSSSQAVSSPISIAILQE  131

5'3' Frame 2
CPNNEELDQFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPI
PCSPEIWELSRFSAVDFSNPPSSSSQAVSSPVSIAILQE

44 R replaced by L, 45 I replaced by D, 111 K replaced by N, 124 I
replaced by V.
```

Figure 3.24: Sequence analysis of *abaI* gene from isolate S11

Contig Sequence:

```
NNNGGNNGTTGN

CTGTCCAAACAATGAAGAACTAGATCAATTTGATAAAGTCGATACAGCTTATGTCGTGGCTCAAGACAGAG
AATCTAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACCTTATTTACTCGGGGAAATATTTCCC
CAACTTCTCAATGGAATGCCTATTCCCTGCTCACCAGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGA
TTTTTCAAATCCACCTTCCTCTAGCAGTCAGGCTGTGTCATCACCGGTCTCAATTGCAATTCTGCAAGAA

GCAATCAACTTTGCAAGAGAACAAGGCGCAAAACAACTCATTACTCTAGACGTACG
```

Blast Analysis (Nucleotide):

```
Score =  464 bits (514),Identities = 276/286 (96%), Gaps = 3/286 (1%)

Query  1    CTGTCCAAACAATGAAGAA-CTAGAT--CAATTTGATAAAGTCGATACAGCTTATGTCGT
57
            |||||||||||||||||| |||||   ||||||||||||||||||||||||||||||||
Sbjct  108  CTGTCCAAACAATGAAGAAACTAGAATCCAATTTGATAAAGTCGATACAGCTTATGTCGT
167

Query  58   GGCTCAAGACAGAGAATCTAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACC
117
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  168  GGCTCAAGACAGAGAATCCAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACC
227

Query  118  TTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAATGCCTATTCCCTGCTCACC
177
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  CTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAATGCCTATTCCCTGCTCACC
287

Query  178  AGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAATCCACCTTCCTCTAG
237
            |||||||||||||||||||||||||||||||||||||||||| || ||||||||||||
Sbjct  288  AGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAAACCGCCTTCCTCTAG
347

Query  238  CAGTCAGGCTGTGTCATCACCGGTCTCAATTGCAATTCTGCAAGAA  283
            ||||||||||||||||||||  ||||||||||||||||||||||||
Sbjct  348  CAGTCAGGCTGTGTCATCACCTATCTCAATTGCAATTCTGCAAGAA  393
```

Protein Analysis:

```
Score =  157 bits (398),Identities = 90/95 (94%), Positives = 91/95 (95%),
Gaps = 1/95 (1%)

Query  1    CPNNEELD-QFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
59
            CPNNEE   QFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
Sbjct  37   CPNNEETRIQFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
96

Query  60   EIWELSRFSAVDFSNPPSSSSQAVSSPVSIAILQE  94
            EIWELSRFSAVDFS PPSSSSQAVSSP+SIAILQE
Sbjct  97   EIWELSRFSAVDFSKPPSSSSQAVSSPISIAILQE  131
```

5'3' Frame 2

```
CPNNEELDQFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPI
PCSPEIWELSRFSAVDFSNPPSSSSQAVSSPVSIAILQE

44 R replaced by L, 45 I replaced by D,111 K replaced by N, 124 I
replaced by V.
```

Figure 3.25: Sequence analysis of *abaI* gene from isolate S53

Contig Sequence:

```
NNNNNGNNNNNN

CTGTCCAAACAATGAAGAACTAGATCAATTTGATAAAGTCGATACAGCTTATGTCGTGGCTCAAGACAGAGAATC
TAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACCTTATTTACTCGGGGAAATATTTCCCCAACTTCT
CAATGGAATGCCTATTCCCTGCTCACCAGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAATCC
ACCTTCCTCTAGCAGTCAGGCTGTGTCATCACCGGTCTCAATTGCAATTCTGCAAGAAGCAATCAACTTTGCAA

GAGAACAAGGCGCAAAACAACTCATTACTCTAGACGTACGA
```

Blast Analysis (Nucleotide):

```
Score =  484 bits (536),Identities = 290/302 (96%), Gaps = 3/302 (0%)

Query  1    CTGTCCAAACAATGAAGAA-CTAGAT--CAATTTGATAAAGTCGATACAGCTTATGTCGT
57
            |||||||||||||||||| |||||   ||||||||||||||||||||||||||||||||
Sbjct  108  CTGTCCAAACAATGAAGAAACTAGAATCCAATTTGATAAAGTCGATACAGCTTATGTCGT
167

Query  58   GGCTCAAGACAGAGAATCTAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACC
117
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  168  GGCTCAAGACAGAGAATCCAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACC
227

Query  118  TTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAATGCCTATTCCCTGCTCACC
177
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  CTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAATGCCTATTCCCTGCTCACC
287

Query  178  AGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAATCCACCTTCCTCTAG
237
            |||||||||||||||||||||||||||||||||||||||||||| || ||||||||||||
Sbjct  288  AGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAAACCGCCTTCCTCTAG
347

Query  238  CAGTCAGGCTGTGTCATCACCGGTCTCAATTGCAATTCTGCAAGAAGCAATCAACTTTGC
297
            ||||||||||||||||||||  |||||||||||||||||||||||||| || |||||
Sbjct  348  CAGTCAGGCTGTGTCATCACCTATCTCAATTGCAATTCTGCAAGAAGCAATTAATTTTGC
407

Query  298  AA  299
            ||
Sbjct  408  AA  409
```

Protein Analysis:

```
Score =  167 bits (423), Identities = 95/100 (95%), Positives = 96/100
(96%), Gaps = 1/100 (1%)

Query  1    CPNNEELD-QFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
59
            CPNNEE   QFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
Sbjct  37   CPNNEETRIQFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
96

Query  60   EIWELSRFSAVDFSNPPSSSSQAVSSPVSIAILQEAINFA  99
            EIWELSRFSAVDFS PPSSSSQAVSSP+SIAILQEAINFA
Sbjct  97   EIWELSRFSAVDFSKPPSSSSQAVSSPISIAILQEAINFA  136


5'3' Frame 2
CPNNEELDQFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSPEIWELSRFSAVDFSNP
PSSSSQAVSSPVSIAILQEAINFA

44 R replaced by L, 45 I replaced by D,111 K replaced by N, 124 I replaced
by V.
```

Figure 3.26: Sequence analysis of *abaI* gene from isolate S54

Contig Sequence:
NNNNNGNNNNNN

```
CTGTCCAAACAATGAAGAACTAGATCAATTTGATAAAGTCGATACAGCTTATGTCGTGGCTCAAGACAGAGAATC
TAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACCTTATTTACTCGGGGAAATATTTCCCCAACTTCT
CAATGGAATGCCTATTCCCTGCTCACCAGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAATCC
ACCTTCCTCTAGCAGTCAGGCTGTGTCATCACCGGTCTCAATTGCAATTCTGCAAGAAGCAATCAACTTTGCAA

GAGAACAAGGCGCAAAACAACTCATTACTCTAGACGTACGA
```

Blast Analysis (Nucleotide):

```
Score =  484 bits (536), Identities = 290/302 (96%), Gaps = 3/302 (0%)

Query  1    CTGTCCAAACAATGAAGAA-CTAGAT--CAATTTGATAAAGTCGATACAGCTTATGTCGT
57
            |||||||||||||||||| |||||   ||||||||||||||||||||||||||||||||
Sbjct  108  CTGTCCAAACAATGAAGAAACTAGAATCCAATTTGATAAAGTCGATACAGCTTATGTCGT
167

Query  58   GGCTCAAGACAGAGAATCTAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACC
117
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  168  GGCTCAAGACAGAGAATCCAATATCATTGGTTGTGCCAGACTACTACCCACCACACAACC
227

Query  118  TTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAATGCCTATTCCCTGCTCACC
177
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  CTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAATGCCTATTCCCTGCTCACC
287

Query  178  AGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAATCCACCTTCCTCTAG
237
            |||||||||||||||||||||||||||||||||||||||||||| || ||||||||||||
Sbjct  288  AGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAAACCGCCTTCCTCTAG
347

Query  238  CAGTCAGGCTGTGTCATCACCGGTCTCAATTGCAATTCTGCAAGAAGCAATCAACTTTGC
297
            ||||||||||||||||||  |||||||||||||||||||||||||| || |||||
Sbjct  348  CAGTCAGGCTGTGTCATCACCTATCTCAATTGCAATTCTGCAAGAAGCAATTAATTTTGC
407

Query  298  AA  299
            ||
Sbjct  408  AA  409
```

Protein Analysis:

```
Score =  167 bits (423), Identities = 95/100 (95%), Positives = 96/100
(96%), Gaps = 1/100 (1%)

Query  1    CPNNEELD-QFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
59
            CPNNEE   QFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
Sbjct  37   CPNNEETRIQFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSP
96

Query  60   EIWELSRFSAVDFSNPPSSSSQAVSSPVSIAILQEAINFA  99
            EIWELSRFSAVDFS PPSSSSQAVSSP+SIAILQEAINFA
Sbjct  97   EIWELSRFSAVDFSKPPSSSSQAVSSPISIAILQEAINFA  136

5'3' Frame2
CPNNEELDQFDKVDTAYVVAQDRESNIIGCARLLPTTQPYLLGEIFPQLLNGMPIPCSPEIWELSRFSAVDFSNP
PSSSSQAVSSPVSIAILQEAINFA

44 R replaced by L, 45 I replaced by D,111 K replaced by N, 124 I replaced
by V.
```

Figure 3.27: Sequence analysis of *abaI* gene from isolate S93

Contig Sequence:

```
CNNNNNGNNGNNCGNCNCAACCAATGNNGATCTANNTCANTTTCGATAAGCTCGATACAGCTTAGGTCGT
GTCTCAAGACTGAGAATCCAATATCANTGGTTGTGCCGTACTACTACCCACCAAAATATCTAATATACTCG
TCGAANN

ATTTCCCCAACTTCTCAATGGAATGCCTATCCCCTGCTCACCAGAAATTTGGGAATTATCAAGGTTTTCAG
CCGTAGATTTTTCAAATCCGCCTTCCTCTAACAGTCAGGCTGTGTCATCACCTGTCTCAATTGCAATTTTG
CAAGAAG

CAATCAACTTTGCAAGAGAACAAGGCGCAAAACAACTCATTACTCTAGACGTACGA
```

Blast Analysis (Nucleotide):

```
Score =  246 bits (272), Identities = 144/149 (96%), Gaps = 0/149 (0%)

Query  1    ATTTCCCCAACTTCTCAATGGAATGCCTATCCCCTGCTCACCAGAAATTTGGGAATTATC
60
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  246  ATTTCCCCAACTTCTCAATGGAATGCCTATTCCCTGCTCACCAGAAATTTGGGAATTATC
305

Query  61   AAGGTTTTCAGCCGTAGATTTTTCAAATCCGCCTTCCTCTAACAGTCAGGCTGTGTCATC
120
            |||||||||||||||||||||||||| |||||||||||| ||||||||||||||||||
Sbjct  306  AAGGTTTTCAGCCGTAGATTTTTCAAAACCGCCTTCCTCTAGCAGTCAGGCTGTGTCATC
365

Query  121  ACCTGTCTCAATTGCAATTTTGCAAGAAG  149
            |||| |||||||||||||| |||||||||
Sbjct  366  ACCTATCTCAATTGCAATTCTGCAAGAAG  394
```

Protein Analysis:

```
Score = 73.2 bits (178), Identities = 46/49 (93%), Positives = 48/49 (97%),
Gaps = 0/49 (0%)

Query  1    FPQLLNGMPIPCSPEIWELSRFSAVDFSNPPSSNSQAVSSPVSIAILQE  49
            FPQLLNGMPIPCSPEIWELSRFSAVDFS PPSS+SQAVSSP+SIAILQE
Sbjct  83   FPQLLNGMPIPCSPEIWELSRFSAVDFSKPPSSSSQAVSSPISIAILQE  131
```

5'3' Frame 2

```
FPQLLNGMPIPCSPEIWELSRFSAVDFSNPPSSNSQAVSSPVSIAILQE

111 K replaced by N, 116 S replaced by N,124 I replaced by V
```

Figure 3.28: Sequence analysis of *abaI* gene from isolate S102

Contig Sequence:

```
NNNNNNNNNTNNCTGTCCAAACAATGAAGAACTAGATCAATTTGATAAAGTCGATACAGCTT
ATGTCGTGGCTCAAGACAGAGAATCCAATATCATTGGTTGTGCCAGAC


TACTACCCACCACACAACCTTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAATGCCTATCCCC
TGCTCACCAGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTTCAAATCCGCCTTCCTCTAACAG
TCAGGCTGTGTCATCACCTGTCTCAATTGCAATTTTGCAAGAA

GCAATCAACTTTGCAAGAGAACAAGGCGCAAAACAACTCATTACTCTAGACGTACGA
```

Blast Analysis (Nucleotide):

```
Score =  307 bits (340),  Identities = 179/185 (96%), Gaps = 0/185 (0%)

Query  1    TACTACCCACCACACAACCTTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAA
60
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  209  TACTACCCACCACACAACCCTATTTACTCGGGGAAATATTTCCCCAACTTCTCAATGGAA
268

Query  61   TGCCTATCCCCTGCTCACCAGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTT
120
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269  TGCCTATTCCCTGCTCACCAGAAATTTGGGAATTATCAAGGTTTTCAGCCGTAGATTTTT
328

Query  121  CAAATCCGCCTTCCTCTAACAGTCAGGCTGTGTCATCACCTGTCTCAATTGCAATTTTGC
180
            |||| |||||||||||| |||||||||||||||||||| |||||||||||||| |||
Sbjct  329  CAAAACCGCCTTCCTCTAGCAGTCAGGCTGTGTCATCACCTATCTCAATTGCAATTCTGC
388

Query  181  AAGAA  185
            |||||
Sbjct  389  AAGAA  393
```

Protein Analysis:

```
Score = 97.4 bits (241), Identities = 58/61 (95%), Positives = 60/61 (98%),
Gaps = 0/61 (0%)

Query  1    LPTTQPYLLGEIFPQLLNGMPIPCSPEIWELSRFSAVDFSNPPSSNSQAVSSPVSIAILQ
60
            LPTTQPYLLGEIFPQLLNGMPIPCSPEIWELSRFSAVDFS PPSS+SQAVSSP+SIAILQ
Sbjct  71   LPTTQPYLLGEIFPQLLNGMPIPCSPEIWELSRFSAVDFSKPPSSSSQAVSSPISIAILQ
130
```

5'3' Frame 3

```
LPTTQPYLLGEIFPQLLNGMPIPCSPEIWELSRFSAVDFSNPPSSNSQAVSSPVS
IAILQE

111 K replaced by N, 116 S replaced by N, 124 I replaced by V
```

Figure 3.29: Sequence analysis of *abaI* gene from isolate S117

Contig Sequence:

```
NNNNNNNNNTGNCNGNNCANACAATGAAGAACTG

AGATCAATTTGATAAAGTCGACACAGCGTATGTCGTTGCTCAAGATAGAGAATCTAACATTATTGGCTGCG
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5'3' Frame 2

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45- I replaced by D, 74- T by I, 87 L by M, 111 K by N, 114 s by T, 116
S by A, 117 S by N, 124 I by V
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Figure 3.30: NCBI Genbank submission of *abaI* gene (Accession number HQ013310)

Acinetobacter sp. S117 *N*-acyl homoserine lactone synthase (*abaI*) gene, partial cds

GenBank: HQ013310.1
FASTA Graphics

Features Sequence

```
LOCUS       HQ013310                264 bp    DNA     linear   BCT 28-AUG-2010
DEFINITION  Acinetobacter sp. S117 N-acyl homoserine lactone synthase (abaI)
            gene, partial cds.
ACCESSION   HQ013310
VERSION     HQ013310.1  GI:304366254
KEYWORDS    .
SOURCE      Acinetobacter sp. S117
  ORGANISM  Acinetobacter sp. S117
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Moraxellaceae; Acinetobacter.
REFERENCE   1  (bases 1 to 264)
  AUTHORS   Anbazhagan,D., Mansor,M., Gracie Ong,S.Y., Yusof,M.Y. and
            Sekaran,S.D.
  TITLE     Identification of an autoinducer synthase gene among biofilm
            forming clinical isolates of Acinetobacter spp. isolated from a
            medical centre in Malaysia
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 264)
  AUTHORS   Anbazhagan,D., Mansor,M., Gracie Ong,S.Y., Yusof,M.Y. and
            Sekaran,S.D.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-AUG-2010) Department of Medical Microbiology, Faculty
            of Medicine, University of Malaya, Petaling Jaya, Kuala Lumpur, KL
            50603, Malaysia
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                     /transl_table=11
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      181 ttcagccgta gatttctcga acccgccaac ctctgctaat caagcagtgt catccccagt
      241 ctctattgct attctgcaag aagc
//
```

