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Potential role of *pseudomonas aeruginosa* in sheep fleece rot and the associated serological response

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POTENTIAL ROLE OF PSEUDOMONAS AERUGINOSA IN SHEEP FLEECE ROT AND THE ASSOCIATED SEROLOGICAL RESPONSE

BY

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A Thesis submitted to the Faculty of Science, University of Wollongong,
in fulfilment for the degree of Doctor of Philosophy.

Department of Biology
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DECLARATION

I declare that this thesis is my own work except where it has been declared that I have had assistance or worked in conjunction with another person. It is being submitted for the degree of Doctor of Philosophy in the University of Wollongong. It has not been submitted for any degree or examination in any other university.

1997.
DEDICATION

To

Mum and Dad

This is for you
ACKNOWLEDGEMENTS

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Barry proof read my first draft; I hope he will recover from the stress quickly. Thank you for all the encouragement that you have given me.

I have not forgotten my babies ... Uyen, Minh, Lee and Kim.
ABSTRACT

Fleece rot is a bacterial infection of the fleece and skin predominantly caused by the bacterium *Pseudomonas aeruginosa* (*P. aeruginosa*). The bacterial infection can attract the blowfly, *Lucilia cuprina* (*L. cuprina*), which lays its eggs at the fleece rot site. The hatching larvae cause a problem known as fly strike or body strike, a debilitating disease which can cause sudden death in affected sheep.

Nine bacterial species have been isolated and identified in the fleece and on the skin of sheep. They remain unimportant until there is an environmental change such as rain and heat. Wetting by rain results in the proliferation of all these bacterial species in the first 24 hours but after this time *P. aeruginosa* predominates in the fleece. The inhibitory activity of *P. aeruginosa* on other bacteria was shown by co-culture of each bacterial species with a *P. aeruginosa* lawn. Pyocyanin, which is produced by *P. aeruginosa*, inhibited the growth of other fleece bacteria *in vitro*. Partial purification of pyocyanin from *P. aeruginosa* culture supernatants revealed that this inhibitory activity was most effective against gram positive bacteria such as *Bacillus cereus*, *Bacillus coagulans* and *Staphylococcus epidermidis*.

Apart from pyocyanin, *P. aeruginosa* produces many extracellular enzymes which may facilitate the invasion of fleece rot. Culture supernatant from the growth media of *P. aeruginosa* contained two proteolytic enzymes. Protease I had a molecular mass of approximately 50 kDa and contains subunits of 25 - 30 kDa. Protease II had a molecular mass of 150 kDa and contains a 50 kDa subunit. Protease I was similar to elastase and protease II appeared to be an alkaline proteinase as previously described in the literature. The production of these proteases from *P. aeruginosa* culture was not only affected by basic media.
ingredients but also by the amount of sheep wool added to the cultural media. Skin biopsies indicated that proteases could cause an acute inflammatory reaction, as assessed by the appearance of haemorrhages and neutrophil infiltration with a mild hyperaemia and oedema after only 2 hours. Skin thickness was found to increase within 4 hours of exposure to these proteases when compared with the controls. This pathological change occurred only when a mixture of both proteases was present but not with either protease alone.

Studies on the sheep serological response to surface membrane proteins of *P. aeruginosa* showed that the outer membrane proteins were more antigenic to sheep when they were subjected to intradermal injection. Evidence is also presented to suggest that the so-called genetically fleece rot "resistant" and "susceptible" sheep have a qualitative and quantitative difference in their serological responses.

Experiments were conducted to introduce *P. aeruginosa* infection with and without *L. cuprina* infestation on the skin of sheep. In all sheep, the serological specificity was higher against the outer membrane proteins purified from *P. aeruginosa* than the inner membrane proteins or the whole cell proteins regardless of the presence or absence of fly strike. These data demonstrated differences in sero-reactivity to bacterial and larval antigen and there was no correlation between antibody levels and intensity of fly strike.

The results in this research contribute further to the understanding of the potential role of *P. aeruginosa* in sheep fleece rot and could assist in the development of strategies and recommendations for the control of fleece rot which in turn will minimise the development of fly strike.
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Chapter 1
GENERAL LITERATURE REVIEW

1.1. Introduction

Fleece rot and fly strike together represent one of the major causes of production loss in the Australian sheep and wool industries. Fleece rot is a pathological condition which occurs after sheep have been exposed to sustained rain. When sheep have been wetted with water for at least five to six days, the physical barriers such as fleece and skin are no longer able to completely protect them. Problems emerge such as fibre weakness induced by *Pseudomonas aeruginosa* (*P. aeruginosa*) activity, disruption of the lipid layer which normally covers the skin surface, thickening of the epidermis, mild oedema of the dermis, serum exudation and discharge of pus onto the skin (Burrell et al., 1982). These problems, together with bacterial odour, attract and support fly strike which is caused by fly larvae of the blowfly, *Lucilia cuprina* (*L. cuprina*). These cause facultative wounds or myiasis which may be often followed by the death of the sheep if left untreated.

Although climatic conditions, especially temperature and moisture, and the level of bacterial activity have been suggested as predisposing factors causing fleece rot and fly strike, other important influences to be considered are the nature of the wax, fleece and skin barriers of the sheep, together with the strength of the host immune defence mechanism and the route of entry of the invader.

The question which needs answering is whether fleece rot and fly strike can be prevented. If fleece rot can be controlled then perhaps fly strike can also be prevented. Because a common feature of fleece rot is the growth of *P. aeruginosa* on the skin surface, an approach leading to the development of a *P. aeruginosa*...
vaccine could be useful in immune therapy.

1.2. Conditions Which Influence the Occurrence of Fleece Rot

Over the years, woolgrowers have referred to the colour change in sheep fleece after prolonged wetting as "water rot", "weather stain" and "dead yolk" (Atkins and McGuirk, 1979; Belschner, 1937b; Holdaway and Mulhearn, 1934; Seddon, 1923). The affected part of the fleece exhibits a very distinct colour such as green, purple, yellow, blue, red or brown. At first, it was thought that the stain was due to external contamination such as leaves or soil.

Fleece rot bands with green and brown wool discolouration caused by *P. aeruginosa*, courtesy Dr. H. Raadsma (University of Sydney).

It is known that susceptibility to fleece rot varies from flock to flock and also from sheep to sheep within the same flock. Mackerras and his co-workers (1944) showed that there were various factors influencing the susceptibility of sheep. The most important of these are:
1. Climatic conditions
2. Sheep characteristics
3. Fleece characteristics
4. Skin characteristics
5. Bacterial activity

1.2.1. Climatic Conditions

Weather plays an important part in predisposing towards fleece rot. Fleece rot is initiated by prolonged wetting of the fleece and skin, especially during hot weather. It occurs in the higher rainfall areas of all states and, in exceptionally wet years, may extend into normally dry areas. Belschner (1937b), who first described these conditions, concluded that heavy rain, over a period of four to six weeks, would lead to an outbreak of fleece rot. Hayman (1953) reported that, provided weather conditions were suitable, outbreaks may occur within a week. Field observations of rainfall and of fleece rot outbreaks over five years at Camden Park Station (near Sydney) showed twelve outbreaks recorded. Ten of these outbreaks coincided with months in which there were eight rainy days per month or rainfall of at least 100 mm (Hayman, 1953).

1.2.2. Sheep Characteristics

1.2.2.1. Moisture

Moisture in the fleece and on the skin of sheep is the most important factor that influences the development of fleece rot and fly strike. The problem emerges initially by the ease with which water can penetrate the fleece to the skin level and subsequently by the increase in the vascular permeability of the skin. When fleece is in a constant state of dampness, accompanied by a warm humid atmosphere, fleece bacteria are encouraged to multiply rapidly (Fraser and
Work on the fragility of sheep skin by Nay and Watts (1977) showed the histological changes associated with the development of fleece rot, such as inflammation, skin thickening with hyperkeratosis and acanthosis. This causes leucocyte infiltration of the epidermis after six hours of wetting, followed by serum exudation onto the skin surface after forty eight hours with mild oedema of the dermis (Hollis et al., 1982).

A study by Burrell et al. (1982) produced evidence that the loss of seropurulent fluid through the epidermis could be due to microabscesses forming in the stratum corneum as a result of the inflammatory response; in some cases, discharge of pus and blood onto the skin surface can be seen. Eggs and newly hatched fly larvae are unable to survive if the skin is dry (Mackerras and Mackerras, 1944).

1.2.2.2. Age of Sheep

Young sheep, regardless of sex, are more susceptible to fleece rot than older sheep. Severe fleece rot and fly strike is normally found in sheep from six to twelve months old. This is probably due to the immaturity of the skin (Belschner, 1937b).

1.2.2.3. Conformation of Sheep

The wither is that part of the back between the second and sixth thoracic vertebrae and extends laterally over the upper margin of the scapulae. There are three types of withers, namely high shoulder blades, broad wither, and "pinch" withers which are narrow over the fourth to sixth ribs. All three are commonly subject to fleece rot and fly strike attacks because the constant movement of the
high shoulder blades has the effect of opening up the fleece on the wither and thus allowing the rain to enter. With broad withers, there is frequently a distinct depression between the tops of the shoulder blades from which water does not drain away readily. With narrow blades, the animal is "pinched" behind the withers, exhibiting an "devil grip" where the moisture is retained (Belschner, 1937b). However, in his survey of over 500 sheep, Belschner mentioned that sheep with wrinkly breeches are not affected with fleece rot.

1.2.2.4. Fleece Characteristics

There have been many investigations on fleece characteristics which have shown that the relative susceptibility of individual sheep of the same age is determined chiefly by:

1. Wax and suint
2. Wool colour, characters, handle
3. Staple arrangement and density

1.2.2.4.1. Wax and suint

Natural fleece consists of three main components:

1. The wax which is a lipid film produced from both the primary and secondary
sebaceous glands.

2. The suint which is a water soluble protein produced from the sweat glands of primary follicles.

3. The wool fibre produced from the primary and secondary follicles.

The relative amounts of each component in the fleece is the result of a complex interaction between the genetic make up of the sheep, its physiological state and the environment in which it is living. Increased fibre density is naturally associated with a higher sebaceous gland density. Increased fibre density is also related to reduced fibre diameter (Salisbury and Barrowman, 1984).

Each fraction of wax and suint is chemically complicated but, as crude extracts, they are useful for measuring the activity of skin glands. Animals in good condition will produce more wax and suint than animals in poor condition, that is, within the limits set by the number and size of the skin gland population. Changes in temperature produce opposite effects on the products of the two types of glands. The sweat gland output rises directly as the heat rises, conversely the sebaceous gland output or the wax fraction tends to increase as the temperature falls.

There has been a controversy about the wax and suint content relationship with resistance or susceptibility to fleece rot and fly strike. Thornberry et al. (1980) and Raadsma (1987a) showed that there was no strong correlation found between occurrence of fleece rot and wax or suint production.
1.2.2.4.2. Wool

Wool colour appears to be an important part in exhibiting fleece rot. The suint pigments are responsible for the colour of "dead yolk" or "golden colour" in fleece (Belschner, 1937b; Paynter, 1961). Greasy wool has been shown consistently to be associated with the susceptibility of sheep to fleece rot (Atkins and McGuirk, 1979; Belschner, 1937b; Holdaway and Mulhearn, 1934; Paynter, 1961; Raadsma, 1987b).

Wool handle is the term for the softness of the wool to touch. A wool with softer handling generally represents the more resistant fleece. Animals showing fleece rot resistance in general possess a distinctive quality of breed and style. It was observed that wool with higher grade, better character, more style and better handling were the qualities pointing to resistance (Belschner, 1937b).

1.2.2.4.3. Staple Arrangement and Density

Belschner (1937b) first used the term density to describe the compactness or
staple arrangement of the fleece over the sheep withers. He described dense fleece as either bulky or slack. Lipson (1978) later regarded the bulky property as being a protective factor against fleece rot. On the other hand, open or slack wool was found to be more commonly affected by fleece rot because these fleeces show a tendency for water to penetrate, allowing direct wetting of the skin (Lipson, 1978). Fraser (1957) also found that a dense fleece which was square or blocky at the tip had a lower moisture content at the skin surface than did open fleece having staples with a pointed tip. This is what Belschner (1937b) described as slack wool and Mackerras (1936) described as fuzzy wool. Coy (1983) demonstrated these features thus:

![Diagram of pointed and blocky staple tips]

A fleece with little or no staple formation has a fairly even distribution of follicles over the whole skin area with a high density, i.e., ninety follicles per square millimetre. A fleece with a thick, well defined staple also has an even distribution of follicles but with relatively lower density (Coy, 1983; Raadsma, 1987a). Pointed tips form an irregular fleece structure, whereas blocked tips give a regular arrangement (Coy, 1983). However, these staple characteristics had no strong correlation with fleece rot (Raadsma, personal communication).
1.2.2.5. Skin Characteristics

The skin surface forms the anatomic boundary between the animal and its external environment. The most important function of skin is to act as a barrier that protects the animal from external agents. The two principal layers are the epidermis and the dermis. The epidermis is composed of the non-living-epidermis or the stratum corneum layer, and the living-epidermis layer.

The epidermal cells are mainly keratinocytes which are produced in the basal layer of the epidermis. Cells differentiate as they migrate outward and gradually die to form flattened squames of inert keratin which together with sweat and lipid comprise the stratum corneum, an outer physical barrier. The living epidermis with inward intercellular movement forms tight junctions. Integrated between the keratinocytes of the epidermis are pigment cells, Langerhans cells, and neurosecretory cells. The living epidermis is an important barrier to bacteria, and also plays an important role in maintaining resistance to fleece rot.

The dermis is composed of collagen, elastin fibres, blood vessels, lymphatics, nerves and dermal cells. The dermis is constructed to resist tearing and prevent bruising. It has homeostatic functions.
Hollis et al. (1982) observed that in wet sheep, an inflammatory response occurred in the upper layers of the dermis causing the release of polymorphonuclear leucocytes during the first few days of wetting. After that mononuclear cells predominate resulting in skin thickening.

1.2.3. Bacterial Activity

Stuart (1894) and Seddon and McGrath (1929) were the first to show that the colour change in fleece was associated with bacteria. Where a distinct colour is present some particular pigment-producing microorganism generally predominates. These include *P. aeruginosa* - responsible for green, purple, brown, yellow or red stain (Belschner, 1937b; Fraser and Mulcock, 1956); *Pseudomonas indigofera* - which may be responsible for blue stain; *Bacillus subtilis* and *Bacillus vulgatus* - pink stain (cited by Mulcock, 1965); *Chromobacteria violaceum* - violet stain; and *Serratia marcescens* - red stain (Seddon, 1937).

Sheep which have fleece rot are commonly identified by a blue green colouration. This is the result of an extracellular pigment, pyocyanin, which is produced by *P. aeruginosa*. Wool discolouration is not the only symptom to appear when bacteria are present in large numbers. In some outbreaks there is gelatinous material near the skin surface that leads to matting of the wool. This sometimes takes the form of many lumpy masses in the fleece which may be confused with dermatophilosis or lumpy wool in its early stages of development. The difference between dermatophilosis and fleece rot is that mycotic dermatitis multiplies mainly in the wool follicles, while the bacteria responsible for fleece rot are found mainly on the surface of the skin (Belschner, 1968).

A field survey of 3110 sheep for fleece rot conducted by Burrell and MacDiarmid cited by Burrell (1988), revealed that of the 40% of sheep which developed fleece
rot, 23% had a green colour while the other 77% were yellow to brown. Bacteriology studies showed 38% yielded \textit{P. aeruginosa} and 28% yielded \textit{P. maltophila}. \textit{P. putida} and \textit{P. stutzeri} were sometimes found together with \textit{P. aeruginosa}. The conclusion could be made that \textit{P. aeruginosa} was the best model for studying fleece rot.

1.2.3.1. Biology of \textit{Pseudomonas aeruginosa}

\textit{P. aeruginosa} was first named as \textit{Bacterium aeruginosa} or \textit{Bacillus pyocyaneus} or \textit{Pseudomonas polycolour}. It is a Gram negative rod ranging in size from 0.5 to 0.8 micron by 1.5 to 3.0 micron. Most have many fine pili with a single polar flagellum. \textit{P. aeruginosa} multiplies rapidly in simple media, usually producing characteristic colonies, pigment and odour. Colonies vary from mucoid and smooth to wrinkled and rough in form, and from a yellowish to greenish colour in culture media (Gilardi, 1991). To encourage the synthesis of its yellow fluorescent pigment Garibaldi (1967) reported that trypticase soy agar with the addition of egg white would give bright yellow colonies. Commercial \textit{Pseudomonas} agar P made by "Difco laboratory" has been available for this purpose.
In routine bacteriology, the properties of *P. aeruginosa* used to confirm identification are:

1. Motile organism
2. Production of pyocyanin and fluorescein
3. Growth at 42 °C
4. Utilisation of glucose, mannitol and xylose
5. Reduction of nitrate to gaseous nitrogen
6. Gelatine and casein hydrolysis
7. Positive for arginine hydrolase, urease

1.2.3.2. Pathogenic features

*P. aeruginosa* is readily found in most moist environments, such as soil, plants, water, in lower and warm blooded animals and the body fluid/tissues in humans. It is also a common pathogen which can cause death in infants with epidemic diarrhoea and is commonly found in patients with burns (MacMillan, 1971) or lung infection (Hoiby, 1982). It is well known that *P. aeruginosa* is a hospital opportunistic pathogen. This organism is of interest in the clinical area as a result of its resistance to many antibiotic therapies.

Although exposure to this organism is unavoidable, human beings exist well with it until various factors that might be categorised as predisposing allow *P. aeruginosa* to cause infection. Predisposing factors in humans include: a) impaired host defence, b) chronic illness, c) damage skin integrity.

Impaired host defence could be mechanical, biological or hereditary. Mechanical impairment occurs in burn patients where damaged skin allows bacteria to colonise the burn sites. Biological impairment and chronic illness such as in immuno-compromised patients, or patients with illnesses such as pneumonia, hypo
or hyperglycaemia and ulcerative diseases provide ideal conditions for *P. aeruginosa* infection thereby increasing morbidity and mortality. Cystic fibrosis is a generalised hereditary disorder which affects a variety of organs such as the lung, intestine and liver. *P. aeruginosa* has been recognised to cause irreversible lung damage in these patients.

