Investigating the Role of Antibodies Against the Biofilm Associated Protein (BAP) of *Acinetobacter baumannii*

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Medical Microbiology

University of Manitoba

Winnipeg

Dedication

To my family and friends whose undying support and encouraging words kept me going for the past two and half years. Your love and confidence pushed me to successfully complete my Masters degree. Thanks for always believing in me!

Acknowledgements

I would like to thank my supervisor Dr. Jody Berry for his extensive knowledge, support and guidance for the duration of my Masters degree. Committee members Dr. G. Zhanel, Dr. M. Alfa and Dr. T. De Keivit for all their encouragement and suggestions that helped my project develop and mature. A very special thank you to the entire Molecular Immunology lab of Cangene Corporation, for without their training, guidance and continuous support my project would never have been completed.

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LIST OF ABBREVIATIONS USED

AHL	**N-ACTYL HOMOSERINE LACTONE**
AI	**AUTO-INDUCER**
BAP	**BIOFILM ASSOCIATED PROTEIN**
BCA	**BICINCHONINIC ACID**
ESBL	**EXTENDED SPECTRUM BETA-LACTAMASE**
ELISA	**ENZYME-LINKED IMMUNOSORBENT ASSAY**
ICU	**INTENSIVE CARE UNIT**
LPS	**LIPOPOLYSACCHARIDE**
MAB	**MONOCLONAL ANTIBODY**
MDR	**MULTI-DRUG RESISTANT**
MIC	**MINIMUM INHIBITORY CONCENTRATION**
NCBI	**NATIONAL CENTRE FOR BIOTECHNOLOGY INFORMATION**
OEF	**OPERATION ENDURING FREEDOM**
OIF	**OPERATION IRAQI FREEDOM**
OMC	**OUTER MEMBRANE COMPLEX**
OMP	**OUTER MEMBRANE PROTEIN**
OMV	**OUTER MEMBRANE VESICLE**
PAB	**POLYCLONAL ANTIBODY**
PAI	**PATHOGENICITY ISLAND**
PBP	**PENICILLIN BINDING PROTEIN**
PDR	**PAN-DRUG RESISTANT**
PIA	**POLYSACCHARIDE INTERCELLULAR ADHESIN**
PNAG	**POLY-β-1,6-LINKED N-ACETYLGLUCOSAMINE**
QS	**QUORUM SENSING**
RND	**RESISTANCE NODULATION-CELL DIVISION**
SDS-PAGE	**SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS**
SSTI	**SKIN AND SOFT TISSUE INFECTION**
XDR	**EXTREME-DRUG RESISTANT**

Abstract

Acinetobacter baumannii is an opportunistic pathogen and can cause severe disease in immune-suppressed and/or injured patients. It is an extreme-drug resistant bacterium with the ability to form biofilms thereby significantly increasing resistance to treatment. Because of the extreme drug resistance and relatively unknown immunological profile of *A. baumannii* new treatment options are needed. *A. baumannii* has been reported to express a Biofilm Associated Protein (BAP); a high molecular weight protein composed of multiple repeat modules and thought to be surface exposed on planktonic bacterium and upregulated in biofilm. While it is unknown if BAP has any role in *in vivo* infection of humans, the repeats of BAP proteins are thought to function in intercellular adhesion to support the mature biofilm and thus represent potential targets for immunotherapeutic intervention. Herein my thesis is aimed at trying to verify that BAP is surface exposed, upregulated in biofilm and to prove a role for BAP in pathogenesis, as well as investigating *A. baumannii* interactions with components of the innate immune system *in vitro*. Consensus synthetic peptides corresponding to the major internal repeats of BAP were designed and conjugated to carrier proteins and recombinant proteins were manufactured to correspond to the non-repetitive N and C terminals of the protein for murine immunization and assay development. Serum from immunized mice was collected and analyzed in ELISA and western immunoblot to determine reactivity with planktonic and biofilm whole organism. Anti-serum to whole bacteria was also tested in opsonisation assays to determine direct killing ability of serum on bacteria *in vitro*. Anti-serum to whole bacteria showed direct killing of the organism *in vitro* when in high concentrations (diluted 1/10), relative to pre-immune serum, but was less effective in lower concentrations

(diluted 1/50). Despite generating antibody reagents to multiple domains and epitopes spanning the published BAP sequence, we were unable to confirm that BAP is expressed by *A. baumannii* as reported by others. However, if BAP is indeed expressed in *A. baumannii* our DNA and immunochemical data collectively suggest that BAP is potentially mosaic in this pathogen.

1 Introduction

1.1 Global importance/impact of *Acinetobacter baumannii*

1.1.1 Nosocomial and Community-acquired infections

Acinetobacter baumannii was initially considered a relatively insignificant pathogen and commonly ignored until the 1960's even when isolated from clinical samples (60). Up until the 1970's this bacterium was mostly detected in hospital-acquired infections after surgery and from the urinary tract of patients in the intensive care unit (ICU), but since the 1980's *Acinetobacter* species have been found increasingly through the ICUs of hospitals all over the world (6, 60). In 2004 the Centers for Disease Control and Prevention stated that *Acinetobacter baumannii* accounted for approximately 80% of reported infections (59) and in a 2009 Canadian study *Acinetobacter baumannii* was ranked as the 20th most common organism identified from hospital ICUs (44). This bacterium can spread epidemically among patients throughout hospitals causing various infections that are hard to treat as most strains are highly resistant to antibiotics and are categorized as multi-drug resistant (MDR) or extreme-drug resistant (XDR) (19). To this day *Acinetobacter baumannii* continues to be an escalating problem with outbreaks reported in North America, Europe, China, Taiwan, Brazil, Japan, and areas as remote as the South Pacific and Tahiti (56). Many of these outbreaks involve strains that are increasingly multi-drug resistant and therefore make *Acinetobacter baumannii* a very successful nosocomial pathogen (31). Although *Acinetobacter baumannii* is predominantly associated with hospital-acquired infections these bacteria have also been shown to be involved in numerous community-acquired infections making it a dynamic and resilient bacterium (23). However, community

acquired *Acinetobacter baumannii* infections seem to be more prevalent in people with higher morbidity and those living in tropical/subtropical climates as it colonizes a higher number of people (3.8%) in these warm and humid environments compared to people living in more temperate areas (0.5%) (1). Approximately 10% of severe community acquired pneumonia and 20% of deaths from bacteraemia are attributed to *Acinetobacter* species in tropical northern Australia (1). Other cases of community acquired infections have been identified in China, Taiwan, the Far East, Oceania and New Guinea. Co-morbidity such as chronic obstructive pulmonary disease, diabetes and renal disease was common in almost all cases as well as excess alcohol consumption and smoking (1, 23).

1.1.2 War and Natural Disaster

Acinetobacter has been responsible for infections in various scenarios. In addition to nosocomial and community-acquired infections *Acinetobacter* species have been extremely successful in taking advantage of natural disasters and colonizing combat inflicted wounds (13). In the aftermath of the 1999 Marmara earthquake in Turkey 84% of trauma victims were hospitalized, 18.6% of the injured had nosocomial infections and 31.2% of these infections were *Acinetobacter baumannii* isolates (54). *Acinetobacter* infections are found even more so in combat associated wounds and have been documented as early as the Vietnam and Gulf wars where it was the most commonly recovered isolate from war wounds and the second most frequent bacterium causing bloodstream infections in the U.S marines with extremity wounds (13). It is now commonly found colonizing wounded soldiers returning from the Iraqi and Afghanistan conflicts (13, 14). Most war isolates past and present are identified as multi-drug resistant

(MDR) and are termed war-zone community acquired pathogens both colonizing and infecting casualties (14). During the Iraq and Afghanistan conflicts, particularly Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) over 29,000 United States Military personnel were wounded in action (51, 53). Over 700 of these personnel who sustained traumatic wounds, majority of these being orthopaedic or extremity injuries, were found to be infected with virulent *Acinetobacter baumannii* (50). Among the wounded soldiers in Iraq and Afghanistan, MDR *Acinetobacter* has been reported to cause deep wound infections, osteomyelitis, respiratory infections and bacteraemia more so than other notable Gram negative bacteria including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* (51, 84). Greater than 30% of combat related injuries in Iraq and Afghanistan have resulted in osteomyelitis due to MDR *Acinetobacter baumannii* which has undoubtedly complicated orthopaedic blast injuries and increased morbidity ((38, 55, 84, 85). Further to this, *Acinetobacter baumannii* are the most common pathogens (33%) associated with infections resulting from blast wounds and are more likely to develop skin and soft tissue infections (SSTI) (55). In a recent study of inpatients admitted to a naval hospital 14% of patients were diagnosed with *Acinetobacter baumannii* – associated skin and soft tissue infection which exhibited cellulitis with progression towards necrotizing infection and hemorrhagic blisters when not treated (13). However, *Acinetobacter* associated SSTIs are relatively uncommon outside the military setting as seen from a four year study which collected and analyzed greater than 1700 bacterial isolates from Latin American medical centres and found that *Acinetobacter* species were the cause of only 4.1% of all SSTIs (66).

The high prevalence of *Acinetobacter* infections in military combat associated wounds has been attributed to several potential sources: (i) the bacterium already colonized the skin before injury, (ii) the bacterium entered the wound at the time of injury from environmental soil contamination, or (iii) the bacterium was acquired after injury during treatment in a field hospital or healthcare facility (65). The cause of infections could be one or a combination of the three above sources but the true origin is not conclusive as of yet.

Clearly *Acinetobacter baumannii* is an extremely important global pathogen not only in both the clinical and community setting, but from a military perspective as well. It deserves attention from clinicians and researchers alike to develop innovative ideas for new and improved treatment options.

1.2 *Acinetobacter baumannii* Microbiology and Epidemiology

1.2.1 Definition, Classification and Clinical importance

Members of the future genus *Acinetobacter* were first described in 1896 by Morax and in the following years by Axenfeld, designating them "Morax-Axenfeld bacilli" for many years. The name *Acinetobacter* from the Greek *akinetos* meaning "unable to move" was proposed in 1954 and adopted in 1969, thus the genus *Acinetobacter*, having only one species at the time, was borne (2). Currently there are 17 named and 14 unnamed *Acinetobacter* species classified under the family Moraxellaceae with only a few recognized as potential pathogens. *A. baumannii*, one of the most frequently involved in severe infections, are Gram negative, non-motile, non- lactose-fermenting, oxidase negative and catalase positive free-living coccobacilli (29). *A. baumannii* is ubiquitous in the

environment and can be found in soil, water, foods and can colonize the skin, throat, occasionally the digestive tract and most moist body areas of healthy individuals with no detrimental effect (13, 17). In the clinical setting however *A. baumannii* can be extremely dangerous, especially to immune compromised patients such as ones undergoing chemotherapy, surgical procedures or ones with underlying diseases (56). Being an opportunistic pathogen, once it comes into contact with immunosuppressed individuals it can cause serious nosocomial infections including severe bacteraemia, urinary tract and catheter-related infections, pneumonia, meningitis, skin and wound infections, osteomyelitis and many other hospital and ICU-acquired illnesses (47).

1.2.2 Risk Factors, Transmission and Control Measures

Acinetobacter infections are occurring at high rates in both the hospital setting and in traumatic war injuries. What makes the increasing rates even more alarming is that many of these infections are multi-drug resistant (MDR) and incredibly difficult to treat. The exact origin and contributing factors of infection have not been specifically identified but there are various themes that have been proposed (47). There are specific risk factors that make a patient more susceptible to *Acinetobacter* infection which differ and overlap for military personnel and civilians: (i) whether or not the patient has had previous invasive procedures and or mechanical ventilation, (ii) selective pressures by antibiotics, especially inadequate antibiotic therapy that has little or no activity against *Acinetobacter* strains which cause the mutations in antimicrobial resistance genes to be selected and enhanced, (iii) prolonged length of stay in the hospital, the patients age, underlying pathologies or multi-system diseases, (iv) environmental contamination issues, such as

contaminated materials, medical equipment, catheters, ventilators and mattresses and (v) battlefield-specific wounds (2, 50, 51). Studies reveal the most common contributing factors for acquisition are contaminated environmental/medical surfaces, non-compliance with infection control guidelines, exposure to invasive medical devices and procedures (50) and prior/mis-use of broad-spectrum antibiotics (22). Being intrinsically resistant to many antibiotics, it should not be surprising that the prior use and selective pressure created by administration of these medications would increase risk of infection and drive the progression towards a MDR phenotype (22). In any bacterial pathogen, decreasing the use of ineffective and unnecessary antibiotic treatments and therefore the selective pressures on bacteria would have resulted in loss of mutated genes encoding for antimicrobial resistance as expression of these genes create an unneeded metabolic expense for bacteria (13).

Like most bacteria, in many cases the transmission of *A. baumannii* is quite preventable if all individuals involved took the proper precautions. *A. baumannii* can be carried on healthcare personnel and transmitted from hands and gowns to patients directly or it can colonize and infect patients that come into contact with contaminated hospital equipment (52); therefore washing of hands and proper sterilization of medical devices are of utmost importance. To a lesser extent it can spread through the air in the scales of skin from colonized patients and in water droplets over short distances (19). Patients who become colonized or infected can then spread the bacteria to other occupants on the ward and contaminate their surrounding environment as *A. baumannii* can survive dry conditions and live on inanimate objects for months (19, 29). The cleaning and disinfecting of infected patients rooms has been shown to halt outbreaks and this clearly emphasizes

the importance of enforcing strict infection control guidelines and strategies which require cooperation of all levels of healthcare personnel (29, 50).

1.3 Mechanisms of Resistance

1.3.1 Multi-drug Resistance

The clinical interest in *A. baumannii* has escalated over the last few decades as it has become increasingly more resistant to many classes of antibiotics designating it a multi-drug resistant (MDR) bacterium (47, 60). MDR is defined as resistance to three or more classes of antibiotics, however there are emerging strains of *A. baumannii* that are resistant to almost all available antimicrobial agents classifying these strains as pan-drug-resistant (PDR) or extreme-drug resistant (XDR) (83). This organism is intrinsically resistant to numerous commonly used antibiotics including penicillin, first and second generation cephalosporins and chloramphenicol thus the choice of appropriate therapy has always been limited as *A. baumannii* is inherently MDR (19, 29). Furthermore, *A. baumannii* has a remarkable capacity for acquiring mechanisms, by mutational changes or acquisition of genetic material, that confer resistance to broad-spectrum beta-lactams, aminoglycosides, fluoroquinolones, and tetracyclines (19, 56) and as a result this organism utilizes all the major resistance mechanisms that are known in bacteria to mediate resistance to antimicrobial agents. *A. baumannii* has even resorted to loss of its lipopolysaccharide (LPS) to confer resistance to certain antimicrobials as was shown in a 2010 study where loss of LPS occurs in a pan-resistant isolate. This is the first report of this spontaneously occurring in a Gram negative bacterium (48).

1.3.2 Beta-Lactamases, OMPs, PBPs and Efflux pumps

Beta-lactam antibiotics which inhibit bacteria by disrupting cell wall synthesis were the gold standard for treating bacterial infections and still are, for the most part, the desired course of therapy for treatment of generic infections (74). Unfortunately, bacteria have evolved complex mechanisms of resistance to counteract the lethal effects of beta-lactam antibiotics, *A. baumannii* being one of several bacteria that has taken this to extremes (56, 57). *A. baumannii* can harbour every class of beta-lactamase enzymes which work by hydrolyzing the beta-lactam ring of these antibiotics rendering them less effective. Beta-lactamases present in *A. buamannii* include naturally encoded class C AmpC-type cephalosporinase and class D OXA-51/69 oxacillinases as well as class A beta-lactamases and extended spectrum beta-lactamases (ESBLs) such as TEM-1, PER-1, CTX-M, SHV-12 and TEM-116 (71). Class B or metallo-beta-lactamases such as the IMP and VIM families are also prominent in *A. baumannii* strains which have caused much concern to clinicians as these beta-lactamases can hydrolyze all beta-lactams and along with the class D beta-lactamases can hydrolyze carbapenems, which were at one time considered the drug of choice for treating *A. baumannii* infections (56, 57, 71).

In addition to the many beta-lactamases harboured by *A. baumannii*, it also utilizes changes in outer membrane proteins (OMPs) and penicillin binding proteins (PBPs) as resistant mechanisms against beta-lactam antibiotics (56). Altering the structure and number of porin proteins which results in decreased permeability to antibiotics and changes in the PBPs which prevent beta-lactam action are common in *A. baumannii* (33, 81). This includes reduced expression or total loss of various OMPs which can result in

resistance to the carbapenems imipenem and meropenem (33). PBP-mediated mechanisms of resistance include acquiring a novel less sensitive enzyme, mutation of an endogenous PBP to decrease the reaction with beta-lactams and up regulation of PBP expression (33, 82). Beta-lactam antibiotics cannot bind as effectively to these altered PBPs and thus cannot inhibit the final cross linking of the peptidoglycan layer in the bacterial cell membrane.

The reduced accumulation of antibiotics in bacteria can be attributed to the combination of slower diffusion due to reduced expression of OMPs and increased expression of efflux pumps which actively pump out toxic substances passing across in the inner and outer cell membranes, such as the many classes of antibiotics *A. baumannii* is resistant to (56, 64, 74). Efflux pumps illustrate a unique phenomenon in drug resistance: that is a single mechanism causing resistance against several different classes of antibiotics. There are specific efflux pumps belonging to distinct families found in various species of bacteria and there is one in particular that is well described in *A. baumannii*, the AdeABC efflux pump (56, 81). The AdeABC efflux pump belongs to the Resistance Nodulation-cell Division (RND) family and pumps out aminoglycosides, cefotaxime, tetracyclines, erythromycin and fluoroquinolones among other antibiotics and is also thought to confer high level of resistance to carbapenems when overexpressed (81). In general the RND family of efflux pumps is the most superior when mediating antibiotic resistance, virulence and fitness of Gram negative bacteria (64). The expression of this efflux pump is controlled by a two-component regulatory system containing a regulator (*adeR*) and a sensor (*adeS*). A single point mutation in the *adeR* or *adeS* genes can cause increased expression of AdeABC and therefore increased expulsion of antibiotics (19, 56, 64, 74, 78).

1.4 Treatment Options

1.4.1 Carbapenems, Colistin and Combination Therapies

The upregulation of intrinsic resistance mechanisms in combination with acquired genetic elements such as resistance islands, which can carry up to 52 resistance genes, and its ability to survive in the environment makes *A. baumannii* an important superbug that requires much attention (64, 78). Its resistance profile has made infections very hard to treat and raises the question of what's next and what available antimicrobials can be utilized? Up until recently, carbapenems such as imipenem and meropenem were considered the last resort drug of choice for treating *A. baumannii* infections, however carbapenem resistant strains are on the rise and therefore these antibiotics are becoming progressively less effective even when used in combination therapies (78, 83). This trend was shockingly apparent in soldiers injured in OIF and OEF such that in a two year period starting in 2005 resistance to imipenem increased from 13% to 46% (55). Further to this, a 2007 surveillance study revealed that resistance rates to imipenem ranged from 38% to 71%, an extreme increase from 20 years ago when imipenem resistance was virtually unheard of (43).

Currently, drugs that show a lower percentage of resistant isolates are colistin, a polymyxin antibiotic, and tigecycline, a glycylcycline antibiotic (78) and combination therapies. Colistin was used in the 1970s to treat infections but due to its high toxicity, low therapeutic index and the development of less toxic therapies it was discontinued (82). However with antimicrobial resistant superbugs on the rise colistin was revisited and has been used in numerous studies to treat MDR *A. baumannii* infections (10, 83).

Nevertheless, similarly to carbapenem resistance, colistin resistance is also on the rise with half the carbapenem resistant clinical strains also resistant to colistin treatment (55, 79). For a more comprehensive review on small molecule antibiotics and *A. baumannii* treatment options see Karageorgopoulos & Falagas, (2008) (29); Vila & Pachon, (2011) (77) and Wroblewska (2006) (82).

1.4.2 Antimicrobial peptides

Novel therapies are clearly needed for treatment of *A. baumannii* infections as this bacterium is incredibly dynamic in terms of resistance. Researchers have been exploring the use of antimicrobial peptides (AMPs) in their quest to find a suitable and proficient therapy to treat MDR infections (55). These host defence peptides are a component of the innate immune system and are evolutionary conserved among all classes of life. They are potential therapeutic agents being similar to potent broad spectrum antibiotics while having increased activity on bacterial membrane disintegration as well as the ability to bind intracellular targets such as DnaK, inhibiting chaperone-assisted protein folding (55). It has been shown *in vivo* that a particular AMP, A3-APO, is able to fight MDR *A. baumannii* infections at similar levels or better than imipenem even when the peptide was administered at much lower doses (55). Furthermore, in a recent study the activity of fifteen different AMPs were tested with colistin-susceptible and colistin-resistant *A. baumannii* strains (78). Of the fifteen AMPs only three, cecropin PI, melittin, and mastoparan showed activity against colistin-susceptible strains. Only mastoparan, a peptide toxin from wasp venom and melittin, the active component in bee venom, showed activity against both colistin-susceptible and colistin-resistant strains having minimum

inhibitory concentrations (MICs) as low as 2-4 mg/L while most of the other AMPs had MICs of 16-25 mg/L or higher (79). Further research and *in vivo* studies are needed to determine the true mechanism of action used by these peptides and whether or not they would be an appropriate treatment for *A. baumannii* infections (79). Nevertheless they do have potential to be antimicrobial agents for MDR *A. baumannii* especially when XDR strains arise.

1.4.3 Vaccines and Antibody Therapy

Scientists have also been looking into different bacterial targets that would be suitable for vaccine development to actively prevent MDR *A. baumannii* infections in particular groups with well-defined risk factors. These groups include patients in long term care facilities, individuals receiving mechanical ventilation and military personnel (41). To date there are no vaccines that have been developed for this organism even though immunization, both passive and active, represents a highly effective strategy for prevention in laboratory animals (13, 43, 83). Vaccines based on whole cell organisms can stimulate an antibody response to multiple bacterial antigens and surface proteins therefore providing protection against a broad range of strains within *A. baumannii* (41). However, immunization of humans with whole bacteria may not be feasible as it raises various safety concerns due to the presence of lipopolysaccharaide (LPS), thus recent studies have explored immunization with proteins from the outer membrane and outer membrane vesicles, commonly called outer membrane complex (OMC) vaccines (13, 13, 42, 43).

Outer membrane vesicles (OMVs) are spherical vesicles made up of outer membrane proteins, periplasmic proteins and LPS which have been suggested to play a role in *A. baumannii* pathogenesis as clinical isolates have been shown to secret OMVs containing putative virulence factors and immune modulating proteins. OMVs are also thought to be involved with quorum sensing, gene transfer and the transport of virulence factors making them appealing targets for developing *A. baumannii* vaccines (42). OMC vaccines have shown potential for treatment and prevention of infections caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* (43) as they elicit polyclonal antibodies against various outer membrane proteins (OMPs) which have demonstrated bactericidal activity and are able to induce protective immunity against infection in animal models (43). Furthermore, preventative treatments such as these have shown so much promise that OMV vaccines have already been developed for research and human clinical trials for numerous Gram negative bacteria including *Helicobacter pylori*, *Neisseria meningitidis* and *Vibrio cholerae* (42).

The potential of these vaccines has been demonstrated in *A. baumannii* as a 2011 animal study revealed that antisera produced by immunization with OMC vaccine was successful in treating established *A. baumannii* infections, including one caused by an XDR isolate (43). Clearly antibodies play a role in protective immunity in experimental infection. The polyclonal antibody response elicited by OMC and OMV vaccines is favourable as there is less chance of developing mutations of target epitopes compared to monoclonal antibody based therapies where a single epitope on a single antigen is targeted (42, 43). Although this study showed that mice produced antigen-specific humoral and cellular responses when immunized with OMC, treatment with vaccine sera seemed to

provide sufficient immunity to *A. baumannii* infection, confirming that antibodies were critical for protection (41, 43). While the importance of a cellular response in the role of protective immunity cannot be ruled out it is safe to say antibody based therapies and vaccination may be alternative strategies for treating *A. baumannii* infections (20, 42).

1.5 Host Immune Response to *A. baumannii* Infection

Although *A. baumannii* infections are prominent problem in both the civilian and military hospital settings there is little known about host defence mechanisms used to regulate infections (4). *A. baumannii* bacteria are able to disseminate rapidly through the body to peripheral organs via the blood. Once bacteria reach organs such as the lungs and spleen they replicate quickly and can develop into severe infection as *A. baumannii* can adhere and invade epithelial cells internalizing in membrane bound vacuoles (8). Under normal conditions the immune system seems to be capable of effectively controlling *A. baumannii* infection and preventing serious illness (58). However, if the immune system is compromised or certain components are removed infection can be extremely lethal (58).

Certain animal studies have demonstrated the importance of neutrophils in host defence against *A. baumannii* pneumonias and sepsis. Normally neutrophils are rapidly shipped to the lungs at the beginning of an infection but depletion of these immune cells prior to challenge with *A. baumannii* resulted in loss of control of bacterial replication at the site of infection and a lethal outcome (4, 58). Further studies have illustrated the important role of toll-like receptor 4 (TLR4) and the pathogen recognition receptor CD14, as the absence of these immune components increased the chance of pneumonia development in murine models (4). TLR4 recognizes bacterial LPS so it is not surprising

that *A. baumannii* LPS stimulates TLR4, in turn eliciting a strong pro-inflammatory cytokine response in murine cells. However, it is well known that the effectiveness of certain types of LPS in stimulating cytokines via TLR4 can differ drastically when applied to human cells compared to murine cells. Thus researchers tested the activity of *A. baumannii* LPS in a human monocytic cell line and discovered that *A. baumannii* endotoxin was able to stimulate elevated levels of both IL-8 and TNF-α at concentrations as low as 0.1ng/mL and 1ng/mL respectively. These results are comparable to trends seen in *E. coli* infection (20). In addition, an *in vivo* murine model of *A. baumannii* lung infection showed release of pro-inflammatory cytokines and chemokines followed by bacterial clearance from the lungs of infected mice. These results plainly underline the importance of inflammatory cytokines in the control and clearing of *A. baumannii* (15).

Other studies have effectively shown contrasting results in that *A. baumannii* induces a poor inflammatory response in human cells. It was shown that airway epithelial cells produce less IL-6 and IL-8 when challenged with *A. baumannii* in vitro compared to other bacterial species, including *Acinetobacter junii*. Further investigation with human macrophages revealed similar results; these cells produced less TNF-α, IL-12p40, IL-8 and IL-10 when challenged with *A. baumannii* strains (15).

Clearly immune interactions with *A. baumannii* have been under-investigated as there is still much to discover about the pathogenicity and virulence of this pathogen. Additional studies are required to determine details of host defence mechanisms and the particulars of how *A. baumannii* interacts with specific components of the immune system.

1.6 Biofilms

1.6.1 Importance, Structure and Function

Bacterial biofilms, once thought to be a rarity, are now known to be fundamental to bacterial survival and a natural lifestyle of many microorganisms in both the clinical and environmental setting (34-36, 75). Detailed studies have revealed that planktonic or free-living growth rarely exists in nature and approximately 99% of microbes on earth form and live within biofilms (18, 80). Biofilms can form on various different surfaces in the natural environment including aquatic and soil ecosystems and are also common in industrial sites such as water piping systems (80). What is of greater concern is the biofilms that have been discovered in the hospital setting including ones formed not only on medical devices such as intubation tubes, catheters, artificial heart valves and prosthetics but living tissues as well (75, 80).

Bacterial biofilms are independent, highly organized communities that are morphologically and physiologically distinct from their planktonic counterparts. Biofilms are aggregates of cells that are attached to a surface and encapsulated in a self-produced hydrated matrix of polysaccharide and protein (34). Biofilm formation and development can arise from a number of environmental cues and occurs through a series of coordinated molecular steps: (i) reversible initial attachment of bacteria to a surface, (ii) proliferation and accumulation of multi layer cell clusters with progression to irreversible attachment, (iii) development of biofilm structure in which cells are encased in exopolymeric substances, (iv) maturation of the biofilm structure and (v) dispersal of colonies to other sites (Figure 1.) (39, 45, 49).

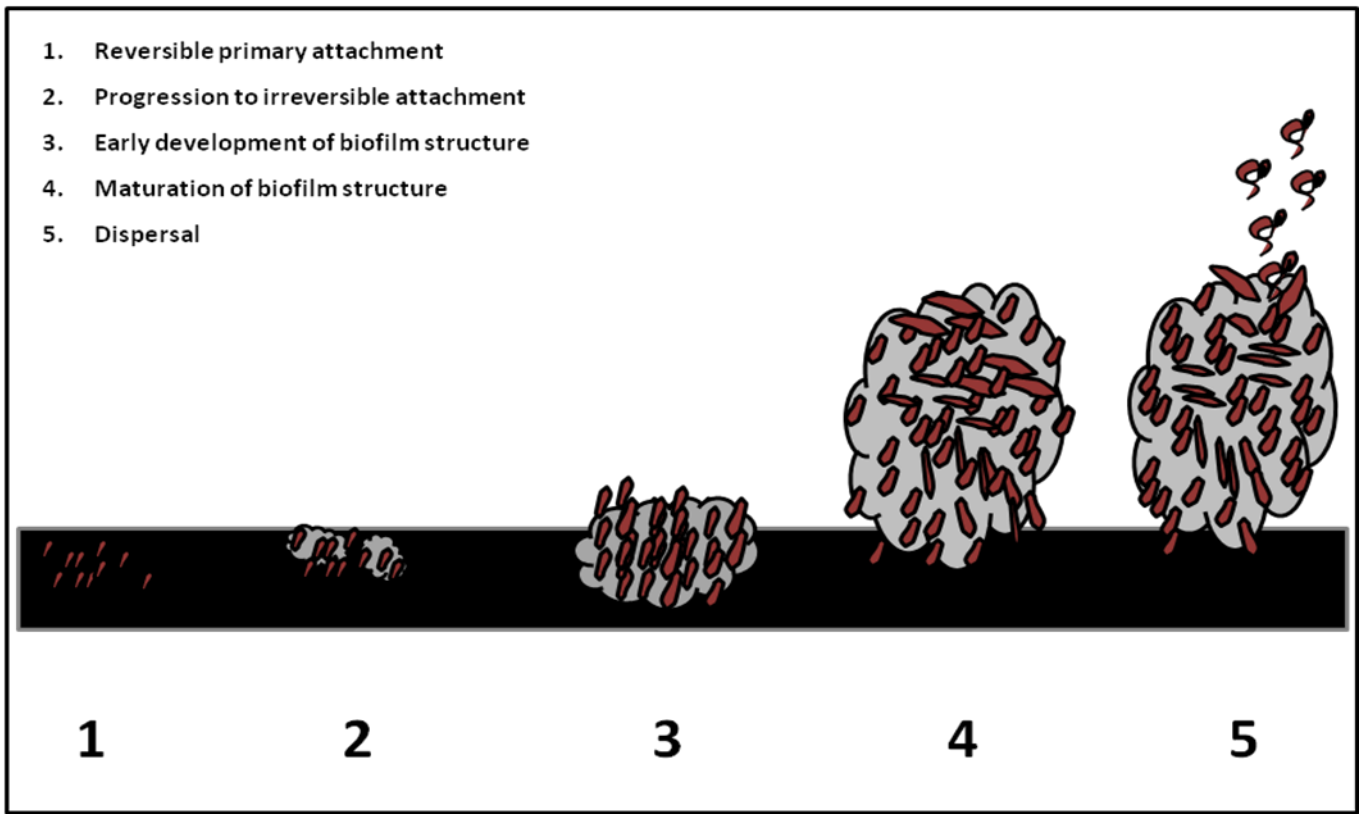


Figure 1. Cartoon representation of the sequence of steps in biofilm formation starting with primary attachment to a surface, progression to irreversible attachment, further proliferation, maturation of multi layer cell structures and finally dispersal of free-living cells from the biofilm structure to colonize other sites. Figure modified from (49).