3.6.3 Cloning of the *abaI* gene (blue-white selection) and construction of the mutant of *abaI* gene (Tetracycline mutant)

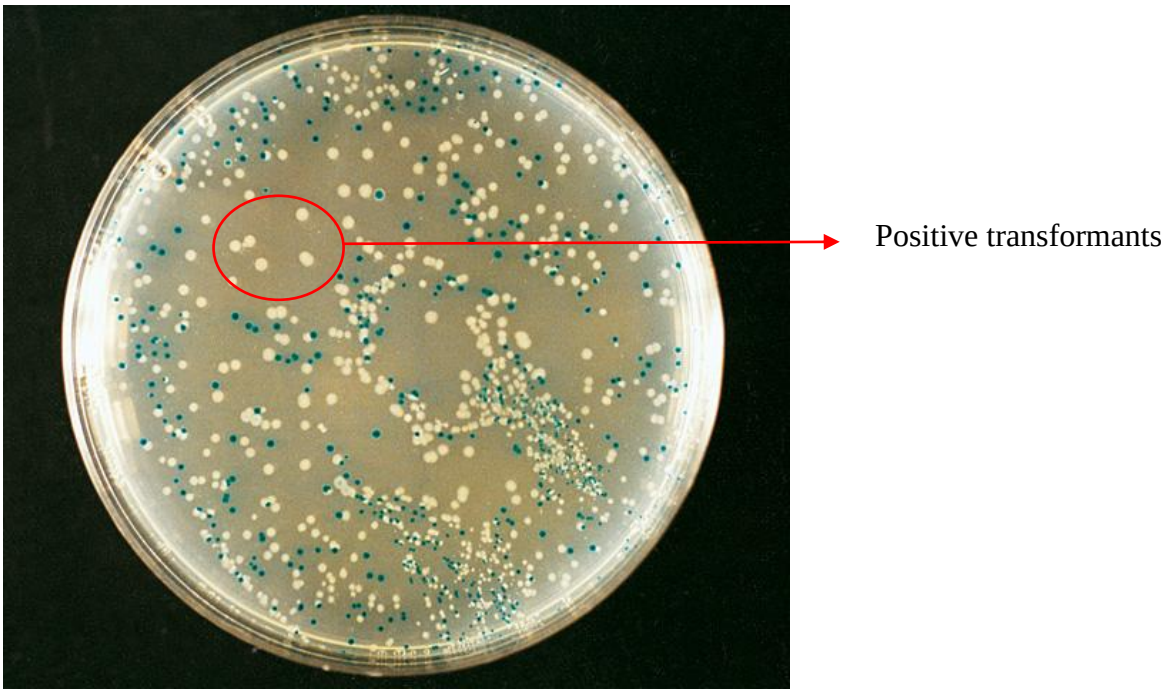
The purified PCR product of *abaI* gene from isolate S117 was cloned into plasmid vector pCR 2.1-TOPO of the TOPO TA cloning kit. The plasmid vector pCR 2.1-TOPO is an activated linearized vector with overhanging 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

The recombinant plasmid was then transformed into chemically competent *E.coli* cells. These cells carrying the wild type *abaI* gene were selected on the plates containing ampicillin as the plasmid has antibiotic resistance marker. Blue-white selection was made in which the light blue and colourless colonies were positive for the *abaI* gene while the dark blue colonies were negative for *abaI* gene (Figure 3.31).

E.coli transformants with the *abaI* gene were confirmed by PCR using the primers specific for *abaI* gene. The gel electrophoretic banding patterns are shown in the figure 3.32. The clones were further confirmed by restriction digestion of the recombinant plasmid with the enzyme EcoRI (Figure 3.33).

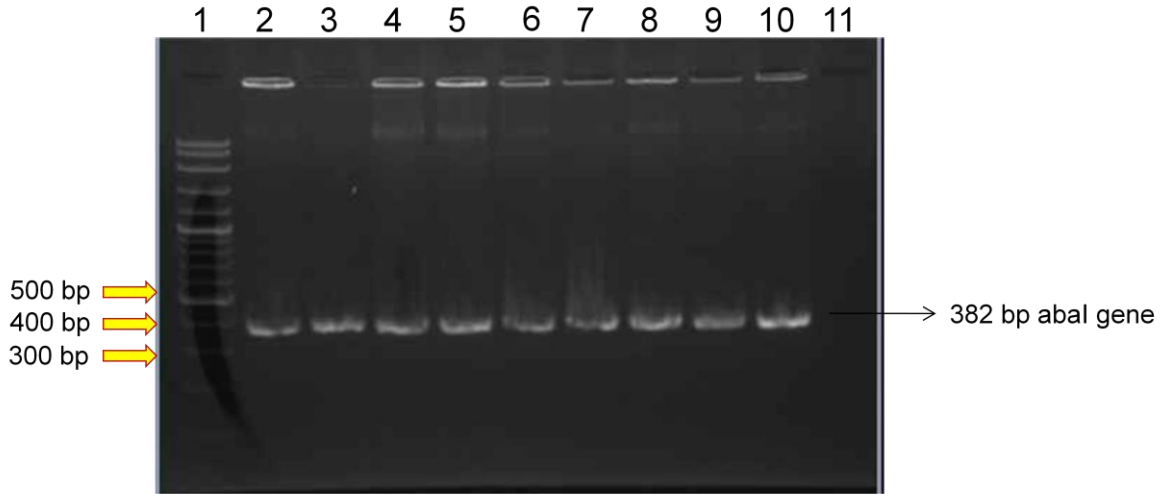
Acinetobacter spp. S117 with mutation in the *abaI* gene (*abaI*::Tc) was created by the introduction of plasmid pUC18.*abaI*::Tc into *Acinetobacter* spp. S117 by conjugation. The positive clones were selectively screened on the LB agar plates containing tetracycline antibiotic markers as seen in the figure 3.34. All the colonies on this selective plate are positive clones with the tetracycline mutant of *abaI* gene. These were confirmed by colony PCR using the primers for tetracycline gene. The electrophoretic banding patterns of the colony PCR is shown in figure 3.35.

Figure 3.31: Blue-white selection of the *E.coli* transformants carrying the *abaI* gene



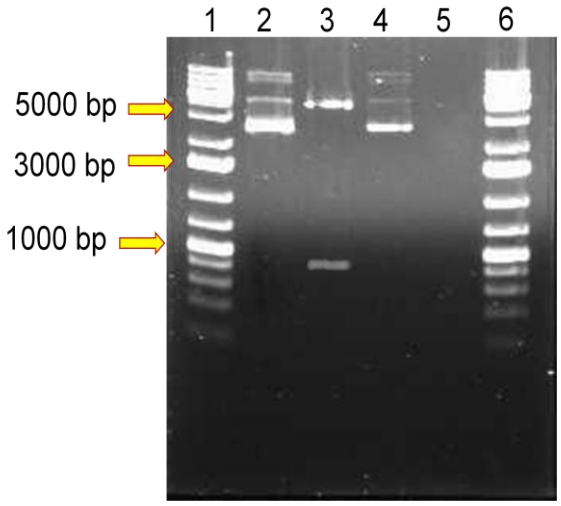
LB Amp plate (blue-white selection based on lacZ disruption)

Figures 3.32: Colony PCR of *E. coli* transformants for confirmation of the presence of *abaI* gene



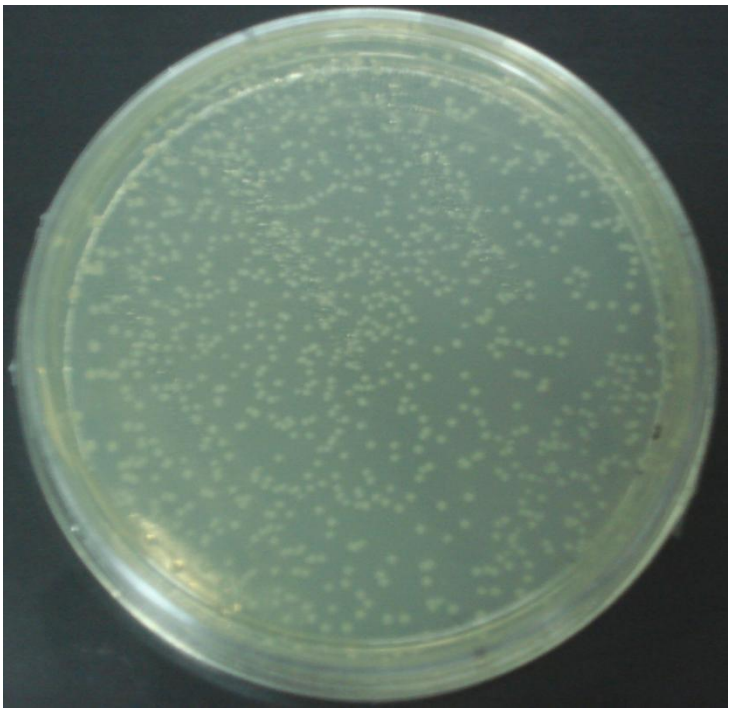
Lane 1: 1000 bp DNA ladder
Lane 2-11: amplicons of colony PCR of *abaI* gene in positive transformants

Figures 3.33: EcoRI fast digestion of the recombinant plasmid DNA



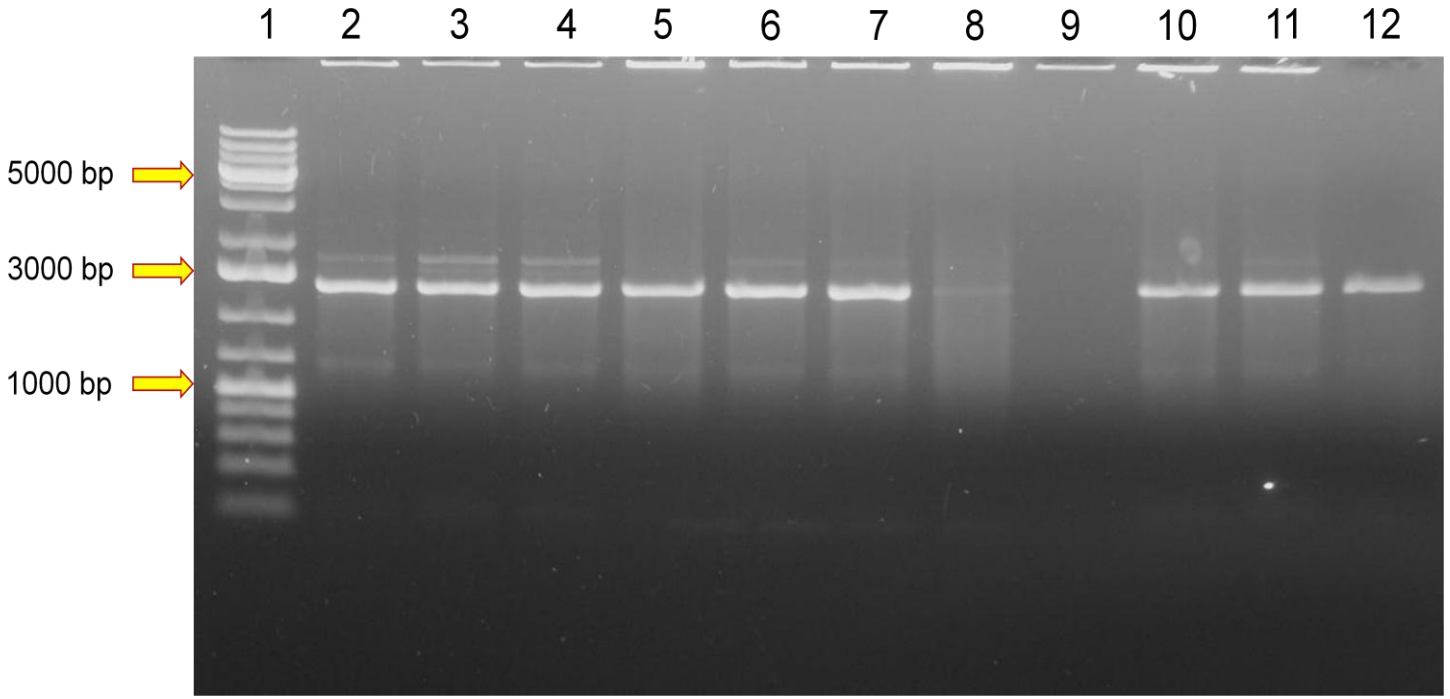
Lane 1 & 6: 1 Kb DNA ladder
Lane 2: Recombinant plasmid DNA
Lane 3: EcoRI digested recombinant plasmid DNA
Lane 4: Uncut recombinant plasmid DNA of the digestion reaction
Lane 5: Negative Control

Figures 3.34: Conjugants with *abaI*::Tc mutant



LB selective plate with tetracycline

Figure 3.35: Confirmation of the *abaI*:Tc mutant by colony PCR for tet gene



Lane 1	1 Kb DNA ladder
Lane 2 to 7	Isolates positive for tet gene
Lane 8 and 9	Isolates negative for tet gene
Lane 10 to 12	Isolates positive for tet gene

3.6.4 Biofilm formation in wild type and mutant *abaI* gene

After the construction of the *abaI* mutant, the wild type *abaI* gene and the *abaI*::Tc mutant were analyzed for AHL production. It was found that the wild type *abaI* produced the same kind of AHL signal produced by S117 in the TLC overlay while its mutant counterpart, *abaI*::Tc mutant failed to produce any detectable signals using the biosensor strain. The examination of the ability of the wild type and the *abaI*::Tc mutant to form biofilms showed that when evaluated after 24 hrs of incubation, there was no signicifant biofilms in both the wild type and the mutant. But, after prolonged incubation for 48 hrs, there was considerable biofilm formation in the wild type *abaI* which was twice (OD-2.25) as much more when compared to its mutant counterpart (OD-0.9) under the same incubation time. Thus, there was inhibition in the biofilm formation in the *abaI*::Tc mutant when compared to the wild type. This is shown in the figure 3.36a.

The mutation complementation experiment with the ethyl-acetate extracts of the culture supernatants from wild type and the *abaI*::Tc mutants showed that the addition of ethyl-acetate extracts from the wild type cells restored the ability of the *abaI*::Tc mutants to form normal biofilms. But, the ethyl-acetate extracts from the *abaI*::Tc mutants failed to restore the biofilm forming capabilities in the *abaI*::Tc mutant (Figure 3.36b).

