

An Investigation of the Role of *Bacillus oleronius* in the Pathogenesis of Rosacea

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Declaration of Authorship

This thesis has not been previously submitted in whole or in part to this or any other University for any other degree and is the sole work of the author.

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Publications

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Abbreviations

AHP	Alkyl hydroperoxide reductase
APS	Ammonium persulfate
BSA	Bovine serum albumin
CK (3, 12, 19)	Cytokeratin
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid disodium salt dehydrate
ECL	Enhanced chemiluminescence
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FPLC	Fast performance liquid chromatography
hCap-18	human cathelicidin antimicrobial protein
HCL	Hydrochloric acid
hEGF	Human Epithelial Growth Factor
H ₂ O ₂	Hydrogen peroxide
H ₂ SO ₄	Sulfuric acid
IAA	Iodoacetamide
IEF	Isoelectric focusing
Ig	Immunoglobulin
IPG	Immobilised pH gradient
kDa	Kilo Dalton
KGM	Keratinocyte growth medium
LC/MS	Liquid chromatography mass spectrometry
MMP	Matrix metalloproteinase

MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO ₃	Sodium hydrogen carbonate
Na ₂ CO ₃	Sodium carbonate
NaOH	Sodium hydroxide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBMNC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PGN	Peptidoglycan
PIPES	Piperazine-N, N' –bis [2-ethanesulfonic acid]
<i>pI</i>	Isoelectric point
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBS	Tris buffered saline
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TLCK	<i>N</i> _α -Tosyl-L-lysine chloromethyl ketone hydrochloride
TMB	3,3',5,5'-Tetramethylbenzidine
TRITC	Tetramethyl rhodamine iso-thiocyanate
v/v	Volume per volume
w/v	Weight per volume

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Abstract

Rosacea is a chronic inflammatory dermatological condition that has been reported to affect at least 3% of the population in Ireland. Symptoms are concentrated around the center of the face and include chronic inflammation, telangiectasias and inflammatory lesions (papules, pustules and plaques). Up to 50% of those suffering from rosacea have symptoms that manifest in the periocular region, including irritation, inflammation of the eyelids (blepharitis), increased vascularisation of the ocular surface and in severe cases inflammation of the cornea (keratitis) can develop leading to sterile ulcers and visual impairment. Individuals with rosacea often have an elevated presence of *Demodex* mites in their skin follicles and eyelashes. *Bacillus oleronius* is a gram negative bacterium isolated from a *Demodex folliculorum* mite from the face of a rosacea patient. Antigens associated with the bacterium (62 and 83 kDa) were isolated and shown to cause immunoreactivity in the serum of papulopustular rosacea patients. A collaboration with Dr. Scheffer Tseng (Ocular Surface Research Centre, Miami) led to the publication of work that proved a strong correlation has between serum reactivity to these *B. oleronius* antigens and eye lid margin inflammation ($p = 0.04$), and ocular rosacea ($p = 0.009$). Proteomics techniques were used to investigate the environmental conditions which lead to antigen production in the bacterium. Another area of focus was the effect of *Bacillus* proteins on Neutrophils and Corneal epithelial cells. Neutrophils are known to play a part in the development of inflammation in rosacea. Our studies showed that there was an increase in neutrophil degranulation, migration and cytokine production following exposure to the bacterial antigens. Corneal epithelial (hTCEpi) cells responded to the bacterial antigens by increasing motility and migration, decreasing proliferation and altering protein production. This work provides strong evidence for the role of *Demodex* associated *Bacillus* proteins in the development of facial and ocular symptoms of rosacea.

CHAPTER 1

Introduction

1.1 Rosacea

Rosacea is typically described as a chronic inflammatory dermatological disorder. The onset of the condition usually occurs between the ages of 30-50 years (Dahl *et al.*, 2002) with approximately 57% of cases diagnosed in the under 50's (Elewski *et al.*, 2010). Rare cases have been reported in children as young as four (Cetinkaya & Akova 2006). Symptoms can progress from 'pre-rosacea' characterised by transient flushing to repeated flushing and inflammation that cause vascular damage (Gupta and Chaudhry 2005). The course of the disease consists of exacerbations followed by periods of remission and can be controlled with treatment and avoidance of certain trigger factors rather than cured. Symptoms are concentrated in the t-zone which consists of the forehead, nose and chin (Figure 1.1), and can extend towards the cheeks, ears and neck. Excess production of sebum (the body's natural oil) may occur in the skin of the t-zone due to the presence of a greater number of sebaceous glands. The role of sebum in the pathogenesis of rosacea is unknown however it has recently been shown that patients with papulopustular rosacea (PPR) have an abnormal sebaceous fatty acid composition (Ni Raghallaigh *et al.*, 2012) these findings indicate the possibility of employing sebum-modifying nonantibiotic treatments.

Reports have suggested the prevalence of rosacea is as high as 10% of the population in Sweden (McAleer *et al.*, 2010) and according to the National Rosacea Society over 16 million Americans are estimated to have rosacea. The study carried out by McAleer *et al.*, (2010) estimated the prevalence of Papulopustular rosacea in Ireland at 2.7%. Table 1.1 is adapted from that study and lists results from six studies carried out in five countries with occurrence in the populations ranging from 0.9% – 10%. Some reports have suggested that occurrence is more frequent in women (Elewski *et al.*, 2010) however some reports claim both genders are equally affected but symptoms can be more severe among men (Fimmel *et al.*, 2008). It occurs among all racial demographics however increased frequency among fair skinned individuals suggests a genetic predisposition and has led to rosacea being referred to as the 'Curse of the Celts'.

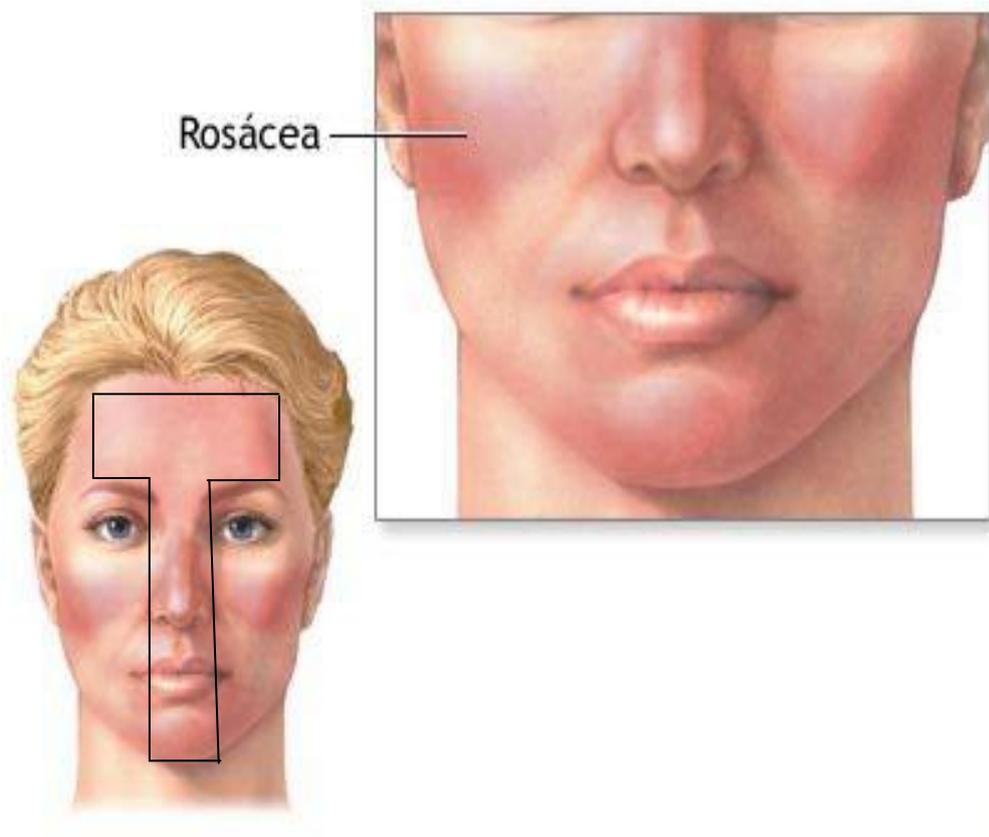


Figure 1.5 Schematic view of the region of the face where rosacea symptoms typically occur.

Symptoms of rosacea are often concentrated in the t-zone region of the face, which is indicated above.

[Taken from the website: www.getacnehomeremedies.info/search/overview-on-rosacea-acne-treatment-causes-and-prevention]

Population	Country	Intention of Study	Population type	Rosacea definition	Prevalence
2341	Denmark (Faroe Islands)	Prevalence of skin Diseases	Households	Not defined	0.09%
809	Sweden	Prevalence of rosacea	Office workers	ETT: redness and telangiectasia of face; PPR: papules and pustules of face	10% (ETT: 8.2%; PPR: 1.8%)
9293	United States	Health survey	Sample of civilian US population	Not defined	1.9%
402	United States	Relationship between childhood stye and rosacea	Database of medical records	Not defined	2.1%
50,237	Greece	Rosacea in dermatology outpatients	Database of dermatology outpatients	Not defined	1.22% (ETT: 0.88%; PPR: 0.34%)
1000	Ireland	Prevalence of papulopustular rosacea (PPR) and its relationship to UV Radiation	500 hospital staff and 500 inhabitants of the Aran Islands	PPR	2.7% (PPR)

ETT = Erythematotelangiectatic Rosacea PPR = Papulopustular Rosacea

Table 1.1 Summary of Rosacea prevalence from six studies. Adapted from (McAleer *et al.*, 2010).

1.2 Diagnostic Features

Despite the fact that rosacea is a common condition that causes visible symptoms that are painful and can lower the self esteem of those affected the pathogenesis of the condition remains elusive. Patients present with a range of symptoms including: cutaneous abnormalities such as flushing, telangiectasia, erythema, papules, pustules, edema ocular lesions and rhinophyma (Crawford *et al.*, 2004). Unfortunately no serologic or diagnostic assays exist for diagnosis of the condition.

According to the standard classification of rosacea reported by the National Rosacea Society the presence of one or more of the primary features affecting the central convexities of the face indicates a diagnosis of rosacea (Dahl *et al.*, 2002). Primary diagnostic features include transient flushing, non-transient erythema (persistent redness of the facial skin), telangiectasia (dilated small blood vessel) or the presence of papules (red dome shaped elevations of the skin) and pustules (skin elevation containing fluid consisting of white blood cells). These symptoms may occur simultaneously or independently and most individuals have underlying extremely sensitive skin.

Secondary features usually accompany the primary features but can occur independently in some individuals. They include stinging sensation, dry skin, elevated red plaques or edema (soft or solid which is exacerbated by inflammation). The most common secondary feature are ocular manifestations occurring in up to 50% of individuals with rosacea (Elewski *et al.*, 2010).

1.3 Rosacea Subtypes

The National Rosacea Society developed a standard classification system that describes four subtypes and one variant (*Granulomatus rosacea*). Those subtypes are Erythematotelangiectatic, Papulopustular, Phymatous and Ocular (Dahl *et al.*, 2002). In 2004 the society published a further report focused on the grading of severity of the condition (Wilkin *et al.*, 2004). Primary symptoms are graded from mild, moderate or severe (0-3) and secondary symptoms are deemed present or absent. This grading system aids the physician in deciding the course of therapy.

1.3.1 Erythematotelangiectatic Rosacea

Type one rosacea (Erythematotelangiectatic rosacea) is characterised by persistent erythema and telangiectases in the central region of the face (Figure 1.2). The erythema or flushing associated with this type of rosacea can last over 10 minutes therefore it can be distinguished from the blushing seen in response to embarrassment. The small dilated blood vessels (telangiectasias) often appear as the condition worsens to grade 2 or 3 (Crawford *et al.*, 2004). People with this subtype of rosacea have extremely sensitive skin that can be rough or scaly and usually report itching, burning and stinging sensations in response to topical application of creams to the skin even those prescribed to treat the condition.

1.3.2 Papulopustular Rosacea

Patients with Papulopustular rosacea often present with symptoms of type one rosacea combined with persistent papules (red elevations of the skin that can be up to 10 mm in diameter) and pustules (smaller elevations containing inflammatory cells that appear as a cloudy fluid). In severe cases of this subtype (grade 3), the papules and pustules can form an inflammatory lesion or plaque (Figure 1.3). This condition was originally described as acne rosacea however the presence of telangiectasias, more persistent erythema and the absence of comedones can assist physicians in distinguishing the two allowing a proper diagnosis of papulopustular rosacea.

1.3.3 Phymatous Rosacea

Phymatous rosacea presents as thickening of the skin accompanied by irregular nodules usually concentrated in the nasal region, rhinophyma (Figure 1.4) but can occur on the ears forehead, cheeks and chin. This condition can occur in conjunction with the first two subtypes but can present spontaneously without any other symptoms. This is the rarest subtype of rosacea and has been reported to be up to 20 times more common in men and at its most severe stage this condition requires surgical correction (Powell, 2005).

1.3.4 Ocular Rosacea

As with the preceding subtypes, ocular rosacea can present in combination with symptoms of types one and two rosacea, it can also occur prior to or spontaneously of any cutaneous manifestations (Oltz & Check 2011). As seen in Figure 1.5, this condition often presents as inflammation of the eyelids (blepharitis or conjunctivitis). Patients also experience burning, stinging or dryness of the eyes, telangiectasias of the corneal surface, blurred vision, cylindrical dandruff of the eyelashes and chronic meibomian gland dysfunction. Keratitis is a severe but uncommon complication that can lead to the formation of nodular scars and sterile ulcers of the corneal surface which cause visual impairment. Figure 1.5 provides an example of the grading system used by physicians (Wilkin *et al.*, 2004). Three cases of ocular rosacea are graded A-C with A representing a mild case and C representing the severe ocular manifestations of rosacea.



Figure 1.6 Erythematotelangiectatic Rosacea

[Taken from Powell, (2005)]

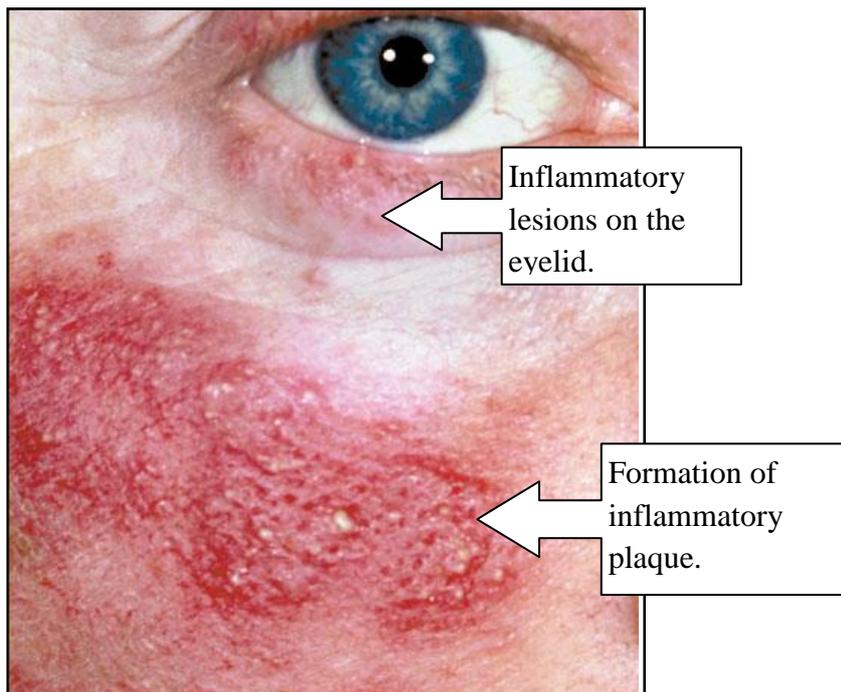


Figure 1.7 Picture of Papulopustular Rosacea symptoms. Severe papulopustular rosacea with moderate ocular involvement.

[Taken from Powell, (2005)]

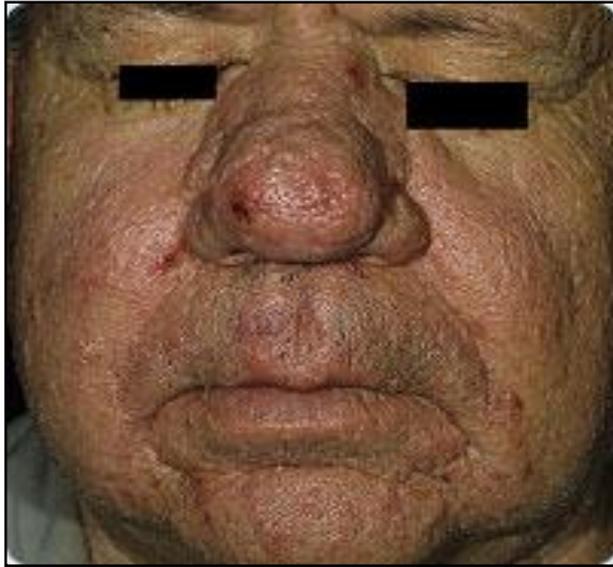


Figure 1.8 Phymatous Rosacea

[Taken from the website www.acne.org/facial-rosacea.html]

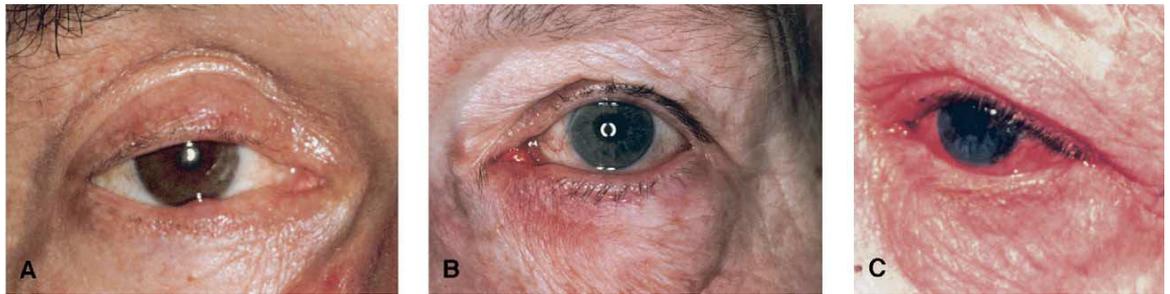


Figure 1.5 Ocular Rosacea graded mild (A), moderate (B) and severe (C).

[Taken from Wilkin *et al.*, 2004]

1.3.5 Rosacea Variants

The National Rosacea Society recognises one variant of the condition called Granulomatous rosacea. This condition is characterised by the appearance of papules or nodules on the cheeks and periorificial areas that are hard, yellow, brown or red in colour and can lead to severe scarring (Dahl *et al.*, 2002). Skin underlying the nodules appears normal unlike in papulopustular rosacea where underlying skin is usually sensitive and prone to erythema. It has been suggested that ultraviolet radiation (UVR) exposure is a more prominent causative factor in granulomatous variant rosacea compared to non-granulomatous rosacea due to a measured up-regulation of matrix metalloproteinases (MMPs), (Jang *et al.*, 2010).

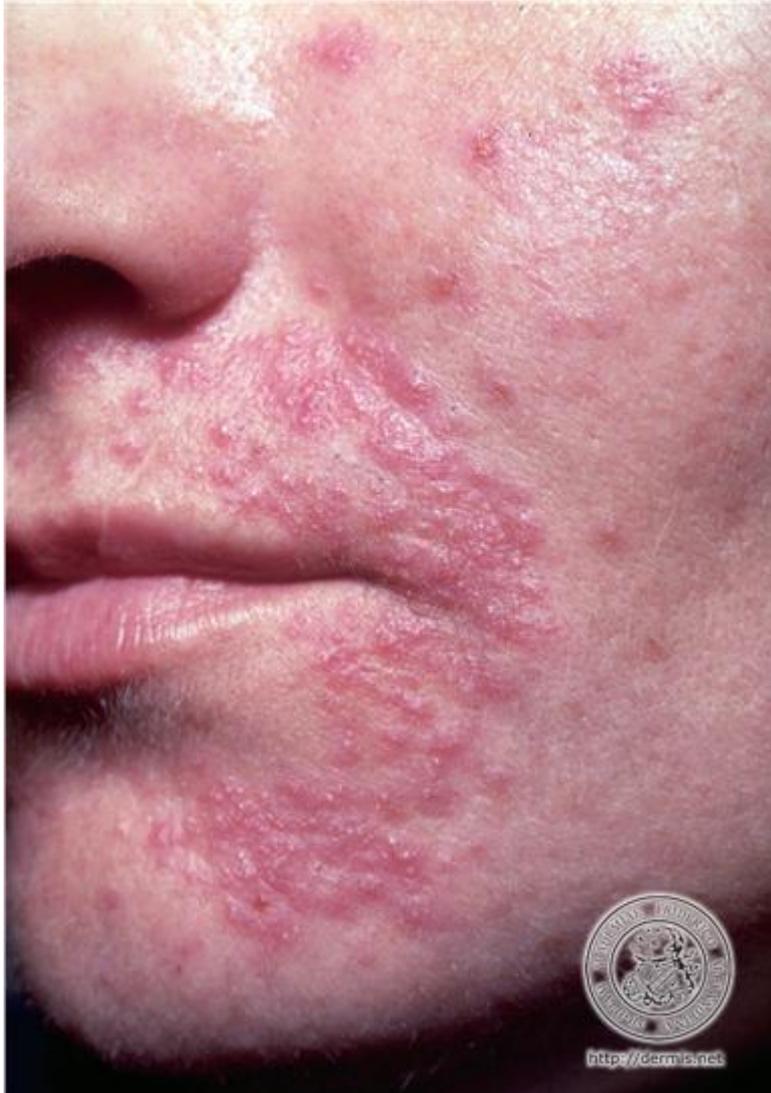


Figure 1.6 Granulomatous Rosacea

[Taken from the website <http://dermis.net/bilder/CD008/550px/img0002.jpg>]

1.4 Factors that trigger rosacea symptoms

The classification and grading of rosacea is now well established (Dahl *et al.*, 2002; Wilkin *et al.*, 2004) however there is no definitive agreement of the causative agents responsible for this condition or its pathogenesis. Factors such as alterations in the innate immune response, vascular changes and the presence of reactive oxygen species within the skin have been suggested as playing a role in the induction and persistence of the condition (Yamasaki & Gallo, 2009). Reports have suggested an underlying genetic susceptibility in those affected by rosacea (Bamford 2001) which is exacerbated by a number of triggers (Gupta & Chaudhry 2005). Individuals that suffer from transient flushing are said to be in the pre-rosacea stage and may benefit from identifying the factors that trigger symptoms so they can be avoided. The study by Gupta and Chaudhry, (2005) includes an extensive list of items known to trigger prolonged blushing or erythema (Figure 1.2) in rosacea patients. The list includes certain foods, alcoholic or hot beverages, skincare products, exposure to the sun, hot temperatures in saunas or humid environments, cold and windy weather, physical exertion or stress. Some of the triggers listed are not easy to avoid such as certain medications, vasodilators and topical steroids, or certain medical conditions, menopause, caffeine withdrawal syndrome or chronic cough (Gupta & Chaudhry, 2005). The paper mentions a survey carried out by the National Rosacea Society of 2083 rosacea patients in relation to avoidance of flare ups of symptoms that says 78% of patients said avoiding trigger factors that aggravate their condition had been effective in decreasing flare-ups (<http://www.rosacea.org/rr/1999/spring/article-3.php>). Figure 1.7, is taken from a review article (Yamasaki & Gallo, 2009) which gives a comprehensive outline of triggers and contributing factors that ultimately cause dysregulation of the immune response leading to the appearance of chronic symptoms.

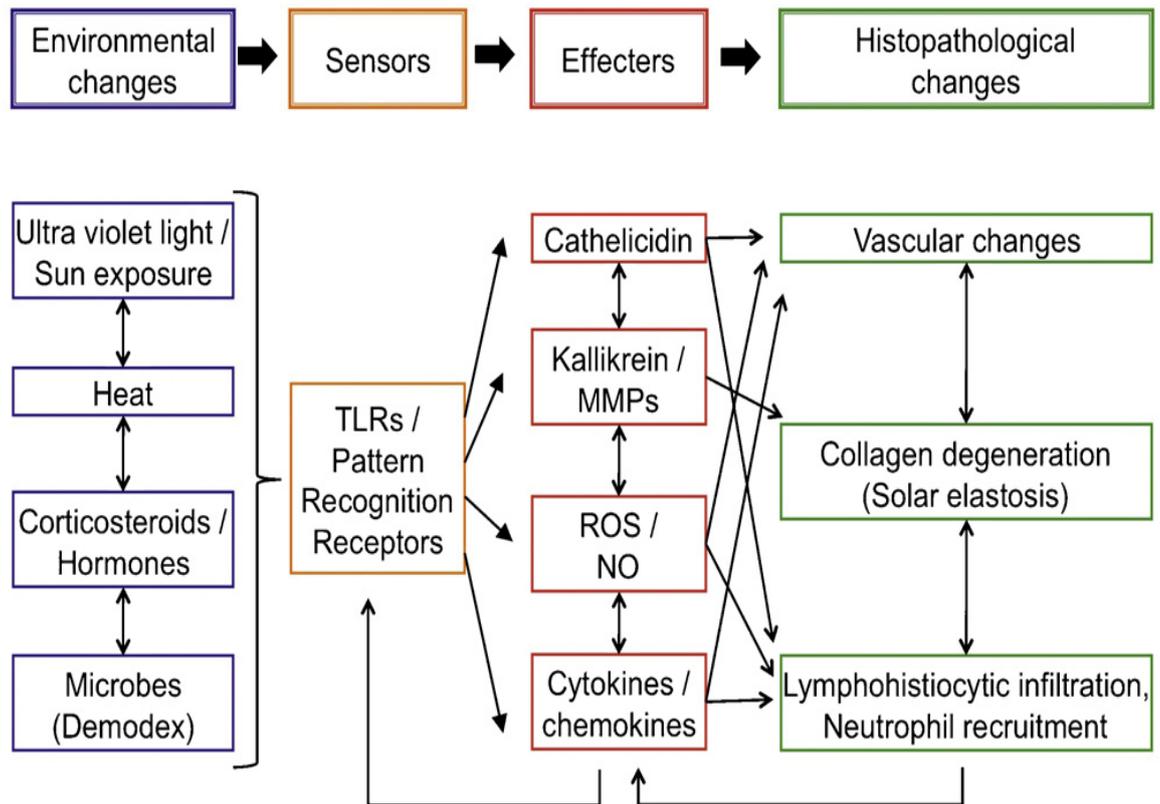


Figure 1.7 Schematic of environmental factors involved in rosacea and the immune changes that are triggered due to exposure to these factors.

[Taken from Yamasaki & Gallo, 2009]

1.5 *Demodex* Mites (*Demodex folliculorum* and *Demodex brevis*)

Demodex mites are dermal mites that colonise human skin and feed on sebum produced in the glands and were first described as a commensal organism in the 1840s (Vu & English, 2011). The German dermatologist Gustav Simon first located the mite upon examination of the contents of acne lesions which prompted interest in the pathogenesis of the mite (Lacey *et al.*, 2011; Fell 1886). There are two species that inhabit humans, *Demodex folliculorum* are usually located in the hair follicles while *Demodex brevis* burrow deeper into the sebaceous and meibomian glands (Bonnar *et al.*, 1993). Both mites were extensively re-examined by Desch and Nutting (1972). The mite body consists of gnathosoma (mouth parts), podosoma with four pairs of evenly spaced legs (in both species) and the opisthosoma containing the abdomen (longer in *D. folliculorum* than *D. brevis*). A finding which may contribute to *Demodex* pathogenesis is that they have no anus (Lacey *et al.*, 2011) therefore ingested material such as microbes are stored and can proliferate in an environment that is sheltered from the host immune system.

Most individuals become colonised in childhood and numbers proliferate around the time of puberty when the sebum level is present in the skin increases (Desch and Nutting, 1972) the prevalence is said to be 80-100% in those over 50 (Vu & English 2011). *D. folliculorum* can be found singly or in groups where as *D. brevis* is most likely to be found as a single specimen (Desch and Nutting, 1972). These mites ingest bacteria along with sebum and follicular epithelial skin cells and have been implicated in the transfer of bacteria from person to person or from one location on the host to another (Powell, 2005; Desch and Nutting, 1972). Their life cycle (Figure 1.8) is said to be around 2.5 weeks (Lacey *et al.*, 2011). At the end of the lifecycle mites could release bacteria into the hair follicle causing a localised innate immune response. Mites reportedly stay alive on their human host for up to 55 hours after death of the host therefore they are useful when determining time of death (Özdemir, 2003).

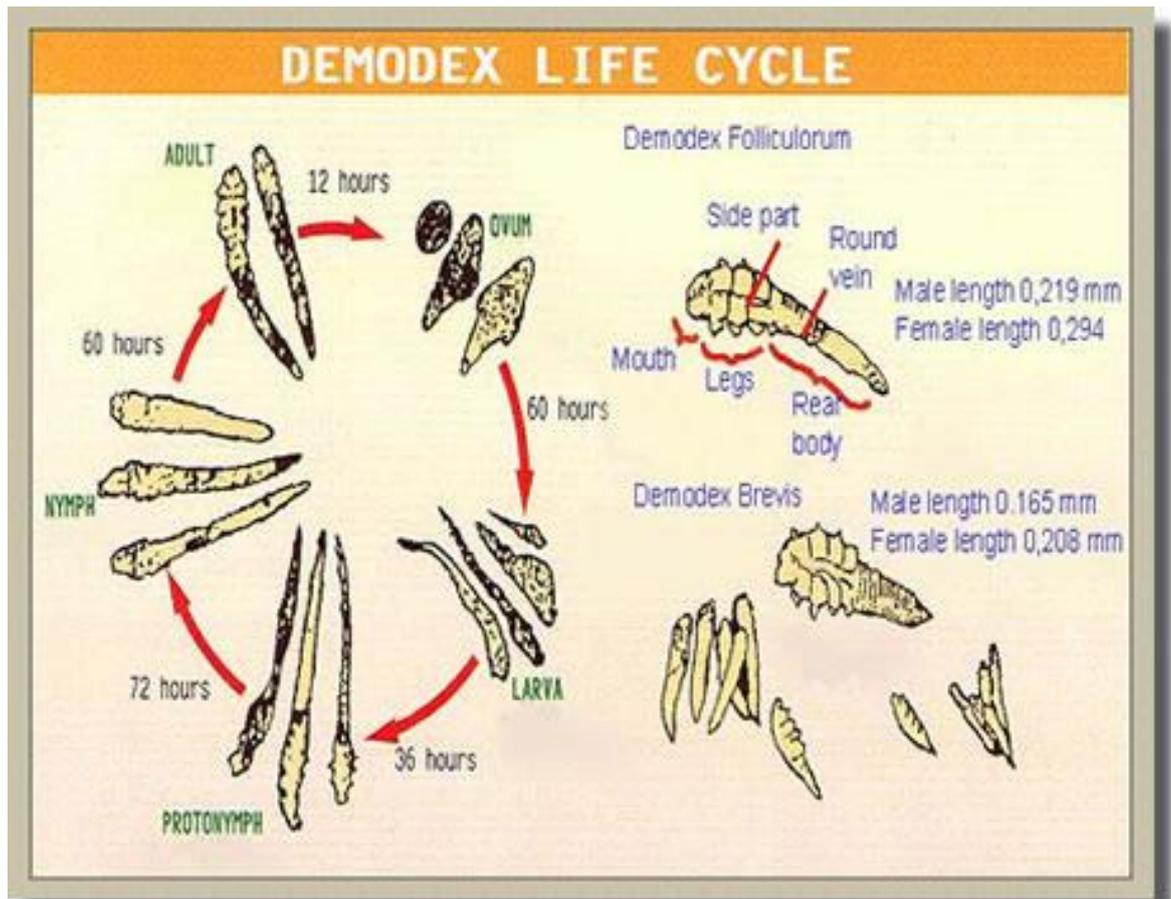


Figure 1.8 Life Cycle of the *Demodex* mite

[Taken from the website www.thejamushop.com/demodex_faq.htm]

1.5.1 The pathogenic properties of *Demodex* mites

Although the pathogenicity of *Demodex* species has been disputed since they are inhabitants of normal skin, localised inflammation is often seen in follicles containing mites particularly when mite density proliferates (Elston, 2010). Presence of *Demodex* has been linked to inflammation, blockage of follicles and foreign body granulomatous reactions (Figure 1.9) (Lazaridou *et al.*, 2011). Since the discovery of these ubiquitous mites they were associated with acne (Fell 1886) and many other dermatological and ophthalmological conditions. The mite acts as a vector for surface microorganisms and endosymbionts, this characteristic is likely to contribute to its pathogenicity (Lacey *et al.*, 2011). *Demodex* mites have been shown to carry *Staphylococcus albus* and transmit these from one hair follicle to another (Norn, 1972). *Demodex* have also been shown to carry *Microsporon canis* (Wolf *et al.*, 1988). The *Demodex* mite or its secretions have been suggested as a causative agent for the inflammation seen in blepharitis (Kim *et al.*, 2011; Liu *et al.*, 2010; Neiberg and Sowka, 2008). The study by Kim *et al.*, (2011) indicated that the elevation of tear cytokines that contributes to lid margin and ocular surface inflammation was caused by increased *Demodex* density. Increased mite density is the causative factor in demodicosis and *Demodex* folliculitis (Hsu, and Lee 2009; Forton *et al.*, 2005; Vu and English, 2011) both of these conditions have a rosacea like appearance.

Demodex mites have been implicated in the induction of rosacea for over 50 years (Ayres & Ayres, 1961). These mites have been seen in follicles and biopsies of rosacea patients (Lacey *et al.*, 2007). Individuals with rosacea have been shown to have a higher density of *Demodex* mites in their skin compared to controls (Bamford 2001; Bonnar *et al.*, 1993) using a cyanoacrylate glue skin surface biopsy test. Sampling of eyelashes (Figure 1.10) is another popular method of counting *Demodex* density and this technique was employed to establish that the mite population is higher in the eyelashes of those with rosacea than those without the condition (Li *et al.*, 2010). Although there is evidence of increased mite density in those with rosacea it has been disputed whether this is a contributing factor to the pathogenesis of the condition or simply a proliferation of mites due to sebum rich conditions (Dhingra *et al.*, 2009).

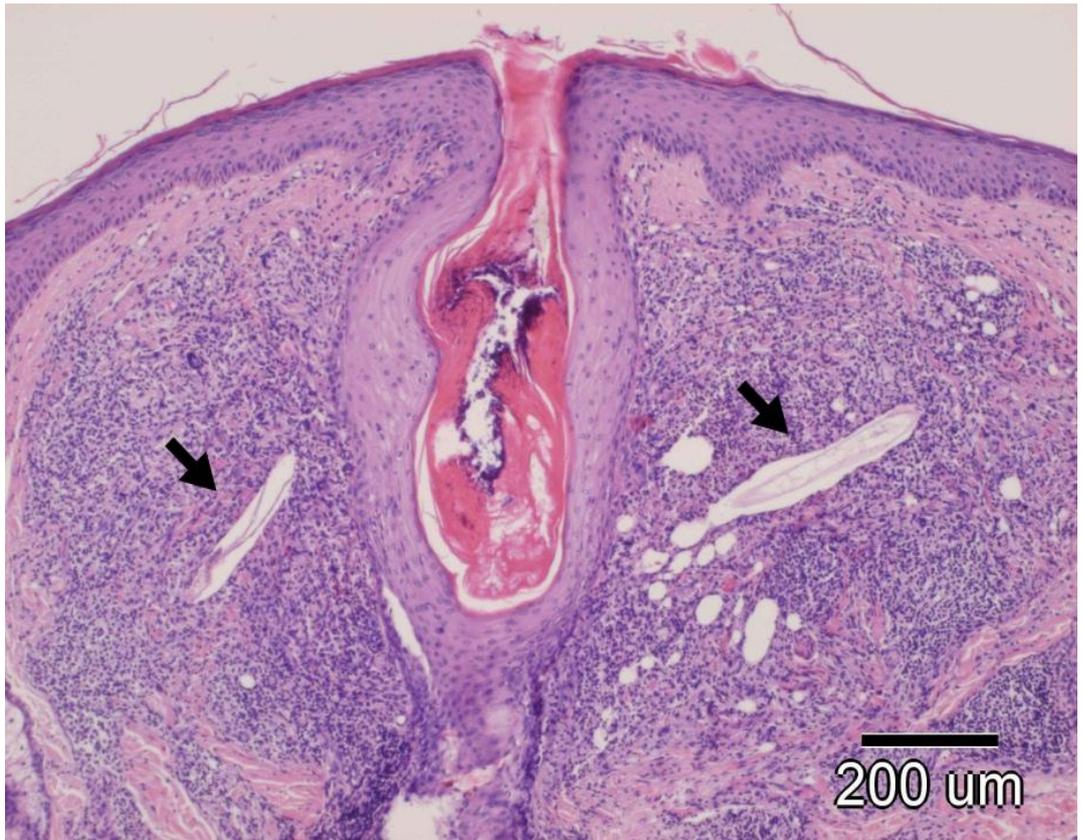


Figure 1.9 *Demodex* mites associated with papules of Granulomatous rosacea

Histopathology of a papule shows inflammatory infiltrate consisting of lymphocytes and neutrophils around a hair follicle with two mites present in the infiltrate indicated by the arrows

[Taken from the website http://dermatology.cdlib.org/134/case_presentations/demodex/lee.html]

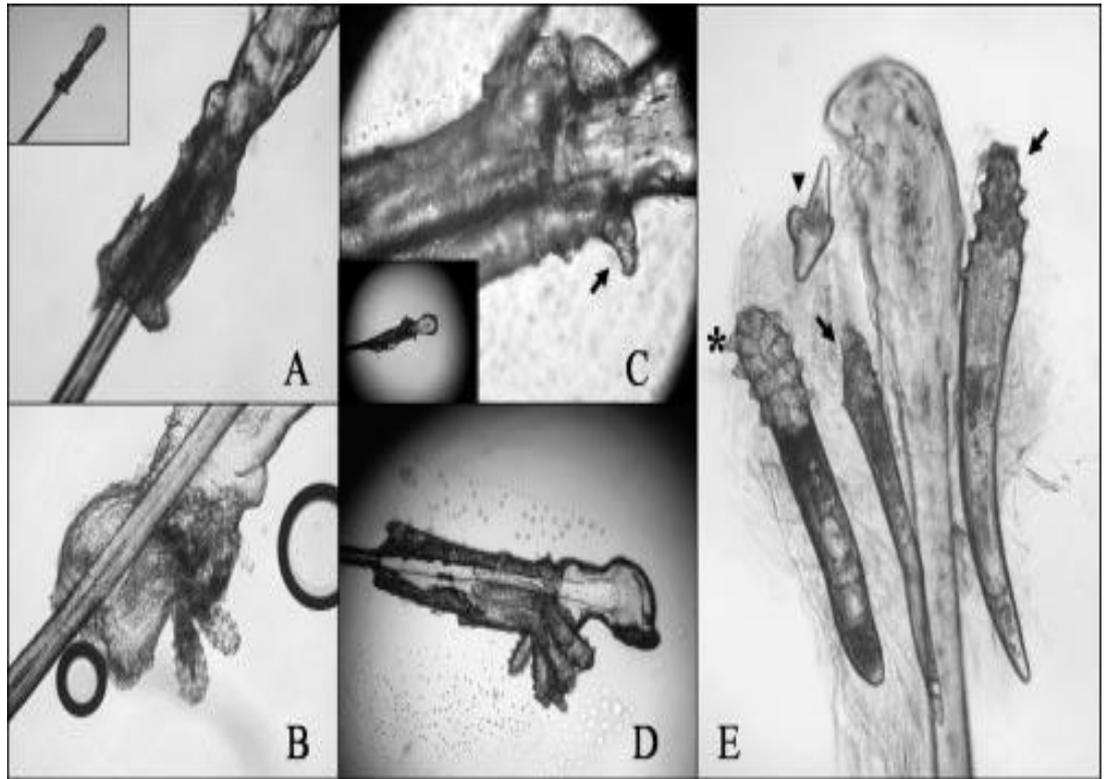


Figure 1.10 *Demodex* mites associated with the eyelashes of rosacea patients

Image **E** shows a family of *D. folliculorum* mites adjacent to the lash follicle, including an egg (*arrowhead*), two larvae which had three to four pairs of poorly developed legs and a slender body (*arrows*), and an adult with four pairs of well-developed legs (*) and a stumpy body.

[Taken from Gao *et al.*, 2005]

1.6 Endosymbiotic bacteria and bacterial antigens in human disease

In a previous study bacterial samples were taken from the faces of individuals with papulopustular rosacea and controls which lead to the isolation of *Bacillus oleronius* (Delaney, 2004). This bacterium was isolated from a micro-dissected *Demodex* mite and was thought to be an endosymbiont of the hindgut of the mite. *Demodex* mites have no excretory anus therefore it was postulated that this bacterium could aid digestion in the mite (Delaney, 2004; Lacey, 2007). Peripheral blood mononuclear cells (PBMC) were isolated from rosacea patients and controls and the T-cell reactivity to *B. oleronius* was present in 72% of rosacea patients and 29% of controls (p=0.01) (Lacey *et al.*, 2007).

Microbes are capable of adapting to niches in hostile environments that are uninhabitable to any other forms of life (Tehei *et al.*, 2004). However the most popular habitat of microbes is within a living host which can be beneficial or detrimental to that host (Moran 1996). Several bacterial species maintain a symbiotic relationship with mites, ticks or insects which cause disease (Hoy and Jeyaprakash, 2005; Mathaba *et al.*, 2002; Oliveira *et al.*, 2006). The study by Oliveira *et al.*, (2006) described the occurrence of sheep scab caused by the mite *Psoroptes ovis* and suggests an association between *Staphylococcus aureus* and the disease causing mite. Bacterial enzymes have been associated with the pathogenic properties of house dust mites such as *Dermatophagoides pteronyssinus* and *Dermatophagoides farina* (Mathaba *et al.*, 2002). *Wolbachia* was investigate as a possible endosymbiotic microorganism in *Demodex* mites but no association was proven (Lazaridou *et al.*, 2011). *Wolbachia* has an endosymbiotic relationship with parasitic filarial nematodes that cause devastating diseases such as elephantiasis and river blindness in over 150 million people in some of the world's poorest communities (Taylor, 2002). The inflammation seen in these conditions is associated with an endotoxin-like activity when upon death of the nematodes the bacteria are released into the blood stream. The bacteria are essential to their nematode host and are thought to be involved in protection from the human immune system via production of catalase (Taylor, 2002).

It is well established that antigens and superantigens produced by *Streptococci* and *Staphylococci* bacteria have been associated with the induction of a variety of diseases such as psoriasis, food poisoning, inflammatory disorders and shock syndromes (Lu, 2003; Nickoloff & Wrone-Smith, 1998; Valdimarsson *et al.*, 1995). Antigenic material isolated from the bacterium *Propionibacterium acnes* has been implicated in the inflammation of acne since T-cell proliferation has been seen in response to bacterial culture supernatant (Jappe *et al.*, 2002). These examples indicate a possible role for micro-organisms in the induction and persistence of these diseases. Therapy that eliminates micro-organisms can have the potential to reduce or clear the symptoms of these conditions.

1.6.1 Role of *Bacillus oleronius* in the induction of rosacea

The success of treating rosacea with antibiotics such as metronidazole, tetracyclines and erythromycin implies a role for bacteria in the condition, however no specific organism has been definitively proven as having a pathogenic role and it has been suggested that these antibiotics may act primarily as anti-inflammatory agents rather than by inhibiting a specific bacterium. *Bacillus oleronius* has been under investigation as a potential contributing factor to the pathogenesis of rosacea for the last 10 years. The bacterium was isolated from a *Demodex folliculorum* mite from a rosacea patient (Delaney, 2004) which produced antigens that induced an inflammatory immune response in 72% of rosacea patients but only 29% of controls ($p = 0.01$) (Lacey *et al.*, 2007). *B. oleronius* was previously isolated from the digestive tract of a termite where it facilitates digestion (Kuhnigk *et al.*, 1995). The bacterium has also proven susceptible to treatment with tetracycline, doxycycline, and minocycline, which are often used to treat rosacea (Lacey *et al.*, 2007). Delaney, (2004) also highlighted the two stimulatory antigens (62 and 83 kDa) which were to become the focus of subsequent studies. A strong correlation has been established between ocular *Demodex* inflammation and serum reactivity to these antigens in patients with ocular rosacea. In addition eye lid margin inflammation ($p = 0.040$) and facial rosacea ($p = 0.009$) were found to correlate with reactivity to these antigens (Li *et al.*, 2010). These two studies (Lacey *et al.*, 2007; Li *et al.*, 2010) suggest a possible role for bacterial antigens in the etiology of rosacea.

1.7 Treatment of Rosacea

Although the etiology of rosacea remains ambiguous, there are a variety of successful treatment options (Powell, 2005). A systematic review of treatments for rosacea was published in 2007, based on data from 29 studies (van Zuuren *et al.*, 2007). Findings of this review revealed that many of the studies investigating rosacea treatments were not well designed and very few assessed the important factor of the quality of life in participants. The review confirmed that systemic and topical antibiotics are the most popular therapies prescribed by physicians. A lot of emphasis has been put on the success of avoidance of ‘trigger factors’ in order to prevent the onset of flushing, erythema and inflammation (Gupta & Chaudhry, 2005). Rosacea patients often have extremely sensitive skin and therefore moisturizers and cosmetics must be chosen carefully and daily use of sunscreen (at least factor 15) is recommended (Pelle *et al.*, 2004). Without the identification of a causative agent in the condition the success of antibiotic treatments has been attributed to their anti-inflammatory properties rather than reducing bacteria (Monk *et al.*, 2011). Many of the agents most commonly used to control rosacea such as tetracyclines, azelaic acid, retinoids and metronidazole inhibit reactive oxygen species (ROS) production and lead to a clearance of symptoms thus suggesting that control of the immune response is an important aspect of treatment (Monk, 2011).

Topical treatments include metronidazole (0.75% gel or 1% cream) which is an effective treatment for moderate to severe rosacea (Crawford *et al.*, 2004). This treatment is proven to inhibit the generation of ROS and reduce papules, pustules and erythema (Elewski *et al.*, 2010), however it had limited effect on telangiectasia. Azelaic acid is another effective topical treatment (15% gel, 20% cream) that has both antimicrobial and anti-inflammatory properties (Elewski *et al.*, 2010). This treatment has also been proven to reduce ROS and can successfully reduce papules, pustules and erythema (Oltz & Check 2011). Sodium sulfacetamide 10% + sulfur 5% has proven effective as a topical emollient, antimicrobial, antifungal and antiparasitic properties have been recorded (Oltz & Check 2011). This treatment can also reduce inflammatory lesions and erythema (Gupta & Chaudhry, 2005). Effective oral antibiotics include doxycycline, minocycline, erythromycin and metronidazole which are generally used at sub-antimicrobial levels for up to 4 months (Powell, 2005). Currently the only FDA

approved oral treatment for papulopustular rosacea is a 40 mg per day anti-inflammatory dose of doxycycline which comes in the form of a slow release capsule (Oltz & Check 2011).

When treating ocular rosacea physicians often prescribe a combination of oral medication and one of the topical antibiotics can also be applied to the lid margin (Elewski *et al.*, 2010). The use of tea tree oil (TTO) for ocular rosacea has also proven successful (Gao *et al.*, 2005; Kheirkhah *et al.*, 2007; Liu *et al.*, 2010). This treatment is proven to reduce *Demodex* mite numbers as well as having antibacterial and anti-inflammatory effects on the eye-lids and corneal surface. The proposed method of treatment is weekly lid scrubs with 50% tea tree oil and a daily lid scrubs with tea tree shampoo for a minimum of six weeks (Gao *et al.*, 2005). This method can eradicate *Demodex* mites from the lashes, decrease inflammation, vascularisation and reduce phlyctenule-like lesions in some cases. Kheirkhan *et al.*, (2007) described a case study of an ocular rosacea patient who underwent amniotic membrane transplant but had a relapse in symptoms. After following the TTO treatment the patient's symptoms and vision had improved (Figure 1.11) and there was no evidence of limbal stem cell deficiency.

In severe cases of phymatous (Wollina 2011) and ocular (Kheirkhah *et al.*, 2007) rosacea surgery is required in the latter stages of treatment. In the case of phymatous rosacea or rhinophyma surgical techniques such as, excision of excess tissue under general anesthesia, dermabrasion or carbon dioxide laser vaporization may be used (Wollina 2011). Advanced cases of ocular rosacea can lead to partial or complete limbal stem cell deficiency. In the case of partial limbal stem cell deficiency amniotic membrane transplantation can effectively restore a stable corneal epithelium. Where limbal stem cell deficiency affects the whole limbus, transplantation of limbal tissue from the individuals healthy eye (autologous) or from a HLA matched allogenic donor or a non-matched cadaver can be used to obtain a stable corneal epithelium (Ahmad *et al.*, 2010; Elewski *et al.*, 2010; Kheirkhah *et al.*, 2007). All of the subtypes of rosacea can incorporate vascular changes such as erythema and formation of telangiectatic vessels direct treatment of these symptoms can be achieve with intense pulsed light (IPL) systems and lasers (Kautz & Kautz, 2008).

Although there are many proven anti-inflammatory agents derived from natural sources these have not been investigated extensively in rosacea (Emer *et al.*, 2011). This study describes the hydrating and anti-inflammatory effects of phenolic compounds derived from colloidal oatmeal which are proven to be UV absorbent and capable of reducing IL-8 in keratinocytes *in vitro*. Nicotinamide or vitamin B3 was also mentioned for its ability to reduce sebum and liquorice extract can inhibit ROS and has soothing properties (Emer *et al.*, 2011). To become established as part of the regular treatment of rosacea these ingredients would need to be researched further and perhaps would serve to augment the effects of the typical antibiotic treatments.

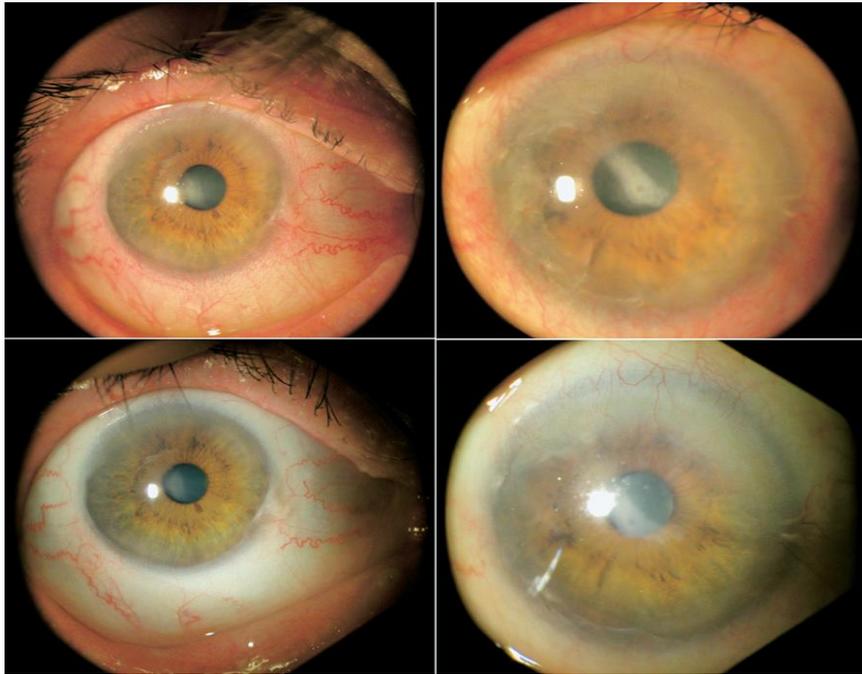


Figure 1.11 Photographs demonstrating a case study of an individual with rosacea and ocular *Demodex* infestation.

Top line represents symptoms before treatment both eyes are affected by peripheral scarring, conjunctival involvement and vascularisation of the corneas.

Bottom line represents the outcome of treatment with Tea Tree Oil; vascularisation and scarring have decreased.

[Adapted from Kheirkhan *et al.*, 2007]

1.8 Neutrophils and the innate immune response

The innate immune response is the first line of defence that the body employs in response to pathogens (Segal 2005). Neutrophils are highly active and motile phagocytes that respond to molecular signals from invading pathogens or damaged host cells by travelling rapidly to sites of infection or damaged tissue to engulf potential threats by phagocytosis (Figure 1.12). Chemokines and cytokines are important components of the innate immune response involved in recruitment and activation of neutrophils. IL-8 is particularly important in the early stages of infection, it binds LPS and its production can be triggered by other cytokines (Kenny and O'Neill, 2008). Neutrophils are recruited to the site of infection (Figure 1.13 & 1.14) and begin to change their morphology in response to cytokines, they express adhesive molecules so they can cling to the walls of blood vessels and enter the site of infection (Appelberg, 2007). TNF- α is also important in the induction of inflammation.

Upon exposure to certain stimulants such as microbes, the neutrophil becomes activated and increases its oxygen uptake leading to a 'respiratory burst' (Manara *et al.*, 1991). The molecular oxygen consumed during this burst is utilised in the oxidative mechanism of killing carried out by the neutrophil (Curnutte *et al.*, 1987). Following the closure of the phagocytic vacuole that internalises the pathogen, NADPH oxidase located on the membrane of the phagosome is activated (Segal, 2005). This mechanism is characterised by the production of reactive oxygen species (ROS) facilitate by NADPH oxidase. The membrane bound NADPH oxidase enzyme causes the single electron reduction of oxygen to superoxide anion (O_2^-) (Curnutte *et al.*, 1987). The superoxide anion is then involved in the generation of ROS such as, hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) or peroxynitrite resulting from an interaction with nitric oxide (NO) (D'Agostino *et al.*, 1998).

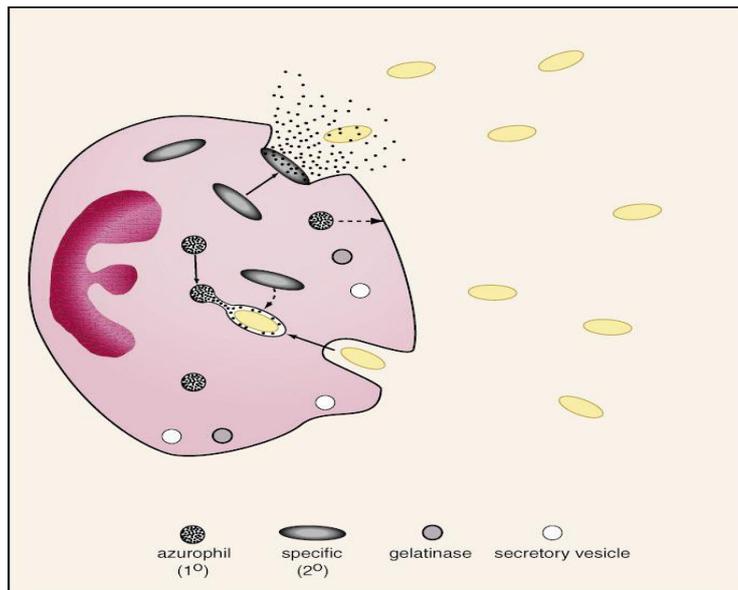


Figure 1.12 Schematic representation of Neutrophil phagocytosing a pathogen

Yellow oval represents pathogen. Neutrophil granules are depicted forming part of the lysosome and releasing antimicrobial peptides into the extracellular space.

[Taken from Levy, 2000]

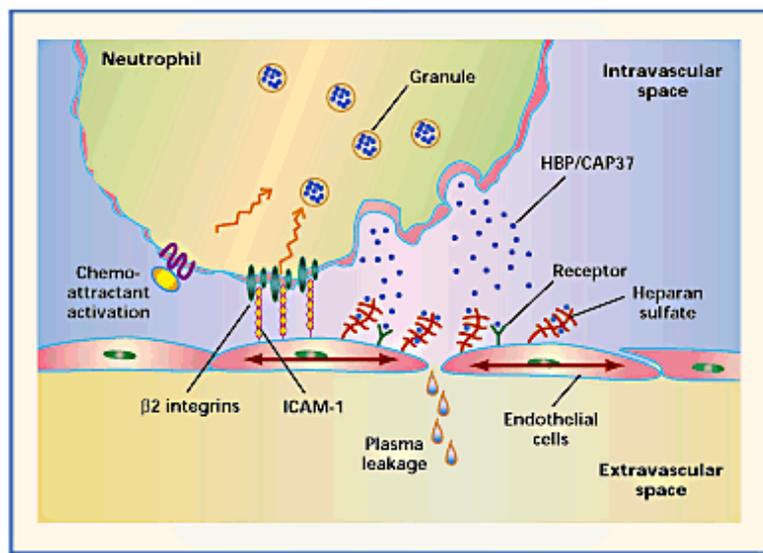


Figure 1.13 Schematic representation of Neutrophil response to pathogens

Recognition of pathogens triggers migration of neutrophils and signaling from cells of the innate immune response that leads to inflammation.

[Taken from the website http://www.nature.com/nm/journal/v7/n10/fig_tab/nm1001-1105_F1.html]

Degradation of the ingested microbe in the phagocytic vacuole by non-oxidative mechanisms due to neutral serine proteases, hydrolytic enzymes and cationic proteins can also occur (Segal 2005; Pham 2008). These proteolytic enzymes are packaged within cytoplasmic granules that are released upon cell activation in the process of degranulation. Degranulation accompanies the process of ROS production following a lag of around 20 seconds (Segal, 2005). Degranulation involves the fusion of membranes and release of the granular contents into the phagosome and the extracellular space (Manara *et al.*, 1991). Neutrophils contain primary azurophilic, secondary, gelatinase and lysosome granules (Figure 1.12) which contribute to the killing of internalised microbes.

There is a vast array of proteins released by neutrophil granules; among them is Myeloperoxidase (MPO), Matrix metalloproteinase 9 (MMP-9) and Human cathelicidin 18 (hCAP-18). These three granule components are investigated in Chapter 4. MPO is a component of primary granules and in the presence of hydrogen peroxide produces hypochlorous acid (Reeves, 2003). MPO comprises approximately 25% of granule protein and around 5% of the whole neutrophil proteome (Segal, 2005). This heme enzyme utilizes hydrogen peroxide to generate hypochlorous acid (HOCl) thus providing a protective mechanism against pathogens and contributes to oxidative injury of host tissue (Jantschko *et al.*, 2005).

Matrix metalloproteinase enzymes (MMPs) are components of the gelatinase granules and are responsible for degradation of extracellular matrix components during inflammation (Lu & Wahl, 2005). MMPs are implicated in many inflammatory diseases (Manicone & McGuire 2008) including rosacea (Jang *et al.*, 2010), where they are implicated in vascularisation in rosacea (Yamasaki & Gallo, 2009). The effectiveness of treatments such as tetracyclines is attributed mainly to anti-inflammatory properties associated to the inhibition of MMP production (Federici 2011).

Human cathelicidin (hCAP-18) or its peptide form LL-37 are associated with the secondary or specific granules of the neutrophil which are readily secreted into the extracellular space (Levy 2000) (Figure 1.12). Cathelicidin promotes the release of IL-8 and therefore the recruitment of neutrophils and has been implicated in the inflammatory processes of psoriasis, atopic dermatitis and rosacea (Morioka *et al.*, 2008).



Figure 1.14 Image representing Neutrophils migrating from the blood vessels to phagocytose and degranulate pathogens in surrounding tissue.

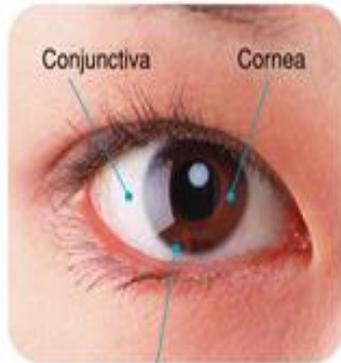
[Taken from the website http://www.rmgh.net/wiki/images/d/dc/Active_neutrophils.jpg]

1.9 Innate immunity of the ocular surface

As a vital sensory organ that is almost continually exposed to our surrounding environment the eyes have a number of defence mechanisms. Mechanical protection from pathogens and debris is provided by blinking and by the action of tears. Tears also contain proteins including bactericidal agents (e.g., IgA, lysozyme, lactoferrin in tear fluids, and all isoforms of human beta defensins) that act directly to kill pathogens (Ueta & Kinoshita, 2010). If pathogens successfully adhere to the ocular surface, the protective role of epithelial cells is activated (Hozono *et al.*, 2006). The ocular surface is covered by two distinct epithelial cell types the conjunctival and corneal epithelia separated by the limbal region (O'Sullivan, 2007) (Figure 1.15). In 1989, Cotsarelis *et al.*, demonstrated that slow cycling cells located in the basal layer of the limbus could be stimulated to proliferate in response to wound healing and suggested that these cells represent a stem cell niche that renews corneal epithelial cells (Cotsarelis *et al.*, 1989). These limbal stem cells are responsible for maintenance and re-population of the corneal epithelium (O'Sullivan & Clynes, 2007; Boulton & Albon, 2004; Ahmad *et al.*, 2010). Limbal stem cells maintain clarity of the cornea by acting as a barrier between conjunctival and corneal epithelia. This function is affected in those who develop limbal stem cell deficiency (Baylis *et al.*, 2011; Ahmad *et al.*, 2010) which can occur in severe cases of ocular rosacea (Kheirkhah *et al.*, 2007).

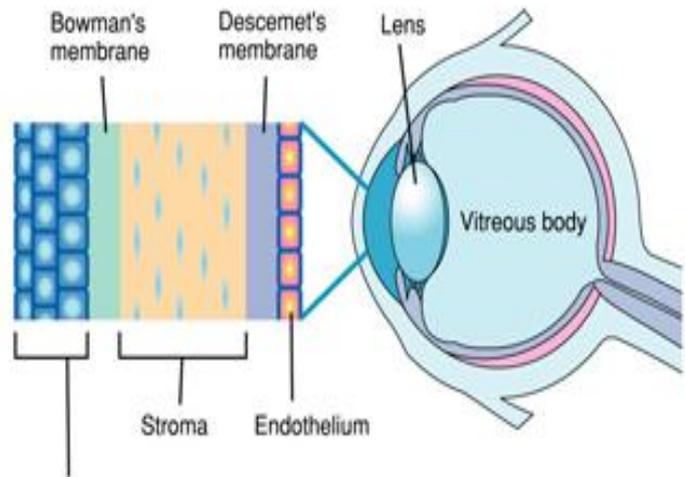
Toll like receptors (TLRs) are found on all epithelial surfaces and act as the cells main method for pathogen recognition through binding of pathogen-associated molecular patterns (PAMPs) (Chang *et al.*, 2006; Philpott *et al.*, 2001). In addition to being expressed on the epithelial surface TLRs are present on the endothelia, antigen presenting cells and lymphocytes (Redfern & McDermott, 2010). In response to recognition of pathogen by the PAMPs the ocular surface epithelium can produce inflammatory cytokines (Lam *et al.*, 2009). Once activated cytokines and chemokines play a role in determining the balance of the acquired immune response. They can up-regulate the acute inflammatory response, recruit immune cells, enhance intracellular killing and are involved in maturation of dendritic cells (McGettrick and O'Neill, 2010; Redfern & McDermott, 2010). In a study of cytokine levels in the tear fluid of individuals with blepharitis it was found that IL-7, IL-12, and IL-17 were increased in those with *Demodex* infestation compared to those patients with non-*Demodex* related blepharitis (Kim *et al.*, 2011).

Structure of human eye



Limbus: Located at the border between the cornea and conjunctiva, this is where corneal epithelial stem cells are found.

Structure of cornea



Epithelium: The outermost layer of the cornea, composed of corneal epithelial cells.

Figure 1.15 The structure of the human eye.

[Taken from the website <http://www.jpte.co.jp/english/business/Regenerative/cultured-corneal-epithelium.html>]

Corneal epithelial cells represent an excellent model for studying the interaction of pathogens with the corneal surface (Hozono *et al.*, 2006; Maltseva *et al.*, 2007). Hozono *et al.*, (2006) investigated the response of corneal epithelial cells to flagellin isolated from pathogenic and non-pathogenic bacteria. Human corneal epithelial cells expressed higher levels of TLR-5 mRNA and protein in response to flagellin isolated from ocular pathogens (e.g. *Pseudomonas aeruginosa*) compared to non-pathogenic bacteria (e.g. *Bacillus subtilis*). Pro-inflammatory cytokines IL-6 and IL-8 were also induced at higher levels in response to pathogenic flagellin. Maltseva *et al.*, (2007) characterised the response of human corneal epithelial cells (exposed to contact lenses) to *P. aeruginosa* supernatant proteins. Corneal epithelial cells normally up-regulated expression of the antimicrobial peptide human β -defensin-2 (hBD-2) in response to *P. aeruginosa*, however in cells that were previously exposed to contact lenses *in vitro* this up-regulation was blocked.

1.10 Results of previous studies

In a previous study bacteria were isolated from the faces of people with papulopustular rosacea and control patients and this led to the isolation of *Bacillus oleronius*, (Delaney, 2004). To establish which bacteria caused immunoreactivity in rosacea patients, peripheral blood mononuclear cells were isolated from rosacea patients and controls and the level of T-cell proliferation was tested. This resulted in 72% of rosacea patients and 29% of controls reacting significantly to *B. oleronius* antigens. This bacterium was isolated from a micro-dissected *Demodex* mite and was thought to be an endosymbiont which aids lipid digestion in the mite which has no excretory anus. The work by Delaney, (2004) also highlighted the two stimulatory antigens (62 and 83 kDa) which were to become the focus of subsequent studies. The study by Lacey, (2007) included the identification of these antigenic proteins by MALDI ToF mass spectrometry, the 62 kDa protein was found to have homology to a protein involved in the phosphoenolpyruvate phosphotransferase sensory system and the 83 kDa protein was found to be homologous to a heat shock protein (Lacey *et al.*, 2007). Upon treatment of human neutrophils with *Bacillus* antigens, there was inhibition of phagocytosis recorded indicating the ability of the bacterium to affect the innate immune response. This work also involved the purification of the 62 kDa antigen by ÄKTA-FPLC, which was used to generate an anti-62 kDa rabbit antibody. The immortalised human sebaceous cell line (SZ95) was employed to study the effects of antibiotics used to treat rosacea, a proteomics approach was used and the effects varied with each antibiotic. This work also attempted to establish a PCR based assay for the presence of *B. oleronius* which showed limited success. Both previous studies (Delaney, 2004; Lacey, 2007) provided evidence that *Demodex* associated bacteria may play a role in the pathogenesis of rosacea and established important techniques for the continued research of the condition.

1.11 Aims of this study

The primary aim of the work presented here was to expand on previous studies by Delaney, (2004) and Lacey, (2007) by investigating the role of *Demodex* associated *Bacillus oleronius* antigens in the development of the chronic inflammatory condition, rosacea.

The aims of this study were:

1. To assess the reactivity of serum taken from individuals with ocular (type 4) and erythematotelangiectatic (type 1) rosacea to the *Bacillus olerionus* antigens by Western blotting.
2. Purification of a 62 kDa stimulatory antigen was performed in a similar fashion to the previous technique by Lacey, (2007) using ÄKTA-FPLC. The product was utilised to investigate the possibility of establishing an ELISA based immunoreactivity assay.
3. To expose human neutrophils to purified *Bacillus* proteins in order to establish the effect that *B. oleronius* has on the innate immune response.
4. To expose human corneal epithelial cells to purified *Bacillus* proteins in order to establish the effect that *B. oleronius* has on the ocular innate immune response.
5. To establish the effect of environmental conditions on the proteomic profile of *B. oleronius* using 2 Dimensional SDS-PAGE and Liquid Chromatography mass spectrometry to establish which growing conditions lead to immunogenic proteins being produced. An anti-62 kDa rabbit antibody was utilised to assess the environmental culture conditions required by *B. oleronius* to produce high quantities of the antigen by Western blotting.

CHAPTER 2

Materials and Methods

2.1 Study Population

2.1.1 Ocular Rosacea Study Population

- Peripheral blood (5 mls) was collected from ocular rosacea patients and healthy controls by staff at the Ocular Surface Center (OSC), Miami, Florida. Participants (n=59, 34 men and 25 women) were age but not gender matched and had a mean age of 60.4 ± 17.6 (range, 17-93) (Li *et al.*, 2010). Blood was centrifuged at 3500 x g for 10 minutes. Serum was transferred to a sterile container under a lamellar flow and stored at -80° C.
- Tear fluid was also collected by staff at the OSC, Miami from participants (n=59). Tear fluid is collected by placing a polyester rod at the conjunctival fornix of the eye. Samples were stored at -80° C.
- Serum and tear fluid samples were prepared at the OSC, Miami and shipped to Ireland on ice then immediately returned to -80° C.
- Serum immunoreactivity was determined (NUI Maynooth) by blind testing serum samples sent from the OSC (Miami) for reactivity to *B. oleronius* proteins.