Biofilms are becoming increasingly recognized as a considerable factor in persistent bacterial infections and are a substantial component of antimicrobial resistance used by numerous bacteria species (72). With approximately 65% of all bacterial infections involving biofilm formation, clinicians are encountering additional complications when treating MDR pathogens since biofilms render bacteria up to 1000 times more resistant to antimicrobials than their free-living forms (45, 75). This is especially the case with non-healing chronic wound infections which are most often found in biofilm form (13, 72). Biofilms provide protection and a mechanically stable environment for the bacteria

enabling them to evade the host immune system as well as protecting them from desiccation and treatment by washing such is done for wound therapy (18, 46, 75). Moreover biofilms provide close cell to cell proximity enabling more horizontal gene transfer and sharing of genetic elements between bacteria. Horizontal gene transfer such as transformation and bacterial conjugation is advantageous to bacteria as this allows increased uptake and sharing of resistance genes among other benefits (13).

1.6.2 Biofilm Resistance Mechanisms

Although biofilms add greatly to antimicrobial resistance, the mechanisms used by non-biofilm bacteria such as target mutations, low cell permeability, efflux pumps and modifying enzymes, do not appear to solely confer resistance in biofilms (72). There are however, four biofilm resistance mechanisms that are proposed to explain the reduced antibiotic susceptibility of bacterial biofilms: (i) slow diffusion or lack of penetration by antibiotics, (ii) decreased growth and altered microenvironment compared to free-living forms, (iii) increased response to environmental stressors and (iv) the presence of bacterial persister cells (70, 72). Several antibiotics slowly diffuse or cannot penetrate the polysaccharide matrix which composes the outer slime layer of these multicellular structures. These include well known drugs such as piperacillin, gentamicin, tobramycin and ampicillin which are not able to penetrate the biofilms formed by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (13, 72, 75). Biofilms have an altered microenvironment and grow at a slower rate than free-living bacteria. The killing capacity of many antibiotics, such as penicillin, depends on bacterial growth or certain types of macromolecular synthesis and therefore these drugs would have little effect where these

processes are halted or reduced. Other antibiotics, such as aminoglycosides, are modulated by oxygen and would not be able to attack bacteria in anaerobic regions of biofilm rendering them less effective (72, 73). The stress responses that allow all bacteria to cope with temperature changes, DNA damage, starvation and other environmental challenges may be increased in biofilm promoting their survival and thus resistance (69). The community of cells within biofilm are thought to be given greater opportunity to express traits related to these adaptive responses as a result of slow growth rate and decreased penetration of antibiotics (72, 75). In addition to antibiotic resistance biofilms also provide defence against chemical disinfectants which is thought to be explained by the presence of persister cells in the biofilm, adding to the resistance profile (70, 72). These hardy, spore-like cells are highly protected and although they constitute a small portion of the population, they occur in much higher frequency in biofilm communities compared to planktonic (72).

The genetic and molecular details of these hypothetical biofilm resistance mechanisms are not fully known and are gradually emerging in research. However, it is thought that the extreme resistance seen in biofilm infections is a combination of many mechanisms which most likely differ among and between bacterial genera, species and strains (34, 72, 73). Regardless of the causes and particulars of biofilm development and resistance, they pose an extreme challenge and expense to public health and human medicine (71, 72).

1.6.3 Extracellular Polysaccharides and GGDEF/EAL Domain Proteins

Although the biofilms produced by various unrelated bacteria have differences in their structure, all bacterial biofilms are proposed to have several functionally conserved components in common (36, 39). These common elements include the production of extracellular polysaccharide matrix, regulation of extracellular components via c-di-GMP, which in turn is dependent on intracellular signalling mediated by proteins containing the GGDEF/EAL domain, and large surface adhesion proteins (34).

Although numerous exopolysaccharides have been described in bacterial biofilms, cellulose, β-1,6-linked *N*-acetylglucosamine or polysaccharide intercellular adhesin (PIA) and poly- β-1,6-linked *N*-acetylglucosamine (PNAG) are among the most common. PIA and PNAG are now known to be structurally and immunologically identical (7, 34) and from this point on will be referred to as PIA/PNAG. PIA/PNAG polysaccharides are manufactured by four homologous proteins named IcaA, IcaD, IcaB and IcaC which are encoded by an organized set of genes in a single operon (*icaADBC*) (34). These polysaccharides play an important role in cell to cell adherence and are also considered a vital virulence factor that provides protection against innate host immunity (7). Within the last few years there have been discoveries of related loci that are genetically and functionally similar to the *icaADBC* operon in that they encode proteins that synthesize similar exopolysaccharides (7). These loci have been described in numerous Gram negative bacteria, but one in particular, the *pgaABCD* operon, is especially well known. PgaABCD proteins produce an unbranched polysaccharide identical to PIA/PNAG which acts as a stabilizer of biofilm formation during diverse growth conditions (7, 34).

In Gram negative bacteria extracellular polysaccharides can be neutral, polyanionic or can contain uronic acids and ketal-linked pyruvates which amplify their anionic properties. Being anionic is advantageous as it allows increased association with divalent cations such as calcium and magnesium, thus strengthening the binding force in developed biofilm (34, 80). The proportion of extracellular polymeric substances in biofilms can range from 50-90%, but composition will often vary depending on several factors including the type of organism, age of the biofilm, levels of oxygen and nitrogen, temperature, pH and available nutrients (75, 80). Since biofilms are very dynamic they can change their composition and adhesion abilities in response to environmental changes and the diverse surfaces they attach to (18, 34, 80).

The GGDEF/EAL domain proteins contain approximately 180 to 240 residues, are present in majority of bacteria and are generally associated with signal sensing and signal transduction (27, 34). They are an important component of bacterial biofilms as they mediate 3'5'-cyclic diguanylic acid (c-di-GMP), a second messenger in intercellular signalling, and are also related to regulation of exopolysaccharide production, both being vital to biofilm formation (9, 63). Proteins containing a GGDEF/EAL domain control intercellular c-di-GMP levels which in turn are thought to control the transition between biofilm and planktonic states. Therefore expression and activity of GGDEF/EAL domain proteins are regulated in response to environmental cues or stressors that would drive towards a biofilm phenotype (9, 34). Decreased levels of c-di-GMP resulting from mutations in genes encoding for GGDEF/EAL domain have been associated with diminished exopolysaccharide production and reduced capacity to form biofilm suggesting even

further that c-di-GMP and GGDEF/EAL domain proteins are a crucial component in biofilm formation (27).

1.6.4 Biofilm Associated Proteins (BAP) and BAP Homologues

Extracellular polysaccharides are generally portrayed as being the most fundamental components of the biofilm matrix and biofilm formation as they are the framework in which microbial cells aggregate. Surface proteins are also a highly conserved component, although since they do not form the bulk of the matrix they have been slightly overlooked and for the most part have been linked solely to the initial attachment of cells to a surface (35). However, extensive research and interest in characterizing bacterial biofilms has led to the finding that large surface proteins are more important than once thought (12, 34, 36). The first member of this group of large surface proteins which all share similar structural and functional features was identified in a *Staphylococcus aureus* isolate from a bovine mastitis infection and was named BAP for Biofilm Associated Protein (11). Since the initial discovery of the first BAP numerous other proteins have been described that exhibit homology to the BAP of *S. aureus* in several diverse bacterial species (Table 1) (35, 36). Proteins belonging to the BAP family share a distinct set of structural features distinguishing them from other biofilm related proteins. BAP and BAP homologues: (i) are situated on the bacterial surface and have a signal sequence for extracellular secretion, (ii) are excessively large with high molecular weight, (iii) have a core domain consisting of tandem repeats whose number varies among different isolates and (iv) confer on bacteria the aptitude for biofilm formation (12, 34, 39). Although members of the BAP family share these structural and functional similarities, their primary

sequence and origin can be fairly diverse (39). For instance, the *bap* gene from *S. aureus* is encoded in a pathogenicity island (PAI) whereas *bhp*, a gene in *S. epidermidis* which encodes a protein highly homologous to BAP, is not associated with a mobile element of any kind (25). Similarly, BapA of *Salmonella enteritidis* and LapA of *Pseudomonas fluorescens* are not known to be linked to any PAIs, but BapA of *Salmonella typhi* is contained within a PAI. What's more is that two related species of bacteria can carry the same *bap* or *bap*-related gene in completely different PAIs, such is the case with *Enterococcus faecalis* and *Enterococcus faecium* (35). It has been suggested that PAIs act as dynamic vehicles for attaining biofilm forming capabilities and reverting back to a free-living lifestyle by the process of horizontal transfer of *bap* genes; this would represent a rapid and flexible means of evolution in bacterial pathogenesis and virulence and requires further exploration (35, 36).

Table 1. The diverse bacterial species harbouring proteins homologous to the BAP of *S. aureus*. Modified from (35, 36)

Protein	Bacterium	~ % homology with BAP	Proposed/known function
BAP	*Staphylococcus aureus*	-	Initial attachment and biofilm formation on abiotic surfaces
Mus 20	*Pseudomonas putida*	29	Initial colonization
BapA	*Salmonella enteritidis*	28	Biofilm formation and host colonization
Bap	*Burkholderia cepacia*	33	Late stages of biofilm formation
Espfm	*Enterococcus faecium*	27	Eukaryotic cell adhesion
Esp	*Enterococcus faecalis*	26	Initial attachment and biofilm formation on abiotic surfaces
LapA	*Pseudomonas fluorescens*	25	Progression to irreversible attachment to a surface
YeeJ	*Escherichia coli*	21	Non-specific adhesion
VP1443	*Vibrio parahaemolyticus*	20	Maturation of biofilm structure
BAP	*Acinetobacter baumannii*	Homology restricted to A-C repeat regions	Cell to cell interaction and maintenance of mature biofilm

The value of these proteins has been defined within the last decade as the mysteries of these once overlooked components of biofilm formation are gradually uncovered. It has been determined that proteins of the BAP family are equally important as extracellular polysaccharides in the process of biofilm formation. They have been shown to promote adhesion to biotic (living) and abiotic (non-living) surfaces and are pertinent components to the bacterial infectious process as they play a role in intercellular adhesion and the accumulation of multicellular clusters within the mature biofilm (12, 13, 34). In a study performed by Cucarella *et al* (2004) interference with the *bap* gene led to a decreased accumulation of exopolysaccharides suggesting that impaired biofilm formation in BAP mutant strains was due to reduced levels of PIA/PNAG polysaccharides (12, 34). Bacterial isolates harbouring both the *bap* gene and the *icaADBC* operon, which is responsible for encoding PIA/PNAG synthesis, were good biofilm producers. Interestingly enough, bacterial strains deficient in the *icaADBC* operon, but positive for *bap*, were still able to generate strong biofilms (12). Therefore, the expression of the BAP protein is sufficient to mediate biofilm formation in the absence of exopolysaccharides, which were once thought to be the key elements in forming biofilms (12, 34, 36).

1.7 Biofilm Formation and Regulation

Biofilm formation is a complex process that relies on the changes and transport of microbial cells and extracellular substances such as polysaccharides and proteins to a surface, followed by irreversible attachment and further proliferation into multi cell layers (49, 80). This process relies on coordination of bacterial cells to communicate their population behaviour through signalling molecules. These small extracellular molecules

are released into the environment and sensed by neighbouring cells who respond to them accordingly. Bacteria use a system termed quorum sensing (QS), in conjunction with two-component regulatory systems and transcriptional regulators, to monitor the concentration of these molecules and coordinate gene expression; therefore permitting planktonic cells to form and live in unity (21, 26).

Generally biofilm formation is regulated and maintained by bacterial QS which can be divided into two types: (i) one mainly involved in intra-species communication and produced and released by Gram negative bacteria, autoinducer-1 (AI-1) and (ii) one related to inter-species interaction mainly associated with Gram positive bacteria, autoinducer-2 (AI-2) (80). For the purpose of this paper, only the AI-1 QS system will be discussed. Quorum sensing allows necessary cell to cell communication and regulation of a specific set of biofilm differentiation and maturation genes that are dependent on cell density for expression; these genes are only activated in response to various environmental signals and once the population density has reached a particular threshold (18, 26, 80). Cell to cell communication and population density are mediated in Gram negative bacteria by the accumulation of AI-1 quorum sensing molecules; these molecules, also called *N*-actyl homoserine lactones (AHLs) (21), are often produced as mixtures of many AHLs and are an important, highly explored class of species-specific autoinducers (18). AHL signal molecules are generated by AHL synthases and act by binding transcriptional regulatory protein receptors thus activating/regulating gene expression in the organism (3). Typically in Gram negative bacteria the AHL QS system is mediated by the LuxI-type synthase and LuxR-type receptor protein families. LuxI-type proteins synthesize AHL molecules which diffuse in and out of the bacterial cell membrane and interact directly with the cognate

LuxR-type receptor proteins (3). Once a particular cell density threshold is reached, the AHL/receptor complex binds to a specific promoter sequence called *lux*-box which triggers a cascade of molecular events resulting in transcription and expression of several QS target genes involved in various behavioural responses (3, 18, 80). Even though there are many similarities among the QS systems utilizing AHL signalling molecules the LuxI-type synthases and the LuxR-type receptors vary considerably depending on the microorganism (18).

Bacterial biofilms pose a significant threat to the treatment of infections caused by pathogenic bacteria, thus it is of vital importance to understand the details behind the mechanisms of biofilm formation. Knowledge of these mechanisms allows research in the development of new strategies which could help in combating biofilm infections. Disruption of the QS system such as blocking autoinducer synthases and cognate receptors and therefore inhibiting bacterial communication is an approach many researchers are looking in to (21, 40). It is thought that resistance to these bacterial communication inhibitors would not occur as rapidly compared to resistance to other forms of treatment because the loss of the QS system does not impede bacterial growth. As a result degradation of AHL/AI signalling molecules and inference with QS represent appealing options for treating bacterial biofilm infections (18, 24). Further research is needed to determine the clinical relevance of these inhibitors, and what specific targets are appropriate for the various biofilm producing bacteria.

1.8 *A. baumannii* biofilm

1.8.1 Common Features

A. baumannii is undoubtedly a significant nosocomial pathogen causing devastating chronic infections in both civilian and military groups (46). Its ability to resist a wide range of antibiotics resulting in a MDR phenotype and its capacity to survive for months on inanimate objects could be due to the fact that this bacterium employs the use of biofilms on abiotic and biotic surfaces (37, 45, 78). These biofilms cause severe infection, particularly ones associated with catheter-related urinary or bloodstream infections and even shunt-related meningitis (26, 45, 78). *A. baumannii* and their biofilms have not been investigated as thoroughly as other biofilm-forming bacteria since this bacterium was highly overlooked up until a few decades ago. However, several distinct characteristics have been discovered that are important in the formation, structure and maintenance of these bacterial biofilms.

Like many bacterial species, *A. baumannii* uses resistant mechanisms synergistically, especially when numerous stressors, such as antimicrobial therapy or environmental pressures, are presented to it (26, 37, 45). It has been shown that *A. baumannii* strains which possess *blaPER-1*, a gene encoding for the PER-1 ESBL, have greater cell adhesiveness and biofilm formation than those that do not have this genetic trait (26, 37). Furthermore, the level of expression of the *blaPER-1* gene was positively correlated with the amount of biofilm formed on plastic and the ability of the bacteria to adhere to human epithelial cells and other biotic surfaces (26, 37, 45). Thus, the functions of the biofilm structures formed by *A. baumannii* include its ability to resist antimicrobial treatment as

well as other stresses from the environment such as limited nutrient availability and dehydration (26).

A. baumannii biofilms are also influenced by other factors including the concentration of free chemical elements in the surrounding environment as both calcium and iron levels have been shown to affect biofilm formation (45). *A. baumannii* grown in an iron starved environment produces a significantly increased amount of biofilm; depleted iron is considered an environmental stressor that induces a biofilm phenotype (69). Growth in these iron deficient conditions also results in the production of iron regulated siderophores which may be linked to the pathogenesis of this bacterium, suggesting the importance of iron acquisition for *A. baumannii* survival (3).

Another factor common in *A. baumannii* biofilm formation is the lack of correlation between cell adhesion and biofilm formation and the hydrophobicity/hydrophilicity of bacterial cells (45). Hydrophobic interactions are involved in the adherence of microorganisms to several diverse surfaces such as plastic, mammalian cells and hydrocarbon substrates (61). In contrast to several other bacterial pathogens, such as *Neisseria meningitidis*, *Listeria monocytgenes* and *Stenotrophomonas maltophilia*, *A. baumannii* biofilm formation does not appear to be dependent on cell or surface hydrophobicity / hydrophilicity as both hydrophobic and hydrophilic strains formed various amounts of biofilm, ranging from almost non-existent to large quantities, on both glass and plastic surfaces (45).

Further studies have demonstrated that *A. baumannii* biofilm formation is related to histidine metabolism as proteins involved in this pathway are up-regulated in the biofilm proteome (5). When *A. baumannii* was cultured with different L-amino acids it was found

that L-His had the greatest capacity to induce biofilm formation compared to the other amino acids including L-Arg, L-Asp, L-Glu, L-Val, L-Ser, and L-Cys. Further to this the different conformational isomers, L-His and D-His, had opposite effects on biofilm. L-His was found to successfully stimulate biofilm formation while D-His seemed to block the process all together. This very specific effect could possibly be advantageous when looking at novel strategies to treat *A. baumannii* biofilms (5).

In addition to the numerous features mentioned above, an important component of *A. baumannii* biofilm formation is the presence of a *pgaABCD* operon, similar to the *icaADBC* operon found in *S. auerus* and *pgaABCD* operon found in *E. coli*, which are responsible for PIA/PNAG synthesis (7, 34). Choi *et al* (2009) determined that this locus is not only present in *A. baumannii* but also essential for producing PNAG polysaccharide for this bacterium. This was clearly evident when the researchers investigated the quantity of polysaccharide produced by *pga* mutants compared to wild-type strains; *pga* mutants were completely unable to produce PNAG whereas wild-types were ample PNAG producers. These results confirm that the *pgaABCD* operon is critical for polysaccharide production in *A. baumannii* biofilms (7).

It has been shown that several other proteins (not discussed) are up-regulated in *A. baumannii* biofilms, each having a specific function that aids the bacteria in forming and maintaining a strong biofilm (Table 2) (69). The level of expression and importance of these proteins and the previously mentioned characteristics of *A. baumannii* biofilms may vary between bacterial strains. However, there is one component that has been found to be essential to *A. baumannii* biofilm development on abiotic surfaces in all strains that have

been investigated to date and that is the *csuA/BABCDE* chaperone-usher pilus system (45, 75).

Table 2. Proteins significantly up-regulated in *A. baumannii* biofilms and proteins only found in *A. baumannii* biofilm cells. Modified from (69).

Protein	Function	Location
Proteins having increased expression in biofilm compared to planktonic		
NAD-linked malate dehydrogenase	Essential in tricarboxylic acid cycle	Unknown
Putative protein (DcaP-like)	Unknown	Outer membrane
Hypothetical protein	Unknown	Outer membrane
Outer membrane receptor protein	Iron transport	Outer membrane
Nucleoside-diphosphate sugar epimerase	Unknown	Unknown
Beta-lactamase PER-I	Resistance to extended-spectrum beta-lactam antibiotics	Periplasm
Exodeoxyribonuclease III	Repair of DNA damage brought on by endogenous oxidative stress	Cytoplasm
Aminoglycoside acetyltransferase (6') type I	Resistance to aminoglycosides	Cytoplasm
Proteins only found in biofilm		
Putative UDP-galactose 4 epimerase (GalE-like)	Catalyzes conversion of UDP-galactose to UDP-glucose. Both important in capsular polysaccharides	Cytoplasm
Phosphoribosylforminino-5-amino-imidazole carboxamide ribonucleotide (ProFAR) isomerise	Involved in histidine biosynthesis	Cytoplasm

1.8.2 *csuA/BABCDE* chaperone-usher pilus system

One of the requirements to form biofilms is the ability to migrate (most often with flagella) to the surface where the biofilm will accumulate (75). As mentioned previously *A.*

baumannii is taxonomically defined as non-motile, lacking flagella and therefore should be far less able to move to sites of biofilm formation (2, 29). However, since these bacteria are good biofilm producers, scientists were prompted to hypothesize additional mechanisms of motility to explain this phenomenon. Research revealed a cellular component required for biofilm formation which presents itself as long filaments evenly dispersed around the cell surface. This distributed filamentous cell formation and absence of movement is indicative of type I pili which function exclusively in adherence to abiotic surfaces (75, 77).

Extensive investigation of this cellular component was able to show that adhesion was caused by a protein encoding six gene operon which was homologous to a chaperone and usher secretion system (75). This entire operon, collectively named *csuA/BABCDE*, was fully sequenced which allowed researchers to discover potential roles of the gene products produced by the operon. The first gene, *csuA/B,* encodes for proteins similar to the type I pili major subunits found in many additional Gram-negative bacteria. The following two genes, *csuA* and *csuB*, display homology to known minor pili subunits, while *csuC* and *csuD* are thought to encode the chaperone and usher components of the system respectively (75, 76). The sixth and final gene in the operon, *csuE*, codes for the putative tip adhesin component of type I pili ((26, 45, 75) and is arguably the most crucial gene in the operon as disruption or mutation of the *csuE* gene results in elimination of pili formation as well as biofilm formation (76). The fact that interference with the pili tip adhesin effectively abolishes biofilm formation suggests that *csuA/BABCDE*-mediated pili are an important element in the initial attachment of bacterial cells to abiotic surfaces and the development of microcolonies within biofilm structures (76).

Research has demonstrated that the *csuA/BABCDE* operon is regulated by a two-component regulatory system which controls expression of pili assembly genes and therefore pili biosynthesis. This regulatory system consists of a sensor kinase, BfmS, and a response regulator, BfmR, which are encoded by *bfmS* and *bfmR* respectively (26). For a comprehensive review of the *csuA/BABCDE* regulatory system see Gaddy & Actis, 2009 and Tomaras *et al* (2008) (26, 74).

Although the *csu-A/BABCDE* operon is essential for *A. baumannii* pili assemblage and adherence to abiotic surfaces, it has been determined that this operon is not required for attachment to biotic surfaces (16, 26). Studies carried out by de Breij *et al* (2009) concluded that *csuE* mutants, which are completely unable to form biofilms on plastic surfaces, were very capable of adhering to biotic surfaces such as bronchial epithelial cells, erythrocytes and *Candida albicans* (yeast) filaments, signifying that *csuA/BABCDE*-mediated pili are not necessary for attachment to biotic cells (16, 75). Furthermore, *csuE* mutant strains were able to adhere to a considerably greater number of epithelial cells than wild-type strains suggesting there might be different types of pili expressed. Interestingly, it was found that certain *A. baumannii* strains produce two morphologically distinct pili: (i) long asymmetrical cell extensions that connect bacterial cells which characterize *csuA/BABCDE*-dependent pili and (ii) short thin pili-like structures that contact the surface areas around bacterial cells termed *csuA/BABCDE*-independent short pili (16). Further studies are needed to verify the particular structural and physiological properties of these short pili and how they relate to the pathogenesis of *A. baumannii* and their biofilms.

1.9 *A. baumannii* Biofilm Associated Protein (BAP)

1.9.1 Discovery and Characterization

It has been well established that *A. baumannii* is capable of forming strong biofilms. It develops multicellular structures by adhering to an abiotic or biotic surface using both *csuA/BABCDE*-mediated pili and *csuA/BABCDE*-independent short pili followed by composition and secretion of exopolysaccharides encoded by the *pgaABCD* operon (7, 16, 26, 75, 76). While these components are undoubtedly important in the maintenance and strengthening of *A. baumannii* biofilm structure, the role of biofilm associated proteins have also been shown to be of great importance in intercellular adhesion and accumulation of multicellular clusters (34-36). Many bacterial species harbour these large surface proteins (Table 1) and *A. baumannii* has been found to encode a putative protein that fits into the biofilm associated protein (BAP) family (39). Studies performed by Loehfelm *et al* (2008) successfully identified and sequenced a gene which encodes for a BAP homologue from a bloodstream isolate of *A. baumannii*. Further characterization revealed this putative protein, like all members of the BAP family, has a high molecular weight, is located on the bacterial surface, has a core domain of tandem repeats and is essential for biofilm development and maturation (39, 59).

Essentially, Loehfelm *et al* (2008) developed a monoclonal antibody (MAb) which recognized a high molecular band they called 'BAP'. They generated MAb to this target by immunizing mice with whole *A. baumannii*. In addition transposon-insertion mutants which were deficient in BAP surface expression were also manufactured. Analysis of common sequences from the flanking transposon insertion sites of the mutants suggested that one common gene was disrupted. Assembly of the full length coding sequence of this

disrupted gene and BLASTP analysis through the NCBI database revealed the coding sequence was similar to *baps* from several bacterial species (39). Moreover, the *A. baumannii* MAb reacted to an epitope on a high molecular weight antigen when tested by immunodot and western blot assays and was conserved among 41% of *A. baumannii* isolates obtained from a military healthcare outbreak. Flow cytometry was then used to determine if the antigen recognized by the MAb was surface exposed; it was found that the MAb was able to bind to the surface of wild-type *A. baumannii*, but not to the mutant strains deficient in BAP surface expression confirming that the antigen was surface accessible and most likely a BAP protein (39). Furthermore, SDS-PAGE analysis revealed a high molecular weight antigen in a single band. This band was cut and analyzed with mass spectrometry to identify internal peptide fragments. Six peptide fragments were identified and each of them were found within the translated *bap* open reading frame on the NCBI database. Additional studies with the transposon-insertion mutants revealed that these *bap* deficient strains were unable to produce sufficient biofilms with equal biovolume and thickness compared to wild-type strains, suggesting an important role in maintaining the mature biofilm (39). Together these results were able to provide adequate evidence that *A. baumannii* contains a BAP protein that is important in biofilm formation, particularly in the maintenance of the mature biofilm structure. Based on cellular location and its involvement with mature biofilm this protein may also be related to cell-cell interactions and adhesion (26).

1.9.2 Structure and Composition

The *A. baumannii* 'BAP' falls into the BAP family because of its high molecular weight and the fact that it has a core of tandem repeats, among other characteristics. The *A. baumannii bap* open reading frame is over 25,000 base pairs and encodes for BAP which is approximately 854kDa, containing 8,621 amino acids making it one of the largest bacterial proteins ever described (39, 59). *A. baumannii* BAP is composed largely of multiple copies of seven repeat units designated A, B, C, D, E, F and G which are flanked by a non-repetitive N (5' end) and C (3' end) terminal (figure 2) (39). For the most part these repeat modules are directly in tandem having no additional amino acids between consecutive repeats. Repeat A consists of 5 copies with 54-99% amino acid homology between copies, repeat B has 22 copies with 72-100% homology, C has 21 copies with 73-100% homology, D has 28 copies with 78-100% homology and repeat modules E, F and G have 2, 2 and 3 copies with 62%, 67% and 36-51% homology respectively (39). Investigation of the primary structure of BAP revealed a large imbalance in the number of acidic and basic amino acids; acidic amino acids outnumber the basic amino acids by 11:1 as there are 1,168 acidic and only 105 basic residues. This may well explain the extremely low isoelectric point of 3 that BAP holds which puts it among the most acidic proteins discovered thus far (39, 59). The tandem repeat domains seem to contribute to the acidity of this protein as this area contains predominantly negatively charged amino acids compared to the non-repeat domains (59). Majority of the amino acids found in BAP are as follows: 1,389 threonine residues (16% of the total), 1,176 alanine residues (14% of the total), 1,109 valine residues (13% of the total), 984 aspartic acid residues (11% of the total) and 184 glutamic acid residues (2% of the total). Interestingly, there is a complete absence of cysteine in the primary structure of BAP, which is also commonly seen in many bacterial toxins (Jody

Berry. Personal communication, 2011), however there is no known explanation as to why this is (39, 59).

Further analysis of the *A. baumannii* BAP by Rahbar *et al* (2010) predicted which residues had the greatest antigenic propensity by determining their surface accessibility. It was found that even though repeat module D is one of the most conserved units it had the lowest level of surface accessibility having 600 of 2,967 residues buried. Other regions with high numbers of residues buried include repeats A5 to G3 (169 out of 921) and G3 to the C- terminal region (144 out of 405). On the other hand repeat regions A1-A4 and B4-C21 tandemly arranged repeats have many more exposed residues and therefore have the highest potential to be antigenic (59).