Figure 3.36a: Biofilm formation of the wild type *abaI* and *abaI*::Tc mutant in microtiter plate

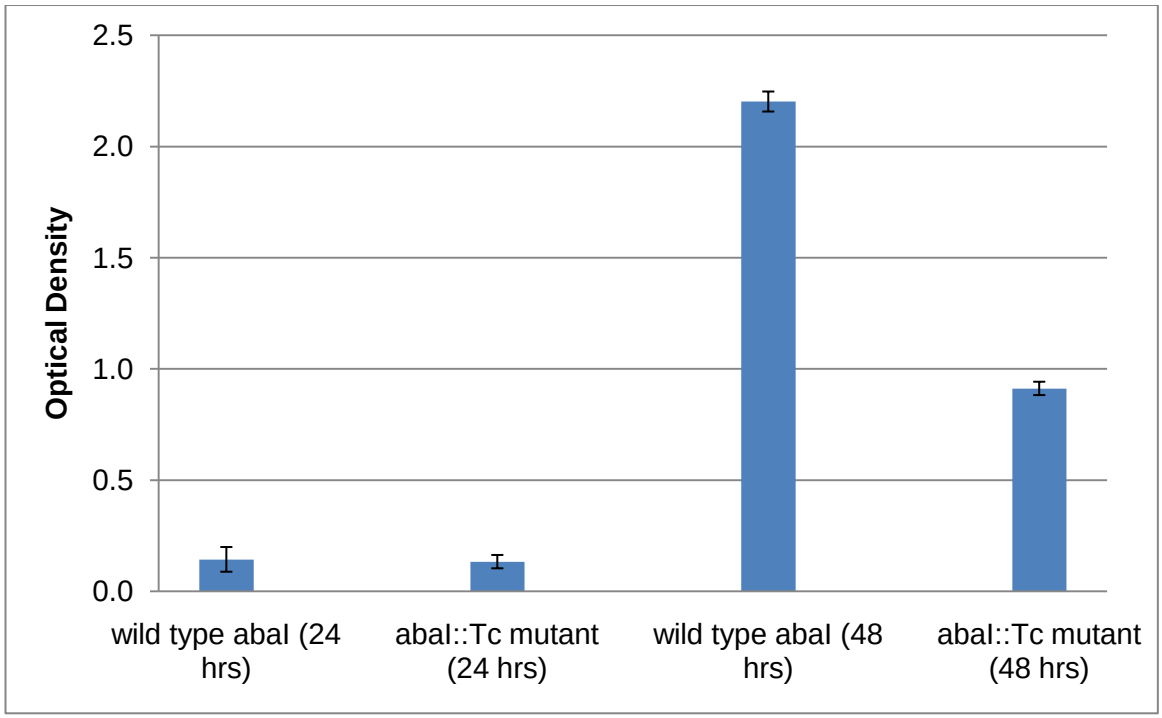
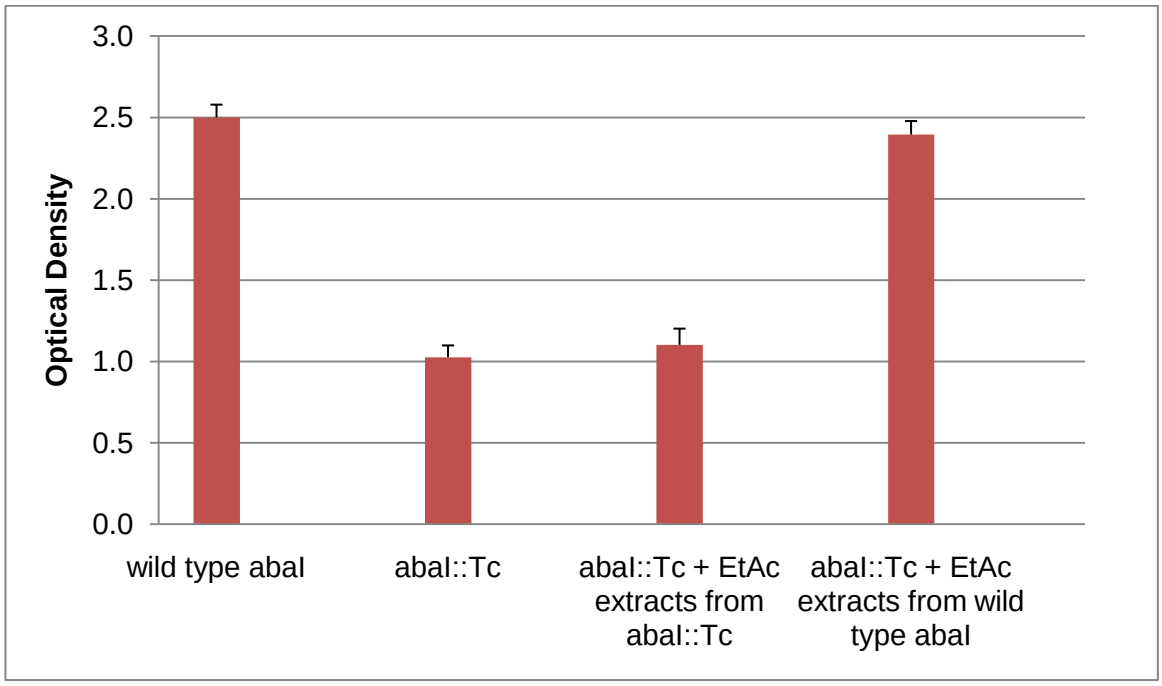


Figure 3.36b: Mutation complementation experiment



3.7 Natural sources as potential quorum quenchers

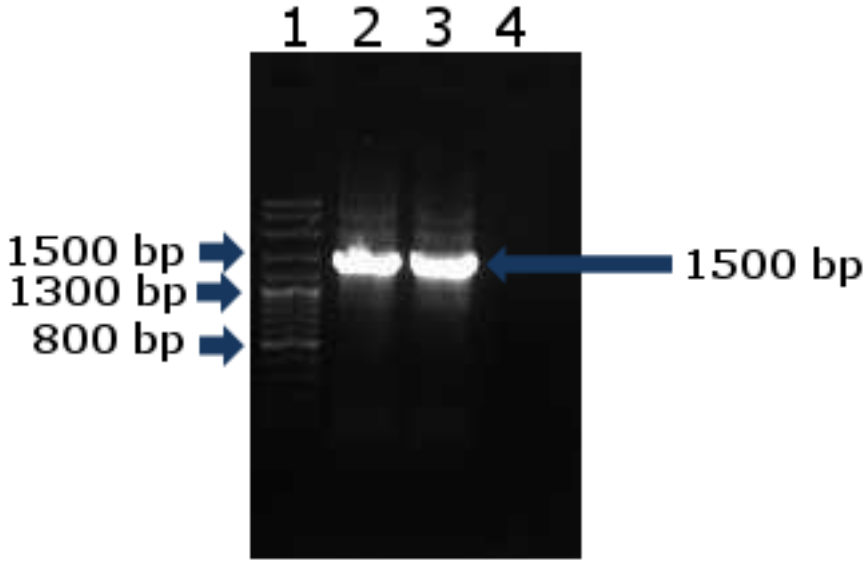
3.7.1 Isolation of soil *Bacilli* and detection of *aiiA* homologue gene

Plating the serially diluted soil samples yielded about 25 bacterial isolates. Of these only two isolates were positive for the genus identification for *Bacillus* using the specific *16S rDNA* primers. The two isolates were identified to be *Bacillus sp.* producing an amplicon length of approximately 1500bp. The gel picture is shown in figure 3.37.

Further sequencing and web-based analysis of the sequenced data revealed that the soil isolates B1 and B2 belong to the genus *Bacillus*. The 16S rDNA sequences (962 nucleotides) of the isolate B1 shared 99% sequence identity with 16S rDNA of *Bacillus thuringiensis* strain Y22 (GenBank accession number JN084160.1) and the 16S rDNA sequences (1005 nucleotides) of the isolate B2 also shared 99% sequence identity with 16S rDNA of *Bacillus sp. akoli* (GenBank accession number JF681181.1).

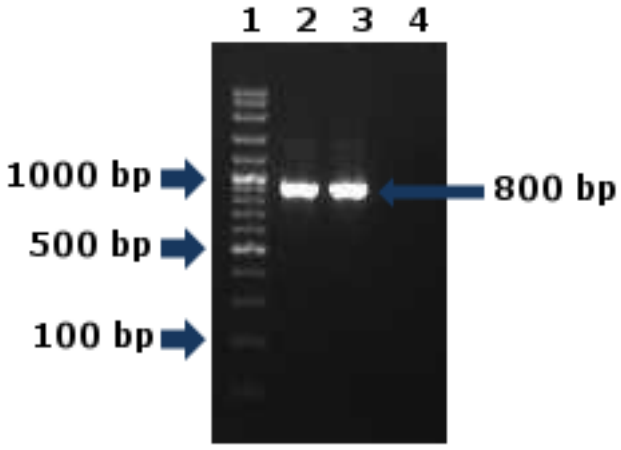
The two isolates identified as *Bacillus sp.* and designated as B1 and B2 were further screened for the presence of the AHL-degrading enzyme *aiiA* homologue gene. PCR specific for this gene produced amplified copies of this gene in B1 and B2. The amplicon size was approximately equal to 800bp which is shown in figure 3.38.

Figure 3.37: PCR amplication of the soil bacterial isolates for the presence of *16S rDNA* gene specific for the genus *Bacillus*.



Lane 1: 1 kb DNA ladder
Lane 2: PCR product of isolate 1
Lane 3: PCR product of isolate B2
Lane 4: Negative control (non-template).
The amplicon size is approximately equal to 1500bp.

Figure 3.38: PCR detection of the soil bacterial *Bacillus* sp. isolates (B1 and B2) for the presence of *aiiA* homologue gene (coding for AHL-degrading enzyme).



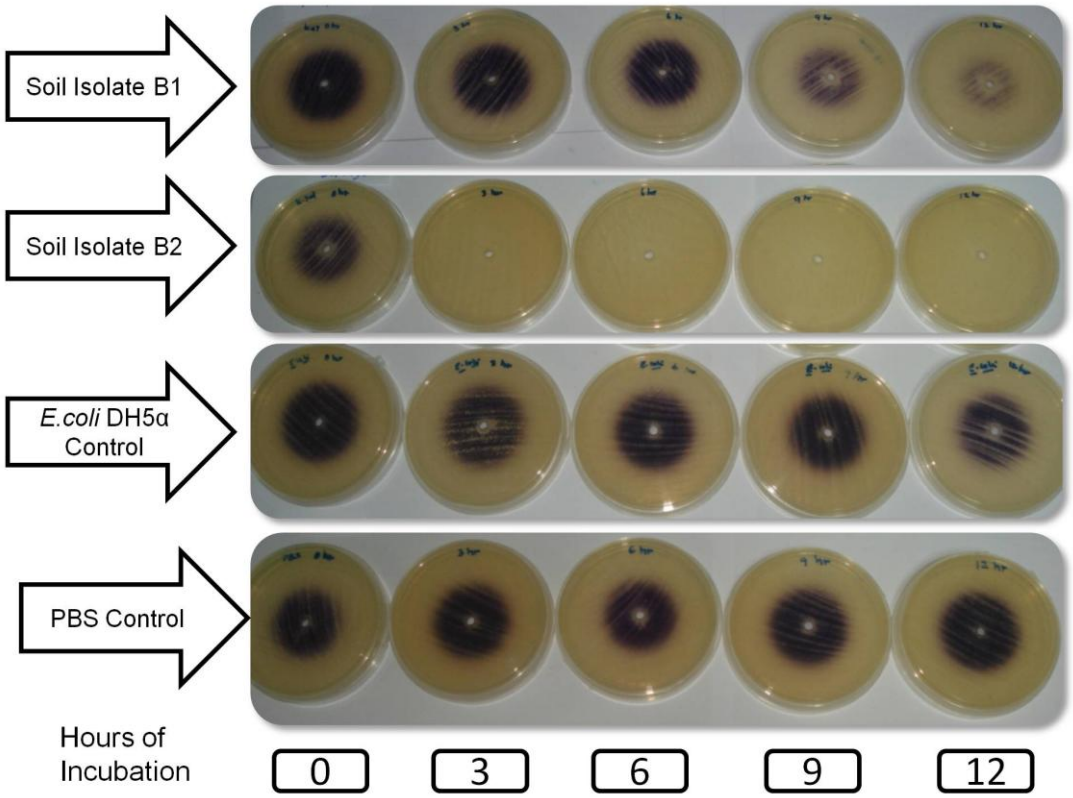
Lane 1: 100bp DNA ladder
Lane 2: PCR product of isolate 1
Lane 3: PCR product of isolate B2
Lane 4: Negative control (non-template).
The amplicon size is approximately equal to 800bp.

3.7.2 AHL inactivation assay of the soil *Bacillus* spp. extracts

We further screened the two *Bacillus* sp. isolates B1 and B2 for their AHL-inactivating activity. Since both of these isolates possessed the *aiiA* gene they also exhibited a strong AHL-degrading activity. Nearly all AHLs used in the bioassay were degraded after incubation with the isolate B1 for 9 hours and after 3 hours in B2. This indicates the rapid degradation of the AHLs. The results are shown in figure 3.39. *Escherichia coli* DH5α and PBS served as the negative controls in which no apparent degradation was observed. Here, *E. coli* DH5α was used as negative control because it does not contain the *aiiA* homologue genes and so it is expected that there would not be any AHL degradation.

The *aiiA* gene amplification by PCR is a preliminary screening for quorum quenching activity. A total of two bacterial isolates containing the *aiiA* gene were selected to confirm their quorum quenching activity by AHL inactivation assay. Using the AHL-inactivation assay, both the isolates showed rapid AHL degradation *in vitro*. The pH of the reaction mixture was buffered to pH 6.5 to avoid alkali lactonolysis because AHLs are easily hydrolyzed under alkaline conditions (Yates *et al*., 2002).

Figure 3.39: Bioassay for the detection of AHL-degrading enzyme activity of the soil *Bacillus* sp. isolates B1 and B2.



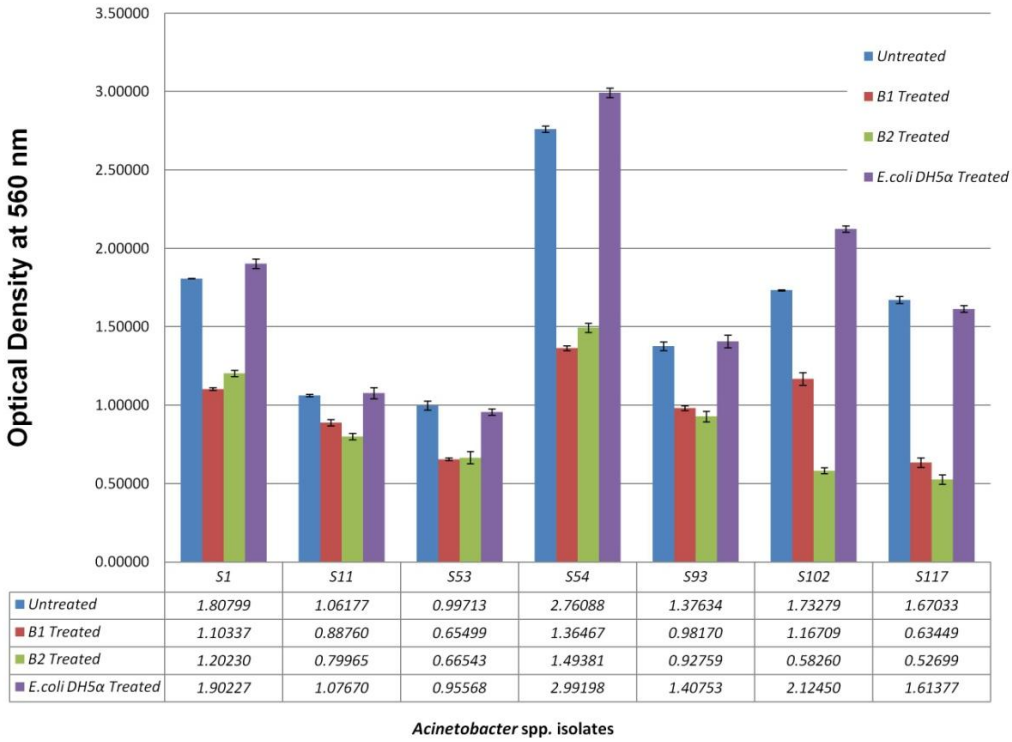
Chromobacterium violaceum strain cv026 was used as a reporter strain for the bioassay to detect exogenous *N*-hexanoyl homoserine lactone (HHL) in the experiment for screening the soil *Bacillus* sp. isolates capable of inactivating acyl homoserine lactones. *Escherichia coli* DH5α and PBS were used as negative controls in which no degradation of AHL was observed. Degradation was evident by the loss of purple pigment in the biosensor strain.

3.7.3 Inhibition of biofilm formation among the clinical *Acinetobacter* spp. isolates

The biofilm forming capabilities of *Acinetobacter* spp. clinical isolates after treatment with the soil *Bacillus* sp. extracts were analyzed. There was considerable inhibition in biofilm formation. After incubation for 24 and 48 hrs (figure 3.40 and figure 3.41 respectively), the treated isolates showed that their biofilm forming capabilities were reduced to half when compared to the untreated isolates. The clinical isolates were treated with extract from *E.coli* DH5α (control), and no inhibition of biofilm formation was noted.

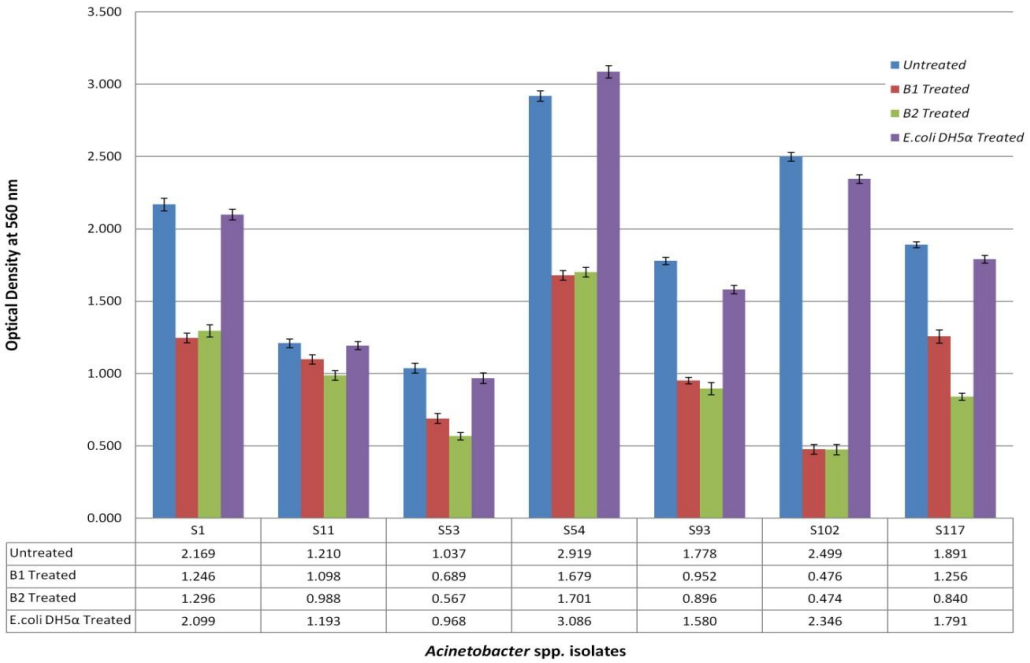
The seven clinical isolates of *Acinetobacter* spp. treated with the extracts from soil *Bacillus* sp. isolates and were also screened for the presence of long chain AHLs and the results reveal that all the seven isolates showed negative results in this screening test (results not shown). The extracts after treatment were also subjected to TLC which also showed the absence of AHLs in all the seven isolates.

