CHAPTER 4

ADHERENCE AND MOTILITY CHARACTERISTICS OF CLINICAL *ACINETOBACTER BAUMANNII* ISOLATES

4.1 Introduction

Three clonal groups designated international clone I, II and III, have been defined and together form the majority of clinical *A. baumannii* strains found in Europe (Section 1.1.2). The existence of international clone I and II *A. baumannii* isolates in Australia has been shown (Post and Hall 2009; Post *et al.* 2010; Runnegar *et al.* 2010), however, no data are available in respect to the prevalence of these widespread lineages throughout Australia. Furthermore, little is known about the survival and persistence strategies of *A. baumannii* or whether these strategies are universally applied in all clinical isolates.

Although, historically the *Acinetobacter* genus is described as non-motile, which is related to the lack of flagella and therefore its inability to swim (Baumann *et al.* 1968), various studies have shown motility of isolates that belong to the *Acinetobacter calcoaceticus-baumannii* complex (Section 1.2.4). Interestingly, reduced iron levels resulted in down-regulation of several genes that encode the type IV pili system (Section 3.2.5), a system that may function in *A. baumannii* motility. Bacterial motility has been linked to increased virulence in various bacteria, such as *P. aeruginosa* and *D. nodosus* (Section 1.2.4.2). Nonetheless, to date, the role of motility in virulence of *A. baumannii* has not been described.

Another factor that may influence the success of *A. baumannii* as a pathogen is its ability to adhere to abiotic surfaces (Section 1.2.2). Initial attachment to abiotic surfaces is the first step for colonisation and subsequent biofilm formation on medical devices, such as ventilator tubing and catheters. Adherence of *Acinetobacter* isolates to abiotic surfaces has previously been examined by a number of groups, and the Csu type I pilus, Bap, OmpA and production of PNAG appears to be involved in this process (Sections 1.2.2.1 and 1.2.2.2). Another critical step in the pathogenesis of *A. baumannii* is the ability to adhere to eukaryotic cells; studies examining adherence to cell lines have revealed a high level of variability between isolates in their binding capacity (Section 1.2.3).

In this study, the clonal groupings of 56 clinical *Acinetobacter* strains isolated from diverse settings were determined and two distinct forms of motility, twitching and swarming, were investigated. Furthermore, the capacity of these isolates to adhere to both abiotic and biotic surfaces is reported. Within the fully sequenced

strains, this phenotypic information was examined in the context of gene content in an attempt to delineate the molecular factors directing these characteristics.