Analysis using three-dimensional structure prediction revealed that repeat modules A-D appear to fold into a seven-stranded beta-sandwich similar to proteins associated with the HYR domain of the immunoglobulin-like fold superfamily (39, 59). Even though HYR contains two conserved cysteine residues which are absent in BAP, the A-D repeat sections contain conserved DTTP and VTATDAAGN amino acid sequences which are indicative of the HYR domain. This domain was first described in eukaryotic proteins involved in cellular adhesion suggesting that the repeats of BAP may play a role in intercellular adhesion and support of the mature biofilm structure (39).

The putative biofilm associated protein of *A. baumannii* has several distinct characteristics that set it apart from other proteins related to biofilm formation. Its enormous size, acidic nature and core of successive repeat modules make it a remarkable protein, worthy of future research. The repeat modules of BAP warrant further

investigation as they represent great potential targets for immunotherapeutic intervention of *A. baumannii* biofilm infections due to their proposed function and involvement in mature biofilm.

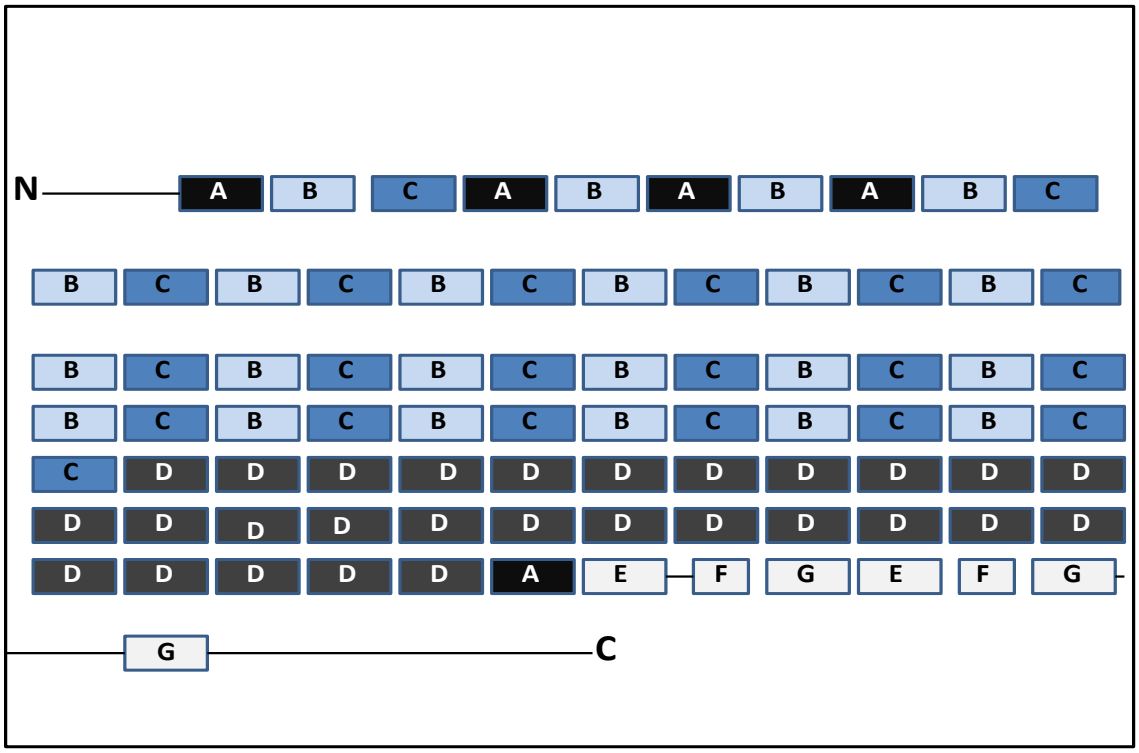


Figure 2. Simplified cartoon representation depicting the linear structure of the BAP of *A. baumannii*. Note the large core domain consisting of tandem repeat modules A-G. Picture modified from (39).

1.10 Summary/Statement of Objectives and Hypotheses

With the exception of the two studies performed by Loehfelm *et al* (2008) (39), and Rahbar *et al* (2010) (59) very little research has been done on the Biofilm Associated Protein (BAP) of *Acinetobacter baumannii*, especially in terms of using this protein as an immunotherapeutic target. Since *A. baumannii* has such an extreme antibiotic resistance

profile and produces strong biofilms which increase the severity of infections by enhancing resistance, it has been a major issue for clinicians, civilian patients and military personnel worldwide. It deserves much attention from researchers to discover novel and superior therapies than those currently on the market today. Ideal treatments include those which are extremely resilient and potent since *A. baumannii* is inherently impervious to many antibiotics, can easily acquire resistance genes from other sources and employs all bacterial mechanisms of resistance. Antibody based vaccines against outer membrane proteins have been investigated for use as future therapies for infections; however antibody therapies which would target biofilm proteins and biofilm formation have not been explored for *A. baumannii*.

This project was initiated to evaluate the effects of polyclonal antibodies (PAbs) produced to specific domains, particularly the repeat modules and non-repetitive N and C terminals, of the Biofilm Associated Protein of *Acinetobacter baumannii* and to determine if these antibodies correlated with biological effects of biofilm formation or structure. Furthermore, antibodies produced to whole *A. baumannii* and how these antibodies affect live bacteria and interact with components of the innate immune system for purposes of investigating its immunological profile were investigated. **We hypothesize that antibodies produced to specific domains of BAP will bind to BAP on the surface of whole bacteria if this protein is surface accessible. Furthermore, if they bind they will interfere with BAP function altering biofilm growth**. **The antibodies raised to whole *A. baumannii* will have inhibitory effects on bacterial growth in conjunction with specific components of the innate immune system such as macrophage cells and complement.** This work describes the development of polyclonal antibodies against whole

organism as well as specific domains of *A. baumannii* BAP and the screening assays used to determine the efficacy of these antibodies.

2 Materials and Methods

2.1 General Rationale of Methods

There are certain problems that are inevitably encountered when working with a protein that is as large as BAP. Due to its considerable size (25,863 bp) it would prove very difficult to amplify and clone the entire protein and therefore challenging to create an effective immunogenic product. It is much easier to work with BAP in divided segments and as a consequence only certain regions were targeted and used for immunogen production. The repetitive structure of BAP made simple PCR cloning problematic and thus oligopeptides were designed and synthesized to correspond to the core containing tandem repeats A-G and were used for murine immunization in hopes of raising antibodies to these domains. Recombinant protein antigens corresponding to the non-repetitive N and C terminal regions of the protein were used for murine immunization and antibody development. These unique terminal regions were also used for primer design for PCR screening of bacterial strains for the presence of full length BAP.

2.2 BAP Target, Bacterial Strains and Culture Conditions

2.2.1 *bap* gene sequence

Loehfelm *et al* (2008) (39) successfully identified and sequenced a *bap* homologue present in *A. baumannii*. The *bap* locus was designated a specific nucleotide sequence accession number and was deposited into the GenBank sequence database which is a

collection of all publicly available DNA sequences provided by the National Centre for Biotechnology Information (NCBI). The NCBI GenBank sequence database was searched for identity using the *A. baumannii bap* accession number, which was published in the work of Loehfelm *et al* (2008), and resulted in access to the full nucleotide sequence of *bap* as well as the full protein sequence. Each repeat and section of BAP was separated in the database to allow easy viewing of each individual protein sequence.

2.2.2 BAP N and C Terminus Primer Design

Oligonucleotide primers were designed using the first 500 base pair (bp) fragment of the 25,863 bp *bap* open reading frame which comprised the N terminus of BAP. The 5' end of this region was used for the forward primer (BapNtermF) and the 3' for the reverse primer (BapNtermR) (Table 3). Oligonucleotide primers were purchased from Applied Biosystems (Foster City, CA. USA).

Oligonucleotide primers were designed from the last 1,216 bp fragment of the *bap* open reading frame which is situated directly after the G3 repeat. The first 31 bp succeeding this repeat were used as the forward primer (F-Bap-C) and the last 31 bp of the entire *bap* open reading frame formed the reverse primer (R-Bap-C) (Table 3). Oligonucleotide primers were purchased from Sigma-Aldrich (Oakville, ON. CA).

2.2.3 *A. baumannii* Strains and Culture Conditions

My work included a total of 18 *A. baumannii* strains. All *A. baumannii* strains were generously provided by past or present collaborators of the Molecular Immunology Lab of Cangene Corporation, save one which was purchased from American Type Culture

Collection (ATCC). Strains labelled B0063658U, B0091943U and B0008996U were collected in the ICU or burn unit of the Hamilton General Hospital and were graciously provided by Dr. Gerry Wright of McMaster University. Strains labelled Swab 4 and Swab 5 were collected from the general surgery ward of the University of Alberta Hospital and the general ICU of the Montreal General Hospital respectively. Swab 4 and Swab 5 were graciously provided by Dr. George Zhanel of the University of Manitoba (Health Sciences Centre, Winnipeg, Manitoba). Strains labelled B1-B11 and B13 were provided by Dr. Gerry Pier of Harvard University and did not come with any supplementary information. The ATCC BAA-1605 strain was cultured in ATCC laboratories (Manassas, VA. USA) after it was isolated from a wounded soldier returning from Afghanistan who was sent to Sunnybrook Health Sciences Centre, Ontario, Canada.

All *A. baumannii* strains were cultured from previously prepared glycerol stocks by inoculating tryptic soy broth (TSB) or Luria Bertani (LB) broth (Fisher) with a sterile loop and growing cultures at 37°C in a shaking incubator (New Brunswick C24) set at 200 rpm. Unless otherwise indicated all bacteria cultures were grown in pure media in the absence of a selective agent.

2.3 Identification of BAP positive *A. baumannii* Strains

2.3.1 Bacterial Screening – PCR Amplification of BAP N Terminal

All bacterial strains were PCR screened using BAP N terminal forward and reverse primers. All strains screening positive for the N terminal of BAP were then PCR screened using BAP C terminal primers. This was used as a method to identify strains with both ends of BAP.

Template DNA from all 18 bacterial cultures was purified using a DNeasy blood and tissue kit (Qaigen) designed for purification of total DNA from Gram negative bacteria. Template DNA was quantified using a NanoDrop-1000 Spectrophotometer (Thermo Scientific). All PCR reactions were performed in thin walled PCR tubes (FisherBrand) in a total volume of 50 µl containing 1µl each of 20µM Nterm forward and reverse primers, 25µl Platinum® blue superMix (Invitrogen) (contains: recombinant *Taq* DNA polymerase, Platinum® anti-*Taq* DNA polymerase antibody, Mg++, dNTPs, glycerol, and blue tracking dye), 18µl of nuclease free water (Ambion) and 5µl of template DNA. Negative controls were prepared by adding water in place of template DNA. Thermal cycling was performed using a MJ Research PTC-200 Thermocycler (GMI) or an Eppendorf Mastercycler PCR machine (Eppendorf) consisting of 1 cycle at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min; succeeded by 72°C for 10 min and 4°C until tubes were removed.

2.3.2 Bacterial screening- PCR Amplification of BAP C Terminal

All PCR reactions were carried out using a similar procedure as described above except Expand High Fidelity PLUS PCR system (Roche) replaced Platinum® blue superMix. This *Taq* produces a higher yield of amplified product and was more suitable for C terminal amplification. Reactions were performed in thin walled PCR tubes in a total volume of 50µl containing 10µl of Expand HiFi PLUS reaction buffer, 1µl of PCR grade nucleotide mix, 0.5µl of Expand HiFi PLUS enzyme blend, 5µl each of 20µM C terminal forward and reverse primers, 5µl template DNA and 23.5µl nuclease free water. Negative controls were prepared by adding water in place of template DNA. Thermal cycling was performed using

a MJ Research PTC-200 Thermocycler or an Eppendorf Mastercycler PCR machine consisting of 1 cycle at 94°C for 2 min followed by 10 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; this was followed by 25 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min +10 sec* (incubation time increased by 10 sec with every cycle), and 1 cycle of 72°C for 7 min and 4°C until tubes were removed.

2.3.3 PCR Analysis

Approximately 20µl of each sample was loaded onto a 1.5% Agarose gel containing 5% ethidium bromide (Fisher) in 1X TAE buffer (Bio-Rad). A low mass DNA ladder was prepared by mixing 1µl of 100 or 1000 bp ladder (New England Biolabs), 1µl of 6x loading dye (New England Biolabs) and 4µl of water and was then loaded into the first well of the gel. Samples were electrophoresed for approx 1hr at 100 V using a Bio-Rad PowerPac High Current system. The gel was visualized under UV light in an Alpha Innotech multi image light cabinet and analyzed using the FluorChem® HD imaging system with AlphaEase image analysis software.

2.4 Immunogen Design – Development of Peptide Antigen

2.4.1 Peptide Design

Synthetic oligo-peptides were designed to correspond to consensus repeat regions of the BAP protein. Since the protein sequences of each repeat are separated on the NCBI database it was possible to generate a consensus of each repeat module. All protein sequences from each individual repeat module (i.e. A1-A5, B1-B22 etc) were inserted into separate protein files of MacVector 10 software. Clustal alignment was performed on each

module to generate a consensus which peptides could be designed from. Peptides were purchased from United Biochemical Research Inc. (Seattle WA. USA). (See appendix I for repeat alignments).

2.4.2 Preparation of Peptides

A small volume (0.5ml) of water was added to each peptide to determine if they readily dissolved in water. Water soluble peptides were dissolved in a calculated volume of nuclease free water (Ambion), dependent on peptide mass, to produce 10mg/ml stocks. Water insoluble peptides were dissolved in DMSO (Sigma) to make 10mg/ml stocks. Peptide stocks were stored at -80°C in 200µl aliquots.

2.4.3 Peptide Conjugation to KLH and BSA Carrier Proteins

Peptides alone are not able to elicit a sufficient immune response due to their small size and thus must be conjugated to specific carrier proteins to yield more effective immunogens. Peptides were conjugated to mcKLH and BSA, for immunization purposes and assay analysis respectively, using an Imject® Maleimide Activated Immunogen Conjugation Kit (Pierce) according to manufacturer's instructions. Briefly: Prepared peptides were thawed on ice while one vial each of lyophilized mcKLH and BSA carrier proteins were reconstituted with nuclease free water by gently pipetting. In microcentrifuge tubes (Fisherbrand) (two per conjugation) 200µl of 10mg/ml peptide, 200 µl of reconstituted carrier protein and 200µl of conjugation buffer were mixed together and incubated for 2 hrs at room temperature. Purification of conjugates was performed by desalting. The peptide-carrier mixture was loaded onto desalting columns and allowed to flow through with any conjugated peptide-carrier remaining trapped in the column.

Conjugated peptides were then eluted with 8-10 aliquots of 0.5ml purification buffer, each fraction was collected in a separate tube. Fractions were measured with a Bio-Rad Smart Spec Plus Spectrophotometer at an absorbance of 280 nm to determine which fractions contained sufficient amount of conjugates. Fractions with a concentration of 0.5µg/ml or higher were kept and pooled for further analysis. Anything with a concentration less than 0.5µg/ml was discarded.

2.4.4 BCA Analysis of Pooled Peptide Conjugates

Total protein concentration of the peptide/KLH/BSA conjugates was determined using a BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. Microtiter plates, used for the assay, were read and analyzed at 562nm using BioTek Synergy 2 plate reader and Gen5 software. Peptide/KLH and peptide/BSA conjugates were labelled with appropriate name and concentration and stored at -20°C in 200µl aliquots until required for immunization.

2.5 Immunogen Design – Development of Recombinant Proteins

2.5.1 Production of DNA Fragments – PCR Amplification of BAP N and C Terminal

N and C terminal fragments were amplified from *A. baumannii* strains which were positive for both BAP N and C terminals. Two reactions per strain were prepared. PCR reactions were performed and analyzed as per sections 2.3.2[a] and 2.3.3.

[a] Expand High Fidelity PLUS PCR system (Roche) was used for amplification of both N and C terminal fragments as it gives a higher yield of amplified product and was suitable for this particular set of experiments.

2.5.2 PCR Fragment Isolation and Purification

PCR amplified BAP N and C terminal fragments of approximately 500 and 1200 base pairs respectively were excised from the agarose gel using a sterile scalpel (Feather). Gel slices were weighed using a Mettler Toledo weigh scale and DNA was purified using a Qiaquick® Gel Extraction Kit (Qiagen) according to manufacturer's instructions. Extracted, purified DNA was quantified using a NanoDrop-1000 Spectrophotometer (Thermo Scientific), labelled and stored at -20°C.

2.5.3 Ligation of PCR product into pET SUMO Vector

Extracted DNA was ligated into a pET SUMO vector using the Champiom™ pET SUMO Expression System (Invitrogen). This expression system uses a small ubiquitin-like modifier (SUMO) that is designed to facilitate cloning of PCR products for expression, purification and generation of recombinant proteins in *E. coli*. The pET SUMO vector includes Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible expression, an N-terminal polyhistidine tag for detection and purification of recombinant fusion proteins, a TA Cloning® site for effective cloning of *Taq*-amplified PCR products and a kanamycin resistance gene for selection in *E. coli* (Champion™ pET SUMO Protein Expression System User Manual, 2004).

Ligation reactions were performed in microcentrifuge tubes in a total volume of 10µl using a 1:1 or 1:3 vector:insert ratio containing 0.5µl to 1µl extracted DNA (as per manufacturer's suggestion), 1µl of 10x ligation buffer, 2µl of 25ng/µl pET SUMO vector, sterile water to total 9µl and 1µl T4 DNA Ligase (all supplied within the pET SUMO

Expression Kit). Tubes were placed in a Thermostat Plus heat block (Eppendorf) set to 15°C and left to incubate overnight.

2.5.4 Transformation of Ligation Product

The ligation product from above was then transformed into One Shot® Mach1-T1 chemically competent *E. coli* cells (Invitrogen) as per commercial instructions. The Mach1-T1 *E. coli* strain is modified from wild-type ATCC #9637 strain and provides a host for stable propagation and maintenance recombinant plasmids (Champion™ pET SUMO Protein Expression System User Manual, 2004). Briefly, each transformation reaction was performed using the following procedure: 2µl of each ligation reaction was added to a vial of Mach1-T1 chemically competent cells and mixed gently. Cells were placed on ice for 15 min and then heat shocked for 30 sec in a 42°C Isotemp 205 water bath and then put back on ice. 250µl of Super Optimal broth with Catabolite repression (S.O.C) medium (Invitrogen) was added to the cells after which vials were capped tightly and placed in a 37°C shaking incubator (New Brunswick C24) set at 200 rpm for 1 hour. Cells were then centrifuged at 13,000 rpm for 2-5 min using an Eppendorf microcentrifuge, the supernatant was discarded and cell pellets were resuspended in 50µl of S.O.C medium. The full 50µl of each transformation reaction was plated out on pre-warmed L.B agar plates containing 100µg/ml kanamycin (Teknova). Plates were placed in a 37°C incubator (Binder) and left to grow overnight. The following day 10 colonies from each plate were picked and re-streaked on L.B agar plates containing 100µg/ml kanamycin and incubated at room temperature.

2.5.5 Analysis of Transformants by Colony PCR Amplification

Even though each transformation reaction produced numerous colonies suggesting that the ligation and transformation reactions were successful, it was important to confirm the presence of the BAP N and C terminal inserts in the SUMO vector, collectively called recombinant fusion proteins.

Template DNA was prepared in the following manner: Each re-streaked colony was given a unique label to allow individual identification. Next, 30µl of sterile water was added to the same number of 600µl microcentrifuge tubes which were labelled to match the individual colonies. A small portion of each re-streaked colony was transferred to the appropriate tube and swirled vigorously in the water to dislodge the bacteria. Tubes were placed in a Thermostat Plus heat block (Eppendorf) set to 94°C for 10 min to burst the *E. coli* cells and release the plasmid DNA into the water. Samples were then cooled on ice for 5 min and centrifuged at 13,000 rpm for 10 min to pellet the *E. coli* cells, leaving the plasmid DNA in the supernatant. Then, 10µl of the supernatant was removed and added to a corresponding thin-walled PCR tube to act as template DNA. The remaining procedure and analysis of the colony PCR reactions were performed as per sections 2.3.1 and 2.3.3 with the following exceptions: 38µl of Platinum® blue superMix (Invitrogen) was added to the PCR tubes but no water.

2.5.6 Extraction and Purification of Plasmid DNA

Transformant colonies which screened positive for BAP N and C terminal inserts were then cultured for subsequent plasmid DNA extraction and purification for sequencing purposes. The pET SUMO vector is a low-copy number plasmid and therefore a larger

quantity of bacterial culture was required to obtain enough plasmid DNA for further analyses.

Small analytical cultures were prepared by scraping a small portion of the re-streaked colonies and inoculating a corresponding 50ml BD Falcon tube (Fisher) containing 15ml of L.B broth and 50µg/ml Kanamycin (Sigma) as a selective agent. Cultures were grown overnight at 37°C in a shaking incubator set to 200 rpm.

Plasmid DNA was extracted and purified from overnight bacterial cultures using a Qiaprep® Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. Extracted and purified plasmid DNA was quantified using a NanoDrop-1000 Spectrophotometer and stored at -20°C for further use.

Glycerol stocks were prepared from the overnight culture for long term storage of samples containing BAP N and C terminal inserts. Two 10% glycerol stocks per sample were prepared by aliquoting 333µl of sterile 30% glycerol to an appropriate number of labelled cryogenic vials (Nalgene) followed by 667µl of overnight culture. Stocks were mixed thoroughly by vortexing and immediately stored at -80°C.

2.5.7 Sequencing Analysis

Plasmid DNA was sent for sequencing to confirm that the BAP N and C terminal inserts were in the correct orientation and in frame with the N-terminal peptide 6xHis tag of the SUMO vector. Plasmid DNA was sequenced using the SUMO Forward and T7 Reverse sequencing primers (Table 3) included in the pET SUMO Protein Expression Kit (Invitrogen). Sequencing samples were prepared by mixing 25ng/µl of plasmid DNA in

sterile water to total 100µl. 3µM primer stocks were prepared by adding 1.2µl of 100µM primers to 38.2µl of sterile water to total 40 µl. Plasmid DNA samples and primers were sent to Beckman Coulter Genomics in Danvers MA., USA for analysis. Sample submission requirements were set by Beckman Coulter Genomics. Sequence data was analyzed using DNASTAR Lasergene 8 software.

Table 3. Primer sequences. BAP N and C terminal fragment primers as well as SUMO forward and reverse primer sequences are seen below.

Primer	Sequence 5' - 3'
BapNtermF	ATGCCTGAGATACAAATTATTGCCAAGGATAATC
BapNtermR	AGGTGCTGAAGAATCATCATTAC
F-Bap-C	ACAGCAGATCCTTGGGCAGATGCTACACTTC
R-Bap-C	TTAGAAAATGATTTGACCATTGTTGATCAAG
SUMO Forward	AGATTCTTGTACGACGGTATTAG
T7 Reverse	CTAGTTATTGCTCAGCGGTGG

2.5.8 Recombinant Protein Expression – Transformation into BL21(DE3) Cells

The Champion™ pET SUMO Protein Expression System controls expression of heterologous genes in *E. coli* by utilizing elements from bacteriophage T7. BL(DE3) One Shot® *E. coli* cells (Invitrogen) are designed for expression of genes regulated by the T7 promoter and therefore provide a suitable host for expression of the pET SUMO constructs (Champion™ pET SUMO Protein Expression System User Manual, 2004).

The plasmid sequences confirmed to have BAP N and C terminal inserts in the correct orientation within the SUMO vector were used for expression studies. Pure plasmid DNA was transformed into BL(DE3) cells using the following procedure: BL(DE3) cells were thawed on ice and 10ng of plasmid DNA in a 5µl volume was added to each vial of cells and stirred gently with a pipette tip. Cells were incubated on ice for 30 min, heat shocked for 30 sec in a 42°C Isotemp 205 water bath and immediately transferred back to ice where 250µl of S.O.C medium was added to each vial. Tubes were capped tightly, taped on their sides and incubated in a 37°C shaking incubator set to 200 rpm for 1 hour. Transformation reactions were then added to 15ml BD Faclon tubes (Fisher) containing 10ml of L.B broth with 50µg/ml kanamycin and grown overnight in a 37°C shaking incubator set to 200 rpm.

2.5.9 Pilot Expression

A pilot expression was performed which allowed us to determine the optimal conditions for expression of the BAP N and C terminal fusion proteins. The pilot expression was done on a time course by taking time points of induced and uninduced cultures.

The pilot expression for each BL(DE3) transformation reaction was performed by inoculating 10ml of fresh L.B broth containing 50µg/ml kanamycin with 500µl of BL(DE3) overnight culture from the previous section. New cultures were incubated at 37°C with 200 rpm shaking for 2 hours until the Optical Density (OD) measured at 600nm was approximately 0.4-0.6 (mid-log phase). Measurements were taken after 2 hours with a Bio-Rad Smart Spec Plus spectrophotometer to confirm the OD600 values were within the appropriate range. The 10ml cultures were then split into two 5ml cultures. Expression

was induced in one of the 5ml cultures by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) to a final concentration of 1mM. This resulted in two cultures per sample; one induced (IPTG+) and one uninduced (IPTG-). A 500µl aliquot was removed from each culture, dispensed in a microcentrifuge tube and centrifuged at 13,000 rpm for 1 min to pellet bacteria. The supernatant was discarded and cell pellets were frozen at -20°C until required for further experiments. These were considered zero time points. Cultures were placed back in a 37°C shaking incubator and removed once every hour for 5 hours to total six time points (t = 0-5). For each time point 500µl aliquots were removed and processed as above.

2.5.10 SDS-PAGE Analysis of Pilot Expression

The frozen cell pellets from each time point of the pilot expression were lysed and subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the optimal incubation time for expression.

Bacterial cell lysis to liberate soluble and insoluble protein factions was achieved using BugBuster® Protein Extraction Reagent Master Mix (Novagen) according to the manufacturer's protocol. To prevent protein degradation BugBuster® Master Mix was mixed with a cocktail of the following protease inhibitors: 1µM Leupeptin, 1µg/ml Pepstatin A and 1mM phenylmethylsulfonyl fluoride (PMSF) (All from Sigma). Small volumes of both soluble and insoluble cell lysate samples were mixed with equal volumes of 4xSDS sample buffer (EMD Biosciences). Lysate/buffer mixtures were placed in a heat block (Eppendorf Thermostat Plus) set to 85°C for 3 min in preparation for SDS-PAGE analysis.

SDS-PAGE analysis was performed using an X Cell Sure Lock Mini-Cell apparatus, 1xMOPS SDS running buffer (diluted from 20x MOPS running buffer) and NuPAGE® Bis/Tris 4-12% Precast gels (All from Invitrogen). The X Cell Sure Lock Mini-Cell apparatus was assembled according to the manufacturer's product manual. NuPAGE® precast gels were removed from the storage pouches, combs were gently removed from the wells and gels were rinsed with distilled water. The gel cassettes were inserted into the buffer chamber and locked into the apparatus with the gel tension lever. The apparatus was filled with 1xMOPS SDS running buffer in preparation for sample loading. 10µl of Precision Plus Protein All Blue Standards molecular marker (Bio-Rad) was dispensed into the first well of each gel; then 20µl of the prepared lysate samples were dispensed into the nine remaining wells of the NuPAGE gel. Samples were electrophoresed at room temperature for approximately 1 hour at 200 V using a Consort, E844 power supply. Gels were removed from cassettes and placed in plastic containers. Protein staining was accomplished using a Colloidal Blue Stain Kit (Invitrogen) by shaking each gel at 60 rpm in a fixing solution containing 20ml distilled water, 25ml methanol (Fisher) and 10ml acetic acid (Fisher) for 10 min at room temperature. This was followed by shaking the gels in a staining solution containing 27.5ml distilled water, 10ml methanol and 10ml Stainer A (included in kit) for 10 min; then adding 2.5ml of Stainer B (also from kit). Gels were stained overnight with shaking using a New Brunswick Excella E5 platform shaker set to 60 rpm. Gel images were visualized and captured using Alpha View Fluorchem Q SA system software, version 3.2.2.0 (Cell Biosciences Inc.).

2.5.11 Expression Scale up of Target Recombinant Fusion Proteins

Individual plasmids encoding N and C terminal fusion proteins were chosen for expression scale up and purification based on the results of the pilot expression analysis.

Expression was scaled up by growing and inducing a 50ml bacterial culture using the same protocol as described in section 2.5.8 with the following exceptions: 50ml of L.B broth containing 50µg/ml kanamycin was inoculated with 1ml of transformation culture. Cultures were grown at 37°C with shaking at 225 rpm for 2-3 hours until the Optical Density (OD) measured at 600nm was approximately 0.5. Expression was induced by adding IPTG to a final concentration of 1mM to the 50ml cultures and incubating them at 37°C with shaking for 4 hours as determined by the pilot expression. Cells were harvested by centrifuging at 3000xg for 10 min at 4°C using a Beckman Coulter, Avanti J-301 floor centrifuge. Cells were stored at -80°C until required for purification.

2.5.12 SDS-PAGE and Western Immunoblot Detection of Recombinant Fusion Proteins

Bacterial cell lysate was prepared using BugBuster® Protein Extraction Reagent Master Mix and analyzed by SDS-PAGE as per section 2.5.10 to confirm that the scaled up expression of N and C terminal protein fragments was successful. Remaining lysate was stored at -20°C. Expression of the N/C terminal-SUMO fusion proteins was also confirmed by western immunoblot analysis according to standard procedures: Concurrently a second set of the samples were ran on a SDS-PAGE gel and then transferred to a nitrocellulose membrane (Bio-Rad). Transfer was accomplished by electrophoresis in an X Cell Sure Lock Mini-Cell transfer apparatus filled with 1x Transfer buffer (diluted from NuPAGE 20x

Transfer buffer (Invitrogen)) at 40 V for 1 hour. The transfer membrane was blocked in 5% skim milk powder (SMP) (Bio-Rad) diluted in 1xTBST (diluted from 10x TBS (Bio-Rad)) and 0.1% Tween 20 (MP Biomedicals) overnight at 4°C. Since the SUMO system creates an N-terminal polyhistidine (6xHis) tag (see appendix II for SUMO layout), detection of proteins was achieved by probing the membrane with an anti-HisG-HRP antibody (Invitrogen) diluted 1:5000 in 1xTBST for 1 hour. In between steps the membrane was washed with 1xTBST three times for 5 min each. The membrane was developed using an Enhanced Chemiluminescence (ECL) Western Blotting Substrate kit (Pierce) according to the manufacturer's instructions. Membrane images were visualized and captured with Alpha View Fluorchem Q SA system software, version 3.2.2.0 (Cell Biosciences Inc.).