Figure 3.40: Biofilm formation in *Acinetobacter* spp. isolates treated with the soil *Bacilli* extracts (24 hrs)



	S1	S11	S53	S54	S93	S102	S117
Untreated	1.80799	1.06177	0.99713	2.76088	1.37634	1.73279	1.67033
B1 Treated	1.10337	0.88760	0.65499	1.36467	0.98170	1.16709	0.63449
B2 Treated	1.20230	0.79965	0.66543	1.49381	0.92759	0.58260	0.52699
E.coli DH5α Treated	1.90227	1.07670	0.95568	2.99198	1.40753	2.12450	1.61377

Figure 3.41: Biofilm formation in *Acinetobacter* spp. isolates treated with the soil *Bacilli* extracts (48 hrs)



	S1	S11	S53	S54	S93	S102	S117
Untreated	2.169	1.210	1.037	2.919	1.778	2.499	1.891
B1 Treated	1.246	1.098	0.689	1.679	0.952	0.476	1.256
B2 Treated	1.296	0.988	0.567	1.701	0.896	0.474	0.840
E.coli DH5α Treated	2.099	1.193	0.968	3.086	1.580	2.346	1.791

Microtitre plate method was done for the biofilm formation and there is considerable decrease in biofilm formation in the treated samples showing that the soil extracts significantly inhibit the biofilm formation in the clinical isolates of *Acinetobacter* spp.

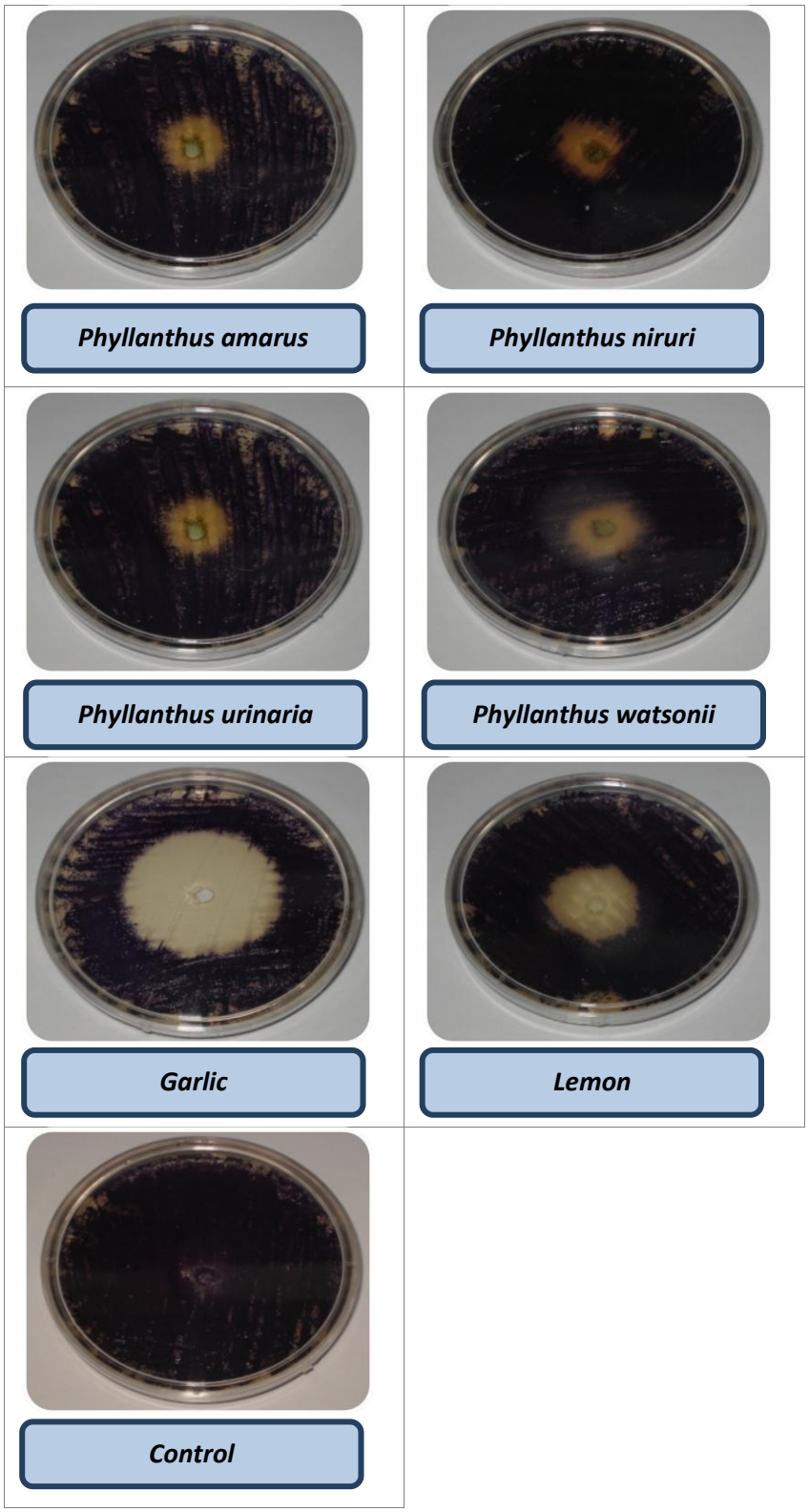
3.7.4 Use of plant extracts to inactivate AHLs among clinical *Acinetobacter* spp. isolates

Four species of *Phyllanthus* extracts, garlic and lemon extracts were evaluated for their quorum quenching activity and were all found to exhibit quorum quenching activity. The halos produced on lawns of the biomonitor strains could be the result of either (i) inhibition of cell growth or (ii) quenching of QS signals. The halo-effect is created by pigmentless (QS interrupted) cells adjacent to the well, and the presence of violacein-producing (QS active) cells further out. In this test, bacterial growth inhibition would result in a clear halo around the well, while a positive result of quorum sensing inhibition is exhibited by a turbid halo harboring pigmentless bacterial cells of the *Chromobacterium violaceum* CV026 monitor strain. To differentiate, the halo was examined under a higher magnification. Measurements were made from the outer edge of the wells to the edge of the zones of anti-QS inhibition. Loss of purple pigment in *Chromobacterium violaceum* is indicative of QS inhibition by the plant products used. The levels of anti-QS activity is calculated based on the diameter of the halo of pigmentless colonies of *Chromobacterium violaceum* CV026 monitor strain. Garlic had the strongest anti-QS activity (20 mm), followed by lemon extract (10 mm), and then *Phyllanthus* spp. extracts (all the four species showed similar colourless colony zone of 5 mm). The varying zones of colourless colonies are shown in figure 3.42.

Analysis of the biofilm forming capabilities of the seven clinical isolates of *Acinetobacter* spp. after treatment with the extracts from *Phyllanthus* spp., lemon, and garlic showed that there was considerable inhibition in biofilm formation. After incubation for 48 hrs (figure 3.43), the treated isolates showed that their biofilm forming capabilities were reduced to half when compared to the untreated isolates. The exact values (A570) of reduction are given in figure 3.43. Treated clinical isolates of *Acinetobacter* spp. were also screened for the presence of long chain AHLs and the results reveal that all the seven isolates showed negative results in this screening test

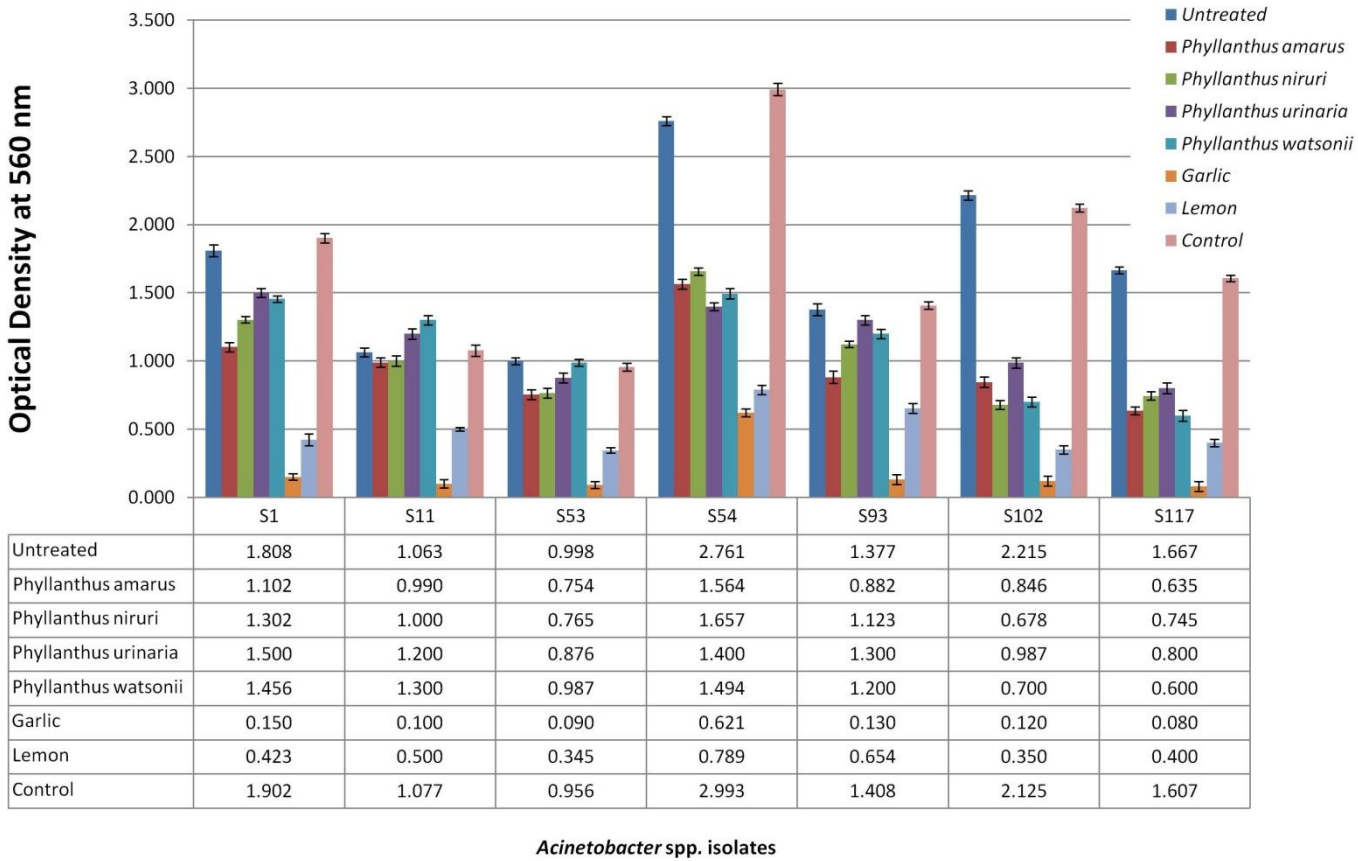
(results not shown). The extracts after treatment were also subjected to TLC which also showed the absence of AHLs in all the seven isolates.

Figure 3.42: Anti-QS activity using *Chromobacterium violaceum* CV026 monitor system.



Anti-QS activity was tested using 20 µl of methanolic extracts of *P. amarus, P. niruri, P. urinaria, and P. watsonii* and 20 µl of ethanolic extracts of garlic and lemon. Sterile DMSO was used as a negative control.

Figure 3.43: Biofilm formation in *Acinetobacter* spp. isolates treated with the extracts from *Phyllanthus species*, garlic and lemon.



	S1	S11	S53	S54	S93	S102	S117
Untreated	1.808	1.063	0.998	2.761	1.377	2.215	1.667
Phyllanthus amarus	1.102	0.990	0.754	1.564	0.882	0.846	0.635
Phyllanthus niruri	1.302	1.000	0.765	1.657	1.123	0.678	0.745
Phyllanthus urinaria	1.500	1.200	0.876	1.400	1.300	0.987	0.800
Phyllanthus watsonii	1.456	1.300	0.987	1.494	1.200	0.700	0.600
Garlic	0.150	0.100	0.090	0.621	0.130	0.120	0.080
Lemon	0.423	0.500	0.345	0.789	0.654	0.350	0.400
Control	1.902	1.077	0.956	2.993	1.408	2.125	1.607

Microtitre plate method was done for the biofilm formation and there is considerable decrease in biofilm formation in the treated samples showing that the extracts significantly inhibit the biofilm formation in the clinical isolates of *Acinetobacter* spp. Garlic which is shown to have maximum anti-QS activity inhibits biofilm formation considerably followed by lemon extracts. All the four *Phyllanthus species* have more or less the same level of activity as quorum quenchers and they also have an effect as biofilm inhibitors.

Chapter 4
Discussion

The extraordinary ability of certain bacteria to develop resistance to antibiotics has been a great concern among clinicians and health care professionals for several years. Antibiotics were first discovered through a providential experiment by Alexander Fleming in 1928. His work eventually led to the large-scale production of penicillin from the mould *Penicillium notatum* in the 1940s. The resistant strains of bacteria began to appear in the late 1940s. Currently, it is estimated that more than 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the antibiotics used routinely to treat them. Antibiotic resistance continues to expand for a multitude of reasons, including over-prescription of antibiotics by physicians, non-completion of prescribed antibiotic treatments by patients, use of antibiotics in animals as growth enhancers, increased international travel, and poor hospital hygiene.

Among the many pathogens which are evolving to be multi-drug resistant *Acinetobacter* is emerging as an important pathogen in traditional and nontraditional healthcare settings. Its ability to infect healthy hosts and its propensity to develop antimicrobial drug resistance has caused concern among the infectious diseases community. Among the *Acinetobacter* genomic species, *Acinetobacter baumannii* is recognized as the species most frequently isolated from patients (Bergogne-Berezin and Towner**,** 1996). *Acinetobacters* have been isolated from various types of opportunistic infections, including septicemia, pneumonia, endocarditis, meningitis, skin and wound infection, and urinary tract infection (Bergogne-Berezin and Towner**,** 1996). The distribution by site of *Acinetobacter* infection does not differ from that of other nosocomial Gram-negative bacteria. In several surveys, the main sites of *Acinetobacter* infection are the lower respiratory tract and the urinary tract. Often, *Acinetobacter* spp. emerged as important pathogens in the ICU setting, and this is probably due to the increasingly invasive diagnostic and therapeutic procedures used in hospital ICUs over the last two decades (Hartstein *et al.,* 1988). As it is a multi-drug resistant organism,

infections are difficult to treat (Falagas and Karveli, 2007), resulting in mortalities of 23% for hospitalised patients and 43% for patients under intensive care (Falagas *et al.*, 2006). Thus, *Acinetobacter* spp. is emerging as an increasingly important multidrug resistant pathogen, spreading in hospitals, and causing severe adverse outcomes. It has become a leading nosocomial pathogen in many hospitals as compared to other non-fermenting Gram-negative bacilli. Therefore, a new strategy in the successful treatment of *Acinetobacter* infections is an absolute necessity.

In this study, 50 strains of *Acinetobacter* spp. isolated between August 2003 and March 2004 from University Malaya Medical Center and confirmed biochemically to belong to the *Acinetobacter baumannii/calcoaceticus* complex were chosen. Among these isolates, 39 of them were carbapenem resistant. Of the 50 strains, 30.6% were isolated from the ICU, 12.2% isolated from geriatric units, 12.2% from patients associated with bone marrow transplant (BMT), 10.2% from the paediatric units and 34.7% from the surgical wards. This suggests that these strains may be a pathogen among immunocompromised patients and may be circulating in the hospital setting. These strains are also suggested to be colonizers rather than direct pathogens as it was mostly non-invasive isolates (89.8%) (Table 3.1). The routine methods of identification were performed and they belong to the *Acinetobacter baumannii/calcoaceticus* complex.