2.1.2 Erythematotelangiectic Rosacea Study Population

- Peripheral blood (5 mls) was collected from erythematotelangiectic rosacea patients and healthy controls attending the Servico de Dermatologia do Centro Hospitalar de VN de Gaia Espinho, Portugal, and shipped on ice to Ireland.
- Participants in the study had Fitzpatrick skin types II-III. Patients (n=26, 9 males, 17 females) with an average age of 48.3 years (range 20-83) and controls (n=21, 9 males and 12 females) with an average age of 54.3 (range 28=80) were given a dermatological examination and had blood taken.
- When the blood samples were received in NUI Maynooth; they were centrifuged at 3500 x g to sediment the clot and the serum layer was

removed under lamellar flow then stored at -80° C before being blind tested for serum immunoreactivity *B. oleronius* proteins.

2.1.3 Neutrophils

Human neutrophils were isolated from healthy volunteers (Ethical permission granted by NUI Maynooth Ethics Committee).

2.2 General Chemicals and Reagents

All chemicals and reagents were of the highest quality and purity. Unless otherwise stated chemicals were purchased from Sigma Aldrich Chemical Co. Ltd, Dorset, UK. Buffers were prepared using distilled H₂O which was purified using a Millipore Milli-Q apparatus.

2.3 Sterilisation Procedure

All equipment, growth media and buffers were sterilised by autoclaving at 121° C with 15 lb per square inch of pressure for 15 minutes. Any chemical or reagent that was unsuitable for autoclaving was filter sterilised using 0.2 µm pore filter discs.

2.4 Culture Media

2.4.1 Nutrient Agar

Nutrient agar (Oxoid) (28 g) was dissolved by stirring in 1 litre of dH₂O. This solution was autoclaved and allowed to cool before pouring into 9 cm petri dishes under sterile conditions. Plates were stored at 4° C until needed.

2.4.2 Nutrient Broth

Nutrient broth (Oxoid) (13 g) was dissolved by stirring in 1 litre of dH₂O. The solution was autoclaved and stored at room temperature until needed.

2.4.3 2XYT Broth

Tryptone (Difco) (16 g), Yeast extract (Oxoid) (10 g) and NaCl (BDH Laboratory supplies, England) (5 g) were dissolved in 1 litre of dH₂O. The solution was autoclaved and stored at room temperature until needed.

2.5 Culture Conditions

All bacterial strains (Table 2.1 below) were maintained on nutrient agar plates and sub-cultured every 6-8 weeks using aseptic techniques. Freshly sub-cultured plates were grown overnight at 30° C or 37° C depending on strain then stored at 4° C. Liquid cultures were grown to stationary phase in nutrient broth (100 ml) at 30° C and 200 rpm, for 24-48 hours depending on strain.

Table 2.1 Bacterial Strains

Strain	Genotype/Origin	Reference
<i>Bacillus oleronius</i>	<i>Demodex</i> mite	Delaney, 2004

2.6 Glycerol Stocks

A bacterial culture was grown over night and 500 µl was mixed with 500 µl of sterile glycerol and stored at -70° C.

2.7 Phosphate Buffered Saline (PBS)

PBS tablets were dissolved in the relevant amount of distilled water according to the manufacturer's guidelines then sterilised by autoclaving.

2.8 Bacillus Lysis Buffer (pH 7.2)

Piperazine (20 mM) and 5 mM NaCl were dissolved in dH₂O and autoclaved. Triton x100 (0.2% v/v) was added and the buffer was stored at 4° C. Protease inhibitors (10 µg/ml of Leupeptin, Pepstatin A, Aprotinin and TLCK) were added on the day of the extraction.

2.9 FPLC Binding Buffer (pH 4.8)

Piperazine (20 mM) and 10 mM NaCl were dissolved in dH₂O, autoclaved and stored at 4° C. The buffer was filtered through a 2 µm disc before application to the AKTA FPLC.

2.10 FPLC Elution Buffer (pH 4.8)

Piperazine (20 mM) and 1 M NaCl were dissolved in dH₂O, autoclaved and stored at 4° C. The buffer was filtered through a 2 µm disc before application to the AKTA FPLC.

2.11 Bradford Protein Assay

2.11.1 Bio-rad dye reagent concentrate (Bio-rad) was diluted 1:5 with dH₂O to give a working solution which can be stored at 4° C overnight. Extracted proteins were diluted in sterile PBS and 20 µl was added to a 1.5 ml cuvette. To this 1ml of the working solution of bio-rad was added and allowed to stand for 5 minutes at room temperature. The protein concentration was read in a spectrophotometer (Eppendorf, Biophotometer) at 595 nm.

2.11.2 When sample volume was low a microplate assay was employed to determine the protein concentration. Bovine serum albumin (BSA) was dissolved to a concentration of 1 mg/ml and stored at -20° C to prepare the standards as outlined in Table 2.2 below.

To read the assay 5 µl of standards and samples were added to a 96 well plate in duplicate followed by 250µl of bio-rad working solution then left to stand at room temperature for 5 minutes. Plates were read at 595nm on a Biotek Synergy HT plate reader.

Table 2.2 Dilution of BSA for protein quantification standards

Tube	Dilution	Reagent
1	1	1 mg/ml BSA stock
2	0.8	32 µl from tube 1+8 µl of lysis buffer
3	0.6	12 µl from tube 1+8 µl of lysis buffer
4	0.4	20 µl from tube 2+20 µl of lysis buffer
5	0.2	10 µl from tube 4+10 µl of lysis buffer
6	0	Lysis buffer only

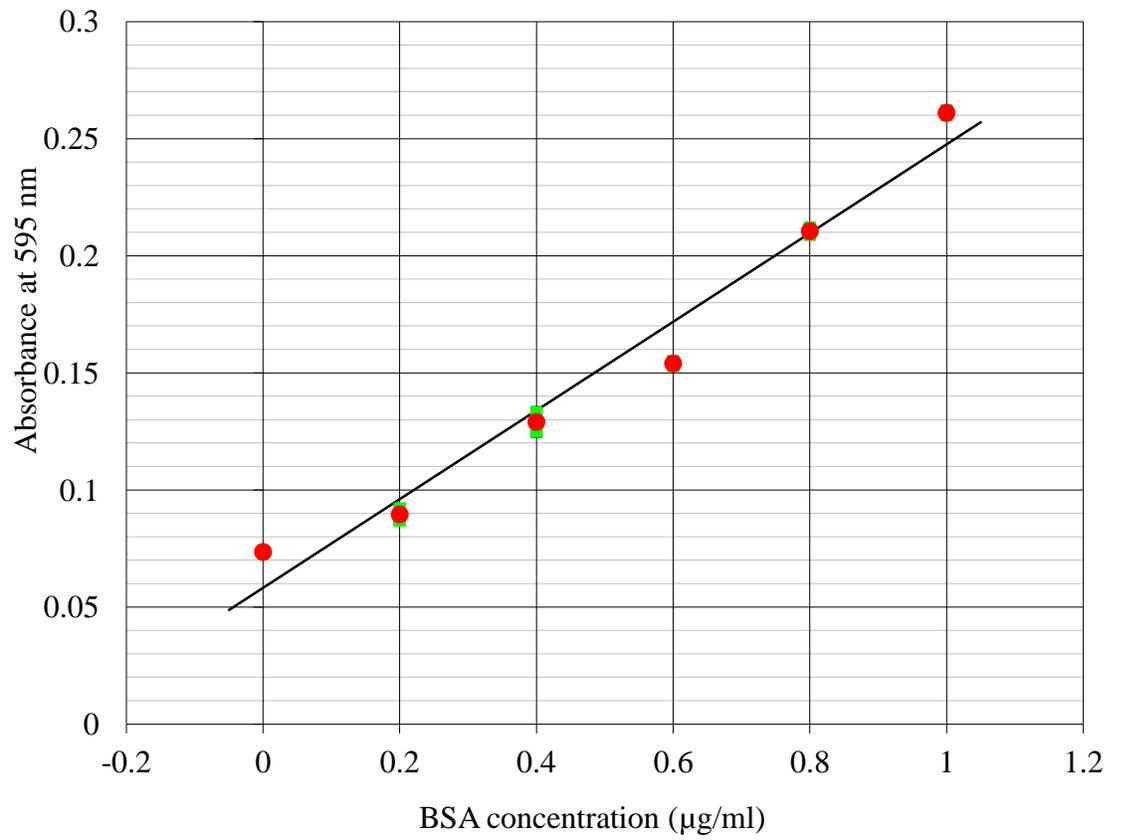


Figure 2.1 Typical standard curve of Bovine Serum Albumin (BSA) for the determination of protein concentration in samples.

2.12 Protein extraction from *Bacillus oleronius*

Liquid medium (100 ml) was inoculated with a loopful of bacterial culture, aseptically taken from a fresh plate and grown over night with shaking (200 rpm) at 30° C. This culture was then used to inoculate fresh media so the effect of varying environmental conditions on protein expression could be assessed. Cells were grown in rich medium (2XYT) and minimal medium (Nutrient Broth) at 30° C and 37°C with and without agitation. NaOH and HCl were used to adjust the pH of nutrient broth to pH6-9. The effect of hydrogen peroxide on the bacterium was also examined by adding 5-10 mM to 100 ml cultures for between 1-24 hours.

Cultures between 100-200 ml were grown to late stationary phase and cells were harvested by centrifugation at 3000x g for 20 minutes (Beckman GS-6 Centrifuge). The supernatant was discarded and cells were washed with PBS. Cells were re-suspended in lysis buffer (section 2.8) and stirred at 4° C for 1hr. The volume of lysis buffer used was dependent on the size of the cell pellet; approximately 1ml per 100ml of starting culture was used.

Sonication was also employed as a method to extract proteins from the cell surface of the bacteria. Cell suspensions were sonicated with 3 x 10 second blasts using a soniprobe sonicator (Bandelin Sonopuls, HD 2200). Suspensions were stored on ice between sonications to avoid excessive overheating of the proteins.

Following treatment with Triton-X 100 or sonication the protein suspension was obtained by centrifuging cells at 6000 x g for 2 minutes at 4° C. The supernatant containing proteins was retained and the protein concentration was determined using Bradford protein assay (section 2.11). Protein samples were acetone precipitated when necessary or alternatively 5X sample buffer was added directly to the protein suspension which was boiled for 3 minutes at 95° C before storage at -20° C until analysis with SDS-PAGE was carried out as described in section 2.17.

2.13 Preparation of bacteria for confocal microscopy

Cells cultured in various environmental conditions were also viewed by confocal microscopy. Cultures (approx 50 mls) were grown overnight or longer in the case of static conditions. Cells were harvested by centrifugation 3000 x g for 20 minutes and washed twice in PBS. Cells were resuspended in fresh PBS (amount dependent on size of pellet) and allowed to adhere to a glass slide by air drying. They were then heat fixed at 37° C for 5 minutes after which slides were immersed in PBS. Cells were fixed with 3.7% formaldehyde for 10 minutes. The slide was immersed in PBS again and the cells were blocked with 1 X TBS-Tween with 1% BSA for two hours. Slides were immersed in PBS before the primary antibody (1:5000 of the rabbit anti bacillus antibody in the blocking buffer) was added and incubated at 4° C overnight. The secondary antibody (TRITC labelled anti-rabbit IgG, Jackson laboratories, USA) diluted 1:200 in the blocking buffer was added for 2 hours in the dark at room temperature. Slides were washed once more with PBS and allowed to dry before a cover slip was applied. Cells were viewed using the Olympus Fluoview 100 confocal microscope.

2.14 Fractionation by Q-Sepharose charge separation

The Triton-X soluble outer membrane proteins of *B. oleronius* (section 2.12) were purified and chromatographed by Q-sepharose charge separation using an AKTA Purifier 100 system (Amersham Biosciences, UK). The starting material was inverted with washed Q-sepharose beads at 4° C for at least 1 hour. This material was loaded to make a column of 2 cm x 5 cm. The column was washed with binding buffer (section 2.9, pH 4.8) for 30 minutes prior to 500 µl fractions being eluted per minute with a 30 ml linear gradient of 1M NaCl in eluting buffer (section 2.10, pH 4.8). Peak fractions containing the antigens of interest were identified by SDS-PAGE and Western blot analysis. Fractions containing the antigens of interest (62 and 83 kDa) were pooled and concentrated using Amicon filters. The semi-purified antigen preparation was then precipitated using acetone and resuspended at a concentration of 1µg/10µl in PBS with added protease inhibitors. The antigen preparation was then ready for use in neutrophil and corneal cell assays.

2.15 Development of ELISA for testing reactivity of Human serum to *B. oleronius* antigens

5X Concentrated Coating Buffer

Sodium Carbonate Na_2CO_3 (9.54 g), Sodium Hydrogen Carbonate NaHCO_3 (34.44 g) and Sodium Azide (0.2 g) and were dissolved in 1 Liter of dH_2O and adjusted to pH 9.3 using 5 M NaOH and stored at 4° C. Immediately before use the buffer was diluted 1:5 with dH_2O and pH was adjusted to 9.6, stored at 4° C for up to 4 days.

Preparation of ELISA plate

Antigen isolated from *B. oleronius* and purified as described in section 2.15 was mixed with the 1X coating buffer to achieve a concentration of 1 $\mu\text{g}/\text{ml}$. This solution was coated onto 96 well plates (100 $\mu\text{l}/\text{well}$) and stored overnight at 4° C. The buffer was aspirated and the plate was washed 4 times with PBS-Tween. Blocking buffer (200 $\mu\text{l}/\text{well}$) consisting of 0.5% (w/v) BSA and 5% (w/v) Sucrose in PBS was added and the plates were incubated at 37° C for 1 hour. After this time the blocking buffer was decanted and the plates were allowed to dry overnight at 37° C.

Assay Procedure

Serum diluted 1:10-1:1000 (100 μl) was added to the wells and incubated for 1 hour at room temperature. Serum was aspirated and wells were washed 4 times with 200 μl of PBS-Tween. Secondary anti-human IgG diluted 1:1000 (100 μl) was added per well. Plate was washed again with PBS-Tween 4 times and blotted well on paper towel to remove all liquid. TMB substrate (50 μl) was added and the blue colour was allowed to develop for 10-15 minutes. The assay was stopped by adding 50 μl of 1 N H_2SO_4 and the optical density of the assay was read at 450 nm on a plate reader.

2.16 Acetone Precipitation

Three times the volume of 100% (v/v) ice-cold acetone was added to protein samples and incubated at -20°C for a minimum of 2 hours. Precipitated protein was centrifuged at $20000 \times g$ for 30 minutes at 4°C . The acetone was discarded and the pellet was allowed to air dry before the protein was re-suspended in 5X sample buffer (section 2.19).

2.17 Sodium Dodecyl Sulphate-Poly acrylamide (SDS-PAGE) Gel Electrophoresis

Stacking Gel Buffer

Tris base (Sigma, 30.25 g) was dissolved in 450 mls of dH_2O . 5M HCl was added until pH6.8 was achieved, the final volume was adjusted to 500 ml with dH_2O . The solution was autoclaved and stored at 4°C .

Separating Gel Buffer

Tris base (Sigma, 90.75 g) was dissolved in 450 mls of dH_2O . HCl solution (5 M) was added until pH8.9 was achieved, the final volume was adjusted to 500 ml. The solution was autoclaved and stored at 4°C .

Sodium Dodecyl Sulphate (SDS) (10%, w/v)

SDS (Sigma, 10 g) was dissolved in 100 ml dH_2O and stored at room temperature.

Ammonium Persulphate (APS)

APS (Sigma, 1 g) was dissolved in 10 ml dH_2O and divided into 500 μl aliquots which were stored at -20°C .

Table 2.3 Constituents of Separating gel for SDS-PAGE electrophoresis.

Reagent	10% Acrylamide	12.5% Acrylamide	15% Acrylamide
30% Acrylamide (ml)	5	6.3	7.5
1.5 M Tris (ml)	3.8	3.8	3.8
10% SDS (μl)	150	150	150
dH₂O (ml)	6	4.8	3.6
10% APS(μl)	75	75	75
Temed (μl)	5	5	5

Table 2.4 Constituents of Stacking gel for SDS-PAGE electrophoresis.

Reagent	5%
30% Acrylamide (ml)	1
0.5 M Tris (ml)	1.5
10% SDS (μl)	60
dH₂O (ml)	3.6
10% APS(μl)	60
Temed (μl)	6

Pouring the SDS-Polyacrylamide Gel

Glass plates (1.0mm) were cleaned with ethanol prior to placement in a gel casting rig. The separating gel was poured between the plates and a layer of 0.1% SDS was sprayed on the top to ensure the top of the gel was straight and to remove air bubbles. The gel was allowed to set for approximately 30 minutes the SDS was removed and the top of the gel washed with dH₂O. Filter paper was used to remove any excess water. The stacking solution was poured on top and a 10 toothed comb was inserted. Once the stacking gel was set the gel was placed in a gel rig (Bio-rad) which was filled with 1X running buffer. The comb was removed slowly and protein samples were loaded into the wells. A pre-stained protein marker (6-175 kDa, New England Biolabs) was run in the first lane of each gel to identify the molecular weights of the protein bands. Gels were run at 60 volts using a Power-pac 300 (Bio-Rad) until the sample had left the stacking gel and increased to 80-120 volts through the separating gel.

2.18 10X running buffer, pH8.9

Tris base (Sigma, 30g) Glycine (Sigma, 144g) and SDS (Sigma, 10g) were dissolved by stirring in 1 litre of dH₂O. The solution should not require adjustment to pH8.9; it was stored at room temperature.

2.19 10X Sample Buffer

SDS (2g), Dithiothreitol (DTT, 1.54g), 200 mM EDTA (pH7), 3 M Tris-HCL (pH 6.7) and 2ml (0.2% Bromophenol blue, 50% Sucrose) were dissolved by stirring in a final volume of 10 mls of dH₂O. The solution was divided into 500 µl aliquots and stored at -20° C.

2.20 Staining of SDS-PAGE Gels

Coomassie Staining Solution

Brilliant Blue R (0.2%, w/v) was dissolved in acetic acid (10%, v/v), methanol (45%, v/v) and dH₂O (45%, v/v)

Detain Solution

Acetic acid (10%, v/v), methanol (20%, v/v) and dH₂O (70%, v/v)

Colloidal Coomassie Fixer

Ethanol (50%, v/v), phosphoric acid (3%, v/v) and dH₂O (47%, v/v)

Colloidal Pre-Incubation Buffer

Ammonium Sulphate (17%, w/v) was dissolved in ethanol (34%, v/v), phosphoric acid (3%, v/v) and dH₂O (46%, v/v)

Colloidal Coomassie Protocol

Gels were placed in the fixing solution for a minimum of 3 hours. Following 2 x 30 minute washes in water the gels were covered in the pre-incubation buffer for 1 hour before a spatula tip of SERVA Blue powder was added. Gels were incubated in this solution overnight or until the proteins were visible. The gels were then washed in distilled water until the background blue stain was removed at this point the gels were ready to be scanned and prepared for Mass spec analysis.

2.21 Two-Dimensional Gel Electrophoresis

IEF Buffer

Urea (8 M), 2 M thiourea, CHAPS (4% w/v), Triton-X 100 (1% v/v) and 10 mM tris base were dissolved in deionized H₂O. The solution was divided into 1 ml aliquots and stored at -20° C. Ampholytes (0.8%, v/v) and DTT (65 mM) were added to the defrosted aliquots on the day of use.

Isoelectric focusing (IEF) in the first dimension

Bacterial protein was extracted, quantified and precipitated using acetone. For 7 cm IPG strips 150µg of protein was resuspended in 125µl of IEF buffer and for 13 cm IPG strips 500µg was resuspended in 250µl of IEF buffer. A small amount of bromophenol blue tracking dye was added to the sample and it was incubated at room temperature for 1 hour. The sample was applied to a ceramic strip holder at the anodic end. The IPG strip (pH 4-7, 7 cm or 13 cm) was placed gel face down into the sample. Dry strip cover fluid (1 ml for 7 cm or 2 ml for 13 cm) was applied over the strip. Proteins were isoelectrically focused using an Ettan IPGphor II focusing system (Amersham biosciences, England) according to the program below.

Table 2.5 Isoelectric Focusing Protocol.

Time	Volts	Mode
8 hours	50 V	Gradient
15 minutes	250 V	Gradient
2 hours	1000 V	Gradient
4 hours	4000 V	Gradient
4 hours	8000 V	Gradient
4 hours	8000 V	Hold

Following IEF, strips were equilibrated in reducing buffer (50 mM Tris-HCL, 6 M Urea, 2% (w/v) SDS, 30 % (v/v) Glycerol, 1% (w/v) DTT, pH 6.8) for 15 minutes at room temperature, followed by equilibration in alkylation buffer (50 mM Tris-HCL, 6 M Urea, 2% (w/v) SDS, 30 % (v/v) Glycerol, 2.5 % iodoacetamide (IAA), pH 6.8) for 15 minutes. Strips were then placed on top of 12.5% SDS-PAGE gels and overlaid with hand warm sealing solution. Gels were electrophoresed on the PROTEAN PLUS Dodeca cell system (Bio-rad) with temperature maintained at 6° C using a cooling system for 20 hours at 100V or until the bromophenol indication layer was at the bottom of the gel. Gels were fixed and stained using Colloidal Coomassie. Gels were produced in triplicate for each treatment and scanned on a HP scanjet 5400C.

2.22 Liquid Chromatography Mass Spectrometry

Destaining Buffer (100mM Ammonium Bicarbonate/Acetonitrile)

Ammonium bicarbonate (0.79 g) was dissolved in 100ml of ddH₂O and diluted 1:1 with acetonitrile. This buffer was made fresh on the day of use.

Digesting buffer

Trypsin (20 µg) was diluted in 100 µl of reconstitution buffer. For every 10 µl of buffer 500 µl of 50mM ammonium bicarbonate was added. If not required the reconstituted trypsin can be stored in 10 µl aliquots at -20° C for up to 1 month.

Extraction Buffer

Formic acid (5%, v/v) and acetonitrile were mixed 1:2 (v/v)

Preparation of excised protein bands and spots for LC/MS analysis (Shevchenko *et al.*, 2006)

All reagents used were HPLC grade and all 1.5 ml microfuge tubes were washed with acetonitrile before use. Colloidal coomassie stained protein bands or spots were chosen from SDS-PAGE gels using ProgenesisTM same spot software. Gels were washed with water before spots/bands were picked using a clean scalpel or 200 µm pipette tip. Gel pieces were placed in pre-washed microfuge tubes. Gel pieces were incubated in 100 µl of destaining buffer for 30 mins (or until stain was removed) shaking at room temperature. Then 500 µl of acetonitrile was added for 10 mins shaking at room temperature. Gel plugs turned white and shrank and most of the coomassie stain was removed at this stage.

Samples were then subjected to in-gel trypsin digestion. Approximately 50µl of trypsin buffer (or enough to cover the gel matrix) was added to the samples and they were stored on ice for 30 mins. If all the trypsin solution was absorbed at this point more was added to cover the matrix. Gel pieces were incubated on ice for a further 90 mins to saturate them with trypsin. Then 20 µl of 50 mM ammonium bicarbonate was added to ensure the

gel pieces did not become dehydrated during the trypsin digestion. Finally to digest the peptides the microfuge tubes were placed in a 37° C air circulating incubator over night.

To extract the peptide digestion products the contents of the microfuge tube after trypsin digestion were removed to a fresh eppendorf. Extraction buffer (100 µl) was added to each tube and incubated for 15 mins at 37° C in a shaker. The supernatant from this extraction was added to the trypsin digestion supernatant. Extracts were dried in a vacuum centrifuge over night. Dried extracts could be stored at -20° C for a few months. Immediately prior to Mass spec analysis, dried peptides were resuspended in 20 µl of 0.1% (v/v) formic acid. The microfuge tubes were placed in a sonication bath for 5 mins and then centrifuged for 15 mins at 7,000 x g at 4° C. Samples were filtered using spin filters in a centrifuge for 2 mins at 7,000 x g and added to acetonitrile washed glass vials (Agilent). Analysis of digested peptides was achieved using an Agilent 6340 Ion Trap Liquid Chromatography Mass Spectrometer.

2.23 Western Blotting

10X Tris Buffered Saline (TBS) pH 7.6

Tris HCl (0.5 M, 157 g) and NaCl (1.5 M, 175.3 g) were dissolved in 2L of dH₂O and the pH was brought to 7.6 with the addition of NaOH pellets.

1X TBS-Tween

10X TBS (100 ml) and tween-20 (500µl) were added to 900 ml dH₂O.

Transfer Buffer

Tris-base (6.06 g) and glycine (28.8 g) were dissolved in 400 ml Methanol and 1600 ml dH₂O.

Blocking Buffer

Dried skimmed milk powder (Marvel, 15 g) and albumin from bovine serum (BSA, 5 g) were dissolved in 1X TBS-Tween (500 ml)

Transfer procedure

Transfer of protein onto nitrocellulose membrane was performed by electro-blotting using a wet blotter (Mini Trans-Blot Cell, Bio-Rad). Prior to transfer Whatman filter paper, nitrocellulose membrane and sponge inserts were soaked in transfer buffer. The blot sandwich was arranged inside the transfer cassette as follows; a sponge insert was placed on the black side of the cassette followed by a piece of filter paper, the nitrocellulose membrane was placed on top and the gel was placed on the membrane followed by more filter paper and the other sponge insert. The sandwich was rolled gently to remove air bubbles. The cassette was placed in the tank along with an ice cooling block and the apparatus was run at 100V for 70 minutes.

To assess the quality of the transfer the membrane was stained using Ponceau-S-Red solution, which stained transferred proteins pink. Non-specific binding was inhibited by incubating the membrane in blocking solution for 1 hour at room temperature or overnight at 4° C.

Membranes were incubated in primary antibody (Table 2.6) overnight at 4° C on an orbital shaker, followed by washing with 1X TBS-Tween for 1 hour changing the TBS frequently. The membrane was incubated in the appropriate anti IgG-HRP labelled secondary antibody for 2 hours at room temperature. The wash step was repeated and antibody labelled protein bands were visualised by enhanced chemiluminescence (ECL) using the Perkin Elmer Supersignal ECL kit as per the manufacturer's instructions. Blots were incubated in the substrate before being covered with an acetate and overlaid with X-ray film (Kodak Biomax MS) and exposed for 1-10 minutes inside a cassette (Kodak). X-ray films were developed in 1:5 developing solution (Polymax RT, Kodak) followed by a rinse with water and then placed in 1:4 fixing solution (Polymax RT, Kodak) and allowed to air dry.

Some blots were also developed using diaminobenzidine tetra-hydrochloride (DAB), where the washed blot was incubated in the DAB solution (1 tablet in 15 mls of 100 mM Tris-HCL (pH 7) and 12 µl of hydrogen peroxide) for 5-10 minutes followed by washing in dH₂O and allowing to air dry.

Table 2.6 Antibody Dilutions

Primary Antibody	Source	Western Blot Dilution	Immuno/Confocal Dilution	Company	Reference
Anti 62 kDa (<i>B. oleronius</i>)	Rabbit	1:5000	1:2000	Generated in DCU	Lacey, (2007)
Patient Serum	Human	1:100	-		
MPO	Rabbit	1:500	-	Genway	
MMP-9	Goat	1:1000	-	-	Dr. Emer Reeves, RCSI
IL-37/Hcap	Rabbit	1:1000	-	-	Dr. Emer Reeves, RCSI
CK19	Mouse	1:1000	1:100	Chemicon	-
GAPDH	Mouse	1:2000	-	Sigma	-
β 1-Integrin	Mouse	1:1000	1:2000	Chemicon	-
Vinculin	Rabbit	1:500	-	Cruz Biotechnology	-
CK3	Mouse	1:1000	1:100	Chemicon	-
CK12	Rabbit	1:1000	1:100	Chemicon	-
E-cadherin	Mouse	1:2000	1:500	Chemicon	-

2.24 Trypan blue exclusion assay

This assay was used to assess the viability of cells. Healthy cells will exclude the dye from passing through the membrane whereas unhealthy cells will allow the dye to penetrate the membrane. Cells were combined with trypan blue solution (0.4%, v/v) in a ratio of 1:1. If the cell number was too high to count a further dilution was made. Cells were mixed thoroughly and counted using a haemocytometer.

2.25 Assessing the effect of *Bacillus* antigens on Neutrophils

Neutrophil extraction buffers

All buffers were allowed to come to room temperature and heparinised (5 µl/ml) to prevent coagulation.

1 X Saline

NaCl (0.9% w/v) dissolved in dH₂O before autoclaving.

2 X Saline

NaCl (1.8% w/v) dissolved in dH₂O before autoclaving.

Dextran sulphate

Dextran (10% w/v) dissolved in 1 X saline before filter sterilising.

2.26 Isolation of human neutrophils

Human neutrophils were isolated from healthy volunteers (Ethical permission granted by NUI Maynooth Ethics Committee), separated from blood collected in 10 ml vacuumed heparinised tubes (BD Vacutainer Systems, Plymouth, UK) by dextran

sedimentation and Ficoll-Hypaque (Axis-Shield PoC AS, Oslo, Norway) centrifugation as previously described (Reeves *et al.*, 2002). Blood (20 ml) was added to 25ml of 1X saline (0.9% (w/v) NaCl in dH₂O) and 5ml of a 10% dextran sulphate salt solution was added to the mix to stimulate sedimentation. Each aliquot was inverted and left standing for 30 minutes. Neutrophils will not sediment therefore the top lighter coloured layer is removed to a fresh 50ml falcon tube. Samples were pooled if required to obtain 40ml in the tube which is then under laid with 5ml of lymphoprep (Axis-Shield) and centrifuged at 200 x g for 10 minutes without the break. Erythrocytes were removed by hypotonic lysis in water and brought back to osmolarity using a 1.8% (w/v) NaCl solution. This step was carried out quickly to prevent lysis of the neutrophils. Neutrophils were centrifuged at 200 x g for 5 minutes. The cells were resuspended in Phosphate Buffered Saline (PBS, pH 7.4) containing 5 mM glucose and used immediately. Viability was assessed using the Trypan Blue exclusion assay (Eichner *et al.*, 1986).

2.27 Neutrophil Migration assay

Neutrophils (1×10^6 /ml) were resuspended in RPMI-1640 media containing 2mM L-glutamine and 5% FCS. Cells were treated with the bacterial protein (2 and 6 μ g/ml of purified antigen or 2 μ g/ml) for one hour along with untreated cells as a control. Treated medium (500 μ l) was applied to a 24 well plate and either a 3 or 8 μ m porous insert (BD Falcon) was added to the well before 500 μ l of the treated cells were placed inside the insert. The plate was incubated for 24 hours in a humidified 5% CO₂ incubator at 37° C. Inserts were removed and washed three times with PBS before staining with crystal violet (0.25% w/v). A cotton bud was used to remove unattached cells from inside the insert and remaining cells adhering to the underside of the insert were allowed to dry before images were taken with an Olympus camera. The cells were then eluted using 33% glacial acetic acid and a colorimetric assay was carried out using a (Biotek plate reader) set at 570nm.

2.28 Quantification of release of MPO MMP-9 and hCAP-18 (LL-37) as markers of degranuation.

Neutrophils ($1 \times 10^7 \text{ ml}^{-1}$) were resuspended in PBS containing 5 mM glucose and protease inhibitors as described. Following coincubation with *Bacillus* antigens (2 and 6 $\mu\text{g/ml}$) in a stirred chamber, an aliquot was removed at $t = 0, 3, 6$ and 9 minutes. Cells were removed by centrifugation (800 x g). The protein content of the supernatant was precipitated and prepared for electrophoresis and immuno-blotting.

Samples were separated by SDS-PAGE (12.5% (w/v) acrylamide), and protein profiles were visualized by colloidal coomassie staining. For Western blotting, the protein was transferred to a nitrocellulose membrane using a wet blotter. Antibodies were applied as outlined in section 2.23 and Table 2.6. Reactivity of degranulated proteins to Myeloperoxidase antiserum, MMP-9 antiserum and IL-37 antiserum were characterised.

2.29 Production of neutrophil culture supernatants for cytokine assays

Isolated cells ($1 \times 10^6/\text{ml}$) in RPMI-1640 media containing 2mM L-glutamine and 5% FCS were distributed into 24 well flat bottomed tissue culture plates (BD Falcon) and incubated for 3, 15 and 24 hrs. Cells were treated with 2 and 6 $\mu\text{g/ml}$ of purified antigen and 2 $\mu\text{g/ml}$ of crude *Bacillus* lysate. Culture supernatants were harvested at the indicated time points and stored at -80°C until assayed.

IL-8 and TNF- α levels were measured using commercial ELISA kits (Human IL-8 ELISA, R&D Systems and Human TNF- α mini ELISA, Peprotech) according to the manufacturer's guidelines.

2.30 General Cell Culture Techniques

2.30.1 Cell Preservation

Long-term storage of cells was carried out using liquid nitrogen (-195°C). Cells in the exponential phase of growth were harvested and resuspended in freezing medium [10% DMSO (v/v), 10% FBS (v/v), 80% DMEM] at a cell density of approximately 1×10^6 cells/ml. 1 ml aliquots of the cell suspension were dispensed into cryovials (Nunc) and frozen in a styro-foam freezing box (Nunc) at -70°C over night. Vials were immediately placed into a liquid nitrogen chamber.

2.30.2 Cell Recovery

Cells were recovered from liquid nitrogen by rapid thawing at 37°C in warmed PBS. Following thawing, cell suspension was dispensed into 9 ml of fresh medium (5% FBS (v/v), 95% DMEM) and centrifuged at $1000 \times g$ for 5 mins. Cells were resuspended in fresh keratinocyte growth medium (Lonza, 500 ml of KGM™2 Basal medium was supplemented with 2 ml Bovine Pituitary extract, 0.5 ml hEGF, 0.5 ml Insulin, 0.5 ml Hydrocortisone, 0.5 ml Transferrin, 0.5 ml Epinephrine and 0.5 ml GA-1000.) and seeded in culture flasks and incubated at 37°C in a humidified atmosphere containing 95 % air, 5% CO_2 .

2.30.3 Cell counting and viability testing

Cell counts were performed using a haemocytometer counting chamber under an inverted microscope. Cellular viability was estimated by exclusion of the vital dye, trypan blue (0.4% w/v). Viable cells exclude the dye while dead cells stain blue. Cell suspension (30 μl) was added to 30 μl of trypan blue and incubated for 5 minutes. A 10 μl aliquot of the solution was loaded into both counting chambers and cells were counted in the four corner squares (each with 16 subsquares). The average count from the two chambers was

multiplied by the dilution factor and finally by 10^4 to account for the chamber volume. In this manner, a value representing the number of cells per volume was obtained.

2.30.4 Harvesting and subculture of cells

The cell line used in this study was adherent and required trypsinization prior to subculture. For sub-culture, the spent medium was removed and the cells were washed with sterile PBS (pH 7.4). Cells were detached by the addition of 2 ml trypsin solution [0.025 % (w/v) trypsin, 0.53mM (w/v) EDTA in PBS, pH, 7.4]. The flasks were then incubated at 37°C until the cells were fully detached. Trypsinization was terminated by the addition of 8 ml of complete medium (DMEM and 5% FBS). Disaggregated cells were harvested by centrifugation at 1000 x g for 5 minutes in a bench top centrifuge. The resultant pellet was resuspended in 2 ml of fresh media (KGM) and seeded into new flasks.

2.31 Assays using Human corneal Epithelial Cells (hTCEpi)

The human telomerase immortalized corneal epithelial cell line (hTCEpi) was generated by infecting primary cultures of human corneal epithelial cells with a retroviral vector encoding telomerase reverse transcriptase (hTERT). The purpose of this cell line was to provide a model for the study of molecular mechanisms involved in human corneal cell differentiation (Robertson *et al.*, 2005). Following a review of corneal epithelial cell culture models the method used to generate the hTCEpi cell line was deemed an efficient way to generate epithelial cell lines (Castro-Muñozledo 2008). The cell line was recently used to investigate the role of plasma membrane calcium-ATPase expression in a wound healing model (Talarico 2010).

hTCEpi cells were maintained in Keratinocyte Growth Medium-2 (KGM-2) supplemented with KGM-2 Single Quot Kit Supplements & Growth Factors (Clonetics-BioWhittaker, Inc., Walkersville, MD), in a humidified 5% CO₂ incubator at 37°C, and passaged every 7 to 10 days.

2.31.1 Toxicity Assay

hTCEpi cells were cultured to approximately 90% confluency and trypsinised, cell concentration was calculated and 4×10^4 cells/ml were added to a 24 well culture dish. Cells were allowed to adhere. The following day they were washed and KMG-2 media (Lonza) was added to the wells. Normal media as well as media with Bovine Serum Albumin (Sigma) (2 and $6 \mu\text{g/ml}$) were employed as controls. Media with increasing concentrations of the antigen preparation ($0.5\text{-}6 \mu\text{g/ml}$) was added to relevant wells and incubated for 5 days at 37°C in a humidified 5% CO_2 incubator. Growth media was supplemented every 2 days. The assay was evaluated by staining with 0.05% crystal violet (w/v). Cells were incubated in the dye at room temperature for 30 minutes then excess dye was washed off with H_2O . Plates were allowed to dry and cells were eluted with $200 \mu\text{l}$ of 33% (v/v) glacial acetic acid. Cells in suspension were transferred to a 96 well plate and read on a spectrophotometer at 570nm.

2.31.2 Wound healing assay

To test the effect of the bacterial antigens on wound healing, hTCEpi cells (6×10^4) were cultured in 6 well plates and supplemented with KGM-2 until a confluent layer was achieved. A scratch wound, the length of the whole well was made across the cell layer using a $200 \mu\text{m}$ pipette tip. The plate was washed with PBS to remove cell debris and then incubated in the presence of the antigen preparation (2, 4, $6 \mu\text{g/ml}$). Cells without any treatment were used as a control. The progress of cell migration into the scratch wound was photographed at 0, 24, 48, 72 and 96 hour time points.

2.31.3 Corneal cell Migration assay

hTCEpi cells were treated with the bacterial protein (2, $6 \mu\text{g/ml}$) for 4 days along with untreated cells as a control. Cells were trypsinized and resuspended at a concentration of $4 \times 10^4/\text{ml}$ in $500 \mu\text{l}$ of the relevant treated media. Treated media ($500 \mu\text{l}$) was applied to a 24 well plate and an $8 \mu\text{m}$ porous insert (BD Falcon) was added to the well before the cells

were placed inside the insert. Cells were incubated for 24 hours in a humidified 5% CO₂ incubator at 37° C. Inserts were removed and washed three times with PBS before staining with crystal violet (0.25% w/v). A cotton bud was used to remove unattached cells from inside the insert and remaining cells adhered to the underside of the insert were allowed to dry before counting.

2.31.4 Invasion Assay

Cells (4×10^4) were treated identically as in the case of the migration assay but were placed in an 8 µm matrigel invasion chamber insert (BD BioCoat). The same staining protocol was followed as for the migration assay outlined above (section 2.31.3)

2.31.5 Adhesion Assay

The lids of 96 well plates were coated with components of the ECM (Collagens I, IV, V, Laminin, Fibronectin and Gelatin). hTCEpi cells were pre-incubated with the antigen preparation (2,4 and 6 µg/ml), trypsinised and applied at a concentration of 1×10^4 cells/ml onto the different ECMs. The cells that adhered were stained using crystal violet dye (0.25% w/v) and counted using a Leica microscope.

2.32 Isolation of protein from corneal cells

hTCEpi cells were exposed to the bacterial antigen (2, 6µg/ml) for five days in 6 well culture dishes. Lysis buffer consisting of 0.25M Tris HCl (pH 7.2) and 2.5% SDS was added in a volume dependent on the consistency of the lysate (approx 500 µl). Following protein determination using Bradford reagent the protein was mixed with Lamelli sample buffer (Biorad) and frozen at -20° C until required.

2.33 Electrophoresis and Western Blotting

To carry out electrophoresis the sample was boiled for 3 minutes and cooled to room temperature before application of 20µg of protein to the well. Gels were transferred to nitrocellulose membrane using a semi dry blotter (Biorad) in Towbin transfer buffer (25mM Tris, 192mM Glycine, 20% Methanol in dH₂O, pH 8.3).

Membranes were then blocked using 1 % dried milk powder (Marvel) in TBS-tween for 1 hour at room temperature. Primary antibodies were diluted according to Table 2.6 and incubated at 4° C overnight.

The relevant horseradish peroxidase-linked secondary antibody diluted 1:1000 was applied and incubated at room temperature for 2 hours. Enhanced chemiluminescence (Perkin-Elmer) was used to develop reactive protein bands and image-J software was used to perform densitometry analysis.

2.34 Gelatin Zymography of MMP-9 activity

This work was contributed by Dr. Clair Gallagher and Dr. Finbarr O'Sullivan, collaborators from the NICB, DCU. SDS-PAGE gels were prepared according to standard procedure with the addition of 0.1% gelatin. Protein was extracted from the antigen treated hTCEpi cells using a denaturing buffer and mixed with the sample buffer but not boiled. Protein (20 µg/ml) was added to each well and separated by eletrophorises. After running the gel was incubated in zymogram renaturing buffer for 30 minutes at room temperature. The gel was then placed in zymogram developing buffer and equilibrated for 30 minutes at room temperature before being incubated overnight at 37 °C. The zymogram was stained with coomassie blue and then destained so the area of protease activity could be visualized.

2.35 Immunofluorescence

hTCEpi cells were cultured at a concentration of 4×10^4 on circular glass coverslips ($0.2 \mu\text{m}$) in 24 well dishes. Antigen treated and control cells were incubated at 37°C in a humidified incubator ($5\% \text{CO}_2$) for 4 days. They were then washed with PBS-tween and fixed in methanol for 5 minutes at -20°C . Methanol was removed and the coverslips were stored at -20°C . Cells were blocked for 1hr at 37°C in 5% goat serum (Biorad) diluted in PBS (Accugene). The primary antibodies were diluted (Table 2.5) in 1% goat serum and incubated at 4°C overnight. Fluorescently labeled secondary antibodies (Alexafluor, Invitrogen) were diluted to 1:1000 and applied out of direct sunlight and incubated for 1 hour at room temperature. DAPI nuclear stain (1:5000) was added for 1 minute and then cells were washed with PBS before the cover slips were mounted onto glass slides using ProLong® Gold antifade reagent (Invitrogen). Images were taken using a Nikon Eclipse microscope and Metamorph software.

2.36 Generation of Primary Corneal cell cultures from Limbal stem cells using 3T3 fibroblast feeder cells

Primary cultures were generated from donor corneal tissue (obtained from a cadaver donor from America) specifically the residual corneo-scleral rings after keratoplasty and maintained in optisol media until arrival at the NICB. Limbal tissue was isolated from either side of the corneo conjunctival junction. This tissue was diced approximately into 1 mm cubes. The tissue was transferred to gelatin coated 6 well plates and treated with a few drops of pre-warmed media. Limbal cell media consisted of, DMEM:F12 (3:1), 10% FCS, 5 $\mu\text{g}/\text{ml}$ Insulin, 10 ng/ml rhEGF, 100 ng/ml Cholera Toxin A Subunit, 0.4 $\mu\text{g}/\text{ml}$ Hydrocortisone, Triiodothyronine ($2 \times 10^{-9} \text{ml}^{-1}$) and irradiated 3T3 cells (2×10^3).

Irradiation of 3T3 fibroblast cells was carried out by Dr. Kishore Reddy Katikireddy (NICB, DCU). The 3T3 fibroblasts were routinely maintained in DMEM supplemented with 5 % fetal calf serum (FCS) (Hyclone, Logan, UT). Cells were trypsinised at 60-70%

confluency, washed and carried in HBSS buffer for irradiation. Inactivation of 3T3 fibroblasts was achieved by lethal irradiation 60 Gray. Irradiated 3T3 cells were stored in liquid nitrogen with freezing medium (10% DMSO). Irradiated 3T3 cells were used for culture of limbal-corneal cells, with density of $2.4 \times 10^4/\text{cm}^2$ cells per well/ insert.

Due to the infrequent availability of primary culture tissue, these experiments were carried out using tissue from a single donor and are therefore considered as technical replicates. The tissue was maintained with medium and irradiated 3T3 cells until a confluent layer of corneal epithelial cells was achieved. At this point the cells were removed from the culture plate surface using trypsin. These primary culture cells were then used for some of the assays outlined using the same procedure as for the immortalized cell line.

2.36.1 Wound healing in Limbal epithelial cells

Limbal epithelial cells (6×10^4) were cultured in 6 well plates and supplemented with conditioned media described above (Limbal cell media) until a confluent layer was achieved. A scratch wound was made in the cell layer across the whole length of the well using a 200 μm pipette tip. The plate was washed with PBS to remove cell debris and then incubated in the presence of the antigen preparation (2 & 4 $\mu\text{g}/\text{ml}$) in the culture medium. Cells without any treatment were used as a control. The progress of cell migration into the scratch wound was photographed at 0, 24 and 48 hour time points.

2.36.2 Preparation of Limbal epithelial cells for Western blotting

Limbal epithelial cells (6×10^4) were seeded to 6 well plates and allowed to adhere over night. The plate was washed with PBS and incubated in the presence of the antigen preparation (2 & 4 $\mu\text{g}/\text{ml}$) in the culture medium. Cells without any treatment were used as a control. After 3 days protein was extracted from the cells using a lysis buffer consisting of 0.25M Tris HCl (pH 7.2) and 2.5% SDS was added in a volume dependent on the

consistency of the lysate (approx 500 μ l). Following protein determination using Bradford reagent the protein was mixed with Lamelli sample buffer (Biorad) and frozen at -20° C until required. Western blotting was carried out as described in section 2.33.

2.37 Statistical Analysis

All experiments were performed on at least three independent occasions. Results presented are mean \pm standard error.

For comparisons between two treatments, a 1-tailed, 2-sample equal variance (homoscedastic) Student's t-test was performed and samples with a p value \leq 0.05 (indicated by *) or \leq 0.01 (indicated by **) were deemed significant.

For the comparison of ocular rosacea patients (group 1) and control (group 2) sera reactivity using ELISA (Figure 3.15 A & B) the data was analyzed using a 2-way anova test.

CHAPTER 3

**Fractionation of the stimulatory proteins
from *Bacillus oleronius* and
characterisation of those proteins which
elicit an immune response in rosacea
patient sera**

3.0 Introduction

Prior to the work by Delaney (2004), the role of *Bacillus oleronius* in the induction of an immune response in rosacea had not been investigated. It was discovered following a screen of several bacterial isolates that *B. oleronius* caused significant ($p=0.01$) PBMC stimulation in 73% of rosacea patients compared to 29% of controls (Lacey *et al.*, 2007). Delaney (2004), used fractionation of the bacterial proteins and Western blotting to identify antigenic proteins including the 62 and 83 kDa proteins that caused immune reactivity in rosacea serum but not controls. Lacey (2007) continued the study of these antigens, using 2D-electrophoresis coupled with MALDI-ToF analysis to characterise their functions. Although *B. oleronius* has not been sequenced, the 62 kDa protein was found to have homology to a protease involved in carbohydrate metabolism and signal transduction and the 83 kDa protein showed similarity to a heat shock protein. It was evident that both these proteins have the potential to be involved in the induction of inflammatory reactions (Lacey *et al.*, 2007).

The aim of the work presented in this Chapter was to (1) analyse the reactivity of sera and tear fluid samples to the whole cell protein lysate of *B. oleronius* using Western blotting, (2) fractionate the bacterial proteins and use LC mass spectrometry in order to identify their possible functions (3) pool antigenic proteins, in particular the 62 and 83 kDa antigens, for investigations using human cells and (4) determine if reactivity to these pooled antigens could be captured in a simple ELISA assay to aid in the diagnosis of rosacea.

3.1 Preparation of *Bacillus* proteins

Bacillus protein extracts were obtained by sonication and use of a detergent (0.2% Triton-X 100) containing buffer to discover which method provided adequate amounts of the stimulatory antigens. Western blotting was performed using a rabbit antibody generated towards fractionated *B. oleronius* proteins that is particularly reactive to the 62 kDa antigen. Following Coomassie staining of the SDS gel the protein extracts (Figure 3.1 A) appear similar. Figure 3.1 B represents an immunoblot probed with positive rosacea sera (pooled from 5 patients). The blot shows increased expression in the 62 kDa protein when cells were subjected to Triton-X detergent extraction

(section 2.12) compared to cells subjected to sonication (2.8 fold) or a combination of both (1.6 fold). The combination of detergent and sonication extraction lead to a higher yield of the 83 kDa protein of 1.4 fold compared to Triton-X and 3.8 fold compared to sonication alone. Subsequently the combination of both methods was used where the cells were suspended in the detergent lysis buffer for 30 mins-1 hr followed by 3 x 10 second blasts of sonication at 20% power. This method was used to obtain the maximum amount of both proteins in the extracts used to test patient sera reactivity.

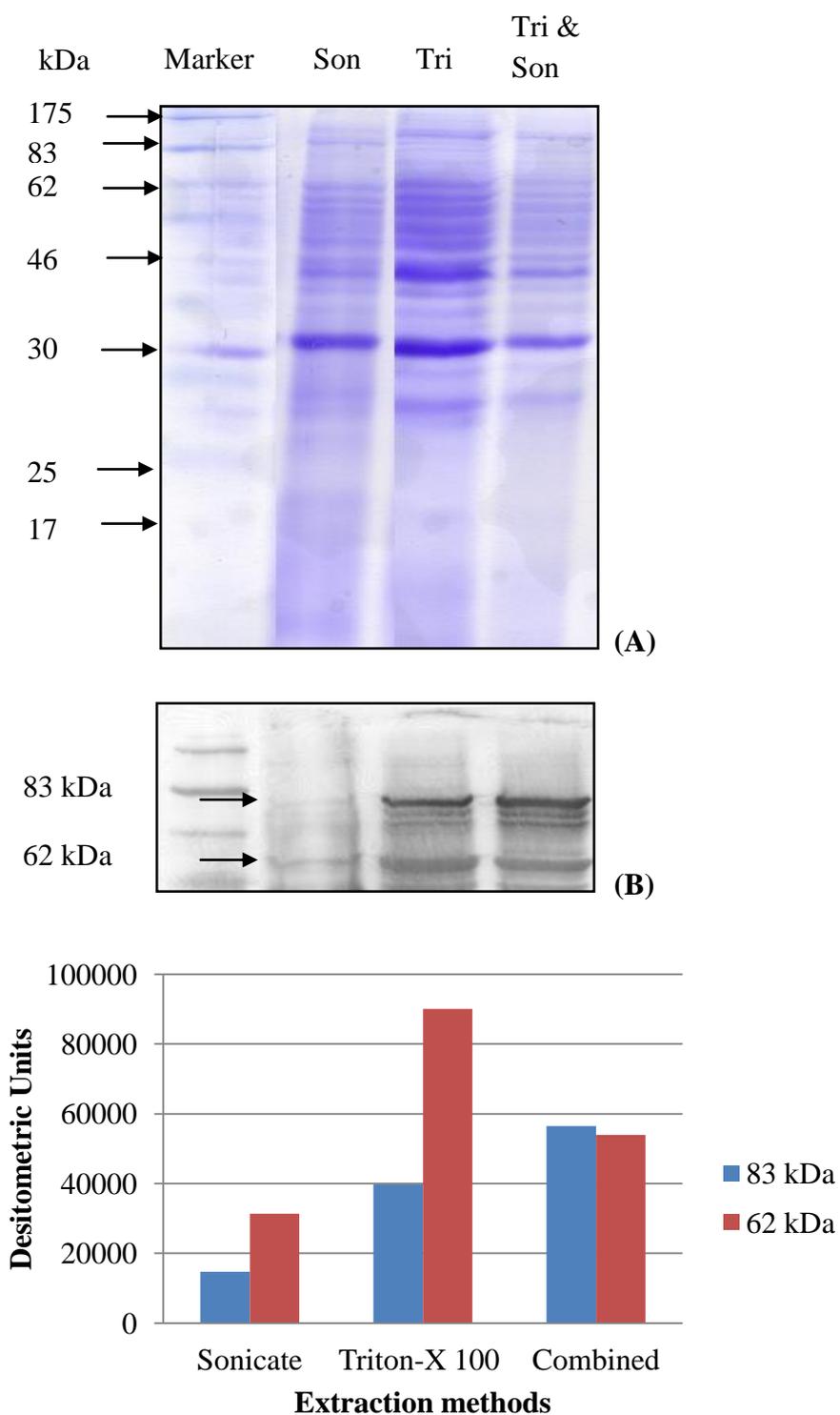


Figure 3.2 SDS-PAGE (A) and Western blot (B) coupled with densitometric analysis of *B. oleronius* protein extracted using different methods

‘Son’ represents the protein extract obtained by sonication alone as described in section 2.12. ‘Tri’ represents the protein extract obtained with the use of a detergent buffer. The third lane of the gel represents a combination of the 2 methods.

3.2 Testing reactivity of individual patient sera to *Bacillus* antigens using Western blotting

A previous study used 16 papulopustular rosacea patient serum samples and 100% showed reactivity to the 62 kDa antigen while the 83 kDa protein induced an immune response in 50% of patients. By comparison 25% of control sera reacted to the 62 kDa antigen and no controls responded to the 83 kDa protein (Lacey, 2007). Therefore it was established that *B. oleronius* produced 2 immune stimulatory proteins that could be detected in a serological assay when tested on the sera of Papulopustular rosacea patients. In this study involving 59 blind tested sera samples included patients with ocular rosacea (Ocular Surface Research Centre in Miami) and 18 blind tested sera samples including patients with erythematotelangiectatic (type one) rosacea (Sengio Dermatologia Portugal). *Bacillus* protein lysate (20 µg/well) was prepared as described in section 2.12, separated by SDS-PAGE and transferred to nitrocellulose membrane. These membranes were cut into individual strips and Western blots were carried out using serum diluted in blocking buffer (1:100). The membranes were then incubated for 2 hours at room temperature in a HRP-linked anti-human secondary antibody diluted 1:1000 in blocking buffer. Reactivity was visualised using DAB substrate (section 2.23). Serum immunoreactivity to *B. oleronius* in some of the ocular rosacea patients is shown in Figure 3.2 A, Figure 3.2 B represents control sera responses. Serum reactivity in a sample of the type one rosacea cases is represented in Figure 3.3 A, while Figure 3.3 B shows a sample of the controls from that study. Western blot analyses from all participants in both studies are presented in the Figures 8.1-8.6.

3.2.1 Testing for reactivity to *Bacillus* antigens using Western blotting assays in Ocular rosacea

Ocular rosacea is difficult to diagnose since symptoms involving the eyes reportedly manifest in up to 20% of cases prior to any cutaneous symptoms (Dahl *et al.* 2002; Jansen & Plewig 1997; Starr & Macdonald 1969; An *et al.* 2005). Diagnosis is achieved by careful examination of the eye however common ophthalmic signs of rosacea such as blepharitis and conjunctival inflammation may be confused with other conditions such as seborrhoeic dermatitis, cylindrical dandruff, blepharitis or

conjunctivitis (Jansen & Plewig 1997; Gao *et al.*, 2005; Neiberg & Sowka 2008). More serious ocular manifestations are rare but can include iritis and keratitis leading to sight threatening opacification of the cornea or limbal stem cell deficiency (Jansen & Plewig 1997; Kheirkhah *et al.* 2007; Oltz & Check 2011). Ocular manifestations occur in up to 50% of cases of rosacea and reports suggest that up to 33% of those may involve corneal symptoms (Starr & a Macdonald 1969). The lack of agreement on the etiology of rosacea indicates that further work is needed to establish a diagnostic assay for the condition.

Results from the study of serum immunoreactivity to 2 stimulatory *Bacillus* proteins formed the basis for a publication (Li *et al.*, 2010) with 66% (14/21) of patients positive for facial rosacea also proving positive for serum immunoreactivity to one or more of the antigens. The 59 patients were subdivided into 2 groups according to serum immunoreactivity, positive (n=21) and negative (n=38) (Figure 3.2 A&B). Comparison of these 2 groups showed that positive serum immunoreactivity was significantly correlated with ocular *Demodex* infestation (p=0.048), facial rosacea (p=0.009), and lid margin inflammation (p=0.040) (Li *et al.*, 2010). Of those individuals in the serum positive group 100% (21/21) had antibodies towards the 62 kDa protein and 61% (13/21) showed serological reactivity to the 83 kDa protein. Sixty eight % (26/38) of those in the negative serum group were controls. Thirty three % (7/21) of the control sera reacted to the *Bacillus* antigens.

Those individuals who show reactivity to *Bacillus* proteins but are negative for rosacea symptoms may have generated antibodies due to previous exposure to the bacterium since it is thought to be an endosymbiont of *Demodex* mites which are ubiquitous in the skin of all adults (Bonnar *et al.*, 1993). The pattern of serum reactivity in Figure 3.2 A (Ocular rosacea patients) is clearly distinguishable from the reactivity seen in Figure 3.2 B (Control sera). Sera from rosacea patients is generally more reactive than control sera and the 62 kDa band is clearly visible in cases 1, 6, 7, 12 & 23. Reactivity to the 83 kDa protein is present in four (cases 1, 6, 7 & 23) of the blots presented here. Cases 16, 17, 20, 21 & 25 represent controls, weak reactivity to the 83 kDa band is seen in cases 16 & 17. Case number 25 showed weak reactivity to both antigens but was still distinguishable as a control.

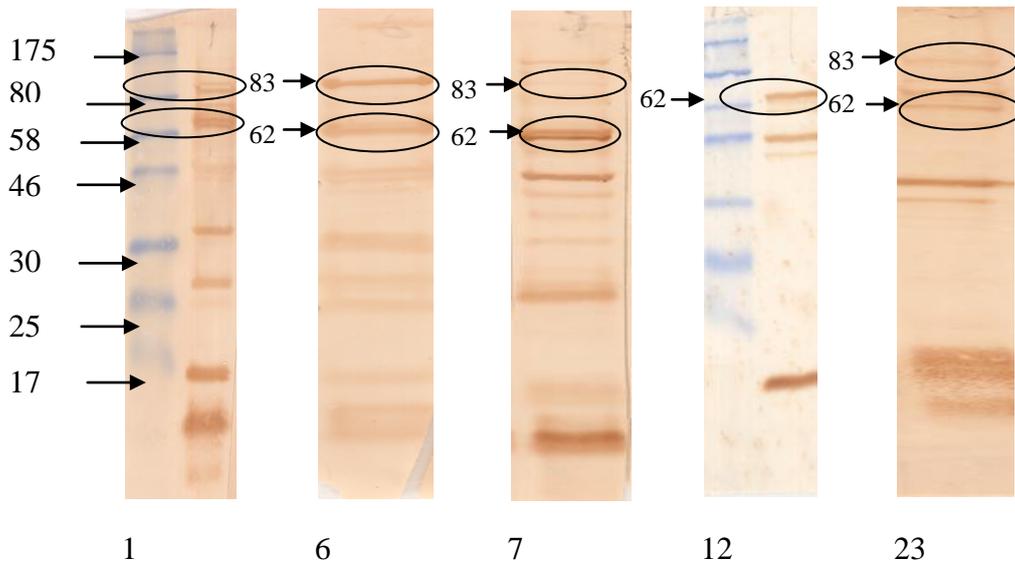


Figure 3.2 A Western blot analysis of sera from ocular rosacea patients to *B. oleronius* protein extract

These blots represent cases where the sera showed positive reaction to one or both of the stimulatory antigens and the person had been diagnosed with ocular rosacea.

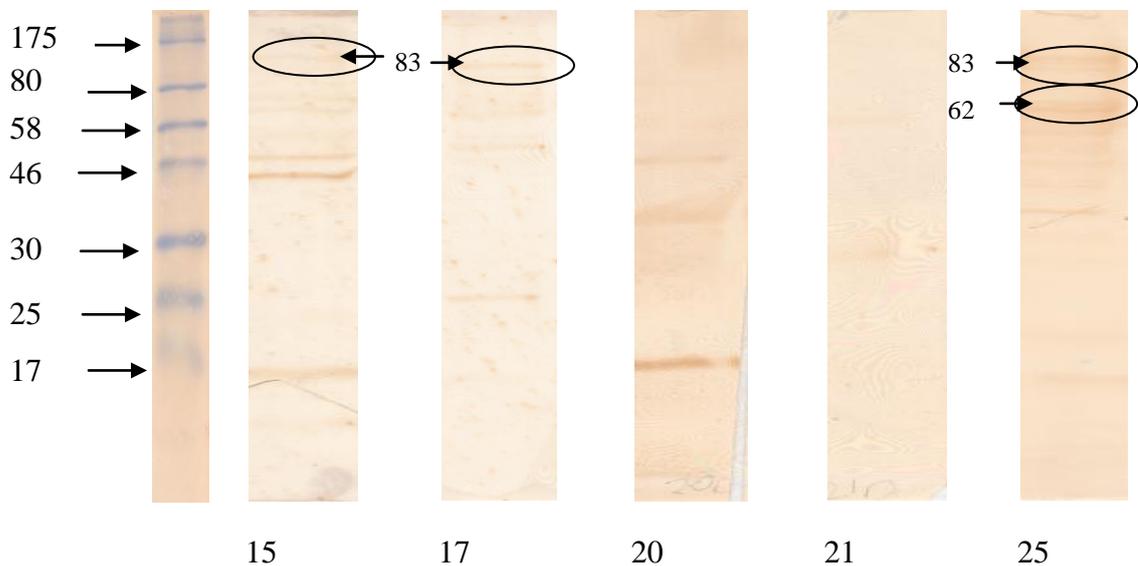


Figure 3.2 B Western blot analysis of control sera to *B. oleronius* protein extract

These blots represent sera from controls that showed no or very weak reactivity to the stimulatory antigens.

3.2.2 Testing for reactivity to *Bacillus* antigens using Western blotting assays in Erythematotelangiectatic rosacea

Erythematotelangiectatic (type one) rosacea is characterised by flushing in the central region of the face rosacea patients describe their flushing to last longer than 10 minutes therefore it differs from the flushing seen in response to embarrassment, exercise, or hot environments (Crawford *et al.*, 2004). Individuals presenting with prolonged flushing and sensitive irritated skin may be assessed for symptoms of rosacea using clinical classification and grading guidelines (Dahl *et al.*, 2002; Wilkin *et al.*, 2004), however no diagnostic assay exists. In this section it was sought to investigate the possibility that a serological reaction to *Bacillus* antigens might occur in type one rosacea patients. Immunoblotting was carried out as described in section 3.2.

Blind testing of control and patient sera was carried out on samples donated from Dr. Nuno Menezes (Servico Dermatologia, Lisbon, Portugal). Following analysis of clinical data the results were divided into two groups, 26 individuals were positive for type one rosacea and 22 were controls. Twentyone serum positive individuals were present among the type one rosacea patients (80%, 21/26). Of these 85.7% (18/21) of sera samples reacted to the 62 kDa antigen, 42% (9/21) reacted to the 83 kDa antigen while six patients (6/21, 28.5%) demonstrated reactivity to both proteins (Figure 3.3 A). Of the 22 samples later revealed to be controls 40% (9/22) showed weak reactivity to the 62 kDa antigen (Figure 3.3 B). Figure 3.3 A presents five rosacea positive cases (A, B, D, E & H) that all show a reactive band at the 62 kDa region, case B is also reactive to the 83 kDa protein. Control sera represented by cases; C, G, N & O (Figure 3.3 B) show no reactivity to either band, while case F is reactive to the 62 kDa protein but is still distinguishable as a control. Data from this study were analysed using a Chi-squared test and a significant correlation ($p=0.004$) between serum reactivity to the two bacillus proteins and the occurrence of type one rosacea was established.

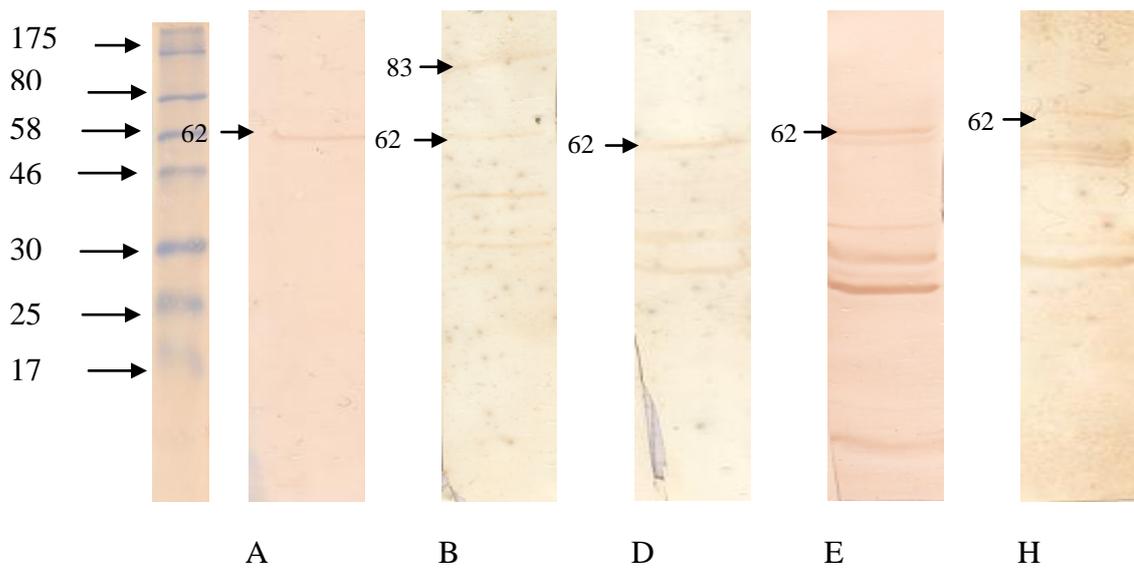


Figure 3.3 A Western blot analysis of sera from type one rosacea patients to *B. oleronius* protein extract

These blots represent cases where the sera showed positive reaction to one or both of the stimulatory antigens and the person had been diagnosed with type one rosacea.

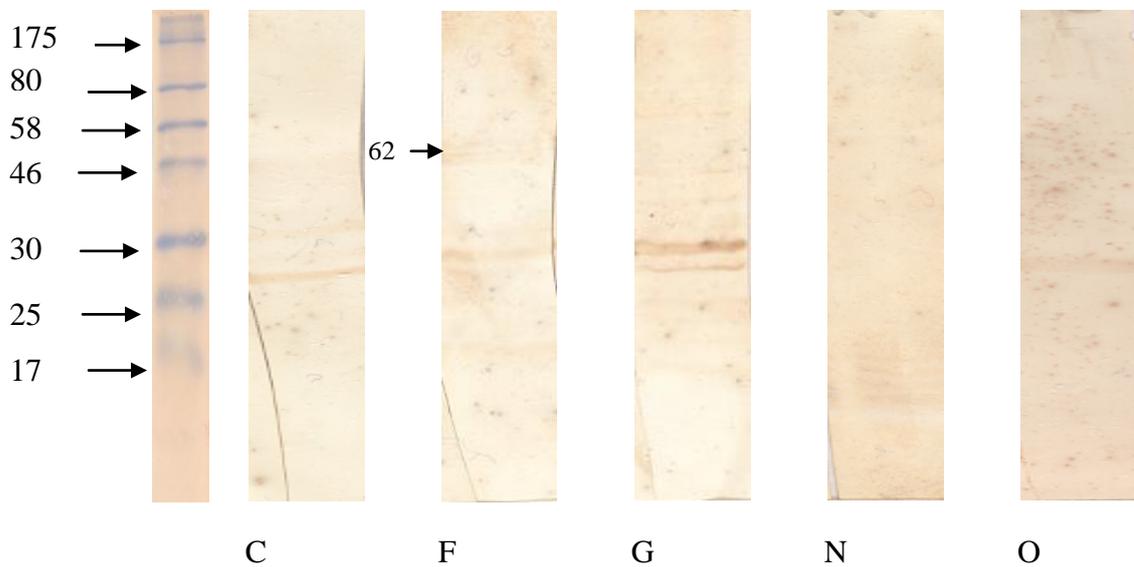


Figure 3.3 B Western blot analysis of control sera to *B. oleronius* protein extract

These blots represent sera from controls that showed no or very weak reactivity to the stimulatory antigens.

Study Population	Positive for Serum Reactivity and rosacea	Negative serum response in rosacea patients	Positive serum response in controls	Negative Serum response from controls
Ocular Rosacea	14/21 (66%)	12/38 (31%)	7/21 (33%)	26/38 (68%)
Erythematotelangiectatic Rosacea	21/26 (80%)	5/26 (19%)	9/22 (40%)	13/22 (59%)

Table 3.1 Summary of Serum reactivity results

Sera were blind tested for reactivity to 2 stimulatory *Bacillus* proteins and predicted positive or negative for rosacea. Following clinical examination patients were diagnosed and a correlation between serological reactivity and rosacea status.

3.3 Testing reactivity of tear fluid to *Bacillus* antigens

Samples received from the Ocular Surface Research Centre, Miami were from controls and patients with some degree of ocular involvement in their rosacea. In conjunction with the serum samples, tear samples were also received and tested for reactivity to the bacterial antigens. Tear fluid was extracted from the conjunctival fornix of the eye using polyester wicks. The samples were sent on ice and stored at -80°C. In order to test the tear fluid the wicks were soaked in DTT sample buffer (section 2.19) to solubilise the proteins. This protein was separated by SDS-PAGE and visualized using colloidal Coomassie blue dye. The sample method did not allow the protein to be quantified however following staining of the gels (Figure 3.4 A) it was evident that there were high levels of extractable protein in the tear fluid.