2.5.13 Purification of Recombinant Fusion Proteins

Lysate samples containing recombinant fusion proteins were purified using ProBond™ Purification System (Invitrogen) according to the manufacturer's instructions. N terminal recombinant proteins were purified under native conditions and C terminal recombinant proteins were purified under hybrid conditions using ProBond™ nickel-chelating resin filled columns according to the protocols in the manufacturer's user manual. All purification flow through, wash and eluted fractions from the columns were analyzed by SDS-PAGE analysis as per section 2.5.10 to determine which fractions contained purified recombinant proteins. Fractions containing purified protein were pooled for further analysis. Western immunoblot analysis as per section 2.5.12 was performed to detect and confirm the presence of purified protein in the pooled samples. Total protein concentration of purified samples was determined using a BCA Protein Assay Kit (Pierce)

according to manufacturer's instructions. BCA plates were read and analyzed at 562nm using a BioTek Synergy 2 plate reader and Gen5 software

2.5.14 Generation of Native protein

Native protein was generated by cleaving the N-terminal peptide containing the 6xHis tag and SUMO protein using SUMO protease (Invitrogen) according to the instructions found in the Champion™ pET SUMO Protein Expression System user manual. Native protein was purified as per section 2.5.13 and analyzed by SDS-PAGE as per section 2.5.10.

2.5.15 Buffer Exchange of Purified Proteins

Purified native and N/C terminal-SUMO fusion proteins were buffer exchanged into PBS and concentrated using a Centriprep Centrifugal Filter Device (Millipore) with an Ultracel® 10 kDa cut-off membrane. Centriprep devices were spun at 2500 x g for 30 min at 4°C in a Beckman Coulter Allegra X12R floor centrifuge.

Total protein concentration of buffer exchanged samples was determined using a BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. BCA plates were read and analyzed at 562nm using a BioTek Synergy 2 plate reader and Gen5 software. Purified proteins were stored at -20°C until required for immunization.

2.6 Preparation of Whole Bacteria Antigen

2.6.1 Planktonic *A. baumannii* Cultures

Planktonic cultures of BAP positive strains were prepared by streaking out glycerol stocks on L.B agar plates (Teknova) and incubating them overnight at 37°C. Single colonies were picked with a sterile loop and used to inoculate 20ml of sterile L.B broth media. Cultures were grown as described in section 2.2.3.

2.6.2 Biofilm *A. baumannii* Cultures

Bacterial biofilms were grown in a device manufactured in house consisting of 600ml beakers, omniwrap autoclave paper and 15ml BD Falcon tubes (Figure 3). Construction of each biofilm device and biofilm growth was accomplished as follows: Autoclave paper was placed on the top of a 600ml glass beaker as to cover the entire open surface and taped tightly around the beaker with autoclave tape. Using a scalpel, star shaped openings were made on the top of the paper to allow insertion of 5-7 plastic tubes. Tubes were inserted into the holes and covered with another layer of autoclave paper which was secured tightly with more autoclave tape. The apparatus was autoclaved on gravity cycle for approximately 1 hour and placed immediately in a sterile biosafety cabinet (BSC). In the BSC the top cover of autoclave paper and one tube were removed from the device and placed on the sterile surface. Next, 340ml of sterile 50% L.B broth (diluted in sterile distilled water to produce media with decreased nutrient content to induce biofilm formation) was added to the beaker through the opening left by removal of one of the tubes. The media was then inoculated with 10ml of culture previously prepared as per

section 2.6.1. Devices were placed in a 37°C incubator with 80-90 rpm slow shaking for 2-5 days to allow biofilm growth on the outer surface of the plastic tubes.

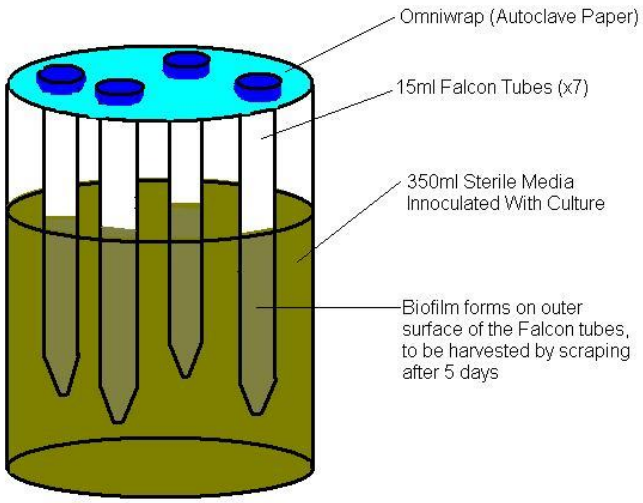


Figure 3. Cartoon representation of in house manufactured biofilm device.

Tubes were inspected for growth on day 2 and biofilm was only harvested if growth was sufficient, otherwise devices were left to incubate. Biofilm was harvested in a sterile BSC by carefully removing each tube, washing them in sterile Dulbecco's Phosphate Buffered Saline (PBS) (Sigma) and scraping off biofilm with a sterile cell scraper (VWR) into tubes containing 20ml fresh sterile PBS.

2.6.3 Concentration and BCA Analysis of Bacterial Cultures

Planktonic cultures prepared as in section 2.6.1 were centrifuged at 3000 x g to pellet bacteria. L.B broth supernatant was discarded and bacteria were resuspended in an equal volume of sterile PBS. This step was not necessary for the biofilm bacteria as it was already in PBS. Both planktonic and biofilm stocks were concentrated approximately 10 times using a Centriprep Centrifugal Filter Devices (Millipore) with an Ultracel® 10 kDa

cut-off membrane according to manufacturer's instructions. Centriprep devices were spun at 2500 x g for 30 min at 4°C in a Beckman Coulter Allegra X12R floor centrifuge.

Concentrated bacteria samples were then analyzed by BCA to determine total protein concentration using a BCA Protein Assay Kit (Pierce) according to manufacturer's instructions. BCA plates were read and analyzed at 562nm using a BioTek Synergy 2 plate reader and Gen5 software. Planktonic and biofilm stocks were aliquoted and stored at -80°C.

2.6.4 Chemical Inactivation of *A. baumannii*

Chemical inactivation of planktonic and biofilm bacteria was performed by adding 5% protocol 1:10 dilution buffered formalin (Fisher) to bacterial stocks and incubating overnight at room temperature on a rotating wheel to ensure good mixing. Bacteria were washed 3 times by centrifuging at 2500 rpm for 25 min, discarding supernatant and resuspending in fresh PBS to remove the formalin. Approximately 20µl of each inactivated sample was spread on L.B agar plates and incubated at 37°C overnight to check for growth. A control plate was also prepared with 20µl of live bacteria and incubated at 37°C overnight. Plates were inspected and compared to the control to ensure formalin inactivation was successful. Inactivated samples were stored at -20°C until required for immunization.

2.7 PAb Development

2.7.1 Immunization with Peptide Antigens

University of Manitoba 6 – 8 week old female Balb/c mice were immunized with peptide/KLH conjugates throughout a 90 day period consisting of a first immunization on day zero followed by a series of boosters on days 28, 48, 63 and 77[b]. Each peptide group contained 4 mice resulting in a total of 20 mice for peptide immunization. All immunizations were administered subcutaneously (S.C) using 1 c.c. syringes and 23 gauge needles (BD). On day zero mice were administered 100µl of a 10µg dose of peptide antigen and PBS in a 1:1 dilution of Complete Freund's Adjuvant (CFA) (Brenntag Biosector). The following boosters contained the same dose as the first immunization and were given in a 1:1 dilution with Incomplete Freund's Adjuvant (IFA) (Brenntag Biosector) with the exception of the final booster, or final push, which was given as a 5µg dose in 50µl straight PBS. Final boosters were administered by the intraperitoneal (I.P) route. All immunizations were performed in accordance with Canadian Council of Animal Care (CCAC) guidelines.

2.7.2 Immunization with Recombinant Proteins

University of Manitoba 6 – 8 week old female Balb/c mice were immunized with recombinant proteins using an accelerated immunization schedule consisting of a first immunization on day zero followed by a booster on day 14 and 28[c]. The N and C terminal-

[b] Dependent on antibody titres determined by ELISA analysis of trial bleeds

[c] Dependent on antibody titres determined by ELISA analysis of trial bleeds

SUMO and native C terminal protein groups contained 2 mice each resulting in a total of 6 mice for recombinant protein immunization. All immunizations were administered intraperitonealy (I.P) using 1 c.c. syringes and 23 gauge needles (BD). The first immunization and subsequent boosters were given as 2µg doses in 100µl of prepared recombinant protein antigen and CpG adjuvant (InvivoGen) in PBS containing 30% v/v Emulsigen (MVP Technologies). The final push was given I.P as a 2µg dose in 50µl straight PBS. All immunizations were performed in accordance with Canadian Council of Animal Care (CCAC) guidelines.

2.7.3 Immunization with Whole Bacteria – Production of Control Antibody

Control antibody was produced by immunizing mice with whole inactivated planktonic and biofilm *A. baumannii* as per section 2.7.1 with the exception that 10µg doses were given in 50µl rather than 100µl. The first immunization was given in a 1:1 dilution with IFA instead of CFA as the antigen was whole bacteria, a strong immunogen, and CFA is only used for antigens suspected to be weakly immunogenic. Planktonic and biofilm groups contained 4 mice each resulting in a total of 8 mice for whole bacteria immunization. All immunizations were performed in accordance with Canadian Council of Animal Care (CCAC) guidelines.

2.7.4 Mice Euthanasia and Serum collection

Mice were euthanized 3 days post final push if they were immunized using Freund's adjuvant and 7 days post final push if they were immunized with CpG-Emulsigen adjuvant. Euthanasia was carried out by anaesthetizing the mice and performing a cardiac puncture for blood collection until death of the animal. Cervical dislocation was done as a secondary

and precautionary measure to ensure mice were dead before bagging them. A certified animal technician performed all anaesthetizing procedures, cardiac punctures and euthanasia.

Blood obtained from the cardiac punctures was dispensed into serum separator tubes (BD) and centrifuged at 13,000 rpm for 1 min. Serum was collected and aliquoted into microcentrifuge tubes and stored at -20°C.

2.8 PAb Efficacy Assays

2.8.1 ELISA Reactivity of Hyperimmune Serum

All Enzyme-linked immunosorbent assays (ELISAs) were performed in maxiSorp 96-well flat bottom plates (NUNC™) and were of the 'indirect' type consisting of 5 steps: (i) The amount of antigen (Ag) needed for the first coat was calculated depending on the number of plates and amount of Ag per well. The amount of Ag per well varied depending on the type of Ag being coated (peptide, recombinant protein or whole bacteria). A calculated amount of Ag was added to PBS (for peptide and recombinant proteins) or carbonate-bicarbonate (for whole bacteria) (Sigma) coating buffer and 60µl was added to each well using a multichannel pipette. The plate was incubated at 4°C overnight allowing the Ag to adhere. Plates were washed 5 times with MillQ® water (resistivity 18.2Ω•cm) using a BioTek ELx405 plate washer. (ii) The plates were blocked with 60µl of blocking buffer consisting of 5% SMP in PBS and incubated at 37°C for 1 hour. Plates were washed as above. (iii) Primary antibody was prepared by diluting mouse anti-serum or naive serum (negative control) in dilution buffer consisting of 2.5% SMP in PBS. 60µl of primary antibody at a starting dilution of 1:100 was added to the first row. The remaining 7 rows

were coated with 60µl of serially diluted antibody; resulting in 2-fold diluted antibody in each subsequent row. Plates were incubated at 37°C for 1 hour and washed as above. (iv) Then 60µl of a 1:2000 dilution of goat anti-mouse IgG-HRP (Southern Biotech) in 2.5% SMP dilution buffer was added to each well as a secondary antibody. Plates were incubated at 37°C for 1 hour and washed as above. (v) Plates were then coated with 60µl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) substrate (Roche) and incubated at room temperature to produce a color change. Plates were inspected for color change at 15, 30 and 60 min. Colour development was measured at 405nm using a BioTek Synergy 2 plate reader and Gen5 software.

2.8.2 Western Immunoblot Reactivity Assays

All western immunoblot assays were performed as described in section 2.5.12 with the following exceptions: All samples were prepared as 15µl loads of 1-5µg of Ag and 5µl of 4x NuPAGE® LDS sample buffer (Invitrogen) diluted in a calculated volume of PBS (dependent on volume of Ag). Samples were heated at 70°C for 10 min in a heat block and the total 15µl was loaded onto NuPAGE® Bis/Tris 4-12% precast gels. Samples were run using the SDS-PAGE protocol as per section 2.5.10. When testing peptide and recombinant protein anti-serum reactivity with whole bacteria, 5µg of bacteria Ag was loaded onto NuPAGE® Novex 3-8%[d] Tris-Acetate gels and ran with 1x Tris-Acetate SDS running buffer (diluted from 20 x NuPAGE® Tris-Acetate SDS running buffer) (both from Invitrogen). Gels were electrophoresed at 150 V for 1.5-2 hours and transfer took place for 1.5 hours at

[d] lower cross linked gels were used as they allow larger proteins such as BAP to run through easier than the higher 4-12% cross linked gels.

40 V. In all cases mouse anti-serum was used as a primary antibody probe at a dilution of 1/250 in half strength blocking buffer (2.5% SMP in 1 x TBST) and goat anti-mouse IgG-HRP diluted 1:6000 in 1 x TBST was used as the secondary antibody probe[e]. All remaining steps were the same as described in section 2.5.12.

2.9 *In Vitro* Assays

2.9.1 Preparation of Bacterial Stocks and CFU/ml Calculations

Bacterial stock 10ml *A. baumannii* cultures were prepared as per section 2.6.1 and centrifuged at 3000 x g to pellet bacteria. L.B supernatant was discarded and bacteria pellets were resuspended in 10ml sterile PBS. Ten 15ml BD Falcon tubes were filled with 9ml of PBS. 10-fold serially dilutions were performed in PBS to produce bacterial dilutions ranging from 10^1 to 10^{10}. 100µl of each dilution was spread on TSB plates (bioMerieux) which colonies were too numerous to count in which case they were labelled TNTC and set aside. The dilution plate with approximately 50-150 colonies was chosen to calculate colony forming units per ml (CFU/ml). The original bacteria/PBS culture was aliquoted, labelled with calculated CFU/ml and stored at -80°C.

[e] Suitable dilutions for anti-serum primary antibody and the secondary antibody were determined from a set of preliminary western immunoblot experiments performed previously. Various amounts of antigen and dilutions of antibody were tested together to determine optimal dilutions for pre-immune (negative control) conditions, positive control conditions and to ensure no background noise occurred from secondary antibody probing.

2.9.2 Complement Dependent Assays

Complement assays were performed in MaxiSorp 96- well flat bottom plates with each sample ran in triplicate. Each sample had a total volume of 60µl consisting of: 10µl of *A. baumannii* bacteria at a concentration of 10^7/ml, various dilutions of whole *A. baumannii* anti-serum or naive mouse serum (negative control), 10µl rabbit complement (Cedarlane) or 10µl DMEM, 10µl of DMEM + 0.6% FBS and DMEM to total 60µl (dependent on antibody volume).

A. baumannii stocks were diluted to 10^7 and incubated with serum, DMEM and DMEM+0.6% FBS in the first row of wells for 10-15 min at 37°C. In this time lyophilized rabbit complement was reconstituted with 0.5ml cold sterile MilliQ® water and 0.5ml DMEM and filter sterilized using a 33mm millex GV 0.22µm filter unit (Millipore) and 1ml syringe (BD). Complement was added to samples designated '**+** complement' and DMEM was added to samples designated '**-** complement'. Samples were gently mixed by tapping the corner of the plate and incubated at 37°C for 1 hour. Next, 40µl of sterile PBS was added to each well bringing the volume to 100µl and diluting bacteria further to 10^6/ml. In the next 4 rows 90µl of PBS was added to the wells and 10-fold serial dilutions were performed to dilute bacteria even further. Then, 50µl of the 10^3 and 10^4 dilutions were plated on TSB plates for bacteriology and incubated overnight at 37°C. Colonies were counted and CFU/ml were calculated for each sample to determine if *A. baumannii* was susceptible to complement and whether whole *A. baumannii* anti-serum activity was complement dependent.

2.9.3 Growth and Maintenance of J774A.1 Macrophage Cell Line

Murine macrophage cell line J774A.1 (ATCC) were grown from a previously prepared Master stock ($1.0x10^7$ cells/ml)[f] in sterile T75 cm^2 Corning® rectangular cell culture flasks (Fisher) using Dulbecco's Modified Eagle Medium (DMEM) (ATCC) with 10% Fetal Bovine Serum (FBS) (Wisent) and 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma) at 37°C with 5% CO_2. Cultures were passaged every 3 days and harvested at a density of $2.0x10^7$ cells/ml on the day macrophage assays were performed.

2.9.4 Macrophage Opsonization Assays

All macrophage assays were performed in MaxiSorp 96- well flat bottom plates with each sample ran in triplicate. Each sample had a total volume of 50µl consisting of: 10µl of *A. baumannii* bacteria at a concentration of 10^8/ml, various dilutions of whole *A. baumannii* anti-serum or naive mouse serum (negative control), 10µl of J774A.1 cells at a concentration of $2.0x10^7$ cells/ml or 10µl of DMEM and PBS to 50µl (dependent on antibody volume).

Bacteria were incubated with serum and PBS in the first row of wells for15 min at 37°C. J774A.1 cells were added to samples designated '**+** macrophage' and DMEM was added to samples designated '**-** macrophage'. Samples were gently mixed by tapping the plate and incubated at 37°C for 3 hours, after which macrophage cells were lysed with 50µl of 0.2% Triton®-X100 (Sigma). Then, 90µl of PBS was added to wells of the next 5 rows

[f] J774A.1 Master and Token stocks previously cultured and froze down by Research Technician Yvonne Kindiak of the Molecular Immunology Lab, Cangene Corporation.

and 10-fold serial dilutions were performed to dilute bacteria. 50µl of the 10^4 and 10^5 dilutions were plated on TSB plates for bacteriology and incubated overnight at 37°C. Colonies were counted and CFU/ml were calculated for each sample to determine if *A. baumannii* was susceptible to macrophage opsonization.

3 Results

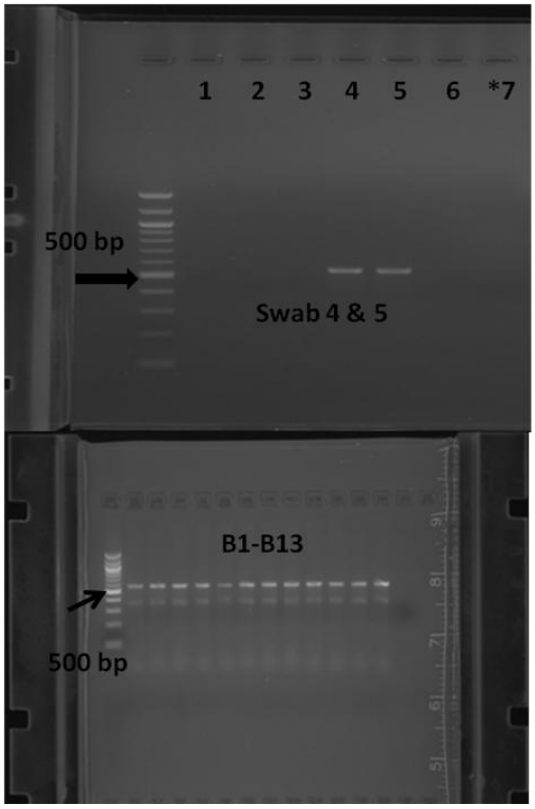
3.1 Identification of BAP Positive Strains - BAP N and C Terminus Amplification

Molecular screening was performed on all 18 *A. baumannii* strains in order to determine which strains contained the *bap* gene and therefore which strains to use for further experimentation and antibody development against whole organism (Table 4). The *bap* gene is much too large (25,863bp) to PCR amplify as attempts were made but proved unsuccessful (data not shown). Therefore the N terminal and C terminal fragments were amplified using BAP N and C terminal oligonucleotide primers (Table 3) to identify BAP positive strains. Strains which screened positive for the N terminus of BAP included Swab 4, Swab 5 and B1-B11, B13. Evidence of this could be seen clearly on agarose gels as bright bands appeared around the 500 base pair mark which is the approximate size of the N terminal fragment (Figure 4A). These strains were then screened for the C terminus of BAP; however analysis revealed that this fragment could only be amplified in bacterial strains Swab 4 and Swab 5, but not the other strains as seen by the two very prominent bands around the 1200 base pair mark (Figure 4B). Therefore, all further experimentation was performed using strains Swab 4 and Swab 5.

Table 4. Strain I.D and details for the 18 isolates of *Acinetobacter baumannii* that were screened for the BAP protein. All supplementary information was supplied by Dr. G. Zhanel and Dr. G. Wright. No information was supplied for strains B1-B11, B13.

Strain I.D	Collection Date	Location	Collection Site	Source	Age of patient	Gender of patient
B0063658U	Nov 5, 2007	Hamilton General Hospital	ICU	*Endotracheal tube* Aspirate	Unknown	M
B0091943U	Jul 24, 2006	Hamilton General Hospital	ICU	Rectal swab	Unknown	M
B0008996U	Jan 21, 2007	Hamilton General Hospital	Burn Trama Unit	Blood – Central line	Unknown	M
Swab 4	Jan 2, 2007	U of Alberta Hospital	General Surgery	Urine	40	M
Swab 5	Apr 15, 2007	Montreal General Hospital	General Unspecified ICU	Blood	72	F
BAA-1605	Jun 30, 2006	Health Sciences Centre, Ont.	Unknown	Wound	Unknown	Unknown
B1-B11, B13	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown

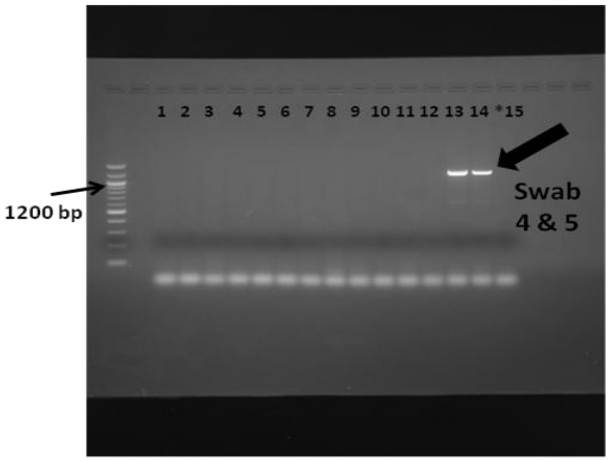
(A)



Strain	BAP
1 - B0063658U	X
2 - B0091943U	X
3 - B0008996U	X
4 - Swab 4	YES
5 - Swab 5	YES
6 - BAA-1605	X
B1	YES
B2	YES
B3	YES
B4	YES
B5	YES
B6	YES
B7	YES
B8	YES
B9	YES
B10	YES
B11	YES
B13	YES

*7 – Negative control

(B)



Strain	BAP
1 - B1	X
2 - B2	X
3 - B3	X
4 - B4	X
5 - B5	X
6 - B6	X
7 - B7	X
8 - B8	X
9 - B9	X
10 - B10	X
11 - B11	X
12 - B13	X
13 - Swab 4	YES
14 - Swab 5	YES

*15 – Negative control

Figure 4. Ethidium bromide stained agarose gel showing PCR amplification of BAP N terminus (A) and C terminus fragments (B) to identify BAP positive strains. Since Swab 4 and Swab 5 screened positive for both the N and C terminals they were used for all futher experiments.

3.2 Generation of Peptide Antigens

3.2.1 Repeat Consensus and Peptides

The tandem repeats present in the core of BAP made it possible to design consensus synthetic peptides. These were designed for repeat modules A, B, C, D and G, but not E and F (Table 5). Since the 5' section of the protein has numerous copies of each repeat module but the 3' section consisting of E, F and G has very few copies (figure 2) which are less conserved, we thought it necessary to design a peptide for only one module of the 3' repeats. Module G was chosen as it is the most conserved of the three. It is most likely that one or several of these repeat modules are exposed to solvent.

Table 5. Peptide consensus and design. Peptides A, B, C, D, and G were manufactured and purchased from United Biochemical Research Inc. Seattle WA. USA. Cysteine residues were added to the amino terminus of each peptide for easy conjugation as the BAP protein as a whole does not contain any of these amino acids.

Repeat Module	Peptide Consensus/Design
A	NH_2-C-A D A D K T I D A K V T F T D A A G N-COOH
B	NH_2-C-A P N A P V L D P I N A T D P V S G T-COOH
C	NH_2-C-N N G D G T W T L A D N T L P-COOH
D	NH_2-C-N Y T V T A A D L A N G Y I T A-COOH
G	NH_2-C-G Q V D Q F T Y T L T D P V T G-COOH

3.2.2 Conjugation, Total Protein Concentration and Immunization

The small size of the synthetic peptides render them less than effective immunogens; therefore conjugation of the peptides to larger molecules is a necessary step to produce an effective immunogen and anti-peptide antibodies. The peptides alone are unable to elicit a sufficient immune response from the mice but when conjugated to good carrier proteins such as Keyhole Limpet hemocyanin (KLH), a non-heme protein found in arthropods and mollusces, they prove quite adequate. Peptides A, B, C, D and G were all conjugated to KLH for immunization purposes and BSA for experimental assays. Conjugates were purified and fractions were quantified using a spectrophotometer to determine which to pool and which to discard. BCA analysis was was used to determine total protein concentration of pooled fractions which was then used in immunization calculations.

For each peptide a group of 4 mice were immunized. All groups responded well to immunization with peptide/KLH conjugates as none of the mice showed any detrimental side effects throughout the immunization period. Immune response and antibody production was verified by testing antibody serum against peptide antigen in ELISA (Figure 5). Serum from each group was tested against 200ng of the corresponding peptide/BSA antigen in ELISA. Results revealed an exceptional antibody titre response compared to the negative control which was naive pre-immune serum tested against each peptide antigen.

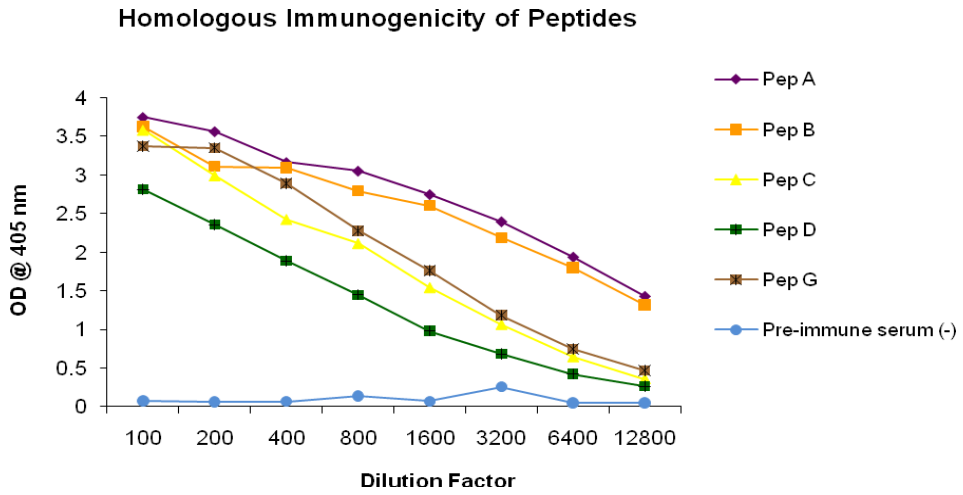


Figure 5. ELISA assay on serum IgG of peptide immunogens testing the efficacy of peptide/KLH conjugates. Peptide anti-serum IgG titre against 200ng/well peptide/BSA antigen compared to naive pre-immune serum IgG titre. Peptide anti-serum was also tested for reactivity against whole bacteria (section 3.6).

3.3 Generation of Recombinant Proteins

3.3.1 N and C Terminal PCR Product and Ligation into SUMO vector

In addition to making antibody reagents to the repeat modules of BAP, we also wanted to produce antibodies to the non-repetitive N and C terminal regions. In order to produce recombinant proteins the cDNA encoding the N and C terminal fragments had to be ligated into the pET SUMO expression vector (refer to section 2.5.3 for information on pET SUMO) and thus DNA fragments of both had to be amplified and purified. Fragments from *A. baumannii* strains Swab 4 and Swab 5 were used for production of recombinant proteins as they were the only two strains to screen positive for both regions of BAP. Two samples of each strain were prepared for agarose gel electrophoresis using BAP N and C

terminal oligonucleotide primers (Table 3) to generate a PCR product for cloning and expression (Figure 6).