The pathological features of *P. aeruginosa* infection together with fleece rot were presented by Nay and Watts (1977), and Burrell *et al.* (1982). According to these researchers the organism can live on fibre protein and causes narrowed stretches of wool fibre. The wool fibre easily breaks at these weakened stretches causing them to withdraw into the region of the wool follicle canals forming "plugs". These plugs move towards the epidermis layer and eventually the wool follicles collapse. The organism may further penetrate the wool follicles producing inflammation with the development of ulcerative lesions. Skin lesions lead to serum exudation that flows over the skin surface. This wetting further enhances *P. aeruginosa* invasion. Increases in the thickness of the epidermis and the dermis will develop. Histopathological changes include mild hyperaemia, oedema and polymorphonuclear cell infiltration of the dermis. There is an increase in the number of mast cells, basophils and mononuclear cells in the epidermis and dermis layers (Burrell *et al.*, 1982). However no evidence has been obtained of invasion of the epidermis and dermis by the bacterium itself. Burrell (1985) suggested that *P. aeruginosa* may release extracellular products onto the sheep skin, causing tissue damage.

### 1.2.3.3. Extracellular factors

#### 1.2.3.3.1. Pyocyanin

*P. aeruginosa* produces a variety of toxins and pigments, some of which may
contribute to the pathogenic properties of the bacteria. *P. aeruginosa* is the only *Pseudomonas* species producing the blue-green pigment, pyocyanin, which is soluble in chloroform as well as water. Pyocyanin is blue in alkaline or neutral media and red in acid media. Pyocyanin has been shown to have some toxic effects on tissue cultures of human epithelial cells (Cruickshank and Lowbury, 1953) and fibroblasts (Schoental, 1941). It can kill mice which have been given a low dose (Stewart-Tull and Amstrong, 1972).

Other extracellular products produced by *P. aeruginosa* include surface slime, exotoxin, endotoxin, proteases, phospholipase and enterotoxin. Some of these are reported to be toxic.

### 1.2.3.3.2. Slime

The slime or alginate of *P. aeruginosa* is also known as capsular exolipopolysaccharide and was originally recognised by Liu (1974). Later it was characterised by Pier et al. (1978). It is reported that it mediates adherence of the mucoid strain and facilitates the attachment of *P. aeruginosa* to tissue surfaces, for example the pulmonary epithelial lining (Costerton et al., 1979). Some strains of *P. aeruginosa* are capable of producing a very thick layer of alginate so that bacterial aggregates resist both phagocytosis by macrophages and clearance by the mucociliary mechanism (Costerton et al., 1979). The role of alginate in sheep fleece rot is still unclear.

### 1.2.3.3.3. Endotoxin

*P. aeruginosa* produces lipopolysaccharide (LPS) or endotoxin. Although it was reported to be not very toxic (Liu, 1974), it has been used to produce anti-LPS antibodies, and a LPS vaccine was shown to be protective in mice and humans (Cryz et al., 1983; Pollack and Young, 1979). There is still a question whether
antibodies produced by a LPS vaccine can protect all sheep with *P. aeruginosa* infection. It is not known whether LPS plays a role in the pathogenesis of fleece rot in sheep.

1.2.3.3.4. Exotoxin A and Exotoxin S

Exotoxin A is a heat labile enzyme originally isolated by Liu *et al.* (1973) from *P. aeruginosa*. Exotoxin A was described as very toxic to mice (Liu *et al.*, 1973), rabbits (Liu, 1974) and dogs (Atik *et al.*, 1968). The effects of exotoxin A in humans were shown by Cross *et al.* (1980) to be as toxic as in animals.

Exotoxin S is more heat stable than exotoxin A. It is also toxic to mice and rats (Woods and Que, 1987; Woods *et al.*, 1988). Both exotoxin A and S are commonly found in human clinical isolates (Woods *et al.*, 1986). Their virulence is reduced when exotoxin deleted mutant strains of *P. aeruginosa* were tested in animal infection models (Nicas and Iglewski, 1985, Woods and Sokol, 1985). There is no evidence so far on whether either exotoxin participates in fleece rot.

1.2.3.3.5. Phospholipase C

Phospholipase C was first discovered by Esselman and Liu (cited by Liu 1979). It was suggested that phospholipase C could only be produced in an environment that could supply a free glycolipid source, such as blood or skin (Liu, 1979). The role of phospholipase C in the pathogenesis of *P. aeruginosa* in animals and humans has been studied (Berk, *et al.* 1987; Berka *et al.*, 1981). Mutant strains of *P. aeruginosa* devoid of the enzyme showed less virulence in mice than the wild type strains (Ostroff, 1989). In 1988, Chin and Watts obtained purified phospholipase C from *P. aeruginosa*. This enzyme is considered to be an important factor in the pathogenesis of *P. aeruginosa* in fleece rot as it was found that lesions caused by phospholipase C are similar to those found in fleece rot.
1.2.3.3.6. Proteases

At least two proteolytic enzymes are produced by *P. aeruginosa*; elastase and alkaline proteinase. Elastase is one of the proteases that can destroy blood vessels (Mull and Callahan, 1965) and damage the wall of small arteries (Diener, et al., 1973). Extracellular alkaline proteases serve as virulence factors in the mouse burnt skin extract of Cicmanec and Holder (1979). According to Holder and Haidaris (1979), the production of extracellular protease and elastase appears necessary for the full virulence of *P. aeruginosa*. It was also found that mutant strains incapable of elaborating extracellular proteases had reduced mortality in the burned mouse model which partial burns to the backs of mice and the subcutaneous inoculation of *P. aeruginosa* at the burn sites (Holder and Haidaris, 1979). Woods et al. (1982) obtained similar results when they used an extracellular protease mutant strain for lung infection in rats.

Hirakata et al. (1995) demonstrated that the production of exoenzyme S and alkaline proteinase contributed to the pathogenicity of bacterial colonisation.

Hay and co-workers (1982) reported a loss of wax and decreased hydrophobic properties of the remaining wax after the development of fleece rot in sheep. It is possible that lipases from *P. aeruginosa* could be involved in wool wax degradation.

1.3. Body strike

Body strike was noticed in Australia at least a century ago, but did not become prevalent until the early 1930s when it emerged as a serious problem for the sheep industry. Its scientific name is cutaneous myiasis which is a condition
produced by the growth of fly larvae which infest the skin of living sheep (Tillyard, 1933). It can involve any part of the body, crutch or tail region (breech strike), head region (poll strike) and around the preputial region (pizzle strike). Most importantly, the shoulder and back regions (fly strike) have been shown to be the most commonly affected sites (Belschner, 1937a, b; Seddon, 1937).

1.3.1. Breech Strike

This includes those cases in which the sheep are struck on the crutch and tail areas. The predisposing conditions for breech strike result from liquid faeces (scouring) and urine saturating the wool. Other factors which influence the incidence of breech strike are:

1. Sex. Ewes are more susceptible than wethers or rams.
2. Age. Young sheep are more affected.
3. Season. Autumn and spring when diet changes.
4. Conformation of the crutch area. Folds or wrinkles of skin aggravate the strike.
5. Tail length. Butted tail sheep are more susceptible than medium or long tail sheep (Belschner, 1937a; Monzu, 1986b)
1.3.2. Poll Strike

Poll strike or head strike is mainly found in rams. It sometimes follows wounds sustained in fighting, with the strike usually occurring on the wool and the skin at the base of the horns (Belschner, 1937a). Raadsma (1987a) reported that head strike was the most common type of strike (55% of all strikes) in ram flocks and up to 10% of all rams can expect to be struck on the head.

1.3.3. Pizzle Strike

This is likely to occur in wethers and young rams, and follows urine soiling and bacterial decomposition in the wool around the prepuce, or the presence of balanitis or “pizzle rot” (Belschner, 1937a).

1.3.4. Fly Strike

Fly strike is sometimes called body strike, and occurs around the back and shoulder regions. More sheep are struck in Australia by L. cuprina than by any other flies. It has been reported that this fly is especially attracted to sheep which have already developed fleece rot and to sheep whose fleece remains persistently wet, either by conditions of high rainfall and/or the presence of skin exudation (Belschner, 1937a; Seddon and Belschner, 1937; Mackerras and Mackerras, 1944).

Under conditions of continued infection with P. aeruginosa, the odour which is produced attracts female flies to lay their eggs at the lesion sites. It is possible that once the larvae are established, they are able, by means of proteolytic enzymes, to invade further into the already injured skin and find an abundant food supply beneath (Bull, 1931). This subjects the affected sheep to severe stress (Shutt et al., 1988) and sometimes leads to sudden death, although the reasons for this are not well understood.
Shoulder

Head<> Tail

Sheep showing skin lesion and wool loss associated with *L. cuprina*.
Courtesy Dr. H. Raadsma, University of Sydney.

1.4. The Fly

1.4.1. Fly Population

Blowflies are present all year round in all agricultural areas of Australia. Their number is extremely low in the very hot summer months. Further, in wet, cold winters, flies often disappear for two or three months (Monzu, 1986a). The distribution of *L. cuprina* is extremely patchy. The greatest concentrations are associated with sheep at the breeding sites.

Field studies have revealed that where there is a high population of gravid females there is a relatively higher number of sheep being struck. Normally the percentage of gravid females ready to oviposit is low, usually about five-
per cent of the total population (Mackerras and Mackerras, 1944).

There are at least five fly species which have been identified in fly strikes around Australia. The primary or first strike species are *L. cuprina*, *Calliphora albifrontalis* and *Calliphora nociva*. However, it is believed that *L. cuprina* is responsible for ninety per cent of fly strikes. Secondary strike species, which require the presence of a primary species before they can strike sheep, are *Chrysomia rufifacies* and *Sarcophagid* species. The tertiary strike species is *Australophyra rostrata* which is a muscid that cannot initiate a strike without action by the primary and secondary species (Monzu, 1986a).

Flies attracted by the odours of rotting wool lay their eggs at the infected site. Moisture and the presence of proteins contribute to the attractiveness of fleece rot lesions as egg laying sites. Female flies require protein sources to develop their ovaries and produce eggs. They can obtain this from wounds or existing strikes on sheep. Each female can lay up to eight hundred eggs at a time. Under natural conditions, females rarely lay more than two egg batches during their lives. The hatching time of the eggs depends upon temperature; 35 hours at 30°C but only 8 hours at 37°C. The emerging larvae then pass through a series of developmental stages called instars. Larvae remain on the sheep from three to seven days, by which time they enter the prepupal stage; that is when they stop feeding and migrate down the flanks of the sheep, eventually dropping off onto the soil during the night. Pupae need only 6 days at 30°C to reach the fly stage, but with cooler temperatures, they take longer to develop (30 days at 15°C).

Temperature and wind are the two most important factors influencing fly activity. If the flies are not active enough, they cannot disperse, mate and lay eggs. The optimum temperature for flies to be active ranges from 25°C to 35°C (Mackerras
Wind also affects fly activity. The wind helps flies to migrate to suitable sites to lay their eggs. Optimum wind strength ranges from nine to thirty kilometres per hour (Mangano, 1986; Monzu, 1986a).

Life cycle of *L. cuprina*, clock face shows time in days [Agnote, Dec1990]
1.4.2. The Larvae

*L. cuprina* eggs develop successfully into larvae on sheep if there is a continual supply of moisture at the infected site. Their general growth pattern develops along three stages of instars. The first instar larvae have poorly developed mouth parts. They feed on serum protein exuded at infected fleece rot sites. The second instar larvae have sufficiently developed mouths to scratch the skin and feed on wounds and skin tissues, thus starting the strike. The strike will expand as the development of the third instar occurs. These instars remain on the sheep for only three to seven days before leaving for the pupae stage, but the damage they cause, such as disrupted skin or more importantly myiasis, is the most traumatic loss caused by fly strike.

Studies on the pathogenicity of the larvae have not demonstrated any potential factors which could directly cause wound formation in sheep. Firstly, proteases produced by larvae of *L. cuprina* have been studied over the years, though without conclusive proof of their involvement. Secondly, immunisation against the excretory/secretory components from the gut of first instars is yet to be revealed (Sandeman, 1990).

1.5. Economic Importance

Fleece rot, by itself, is of only minor economic importance but in conjunction with fly strike it assumes major economic importance. Fleece rot, and the bacterial stain produced, reduces wool production and quality and therefore reduces the value of the fleece. The bacterial stain usually does not scour out and has been classified as one of the main faults which wool buyers look for in wool from high rainfall areas (Jefferies, 1961).

However, active fleece rot lesions lead to fly strike which results in the greatest
losses to the Australian sheep industry. If uncontrolled, fleece rot and fly strike can lead to loss of wool quality and quantity, reduce live weight gain, cause loss of fertility and the sudden death of sheep.

In 1985, Beck et al. of the Broadacre Economics Research Station estimated that the sheep fly strike cost Australia 250 million dollars a year. Included in this estimation is the cost of control measures such as management techniques, chemical prevention and control treatments, the value of labour, materials, repairs and capital depreciation as well as production losses.

1.6. Management Factors

Several management factors are important in determining whether sheep develop fleece rot and fly strike. All of these factors involve altering the structure or conformation of the fleece and skin. They include stress, nutrition and mechanical damage.

1.6.1. Stress

Stress may be due to sudden changes of weather or droving and dogging of very hot or wet sheep, and may aggravate the development of fleece rot (Murray, 1979).

1.6.2. Nutrition

The fleece of underfed young sheep tend to open up more along the backline, allowing water and dust to enter and penetrate into the fleece. On the other hand, fat sheep develop a groove along the backline which has a tendency of increasing their susceptibility to fleece rot (Murray, 1979).

1.6.3. Mechanical Damage
The staple tip acts as a physical barrier against water penetration. Disruption of the staple tips can result from opening of the fleece for inspection, branding or even trampling by dogs' feet during yarding. Damage of this type leads to a susceptible condition.

Much also has been learned about shearing methods and shearing times. In 1953, Hayman suggested that shearing may be used as a management factor to prevent fleece rot and fly strike. In practice, shearing the fleece from a sheep usually removes the fleece rot bands and scabs of dermatophilosis and therefore removes these pre-disposing conditions. Leaving scabs intact on the skin, or skin damage caused by shearing can increase the risk of fleece rot spread (Mangano, 1986).

1.7. Control

1.7.1. Prevention

Control programs which have been used to combat fly strike are:

1. Selection of lambs against fleece rot and fly strike.
2. Timing of annual shearing.
3. Management of pastures to reduced scouring and long grass problems.

Sheep with short wool dry out rapidly and so may escape outbreaks of disease. It was therefore suggested by Hayman (1953) that different shearing times should be used for different climatic areas. For instance, in heavy rainfall areas (e.g. Tasmania), shearing in early spring may decrease the risk, while in other areas where late summer and autumn temperatures and rainfall are high (east and west coasts), shearing in early summer can minimise the risks of fleece rot.
If wool growers carry out the most appropriate practices then sheep are considered to have the ability to survive under the pressures applied by fleece rot and fly waves within that environment (Perry, 1979). However if vaccination against the causal organisms was feasible an additional control method would be available. Other means of chemical and biological control of bacteria and flies would also be advantageous control mechanisms.

1.7.2. Selection of Fleece rot Resistant Sheep

Wool growers maximise their returns from wool by optimising fleece characteristics such as fineness, colour, length etc. However, research has extended this list to include a relatively larger number of factors that may influence the resistance of animals; these are known as breeding indicators (Raadsma, 1987a). There are two methods available for reducing the incidence of fleece rot, and therefore reducing fly strike in flocks.

1. Direct selection is based on visual estimation and culling of struck sheep after fleece rot and fly strike challenge.

2. Indirect selection is based on the selection of genetically stable and dominant characteristics of the wool, such as colour, diameter and staple arrangement.

1.7.2.1. Direct Selection

This is a more practical procedure which has been performed by selected sheep breeders through the exclusion or culling of sheep which become struck, or which develop fleece rot. The aim is to reduce outbreaks in sheep breeding areas. A similar procedure occurs at research stations such as Trangie. In this selection process, sheep are exposed to large amounts of moisture in an artificial wetting regime, and animals which are susceptible will develop fleece rot and fly strike lesions and then be suitable for culling (Atkins and McGuirk, 1979). The
conformation of susceptible sheep is also studied and used as a basis of direct selection.

1.7.2.2. Indirect Selection

Indirect selection has been proposed as an alternative option to reduce the susceptibility of sheep to fleece rot and fly strike. If the genetic correlation between susceptibility to fleece rot and fly strike is unity, then indirect selection for reduced incidence of fleece rot is likely to be more effective in reducing fly strike than direct selection against fly strike itself. However, before an indirect selection trait can be used it is important that the following criteria be met (Raadsma, 1987a), such that it must:

1. Have a high genetic correlation with fleece rot.
2. Have a high heritability.
3. Have a wide range of expression.
4. Allow measurement independent of prevailing conditions.
5. Be simple and economical to measure.
6. Preferably not have an undesirable genetic association with major production traits.

However, selection based on these procedures may be difficult in commercial flocks. Nevertheless, at the Trangie Agriculture Research Centre, Raadsma and his co-workers have established the following criteria that are likely to be indicators of fleece rot resistance by undertaking comparisons of relatively resistant and susceptible flocks.

1. High wool brightness.
2. Good character and handle.
3. Regular, defined staples with square tips.
4. Large fibre diameter.
5. Low follicle density.
6. Low moisture content.

The most consistent associations with the selection which have been recorded by Raadsma (1987a) for resistant sheep are the bright greasy wool colour score, large fibre diameter and clean fleece weight which indicates low moisture content. However, for each of the traits where a difference between affected and unaffected animals was recorded, at least one study reported no difference or a reversal in trend for that particular trait. These inconsistencies could indicate real variation in the relationship between fleece traits and susceptibility to fleece rot because of differences in age, breed, strain or flock. The inconsistency could also be due to variation in the time at which the fleece traits were measured in relation to fleece rot development, variation in the method of fleece rot challenge (natural, induced, type and pattern of rainfall) or differences in the method used to classify animals as "resistant" and "susceptible" (Raadsma, 1987a).

Further studies by O'Meara and co-workers (1992; 1995) on the skin inflammatory responses of sheep genetically selected for resistance to both fleece rot and fly strike sheep indicated that serum protein exudation from sheep with infected wounds could also be used as a potential tool for indirect selection.

1.7.3. Chemical and Biological Control

Chemical and biological control of bacteria and flies fall into three areas. The first is to treat the sheep with chemicals or insecticides. The second is to suppress fly numbers by genetic control. The third is immunisation to develop immunity against *P. aeruginosa* and fly larval antigens.