It was decided that Western blotting analysis would be carried out so the proteins were transferred to a nitrocellulose membranes. For this experiment an anti-*B. oleronius* antibody generated in rabbit was used for the primary incubation. Normal Western blotting procedure was followed and the membranes were developed using ECL chemiluminescence reagents (Perkin Elmer). Although gels revealed sufficient proteins there was minimal success in detecting reactivity to *Bacillus* antigens from rosacea positive patients with this method. Figure 3.4 B shows six representative cases where faint traces of reactivity to the *Bacillus* antibody were detected in the tear fluid. In cases 16, 18, 22, 23 & 40 reactive bands were identified in tear fluid at the 62 kDa region and case number 45 showed reactivity at the 83 kDa region. All six individuals were positive for ocular rosacea (Figure 6.4 C) and four (22, 23, 40 & 45) had initially tested serum positive showing reactivity to both *Bacillus* proteins. The remaining 2 (16 & 18) had weak reactivity and were labelled as possible patients.

Since up to 90% of ocular rosacea patients show no facial symptoms, diagnosis of the disease can be difficult. An *et al*, (2005) proposed a mass spectrometry based assay for detection of oligosaccharides which were more abundant in the tear fluid of those with ocular rosacea than controls. Western blotting of tear fluid proved to be an unreliable detection method for the detection of the stimulatory bacterial antigens. The accuracy of a tear fluid based assay for the detection of bacterial antigens in the tear

fluid of rosacea patients could be improved with the use of a more sensitive technique such as Mass spectrometry or ELISA.

3.3.1 Summary

Western blotting analysis has confirmed the presence of antibodies that are reactive to *B. oleronius* proteins (62 and 83 kDa) in the serum and tear fluid of rosacea patients. In a study of individuals with ocular rosacea 66% of patients had reactivity to one or both of the stimulatory proteins in their serum and 68% of controls showed a negative response. Reactivity of patient tear fluid using a rabbit antibody for the 62 kDa protein was detected in 6 individuals who were positive for ocular rosacea. In a study of individuals with erythmatotelangiectatic rosacea 92% of patients showed a positive serum response while 100% of controls showed a negative serum response. These results indicate the possible use of *Bacillus* proteins in the diagnostic detection of rosacea.

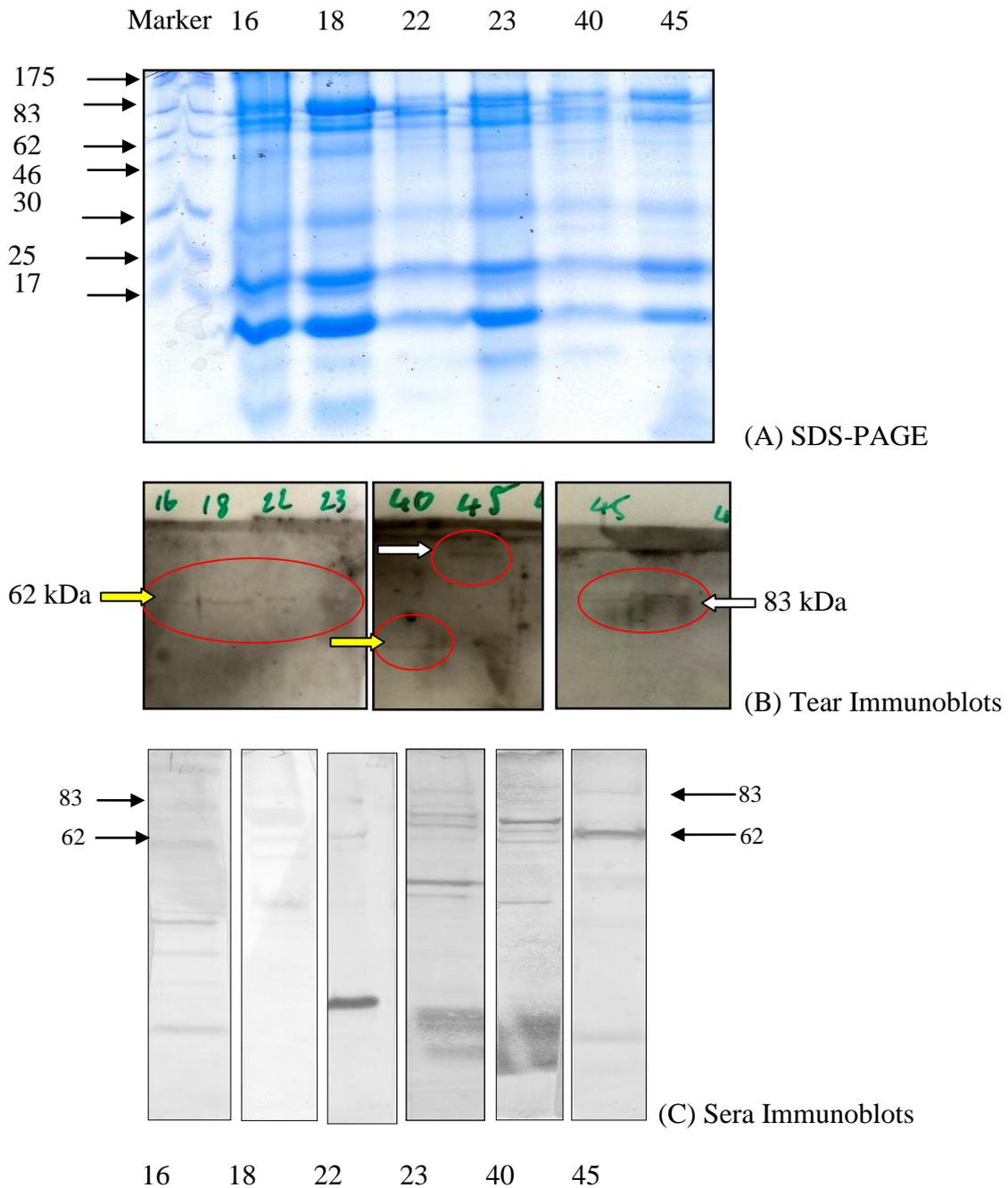


Figure 3.4 SDS-PAGE (A) and Western blot (B) analysis of tear fluid proteins

Reactive bands were identified in tear fluid samples from 6 ocular rosacea patients (case numbers 16, 18 22, 23, 40 & 45).

(C) Serum reactivity in patients which showed reactivity in their tear fluid

These blots represent cases where the sera showed positive reaction to both of the stimulatory antigens (62 & 83 kDa) and had some level of tear fluid reactivity.

3.4 Fractionation of *B. oleronius* proteins by Q-Sepharose charge separation using Fast Performance Liquid Chromatography (FPLC)

When rosacea patient sera was tested for reactivity using crude *B. oleronius* extracts there was reactivity to two antigenic proteins (62 and 83 kDa) in particular. Fractionation of *B. oleronius* protein was carried out (Lacey, 2007) in order to generate an antibody against the antigenic proteins of the bacterium. This antibody was raised in rabbit and is primarily reactive to the 62 kDa protein. It was postulated that this protein was more stable than the 83 kDa antigen during the fractionation process and therefore present at a higher concentration in the protein mixture used for inoculation to generate the antibody. The 62 kDa protein is reactive in a higher amount of rosacea patients compared to the 83 kDa protein (section 3.2).

The aim of this section was to fractionate the reactive proteins for use in assays with human cells and to investigate the potential to generate a diagnostic assay. Large amounts of bacteria (up to 5 Litres) were grown in rich medium (2XYT broth) at 30°C and 200 rpm, for several days to give a dense culture. Cells were then inverted with a lysis buffer containing 0.2% Triton-X 100 detergent for at least one hour at 4°C to ensure minimal protein degradation occurred. The cells were also subjected to sonication to ensure the maximum protein concentration was obtained in the lysate (section 2.12).

Fractionation was performed using the ÄKTA Purifier 100 system (Amersham Biosciences) and a Q-sepharose column, the system is automated and monitored by the Unicorn 5.01 programme. It was previously established (Lacey, 2007) that *Bacillus* proteins bound favourably to the positively charged Q-sepharose beads and that inverting the beads in the lysate prior to preparation of the column gave maximum binding. The column was attached to the ÄKTA system so that the beads (5-10 mls) could be equilibrated prior to mixing with the lysate, which was achieved by pumping binding buffer (section 2.9) through the column at 1 ml/minute with a 0% gradient for five minutes, then elution buffer (section 2.10) was pumped at the same flow rate with 100% gradient for five minutes, followed by a repeat wash with the binding buffer for five minutes. At this point the beads were removed from the column and inverted with

the filter sterilised protein mixture at 4 °C for at least 1 hour. This process was continued overnight in some cases.

The beads were loaded back onto the column which was reattached to the ÄKTA system. At this point the protein sample that remained was injected back onto the column at a rate of 0.5 ml/min followed by the binding buffer (section 2.9) until the absorbance reading returned to zero. The elution buffer containing 1 M NaCl was washed through the column to elute the protein (section 2.10) and 0.5 ml fractions were collected per minute by an automatic fraction collector. Figure 3.5 (A) represents a chromatograph generated by the Unicorn 5.01 computer programme from one run of the ÄKTA system. In the magnified image (Figure 3.5 B) the blue peak corresponding to the UV 280 absorbance reading represents protein being eluted from the column and the vertical red lines represent the fractions as they are labelled on the fraction collector.

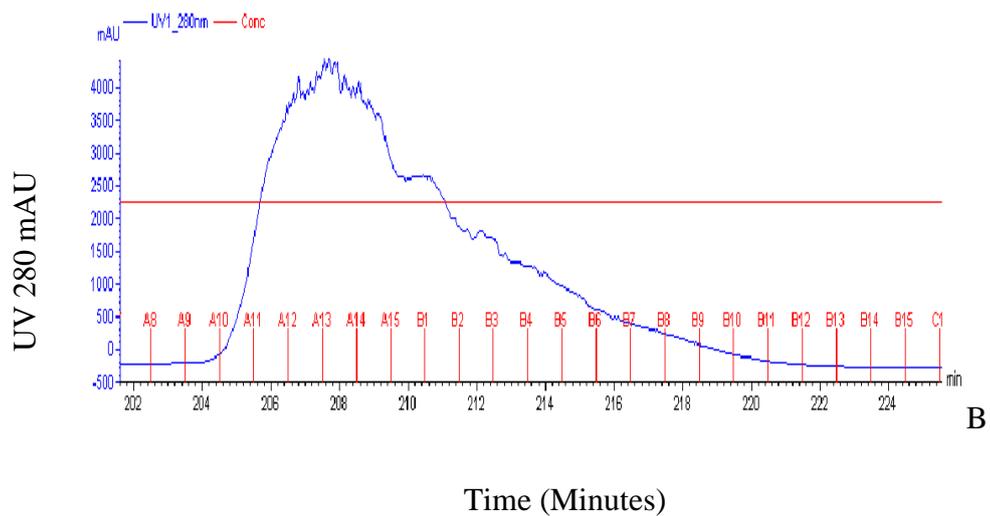
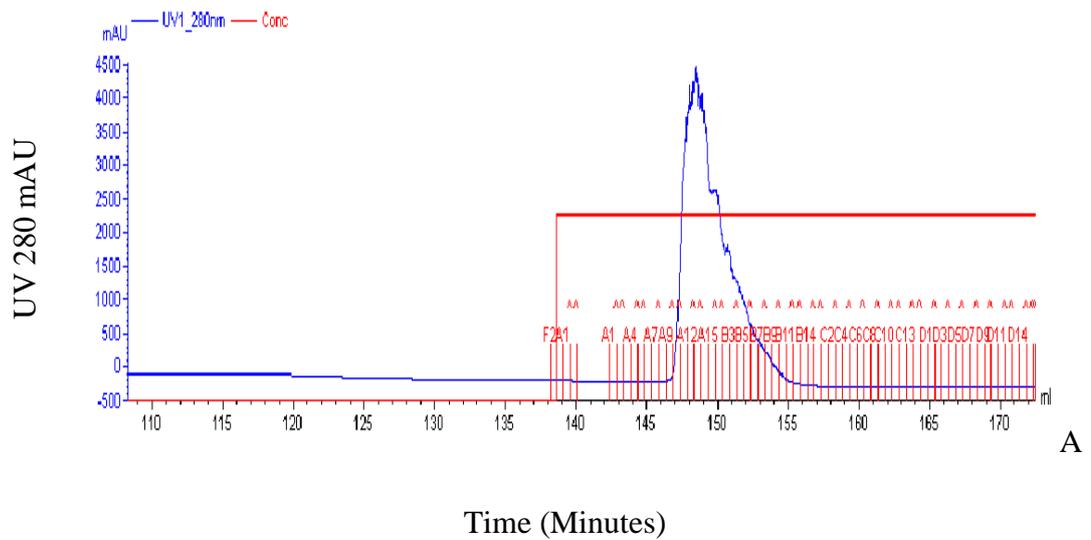


Figure.3.5 Chromatographs from ÄKTA-FPLC fractionation of *B. oleronius* protein

- (A) Represents a chromatograph generated by the Unicorn 5.01 computer programme as the anion exchange purification was performed.
- (B) Represents the protein eluted (blue peak) from the column and the fractions (500 µl/min) collected (vertical red lines). All fractions were analysed by SDS-PAGE and those corresponding to the peak contained the highest concentration of protein.

3.4.1 SDS-PAGE and Western blot analysis of the FPLC fractions

In order to identify the presence of the antigens of interest, 50 µl was taken from each fraction and mixed 1:1 with 10 x sample buffer (section 2.19) and boiled before application to 1D SDS gels. Gels were duplicated so one could be stained with Coomassie while the other was transferred to nitrocellulose membrane for Western blotting. The figures below (3.6-3.8) represent the results of one fractionation procedure. This procedure was carried out multiple times to obtain the semi purified protein product. The wash was collected after the column was attached during the equilibration with binding buffer and there was minimal protein in this lane (Figure 3.6 A & B) of the gel because the protein was bound to the Q-sepharose beads on the column. The sample was re-applied to the column after inversion with the beads and the flow through was collected at this point. Samples of the wash and flow through (lanes 1 and 2) were applied to the SDS gel to ensure that large amounts of protein were not being lost as waste during the procedure. The *B. oleronius* cells post-protein extraction were applied to lane 3 (Figure 3.6 A & B) there was some residual protein seen on the gel and Western blots however the yield of protein in the fractions was also high. Cells were sometimes retained and stored at -20 °C for subsequent fractionations.

The fractions (F11-F19) represented in Figure 3.7 A & B contain multiple proteins. At fraction 17 the 62 kDa band becomes the predominant protein in the SDS gels and in Figure 3.8 (A) there appears to be a single band present in the fractions. The Western blot analysis reveals other proteins or perhaps breakdown products of that protein are also present (Figure 3.8 B). Western blotting can be more sensitive therefore the 83 kDa protein does not appear to be present on the gel (Figure 3.8 A) however is visible in some fractions on the blot (Figure 3.8 B). Fractions containing the proteins of interest were pooled, in this case fractions 17-54, then concentrated using amicon filters, re-suspended at a concentration of 1 µg/10 µl and stored at -20 °C until required. 1D SDS-PAGE analysis was used to identify the fractions containing proteins of interest each time more protein was needed for the assays to ensure the make-up of the 'purified preparation' remained constant and assays were reproducible. Figure 3.9 represents the *Bacillus* protein extract prior to ÄKTA purification and the product the results from pooling fractions that show reactivity to the 62 kDa antibody.

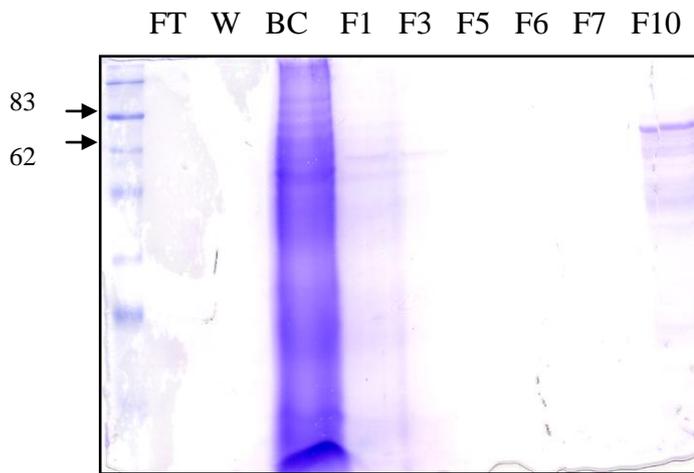


Figure 3.6 (A)

SDS-PAGE analysis of flow through, wash, bacteria cells and Q-sepharose fractions 1-10

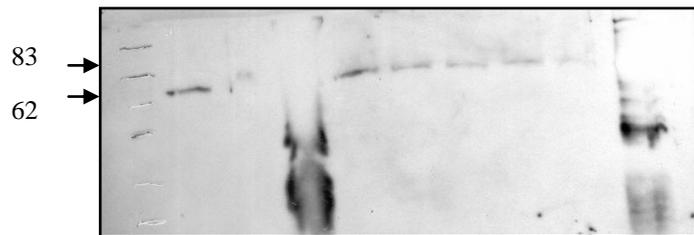


Figure 3.6 (B)

Western blot analysis of flow through, wash, bacteria cells and fractions 1-10

Probed with rabbit antibody

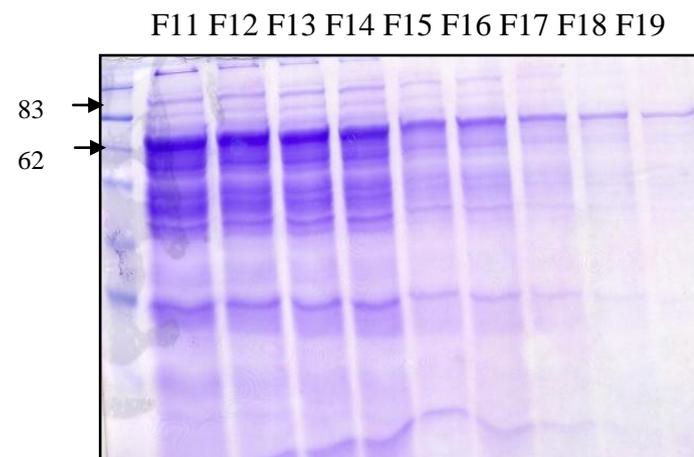


Figure 3.7 (A)

SDS-PAGE analysis of Q-sepharose fractions 11-19

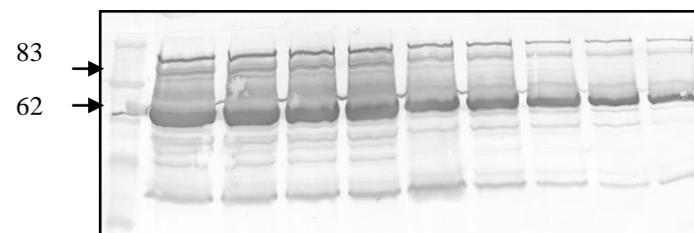


Figure 3.7 (B)

Western blot analysis fractions 11-19

Probed with rabbit antibody

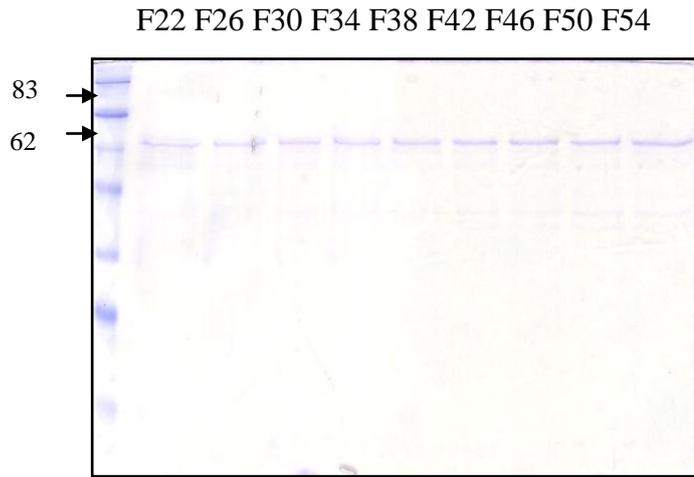


Figure 3.8 (A)

SDS-PAGE analysis of Q-sepharose fractions 22-54

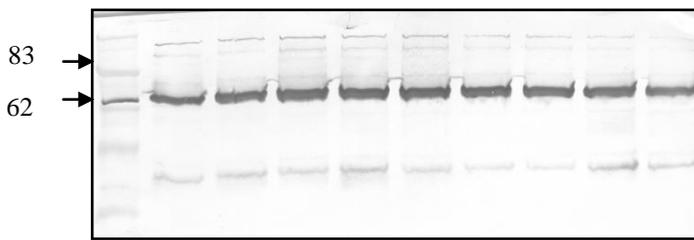


Figure 3.8 (B)

Western blot analysis of fractions 22-54

Probed with rabbit antibody

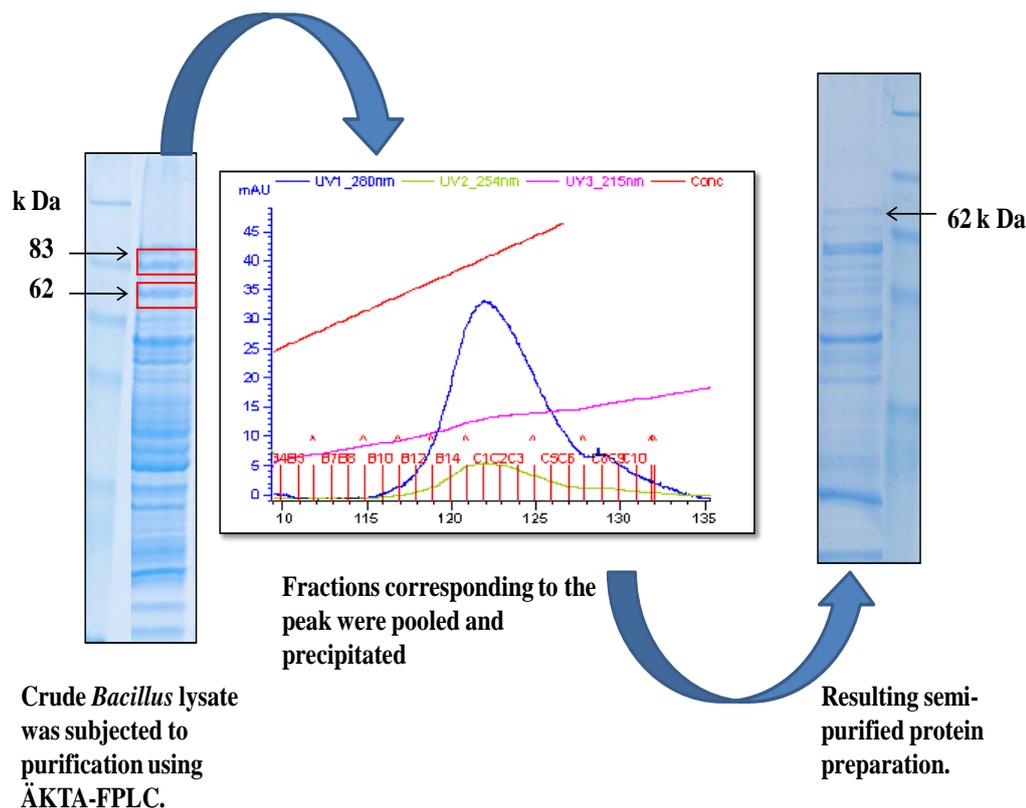


Figure 3.9 Representation of ‘Crude lysate’ and ‘Purified protein preparation’ following ÄKTA fractionation

Bands indicated in the crude lysate at the 62 and 83 kDa regions were excised and subjected to LC/MS analysis (Table 3.2).

All bands present in the purified preparation were excised and subjected to LC/MS as indicated in Figure 3.10 and Table 3.3.

ID	Protein	Species	Accession	Mass	pI	Z-score	Coverage %
62	groEL chaperonin	<i>Bacillus subtilis</i>	1906220B	57470	4.75	375	15%
83	aconitate hydratase	<i>Bacillus sp. SG-1</i>	ZP_01858967	99213	4.94	358	9%.

Table 3.2 Proteins identified by LC/MS corresponding to the two stimulatory *Bacillus* proteins

3.4.2 Liquid Chromatography Mass Spectrometry analysis of fractionated proteins

It has been previously demonstrated that *B. oleronius* produces proteins that cause serum immune reactivity in rosacea patients (Lacey *et al.* 2007; Li *et al.* 2010). To analyse the effect that this bacterium has on human neutrophil and corneal epithelial cells a protein lysate was prepared and purified using ÄKTA FPLC (section 3.4.1). Figure 3.9 shows the SDS-PAGE profile of bacterial protein 'crude lysate' before and the 'purified protein' after fractionation (section 3.4). Protein bands from the 62 kDa and 83 kDa regions of the crude lysate (Figure 3.9) were excised and subjected to Liquid Chromatography Mass Spectrometry and the identified proteins are presented in Table 3.2. The antigenic protein at the 62 kDa region showed homology to a groEL chaperonin protein from *Bacillus subtilis* (accession number 1906220B) with a score of 375 and sequence coverage of 15%. This protein is said to be located in the cytoplasm and is a member of the chaperonin family of heat shock proteins (Hsp) expressed in many bacterial species. Chaperonins assist the folding of newly synthesized polypeptides and its cofactor GroES may be required for proper folding (Khor *et al.* 2004). The 83 kDa band was identified as aconitate hydratase from *Bacillus sp. SG-1* (accession number ZP_01858967) with a score of 358 and sequence coverage of 9%. This is an enzyme of the tricarboxylic acid (TCA) cycle.

The protein bands present in the 'purified preparation' (Figure 3.9 & 3.10) were also excised from the gel, trypsin digested (section 2.22) and analyzed by LC/MS. Peptides were identified by peptide mass finger printing using the Matrix Science Mascot Ions search engine. The proteins are identified in Table 3.4. Band one showed homology to Kat E1 and band three was identified as vegetative catalase both of these proteins have enzymatic properties that function to protect the bacterial cells against reactive oxygen species (ROS). Catalase converts hydrogen peroxide water and oxygen. H_2O_2 is a component of ROS produced by the host immune system in response to pathogens. Catalase from *Mycobacterium tuberculosis* has previously been described as a tissue antigen indicated in the induction of granulomatous inflammation in sarcoidosis (Song *et al.*, 2005). Band two was identified as a hypothetical protein involved in peptidoglycan synthesis. Peptidoglycan is a component of the bacterial cell wall and has been implicated as a pathogen associated molecular molecule (PAMP) which are recognised by cells of the innate immune response (Vance *et al.*, 2009). Only a select group of bacterial molecules act as PAMPs however sensing of bacterial replication or

local alterations in host amino acid and nutrient levels can act as triggers for an immune response (Vance *et al.*, 2009). Band four was homologous to aminopeptidase an enzyme that catalyses the removal of amino acids from the N-terminus of peptides and proteins. Aminopeptidases are involved in the ATP-dependent steps during cytosolic protein degradation in bacteria (Chandu 2003). Band five was identified as an elongation factor which catalyses the GTP dependent ribosomal translocation step during elongation. Bacterial elongation factors have been implicated in interactions with the human immune system via TLR signalling (Kyungwoo, 2011; Nieves *et al.*, 2010; Sharma *et al.*, 2011). Band six was identified as alanine dehydrogenase which is involved in the generation of pyruvate and NADH during respiration. Band seven was homologous to malate dehydrogenase; which catalyses the oxidation of malate to oxaloacetate. Enzymes such as those corresponding to bands 6, 7 and 10 involved in metabolism and respiration within bacterial cells may cause localised changes as they obtain nutrients for cell growth within the host tissue and this may contribute to the stimulation of an immune response (Vance *et al.* 2009). Band eight was identified as flagellin which is produced by bacterial cells to aid flagellar motility. Flagellin is recognised by the host immune response as a PAMP and it can stimulate the production of cytokines via interactions with TLR-5 (Vance *et al.* 2009; Hozono *et al.* 2006). Superoxide dismutase (SOD) was identified as the protein corresponding to band nine and it is involved in protecting the bacterial cells from oxidative stress. Band 10 was identified as alkyl hydroperoxide reductase (AHP) which, is involved in the maintenance of the redox environment within bacterial cells. SOD and AHP are enzymes which can protect bacterial cells against the host immune responses production of oxygen radicals (Cabiscol *et al.*, 2000; Seaver *et al.*, 2001). Both AHP and SOD from *Helicobacter pylori* have been implicated as antigenic proteins (Every *et al.*, 2011; Olsen *et al.*, 2000; Pourakbari *et al.*, 2011).

3.4.3 Summary

B.oleronius proteins were successfully fractionated and analysed by SDS-PAGE in order to identify fractions which contained proteins reactive to the 62 kDa rabbit antibody. These fractions were then pooled and protein bands present in the preparation were trypsin digested and subjected to LC/MS in order to identify the types of proteins

present. Since this protein preparation was to be exposed to human neutrophils and corneal epithelial cells, the ability of individual proteins in the preparation to interact with these cell types was investigated. The purified protein was also used in an to investigate the possibility of developing a diagnostic ELISA for reactivity to *B. oleronius* proteins in sera from individuals with rosacea.

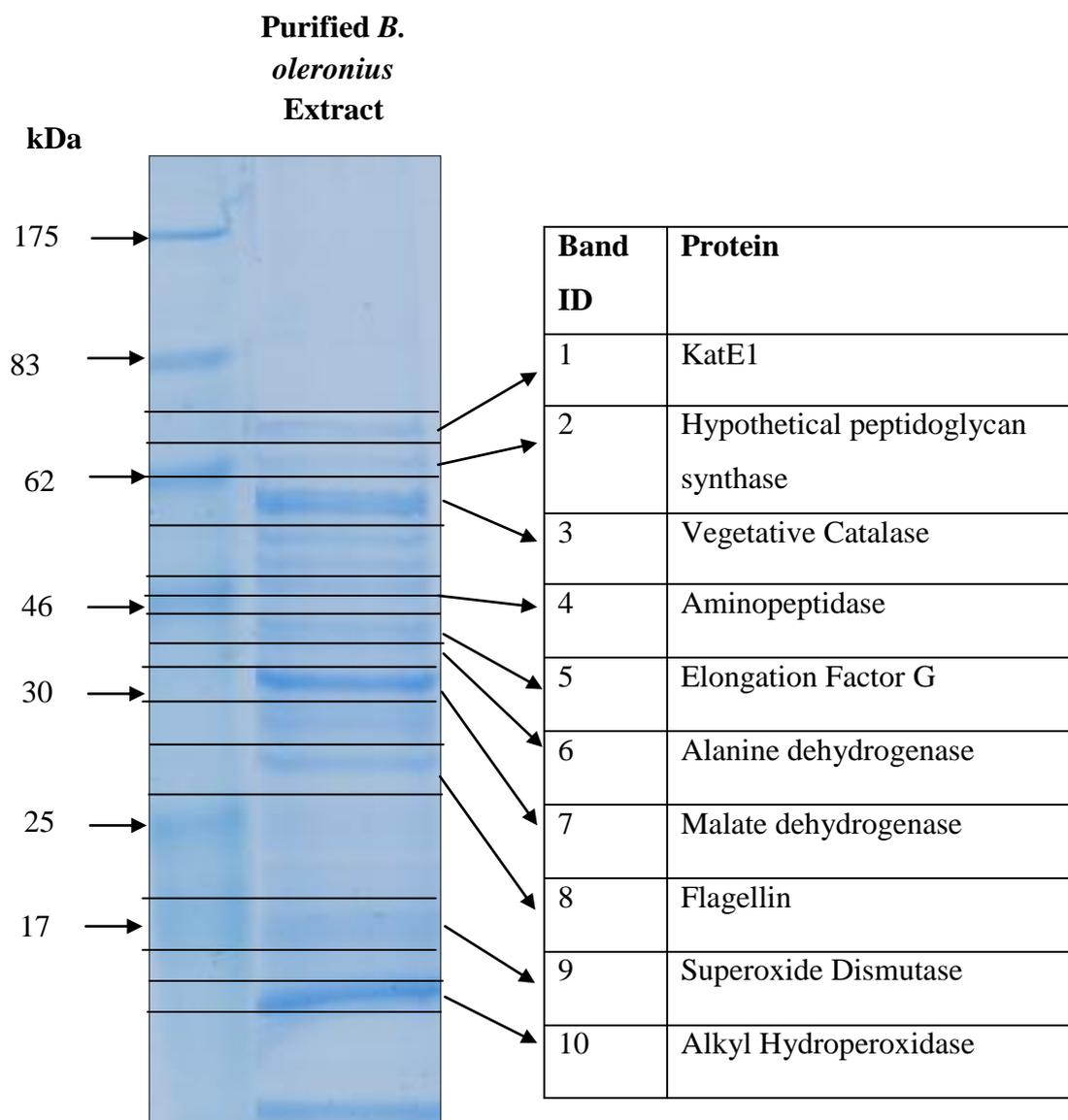


Figure 3.10 SDS-PAGE separation of ‘Purified protein preparation’ following ÄKTA fractionation

Table 3.3 Proteins identified by LC/MS corresponding to the 10 bands indicated in Figure 3.10

Proteins identified in Figure 3.10 and Table 3.3 are listed in further detail in Table 3.4.

Band ID	Protein	Accession	Mass	pI value	Mascot Z-score	Coverage %	Uniprot Function
1	KatE1	YP_093702	54541	5.48	181	7	Response to oxidative stress
2	Hypothetical Protein Noc_2222	YP_344212	40113	5.63	71	4	Peptidoglycan Biosynthetic process
3	Vegetative Catalase 1	YP_081190	54801	6.03	287	22	Decomposes hydrogen peroxide
4	aminopeptidase	YP_081153	48162	8.63	272	10	Catalyzes the removal of amino acids
5	Elongation factor G	YP_077397	76499	4.83	189	9	Tanslocation

Table 3.4 ‘Purified Proteins’ from *Bacillus oleronius* identified by LC-MS

Band ID	Protein	Accession	Mass	pI value	Mascot Z-score	Coverage %	Uniprot Function
6	alanine dehydrogenase	ZP_01859623	39777	5.04	74	6	Metabolism
7	malate dehydrogenase	YP_080204	33634	4.99	380	40	Metabolism
8	Flagellin	BAB58982	26512	5.09	189	20	Ciliary or flagellar motility
9	Superoxide dismutase	YP_079829	22530	5.33	171	25	Destroys oxygen radicals
10	Alkyl Hydroperoxide Reductas	YP_081364	20874	4.66	237	27	Maintenance of redox environment

Table 3.4 (Continued) ‘Purified Proteins’ from *Bacillus oleronius* identified by LC-MS

3.5 Use of fractionated proteins to investigate of the possibility of establishing an ELISA based assay for rosacea

The Enzyme-Linked Immunosorbent Assay (ELISA) technique was developed in the 1970s by Peter Perlman and Eva Engvall (Engvall & Perlmann 1971). Since then it has become a extremely important technique in the research and clinical setting (Lequin 2005) and can be used to test an large number of protein interactions. In the assay format proposed in this work the purified *B. oleronius* protein (1 µg/ml) was coated to a 96 well plate and its antigenic potential to bind antibodies in rosacea patient and control sera was analysed using a tetramethylbenzidine (TMB) substrate to detect horseradish-peroxidase bound to the secondary anti-human IgG antibody (diluted 1 in 2000). Reactivity was read using a spectrophotometer at 450 nm.

3.5.1 Investigation of reactivity of Rabbit 62 kDa antibody to purified *B. oleronius* protein in an ELISA format

In order to investigate the potential to develop an assay for use as a diagnostic aid, the fractionated protein was used to coat 96 well plates to form the basis for an ELISA test as described in section 2.15. Firstly the specificity of the bacterial protein was investigated (Figure 3.11). A serial dilution of the anti-62 kDa antibody (Lacey *et al.*, 2007) was made in 1% PBS-tween starting with a neat sample of the antibody then adding a dilution factor of 10 until a 1/100000 was reached, a detectable signal was achieved with the 1/1000 dilution and the more concentrated samples caused a signal to high to be read on the spectrophotometer. Increased binding of the antibody to the protein was detected as the dilution factor of the antibody decreased which signified that the protein used to coat the ELISA plate was reactive with the anti 62 kDa antibody. A 1% BSA solution made in PBS was added to the plate to test for unspecific binding to the *Bacillus* protein which was minimal in this assay when compared to the signal observed in when the rabbit antibody was tested. To further investigate the unspecific binding of the 62 kDa antibody to BSA an assay comparing a *Bacillus* protein coated plate and a 1% BSA coated plate was performed (Figure 3.12). However the unspecific binding of the antibody to 1 % BSA increased as the dilution factor decreased.

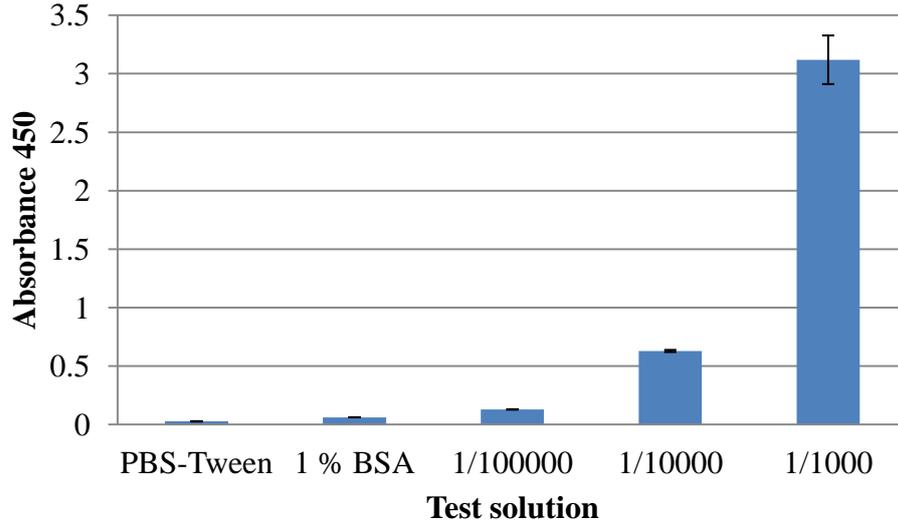


Figure 3.11 Reactivity of the Anti-62 kDa rabbit antibody to the *Bacillus* protein used to coat ELISA plates.

The results show binding of the anti-62 kDa antibody to the protein on the plate increased as the dilution factor decreased.

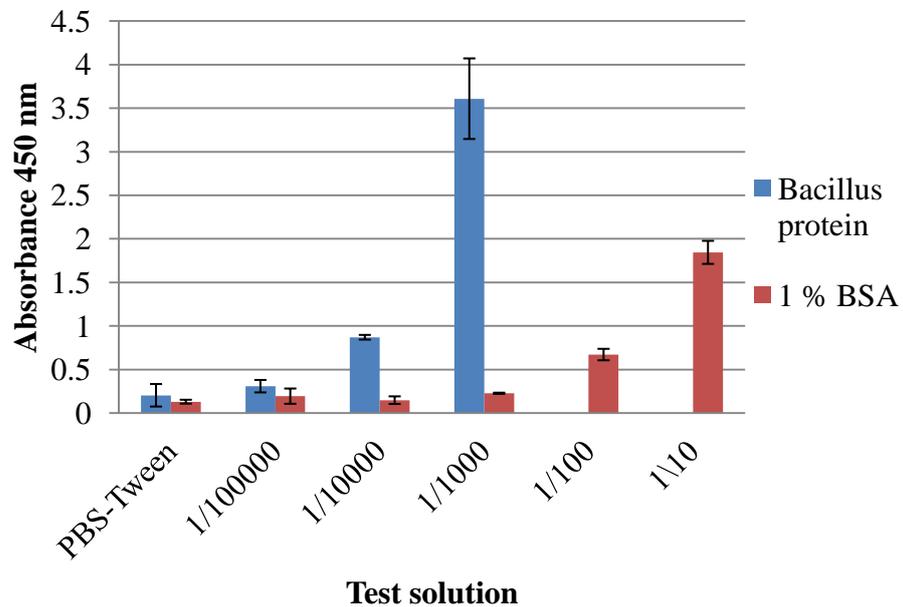


Figure 3.12 Reactivity of the Anti-62 kDa rabbit antibody to the *Bacillus* protein used to coat ELISA plates.

The results show binding of the anti-62 kDa antibody to the protein on the plate increased as the dilution factor decreased. However the unspecific binding of the antibody to 1 % BSA also increased as the dilution factor decreased.

3.5.2 Investigation of reactivity of rosacea patient sera and control sera to purified *B. oleronius* protein in an ELISA format

It was decided to investigate the response of rosacea patient and control sera in a number of formats to investigate if the specificity would be improved compared to the rabbit antibody assay. The format of the test remained the same as that described in section 2.15 and section 3.5.1 where 1 µg/ml of the fractionated protein was coated to a 96 well plate and binding of serum antibodies was detected using TMB. Initial results proved encouraging as there was a statistically significant increase of 2 fold ($p=0.023$) in binding of serum antibodies to antigen coated plates when rosacea positive sera (Figure 3.13) was compared to control sera (Figure 3.14), these results were observed when a 1/1000 dilution of the serum was used. Patient sera diluted 1/100 showed increased binding compared to controls of 1.4 fold however this was not statistically significant ($p=0.09$).

There was also no significant difference between binding of patient and control sera to the BSA coated wells. The serum immunoblot in Figure 3.14 indicates two issues that could be detrimental to the ELISA assay format performed in this work. The red ovals (Figure 3.14) indicate (1) that sera that can be correctly distinguished as control with the immunoblot due to the absence of the 62 kDa band could still show reactivity to the 83 kDa band and (2) the protein preparation did not contain the 62 kDa protein alone (section 3.4, Table 3.4). Therefore the strong reactivity indicated to bands below the 62 kDa region could have caused detection of reactivity from control sera with the ELISA. There was an increase of 3.4 fold ($p=0.01$) detected in case 3 compared to case 10 diluted 1/1000 and an increase of 3.2 fold ($p=0.02$) detected at the 1/100 dilution.

It was decided to investigate the use of 1% BSA as a diluent for the sera to try to decrease the unspecific binding thought to be causing increased reactivity in some control cases. Patient sera did not show a statistically significant increase in binding to *Bacillus* protein ($p=0.073$) or BSA (0.044) when compared to controls. The highest absorbance recorded among the patient sera was in sample 7 (OD 450 = 0.9) (Figure 3.14 B) and this was reduced only slightly with the used of the BSA diluent compared to in Figure 3.13 where an OD 450 of 1 was recorded. Sample 2 showed the highest OD in the controls when this was compared to patient sample 7 a 3 fold ($p=0.013$) increase was recorded.

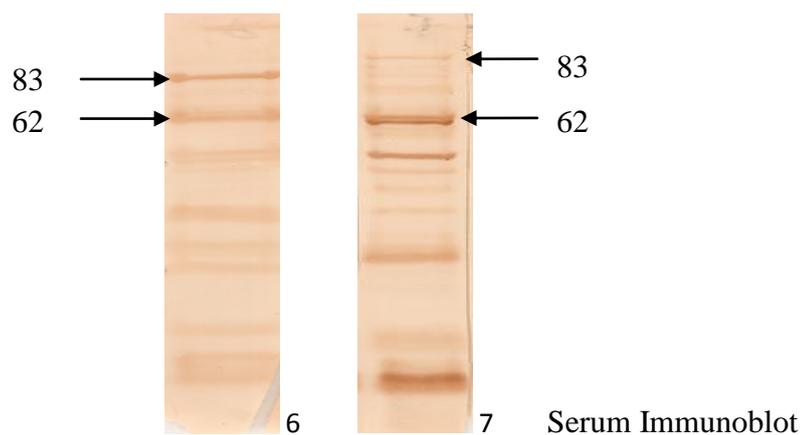
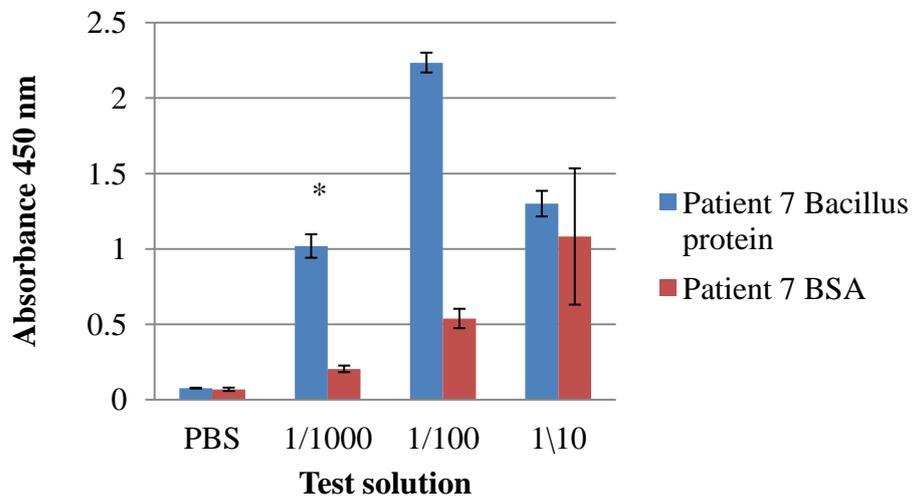
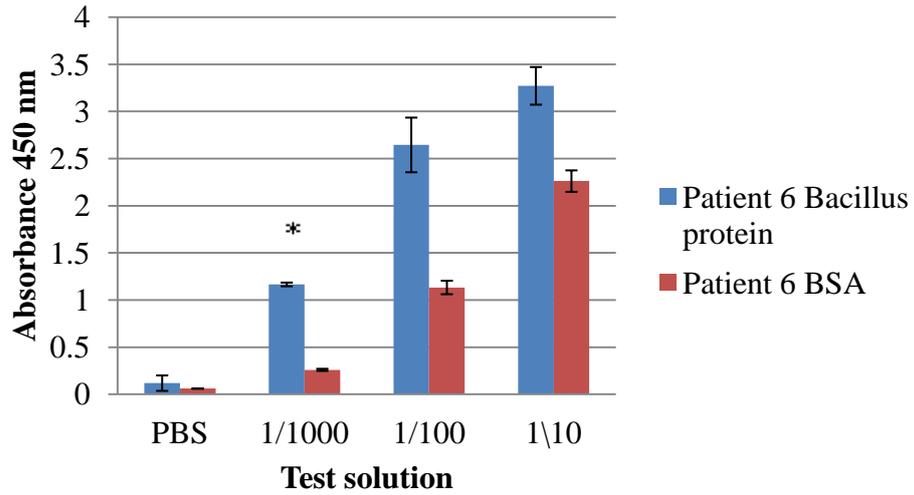


Figure 3.13 Reactivity of two serum positive rosacea patients (case 6 & 7) to purified *Bacillus* protein in an ELISA assay

Detection of patient sera antibodies bound to *Bacillus* protein was 2 fold ($p=0.023^*$) increased in patient sera compared to control sera (Figure 3.14) diluted 1/1000.

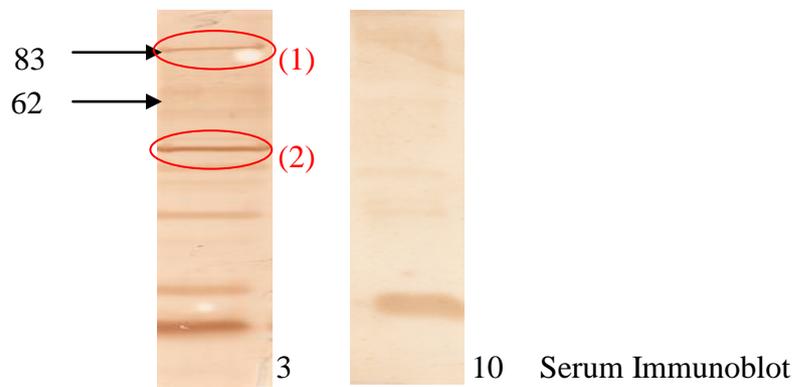
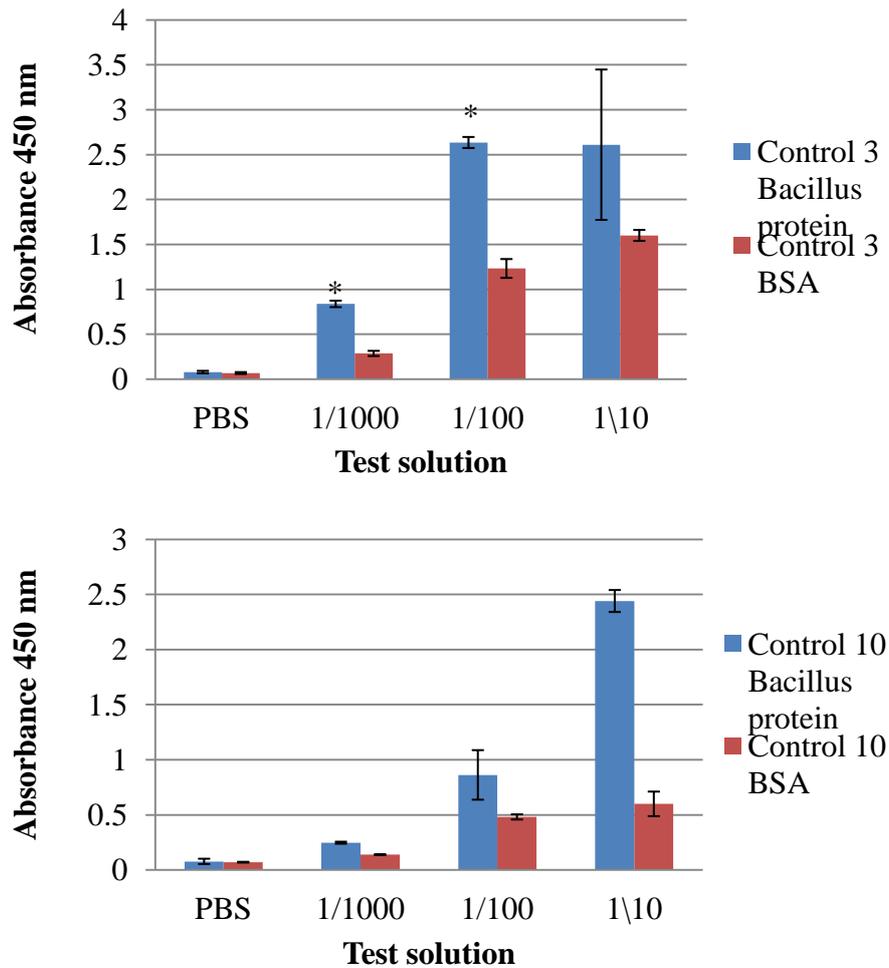
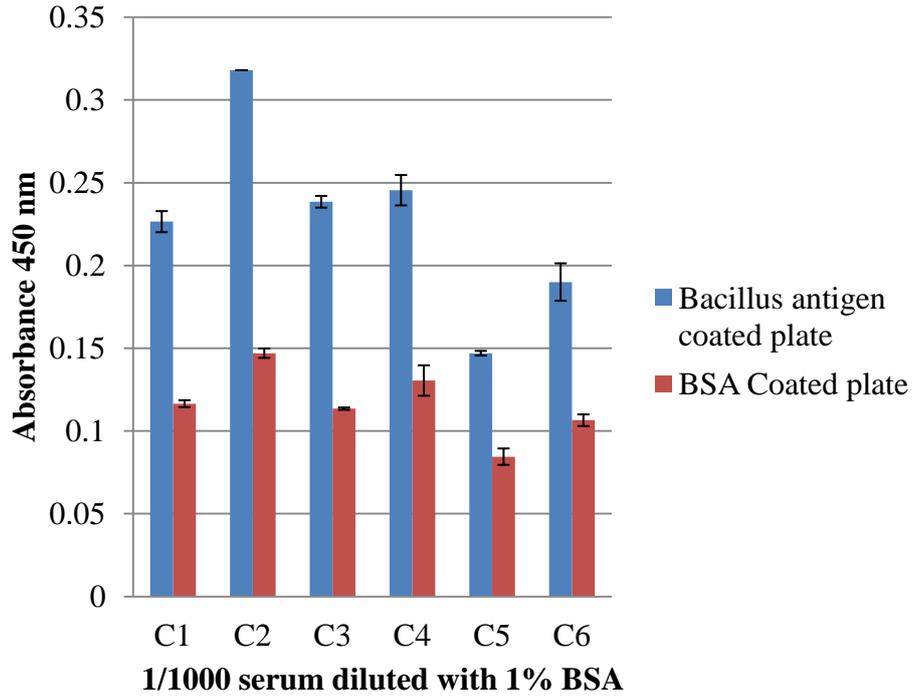
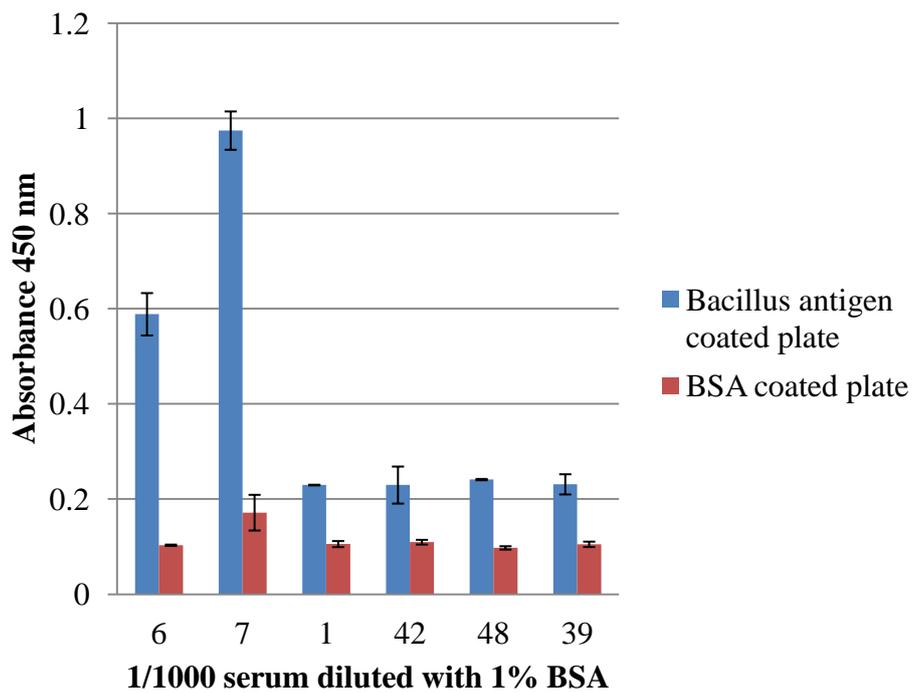


Figure 3.14 Reactivity of two controls (case 3 & 10) to purified *Bacillus* protein in an ELISA assay

There was no significant difference when a comparison was made between binding of patient and control sera to BSA with 1/100 or 1/1000 dilutions of sera. Possible causes for increased reactivity in control case 3 compared to control 10 are highlighted in the red circles (1 & 2). In this case control sera showed reactivity to the 83 kDa (1) protein and to a band (2) below the 62 kDa protein which may be present in the semi-pure protein preparation. There was an increase of 3.4 fold ($p=0.01^*$) detected in case 3 compared to case 10 diluted 1/1000.



A



B

Figure 3.14 Reactivity of six control (A) and six rosacea patient (B) sera diluted in 1% BSA to purified *Bacillus* protein and BSA in an ELISA assay

Patient sera did not show a statistically significant increase in binding to *Bacillus* protein ($p=0.073$) or BSA (0.044) when compared to controls.

Despite the promising initial results that prove this assay is capable of detecting reactivity in rosacea patient sera the high reactivity detected among some of the controls suggests the assay is not suitable as a diagnostic in its current format. Following trials to identify the optimum diluents with a small amount of samples, the assay was tested on 49 sera samples from the ocular rosacea study (Li *et al.*, 2010). All sera were diluted 1/100 in PBS-T with 1% BSA and divided into group 1 (Positive serum reactivity with Western blot) and group 2 (Control sera). The ELISA was carried out as described in section 2.15. It is evident in Figures 3.15 A & B that some of the sera obtained from rosacea patients was only weakly reactive in this assay while some of the controls showed strong reactivity to proteins present on the ELISA plates. The average reactivity at OD 450 recorded from the pooled positive samples was 0.933 while the average absorbance detected from the control sera was 0.869 there was no statistical significance between the 2 groups ($p=0.27$). Proof that *B. oleronius* antigens could be used as a diagnostic marker remains unclear however the use of a completely pure sample of the 62 kDa antigen obtained with recombinant techniques could potentially improve the outcome of the assay.

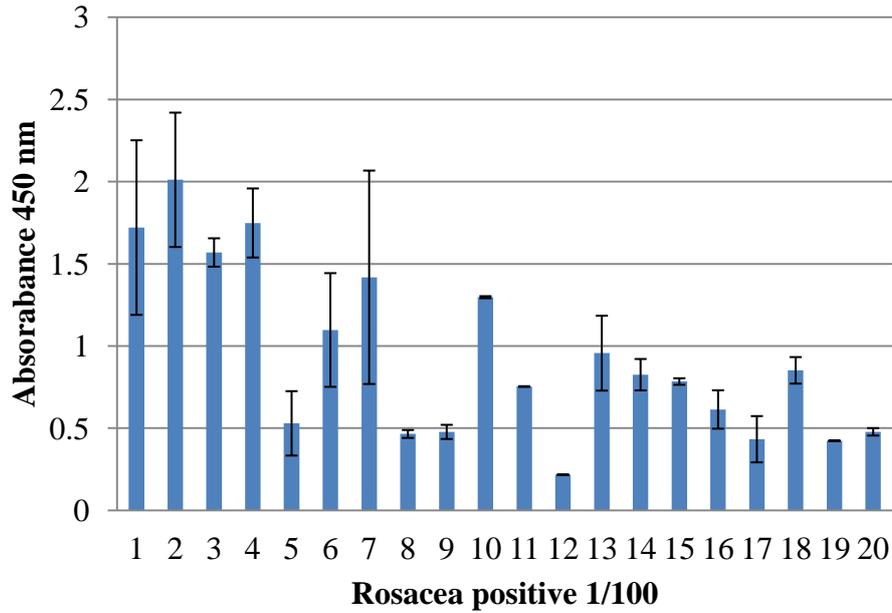


Figure 3.15 (A) ELISA representing reactivity of sera samples obtained from Ocular Rosacea (Group 1) patients to the ‘purified’ *Bacillus* protein.

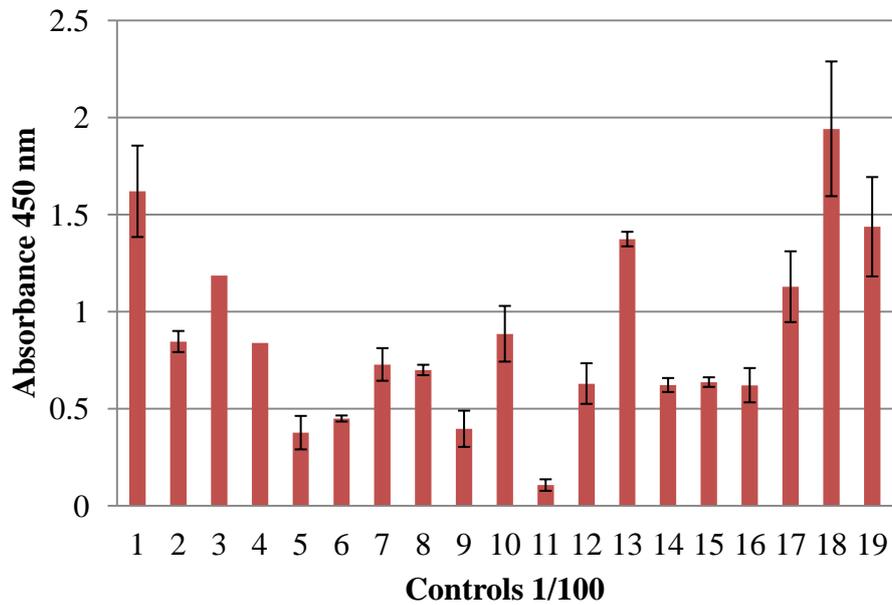


Figure 3.15 (B) ELISA representing reactivity of sera samples obtained from controls (Group 2) to the ‘purified’ *Bacillus* protein.

A fold change of 1.07 was detected using a 2-way anova test between the average absorbance of the controls (0.0869) compared to the average absorbance of the rosacea patients (0.933) (p=0.027).

3.6 Discussion

In this Chapter the Western blot method employed previously by Delaney, (2004) and Lacey, (2007) was used to blind test serum samples for reactivity to *Bacillus oleronius* antigens. In section 3.4.2 the 62 kDa protein was identified as a groEL chaperonin protein from GroEL is analogous to the human chaperonin hsp60 which is said to have pro-inflammatory cytokine like properties and has a role in folding of essential oxygen scavenging enzymes such as manganese superoxide dismutase (Khor *et al.*, 2004). Bacterial Hsps are known to have antigenic properties and proteins homologous to GroEL have been identified in, *Escherichia coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and *Burkholderia pseudomallei* (Shinnick *et al.*, 1988; Woo *et al.*, 2001). The 83 kDa band was identified as aconitate hydratase. The function of this protein is in the utilization of glucose to obtain energy for the cell the TCA cycle is active in times of low glucose availability and is repressed by excessive glucose (Hecker *et al.*, 2008). Aconitate hydratase has been identified in *Salmonella enterica* cells responding to H₂O₂ exposure (Kim *et al.*, 2010).

Serological reactivity to these proteins in rosacea patients indicated prior exposure to *B. oleronius*. Sera samples were analysed for reactivity to stimulatory *Bacillus* proteins and after establishing the presence of antibodies towards the 62 or 83 kDa proteins, the identity of the serum was predicted as either control or patient. Collaborating clinicians then revealed the case numbers for the sera that originated from patients and controls and correlations were recorded. In a study of individuals with ocular rosacea 66% of patients had reactivity to one or both of the stimulatory proteins in their serum and 68% of controls showed a negative response. In a study of individuals with erythematotelangiectatic rosacea 92% of patients showed a positive serum response while 100% of controls showed a negative serum response. When results of both the ocular rosacea (section 3.2.1) and the erythematotelangiectatic rosacea (section 3.2.2) studies were considered 76% of patients were serum positive and 72% of controls were serum negative. In the case of at least some of the serum negative cases that were later revealed to be rosacea patients it has been postulated that these patients may have received treatments that reduced the density of *Demodex* in their skin and therefore the presence of bacteria. These results indicate a positive correlation between serum immune response to *B. oleronius* proteins in rosacea patients. Results

from the ocular rosacea study involving 59 individuals were published in Li *et al.*, (2010) and significant correlations between positive serum immunoreactivity and facial rosacea ($p=0.009$) and ocular *Demodex* infestation ($p=0.048$) were reported. Lid margin inflammation was graded in patients and a significant correlation between it and positive serum reaction was recorded ($p=0.040$). Patients in the study with facial rosacea were also found to have significantly higher *Demodex* counts than those without ($p=0.014$) (Li *et al.*, 2010). These findings link serum reactivity to the 62 and 83 kDa *Bacillus* antigens to ocular *Demodex* infestation and ocular surface inflammation for the first time and add to the evidence that bacterial antigens may be responsible for the dysregulated immune response and chronic inflammation seen in rosacea.

Detection of specific proteins expressed in human tear fluid has been suggested as an indicator of ocular surface disease (Cheng *et al.*, 1996; Spurr-Michaud *et al.*, 2007; An *et al.*, 2005; Määttä *et al.*, 2006; Pong *et al.*, 2010). The protein hemopexin was detected at increased levels in the tear fluid of patients with the ocular inflammatory disease vernal keratoconjunctivitis (VKC) compared to controls. Analysis of tear fluid using 2D-electrophoresis and ELISA were used in the study (Pong *et al.* 2010). Elevated tear fluid levels of MMP-8 have been detected in rosacea patient compared to controls (Kari & Kari, 2005) using immunofluorometric and immunoblotting assays. Analysis of reactivity of tear fluid to *Bacillus* antigens was difficult to assess using the Western blot method. Although reactivity was found in 6 cases (numbers 16, 18, 22, 23, 40 & 45) that were all later revealed to be patients (Figure 3.4), these results were not reproducible due to the small amount of sample available. Cases 22, 23, 40 and 45 were all positive for both the 62 and 83 kDa antigens using the immunoblotting technique and had been predicted as patients. Cases 16 and 18 showed weak reactivity to only the 62 kDa antigen using immunoblotting and were labelled as controls however upon clinical examination these individuals were diagnosed as rosacea positive. This result may indicate that detection of *B. oleronius* proteins in the tear fluid could occur prior to the detection of the proteins in patient sera. Although the Western blotting technique employed for this work was not successful in detecting all patient samples, an altered assay format for the testing of *B. oleronius* proteins in ocular rosacea patient tear fluid such as an ELISA could be useful.

Due to the work outlined in this Chapter and the previous work by Delaney (2004) and Lacey (2007) there is evidence that bacterial antigens contribute to the inflammation seen in rosacea. In order to further analyse which bacterial antigens could be inducing the immune response, *B. oleronius* proteins were subjected to Liquid Chromatography Mass Spectrometry (section 3.4). Bacterial outer membrane proteins were solubilised using a lysis buffer containing Triton-X 100 detergent coupled with sonication. The proteins were then fractionated (section 3.4.1), separated by SDS-PAGE and individual bands were excised and their peptide make up analysed by LC-MS (section 3.4.2). Previous studies (Delaney, 2004; Lacey, 2007) had identified the 62 and 83 kDa proteins as causing serum reactivity in rosacea patients and an antibody that was reactive to these proteins was generated. Therefore the fractionated proteins were blotted for reactivity to this antibody (Figure 3.6 A-3.8 B) in order to identify which fractions should be pooled for use in human cell and serum ELISA assays.

Streptococcal antigens or superantigens have been implicated in inflammatory guttate psoriasis (Leung *et al.*, 1995), it is thought that the increase infiltration of T-cells observed in psoriasis lesions is mediated by these bacterial super antigens. Antigenic material isolated from the bacterium *Propionibacterium acnes* has been implicated in the inflammation of acne since T-cell proliferation has been seen in response to bacterial culture supernatant (Jappe *et al.*, 2002). LC/MS analysis revealed that a number of the *Bacillus* proteins identified were capable of interacting with the host immune response and some had been implicated as serum antigens in human disease caused by bacteria. Flagellin was one of the proteins identified and is a known ligand for TLR-5 and therefore can stimulate neutrophils to produce cytokines such as IL-8 and TNF- α and contribute to the induction of inflammation (Vance *et al.*, 2006). The human immune system is designed to recognize a number of molecules produced by microbes that are termed pathogen-associated molecular patterns (PAMPs). Recognition of these molecules leads to stimulation of the innate and eventually the adaptive immune responses. However PAMPs are not always associated with pathogenic microbes (Cookson *et al.*, 2001; Gallo & Nizet, 2008). Peptidoglycan recognition is a trigger for the innate immune response (Dziarski 2004) a hypothetical Noc protein identified in the 'purified antigen preparation' was identified as a peptidoglycan synthesis protein. A study by Travis (2000), has highlighted the use of drugs to disrupt metabolic pathways as a new target for treatment of pathogenic bacteria. Several

enzymes involved in metabolic pathways were identified by the LC/MS analysis including; aminopeptidase, alanine dehydrogenase, malate dehydrogenase these proteins could contribute to the induction of inflammation by altering the local homeostasis of the pilo-sebaceous unit within the host (Vance et al. 2009).

A number of the proteins identified were involved in the bacterial response to oxidative stress, such as Kat E1, vegetative catalase 1, superoxide dismutase (SOD) and alkyl hydroperoxide reductase (AHP). Increased production of ROS has been implicated in rosacea patient skin (Tisma *et al.*, 2009; Yamasaki & Gallo, 2009) therefore it is possible that bacteria such a *B. oleronius* present in the pilo-sebaceous unit could produce increased levels of these oxidative response proteins thus triggering an antigenic response. A study by Jeavons *et al.*, (1998) describes the isolation of proteins from *Penicillium marneffe* which caused immunoreactivity in human sera one of which had homology to catalase. Catalase from *Mycobacterium tuberculosis* has also been detected as an antigenic protein in individuals affected by sarcoidosis a disease which is characterized by granulomatous inflammation (Song *et al.*, 2005). SOD from *Mycobacterium paratuberculosis* has been suggested as an antigen that could be used as a potential vaccine for cattle (Mullerad *et al.*, 2002) due to detection of serum reactivity in mice infected with the bacterium. Proteins such as SOD and AHP have been suggested as vaccine components for *Helicobacter pylori* infection which can cause gastric cancer (Every et al. 2011; Pourakbari et al. 2011; I. Olsen et al. 2000). These proteins represent good targets in this case because they are essential to the survival of the cell in the hostile environment of the stomach (Every et al. 2011). The use of AHP protein in an assay for the detection of *H. pylori* infection in human stool was investigate using a purified recombinant rabbit antibody which detect the presence of a 26 kDa antigen in stool of 83.3% (40/48) positive samples (Pourakbari *et al.*, 2011).