4.1 Biofilm formation by *Acinetobacter* spp. clinical isolates

Survival in the hospital environment is one of the important challenges faced by microorganisms and they have in the course of time evolved various mechanisms to tolerate these adverse conditions. Nosocomial pathogens have to survive from the antimicrobial agents, disinfectants and also nutrient depletion. Among all the pathogenic bacteria, *Acinetobacter* spp. is the microorganism that has adapted

efficiently to overcome these adverse conditions. A high intrinsic resistance to antibiotics, disinfectants and its ability to acquire new resistance genes prevents the elimination of this pathogen from the hospital environment. The ability of this organism to resist desiccation is the key factor that facilitates the persistence of *Acinetobacter* spp. for longer periods on inanimate objects. Biofilm formation is a possible explanation for its survival under desiccation and it could be responsible for the device-related infections. The biofilm structures could protect these organisms under adverse conditions, than existing as planktonic forms.

Most *Acinetobacter* spp. research to date has focused on cataloguing and understanding the variety of antimicrobial resistance genes and mechanisms found within the species (Bonomo and Szabo, 2006; Navon *et al*., 2007; Tsakris *et al.,* 2006; Vila *et al*., 2007). Apart from the molecular mechanisms involving the drug-resistant genes, it has been shown that *A.baumannii* forms biofilm with enhanced antibiotic resistance (Vidal *et al*., 1997; Vidal *et al*., 1996). Biofilm formation is a trait closely related to pathogenicity. According to a public announcement from the US National Institutes of Health more than 80% of all microbial infections involve biofilms (Ren *et al.,* 2005; Rasmussen *et al.,* 2006).

In this study, biofilm formation was analysed in 50 clinical *Acinetobacter* spp. isolates collected from University of Malaya Medical Centre. Thirty of the clinical isolates (60%) were able to significantly ($p < 0.5$) form biofilm structures in 96-well polystyrene microtiter plate under prolonged period of incubation (48 hrs); the experiment was done in triplicates. In a previous study, it was shown that 63% of *A. baumannii* clinical isolates formed solid-liquid interface-biofilm (Rodriguez-Baño *et al*., 2008). Similar results were obtained in a study done by Marti *et al*., 2011. They have also shown that 63% of *A. baumannii* clinical isolates formed solid-liquid interface-biofilm.

Formation of these sessile biofilm communities and their inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections. Biofilms represent microbial societies with their own defense and communication systems. The current view of biofilm infections leads to the realization that their effective control will require a concerted effort to develop therapeutic agents that target the biofilm phenotype and community signaling–based agents that prevent the formation, or promote the detachment, of biofilms (Costerton *et al.,* 1999). While the distribution of biofilms is quite ubiquitous, many fundamental aspects of their physiology remain unknown. Several molecules have been associated with the coordination of activities of microorganisms within a community. These include AHL, oligopeptides, amino acids such as glutamate and aspartate, and fatty methyl esters. It is conceivable that one or more of these molecular signals may impact biofilm physiology **(**Loehfelm *et al*., 2008; McLean *et al*., 1997**).**

Genetic analysis of biofilm formation has led to the proposal that extracellular signals and quorum sensing regulatory systems are essential for differentiated biofilms. Although such a model fits the concept of density-driven cell–cell communication and appears to describe biofilm development in several bacterial species and conditions, biofilm formation is multifactorial and complex. Hence, differentiated biofilms may also be the net result of many independent interactions, rather than being determined by a particular global Quorum Sensing system (Kjelleberg and Molin**,** 2002).

4.2 Identification of QS signal molecules among clinical *Acinetobacter* spp. isolates

QS is a regulatory mechanism which enables bacteria to make collective decisions with respect to the expression of a specific set of genes. These may include genes involved in biofilm formation and virulence. They play a key role in orchestrating the expression of exoproteases, siderophores, exotoxins and several secondary metabolites,

and participate in the development of biofilms. It has been shown that in contrast to the other QS systems, the AHL-mediated QS signalling system appears to control genes essential for colonisation of eukaryotes across a large number of bacterial species. This process is facilitated by bacterial biofilms (Costerton *et al.,* 1999). The high density of bacteria within biofilms has led to the speculation that quorum-sensing genes and AHL production may be fundamentally associated with biofilm physiology. At least in some bacteria, mutations in autoinducer synthesis lead to the formation of biofilms with abnormal structures (Niu *et al.,* 2008). The presence of AHL activity in naturally occurring aquatic biofilms has already been reported (McLean et al., 1997). Reports have also suggested that the autoinducer (AI), quorum sensing signal molecules may play an important role in biofilm formation (Camilli and Bassler, 2006; Domka *et al.,* 2007). Cell-to-cell signalling is often mediated by the production of *N*-acyl-homoserine lactone (AHL) signalling molecules (Fuqua *et al.*, 1996).

In this study, we report the detection of AHL signal molecules among the 50 biofilm forming clinical isolates of *Acinetobacter* spp. using the *C. violaceum* CV026 biosensor monitor system. The CV026 induction assay which detects the presence of short-chain AHLs revealed that none of our isolates produced short-chain AHL molecules. So, we further carried out the CV026 inhibition assay, in which the induced CV026 will be inhibited in the presence of the long-chain AHLs produce by the clinical isolates. The results of this assay showed that 7 of the biofilm forming isolates significantly produced long-chain AHL molecules. We present here the first report of biofilm formation and long chain AHL molecules in *Acinetobacter* samples from our medical center. The method presented here for screening pure cultures of *Acinetobacter* spp. for AHL production using the *Chromobacterium violaceum* CV026 monitor system was developed and shown to be rapid and does not require sterile supernatants or AHL extractions from cultures. It also has the advantages of only requiring monitor strains

and the ability of the strains tested to grow on LB media. Six strains can be tested on the same plate simultaneously. The only limitation of this assay is that the detection limit lies within the scope of the signal monitor system CV026 that was used.

AHLs can be partially characterized by TLC on C18 reversed phase plates. This organic extraction procedure increases many-fold the sensitivity of biosensors (McClean *et al*., 1997; Shaw *et al*., 1997; Schaefer *et al*., 2000). Separation by TLC coupled with detection by AHL biosensors gives a rapid and direct visual index of the AHL(s) produced by the tester bacteria. Previous studies have shown AHL production by *Acinetobacter* spp. by TLC (Gonzalez *et al*., 2001). In *A. calcoaceticus* BD413 supernatants four compounds were detected in a time-dependent manner, and maximal activity was reached at stationary phase (Gonzalez *et al*., 2001). In a variety of gram-negative bacteria, it has also been demonstrated that biofilm development can be dependent on AHL signaling (Davies *et al*., 1998; Huber *et al*., 2001; Lynch *et al*., 2002).

In this study, thin layer chromatography bioassay was thus used to confirm the presence of AHL molecules and semi-qualitatively identify the AHL molecules. TLC analysis has revealed that our clinical isolates produced C_{10} and C_{12} AHLs. All these were predicted based on their R*f* values and in comparison with the standard DHL (Decanoyl homoserine lactone and dDHL (Dodecanoyl homoserine lactone). Kang *et al.* (2010) showed by TLC bioassay that *Acinetobacter* sp. DR1 strain generated three putative acyl homoserine lactones, while the DR1R produced only one signal and Niu *et al*. (2008) similarly detected the presence of three different AHLs in *Acinetobacter baumannii* strain M2. In *A. calcoaceticus* BD413 supernatants, Gonzalez *et al.* (2001) detected four compounds by TLC in a time-dependent manner, and maximal activity was reached at stationary phase.

The AHL biosensors can also be used for quantifying AHLs by measuring the activity of the reporter system present in the biosensor bacterial strain. This is useful for studying regulation of AHL synthesis and for identifying strain-level differences in AHL production. In order to quantify accurately one must determine, using the synthetic AHL, the minimal amount of AHL required for a response as well as the amount necessary for a saturated response in order to plot the linear dose response.

In our study, we semi-quantitatively determined the amount AHLs produced by the 7 clinical isolates designated as S1, S11, S53, S54, S93, S102, S117 using the well-diffusion assay. Based on the standard curves, extraction efficiencies were calculated for DHL and HHL using both acidified and non-acidified ethyl acetate. Approximately twice as much AHL was extracted when using acidified ethyl acetate as compared to non-acidified. This is consistent with the results shown by Shaw *et al*., 1997. Extraction efficiencies were tested on supernatants from *Acinetobacter* strains and it was observed that acidified extractions gave better yields as compared to the non-acidified extractions. This revealed that a minimum of approximately 2×10^{-9} moles and a maximum of 6×10^{-9} moles of AHLs were detected among the clinical isolates of *Acinetobacter* spp. The presence of such high levels of AHLs in these clinical isolates could be a key factor that aids in their biofilm development, which in turn confers resistance to these organisms in adverse environments.

AHLs cannot be unambiguously identified using TLC. However, their chromatographic properties can be used to assign tentative structures, as R*f*s calculated for the samples can be compared with R*f*s of AHL standards. AHL structures are unequivocally determined on the basis of spectroscopic properties (Schaefer *et al*., 2000) including Mass Spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). We accurately quantified and determined the structures of the AHLs produced by the clinical *Acinetobacter* spp. isolated by Mass Spectrometry. Each sample was

analysed using a Precursor Ion method monitoring for the following common fragments: 102.1, 74.1, 71.1. These fragments had been observed to be fairly common when performing optimization studies on the standards (β-ketocaproyl, hexanoyl, heptanoyl, octanoyl and decanoyl). By monitoring for these fragments any AHL present was identified. Mass spectrometric analysis confirmed the presence of C_{10} AHLs in the tested 5 isolates (S1, S11, S54, S93 and S117) and AHLs with chain lengths C_{12} in other 2 isolates (S53, S102). Of the masses that were reported the only ones that seem to make sense are 284 and 282 as an AHL with a 12 carbon acyl chain, with the lower mass (282) containing a single alkene. An alkene could be anywhere along the chain length, however it can be rationalized that it could be a dehydration product from a beta-hydroxy derivative. The retention time at a later time than the decanoyl standard fits well. These masses were found in samples 53 and 102, which confirms the TLC report. The TIC (trace ion current) trace of each sample (1, 11, 53, 54, 93, 102, 117) with overlaid common precursor ions are shown in the results section. In a previous study by Niu *et al*. (2008), AHL signals directed by *Acinetobacter baumannii* strain M2 were identified by mass spectrometry as being C12 and in another study by Chan *et al*. (2011), it was shown that AHLs produced by *Acinetobacter* spp., upon analysis by liquid chromatography (LC) coupled to hybrid quadruple-linear ion trap mass spectrometry (LC-MS/MS), revealed the presence of C12. These findings further confirm results of our study.

4.3 Investigation of the gene involved in QS and construction of its mutant as a QS control strategy

Quorum sensing signal molecules are autoinducers, as they induce their own genes in a positive feedback loop. An autoinducer synthase gene in *A.baumannii* that was designated as *abaI* has already been identified and characterised. The deduced AbaI protein was highly similar to the members of the LuxI family. It has been shown that

this autoinducer synthase was required for normal biofilm development (Niu *et al.*, 2008). In our study we have identified and analysed *abaI* gene which encodes a similar protein. Polymerase Chain Reaction (PCR) for *abaI* gene, produced amplicons of 382 bp in all the 7 isolates (isolate no.S1, S11, S53, S54, S93, S102, S117) that produced the QS signal molecules. The amplicon of the *abaI* gene from all these isolates were further sequenced and analysed. Isolate S117 showed the maximum differences in the nucleotides and thus the aminoacid sequence of the autoinducer synthase protein when compared with the already reported *abaI* gene sequences (Table 3.19). Hence the *abaI* gene from S117 was chosen for all subsequent experiments. The BLAST analysis program revealed that the *abaI* gene identified in *Acinetobacter* spp. isolate S117 is 92% identical to the corresponding protein reported in recently published *A. baumannii* ATCC 17978 genome (Smith *et al.,* 2007). The AbaI protein from our isolate S117 shared 91% identity and 97% similarity with the already reported AbaI protein of *A. baumannii* M2 strain (Niu *et al.,* 2008). Further, the BLAST analysis also revealed that the AbaI protein exhibited 61% identity and 79% similarity to the acyl homoserine lactone synthase of *Halothiobacillus neapolitanus* c2, 65% identity and 75% similarity to the autoinducer synthesis protein of *Acidithiobacillus ferrooxidans* ATCC 53993, 47% identity and 67% similarity with autoinducer synthatase family protein of *Burkholderia mallei* ATCC 23344 and 49% identity and 63% similarity with *N*-acyl homoserine lactone synthase from *Pseudomonas fluorescens*. The similarity of the identified AbaI protein from S117 to various species of *Burkholderia, Pseudomonas fluorescens* and *Acidithiobacillus ferrooxidans* suggests a common origin of these genes. Niu *et al*., also identified the *abaI* gene in a clinical isolate *A. baumannii* strain M2 and when compared to the recently published *A. baumannii* ATCC 17978 genome, this AbaI protein from *A. baumannii* strain M2 was 96% identical to the corresponding protein (GenBank accession number NC_009085.1). However, the AbaI protein

identified from strain M2 was predicted to be 56 amino acids shorter at the amino terminus. Using the BLAST analysis program, the AbaI protein exhibited 54% identity and 69% similarity to the AfeI protein of *Acidithiobacillus ferrooxidans* and to LuxI proteins from *Burkholderia cepacia* (46% identity and 64% similarity) and *Ralstonia solanacearum* (41% identity and 65% similarity).

In this study, we have further cloned the *abaI* gene from *Acinetobacter* spp. isolate S117 into a plasmid vector, further mutated this gene by insertional inactivation and a *abaI*::Tc mutant of the gene was created. The *abaI*::Tc mutant was shown to have inhibition in the biofilm formation. This shows that the *abaI* gene product was required for biofilm formation. The differences between the wild type and the *abaI*::Tc mutant clearly showed that *abaI* directed AHL pathway is required for efficient biofilm formation. In a similar study, the ability of the wild type and the isogenic *abaI*::Km mutant to form biofilms was examined in wells of a microtiter plate and it was observed that there is inhibition in biofilm formation (Niu *et al*., 2008).

The above mentioned results in our study have revealed that it is likely that QS regulation is important for biofilm development. In a given setting, the biofilm associated community will exploit all available adaptive mechanisms and the corresponding network of regulatory activities (including QS) and it is not possible to unequivocally assign a specific determining regulatory factor for the structure/function relationship of the community. Further identification of the exact AHL signals and thus their gene sequences encoding them will allow the identification of signal antagonists that inhibit biofilm development. These antagonists may also reduce the ability of *Acinetobacter* sp. to survive on environmental surfaces for extended periods, a key component of its ability to form biofilms and persistence in intensive care wards.

4.4 Preliminary investigations into possible natural sources as potential quorum quenchers

Quorum sensing signal molecules in many of the organisms control many phenotypic traits, such as bioluminescense (Nealson and Hastings, 1979), antibiotic production (McGowan *et al.,* 1995), and virulence factor synthesis (Pearson *et al.,* 1997). In the plant pathogen *E. carotovora*, virulence related exoenzyme genes are regulated through the quorum sensing system (Jones *et al.,* 1993).

The continuing search for new and novel antimicrobials and antipathogenic agents has focused on exploiting the fact that plants surviving in an environment with high bacterial density have been seen to possess protective means against infections (Cos *et al.,* 2006). Using this argument, researchers are increasingly looking at herbal products in the quest for new therapeutic and antipathogenic agents which might be nontoxic inhibitors of quorum sensing, thus controlling infections without encouraging the appearance of resistant bacterial strains (Hentzer and Givskov, 2003). Current literature estimates that 10% of all terrestrial flowering plants on earth have been used by different communities in treating diseases, however, only around 1% have gained recognition and validation (Lewis and Ausubel, 2006). Controlled studies indicate the great potential of phytochemicals to be the richest reservoir of new and novel therapeutics (Kumar *et al.,* 2006). Searching the literature, it is surprising to find very few works discussing plant extracts and their antiquorum sensing activities. It is believed that plant extracts with well documented antimicrobial activities could possess antipathogenic as well as antivirulent activities, which may not be linked to the growth and inhibition of the microorganism (Vattem *et al.,* 2007). The antiquorum sensing activity of herbal plants is very poorly investigated and it is very likely that it will be found that the antimicrobial efficacy is mediated by quorum sensing inhibition.