1.7.3.1. Chemical control
Preventive control of fly strike by insecticides does not reduce the attractiveness of the sheep to the fly. Most commercial products are aimed at protecting the sheep against the strike by directly killing fly larvae as they hatch or as they develop.

Insecticides can also be used to either treat or dress the struck sheep or they can be used to reduce fly activity. Struck sheep should be treated by closely clipping all the wool from the affected areas and around the wound and then applying dressing or insecticides around the bare areas. There are two types of dressing available; non-irritant formations based on dieldrin, diazinon or orthodichlorobenzene and the irritant type of dressing based on mineral oil, arsenin. The irritant type tends to delay the healing, thereby predisposing the area to restrike (Graham, 1959).

Chemical application is normally done by the jet method. This consists of forcing at high pressure, by means of a jet, some fluid into the wool and onto the skin of the sheep. The jetting of sheep over the shoulder and withers as a protection against strike is carried out prior to or during the fly wave was first mentioned by Belschner (1937a). By the use of suitable insecticides, the occurrence and extent of strike may be reduced for a period of up to two to three months (Shanahan, 1965).

In the past there were various types of insecticides available in Australia. Sodium arsenite or calcium arsenite was first considered by McCullock in 1932. However, arsenite has never been used as an effective agent against fly strike because of the possibility of scalding and toxicity to the sheep (Hughes and Shanahan, 1979). Since the development of cyclodiene (Shanahan, 1953), and later organophosphate (Skerman and Pryor, 1957) and carbamate fly protectants
(Harrison, 1967), graziers had products that could protect sheep during the normal wave of fly strike. However, the development of resistance by flies to insecticides soon altered the situation (Hart et al., 1979).

Four organochlorines-DDT, BHC, deldrin and aldrin were introduced in Australia during the early 1940s. Early in the 1950s when resistance arose to organochlorines, organophosphates were introduced, such as Diazinon, Fenthion ethyl, Chlorfenvinphos and Propeaphos. However, resistance to these emerged after a decade. In the late 1960s, carbamate (Butacarb) was a substitute to overcome organophosphate resistance; unfortunately resistance to this chemical was also demonstrated several years later (Shanahan and Hughes, 1979).

Friedel et al. (1988) showed that cyromazine could inhibit the development and ecdysis of L. cuprina larvae cuticles in vitro. Later, Gruss (1988); Kotze and Reynolds (1993) showed that cyromazine could protect sheep from fly strike by inhibiting adult fly development.

However, Ivermectin has been reported to be the most effective chemical to control flies at the present time (Levot, 1995; Thompson et al., 1994), even though it was introduced in the early 1980s as an antiparasitic agent in cattle (Armour et al., 1980) and in horses (Schroder and Swan, 1982; Torbert et al., 1982).

1.7.3.2. Genetic control

Whitten (1974) described two categories of genetic controls which have been developed to induce genetic death in generations following the release of the flies.

1. Genetic manipulation or displacement of the field populations with strains of
flies carrying conditional lethal mutations which render the flies susceptible to other control methods (re-use of insecticides). In this system the male flies would have their chromosomes reconstructed before their release; the female larvae-determining chromosome having been altered to encode insecticide susceptibility. Therefore the hybrid larvae can be eliminated in the early stages of instar development due to insecticide susceptibility.

2. Suppression of pest numbers through the introduction of high levels of genetic death into the population. In this system, both female and male flies are chromosome reconstructed for autocidal control before their release. Their chromosomes have been rearranged so that hybrids between them and untreated flies do not survive (Whitten and Foser, 1974). A trial was carried out on Flinders Island to assess the use of this genetic control. It involved producing hybrids of the translocated male y-chromosome which causes eye problems that affects determination of the correct species for mating; the second generation of the cross bred reproduction less efficiently resulting in fewer flies (Davidson, 1989).

1.7.3.3. Vaccination

Antimicrobial therapy and immunotherapy against P. aeruginosa in humans have been intensively carried out around the world since Fox and Lowbury (1953a) mentioned that humans could produce antibodies against P. pyocyanea infection. Alexander et al. (1971) developed a LPS vaccine for burn patients and it was reported to be effective. Since then, it has been used in conjunction with antibiotics. However, this LPS vaccine is being re-evaluated since its efficacy has declined (Cryz et al., 1989). It has been suggested that a LPS base-toxin A conjugate vaccine might be more effective (Cryz et al., 1989; 1991).

The outer membrane protein F of P. aeruginosa (which is a conserved membrane protein) was suggested as an alternative base for a vaccine (Gilleland et al.,
A vaccine has been developed to preserve pulmonary function and reduce lung infections in a rat infected model (Gilleland et al., 1988; Fox et al., 1994). In 1995, Vonspecht and co-workers demonstrated that active and passive immunisation of immunodeficient mice with recombinant *P. aeruginosa* outer membrane protein F and outer membrane protein I fusion proteins resulted in a decrease in mortality to challenge. However, as yet, no human trial has been carried out of a vaccine of this nature. Recently, Doring and co-workers (1995) were able to demonstrate that *P. aeruginosa* flagella vaccine could provide some protection in treating human with *P. aeruginosa* related bronchitis.

There is still a controversy as to whether bacterial alginate can be used as a vaccine following Woods and Bryan (1985) suggestion that it could provide some protection in rats. Further study by Pier et al. (1990) was unable to confirm that alginate was very immunogenic in humans and it is therefore unknown whether it might protect or not (1990).

A study of sheep was carried out by Burrell (1985) using *P. aeruginosa* culture supernatant as a vaccine. He was able to protect sheep from both experimentally induced and natural fleece rot. However this investigation could not indicate which components were more or less immunogenic, or protective.

Other vaccines have been based on using fly larvae. Sandeman and Carnegie (1984) and Eisemann et al. (1990) have shown that sheep can produce antibodies against fly larvae. It is possible that sheep which produce antibodies to larval antigens may inhibit larvae survival. Sheep with a higher protective response are expected to produce relatively higher levels of antibodies against protective larval antigens (Jenkinson et al., 1979).
1.8. Controversies

Over the last few years there have been several controversies in sheep fleece rot and fly strike research. These include the relative role of bacterial species other than \textit{P. aeruginosa} in fleece rot, and the existence or otherwise, of immunity.

1.8.1. Bacterial Involvement

Work by MacDiarmid and Burrell (1986) showed that \textit{P. maltophilia} is found frequently in fleece rot affected sheep. London and Griffith (1984) isolated nine \textit{Pseudomonas} species from fleece rot affected wool. It is therefore possible that several bacterial species participate in the development of fleece rot which leads to fly strike.

The factors that lead to \textit{P. aeruginosa} predominating in the fleece ecosystem are still not clear. Merritt and Watts (1978b) suggested that \textit{P. aeruginosa} produces some bacteriostatic agent which could effectively control other bacteria in the fleece and skin environment, though this hypothesis needs to be confirmed.

1.8.2. Sheep Immunity

1.8.2.1. Humoral Immunity

Sheep immunity should be of particular relevance in assessing natural resistance against any opportunistic infections. The presence of immunoglobulin A, G and M on the skin of sheep suggests the existence of a defence immune system in sheep similar to that suggested for the skin in humans (Jenkinson et al., 1979).

Burrell (1985) suggested that a reduction in the severity of fleece rot dermatitis by the immunisation of sheep with \textit{P. aeruginosa} as an antigen was possible because the reduction of dermatitis would render the sheep less attractive to flies.
and fly strike. It is possible that immunised sheep produce some antibodies against the extracellular products or surface components of *P. aeruginosa*. Evaluating this serological response in healthy sheep or those with well developed fleece rot may contribute to the development of an effective vaccine.

Using *in vitro* procedures, Colditz and Eisemann (1994) examined whether larval growth was affected by various humoral serum mediators, such as histamine, bradykinin, platelet activating factor, serotonin, complement, leukotriene B4, interleukin 1β, interleukin 8, interferon γ and tumour necrosis factor α. None of them showed any effects on *L. cuprina* larvae. According to Colditz et al. (1994), none of those inflammatory factors showed a difference in the neutrophil accumulation in the inflammed skins of sheep bred for resistance and susceptibility to fleece rot and fly strike.

It has also been suggested that fleece rot resistant sheep have a better protective immune response than susceptible sheep. Results by O'Meara et al. (1995) suggested that serum IgG might contribute to bacterial clearance in fleece rot resistant sheep. On the other hand, Colditz et al. (1994) suggested that IgE secreting cells in skin may contribute toward the immunity of those sheep.

### 1.8.2.2. Skin Associated Lymphoid Tissues (SALT)

The skin of sheep is only a few millimetres thick, but it is the body's largest organ and within it a variety of highly specialised cells are organised into intricate structures and subsystems. Skin is not only a physical and mechanical barrier but also functions as a general defence system, with the immune system evolving as a complementary line of defence (Bos and Kapsenberg, 1986). Streilein (1983; 1990) used the term "skin associated lymphoid tissues" to describe this system which consists of numerous components:
1. Langerhans cells along with dendritic cells and macrophages each with their antigen-accepting, processing and presenting properties to lymphocytes in situ or in draining lymph nodes.

2. Epidermotropic recirculating T lymphocyte subpopulations responsible for infiltrating the epidermis, providing cognate antigen recognition.

3. Keratinocytes, comprising the great bulk of the epidermis, creating a microenvironment that is favourable to antigen uptake and recognition by lymphocytes through the production of epidermal thymocyte-activating factors.

4. Skin draining peripheral lymph nodes integrate this multicellular system.

Reduced competency of any of the components of the SALT could occur and contribute to decreased immunity to skin bacteria. For example, there could be genetic impairment of antigen presentation by Langerhans cells, or a reduction in the numbers and varieties of antigen specific recirculating lymphocytes (Streilein, 1983). This may lead to an impairment of the skin immune protection system.

Studies of the SALT of both intact and damaged skin in sheep have not been well documented. Chin and Watts (1992) did a comparative study of the skin immune response to intradermal sensitisation and challenge with *P. aeruginosa* in sheep that were bred for fleece rot and fly strike. O'Meara et al. (1992) did a similar study but using *L. cuprina* excretory and secretory products as antigens. Both studies showed there was a larger skin inflammatory response in resistant than in susceptible sheep which indicated that the resistant sheep might have an advantage/protective property over the susceptible sheep.
1.9. Aims

Whether *P. aeruginosa* is the sole pathogenic microorganism causing fleece rot is subject to debate and why this organism tends to dominate is not known. Evidence suggests that if the fleece rot lesion does not progress beyond the stage where there is extensive bacterial growth then serum exudation will not occur and there will be less chance of the sheep being struck by *L. cuprina*. The potential role of extracellular enzymes in this lesion process requires clarification.

Hence the aims of the current research were to:

1. Investigate the potential role of *P. aeruginosa* during fleece rot development.
2. Investigate the mechanism by which *P. aeruginosa* becomes dominant in fleece rot.
3. Identify the potential roles of *P. aeruginosa* extracellular products in the development of fleece rot.
4. Define the specific antigenic proteins of *P. aeruginosa* and *P. maltophilia* outer membranes and their capacity to elicit a serological response.
5. Examine the antibody response of sheep that are experimentally affected with fleece rot and fly strike.
Chapter 2

GENERAL MATERIALS AND METHODS

2.1. Bacteria

2.1.1. Sample collection

The collection of fleece bacteria from normal and fleece rot-affected sheep was obtained by skin and fleece washing. Each sample was collected from an area of approximately 4.9 cm² using a flat based funnel of 2.5 cm diameter. Sterile saline (5 ml) was placed in the funnel and the area formed by the parting of the fleece (skin and fleece) was given a vigorous wash by sucking up and down with a sterile plastic pipette. The suspension was then collected in sterile plastic tubes and immediately taken to the laboratory. It was then diluted in sterile saline 10, 100 and 1000 fold before spreading 100 μl aliquots of each dilution over Columbia blood agar plates (Appendix 2). The plates were incubated aerobically for 48 hours at 37°C for further isolation and identification of the resultant bacteria.

2.1.2. Identification

Bacterial identification to determine the complete range of species in sheep fleece and skin was based on morphology and routine biochemical tests carried out according to the method devised by Cowan and Steel (1975). A routine set of standardised procedures was followed using primary and secondary tests.

For the primary tests, the selected colonies were further subcultured onto another Columbia blood agar plate and then tested for haemolysis, anaerobic growth, Gram stain, motility, glucose requirement, oxidation and fermentation (Hugh and Leifson) tests, triple sugar iron agar utilisation, indole formation, catalase,
oxidase, urease and o-nitrophenol galactosidase production. Preliminary identification of the family and genera of the bacteria species were normally available at this stage.

Secondary tests were required for possible species identification. Routine biochemical, conventional sugar utilisation and enzyme activity testing as described in Appendix 3 were carried out in the laboratory. The primary and the secondary tests led to a strong presumptive identification (Cowan and Steel, 1975).

All bacteria were stored as pure cultures for short term (in agar vials) or long term (at -80°C) maintenance as described in Appendix 3.

2.2. Fly larvae

All *L. cuprina* larvae used for experiments were propagated and maintained by the Merck Sharp and Dohme (MSD) fly breeding unit, Ingleburn, NSW and were donated to our laboratory from MSD on the day for the antigen preparation.

2.3. Animals

Sheep: All sheep were Merino more than 6 months old and were housed indoors on a wire mesh floor at either the Department of Agriculture (Glenfield) or the Elizabeth Macarthur Agriculture Institute for one week before used in experiments. All pens were cleaned daily. The sheep were provided with lucerne pellets and fresh water daily.

Rabbits: The New Zealand white rabbits used were more than 6 months old and obtained from the rabbit breeding house at either the Department of Agriculture
(Glenfield) or the Elizabeth Macarthur Agriculture Institute. They were held in individual cages and provided with dried pellets and water daily.

Immunisation: A standard immunisation procedure was carried out for all animals throughout the whole project. The first immunisation was a intramuscular injection of an emulsion of antigen at a predetermined concentration together with complete Freund's adjuvant. Two weeks later the same dose of antigen with incomplete Freund's adjuvant (IFA) was administered subcutaneously. If the antibody titre was still inadequate after the second dose, a subcutaneously injection of the same dose in IFA was followed to boost the immune response.

2.4. Serum collection

Blood collected from rabbits was taken from the marginal ear vein. In order to prevent haemolysis of the samples it was essential to obtain a free flow of blood and this was achieved by gently milking the earlobe of the rabbits. Before and after bleeding, the region of needle entry was sterilised with 70% ethanol to minimise the possibility of contamination and infection. Blood samples from sheep were taken from the jugular vein using Vacutainer tubes (Becton Dickinson). Blood was allowed to clot at room temperature for 2-3 hours. Clotted blood was then centrifuged for approximately 10-15 minutes at 250 g in a benchtop centrifuge.

All sera collected were then diluted with sterile tris-saline glycerol merthiolate (Appendix 2) to a final concentration of 1/5 or 1/10 and were stored at -20°C unless otherwise specified.
2.5. Enzyme Linked ImmunoSorbent Assays (ELISA)

This is a simple method to detect and measure antibodies in serum or antiserum samples. In principle, antigen is noncovalently attached to each well of plastic microtitre plates. For this project, either whole cell bacteria or soluable proteins were used as antigens.

(a) Bacterial cultures in Luria Bertanie broth (Appendix 2) were washed twice with sterile saline and the concentration of cell suspension adjusted spectrophotometrically to A650 units (A650) and stored at -20°C.

The final volume of cell suspension was made in tris-saline-methanol pH7.2 (Appendix 2) to 0.04 A650 and 50 µl added to each well of microtitre plates (Flow Lab.). Microtitre plates were allowed to dry in a fan forced oven (Chin and Plant, 1989) and 50 µl of 1% (w/v) skim milk in tris-saline pH7.2 (Appendix 2) was added to each well to block non-specific binding sites. Microtitre plates were then incubated at 37°C for one hour. Excess skim milk was flicked off and a washing cycle followed. This washing cycle comprised two washes with deionised water, the filling of wells with tris-saline-tween 20 pH7.2 (Appendix 2) followed by incubation for 5-10 minutes at room temperature and two more washes with deionised water.

(b) Soluble antigens such as proteins were diluted in 50 mM bicarbonate buffer (pH 9.6) to 10 µg/ml and aliquots were added to wells. The microtitre plates were incubated overnight at 4°C. Excess antigens were removed by a washing cycle as described above.

Antisera were diluted in 1% skim milk tris-saline to the appropriate concentration before being added to the antigen-coated well. 50 µl of diluted antiserum was
delivered to each well and plates were incubated for 2 hours at 37°C. Excess antibodies were removed by a washing cycle as described above.

The relevant second antibody, conjugated to horseradish peroxidase, was added to each well in 50 µl volumes followed by incubation at 37°C for 2 hours. Excess second antibodies were washed off and substrate was added. The substrate, o-phenylene diamine (OPD), was made as described in Appendix 2 and 50 µl of OPD/H₂O₂ was added to each well. The enzyme catalysed reaction was allowed to proceed for up to 15 minutes at room temperature after which the reaction was stopped by adding 25 µl of 2.5 M sulphuric acid. The absorbance at 492 nm was measured. Control negative and positive were always included in each assay. The control positive was serum collected from immunised animals and the control negative was the pre-immunised serum.

2.6. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

In this study electrophoresis was carried out under reducing conditions as described by Laemmli (1970). The procedure is given in Appendix 3.

2.7. Polyacrylamide gel electrophoresis (PAGE)

Native polyacrylamide gel electrophoresis was also used. The method for gel preparation was the same as described for SDS-PAGE, except that SDS was omitted. Samples were prepared in a similar manner to the reduced samples (Appendix 4) except that the β mercaptoethanol was replaced with electrophoresis running buffer in the preparation method and without the heating step. The native gel system was used to separate proteolytic enzymes.
2.8. Western Immunoblotting

The transfer of proteins from gel onto membranes was accomplished as described in Appendix 4.