4.2 Results and Discussion

4.2.1 Strain selection and clonality

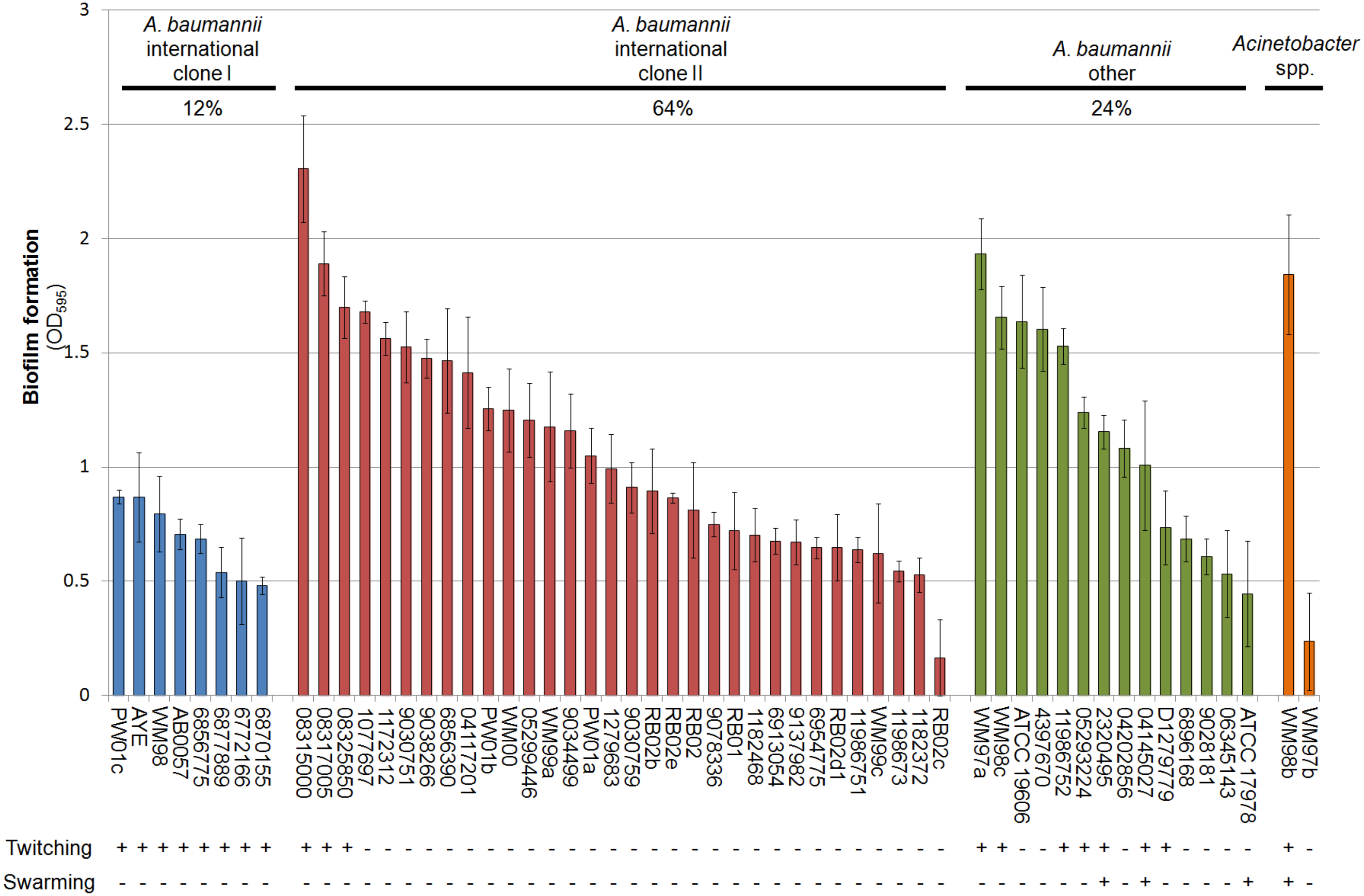
A total of 52 randomly selected Australian clinical *Acinetobacter* strains were used in this study of which 50 were *A. baumannii* isolates, one *Acinetobacter* genome species 13TU (WM98b) and one *Acinetobacter* genome species 3 (WM97b) (Table 2.2). Four non-Australian *A. baumannii* strains were also included in the characterisation; AB0057 (Adams *et al.* 2008), AYE (Fournier *et al.* 2006), ATCC 19606 and ATCC 17978 (Smith *et al.* 2007). The site of isolation of the strains has been listed in Table 2.2.

The clonal groupings amongst clinical *A. baumannii* strains were investigated using an established allele specific multiplex PCR (Section 2.3.1) (Turton *et al.* 2007). This showed that 12% of the *A. baumannii* isolates studied belonged to international clone group I (n=6), 64% to international clone group II (n=32) and 24% were found to not be part of either of these clonal lineages (n=12) (Figure 4.1). No strains were found to belong to international clonal lineage III. This is the first study on the prevalence of the international clones in Australia. The epidemiological grounds for the low prevalence or absence of the international clone III isolates in Australia remain to be determined.

More than 46% of the *A. baumannii* isolates were respiratory tract related, including isolates from sputum (n=16), tracheal aspirate (n=7) or bronchial lavage (n=1) (Table 2.2). Also included were eleven isolates from wound or pus samples, four from urine/catheters, three from blood, two from the rectum, one from bile and one vaginal isolate (Table 2.2).

4.2.2 Motility of *A. baumannii*

It was found that three non-international clone type *A. baumannii* strains (2320495, 04145027 and ATCC 17978) and the *Acinetobacter* genome species 13TU strain WM98b had the ability to migrate on semi-solid agars (Figure 4.1). This form of surface translocation was designated as swarming, as proposed by Kaiser (2007).