Elongation factor G was also among the identified proteins and several studies have reported proteins with elongation and translational functions in the ribosome as antigenic (Shin *et al.*, 2007; Shin *et al.*, 2009). An elongation factor (EF-Tu) from *Francisella novidica* which causes severe pulmonary disease was detected in the serum of mice vaccinated with an attenuated form of the bacteria suggesting an antigenic role for this protein (Sharma et al. 2011). The study also described the ability of a recombinant EF-Tu from *F. novidica* to induced macrophages to produce inflammatory cytokines in a TLR4 dependent manner suggesting an immune stimulatory role for the

protein. A study of the pathogenic bacterium *Burkholderia pseudomallei* has also identified EF-Tu as a potential vaccine to protect against melioidosis which is endemic in Southeast Asia (Nieves et al. 2010).

The identification of specific antibodies in patient serum samples can provide a tool for the diagnosis of disease (Engvall & Perlmann 1971). As mentioned previously the exact etiology of rosacea is not fully understood and diagnosis of the condition can also be difficult due to the similarities to other conditions such as acne, dermatitis and conjunctivitis. Oltz & Check, (2011) contains a Table of the differential diagnoses for rosacea that lists 16 of the conditions that can be mistaken as rosacea. If patients are misdiagnosed with one of these conditions and do not receive prompt treatment for ocular rosacea they are at risk of developing serious symptoms that can lead to ulceration of the cornea and visual impairment. To investigate the potential to provide a diagnostic assay for rosacea Fast Performance Liquid Chromatography was employed to fractionate immune stimulatory proteins in particular the 62 kDa protein (Table 3.2). Fractionated *Bacillus* proteins (Table 3.4) were coated onto 96 well plates so that an ELISA protocol could be employed to analyse the response of serum to the antigens. As discussed previously several of the proteins identified in section 3.4 have the potential to interact with components of the immune response. A protein homologous to GroEL (detected in this work at the reactive 62 kDa region) has been indicated as a possible marker for *Burkholderia pseudomallei* (Woo et al. 2001) in individuals suffering from melioidosis.

ELISAs provide a more quantitative analysis technique than Western blotting and only 1 µg/ml of the antigen preparation was required to coat the plates (section 2.15). Initial tests showed reactivity with the rabbit antibody generated towards the 62 kDa *B. oleronius* protein (Lacey, 2007) proving that the ELISA format was suitable for binding of the antigen (ie: the *Bacillus* protein preparation section 3.4) to the antibody (Figure 3.11 & 3.12). When this was established testing of human serum was carried out (Figures 3.13 – 3.15). When human serum was tested for reactivity in the ELISA format it was difficult to distinguish patients from controls in some cases. Diagnostic ELISAs usually employ recombinant monoclonal proteins while the assay described in this work was performed using a protein preparation purified by fractionation that contained multiple potentially serum reactive proteins. In the clinical setting such a test could potentially provide a distinction between ocular rosacea and other ocular inflammatory

conditions such as blepharitis. The results described in this work suggest that a more refined ELISA format could potentially provide a useful diagnostic tool.

CHAPTER 4

**An investigation of the effect of
Demodex-associated *Bacillus oleronius*
proteins on human neutrophils**

4.0 Introduction

Rosacea is a chronic inflammatory disorder that involves lack of regulation of the innate immune system, but the exact etiology of the condition is not fully understood (Crawford *et al.*, 2004). According to the National Rosacea Society over 16 million Americans suffer with rosacea and 70% of patients surveyed in 2006 reported low self esteem due to the condition (Fimmel *et al.*, 2008). Symptoms usually occur around the central region of the face and vary across the four subtypes (papulopustular, erythematotelangiectatic, ocular and phytamous) of the condition (Wilkin *et al.* 2004). Patients usually present with one of the following; erythema (transient or persistent), papules and pustules or telangiectasia, in conjunction with secondary features such as edema, burning and stinging skin or ocular manifestations (Dahl *et al.* 2002). A better understanding of the molecular mechanisms involved in the condition is emerging and the action of neutrophils has been linked to the development of many symptoms observed in this condition (Yamasaki & Gallo, 2009).

In this Chapter the effects of *Demodex*-associated *Bacillus* antigens on neutrophils were examined. *Demodex* are saprophytic mites located in the pilosebaceous unit of the skin and the eyelashes. Two species are found to inhabit the human skin, *Demodex folliculorum* in the hair follicles and eyelashes and *Demodex brevis* deeper in the sebaceous glands and meibomian glands of the eyes (Kheirkhah *et al.*, 2007). There is support in the literature for a correlation between increased mite density and the occurrence of rosacea (Bonnar *et al.*, 1993; Gao *et al.*, 2005; Li *et al.*, 2010). However these mites remain non-pathogenic to most people and there is no definitive proof that the high level of *Demodex* in rosacea patients is an etiological correlation rather than a coincidence (Dhingra *et al.*, 2009). It could be the case that the skin of individuals with rosacea is favourable for the proliferation of these mites and that in turn leads to the high density which could lead to a pathogenic role in rosacea.

Previous work by Delaney (2004) lead to the isolation of a bacterium from a *Demodex* mite from the face of a papulopustular rosacea patient. This bacterium, *Bacillus oleronius*, produces antigens that can initiate peripheral blood mononuclear cell (PBMC) proliferation in 73% of rosacea patients compared to 29% of controls ($p = 0.0105$) (Lacey *et al.*, 2007). Interestingly the growth of this bacterium was found to be

susceptible to metronidazole and tetracycline which are used in the treatment of rosacea (Lacey *et al.*, 2007). Proteins expressed by *Bacillus oleronius* were examined using Liquid Chromatography-Mass Spectrometry (Section 3.4.2). The function of these proteins was outlined (Table 3.2) and the antigenic potential of many of these proteins was discussed (Section 3.6).

Bacterial superantigens have previously been implicated in the induction of the inflammatory skin disorder psoriasis (Leung *et al.*, 1995). Many reports have focused on the possibility that microbes lead to the exacerbation of the innate immune response seen in rosacea patients (Elewski *et al.*, 2010; Whitfeld *et al.*, 2011, Yamasaki *et al.*, 2009). Bacteria such as *Staphylococcus epidermidis* and *Helicobacter pylori* have been discussed as possible contributors to the exacerbation of inflammation in rosacea (Whitfeld *et al.*, 2011, Utaş, *et al.*, 1999). Interestingly *S. epidermidis* isolated from rosacea patients were consistently β -hemolytic while bacteria from controls were non-hemolytic when cultured on blood agar (Dahl *et al.*, 2004). In addition, this bacterium has been isolated from the pustules of patients with papulopustular rosacea and from the eyelids of patients with ocular rosacea indicating a possible role for this bacterium in the induction of these conditions (Whitfeld *et al.*, 2011).

The evidence for microbial involvement in the condition is augmented by investigating the typical treatments that are effective against the four subtypes of rosacea. Traditional therapies for treatment of rosacea include tetracyclines, metronidazole and doxycycline which are well known antibiotics but also have highly effective anti-inflammatory properties (Bartholomew *et al.*, 1982; Monk, *et al.*, 2011; van Zuuren, *et al.*, 2007). Tetracyclines are known inhibitors of matrix metalloproteinases (MMPs), over expression of which has previously been linked to rosacea (Jang, *et al.*, 2010; Monk *et al.*, 2011). Treatment with these drugs can also reduce inflammation by inhibition of neutrophil chemotaxis and regulation of cytokine production. Another primary treatment for rosacea is azelaic acid which can be applied as a 15% gel to relieve inflammation by reducing reactive oxygen species (ROS) produced by neutrophils (Oltz & Check 2011).

Neutrophils are part of the first line of defence of the body against harmful stimuli. The innate immune response is delicately balanced in healthy individuals, however over expression of its components have been implicated in a range of

inflammatory conditions such as psoriasis and atopic dermatitis (Bevins & Liu, 2007). Neutrophils are granular leukocytes that can engulf and kill microorganisms (Vance *et al.*, 2009). Their killing mechanism can prove harmful to the surrounding tissue due to the release of ROS which are generated during the oxidative burst triggered by activation of NADPH oxidase (Curnutte *et al.*, 1987; Boh, 1996; Segal, 2005). Higher levels of ROS have been reported in rosacea skin and these free radicals can damage the skin by decreasing collagen synthesis and mediating cytokine release (Monk *et al.* 2011). Infiltration of neutrophils followed by the accumulation of pro-inflammatory mediators such as cytokines and reactive oxygen species along with proteolytic enzymes lead to the development of inflammatory lesions (Elewski *et al.* 2010). The tissue damage caused in the area surrounding the inflammation could induce erythema and angiogenesis, which contributes to the development of telangiectasia, one of the main symptoms of rosacea.

The aim of the work presented here was to investigate the response of neutrophils to antigens from *B. oleronius* as it was hypothesized that this *Demodex*-associated bacterium could be involved in the induction of rosacea. The goal was to elucidate whether these antigens could stimulate neutrophils and so lead to the localised inflammation that is key to rosacea pathogenesis. Throughout the work described here phorbol myristate acetate (PMA) was employed at a concentration of 2 µg/ml as a positive control. PMA is capable of modulating NADPH oxidation in human neutrophils (Van Epps *et al.*, 1985) which facilitates the uptake of oxygen and the generation of superoxide radicals during phagocytosis and degranulation. Therefore comparisons could be made between activated neutrophils and neutrophils exposed to the *Bacillus* proteins. The purification of *Bacillus* proteins is described in Chapter 3 and LC/MS identification of 'purified proteins' in this preparation was outlined in Table 3.4.4. When exposure to 'crude protein' is mentioned this refers to the protein extracted from *B. oleronius* prior to fractionation.

4.1 The effect of *Bacillus* antigens on human neutrophil migration

Neutrophils are motile cells that migrate toward the site of infection or tissue damage in the body in response to a number of signals including cytokines. Blood vessels become dilated during an inflammatory response which allows blood flow to slow down and ligands are expressed on the endothelial cells on the surface of the vessels that attract surface molecules on the neutrophils. The result of these changes is the recruitment of neutrophils into the tissue (Hague & Jones 2008). For this experiment neutrophils were isolated (section 2.26) from whole blood of healthy donors and pooled in order to examine the effects of the *Bacillus oleronius* protein extract (section 2.14) on cell migration using porous membranes to similar to the endothelial surface. The cells were treated with 2 or 6 µg/ml of the purified *Bacillus* protein (section 3.4.2, Table 3.3) or 2 µg/ml of the crude protein and incubated at 37 °C with 5% CO₂ for 1 hour. During this incubation period 24 well culture plates were set up as described in section 2.27 before addition of the treated cells and further incubation for 24 hours. After 24 hours the cell migration was quantified using a crystal violet dye elution method described in section 2.27. Cells were also photographed using an Olympus camera.

Results from experiments that used both 3 and 8 µm porous inserts showed increased migration of neutrophils across the membrane when treated with the *Bacillus* protein compared to control cells. Initial results (Figure 4.2) were promising, in images A-F it can be seen that there are more cells present on the membrane following treatment with the bacterial antigens. In this case the assay was incubated for only 1 hour and the 8 µm inserts were used, when the cells were eluted there was a significant increase in the amount of migrated cells $p < 0.05$ with two of the antigen treatments (4 µg/ml purified antigen and 1 µg/ml crude antigen). It was decided that the assays would be incubated over night and Figure 4.2 shows that the number of cells migrating increased more uniformly as the treatment concentration increased. However the assay was not completely reproducible and one reason for this may be the elution of crystal violet that was staining the insert rather than the cells may have lead to an increase in the absorbance reading. The 2 µg/ml purified treatment caused an average increase of 2 fold ($p = 0.031$) across 3 replicates of the assay which was statistically significant. Images of the stained cells before elution show increased numbers of cells had migrated following treatment with the *Bacillus* antigens.

In order to examine if the lack of reproducible result was due to the pore size being too big for the cells a 3 μm porous insert was employed. Results represented in Figure 4.3 show migration of treated cells compared to controls was significantly increase by 4.3 fold ($p = 0.02$) and 6 fold (0.001) respectively following exposure to 2 $\mu\text{g/ml}$ and 6 $\mu\text{g/ml}$ of purified antigens and a 7 fold increase was recorded following exposure to 2 $\mu\text{g/ml}$ (0.027) of the crude antigens. The images in Figure 4.3 also compliment the statistical analysis by showing increased numbers of migrated cells on the membranes following treatment with the bacterial protein.

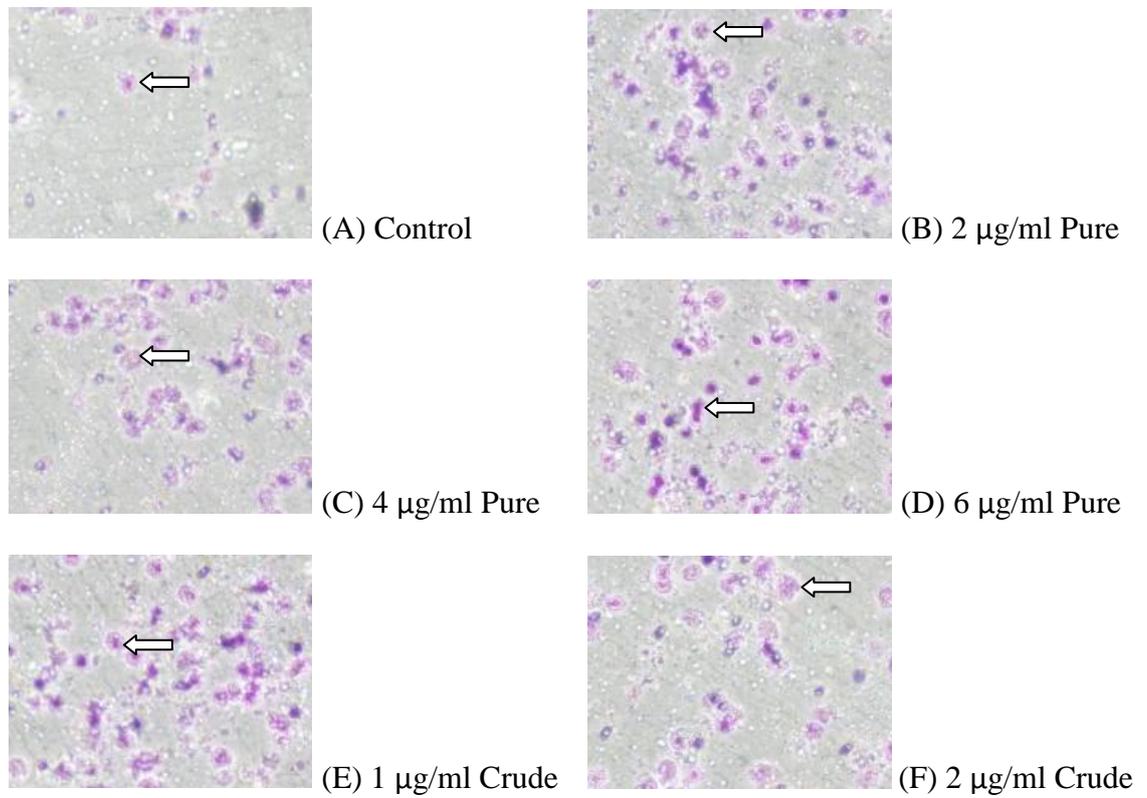


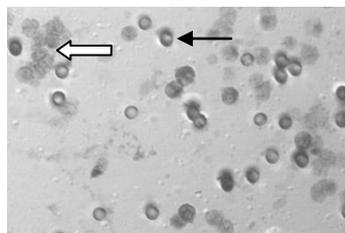
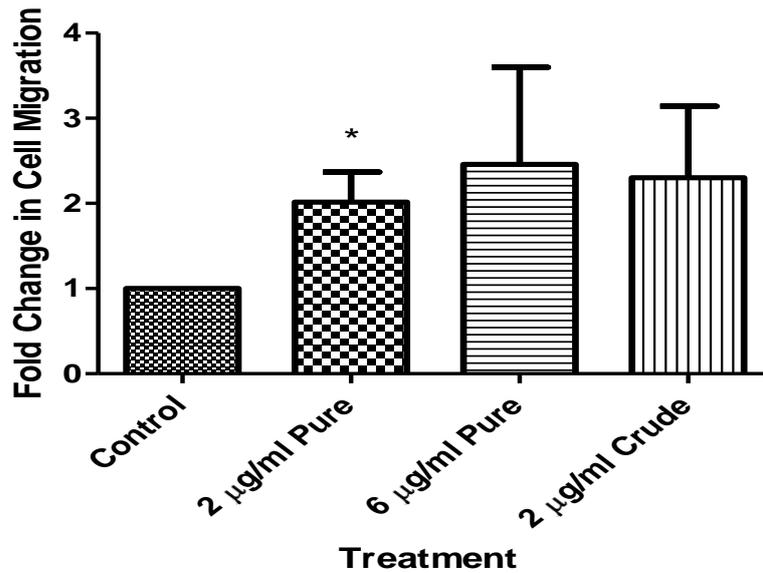
Figure 4.2 Migration of neutrophils through 8 µm porous membrane following treatment with *Bacillus oleronius* proteins after 1 hour

(A-F) Images of stained neutrophils (denoted by the white arrow) that had migrated through the porous membrane viewed at x 20 magnification.

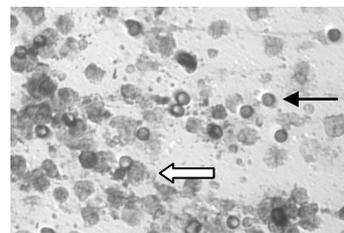
In the control image (A) few neutrophils have migrated through the membrane.

Images (B-D) represent cells exposed to 2, 4 & 6 µg/ml of the purified protein. The amount of migrated cells has increased.

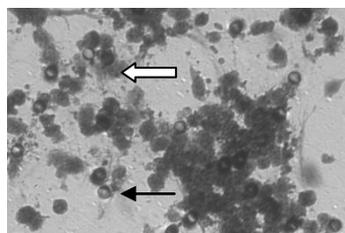
Images (E & F) represent cells exposed to 1 & 2 µg/ml of the crude protein extract, migration of these cells has increased compared to the control image.



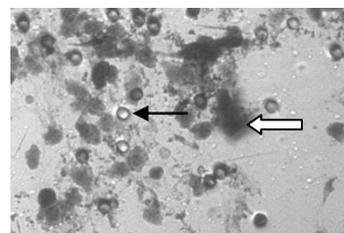
(A) Control



(B) 2 µg/ml Pure



(C) 6 µg/ml Pure



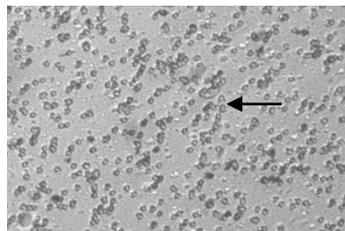
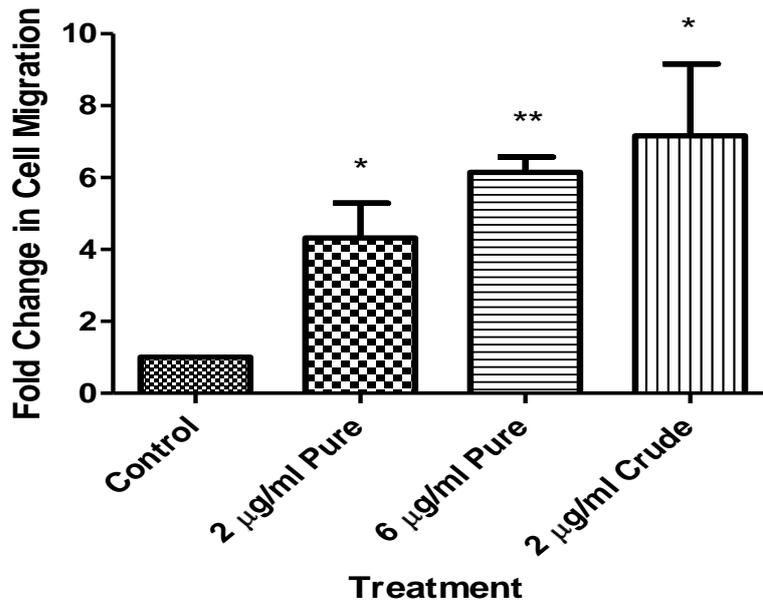
(D) 2 µg/ml Crude

Figure 4.2 The effect of *B. oleronius* proteins on migration of human neutrophils through 8 µm porous membranes after 24 hours

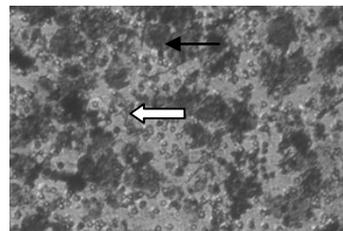
(A-D) Images of stained neutrophils (white arrows) that had migrated through the 8 µm membrane and the pores visible on the membrane (black arrows) viewed at x 40 magnification.

The graph represents fold change in number of crystal violet stained cells eluted using 33% (v/v) glacial acetic acid where * denotes $p < 0.05$ and ** denotes $p < 0.01$.

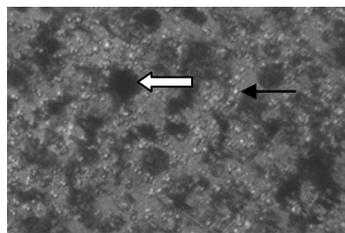
These data represent 3 biological repeats of the assay.



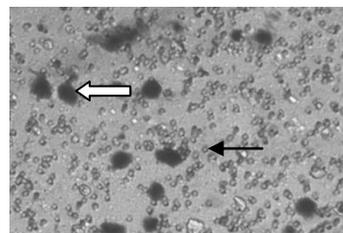
(A) Control



(B) 2 µg/ml Pure



(C) 6 µg/ml Pure



(D) 2 µg/ml Crude

Figure 4.3 The effect of *B. oleronius* in the migration of human neutrophils through 3 µm porous membranes after 24 hours

(A-D) Images stained neutrophils (white arrows) that had migrated through the 3 µm membrane and the pores visible on the membrane (black arrows) viewed at x 40 magnification.

The graph represents fold change in number of crystal violet stained cells eluted using 33% (v/v) glacial acetic acid where * denotes $p < 0.05$ and ** denotes $p < 0.01$.

These data represent 3 biological repeats of the assay.

4.2 The effect of *Bacillus* antigens on human neutrophil degranulation

When neutrophils become activated they undergo an oxidative burst which increases their uptake of oxygen leading to the generation of reactive oxygen species (ROS), this process is accompanied by degranulation (Manara *et al.*, 1991). Degranulation is the process by which leukocytes (neutrophils in particular) release the contents of the granules contained in their cytoplasm. Neutrophils have four types of granules (secretory, gelatinase, specific, azurophil) each containing a variety of enzymes, anti-bacterial peptides, complement molecules and membrane receptors (Nuutila *et al.* 2009). Degranulation can be triggered in many ways such as hypoxia, cellular stress, formyl peptides produced by invading microorganisms (Deree *et al.*, 2006; Lekstrom-Himes *et al.*, 2005), isolation of the neutrophil, temperature changes or a difference in anti-coagulant type (Nuutila *et al.*, 2009). Upon stimulation of the neutrophil the granules translocate and fuse to the phagosomal or plasma membrane where the contents are released (Lacy 2006).

The aim in this section was to examine whether neutrophil degranulation was induced by treatment with the antigenic *Bacillus* proteins, thus adding to the body of evidence that suggests neutrophils and the array of proteins and proteolytic enzymes they produced play a critical role in inducing symptoms of rosacea such as erythema, edema and telangiectasia.

4.2.1 Analysis of the effect of *Bacillus* antigens on MPO production by human neutrophils.

Myeloperoxidase (MPO) is contained primarily in the azurophil granules of neutrophils; it is an enzyme that produces hypochlorous acid from hydrogen peroxide and chloride anion. Azurophil granules combine with the phagocytic granules in order to kill microbes that have been engulfed (Bellocchio *et al.*, 2004), MPO has not been previously linked to tissue damage in rosacea however it has the potential to contribute to a pro-inflammatory state in the tissue (Wang *et al.*, 2008). In order to evaluate the effect of *Bacillus* antigens on MPO production a stirred chamber based assay was used. Healthy control neutrophils were exposed to the *B. oleronius* antigen preparations and cell free supernatants were isolated at 0, 3, 6 and 9 minute time points and resolved by 1-D SDS-PAGE. Western blot analysis (Section 2.28) of these supernatants demonstrated there was an increase in the levels of MPO suggesting that neutrophils degranulate in response to the bacterial antigens. Reactivity to the MPO antibody increased by 3.9 fold following 6 minutes of treatment with 2 µg/ml of the bacterial antigen (p=0.003). When the crude lysate (2 µg/ml) was tested a 4.27 fold increase was seen at the zero minute time point compared to control cells (p=0.04).

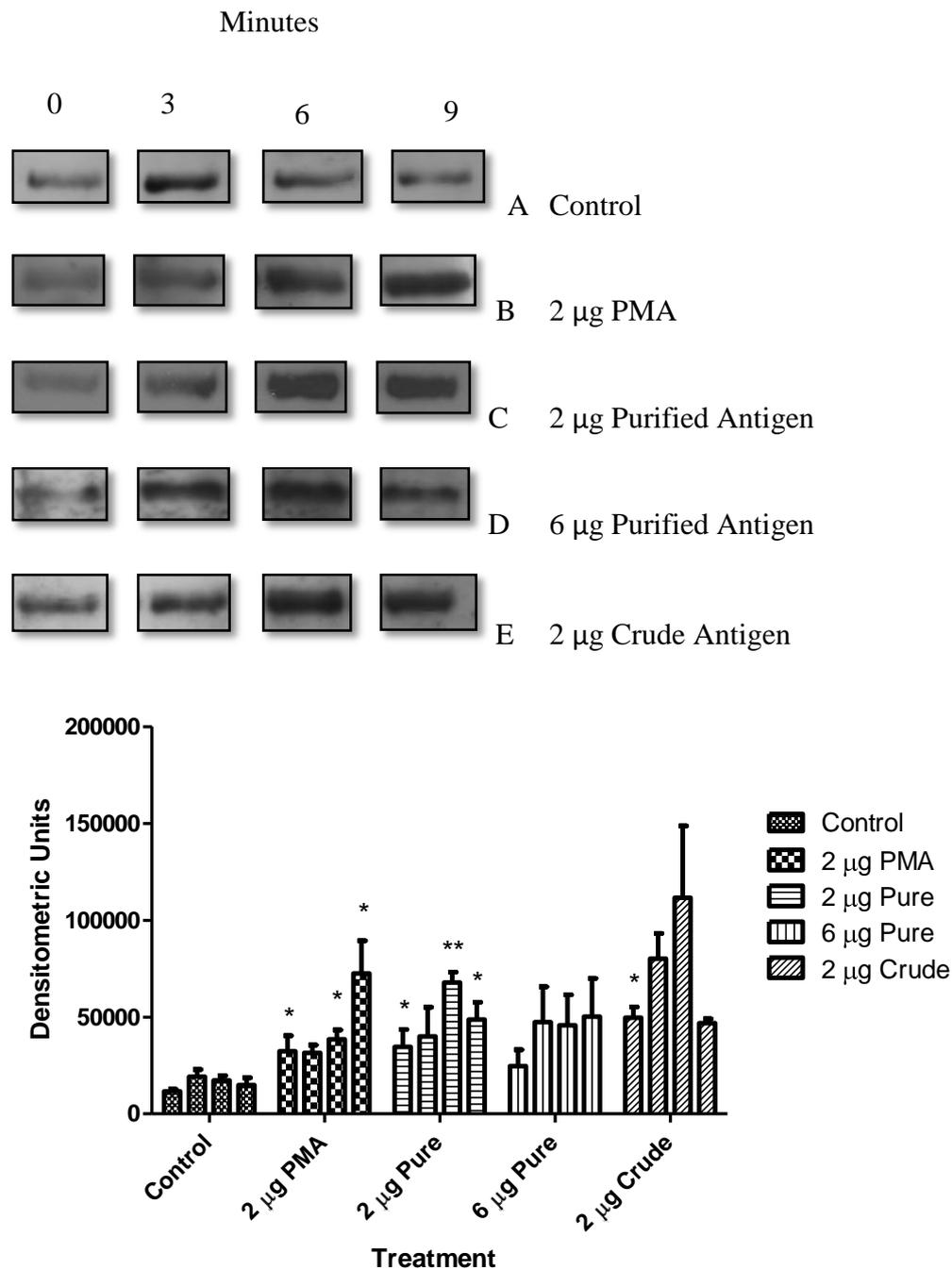


Figure 4.4 Western blot analysis of secreted MPO

A-E Western blots of Neutrophil secreted proteins at time points 0-9 minutes following treatment with purified or crude *Bacillus* extract at 2 or 6 μ g/ml.

The graph represents densitometric analysis of the blots using Image J software. There is a significant increase in reactivity to the MPO antibody in 2 μ g/ml purified and crude treatments over the 9 minutes ($p = 0.043$, $p = 0.098$ respectively).

These data represent 3 biological repeats of the assay.

4.2.2 Analysis of the effect of *Bacillus* antigens on MMP-9 release by human neutrophils.

In a study by McAleer, (2010) it was stated that there was no correlation between the prevalence of rosacea and UV damage. However UV damage was linked to many of the pathological changes seen in rosacea through ROS mediated increase of matrix metalloproteinases (MMPs) production in keratinocytes (Shin *et al.*, 2008; Yamasaki & Gallo, 2009). ROS released by infiltrated neutrophils destroys collagen by inactivation of MMP inhibitors (Bakar *et al.*, 2007; Monk *et al.*, 2011). MMP-9, also known as gelatinase B, it is produced in gelatinase granules of the neutrophil and is responsible for the degradation of collagens IV and V (Murphy & Gavrilovic 1999). It is also the primary matrix degrading enzyme produced by the corneal epithelium and it has been detected in higher than normal levels in the tear fluid of ocular rosacea patients (Kari *et al.*, 2006). Degradation of the collagen of the connective tissue leads to weakening of the vasculature and further recruitment of neutrophils which could potentially contribute to the edema and erythema seen in rosacea. MMP-9 is directly involved in the induction of angiogenesis (Ardi *et al.*, 2007), up-regulation of this process can lead to telangiectasia which is a characteristic symptom of rosacea. The enzyme is also capable of converting IL-8 and TNF- α to their active forms (Manicone & McGuire 2008).

Neutrophil supernatants were generated as previously described (Section 2.28) and probed for reactivity to an MMP-9 antibody raised in goat in the same fashion as the MPO analysis was performed (section 4.2.1). An increase was detected in the levels of MMP-9, a marker secreted by neutrophils during degranulation, when neutrophils were treated with the *Bacillus* antigens (Figure 4.5). There was a 2.9 fold increase in release of MMP-9 from neutrophils treated with 2 $\mu\text{g/ml}$ of the purified antigen after 3 minutes when compared to the controls ($p=0.001$). Following 9 minutes of treatment with 6 $\mu\text{g/ml}$ of the antigen there was a 2.7 fold increase ($p=0.002$). These results indicate that exposure of neutrophils to the crude or purified *B. oleronius* antigen preparations leads to significantly elevated rates of degranulation.

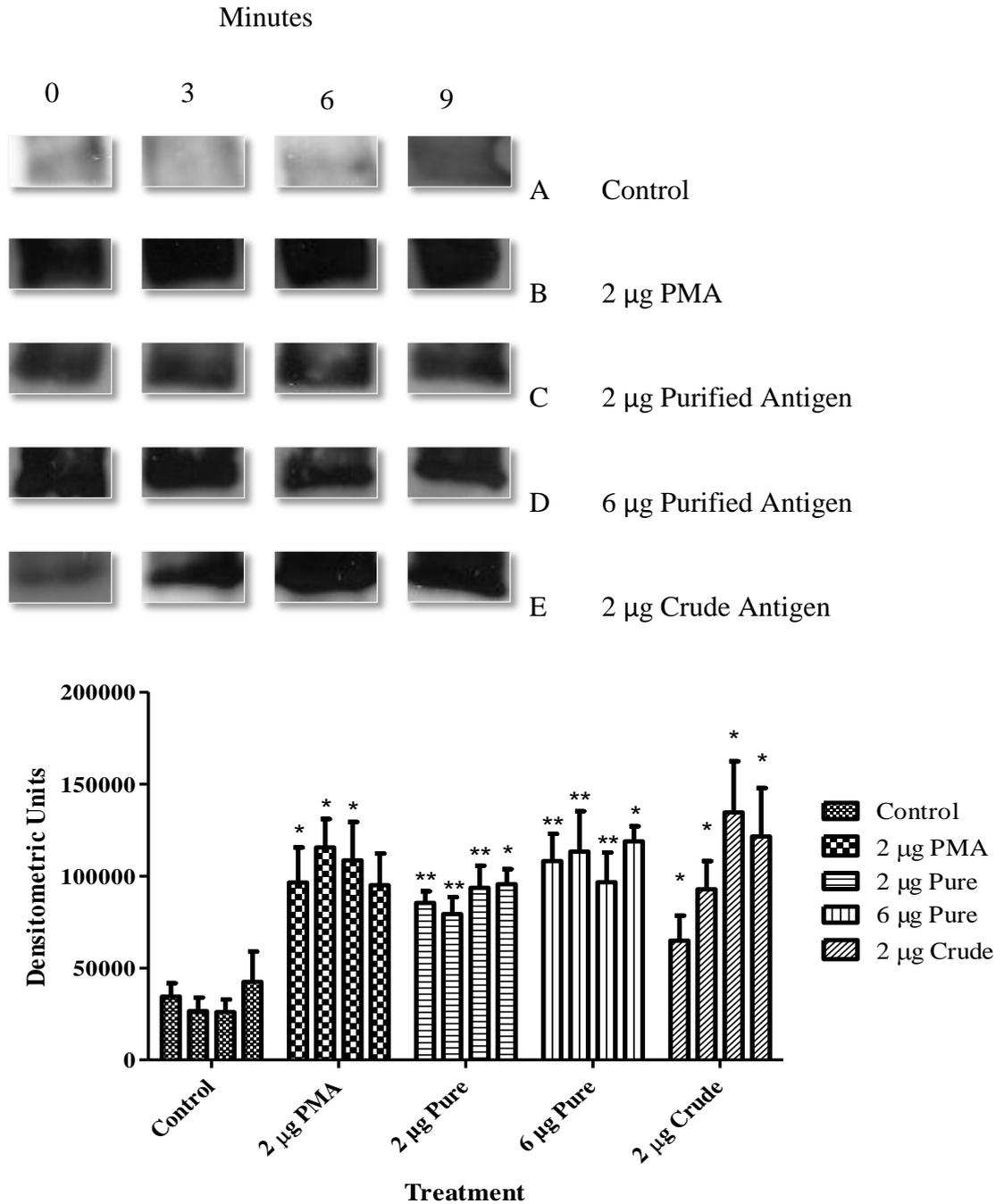


Figure 4.5 Western blot analysis of secreted MMP-9

A-E Western blots of Neutrophil secreted proteins at time points 0-9 minutes following treatment with purified or crude *Bacillus* extract at 2 or 6 µg/ml.

The graph represents densitometric analysis of the blots using Image J software. There is a significant increase in reactivity to the MMP-9 antibody in the control compared to 2 and 6 µg/ml purified and 2 µg crude treatments over the 9 minutes where * denotes that the treatment means are significantly different at $p < 0.05$ and ** denotes highly significant difference at $p < 0.01$.

These data represent 3 biological replicates of the assay.

4.2.3 Analysis of the effect of *Bacillus* antigens on hCAP-18 release by human neutrophils.

Neutrophil supernatants were employed to examine the level of hCAP-18 produced in response to *B. oleronius* antigens. Cathelicidin has been implicated in the pathogenesis of rosacea in recent studies (Ogawa, 2005; Yamasaki, *et al.*, 2007). Cathelicidins are produced in neutrophils and are important components of the innate immune response across many species where they have anti-microbial and pro-inflammatory roles. In a study by Yamasaki (2007) it was demonstrated that the expression of human cathelicidin (hCAP 18) was increased in the skin of rosacea patients compared to controls. Cathelicidin is cleaved into an active peptide (LL-37) by the stratum corneum tryptic enzyme, which was also increased in the skin of rosacea patients compared to controls. To verify that the changes in rosacea skin could be caused by increased levels of hCAP-18 and its active peptide human keratinocytes were treated with LL-37 and increased secretion of IL-8 was observed. Sub-cutaneous injection of LL-37 caused induction of erythema, vascular dilation and neutrophil infiltration in mice, which are all characteristics of rosacea (Yamasaki *et al.*, 2007).

Neutrophil supernatants were generated as previously described (Section 2.28), resolved by 1D SDS-PAGE, transferred to a nitrocellulose membrane and probed for reactivity to a hCAP-18 antibody raised in rabbit. As shown in Figure 4.6 there is a clear increase in the reactivity to the hCAP-18 antibody following treatment with the bacterial antigens. At the zero minute time point when 2 µg/ml of purified antigen was used there was a 3.8 fold increase of hCAP-18 release compared to control cells ($p=0.002$), following 6 minutes of incubation with the antigens a 2.4 fold increase was noted ($p=0.037$). These results suggest that the *Bacillus* antigens induce hCAP-18 to be released from neutrophils as part of a degranulation response.

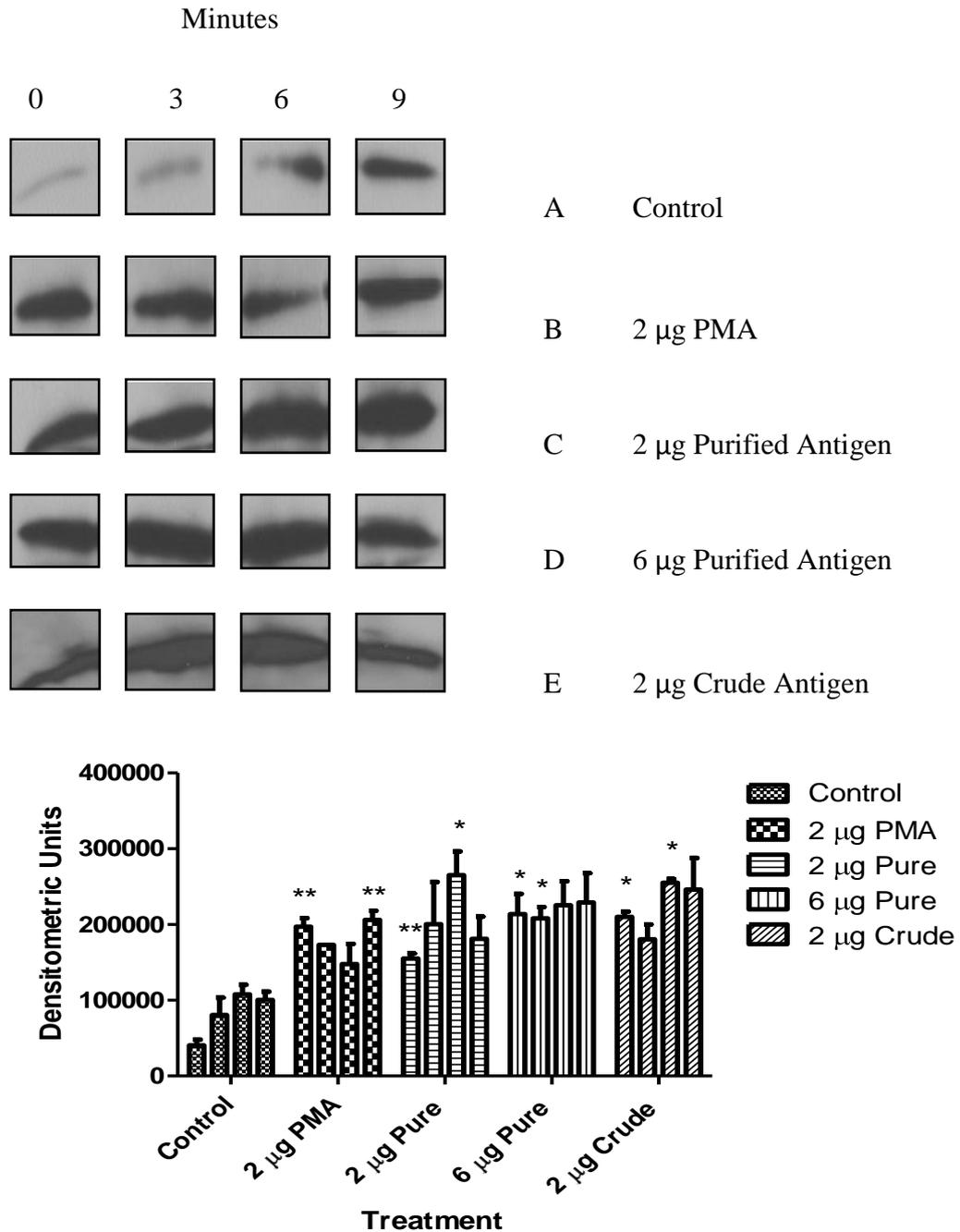


Figure 4.6 Western blot analysis of secreted hCAP-18

A-E Western blots of Neutrophil secreted proteins at time points 0-9 minutes following treatment with purified or crude *Bacillus* extract at 2 or 6 µg/ml.

The graph represents densitometric analysis of the blots using Image J software. There is a significant increase in reactivity to the hCAP-18 antibody in the control compared to 2 and 6 µg/ml purified and 2 µg crude treatments over the 9 minutes where * denotes that the treatment means are significantly different at $p < 0.05$ and ** denotes highly significant difference at $p < 0.01$.

These data represent 3 biological replicates of the assay.

4.3 The effect of *Bacillus* antigens on cytokine production by human Neutrophils

Cytokines are produced in response to the detection of infection and injury, their presence in the blood is one of the key factors that mediate neutrophil migration to the affected area during an inflammatory response (Retini *et al.*, 1996), this is usually a tightly regulated response (Yamasaki & Gallo, 2009). Cytokines have previously been detected in elevated levels in the tear fluid of individuals with *Demodex* blepharitis (Kim *et al.*, 2011) which is an inflammatory condition exacerbated by elevated levels of *Demodex* mites in the eyelash follicles and the meibomian glands. Conversely the disruption of cytokine production has been implicated in causing susceptibility to infection in alcoholic patients (Taieb *et al.*, 2002). Decreases in IL-8 and TNF- α were recorded when neutrophils were treated with ethanol. Increased cytokine levels have been implicated in disease such as psoriasis (Blaschke *et al.*, 2000), following quantitative RT-PCR analysis of tissue sections IL-8 and TNF- α cytokines were found to be elevated compared to the level detected in normal skin. TNF- α mediates the keratinocyte proliferation and angiogenesis in psoriasis (Hitoshi *et al.*, 1997; Duan *et al.*, 2001) and induces the production of IL-8. TNF- α inhibiting drugs have been developed to treat psoriasis (Ahdout *et al.*, 2010), since TNF is a potent stimulator of the inflammatory response inhibiting its production is a viable treatment however many patients report adverse side effects to the drugs.

In order to determine the effects of *Bacillus* antigens on cytokine production, neutrophils (1×10^6 cells/ml) were seeded into 24 well plates and incubated in RPMI-1640 culture medium containing 5% (v/v) FCS and 2 mM L-glutamine with the relevant concentration of bacterial protein. Culture medium was collected at 3, 16 and 24 hour time points and any cells present were removed by centrifugation (Section 2.29) before the media were employed in a sandwich-ELISA protocol. Chemoattractant (IL-8) and pro-inflammatory (TNF- α) cytokines were quantified (Figures 4.7 & 4.8). These cytokines have the potential to contribute to the chronic inflammation seen in rosacea in a similar way to the pathogenesis of psoriasis; by recruiting neutrophils, inducing angiogenesis and maintaining the inflammation by inducing further cytokine production.

4.3.1 Evaluating IL-8 production by Neutrophils in response to *Bacillus* proteins

Interleukin 8 (IL-8) is a proinflammatory cytokine produced by neutrophils in response to pathogen detection, the movement of neutrophils is mediated predominantly by this cytokine (Teranishi *et al.*, 1995). The bacterium *Propionibacterium acnes* has been implicated in the inflammatory stage of acne vulgaris, this bacterium can induce the production of IL-8 in keratinocytes leading to the infiltration of neutrophils (Grange *et al.*, 2009). There was a significant increase in IL-8 production by neutrophils (Figure 4.7) treated with the bacterial antigens in this study, *in vivo* this could lead to similar effects to those seen in other chronic inflammatory disorder such as psoriasis. Following an exposure time of 3 hrs the highest (4 fold, $p=0.01$) increase of IL-8 production was recorded in cells exposed to 6 $\mu\text{g/ml}$ of bacterial protein. Neutrophils exposed to 6 $\mu\text{g/ml}$ of the pure protein produced a 3.4 fold ($p=0.022$) higher concentration of IL-8 compared to cells incubated in untreated medium. At the 24 hr time point an increase in production of IL-8 of 4.2 fold ($p=0.003$) was recorded. Reports have shown increased production of IL-8 by PBMCs in psoriasis patients (Teranishi *et al.*, 1995) leading to infiltration of neutrophils and keratinocytes to generate the inflammatory lesion and plaques that are characteristic on rosacea skin.

4.3.2 Evaluating TNF- α production by Neutrophils in response to *Bacillus* proteins

Neutrophil supernatants were further employed to assess the level of TNF- α produced in response to the bacterial proteins. TNF- α is a pro-inflammatory cytokine produced by cells involved in innate immunity, it induces the production of other cytokines including IL-8 (Teranishi *et al.*, 1995). ROS which are said to be increased in rosacea lesions compared to control skin (Bakar *et al.*, 2007) can mediate the release of TNF- α in human keratinocytes. The greatest increase in TNF- α production was evident in neutrophils exposed to 2 $\mu\text{g/ml}$ crude *Bacillus* antigen preparation for 3 hours (2.9 fold increase $p=0.0008$) or 16 hours (2.8 fold increase $P=0.0002$) (Figure 4.8). These results indicate that exposure of neutrophils to crude or purified material from *Bacillus* lysate leads to cytokine production; which *in vivo* could have the effect of recruiting neutrophils to the sites of antigens exposure and provoking inflammation.

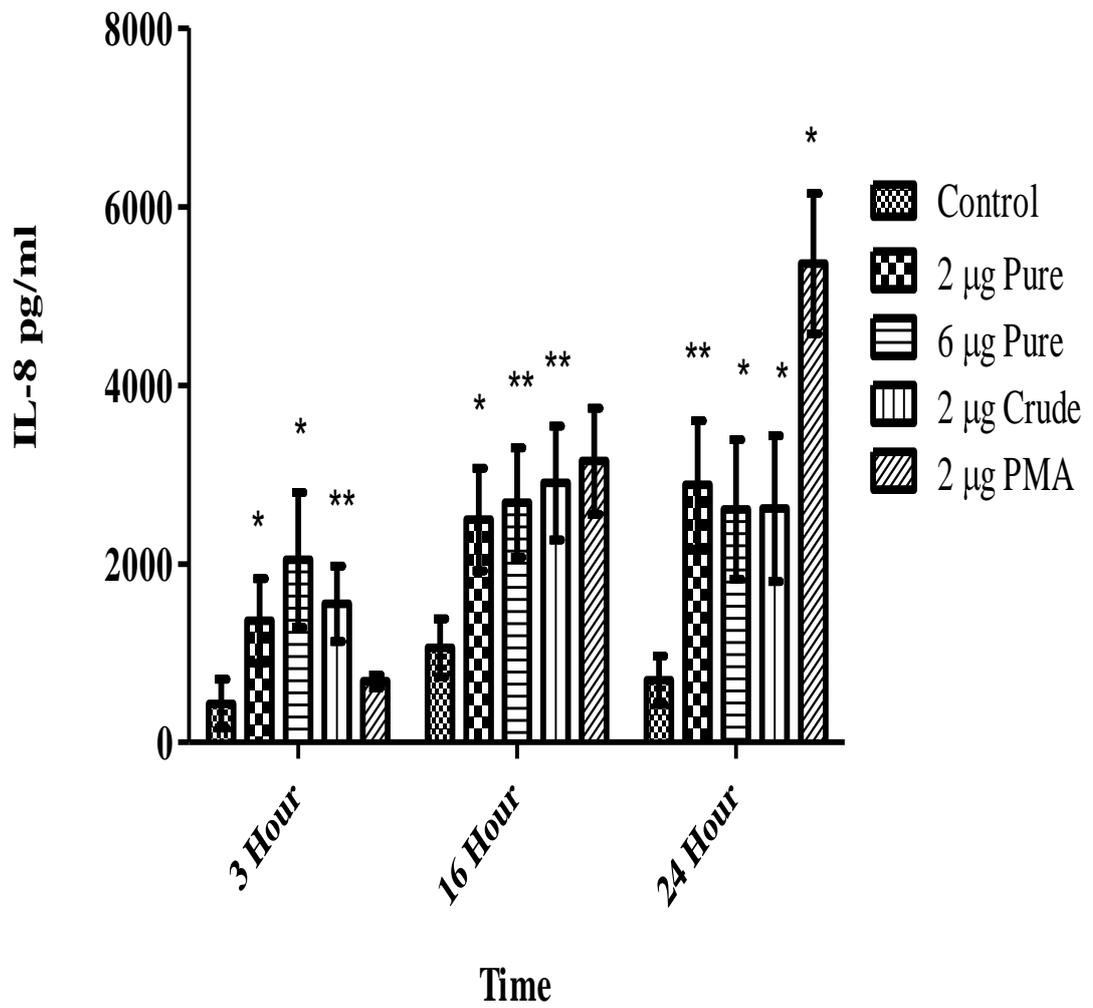


Figure 4.7 The effect of *B. oleronius* proteins on IL-8 secretion by human neutrophils

The results are expressed as pg/ml of cytokine and represent three biological replicates of the assay where $p \leq 0.05$ is denoted by * and ** is $p \leq 0.01$.

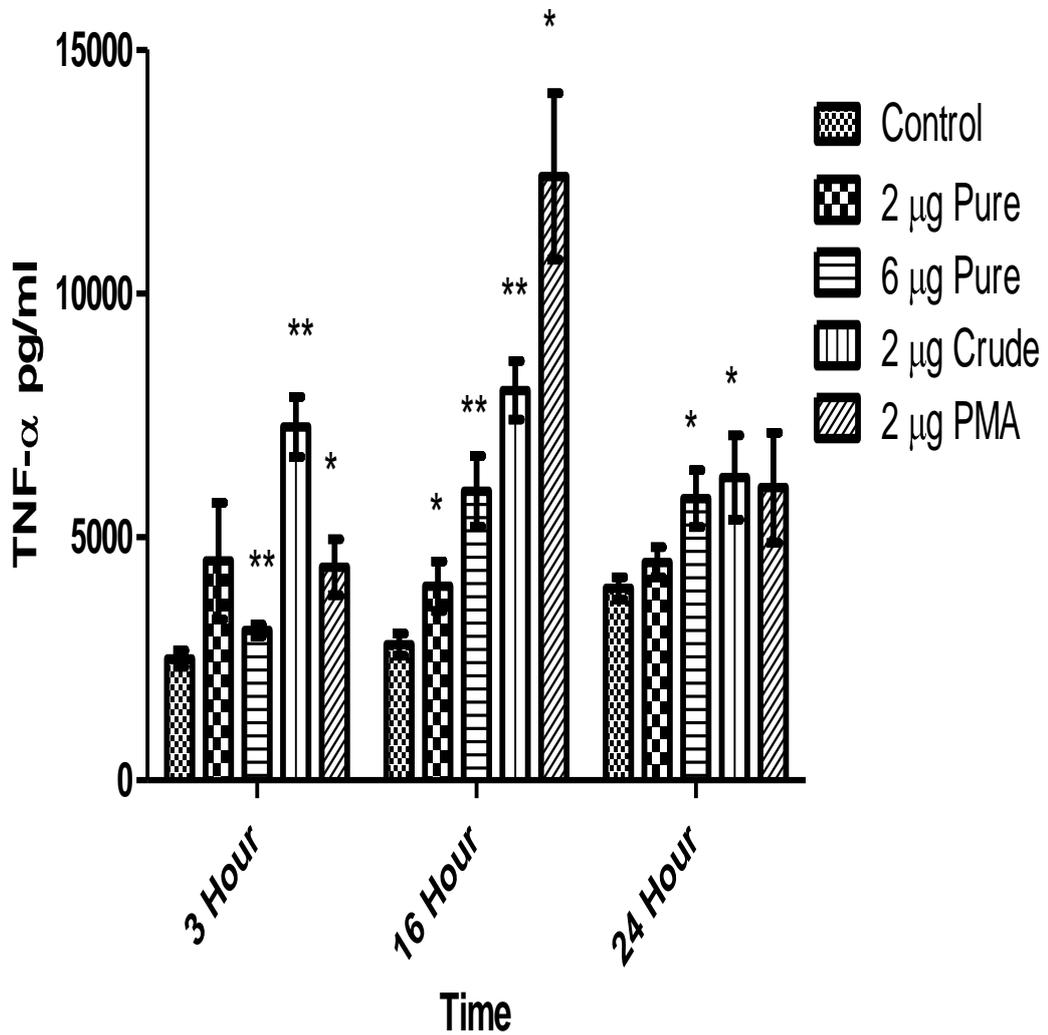


Figure 4.8 The effect of *B. oleronius* proteins on TNF- α secretion by human neutrophils.

The results are expressed as pg/ml of cytokine and represents three biological replicates of the assay where $p < 0.05$ is denoted by * and ** is $p < 0.01$.

4.4 Discussion

Recently the *Demodex* associated bacterium *Bacillus oleronius* has been shown to induce serum immunoreactivity in papulopustular (Lacey *et al.*, 2007) and ocular (Li *et al.*, 2010) rosacea patients. An elevation in the density of *Demodex* mites has been reported in both facial (Diazperez, 1994; Bonnar *et al.*, 1993) and ocular (Gao *et al.*, 2005; Czepita *et al.*, 2007; Kheirkhah *et al.*, 2007) rosacea. Common effective treatments for rosacea such as doxycycline do not eradicate mites from the face (Monk *et al.*, 2011) however it is possible that these antibiotics reduce the bacterial associated with the mites therefore relieving the symptoms. Tea tree oil (TTO) is an effective treatment which can eradicate *Demodex* mites in patients with cylindrical dandruff (Gao *et al.*, 2005) and ocular rosacea (Kheirkhah *et al.*, 2007). TTO has multiple benefits in that treatments that utilize this oil have acaricide, antimicrobial, antifungal and anti-inflammatory properties (Gao *et al.*, 2005). The focus of this Chapter was to investigate the effects of proteins isolated from the *Demodex* associated bacterium on the host's immune response by characterizing the effect on human neutrophils.

B. oleronius protein was purified using ÄKTA fast performance liquid chromatography to obtain a semi-purified preparation. This purified protein along with a crude lysate was exposed neutrophils in a range of assays to investigate the potential scenario that the cells migrate towards areas where this antigen may be present in the skin of rosacea patients (ie: the pilo-sebaceous units, eyelash follicles and tear fluid) leading to the production of cytokines and degranulation of proteins that induce inflammation and tissue damage. Following LC-Mass Spectrometry analysis it was found that the purified *Bacillus* protein preparation contained a wide range of proteins (section 3.4.2, Table 3.2) which could potentially interact with and activate the immune response in rosacea patients. Neutrophils are one of the most important cellular components of the innate immune response (Segal, 2005). It has been discussed at great length how dysregulation of the innate immune response is central to the pathogenesis of this rosacea (Dahl *et al.*, 2002; Lacey *et al.*, 2007; Li *et al.*, 2010; Powell, 2005; Yamasaki & Gallo, 2009).

The production of reactive oxygen species (ROS) has been implicated in the pathogenesis of rosacea (Yamasaki & Gallo, 2009), neutrophils produce ROS during the phagocytosis and killing of microorganisms (Reeves, 2003). Neutrophils can recognise

and respond to specific bacterial components (Vance et al. 2009) some of which were present in the protein preparation exposed to the neutrophils in this work. Among those was flagellin the protein monomer expressed in most species of bacteria which interacts with TLR-5 (Tapping 2009). TLR-5 signalling triggers activation of pathways that lead to the activation of NF- κ B and other transcription factors involved in regulation of the immune response and stimulation of neutrophils (Hayashi *et al.*, 2003). Therefore recognition of bacterial flagellin could be responsible the increased production of IL-8 and TNF- α cytokines (Figures 4.7 & 4.8) and the increase in expression of degranulation proteins (Figures 4.4 – 4.6) observed in response the *B. oleronius* protein preparation. A protein involved in peptidoglycan synthesis was also identified (Section 3.4, Table 3.4), recognition of peptidoglycan by neutrophils has been implicated in the induction of inflammation (Weiss 2003; Dziarski 2004). Tertiary neutrophil granules contain a peptidoglycan recognition protein which functions as an antibacterial granule protein (Dziarski 2004). Elevated expression of degranulation proteins MPO, MMP-9 and hCap-18 were detected in response to the bacterial protein preparation (section 4.2). Peptidoglycan can also be recognised by TLR-2 and therefore could potentially contribute to the induction of inflammation in the tissue (Tapping 2009).

Exposure of neutrophils to pro-inflammatory cytokines such as IL-8 and TNF- α can prime them for rapid degranulation (Nuutila *et al.*, 2009). Neutrophils contain granules in their cytoplasm which produce cytotoxic super oxide species and antimicrobial peptides. The process of degranulation can be stimulated in many ways and leads ultimately to the release of the granular contents into the phagocytic vacuole or the surrounding tissue (Levy 2000). The level of MPO secreted by neutrophils in response to the bacterial antigens was quantified using Western blotting and revealed to be significantly increased (section 4.2.1). Western blotting revealed that MPO (Figure 4.4) along with MMP-9 (Figure 4.5) and hCAP-18 (Figure 4.6) protein secretion increased when cells were treated with the antigen compared to control cells. These proteins have the potential to cause tissue damage in the skin and MMP-9 and hCAP-18 have been implicated in the pathogenesis of rosacea in previous studies (Bamford, 2001; Kari, 2006; Monk *et al.*, 2011; Yamasaki *et al.*, 2007). Studies using epithelial cell lines and sebaceous cell lines have been performed in relation to rosacea. Yamasaki *et al.*, (2007) stated that treating human keratinocytes with LL-37 (hCAP-18s active peptide) caused increased release of pro-inflammatory cytokines such as IL-8 by the

cells. LL-37 can also induce neutrophil chemotaxis of adaptive immune cells and angiogenesis which are processes that contribute to the pathogenesis of rosacea (Schauber & Gallo, 2009). The human cathelicidin peptide hCAP-18 is expressed in neutrophils and the keratinocytes of inflamed skin (Levy 2000). It has been reported that abnormal cathelicidin which caused increased inflammation was detected in the skin of rosacea patients compared to controls (Schauber & Gallo, 2009; Yamasaki *et al.*, 2007). Increased stratum corneum tryptic enzyme (SCTE) has been detected in the skin of rosacea patients which is thought to contribute to the processing of the abnormal cathelicidins (Yamasaki *et al.* 2007). Inflammatory neutrophils provide an important source of MMP-9 in the process of angiogenesis in tumour progression (Ardi *et al.* 2007) angiogenesis is a process that occurs in the skin of rosacea patients and contributes to the erythema which causes the characteristic redness in the central region of the face (Elewski *et al.* 2010).

Cytokines are also a contributing factor in relation to neutrophil migration; they are produced by neutrophils and epithelial cells in response to pathogen recognition or tissue damage in an area of inflammation (Okubo & Koga 1998; Ramos 2003). Exposure of human neutrophils to bacterial peptidoglycan from *Bacterioides fragilis* was shown to induce an increase in the migratory response of the white blood cells (Sperry & Burns 1987). IL-8 has a major role in the recruitment of neutrophils and in this study there was a significant increase in IL-8 (Figure 4.7) production by neutrophils exposed to *B. oleronius* protein. In section 4.5.1 it was demonstrated how the increase in IL-8 production by neutrophils occurred in response to each concentration of antigen tested across a 24 hour period. TNF- α is involved in the induction of inflammation and has previously been implicated in chronic inflammatory disorders such as psoriasis (Hitoshi *et al.*, 1997; Okubo & Koga, 1998; Deeva *et al.*, 2010). Production of this cytokine was also significantly increased in response to bacterial antigen treatment (Figure 4.8). Again the effect was observed across all concentrations of antigen for a 24 hour period; detection was highest at the 16 hour time point and appeared to drop by 24 hours. Both of these signaling molecules could be produced initially at low levels *in vivo* followed by infiltration of higher levels of neutrophils and increased cytokine production in response to the bacterial antigens adding to the potential for a chronic inflammatory state to develop in the tissue.

Skin temperature is reported to be increased in people with rosacea (Whitfield *et al.*, 2011), this is said to be due to the long history of flushing and the altered vasculature which in turn leads to increased blood flow to the skin affected by rosacea. It is possible that the increased blood flow reported could predispose the skin of rosacea patients to having increased presence of immune cells such as neutrophils. To determine if *Bacillus* antigens had the potential to increase the migration of neutrophils, cell culture inserts (BD Falcon) with 3 and 8 μm polyethylene terephthalate (PET) membranes were used to set up migration assays (section 4.1). There was an increased migratory response in cells treated with the *Bacillus* protein compared to control cells as indicated in Figures 4.1-4.3. This was measured visually by taking images of the membranes with the migrated cells (which had been stained with crystal violet dye) intact and simply comparing the number of cells by eye (Figures 4.1-4.3). When using the 3 μm inserts, a mean increase of 4.3 fold ($p = 0.02$) was observed when cells were treated with 2 $\mu\text{g}/\text{ml}$ of the purified antigens this rose to a 6.1 fold increase ($p=0.001$) with 6 $\mu\text{g}/\text{ml}$ and a 7 fold increase when 2 $\mu\text{g}/\text{ml}$ of the crude lysate ($p=0.027$) was used. The outcome of this reaction to *Bacillus* antigens *in vivo* could lead to infiltration of high numbers of neutrophils in the tissue surrounding the pilosebaceous unit and the lash follicles where the *Demodex* and their associated bacteria are present. Eventually this could lead to tissue damage in those areas due to the destructive nature of neutrophils.

Neutrophils have been used extensively to study chronic inflammatory disorders (Bellocchio *et al.*, 2004; Hartl *et al.*, 2008; Morioka *et al.*, 2008; Hayes *et al.*, 2011). In a study by Wang *et al.*, (2008) a recombinant *Helicobacter pylori* neutrophil activating protein (HP-NAP) was produced and used to assess the level of MPO activation in neutrophils. *H. pylori* has been implicated in chronic gastrointestinal inflammatory disorders and the ability of this protein to induce MPO production was suggested as playing a role in causing the tissue damage seen in the condition. In this Chapter an increase in MPO production following exposure of neutrophils to the bacterial protein was observed (section 4.2.1). The pathogenesis of cystic fibrosis is characterized by chronic inflammation of the lungs and increased infiltration of neutrophils, which are unsuccessful at clearing microbial infections (Hayes *et al.*, 2011). In a study of chronic inflammatory lung disease and rheumatoid arthritis (Hartl *et al.*, 2008) it was shown that infiltrated neutrophils expressed more chemokine receptors on their surface than

circulating neutrophils. Increased migration of neutrophils in response to *B. oleronius* protein was observed *in vitro* (section 4.1) therefore the presence of this bacterium in the pilo-sebaceous unit could induce infiltration of neutrophils in the surrounding area. Infiltration of neutrophils has been observed in the inflammatory lesions of granulomatous rosacea patients (Jang *et al.*, 2010; Sánchez *et al.*, 2008).

The work outlined here has revealed some of the cellular changes that occur in neutrophils treated with *Bacillus* antigens. *Demodex* mites reside in the pilosebaceous units of the pores and in the eyelash follicles. Upon death the mites may cause local inflammation by release of bacterial endosymbionts (eg: *Bacillus oleronius*) and their associated antigens into the surrounding tissue. It is possible that *Bacillus* antigens may be present at increased concentrations in the skin of people with rosacea due to the presence of higher numbers of *Demodex* mites. It was found that neutrophils treated with the *Bacillus* antigens showed increased cell motility, increased secretion of tissue damaging degranulation proteins (MPO, MMP-9 and hCAP-18) and up-regulation of cytokine (IL-8 and TNF- α) production. All of the cellular changes in response to the *Bacillus* antigens have been previously implicated in inflammatory responses, many have overlapping functions. The neutrophil responses described in this study provide strong evidence that *Bacillus oleronius* antigens could potentially contribute to the pathogenesis of rosacea.

CHAPTER 5

An investigation of the effect of *Demodex* associated *Bacillus oleronius* proteins on the corneal epithelial cell line hTCEpi and on primary limbal epithelial cells

5.0 Introduction

The National Rosacea Society established a classification system in 2002 (Wilkin *et al.*, 2002) where the subtype under examination in this work was termed ocular rosacea. Some reports have stated that ocular involvement occurs in up to 50% of patients with rosacea and that ocular symptoms can manifest prior to any cutaneous symptoms (Oltz & Check, 2011). As mentioned previously the pathogenesis of rosacea is ambiguous; there are many possible contributing factors to this condition such as elevated *Demodex* mite density (Bonnar *et al.*, 1993; Dhingra *et al.*, 2009; Diazperez, 1994; Gao *et al.*, 2005; Kheirkhah *et al.*, 2007), bacterial components (*S. epidermidis* and *B. oleronius*) (Lacey *et al.*, 2007; Li *et al.*, 2010; Whitfeld *et al.*, 2011) and an underlying irregular innate immune response (eg: production of abnormal cathelicidin) (Yamasaki & Gallo, 2009). Ocular rosacea presents with a range of symptoms including irritated dry eyes, blepharitis, neo-vascularisation of the corneal surface and in severe cases complications such as corneal erosion and keratitis can develop (Dahl *et al.*, 2002; Ramamurthi *et al.*, 2006; Wilkin *et al.*, 2004). Corneal thinning, development of phlyctenules, nodular infiltrates and ulcerations have all been observed as corneal complications linked to rosacea (Oltz & Check 2011; Kheirkhah *et al.* 2007; Ramamurthi *et al.* 2006) these can eventually cause scarring and corneal perforation leading to visual impairment. Differential diagnosis for rosacea include conjunctivitis and blepharitis treatment for these conditions without recognition of the underlying rosacea is often unsuccessful (Oltz & Check, 2011).

The ocular surface is in constant contact with commensal bacteria however excessive inflammation is avoided due to an apparent difference between mucosal surfaces that are in contact with commensals and the conventional immune response to pathogens (Ueta & Kinoshita 2010; Graham *et al.* 2007; Redfern & McDermott 2010). Corneal epithelial cells play a critical role in protecting the ocular surface from infection and are very selective in their response to microbial components (Kumar *et al.*, 2007; Ueta & Kinoshita, 2010). The selective response is mediated by toll like receptors (TLRs) (Chang *et al.*, 2006; Redfern & McDermott, 2010) which can induce the production of cytokines. It was recently reported that mRNA expression of TLRs 1-10 (excluding TLR-8) was found in corneal epithelia cells and that corneal epithelial cells respond to ocular pathogenic but not non-pathogenic flagellin via TLR-5 (Chung *et al.*,

2009; Hozono *et al.*, 2006; Ueta & Kinoshita, 2010). Rosacea can manifest as an inflammatory ocular surface disease affecting the eyelids, conjunctival and corneal regions (Oltz & Check, 2011). The ocular surface epithelium could potentially be producing cytokines such as IL-8 and TNF- α in response to *Bacillus oleronius* components which initiate an immune response that becomes exaggerated and chronic as the condition progresses. Efficient distinction between pathogen and commensal is essential to avoid destructive inflammation of the corneal surface and this property may be impaired in those with ocular rosacea.

Ulceration of the corneal surface in rosacea patients has been linked to *Demodex* infestation and has development of ulcers can potentially lead to visual impairment (Oltz & Check 2011; Wilkin *et al.* 2004; Elewski *et al.* 2010; Kheirkhah *et al.* 2007). Ulceration often begins with the development of phlyctenular keratoconjunctivitis which is usually considered more severe when it affects the cornea (Neiberg & Sowka, 2008). It has been reported that 5-30% of patients with facial rosacea have corneal complications such as phlyctenule-like lesions (Bartholomew *et al.*, 1982; Kheirkhah *et al.*, 2007). The formation of phlyctenular lesions combined with neovascularisation, usually originates in the limbal region of the eye and can infiltrate the cornea (Neiberg & Sowka, 2008). Even in cases where treatment is sought, ulcers can develop on the cornea potentially leading to opaque scarring and perforation that can be sight threatening (Du & Oral, 2005; Monk *et al.*, 2011; Oltz & Check, 2011).