2.9. Skin biopsies and Haematoxylin/Eosin staining

Skin biopsies were trimmed and immersed in formalin buffer (Appendix 2) and the tissues were processed and sectioned courtesy of the Regional Veterinary Laboratory at the Elizabeth MacArthur Institute. The detailed procedure is described in Appendix 5.
Chapter 3

FLEECE BACTERIA: IN VITRO GROWTH INTERACTION

3.1. Introduction

Representatives of nine species of bacteria are obtained from skin and fleece swabs during the first 12 - 24 hours of skin wetting, including the common Gram positive bacteria such as Bacillus spp., Staphylococcus spp., and Gram negative bacteria such as Pseudomonas spp. After this time, P. aeruginosa predominates in the majority of isolates from fleece rot lesions (Chin and Watts, 1992; Merritt and Watts 1978a, b). Table 1 shows fleece bacteria before and after fleece rot incidence. These measurements were done before and after two days of heavy rainfall at Glenfield station. The results are the mean of total colony forming unit of each bacterial species per cm² of sheep skin. [Courtesy Drs. J. Chin and J. Watts].

<table>
<thead>
<tr>
<th>Fleece bacteria</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas hydrophila</td>
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<td>$&lt;10^2$</td>
</tr>
<tr>
<td>Alkaligenes faecalis</td>
<td>$10^2-10^3$</td>
<td>$&lt;10^2$</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>$10^2$</td>
<td>$&lt;10^2$</td>
</tr>
<tr>
<td>Bacillus coagulans</td>
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<td>$&lt;10^2$</td>
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<td>$&lt;10^2$</td>
</tr>
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<td>Proteus mirabilis</td>
<td>$10^2$</td>
<td>$&lt;10^2$</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>$10^2-10^4$</td>
<td>$10^7$</td>
</tr>
<tr>
<td>Pseudomonas alkaligenes</td>
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<td>$10^3$</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>$10^2$</td>
<td>$&lt;10^2$</td>
</tr>
</tbody>
</table>
It is not known why *P. aeruginosa* is so pre-eminent in wet fleeces. However, dominance by a particular bacterial species in specific micro-ecological niches such as the buccal cavity (Van der Hoeven and Rogers 1979; Weerkamp 1977) and the intestinal tract (Guiot 1982, Nuet et al., 1985) is well documented. For example, the ability of *Streptococcus* spp. to adhere to dental enamel contributes to an ecological advantage of this group in causing dental carries. Similarly, *Bacteroides* spp. predominate in the human intestinal tract in accordance with the availability of natural substrates such as polysaccharides (Guiot 1982; Nuet et al. 1985; Van der Hoeven and Rogers 1979; Weerkamp 1977). In 1995, studies by Vindenes and Bjerknes on the microbial colonisation of large wounds in humans showed that it took only a week or two for *P. aeruginosa* to outgrow Gram positive bacteria such as *Staphylococcus* and *Streptococcus*. However the cause was not determined. In none of these examples however has the ascendancy of one species, such as *P. aeruginosa*, so predominated the ecological microenvironment to the almost total exclusion of all others as appears to be the case in fleece rot.

This led to two main questions: (1) why *P. aeruginosa* dominates; and (2) how this organism establishes itself in the fleece. There is the possibility that inhibition factors such as pyocyanin might be involved, though relatively little attention has been given to this area in the study of fleece rot.

This chapter seeks to investigate the interactions of fleece bacteria in vitro, and the possible factors which allow *P. aeruginosa* to successfully compete with other bacterial species.
3.2. Materials and Methods

3.2.1. Bacterial isolation and identification

All the fleece bacterial isolates studied for this lawn experiment were primarily isolated and kindly donated by previous researchers (Drs. J. Watts and J. Chin). Bacteria were recovered from the skin washings of sheep before and after the natural induction of fleece rot at the Glenfield Agriculture station. After sampling, the skin washings were streaked onto Columbia blood agar plates that were incubated and subcultured to generate single representative colonies. All isolated bacteria were identified to species level based on morphology and routine biochemical and conventional sugar reaction, according to the methods of Cowan and Steel (1965). Bacteria were maintained in stab culture (Appendix 3) between each isolation.

All bacterial isolates were grown at 37°C in either Luria Bertoni broth (LB) or Luria Bertoni agar (LA) as described in Appendix 2.

3.2.2. Bacterial interactions or lawn experiments

Bacterial interactions were assessed by the author using colony growth inhibition of the test species on a lawn of opposing inhibitor bacteria. Growth of fleece bacteria in the presence of *P. aeruginosa* lawn was initially studied. In fleece rot, *P. aeruginosa* normally takes 24 - 48 hours to establish in the fleece. Thus 48 hours incubation was applied. LA plates were first spread with $10^5$ bacteria/100 μl of *P. aeruginosa*, and four replicate drops of 1 μl containing $10^3$ colony forming units (cfu) of the test bacteria were then dropped onto this bacterial lawn; the control plates had no *P. aeruginosa* bacterial lawn. After 48 hours of incubation at room temperature the mean colony diameters of test species in the presence and absence of the *P. aeruginosa* lawn were measured and calculated.
percentage growth of all bacteria in the absence of \textit{P. aeruginosa} was presumed to represent 100\%. The percentage reduction of colony diameters by \textit{P. aeruginosa} relative to the percentage of the control (\% Control) was expressed as percentage inhibition (\% Inhibition). To see whether this inhibitory effect of \textit{P. aeruginosa} was unique, three other bacterial species were used as lawns to test for a comparable inhibiting effect- \textit{Pseudomonas alkaligenes}, \textit{Aeromonas hydrophila} and \textit{Alkaligenes faecalis}. Those were chosen because preliminary experiments had shown that they were least inhibited by co-culture with \textit{P. aeruginosa}.

3.2.3. Purification of pyocyanin from Luria Bertanie broth medium

Pyocyanin was separated by a modification of the method described by Chang and Blackwood (1969). \textit{P. aeruginosa} was grown in LB broth for 18 hours on a rotary shaker at 37° C and 200 rpm. Bacteria were removed by centrifugation at 10,000g for 20 minutes at 21° C in a Sorvall RC-5 ultracentrifuge. The culture supernatant was extracted with a tenth volume of chloroform by vigorous shaking for 10 minutes. The chloroform layer containing pyocyanin was allowed to separate before recovery with the aid of a separating funnel. The crude pyocyanin extract was filtered through Whatman No. 41 filter paper and allowed to evaporate to a small volume in a rotary evaporator. One half of the concentrated extract was used to test the purity by the absorption spectra as outlined below. The other half was used for the yield determination, ie., it was transferred to four preweighed eppendorf tubes and allowed to dry in a 37° C fan oven. The crystallised pyocyanin appeared as a dark blue-green powder. The weight was determined after dryness was completed. The concentration of pyocyanin was calculated using the procedure of Flood \textit{et al.} (1972).

3.2.4. Isolation of pyocyanin from sheep fleece

Samples of blue green coloured wool were collected from six naturally infected
fleece rot affected sheep. The blue green pigment was found as a single band about 15-20 mm wide in the fleece. Wool bearing the intense blue-green bands was removed and weighed. The pyocyanin pigment was removed by washing the fleece with chloroform. The fleece suspension was agitated by stirring for three hours and then squeezed through a 20 ml syringe until all the blue colour was removed. The blue extract was then filtered through Whatman No. 41 filter paper and subsequently through a 0.45 µm Millipore filter (Millipore Pty Ltd, Australia) to remove all particles and wool wax. The dry weight was determined by allowing the extract to dry in a 37°C oven. The average yield of pyocyanin per gram of blue-green wool was determined as described for the pyocyanin purification (Section 3.2.3).

3.2.5. Absorption spectra of pyocyanins

The purity of pyocyanin in the extracts from culture media and from the fleece rot wool was determined by absorption spectra. The pyocyanin extracts were dissolved in chloroform at an estimated concentration of 20 µg/ml and chloroform was used as reference. The absorption spectra were measured using an LKB Biochrom-Ultraspec 4050 spectrophotometer (Appendix 1), at 5 nm intervals from 220 nm to 800 nm. The actual absorbance of pyocyanin was calculated relative to the reference absorbance by subtracting the chloroform reference absorption from that of the pyocyanin solution.

3.2.6. Pyocyanin inhibition test

The effect of P. aeruginosa pyocyanin on the growth of other fleece bacteria was examined as follows: a 96 well microtitre plate (Flow Lab., Australia) was filled with 200 µl of test bacteria at 4 x 10³ cfu/ml in LB broth containing a final concentration of 200 µg/ml and 20 µg/ml (1 mM and 0.1 mM respectively) pyocyanin. The pyocyanin was pre-dissolved in minimal volume of methanol
before adding to LB; control wells were LB containing an equivalent amount of methanol but no pyocyanin. Each test was done in triplicate. Plates were incubated aerobically at 37°C with occasional shaking. Duplicate 5 μl aliquots were removed after 0, 1, 2 and 4 hours of incubation and spread onto fresh LA plates to detect the numbers of cfu. A total of eight fleece bacterial species were used in this test. *P. aeruginosa* was also included for this test.

### 3.2.7. Statistical analysis

Data were collected from replicates of the control (no bacterial lawn nor pyocyanin) and the test (on bacterial lawn or in the presence of pyocyanin). The statistical significance of the growth inhibition of any test species was determined by analysis using Student *t*-test conducted on the raw data. The relative inhibition was evaluated by calculating the percentage inhibition as follows

For the lawn experiment:

\[
\text{% Inhibition} = 100 - \left[ \text{Mean}_{\text{cd}} \text{ (with lawn)} + \text{Mean}_{\text{cd}} \text{ (without lawn)} \right] \times 100
\]

For the pyocyanin experiment:

\[
\text{% Inhibition} = 100 - \left[ \text{Mean}_{\text{cfu}} \text{ (with pyocyanin)} + \text{Mean}_{\text{cfu}} \text{ (without pyocyanin)} \right] \times 100
\]

[cd: colony diameter; cfu: colony forming unit]

### 3.3. Results

#### 3.3.1. Bacterial isolates

Drs J. Watts and J. Chin had identified a total of nine bacterial strains from the wet fleece and skin of sheep. These bacteria were isolated before and after two days of heavy rain at Glenfield station. There were only three Gram positive species *Bacillus cereus*, *Bacillus coagulans* and *Staphylococcus epidermidis*. The
Gram negative bacteria comprised *Aeromonas hydrophila*, *Alkaligenes faecalis*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Pseudomonas alkaligenes*. The bacteria had been kept at -80 °C as well as at room temperature in stab culture (Appendix 3). The nature of bacteria isolated before and after the onset of fleece rot was also documented by previous workers, Drs. J. Watts and J. Chin. It is believed that these bacterial species were not the only fleece bacteria found in sheep fleece (Dr. J. Chin, personal communication). Other bacterial species were isolated and identified by the author such as *Acinetobacter* spp., *Corynebacterium* spp. and *Staphylococcus aureus*. However they were not used for this experiment.

*P. aeruginosa* isolated from sheep fleece rot produces a strong blue green pigment known as pyocyanin. This pigment can be seen in broth or agar cultures after 24 hours of incubation at 37 °C. It is highly mucoid with a colony appearance similar to those as seen in Section 1.2.3.1. Figure 1 shows an electron micrograph of *P. aeruginosa* after being recovered from an LA plate after overnight growth. The organism was washed with phosphate buffer saline, stained with negative stain Tungsten phosphate for 2-3 minutes before being coated onto a Gold grid for viewing. A different *P. aeruginosa* strain with flagella and pili was used as a control. Electron micrography indicates that *P. aeruginosa* isolated from sheep fleece rot does not have a flagellum nor pilus. This confirmation allowed elimination of the necessity of studying flagella and pili of the current *P. aeruginosa* isolates.
Figure 3.1: Electron micrograph of *P. aeruginosa* isolated from fleece rot. (X30,000). Note the formation of the exopolysaccharide layer surrounding cell of a mucoid fleece rot bacterium growing in a solid agar medium, after it had been washed once with phosphate buffer saline, stained with Tungsten phosphate.
3.3.2. Studies of growth inhibition by lawn experiments

All fleece bacterial isolates grown in LB broth overnight were adjusted to $10^6\text{cfu per ml.}$ All nine bacterial species including *P. aeruginosa* were first tested on *P. aeruginosa* lawns. Appendix 6.1 shows the percentage growth and inhibition of bacteria in the presence and absence of *P. aeruginosa* lawn.

Figure 3.2 shows that *P. aeruginosa* inhibited the growth of all other fleece bacteria. The results particularly showed that *P. aeruginosa* inhibited the growth of Gram positive far more than Gram negative species. There was at least 70% inhibition in the case of *Bacillus* and *Staphylococcus* species. The three least inhibited bacteria were *Aeromonas hydrophila*, *Alkaligenes faecalis* and *Pseudomonas alkaligenes* with less than 40% inhibition. Culture of *P. aeruginosa* on lawn of itself did not show a significant effect; the 5% growth inhibition of *P. aeruginosa* noted was most likely due to nutrient advantage of the first bacteria in the *P. aeruginosa* lawn which were seeded before the second *P. aeruginosa* was dropped on.

Figure 3.3 shows a typical result of a lawn experiment to test the inhibitory effect of *P. aeruginosa* on another bacterium on a LB agar plate. Parallel controls and tests indicated that the colony diameters of some bacterial species decreased in the presence of *P. aeruginosa*, as seen for species 3 and 4. In contrast, a complete inhibition of species 1 was seen, as no colonies were visible in the test lawn plate and similarly a minimal growth for species 2 was demonstrated. All eight fleece bacterial species showed some level of inhibition by *P. aeruginosa*. There was no evidence from the control plates that one test bacterial species could affect the growth of the other species.

To observe whether other bacteria suppressed growth of one or more isolates, as shown in figure 3.2, the three bacteria species least inhibited by *P. aeruginosa* were
selected for use in lawn; that is, *Aeromonas hydrophila*, *Alkaligenes faecalis* and *Pseudomonas alcaligenes*. An identical procedure to that described for the *P. aeruginosa* experiment was employed. The bacteria for the lawn were spread on LB agar plates and the test bacteria drops applied. Colony growth of test bacteria on the lawn and the controls were measured for inhibition effects. In all three experiments, the colony diameters of the test bacteria showed less than 20% difference between the control and the tests. Percentage growth and inhibition of each species on agar plates were calculated as shown in Appendix 6.2, 6.3 and 6.4.

Figures 3.4a, 3.4b and 3.4c show the extent of inhibition of fleece bacteria by the lawn tests. The results of all four lawn experiments indicate clearly the supremacy of *P. aeruginosa* in suppressing other fleece bacteria and the lack of effect of the other species on *P. aeruginosa*. *Aeromonas hydrophila*, *Alkaligenes faecalis* and *Pseudomonas alcaligenes* have a limited impact on the growth of other bacterial species. This could be seen by the similarity of the colony diameters of all the test bacteria on the control plates that had no bacterial lawns and the test plates that had one of those three bacterial lawns. It was most obvious when the swarmming property of *Proteus mirabilis* was seen in those lawns but not on the *P. aeruginosa* lawns.

3.3.3. Absorption spectra of Pyocyanin

Figure 3.5 shows the absorption curves from the ultraviolet to the visible regions of pyocyanin samples isolated from wool (in vivo) and from culture media (in vitro). The curves illustrate a similar absorption for pyocyanin isolated from the two different origins, suggesting that the pyocyanin produced in the culture medium is similar to the product derived from the fleece rot-stained wool. Both spectra show a high absorption between 220 nm and 320 nm, probably due to the solvent absorption; a low absorption between 330 nm - 360 nm wavelength and an
absorption peak from 360-390 nm indicates the presence of pyocyanin.

Figure 3.2: Growth inhibition of fleece bacterial isolates by *P. aeruginosa* lawn.

Data represent the percentage inhibition of fleece bacteria growing on a *P. aeruginosa* lawn compared to controls. *P. aeruginosa* is added as a control.

When comparing the growth of treatments and controls all species had probability values of < 0.05 except *P. aeruginosa*.

**Abbreviation:**

- A.hy. : *Aeromonas hydrophila*
- A.fa. : *Alkaligenes faecalis*
- B.ce. : *Bacillus cereus*
- B.co. : *Bacillus coagulans*
- E.ae. : *Enterobacter aerogenes*
- P.ae. : *Pseudomonas aeruginosa*
- P.mi. : *Proteus mirabilis*
- P.al. : *Pseudomonas alkaligenes*
- S.ep. : *Staphylococcus epidermidis*
Figure 3.3: Representative example of the inhibition of growth of four species of fleece bacteria by a *P. aeruginosa* lawn. The left (L) plate is the control that has no bacterial lawn and the right (R) plate is the test plate where the effect of a *P. aeruginosa* lawn on the growth of the same bacterial spp. after 48 hours incubation is manifested. This is a typical result showing different degrees of inhibition of the four bacterial species. Note that the growth of species 1 (spp.1) is completely inhibited, while there is minimal growth of spp.2 and partial growth of spp.3 and spp.4.
Figure 3.4: Growth inhibition of fleece isolates by different bacterial lawns; 4(a) organisms were cultivated on *P. alkaligenes* lawns; 4(b) on *A. faecalis* lawns and 4(c) on *A. hydrophila* lawns. Data represent percentage inhibition of each species on a respective bacterial lawn compared to control colonies growing free of lawns. All data had a probability value $> 0.05$. See Figure 3.2. for abbreviation.
Figure 3.5: Absorption spectra of the pyocyanin (0.1 mM; 20 μg/ml) isolated from fleece \textit{(in vivo)} and culture media \textit{(in vitro)}. Curves were calculated from the chloroform as reference at every 5 nm interval. Insert is the whole spectrum measured between 220 nm - 480 nm.
3.3.4. Bacterial inhibition by Pyocyanin

To assess the possibility of an antagonistic effect of pyocyanin on bacterial growth, pyocyanin was added to well cultures of fleece bacteria. Two different concentrations of pyocyanin (20 \( \mu g/ml \approx 0.1 \text{ mM} \) and 200 \( \mu g/ml \approx 1 \text{ mM} \)) were added to wells containing each bacterial species in LB broth. The results of bacterial growth were first calculated as percentage growth from the average cfu per 5 \( \mu l \) of triplicates in the presence of pyocyanin after 0, 1, 2, 4 hours incubation relative to the cfu of the control. The results were further calculated for percentage inhibition. In all the control assays which had no pyocyanin, a rapid increase in cfu was obtained for all bacterial species. The effect of different concentrations of pyocyanin on fleece bacteria at different time points is summarised in Table 3.2. The addition of pyocyanin to \textit{Alkaligenes faecalis} showed to be affected after 2 hours of incubation. \textit{Enterobacter aerogenes} did not have any of its effect over within 1 hour of incubation; whereas \textit{Bacillus coagulans}, \textit{Bacillus cereus} and \textit{Staphylococcus epidermidis} reached an inhibitory state within one hour at a significant level. The decrease of bacterial growth was strong among the Gram positive bacterial isolates, ie., \textit{Staphylococcus epidermidis}, \textit{Bacillus coagulans} and \textit{Bacillus cereus} after 2 hours of incubation. All bacteria displayed a reduction of growth in the presence of pyocyanin and higher pyocyanin concentrations (1 mM) had a greater inhibitory effect than the lower concentration (0.1 mM). Appendix 6.5 shows the cfu count at each time point. Figure 3.6 (derived from Table 3.2) depicts the results after a 4 hour exposure to pyocyanin purified from a culture supernatant of \textit{P. aeruginosa}. There is inhibition in the growth of all fleece bacteria except \textit{P. aeruginosa}. The inhibition is greatest for \textit{Bacillus} and \textit{Staphylococcus} bacteria. Gram negative bacteria were shown to be less affected. Pyocyanin does not reduce the proliferation of \textit{P. aeruginosa} itself significantly. The results were essentially similar to those of the lawn experiments where fleece bacteria were tested on \textit{P. aeruginosa} lawns.
This result suggests that pyocyanin may be one of the factors contributing to the inhibition process measured in the bacterial lawn experiments.