Biofilm formation (OD595)
A. baumannii international clone I
12%
A. baumannii international clone II
64%
A. baumannii other
24%
Acinetobacter spp.
PW01c
AYE
WM98
AB0057
6856775
6877889
6772166
6870155
08315000
08317005
08325850
1077697
1172312
9030751
9038266
6856390
04117201
PW01b
WM00
05299446
WM99a
9034499
PW01a
1279683
9030759
RB02b
RB02e
RB02
9078336
RB01
1182468
6913054
9137982
6954775
RB02d1
11986751
WM99c
1198673
1182372
RB02c
WM97a
WM98c
ATCC 19606
4397670
11986752
05293224
2320495
04202856
04145027
D1279779
6896168
9028181
06345143
ATCC 17978
WM98b
WM97b
Twitching
Swarming

Figure 4.1: Characterisation of *Acinetobacter* isolates

The graph represents the level of biofilm formation (OD_{595}), which was investigated in a 96-well microtiter tray using a crystal violet stain method (Section 2.3.3). The error bars represent the standard deviation. All 56 strains were sorted based on species, clonality and biofilm formation; *A. baumannii* international clone I (blue), *A. baumannii* international clone II (red), non-international (other) *A. baumannii* isolates (green) and *Acinetobacter* spp. (orange). Solid MH media (1% agar) and semi-solid LB media (0.25% agar) were used to investigate twitching and swarming motility, respectively (Section 2.3.4). Details about the site of isolation and the source of the strains can be found in Table 2.2.

Swarming motility was further investigated on different media, LB, MH and M9, and at varying temperatures, 25°C, 30°C and 37°C. All swarming strains displayed a more pronounced motile phenotype on semi-solid LB media incubated at 37°C. It was also determined that swarming occurred at a higher rate on media with lower agar percentages (data not shown). The lowest tested concentration of agar was 0.25%, which was used to investigate swarming by the *Acinetobacter* strains in our collection (Section 2.3.4; Figure 4.1).

Various other *A. baumannii* strains, including AYE and AB0057, showed no motility on semi-solid media, however, these strains migrated in the medium-plastic interface of solid media, referred to as twitching motility (Semmler *et al.* 1999). Therefore, all strains were investigated for twitching on both LB and MH media (Figure 4.1). Although some strains had the ability to twitch in LB media, a greater proportion of strains were able to twitch in MH media, no strains were found which could only twitch in LB media. Twitching of various representative strains was studied at temperatures of 25°C, 30°C and 37°C and using varying agar percentages, 0.25%, 0.5%, 0.75% and 1% (Section 2.3.4). These results revealed that twitching occurred at an optimal rate in MH containing 1% agar that was incubated at 37°C. These conditions were used as the standard method for investigation of twitching of *Acinetobacter* species as shown in Figure 4.1. All eight international clone I isolates showed a twitching zone of more than 10 mm (defined to be the minimum in this study). Of the strains which exhibited twitching motility, only a subset also displayed swarming motility, and vice versa (Figure 4.1), highlighting that twitching and swarming represent two distinct phenotypes in *Acinetobacter*.

4.2.3 Adherence to abiotic surfaces and biofilm formation

The ability of *Acinetobacter* to adhere to abiotic surfaces was investigated using a microtiter plate biofilm assay (Section 2.3.3). A substantial difference in biofilm levels between strains was observed (Figure 4.1). International clone II strain 08315000 showed more than 10-fold higher levels of biofilm than international clone II strain RB02c. Analysis of the biofilm data using a two-tailed student *t*-test revealed that international clone I isolates formed less developed biofilms compared to international clone II and non-international clone isolates, $p<0.005$ and $p<0.05$, respectively. No significant difference in biofilm formation was observed between international clone II and non-international clone isolates. Whether these significant differences play an important role in persistence in the hospital environment and

potentially increased dissemination levels requires further examination. A link between the site of isolation of the specimen and the level of biofilm formation was not observed.