Matrix degenerating enzymes have been implicated in the formation of non-infectious ulcers (Fini *et al.*, 1998; Li *et al.*, 2001; Yamasaki *et al.*, 2007) such as those seen in severe cases of rosacea. Matrix metalloproteinase 8 (MMP-8) and MMP-9 have previously been detected at an increased concentration in the tear fluid of ocular rosacea patients (Kari & Kari, 2006). Many reports have suggested that the success of tetracyclines for the treatment of ocular rosacea is largely due to their anti-inflammatory properties and ability to block the production and activation of MMPs (Du & Oral, 2005; Gupta & Chaudhry, 2005; Kari & Kari, 2006; Monk *et al.*, 2011; Oltz & Check, 2011). Infiltration of inflammatory cells such as neutrophils has been implicated in the formation of ulcers, these cells are reported to increase in numbers at the stromal matrix that underlies the corneal epithelium (Fini *et al.*, 1998). It was previously shown in Chapter four that neutrophils treated with proteins extracted from *Bacillus oleronius* can induce the production of MMP-9 at an increased level during degranulation when

compared to untreated control cells. This result along with the reports of increased neutrophil infiltration could potentially lead to a scenario where the stromal matrix is exposed to high levels of tissue degrading enzymes, which induces vascularisation and ulceration if the chronic inflammation remains untreated.

Severe cases of keratitis and ulceration can potentially lead to limbal stem cell deficiency in rosacea patients (Holland & Schwartz 2002). The corneal epithelium is maintained by stem cells located in the limbus which is located between the conjunctival and corneal epithelial tissues (Baylis *et al.*, 2011; Kolli *et al.*, 2010; Notara & Daniels, 2008). The stem cells are maintained in a niche at the inferior and superior regions of the limbus where they are supported by their proximity to the nutrient rich stromal region. As mentioned previously there have been reports indicating that the stroma is damaged as ocular rosacea progresses, this damage may contribute to limbal stem cell deficiency (Notara & Daniels 2008), which could potentially lead to visual impairment in rosacea patients.

It has been described in Chapter four how proteins extracted from *Bacillus oleronius* were isolated (section 3.4.) and exposed to human neutrophils. In this Chapter the same extract was exposed to the corneal epithelial cell line (hTCEpi) and limbal epithelial cells. Limbal epithelial stem cells were isolated and cultured on gelatine coated plates to generate primary limbal epithelial cells for use in assays. Recently, a strong correlation has been established between serum reactivity to these *Bacillus* proteins and eye lid margin inflammation ($p = 0.04$), and ocular rosacea ($p = 0.009$) (Li *et al.*, 2010). The aim of this work was to elucidate if the cellular changes caused by exposure to the bacterial proteins could contribute to the symptoms seen in ocular rosacea. It was hypothesised that corneal cells *in vivo* could potentially be exposed to *B. oleronius* proteins due to the presence of increased *Demodex* mites in the lash follicles of individuals with rosacea. Therefore the surrounding tissue and tear fluid could potentially contain immune triggering levels of *B. oleronius* antigenic proteins, thus contributing to the symptoms of rosacea.

5.1 The use of immortalized human corneal epithelial cell line (hTCEpi) as a model for the investigation of the role of *Bacillus* proteins in the development of ocular rosacea

The hTCEpi cell line was generated by infecting primary cultures of human corneal epithelial cells with a retroviral vector encoding telomerase reverse transcriptase (hTERT). The purpose of this cell line was to provide a model for the study of molecular mechanisms involved in human corneal cell differentiation (Robertson *et al.*, 2005). Following a review of corneal epithelial cell culture models the method used to generate the hTCEpi cell line was deemed an efficient way to generate epithelial cell lines (Castro-Muñozledo 2008). The cell line was recently used to investigate the role of plasma membrane calcium-ATPase expression in a wound healing model (Talarico, 2010). Ocular symptoms are characteristic in up to 50% of rosacea patients so in this study we employed the corneal epithelial cell line to study the potential cellular changes that could contribute to the symptoms of ocular rosacea.

5.2 The effect of *Bacillus* proteins on growth of hTCEpi cells

Prior to studying the response of the hTCEpi cells to the *Bacillus* antigens more closely the toxicity of the antigen was assessed. Since the potential concentration of antigen present *in vivo* was not known, a concentration gradient from 0.5 µg – 6 µg/ml of antigen diluted in KGM-2 media was tested. The experiment was carried out in triplicate and 3 wells of a 24 well plate were assessed for each treatment. The growth of hTCEpi cells in the presence of the bacterial antigen was quantified using the crystal violet dye elution method as described in section 2.31.1 Results were expressed as a percentage of growth in comparison to growth of the control cells (Figure 5.1). As depicted in Figure 5.1, there was a statistically significant decrease in cell growth at the lowest antigen concentration where the cells dropped to 75% growth on average. BSA was employed as a control to ensure that addition of an arbitrary protein did not elicit the same effects seen with addition of the antigen. After 5 days of exposure to an antigen concentration of 2 µg/ml cell growth was inhibited by 26 % ($p = 0.018$) and at an antigen concentration of 6 µg/ml cell growth was inhibited by 75 % ($p = 0.0003$). It

was therefore decided that 2, 4 and 6 $\mu\text{g}/\text{ml}$ of the purified protein preparation (section 3.4.2, Table3.3) would be used and in some cases 2 μg of the crude lysate would be employed.

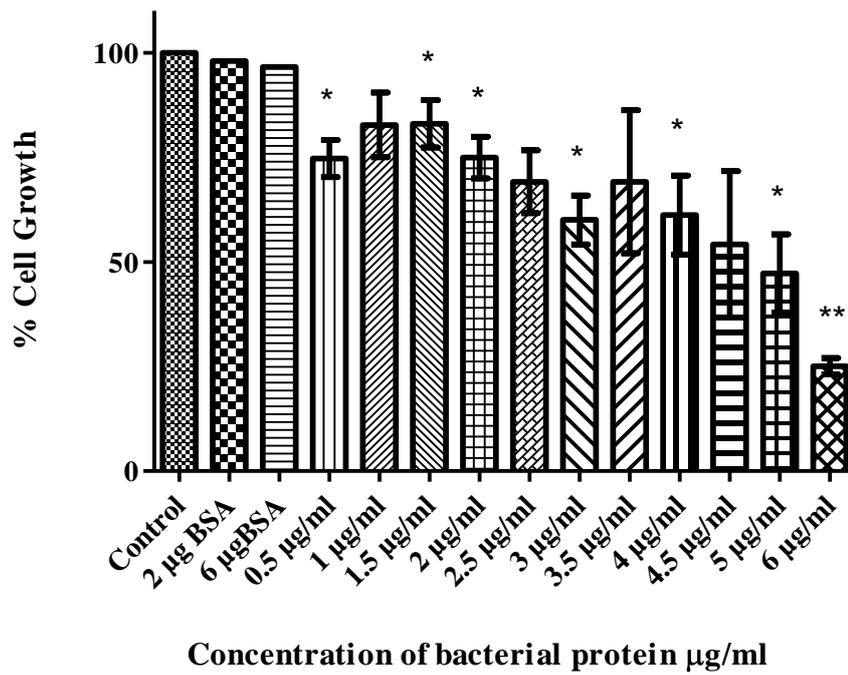


Figure 5.23 The effect of *Bacillus* antigens on the growth of the hTCEpi cell line

The results presented here are a combination of three replicates with statistical significance calculated by comparing treatments to control. $P < 0.05$ is denoted by * and $P < 0.01$ by **.

5.3 The effect of *Bacillus* protein on wound healing in hTCEpi cells

In order to examine the wound healing response the hTCEpi cells were grown to confluency in 6 well culture plates. The assay was carried out as described in section 2.31.2. Scratch wounds were generated using a 10-200 µl pipette tip. Results for these assays (Figure 5.2) showed that closure of the scratch wound was slower in control cells than in cells that were exposed to the *Bacillus* protein. However growth of the hTCEpi cells was seen to decrease in response to increasing concentrations of the proteins (Figure 5.1). Therefore the results are in agreement with previous studies using animal models that have shown corneal epithelial cells migrate to close superficial wounds (Jumblatt & Neufeld 1986). The results depicted in Figure 5.2, suggest that the proteins used to treat the corneal cells may be causing the ocular surface epithelium to migrate at the leading edge of the wound. Therefore the cells may become more motile and *in vivo* this could contribute to the loss of corneal surface integrity and in a chronic condition could lead to the formation of sterile ulcers. The production of the collagen degrading enzyme MMP-9 is increased in the tear fluid of rosacea patients (Kari & Kari, 2006). Over production of this protein by corneal epithelial cells at the leading edge of corneal surface wound has been linked to cell migration and sterile ulcer formation in humans (Li *et al.*, 2001).

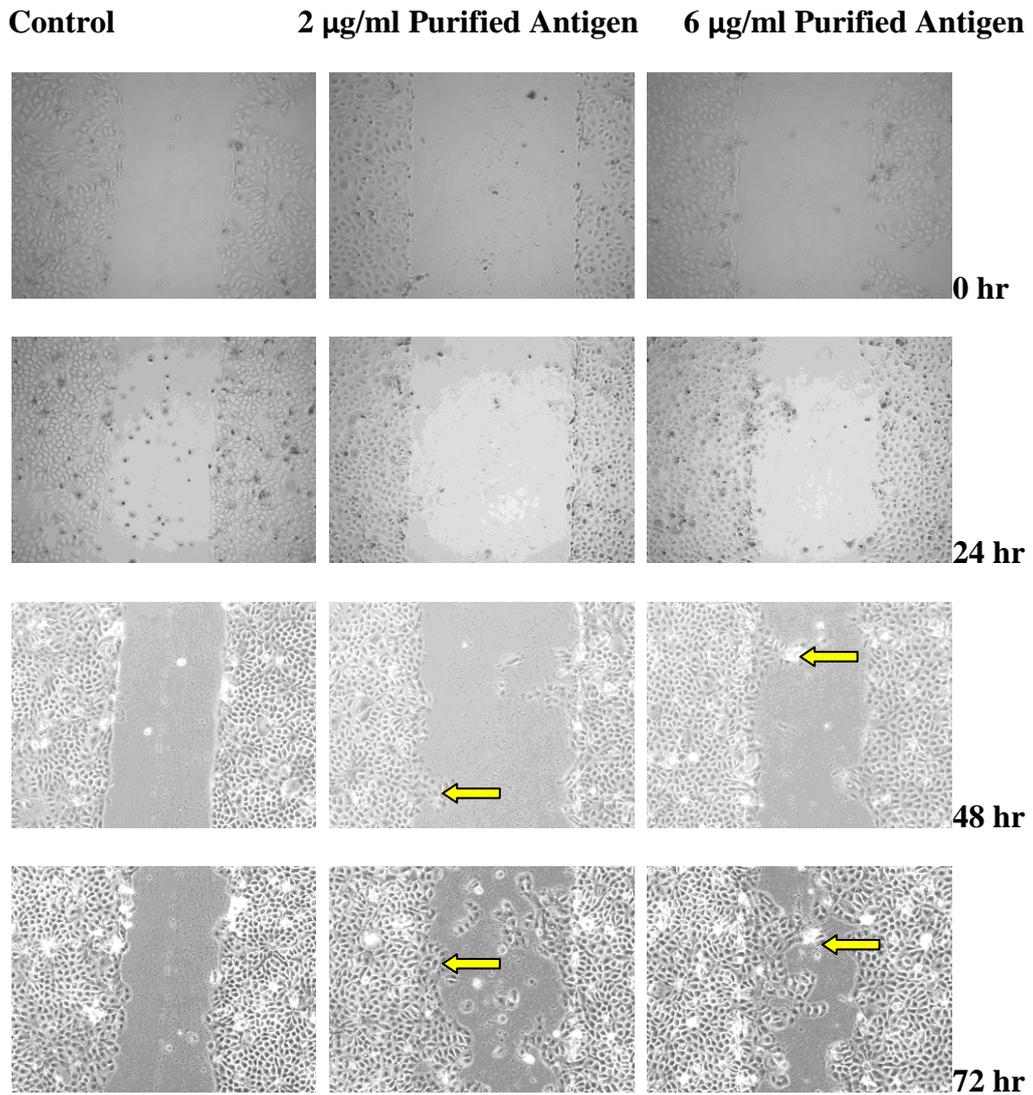


Figure 5.24 The effect of *Bacillus* proteins on the healing of a scratch wound in hTCEpi cells

Images are representative of 5 repeats of this assay. Migration of cells at the leading edge of the wound was increased in cells treated with the antigen compared to control cells.

5.3.1 Investigating the effect of *Bacillus* antigens on wound healing using Immuno fluorescence microscopy

In this section it was sought to establish if there was up-regulated expression of certain protein markers at the leading edge of the wound where cells were migrating (Figures 5.3 and 5.4). This was achieved by setting up a scratch wound assay as described in section 2.31.2 and a glass slide was placed in the culture dish before the cells were seeded. Several markers involved in cell-cell interactions, cell-matrix interactions and cell movement were examined. E-cadherin is a transmembrane protein associated with cell-cell junctions and actin containing filaments of epithelial tissues. Down regulation of cadherins has been implicated in some cancers because it causes cells to become less adherent to each other and to the basement membrane and this can result in increased motility that can lead to metastasis (Wijnhoven *et al.*, 2000). Phalloidin is a mycotoxin derived from *Amanita phalloides* that is fluorescently tagged using rhodamine. Corneal epithelial cells were first incubated with the E-cadherin antibody then counter stained with phalloidin which binds to F-actin filaments (Verderame *et al.*, 1980). Actin is a major component of the cytoskeleton therefore this staining allowed the visualization of the shape of the cells and at the leading edge of the wound. The actin filaments can be seen extending as the cells migrate in response to the bacterial antigens. Figure 5.3 highlights the movement of the cells when treated with the antigen. The actin filaments are stained using phalloidin (red) and the cellular junctions using E-cadherin (green) while the nucleus is stained with DAPI (blue).

5.4 The effect of *Bacillus* protein on migration of hTCEpi cells

Migration assays were performed using 8 μm porous inserts as described in section 2.31.3. When hTCEpi cells were exposed to increasing concentrations of the bacterial antigens, migration through the porous membrane increased. The images (Figures 5.5) show higher numbers of cells stained on the membranes following treatment with the bacterial protein than in the untreated controls. Migration increased 3.6 fold ($p=0.001$) in response to 2 $\mu\text{g}/\text{ml}$ of the antigen and by 14.5 fold in response to 6 $\mu\text{g}/\text{ml}$ treatment ($p=0.0001$) when compared to control cells (Figure 5.6).

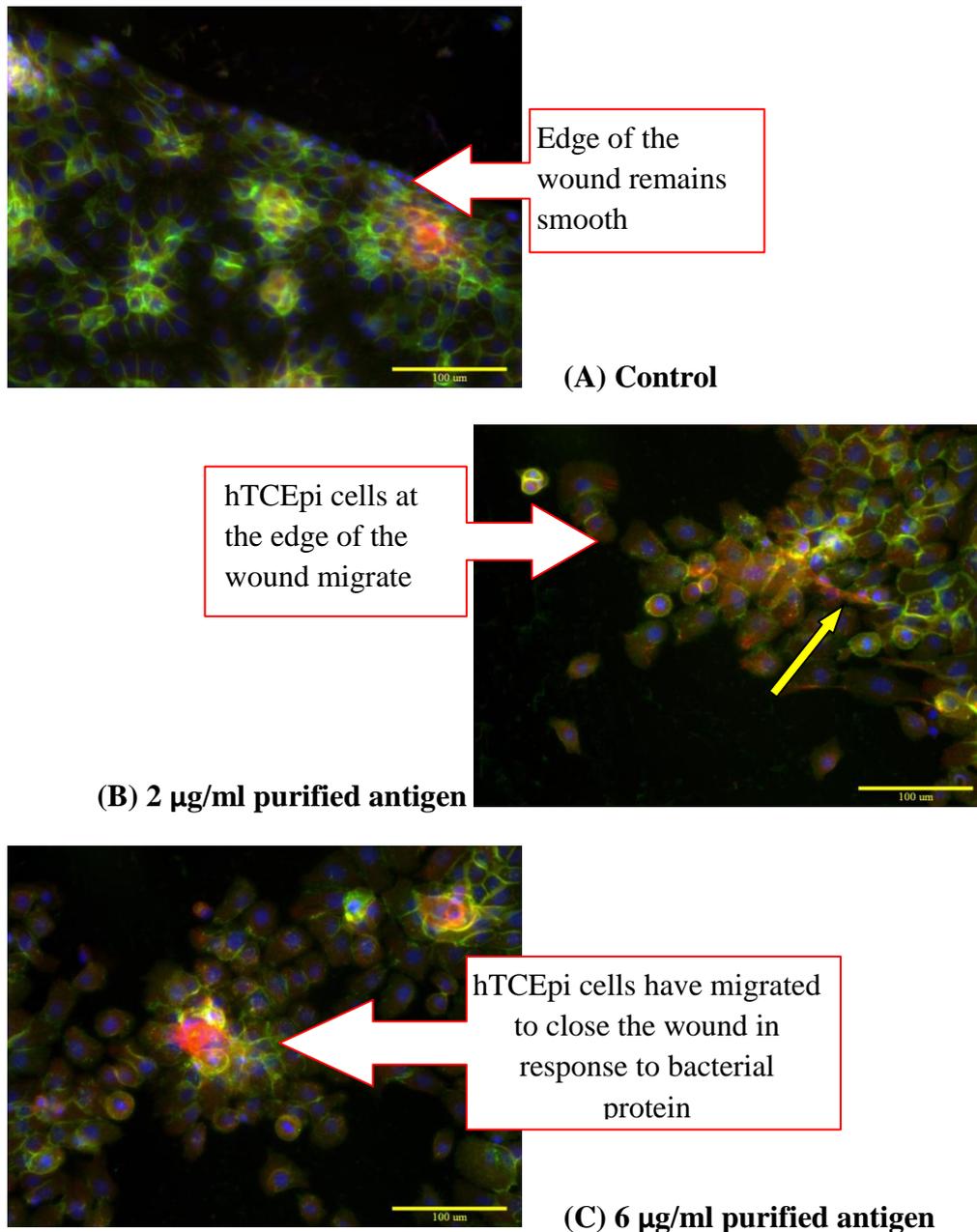


Figure 5.25 Immunofluorescence of wound healing in hTCEpi cells stained with E-cadherin (green), counterstained with Phalloidin (red) and DAPI (blue), following 72 hours incubation in media supplemented with the *Bacillus* antigens

- (A) Represents untreated cell response to wound healing
- (B) Represents hTCEpi cells treated with 2 µg/ml of bacterial protein. The yellow arrow indicates the actin filaments (stained with phalloidin) stretching as the cells migrate
- (C) Represents hTCEpi cells treated with 6 µg/ml of bacterial protein that have migrated to close the scratch wound

Scale bar is 100 µm

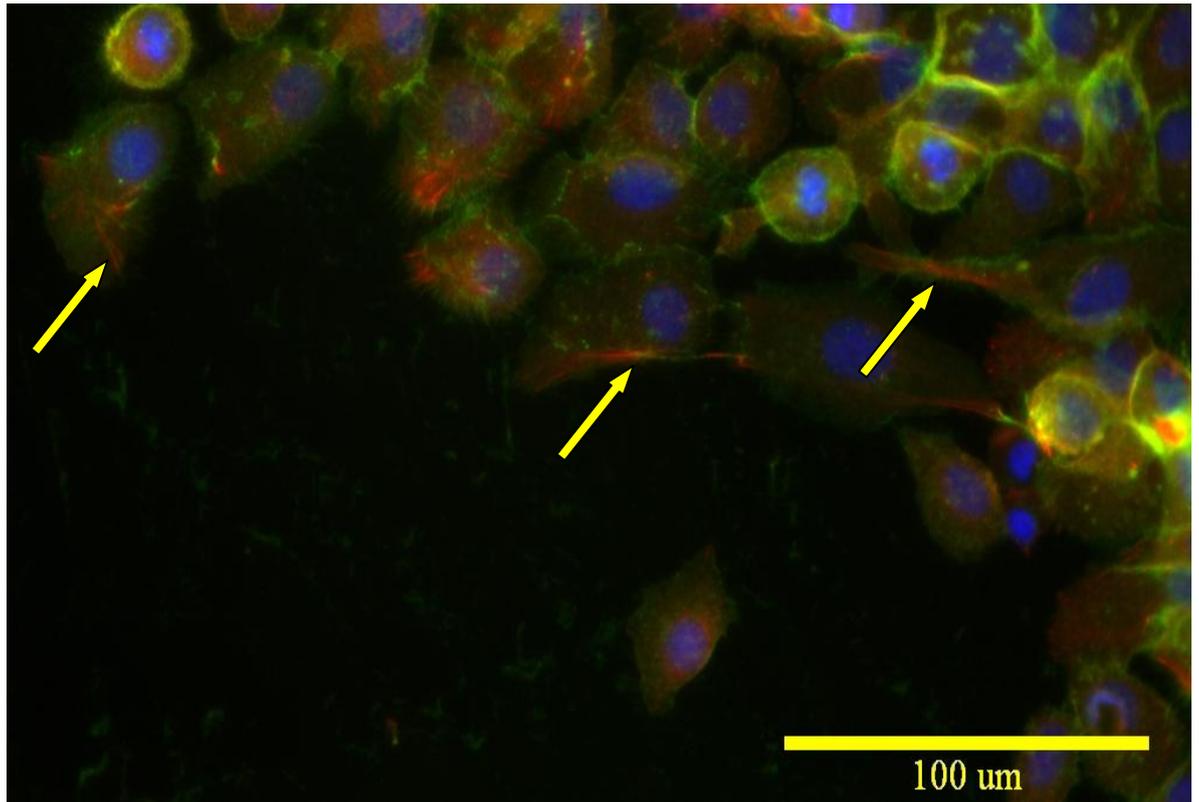


Figure 5.26 Magnified image of E-cadherin (green) stained cells, counter stained with Phalloidin (red).

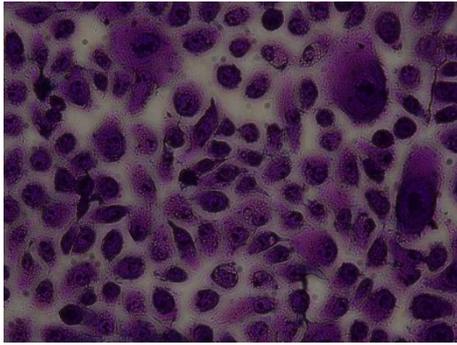
The yellow arrow indicates the actin filaments extending as the hTCEpi cells migrate to close the scratch wound

5.5 The effect of *Bacillus* protein on invasion of hTCEpi cells

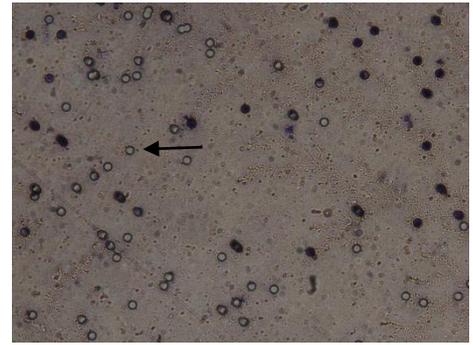
Cell invasion was tested as described in section 2.31.4 using similar 8 µm porous inserts that are coated with a matrigel that replicates the basement membrane matrix. Invasive cells detach from the matrix migrated through the gel and the 8 µm pores so the cells can be stained and counted. Results of these assays revealed an increase in hTCEpi cell invasiveness of 1.7 fold (p=0.003) in response to 2 µg/ml of the antigen and by 1.8 fold (p=0.01) in response to 6 µg/ml when compared to control cells (Figure 5.7). Invasion assays indicate a more active response than that seen in migration assays since the cells pass through the barrier of the matrix. This result considered together with the results of the migration assays indicate a possible role for the bacterial proteins in corneal erosion (Lin & Kurpakus-Wheater 2002; Yamaguchi *et al.* 2011; Markoulli *et al.* 2011).

5.6 The effect of *Bacillus* protein on adhesion of hTCEpi cells

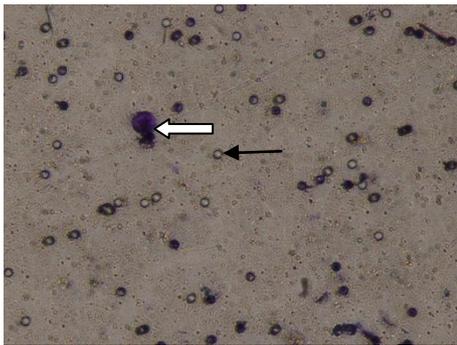
Cell adhesion assays were performed as described in section 2.31.5, using a variety of components of the ECM (Collagens I, IV, V, Laminin, Fibronectin and Gelatin). These proteins were coated onto the lid of a 96 well plate. Cells were pre-incubated with antigen preparation, trypsinised and applied at a concentration of 1×10^4 cells/ml onto the different ECMs. The cells that adhered were stained and counted. The hTCEpi cells adhered consistently at an increased density to collagen I (Figure 5.8) following exposure to 2 and 6 µg/ml of bacterial antigens, however student t-test revealed no statistical significance. There was a consistent decrease in adherence to laminin of 1.7 fold (p=0.002) following exposure to 2 µg/ml of bacterial antigens, adherence was decreased only 1.26 fold following exposure to 6 µg/ml of the antigens. Adherence to collagen IV and gelatin was increased by 1.47 fold (p=0.01) and 1.5 fold (p=0.004), respectively following exposure to 6 µg/ml of antigens. Adherence of hTCEpi cells to fibronectin and collagen V both decreased significantly following exposure to 2 µg/ml and then increased slightly compared to control cells when exposed to 6 µg/ml of *Bacillus* antigens.



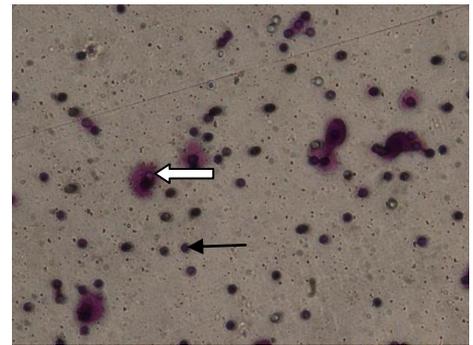
Control stained cells before migration assay



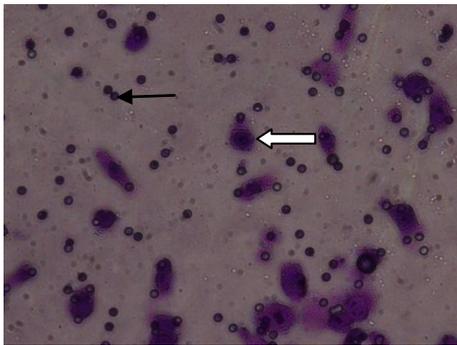
Untreated Control



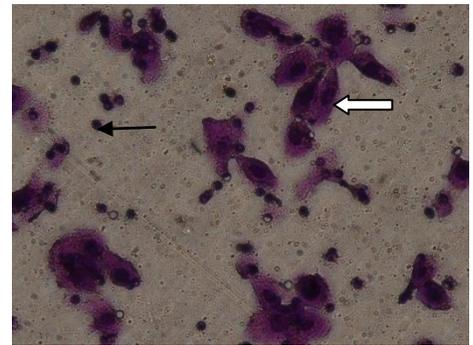
BSA treated control



2 µg/ml antigen



4 µg/ml antigen



6 µg/ml antigen

Figure 5.27 Images of porous membranes with hTCEpi cells that have migrated through 8 µm pores

Corneal epithelial cells stained with crystal violet dye and some examples are labeled with white arrows, while the pores of the inserts can be seen marked by black arrows. Images were taken at 40 x magnification.

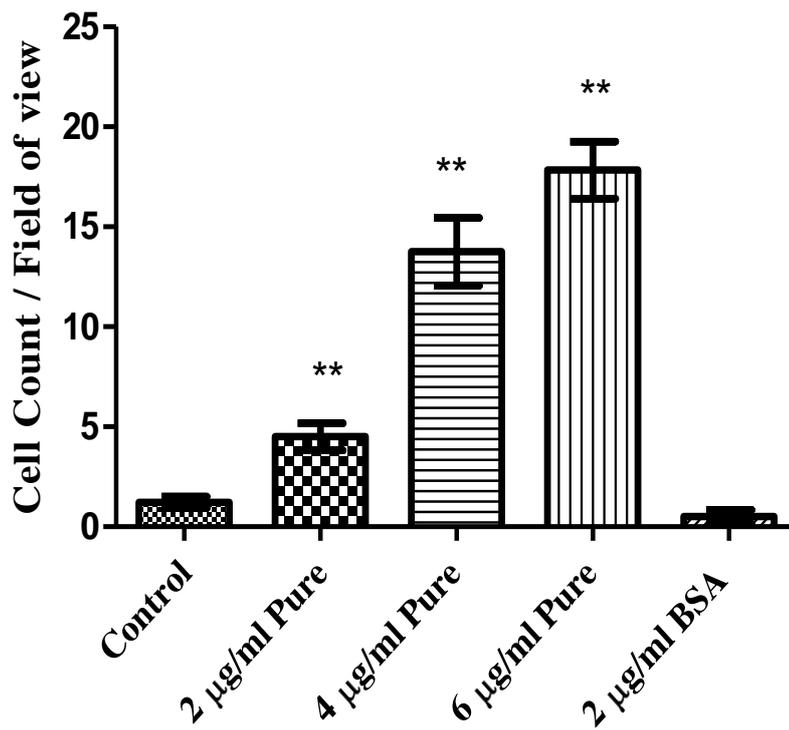


Figure 5.28 The effect of *B. oleronius* antigen on the migration of hTCEpi cells through 8 µm porous membranes

These data are representative of 3 repeats of the assay where * denotes $p < 0.05$ and ** is $p < 0.01$.

Membranes were viewed at 40 x magnification and 3 x fields of view were counted per treatment for each replicate in the assay.

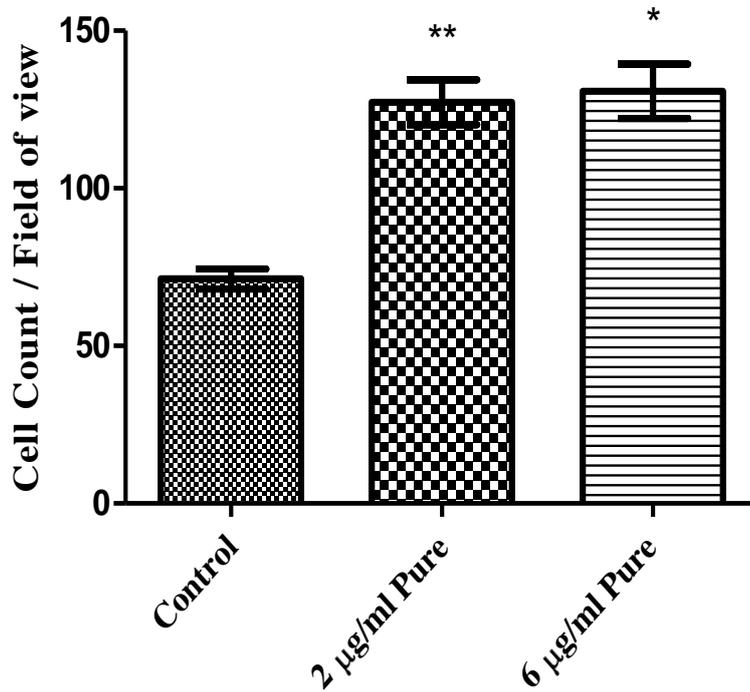


Figure 5.29 The effect of *B. oleronius* antigen on the invasion of hTCEpi cells through a matrigel membrane

These data are representative of two replicates of the assay where * denotes $p < 0.05$ while ** in $p < 0.01$.

Membranes were viewed at 40 x magnification and 3 x fields of view were counted per treatment for each replicate in the assay.

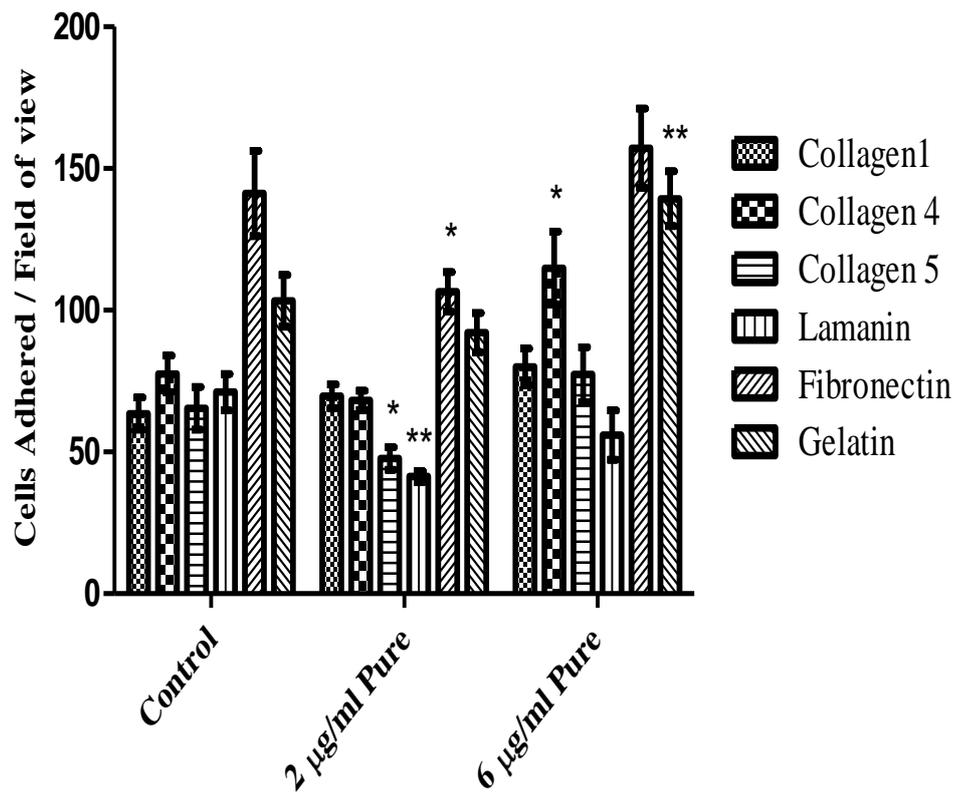


Figure 5.30 The effect of *B. oleronius* antigens on the adhesion of hTCEPi cells to extracellular matrix components

These data are representative of a single assay where technical replicates were used to perform statistical analysis and * denotes $p < 0.05$, while ** is $p < 0.01$.

5.7 Summary

Initial results revealed that the proteins extracted from *Bacillus oleronius* inhibited growth of hTCEpi cells and increased movement of the cells. Cell growth decreased as the concentration of protein extract in the media increased (Figure 5.1). The scratch wound assay (Figure 5.2-5.4) revealed that the cells were migrating at an increased rate at the leading edge of the wound following exposure to the antigens. Plate assays using porous inserts that examine migration (Figure 5.5-5.6) and invasion (Figure 5.7) also added to the evidence that hTCEpi cells become more motile in response to treatment with *Bacillus* proteins. Adhesion assays showed a consistent decrease of hTCEpi cell adherence to laminin of 1.7 fold ($p=0.002$) following exposure to 2 $\mu\text{g/ml}$ of bacterial proteins. Altered migration, wound healing, invasion and adhesion to the basement membrane are factors that would potentially contribute to corneal cell erosion (Lin & Kurpakus-Wheater 2002; Yamaguchi *et al.* 2011; Markoulli *et al.* 2011). Corneal erosion is linked to the development of microbial keratitis in contact lens wearers (Markoulli *et al.*, 2011). It is possible that the presence of bacterial antigens in the tear fluid of individuals with ocular rosacea could cause an inflammatory response in corneal epithelial cells that may lead to corneal erosion.

5.8 The effect of *Bacillus* antigens on protein expression in the hTCEpi cell line

The effect of *B. oleronius* antigens on the expression of certain proteins in both hTCEpi cell line and primary limbal epithelial cells derived from limbal stem cell tissue was assessed using Western blotting and immunofluorescence microscopy. As described in section 2.32 and protein was extracted from the cells after incubating for five days with varying concentrations of the antigen. This protein extract was separated by one dimensional SDS-PAGE and transferred to nitrocellulose membrane in preparation for Western blotting. Several antibody markers were employed to investigate possible changes in protein expression. Glass slides were placed in 24 well culture dishes prior to the hTCEpi cells being seeded in order to investigate the proteins using immunofluorescence microscopy. As described in section 2.34 cells were incubated in the presence of the *Bacillus* proteins or in KGM media alone for the

controls for four days. After this time the medium was removed and ice cold methanol or acetone (depending on which antibody was to be used) was added to fix the cells for 10 minutes. At this point the slides could be stored at -20°C until the staining procedure was carried out as described in section 2.34. Goat anti-serum was employed as a blocking agent and as a diluent for the primary and secondary antibodies. Primary antibodies were generally incubated overnight (Table 2.6 Antibody dilutions) at 4°C. For samples where two primary antibodies were applied, the second was added for 1 hour at 37°C. Following wash steps using PBS-tween, the secondary antibodies (Alexafluor) were diluted 1:1000 and applied for 1 hour at room temperature in the dark. At this point slides were washed again and mounted to cover slips then images were obtained using a Leica microscope.

5.8.1 The effect of *Bacillus* antigens on the production of Cytokeratin 3 and 12

Cytokeratins 3 and 12 are type II keratins and are expressed together in the corneal epithelium. Expression of these proteins was examined following treatment of the hTCEpi cells with the bacterial antigens by Western blotting and immunofluorescence microscopy. Each row of images (A-C) represents one replicate of immunofluorescence protocol described in section 2.32. There appeared to be a decrease in the intensity of keratin 3 staining in cells that were treated with the bacterial antigen as seen in Figure 5.9 A & B. However on subsequent occasions the staining intensity seemed to be similar in control and treated cells (Figure 5.9 C). Western blotting analysis showed a slight decrease in expression of cytokeratin 3 following exposure, however student T-test revealed no significant change in expression of following exposure to the antigens (Figure 5.10).

Immunofluorescence images of corneal cells stained for keratin 12 appeared to show little change in staining intensity and the antibody binding seemed non-specific (Figure 5.11). The staining could potentially have been improved by investigating further fixing agents for the slides or altering the antibody. Western blotting with this antibody also revealed a slight decrease in expression of this protein following exposure to the antigens, however no significant change in expression across the treatments (Figure 5.12) following student T-test analysis.

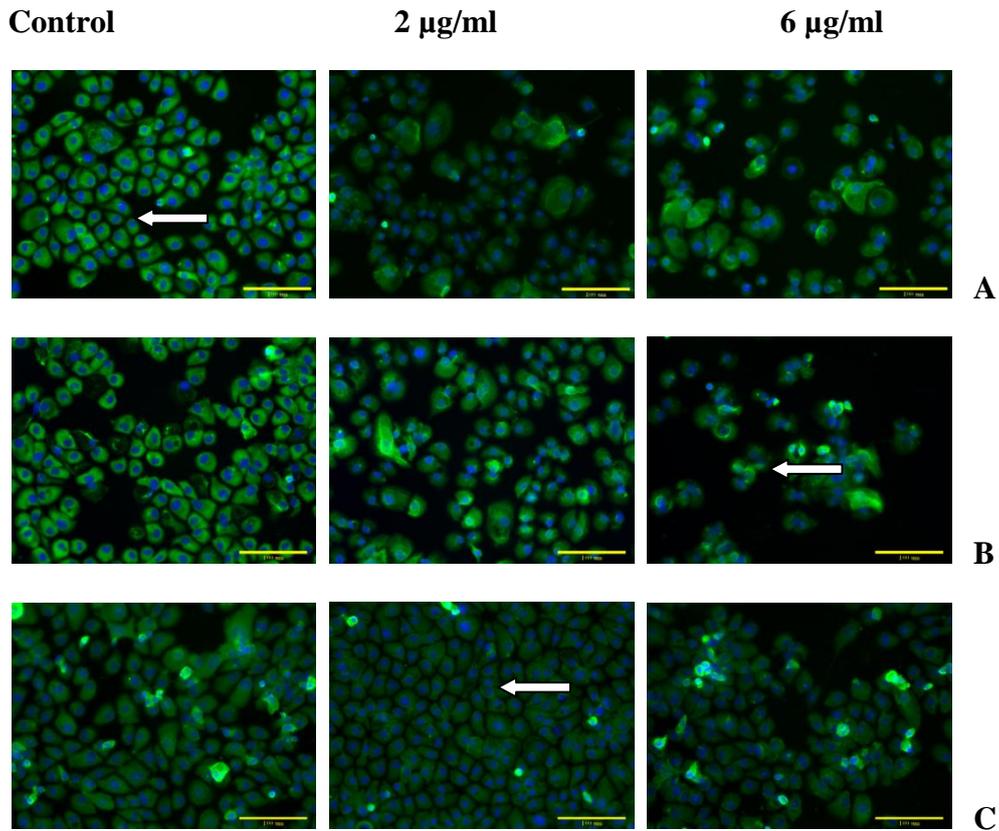


Figure 5.31 Cytokeratin 3 staining of hTCEpi cells exposed to bacterial protein nuclear material stained blue using DAPI

The white arrows highlight some of the changes described in A-C below.

- (A) Expression of CK3 on the surface of untreated hTCEpi cells is bright and uniform throughout the culture.
- (B) Cells exposed to the bacterial protein appear to show decreased expression and irregular shape particularly in the 6 $\mu\text{g/ml}$ treatment.
- (C) CK3 expression appears decreased even in treated cells that appear to have grown well when compared to controls.

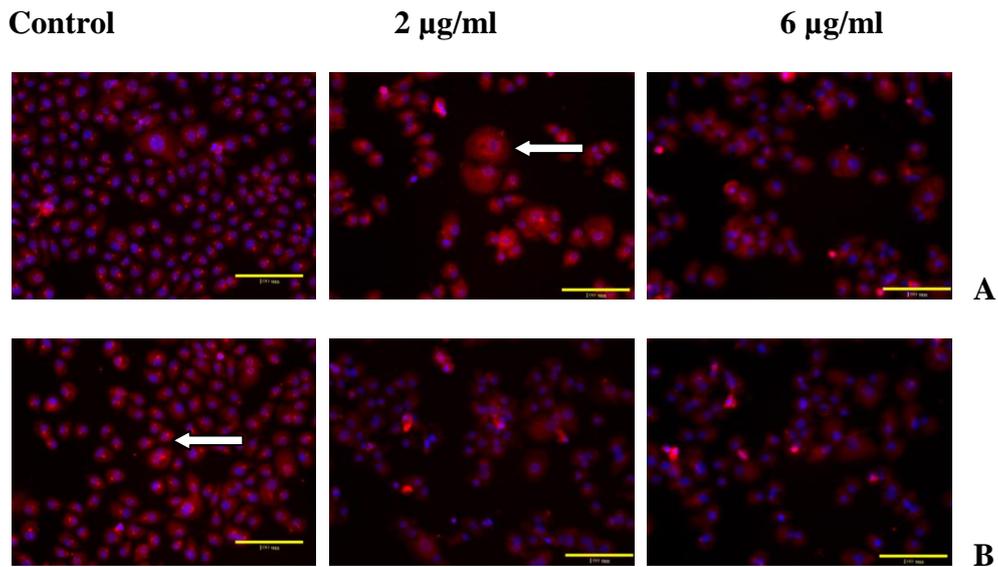


Figure 5.32 Cytokeratin 12 staining of hTCEpi cells exposed to bacterial antigen nuclear material stained blue using DAPI

The white arrows highlight some of the changes described in A-B below.

- (A) Staining intensity of CK12 appears to increase in the 2 $\mu\text{g/ml}$ treatment compared to control cells.
- (B) In this case CK12 appears increase in the control cells compared to those exposed to the bacterial protein.

Investigations with this antibody were not conclusive however the irregular shape and decreased growth of cells exposed to the bacterial protein is indicated here.

Scale bar is 100 μm

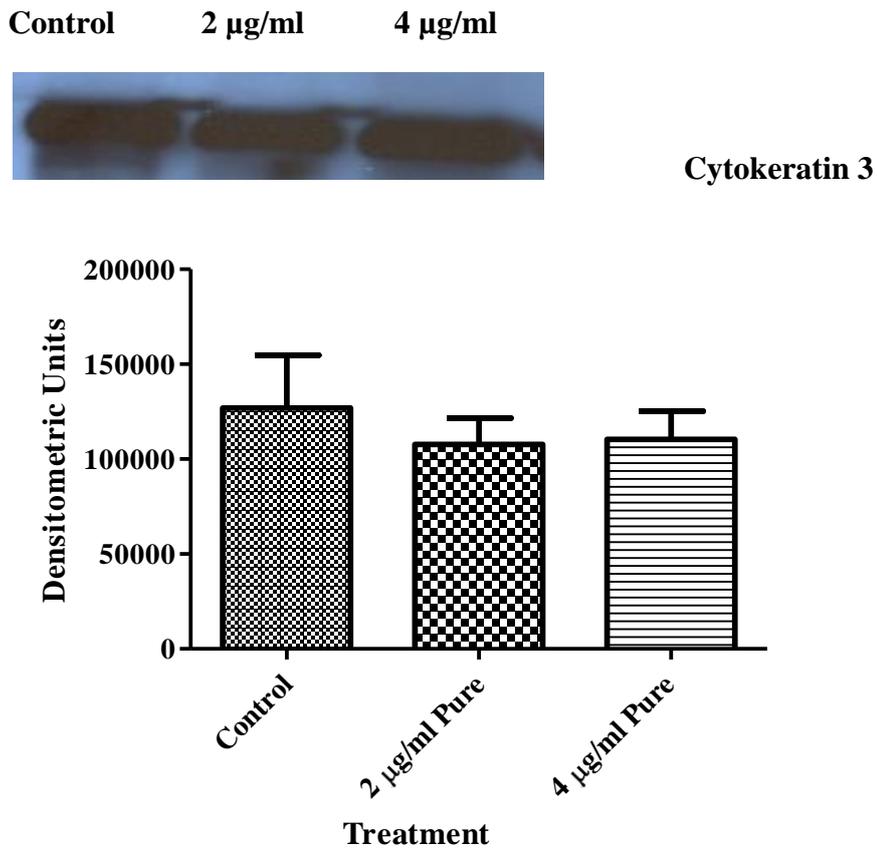


Figure 5.33 Western blotting for Cytokeratin 3 expression of hTCEpi cells treated with bacterial protein

The densitometric analysis indicates decreased expression of CK3 in hTCEpi cells exposed to 2 & 4 $\mu\text{g/ml}$ of bacterial protein however the decrease was not significant.

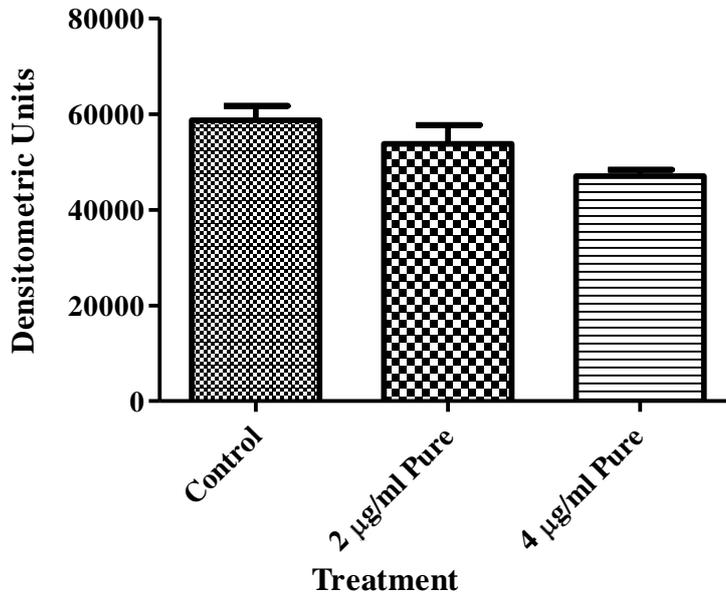
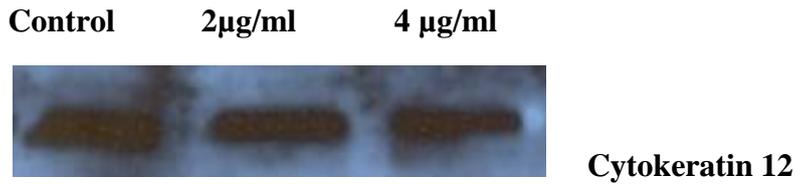


Figure 5.34 Western blotting of hTCEpi cells treated with bacterial antigen stained for Keratin 12 expression

Densitometric analysis suggests a decrease in CK12 expression however it was not significant.

5.8.2 The effect of *Bacillus* antigens on the expression of Cytokeratin 19

Cytokeratin 19 is a type I keratin and the smallest unpaired acidic keratin and it is not uniformly expressed across all corneal epithelial cells in the hTCEpi. CK19 is said to be a marker for conjunctival epithelial cells *in-vivo* (Sacchetti *et al.* 2005). Figure 5.13 A-C represents the expression of CK19 using immunofluorescence microscopy. Across all the treatments there are incidences where the nucleus is clearly stained with DAPI (blue) and no CK19 expression is present. Due to the sporadic expression of this protein even in controls it was difficult to judge if there was a change in the intensity in cells exposed to the bacterial proteins compared to controls. Keratin 19 appeared to be increased and decreased in various blots however proof of a significant change either way was not achieved by reproducible blots (Figure 5.14). Clinical cytological examination that reveals CK3 and CK19 expression on the ocular surface is indicative of limbal stem cell deficiency since this expression pattern is indicative of mixed corneal and conjunctival epithelia (Sacchetti *et al.* 2005).

5.8.3 The effect of *Bacillus* antigens on proteins involved in cell motility (E-cadherin, β 1-integrin and Vinculin)

Since increased migration was caused by treatment with the bacterial antigens it was decided that proteins involved in cell movement and interaction would be investigated. E-cadherin, β 1-integrin and vinculin antibodies were employed. E-cadherin is involved in cell-cell interactions and can be seen here staining brightly at the junctions between the cells. Figure 5.15 also depicts E-cadherin counter stained with p63 a nuclear protein that encodes transcription factor. There did not appear to be a difference in the staining intensity following treatment with the bacterial antigen this was also confirmed with Western blotting. β 1-integrin is involved in cell binding to the extracellular matrix components such as fibronectin, laminin and collagens it is therefore involved in cell motility, adhesion and signaling. This protein appeared to decrease by approximately 1.25 fold ($p=0.01$) in expression following exposure to the bacterial antigens as seen in Figure 5.17, using Western blotting and immunofluorescence investigations. Decreased expression of this protein induced migration of the cells since the cells become less adherent to the components of the ECM.

Cell movement and interaction with the extracellular matrix was likely affected by a range of transient cellular changes in response to these antigens. One protein that appeared to have a significant increase in expression was vinculin. The expression of this protein was examined by Western blotting only and it is clearly seen in Figure 5.18, that there is an increase in expression following treatment with the bacterial antigens. Reactivity to the vinculin antibody increased by approximately 2.05 fold ($p=0.0026$) and 2.7 fold ($p=0.0009$) in response to exposure to 4 $\mu\text{g/ml}$ and 6 $\mu\text{g/ml}$ of the purified antigen preparation respectively. Vinculin is a membrane cytoskeletal protein involved in linkage of integrins to the actin filaments of the cytoskeleton and is involved in anchoring F-actin to the membrane.

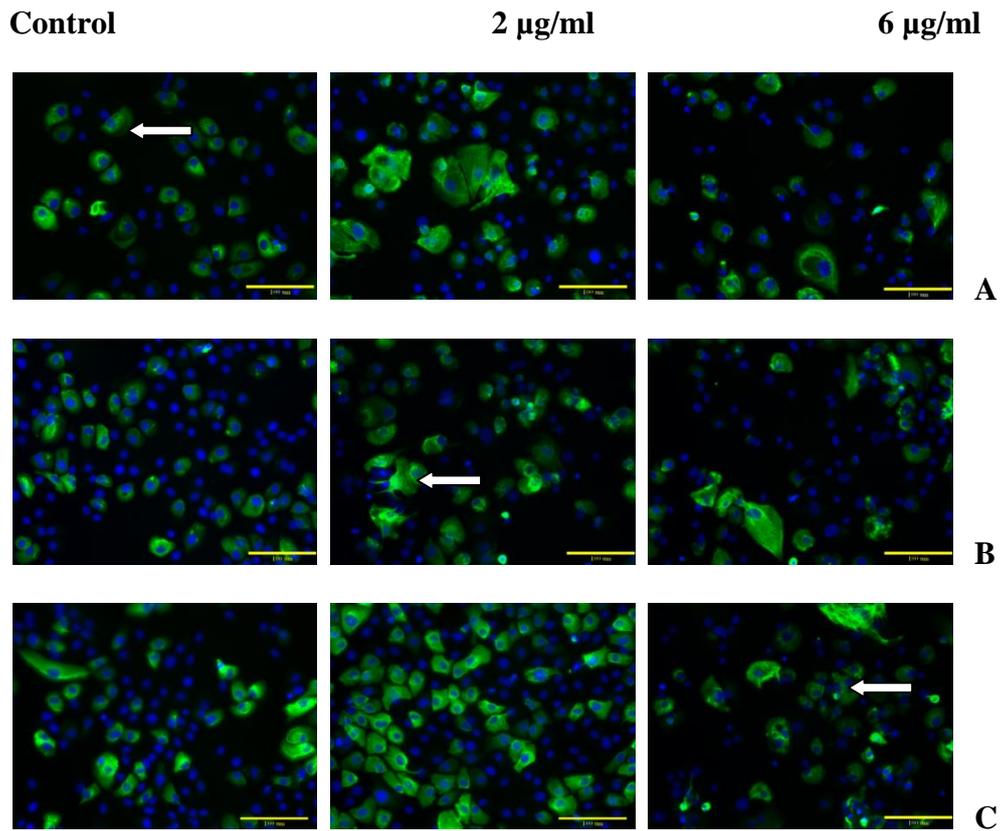


Figure 5.35 Cytokeratin 19 staining of hTCEpi cells treated with bacterial antigen

The white arrows highlight some of the changes described in A-C below.

- (A) CK19 is expressed sporadically in control cells
- (B) Expression appeared to increase in cells exposed to 2 $\mu\text{g/ml}$ of *Bacillus* protein
- (C) Expression appeared increased in the 2 $\mu\text{g/ml}$ but not 6 $\mu\text{g/ml}$ treated cells compared to controls

Scale bar is 100 μm

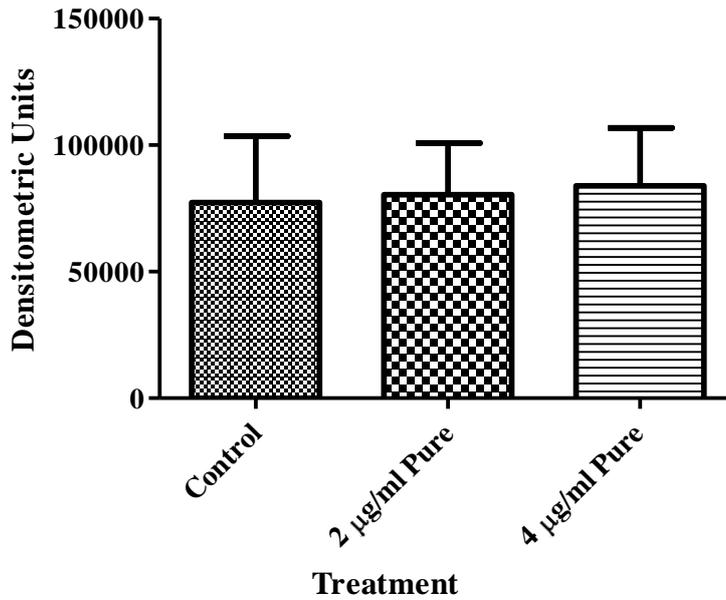
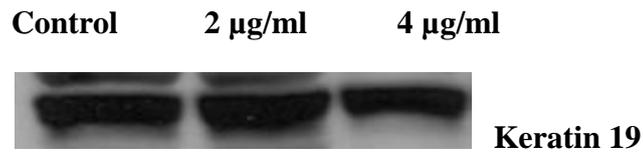


Figure 5.36 Western blotting of hTCEpi cells treated with bacterial antigen stained for anti-Keratin 19 antibody expression

There was no significant change in CK19 expression across all replicates.

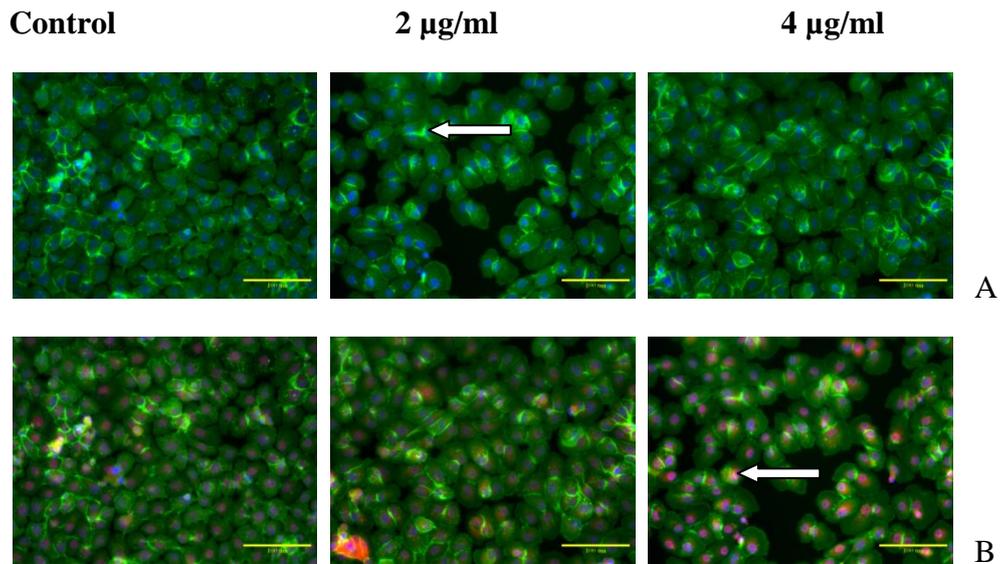


Figure 5.37 E-cadherin/p63 staining of hTCEpi cells treated with bacterial antigen

The white arrows highlight some of the changes described in A-B below.

- (A) There does not appear to be a difference in expression of E-cadherin (green staining) following exposure to the bacterial proteins
- (B) P63 staining is red and localized to the nucleus

Scale bar is 100 μm

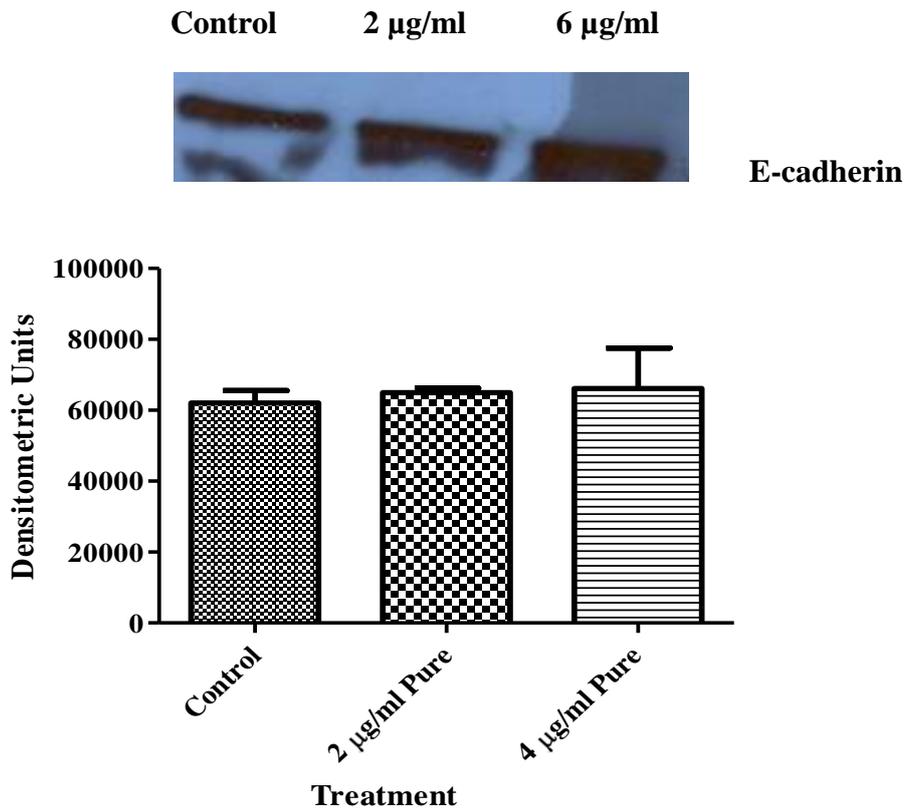


Figure 5.38 Western blotting of hTCEpi cells treated with bacterial antigen stained for E-cadherin expression

E-cadherin is a protein involved in cell-cell interactions and its expression remained unchanged following exposure to bacterial proteins

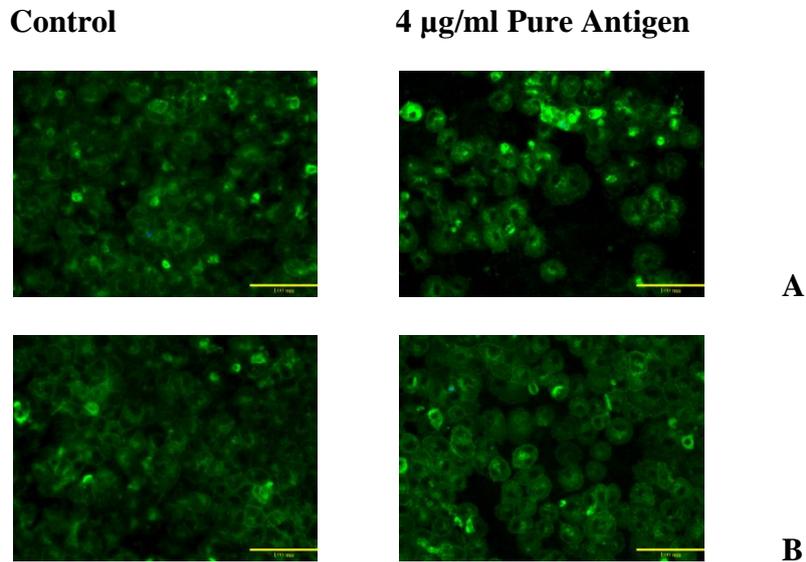
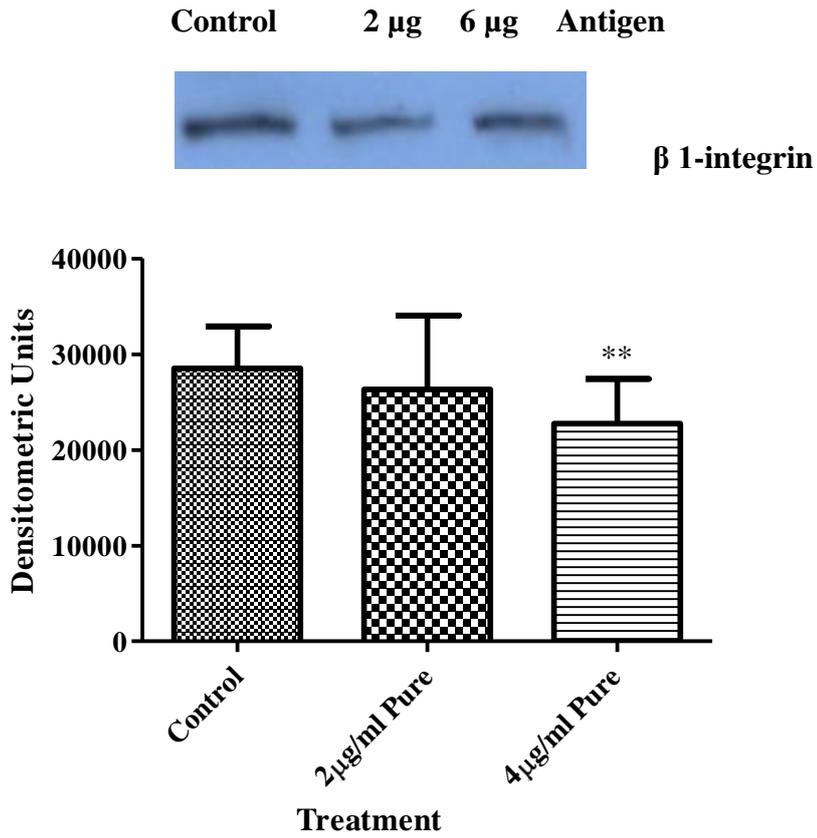


Figure 5.39 Immunofluorescence images and Western blotting of $\beta 1$ -integrin staining of hTCEpi cells treated with bacterial antigen

Densitometric analysis revealed a fold change in expression of $\beta 1$ -integrin of 1.25 ($p=0.01$) following exposure to 4 $\mu\text{g/ml}$ of the bacterial proteins. $p \leq 0.01$ is denoted by **. Immunofluorescence did not indicate a change in expression of the $\beta 1$ -integrin in treated cells compared to controls. Scale bar is 100 μm

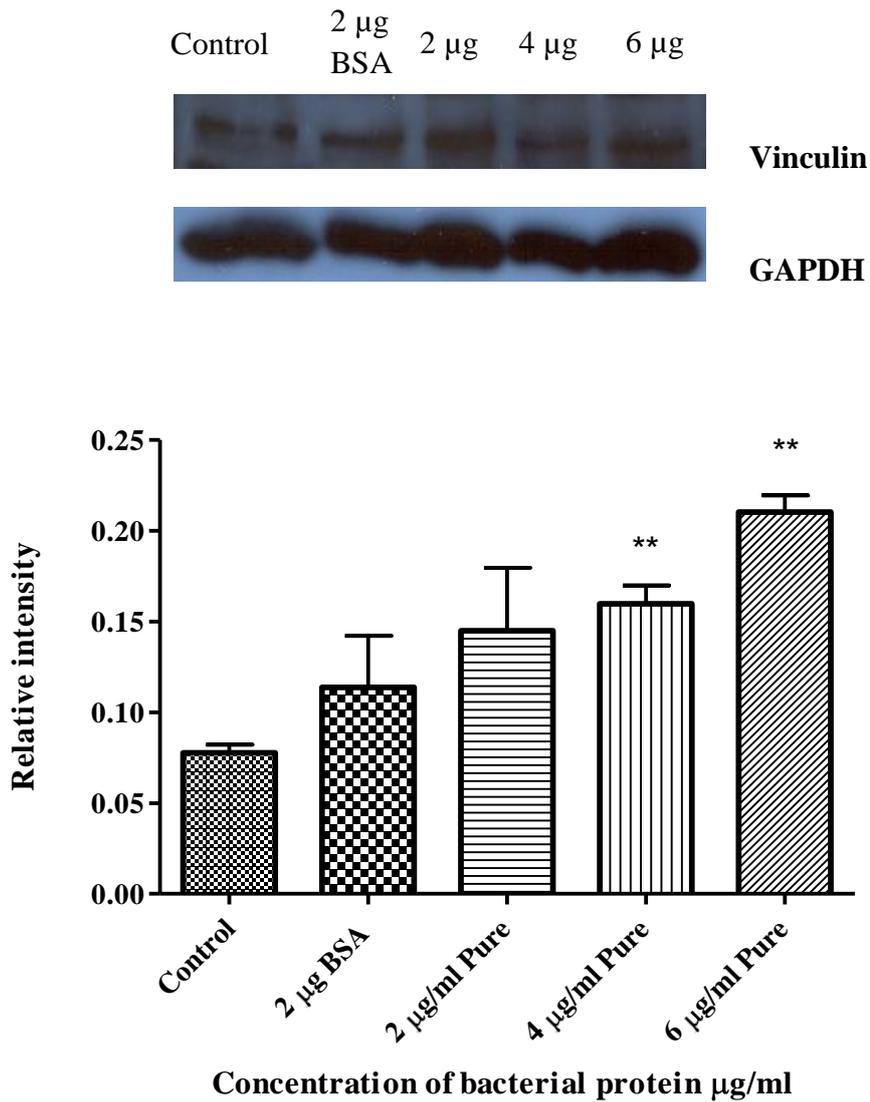


Figure 5.40 Western blot of Vinculin expression in hTCEpi cells following antigen treatment

Graph represents densitometric analysis of intensity of vinculin expression relative to the GAPDH loading control, with significant differences denoted by ** were $p < 0.01$

There was a 2 fold ($p = 0.026$) increase in expression of vinculin in cells exposed to 4 µg/ml and a 2.7 fold ($p = 0.0009$) increase in expression following exposure to 6 µg/ml of *B. oleronius* protein.