<table>
<thead>
<tr>
<th>Time \ [Pyo]</th>
<th>A. hydrophila</th>
<th>A. faecalis</th>
<th>B. cereus</th>
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<tbody>
<tr>
<td></td>
<td>0 0.1mM 1mM</td>
<td>0 0.1mM 1mM</td>
<td>0 0.1mM 1mM</td>
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<td>0 0 0</td>
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<tr>
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<table>
<thead>
<tr>
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<th>B.coagulans</th>
<th>E. aerogenes</th>
<th>P. aeruginosa</th>
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<tbody>
<tr>
<td></td>
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Table 3.2: Organisms were cultured in LB broth containing pyocyanin at final concentrations of 0.1mM and 1 mM. Triplicate aliquots of each treatment were cultured on LB agar at the time shown. The percentage growth inhibition for each treatment was calculated from averages with respect to control cultures (No pyocyanin). Negative values represent inhibition, positive values indicate higher counts than the control.
Figure 3.6: Inhibition of fleece bacteria in the presence of pyocyanin (1 mM).

Data represent the percentage inhibition of bacterial growth in triplicates by pyocyanin after 4 hour. Abbreviation: see figure 3.2.
3.4. Discussion

Many studies have focused on bacterial antagonism of other bacterial species in the mouth and intestines of humans, and also in rats. Many factors are involved in the predominance of specific bacteria (Neut et al., 1985). The predominance of the specific strains of *Streptococcus mutans* in the mouth is considered to be due to the production of a bacteriocin which acts against other bacterial species of oral plaque-associated microflora (Van der Hoeven and Rogers, 1979: Weerkamp et al., 1977). In the human intestinal tract, *Klebsiella pneumoniae* and *Escherichia coli* were reported as the predominant species due to microcin activity (Freter, 1962). Among the diverse ecological outcomes found in vivo, *P. aeruginosa* colonises the lungs of cystic fibrosis patients due to the production of pyocin which inhibits the growth of other bacteria (Govan, 1986). Pyocin has been reported to be a bactericidal substance produced by *P. aeruginosa*. However its induction in vitro requires specific culture media containing Mitomycin C (Jones et al., 1973). Pyocin was reported to be an unstable marker; changes in bacterial metabolism may result in a change in pyocin production (Farmer and Herman, 1974). Thus pyocin was not ideal for this study.

Studies simply looking at the number of bacteria present in the fleece at any one time can be misleading, as there are many environmental conditions which can influence the proliferation of fleece bacteria including the wet and dry condition of the fleece, and the climate. While laboratory conditions are artificial they do enable the potential inhibitory effects of one species against another to be studied. Hence, the lawn experiment was used here to test the hypothesis that one bacterium, *P. aeruginosa*, selectively inhibited other bacteria found in the fleece. The positive result could support the contention that *P. aeruginosa* is important in fleece rot development.
Other in vitro studies have shown that *P. aeruginosa* inhibits a range of other bacterial species, such as *Vibrio cholerae*, *Staphylococcus aureus* (Schoental, 1941) and *Proteus species* (Knight et al., 1979). Machan et al. (1991) reported that the colonisation of *P. aeruginosa* in human sputum of cystic fibrosis was related to the absence of *Staphylococcus aureus*. In their studies, pyocyanin showed its effective growth inhibition against not only *Staphylococcus aureus* but also *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae* and *Acinetobacter* spp. An abstract citation of Bai (1991) pointed out that the potential bactericidal effect of pyocyanin on other bacterial species such as *E. coli*, *Proteus vulgaris*, *Citrobacter freundii* and *Staphylococcus epidermidis* was dose dependent. Recently, an abstract citation by Li et al. (1995) further added *Samonella infantis* to the list of bacteria which were affected by pyocyanin. The author also mentioned that pyocyanin was necessary for the growth inhibition since they found that the pigmented strains of *P. aeruginosa* were much stronger in their antimicrobial activity than the non-pigmented strains (Li et al., 1995).

The study in this chapter was designed to examine the impact of fleece bacterial isolates on each other and there was a statistically significant inhibition on all other fleece bacteria by *P. aeruginosa* (Figure 3.2). As was shown in figure 3.4.a, b and c inhibitory activity was not demonstrated by three fleece bacteria; *Pseudomonas alkaligenes*, *Alkaligenes faecalis* and *Aeromonas hydrophila* had no significant effect on the growth of other fleece bacterial isolates. Thus, it was suggested that *P. aeruginosa* has some direct effect on the growth of other bacterial species, and this appears to represent specific growth competition rather than nutrient competition.

From a consideration of the changes in fleece bacteria population during fleece rot development, Merritt and Watts (1978b) suggested that an extracellular
product of *P. aeruginosa* might be responsible for the inhibitory affect on other fleece bacteria.

Pyocyanin or N-methyl-1-hydroxyphenazine is an extracellular pigment produced by *P. aeruginosa*. It can be extracted from culture supernatant by chloroform (Chang and Blackwood, 1969). In its neutral state it has a blue-green colour. Its colour changes to red below pH4 and to deep blue at pH > 10. According to Flood *et al.* (1972), pyocyanin has a melting point between 132-133°C, a yield of 15 - 55 µg/ml from pure culture, a molecular mass of 210 and the formula is C$_{13}$H$_{10}$ON$_{2}$. Crystallised pyocyanin can be redissolved in water or methanol.

![Pyocyanin molecule](image)

Pyocyanin is reported to be active against lymphocytes in humans (Nutman *et al.*, 1987; Rubbin *et al.*, 1983) by the suppression of DNA synthesis and IL-2 production. Pyocyanin can impair mitochondrial respiration by inhibiting the uptake of oxygen (Armstrong, 1970; 1971) and is very toxic to human epidermis skin tissue (Cruickshank and Lowbury, 1953) and to the mouse liver tissue (Stewart-Tull and Armstrong, 1972). It inhibits ciliary motility, caused epithelial cell disruption in rabbit respiratory cilia (Hingley *et al.*, 1986) and slowed down the tracheal mucus velocity in guinea pigs allowing *P. aeruginosa* to colonise easily in the respiratory tract (Munro *et al.*, 1989). Pyocyanin can inhibit prostacyclin release from arterial endothelial cells, thus causing vasculitis of the small pulmonary arteries (Kamath *et al.*, 1995). The possibility that pyocyanin was the inhibiting factor that independently suppresses other fleece bacterial
proliferation in vitro was investigated.

In the current study, this pigment was easy to generate at approximately 10 μg/ml from bacteria grown in LB broth, and could be stored in the crystalline form for up to 3 months in the dark. Green wool had a high concentration of pyocyanin (100 μg/g wool). A preliminary experiment of pyocyanin inhibition had shown that at 200 μg/ml, pyocyanin completely inhibited the replication of *Bacillus* species and *Staphylococcus epidermidis* after 2 hours. Considering the amount of pyocyanin recovered per gram of wool under in vivo conditions, this concentration would be unlikely to be reached in wet fleece. Hence another type of experiment was set up to study bacterial growth under two different concentrations of pyocyanin, 20 μg/ml and 200 μg/ml. It was shown that a concentration of pyocyanin of 20 μg/ml significantly inhibited *Bacillus coagulans* and *Staphylococcus epidermidis* after 4 hours; Gram negative fleece bacteria were also inhibited by pyocyanin at 20 μg/ml. There was no significant difference in *P. aeruginosa* growth at this concentration compared to controls. This experiment clearly suggests a mechanism for the growth inhibition phenomenon seen in the lawn experiment. In vivo, several bacteria may be able to replicate on wet skin. However, once reasonable numbers of *P. aeruginosa* exist, the pyocyanin concentration will rise and this could then antagonise the growth of other species.

Results from this chapter were first presented to the Australian Society for Microbiology Conference held in 1988 in Canberra.
4.1. Introduction

Sheep with fleece rot suffer damage to the wool fibre, the wax layer and the dermal layer which can be easily seen after the phase of rapid multiplication of *P. aeruginosa* in the fleece (Merritt and Watts, 1978b). However, even when fleece rot is well established, *P. aeruginosa* does not penetrate the epidermis. Controversies persist as to whether extracellular products from *P. aeruginosa* can be invasive or pathogenic in sheep. No role for pyocyanin has been determined in the lesion. Other molecules of interest in the fleece rot lesion are extracellular enzymes which have attracted interest since Kapur and Shrini (1987) reported that strains of *P. aeruginosa* producing less elastase and proteases were less virulent than pathogenic strains in humans.

A number of studies have looked at extracellular molecules released during *P. aeruginosa* infection. Exotoxin A is described as the most toxic substance known to be produced by *P. aeruginosa* (Liu, 1974; Pavlovskis and Wrestlind, 1989). Phospholipase C (Chin and Watts, 1988) has also been shown to be one of the virulence components associated with *P. aeruginosa* infection, a condition that is similar to fleece rot dermatitis. *P. aeruginosa* produces several proteases; including elastase and alkaline proteinase which have similar enzymatic activities (Holder, 1985). These proteases have attracted special attention in fleece rot, since Kawaharajo et al. (1975a) reported that the injection of *P. aeruginosa* alkaline proteinase and elastase into the skin of rabbits would induce haemorrhagic lesions within minutes. Further, Kharazmi et al. (1984) reported that
*P. aeruginosa* alkaline proteinase and elastase could inhibit human neutrophil chemotaxis, that is these enzymes, released in localised infections such as fleece rot, may influence sheep immunity. However, none of these studies have evaluated the impact of sheep wool on production or enzymatic activity. It is likely that *P. aeruginosa* produces proteases that degrade wool fibre proteins and other available proteins on the skin, and that the impact of proteases and phospholipase C on the skin layer could result in the dermatitis associated with fleece rot.

To date little attention has been given to the local immune response in sheep due to extracellular products of *P. aeruginosa*. The objectives of this present study were: (1) to develop techniques for separating proteases, (2) to identify the proteases produced by *P. aeruginosa* in *in vitro* and *in vivo* conditions; (3) to show the importance of sheep wool in enzyme production; (4) to study the role of extracellular proteases in fleece rot development and (5) to examine the serological response of sheep against proteolytic enzymes of *P. aeruginosa* in fleece rot affected sheep.

### 4.2. Materials and Methods

#### 4.2.1. Culture media

The following media, as described in Appendix 2 were used:

* Luria Bertani broth (LB)
* Trypticase soy broth (TSB)
* Low nutrient medium broth (LNM)
* Minimal nutrient medium broth (MNM)

All media were prepared in 200 ml Erlenmeyer flasks containing 25 ml culture medium. Each medium was prepared in 3 flasks. After being autoclaved, sterile sheep wool was added to the media at 1% and 5% (w/v) while the control was
without wool. The wool was originally removed from the sheep's back by shaving. It was brought to the laboratory where dirty tip ends were discarded before packing a small quantity in aluminium alfoil for autoclaving. This would eliminate any potential bacteria that could possibly were found in the fleece. After sterilisation, wool appeared as brownish colour, however it was still intact. This was weighed and added to media accordingly.

4.2.2. Growth condition

*P. aeruginosa* was grown on LB agar plate. Two or three single colonies were transferred to a 2 ml LB as starter culture. This culture was shaken for 6 hrs at 37 °C. To standardise numbers of inoculant cfu from each batch of inoculation, an aliquot of 100 μl of the starter culture containing approximately $10^6$ cfu was then inoculated into the test and control 25 ml broths in 200 ml flasks.

After incubation at 37°C for 18 hours on a rotary shaker at 100 g, 1 ml aliquots of all bacterial suspensions were removed for cfu determination. Cells from the remaining 24 ml cultures were then removed by centrifugation (10,000 g, 10 min, 4°C on Sorvall RC-5 ultracentrifuge) and the resultant culture supernatant filtered through 0.22 μm cellulose acetate filters before these filtrates were used for testing in the presence of proteolytic enzymes.

4.2.3. Measurement of colony forming units (cfu)

Each sample was serially diluted in LB broth and plated on LB agar plates for viable counts. Triplicate aliquots of the 10 μl of $10^{-5}, 10^{-6}$ and $10^{-7}$ dilutions were spread on LB agar plates and incubated for 4-5 hours at 37 °C. Colonies were counted using a magnifying glass while the colonies were still small and separated and the average yield of cfu was calculated per μl of culture medium.
4.2.4. Polyacrylamide gel analysis

Proteolytic enzymes were separated electrophoretically on polyacrylamide slab gels at pH 8.8 (Kortt et al., 1983) under non-reducing conditions as described in Chapter 2 (Section 2.7). Proteolytic activity was detected by placing the 7.5% polyacrylamide gel on a layer of agarose-gelatine gel (Appendix 2) and allowing hydrolysis of the gelatine to occur. After 3 hours at 37°C in a humid chamber, the non-hydrolysed gelatine was precipitated by flooding with saturated ammonium sulphate (pH 7.2). This overlayed zymogram was placed against a black backing to visualise the black bands indicating the presence of proteases. This allowed a quick identification of proteolytic isoenzymes. Identical gels with and without SDS were set up with molecular weight standards to double check the sizes of proteases.

4.2.5. Protease purification

*P. aeruginosa* was grown in 50 ml of sterile LB broth in 200 ml Erlenmeyer flasks. After 18 hours, bacteria were removed by centrifugation (10,000 g, 10 min, 4°C). The supernatant fluid was filtered through a 0.22 μm cellulose acetate filter to remove all the remaining bacteria and was concentrated to one fifteenth of its volume in dialysing tubing (with 10 kDa molecular weight cut off pore size) against Aquacide (Boehringer-Manheim). The resulting solution was designated as crude culture supernatant (CS). Polyacrylamide gel electrophoresis was carried out at a constant voltage of 200 Volts. Rapid identification of proteases was carried out by overlaying the gel with agarose-gelatine agar (Appendix 2) substrate for 3 hours at 37°C. A clear zone in the gelatine substrate after ammonium sulfate precipitation of the proteins indicated the enzyme locations. By matching the overlayed zymogram and the native polyacrylamide gel, two major enzyme strips were removed from the polyacrylamide gel and then minced by mortar and pestle for further purification.
Two different procedures were subsequently employed. Electroelution was the first procedure used in which the minced gel was placed inside electroelutor plastic tube (in the upper chamber) and electrophoresed vertically at a constant voltage of 100 Volts for 3 hours in tris-glycine (pH8, Appendix 2) as running buffer. The proteases were eluted into a dialysing tubing at the bottom chamber. Electroeluted proteases were concentrated using Aquacide. However the enzyme recovery by this electroelution method was low, and thus a second procedure was developed to improve the yield. In this case, the minced gel was sonicated in the presence of 25 mM tris pH8 for 5 minutes at 4°C. Each enzyme filtrate was collected by filtration using 0.22 μm cellulose acetate membrane under vacuum and concentrated by Aquacide to nominal volumes which would be used later for quantitative comparisons on protease yields. The protein concentration of the protease preparation was determined by a modified Lowry procedure (Markwell et al., 1978).

4.2.6. Characterisation of proteases

4.2.6.1. Measurement of proteolytic activity

Preliminary determination of the optimum pH for enzyme assays was done by assaying the crude culture filtrate in 50 mM tris buffer over a range of pH from 5-10 (results not shown). It was found that the optimum pH for gelatine hydrolysis was between pH7.5 - 8. Thus, all the following proteolytic assays were carried out in 50 mM tris buffer with 1 mM CaCl₂ at pH 7.8. Enzyme activities were measured semi-quantitatively by using the gelatine agar diffusion method and protease assay based on the method of Johnson et al. (1967).

4.2.6.1.a. Rapid screening of protease production was carried out by the radial diffusion gel method, by measuring the proteolytic activity diffused in gelatine
agar. Replicates of 15 µl (containing 1-2 µg proteases) of crude culture supernatant or purified protease were loaded into round wells with 4 mm diameters. Controls contained phosphate buffer. After 3 hours of incubation at 37 °C, the agar was flooded with saturated ammonium sulfate pH 7.2 and a clear zone indicated protease activity. These cleared zones were measured in two directions and the proteolytic activity calculated. One unit of proteolytic activity is the amount of enzyme required to clear an area of 1 mm² of gelatine agar.

4.2.6.1.b. Proteolytic activity was also determined by bovine serum albumin (BSA) digestion. This assay has been utilised to measure the K_m and to determine enzymic properties of both proteases. This was enhanced by experiments requiring multiple analysis with various pH, incubation time, concentration of enzymes and BSA. Each of the reaction components was evaluated to ensure the optimal concentrations of enzyme and substrate.

The assays were carried out in acid washed (10 mM Nitric acid) glass tubes in triplicate. The final reaction volume was 1 ml consisting of 50 mM tris buffer (pH 7.8); 1 mg of BSA in tris buffer with 1 mM CaCl_2, and 0.1 mg of protease. The control assays had no enzymes. The reactions were performed for 3 hours at 37 °C on an orbital shaker at 100 g and were terminated by the addition of 0.1 ml of 20 % (w/v) trichloroacetic acid and incubation for a further 30 minutes. Unhydrolysed BSA was precipitated by centrifugation at 2,000 g for 15 minutes in a benchtop IEC (International equipment, USA) centrifuge. The amount of amino acid in the supernatant released from hydrolysed BSA was determined by measuring absorbance at 280 nm against tris buffer as a blank.