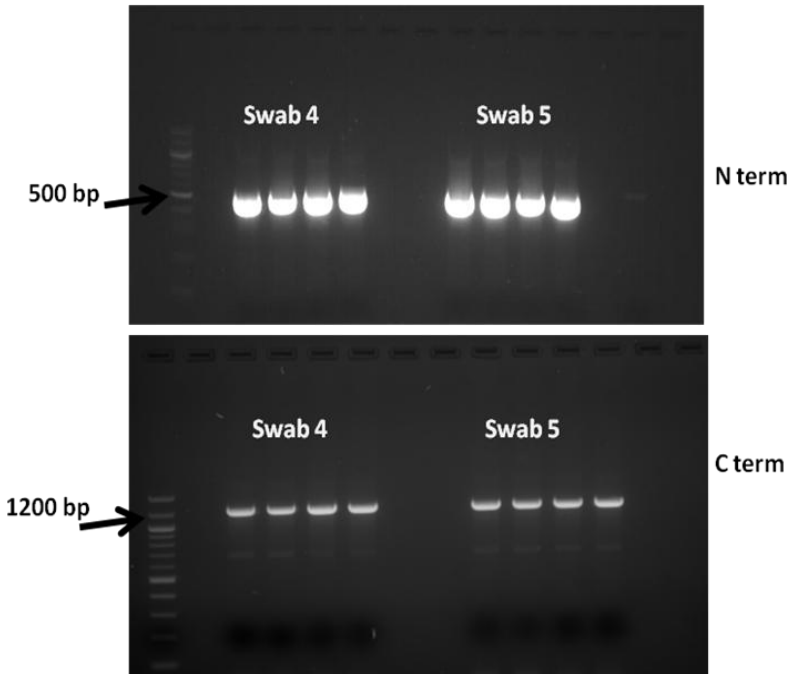


Figure 6. Ethidium bromide stained agarose gel showing PCR amplification of BAP N and C terminal fragments for ligation. Two PCR reactions per BAP positive strain (Swab 4 (S4) and Swab 5 (S5)) were prepared with both N and C terminal primers to ensure there would be sufficient DNA for gel extraction. Bright, prominent bands were visible around the 500 and 1200 bp mark; the approximate sizes of the N and C terminals respectively. DNA bands were excised from agarose gels and purified for subsequent ligation into the SUMO vector.

Purified N and C terminal DNA was then ligated into the pET SUMO vector which has a TA cloning site for effective cloning of *Taq*-amplified PCR products (Refer to appendix II for vector map and sequence containing TA cloning site).

3.3.2 Colony PCR Amplification of Successful Mach-1-T1 Transformants

Following the ligation the plasmids were transformed into Mach-1-T1 Competent *E. coli* cells (refer to section 2.5.4 for information on Mach-1-T1 cells). Transformations were grown on L.B agar plates containing 100μg/ml kanamycin as the pET SUMO vector contains a kanamycin resistance gene for selection in *E. coli*. Ten N terminal colonies and 20 C terminal colonies were analyzed by colony PCR amplification to determine if N and C terminal inserts were present in the vector (Figure 7A, B). The N terminal insert was present in all transformants of Swab 4 and Swab 5 except sample Swab 5 colony #4 as seen by the bright, prominent bands around the 500 base pair mark of the molecular marker and absence of a band in lane 10 (Figure 7A). The C terminal insert was present in 11 of the 20 chosen colonies and 8 were chosen for further analysis based on brightness and general appearance of the DNA bands in the original gel (Figure 7B).

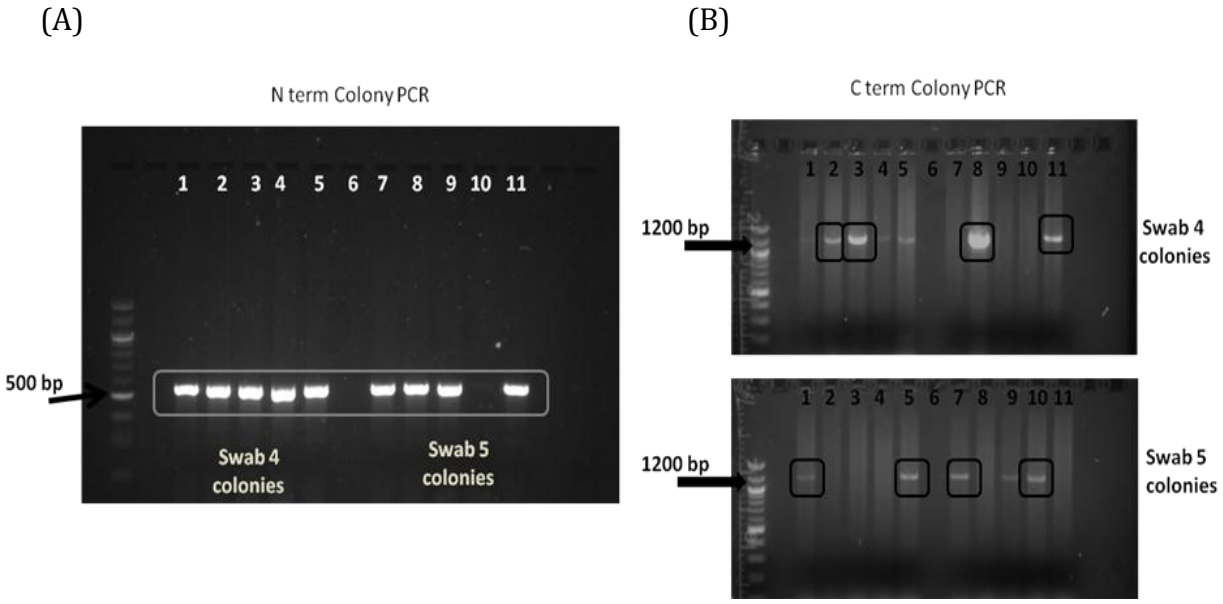


Figure 7. Ethidium bromide stained agarose gel showing colony PCR amplification of N terminal (A) and C terminal (B) transformants. Colony PCR using designed N and C terminal oligonucleotide primers confirming the presence of BAP N and C terminal inserts in the SUMO vector. (A)N terminal insert was present in 9 of the 10 chosen Swab 4 (S4) and Swab 5 (S5) colonies. Lanes 1-11 are as follows: (1) S4 colony #1, (2) S4 #2, (3) S4 #3, (4) S4 #4, (5) S4 #5, (6) negative control, (7) S5 #1, (8) S5 #2, (9) S5 #3, (10) S5 #4, (11) S5 #5. All 9 samples were used for further analysis. (B) C terminal insert was present in 11 of the 20 colonies and 8 were chosen for further analysis (boxes). Top gel, lanes 1-11 are as follows: (1) S4 1:1#1, (2) S4 1:1#2, (3) S4 1:1#3, (4) S4 1:1#4, (5) S4 1:1#5, (6) negative control, (7) S4 1:3#1, (8) S4 1:3#2, (9) S4 1:3#3 (10) S4 1:3#4, (11) S4 1:3#5. Swab 5 samples are seen on the bottom gel and are labelled similarly. The 8 samples chosen for further analysis were: S4 1:1 colonies #2 and 3, S4 1:3 colonies #2 and 5, S5 1:1 colonies #1 and 5 and S5 1:3 colonies #1 and 4. These bands are visible around the 1200 base pair mark of the molecular marker. (1:1 and 1:3 represent the vector:insert ratio).

3.3.3 Plasmid DNA and Sequence Analysis

The 9 N terminal colonies and 8 C terminal colonies were re-streaked to be used in further analysis. Plasmid DNA from the colonies was extracted, purified and quantified before it was sent to Beckman Coulter Genomics for sequence analysis. Two sequence reactions per sample were performed; one forward reaction using SUMO forward primer and one reverse reaction using T7 reverse primer (See table 3 for primer sequences).

Initial sequence analysis revealed good data for all samples. When analyzing sequence reactions Beckman Coulter genomics uses a phred score to assign the quality of each peak and therefore a pass or fail to each reaction. Phred is a base calling computer program for identifying nucleobases from DNA sequence traces; it reads DNA sequence files and analyzes the peaks around each base and assigns a quality 'Phred score' to each identified base. In the case of our sequence data each reaction required a score of phred20 quality or higher to pass; meaning that the accuracy of the base identification had to be 99% or higher (http://www.phrap.com/phred/#qualityscores).

All forward reactions passed having a phred20 quality or higher, however only one reverse reaction passed with the others failing. Reverse reactions were re-sequenced in another attempt which was successful as all reactions passed the second time. Analysis of the sequence data using DNASTAR Lasergene 8 software revealed that 2 of the 8 plasmids containing the C terminal and 6 of the 9 plasmids containing the N terminal had inserts in the correct orientation. BLAST analysis of these samples through the NCBI database revealed high sequence homology with *Acinetobacter baumannii* genomes and *A. baumannii bap* genes on the database. Furthermore, the sequences of the N and C terminals showed very few variations between strains Swab 4 and Swab 5. (See appendix III for sequence analysis and BLAST results).

3.3.4 Expression of N and C terminal Recombinant Proteins

All N and C terminal samples which had correct inserts within the vector were used for preliminary pilot expression studies. Plasmid DNA from 8 samples were transformed into BL21(DE3) *E. coli* cells, grown overnight and then used to inoculate fresh culture

which was incubated until bacteria reached mid-log phase and had an O.D measurement of approximately 0.4 - 0.6 or higher at 600nm. The 8 cultures were split in two and protein expression was induced by adding 1mM IPTG to one of the cultures while the other was kept as the original. Next, 500µl aliquots were removed once every hour for 5 hours from both induced and uninduced cultures and centrifuged to pellet bacteria. Proteins were liberated from bacterial cell pellets using BugBuster® reagent which replaces mechanical methods such as sonication by gently perforating the bacterial cell wall to release soluble and insoluble fractions of proteins. Soluble and insoluble lysate samples were analyzed by SDS-PAGE to determine which fraction target proteins were present in and the optimal conditions for expression (Figure 8).

The N terminal of BAP has an original molecular weight of approximately 19.6kDa and the C terminal has an approximate weight of 44.6kDa. However, expression of target proteins with the N-terminal peptide containing the 6xHis tag and SUMO fusion protein resulted in an increase in size of the fragments by approximately 13kDa. Therefore, recombinant N and C terminal fusion proteins were seen on gels as approximately 33 and57kDa bands respectively. Initially two of the N terminal samples (S4 #1 and #2) and the two C terminal (S5 1:1 #1 and S5 1:3 #4) pilot expression samples were analyzed. Results revealed that the N terminal fragment was exclusively in the soluble protein fraction as seen by the increasing intensity of the bands just under the 37kDa mark in the 'soluble gels' and lack of induced bands in the 'insoluble' gels (Figure 8A). The C terminal was mostly in the insoluble protein fraction as seen by increasing intensity of bands just above the 50kDa mark, but could also be seen slightly in soluble fractions (Figure 8B).

The remaining soluble lysate of the N terminal BAP fragment samples were analyzed and confirmed that protein was in fact present in the soluble fraction (data not shown). Observation of each time point band revealed that 4-5 hours of growth was appropriate for optimal expression. The final time point (t=5) of the uninduced culture (IPTG -) for all samples was analyzed concurrently for comparison purposes.

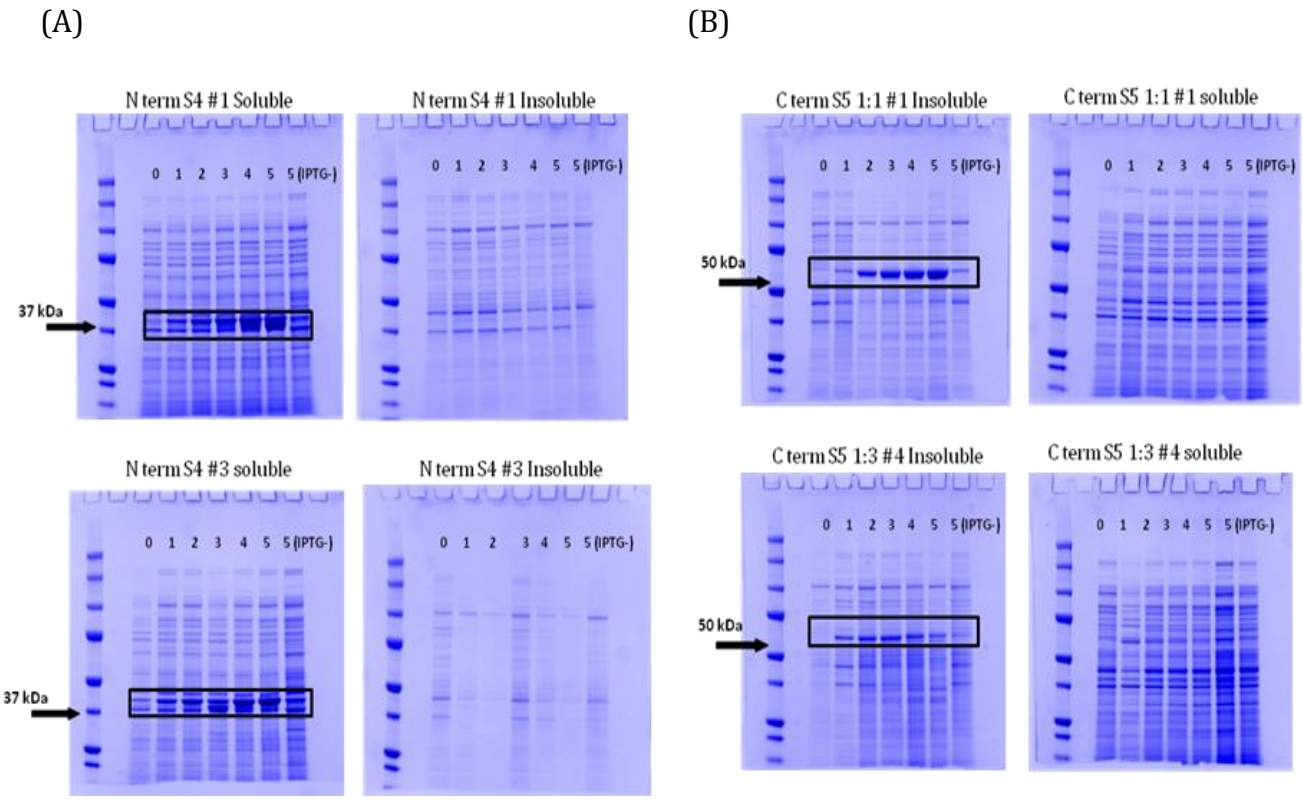


Figure 8. Colloidal Coomassie stained SDS -4-12% polyacrylamide gels of the preliminary pilot expression of recombinant proteins in SUMO vector. Analysis revealed N terminal samples S4#1 and #2 (A) are in the soluble protein fraction (left) seen by the increasing intensity of bands around the 37kDa mark (boxes). The two C terminal samples S5 1:1#1 and S5 1:3 #4 (B) can be seen in the insoluble fraction (left) by the increasing bands around the 50kDa mark (Boxes). This increase in intensity of bands is evidence of induced expression of target proteins over time. Lanes 0-5 represent time points 0hrs-5hrs of the induced culture (IPTG+). The last lane labelled 5(IPTG -) is time point 5 of the uninduced culture. The smaller size of these bands compared to the others is further proof that target proteins were expressed by IPTG.

Next, one representative N and C terminal fusion protein were arbitrarily chosen for expression scale up based on the results of the pilot expression analysis. Only one sample from each recombinant protein was scaled up as only one was needed for subsequent purification and immunization and results of sequence analysis suggested there was little difference between samples and strains, therefore expressing all samples would be redundant. Expression of N terminal S5#2 and C terminal S51:1 #1 was scaled up to a 50ml culture and grown for 4 hours as determined from the pilot expression. SDS-PAGE analysis of N terminal soluble lysate and C terminal insoluble and soluble lysate was performed which confirmed the success of the scale up as seen by large protein bands of appropriate molecular weights (Figure 9A). Concurrently western immunoblot analysis was performed to detect recombinant proteins via 6xHis tag using anti-HisG-HRP antibodies (Figure 9B) to further confirm expression. Results confirmed that the N terminal BAP fragment is expressed in the soluble fraction and the C terminal BAP fragment is expressed in the insoluble fraction.

(A) (B)

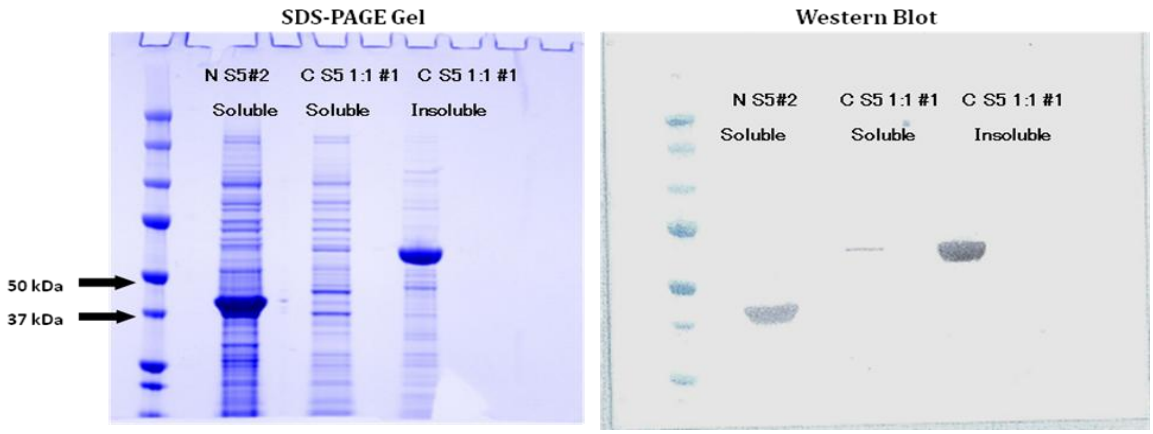


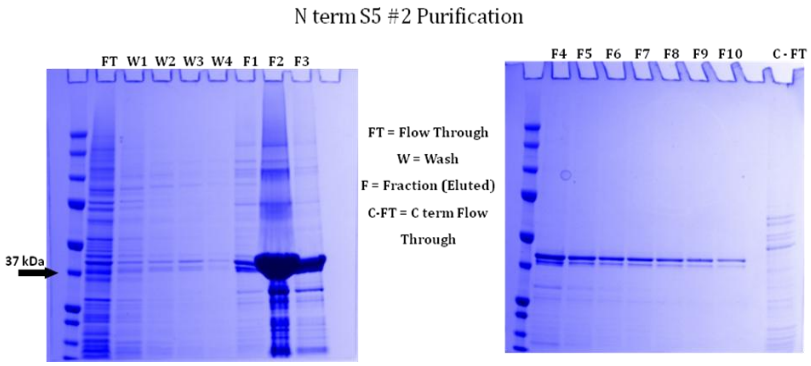
Figure 9. Scaled up expression of recombinant proteins. Colloidal Coomassie stained SDS - 4-12% polyacrylamide gel (A) and western immunoblot (B) demonstrating success of scaled up expression and detection of recombinant proteins containing 6xHis tag by anti-HisG-HRP antibodies. Majority of the C terminal protein can be seen in the insoluble fraction and thus was used in all further experiments while soluble fractions were discarded.

3.3.5 Purification of N and C terminal Recombinant Proteins

Bacterial lysate containing recombinant proteins from the scaled up expression was then purified using the ProBond™ Purification System. Nickel column purification of the N terminal sample was performed under 'native' conditions as it was determined to be soluble and we wanted to preserve protein activity. However, since the C terminal was insoluble but we still wanted to preserve protein activity it was purified under 'hybrid' conditions as per commercial instructions. Therefore C terminal was prepared and bound in the column as an insoluble protein but was washed and eluted using buffers typically used for soluble proteins. N terminal columns were washed 4 times and protein was eluted in ten 1ml fractions whereas C terminal columns were washed 8 times, but protein was eluted and collected in the same manner. All wash and eluted fractions from N and C

terminal recombinant protein purifications were analyzed by SDS-PAGE (Figure 10A, B) and fractions containing purified protein were pooled as determined by results. Sample flow through was also analyzed to confirm that protein was no longer in the flow through but bound and eluted from the column. As seen in figure 10A all N terminal eluted fractions contained purified protein with fractions 2 and 3 containing the most significant amount, nonetheless all fractions were pooled. C terminal fractions 1-4 contained the majority of purified protein (Figure 10B) however, protein was present in all fractions and thus all fractions were pooled as well.

(A)



(B)

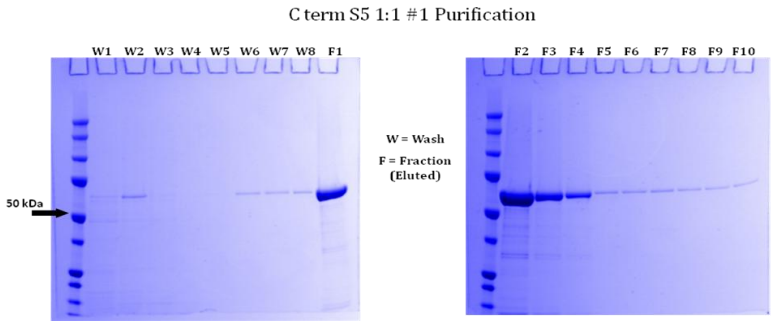
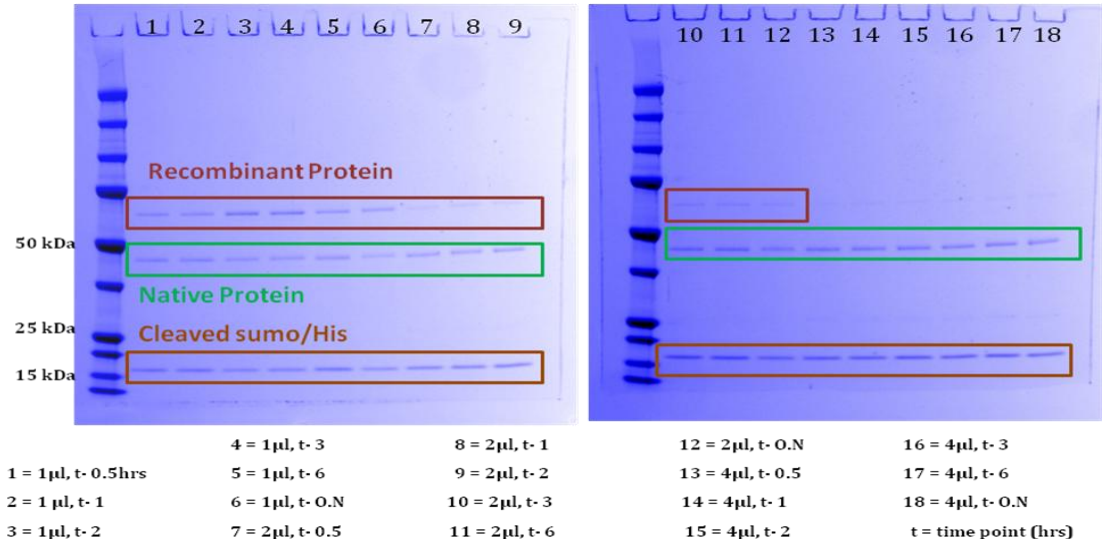


Figure 10. Purification of recombinant proteins. Colloidal Coomassie stained SDS -4-12% polyacrylamide gels of N terminal (A) and C terminal (B) recombinant protein purification. Purified protein is seen in eluted fractions and not the flow through or wash fractions.

3.3.6 Generation and purification of Native N and C terminal Protein

Generation of native N and C terminal protein was to be accomplished by cleaving the N-terminal peptide containing the 6xHis tag and SUMO from the N/C- SUMO fusion proteins with SUMO protease included in the pET SUMO protein expression kit. Initial cleavage reactions were incubated at 30°C with 2µg of fusion protein and 1, 2 and 4µl of protease. Aliquots were taken at time points 0.5, 1, 2, 3, 6 hours and overnight and analyzed by SDS-PAGE to determine the optimal time which allowed the greatest percentage of cleavage; however cleavage proved difficult as 4 separate reactions were attempted with only one being successful in which native C terminal was generated (Figure 11A). Cleavage was deemed successful when a greater portion of native protein was seen compared to recombinant protein which was determined by the molecular weight of the proteins seen on the polyacrylamide gels. C terminal recombinant and native proteins are approximately 57 and 44.6kDa respectively, while cleaved SUMO/6xHis-tag is approximately 13-15kDa as seen in Figure 11A. Unfortunately, native N terminal protein could not be generated in any of the cleavage reactions, even when attempted in an additional reaction at 4°C. Native N terminal protein should be approximately 19kDa which was not seen on the resulting SDS-PAGE gels (Figure 11B).

(A)



(B)

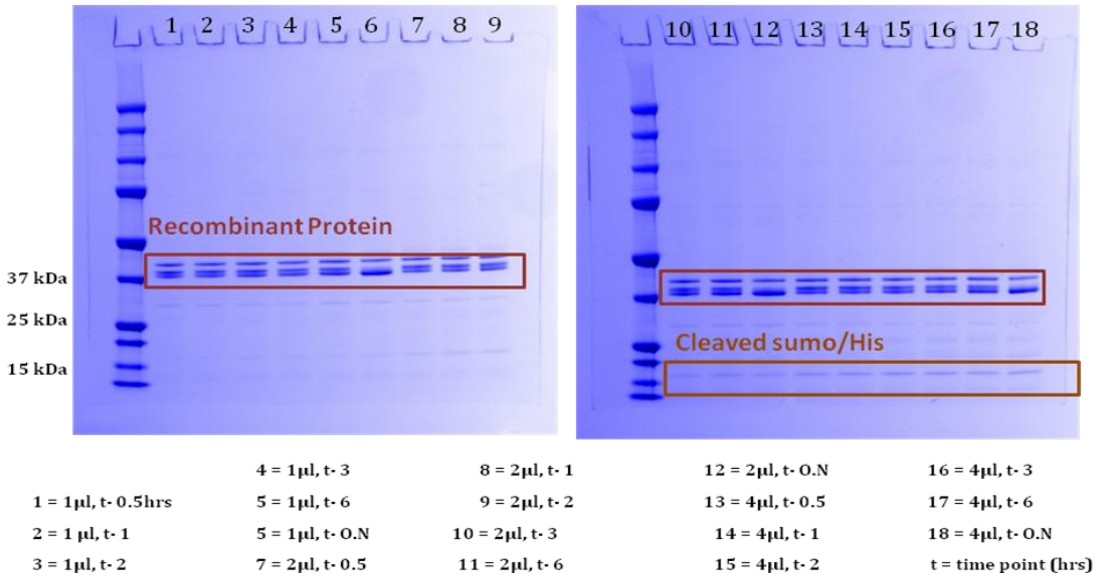


Figure 11. Preliminary cleavage reactions to generate native protein. Colloidal Coomassie stained SDS -4-12% polyacrylamide gels demonstrating the generation of native C terminal protein (A) and the unsuccessful cleavage of SUMO from N terminal protein (B). The highest percentage of native C terminal protein was generated when 4µl of SUMO protease was used, with cleavage occurring independently of incubation time at this volume. However, cleavage could not be accomplished for N terminal protein, regardless of the volume of protease or incubation time. Native C terminal was scaled up and purified for further experimentation whereas N terminal was left in the recombinant fusion protein state.

Once the appropriate conditions were determined the cleavage reaction was scaled up as to generate a greater volume of native C terminal protein. The entire reaction was scaled up by 20 and incubated overnight to ensure the greatest amount of cleavage and then purified in the same manner as recombinant proteins with ProBond™ Purification nickel columns. However, instead of being present in the eluted fraction, native protein remained in the flow through while cleaved SUMO remained in the resin. All fractions were again analyzed by SDS-PAGE including the cleavage reaction, the flow through containing native protein and eluted fractions containing SUMO as well as all wash fractions. Analysis revealed the scaled up cleavage reaction was a success as native protein was present in the flow through and SUMO was present in the eluted fractions (Figure 12).

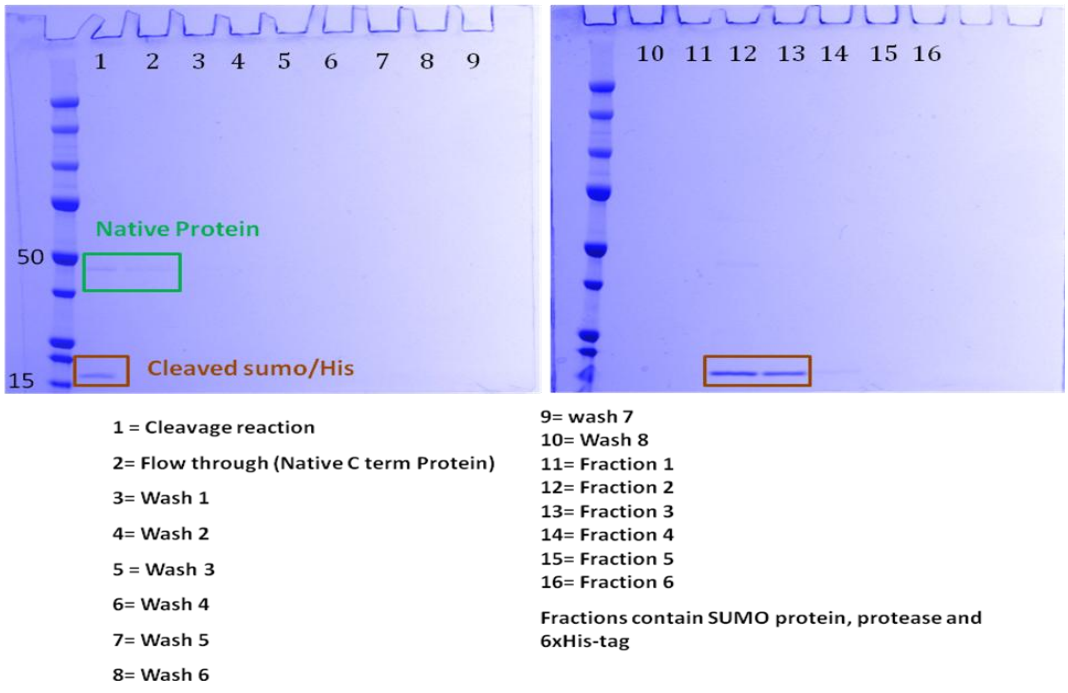


Figure 12. Colloidal Coomassie stained SDS -4-12% polyacrylamide gels representing success of scaled up cleavage reaction resulting in native C terminal protein. Although native protein bands are poorly visible above, original gels had good clarity.

3.3.7 Buffer Exchange and Total Protein Concentration of Purified Proteins

Pooled purified recombinant proteins and native C terminal protein were buffer exchanged out of elution and binding buffer into neutral PBS (pH 7.2) and analyzed by BCA to determine total protein concentration. BCA analysis revealed that N terminal S5#2 had a greater amount of total protein (922.41µg/ml) than C terminal S51:1#1 (197.22 µg/ml) which was not an unexpected result as N terminal gels clearly have a greater amount of protein than C terminal gels as seen in figure 10. However, the native C terminal protein concentration (132.20µg/ml) was quite comparable to the recombinant C terminal protein concentration.