4.2.4 Adherence to eukaryotic cell surfaces

Two distinct eukaryotic cell types were used to examine adherence, and potentially invasion and intracellular replication, of a selected number of *A. baumannii* isolates (Section 2.3.6). Detroit 562 human nasopharyngeal cells were chosen in order to mimic adherence/carriage of *A. baumannii* strains in the nasal pharyngeal cavity. The second cell line employed was A549 human type 2 pneumocytes, that has previously been used to mimic adherence to the human lung and as such represents a potential model for pneumonia caused by *A. baumannii* (Section 1.2.3). The *A. baumannii* isolates selected for cell adherence studies displayed differential abiotic surface adherence and motility characteristics. These studies also included a number of previously studied and published strains. Similar to the results on abiotic adherence, there were significant differences between *Acinetobacter* strains in their capacity to adhere to eukaryotic cells (Figure 4.2). For example, differences of more than 17-fold were seen between ATCC 19606 and WM99c when investigating binding to A549 cells. A more than 60-fold difference in adherence to Detroit 562 cells was observed between strains D1279779 and WM97a. Examination of the ability of differing clonal groups to adhere to the eukaryotic cells revealed no clonal specific trends. A significant difference between binding to A549 and Detroit 562 cells was observed for *A. baumannii* strains D1279779 and ATCC 17978 ($p<0.05$, two-tailed student *t*-test). Both of these *A. baumannii* strains showed a higher level of adherence to lung epithelial cells compared to nasopharyngeal cells. All other strains examined had similar levels of binding to the two distinct epithelial cell lines.

4.2.5 Genomic analysis of *A. baumannii* motility and adherence features

Genomic comparison may prove useful for the identification of the molecular mechanisms involved in the characteristics studied here. The complete genome of a number of *A. baumannii* strains has been sequenced and six of these fully sequenced strains were included in this study. Although limited information is available on the molecular mechanism, type IV pili may play a role in *A. baumannii* motility

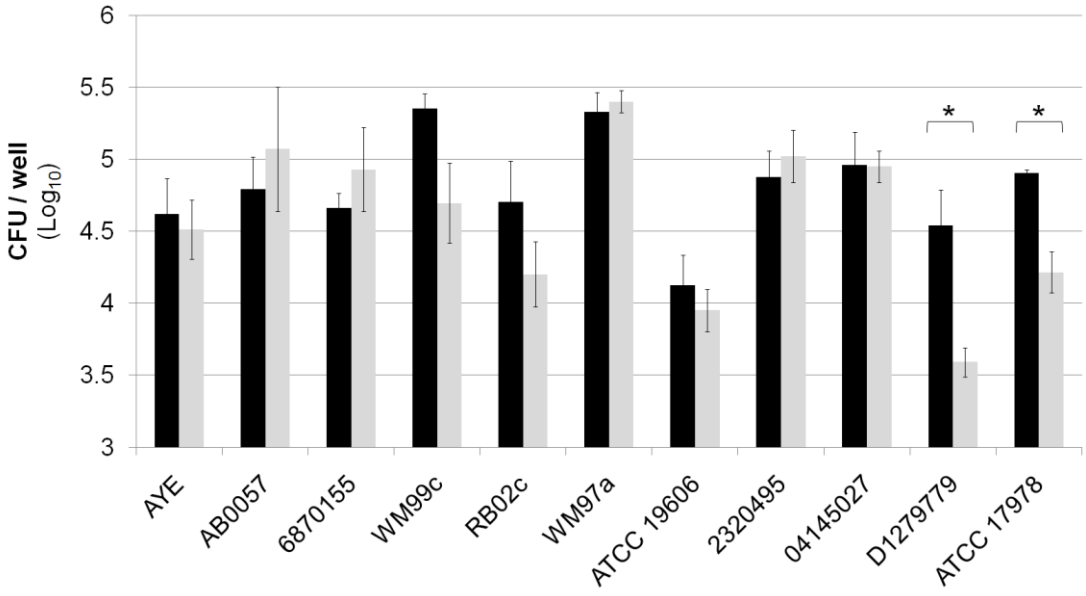


Figure 4.2: Adherence of *A. baumannii* strains to eukaryotic epithelial cells

Depicted are the colony forming units (CFUs) in Log_{10} values of *A. baumannii* cells that adhered to A549 cells (black) and Detroit 562 cells (grey) (Section 2.3.6). Strains D1279779 and ATCC 17978 showed significantly lower binding to Detroit 562 cells compared to A549 cells ($p<0.05$) and are indicated by an asterisk. Statistical analysis was carried out using a two-tailed student *t*-test. Error bars show the standard error of the mean.