4.2.6.2. Mean of proteolytic activity

Recovery of cfu/ml of *P. aeruginosa* from different cultures prepared in the
presence or absence of wool was compared with the recovery of proteolytic activity from the same cultures. The proteolytic activity for gelatine substrate was determined by the radial diffusion method, and calculated on the basis of cleared area per fixed volume of culture supernatant or purified enzymes.

4.2.7. Protein concentration of culture supernatant

Preliminary experiments showed that culture supernatant prepared from LB contained more protease activity than the other three media (TSB, LNM, MNM) thus investigations were performed on the purified proteases from LB culture supernatant only. *P. aeruginosa* culture supernatant were dialysed in PBS overnight at 4°C before assaying for total protein by a modified Lowry procedure (Marwell *et al.*, 1978). The controls included dialysed LB broth.

4.2.8. Serological analysis

4.2.8.1. Preparation of antiserum

A standard procedure for immunisation was carried out in rabbits and sheep using purified enzyme I and II as antigens. Preliminary purification indicated that protease I and II had molecular mass of 150 kDa and 50 kDa respectively. A 1 ml mixture of equal parts of complete Freund's adjuvant and mixed proteases I and II was injected intramuscularly into rabbits (100 µg of mixed proteases) and sheep (1mg). At two week intervals, a similar dose of pooled enzymes in a equal volume of incomplete Freund's adjuvant was administered subcutaneously at 4 different sites. Blood was collected from the rabbits by the marginal ear vein and from sheep by the jugular vein. Bleed 0 designated the pre-immunised serum and bleed 1 the sera collected one week after the third vaccination. These sera, which were prepared as described in Chapter 2 (Section 2.5) were used in ELISA, immunoblots and neutralization assays.
4.2.8.2. Antibody neutralisation assays

Enzyme inhibition assays were carried out to examine if antibodies were produced which could inactivate the proteolytic activity. Incubation of dialysed *P. aeruginosa* culture supernatant with antiserum (final dilution 1/2, 1/4, 1/8 and 1/16) was carried out for 3 hours at 37°C with occasional shaking. Duplicate 10 μl aliquots of the mixture were subsequently removed and tested for proteolytic activity by the radial diffusion method in gelatine agar plate. A similar treatment of a mixture substituting PBS was used as a control.

4.2.8.3. ELISA

ELISA was carried out to measure the anti-protease antibody reactivity in rabbits and sheep. Sera were collected from rabbits and sheep which were immunised with combined protease I and II and stored at -20°C. Plates were coated with 50 μl per well of dialysed culture supernatant, protease I and protease II in coating buffer pH 9.6 with 50 μg/ml (Appendix 2) in flat bottom microtitre plates (Flow lab.) and after overnight incubation at 4°C, the plates were then washed following the ELISA method as described in Chapter 2 (Section 2.5). All sera to be tested were diluted 1 in 50 with 1% skim milk in tris-saline (TS-skim milk). Pre-immunised sera were used as negative control on the same plate. The second antibody (monoclonal anti ruminant antibody) conjugated to peroxidase (HRP-ARIMab) was diluted in 2,000 in TS-skim milk. H₂O₂/OPD (Appendix 2) was used as substrate. The absorbance was read at 492 nm, after 10 minutes reaction and acid H₂SO₄ termination.

4.2.8.4. Immunoblots

Electrophoresis of non dialysed culture supernatant were carried out in a mini Hoefer gel system under reducing conditions as described in Appendix 4. Culture supernatant were heated at 100°C for 5 minutes in the presence of β-
mercaptoethanol (Appendix 2) before being loaded onto 12% [w/v] acrylamide running gels with 4% [w/v] stacking gels. Electrophoresis was carried out at a constant voltage of 150 Volts until the bromophenol blue tracking dye had reached the bottom of the gel.

Proteins were transferred from gels to nitrocellulose membrane with a blotting apparatus for 3 hours at 60 V (Towbin et al., 1979) as described in Appendix 4. The nitrocellulose membranes were incubated with gelatine blocking agent (Appendix 2) followed by rabbit or sheep antisera, and with anti-rabbit immunoglobulin- or anti-ruminant immunoglobulin- conjugated alkaline phosphatase at 21°C (Appendix 4). Membranes were reacted with nitroblue tetrazolium/ 5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) or alkaline phosphatase colour development reagent (Appendix 2).

4.2.9. Enzyme inhibition studies

To see if the proteases share the same catalytic activity, several enzyme inhibitors were tested for their ability to decrease the proteolytic activity of the purified proteases (see Table 4.1). In the assay, a duplicate of 100 μg of purified protease in 100 μl of tris-HCl pH 7.8 (Appendix 2), was pre-incubated in duplicate with an appropriate concentration of inhibitor (buffer was used for the control) for 2 hours at 37 °C. The enzyme activities were determined by gel diffusion assays in gelatine agar as described in Section 4.2.3. Clearing zone diameters were measured after 3 hours of incubation at 37 °C. The remaining proteolytic activity was calculated relative to the control which had no inhibitors.
Table 4.1: Proteolytic inhibitors and their final concentrations used in inhibition assays of *P. aeruginosa* proteases.

4.2.10. In vivo studies

4.2.10.1. Skin thickness

Two merinos that did not have any fleece rot were used in this study. They were clinically normal animals aged between 16 to 20 months. Anti-protease activity in the pretreatment-serum was tested negative in ELISA test. For the purpose of skin thickness measurement and histopathological examination, the sheep's back was shaved to skin level with clippers. Skin was marked with colour pens to aid the accurate identification of injection and measurement sites.

The first experiment was designed to examine the possible pathological effects of
enzyme I or II in the sheep skin by intradermal injection over the range of 1μg/ml, 10μg/ml and 100 μg/ml. The second experiment was to study the toxicity effects of combined enzymes I and II at 100 μg/ml, 1 mg/ml and 10 mg/ml at each site. Six replicates of each test were done by intradermal injection with a total 100 μl replicate volume for each dose at the marked spots. Sterile saline (0.15 M) was used as a negative control. Immediately after injection, the skin thickness was measured with spring callipers. Skin thickness was subsequently measured after 2, 4, 24 and 48 hours at the injection site. The mean values and the standard errors were obtained for the Student's t-test.

4.2.10.2. Skin tissue

After the skin thickness measurement, skin punch biopsies were taken from each site after 2 hours and 24 hours, a 6 mm diameter of skin biopsy was removed under local anaesthesia. This was done by an experienced veterinary officer. The tissues were fixed in 10 % formalin and processed using the paraffin wax embedding procedure. Sectioning and staining of skin tissues are described in Appendix 5.

4.3. Results

4.3.1. The effect of wool on the growth and enzyme production of P. aeruginosa

Four different bacterial media, LB, TSB, LNM and MNM (Section 4.2.1) were used for comparison of growth and enzyme production, and the impact of the presence of sheep wool on this enzyme production. Wool possibly represents an important nutrient source for bacteria during fleece rot development.

The nutrient composition of the media varied from high in the case of LB, decreasing in order from TSB, LNM to MNM. Each medium was prepared
under three conditions with or without sterile wool (Section 4.2.1).

The quantity of total protease activity in culture supernatant was calculated by the proteolytic activity on the gelatine substrate as shown in Table 4.2. The results show that the yields of proteolytic enzymes were increased when the nutrient in the growth medium was higher, that is more activity was found in LB and TSB and less activity in LNM and MNM. Also, enzyme production was increased when more wool was added, that is there was more proteolytic activity in 5 % wool medium than the 1 % wool medium.

Since there was a difference in the enzyme activity between the media, the question then arises as to whether there was a difference in cell viability in the different cultures. The mean cfu from each bacterial culture was determined for the bacterial growth studies. Three replicate 10 µl aliquots from each bacterial culture prepared with and without wool were removed and spread onto LB agar. The cfu per microlitre from LB and TSB cultures show higher numbers than from LNM and MNM cultures (Table 4.3). There is a difference in the numbers of *P. aeruginosa* with respect to the amount of wool added to the media. The results of viable bacterial counts clearly indicated that the sterile wool added to the media did not affect the sterility of the bacterial cultures and that the increase of bacterial growth correlated with increasing yields of proteolytic enzymes. Thus, a difference in enzyme production was directly attributable to the presence of wool.
Table 4.2: Effect of wool on the protease production from different culture supernatant from difference media. The media used ranged from richly nutrition to minimal, and were supplemented as shown with autoclaved wool (w/v). The results are the mean hydrolysing unit ± S.E. of culture supernatant tested in triplicates. A hydrolysing unit was calculated as the proteolytic activity that hydrolyses 1 mm² of gelatine area.

<table>
<thead>
<tr>
<th></th>
<th>0% Wool</th>
<th>1% Wool</th>
<th>5% Wool</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>5.50 ± 0.2</td>
<td>6.94 ± 0.34</td>
<td>10.7 ± 0.25</td>
</tr>
<tr>
<td>TSB</td>
<td>3.01 ± 0.18</td>
<td>5.88 ± 0.12</td>
<td>8.30 ± 0.28</td>
</tr>
<tr>
<td>LNM</td>
<td>0.72 ± 0.06</td>
<td>2.80 ± 0.17</td>
<td>5.02 ± 0.19</td>
</tr>
<tr>
<td>MNM</td>
<td>0.16 ± 0</td>
<td>1.16 ± 0.07</td>
<td>1.81 ± 0.14</td>
</tr>
</tbody>
</table>

Table 4.3: Effect of increasing autoclaved wool supplement on the cell yields (cfu) in different culture media. The results are the mean cfu per μl of each bacterial culture which were serially diluted and plated in triplicate. ND: Not detected at 10⁻⁵ dilution. See Table 4.2 for abbreviations.
4.3.2. Identification of native and subunits of proteases

Since the enzyme protein bands were not easily visualised by Coomassive blue staining after electrophoresis, an overlayed zymogram was used to detect the presence of the purified enzymes. Culture supernatant and purified proteases were separated by native polyacrylamide gel (PAGE) as described in Appendix 4. The result was an overlayed zymogram which gave clear bands on the white background. Clear bands indicate the location of the proteases on the original PAGE gels as well as the enzyme activities.

The zymogram (Figure 4.1) indicated that *P. aeruginosa* culture supernatant contained several biological activities. At least two major protease isoenzyme bands can be identified by visualisation of the zymogram. Preliminary screening of all 12 cultures (LB, TSB, LNM and MNM with and without wool) showed the same enzyme patterns on zymograms. However for the same volume, the filtrate from cultures containing 5% wool showed larger bands than cultures containing 1% and no wool. This indicates an increase in levels of proteolytic activity in the cultures with a greater concentration of wool and organisms.

The first set of experiments was to determine the molecular weights of native enzymes. These were estimated by comparing zymogram profiles with relative molecular weight standards obtained from another identical PAGE gel. SDS PAGE was also used to confirm the molecular weights of standards and proteases. Protease I appears to have a molecular weight of 150 kDa and protease II of 50 kDa (Figure 4.1).

A second set of experiments was to determine the molecular sizes of the subunits of the enzymes. The purified proteases were treated with SDS and β-mercaptoethanol and run on SDS-PAGE (Appendix 4), which was then stained
with Coomassie blue. The relative molecular sizes of subunits from both proteases were identified. Protease I possessed 50 kDa subunits and protease II 32 kDa subunits (Figure 4.2). The difference in migration of protease I under the two conditions may be due to the fact that the native enzyme protein had a tertiary structure which could influence the migration so that it could move faster in the native gel.

The proteolytic enzyme activity also has been shown to be SDS resistant. Native proteases run on SDS-PAGE were still capable of hydrolysing overlayed with gelatine substrate. Both crude culture supernatant and purified proteases resulted in the same activity and position after running on a acrylamide gel with and without SDS incorporation, that is the addition of SDS did not alter the electrophoretic mobility (refer to Fig 4.1).
Figure 4.1: Zymogram of protease activity in gelatine agarose gel as detected by hydrolysis after 7% PAGE under nonreducing conditions with SDS. The enzymes were visualised by gelatine hydrolysis after the enzymes had been separated by electrophoresis in a polyacrylamide gel. Lane CS depicts two majors proteases with 150 kDa and 50 kDa. Lane I and II depict purified protease I and protease II after separation. High molecular weight standards in kDa are indicated on the left.
Figure 4.2: SDS-PAGE stained with Coomassie blue of crude culture supernatant (CS), purified protease I and II under reducing conditions (heat treated for 5 minutes in β-mercaptoethanol). Protease I shows a 50 kDa subunit and protease II shows a 32 kDa subunit. Purified proteases were prepared after being separated on PAGE, sonication and electroelution and reduction treatment. Molecular weight standards in kDa are indicated on the left.
4.3.3. Enzyme Activity

A semi quantitative measurement for enzyme activity after purification was carried out using gelatine hydrolysis. Table 4.4 suggested that both purified protease I and II showed similar substrate hydrolytic activity against gelatine. Similar results were also found when proteases were incubated with bovine serum albumin BSA (Figure 4.3).

The relative proteolytic activity of both purified proteases and dialysed culture supernatant on BSA was determined as described in Section 4.2.2. The absorption at 280 nm of the supernatant was measured as the amount of amino acid released from BSA by proteolytic activity. Preliminary experiments established the optimal condition for BSA hydrolysis reactions. The optimum conditions for both enzymes were 1 mg of substrate, 0.1 mg of enzyme, pH in the range of 7-8 and temperature 37 °C (results not shown). Using these conditions, the $K_m$ were determined. To obtain $K_m$ the Michaelis-Menten equation was applied.

$$v = V [S] + (K_m + [S])$$

For protease I, the $K_m$ is $0.9 \times 10^{-3} \text{M}$, for protease II the $K_m$ is $0.95 \times 10^{-3} \text{M}$ and for crude culture supernatant a value of $1 \times 10^{-3} \text{M}$ for enzyme activity was obtained. Figure 4.3 is a double reciprocal plot of $1/v$ vs $1/[S]$ over a range of different concentrations of BSA.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enz Activity (mm²/min)</th>
<th>Total Proteins (mg/ml)</th>
<th>S.A. (mm²/min/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>2.35 ± 0.08</td>
<td>14.8</td>
<td>(10.69 ± 0.36) 10⁻³</td>
</tr>
<tr>
<td>I</td>
<td>1.77 ± 0.09</td>
<td>14.0</td>
<td>(8.43 ± 0.45) 10⁻³</td>
</tr>
<tr>
<td>II</td>
<td>2.19 ± 0.07</td>
<td>16</td>
<td>(9.15 ± 0.32) 10⁻³</td>
</tr>
</tbody>
</table>

Table 4.4: The specific activity (SA) of dialysed culture supernatant (CS), proteases I and II using gel diffusion method. Duplicates of 15 ul of enzyme (known concentrations) was pipetted into wells of gelatine agar plates (4 mm diameter well). After 3 hour incubation and ammonium saturation, clearing area was determined. Results indicate the mean ± S.E.
Figure 4.3: Reciprocal plot of the kinetics of proteases and culture supernatant. The plot is of $1/v$ vs $1/[S]$, where $[S]$ was a series of different concentration ($10^{-2}$ M) of BSA. Results show a $K_m$ for CS is $1 \times 10^{-3}$M, for protease I is $0.90 \times 10^{-3}$M and protease II is $0.95 \times 10^{-3}$M.
4.3.4. Protease inhibition studies

To investigate the effect of various inhibitors on the enzyme activity, purified enzymes and culture supernatant were pre-incubated with inhibitors before gelatine diffusion assays. Table 4.5a and 4.5b show the percentage of proteolytic activity remaining after the \textit{P. aeruginosa} enzymes were treated with two different concentrations of the same inhibitor. The activity of enzymes without inhibitors was considered to be 100%. The buffer itself was used as a negative control. The results indicate that the inhibition of enzyme activity was related to the dose of inhibitors. The enzyme activity of both protease I and II was markedly inhibited by the presence of 20 mM EGTA and EDTA (inhibitors of metalloproteases). The activity of the proteases were slightly inhibited by PMSF (an active site, irreversible inhibitor of serine proteases). Copper ions (inhibitor of Ca or Zn cation cofactors) inhibited proteases I and II by replacing Ca with Cu. Other common inhibitors such as benzamidine, lysozyme, DNases, RNase and proteinase-K did not affect protease I and II activity. In fact, pure proteinase-K increased the proteolytic activity. This possibly was due to the effect of additional proteolysis, i.e., proteinase can also hydrolyse gelatine, or a synergistic effect of proteinase-K hydrolysing the gelatine substrate, together with the proteases present in the assays.