5.8.4 The effect of *Bacillus* antigens on MMP-9 expression and activity

Matrix metalloproteinases are a family of enzymes involved in degradation of extracellular matrix components such as collagens, fibronectin and laminin. MMP-2 and 9 in particular are implicated in ocular surface diseases. Western blotting (Figure 5.19) revealed an increase in expression of this antibody when cells were exposed to 2 $\mu\text{g/ml}$ of *Bacillus* antigens of 1.34 fold ($p=0.033$). Zymography is an electrophoresis technique used to study the activity of MMPs, in this incidence gelatin was used as the substrate. The bright bands shown in Figure 5.20 represent the amount of gelatin substrate that has been digested therefore when the unstained area of the gel increases and this reflects an increase in the MMP activity. MMP-9 or gelatinase activity increased by an average of 1.96 fold ($p=0.043$) when cells were exposed to 4 $\mu\text{g/ml}$ of the bacterial antigens compared to untreated control cells.

5.9 Summary

The Western blotting and immunofluorescence work presented here has established that exposure to *Bacillus* antigens affects protein expression in cultured hTCEpi cells. While the expression of some of the markers for corneal epithelial cells such as the cytokeratins remained relatively unchanged, there were significant alterations to expression of proteins involved in cell migration and gelatin degradation. β 1-integrin expression was decreased (Figure 5.17) in cells exposed to the antigens compared to control cells and vinculin expression was increased (Figure 5.18). MMP-9 expression and activity also increased (Figures 5.19 & 5.20) and previously it was shown that treated cells adhered less to laminin coated plates compared to control cells (Figure 5.8), this may be due to the degradation of laminin by MMPs. These results give an insight into the proteomic changes that are influencing cell migration, tissue remodeling and angiogenesis of telangeiectastasis. In severe cases patients with ocular rosacea can develop keratitis which is an inflammatory condition of the cornea. This Chapter provides evidence that the effects caused by the *Bacillus* antigens have the potential to contribute to the development of the serious symptoms of rosacea.

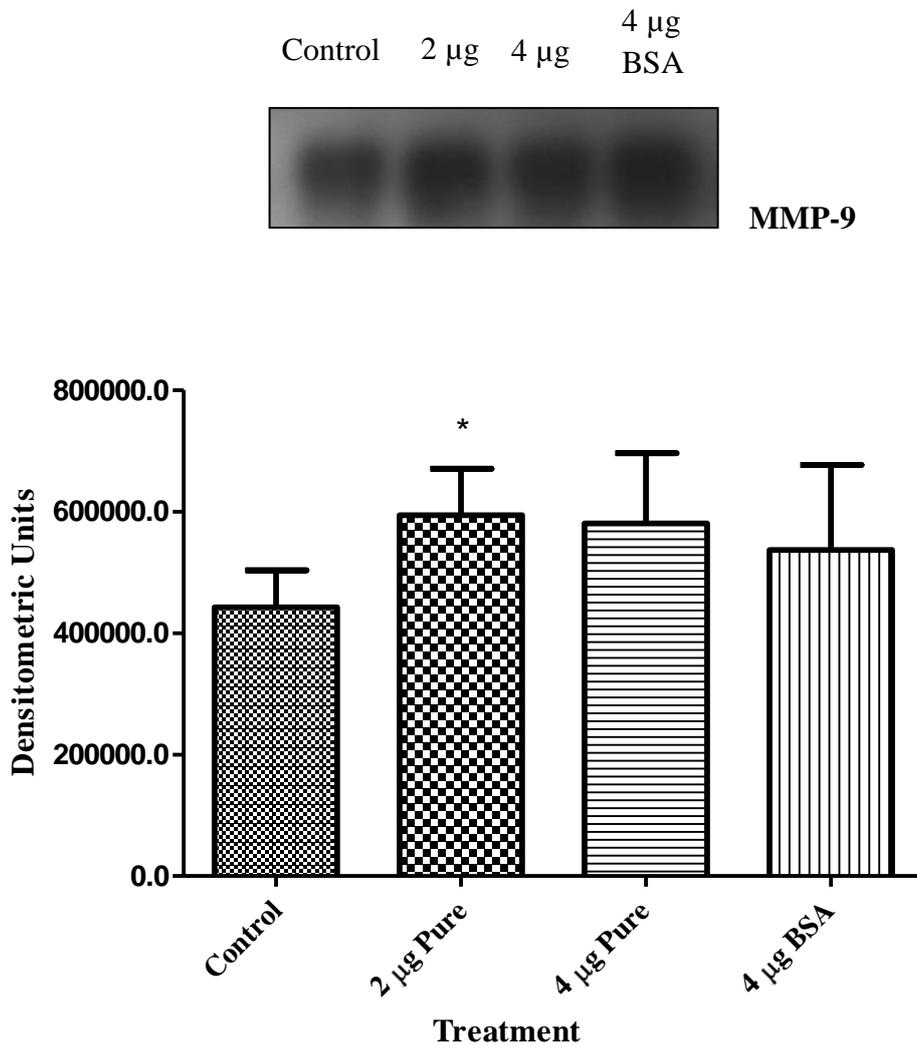


Figure 5.41 Western blot of MMP-9 expression in hTCEpi cells following antigen treatment

Graph Represents densitometric analysis of the blots with significant differences denoted by * were $p < 0.05$

MMP-9 protein expression was significantly increased by 1.34 fold ($p = 0.033$) in corneal cells exposed to 2 µg/ml of *B oleronius* proteins

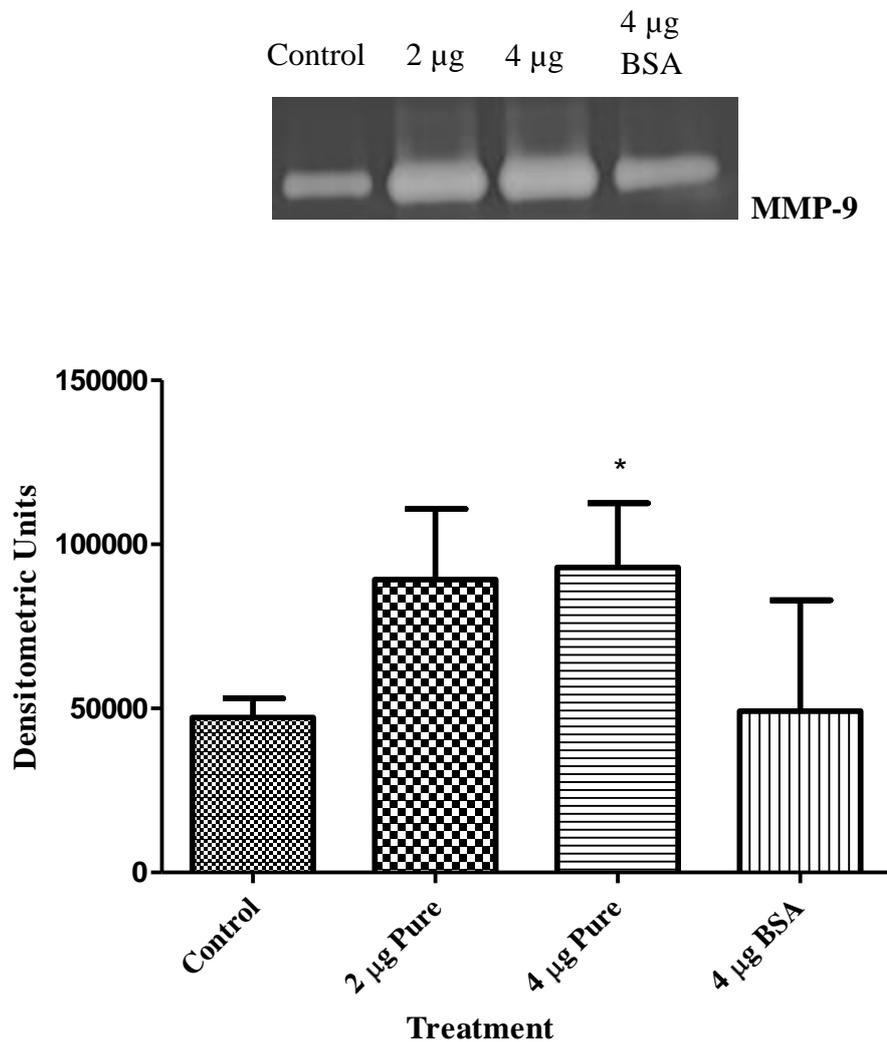


Figure 5.42 Zymogram of MMP-9 activity in hTCEpi cells following antigen treatment

Graph Represents densitometric analysis of the blots with significant differences denoted by * were $p < 0.05$

MMP-9 protein activity was significantly increased by 1.96 fold ($p = 0.043$) in corneal cells exposed to 2 µg/ml of *B. oleronius* proteins

5.10 Isolation of limbal stem cells and generation of primary limbal epithelial cell cultures

Limbal stem cells are located at the outer most ring of the cornea and are essential to the maintenance and regeneration of the corneal surface (Notara & Daniels, 2008). When corneal epithelial cells are shed, limbal epithelial cells migrate from the periphery towards the centre of the cornea and from the basal layers of the corneal epithelium towards the surface thus replenishing the corneal epithelial surface. In severe cases of ocular rosacea limbal stem cell deficiency has been reported (Kheirkhah *et al.*, 2007). In this condition there is a recurrent loss of corneal epithelial cells and the limbal stem cells become gradually ineffective at regenerating the corneal surface (Kolli *et al.*, 2010). This leads to a situation where conjunctival cells begin to re-epithelialize the corneal surface, the consequences of this are the neo-vascularization and ulceration of the corneal epithelium. The corneal surface becomes opaque and visual acuity is challenged.

Culture of limbal stem cells and transplantation of limbal epithelial cell sheets was first established as a technique for the treatment of limbal stem cell deficiency in 1997 (Pellegrini *et al.*, 1997). This technique has been extensively investigated and advanced in recent years (Baylis *et al.*, 2011). In this section it was sought to use cultured limbal epithelial cells in assays to investigate the potential effect that *Bacillus oleronius* proteins might have on these cells. Donor limbal tissue was obtained and small pieces of the tissue were dissected and placed in gelatin coated culture dishes. Growth medium was added and the tissue explants were incubated overnight to allow them to adhere. Culture medium was supplemented with irradiated 3T3 cells, this is an established protocol which aids the growth of epithelial cells by providing key growth factors to the cells. In Figure 5.18A the dark area in the corner of the image is the explant of limbal tissue and epithelial cells can be seen growing out from the tissue. Once the epithelial cells began to grow successfully they could be maintained with media and expanded for use in the assays.

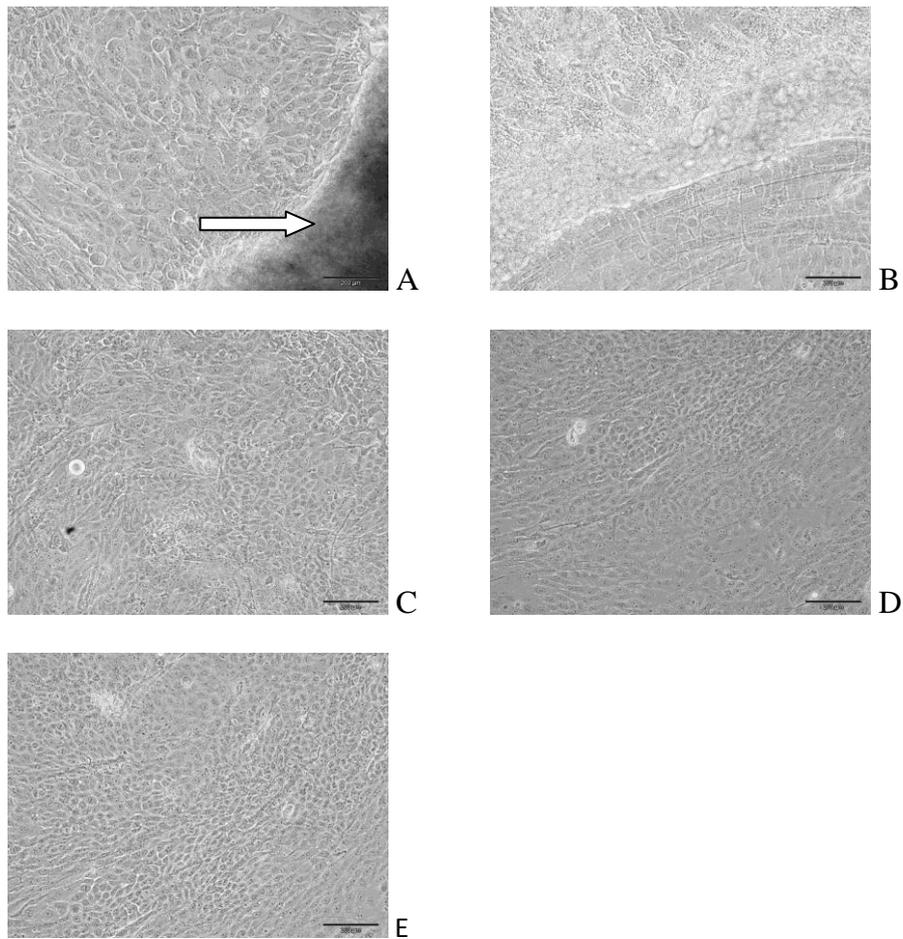


Figure 5.43 Primary human limbal epithelial cells growing out from explants of limbal stem cell tissue

- (A) Limbal epithelial cells are growing out from the tissue explant indicated by the arrow
- (B) Cells begin to proliferate faster until a confluent layer (C-E) is formed. At this point cells were trypsinised and used for investigations of wound healing and protein expression.

Scale bar is 200 μm

5.10.1 The effect of *Bacillus* proteins on wound healing in primary corneal epithelial cells

When the limbal epithelial cultures grew to a confluent layer the cells were removed using trypsin and re-seeded into 6 well culture dishes (3 wells per treatment) in order to set up wound healing assays similar to those that were carried out with the immortalized cell line. Cells were nourished until they reached approximately 100 % confluence (Figure 5.20) then scratch wounds were made using 200 μ l pipette tips as described in section 2.35.1. Figure 5.23 represents the wound healing response of the limbal cells following exposure to bacterial antigens compared to control cells that were incubated in untreated growth medium. The outcome of this assay was different to that of the cell line assay where cells exposed to the bacterial antigens migrated at an accelerated rate to close the wounds compared to the control cells (Figure 5.2).

In the case of the primary cultures there was closure of the scratch wounds at around the same rate in treated and control cells. In all 3 replicate wells (2 are shown) the wounds were almost completely closed at 24 hours. Since there was limited availability of the limbal tissue these assays could not be repeated however it was suggested that capturing the images at shorter time intervals may have revealed changes in the rate of wound closure.

5.10.2 The effect of *Bacillus* proteins on protein expression in primary corneal epithelial cells

The aim of this section was to expose the primary limbal epithelial cells to the bacterial antigens then assess the effect using Western blotting. Primary corneal epithelial cells were treated with the antigen for four days as described in section 2.35.2. Samples were resolved on 1D 10% SDS-PAGE gels and transferred to nitrocellulose membranes in preparation for Western blotting with a variety of antibodies (Table 2.6). All desitometric graphs represent 3 technical replicates of the blots however since there was a limited amount of this tissue, the treated cells could only be obtained once. Of the markers tested (Keratin3, Keratin 19, E-cadherin and p63) only Keratin 19 exhibited a significant increase in expression. When exposed to 2 μ g/ml of the antigen, expression of keratin 19 increased 1.24 fold on average ($p=0.005$), this increase was not consistent

as the higher concentration of antigen (6 µg/ml) caused only a 1.14 fold increase compared to control cells.

Therefore, for the purpose of examining the potential effect of the *Bacillus* antigens on the ocular surface these markers did not give a significant insight into how cellular changes could contribute to the progression of rosacea. Elevated cytokeratin 19 expression was found in the sera of patients with inflammatory and vascular lung disorders (Fujita *et al.*, 1999). The results shown here implicate that there is more scope to continue the study of *B. oleronius* antigen effects on primary cells.

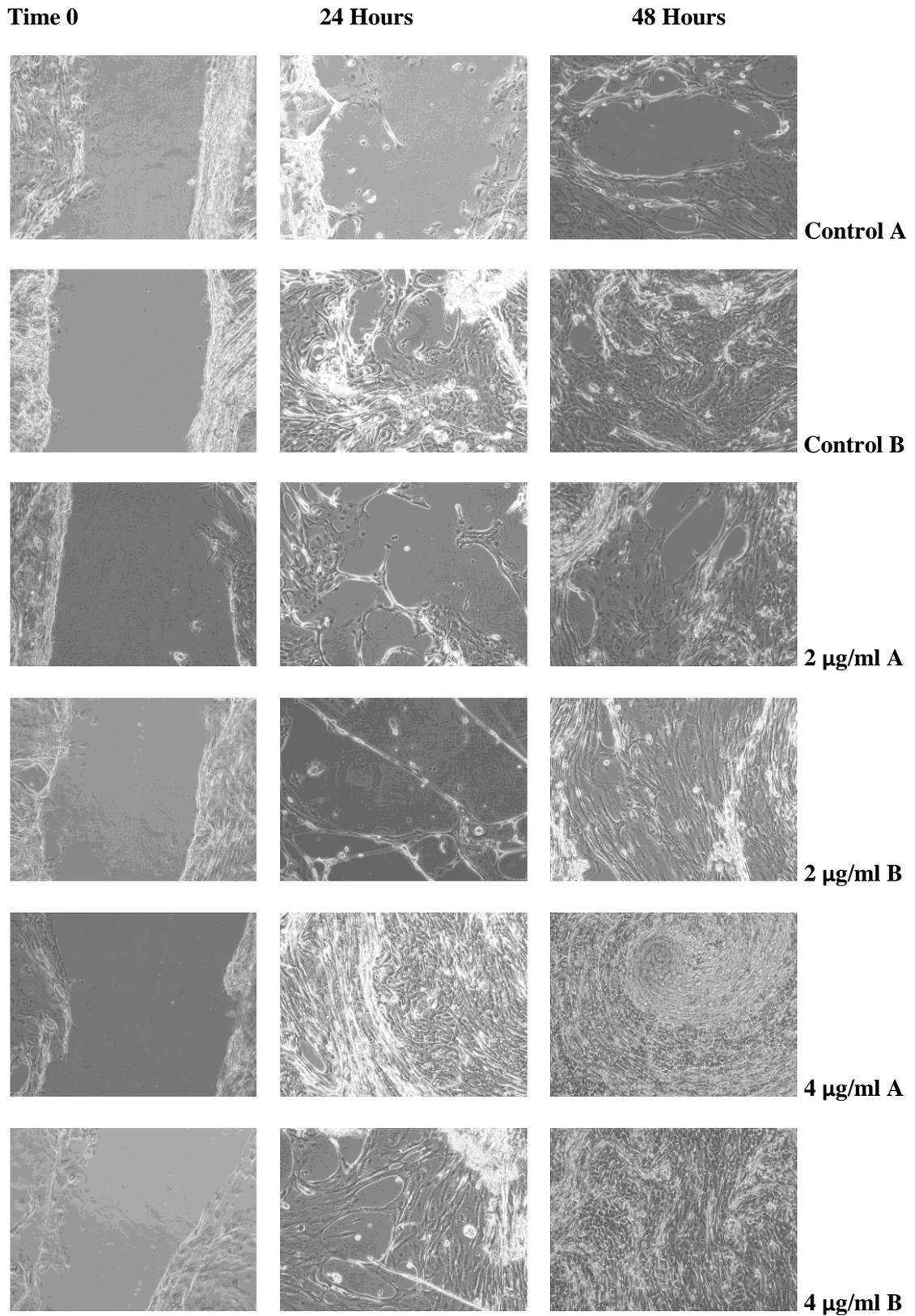


Figure 5.44 Limbal epithelial cell scratch wound assay

Migration occurs at a similar rate in control cells and those exposed to the bacterial proteins

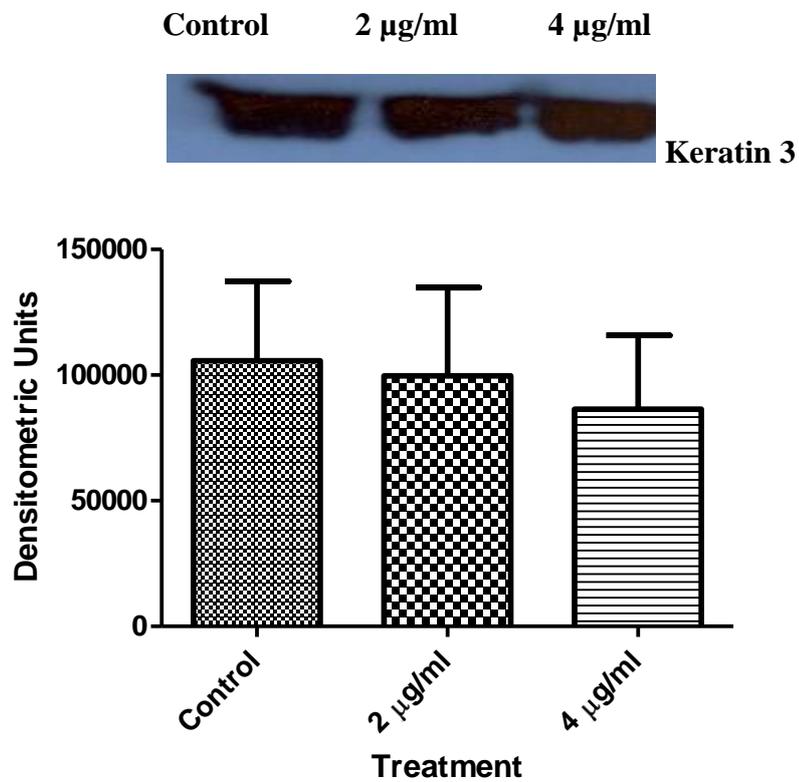


Figure 5.45 Western blotting analysis of Keratin 3 expression in primary limbal epithelial cells

Expression of CK3 was decreased in cells exposed to *B. oleronius* protein compared to control cells however a student T test did not indicate a significant difference.

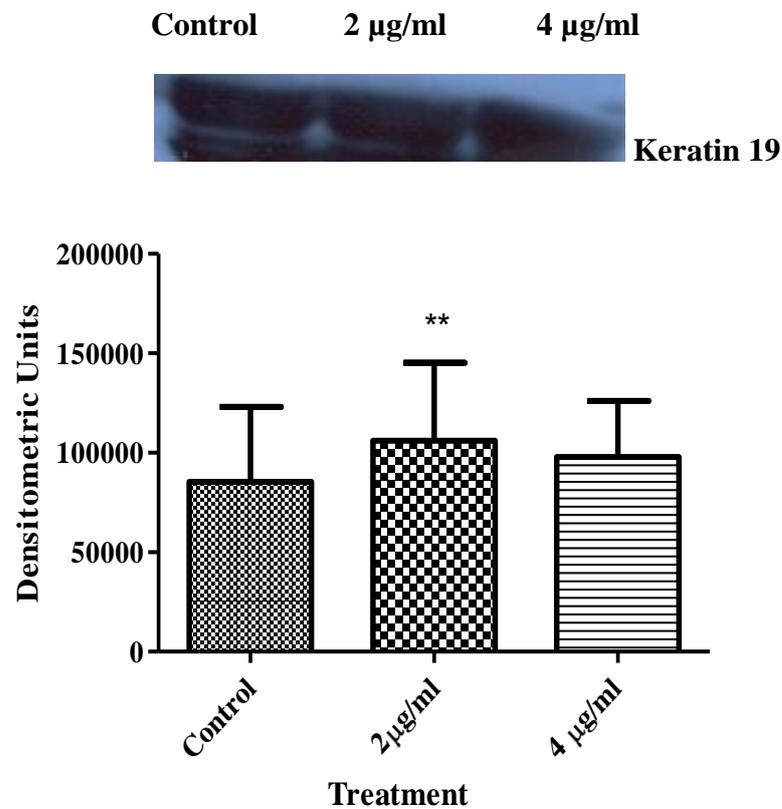


Figure 5.46 Western blotting analysis of Keratin 19 expression in primary limbal epithelial cells

Limbal epithelial cells exposed to 2 µg/ml of *B. oleronius* protein showed increased expression of CK19 (1.24 fold, $p=0.005$)

Significance is indicated by ** where $p \leq 0.01$

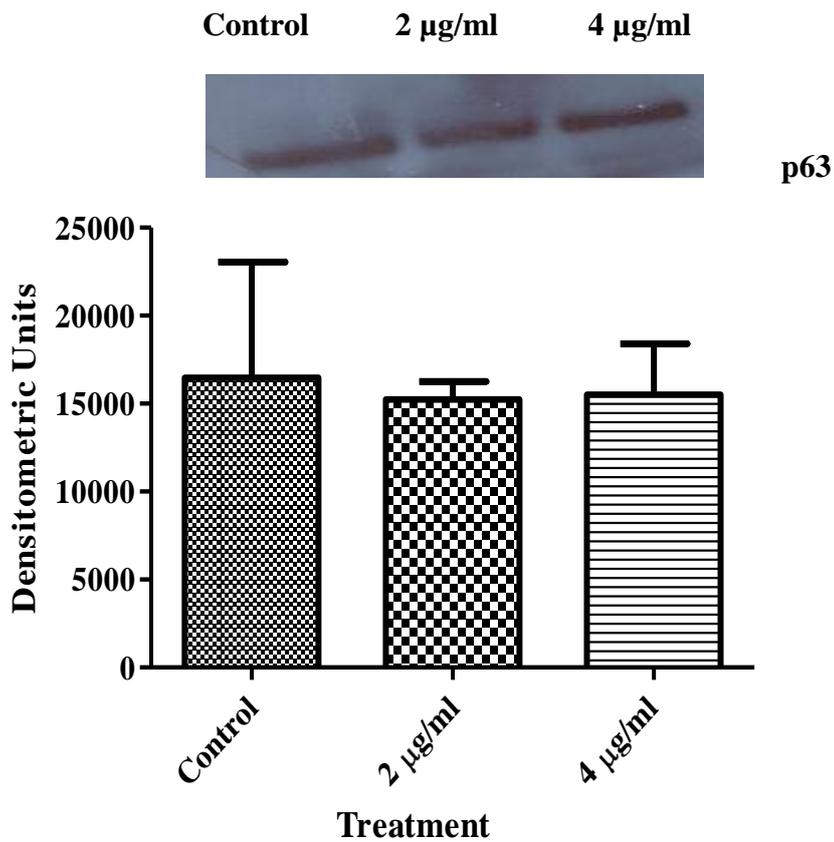


Figure 5.47 Western blotting analysis of p63 expression in primary limbal epithelial cells

Expression of p63 was decreased in cells exposed to *B. oleronius* protein compared to control cells however a student T test did not indicate a significant difference.

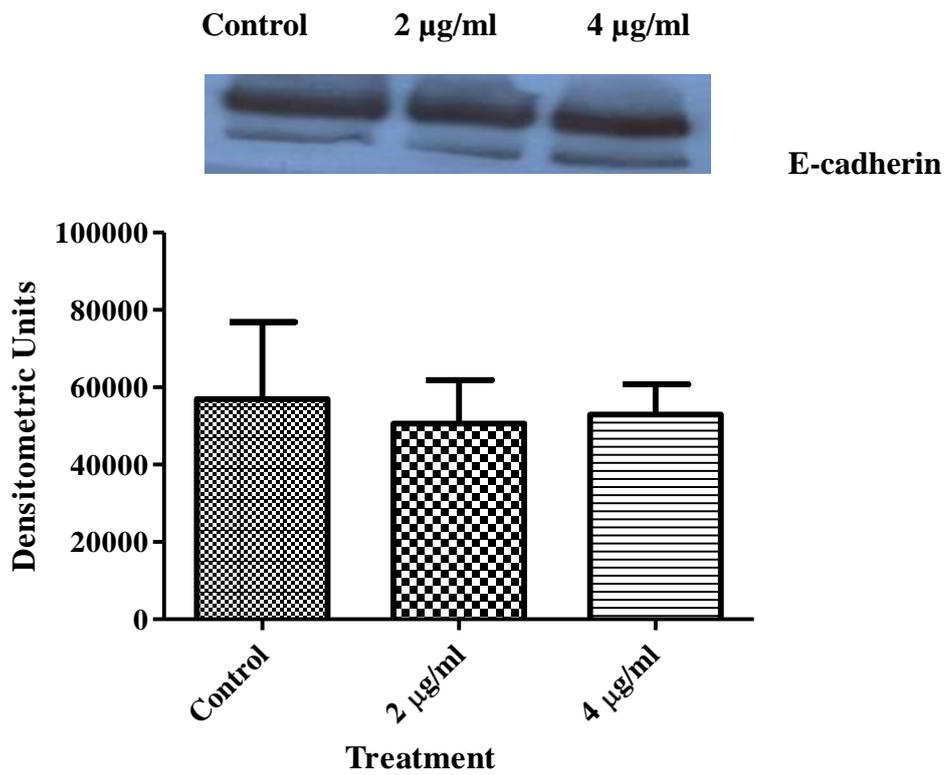


Figure 5.48 Western blotting analysis of E-cadherin expression in primary limbal epithelial cells

Expression of E-cadherin was decreased in cells exposed to *B. oleronius* protein compared to control cells however a student T test did not indicate a significant difference.

5.11 Discussion

The focus of this Chapter was to study the effect of *Demodex* associated bacterial antigens on a corneal epithelial cell line (hTCEpi) and to a limited extent on primary limbal epithelial cells. It has been discussed at great length how dysregulation of the innate immune response is responsible for the progression of rosacea (Dahl *et al.*, 2002; Lacey *et al.*, 2007; Li *et al.*, 2010; Powell, 2005; Yamasaki & Gallo, 2009). Corneal epithelial cells make up an important part of the innate immune response as they provide a barrier that protects the eye from physical and environmental damage. Studies using epithelial cell lines and sebaceous cell lines (Lacey *et al.*, 2007) have been carried out in relation to rosacea. Yamasaki *et al.*, (2007) stated that treating human keratinocytes with LL-37 caused increased release of IL-8 by the cells. *Bacillus oleronius* antigens may be present at increased concentrations in the skin due to the presence of higher numbers of *Demodex* mites in people with rosacea (Diazperez, 1994). *Demodex* mites reside in the pilosebaceous units of the pores and in the eyelash follicles. Upon death the mites may cause local inflammation by release of bacterial endosymbionts into the surrounding tissue and may be present in the tears and on the corneal surface. Therefore the hTCEpi cell line was chosen as a suitable model for the ocular surface and experiments were carried out to discover the effects that the bacterial proteins caused in these cells.

In order to design appropriate experiments with these cells the toxicity of the antigen to the growth of the cells was assessed. As depicted in Figure 5.1, cell growth decreased in the presence of bacterial proteins. There was a statistically significant decrease of 26 % in cell growth following exposure to 2 µg/ml of the bacterial antigen and cell growth dropped by 75 % when the concentration of antigen was increased to 6 µg/ml (Figure 5.1). The wound healing response of the cells was assessed since there are many factors that contribute to corneal wound healing, therefore the changes observed in this response could provided information as to which aspects of the corneal epithelial cells to investigate. As seen in the images (Figure 5.2) there was an increase in migration of cells at the leading edge of the wound following treatment with the antigen compared to control cells. Since the toxicity assay had shown a reduction in cell growth due to incubation with the antigens it was speculated that increased cell motility

caused by changes in protein synthesis rather than increased cell proliferation (Zieske *et al.*, 1989) was responsible for this result.

Epidermal growth factor (EGF) is an essential mediator of adhesion, migration and wound healing in corneal epithelial cells and reactive oxygen species (ROS) generation were shown in the study by Huo *et al.*, (2009) to be mediators of EGF in relation to wound healing. The research reported that control cells were slow to close the scratch wound while cells treated with EGF completely closed the wound within 20 hours. However by inhibition of ROS production using an antioxidant there was a return to the control phenotype. The experiment depicted in Figure 5.2 revealed a less dramatic phenotype to that seen in the study by Hou *et al.*, (2009) however there is increased migration of the hTCEpi cells at the leading edge to close the wound in response to exposure to the *Bacillus* antigen preparation. In Chapter 4 it was shown that there was increased motility of neutrophils and increased IL-8 production which would lead to the infiltration of neutrophils to the surrounding tissue and this in turn could potentially lead to an increase in inflammatory mediators in localized areas of the dermal and ocular tissue. There is potential that corneal epithelial cells could be exposed to these antigens *in vivo* leading to an accelerated rate of migration and shedding of corneal epithelial cells. Chronic inflammation and loss of corneal epithelium induces conjunctival epithelial cells to migrate to the corneal surface and contributes to ulceration of the cornea (Kolli et al. 2010). It would therefore be interesting to investigate the response of conjunctival epithelial cells following exposure to these antigens.

Following the changes observed with live cell imaging it was decided that the wound healing response would be investigated using immunofluorescence microscopy. Figures 5.3 and 5.4 show cells stained with E-cadherin and phalloidin, there does not appear to be any significant change in intensity of expression of these proteins. However the images do provide a clear view of the migration of the cells in responses to the bacterial antigens, with Figure 5.4 showing a zoomed in view of the shape of the cells and their actin filaments extending as they migrated. The effect of the bacterial antigens on migration was further investigated using another plate assay format, where cell movement through a porous insert was assessed (section 5.4). The results of this assay proved that incubating the cells in the presence of the bacterial antigen caused more cells to migrate through the membrane of the insert while untreated cells did not migrate at all in some replicates. Migration increased 3.6 fold ($p=0.001$) in response to 2

$\mu\text{g/ml}$ of the antigen and by 14.5 fold in response to 6 $\mu\text{g/ml}$ treatment ($p=0.0001$) when compared to control cells (Figure 5.6). Invasion of cells into gel inserts composed of extracellular matrix was also investigated, and again there was an increase in the number of antigen treated cells migrating through the insert compared to control cells. Results of these assays revealed an increase in hTCEpi cell invasiveness of 1.7 fold ($p=0.003$) in response to 2 $\mu\text{g/ml}$ of the antigen and by 1.8 fold ($p=0.01$) in response to 6 $\mu\text{g/ml}$ when compared to control cells (Figure 5.7). Adhesion of cells is another consideration when investigating the wound healing responses. As seen in the graph depicting stained cells where one field of view per well was counted and there were 8 replicate wells for each ECM (Figure 5.8). Student T-tests (GraphPadPrism) revealed adherence to collagen IV and gelatin was increased by 1.47 fold ($p=0.01$) and 1.5 fold ($p=0.004$), respectively following exposure to 6 $\mu\text{g/ml}$ of antigens. Adherence of hTCEpi cells to fibronectin and collagen V both decreased significantly following exposure to 2 $\mu\text{g/ml}$ and then increased slightly compared to control cells when cells were exposed to 6 $\mu\text{g/ml}$ of antigens. There was a consistent decrease in adherence to laminin of 1.7 fold ($p=0.002$) following exposure to 2 $\mu\text{g/ml}$ of bacterial antigens, adherence was decreased only 1.26 fold following exposure to 6 $\mu\text{g/ml}$ of the antigens. Both invasion and adhesion assays could benefit from further replications and perhaps variation of the parameters to further investigate these results.

Preliminary investigations into the effect of *Bacillus* antigens on protein production in the hTCEpi cell line were carried out with antibodies targeting cytokeratins which are involved in differentiation and proliferation (section 5.6.1-5.6.2). Cytokeratins 3 and 12 are structural proteins that are expressed in the corneal epithelium. Immunofluorescence images (Figures 5.9 & 5.10) and Western blots (Figures 5.11 & 5.12) appeared to show a slight decrease in the expression of these proteins following exposure to the antigens however statistical analysis revealed no significant changes in expression. Keratin 19 which is expressed in several epithelial cell types and strongly expressed in the limbal region of the eye, remained unchanged in the control and antigen treated cells (Figure 5.13 & 5.14). E-cadherin is involved in cell-cell interactions (Suzuki *et al.*, 2003) and its expression also remained relatively unchanged (Figure 5.15 & 5.16) following exposure to the bacterial antigens.

Since cell migration increased following exposure to these bacterial antigens it was decided to investigate some proteins involved in cell shape movement, such as β 1-integrin and vinculin. The expression of β 1-integrin appeared to decrease an average of 1.25 fold ($p=0.01$) in expression following exposure to the bacterial antigens as seen in the Western blot shown in Figure 5 17. Reactivity to the vinculin antibody increased an average of 2.05 fold ($p=0.0026$) and 2.7 fold ($p=0.0009$) in response to exposure to 4 μ g/ml and 6 μ g/ml of the purified antigen preparation. β 1-integrin interacts with components of the ECM (Suzuki *et al.*, 2003) therefore reduced expression of this protein could cause cells to become more motile because they are less tightly bound to the ECM. Vinculin is a membrane cytoskeletal protein involved in anchoring of F-actin to the basement membrane. Studies have shown that vinculin expression is increased in migrating rat corneal epithelial cells in response to wound healing by up to 27 fold (Zieske *et al.*, 1989). Western blotting for this marker was carried out after experiments on wound healing had been completed however it is likely that this marker may have been seen to be increased at the leading edge of the wound by fluorescent microscopy.

Matrix metalloproteinases are a family of proteins that are involved in normal processes such as reproduction and remodeling of tissue, they are also involved in pathogenic processes such as angiogenesis and wound healing (Li *et al.*, 2001). These enzymes have been linked to the pathogenesis of rosacea (Yamasaki & Gallo 2009). There is evidence that the production of MMPs is up regulated by a number of factors including exposure to UV radiation, presence of reactive oxygen species and inflammation (Lu & Wahl 2005; Jang et al. 2010; Manicone & McGuire 2008). In Chapter 4 (section 4.4.2) it was highlighted how exposure of neutrophils to *Bacillus* antigens can up-regulate the expression of MMP-9 during degranulation. In this Chapter (section 5.6.4) MMP-9 was seen to be produced at higher levels and also to be more active in corneal epithelial cells exposed to the bacterial antigens compared to control cells. Western blotting (Figure 5.19) revealed an increase in expression of the enzyme when exposed to 2 μ g/ml of *Bacillus* antigens of 1.34 fold ($p=0.033$). MMP-9 or gelatinase activity increased by an average of 1.96 fold ($p=0.043$) when cells were exposed to 4 μ g/ml of the bacterial antigens compared to untreated control cells. Studies have shown that MMP-8 and 9 are elevated in the tear fluid of ocular rosacea patients (Kari & Kari, 2006). This study also highlights the ability of tetracyclines such as those

used to treat ocular rosacea to inhibit the production of these enzymes (Kari & Kari, 2006).

In Figure 5.21 primary limbal epithelial cells were successfully cultured from donor limbal tissue on gelatin coated plates supplemented with irradiated 3T3 cells. Limbal epithelial stem cells represent a niche that is promising in terms of regenerative medicine. People who become deficient in these cells through injury or disease suffer painful damage to the cornea and can eventually lose their sight. Advanced transplantation techniques have been developed using cells grown on amniotic membranes to optimize the healing potential of the grafts (Baylis *et al.*, 2011). The limbal region of the eye is located between the cornea and the conjunctiva and contains stem cells which replenish the corneal tissue as corneal epithelial cells are shed. The limbus also provides a barrier to the vascularisation of the cornea (Notara & Daniels, 2008), when this barrier is disturbed scarring and ulceration of the corneal surface occurs. Due to the nature of how samples of this tissue are obtained, the amount of experiments that could be carried out was limited. Wound healing assays carried out with these cells were inconclusive and there was an increase in only one of the protein markers that were tested which was cytokeratin 19. The responses recorded in the hTCEpi cell line provide evidence that a more comprehensive study of limbal cells and possibly including conjunctival cells could provide further evidence that *Bacillus* antigens have a role in the pathogenesis of ocular rosacea.

The work outlined here has shed light on some of the significant cellular changes that occur in response to *Bacillus* antigens on the cornea. Corneal epithelial cells became more motile and protein expression was affected. The antigen becomes toxic to the epithelial cells over time as the concentration increases and build up of this antigen *in vivo* could have a similar effect by damaging the surrounding cells. Corneal and limbal epithelial cells are components of the innate immune response and the changes induced in these cells by the *B. oleronius* antigens could potentially contribute to the chronic inflammation that leads to serious complications for ocular rosacea patients. This work adds to the growing evidence that *Demodex* mites and bacteria that are usually harmless can contribute to the pathogenesis of rosacea.

Chapter 6

A proteomic approach to investigating the effect of environmental conditions and exposure to hydrogen peroxide on protein production by *Bacillus oleronius*

6.0 Introduction

Bacillus oleronius was first isolated from the hindgut of the termite *Reticulitermes santonensis* (Feytaud). According to Kuhnigk *et al.*, (1995) the rod-shaped bacterium stained Gram negative and was capable of forming endospores. *Bacillus* bacteria are ubiquitous in the environment and are commonly found in the soil (Machado *et al.*, 1998). *Bacillus oleronius* was recently isolated as a contaminant from a selection of dairy farms in Europe (Heyndrickx *et al.*, 2011). *Bacillus* species are not often the cause of serious disease with the exception of *B. anthracis* which can cause lethal infections and has been used as a biological weapon (Crawford *et al.*, 2006) and *B. cereus* which can cause food poisoning (Carlin *et al.* 2010). Many species of bacteria have been identified as opportunistic pathogens therefore they colonise humans without causing disease until the host becomes immune compromised and bacteria can initiate factors that cause infection.

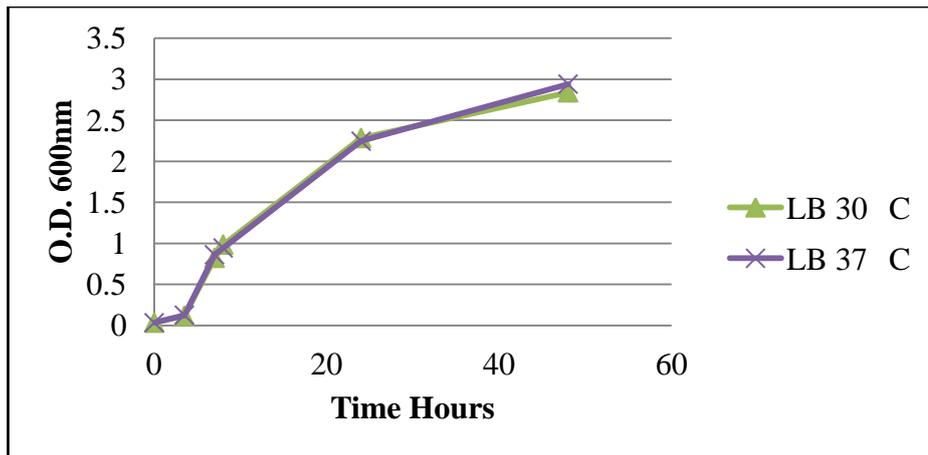
Delaney (2004) isolated *B. oleronius* from a *Demodex* mite from the face of a papulopustular rosacea patient. It was postulated that the bacterium was located in the gut of the *Demodex* and that it may play a role in digestion within the mite. It has been discussed in Chapter 3 that the bacterium elicits an immune response from rosacea patient serum. The focus of this Chapter was to investigate if any *B. oleronius* proteins were up or down regulated in response to the culture conditions of the bacterium. To perform this study bacterial cultures were grown in minimal medium compared to rich medium, at 30 °C or to 37 °C, static or to aerated and at pH ranging from 5-8. Cultures were also exposed to hydrogen peroxide since it is a component of the Reactive Oxygen Species (ROS) response mounted by neutrophils during inflammation. Proteins were extracted from the cultures using lysis buffer (section 2.8) containing Triton-X 100. Lysates were solubilised and subjected to 1D SDS-PAGE, 2D SDS-PAGE and Western blotting. 2D gels were analysed using proteomics same spots technology and spots of interest were picked and digested for Liquid Chromatography Mass Spectrometry analysis. This work has potential to provide information on which environmental conditions in the skin may cause immunogenic proteins to be produced.

6.1 Growth response of *Bacillus oleronius* to alterations of environment

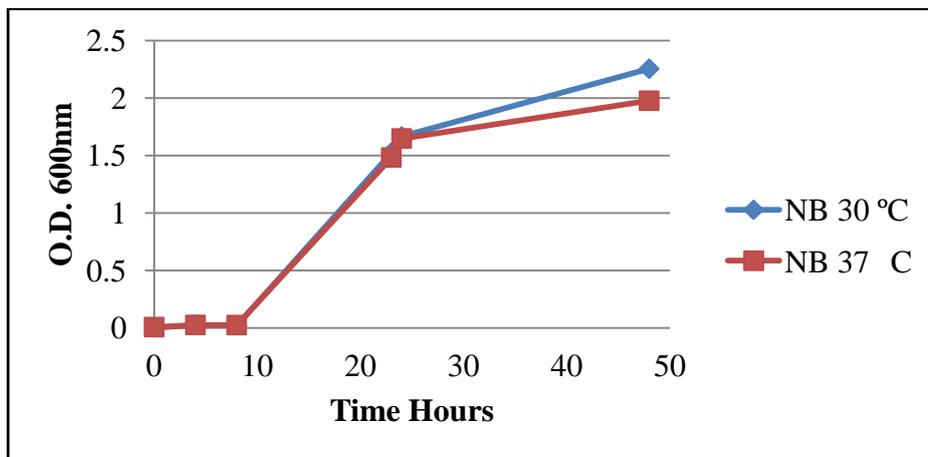
Previous work by Lacey (2007) characterised *Bacillus oleronius* as a slow growing bacterium. It was found that when the bacterium was subcultured from solid nutrient agar to liquid nutrient broth medium at 30 °C and 200 rpm, approximately 48 hours were required to obtain a dense stationary phase culture. Here we sought to re-establish the growth response to a number of culture conditions. Bacteria were subcultured from nutrient agar to 100 mls of nutrient broth and cultured overnight. In order to examine various growth conditions, 1ml of this culture was transferred into 100 ml of the media to be examined (LB broth, Nutrient broth or 2XYT broth). The pH, culture temperature and agitation of the media were also altered. Aliquots of the cultures (1 ml) were taken at 2 hourly intervals and the optical density at 600 nm (O.D. 600) was recorded (Figures 6.1-6.3)

6.1.1 Growth response of *Bacillus oleronius* to nutrient rich and nutrient poor media

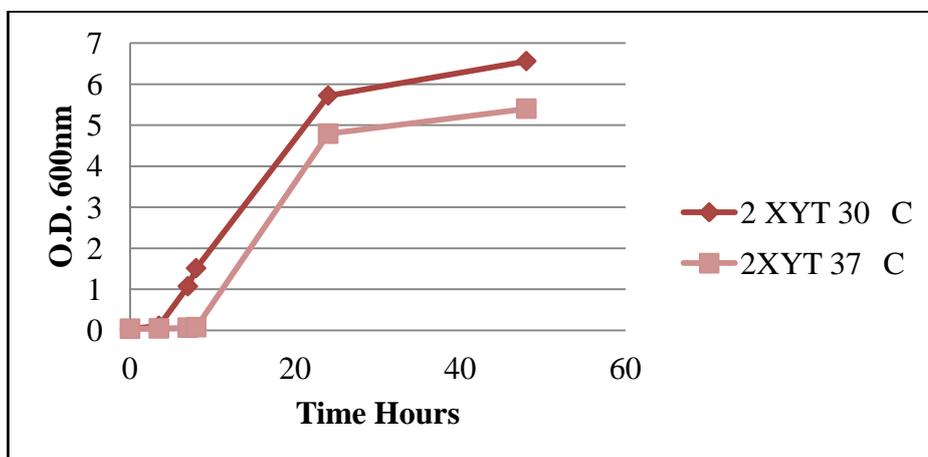
The growth of *B. oleronius* was examined in Nutrient broth (NB), Luria broth (LB) and 2XYT broth at 30 °C and 37 °C. Nutrient broth is a minimal medium, Figure 6.1 (B) demonstrates how the bacteria grows slowly in this medium however the growth rate is slightly higher at 30 °C than 37 °C. The growth rate increased when the bacterium was cultured in LB broth which is composed of a slightly higher nutrient content. The most nutrient rich medium was the 2XYT broth consisting of twice the amount of tryptone and yeast extract per litre as the nutrient broth. At around 10 hours into the culture period bacteria in this broth began to outgrow the others. Following 24 hours of incubation at 30 °C the growth rate had doubled in the 2XYT (O.D. 600 = 5.72) compared to the LB (O.D. 600 = 2.28) and NB (O.D. 600 = 1.56) broths (Figure 6.1 A-C).



(A)



(B)



(C)

Figure 6.1 Growth curve for *Bacillus oleronius* at 30 °C and 37 °C in LB (A), NB (B) and 2XTY (C) broths

6.1.2 Growth response of *Bacillus oleronius* to temperature

Bacterial species can withstand a diverse range of temperatures, some have adapted to the most extreme climates on earth below 0 °C and above 100 °C (Tehei *et al.* 2004). Figure 6.1 (B) represents the comparison between 30 °C (O.D 600 = 2.25 at 48 hrs) and 37 °C (O.D. 600 = 1.97 at 48 hrs) it is clear that bacterium grows preferentially at the lower temperature in the nutrient poor medium. A recent study described strains of *B. oleronius* that were isolated from dairy farms, the study mentions that the bacterium can grow over a broad temperature range of 30 °C – 50 °C and that the organism is capable of forming endospores that can withstand high temperatures (Heyndrickx *et al.* 2011).

Human pathogens generally thrive at 37 °C however in this case *B. oleronius* cultured in both minimal and nutrient rich media grows consistently better at 30 °C (Figures 6.1 A-C and 6.3 A-C). The temperature of the skin surface may be slightly lower than normal body temperature and the availability of nutrients may be more variable since the skin (the habitat of *Demodex* mites and their associated bacteria) is exposed to the elements. This work shows that *B. oleronius* can thrive at a range of temperatures and nutrient levels.

6.1.3 Growth response of *Bacillus oleronius* to pH 5-8

Most bacterial growth media are around pH7 and bacteria generally adapt to and grow in a wide pH range. In order to investigate the effect of pH on growth of *B. oleronius*, the pH range of the medium was adjusted by addition of 1 M NaOH or 1 M HCl. In this case nutrient broth at 30 °C was adjusted to pH 5, 6, 7 and 8. It can be seen in Figure 6.2 that this bacterium does not adjust well to the more acidic culture media at pH 5 – 6. The growth rate begins to increase at 10 hrs in the media at pH 7 and 8, the cells begin to divide exponentially. At the 24 hrs there is very little difference between the growth rates in the neutral (O.D. 600 = 1.24) and the more alkaline (O.D. 600 = 1.34) media. The growth begins to lag at this point as the nutrients in the media are depleted, by 48 hrs the cell density in the pH 7 medium is slightly higher (O.D. 600 = 1.54) while the density reading in the pH 8 medium has dropped (O.D. 600 = 1.23). This work indicates that the bacterium can survive a more alkaline pH.

6.1.4 Growth response of *Bacillus oleronius* to oxygen content of media

Bacillus oleronius is a gram negative aerobic bacterium. The organism is catalase positive (Heyndrickx *et al.* 2011) therefore it can respond to oxidative stress through the breakdown of hydrogen peroxide. The growth response of the bacterium to oxygen content in the medium was examined by placing the cultures in aerated 'shacking' incubators or static incubators. The three media were tested at 30 °C and 37 °C. As expected the bacteria grew more favourably in the aerated conditions over the 48 hours the highest growth rate was recorded in the 2XYT culture (Figure 6.3 C) at 37 °C and 200 rpm. (O.D. 600 = 2.92). The highest growth rate in the static cultures at 48 hrs was recorded in the nutrient broth at 37 °C (Figure 6.3 (B), O.D. 600 = 0.83). There was a sharp increase in growth recorded in the static LB culture at 37 °C (O.D. 600 = 1.73) however this may have been due to some aeration of the culture and the growth rate eventually fell back in line with the other static cultures. Although the cells did not thrive over the 48 hour time frame there was some growth recorded in the static cultures and this proves that the bacterium can withstand low oxygen levels. Growth curves were not recorded past 48 hours however when cells were later cultured for extended periods of up to 5 days in static conditions cultures that provided cell density sufficient to perform protein extractions were achieved. These cultures tended to form clumps or films in the flasks which is more representative of how bacteria grow in our ecosystem.

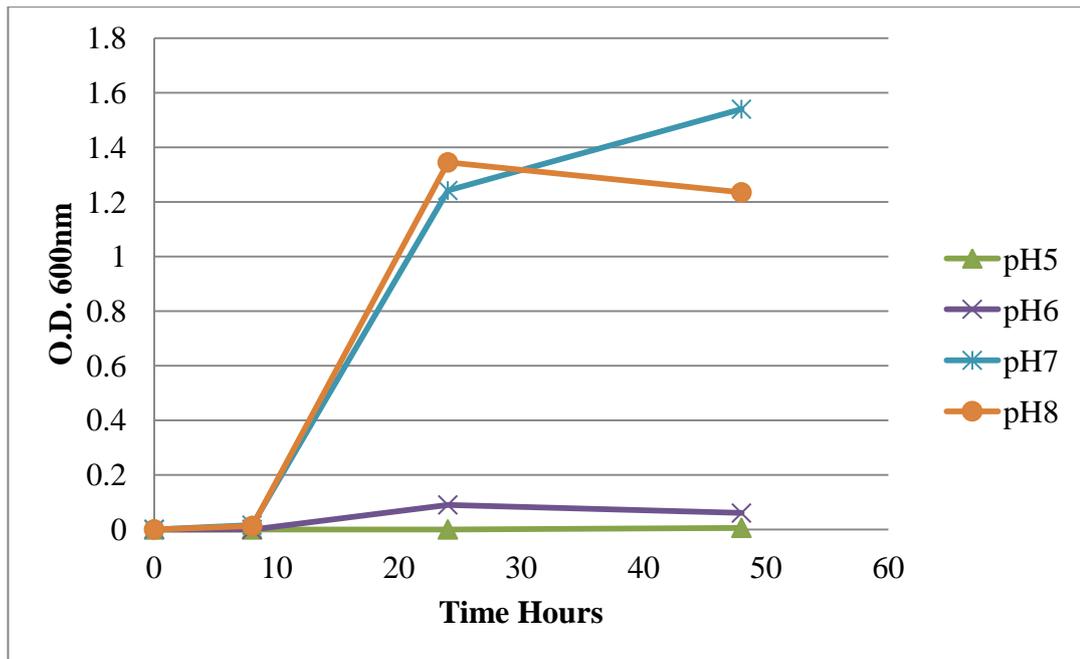


Figure 6.2 Growth curve for *Bacillus oleronius* at 30 °C in Nutrient broth with altered pH 5-8

This graph shows the comparison of growth rate in acidic and alkaline media.

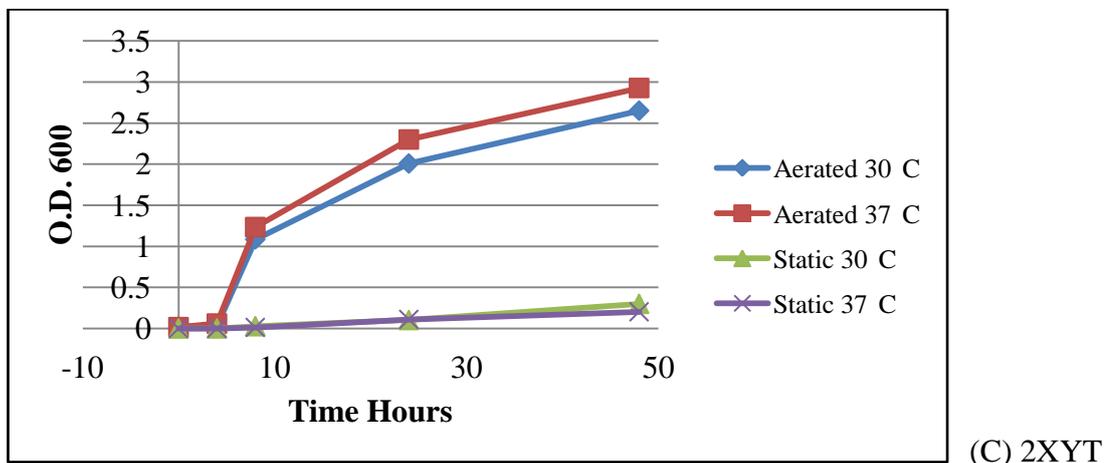
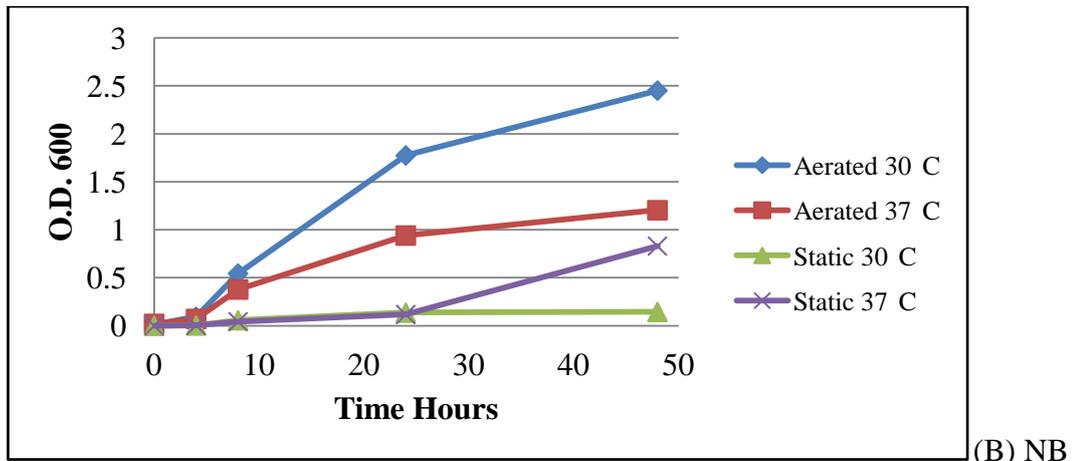
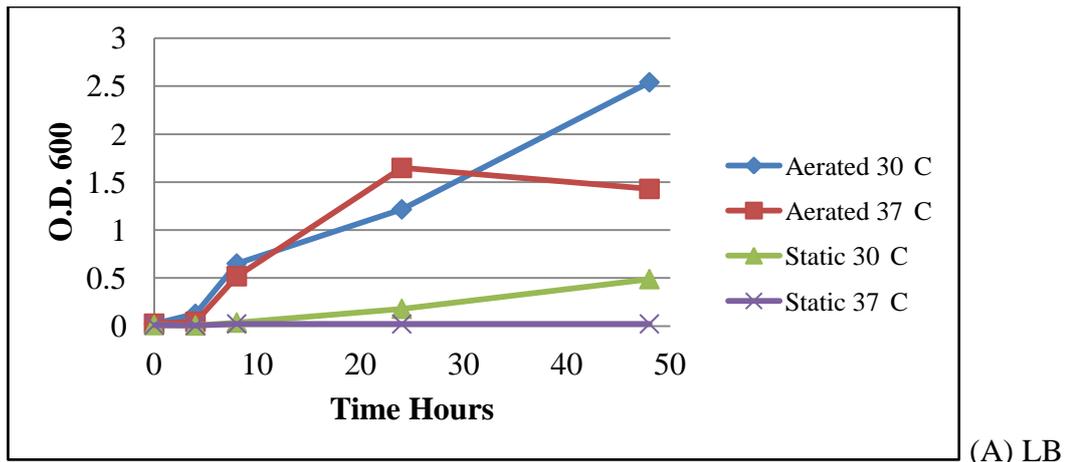


Figure 6.3 Growth curve for *Bacillus oleronius* at 30 °C and 37 °C, in aerated or static medium in LB (A), NB (B) and 2XYT (C) broths

6.1.5 Summary

In this section it was established that *Bacillus oleronius* grew fastest at 30 °C, pH 7 in nutrient rich 2XYT broth under aerated conditions (Figure 6.1 & 6.3). It was also established that the bacterium could survive a broad range of growth conditions which is beneficial for the organism in a competitive environment which could be encountered in the gut of a termite or *Demodex* mite. Throughout the experiments (section 6.1 – 6.1.4) the organism grew in a range of temperatures, pH and in very low oxygen levels. This bacterium is also capable of forming spores (Heyndrickx *et al.* 2011) when the environment becomes unfavourable and has the potential to re-establish itself when the nutrient levels increase and conditions become more favourable. This organism can certainly survive a broad range of environmental conditions in culture and therefore can likely withstand conditions in the gut of the mite and on the surface of the human skin.

6.2 Factors affecting protein expression by *Bacillus oleronius*

In the previous section it was established that *B. oleronius*, like many bacterial species, can thrive in a broad range of environmental conditions. In this section it was sought to discover how protein production was affected by the different growth conditions. The bacterium was cultured under various conditions then prepared for protein extraction (section 2.12). Bacterial proteins were solubilised and separated by 1 dimensional SDS-PAGE. Gels were duplicated so that Western blots could be performed with the 62 kDa anti-rabbit antibody and with pooled patient sera. Statistical analysis was carried out on blots probed with the 62 kDa antibody using student T-test. Statistical significance is denoted by * when $p \leq 0.05$ and ** when $p \leq 0.01$. Whole cells were also fixed and incubated in the anti-62 kDa antibody and viewed using confocal microscopy in order to establish the location of the immune reactive antigens. Another aim was to discover whether culture conditions could alter the amount of stimulatory antigens in particular the 62 kDa antigen being produced by the bacterium in order to use these conditions when fractionating the bacterial proteins (Chapter 3).

6.2.1 The effect of culture media and temperature on the 1-Dimensional proteomic profile of *Bacillus oleronius*

Cells were cultured in Nutrient broth and 2XYT broth at 30 °C or 37 °C in order to analyse their proteomic profiles in these conditions. It is clear (Figure 6.4 A & B) that the expression of proteins varies depending on the culture conditions. Cells cultured in nutrient broth at 30 °C with aeration show a 2.1 fold increase in expression of the 62 kDa protein compared to the lowest expression seen in cells cultured in 2XYT broth at 30 °C with aeration. Expression of the 83 kDa protein is also highest in cells cultured at 30 °C in nutrient broth; there is a 2 fold increase compared to the lowest expression seen in cells cultured at 37 °C with aeration in 2XYT medium. Interestingly the bacterium shows the fastest growth rate when cultured in the 2XYT broth however this medium appears to produce the least serological reaction from rosacea positive patients. It is indicated that the bacterium when grown in nutrient poor broth or under low oxygen conditions produces higher levels of the proteins known to cause an immune reaction in rosacea patients (Lacey *et al.*, 2007). The 2-dimensional proteomic analysis in section 3.6 indicates that cells grown under oxygen and nutrient poor conditions express proteins capable of interacting with the immune response.

Expression of the 62 kDa protein was examined using a rabbit anti 62 kDa antibody (Figure 6.4). There was no significant difference between cells cultured in nutrient broth at both temperatures. Upon comparison of expression in cells grown in 2XYT broth at 30 and 37 °C there was a 1.7 fold increase in expression of the 62 kDa protein at 30 °C ($p=0.01$). Cells cultured in 2XYT broth at 30 °C with aeration show a 1.3 fold ($p=0.029$) increase in expression of the 62 kDa protein compared to cells cultured in low oxygen. A comparison between cells cultured in optimum growth conditions (2XYT broth, 30 °C, with aeration) expressed 1.3 fold ($p=0.04$) more of the protein than cells cultured in the slowest growth conditions (Nutrient broth, 30 °C, Static).

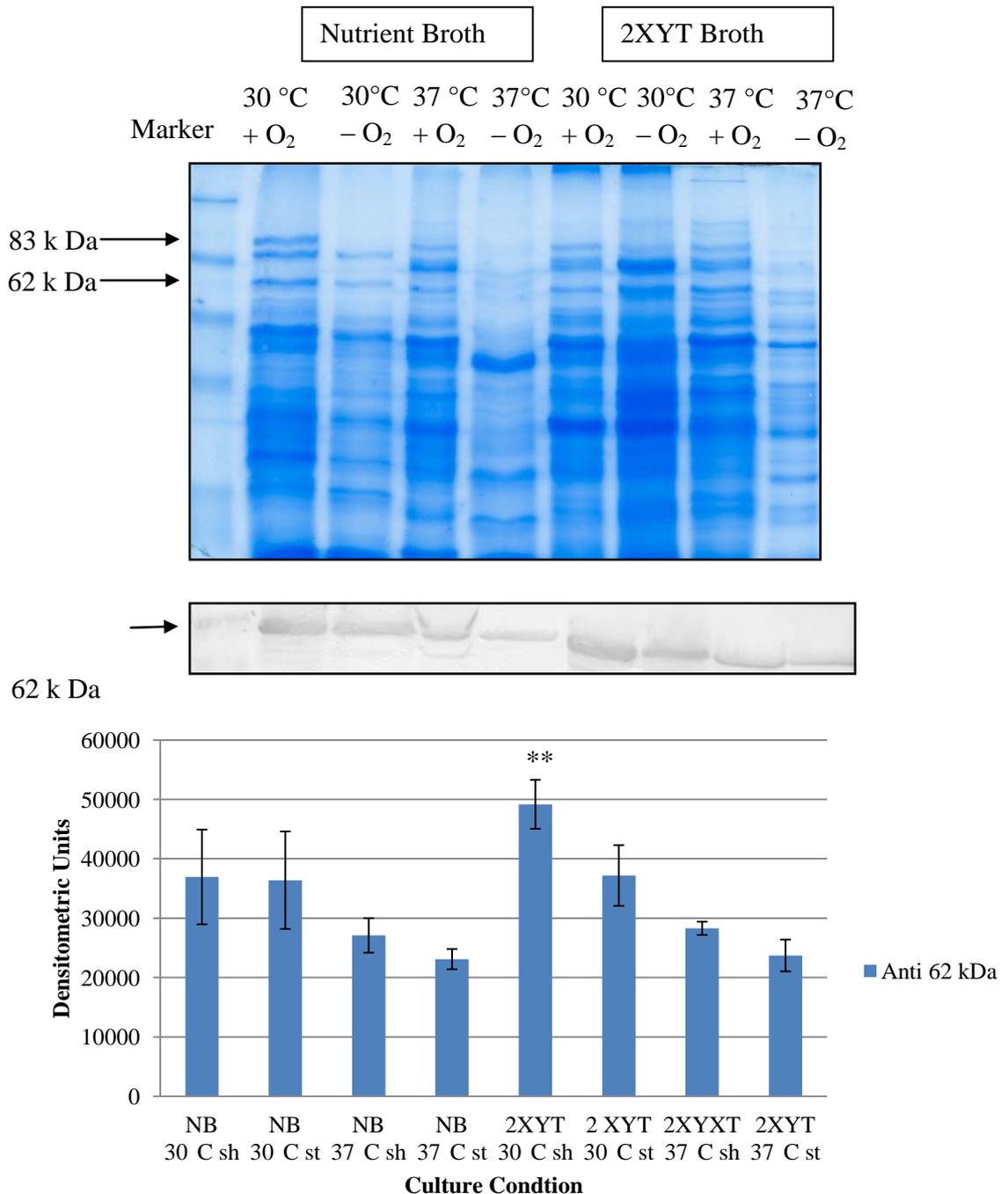


Figure 6.4 (A) SDS-PAGE and (B) Western blot probed with rabbit anti-62 kDa antibody coupled with Densitometric analysis

B. oleronius was cultured in Nutrient broth and 2XYT at 30 °C and 37 °C. Cultures aerated by shaking are denoted by (O₂+), and static cultures are denoted by (O₂-). Statistically significant increased expression of the 62 kDa protein was found when comparing cells grown in 2XYT broth at 30 °C with aeration to cells cultured at 37 °C with aeration (p=0.01 **) and 30 °C static (p=0.029 *) in the same medium and cells grown with low oxygen at 30 °C in nutrient broth (p=0.04 *).

6.2.2 Effect of minimal oxygen on the 1D proteomic profile of *Bacillus oleronius*

In section 6.1.4 it was discovered that the bacterium could grow in conditions when culture flasks were kept static therefore reducing the amount of oxygen available to the cells. In this section it was sought to discover how these culture conditions effected the expression of the 62 kDa protein using the rabbit antibody. Figures 6.4 (A) and 6.5 (A) represent *B. oleronius* cultures grown under low and high oxygen levels. In both cases the SDS-PAGE gels reveal that higher numbers of protein bands were seen in the aerobic compared to the anaerobic environment. Figure 6.5 (B) represents a Western blot of the reactivity of the 62 kDa antibody to the bacteria grown in nutrient rich and poor media at 30 °C and under low or high oxygen conditions. This investigation revealed that the bacterium produces the highest levels of the serum reactive 62 kDa protein when grown in aerobic conditions in rich medium at 30 °C therefore this condition was used to compare to the others. There was a 3.1 fold ($p=0.02$) increase in expression in cells grown in optimum conditions compared to cells grown in static culture, a 1.9 fold ($p=0.04$) increase compared to cells grown in nutrient broth under aerated conditions and a 3.3 fold ($p=0.02$) increase in cells grown in nutrient broth under static conditions.