(Sections 1.2.4.3 and 3.2.5). Comparative genomic analysis using Mauve (Section 2.7.2) showed that the genes encoding different subunits or regulators of the type IV pili were present in all fully sequenced *A. baumannii* isolates included (data not shown). Furthermore, most proteins encoding structural subunits of the type IV pili showed a high level of conservation (e.g. PilB and PilD), except for PilA, the major fimbrial subunit. In *P. aeruginosa*, a distinctive type of PilA has been linked to an enhanced twitching phenotype and virulence (Stewart *et al.* 2011). In this study, amino acid sequence analysis revealed the presence of different *A. baumannii* PilA groups (Figure 4.3). The isolates within these PilA groups were clonally related and exhibited the same motility characteristics, e.g. the international clone I isolates shared a highly similar PilA amino acid sequence and all exhibited a twitching phenotype. Interestingly, the PilA sequences from other motile bacterial species clustered with PilA from the motile *A. baumannii* isolates, e.g. the *P. aeruginosa* and *D. nodosus* PilA shared the highest homology levels with PilA from international clone I isolates and *X. fastidiosa* PilA with that from ATCC strain 17978 (Figure 4.3).

Linking adherence phenotypes to genotypes was attempted, as multiple adherence mechanisms have been identified as described in Sections 1.2.2.2 and 4.1. The gene encoding Bap (A1S_2724) showed major variation between *Acinetobacter* species. The length can vary dramatically between species, from approximately 1 Kb to over 20 Kb for strains ATCC 17978 and AB307-0294, respectively; nucleotide sequence homology is generally lower than 50% (data not shown). However, no direct link between adherence characteristics and sequence homology could be established using the fully sequenced *A. baumannii* isolates studied here, such as AYE and WM99c. The *pgaABCD* cluster responsible for production of PNAG (Choi *et al.* 2009), and *ompA* (Gaddy *et al.* 2009) displayed a high level of conservation between the investigated strains, therefore, sequence differences that may be linked to a phenotype could not be observed. In total, four different type I pili clusters were identified in the six sequenced strains included in this study; AB57_1744-1747, AB57_2565-2570 (*csu* cluster) (Tomaras *et al.* 2003), AB57_2420-2423 and AB57_2003-2007 (Table 4.1). The locus-tags of *A. baumannii* strain AB0057 were used, as strain ATCC 17978 does not possess all clusters. The *csu* gene cluster was found to be well conserved between the strains investigated; however, *csuB* of strain ATCC 17978 contained a single nucleotide insertion, which resulted in a truncation