4.3.5. Antigenicity studies

Routine immunisation (Chapter 2, Section 2.3) of purified protease I and II into rabbits (total 100 \textmu g/dose) and sheep (1mg/dose) did not induce a high antibody response in either rabbits or sheep. The ELISA O.D values (Figure 4.4a and 4.4b) indicate the antibody titre in sera from immunised sheep was lower than that obtained by immunising rabbits. Whether low responsiveness results from the dose or frequency of protease immunisation, or an inability to induce high serological response is uncertain.
<table>
<thead>
<tr>
<th></th>
<th>final concentration</th>
<th>II</th>
<th>I</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA</td>
<td>1 mM</td>
<td>78.2</td>
<td>86.7</td>
<td>77</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM</td>
<td>69</td>
<td>60</td>
<td>74.7</td>
</tr>
<tr>
<td>PMSF</td>
<td>1 mM</td>
<td>98.3</td>
<td>95.8</td>
<td>94.1</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>1 mM</td>
<td>82</td>
<td>90.9</td>
<td>72</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>1 mg/ml</td>
<td>103</td>
<td>99.3</td>
<td>101.1</td>
</tr>
<tr>
<td>DNase</td>
<td>1 mg/ml</td>
<td>102</td>
<td>106</td>
<td>90</td>
</tr>
<tr>
<td>RNase</td>
<td>1 mg/ml</td>
<td>100</td>
<td>102.7</td>
<td>96.5</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1 mg/ml</td>
<td>100</td>
<td>95.1</td>
<td>100</td>
</tr>
<tr>
<td>Protease K</td>
<td>1 mg/ml</td>
<td>153</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>none</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4.5(a): Percentage proteolytic activity remaining after proteases received inhibitor treatment at low concentration, relative to controls. These results activity of enzymes after 2 hour incubation with inhibitors at the indicated concentration, measured by the hydrolysis of gelatine in diffusion gels. Values > 100% represent no inhibition. Data were collected from gel diffusion. Mean of duplicate tests shown. Abbreviations: see Table 4.1.
<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>II</th>
<th>I</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA</td>
<td>20 mM</td>
<td>38.8</td>
<td>42.8</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>20 mM</td>
<td>27.7</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>PMSF</td>
<td>20 mM</td>
<td>83</td>
<td>71</td>
<td>70</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>20 mM</td>
<td>33</td>
<td>35.7</td>
<td>35.2</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>5 mg/ml</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>DNase</td>
<td>5 mg/ml</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>RNase</td>
<td>5 mg/ml</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>5 mg/ml</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Protease K</td>
<td>5 mg/ml</td>
<td>194</td>
<td>214</td>
<td>205</td>
</tr>
<tr>
<td>Control</td>
<td>none</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4.5(b): Percentage proteolytic activity remaining after protease received inhibitor treatment at higher concentration. See Table 4.5(a) for Abbreviations.
Figure 4.4a: Serological response in 2 rabbits after proteases (I & II) immunisation. Results represent the absorbance measured at 492 nm (ELISA O.D. ± S.E.) for anti-protease antibody in the preimmunised serum (open bars) and serum collected one week after the third immunisation (solid bars). Pure protease I, protease II and culture supernatant (CS) were used as antigens, and samples were tested in parallel together on the same plate.
Figure 4.4b: Anti-protease and culture supernatant serum antibody response of 2 sheep before immunisation (open bars) and one week after the third immunisation (solid bars) with dialysed culture supernatant or proteases I & II used as antigens on the same plate, assessed by ELISA and recorded as O.D at 492 nm ± S.E.
4.3.6. Antiserum neutralization of proteases

Antisera from two rabbits were tested to see if the animal anti-protease antibodies could inhibit enzyme activity. Assays comprised pre-treatment of crude culture supernatant for 3 hours at 37°C with serial dilutions of either rabbit or sheep sera. Enzyme neutralization by antibody was determined by measuring proteolytic activity in gelatine agar plates after 3 hours at 37°C. Preliminary screening of the antisera showed similar results for the sera from rabbit 1 and 2. Similarly, sera from sheep 1 and 2 gave similar results. The following results are from one of the representative animals from each group.

Figure 4.5.A documents the remaining proteolytic activity of crude culture supernatant reacted with various rabbit antibody concentrations using antiserum from a protease-immunised animal. The progressive increase of clearing areas with decreasing antibody concentration is apparent (plate A) and there was no different in clearing area as seen in the control (plate B).

When crude culture supernatant was incubated with sheep serum, similar results were noted. Table 4.6 summarises the remaining proteolytic activities when rabbit or sheep serum had been incorporated in the assays. 100% designate the enzymes as not being neutralised. The results from protease radial gel diffusion indicated that the residual proteolytic activity was generally much higher in the presence of sheep serum compared to rabbit serum. ELISA and Western blots were performed to determine whether there are less anti-protease antibodies produced by sheep after immunisation compared to rabbits.
Figure 4.5: Representative radial gel diffusion analysis of rabbit anti-protease antiserum incubated with crude *P. aeruginosa* culture supernatant. Plate (B) is the control in which mixtures of culture supernatant were incubated for 3 hours, 37°C with PBS. Plate (A) was prepared with identical culture supernatant content but rabbit sera from animals immunised with proteases were introduced at different concentrations (final serum concentration) as indicated.

Abbreviations: Ab: antibody only; CS: crude culture supernatant, PBS: phosphate buffer only.
Table 4.6: Remaining activities of culture supernatant proteolytic enzymes in culture supernatant after antibody neutralisation. The results were determined by radial gel diffusion using mixtures of serial dilutions of rabbit/sheep serum with constant amount of crude culture supernatant and incubation for 3 hours at 37°C. The antiserum was derived from protease-immunised animals. The control was crude culture supernatant and PBS and contained 100% activity.

<table>
<thead>
<tr>
<th>Concentration of serum (vol/vol)</th>
<th>Rabbit</th>
<th>Sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>20%</td>
<td>35%</td>
</tr>
<tr>
<td>25%</td>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td>12.5%</td>
<td>80%</td>
<td>90%</td>
</tr>
<tr>
<td>0</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
4.3.7. Immunoblots

The antibody response of sheep and rabbits immunised with protease I and II mixture was also studied in Western blots. To test the specificity of binding to protease subunits, both protease I and II subunits were used for screening against rabbit and sheep antisera. Preliminary Western blots of native enzymes which had been separated on native PAGE gels did not show strong signals which could be visualised. Further experiments on enzyme separated under reducing condition, ie., on SDS-PAGE with β-mercaptoethanol treatment (Appendix 4), however showed a better results. On each lane, 10 µg of treated culture supernatant or purified enzyme were run before transfer to nitrocellulose membrane. As shown in Figure 4.6a rabbit antibodies detected more than two components in the culture supernatant. Those bands were mainly located between 50-32 kDa; the intensity of 50 kDa indicates protease I was the stronger protein being recognised by rabbit serum. This was confirmed in the next lane which contained the purified protease I. However, an identical amount of protease II loaded in lane 3 did not show the specificity at 32 kDa as expected. Instead, there was a stronger band at 50 kDa and the 32 kDa appeared very faint. This suggested that a) protease I could be more immunogenic than protease II when presented to rabbits or b) the cross reactivity in the protease II lane could be the result of cross contamination with a small amount of protease I during the antigen preparation process and it could not be seen on the SDS PAGE gel.

Similarly with the sheep antisera, there was a stronger reaction to protease I than protease II (Figure 4.6b).
Figure 4.6a: Immunoblot (L) and the Coomassie blue stained SDS PAGE gel (R) from which the blot was made after transfer and blocking, the blot was probed with antisera from rabbit immunised with protease I and II. Lane CS: culture supernatant indicates antibody binding to several components between 50 - 32 kDa but strongest with the 50 kDa band; lane I: purified protease I subunit indicates only one band at 50 kDa; lane II: purified protease II indicates antibody binding to two proteins with 50 kDa and 32 kDa. Molecular weight standards in kDa as indicated between SDS PAGE and immunoblot.
Figure 4.6b: Immunoblot of CS, protease I and II against immunised sheep serum produced more than 2 bands in the CS lane. 50 kDa and 32 kDa depicted in CS lane. A strong 50 kDa band appeared in protease II lane, but a very weak 32 kDa (due to non-photographic reproducibility) was seen in protease I.
4.3.8. Effects of proteases on the sheep skin

The effect of proteases on sheep skin was studied by measuring skin thickness after intradermal injection of various doses of enzyme mixture (0.01 mg, 0.1 mg and 1 mg per inoculum) versus a control saline injection. Skin thickness from two sheep was monitored at 0 time (immediately after the injection), 2 hrs, 4 hrs, 24 hrs and 48 hrs. The results are expressed as the percentage increase in skin thickness in each sheep at different time points (Figure 4.7). Similar results were obtained when different doses were applied, that is a linear increase of skin thickness was seen up to 4 hours after inoculation compared with the controls. The higher dose inoculum gave greater skin thickness, that is the 1 mg inoculum showed larger swelling than the 0.1 mg and 0.01 mg. Two days after the inoculation, the skin thickness was returning to normal. However, higher doses may require longer to return to normal because, after 48 hours, the skin thickness was still relatively thicker than the smaller doses. The results suggested that proteases are active in sheep skin.

Figure 4.7 shows that proteases cause some effects in sheep skin thickness as early as 2 hours after injection \((p<0.05)\) and sequential biopsies after 2 hours (Figure 4.8) demonstrated infiltration of polymorphonuclear leucocytes and occasionally mononuclear cells into the dermis. (Pathological work was performed by Dr Ron Gogolewski) It was found that protease I or II alone did not have any impact on tissue lesion while the mixture of the two proteases produced a more haemorrhagic lesion and oedema in the dermis. Formation of thrombosis and degeneration of blood vessels in the dermis were found when this enzyme mixture was increased up to 1mg inoculum. The pathological findings were similar at 24 hours in both sheep.

The results also indicate that proteases from \(P. aeruginosa\) when injected together
are tissue damaging and capable of disrupting the skin layer. In natural infections with *P. aeruginosa* these enzymes may therefore injure the skin barrier early in infection. Table 4.7 summarises the pathological conditions of all the skin biopsies that were collected from two sheep.

<table>
<thead>
<tr>
<th>Enzyme inoculated</th>
<th>Quantity</th>
<th>Pathological Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease I</td>
<td>1 µg</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>10 µg</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>100 µg</td>
<td>None</td>
</tr>
<tr>
<td>Protease II</td>
<td>1 µg</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>10 µg</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>100 µg</td>
<td>None</td>
</tr>
<tr>
<td>Protease I &amp; II</td>
<td>100 µg</td>
<td>Mild haemorrhagic, oedema</td>
</tr>
<tr>
<td></td>
<td>1 mg</td>
<td>haemorrhagic, oedema,</td>
</tr>
<tr>
<td></td>
<td>10 mg</td>
<td>haemorrhagic, oedema and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>neutrophil infiltration</td>
</tr>
</tbody>
</table>

Table 4.7: Summaries of pathological appearance from skin biopsies under light microscope. None indicates no damage. Haemorrhagic indicates the accumulation of red blood cell after the protease inoculation. Oedema appears as abnormal swelling of the skin tissues.
Figure 4.7: Increase of skin thickness in 2 sheep after intradermal inoculation with three different doses of protease mixture (100μl/inoculation) at different sites. Percentage increases relative to the controls (sterile saline only) from six sites.

- - - : 1 mg/ml protease I and II (combined in equal concentration)
- - - : 0.1 mg/ml protease I and II
- - - : 0.01 mg/ml protease I and II

*: Control (saline) are shown.
Figure 4.8: Light micrographs of sheep skin tissue (vertical section) after intradermal inoculation with 1 mg proteases (I & II) after 2 hr (C) and 24 hr (D) compared with the controls after 2 hr (A) and 24 hr (B). Skin tissues of both control and test biopsies were collected from the same sheep. As seen in Figure D the proteases induced a considerable increase in neutrophil infiltration at the dermis as well as the epidermis layer as shown with arrows; with haemorrhage is showing at the top right corner. Similar pathological appearance was also seen in figure C but with less intensity. The saline however did not seem to induce any response (X 25, Haematoxylin and Eosin staining).
4.4. Discussion

Margaretten et al. (1961) were the first to note that *P. aeruginosa* can produce proteolytic enzymes which could cause extensive vascular lesions in humans with cases of *Pseudomonas* septicaemia. They suggested that the elastolysis of arteries was possibly due to elastase activity. It is well known that sheep skin is also composed of collagen and elastin fibres (Mitchell et al., 1985). Thus, skin is possibly a good source of nutrients for elastase activity.

*P. aeruginosa* infections are frequently found in burned skin wounds in human (Nathan et al., 1973). According to Carney et al. (1973), proteolytic enzymes contribute towards bacterial invasion of burnt skin in mice, with bacterial proliferation on the burned skin surfaces proceeded by their infiltration and multiplication in the hypodermis. Kawaharajo et al. (1975a, b) confirmed that proteolytic enzymes could also cause haemorrhage and ulcerating lesions in the skin of rabbits similar to those in humans. *P. aeruginosa* was able to cause corneal destruction in humans using contact lenses (Sugar, 1994). Works by Barletta et al. (1996) further confirmed that proteases from *P. aeruginosa* accelerate the destruction of corneas in rabbit models. In contrast, by adding protease inhibitors to the *P. aeruginosa* culture supernatant, this destruction was delayed (Barletta et al., 1996). Although there are many other studies of *P. aeruginosa* proteolytic enzymes either in humans or animals (Mull and Callahan, 1965; Diener et al., 1973; Holder and Haidaris, 1979; Woods et al., 1982), the role of proteolytic enzymes in the sheep environment is still not clear at the present time.

The nutrient requirements of *P. aeruginosa* are extremely simple; the bacterium can grow in almost any environment and requires minimum carbon sources and energy for growth (van der Kooij et al., 1982). However, nutrients can influence enzyme production. For example, *P. aeruginosa* secretes exotoxin A which was
first described as a virulence factor by Liu (1966b). Morihara et al. (1981) validated the properties of the exotoxin and confirmed that *P. aeruginosa* exotoxin A is not stable in the presence of protease. In addition, culture medium for exotoxin A production requires many supplements, such as glycerol, nucleic acid and monosodium glutamate.

The production of proteases from *P. aeruginosa* in different culture media (with and without wool) was examined in this study. The results clearly confirmed that with *P. aeruginosa*, the more nutrients available the greater the protease production (Tables 4.2 and 4.3). The results also indicate that proteolytic enzymes could be produced in high concentrations from media containing no glucose.

In the preliminary experiments using skin washing it was found that proteases could be recovered from the skin and fleece 24, 48 and 72 hours following skin infection with live *P. aeruginosa*, confirming that proteases can co-exist in the fleece during *P. aeruginosa* infection (results not shown because proteases had identical patterns as shown in Fig 4.1). This result supports the hypothesis that colonisation of *P. aeruginosa* in the fleece results in the production of proteolytic enzymes which may contribute to the fleece rot dermatitis condition in sheep.

The relationship between sheep wool and enzyme production *in vitro* was assessed. This is the first report of the influence of wool on the levels of extracellular proteases. As more wool was added the protease production increased over 18 hours of incubation time in culture flask suggesting that sheep wool could be acting as a stimulator for enzyme production. Protease production was poor when *P. aeruginosa* was cultured in very low nutrient medium such as MNM, but when wool was added the protease production was markedly
increased. When bacterial numbers were counted, it became apparent that the addition of wool, while increasing enzyme levels, also enhanced bacterial growth, and that the higher bacterial number was probably the cause of the higher enzyme levels. However the clear demonstration of protease production suggests its involvement in fleece rot development.

The characteristic pathological feature of fleece rot includes some damage to the wool. It is proposed, firstly, that the production of *P. aeruginosa* proteases in the fleece may provide favourable conditions for this bacterium to grow better in the fleece and, secondly wool wax, sweat and wool fibre proteins possibly are the primary sources of nutrients for the bacteria in the fleece environment. Thus, through their existence of these nutrients in the fleece, *P. aeruginosa* thrives on the nutrient available in its surroundings and secretes products such as extracellular enzymes which appear to cause further damage to the skin.

Two isoenzymes of separated proteins can be identified using gelatine hydrolysis from the *P. aeruginosa* cultures and from washing of the skin of infected sheep. Crude culture supernatant produced cleared bands at approximately 150 kDa and 50 kDa under native conditions. The native enzymes were not readily detected by Coomassie blue stain in the PAGE gel. SDS-PAGE of denatured proteases detected subunits with a molecular weight of 50 kDa for protease I and 32 kDa for protease II.

When native enzymes were injected into rabbits and sheep, antibodies could be detected by ELISA and Western blots indicating that the enzymes are immunogenic. However, the antisera preparations from both rabbits and sheep showed that protease I was more antigenic than protease II. The antibody inhibition assay was also undertaken in which the enzyme activity was
inhibited/neutralised by antisera from both immunised rabbits and sheep. This suggests that proteases could trigger the antibody response against the enzymatic sites when it was introduced to the animals.

The data indicate the two proteases were similar in their affinity for substrates, with a pH optimum of pH7-8, and a requirement for calcium as a cofactor. The differential level of inhibition by proteinase inhibitors, such as EGTA, EDTA, PMSF and CuSO₄ is not significant. Protease I could be differentiated from protease II by β-mercaptoethanol treatment.

The question arises as to how the enzymes recovered in this study relate to the enzymes purified from *P. aeruginosa* in other studies. *P. aeruginosa* elastase has been identified but its molecular weight varies in different reports depending on the technique used. Using SDS-PAGE, the molecular weight of elastase was initially estimated to be 32-34 kDa (Jensen et al., 1980). In 1989, Fukushima succeeded in isolating a clone containing a gene for *P. aeruginosa* elastase. The mature elastase consists of 301 amino acids with a molecular mass of 32 kDa (Fukushima et al., 1989). The enzyme also exists as an inactive precursor with a molecular weight of about 36 kDa associated with 20 kDa proteins and is found in the periplasmic membrane (Kessler and Safrin, 1988). Because of the similarity of molecular weight and its activity, it is possible that the protease II identified in this study, with a molecular weight of 30-32 kDa subunit on SDS-PAGE, is elastase.

The molecular weight of 48.4 kDa described by Inoue et al. (1963) as a proteinase with its optimal pH for hydrolysis of various protein substrates at pH7-9 was named alkaline proteinase by Morihara (1963). In this study, the molecular weight of protease I was estimated at 50 kDa which has an optimum pH at 8.
Thus it is possible that protease I is alkaline proteinase.

Doring et al. (1981) was the first to report that *P. aeruginosa* elastase could degrade human immunoglobulin G (cited by Holder, 1985). Later Holder and Wheeler (1984) demonstrated that mouse plasma IgG declined after *P. aeruginosa* infection. Evidence has been presented to indicate that elastase and alkaline proteinase participate in the pathogenicity of *P. aeruginosa* infection in humans. Both enzymes have been found to associate with the inactivation of blood plasma $\alpha_1$-proteinase inhibitor and $\alpha_2$-macroglobulin (Heimburger, 1974), and complement components (Schultz and Miller, 1974) in humans.

A major question which arises is whether the proteases produced, possibly elastase and alkaline proteinase, play any role in the skin damage found in fleece rot, because fleece rot can be diagnosed by its characteristic skin lesions and superficial colonisation with *P. aeruginosa*. It is well known that invasion of burnt skin in animal experiments is accelerated by proteases (Carney et al., 1973) and exotoxin (Saelinger et al., 1977). Phospholipase C was reported to be one of the virulence factors causing fleece rot dermatitis (Chin and Watts, 1988). In fleece rot, even when *P. aeruginosa* well established itself in the fleece and on the skin, it does not penetrate the skin further than the epidermis. Clearly, applying protease preparations onto the sheep skin is one way of directly investigating the effects of *P. aeruginosa* proteases. Hence in this study, the intradermal inoculation of purified proteases was performed and subsequent pathological changes in the skin studied. Preliminary results of injection with protease I or II alone did not provide any encouraging results, i.e., histologically, no lesions were characterised when either protease (but not both) was injected into the skin at a concentration of 100 $\mu$g/dose. By contrast, damage could be detected when a mixture of both proteases were given, i.e., a typical mild cellular infiltration
would appear that naturally occurring *P. aeruginosa* could produce both protease I and II simultaneously, and that they could act together to stimulate fleece rot development.