Anti- QS activity has also been shown in a number of southern Florida seaweeds and a few terrestrial plants (Allison *et al.,* 2006). However, so far, only a handful of higher plants have been studied, and nothing has been published with regard to anti-QS activity in medicinal plants. The plant of the genus *Phyllanthus* belongs to the family *Euphorbiaceae* and has been reported to have pharmacological effects such as antiviral activity, anti-bacterial activity, anti-hepatotoxic or liver-protecting activity as well as anti-tumour and anti-carcinogenic properties. The plant genus *Phyllanthus* has thus been shown to be of medical importance. The anti-QS properties of these plants have not been reported before. So we sought to study the quorum quenching properties of four *Phyllanthus* species (*P. amarus, P. niruri, P. urinaria, and P. watsonii*). The anti-QS properties of garlic extract have been well studied before and it has a strong anti-QS property. Garlic extract with strong anti-QS property was used to quench the QS system in clinical extracts of *Acinetobacter spp*. The extracts of lemon and *Phyllanthus* spp. were also studied for their quorum quenching property. The anti-QS activity was evident with the growth of the biosensor reporter strain with colourless colonies rather than the zone of clearance. In all the extracts, there was no growth inhibition as there was a turbid halo of colourless colonies. In all the above biofilm inhibition analysis, the concentrations of the extracts used were also found to have no impact on the growth of the cultures. This indicates that the biofilm inhibition is probably due to the QS inhibition rather than growth inhibition.

Quorum quenching employing the AHL-degrading enzyme can be successfully used to attenuate the pathogenicity in highly virulent organisms resulting in disruption of all the traits associated with quorum sensing. There are various reports on the presence of AHL-degrading enzymes in *Bacillus* sp. Thus, we sought to isolate spore forming *Bacillus* sp. from the Malaysian soil. The heat treatment prior to isolation by serial dilution probably killed all the numerous vegetative forms of bacteria in the soil,

with which screening would have been more difficult. Sequencing and web-based analysis of the sequenced data revealed that the soil isolates B1 and B2 belong to the genus *Bacillus*. PCR for *aiiA* homologue gene, which is a reported AHL-degrading enzyme, for the 2 isolates confirmed that they belong to the genus *Bacillus*. Through the AHL-inactivation bioassay, it was confirmed that there was AHL degrading activity in the extracts from the soil *Bacillus* sp. isolates. But it was found that there was varying degrees of degrading activities exhibited by the isolates. Isolate B1 degraded almost all the AHLs used in the assay in 9 h, whereas B2 degraded in just 3 h treatment. After the confirmation of the AHL degrading capacity of the soil *Bacilli* extracts, we sought to use these extracts to disrupt quorum sensing in clinical isolates of *Acinetobacter* spp. Recently, a similar AHL-degrading enzyme gene (*aiiA*) from *Bacillus sp.* strain 240B1 was identified (Dong *et al.,* 2000). Transgenic tobacco and potato expressing the bacterial gene *aiiA* inactivated exogenous AHL and reduced the virulence of gram-negative *E. carotovora* (Dong *et al.,* 2001). Also they cloned a novel lactonase gene from a *Bacillus* sp. This gene designated as *aiiA* encodes an enzyme that can degrade the AHL molecules. Expression of *aiiA* gene in transformed *Erwinia carotovora*, that causes soft rot disease in many plants, reduces the release of AHL, decreases extracellular pectrolytic enzyme activities, and attenuates pathogenicity for potato, egg-plant, celery, carrot, cauliflower, Chinese cabbage, and tobacco (Dong *et al.,* 2000). An isolate of *Variovorax paradoxus* has been reported to use AHL molecules as energy and nitrogen sources, indicating that AHL-degrading enzymes are present in this organism (Leadbetter and Greenberg, 2000).

After the quorum quenching properties of all these plant extracts and soil *Bacillus* sp., were analysed, they were used to treat the seven biofilm forming clinical isolates of *Acinetobacter* spp. These clinical isolates have already been screened for the production of quorum sensing signal molecules and their biofilm forming capabilities analysed. In

the tests done to attenuate the pathogenicity of *Acinetobacter* spp. using the extracts from soil bacilli, the extracts could effectively degrade the AHLs produced and also inhibit the biofilm formation to a considerable extent. To our knowledge, this is the first report of the use of soil bacilli extracts to inhibit the biofilm formation in clinical isolates of *Acinetobacter* spp.

Since the *aiiA* gene product from *Bacillus* sp. and the plant extracts tested are found to interfere with the quorum sensing system, these extracts can be used to effectively attenuate the pathogenic organisms whose virulence is associated with the quorum sensing system. From our present study we have proved that the AHL degrading enzyme *aiiA* from the soil *Bacillus* sp. and the plant extracts of *Phyllanthus* spp., garlic bulb and lemon efficiently inhibited the biofilm formation in the clinical isolates of *Acinetobacter* spp. This makes the recombinant protein of *aiiA* gene or the extract from the *Bacillus* sp. producing these proteins and the extracts from the tested plant sources to be promising agents that could attenuate the pathogenicity in any organism producing quorum sensing signal. The use of all these extracts is only first step. After further fractionation and purification of the extracts, the chemical nature of the QS-inhibiting compound should be known before an application.

Chapter 5
Conclusion

In conclusion, the increasing emergence of multidrug resistance in *Acinetobacter* spp. has urged the scientific community to find alternatives to antibiotics as antimicrobial agents. This has led to the development of novel strategies in controlling *Acinetobacters.* One approach is to attenuate the pathogenicity, rather than killing the pathogen. This is called quorum sensing inhibition or quorum quenching. Using a microtitre-plate assay it was shown that 60% of the 50 *Acinetobacter* spp. isolates significantly formed biofilms under prolonged period of incubation. Further detection showed that 7 of these biofilm forming strains designated as S1, S11, S53, S54, S93, S102, and S117, produced long chain signal molecules. On analysis of the biofilm forming capabilities of these isolates, they all form biofilms significantly. We present the first report of long chain AHL molecules in *Acinetobacter* samples from our medical center.

Thin layer chromatography bioassay confirmed that five of these isolates produced *N*-decanoyl homoserine lactone and two isolates produced acyl-homoserine lactone with a chain length C_{12}. The data are consistent with the presence of quorum sensing signal molecules among the biofilm forming clinical isolates of *Acinetobacter* spp. Mass spectrometric analysis further confirmed the TLC results. Quantification of the QS molecules by well diffusion assay and mass spectrometric analysis revealed that a minimum of approximately 2×10^{-9} moles and a maximum of 6×10^{-9} moles of AHLs were detected among the clinical isolates of *Acinetobacter*.

In our study we have identified and analysed *abaI* gene which encodes AbaI protein. The BLAST analysis revealed that the AbaI protein has only 91% identity with the already reported protein, but still retains its function as a quorum sensing autoinducer synthase. The identified AbaI protein from S117 was found to be similar to various species of *Burkholderia*, *Pseudomonas fluorescens* and *Acidithiobacillus ferrooxidans* suggests a common origin of these genes.

A mutant of the cloned *abaI* gene was created and shown to inhibit biofilm formation. The differences between the wild type and the *abaI*::Tc mutant indicated that *abaI* directed AHL pathway is required for efficient biofilm formation.

Since there are various reports on the presence of AHL-degrading enzymes in *Bacillus* sp., we sought to isolate spore forming *Bacillus* sp. from the Malaysian soil. These extracts were found to disrupt quorum sensing in clinical isolates of *Acinetobacter* spp. by degrading the AHLs produced and also by inhibiting the biofilm formation to a considerable extent. To our knowledge, this is the first report of the use of soil bacilli extracts to inhibit the biofilm formation in clinical isolates of *Acinetobacter* spp.

Preliminary investigations into other sources of anti-QS activity were noted with lemon extract, garlic bulb and *Phyllanthus* spp., with garlic having the strong quorum quenching activity. All extracts used indicated that there was a considerable inhibition in biofilm formation among the clinical isolates tested.

In conclusion, this study has revealed that it is likely that QS regulation is important for biofilm development. The use of quorum sensing signal blockers to attenuate bacterial pathogenicity is therefore highly attractive, particularly with respect to the emergence of multi antibiotic resistant bacteria. Since the *aiiA* gene product from *Bacillus* sp. and the plant extracts tested are found to interfere with the quorum sensing system, they can be used to effectively attenuate the pathogenic organisms whose virulence is associated with the quorum sensing system.

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APPENDIX – 1

Gram staining reagents

1. Crystal violet:

Solution 1	Crystal violet	2 g
	Ethanol 95 %	20 ml
Solution 2	Ammonium oxalate	0.8 g
	Distilled water	80 ml
Solution 1 and 2 were mixed and allowed to stand for at least 24 hours, and filtered before use.		

2. Lugol's Iodine:

Potassium iodide	2 g
Iodine	1 g
Distilled water	300 ml

3. Diluted carbol fuschin:

Stock solution	Safranine O	2.5 g
	Ethanol 95 %	100 ml
Working solution	Stock solution	10 ml
	Distilled water	90 ml

Media preparation

1. Mueller Hinton Agar:

Beef infusion	300 g
Casein	17.5 g
Starch	1.5 g
Agar	17 g
Distilled water	1000 ml
• Sterilize by autoclaving.	
• Blood may be added as described for blood agar.	

2. Nutrient agar (NA):

Nutrient agar (Oxoid, UK)	28g
Double-distilled water	1000 ml

3. Luria-Bertani (LB) broth:

Luria-Bertani (Difco, France)	25g
Double-distilled water	1000 ml

- Sterilized by autoclaving.
- **LB agar** plates were prepared by adding 15 g/L of agar to the the above mixture.
- **LB ampicillin** plates were prepared by adding 50 µg/ ml ampicillin to sterilized LB agar medium.
- **LB tetracycline** plates were prepared by adding 20 µg/ ml tetracycline to sterilized LB agar medium.

4. 40% Luria-Bertani glycerol stock:

40% Glycerol (Sigma, USA)	30 ml
LB broth	100 ml

5. TY broth:

Bacto tryptone	8 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1000 ml

- Sterilized by autoclaving.
- TY agar plates were prepared by adding 15 g/L of agar to the the above mixture.

Standard biochemical reagents

1. Catalase reagent:

Hydrogen peroxide	3 %
• Stored in refrigerator at 4°C	

2. Mc Farland 0.5 Turbidity Standard:

0.048 M $BaCl_2$	0.5 ml
0.35 M NH_2SO_4	99.5 ml

3. Saline:

Sodium chloride	0.9 g
Distilled water	100 ml
• Sterilized by autoclaving	

4. Tris-acetate EDTA (TAE) buffer (50X) :

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA	18.61 g
Distilled water	1000 ml
• Sterilized by autoclaving.	

5. Tris-borate EDTA (TBE) buffer (10X) :

Tris base	121.2 g
Boric acid	61.8 g
EDTA	0.745 g
Distilled water	1000 ml
• Sterilized by autoclaving.	

6. Tris-EDTA (TE) buffer :

1 M Tris-HCL, pH8.0	1ml (final concentration = 10mM)
0.5 M EDTA	200 µl (final concentration = 1mM)
Distilled water	100ml
• Sterilized by autoclaving.	

7. 0.5 M EDTA, pH7.0, 7.5, or 8.0

Disodium EDTA.$2H_20$	18.61 g
Distilled water	60 ml
• The pH was adjusted as desired with 1 M NaOH and then made upto 100 ml with distilled water.	
• Sterilized by autoclaving.	

8. 10 mM Tris-HCL, pH 7.5

Tris base	0.12 g
Distilled water	80 ml
• The pH is adjusted as desired with concentrated HCL and then made upto 100 ml with distilled water.	
• Sterilized by autoclaving.	

9. Phosphte-buffered saline (PBS)

NaCl	10 g
KCL	0.25 g
Na_2HPO_4	1.43 g
KH_2PO_4	0.25 g
• Adjusted to pH7.3	
• Distilled water 1000 ml	
• Sterilized by autoclaving.	

APPENDIX - 2

Table A1.1: Selected compounds with observed mass, retention time and observed fragment ion in sample 1.

Sample	Compound	Mass	RT	Fragment
1	1	274.7	4.94	102.1
		547.8		102.1
		274.7	4.94	74.1
		547.8		74.1
		274.6	4.94	71.1
	2	256.7	5.59	102.1
		512		102.1
		256.7	5.59	74.1
		512		74.1
		256.7	5.59	71.1
		512		71.1
	3	512	7.51	102.1
		316.6		102.1
		297.7		102.1
		256.7		102.1

Table A1.2: Full list of compounds found in Sample 1

Cpd	Area	Base Peak	Height	m/z	RT
1	45958	202.4	14557	102.1	0.576
2	19034	236.3	2947	74.1	0.639
3	284445	316.8	70290	102.1	0.654
4	15704	317.5	3337	102.1	0.791
5	6876	250.5	895	74.1	1.104
6	16861	198.5	3239	71.1	1.171
7	2560	226.6	443	74.1	1.251
8	4762	240.3	688	74.1	1.499
9	1861	316.3	657	102.1	1.735
10	11325	240.4	1703	74.1	1.799
11	823	148.3	294	102.1	1.815
12	52631	212.5	5745	71.1	1.934
13	1838	256.6	568	74.1	1.992
14	149771	212.7	17487	71.1	2.25
15	81124	246.4	11695	71.1	2.998
16	26014	430.7	4415	102.1	3.935
17	11394	246.3	3335	74.1	4.102
18	74870	471.9	20761	102.1	4.331
19	27975	274.5	6777	102.1	4.545
20	71846	274.5	17183	74.1	4.55
21	9752	274.7	2769	71.1	4.558
22	54164	228.5	14395	74.1	4.693
23	40098	228.3	9760	102.1	4.704

Table A1.2 Continued

Cpd	Area	Base Peak	Height	m/z	RT
24	242439	274.7	64396	102.1	4.838
25	160177	274.9	41271	71.1	4.841
26	1103225	274.7	291003	102.1	4.94
27	3554355	274.7	916483	74.1	4.941
28	540954	274.6	135572	71.1	4.941
29	25599	242.6	6332	71.1	5.17
30	11685	242.4	3312	102.1	5.171
31	55340	274.5	7700	74.1	5.174
32	3151191	256.7	694673	74.1	5.594
33	3492433	256.7	773989	71.1	5.595
34	6994584	511.9	1765250	102.1	5.595
35	133074	256.5	24117	102.1	5.77
36	43789	256.6	7724	74.1	5.936
37	4714	256.5	2595	102.1	6.036
38	2517	256.6	1315	71.1	6.045
39	18950	256.6	4127	74.1	6.053
40	24217	256.6	4900	74.1	6.101
41	6901	256.9	2416	102.1	6.117
42	136	256.6	256	71.1	6.123
43	9346	256.7	2874	74.1	6.214
44	4543	256.6	1817	71.1	6.246
45	44251	284.5	12183	74.1	6.343
46	54276	284.5	13956	102.1	6.344
47	22971	284.6	8057	71.1	6.344
48	-4650	256.4	-265	71.1	6.428
49	38299	233.6	11893	71.1	6.553
50	11263	256.5	2295	74.1	6.583
51	1854	256.7	1256	71.1	6.647
52	4370	228	1544	102.1	6.847
53	23084	256.5	2432	74.1	6.873
54	13446	256.6	4297	71.1	6.944
55	41760	415	10485	71.1	7.035
56	-9994	391.7	-2775	71.1	7.18
57	2880	372.8	1109	74.1	7.195
58	6399	273.5	2784	71.1	7.266
59	13105	256.2	2522	74.1	7.328
60	165259	256.7	41900	74.1	7.512
61	130831	256.8	37192	71.1	7.516
62	362135	256.7	90958	102.1	7.516
63	6776	256.3	2954	102.1	7.625
64	9966	257	3141	74.1	7.628
65	28830	282.8	9068	71.1	7.654
66	13401	353.9	2615	74.1	7.716
67	3421	228.6	1341	102.1	7.871
68	60	391.6	116	71.1	7.908
69	43526	391.8	10269	71.1	7.998

Table A1.2 Continued

Cpd	Area	Base Peak	Height	m/z	RT
70	39557	284.4	10687	102.1	8.187
71	4274	284.5	2094	71.1	8.189
72	19699	284.7	4941	74.1	8.19
73	680	419.6	647	71.1	8.243
74	-22912	419.8	-336	71.1	8.377
75	2033	256.8	1002	71.1	8.603
76	3955	256.5	1127	102.1	8.819
77	21395	391.7	3232	71.1	8.915
78	7699	391.7	2327	71.1	9.038
79	17050	447.5	3496	71.1	9.22
80	35503	445.1	7833	71.1	9.34
81	17955	448	4050	71.1	9.507

Table A1.3: Selected compounds with observed mass, retention time and observed fragment ion in sample 11.