3.4 Production of Whole *A. baumannii* Antigen

Clinical isolates Swab 4 and Swab 5 were grown up as planktonic and biofilm stocks, both being good biofilm producers. However, since sequencing analysis determined little difference in N and C terminal proteins of BAP between these strains, Swab 4 was used for remaining experimental procedures and immunizations.

Swab 4 planktonic and biofilm bacterial stocks were buffer exchanged into PBS and total protein concentration was determined by BCA analysis to use in immunization and assay calculations.

3.5 Development of Immune Sera

Ten groups of mice were immunized with prepared antigen; 1 group each for peptides A-G (5 total), 1 group each for recombinant and native proteins (3 total), and 1 group each for planktonic and biofilm bacteria (2 total). All mice responded well to

immunization and only one was put down before serum collection due to a coincidental infection at the site of injection. A few groups developed bumps at the site of injection, however these did not affect normal healthy behaviour as mice retained their appetite and remained active. No other health issues or problems were observed throughout the immunization period.

3.6 Immune Sera Efficacy Assays

3.6.1 Control Antibody – Whole Organism Anti-Serum

Mice received 10µg doses of whole formalin inactivated Swab 4 planktonic or biofilm bacteria throughout a 90 day immunization period. On approximately day 55, trial bleeds were taken from the mice to determine if they were producing an immune response to the bacteria. Approximately 100µl of serum was obtained from the tail vein of each mouse and pooled for testing against 800ng/well whole bacteria in ELISA. Results revealed high antibody titre suggesting mice were responding well to bacteria antigens (Figure 13A, B). Mice seemed to respond better initially to biofilm bacteria as they produced a higher serum IgG antibody titre to biofilm antigen (Figure 13B) compared to planktonic bacteria antigen (Figure 13A), although this could be due to assay differences and/or coating differences between bacterial phenotypes (i.e. Biofilm may stick and coat better to the ELISA plate than planktonic bacteria).

(A) (B)

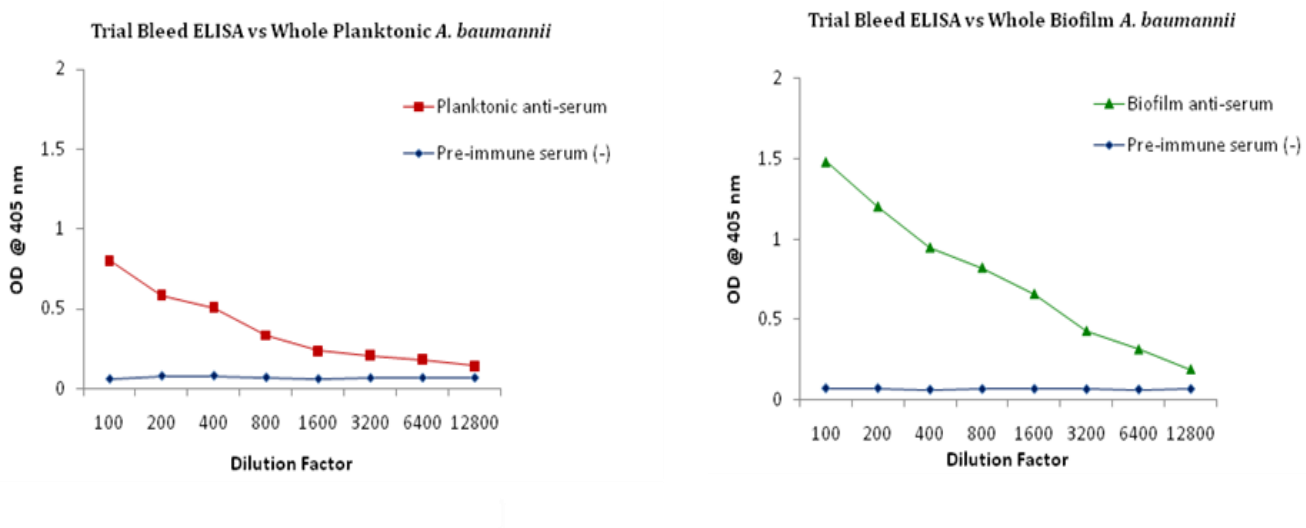


Figure 13. Trial bleed antibody titre of planktonic anti-serum against 800ng/well whole planktonic bacteria (A) and antibody titre of biofilm anti-serum against 800ng/well whole biofilm bacteria (B) compared with pre-immune naive serum. Positive results are defined as OD readings greater than 4x the average of the negative control.

Immunizations continued for the remainder of the schedule to increase antibody response from the mice. At the end of the immunization period mice were euthanized and exsanguinations were performed to collect all blood. Exsanguination serum was tested against 800ng/well of whole bacteria in ELISA to confirm high serum IgG antibody titre and to determine if planktonic and biofilm sera was cross reactive (i.e. Do planktonic antibodies bind biofilm and vice versa?). ELISA analysis revealed high titre response and also cross reactivity of antibodies. Planktonic and biofilm immune serum reacted with both planktonic and biofilm bacteria (Figure 14A, B) with planktonic anti-serum reacting more strongly with both types of bacteria than biofilm anti-serum.

(A) (B)

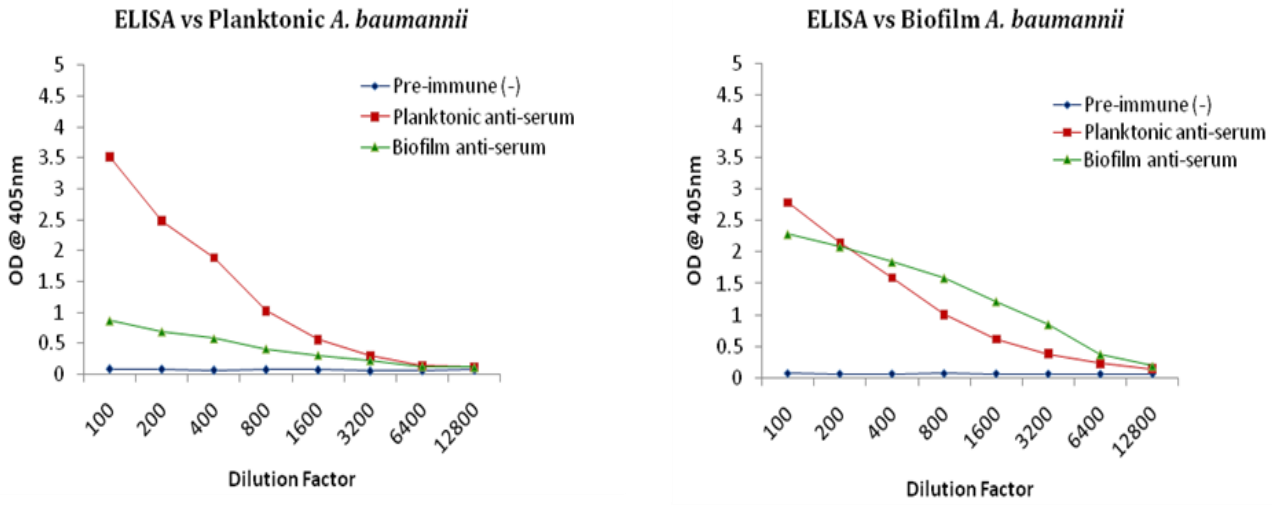


Figure 14. Antibody titre of planktonic and biofilm anti-serum against 800ng/well whole planktonic bacteria (A) and antibody titre of planktonic and biofilm anti-serum against 800ng/well whole biofilm bacteria (B) compared with pre-immune naive serum. Positive results are defined as OD readings greater than 4x the average of the negative control.

Next, whole organism anti-serum was tested in western immunoblot reactivity assays to determine if sera could detect bacterial proteins. Two samples each of 5µg of whole planktonic and biofilm bacteria were loaded onto polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was cut in two with one half probed with 1/250 diluted planktonic anti-serum and the other probed with 1/250 diluted biofilm anti-serum. Development of blots using ECL revealed both types of immune sera were able to detect proteins in both life forms of bacteria (Figure 15). Majority of proteins were detected by both sera; however certain proteins seemed to be specific to the individual bacterial phenotypes as they were detected by only one type of anti-serum but not the other. Both this experiment and ELISA data suggested whole organism anti-sera would be a sufficient control antibody in further experiments.

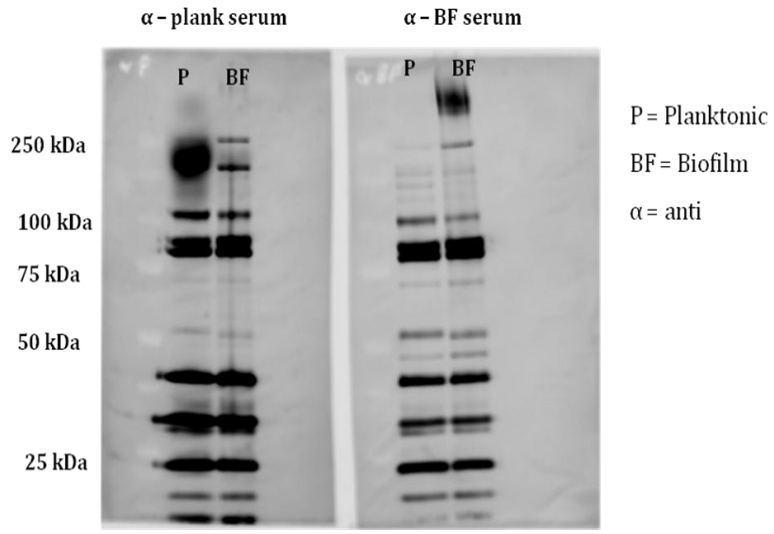
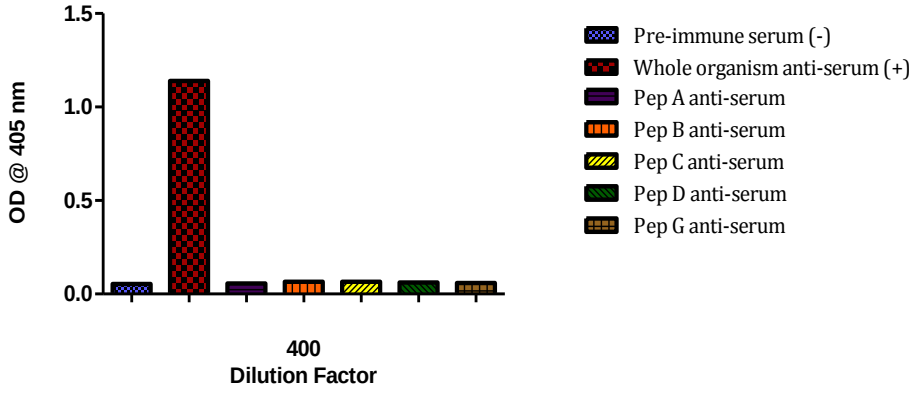


Figure 15. ECL developed western immunoblot demonstrating the reactivity of whole planktonic and biofilm anti-serum with whole *A. baumannii* bacteria. One half of the membrane was probed with planktonic anti-serum (left) and the other with biofilm anti-serum (right) illustrating protein specificity between free-living and biofilm phenotypes.

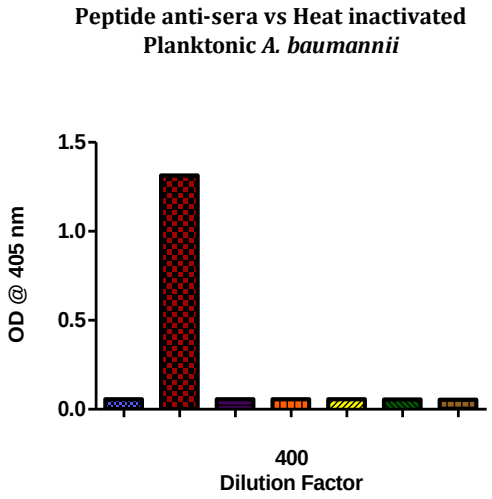
3.6.2 Peptide Anti-Serum

Synthetic peptide anti-serum was tested against the homologous peptide antigen and it was evident that mice produced a strong immune response (Figure 5). However, peptide anti-sera was also tested against whole bacteria to determine which BAP repeats, if any, were surface exposed on live and heat inactivated planktonic *A. baumannii* as well as biofilm *A. baumannii*. ELISA analysis showed little to no reactivity between peptide anti-sera and whole organism, regardless of whether bacteria was live, heat inactivated, planktonic or biofilm as OD readings were similar to negative controls (Figure 16A, B, C).

(A)



(B)



(C)

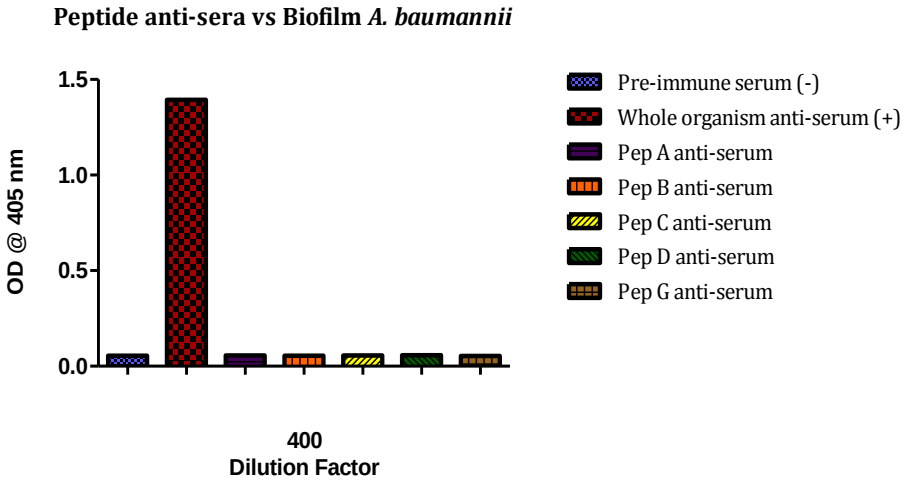


Figure 16. Reactivity of peptide anti-sera against 1µg/well of live planktonic (A), heat inactivated planktonic (B) and biofilm (C) *A. baumannii* bacteria in ELISA compared to pre-immune and serum whole organism anti-serum. Positive OD readings are defined as measurements greater than 4x the average of the negative control. Peptide sera did not react with whole bacteria as OD readings were similar to pre-immune serum.

Western immunoblot analysis was performed to confirm that peptide anti-sera did not react with whole bacteria by loading 5µg of whole planktonic and biofilm bacteria onto polyacrylamide gels and transferring to nitrocellulose membranes. Membranes were cut,

each section containing transferred planktonic and biofilm proteins. Membranes were probed with peptide A-G anti-sera diluted 1/250, pre-immune serum as a negative control or whole organism anti-sera as a positive control and goat anti-mouse IgG-HRP was used as a secondary detection antibody. Similar to ELISA results, western immunoblot analysis revealed that peptide anti-sera did not react with whole bacteria regardless of phenotype (Figure 17).

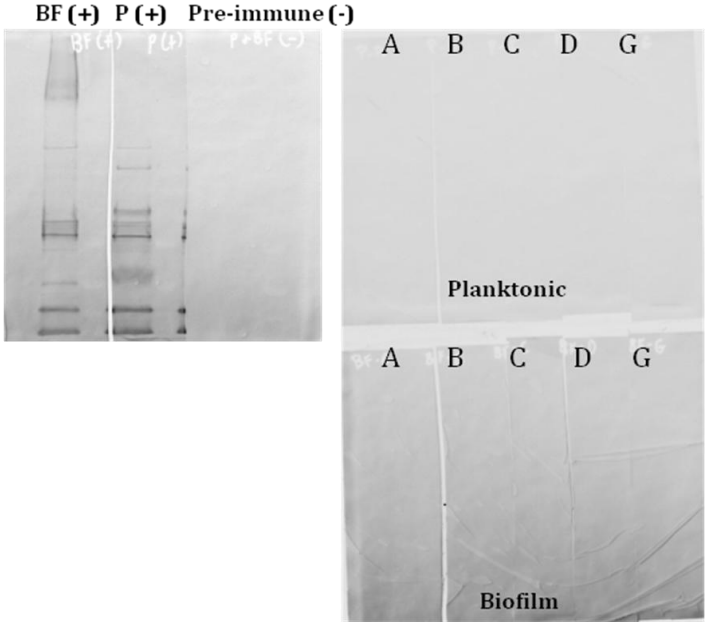


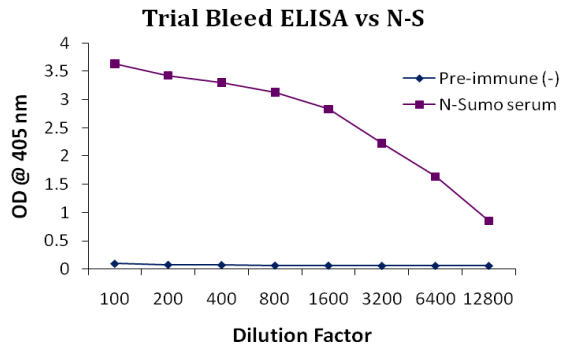
Figure 17. Western immunoblot analysis demonstrating the lack of reactivity of peptide anti-sera with whole planktonic and biofilm *A. baumannii* (right) compared to blots probed with pre-immune serum (-) and whole organism anti-serum (+) (left). Goat anti-mouse IgG-HRP was used as a secondary detection antibody.

3.6.3 Recombinant Protein Anti-Serum

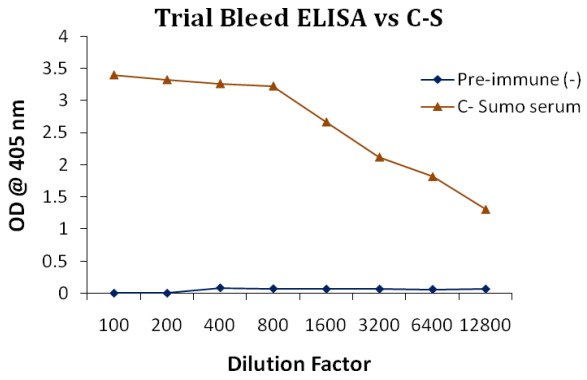
Mice were immunized with recombinant proteins (N terminal-SUMO and C terminal-SUMO) and native protein (C terminal) using an accelerated immunization schedule, however, trial bleeds were still performed to determine if mice were building an

immune response to the proteins. Approximately 100µl of serum was obtained from the tail vein of each mouse from each group and pooled for testing against 800ng/well of the corresponding protein in ELISA. Results revealed that the mice were producing an excellent immune response to the protein antigens (Figure 18A, B, C).

(A)



(B)



(C)

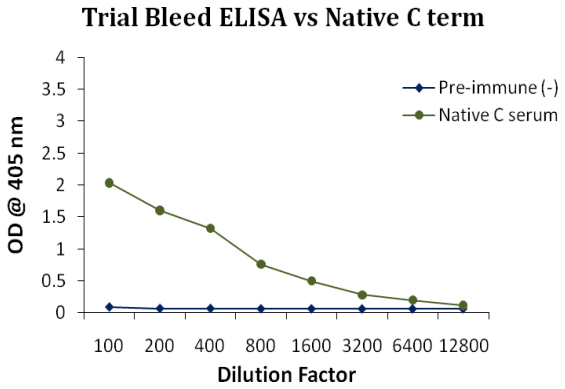
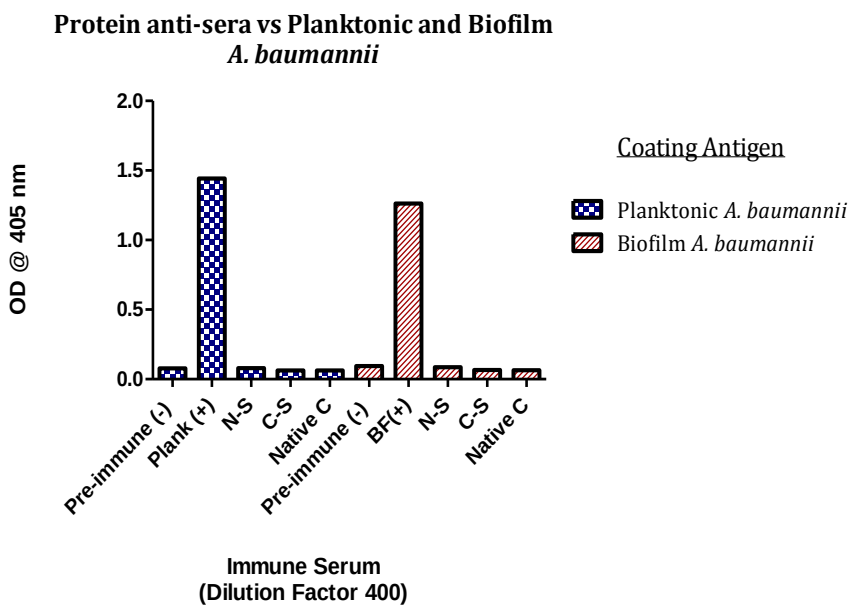


Figure 18. Trial bleed antibody titre of N terminal-SUMO (N-S) recombinant protein anti-serum (A), C terminal-SUMO (C-S) recombinant protein anti-serum (B) and native C terminal protein anti-serum against 800ng/well of the corresponding protein compared with pre-immune naive serum. All groups responded well to protein antigens as seen by high titre response at low serum dilutions. Positive results are defined as OD readings greater than 4x the average of the negative control.

Results of the trial bleed analysis determined mice were responding well to the protein antigens and therefore mice were administered their final immunization and euthanized one week later. Exsanguinations were performed to collect all blood and serum was analyzed in ELISA against 800ng/well whole planktonic and biofilm *A. baumannii*. Similar to peptide anti-sera results, protein anti-serum did not seem to be reactive with whole bacteria regardless of bacterial phenotype (Figure 19A). Mouse anti-serum from animals immunized with native C terminal fragment alone did not react with whole bacteria (Figure 19A) but did react with recombinant C terminal-SUMO protein (data not shown) suggesting mice did produce antibodies to the terminal fragments and not just SUMO.

Protein serum was also tested for reactivity in western blot to confirm the lack of reactivity with whole bacteria. 5µg of planktonic and biofilm bacteria were loaded onto polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were cut, each section containing transferred planktonic and biofilm proteins, and probed with protein anti-sera diluted 1/250, pre-immune serum as a negative control or whole organism anti-sera as a positive control. Goat anti-mouse IgG-HRP was used as a secondary detection antibody. Similarly protein anti-sera did not react with whole bacteria regardless of bacterial phenotype (Figure 19B).

(A)



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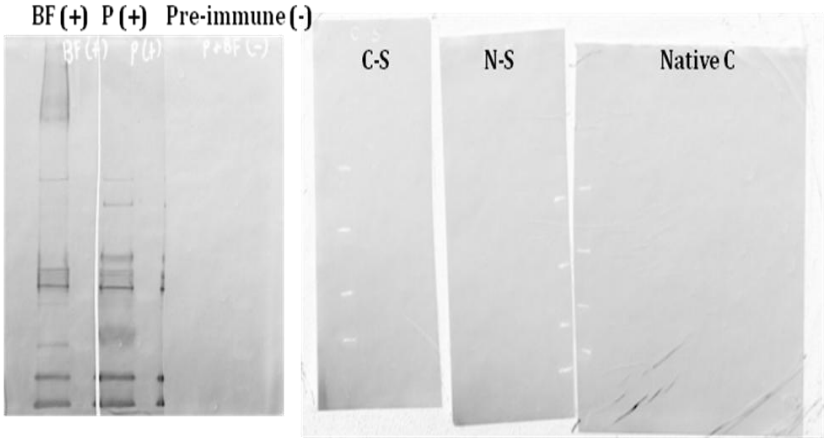

Figure 19. Reactivity of protein anti-sera against 800ng/well live planktonic and biofilm *A. baumannii* bacteria in ELISA (A) and 5µg live planktonic and biofilm bacteria in western immunoblot (B) compared to pre-immune serum and whole organism anti-serum. ELISA positive OD readings are defined as measurements greater than 4x the average of the negative control. Protein sera did not react with whole bacteria as OD readings were similar to pre-immune serum (A). Similarly no proteins were detected on the blots as seen by lack of bands when probed with C-S, N-S and native C anti-serum. Goat anti-mouse IgG-HRP was used as a secondary detection antibody (B). Plank (planktonic), BF (biofilm), N-S (N terminal-SUMO recombinant fusion protein), C-S (C terminal-SUMO recombinant fusion protein), Native C (native C terminal protein).

3.7 *In Vitro* Assays - *A. baumannii* and Innate Immune Components

3.7.1 Complement

In vitro assays were performed to determine if *A. baumannii* whole organism serum was bactericidal or bacteriostatic against *A. baumannii* and if so, was the serum able to act alone or was it complement dependent. All complement assay samples were performed in triplicate with each one being plated for bacteriology. Colony counts were calculated as colony forming units per millilitre (CFU/ml) to establish which assay conditions (amount of serum, addition of complement?) were best for decreasing bacterial growth. All samples were incubated with *A. baumannii* strain Swab 4 and whole organism Swab 4 anti-sera except for controls (Table 6). Negative controls were samples incubated with no serum or pre-immune serum and positive controls were *A. baumannii* ATCC strain 19606 incubated with 19606 rabbit anti-serum and complement as well as Swab 4 incubated with 1/10 diluted Swab 4 whole organism anti-serum. Colony counts of each sample revealed that Swab 4 anti-serum diluted 1/50 or greater did not have bactericidal or bacteriostatic effects on *A. baumannii* bacteria regardless of whether complement was present or not, however, serum diluted 1/10 (positive control) seemed to be very effective at arresting bacterial growth as seen by the log reduction in CFU/ml relative to the negative control (Figure 20).

Table 6. Complement assay experimental outline illustrating components of each sample. Sample 1 and 2 represent the negative controls while samples 3 and 4 represent the positive controls. Each sample was performed in triplicate and plated separately.

Samples

Component	1	2	3	4	5	6	7	8	9	10	11	12
DMEM (µl) to equal 60µl												
DMEM+ 0.6% FBS (µl)	10	10	10	10	10	10	10	10	10	10	10	10
A. baumannii 10^6(µl)	10	10	10 19606	10	10	10	10	10	10	10	10	10
Whole organism anti-sera (µl)	-	Pre-immune 1/50	Anti-19606 1/50	Serum 1/10	Serum 1/50	Serum 1/100	Serum 1/200	Serum 1/500	Serum 1/50	Serum 1/100	Serum 1/200	Serum 1/500
Complement (1:1) (µl)	-	-	10	-	-	-	-	-	10	10	10	10

Negative Controls | Positive

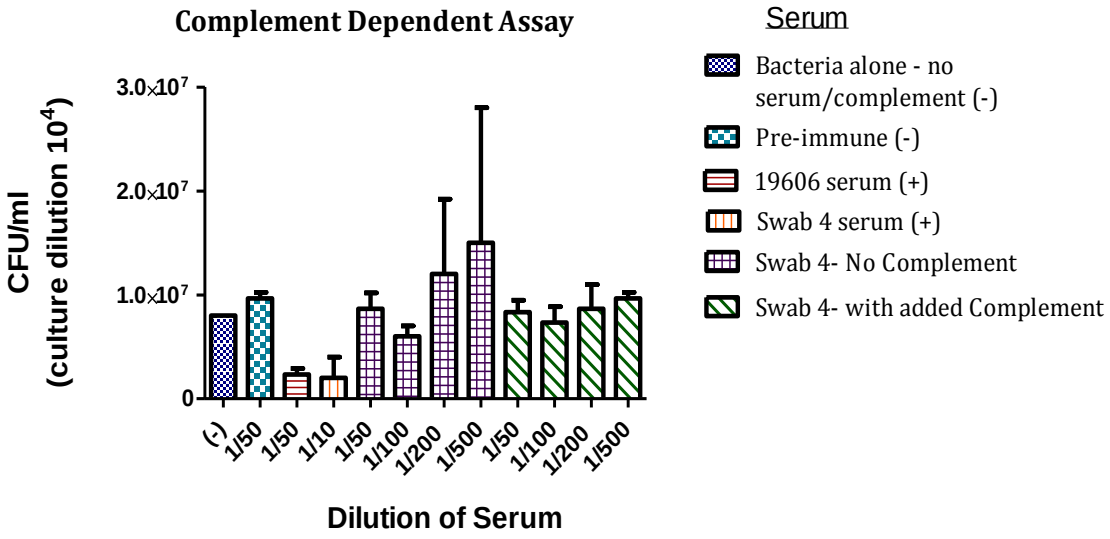

Figure 20. Results of complement/serum killing assay. Comparing effects of complement and various dilutions of Swab 4 anti-serum on *A. baumannii* growth. 1/50, 1/100, 1/200, 1/500 serum dilutions were performed with and without complement, all samples were tested in triplicate. Little difference in CFU/ml was observed between samples incubated with and without complement compared to the negative controls. However, positive controls showed a log decrease in CFU/ml compared to negative controls suggesting that immune serum in larger volumes may be effective against bacterial growth.

3.7.2 Macrophage

A second *in vitro* assay was performed to determine if *A. baumannii* was susceptible to antibody opsonization via macrophage cell line. We wanted to determine if bacterial growth decreased due to ingestion by macrophage cells when the bacteria were marked or flagged by antibodies contained in whole organism anti-sera. All macrophage assay samples were performed in triplicate with each one being plated for bacteriology. Colony counts were calculated as colony forming units per millilitre (CFU/ml) to establish which assay conditions (amount of serum, addition of macrophage cells?) were best for decreasing bacterial growth. All samples were incubated with *A. baumannii* strain Swab 4 and whole organism Swab 4 anti-sera except for controls (Table 7). Negative controls were samples incubated with no serum or pre-immune serum and positive controls were *A. baumannii* ATCC strain 19606 incubated with 19606 rabbit anti-serum as well as Swab 4 incubated with 1/10 diluted Swab 4 whole organism anti-serum. Colony counts of each sample revealed that Swab 4 anti-serum diluted 1/50 or greater did not have a great effect on *A. baumannii* bacterial growth compared to positive controls, as serum diluted 1/10 seemed to be very effective at decreasing bacterial growth which is seen by the log reduction in CFU/ml (Figure 21). Furthermore, the addition of macrophage cells did not seem to hinder bacterial growth in any way; in fact an increase in colonies was noted in samples which had been incubated with J774A.1 macrophage cells.