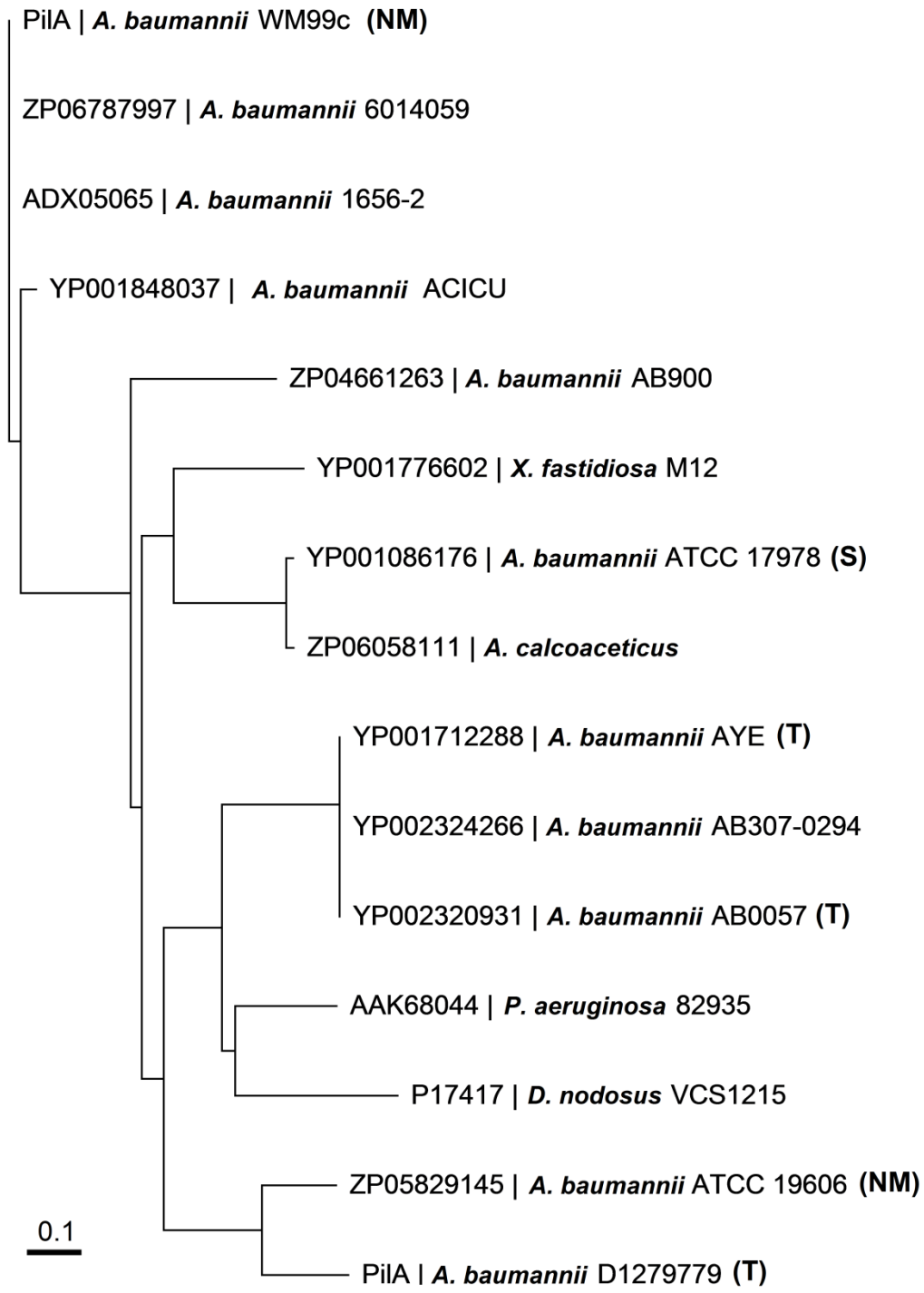


Figure 4.3: PilA similarity analysis

PilA amino acid sequences of various sequenced *Acinetobacter* strains were investigated. The PilA sequences from other bacterial species that showed a high level of similarity, identified by Blastp searches (Section 2.7.2), *viz. P. aeruginosa*, *X. fastidiosa* and *D. nodosus*, were also included. The sequences were aligned using ClustalW2 and a tree was generated using the neighbour-joining clustering method (Section 2.7.1). Six strains were investigated for their motility phenotype; twitching (T), swarming (S) or non-motile (NM) (Section 4.2.2).

Table 4.1: Presence of type I pili cluster in fully sequenced *A. baumannii* isolates

AB0057 [a]	**ATCC 17978** [a]	**ATCC 19606** [a]	**AYE** [a]	**WM99c** [a]	**D1279779** [a]
AB57_1744 -1747	A1S_1507-1510	Y	ABAYE2138 -2133 [d]	Y	Y
AB57_2003 -2007	N	Y	ABAYE1856 -1860	Y	N
AB57_2565 -2570	A1S_2213-2218 [b]	Y	ABAYE1319 -1324	Y	Y
AB57_2420 -2423	A1S_2088-2091 [c]	Y [c]	ABAYE1470 -1473	N	Y

[a] Presence determined by whole genome (accession numbers are provided in Table 2.2) alignment using Mauve (Section 2.7.2); N = not detected; Y = present, but no locus-tags.

[b] Frame-shift detected in *csuB*, resulting in truncation of CsuB and potential translational complications of the down-stream genes.

[c] Frame-shift detected in first open reading frame of the operon, resulting in expression of non-functional type I pili.

[d] Non-functional as a result of disruption by an insertion element.

of the open reading frame. Furthermore, the gap between *csuB* and *csuC* increased from 5 bp to 96 bp. Although transcription is unlikely to be influenced by a single nucleotide insertion, the increase between the *csuB* and *csuC* ORFs may affect translation of *csuC* and other downstream genes. Interestingly, this strain showed the lowest level of binding to abiotic surfaces of all *A. baumannii* strains investigated, with the exception of strain RB02c (Figure 4.1). The first ORF of the AB57_1744-1747 and AB57_2420-2423 polycistronic gene clusters contained homopolymeric tracts of varying lengths, and were therefore reanalysed by Sanger sequencing (Section 2.4.7) using the AB57_1747 and AB57_2423 oligonucleotides (Table 2.4). Sequence differences were rebutted for AB57_1744 using Sanger sequencing, however, strains ATCC 17978 and ATCC 19606 appeared to have an additional thymine in AB57_2423, which resulted in a frame-shift (data not shown). Nevertheless, even with this additional information, no direct correlation could be determined between the presence of type I pili clusters AB57_1744-1747, AB57_2420-2423 or AB57_2003-2007 and adherence to either biotic or abiotic surfaces.