Sample	Compound	Mass	RT	Fragment
11	1	317.6	1.36	102.1
	2	274.7	4.92	102.1
		274.8		74.1
		318.9		74.1
		548.1		74.1
		274.8		71.1
		318.9		71.1
		548.1		71.1
	3	256.6	5.57	102.1
		512		102.1
		256.7		74.1
		300.9		74.1
		512		74.1
		256.7		71.1
		334.7		71.1
		512		71.1

Table A1.4: Full list of compounds found in Sample 11

Cpd	Area	Base Peak	Height	m/z	RT
1	50031	202.3	16617	102.1	0.581
2	14179	166.3	2817	74.1	0.592
3	310785	302.7	70943	102.1	0.661
4	24679	262.7	3622	71.1	0.872
5	51353	262.4	7091	71.1	1.062
6	388369	316.9	47834	102.1	1.132
7	18349	198.6	4289	71.1	1.204
8	717841	317.6	53203	102.1	1.364

Table A1.4 Continued

Cpd	Area	Base Peak	Height	m/z	RT
9	11915	212.2	2709	71.1	1.879
10	69887	212.5	10850	71.1	2.031
11	131166	212.6	16693	71.1	2.373
12	96352	246.6	14417	71.1	3.049
13	5515	430.7	1604	102.1	3.964
14	34977	246.4	10235	74.1	4.111
15	79161	472.1	20872	102.1	4.314
16	149095	274.8	42237	74.1	4.526
17	36085	274.3	9001	102.1	4.531
18	81401	228.8	23093	74.1	4.681
19	77049	228.4	20382	102.1	4.685
20	380858	274.7	103714	102.1	4.829
21	200700	274.7	55514	71.1	4.833
22	771833	274.7	185304	71.1	4.92
23	4797493	274.8	1141941	74.1	4.921
24	1672850	274.5	385988	102.1	4.922
25	25973	242.4	6418	102.1	5.139
26	43633	242.7	12818	71.1	5.146
27	75639	242.5	14794	74.1	5.152
28	9869517	511.9	2210319	102.1	5.571
29	4354282	256.7	842996	74.1	5.572
30	4249202	256.7	859222	71.1	5.573
31	180293	256.6	28727	102.1	5.769
32	89168	256.8	11648	71.1	5.935
33	72257	256.7	9854	74.1	5.982
34	33651	256.6	9433	71.1	6.01
35	59394	256.3	9384	74.1	6.056
36	2355	256.4	1788	102.1	6.128
37	44040	256.6	8266	71.1	6.169
38	-8684	256.6	-612	102.1	6.208
39	24011	256.4	5767	74.1	6.227
40	50315	256.7	9105	71.1	6.253
41	79748	284.6	22472	102.1	6.346
42	82579	284.8	19089	74.1	6.346
43	80145	284.5	17138	71.1	6.35
44	15065	256.5	4380	74.1	6.461
45	20242	256.5	4087	74.1	6.533
46	94792	233.5	20889	71.1	6.577
47	-713	256.4	289	102.1	6.586
48	17706	256.4	3282	74.1	6.671
49	2460	256.3	987	102.1	6.686
50	15403	256.5	2913	71.1	6.73
51	13310	256.5	3889	71.1	6.79
52	5778	256.6	1419	74.1	7.035
53	115635	256.3	18964	71.1	7.057
54	5668	256.4	1325	102.1	7.1

Table A1.4 Continued

Cpd	Area	Base Peak	Height	m/z	RT
55	3582	256.5	1376	74.1	7.123
56	66810	256.5	9653	71.1	7.311
57	38795	256.9	8514	74.1	7.577
58	46938	256.7	12759	102.1	7.577
59	54472	256.8	9238	71.1	7.578
60	71331	282.7	17823	71.1	7.725
61	6126	270.8	1472	102.1	7.823
62	7014	282.6	3094	71.1	7.824
63	27165	391.8	3763	71.1	8.999
64	5200	256.4	1194	74.1	9.926

Table A1.5: Selected compounds with observed mass, retention time and observed fragment ion in sample 53.

Sample	Compound	Mass	RT	Fragment
53	1	317.7	1.45	102.1
	2	212.6	2.37	71.1
	3	246.6	3.02	71.1
	4	471.1	4.34	102.1
	5	274.8	4.85	102.1
		318.8		102.1
		274.7		71.1
	6	318.8	4.99	74.1
				102.1
	7	300.1	5.51	74.1
				102.1
	8	256.9	7.54	74.1
		297.9		74.1
		256.7		102.1
		297.5		102.1
		256.8		71.1
		297.6		71.1
	9	282.8	7.68	71.1
	10	284.6	8.23	102.1
		327		102.1

Table A1.6: Fill list of compounds found in Sample 53

Cpd	Area	Base Peak	Height	m/z	RT
1	43890	202.8	13355	102.1	0.581
2	22774	166.4	3602	74.1	0.635
3	254927	316.8	66390	102.1	0.66
4	12229	262.5	3382	71.1	0.805
5	6058	317.7	1580	102.1	0.83

Table A1.6 Continued

Cpd	Area	Base Peak	Height	m/z	RT
6	39450	317.3	7287	102.1	1.028
7	117562	198.5	12747	71.1	1.066
8	176770	317.7	19408	102.1	1.453
9	83296	212.5	9399	71.1	2.026
10	185159	212.6	18245	71.1	2.374
11	76482	246.6	9795	71.1	3.026
12	11412	430.8	2421	102.1	3.948
13	95914	471.7	26614	102.1	4.337
14	4056	272.8	1305	74.1	4.681
15	4968	272.4	1310	102.1	4.684
16	213392	274.8	56340	102.1	4.85
17	163707	274.7	41883	71.1	4.856
18	170850	318.8	46349	74.1	4.989
19	57487	318.7	16565	102.1	4.992
20	241296	300.9	67826	74.1	5.515
21	442315	300.7	124661	102.1	5.518
22	7955	256.6	1600	74.1	5.641
23	21461	362.8	5212	102.1	5.773
24	33115	322.9	3849	71.1	6.352
25	22784	233.8	5955	71.1	6.571
26	5291	363.6	2021	71.1	6.656
27	58571	458.8	14753	71.1	7.039
28	10536	273.7	3286	71.1	7.286
29	141583	256.9	36794	74.1	7.54
30	278264	256.7	72668	102.1	7.543
31	143466	256.8	37271	71.1	7.543
32	56711	282.8	15013	71.1	7.681
33	5239	327.2	1387	71.1	8.119
34	42404	284.6	11250	102.1	8.234
35	8627	284.6	2394	74.1	8.237
36	18206	284.5	5232	71.1	8.239
37	4246	256.3	664	74.1	8.333
38	1758	186.8	579	102.1	8.423
39	1816	737.9	455	74.1	8.54

Table A1.7: Selected compounds with observed mass, retention time and observed fragment ion in sample 54.

Sample	Compound	Mass	RT	Fragment
54	1	274.7	4.59	102.1
		548.2		102.1
		274.8		74.1
		274.8		71.1
		547.9		71.1
	2	256.8	5.26	74.1
		512		74.1
		256.8	5.26	71.1
		512		71.1
		256.8	5.26	102.1
		512		102.1

Table A1.8: Full list of compounds found in Sample 54.

Cpd	Area	Base Peak	Height	m/z	RT
1	216602	302.8	48232	102.1	0.657
2	16285	233.8	2088	74.1	0.657
3	8061	145.1	1752	71.1	0.725
4	2703	145.5	962	71.1	0.873
5	2142	262.3	1214	71.1	0.974
6	11071	198.7	2680	71.1	1.097
7	54624	212.6	7391	71.1	1.992
8	52964	246.6	11246	71.1	2.884
9	11267	202.3	3432	71.1	3.536
10	14207	430.7	2909	102.1	3.639
11	12418	246.7	3230	74.1	3.804
12	34824	471.7	9586	102.1	3.984
13	9933	274.6	3105	71.1	4.199
14	96116	274.7	24481	74.1	4.207
15	20365	274.7	5845	102.1	4.211
16	5326	230.4	1757	71.1	4.353
17	30065	228.7	8507	74.1	4.376
18	143334	274.7	38381	102.1	4.481
19	94608	274.8	25394	71.1	4.482
20	729988	274.7	193182	102.1	4.598
21	2528103	274.8	670925	74.1	4.599
22	374769	274.8	98654	71.1	4.6
23	20624	242.6	5192	71.1	4.824
24	24547	274.5	2531	74.1	4.829
25	5326	275.2	1467	74.1	5.009
26	2250096	256.8	549485	74.1	5.258
27	2305620	256.7	545552	71.1	5.259
28	4745387	511.9	1247980	102.1	5.259
29	21279	256.6	6403	102.1	5.43

Table A1.8 Continued

Cpd	Area	Base Peak	Height	m/z	RT
30	36140	256.6	6704	74.1	5.459
31	38159	256.5	5487	102.1	5.543
32	36488	256.4	4511	71.1	5.651
33	15328	256.7	2928	74.1	5.673
34	12646	256.4	2463	102.1	5.791
35	10505	256.5	2412	74.1	5.803
36	10062	256.6	2204	71.1	5.814
37	12027	256.5	3435	71.1	5.944
38	21121	284.3	4671	71.1	6.011
39	25247	284.8	5317	74.1	6.018
40	16802	284.4	3432	102.1	6.022
41	15875	256.2	2242	102.1	6.113
42	21617	300.8	4780	71.1	6.125
43	8123	256.4	1495	74.1	6.16
44	27113	233.3	5247	71.1	6.234
45	7239	256.5	1951	102.1	6.236
46	3161	256.8	569	102.1	6.326
47	2266	256.6	633	102.1	6.426
48	5974	256.6	1428	102.1	6.603
49	7454	256.5	2307	74.1	6.623
50	58603	256.3	8793	71.1	6.703
51	1187	256.4	513	74.1	6.757
52	9886	301.1	1905	71.1	6.944
53	4846	256.4	1280	74.1	7.007
54	34221	256.8	5720	71.1	7.186
55	18728	256.8	5555	102.1	7.194
56	19622	256.3	5665	74.1	7.197
57	31478	282.8	8010	71.1	7.338
58	6319	284.5	1621	102.1	7.814

Table A1.9: Selected compounds with observed mass, retention time and observed fragment ion in sample 93.

Sample	Compound	Mass	RT	Fragment
93	1	274.7	4.59	102.1
		318.8		102.1
		548.1		102.1
		274.7		71.1
		548.1		71.1
		274.7		74.1
		548.1		74.1
	2	256.7	5.58	102.1
		511.9		102.1
		256.7		71.1
		511.9		71.1
		256.7		74.1

Table A1.9 Continued

		511.9		74.1
	3	256.6	7.51	71.1
		298.5		71.1
		256.6		74.1
		298.5		74.1

Table A1.10: Full list of extracted compounds found in Sample 93

Cpd	Area	Base Peak	Height	m/z	RT
1	27724	202.6	9127	102.1	0.579
2	169827	302.7	42407	102.1	0.661
3	4808	262.6	1589	71.1	0.771
4	10501	262.3	3174	71.1	1.027
5	38426	212.6	5263	71.1	1.986
6	111962	212.6	11541	71.1	2.376
7	55880	246.5	9035	71.1	3.047
8	6482	333.1	2014	102.1	3.357
9	10619	202.3	2677	71.1	3.833
10	8214	464.8	1318	102.1	4.002
11	6104	246.4	1805	74.1	4.122
12	27754	471.6	8185	102.1	4.341
13	80281	274.6	22689	74.1	4.575
14	15800	274.5	4602	102.1	4.576
15	32663	228.8	9409	74.1	4.719
16	13436	228.5	4078	102.1	4.724
17	178462	274.7	51181	102.1	4.847
18	121957	274.6	35324	71.1	4.854
19	886608	274.7	232971	102.1	4.947
20	399938	274.8	103651	71.1	4.947
21	2853377	274.8	748862	74.1	4.947
22	25666	274.6	5678	74.1	5.086
23	9182	274.8	2572	74.1	5.146
24	5463	242.6	1701	102.1	5.159
25	25726	242.4	7216	71.1	5.161
26	5782247	511.9	1456528	102.1	5.581
27	2616145	256.7	604405	71.1	5.581
28	2830367	256.8	656945	74.1	5.581
29	63870	256.4	6840	102.1	5.794
30	39281	256.6	7723	74.1	5.8
31	21213	256.6	4823	74.1	5.917
32	16219	256.6	3981	102.1	5.918
33	1557	256.4	1039	71.1	5.941
34	2713	256.6	1298	71.1	6.015
35	17454	256.7	3211	102.1	6.034
36	29429	256.6	5043	74.1	6.05
37	708	256.4	646	71.1	6.111
38	12013	256.3	3289	74.1	6.125

Table A1.10 Continued

Cpd	Area	Base Peak	Height	m/z	RT
39	22808	376.4	5021	102.1	6.129
40	6722	256.5	2221	71.1	6.204
41	14871	256.6	2525	74.1	6.246
42	11491	256.5	3317	102.1	6.279
43	50378	284.7	8207	74.1	6.348
44	9739	284.5	3275	71.1	6.351
45	48036	284.6	7264	102.1	6.356
46	6942	233.2	2287	71.1	6.574
47	2255	256.6	887	71.1	6.739
48	26035	415	6712	71.1	7.019
49	11361	273.5	2936	71.1	7.252
50	279599	256.6	72187	102.1	7.508
51	144145	256.6	33238	71.1	7.509
52	168955	256.8	40106	74.1	7.509
53	27531	282.8	7830	71.1	7.672
54	6231	256.5	1672	74.1	8.051
55	6058	284.8	2210	71.1	8.242
56	54495	284.8	14893	102.1	8.245
57	18296	284.6	4449	74.1	8.249

Table A1.11: Selected compounds with observed mass, retention time and observed fragment ion in sample 102.

Sample	Compound	Mass	RT	Fragment
102	1	317.6	1.4	102.1
	2	212.5	1.93	71.1
	3	212.5	2.39	71.1
	4	246.7	3.04	71.1
	5	274.8	4.83	102.1
		318.5		102.1
		274.8		71.1
		230.7		71.1
	6	318.8	4.98	74.1
	7	301	5.58	74.1
		301		102.1
	8	363.1	5.79	102.1
	9	256.8	7.52	71.1
		297.8		71.1
		256.8		102.1
		297.8		102.1
		256.8		74.1
		297.8		74.1
	10	284.7	8.16	102.1
		325.9		102.1
		284.6		74.1
		326.1		74.1
		284.9		71.1

Table A1.12: Full list of extracted compounds found in Sample 102.

Cpd	Area	Base Peak	Height	m/z	RT
1	73732	202.4	25006	102.1	0.582
2	17748	116.6	2782	71.1	0.637
3	14324	208	2578	74.1	0.641
4	364438	316.8	91796	102.1	0.658
5	9675	418.2	1751	102.1	0.82
6	11048	262.3	2763	71.1	0.844
7	23320	262.5	4913	71.1	0.936
8	44144	198.4	7657	71.1	1.088
9	30876	198.4	6916	71.1	1.194
10	170543	317.6	17612	102.1	1.406
11	36767	212.5	6237	71.1	1.928
12	7123	212.6	2348	71.1	2.038
13	115900	212.5	11057	71.1	2.389
14	54700	246.7	8981	71.1	3.037
15	7070	202.6	1880	71.1	3.819
16	14128	430.9	2554	102.1	3.952
17	7997	236.7	2027	71.1	3.961
18	49885	471.7	13022	102.1	4.31
19	4856	318.3	1459	102.1	4.592
20	177913	274.8	46671	102.1	4.835
21	131016	274.8	34834	71.1	4.84
22	25007	318.8	7135	74.1	4.987
23	14812	318.7	4709	102.1	4.988
24	6370	362.8	1001	74.1	5.289
25	7202	336.5	1393	102.1	5.297
26	106435	301	30184	74.1	5.518
27	140314	300.8	39519	102.1	5.518
28	1581	374.9	572	74.1	5.644
29	29173	363.1	7981	102.1	5.793
30	2075	398.9	536	74.1	6.04
31	18390	376.7	5212	102.1	6.178
32	6291	305.5	1622	71.1	6.311
33	7196	441.3	2229	102.1	6.354
34	8515	256.9	1504	71.1	6.422
35	2077	101.8	717	74.1	6.453
36	16666	233.8	3556	71.1	6.603
37	4315	479.1	700	74.1	6.714
38	2806	469.2	826	102.1	6.717
39	6361	423.7	1400	71.1	6.745
40	17956	228.8	3088	71.1	6.888
41	33763	459	7636	71.1	7.066
42	12282	273.2	3230	71.1	7.29
43	149775	256.8	39753	71.1	7.519
44	341476	256.6	86801	102.1	7.519

Table A1.12 Continued

Cpd	Area	Base Peak	Height	m/z	RT
45	166252	256.7	42696	74.1	7.521
46	12179	282.6	3410	71.1	7.641
47	10585	256.3	2314	102.1	7.693
48	4955	282.3	1361	71.1	7.724
49	52867	284.7	14877	102.1	8.158
50	23433	284.6	6219	74.1	8.167
51	14176	284.9	4069	71.1	8.171
52	3876	338.4	1307	71.1	8.977

Table A1.13: Selected compounds with observed mass, retention time and observed fragment ion in sample 117.