Table 7. Macrophage assay experimental outline illustrating components of each sample. Sample 1 and 2 represent the negative controls while samples 3 and 4 represent the positive controls. Each sample was performed in triplicate and plated separately.

Samples

Component	1	2	3	4	5	6	7	8	9	10
A. baumannii 10^7(µl)	10	10	10	10	10	10	10	10	10	10
J774A.1 Macrophage cells $2x10^7$	-	-	-	-	-	-	-	10	10	10
Whole organism anti-sera (µl)	-	Pre-immune 1/50	Anti-19606 1/50	Serum 1/10	Serum 1/50	Serum 1/100	Serum 1/200	Serum 1/50	Serum 1/100	Serum 1/200
PBS to equal 50µl										
DMEM (µl)	10	10	10	10	10	10	10	-	-	-

Negative Controls | Positive

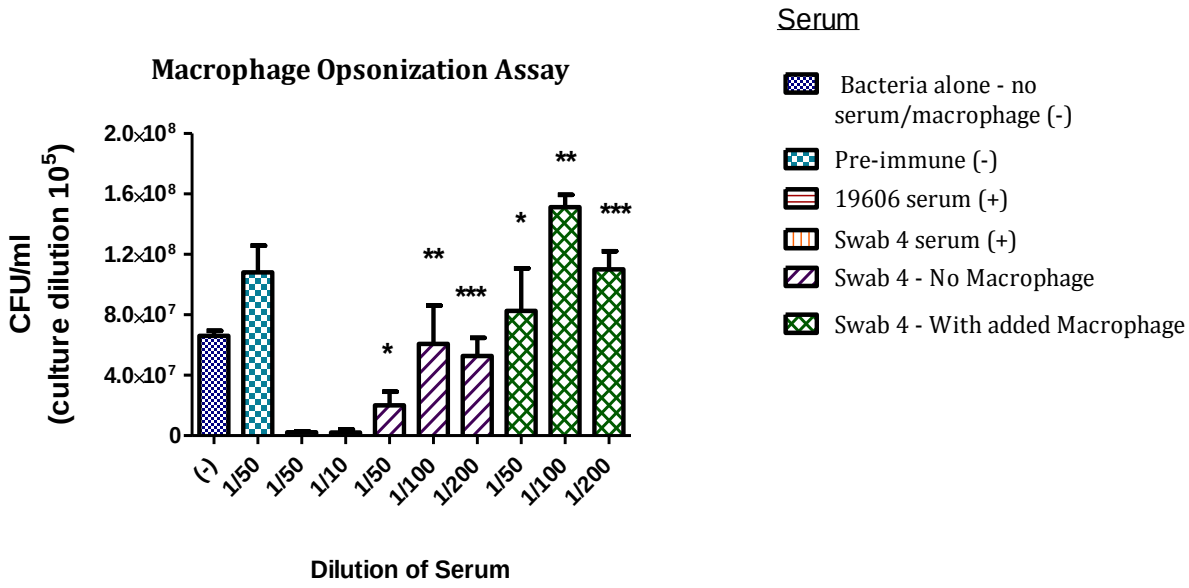

Figure 21. Results of macrophage killing assay comparing effects of macrophage cell line J774A.1 and various dilutions of Swab 4 anti-serum on *A. baumannii* growth. 1/50, 1/100, 1/200 serum dilutions were performed with and without macrophage, all samples were tested in triplicate. Little difference in CFU/ml was observed between samples incubated with and without macrophage compared to the negative controls. However, a greater number of colonies were counted in samples that were incubated with macrophage. Colony counts of samples incubated with and without macrophage with various dilutions of serum were significantly different when compared by t-test (* $P < 0.05$, ** $P < 0.05$, *** $P < 0.05$).

4 Discussion

4.1 Introduction

This thesis describes the specific processes undertaken to determine if a putative biofilm associated protein (BAP) is expressed upon the surface of the opportunistic pathogen *Acinetobacter baumannii*. Moreover, the analysis looked at surface expression on specific bacterial life forms (planktonic and biofilm) as well as for any expression at all. To do this, specific polyclonal antibody reagents were generated to whole organism and to synthetic peptides corresponding to the internal repeat domains and recombinant non-repetitive regions of the biofilm associated protein of *Acinetobacter baumannii*.

Several factors contribute to the success of *A. baumannii*; however biofilm formation is arguably the most significant as it allows for persistent survival and increased resistance to antimicrobials (13, 26, 45). Very few studies have investigated *A. baumannii* biofilms, particularly proteins involved in biofilm formation and development. The putative BAP protein which is a component allegedly involved in maintenance of the mature biofilm and cell to cell interactions is highly under explored in this bacterium (26, 39). Works by Loehfelm *et al* (2008) (39) and Rahbar *et al* (2010) (59) have described the efforts by these researchers to discover structural and functional components of this large molecular weight protein; however other than these studies very little is known about *A. baumannii* BAP. Furthermore, since this bacterium was once overlooked as an important pathogen (37), little is known about its pathogenic mechanisms and the interactions of *A. baumannii* with the immune system (8).

4.2 *A. baumannii* Immune Serum ineffective *in vitro*?

Hyperimmune sera generated from immunizing mice with whole inactivated *A. baumannii* Swab 4 strain was incubated with live bacteria to determine its bactericidal potential *in vitro* and whether or not bactericidal properties were dependent on added complement or increased by the addition of a murine macrophage cell line. Specific anti-serum to whole bacteria has an apparent effect at high serum concentrations (1/10) as seen in figures 20 and 21, but had little effect on bacterial growth when diluted to 1/50 or higher compared to samples incubated with no serum or pre-immune serum (negative controls).

Our assays represent conflicting results with previous murine studies which were able to show that *A. baumannii* inactivated whole cell serum was protective against *A. baumannii* infection *in vivo* when used in passive and active vaccination (41). Since *A. baumannii* serum has been shown to be effective at decreasing bacterial loads *in vivo* we expected to see a similar result *in vitro* which represents a more controlled environment; however our opposite findings could be due to the added components of the immune system which are inevitably present in an *in vivo* system. It is likely the complicated mechanisms and various components of the immune system that are triggered after vaccination (which are lacking in an *in vitro* system) had a large part in decreasing bacterial load and increasing protection against infection. To test this theory, we incubated bacteria and the same dilutions of serum with rabbit complement or J774A.1 macrophage cells. Again results revealed very little difference between samples incubated with added complement and macrophage cells compared to negative controls (Figures 20 and 21).

Samples incubated with macrophage cells actually resulted in a greater number of colonies compared to samples incubated without macrophage (Figure 21). It has been shown that *A. baumannii* can invade epithelial cells and survive within membrane bound vacuoles effectively avoiding host immune pressures (8). Therefore, it is possible that these bacteria are engulfed by macrophage cells but are somehow able to evade digestion within the cell or have adapted a mechanism to avoid phagocytosis all together, as many intercellular pathogens have developed mechanisms to escape phagolysosomal fusion and degradation (28). Further studies are required to elucidate specific details of the interactions between *A. baumannii* and macrophage cells, but are beyond the scope of this thesis.

Since complement is inherently present in serum and is known as an effective component of innate immunity in the killing of bacterial pathogens (32), it was thought the addition of extra complement would result in a significant decrease in bacterial colonies; however samples incubated with added rabbit complement did not differ from samples incubated with serum alone or from negative controls (Figure 20). A possible explanation for these results could be the fact that the bacteria were utilizing a resistance mechanism developed to escape complement-mediated lysis. This has been noted for several pathogenic bacteria and is consistent with observations by others with certain strains of *A. baumannii* (30, 32). It has been proposed that after invading susceptible hosts, *A. baumannii* avoids complement attack through the interactions between factor H, a regulator of complement activation, and bacterial outer membrane proteins (Omps), particularly AbOmpA. AbOmpA is the main complement regulator-acquiring protein which is essential for complement resistance. The binding of factor H to the bacterial surface via AbOmpA allows the organism to escape complement killing and also contributes to

dissemination and persistence of *A. baumannii* infections (30). Furthermore, complement resistance in *A. baumannii* has been linked to biofilm formation with serum resistant strains producing large amounts of biofilm and serum sensitive strains producing far less biofilm (32). Since swab 4 is a good biofilm producer it is likely that this strain uses mechanisms to evade complement. Additional studies are needed to confirm if the particular strain used in this study employs complement resistance mechanisms.

Decreased bacterial CFU may be correlated with titres of serum IgG. Positive controls which consisted of *A. baumannii* Swab 4 incubated with 1/10 diluted Swab 4 anti-sera and *A. baumannii* ATCC strain 19606 incubated with 1/50 diluted19606 rabbit anti-sera were found to significantly decrease bacterial CFU/ml compared to negative controls. This suggests that bacterial load can be decreased simply by higher specific serum IgG concentration. These results concur with serum killing studies (30, 32) which demonstrated a significant decrease in bacterial survival of certain *A. baumannii* strains when serum concentration was increased. Further studies are required to determine whether experiments using even lower serum dilutions than 1/10 produce the same results as above.

Further reasons as to why *A. baumannii* immune serum was not as effective against bacteria *in vitro* could be due to the capsular material produced by this pathogen. It has been shown that *A. baumannii* strains which have a capsule positive phenotype have optimized survival in human serum studies and *in vivo* rat soft tissue infection models suggesting that capsule plays a significant role in protecting the organism (62). Therefore

it is possible the strains used in this study were capsule-positive and thus had an additional resistance mechanism against complement-mediated lysis and macrophage opsonization.

4.3 Reactivity of BAP Peptide Anti-Sera

Recently an *A. baumannii* protein has been discovered which fits into the BAP family, showing homology to the original BAP discovered in *Staphylococcus aureus*. Although little is known about this *A. baumannii* BAP it has been proposed that its involvement is in maintaining the mature biofilm with the internal repeats functioning in intercellular adhesion interactions between neighbouring bacteria (39). The hypothesized function of the internal repeats of BAP makes them attractive immunotherapeutic targets and prompted us to investigate whether or not antibodies generated against these regions could bind BAP on whole bacteria and thus alter biofilm structure.

Antibodies were generated by immunizing mice with synthetic oligo-peptides corresponding to the internal repeats of BAP. Multiple regions were targeted to ensure we would generate a comprehensive range of antibodies that would hit BAP if it were surface exposed. It was determined that mice produced an excellent immune response to the peptides as seen by high antibody titre in serum (Figure 5), however testing the peptide anti-sera against whole planktonic and biofilm bacteria produced negative results. Serum was tested with *A. baumannii* Swab 4 which screened positive for both the N and C terminal of BAP, suggesting its presence in this strain, but ELISA and western immunoblot analysis revealed no reactivity between peptide A-G serum and whole bacteria regardless of phenotype or whether bacteria was live or heat inactivated/denatured (Figure 16 and 17).

This suggests these peptide epitopes are not exposed on the surface of our strain of *A. baumannii*.

In silico analysis of *A. baumannii* BAP has revealed that repeat modules A, B and C have greater than 80% of their residues exposed and therefore have high antigenic propensity, (59) so it was surprising that antibodies raised to these regions did not react with these areas on whole bacteria. However, primary sequence of all BAP repeats can differ considerably between species (35, 36) so it is plausible that they also vary between strains resulting in an increase or decrease of exposed residues. Furthermore, the exposed and hidden residues in each region were determined from *predicted* secondary and tertiary structures of the repeat regions (59) and thus the exact native conformation of the entire protein is not fully known. While it is possible that the BAP protein folds in a way which conceals one or several of the internal repeats, it is very unlikely that every module and all repeats within the same module are hidden, if indeed BAP is on the surface. Moreover, the consensuses of the repeat regions we used to design our peptides may be sections with low antigenicity. Although failure of the western immunoblot assay (Figure 17) strongly suggests these BAP epitopes are not present in our strain of *A. baumannii* or are below our lower limit of detection.

4.4 Reactivity of BAP N and C Terminal Recombinant Protein Anti-Sera

Attempts to clone and amplify the entire BAP protein failed as we encountered numerous difficulties due to the massive size of this protein. BAP is 25,863bp (854kDa) in size (39) and therefore finding appropriate parameters for successful PCR amplification and expression would prove unproductive and time consuming. Therefore the N and C

terminal portions of BAP were used to generate recombinant proteins for murine immunization in addition to the peptides designed from the internal repeats; in doing this we were able to produce multiple antigens for immunization which were a good representation of the entire protein. Similar to our hypothesis about antibodies raised to the internal repeats of BAP, we also assumed that antibodies raised to the N and C terminal fragments would bind BAP on whole bacteria and somehow interfere with the function of this protein.

Antibody to the N and C terminal fragments of BAP did not react with whole *A. baumannii* or denatured bacteria. The N and C terminal antibodies were generated by manufacturing recombinant fusion proteins by cloning amplified BAP N and C terminal fragments into a pET SUMO expression vector and immunizing mice with purified forms of these proteins. It was determined that the mice responded well to the immunizations as high antibody titre was seen in trial bleed ELISAs (Figure 18); however, similarly to the peptide sera, little reactivity was seen when tested with whole bacteria in ELISA and western immunoblot regardless of phenotype (Figure 19). These results raise the question of whether BAP is expressed by the particular *A. baumannii* strain used in this study. PCR screens showed the presence of genetic elements of BAP and BLAST analysis of recombinant proteins revealed homology to BAPs and *A. baumannii* genomes, both suggesting clinical strain Swab 4 contains the BAP protein. However, fragments were undetectable in other the assays, possibly due to folding of the protein; nonetheless, even if recombinant fragments were misfolded they still should have showed up in western immunoblot analysis since proteins are denatured for this assay. Alternatively, the expression of BAP is below our lower limit of detection.

The exact orientation in which *A. baumannii* BAP is exposed on the surface, which domains are exposed, or if it is surface exposed at all are still issues that require investigation. Loehfelm *et al* (2008) (39) were apparently able to identify a surface exposed epitope of BAP in 41% of isolates from an *A. baumannii* outbreak; however these findings do not tell us the specific interactions between BAP and the cell surface or the location of where the entire protein resides, just simply that a single epitope is surface exposed. Several approaches in bioinformatics have been used to predict the subcellular localization of *A. baumannii* BAP and although data suggests it is in fact an extracellular protein secreted by a type one secretion system, a transmembrane anchoring region was absent, and thus how BAP interacts with the cell wall is still uncertain (59). These findings may provide some insight into our results: Our data suggests that the seven areas of BAP (N and C terminal and 5 of the repeat regions) that we raised polyclonal antibodies (PAbs) against are not accessible for antibody targeting and are therefore either hidden within the protein, not accessible due to orientation of the protein on the cell surface or are below our lower limits of detection. Further research is required to determine the exact relationship between BAP and the bacterial cell wall and thus whether the terminal regions of this protein are accessible as immunotherapeutic targets.

4.5 Future Directions

Clearly there is still much to learn about *A. baumannii* BAP as well as the pathogenesis of this bacterium and its interactions with host defence mechanisms. Further studies with whole organism, peptide and recombinant protein anti-sera produced in this study are required to verify the true value of polyclonal antibodies against *A. baumannii*

and its biofilm associated protein. Several experiments have yet to be performed but include further efficacy assays such as: (i) Testing sera as a component in the biofilm growth device to establish how sera effects biofilm growth of *A. baumannii* strains, (ii) Reactivity assays with whole cell lysate and extracted outer membrane protein lysate of BAP positive *A. baumannii* strains, (iii) Immunofluorescence antibody labelling experiments and (iv) killing efficiency of serum in high concentrations with and without macrophage and complement to confirm the precise effects of whole organism anti-serum on clinical strain Swab 4 and additional *A. baumannii* strains.

Additional attempts to clone the entire *A. baumannii* BAP protein may prove beneficial for monoclonal antibody development and targeting of the entire protein which may allow further insight into its exact role in biofilm formation and maintenance. However, this may not be feasible and therefore researching and discovering new BAP targets with immunotherapeutic potential are of great importance.

4.6 Conclusion

This thesis raised many interesting questions about the BAP protein of *A. baumannii* despite not confirming expression of the protein. DNA and immunochemical data collectively suggest that BAP is potentially mosaic in *A. baumannii*. Many surface proteins of pathogenic bacteria undergo antigenic variation mechanisms such as *Neisseria gonorrhoeae*, or phase vary the expression completely (67, 68). Further to this our data also indicates that BAP is not expressed, not surface accessible, below our lower limits of detection or possibly completely absent in some strains of this bacterium. Given we have used multiple antibody reagents spanning the domains and repeat regions of the published

BAP sequence, not all of which could be folded away from solvent, we can conclude that the BAP is not expressed as published previously by Loehfelm *et al* (2008) (39). While the relevance of BAP to *in vivo* infections of humans is unknown if there is any, given our negative results and the potential that BAP is involved in biofilm formation we can conclude that BAP is not consistently expressed from strain to strain and is likely mosaic. This clearly warrants further examination.

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Appendix I

BAP REPEAT ALIGNMENTS

Red boxes indicate consensus sequence used to design synthetic oligo-peptides.

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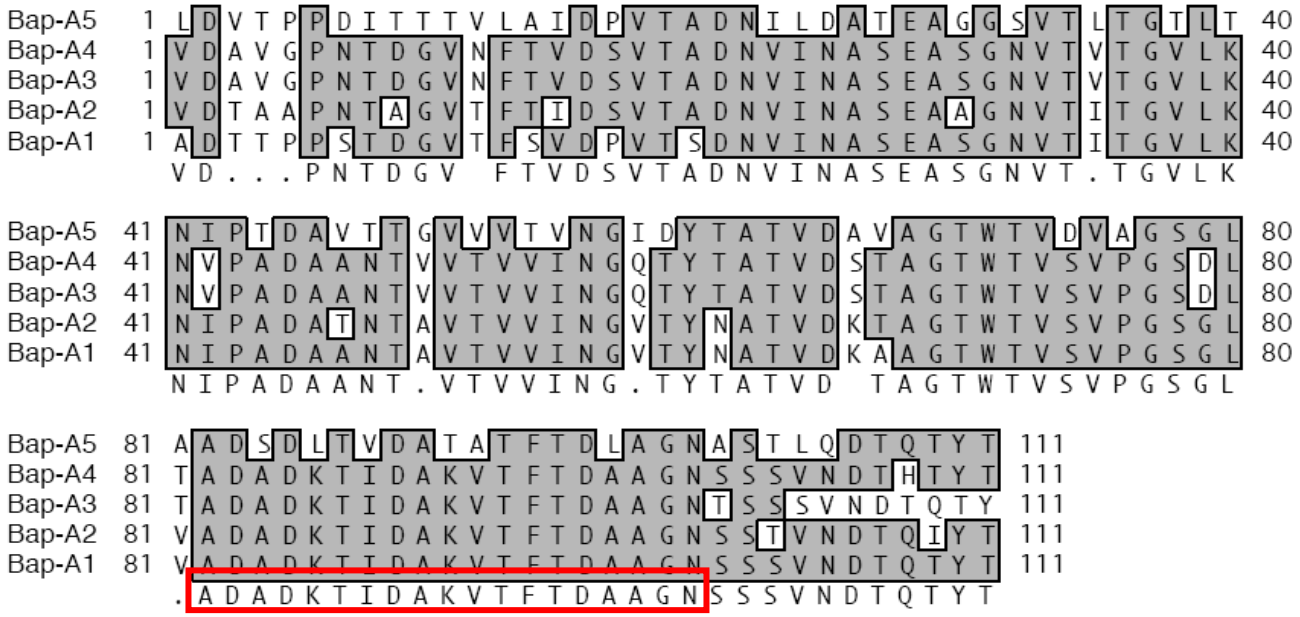

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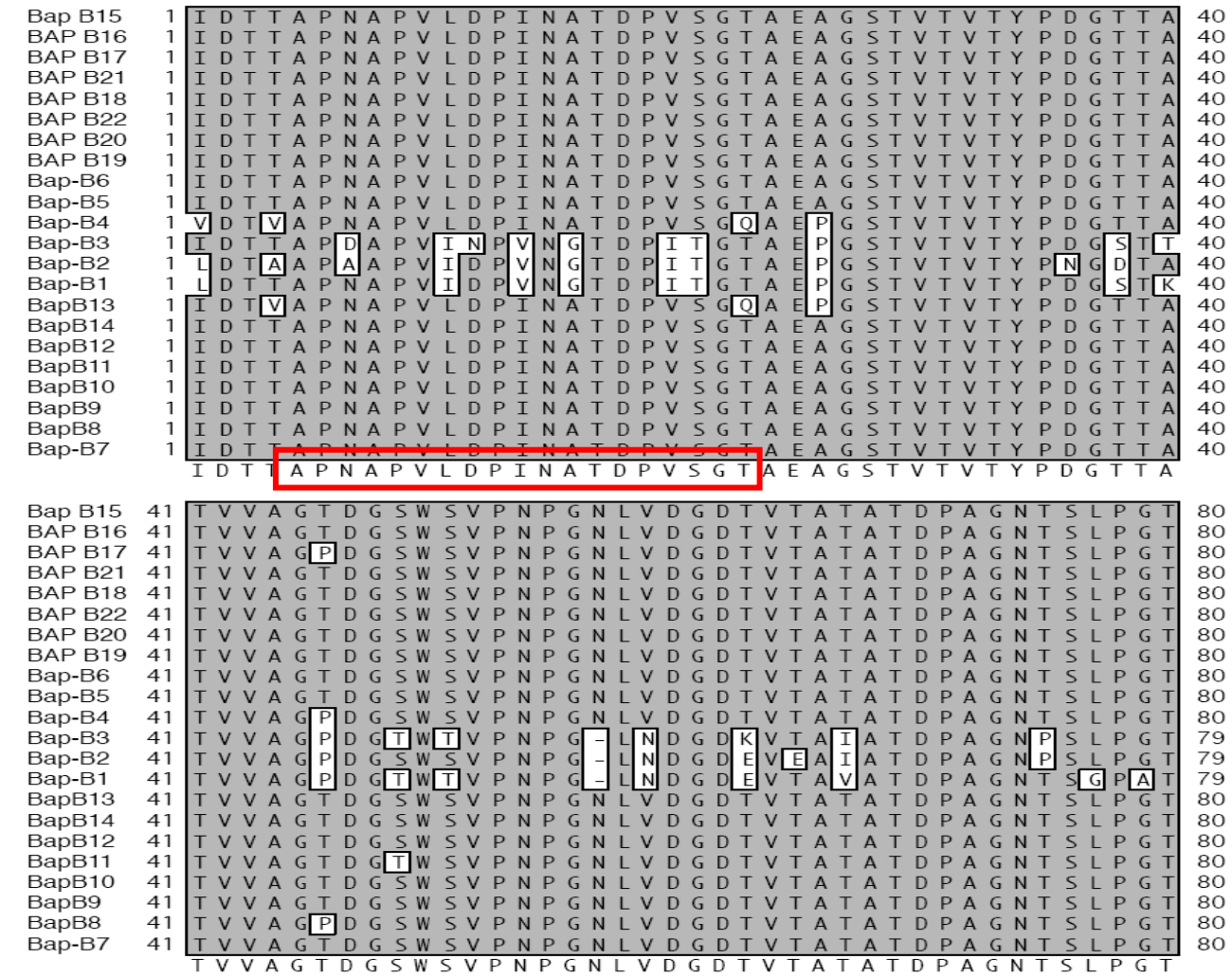
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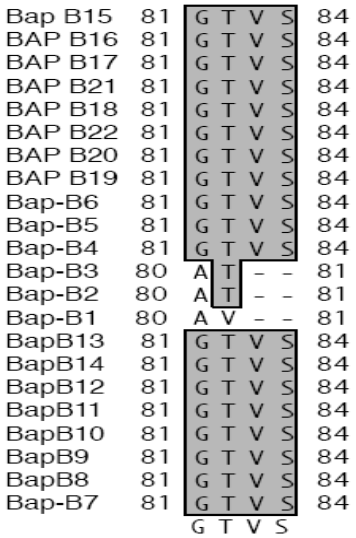
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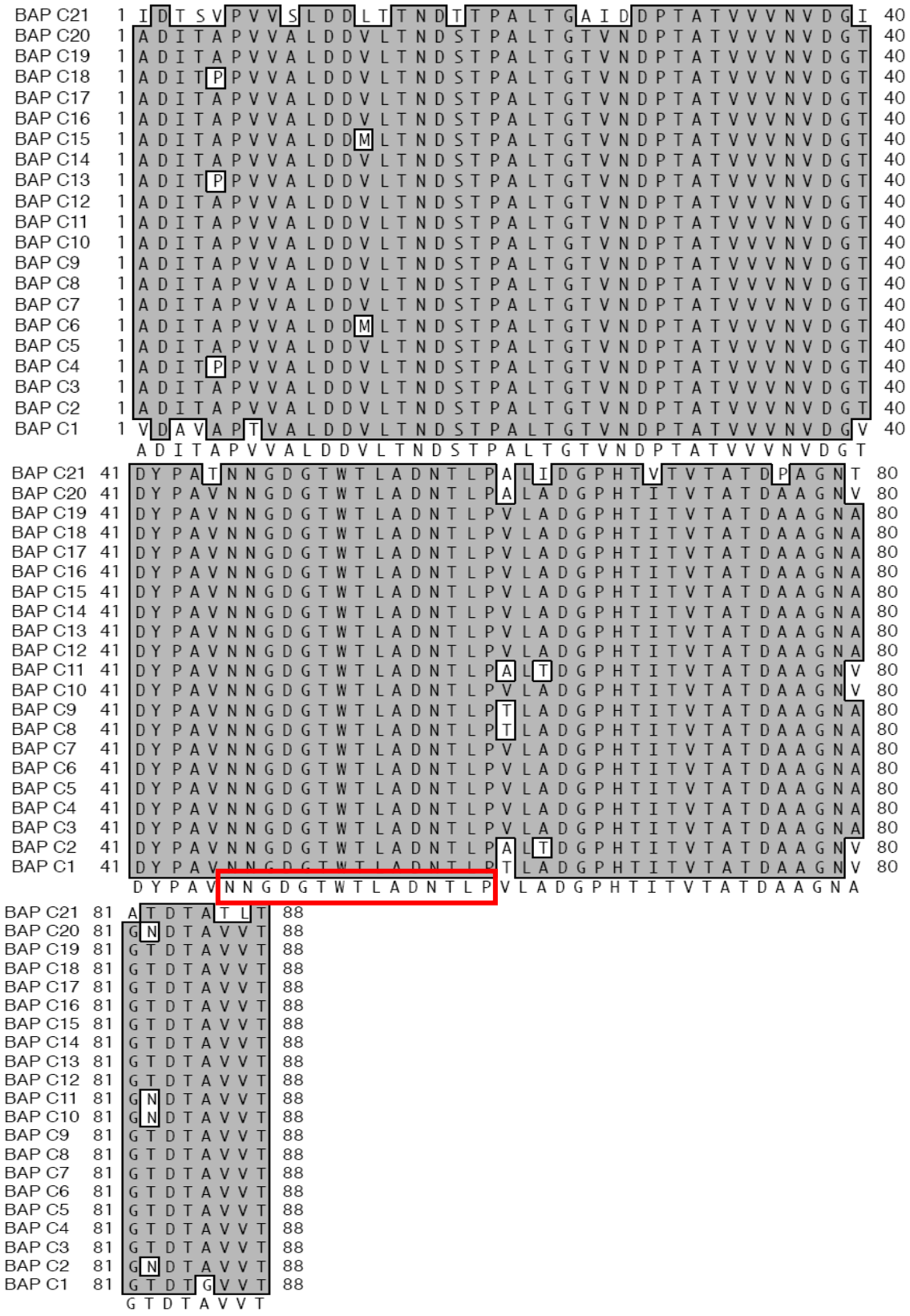
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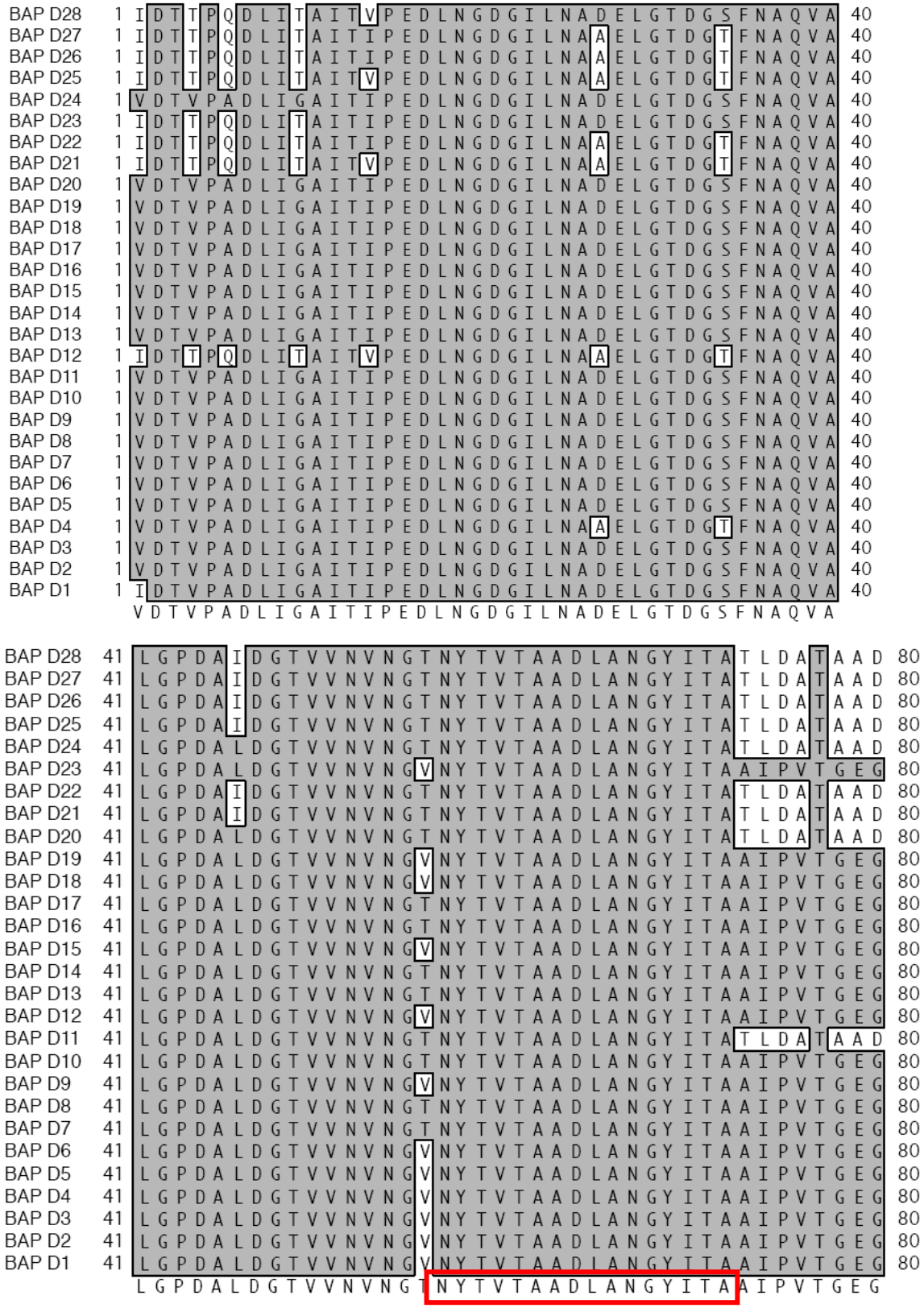
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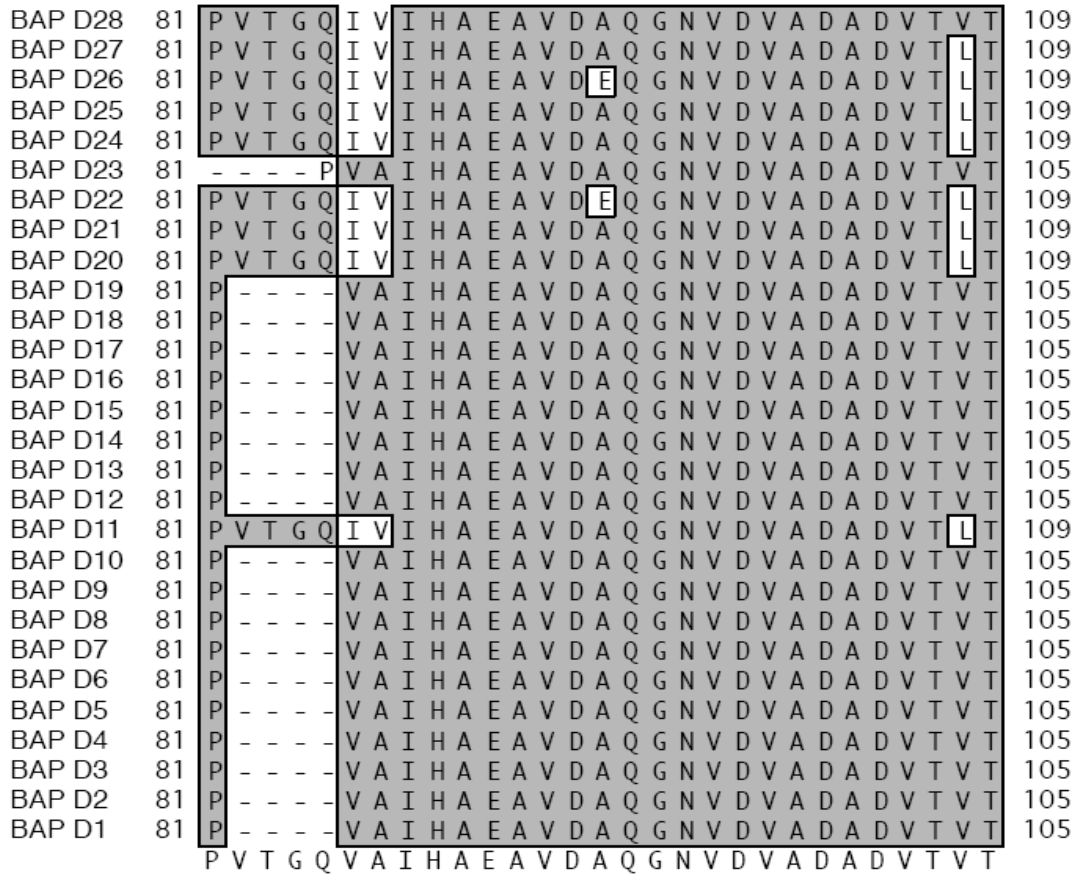
Module D continued