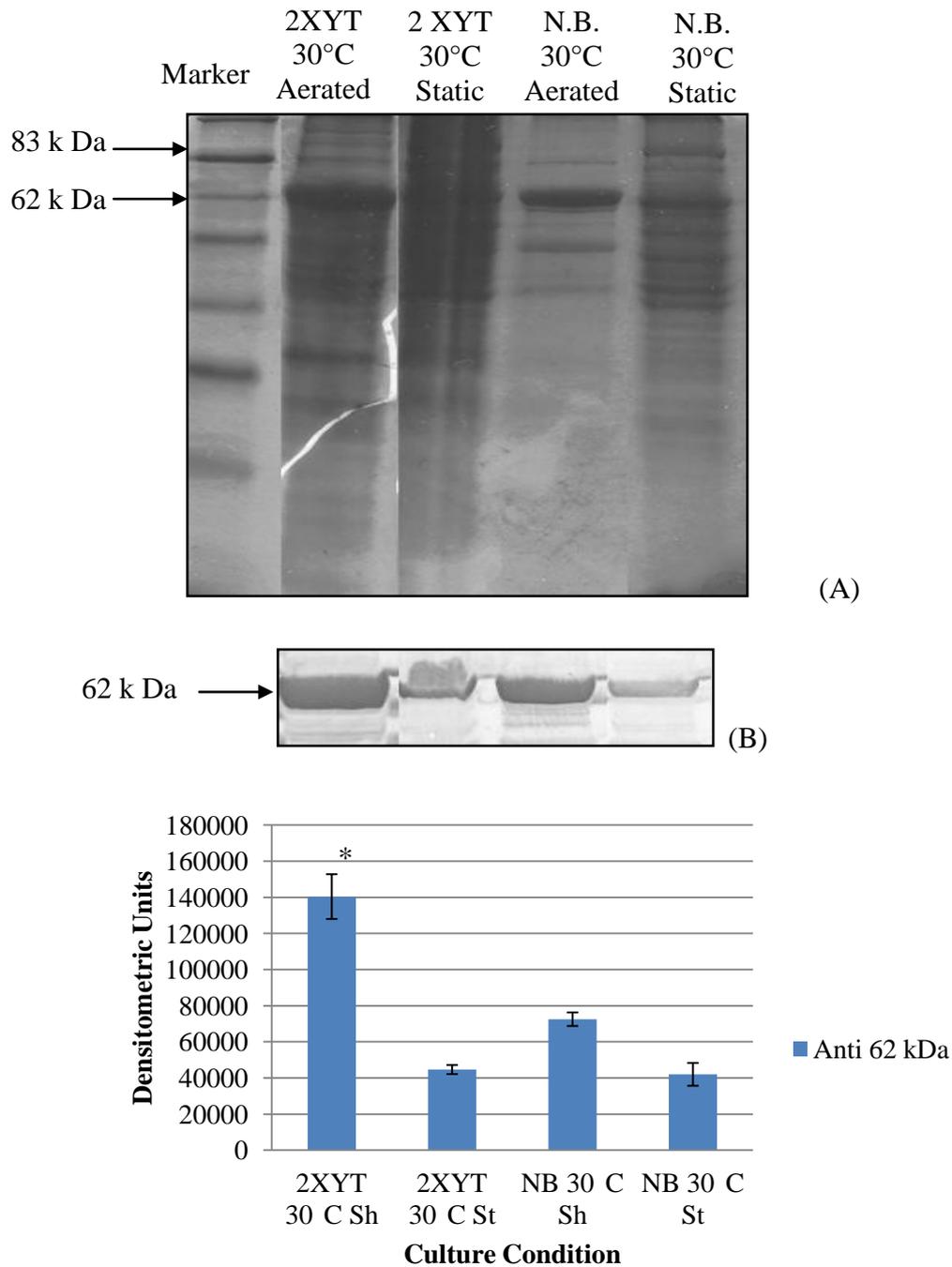


Figure 6.5 (A) SDS-PAGE and (B) Western blot probed with rabbit anti-62 kDa antibody coupled with Densitometric analysis

B. oleronius was cultured in Nutrient broth 30 °C in aerated and static conditions. Cells increased expression of the 62 kDa protein at a statistically significant level when cultured in 2XYT broth at 30 °C with aeration compared to 2XYT without aeration ($p=0.02$), nutrient broth with aeration ($p=0.04$) and nutrient broth without aeration ($p=0.02$).

6.2.3 Effect of medium pH on the 1D proteomic profile of *Bacillus oleronius*

In this section it was sought to discover if *Bacillus* cells cultured at different pH levels. Cells were cultured in nutrient broth adjusted to pH 6, 7, 8 and 9 at 30 °C. The normal pH of nutrient broth is pH 7 while the pH profile of human skin can range from pH 6-8 (Wagner *et al.*, 2003). The growth rate of *B. oleronius* was optimal at pH 7-8 (Figure 6.2). The bacterium was isolated from the hindgut of a *Demodex* mite which is thought to be a vector (Wolf *et al.*, 1988) for the bacterium. It is hypothesized that the bacterium is deposited by the mite and proliferates in the skin of rosacea patients. Therefore it was investigated whether *B. oleronius* produced the stimulatory 62 kDa protein over a pH range 6-9 that it may encounter in the gut of the mite or the skin of rosacea patients. Bacterial cells cultured at pH 8 showed a statistically significant increase of expression (1.7 fold) of the 62 kDa protein ($p = 0.038$) when compared to cells cultured at pH 7. Cells cultured at pH 8 also showed an increase in expression of 1.7 fold ($p=0.013$) compared to cells cultured at pH 9 (Figure 6.6).

6.2.4 Examining the effect of culture conditions of protein expression using confocal microscopy

Lacey, (2007) previously identified the location of the stimulatory 62 kDa protein on the surface of *B. oleronius* cells and concentrated in structures thought to be the endospores. In this section it was sought to examine if the expression of the 62 kDa antigen on the surface of *B. oleronius* varied under different growth conditions. Bacteria were cultured in Nutrient broth under aerobic and anaerobic conditions at pH 6, 7 and 8. Cells were then prepared for viewing by confocal microscopy as described in section 2.13. Cells were heat fixed to glass slides and fixed with formaldehyde (3.7%), following wash steps and blocking with 1% BSA in PBS, cells were incubated in the rabbit anti-62 kDa antibody at 4 °C overnight. A TRITC labelled anti-rabbit secondary antibody was employed. Cells cultured under anaerobic conditions appear to express elevated levels of the 62 kDa protein on their surface with pH 6, 7 and 8 showing similar levels of expression (Figure 6.7).

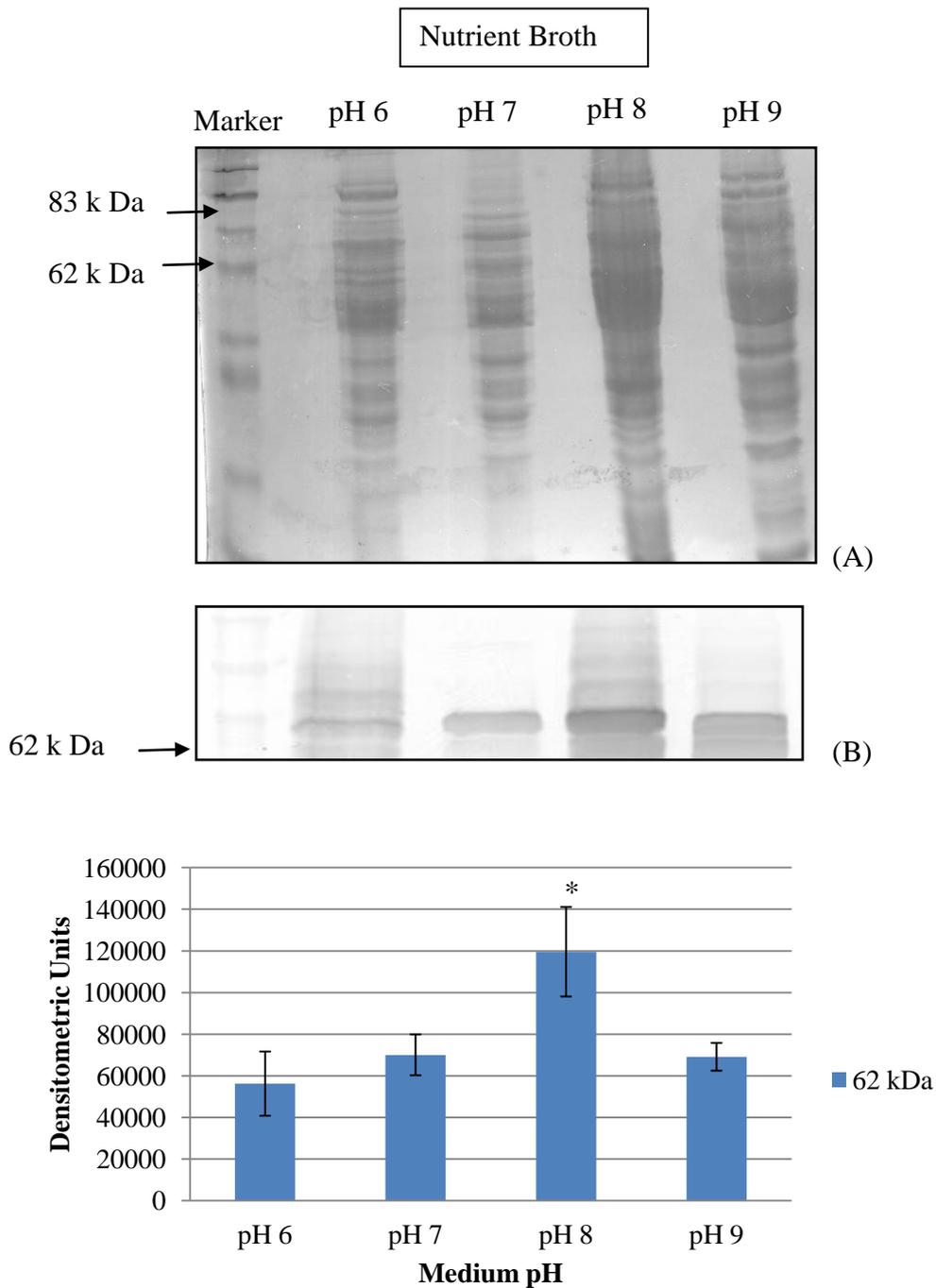
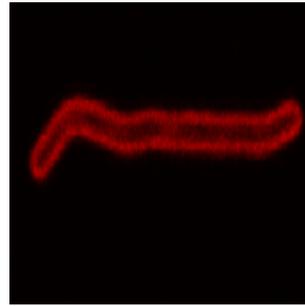
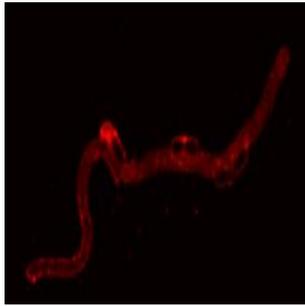


Figure 6.6 (A) SDS-PAGE and (B) Western blot probed with rabbit anti-62 kDa antibody coupled with Densitometric analysis

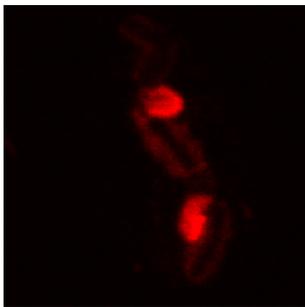
B. oleronius was cultured in Nutrient broth adjusted to pH 6, 7, 8 and 9 at 30 °C. Nutrient broth when made according to instructions is pH 7; therefore all conditions were compared to pH 7. Cells cultured at pH 8 showed a statistically significant increase of expression of the 62 kDa protein ($p = 0.038$).

Nutrient Broth Aerobic Cultures

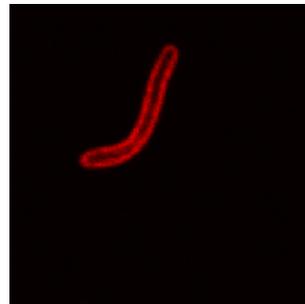
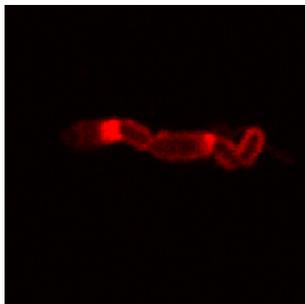
Nutrient Broth Anaerobic Cultures



pH 6



pH 7



pH 8

Figure 6.7 Confocal fluorescence images of *B. oleronius* (X 60).

Cells were incubated in the antibody generated towards the 62 kDa antigen and probed with TRITC labeled secondary antibody. These images indicate that the bacterium expresses more of the 62 kDa protein on its surface when cultured in nutrient and oxygen poor conditions.

6.2.5 Summary

Investigation of the one dimensional proteomic profile of the bacterium grown in various environmental conditions has established that the stimulatory antigens (62 kDa and 83 kDa) are present under all of the conditions. In this section it has been shown that *B. oleronius* can adapt to changes in environment and continue to produce antigens that cause immune reactivity in human rosacea serum samples. By culturing the bacterium in the nutrient rich 2XYT medium at pH 7, 30 °C with aeration of the culture and investigating the level of 62 kDa antibody produced with the rabbit antibody it appears that these conditions cause the bacteria to produce the highest level of the 62 kDa antigen (Figure 6.4 B & 6.5 B). However when Western blots were performed using human sera; reactivity to the stimulatory antigens appeared higher in conditions that could be stressful to the bacterium (Figure 6.4 B). The reactive band in the 62 kDa region appeared more intense when the bacterium was cultured in conditions that caused slow growth (section 6.1-6.5) such as at 30 °C in the nutrient poor medium or at 37 °C in the rich medium with low oxygen. The expression of the 62 kDa protein in poor nutrient and oxygen conditions is also represented in the confocal images (Figure 6.7). The ability of this bacterium to proliferate and produce immunogenic proteins under varied environmental conditions could be a contributing factor to the difficulty in treating rosacea effectively.

6.3 Examining the effect of environmental conditions on the 2 Dimensional proteomic profile of *Bacillus oleronius*

It was previously established that this bacterium adapted to a broad range of environmental conditions (section 6.1-6.1.5) and that variation of the growth conditions can bring about changes in the proteomic profile (section 6.2-6.2.4). There are limitations to one dimensional analysis therefore it was decided that two dimensional proteomic techniques were required. The technique of 2-Dimensional Electrophoresis was first described using bacterial cells (O'Farrell, 1975), it involves the separation of proteins according to isoelectric point by isoelectric focusing in the first dimension, and according to molecular weight by sodium dodecyl sulfate electrophoresis in the second dimension. In this section bacterial proteins were extracted as previously described

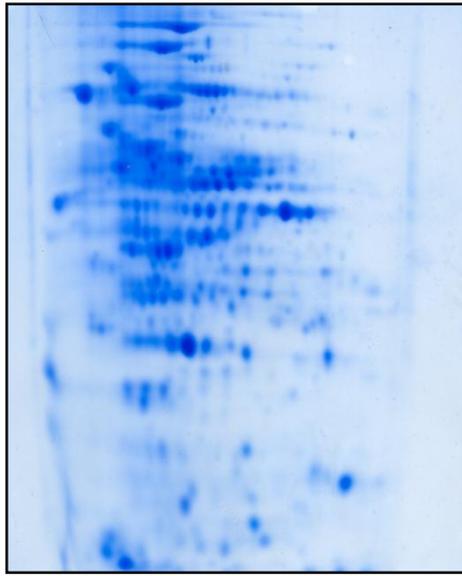
(section 2.12), re-suspended in IEF buffer at a concentration of 500 µg/250 µl and applied to a pH gradient 4-7 IPG strip then subjected to isoelectric focusing (section 2.21) and separated by 12.5% SDS-PAGE (section 2.17) followed by Colloidal Coomassie staining, (section 2.20) and Progenesis SameSpot™ analysis. 2D SDS-PAGE gels were produced in triplicate for each condition being investigated. Experiments were designed to compare gels from 2 growth conditions to each other and the SameSpot™ software automatically generated statistically ordered list of spots from a one way ANOVA analysis. Progenesis SameSpot™ analysis allowed the identification of protein spots of interest which were excised and subjected to trypsin digestion prior to LC/MS analysis (Section 2.22).

6.3.1 Comparison of the 2 Dimensional proteomic profiles of *Bacillus oleronius* grown at 30 °C compared to 37 °C

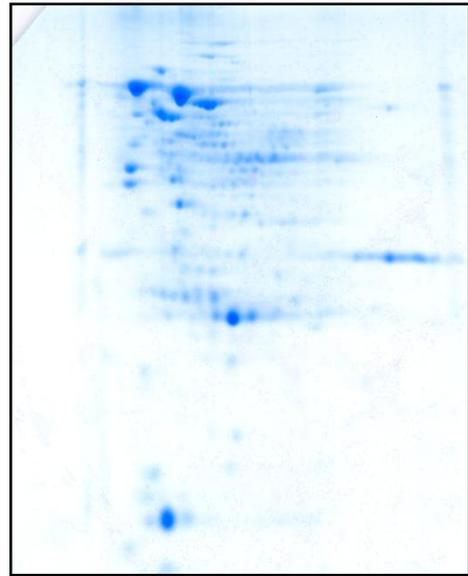
In order to perform this analysis *B. oleronius* cultures were grown in nutrient broth at 30 °C or 37 °C and aerated by shaking at 200 rpm. Bacterial protein (500 µg) was prepared as described (section 2.12), gels were carried out in triplicate and subjected to Progenesis SameSpot™ analysis. The analysis identified 47 spots (Figure 6.9 (C)) that were altered, 11 of these spots showed a statistically significant change in volume ($p \leq 0.05$). Six spots in total were identified by LC/MS from this analysis (Figure 6.9) and 4 of those were statistically significant while 2 were borderline significant ($p < 0.09$). Identified proteins are presented in Table 6.1 with the spot ID assigned by the Progenesis analysis (Reference image).

Spot 145 demonstrated homology to superoxide dismutase from *Bacillus* sp. m3-13 and expression of this enzyme was 2.3 fold higher ($p = 0.017$) in cultures grown at 30 °C. Superoxide dismutase (SOD) catalyzes the conversion of the superoxide radical ($O_2^{\cdot-}$) to H_2O_2 and O_2 (Areekit *et al.*, 2011). This provides a protective function in the cell against superoxide radicals which could be encountered by the bacterial cells when challenged by their host immune system. Spot 50 was homologous to ptsH, a phosphocarrier protein HPR from *Staphylococcus carnosus* which is a component of the phosphoenolpyruvate-dependent sugar phosphotransferase system (sugar PTS). Bacteria cultured at 37 °C demonstrated a 2.5 fold increase ($p = 0.031$) in expression of this protein compared to the 30 °C cultures. Spot 241 represented the other protein that

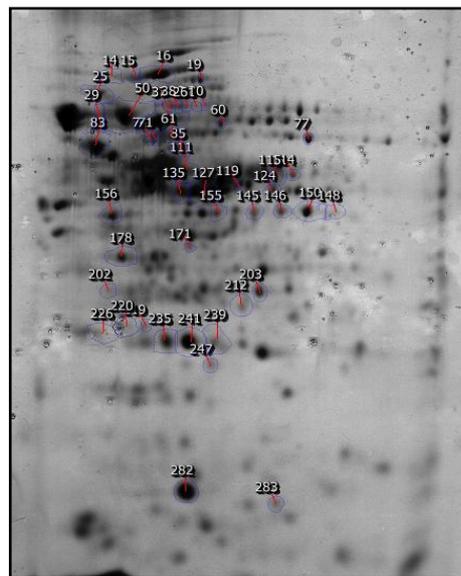
showed increased expression at 37 °C which was homologous to an ATP-dependent Clp protease proteolytic subunit from *Bacillus* sp. SG-1. This enzyme is vital for the cellular homeostasis through the regulation of metabolic enzymes and removal of damaged peptides (Porankiewicz *et al.*, 1999).



(A) Nutrient Broth 30 °C



(B) Nutrient Broth 37 °C



(C)

Figure 6.8. *B. oleronius* cultured at (A) 30 °C and (B) 37 °C in Nutrient broth
(C) Reference image (from 30 °C culture) used for Progenesis™ SameSpots analysis.
Spots that were significantly changed were excised and are represented in Figure 6.9.

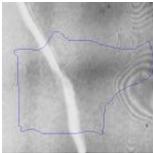
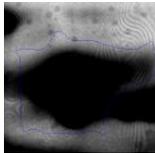
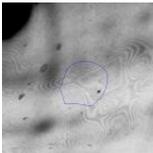
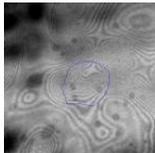
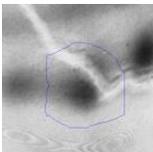
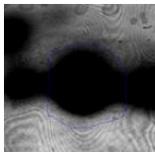
<u>Spot</u>	<u>IdentifiedProtein</u>	<u>Treatment</u>		<u>Average volume</u>		<u>Fold expression</u>	
		<u>NB 30 °C</u>	<u>NB 37 °C</u>	<u>30 °C</u>	<u>37 °C</u>	<u>FC</u>	<u>p value</u>
50.	ptsH			2.20	5.56	2.5	0.031
145.	Superoxide dismutase			3.01	1.31	2.3	0.017
241.	ATP-dependent Clp protease proteolytic subunit			1.61	3.10	1.9	0.048

Figure 6.9 Individual analysis of protein spots which showed a change in expression when grown at 30 °C or 37 °C.

Progenesis™ SameSpot analysis was used to calculate the average volume, fold change and statistical significance values. LC/MS followed by Mascot analysis were employed for protein identification and proteins are presented in Table 6.1.

FC=Fold Change

Spot ID	Protein	Accession	Mass	PI Value	Mascot Z-Score	Coverage %	Uniprot Function
50	ptsH	AAA26663	4767	4.09	76	26	Involved in the phosphorylation of incoming sugar substrates
145	Superoxide dismutase	ZP_07708887	22492	5.17	103	25	Enzyme that catalyzes superoxide into oxygen and hydrogen peroxide
241	ATP-dependent Clp protease proteolytic	ZP_01860338	22647	4.94	156	12	Proteolytic enzyme regulates metabolism and homeostasis

Table 6.1 Summary of proteins identified by LC/MS analysis.

Proteins presented in Figure 6.9 were identified using Mascot and functions were found through Uniprot.

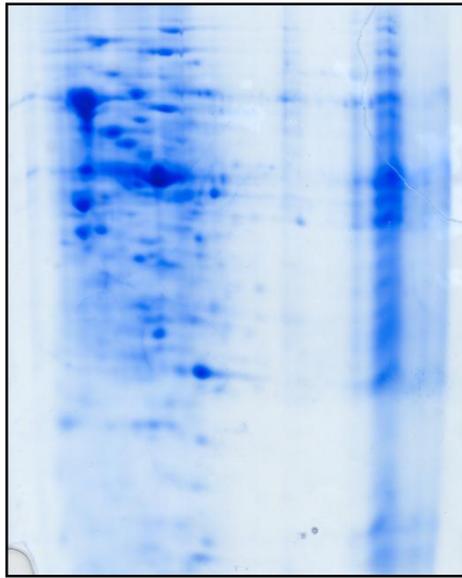
6.3.2 Comparison of the 2 Dimensional proteomic profiles of *Bacillus oleronius* grown in Nutrient Broth compared to 2XYT Broth

B. oleronius cultures were grown at 30 °C and aerated by rotation at 200 rpm in either Nutrient broth (minimal medium) or 2XYT broth (rich medium). Bacterial protein (500 µg) was prepared as described (section 2.12), gels were carried out in triplicate and subjected to Progenesis SameSpot™ analysis. The analysis identified 45 spots (Figure 6.10) that were altered in expression, 14 of these spots showed a statistically significant change in volume ($p \leq 0.05$). Six spots were identified by LC/MS from this analysis (Figure 6.11) and five of those were statistically significantly altered in expression while one was borderline significant ($p \leq 0.1$). Identified proteins are presented in Table 6.2 with the spot ID assigned by the Progenesis analyses.

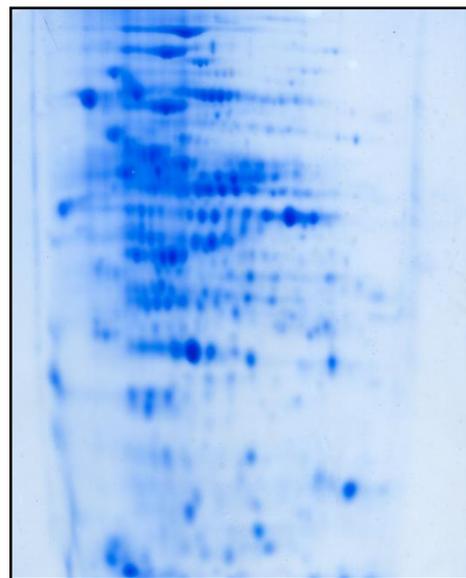
Spot 54 was homologous to a dihydrolipoamide dehydrogenase from *Bacillus* sp. SG-1, this enzyme was increased 1.8 fold ($p = 0.032$) when the bacterium was cultured in nutrient poor compared to nutrient rich medium. This protein is a component of the pyruvate dehydrogenase system which provides the link between glycolysis and the citric acid cycle (Mande *et al.*, 1996). Therefore this protein is crucial for the process of respiration within the cells and the increase noted could reflect the cells adapting to the minimal nutrient conditions. Spot 207 was also increased in the cells cultured in nutrient poor media by 3.6 fold ($p = 0.035$). Mascot analysis revealed homology to naphthoate synthase which was described previously as also having involvement in cellular respiration (Ramchandra & Sturm 2010) and oxidative stress response (section 6.3.1).

Expression of a cell division protein FtsZ from *Bacillus* sp. NRRL B-14911 (Spot 167) was increased 3.2 fold ($p = 0.011$) in cells cultured in nutrient broth. This protein is essential for bacterial cell division (Erickson *et al.*, 1996) and the upregulation seen in cells in the poor medium may indicate that the cells are growing slower due to the lack of nutrients. Cells in the nutrient rich medium initially grow rapidly and cell division may have slowed due to depletion of nutrients. Spot 173 also showed a 2.5 fold increase in expression ($p = 0.046$) in cells cultured in nutrient poor medium and was identified as being homologous to an Elongation Factor Tu from *Bacillus coahuilensis* m4-4. This elongation factor and in highly conserved across bacterial species and is

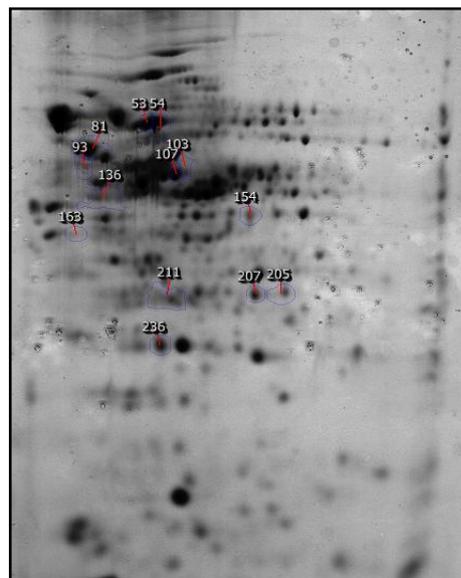
involved in protein synthesis. Immune reactivity to these proteins has been reported in several bacterial species (Kyungwoo, 2011; Nieves *et al.*, 2010; Sharma, *et al.*, 2011) such as the Gram negative *Bacillus*, *Burkholderia pseudomallei*. Spot 242 was homologous to a hypothetical protein HMPREF1013_01383 from *Bacillus* sp. 2_A_57_CT2. Expression of this protein was increased 3.2 fold ($p = 0.054$) in cells cultured in nutrient poor medium. This protein is involved in the assembly of flagellar filaments which allow the cells increase their motility.



(A) 2XYT Broth 30 °C



(B) Nutrient Broth 30 °C



(C)

Figure 6.10. *B. oleronius* cultured at 30 °C in (A) 2XYT broth and (B) Nutrient broth

(C) Reference image (from nutrient broth culture) used for Progenesis™ SameSpots analysis. Spots that were significantly changed were excised and are represented in Figure 6.11

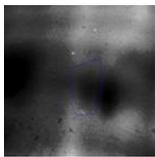
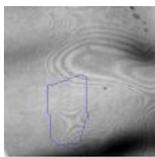
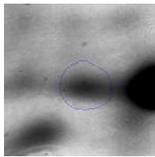
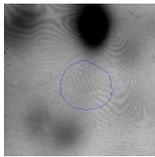
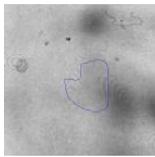
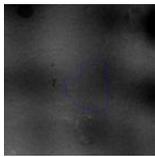
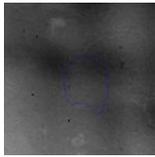
<u>Spot</u>	<u>IdentifiedProtein</u>	<u>Treatment</u>		<u>Average volume</u>		<u>Fold expression</u>	
		<u>NB 30 °C</u>	<u>2XYT 30 °C</u>	<u>NB</u>	<u>2XYT</u>	<u>FC</u>	<u>p value</u>
54.	Dihydrolipoamide dehydrogenase			9.26	5.09	1.8	0.032
207.	Naphthoate synthase			4.71	1.32	3.6	0.035
167.	Cell division protein FtsZ			3.40	1.07	3.2	0.011
173.	Elongation Factor Tu			2.59	1.03	2.5	0.046

Figure 6.11 Individual analysis of protein spots which showed a change in expression when grown in 2XYT and Nutrient broth.

Progenesis™ SameSpot analysis was used to calculate the average volume, fold change and statistical significance values. LC/MS followed by Mascot analysis were employed for protein identification and proteins are presented in Table 6.2.

Spot ID	Protein	Accession	Mass	PI Value	Mascot Z-Score	Coverage %	Uniprot Function
54	Dihydrolipoamide dehydrogenase	ZP_01862040	49716	4.93	173	10	Component of the pyruvate dehydrogenase system involved in metabolism
207	Naphthoate synthase	ZP_01859915	30230	5.51	283	22	Enzyme involved in ubiquinone synthesis and respiration
167	Cell division protein FtsZ	ZP_01172493	40805	5.09	172	19	Essential for cell division and involved in GTP binding
173	Elongation Factor Tu	ZP_03224888	43488	4.76	131	7	GTP binding translation factor

Table 6.2 Summary of proteins identified by LC/MS analysis.

Proteins presented in Figure 6.11 were identified using Mascot and functions were found through Uniprot.

6.3.3 Comparison of the 2 Dimensional proteomic profiles of *Bacillus oleronius* grown in minimal conditions compared to its optimum growth conditions

In order to perform this analysis *B. oleronius* cultures were grown at 37 °C and aerated by shaking at 200 rpm in 2XYT broth (optimum growth conditions) or at 30 °C in static (oxygen poor) conditions in nutrient broth (minimal growth conditions). Bacterial protein (500 µg) was prepared as described (section 2.12), gels were carried out in triplicate and subjected to Progenesis SameSpot™ analysis. The analysis identified 26 spots (Figure 6.12 C) that were altered, 13 of these spots showed a statistically significant change in volume ($p \leq 0.05$). Eight of these spots were identified by LC/MS from this analysis (Figure 6.13 A & B). Identified proteins are presented in Table 6.3 with the spot ID assigned by the Progenesis analyses.

Four of these proteins showed increased expression in cells that were cultured under optimum conditions in nutrient rich 2XYT broth at 37 °C with 200 rpm. The first, spot 22 was identified as a superoxide dismutase form *Bacillus* sp. m3-13 and was increased 1.6 fold ($p = 0.002$). This enzyme is expressed in response to oxidative stress. Spot 28 which was homologous to a transketolase from *Bacillus* sp. SG-1, increased 2.4 fold ($p = 0.025$) is involved in carbohydrate transport and metabolism. Spot 101 showed homology to a putative secreted protein from *Listeria ivanovii* FSL F6-596 which was increased by 1.9 fold ($p = 0.039$) and spot 106, which was homologous to Flagellin was increased 2.8 fold ($p = 0.003$) in cells grown in nutrient rich medium. Both proteins have functions in the assembly of flagellar filaments involved in cell motility.

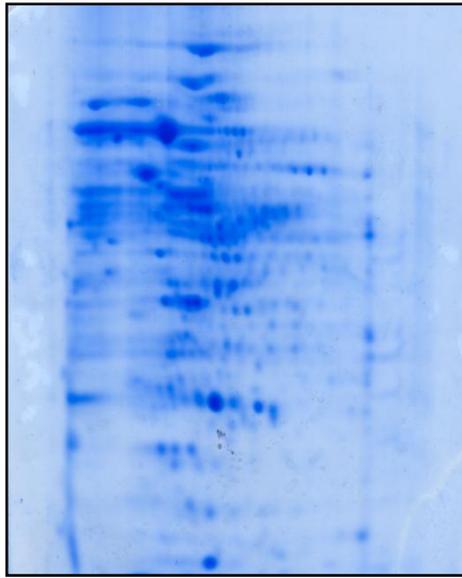
The remaining four proteins showed increased expression in cells that were cultured under nutrient poor conditions in nutrient broth, in a static incubator at 30 °C. Spot 261 was identified as a hypothetical protein HMPREF1013-01383 from *Bacillus* sp. 2_A_57_CT2 which was increased 2.4 fold ($p = 0.033$).

Spot 7 was identified as an aconitate hydratase from *Bacillus* sp. SG-1. This enzyme involve in the TCA cycle was increased 1.6 fold ($p = 0.050$). Spot 268 was identified as a Hag protein from *Bacillus* sp. NRRL B-14911 and its expression was increased 1.6 fold ($p = 0.015$). These proteins are involved in the assembly of flagellar filaments. Spot 260 was homologous to alkyl hydroperoxide reductase (small subunit) from *Bacillus licheniformis* ATCC 14580 and its expression increased 2.4 fold ($p =$

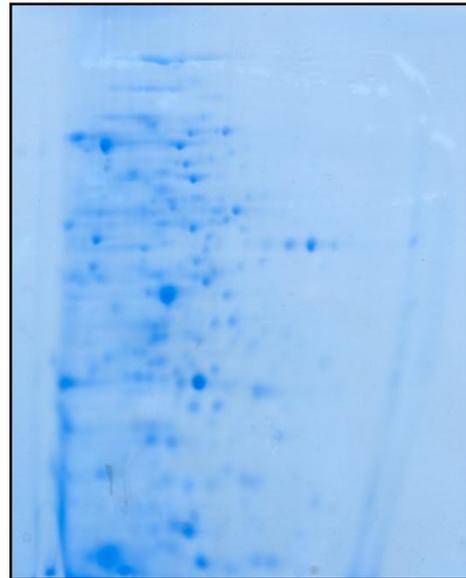
0.028) in cells cultured in minimal conditions. Its function is in protecting cells from oxygen radicals and maintaining homeostasis.

6.3.4 Summary

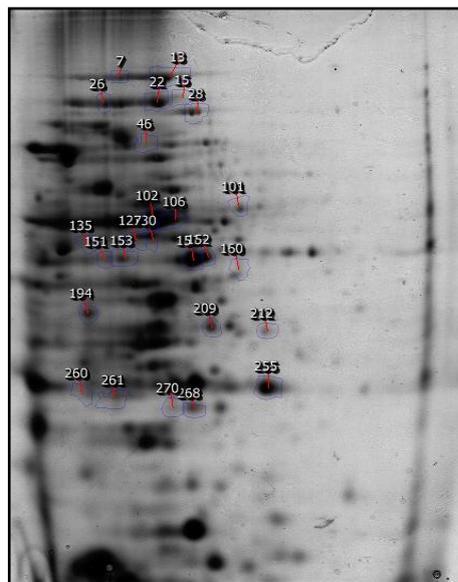
Gel electrophoresis combined with LC Mass Spectrometry were employed to investigate the potential proteomic changes that allow *B. oleronius* to adapt to a broad range of environmental conditions. Among the proteins identified several were found to be involved in respiration and metabolism, others were involved in cell motility and response to oxidative stress. This analysis has proven that the bacterium alters the expression of key proteins in response to its environment; several of these proteins have antigenic potential. Similar proteomic adaptations may occur as the bacteria adjust to varying habitats from the gut of the *Demodex* mite to the sebum rich environment of the pilosebaceous unit.



(A) 2XYT 37 °C Aerobic



(B) Nutrient Broth 30 °C Anaerobic



(C)

Figure 6.12 *B. oleronius* cultured in (A) optimum growth conditions and (B) minimal growth conditions.

(C) Reference image (from optimum growth culture) used for Progenesis™ SameSpots analysis. Spots that were significantly changed were excised and are represented in Figure 6.13

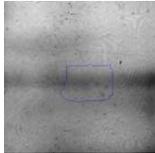
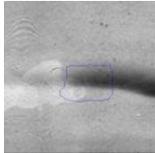
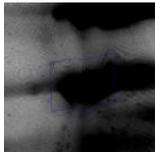
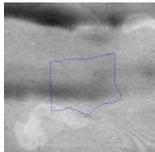
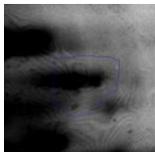
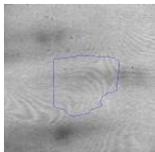
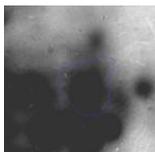
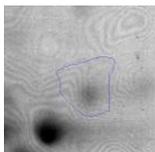
<u>Spot</u>	<u>IdentifiedProtein</u>	<u>Treatment</u>		<u>Average volume</u>		<u>Fold expression</u>	
		<u>2XYT 37 °C Sh</u>	<u>NB 30 °C St</u>	<u>2XYT</u>	<u>NB</u>	<u>FC</u>	<u>p value</u>
7.	Aconitate hydratase			3.16	4.91	1.6	0.050
22.	Superoxide dismutase			1.35	0.82	1.6	0.002
28.	Transketolase			9.8	4.01	2.4	0.025
101.	Putative secreted protein			7.42	3.83	1.9	0.039

Figure 6.13 (A) Individual analysis of protein spots which showed a change in expression when grown in optimum or minimal growth conditions.

Progenesis™ SameSpot analysis was used to calculate the average volume, fold change and statistical significance values. LC/MS followed by Mascot analysis were employed for protein identification.

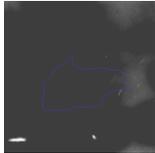
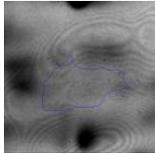
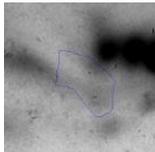
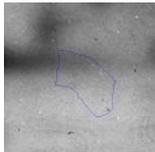
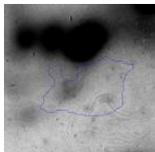
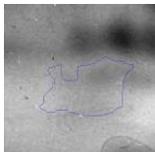
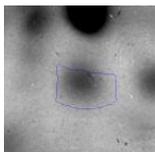
<u>Spot</u>	<u>IdentifiedProtein</u>	<u>Treatment</u>		<u>Average volume</u>		<u>Fold expression</u>	
		<u>2XYT 37 °C Sh</u>	<u>NB 30 °C St</u>	<u>2XYT</u>	<u>NB</u>	<u>FC</u>	<u>p value</u>
106.	Flagellin			4.20	1.165	2.8	0.003
260.	Alkyl hydroperoxide reductase			2.86	6.99	2.4	0.028
261.	Hypothetical protein			5.67	13.5	2.4	0.033
268.	Hag protein			4.02	6.50	1.6	0.015

Figure 6.13 (B) Individual analysis of protein spots which showed a change in expression when grown in optimum or minimal growth conditions.

Progenesis™ SameSpot analysis was used to calculate the average volume, fold change and statistical significance values. LC/MS followed by Mascot analysis were employed for protein identification.

Spot ID	Protein	Accession	Mass	pI Value	Mascot Z-Score	Coverage %	Uniprot Function
7	Aconitate hydratase	ZP_01858967	99213	4.94	675	15	TCA cycle enzyme
22	Superoxide Dismutase	ZP_07708887	22492	5.17	131	25	Catalyse the conversion of superoxide radicals to H ₂ O ₂ and O ₂
28	Transketolase	ZP_001861729	72950	5.11	133	4	Carbohydrate transport and metabolism
101	Putative secreted protein	ZP_07873717	12671	9.12	67	17	Unknown
106	Flagellin	NP_228567	41662	5	260	6	Flagellar motility
260	Alkyl hydroperoxide reductase	YP_081364	20874	4.66	126	22	Antioxidant properties involved in cell redox homeostasis
261	Hypothetical protein	ZP_08004778	44170	4.95	185	17	Bacterial flagellin
268	Hag protein	ZP_01173483	80767	4.71	105	3	Flagellin N terminal helical region

Table 6.3 Summary of proteins identified by LC/MS analysis.

Proteins presented in Figure 6.13 (A & B) were identified using Mascot and functions were found through Uniprot.

6.4 Investigation of the proteomic profile of *Bacillus oleronius* in response to hydrogen peroxide

In this section a proteomic approach was employed to investigate the response of *B. oleronius* cells to hydrogen peroxide. Hydrogen peroxide is one of a number of Reactive Oxygen Species (ROS) produced by neutrophils during the oxidative response to pathogens (Manara *et al.*, 1991). A study by Timsa *et al.*, (2009) has reported that serum peroxide levels are significantly higher in individuals with rosacea compared to control subjects and that rosacea patients had significantly lower antioxidant levels. Therefore the aim of adding hydrogen peroxide to the culture media was to identify protein changes that could potentially occur *in vivo* when the bacterium is challenged by the host immune response. ROS have been implicated in the pathogenesis of rosacea (Yamasaki & Gallo 2009). This analysis was performed by culturing *B. oleronius* cells over night before adding 10 mM of hydrogen peroxide to the cultures for varying lengths of time (1, 2, 4 and 24 hrs).

6.4.1 2-Dimensional electrophoresis and LC/MS analysis of *Bacillus oleronius* whole cell lysate which was exposed to hydrogen peroxide

It was decided that to understand if any proteins were altered in response to hydrogen peroxide a 2 dimensional electrophoresis approach should be investigated. Bacteria were cultured over night before adding 10 mM of hydrogen peroxide to the cultures for varying lengths of time (1, 2, 4 and 24 hrs). Protein (150 µg) was extracted (section 2.12), applied to 7 cm IPG strips and separated by SDS-PAGE (section 2.17) on 10 cm 12.5% SDS gels. Figures 6.14 (A & B) represent the proteomic profiles of control cells and cells exposed to hydrogen peroxide. Gels were performed in triplicate and SameSpot™ software automatically generated statistically ordered list of spots from a one way ANOVA analysis. Progenesis SameSpot™ analysis allowed the identification of protein spots of interest which were excised and subjected to trypsin digestion prior to LC/MS analysis (Section 2.22).

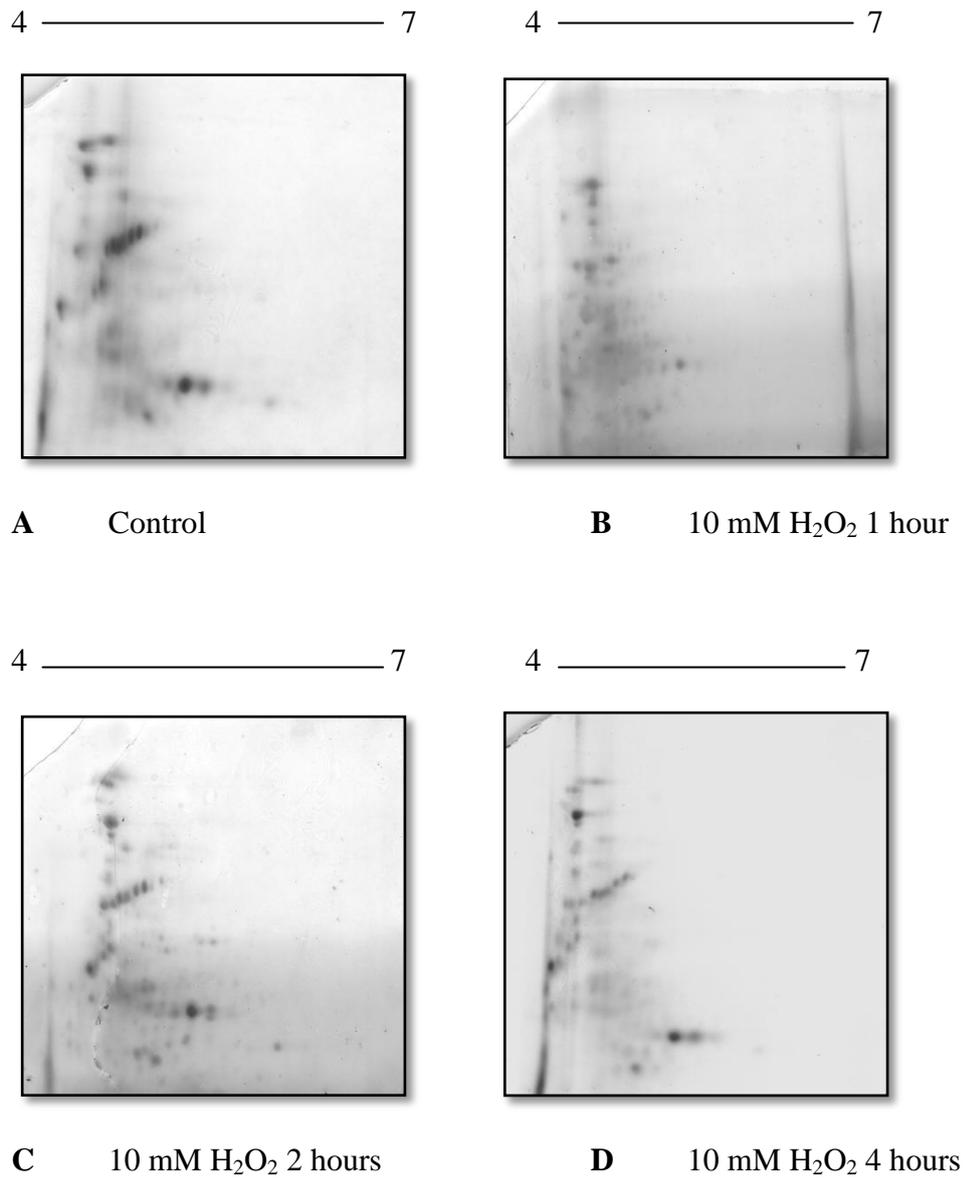


Figure 6.14 *Bacillus oleronius* protein profile following exposure to hydrogen peroxide

(A) Represents the untreated *B. oleronius* profile protein, (B-D) are the profiles following exposure to 10 mM H₂O₂ for 1, 2 and 4 hours

Liquid Chromatography Mass Spectrometry analysis identified 24 spots (Figure 6.15) that showed altered expression, 5 of these spots showed a statistically significant change in volume ($p \leq 0.05$). These spots were identified by LC/MS from this analysis (Figure 6.15 & 6.16). Identified proteins are presented in Table 6.3 with the spot ID assigned by the Progenesis analyses. Only one spot identified as a hypothetical protein from *Bacillus* sp. 2_A_57_CT2 (spot 36) showed an increase in expression of 1.8 fold ($p = 0.011$) in cells exposed to hydrogen peroxide compared to untreated cells. This protein has been described previously as being involved in the assembly of flagellin fillaments.

The protein corresponding to spot 18 all showed decreased expression in cells exposed to 10 mM hydrogen peroxide for 2 and 4 hours compared to untreated cells and these proteins are also involved in the assembly of flagellin fillaments and cell motility. Spot 18 showed homology to a flagellin from *Bacillus* sp. Kps3 and its expression decreased 1.9 fold ($p = 0.004$) following exposure to hydrogen peroxide. Spot 11 showed homology to an enzyme involved in the glycolysis pathway of respiration, phosphopyruvate hydratase from *Bacillus* sp. SG-1 which decrease 1.9 fold ($p = 0.007$) following exposure to 10 mM hydrogen peroxide. Spot 23 was identified as a glyceraldehyde-3-phosphate dehydrogenase GAPDH from *Geobacillus thermodenitrificans* NG80-2 and its expression decreased 2.2 fold ($p = 0.011$) in cells exposed to hydrogen peroxide. GAPDH is another enzyme involved in glycolysis. Spot 24 was identified as a hypothetical protein BB14905_07369 from *Bacillus* sp. B14905 which decreased 2.9 fold ($p = 0.003$). This protein is involved in growth inhibition.

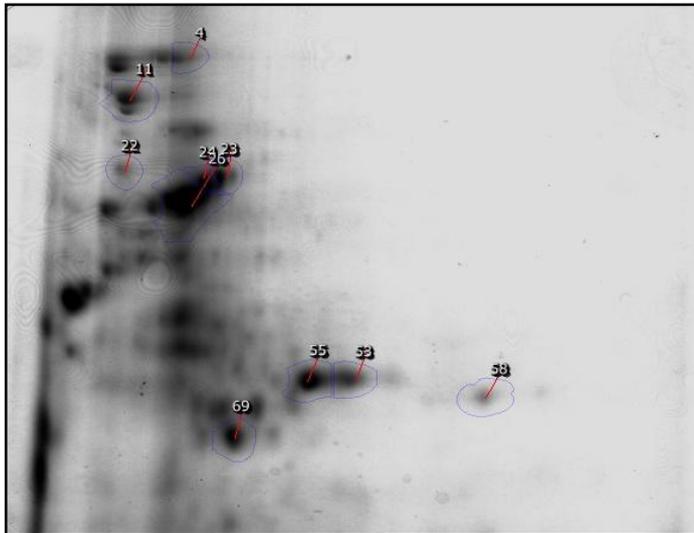
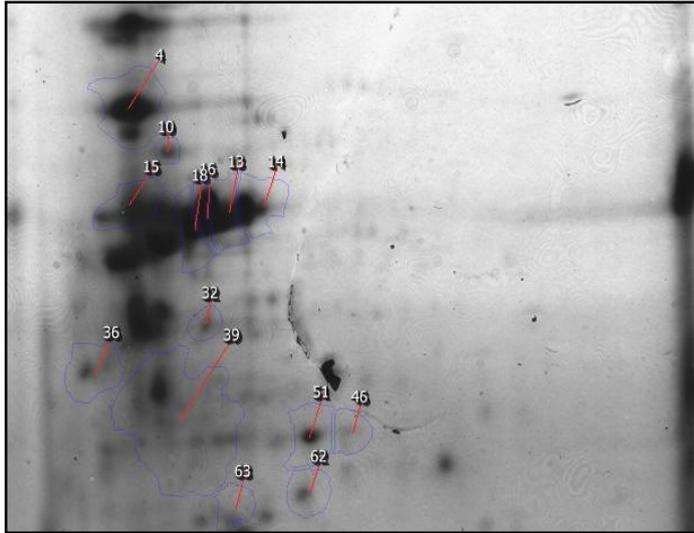


Figure 6.15 Spots indicated to have altered expression by Progenesis Same Spot™ analysis

Spots which showed significant ($p \leq 0.05$) alterations in expression were excised from a gel representing untreated bacterial cells. These spots are comparatively analysed in Figure 6.17 and represented on Table 6.4.

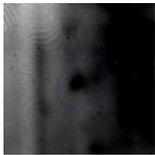
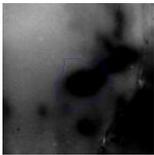
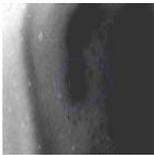
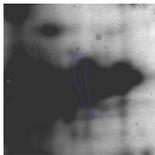
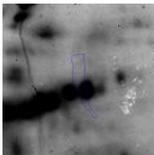
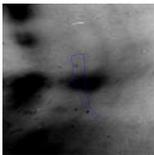
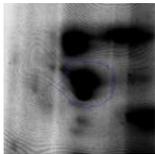
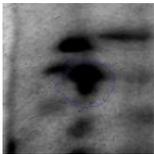
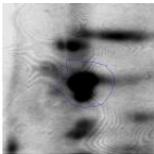
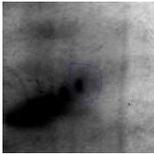
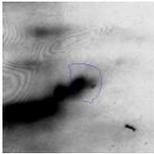
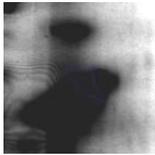
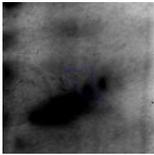
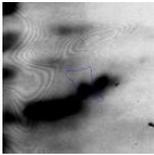
Spot	Identified Protein	Treatment			Average volume				p value
		Untreated	10 mM H ₂ O ₂ 2 hrs	10 mM H ₂ O ₂ 4 hrs	UT	2hrs	4hrs	FC	
36.	Hypothetical Protein				2.3	3.72	4.15	1.8	0.011
18.	Flagellin				3.07	1.59	2.50	1.9	0.004
11.	Phosphopyruvate hydratase				6.44	5.72	1.07	1.9	0.007
23.	GAPDH				3.15	1.44	2.76	2.2	0.011
24.	Hypothetical protein				2.55	0.87	1.86	2.9	0.003

Figure 6.16 Individual analysis of protein spots which showed a change in expression when grown in optimum or minimal growth conditions.

Progenesis™ SameSpot analysis was used to calculate the average volume, fold change and statistical significance values. LC/MS followed by Mascot analysis were employed for protein identification.

Spot ID	Protein	Accession	Mass	pI Value	Mascot Z-Score	Coverage %	Uniprot Function
18	Flagellin	BAH8035	40089	4.80	101	10	Bacterial flagellar motility
36	Hypothetical protein	ZP_08004778	44170	4.95	201	13	Bacterial flagellin N terminal helical region
11	Phosphopyruvate Hydratase	ZP_01862097	46717	4.68	859	40	Enzyme component of Glycolysis pathway
23	Glyceraldehyde-3-Phosphate Dehydrogenase	YP_001126741	37612	6.64	159	8	Enzyme catalyzes the sixth step of glycolysis and thus release energy and carbon molecules
24	Hypothetical protein	ZP_01723688	12811	6.73	69	13	PemK like protein which is a growth inhibitor

Table 6.4 Summary of proteins identified by LC/MS analysis.

Proteins presented in Figure 6.16 were identified using Mascot and functions were found through Uniprot.

6.5 Discussion

It has been hypothesised that the *Demodex* mite and its associated bacteria contribute to the chronic inflammation seen in rosacea patients due to the fact that several studies have reported higher *Demodex* levels in rosacea skin compared to control skin (Diazperez, 1994; Gao *et al.*, 2005; Kheirkhah *et al.*, 2007; Li *et al.*, 2010). *Demodex* mites feed on sebum produced by the host and also ingest bacteria (Elston 2010; Wolf *et al.* 1988), the mites have no anus and therefore when their life span of several weeks ends they harbour large amounts of bacteria. In people that are sensitive to the triggers associated with rosacea this deposition of bacteria may cause localised inflammation (Hsu *et al.*, 2009). The classic treatment for rosacea involves the use of systemic and topical antibiotics (Federici 2011) however the effectiveness of the treatment was largely thought to be due to the anti-inflammatory properties of the drugs rather than their ability to act on bacteria (Federici 2011; Monk *et al.*, 2011). Treatments that reduce the number of *Demodex* mites in the skin such as tea tree oil clear symptoms successfully (Kheirkhah *et al.*, 2007), however the condition cannot be completely cured and often occurs in patterns of flare ups and remissions. Upon consideration of the literature our hypothesis is that antibiotics can reduce *Demodex* numbers by killing symbiotic bacteria and decreasing the bacteria on which they feed. However some mites may remain or the host could become re-populated with mites, which potentially re-establish the pattern of localised inflammation in the hair follicles that becomes chronic over time causing vascular and tissue damage.

The role of *Bacillus oleronius*, a bacterium isolated from a *Demodex* mite in the induction of rosacea has been under investigation for 10 years (Delaney, 2004; Lacey, 2007). Researchers have also begun investigating the role of other bacteria (*S. epidermidis* and *H. Pylori*) in the condition (Whitfeld *et al.*, 2011; Dahl *et al.*, 2004; Utaş *et al.*, 1999; Lazaridou *et al.*, 2011). The aim of this section of work was to investigate *B. oleronius* growth and protein production in response to various environmental conditions. It has been established that *Bacillus oleronius* can produce proteins which cause serum reactivity in rosacea patients (Delaney, 2004; Lacey *et al.*, 2007). This section of work has shown that the bacterium can grow and produce these proteins across a range of culture conditions (section 6.1-6.1.4 & section 6.2-6.2.5). It has not been proven whether the bacterium encounters these conditions *in vivo* on the

surface of the skin, however these results provide an insight into the capability of the bacterium to adapt to conditions that may be present *in vivo*.

B. oleronius is thought to be associated with the hindgut of the *Demodex* mite and after the death of the mite the bacterium could be deposited in the pilosebaceous unit, the temperature, pH and oxygen availability in these environments may differ slightly. *B. oleronius* could potentially evade the host immune response since it is initially located in the gut of the *Demodex* mites (Finlay & McFadden, 2006). Following the death of mites in the hair follicle there could be a localized proliferation in *B. oleronius* and other bacteria within the pilosebaceous unit. The fastest growth rate was seen in cells cultured at 30 °C in 2XYT broth with aeration. This condition also induced a 3.3 fold (p=0.02) increase in expression of the 62 kDa stimulatory protein (probed with the rabbit anti-62 kDa antibody) compared to cells cultured at 30 ° in minimal nutrient and oxygen conditions.

One dimensional gels and Western blots (Figures 6.4-6.7) provided evidence that the stimulatory 62 and 83 kDa antigens are produced under stressful environmental conditions such as low oxygen or nutrients. There was a 2.1 fold increase in production of the 62 kDa protein in the blot probed with rosacea sera when cells were cultured in nutrient broth at 30 °C compared to 2XYT broth at 30 °C. The immunoblot represented in Figure 6.4 indicated that cells in low nutrient and oxygen conditions at 37 °C expressed 1.8 fold more of the 62 kDa protein than cells in optimum growth conditions. Culture of cells at 30 °C in low nutrient medium also produced a 2 fold increase in expression of the 83 kDa protein than those cultured at 37 °C in nutrient rich medium. The effect of culture temperature on protein expression in *B. oleronius* cells indicated that the bacterium produced increased amounts of protein at 37 °C. However Western blotting revealed that the stimulatory antigens are produced at both temperatures, therefore the bacterium could cause an immune response in a wide range of environmental conditions. Research carried out by Dahl *et al.*, (2004) investigating the role of *Staphylococcus epidermidis* in rosacea discovered that that bacterium secreted more proteins when cultured at 37 °C compared to 30 °C. The paper also suggests that the temperature of the skin of rosacea patients is generally higher than controls (Dahl *et al.*, 2004) due to flushing and inflammation. The group did not look for a specific stimulatory protein however the findings add to the evidence that bacterial components are contributing to rosacea. The response of *B. oleronius* to culture in a pH range of 6-9

indicated that cells can tolerate these conditions although slow growth was recorded in culture media at pH 6 (Figure 6.2). The immunoblot presented in Figure 6.6 indicated that cells cultured at pH 8 produced 1.7 fold more of the 62 kDa protein than those cultured at pH 7 ($p=0.038$) and pH 9 ($p=0.013$). The pH of human skin is said to range from pH 6-8 (Wagner *et al.*, 2003) Further investigation into the pH of rosacea skin during exacerbation of the condition may give insight into the significance of the finding that this bacterium can proliferate at an alkaline pH.

The environmental conditions which bacteria are exposed to have a great impact on the proteins they produce, which can mean they become more virulent or better able to evade the host immune response (Finlay & McFadden 2006). Studies have shown that bacteria can produce a wide and varied range of proteins with non-specific functions in response to stress (Carlin *et al.*, 2010; Hecker *et al.*, 1996). In order to examine the proteomic changes further 2D electrophoresis and LC/MS were employed. When the significantly increased protein spots from the comparison of culturing at 30 °C and 37 °C were compared, four of them were increased at 30 °C and two at 37 °C. Growth curves have suggested that *B. oleronius* grows preferentially at 30 °C in minimal medium however it is a slow growing bacterium. Naphthoate synthase and superoxide dismutase (SOD) were both increased at 30 °C and have been described as stress response proteins (Ramchandra & Sturm 2010; Areekit *et al.*, 2011). Naphthoate synthase is a key enzyme in the production of menaquinone in and has a role in the anaerobic biosynthesis of pyrimidines (Ramchandra & Sturm 2010). Lactate dehydrogenase, an enzyme involved in glycolysis and a TetR family transcriptional regulator were also up-regulated at 30 °C. Components of the glycolysis pathway are increased in glucose rich conditions (Hecker *et al.*, 2008) therefore the increased expression of lactate dehydrogenase by 3.9 fold ($p=0.052$) in cells cultured at 30 °C in minimal medium compared to cells at 37 °C may represent the cells utilising their nutrient source preferentially at this temperature.

Two metabolic enzymes, PtsH a component of the phosphoenolpyruvate-dependent sugar phosphotransferase system and ATP-dependent Clp protease involved in cellular homeostasis were increased in cells cultured at 37 °C. PtsH expression was increased 2.5 ($p=0.031$) fold in cells cultured at 37 °C in nutrient broth compared to cells cultured at 30 °C; Lacey (2004) previously identified a stimulatory protein from the 62 kDa region as having homology to ptsH. This protein has a known ability to

stimulate serological reactivity in rosacea patients (Lacey *et al.*, 2007) and individuals with rosacea are said to have increased skin temperature compared to control skin due to inflammation and vasodilation (Dahl *et al.*, 2004).

When the minimal medium was compared to the nutrient rich medium five of the significantly altered spots showed increased expression in the nutrient broth compared to one being up-regulated in the 2XYT. Proteins increased in the nutrient poor medium were also identified as being involved in metabolism and growth such as naphthoate synthase, dihydrolipoamide dehydrogenase, cell division protein FtsZ and elongation factor Tu. Proteins such as those essential to bacterial cell growth and metabolism often have antigenic properties (Kyungwoo 2011; Nieves *et al.*, 2010; Sharma *et al.*, 2011). A hypothetical protein identified as being involved in the assembly of flagellar filaments was also increased (3.2 fold, $p=0.05$) in the nutrient poor conditions compared to the nutrient rich. Flagellins aid the motility of bacterial cells and are often recognised by the host immune response as virulence factors or triggers (Gao *et al.*, 2010; Hozono *et al.*, 2006; Kumar *et al.*, 2007).

Comparison between optimum and minimal growth conditions as observed in the growth curve resulted in four significantly increased proteins being identified from each growth condition. Optimum growth was achieved in the 2XYT broth at 37 °C and 200 rpm, proteins that were increased under these conditions were, transketolase, involved in carbohydrate metabolism, a putative secreted protein (function unknown) and two proteins involved in assembly of flagellar filaments. Culture in the minimal growth conditions lead to increased expression of aconitate hydratase, superoxide dismutase (SOD), alkyl hydroperoxide reductase (AHP) and a Hag protein. SOD and AHP are involved in cellular stress responses by protecting the bacterium from harmful oxygen radicals (Kim *et al.*, 2010). Cells cultured at 30 °C expressed 2.3 fold ($p=0.017$) more SOD than cells cultured at 37 °C. Expression of SOD was also increased 1.6 fold ($p=0.002$) in cells cultured in low nutrient and oxygen conditions than cells cultured in optimum conditions. Increased expression of SOD and other stress proteins reflects the bacterial cells response to the environment. The deposition of *B. oleronius* in the pilosebaceous unit by the *Demodex* mite could also be perceived as a stress inducer for the bacterium. Purified bacterial SOD has been proven to induce an immune response in mice involving the increased production of cytokines TNF- α and IL-6 (Mullerad *et al.*, 2002).

Many bacterial products can stimulate the human immune response (Philpott *et al.*, 2001; Tapping, 2009). Exposure of microorganisms to hydrogen peroxide is one of the ways in which the human immune system retaliates against invading pathogens (Manara *et al.*, 1991). Neutrophils are a major component of the first line of defence that is the innate immune response (Segal 2005). Bacteria have adapted to this response by producing enzymes such as superoxide dismutase which catalyses harmful oxygen radicals into hydrogen peroxide and water. Five proteins that showed significantly decreased expression in H₂O₂ treated cells compared to untreated bacteria were presented in Table 6.4.

One hypothetical protein was revealed to have a function linked to flagellin assembly, flagellins are well known to trigger the human innate immune response via TLR signalling (Tapping 2009). In an investigation by Honzo *et al.*, (2006) it was found that flagellins of pathogenic and non-pathogenic bacteria induced a differential response from human corneal epithelial cells (HCEC). Expression of TLR5 was increased in HCEC in response to flagellin derived from *P. aeruginosa* which has been implicated in the pathogenesis of bacterial keratitis among contact lens users (Hozono *et al.*, 2006; Cheng *et al.*, 1996). Signalling through TLR5 induces the production of TNF- α and IL-8. In chapter 4 evidence that *Bacillus* proteins induced the production of IL-8 at an increased rate in exposed neutrophils compared to control cells.

The protein corresponding to spot 18 is also involved in flagellin assembly and cell motility and this spot showed decreased expression following 2 hrs of exposure to 10 mM hydrogen peroxide (fold change 1.9, p=0.004) however production was decreased by a fold change of only 1.2 after 4 hrs of exposure. Flagellins are proteins that are recognized as antigens by host cells in order to trigger a reaction from the immune system (Honzono *et al.*, 2006). Therefore the decrease in these proteins may represent the bacterial cells attempts to evade the host immune defence in response to the hydrogen peroxide exposure. Further down-regulated proteins identified were phosphopyruvate hydratase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) both involved in metabolic pathways that provide energy to the cells. The decrease of respiratory proteins following the challenge of H₂O₂ may be also be due to the cells slowing down production of proteins that could potentially be seen as antigens. A hypothetical protein involved in growth inhibition was identified along with a DNA polymerase. These proteins along with several of the others showed decreased

expression at 2 hours but expression began to increase again by 4 hours of treatment indicating that the cells have mounted a response to the challenge of the oxygen radicals and begun to grow and respire again.

The work presented in this section has highlighted the ability of *B. oleronius* cells to adapt to a broad range of environmental conditions. The bacterium may increase certain proteins in response to environmental stresses such as starvation, nutrient depletion, low oxygen or exposure to hydrogen peroxide. Western blots carried out with rosacea patient serum showed many reactive bands as seen in the individual blots in Chapter 3. Proteins spots that are increased in the various environmental conditions may correspond to these reactive bands. There is a clear need for a better understanding of the immune response in rosacea patients. The ability of this bacterium to produce antigenic proteins under varied environmental conditions has potential implications for the treatment of the condition.

Chapter 7

General Discussion

7.0 Discussion

The onset of a chronic skin disorder in such a highly visible area as the face can have a dramatic impact on the self esteem of an individual. Rosacea involves chronic inflammatory and vascular symptoms that can become progressively worse (Yamasaki & Gallo, 2009). The phenotypic characteristics of rosacea have previously been associated with excessive alcohol consumption leading to the term ‘whiskey nose’ being used to describe the thickening of the skin on the nose seen in rhinophyma. Some forms of rosacea can cause sudden erythema or prolonged blushing that has many triggers that the patient must try to avoid (Gupta & Chaudhry, 2005). Psoriasis is also a chronic inflammatory skin disorder that has a prevalence of around 2% in Ireland and similarly prevention of flare ups can be achieved by avoidance of triggers (Tonel & Conrad 2009). Like rosacea (Bamford 2001) it has been suggested that genetic predisposition is linked to the onset of psoriasis (Valdimarsson *et al.*, 1995) however the cause of the condition remains unknown. Despite the growing number of patients presenting with the four subtypes of rosacea (over 16 million in the USA and up to 2% of the Irish population) and the vast amount of research in the area there is no definitive evidence or clinical diagnostic marker for a specific causative factor. There is no cure for rosacea, the condition is managed by avoidance of triggers and treatment of flare-ups with anti-inflammatory therapies (Elewski *et al.*, 2010; Gupta & Chaudhry, 2005; van Zuuren *et al.*, 2007). Both rosacea and psoriasis represent inflammatory skin disorders with ocular manifestations (Oltz & Check, 2011; Rehal *et al.*, 2011) and both are notoriously difficult to clear and often require multiple forms of treatment depending on the stage of the condition. Early diagnosis and efficient treatment are extremely important in the management of chronic disease and the lack of diagnostic markers combined with the elusiveness of the causative factors in relation to rosacea represent problems that affect prompt treatment and patient care (Dahl *et al.*, 2002; Powell, 2005; Wilkin *et al.*, 2004).

Demodex mites (Lacey *et al.*, 2011) and many species of bacteria (Krutmann, 2009) are part of the normal skin flora however it is hypothesized by several research groups that both may play a role in the pathogenesis of rosacea (Yamasaki & Gallo, 2009). The *Demodex* mite has been suspected as playing a role in dermatological conditions since its discovery in 1842 (Bonnar *et al.*, 1993; Desch & Nutting, 1972; Fell, 1886; Lacey *et al.*, 2011). *D. folliculorum* has been described as a

bacterial vector (Wolf *et al.*, 1988) and increased mite density has been reported in the pilosebaceous units (Aylesworth & Vance, 1982; Bonnar *et al.*, 1993; Norn, 1970) and eyelashes (Gao *et al.*, 2005; Kheirkhah *et al.*, 2007) of rosacea patients. Therefore as the density of mites increases in the skin of individuals with rosacea, the density of bacteria would also increase. Bonnar *et al.*, (1993) reported that there was no significant reduction in *Demodex* mites in the skin of rosacea patients following one month of treatment with tetracycline although symptoms were cleared. This may suggest that the drug attacked the bacteria associated with the mite rather than having a direct effect on the mite. Treatments such as acaricides and Tea Tree oil which reduce mite numbers can also alleviate symptoms of rosacea (Bonnar *et al.*, 1993; Gao *et al.*, 2005).