4.3 Conclusions

The Australian clinical *A. baumannii* isolates showed a similar clonal distribution to that found in Europe, *viz.* a high prevalence of international clone I and II strains (Peleg *et al.* 2008). However, no international clone III isolates were identified in this study. Since bacterial motility is a known virulence factor in numerous bacterial species (Alarcon *et al.* 2009; Han *et al.* 2008; Proft and Baker 2009), the motility potential of 56 clinical isolates was examined. The motility phenotypes in this study were determined using the general classifications for both swarming and twitching (Kaiser 2007; Semmler *et al.* 1999). All international clone I isolates showed significant twitching. A number of other twitching isolates, not part of this clonal lineage, had the ability to form well developed biofilms compared to the international clone I isolates, with the exception of *A. baumannii* strain D1279779. Interestingly, this relatively poor biofilm former (OD_{595}<1) also showed a small twitching zone (approximately 12 mm). Swarming motility was observed in three non-international clone isolates, including *A. baumannii* strain ATCC 17978.

Studies using MH and LB media showed that twitching and swarming phenotypes are largely medium dependent. Furthermore, twitching and swarming

were demonstrated to be distinct characteristics, as many twitchers did not swarm, and *A. baumannii* strain ATCC 17978 swarmed, but did not twitch. The pathogenic significance of *A. baumannii* motility remains unknown, as a correlation between the source of isolation and motility characteristics was not observed.

PilA showed a high degree of amino acid sequence conservation within twitching isolates, indicating that type IV pili may play a role in motility in this species, as described in Section 3.2.5. The PilA sequence dependent motility characteristics may influence the level of virulence, as observed in *P. aeruginosa* (Stewart *et al.* 2011). Examination of biofilm formation showed that there was a significant difference between international clone I and II isolates, correlating with another study (de Breij *et al.* 2010). A significant difference ($p<0.05$) was also observed between international clone I and non-international clone isolates, indicating that in general international clone I isolates are limited in their ability to form biofilms. The site of isolation, e.g. blood or respiratory tract, was not associated with biofilm formation characteristics. Therefore, the significance of biofilm formation in *A. baumannii* disease potential also remains largely unknown.

The adherence of selected *A. baumannii* isolates to eukaryotic cells of nasopharyngeal (Detroit 562) and alveolar (A549) origin was determined. Not only were significant differences observed between strains, two isolates, D1279779 and ATCC 17978, showed significantly lower adherence to nasopharyngeal cells compared to lung epithelial cells. This indicated that these two stains may have a greater ability to cause disease than to persist as a carriage strain in the upper respiratory tract.

Comparison of the ability to form biofilms and eukaryotic cell adherence revealed no relationship between these two phenotypes in the strains tested. This suggested that the mechanism of adherence to either abiotic or biotic surfaces appears to be different and draws a parallel with the results from other studies (de Breij *et al.* 2010; Lee *et al.* 2008). Moreover, previous studies have shown that adherence to abiotic surfaces is in part mediated by the *csu* type I pili cluster in *A. baumannii* strain ATCC 19606 (Tomaras *et al.* 2003), however, in a subsequent study using the same *csu* knockout strain, no difference was observed in the ability to bind bronchial cells (de Breij *et al.* 2009). The data presented here corroborate the previously published reports on biotic and abiotic adherence. McQueary and Actis

(2011) did not observe an association between the presence of Csu type I pili and the adherence characteristics of a selection of *A. baumannii* strains. However, using Sanger sequencing in this study, a potentially critical mutation was found in the *csuB* ORF of strain ATCC 17978, a strain displaying lower levels of binding to abiotic surfaces compared to the other fully sequenced strains. No direct correlation could be established between the presence or absence of other type I pili clusters and adherence.

Overall, these studies demonstrated the significant diversity in phenotypic characteristics of clinical *Acinetobacter* isolates. Comparative analyses of the type IV pili genes between the sequenced strains examined revealed a potential role in motility. However, further investigation is required to fully delineate the mechanisms of motility and adherence in *A. baumannii* and the role of these phenotypes in promoting virulence of this important pathogen.