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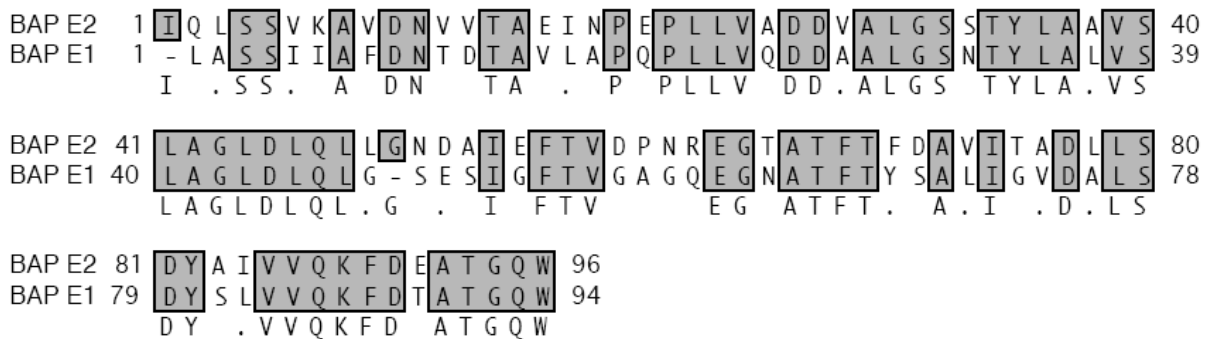
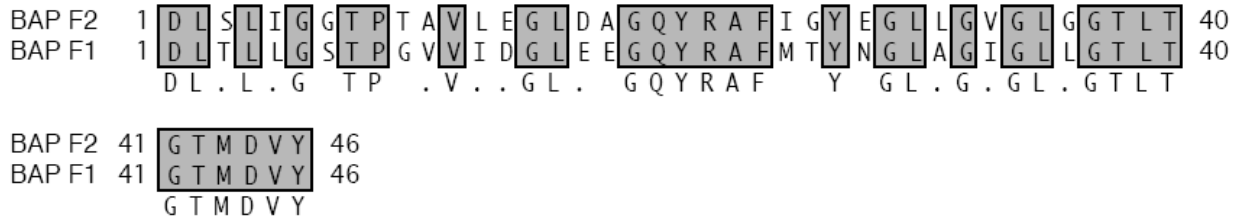


F1-F2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BAP F1 1 DLTLLGSTPGVVIDGLEEGQYRAFMTYNGLAGIGLLGTLT 40
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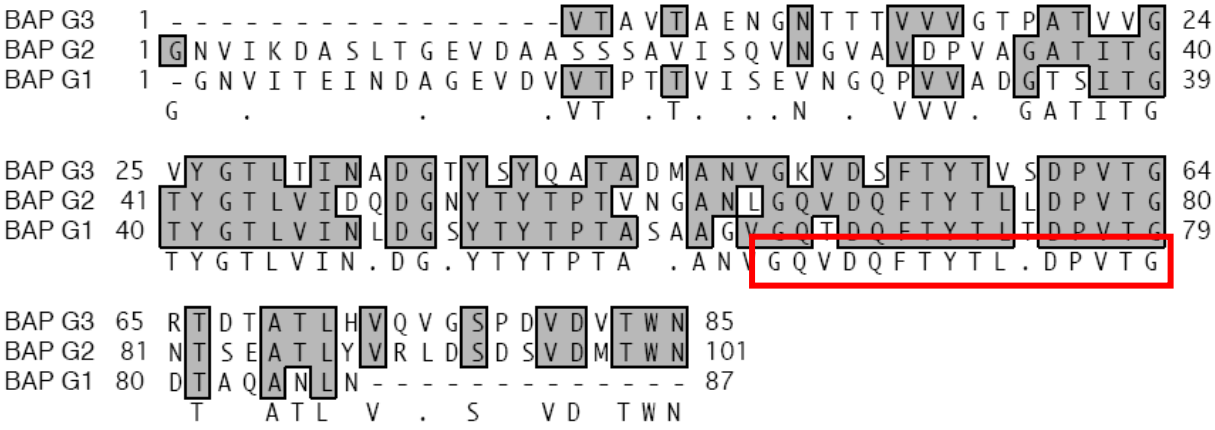
BAP F1 41 GTMDVY 46
GTMDVY

G1-G3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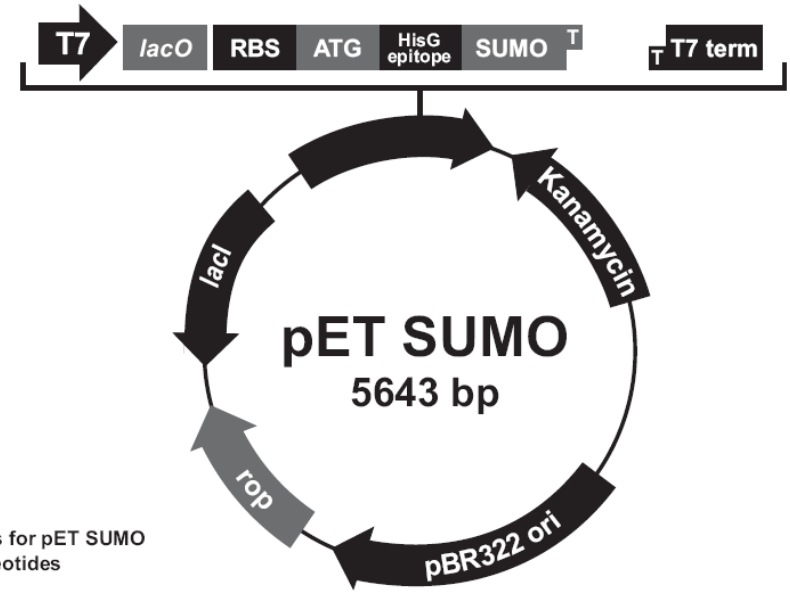
BAP G1 1 -GNVITEINDAGEVDVVTPTTVISEVNGQPVVADGTSITG 39
G .. .VT .T. ..N . VVV. GATITG
BAP G3 25 VYGTLTINADGTYSYQATADMANVGKVDSFTYTVSDPVTG 64
BAP G2 41 TYGTLVIDQDGNYTYTPTVNGANLGQVDQFTYTLLDPVTG 80
BAP G1 40 TYGTLVINLDGSYTYTPTASAAGVGQTDQFTYTLTDPVTG 79
TYGTLVIN.DG.YTYTPTA .ANVGQVDQFTYTL.DPVTG
BAP G3 65 RTDTATLHVQVGSPDVDVTWN 85
BAP G2 81 NTSEATLYVRLDSDSVDMTWN 101
BAP G1 80 DTAQANLN---------- 87
T ATL V . S VD TWN

Appendix II

pET SUMO Vector Map



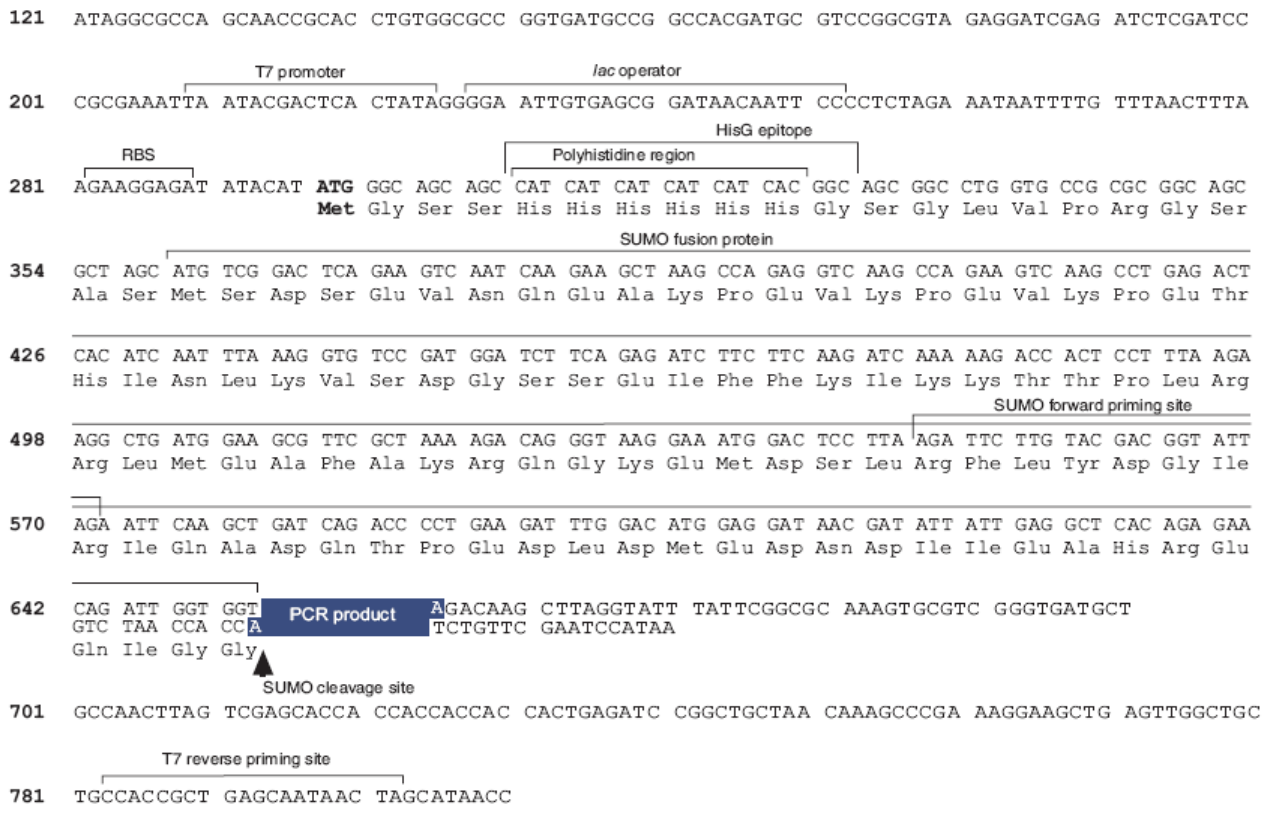
Comments for pET SUMO
5643 nucleotides

T7 promoter: bases 209-225
lac operator (lacO): bases 228-252
Ribosome binding site (RBS): bases 282-288
Initiation ATG: bases 297-299
HisG epitope: bases 309-329
SUMO ORF: bases 360-653
SUMO forward priming site: bases 549-571
TA Cloning site: bases 653-654
T7 reverse priming site: bases 783-802 (C)
T7 terminator: bases 744-872
Kanamycin resistance gene: bases 1431-2246 (C)
pBR322 origin: bases 2342-3015
ROP ORF: bases 3383-3574
lacI ORF: bases 4383-5474 (C)

(C) = complementary strand

Taken from the Champion™ pET SUMO Protein Expression System User Manual, Page 29 (Invitrogen). (Rev. Date: June 18, 2010. Manual part no. 25-0709)

TA Cloning Site



Taken from the Champion™ pET SUMO Protein Expression System User Manual, Page 7 (Invitrogen). (Rev. Date: June 18, 2010. Manual part no. 25-0709)

Appendix III

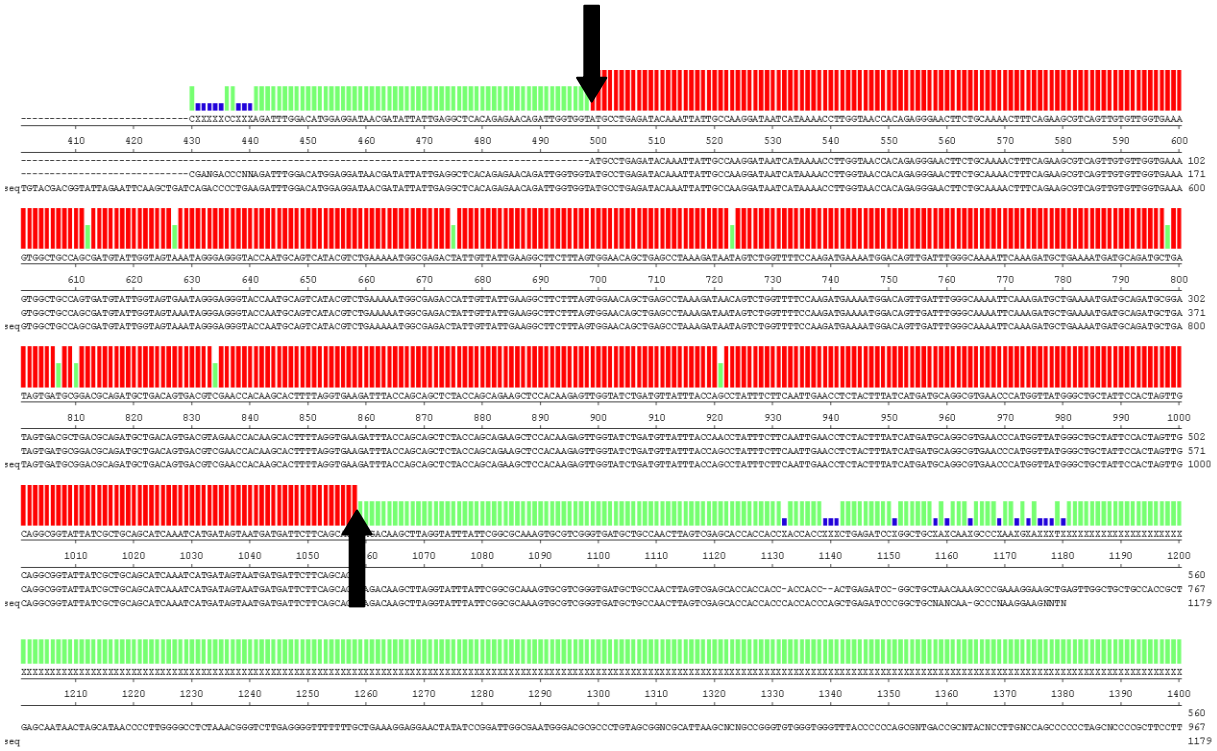
BAP N terminal insert in SUMO vector

Alignment of the BAP N terminal sequence from the NCBI database with plasmid DNA sample N terminal S5#2 forward and reverse sequence reactions (aligned in that order). Arrows indicate start and end of N terminal insert, while 'tails' on either end represent the vector. Red color indicates 100% identity between all 3 sequences and green indicates one base pair difference between all 3 sequences. The following is a representation of all BAP N terminal sequence samples

NCBI BAP N terminal sequence

S5#2 in SUMO sequence (forward reaction)

S5#2 in SUMO sequence (Reverse reaction)



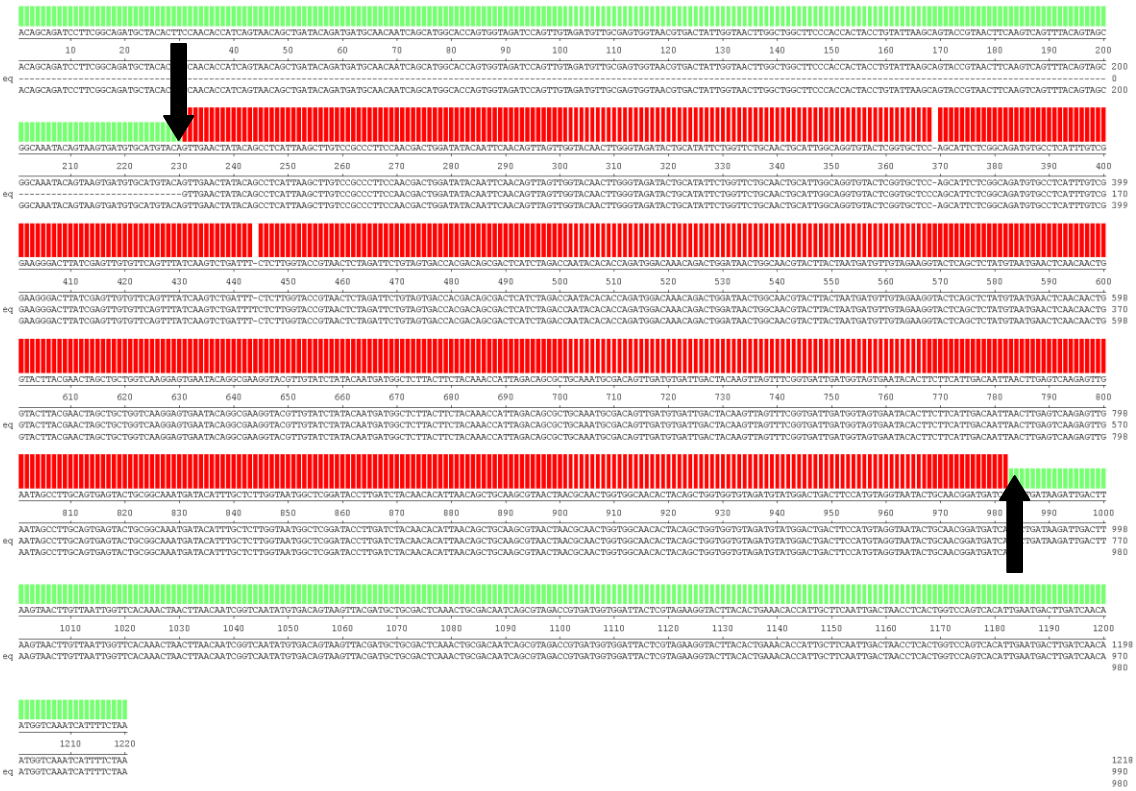
BAP C terminal insert in SUMO vector

Alignment of the BAP C terminal sequence from the NCBI database with plasmid DNA sample C terminal S5 1:1 #1 forward and reverse sequence reactions (aligned in that order). Arrows indicate start and end of C terminal insert, while 'tails' on either end represent the vector. Red color indicates 100% identity between all 3 sequences and green indicates one base pair difference between all 3 sequences. The following is a representation of all BAP C terminal sequence samples.

NCBI BAP C terminal sequence

S5 1:1#1 in SUMO sequence (forward reaction)

S5 1:1#1 in SUMO sequence (Reverse reaction)



BAP N terminal insert in SUMO vector – Plasmid sequence BLAST analysis

The following is a representation of all BAP N terminal samples (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP002522.1	Acinetobacter baumannii TCDC-AB0715, complete genome	1009	1009	76%	0.0	99%
CP001921.1	Acinetobacter baumannii 1656-2, complete genome	1009	1009	76%	0.0	99%
CP000863.1	Acinetobacter baumannii ACICU, complete genome	1009	1009	76%	0.0	99%
CP000521.1	Acinetobacter baumannii ATCC 17978, complete genome	1009	1009	76%	0.0	99%
CP001172.1	Acinetobacter baumannii AB307-0294, complete genome	973	1946	76%	0.0	98%
CU459141.1	Acinetobacter baumannii str. AYE, complete genome	973	1057	82%	0.0	100%
EU117203.1	Acinetobacter baumannii biofilm-associated protein (bap) and hypothetical protein genes, complete cds	973	973	76%	0.0	98%
CP002177.1	Acinetobacter calcoaceticus PHEA-2, complete genome	717	717	75%	0.0	88%
CP002080.1	Acinetobacter sp. DR1, complete genome	666	666	76%	0.0	86%
HM765513.1	Acinetobacter baumannii clone gDNA7 biofilm-associated protein-like gene, partial sequence	661	661	55%	0.0	97%
HM765514.1	Acinetobacter baumannii clone eDNA7 biofilm-associated protein-like gene, partial sequence	437	437	38%	4e-119	95%
BK006938.2	TPA: TPA_inf: Saccharomyces cerevisiae S288c chromosome IV, complete sequence	105	105	7%	3e-19	100%
FN393063.1	Saccharomyces cerevisiae EC1118 chromosome IV, EC1118_1D0 genomic scaffold, whole genome shotgun sequence	105	105	7%	3e-19	100%
NM_001180818.1	Saccharomyces cerevisiae S288c Smt3p (SMT3) mRNA, complete cds >gb|AY558174.1| Saccharomyces cerevisiae clone FLH110992.01X YDR510W gene, complete cds	105	105	7%	3e-19	100%
U33057.1	Saccharomyces cerevisiae chromosome IV cosmids 8166, 9787, 9717, and lambda 3073	105	105	7%	3e-19	100%
U27233.1	Saccharomyces cerevisiae suppressor of MIF2 Smt3p (SMT3) gene, complete cds	105	105	7%	3e-19	100%
GU456634.1	Synthetic construct His-SUMO-FGF21 gene, complete cds	100	100	7%	1e-17	98%
GQ870263.1	Plant RNAi vector pRNAi-LIC, complete sequence	95.1	95.1	7%	6e-16	100%
EU040203.1	Expression vector pLIC-C-TAP, complete sequence	95.1	95.1	7%	6e-16	100%
EF205336.1	Expression vector pASS2SUL, complete sequence	95.1	95.1	7%	6e-16	96%
EF205335.1	Expression vector pASHSUL, complete sequence	95.1	95.1	7%	6e-16	96%
EF205334.1	Expression vector pETS2SUL, complete sequence	95.1	95.1	7%	6e-16	96%
EF205333.1	Expression vector pETHSUL, complete sequence	95.1	95.1	7%	6e-16	96%
HQ700955.1	Expression vector XNWT_GW, complete sequence	93.3	141	6%	2e-15	100%
HQ700954.1	Expression vector NWTX_GW, complete sequence	93.3	141	6%	2e-15	100%
HQ700953.1	Expression vector NX33_GW, complete sequence	93.3	141	6%	2e-15	100%
HQ700952.1	Expression vector NX32_GW, complete sequence	93.3	141	6%	2e-15	100%
HQ700951.1	Expression vector XN21_GW, complete sequence	93.3	141	6%	2e-15	100%
HQ646606.1	Expression vector XN22_GW, complete sequence	93.3	141	6%	2e-15	100%
HQ646605.1	Expression vector pMetYC_GW, complete sequence	93.3	141	6%	2e-15	100%
HM623914.1	Binary vector pOSCAR, complete sequence	93.3	93.3	6%	2e-15	100%
HQ416902.1	Cloning vector pBB-CmR-ccdB, complete sequence	93.3	93.3	6%	2e-15	100%
GQ231553.1	Cloning vector pDONRamp, complete sequence	93.3	141	6%	2e-15	100%
FN377812.1	Cloning vector pKIGW containing genes for GUS, LhGR, nptII and ccdB	93.3	141	6%	2e-15	100%
EU360963.1	Cloning vector pDONR-A-Hyg, complete sequence	93.3	141	6%	2e-15	100%
EU360889.1	Cloning vector pDonrCbx, complete sequence	93.3	141	6%	2e-15	100%
EU181460.1	Cloning vector pEZY45, complete sequence	93.3	141	6%	2e-15	100%
EU181459.1	Cloning vector pEZY202, complete sequence	93.3	141	6%	2e-15	100%
EF025687.1	Cloning vector pQLinkGD, complete sequence	93.3	141	6%	2e-15	100%
EF025686.1	Cloning vector pQLinkHD, complete sequence	93.3	141	6%	2e-15	100%
AY551267.1	Cloning vector pWormgate2, complete sequence	93.3	282	6%	2e-15	100%
AY423863.1	Cloning vector pMK2010, complete sequence	93.3	141	6%	2e-15	100%
AY568055.1	Cloning vector pAGRIKOLA, complete sequence	93.3	282	6%	2e-15	100%
AF489904.1	Cloning vector pHellsgate 8, complete sequence	93.3	282	6%	2e-15	100%

BAP C terminal insert in SUMO vector – Plasmid sequence BLAST analysis

The following is a representation of all BAP C terminal samples
http://blast.ncbi.nlm.nih.gov/Blast.cgi

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP001172.1	Acinetobacter baumannii AB307-0294, complete genome	2185	2185	100%	0.0	99%
CP001182.1	Acinetobacter baumannii AB0057, complete genome	2185	2185	100%	0.0	99%
CU459141.1	Acinetobacter baumannii str. AYE, complete genome	2185	2185	100%	0.0	99%
EU117203.1	Acinetobacter baumannii biofilm-associated protein (bap) and hypothetical protein genes, complete cds	2185	2185	100%	0.0	99%
CP002177.1	Acinetobacter calcoaceticus PHEA-2, complete genome	1443	1443	100%	0.0	86%
CP002522.1	Acinetobacter baumannii TCDC-AB0715, complete genome	69.8	69.8	7%	4e-08	76%
CP001921.1	Acinetobacter baumannii 1656-2, complete genome	69.8	69.8	7%	4e-08	76%
CP000863.1	Acinetobacter baumannii ACICU, complete genome	69.8	69.8	7%	4e-08	76%
CP000521.1	Acinetobacter baumannii ATCC 17978, complete genome	57.2	57.2	5%	3e-04	78%
AK353336.1	Thellungiella halophila mRNA, complete cds, clone: RTFL01-40-N13	44.6	44.6	3%	1.7	87%
CR380954.1	Candida glabrata strain CBS138 chromosome H complete sequence	44.6	44.6	2%	1.7	88%
AL110498.1	Caenorhabditis elegans YAC Y64G10A, complete sequence	44.6	44.6	3%	1.7	83%
FQ311470.1	Sporisorium reilianum SRZ2 chromosome 5 complete DNA sequence	42.8	42.8	2%	5.8	92%
AM455685.2	Vitis vinifera contig VV78X229901.7, whole genome shotgun sequence	42.8	42.8	3%	5.8	84%
DQ377302.1	Bacillus thuringiensis serovar muju strain IEBC-T49 001 flagellin (hag) gene, partial cds	42.8	42.8	5%	5.8	75%
AL035087.20	Human DNA sequence from clone RP4-626B19 on chromosome 6q16.1-21 Contains the 3' end of the PRDM13 gene for PR-domain zinc-finger protein 13, and three CpG islands, complete sequence	42.8	42.8	2%	5.8	92%
BX005134.5	Zebrafish DNA sequence from clone CH211-162G1 in linkage group 12, complete sequence	42.8	42.8	2%	5.8	87%