Sample	Compound	Mass	RT	Fragment
117	1	274.7	4.92	71.1
		548		71.1
		274.8		74.1
		274.7		102.1
		548		102.1
	2	256.7	5.61	71.1
		512		71.1
		256.7		74.1
		512		74.1
		256.7		102.1
		512		102.1
	3	256.7	7.52	71.1
		297.8		71.1
		256.6		74.1
		298		74.1
		256.7		102.1
		297.8		102.1
	4	284.6	8.23	102.1
		325.6		102.1

Table A1.14: Full list of extracted compounds found in Sample 117

Cpd	Area	Base Peak	Height	m/z	RT
1	5603	166.4	1129	74.1	0.618
2	168222	316.8	39971	102.1	0.661
3	4560	198.3	1460	71.1	1.103
4	63320	317.4	12592	102.1	1.125
5	54710	317.5	6837	102.1	1.274
6	54526	317.6	9838	102.1	1.395
7	36107	317.5	7979	102.1	1.481
8	25948	212.5	4189	71.1	2.016
9	51412	212.6	7015	71.1	2.362

Table A1.14 Continued

10	20079	246.6	3230	71.1	3.011
11	18479	430.6	3061	102.1	3.962
12	5592	246.7	1685	74.1	4.114
13	17721	471.8	5142	102.1	4.298
14	25741	274.6	7353	74.1	4.512
15	9489	274.8	2683	102.1	4.526
16	7503	228.3	2363	102.1	4.676
17	15990	228.3	4167	74.1	4.688
18	61913	274.8	17692	71.1	4.803
19	95698	274.6	27336	102.1	4.804
20	233496	274.7	61350	71.1	4.924
21	1766607	274.8	466540	74.1	4.924
22	496799	274.7	138242	102.1	4.925
23	14214	274.5	5368	74.1	5.042
24	12583	242.6	3374	74.1	5.141
25	6224	242.4	2094	71.1	5.156
26	2192878	256.7	535895	71.1	5.614
27	4256487	512	1115337	102.1	5.615
28	2005486	256.7	490656	74.1	5.615
29	64839	256.8	11857	71.1	5.761
30	37136	256.7	7821	74.1	5.808
31	16801	256.2	2857	102.1	5.885
32	15672	256.4	3369	71.1	5.923
33	12732	256.6	1560	74.1	6.046
34	10629	256.8	2726	71.1	6.074
35	3931	376.8	1206	102.1	6.126
36	14261	256.3	2865	71.1	6.192
37	3690	440.8	1261	102.1	6.282
38	11543	284.7	3144	102.1	6.361
39	27642	284.9	7224	74.1	6.363
40	15017	284.3	3575	71.1	6.369
41	6762	233.8	1610	71.1	6.594
42	28120	353	5634	71.1	7.046
43	154647	256.7	40698	71.1	7.523
44	106131	256.6	29651	74.1	7.523
45	270043	256.7	71954	102.1	7.524
46	16417	282.6	4594	71.1	7.665
47	61625	284.6	17856	102.1	8.224
48	23424	284.6	7872	71.1	8.229
49	30777	284.7	8534	74.1	8.232
50	14347	338.9	4105	71.1	9.077
51	25163	428.1	4624	71.1	9.243

PUBLICATIONS & PRESENTATIONS

Scientific Publications:

Anbazhagan D, Mansor M, Yan GOS, Md Yusof MY, Hassan H, *et al*. (2012) Detection of Quorum Sensing Signal Molecules and Identification of an Autoinducer Synthase Gene among Biofilm Forming Clinical Isolates of *Acinetobacter* spp.. PLoS ONE 7(7): e36696. doi:10.1371/journal.pone.0036696

Deepa Anbazhagan, Wang SM, Marzida M, Gracie OSY, Mohd Yasim Y, Shamala Devi S. (2011) Development of Conventional and Real-time multiplex PCR assays for the detection of Nosocomial Pathogens. Brazilian Journal of Microbiology *42*(2): 448-458

Deepa Anbazhagan, Geethanjali GK, Marzida M, Gracie OSY, Mohd Yasim Y, Shamala Devi S. (2010) Multiplex Polymerase Chain Reaction (PCR) Assays for the detection of Enterobacteriaceae in clinical samples. African Journal of Microbiology Research *4*(11): 1186-1191

Wong Eng Hwa, Geetha S, Marzida Bt M, Gracie OSY, **Deepa Anbazhagan** and Shamala Devi S. (2010) Iron regulated outer membrane proteins (IROMPs) as potential target for antimicrobial therapy against carbapenem-resistant *Acinetobacter* spp. isolated from University Malaya Medical Centre. Indian Journal of Medical Research 131: 578-583

Wong EW, Mohd Yusof MY, Bt Mansor M, **Anbazhagan D**, Ong SY, Sekaran SD. (2009) Disruption of adeB gene has a greater effect on resistance to meropenems than adeA gene in *Acinetobacter* spp. isolated from University Malaya Medical Centre Singapore Medical Journal *50*(8): 822-826

Manuscripts submitted:

Deepa Anbazhagan, Marzida M, Gracie OSY, Mohd Yasim Y, Shamala Devi S. (2012) Inhibition of quorum sensing and biofilm formation in clinical isolates of *Acinetobacter* spp. by extracts from soil *Bacillus* species, *Phyllanthus* spp., lemon and garlic. Current Microbiology (under review).

Oral Presentations:

- **Deepa Anbazhagan,** Shamala Devi Sekaran (2009) Detection of Quorum Sensing Signal Molecules among Biofilm forming clinical isolates of *Acinetobacter* spp. The 14th Biological Sciences Graduate Congress, 10^{th} -12^{th} December 2009, Chulalangkorn University, Thailand.

Poster presentations:

- **Deepa Anbazhagan,** Marzida Bt. Mansor, Gracie Ong Siok Yan, Mohd Yasim Mohd Yusof, Shamala Devi Sekaran (2012) Inhibition of Quorum Sensing and biofilm formation in clinical isolates of *Acinetobacter* spp. by soil *Bacillus* species extracts". National Postgraduate Seminar (NPS2012), 11th July 2012. University of Malaya, Malaysia.

- **Deepa Anbazhagan,** Marzida Bt. Mansor, Mohd Yasim Mohd Yusof, Shamala Devi Sekaran (2010) Quorum Sensing and Quorum Quenching as a means to attenuate the pathogenecity of human *Acinetobacter* spp. isolates in Malaysia. Universiti Malaya Innovation and Creativity Expo 2010, 1st -3rd April 2010, University of Malaya, Malaysia.

- **Deepa Anbazhagan,** Marzida Bt. Mansor, Mohd Yasim Mohd Yusof, Shamala Devi Sekaran (2009) Detection of Quorum Sensing Signal Molecules among Biofilm forming clinical isolates of *Acinetobacter* spp. International Congress of Malaysian Society for Microbiology (ICMSM), 1st -4th December 2009, Penang, Malaysia.

Awards and Recognitions:

- **Merit Award for Poster Presentation**, ICMSM, Penang (2009)

- **Travel Award**, Chulalangkorn University, Thailand (2009)

- **Graduate Research Assistantship Scheme (GRAS),** University of Malaya (2009, 2010, 2011)

Presented at the 14th Biological Sciences Graduate Congress, 10th -12th December 2009, Chulalangkorn University, Thailand.

Detection of Quorum Sensing Signal Molecules among Biofilm forming clinical isolates of *Acinetobacter* spp.

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Quorum Sensing is a term that describes an environmental sensing system that allows bacteria to monitor their own population density. Many gram negative bacteria use *N*-acyl-homoserine lactones (AHLs) as Quorum Sensing (QS) signal molecules. This cell density dependent regulation of gene expression contributes significantly to the size and development of the biofilm. In this study, we sought to find out if the biofilm formation among clinical isolates of *Acinetobacter* spp. is under the control of autoinducing QS molecules.

Biofilm formation among 50 clinical isolates of *Acinetobacter* spp. was assessed by microtitre plate method and the statistical analysis showed 60% of the isolates significantly formed biofilms. The production of AHL molecules among seven of these isolates were detected using *Chromobacterium violalecim* cv026 biosensor system. Partial characterisation of autoinducers was carried out by thin layer chromatography (TLC) bioassay. Mass Spectrometric analysis to structurally characterise the type of AHL molecules further revealed that some of the strains produce *N*-decanoyl homoserine lactone signal molecules.

The presence of QS signal molecules among *Acinetobacter* spp. is of great significance as they aid in Biofilm formation which in turn confers various properties of pathogenecity including drug resistance. Further understanding of the rate of the QS mechanism in the regulation of pathogenecity may be used to elucidate new Quorum-Quenching strategies to control cell-cell communication and thus attenuating the pathogenecity in multidrug resistant clinical isolates. Consequently, a drug capable of blocking QS is likely to increase the susceptibility of these pathogenic organisms to host defences and its clearance from the host.

Presented at the National Postgraduate Seminar (NPS 2012), 11th July 2012, University of Malaya, Malaysia.

Inhibition of quorum sensing and biofilm formation in clinical isolates of *Acinetobacter* spp. by soil *Bacillus* species extracts

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Introduction

In many of the Gram-negative bacteria, quorum sensing (bacterial communication) is mediated by a group of widely conserved signal molecules called *N*-acylhomoserine lactones (AHLs), known as autoinducers. These autoinducers are involved in the regulation of diverse biological functions, including expression of virulence genes in many pathogenic bacteria. Many soil bacteria are capable of interfering with the quorum sensing system by enzymatic degradation of AHLs, a process referred to as quorum quenching. Here we report the identification of two soil isolates with a strong AHL-inactivating enzyme activity.

Methods

The soil isolates were subjected to preliminary taxonomic analysis. The extracts from soil isolates of the genus *Bacillus* were further subjected to AHL- inactivation assay to analyse their capability to degrade synthetic *N*-hexanoyl homoserine lactone (HHL). Polymerase chain reaction (PCR) was carried out to detect the presence of *aii*A homologue gene. Strong biofilm-forming clinical isolates of *Acinetobacter* spp., which were capable of producing quorum sensing signal molecules, were tested for the inhibition of their biofilm forming capabilities after treatment with the extracts from these soil bacilli with quorum quenching activity.

Results

Morphological phenotypes and *16s rDNA* sequence analysis showed that these isolates probably belong to the genus *Bacillus*. The AHL-inactivation assay showed that the two isolates rapidly degrade the synthetic *N*-hexanoyl homoserine lactone (HHL). We also identified the *aii*A homologue gene in both the *Bacillus* sp. isolates by PCR analysis. Results showed that the soil bacillus extracts were capable of inhibiting the biofilm formation and also degrade the AHLs produced by the clinical isolates of *Acinetobacter* spp.

Conclusion

Our results indicate that the AHLs -inactivation approach represents a promising strategy for prevention of diseases in which virulence is regulated by quorum sensing.

Key words
AHL- degrading enzymes, Quorum quenching, Biofilms.

Presented at the Universiti Malaya Innovation and Creativity Expo 2010, 1st -3rd April 2010, University of Malaya, Malaysia.

Quorum Sensing blockade as a novel strategy for attenuating Pathogenecity in *Acinetobacter* spp.

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One of the most fascinating discoveries in microbiology is that bacteria communicate with each other. Bacterial communication is called 'Quorum Sensing'(QS) because it is a density-dependent process that functions when a population is of sufficient size. It involves the production of diffusible signal molecules that co-ordinate gene expression. Quorum Sensing systems are widespread and modulate many physiological processes in bacteria associated with humans, plants, animals, soils, marine and fresh waters. Understanding the role of quorum sensing in pathogenecity offers the opportunity to develop novel approaches to combat human, animal and plant diseases. There is increasing evidence that animals and plants 'listen' to bacterial signals and utilise these signals in complex ways. Some plants and animals produce molecules that mimic quorum sensing molecules and so confuse regulation in bacteria; blocking of cell-to-cell signalling may have evolved as a successful strategy to resist infection by pathogenic bacteria.

The most obvious alternative to antibiotic-mediated killing or growth inhibition would be to attenuate the bacteria with respect to pathogenecity. The regulation of virulence via quorum sensing confers a strategic advantage over host defences. The use of quorum sensing signal blockers to attenuate bacterial pathogenecity, rather than bacterial growth, is therefore highly attractive, particularly with respect to the emergence of multi-antibiotic resistant bacteria. Characterisation of the crystal structures of several types of quorum-quenching enzymes has provided valuable information to elucidate the catalytic mechanisms, as well as clues for future protein tailoring and molecular improvement.

Acinetobacter is frequently isolated in nosocomial infections and is especially prevalent in intensive care units, where both sporadic cases as well as epidemic and endemic occurrence is common. The spectrum of antibiotic resistances of these organisms together with their survival capabilities makes them a threat to hospitals as documented by recurring outbreaks. Thus a study on their mode of drug resistance and ways to block these mechanisms becomes necessary.

Objectives of the proposed project

The proposed project aims to investigate the various QS molecules involved in *Acinetobacter* and also study the quorum quenching strategies that can effectively block QS and thus attenuate *Acinetobacter* pathogens.

Key objectives:

1. To prove that the *Acinetobacter* spp. isolated from nosocomial infections have QS mediated virulence.
2. To perform various assays to identify, quantify and characterise the QS molecules (*N*-acyl homoserine lactones (AHL) in gram negative organisms)
3. To study the QS molecules and construct analogues to QS molecules which can be used to block the QS mediated processes.
4. To use the molecular techniques to elucidate the QS molecule binding sequences in the DNA and thus create sequence homologues which can be used for quorum quenching.
5. To study about the effects of natural sources as potential Quorum quenchers.

A few of the possible mechanisms for exploiting the regulatory mechanisms of pathogenic bacteria employing QS systems can definitely be elucidated at the end of this proposed research. This approach will definitely be helpful in formulating drugs that are capable of attenuating not only human pathogens, but also control crop diseases as well as manipulate plant-microbe interactions for improved crop production in the future. This research would for sure lay foundation for novel advances in chemotherapy in the future.

Presented at the International Congress of Malaysian Society for Microbiology (ICMSM), 1st -4th December 2009, Penang, Malaysia.

DETECTION OF QUORUM SENSING SIGNAL MOLECULES AMONG BIOFILM FORMING CLINICAL ISOLATES OF *Acinetobacter* spp.

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Quorum Sensing is a term that describes an environmental sensing system that allows bacteria to monitor their own population density. Many gram negative bacteria use *N*-acyl-homoserine lactones (AHLs) as Quorum Sensing (QS) signal molecules. This cell density dependent regulation of gene expression contributes significantly to the size and development of the biofilm. In this study, we sought to find out if the biofilm formation among clinical isolates of *Acinetobacter* spp. is under the control of autoinducing QS molecules.

Biofilm formation among 50 clinical isolates of *Acinetobacter* spp. was assessed by microtitre plate method and the statistical analysis was done using SPSS software which showed that 60% of the isolates significantly formed biofilms. The production of AHL molecules among seven of these isolates were detected using *Chromobacterium violaceum* CV026 biosensor system. AHLs from selected strains were profiled by thin layer chromatography. The simultaneous use of monitor strains in the top-layer was necessary for the detection of the AHLs. An agar well-diffusion assay based on *Chromobacterium violaceum* CV026 was used for quantifying AHLs from the bacterial supernatants. Partial characterisation of autoinducers by thin layer chromatography (TLC) bioassay showed that some of the strains produce *N*-decanoyl homoserine lactone signal molecules.

The presence of QS signal molecules among *Acinetobacter* spp. is of great significance as they aid in Biofilm formation which in turn confers various properties of pathogenecity including drug resistance. Further understanding of the rate of the QS mechanism in the regulation of pathogenecity may be used to elucidate new Quorum-Quenching strategies to control cell-cell communication, thus attenuating the pathogenecity in multidrug resistant clinical isolates. Consequently, a drug capable of blocking QS is likely to increase the susceptibility of these pathogenic organisms to host defences and its clearance from the host.