Although antibiotics have been the main treatment for rosacea for many years (van Zuuren *et al.*, 2007) bacterial involvement has never been proven and the efficacy of this treatment procedure has largely been attributed to the anti-inflammatory effect of the drugs (Monk *et al.*, 2011). Since the discovery of *Bacillus oleronius* in the *Demodex* mite of a rosacea patient (Delaney, 2004) the possible role of this bacterium in the induction of rosacea has been under investigation (Lacey *et al.*, 2007; Lacey *et al.*, 2009; Li *et al.*, 2010). Bacteria such as *Staphylococcus epidermidis* (Dahl *et al.*, 2004; Whitfeld *et al.*, 2011) and *Helicobacter pylori* (Lazaridou *et al.*, 2011; Utaş *et al.*, 1999) have also been investigated as contributing factors in the development of rosacea. Whitfeld *et al.*, (2011) isolated *S. epidermidis* from the pustules of 9 of 15 patients with papulopustular rosacea while no swabs taken from non-affected areas of the skin contained the bacterium ($p = 0.0003$). *S. epidermidis* was also isolated from the eyelid margins of 4 of 15 patients with papulopustular rosacea while no pure growth was isolated from the eyelids of age- and sex-matched control subjects ($p = 0.05$). The study also found that this bacterium was susceptible to antibiotics commonly used to treat rosacea such as tetracycline (Whitfeld *et al.*, 2011). Dahl *et al.*, (2004) investigated the effect of temperature on *S. epidermidis* and found that the bacterium secreted more proteins when cultured at 37 °C than at 30 °C. It was suggested that the skin of rosacea patients is thought to be increased compared to control skin and this increase in temperature would increase the amount of secreted bacterial protein present in the skin (Dahl *et al.*, 2004). It was also found that *S. epidermidis* isolated from rosacea patients was consistently β -hemolytic, whereas bacteria from control subjects were non-hemolytic which is a characteristic virulence factor (Wilkin *et al.*, 2004).

B. oleronius is the only bacteria isolated to date that potentially contributes to the development of rosacea for which serological markers have been identified (Lacey 2007; Li *et al.* 2010). In Chapter 3 of this study it was shown that two *B. oleronius* proteins (62 and 83 kDa) previously identified by Lacey *et al.*, (2007) cause serological reactivity in individuals with ocular and erythematotelangeictatic (type one) rosacea. Following blind testing of 59 sera samples from patients with ocular rosacea and controls (Li *et al.*, 2010), there was 66% correlation between serum immunoreactivity and occurrence of rosacea. In sera that showed no reactivity to the stimulatory proteins 78% were found to be from controls. Following a study of sera from type one rosacea and controls, 92% of those who showed serum reactivity were positive for rosacea and there was one rosacea patient that did not show serum reactivity to either antigen. None of the controls (5/5) showed serum reactivity. The type one rosacea study has the potential to be strengthened with the addition of more samples. The results of both patient sera studies along with the previous results from a papulopustular study (Lacey, 2007) strengthen the case that *B. oleronius* has a potential role in the induction of rosacea.

The potential to detect reactivity to *B. oleronius* antigens in tear fluid was also investigated. This proved quite difficult with the Western blotting technique employed and only 6 samples showed weak binding with the anti-62 kDa antibody. Tear fluid from case number 45 showed reactivity in the 83 kDa region while cases 16, 18, 22 and 23 were reactive to the 62 kDa protein. In the 6 cases where tear fluid reacted to the *B. oleronius* rabbit-62 kDa antibody; all showed serum immunoreactivity to both the 62 and 83 kDa proteins. Detecting the presence of *B. oleronius* antibodies in tear fluid could prove a useful diagnostic assay perhaps by changing to an ELISA format such as that used to detect the presence of *Pseudomonas aeruginosa* in the tear fluid of contact lens users (Cheng *et al.*, 1996). There is also potential to detect specific proteins in tear fluid using LC/MS (An *et al.*, 2005).

The stimulatory antigens were previously identified by MALDI-ToF mass spectrometry by Lacey, (2007). The 62 kDa antigen showed the strongest homology to a Phosphoenolpyruvate phosphotransferase sensory system (PTS) involved in the metabolism of carbohydrates. The 83 kDa protein showed homology to a Heat shock chaperon protein involved in stress responses. In this study the identification of these proteins was repeated by LC/MS on tryptic digests of bands corresponding to the 62 and

83 kDa regions. The immune stimulatory protein at the 62 kDa region showed homology to a groEL chaperonin protein from *Bacillus subtilis*. This protein is known to elicit serological reactivity in patients affected by several conditions involving bacteria (Shinnick *et al.*, 1988; Qoronfleh *et al.*, 1998; Woo *et al.*, 2001; Mayr *et al.*, 1999). GroEL is conserved across many species of bacteria and has been implicated as an antigenic marker in the serum of individuals infected with *Burkholderia pseudomallei* which causes melioidosis a serious human disease that is endemic in Southeast Asia (Woo *et al.*, 2001). The condition has acute and chronic phases and the GroEL protein identified was suggested as a diagnostic marker for the infection. *Chlamydia trachomatis* can cause the chronic inflammatory disease trachoma which can lead to blindness and an antigenic protein homologous to GroEL has been identified as causing serum reactivity in patients with the condition (Morrison *et al.*, 1989). A recombinant protein clone of this antigen elicited an ocular inflammatory response in guinea pigs (Morrison *et al.*, 1989). The 83 kDa band was identified as aconitate hydratase from *Bacillus sp. SG-1*. Aconitate hydratase is a TCA cycle enzyme involved in metabolism of carbohydrates, this pathway is particularly active when cells are in low nutrient conditions (Michael *et al.*, 2008; Rosenkrands *et al.*, 2002) which could reflect the response of the bacteria to the change in nutrient levels from the hindgut of the *Demodex* mite to the pilosebaceous unit.

Anion exchange with Q-sepharose charge separation combined with fractionation using ÄKTA-Fast Protein Liquid Chromatography was employed to separate *B. oleronius* protein. This method allowed the isolation of fractions that contained a 'semi-pure' preparation of the 62 kDa stimulatory protein. In Chapters 4 and 5, neutrophils and corneal epithelial cells were exposed to this protein preparation in order to determine what effect it had on cells of the innate immune system thought to be affected by the progression of rosacea (Yamasaki & Gallo 2009; Kheirkhah *et al.*, 2007). The protein preparation was resolved by 1D SDS-PAGE and all bands present were excised and subjected to LC/MS in order to assign functionality to the proteins being exposed to the human cells. Flagellin which is known to be a ligand for TLR-5 was present in the protein preparation. Signalling through the toll like receptor (TLR) pathways leads to production of cytokines such as IL-8 (Vance *et al.*, 2009, Hozono *et al.*, 2006), which was shown to be increased in neutrophils treated with the bacterial proteins. A peptidoglycan synthesis protein (Hypothetical protein Noc_2222) was

identified and peptidoglycan recognition is a trigger for the innate immune response (Dziarski 2004). Elongation factor G was also among the identified proteins and several studies have reported proteins with elongation and translational functions in the ribosome as antigenic (Shin, *et al.*, 2007, Shin *et al.*, 2009). A protein involved in metabolism (oligopeptide ABC transporter periplasmic protein) was identified as having a function in the movement of substances in and out of the cell. A study by Travis (2000), has highlighted the use of drugs to disrupt metabolic pathways as a new target for treatment of pathogenic bacteria. A study by Jeavons *et al.*, (1998) describes the isolation of proteins from *Penicillium marneffeii* which caused immunoreactivity in human sera one of which had homology to catalase. Catalase was among a number of proteins released in response to oxidative stress such as Kat E1, vegetative catalase, superoxide dismutase and alkyl hydroperoxide reductase (Mostertz & Hecker, 2003). Recognition of pathogen-associated molecules (PAMPs) leads to stimulation of the innate and eventually the adaptive immune responses. *Bacillus oleronius* is thought to be a commensal or opportunistic pathogen (Lacey *et al.*, 2011). Several of the proteins described may have the ability to stimulate the immune response since PAMPs are not exclusively associated with pathogenic microbes (Cookson *et al.*, 2001; Gallo & Nizet, 2008). Some of the proteins mentioned here were identified in Chapter 6 when protein expression in *B. oleronius* cultured under varied environmental conditions was investigated.

In Chapter 3 the potential to develop a serological diagnostic assay was also investigated. As previously discussed examination of serum with crude *B. oleronius* protein preparations by Western blotting revealed a 66% correlation between serum reactivity and the occurrence of ocular rosacea. Therefore it was decided that the 'semi-pure' *B. oleronius* protein would be used in an ELISA diagnostic assay. However the assay design proved unsuccessful when 20 positive rosacea patients were compared with 19 control samples. All sera (diluted 1/100) bound to the proteins present on the plate and 14/20 rosacea patient samples recorded absorbance over 0.5 (O.D.=450) while 15/19 samples proven to be controls using the Western blotting method recorded the same level of absorbance. More of the sera samples from patients (n=7) recorded absorbance over 1 compared to controls (n=6) and when the cut off was raised to 1.5 (O.D.=450) only 2 control patients showed this level of reactivity compared to 4 of the patient sera samples. ELISA assays represent an easy, reproducible and fast technique

for the detection of antigens or antibodies (Diniz *et al.*, 1999). The development of a diagnostic assay for rosacea has not yet been achieved and with some alterations this technique could prove a useful diagnostic tool.

Lacey *et al.*, (2007) demonstrated that *B. oleronius* produced antigens capable of stimulating peripheral blood mononuclear cells (PBMC) proliferation in 16 of 22 (73%) patients with rosacea but only five of 17 (29%) control subjects ($P = 0.0105$). In Chapter 4 the effect of the 'semi pure' *Bacillus* protein preparation of human neutrophils was examined. As an integral part of the innate immune response (Segal 2005), neutrophils are essential for the detection and destruction of potential pathogens. Neutrophils are involved in the induction of inflammation and degranulation (Manara *et al.*, 1991; Pham 2008). Inflammation is a protective mechanism of the immune response involving epithelial cells and circulating white blood cells (Hozono *et al.*, 2006; Philpott *et al.*, 2001; Ueta & Kinoshita, 2010) which leads to induction of signalling pathways through Toll like receptors (TLRs) (Bellocchio *et al.*, 2004). However when this response is not regulated it becomes chronic and factors that usually contribute to the killing of invading microbes can begin to damage host cells (Bevins & Liu, 2007).

In Chapter 4 it was discovered that *B. oleronius* antigens have the ability to stimulate the production of IL-8 and TNF- α in isolated human neutrophils. TNF- α is essential to the induction of inflammatory responses (Teranishi *et al.*, 1995) and IL-8 is a chemotactic cytokine (Ramos 2003) that encourages the movement of white blood cells to the site of inflammation, therefore induction of these cytokine could contribute to the chronic nature of the condition (Yamasaki & Gallo, 2009). The induction of these cytokines was likely caused by the recognition of the bacterial protein components being potentially pathogenic (Vance *et al.*, 2009). Many of the *B. oleronius* proteins identified were shown to have antigenic properties when their functions were investigated (section 3.4.2, Table 3.4) and therefore have the potential to induce cytokine production in neutrophils. The highest increase in IL-8 production of 2.7 fold ($p=0.0027$) was observed following 16 hrs of exposure to 2 $\mu\text{g/ml}$ of the crude *Bacillus* protein extract. The crude extract (2 $\mu\text{g/ml}$) also elicited the highest concentration of TNF- α production in human neutrophils, at 3 hrs a 2.9 fold ($p=0.0008$) fold increase was noted and at 16 hrs a 2.8 fold ($p=0.0002$) increase was seen. The potential for these antigens to induce significantly increased concentrations of IL-8 and TNF- α compared

to control neutrophils could contribute to the overall dysregulation of the immune system that induces rosacea symptoms (Yamasaki & Gallo, 2009).

Increased migration of neutrophils in response to *Bacillus* protein exposure was also detected with an assay using a porous insert. When a 3 µm porous insert was employed and neutrophils were exposed to 2 µg/ml of the 'semi-pure protein preparation' a 4.3 fold (p=0.02) increase in migrated cells was seen compared to control samples. This increase rose to 7 fold (p=0.027) when 2 µg/ml of the crude extract was used compared to control cells. Increased IL-8 production by the treated neutrophils *in-vitro* and the migration assay adds to the theory that *in-vitro* migration is being induced in response to the *Bacillus* proteins which suggests that migration of neutrophils could potentially occur *in-vivo*. Infiltration of neutrophils is part of the inflammatory response (Gambero *et al.*, 2004) and has been previously linked to the pathogenesis of rosacea (Crawford *et al.*, 2004).

If the infiltration of neutrophils is not regulated by the immune response there is potential for inflammation to become chronic (Hartl *et al.*, 2008; Mizgerd *et al.*, 1999). The main function of neutrophils is phagocytosis of pathogens which is followed by degranulation (Lacy 2006), this process sometimes involves the release of granule contents into the surrounding tissue (Levy 2000). In Chapter 4, Western blotting was employed to detect degranulation proteins secreted by neutrophils in response to exposure to the bacterial antigens. Myeloperoxidase (MPO), matrix metalloproteinase 9 (MMP-9) and cathelicidin (hCAP-18) are antimicrobial components of neutrophil granules which are released into lysosome vacuoles which contain pathogens engulfed by the neutrophil (Curnutte *et al.*, 1987; Manara *et al.*, 1991). Increased secretion of all three proteins was detected in neutrophil cells exposed to the *Bacillus* antigens compared to controls. The highest fold change in MPO (3.9, p=0.0003) was observed when neutrophils were exposed to 2 µg/ml of the crude protein extract for 6 minutes in a stirred chamber. MMP-9 secretion was detected to be 2.9 fold (p=0.0001) following 3 minutes of exposure to 2 µg/ml of the 'semi-pure protein' compared to control cells. MMPs are involved in processes such as tissue remodelling and neo-vascularisation (Manicone & McGuire 2008; Pflugfelder *et al.*, 2005). These enzymes are involved in the degradation of ECM components and have been linked to inflammation in granulomatous rosacea (Jang *et al.*, 2010). Cathelicidin peptides have vasoactivating and pro-inflammatory functions and are implicated in the induction of rosacea

(Yamasaki & Gallo, 2009). Detection of this protein was increased 2.4 fold ($p=0.037$) when neutrophils were exposed to 2 $\mu\text{g/ml}$ of the 'semi-pure' protein for 6 minutes compared to control cells.

In Chapter 5 the effect of *Bacillus* proteins on the corneal epithelial cell line (hTCEpi) and on primary limbal epithelial cells was investigated. Corneal epithelial cells function as a protective barrier of the ocular surface and form part of the innate immune response (Ueta & Kinoshita 2010). Rosacea patients manifest ocular symptoms in up to 50% of cases (Oltz & Check 2011). In the most severe cases of ocular rosacea, sight diminishing ulcers can form on the corneal surface resulting in the need for surgical correction in some cases (Oltz & Check, 2011; Kheirkhah *et al.*, 2007). In Chapter 3 the detection of *B. oleronius* proteins in the tear fluid of 6 people with ocular rosacea was observed therefore it is possible that the presence of *Demodex* mites and this associated bacterium in the eyelash follicles and meibomian glands could induce inflammation of the corneal surface observed in severe cases of ocular rosacea.

The growth of hTCEpi cells in response to the potentially antigenic proteins was assessed first. There was a significant inhibition of 26% ($p = 0.018$) in corneal cell growth following 5 days of antigen exposure (2 $\mu\text{g/ml}$). Growth was inhibited by 75% following exposure to 6 $\mu\text{g/ml}$ of the 'semi pure' protein. The slowing of epithelial cell growth indicates that the protein had a toxic affect on the corneal cells over time (Huo *et al.*, 2009). The next aim in this Chapter was to investigate the wound healing response of hTCEpi cells to the bacterial proteins (2 & 6 $\mu\text{g/ml}$). Images presented in section 5.3 revealed that the cells were closing the wound more rapidly following exposure to the proteins. Therefore it was thought that cells were migrating at an increased rate at the leading edge of the wound following exposure to the antigens (Zieske *et al.*, 1989). A number of experiments were then designed to examine the effect of the bacterial proteins on cell movement.

Migration was examined in a similar assay to that used to test neutrophil responses. Corneal cells were placed inside porous inserts (8 μm) and incubated in KGM-2 medium containing bacterial protein (2, 4 & 6 $\mu\text{g/ml}$), an increase in migration was noted following exposure to the proteins. Migration increased 3.6 fold ($p=0.001$) in response to 2 $\mu\text{g/ml}$ of the antigen and by 14.5 fold in response to 6 $\mu\text{g/ml}$ treatment ($p=0.0001$) when compared to control cells. hTCEpi cell invasion was examined using a

similar technique and was shown to increase by 1.7 fold ($p=0.003$) in response to 2 $\mu\text{g/ml}$ of the antigen and by 1.8 fold ($p=0.01$) in response to 6 $\mu\text{g/ml}$ when compared to control cells. These assays along with the wound healing assay indicate increased motility of corneal epithelial cell following exposure to the bacterial proteins. Adhesion assays showed a consistent decrease in adherence to laminin of 1.7 fold ($p=0.002$) following exposure to 2 $\mu\text{g/ml}$ of bacterial proteins. Altered migration, wound healing, invasion and adhesion to the basement membrane are factors that would potentially contribute to corneal cell erosion (Lin & Kurpakus-Wheater 2002; Yamaguchi *et al.* 2011; Markoulli *et al.*, 2011). Corneal erosion is linked to the development of microbial keratitis in contact lens wearers (Markoulli *et al.*, 2011). It is possible that the presence of bacterial antigens in the tear fluid and on the ocular surface cause an inflammatory response in corneal epithelial cells that can lead to corneal erosion

In Chapter 5 the expression of a number of proteins in corneal epithelial cells in response to the *Bacillus* antigens were also investigated. Several of the proteins investigated by immunofluorescence imaging and Western blotting revealed no change in response to these antigens. Cytokeratins 3, 12 and 19 expressions were not altered in treated compared to control cells, neither was the cell-cell interaction protein E-cadherin. In line with the increase in migration there was an increase in vinculin expression recorded along with a decrease in expression of $\beta 1$ -integrin. Vinculin is a membrane cytoskeletal protein that interacts with the basement membrane (Wu *et al.*, 1995) and it was increased 2.7 fold ($p=0.0009$) in response to exposure to 6 $\mu\text{g/ml}$ of the protein preparation. Increased vinculin expression has been demonstrated in migrating cells at the leading edge of a corneal wound in rat cells (Zieske *et al.*, 1989). Matrix metalloproteinases have been implicated in degradation of the stromal basement membrane over which the cell migrates (Fini *et al.*, 1996). The presence and activity of MMP-9 in corneal epithelial cell exposed to *Bacillus* antigens was investigated. An increase of 1.34 fold ($p=0.03$) in MMP-9 expression was recorded following exposure to 2 $\mu\text{g/ml}$ of bacterial protein. MMP-9 activity increased by 1.96 fold ($p=0.046$) following exposure to 4 $\mu\text{g/ml}$ of the proteins. Exposure of hTCEpi cells to the *Demodex*-associated bacterial proteins caused increased cell movement which could potentially cause an increased loss of corneal cells through re-epithelialization. Recurrent movement of the cells coupled with increased production of MMP-9 which degrades components of the basement membrane can contribute to the formation of

corneal ulcers (Fini *et al.*, 1998; Fini *et al.*, 1996; Suzuki *et al.*, 2003). The presence of MMPs has previously been detected at increased concentrations in the tear fluid of ocular rosacea patients (Kari & Kari, 2006).

In Chapter 5 the limbal epithelial cells (LECs) were cultured using limbal stem cell tissue explants. The limbal region of the eye harbours stem cells which service the corneal epithelial surface (Ahmad *et al.*, 2010). A limited amount of limbal tissue was available therefore results from this section are inconclusive and the need for further investigation in this area is indicated. LECs were investigated in a wound healing assay and cells indicated a growth response similar to that seen in the hTCEpi cell line in that the cells exposed to the bacterial proteins tended to close the wound faster than control cells. Immunoblots were performed by exposing LECs to *Bacillus* proteins (2 & 4 µg/ml) for four days then obtaining the protein extract from the limbal cells. Most of the markers tested showed no significant change in response to the antigens. Cytokeratin 19 expression was increased 1.24 fold (p=0.005) following exposure to 2 µg/ml of bacterial protein, however these results are represented from technical replicates. Further investigation is necessary to indicate if any significant changes are induced in these cells by *B. oleronius* proteins.

In Chapter 6 it was sought to examine the effect of culture conditions on protein production in the bacterium *B. oleronius*. It has been established throughout this body of work that this bacterium produces proteins capable of inducing an inflammatory immune response (Lacey *et al.*, 2007; Li *et al.*, 2010). The growth response of the bacterium to various culture conditions was assessed first and it was found that this bacterium could adapt to many environments. Two-Dimensional proteomic analysis was coupled with LC/MS to allow a more detailed comparison of the proteins with altered expression in the different growth environments (Michael *et al.*, 2008). Following comparison of protein production at 30 °C and 37 °C in minimal nutrient broth, four proteins were found to be increased at 30 °C and two at 37 °C. Growth curves have suggested that *B. oleronius* grows preferentially at 30 °C in minimal medium. Naphthoate synthase and superoxide dismutase (SOD) were both increased at 30 °C and have been described as stress response proteins (Ramchandra & Sturm 2010; Areekit *et al.*, 2011). SOD was also identified in the protein preparation used to treat the neutrophil and corneal cells. SOD is produced by bacterial cells in response to reactive oxygen species (ROS) in the host tissue. ROS are elevated in rosacea patient skin and

the anti-inflammatory drugs used to treat the condition are known to target these molecules (Yamasaki & Gallo, 2009). Lactate dehydrogenase, an enzyme involved in glycolysis and a TetR family transcriptional regulator were also up-regulated at 30 °C.

PtsH a component of the phosphoenolpyruvate-dependent sugar phosphotransferase system showed increased expression in cells cultured at 37 °C, this enzyme was previously identified as a stimulatory protein spot from the 62 kDa region (Lacey *et al.*, 2007). Growth curves have shown that *B. oleronius* cultured in nutrient broth at 37 °C grow slower than those at 30 °C. One dimensional electrophoresis has shown that this bacterium tends to produce more proteins at 37 °C. A study performed by Dahl *et al.*, (2004) investigated protein secretion by *S. epidermidis* at 30 °C and 37 °C and found that protein production was increased at 37 °C. They also postulate that skin temperature might regulate the protein secretion of these proteins and the skin of rosacea patients is thought to be elevated compared to control subjects. Western blotting analysis using both the rabbit anti-62 kDa antibody and human serum indicated that aerated cultures at 30 and 37 °C produce similar levels of the stimulatory protein.

Both 1D and 2D techniques were employed to make a comparison between nutrient rich (2XYT broth) and nutrient poor (nutrient broth) media. Upon identification of the significantly up-regulated proteins most were found in cells cultured in nutrient poor medium. Proteins involved in metabolism and growth such as naphthoate synthase, dihydrolipoamide dehydrogenase, cell division protein FtsZ and elongation factor Tu were all upregulated in cells grown at 30 °C in nutrient poor medium compared to those cultured at 30 °C in 2XYT broth. Proteins such as those essential to bacterial cell growth and metabolism often have antigenic properties (Kyungwoo 2011; Nieves *et al.*, 2010; Sharma *et al.*, 2011). Growth curves revealed that *B. oleronius* grew more rapidly in the rich medium and production of the 62 kDa stimulatory antigen is similar in both media under aerated conditions when probing with the rabbit antibody. However by immunoblotting with rosacea patient sera it can be seen that the bacterium produced more stimulatory proteins in nutrient poor conditions than in nutrient rich conditions at 30 °C. Production of flagellin was also increased in nutrient poor conditions at 30 °C, this was another protein identified in the protein preparation used to in cell culture assays. Flagellins aid the motility of bacterial cells and are often recognised by the host immune response as virulence factors or triggers (Gao *et al.*, 2010; Hozono *et al.*, 2006; Kumar *et al.*, 2007).

Following consideration of the growth curves it was noted that culture of *B. oleronius* at 30 °C nutrient broth in a static incubator gave the slowest growth and culture at 37 °C in 2XYT medium with aeration gave the fastest growth. Upon examination of the expression of the stimulatory 62 kDa antigen under these conditions it was seen (with the use of the rabbit anti-62 kDa antibody) that optimum growth conditions produced higher amounts of this protein. Similar results were noted upon blotting with rosacea patient sera however cells grown in static culture did produce a visible reactive band at the 62 and 83 kDa regions. Proteins identified to have increased expression when cells were cultured in optimum conditions were, transketolase, involved in carbohydrate metabolism, a putative secreted protein (function unknown) and two proteins involved in assembly of flagellar filaments. Culture in the minimal growth conditions lead to increased expression of aconitate hydratase, superoxide dismutase (SOD), alkyl hydroperoxide reductase (AHP) and a Hag protein. SOD and AHP are involved in cellular stress responses by protecting the bacterium from harmful oxygen radicals (Kim *et al.*, 2010) and these proteins were also identified in the protein preparation used to treat cells of the innate immune system.

It has been postulated that the proliferation of *Demodex* mites in the pilosebaceous unit and the eyelash follicles known to occur in rosacea patients (Bonnar *et al.*, 1993) leads to increased density of the bacteria for which these mites act as vectors (Wolf *et al.*, 1988; Lazaridou *et al.*, 2011). The *Demodex*-associated bacterium *Bacillus oleronius* and its associated stimulatory proteins could potentially induce an immune response (Delaney, 2004; Lacey *et al.*, 2007; Lacey, 2007; Li *et al.*, 2010) in the tissue surrounding the pilosebaceous unit meibomian glands. The work outlined here has shed light on the potential cellular responses of cells of the innate immune response to these bacterial proteins. Many questions remain to be answered about the etiology and pathogenesis of rosacea, this is an active area of research with many groups worldwide focused on elucidating the molecular and cellular mechanisms involved in the progression of the condition.

The work discussed here indicates the potential for further investigation of the role of *Demodex* and the bacterium *Bacillus oleronius* in the pathogenesis of rosacea. Further investigation into producing a diagnostic assay perhaps with the use of recombinant anti 62 kDa antigen could be beneficial. ROS production through the NADPH oxidase pathway in response to *Bacillus* antigens could provide further insight

into the pathogenesis of the bacterium. The effect of *B. oleronius* on corneal epithelial cells could be further investigated with the addition of an air-lifted cell model. The use of other ocular surface cell lines such as conjunctival epithelial cells and more extensive investigations using limbal epithelial cells could also provide useful information. Further investigations employing sebaceous gland cells or other skin cells in air-lifted models could provide more insight into the cutaneous effects caused by the bacterial antigens.

Appendix

Immunoblot analysis of sera from ocular rosacea patients, erythematotelangiectatic rosacea patients and controls. These blots correspond to the data in Chapter 3, section 3.2.1 & 3.2.2. The data is summarised in Table 3.1.

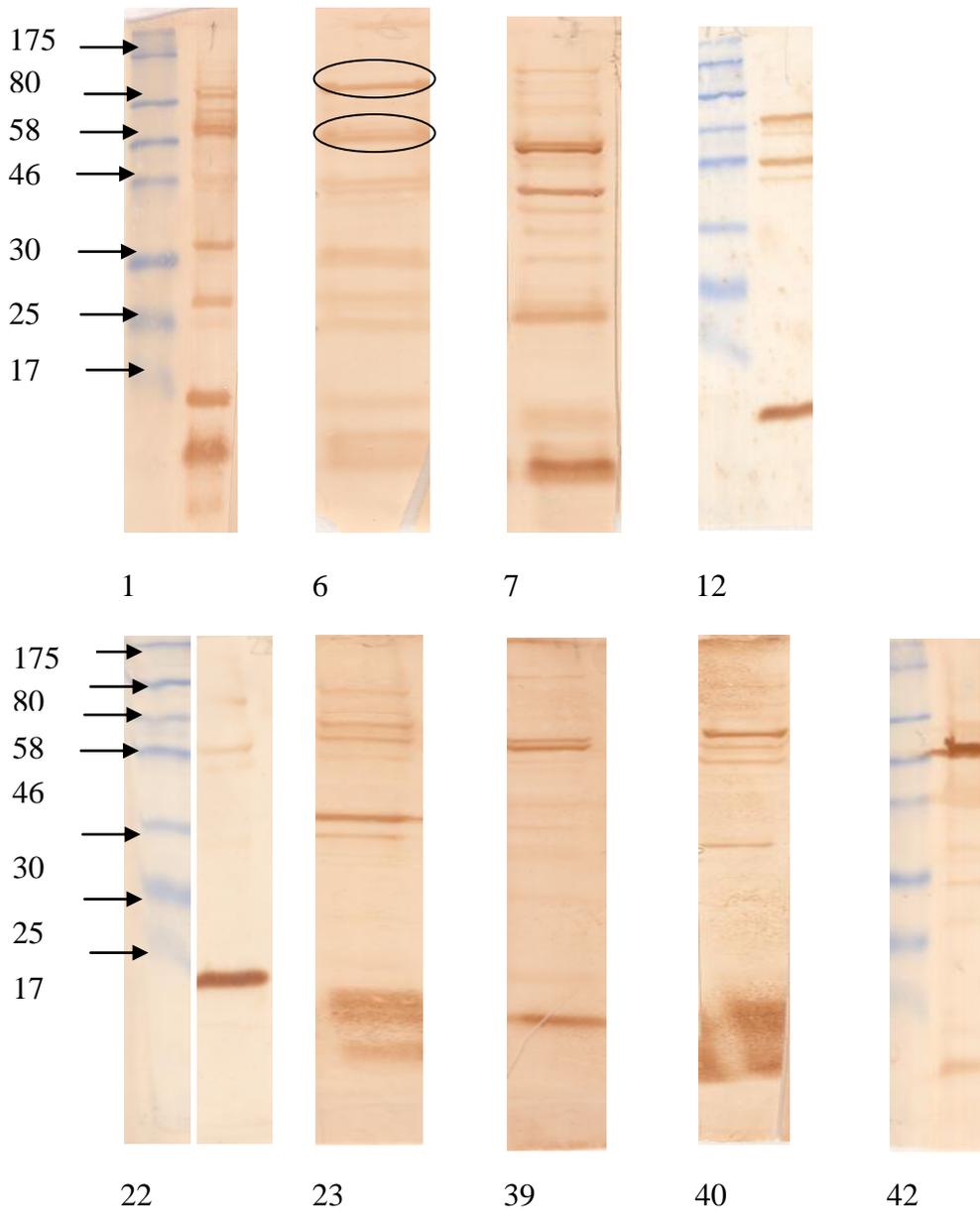


Figure 8.1 A Western blot analysis of sera from rosacea patients to the *B. oleronius* protein extract

These blots represent cases where the sera showed positive reaction to one or both of the stimulatory antigens and the person had been diagnosed with ocular rosacea.

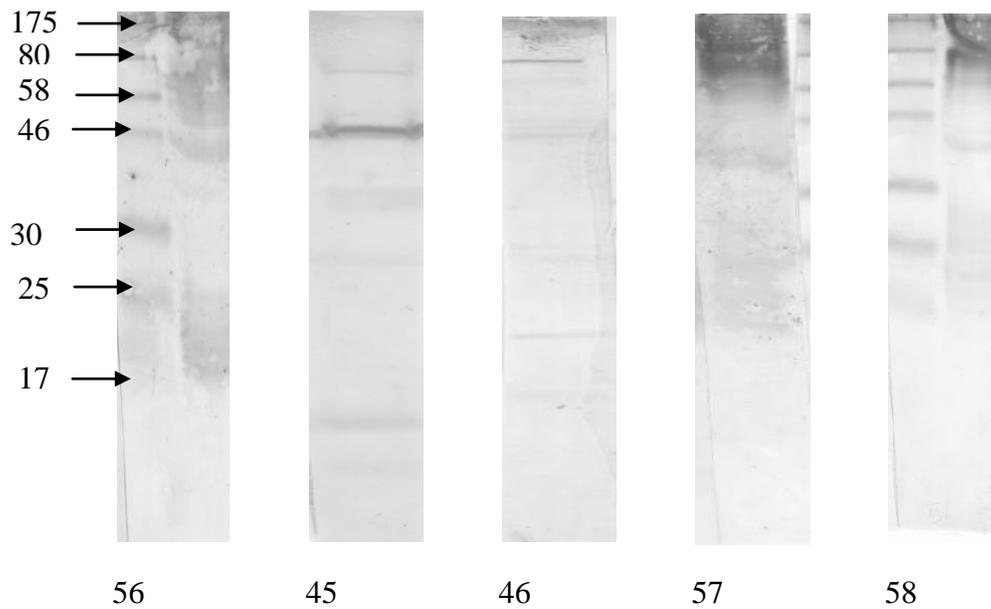


Figure 8.1 B Western blot analysis of sera from rosacea patients to the *B. oleronius* protein extract

These blots represent cases where the sera showed positive reaction to one or both of the stimulatory antigens and the person had been diagnosed with ocular rosacea.

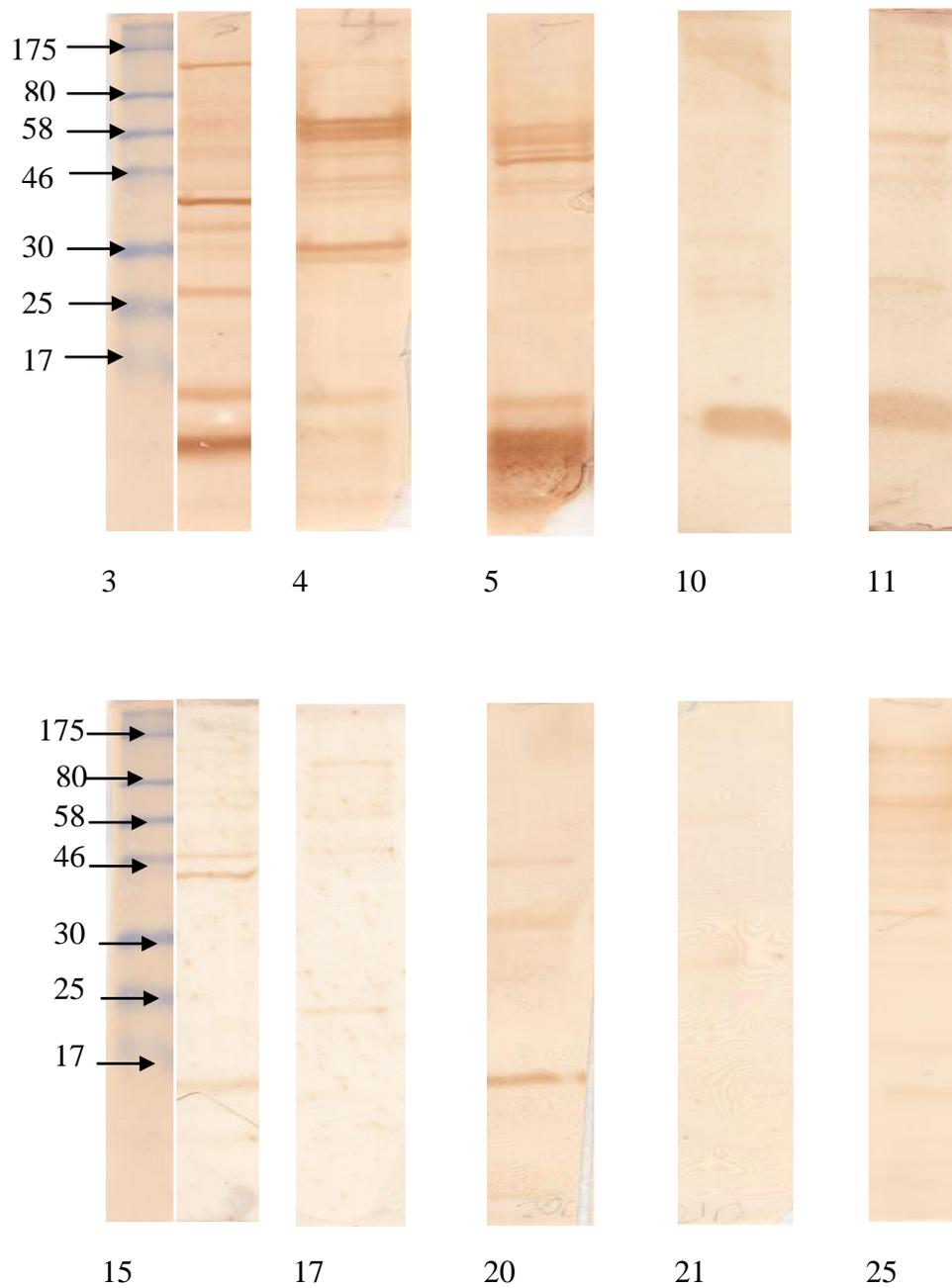


Figure 8.2 A Western blot analyses of control sera to the *B. oleronius* protein extract

The blots represented here are control samples that showed no or very weak reactivity to the stimulatory antigens. (Ocular Rosacea study)

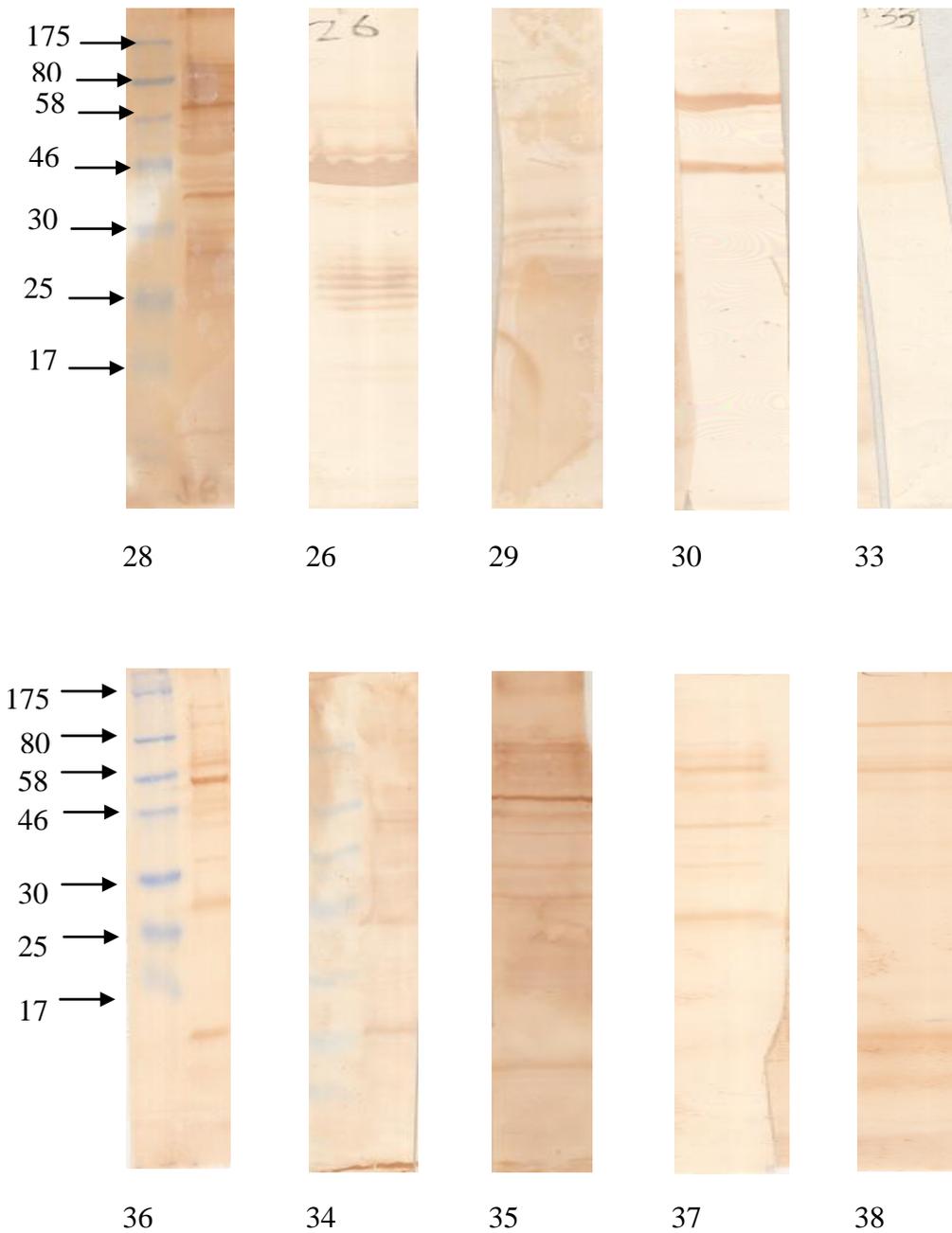


Figure 8.2 B Western blot analysis of control sera to the *B. oleronius* protein extract

The blots represented here are control samples that showed no or very weak reactivity to the stimulatory antigens. (Ocular Rosacea study)

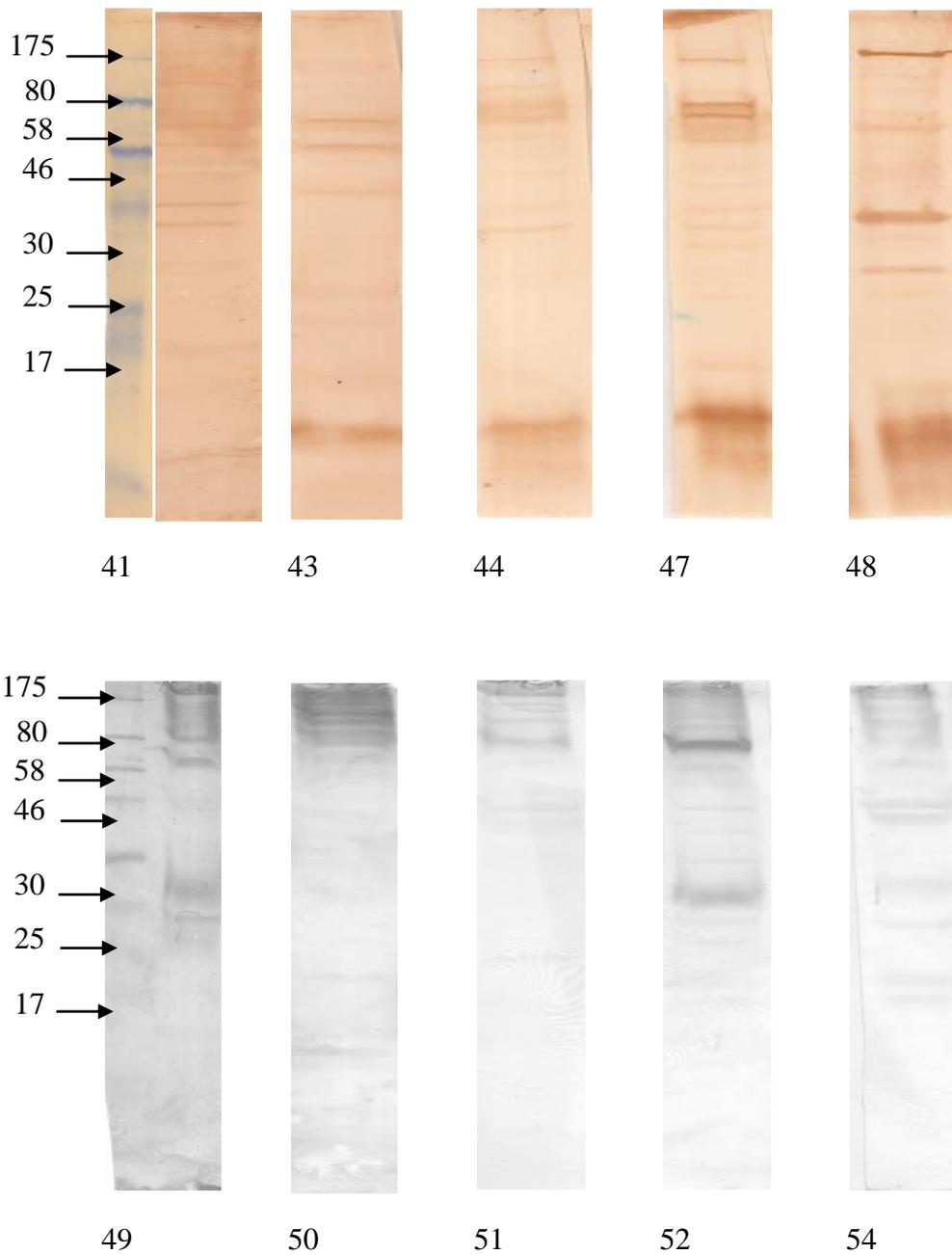


Figure 8.2 C Western blot analysis of control sera to the *B. oleronius* protein extract

The blots represented here are control samples that showed no or very weak reactivity to the stimulatory antigens. (Ocular Rosacea study)

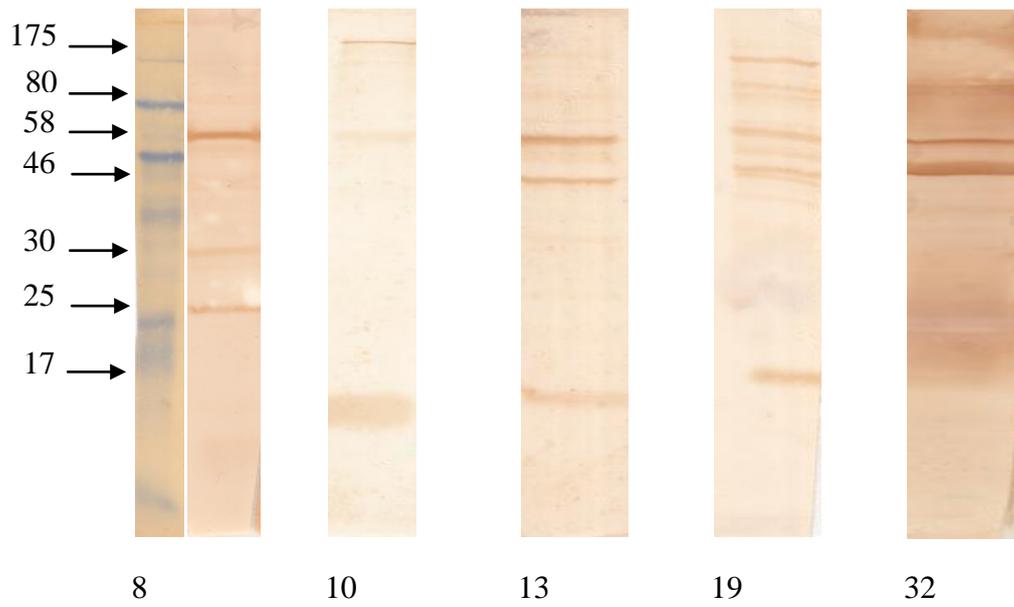


Figure 8.3 Western blot analysis of sera from rosacea patients to the *B. oleronius* protein extract

The blots represented here showed reactivity to one or both of the stimulatory antigens but where later revealed to be controls. (Ocular Rosacea study)

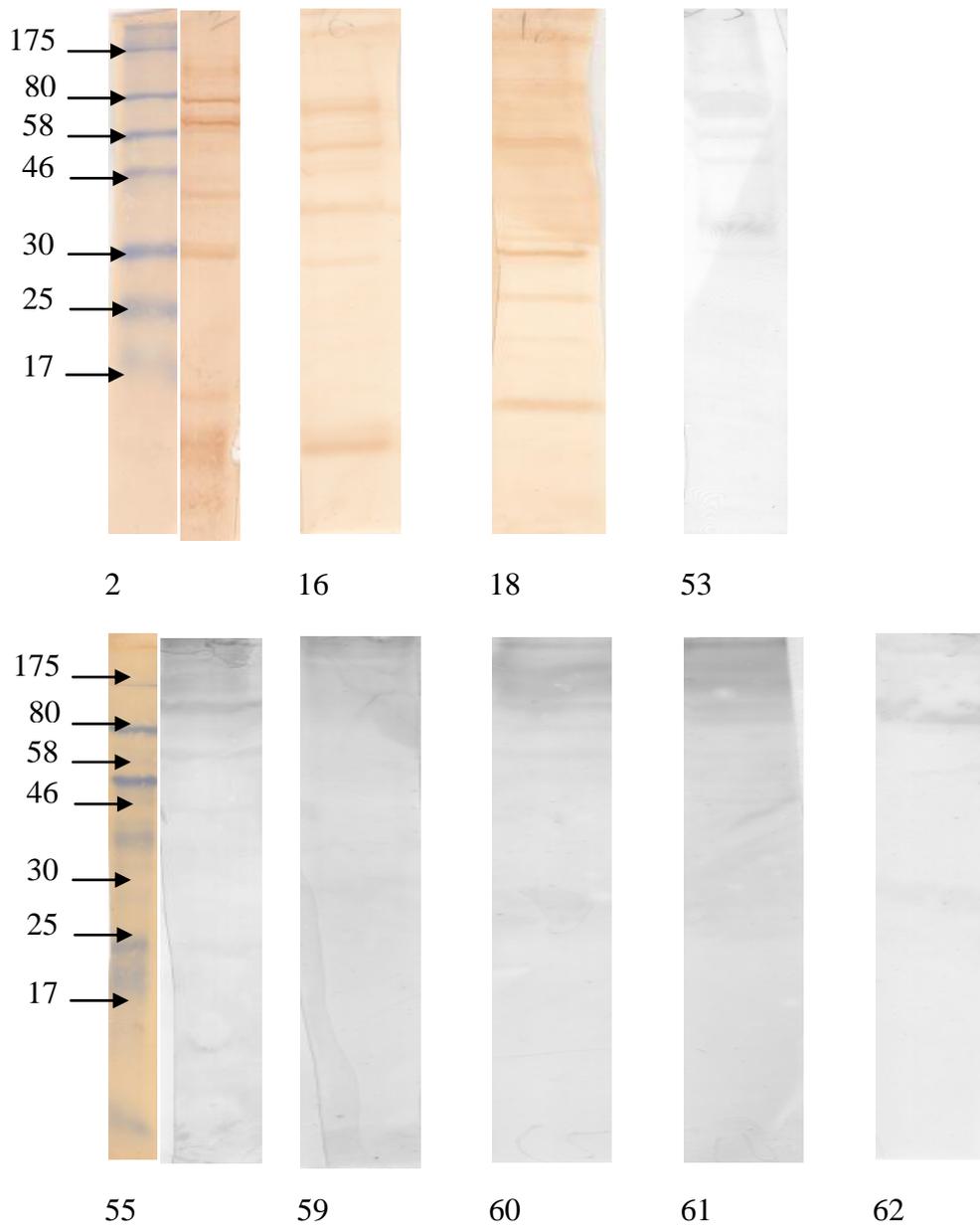


Figure 8.4 Western blot analysis of sera from rosacea patients to the *B. oleronius* protein extract

The blots represented here showed weak reactivity to one or both of the stimulatory antigens, they were labelled as controls and where later revealed to be positive for ocular rosacea. (Ocular Rosacea study)

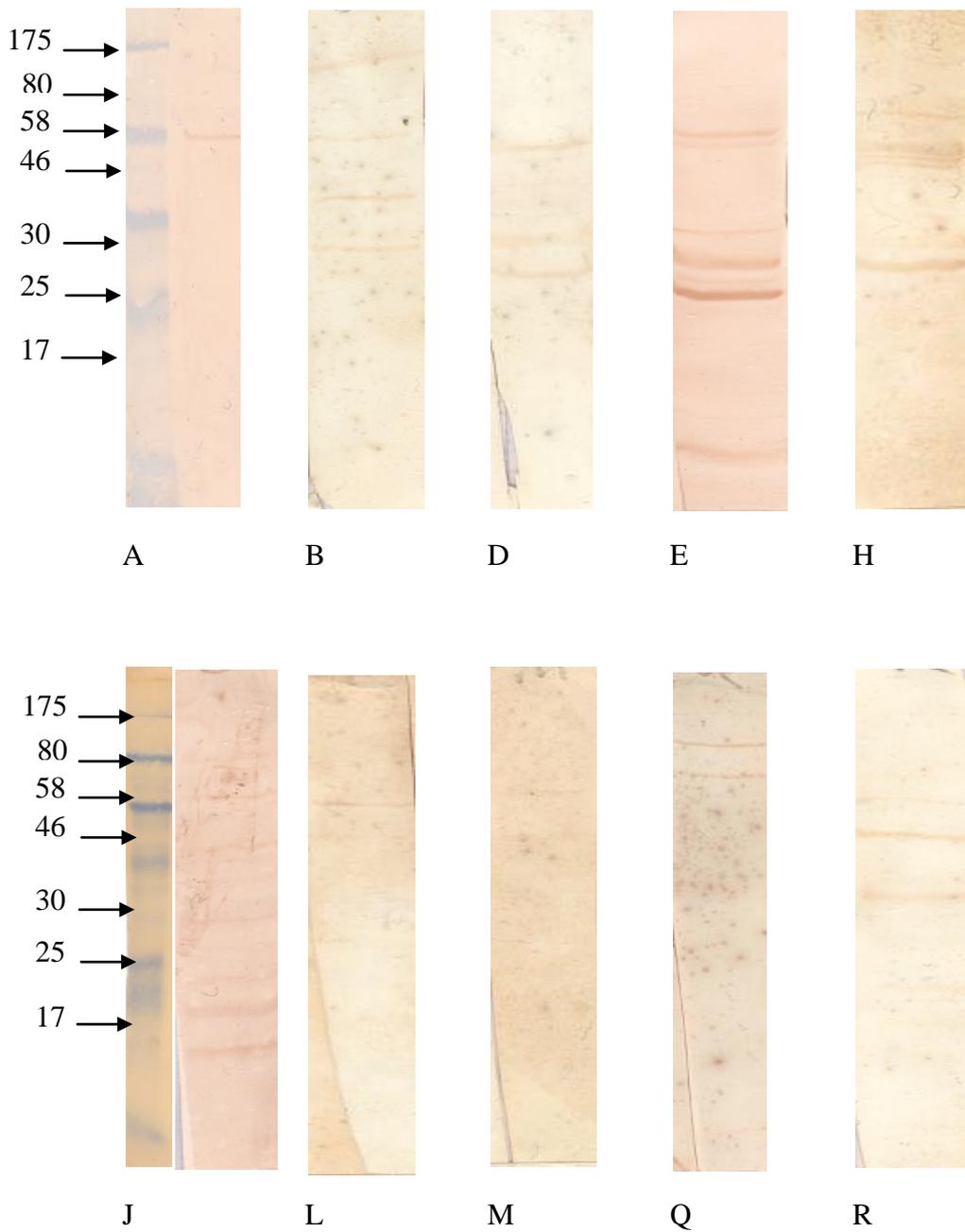


Figure 8.5 A Western blot analysis of sera from type one rosacea patients to the *B. oleronius* protein extract

These blots represent cases where the sera showed positive reaction to one or both of the stimulatory antigens and the person had been diagnosed with type one rosacea.

(Erythematotelangiectatic Rosacea study)

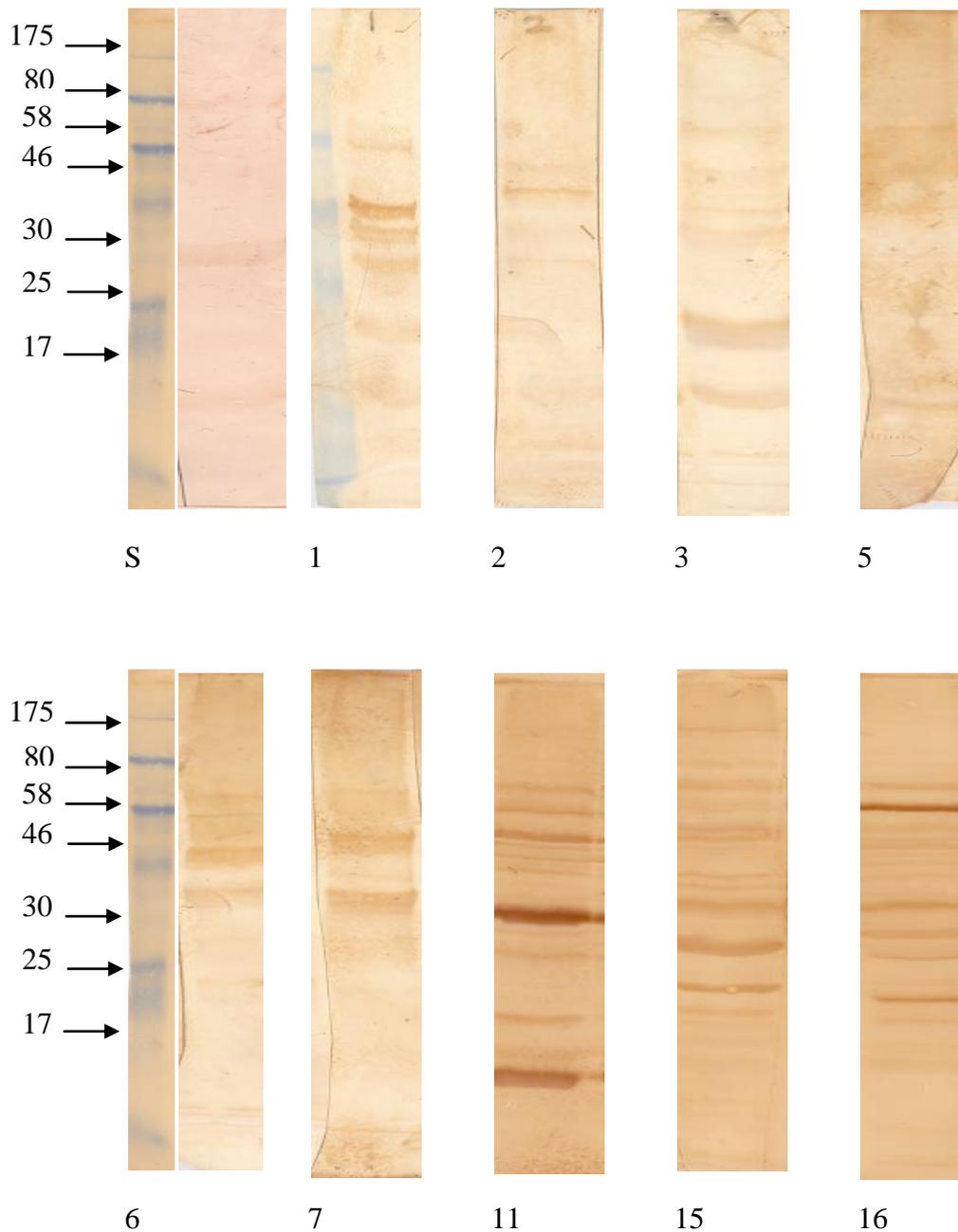


Figure 8.5 B Western blot analysis of sera from type one rosacea patients to the *B. oleronius* protein extract

These blots represent cases where the sera showed positive reaction to one or both of the stimulatory antigens and the person had been diagnosed with type one rosacea.

(Erythematotelangiectatic Rosacea study)

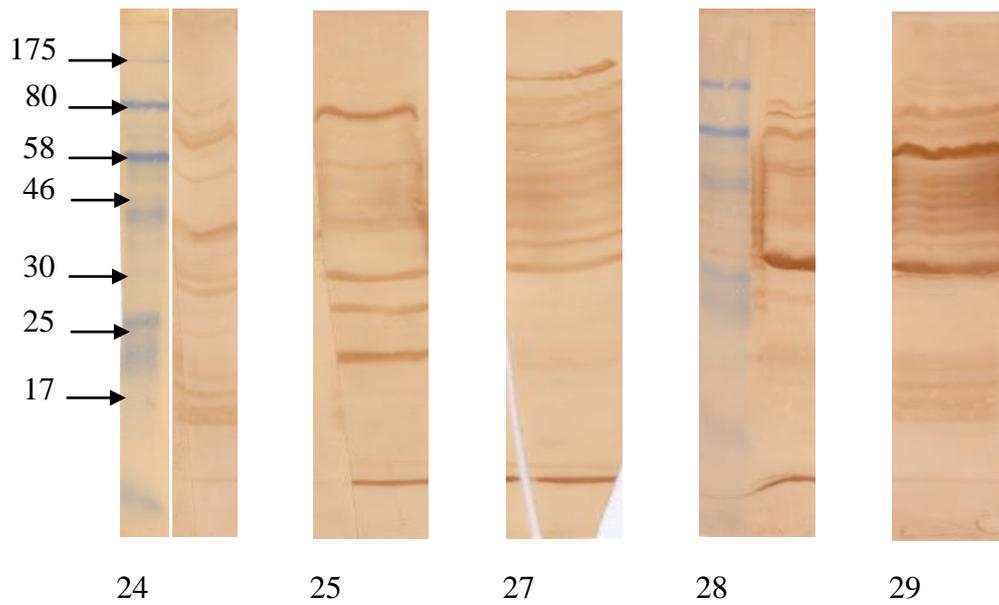


Figure 8.5 C Western blot analysis of sera from type one rosacea patients to the *B. oleronius* protein extract

These blots represent cases where the sera showed positive reaction to one or both of the stimulatory antigens and the person had been diagnosed with type one rosacea.

(Erythematotelangiectatic Rosacea study)

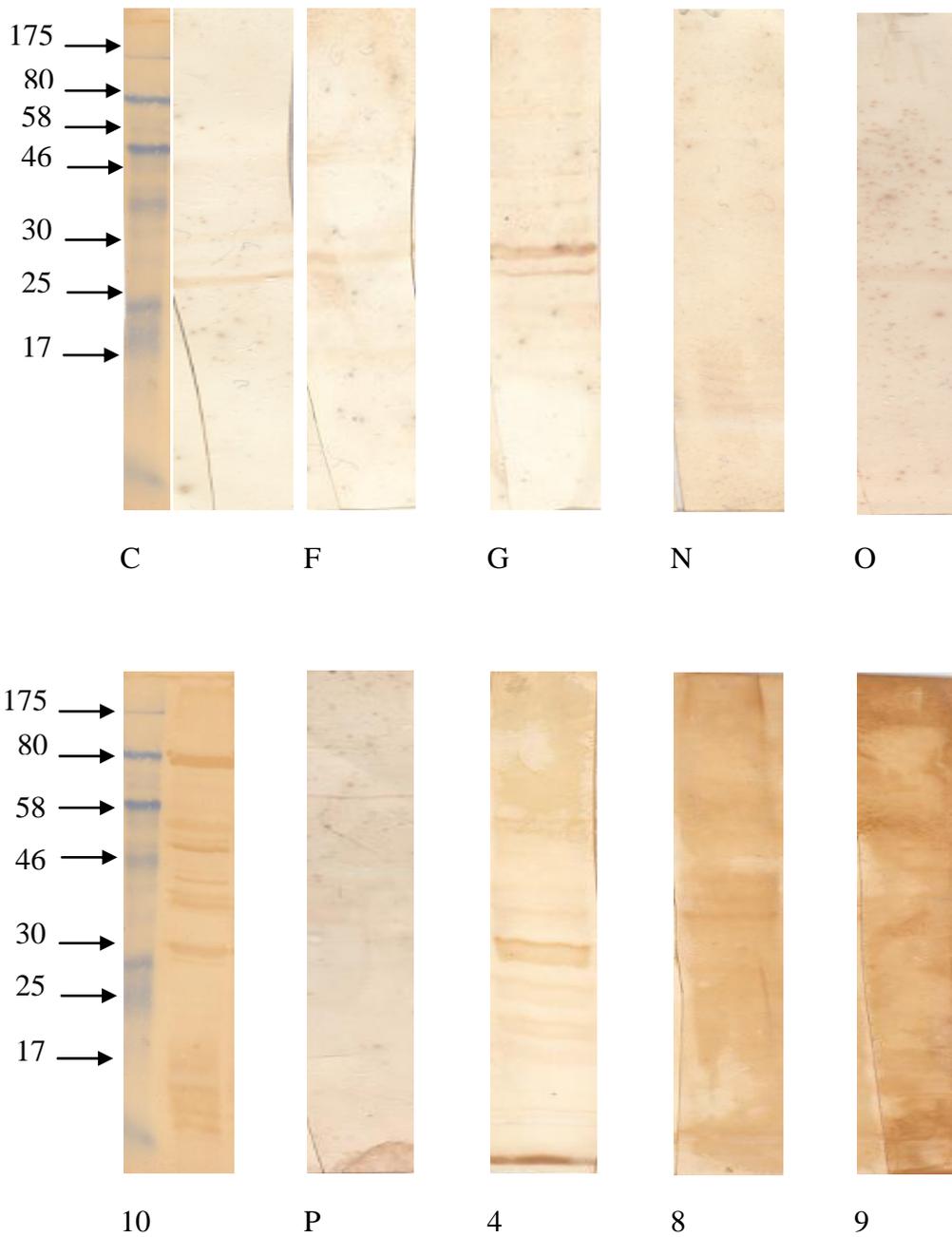


Figure 8.6 A Western blot analysis of control sera to the *B. oleronius* protein extract

The blots represented here are control samples that showed no or very weak reactivity to the stimulatory antigens. (Erythematotelangiectatic Rosacea study)

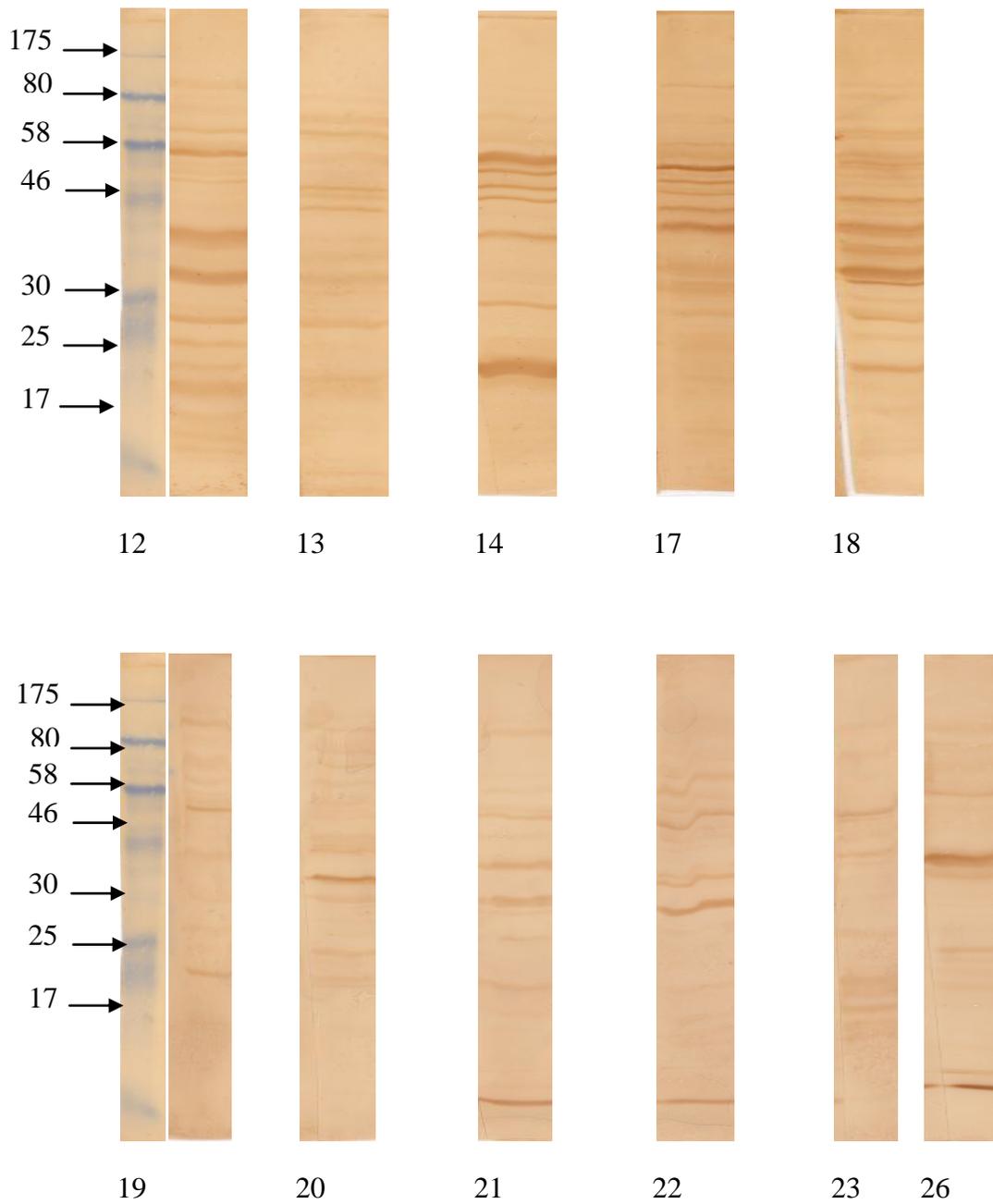


Figure 8.6 B Western blot analysis of control sera to the *B. oleronius* protein extract

The blots represented here are control samples that showed no or very weak reactivity to the stimulatory antigens. (Erythematotelangiectatic Rosacea study)

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