While encouraging correlations have been observed, the available evidence is still insufficient to decide whether these enzymes appear to be candidates for mediating the haemorrhagic and ulcerating lesions in the skin of sheep.

Three conclusions can be drawn from this chapter:

1. Extracellular protease production by *P. aeruginosa* could be increased by the presence of sheep wool *in vitro*. Thus, the fleece environment could influence bacterial growth and the release of various enzymes which could facilitate the mechanical breaching of the skin leading to fleece rot formation.

2. Evidence has been presented of the relationship between the proteases and skin inflammation which strongly suggests that *P. aeruginosa* proteases are important contributors to fleece rot dermatitis.

3. Further study on quantification of proteases produced by *P. aeruginosa* on sheep skin, and of antibody response against proteases in affected sheep are recommended.
Chapter 5

CHARACTERISTICS OF MEMBRANE PROTEINS FROM

PSEUDOMONAS AERUGINOSA AND PSEUDOMONAS MALTOPHILA

5.1. Introduction

If P. aeruginosa does breach the skin barrier through the activity of its proteases then systemic antibodies and/or skin localised antibodies against outer membrane proteins of P. aeruginosa may prove effective as a defence mechanism in sheep. Outer membrane proteins of P. aeruginosa have been reported to be a good vaccine candidate in P. aeruginosa infected mice (von Specht et al., 1995), in rats (Fox et al., 1994) and in humans (von Specht et al., 1994, Abstract citation).

Pseudomonas species are gram negative bacteria. The cell envelope includes two separate bilayers called the inner and outer membrane. The outer membrane is presumed to have fewer proteins than the inner membrane with a distribution that varies from random to organised in closely packed patches. Traditionally, the outer membrane proteins (OMV) could be separated from inner membrane proteins (IMP) by various methods after French pressure cell treatment which breaks the cell membrane into small fragments (Thornley et al., 1973) or by cell lysis (Miura and Mizushima, 1968). Complete solubilisation of the inner membrane proteins either by Triton X-100 in the presence of Mg\(^{2+}\) (Schnaitman, 1971) or by Sarkosyl (Filip et al., 1973) has been reported. Sucrose gradient centrifugation at high speed can then separate the inner from outer membrane proteins. Recently, the organic phase partitioning method has been used successfully to extract integral hydrophobic membrane tissues and associated proteins (Brunden et al., 1987). This procedure was also utilised.

While P. aeruginosa is the major focus for study, other reports (London and
Griffith, 1984) have suggested the possible involvement of many other Pseudomonas species and it is conceivable that the early stages of fleece rot formation may be caused by the synergistic interaction of Pseudomonas complexes. MacDiarmid and Burrell (1986) specially mentioned the involvement of *P. maltophilia* in association with *P. aeruginosa* in fleece rot. *P. maltophilia* is the second most common bacterium isolated in human clinical laboratory studies after *P. aeruginosa* (Gilardi, 1991). Thus the method for outer membrane protein preparation was tested on *P. maltophilia* as well as *P. aeruginosa*.

Subsequently experiments were designed to study the possibility of outer membrane proteins conferring protective immunity. Firstly, possible induction of systemic antibodies in sheep which were genetically selected as fleece rot resistant as compared to susceptible groups, was ascertained. Secondly, the antigenicity of purified outer membrane proteins (from *P. aeruginosa*) in sheep and rabbits was determined. These proteins were prepared using chloroform-methanol extraction and sucrose gradient centrifugation.

5.2. Materials and Methods

5.2.1 Source and growth of *P. maltophilia* and *P. aeruginosa*.

Both *P. aeruginosa* and *P. maltophilia* were originally isolated from fleece rot lesions in sheep with the *P. maltophilia* being kindly donated by Dr. Calvin J. London. The bacteria were grown in 250 ml Luria Bertani broth in a 2 litre flask which was shaken at 150 g overnight at 37 °C. Bacteria were harvested by centrifugation at 10,000 g in a Sorvall RC-5 ultracentrifuge for 10 minutes at 4 °C and washed twice with 50 ml sterile saline. Yields of bacteria was usually about 2 - 3g from each flask.
5.2.2. Preparation of membrane fractions

Bacteria were washed and cell pellets were resuspended at 1g wet weight in 5 ml of extraction buffer 10 mM Hepes pH7.2 (Appendix 2) containing 10% (w/v) sucrose and 2 mM phenyl methyl sulphonyl fluoride. They were then passed twice through a french pressure cell (Anerpac) at 12,000 psi. Cell debris and unbroken cells were removed by centrifugation at 15,000 g for 10 minutes. The supernatant was then carefully layered onto 5 ml of 20% (w/v) sucrose in Hepes in a 60 Ti tube (Beckman centrifuge). Ultracentrifugation for 90 minutes at 35,000 g resulted in the formation of gelatinous membrane complexes (35 kp) at the bottom of the tubes. These complexes contain the inner and outer membrane protein fractions.

a. Sarkosyl or sodium lauryl sarcosinate extraction

Inner membrane proteins IMP were extracted from the 35 kp pellet with Sarkosyl in 10 mM Hepes pH7.2 (2% [w/v]). The extract was then loaded onto a discontinuous sucrose gradient composed of 5 ml layer of 20, 40, and 60% (w/v) sucrose in 10 mM Hepes pH7.2 in a 60 Ti tube. The samples were centrifuged for 90 minutes at 35,000 g at 4 °C in a Beckman ultracentrifuge. Sarkosyl solubilised IMP remained at the top of the gradient while outer membrane proteins (OMV) band was at the 40-60% sucrose interface. Both inner and outer membrane fractions were removed with a sterile Pasteur pipette and subsequently dialysed against 10 mM Hepes pH7.2 overnight at 4 °C.

b. Organic phase partitioning with chloroform-methanol

The method of Brunden et al. (1987) was adopted in this chapter. 4% (w/v) sodium dodecyl sulphate (Appendix 2) in 10 mM Hepes pH7.2 was added to the 35 kp pellet (containing IMP and OMV) and the mixture was sonicated 4 times with 15 second pulses using a sonicate probe. The brownish sonicate was then
mixed with an equal volume of chloroform-methanol (2:1 v/v) and vortexed vigorously for 2-4 minutes followed by centrifugation at 10,000 g in 60 Ti tubes resulting in the formation of a white milky band between the organic and aqueous phase. This interface band (chloroform-methanol extract proteins or CMx proteins) was removed and solubilised in an equal volume of glacial acetic acid. The pH was adjusted upwards by dialysing against several changes of 10 mM Hepes pH7.2 before proteins were used for further characterisation.

5.2.3. Production of anti CMx protein-antisera

Rabbits were immunised against the interface proteins (CMx proteins). Two rabbits were immunised against CMx proteins of *P. maltophilia* while two rabbits were immunised against CMx proteins of *P. aeruginosa*. In both cases, a suspension of an equal volume of complete Freund's adjuvant and CMx proteins containing 1 mg proteins were intramuscularly injected into the rabbits at six sites. On day 14, a similar dose of CMx proteins emulsified in an equal volume of incomplete Freund's adjuvant was administered subcutaneously. After another 14 days, final injection of the incomplete Freund's adjuvant-CMx proteins was given intramuscularly. Blood was collected from the marginal ear vein before immunisation and one week after the final injection sera from clotted blood were clarified after centrifugation and stored at -20 °C.

5.2.4. Outer membrane protein immunisation

Merino sheep were bred for resistance and susceptibility to fleece rot at Trangie research station and transferred to Glenfield station (near Sydney). Three sheep from each group were used for this study. Outer membrane proteins (OMV) were obtained using the Sarkosyl extraction method. Each sheep was immunised with 2 mg of purified outer membrane proteins in 1 ml of 10 mM Hepes pH7.2 buffer emulsified in an equal volume of complete Freund's adjuvant. The
emulsion was injected intradermally in 10 sites along the back. Two weeks later, 2 mg of proteins emulsified in incomplete Freund's adjuvant was injected into the same sites, and this procedure repeated every two weeks thereafter for 6 weeks. Blood was collected before the immunisation and every week after the injection. Preliminary ELISA indicated whether the last immunisation was required. This was done by using the last bleed for ELISA against *P. aeruginosa* whole cell antigen. If the ELISA optical density (ELISA O.D.) reached the optimum limit, then the sheep no longer required more immunisation. Sera from each sheep were used in ELISA and immunoblots for detection of serological response using whole cells lysate (WC), CMx, IMP and OMV as antigens.

5.2.5. SDS-PAGE

Protein contents in all membrane fractions were estimated by the modified Lowry procedure (Markwell *et al*., 1978) before being subjected to the SDS-PAGE. All samples including bacterial whole cells were treated with reducing mixture (Appendix 2) containing β-mercaptoethanol and were heated at 100 °C for 5 minutes. 10 μl from each of these suspensions was loaded onto 12% SDS-PAGE. Low molecular weight standards were also treated similarly and loaded onto each gel. Electrophoresis was carried out at a constant voltage of 150 volts until the bromophenol blue tracking dye had moved to the bottom of the gel (Appendix 4).

5.2.6. Immunoblots

Proteins were electrophoretically transferred from the SDS-PAGE to nitrocellulose membrane in a Hoefer trans-blot apparatus under conditions essentially similar to those described in Appendix 4. Immunoblots were also incubated in high salt tris-saline-Tween20 pH 8.9 (Appendix 2) overnight at room temperature to block the non specific binding sites. Blots were washed in normal
tris-saline-tween 20 pH7.2 (TS-Tw20) buffer and further blocked in 1.5% (w/v) gelatine with TS-Tw 20 for one hour. Immunoblots were reacted with rabbit or sheep antisera diluted in normal TS-Tw20 for two hours at room temperature on a shaker. Rabbit or sheep antibodies, which bound to bacterial proteins on the nitrocellulose membrane, were located by reaction with a second antibody conjugated to horse radish peroxidase (HRP-ARIMab) followed by diamino benzidine (DAB)/H₂O₂ substrate.

5.2.7. ELISA

Whole cells of *P. aeruginosa* and *P. maltophilia* were washed and quantified to 0.04 A650 unit (Chapter 2). Triplicate samples of 50 μl were coupled onto the microtitre plate; all other soluble antigens, ie., IMP, OMV and CMx (20 μg/ml) in coating buffer (Appendix 2) were also coupled onto the same plate (Chapter 2). An ELISA procedure was carried out as described in Chapter 2.

5.3. Results

5.3.1. SDS-PAGE analysis

The Coomassie blue stained SDS-PAGE profile of whole cell WC lysates, Sarkosyl extracted IMP, OMV resolved by sucrose gradient centrifugation and interface proteins CMx of both *P. maltophilia* and *P. aeruginosa* are depicted in figure 5.1a and 5.1b.

Figure 5.1a represents the SDS-PAGE with Coomassie blue stain obtained from *P. aeruginosa*. The lane containing polypeptides in WC lysate showed similar patterns to polypeptides in the IMP lane. The inner membrane polypeptides extracted by Sarkosyl were very heterogenous in size distribution while the outer membrane polypeptides could be divided into six major bands corresponding to...
67, 46, 44, 39, 25 and 21 kDa respectively. The six major CMx protein bands were identical to the polypeptides present in the OMV fraction resolved by sucrose gradient centrifugation.

Figure 5.1b shows the Coomassie blue staining patterns obtained from \textit{P. maltophila}. Sarkosyl extracted a large number of polypeptides from the IMP fraction. The OMV fraction included only four major polypeptides corresponding to 87, 43, 24 and 23 kDa. These four major bands were identical to the polypeptide in the CMx protein preparation. The chloroform-methanol extraction appeared to enhance the recovery of some polypeptides compared to the sucrose separations, i.e., with the same loading in both lanes, the minor polypeptides in CMx showed less intensity than those in OMV lane.

In membrane protein preparations for both bacteria, the protein content of OMV in CMx fractions were consistently better (4 - 4.5 mg g$^{-1}$ wet weight of the cell) than that obtained by Sarkosyl extraction (2 - 2.5 mg g$^{-1}$ wet weight of the cells).
Figure 5.1: SDS-PAGE analysis of bacterial membrane protein preparations (Coomassie blue stain). Figure (1a) represents results from *P. aeruginosa* and (1b) from *P. maltophila*. Lanes labelled IMP (inner membrane proteins) and OMV (outer membrane proteins) were from the Sarkosyl method and CMx proteins were outer membrane proteins from the chloroform-methanol method. Whole cell (WC) lysate indicates the total proteins released from bacterial whole cell. Molecular weight standards (in kilodalton) are indicated on the left of each gel.
5.3.2. ELISA reactivity to membrane proteins using rabbit antisera

Rabbits were immunised against CMx proteins from *P. aeruginosa* (rabbit PA) and *P. maltophilia* (rabbit MALTO). Tests for the serological reactivity of Sarkosyl extracted IMP, OMV and CMx proteins from both preparations of *P. aeruginosa* and *P. maltophilia* against these rabbit antisera were carried out. Bacteria whole cells were also used in the ELISA as antigen to see if the extracted membrane proteins occupy the bacterial surface in situ. *P. maltophilia* and *P. aeruginosa* were fixed with tris-saline-methanol (TS-methanol, Appendix 2) and then coated onto the wells (Chapter 2); other antigens include IMP, OMV and CMx. The results obtained from ELISA (Figure 5.2a, 5.2b) show that both pre-immunised sera were unable to bind to any antigens. However, a high antibody titre was obtained one week after the third immunisation with a higher level of reactivity against both OMV and CMx, and a lower activity against IMP and the WC lysate. Figure 5.2a showed that using the same concentration of antigens in the same assay, the reactivity was lower against IMP extracted from *P. maltophilia* than to CMx preparations from this bacterium. However, this was not so obvious for *P. aeruginosa*. Therefore, the data in both Figure 5.2a and 5.2b show that both rabbit antibodies are directed against outer membrane proteins.

Taking all the above results together suggests that: (1) the outer membrane proteins are immunogenic and (2) both outer membrane proteins prepared by Sarkosyl method and chloroform-methanol display similar patterns of reactivity against rabbit antibodies.
Figure 5.2a: ELISA for detection of antibody levels in the sera of rabbit immunised with CMx proteins from P. maltophilia against different membrane protein preparations. ELISA antibody activity is detectable by the 3rd immunisation with CMx proteins (black bars) and the pre-immunised serum (grey bars) showing only the background level. Data are mean absorbance measured at 492 nm ± standard error of triplicate in each bleed. Abbreviations: O.D.: optical density; WC: P. maltophilia whole cell; IMP: inner membrane proteins; OMV: outer membrane proteins and CMx: outer membrane proteins from chloroform-methanol extraction.
Figure 5.2b: ELISA for detection of antibody levels of rabbit anti-*P. aeruginosa* CMx against different membrane preparations from *P. aeruginosa*, prior to CMx immunisation (grey bars) and after 3rd immunisation (black bars). Data are mean absorbance measured at 492 nm ± standard error of triplicate in each bleed. See figure 5.2a for abbreviations.
5.3.3. ELISA reactivity to membrane proteins using sheep antisera

ELISA analysis using sera demonstrated that all sheep responded to all membrane protein antigens of *P. aeruginosa*. Even though sheep were immunised with OMV, sera were also tested for activity against IMP and WC of *P. aeruginosa*. Data showed that sheep antibodies had a high binding to all antigens after one week (green bars) and two weeks (blue bars) of the 3rd immunisation compared with the pre-immunised serum (red bars) as shown in Figure 5.3a, 5.3b and 5.3c. The IMP (Figure 5.3b) displayed high reactivity with sheep antibodies which indicates that the OMV proteins prepared by the Sarkosyl method were either contaminated with IMP proteins or that some proteins are common in epitopes when presented to the sheep antibodies. Overall, the genetically resistant sheep had a greater serological response to outer membrane proteins of CMx preparations (Figure 5.3c) than the proteins in the IMP (Figure 5.3b) and WC lysate (Figure 5.3a). Although three sheep from each group were used, the ELISA analysis showed that the antibody titres from three resistant sheep immunised with outer membrane proteins were consistently higher than these obtained from the three susceptible sheep against all antigens. There was also variation between the sera in terms of collection time after immunisation in that sera collected one week (green bars) after the third immunisation had a lower ELISA O.D. compared to the sera which were collected after two weeks (blue bars) of the third immunisation. This is a good sign for designing a vaccine. One should keep in mind that a good vaccine must be able to develop a good immunologic memory in the host, that is, after the first immunisation; subsequently, a similar dose of the vaccine should induce a higher antibody level. A more effective protection should maintain high serum level of antibodies for a longer period and this is shown by the higher level of serum antibody in all sheep after two weeks of immunisation. These ELISA results are encouraging and it suggested, firstly that OMV are more antigenic than the IMP and WC preparation and secondly the antibody response is mounted towards the outer membrane proteins of *P. aeruginosa* in all sheep.
Figure 5.3a: ELISA for detection of antibody levels in sera from fleece rot resistant (U2, U3, U4) and fleece rot susceptible (D9, D10, D11) sheep to *P. aeruginosa* whole lysate (WC) as antigen. All sheep were immunised with OMV of *P. aeruginosa*. Data represent pre-immunised sera (red bars), one week (green bars) and two week (blue bars) after 3rd immunisation with OMV. Data are mean O.D. measured at 492 nm ± standard error of triplicate from each bleed.
Figure 5.3b: ELISA for detection of antibody levels in sera from fleece rot resistant (U2, U3, U4) and fleece rot susceptible (D9, D10, D11) sheep to *P. aeruginosa* inner membrane proteins (IMP) as antigen. Data represent pre-immunised sera (red bars), one week (green bars) and two week (blue bars) after 3rd immunisation with OMV. Data are mean O.D. measured at 492 nm ± standard error of triplicate from each bleed.
Figure 5.3c: ELISA for detection of antibody levels in sera from fleece rot resistant (U2, U3, U4) and fleece rot susceptible (D9, D10, D11) sheep to outer membrane proteins (CMx) from *P. aeruginosa* as an antigen. Data represent pre-immunised sera (red bars), one week (green bars) and two week (blue bars) after the 3rd immunisation with OMV. Data are mean O.D. measured at 492 nm ± standard error of triplicate from each bleed.
5.3.3. Immunoblots

The whole cell and membrane proteins were used as antigens in immunoblots for screening rabbit antisera from animals immunised with CMx of *P. maltophilia* and *P. aeruginosa*. The result is shown in figure 5.4a and 5.4b, using rabbit antisera and membrane antigens from *P. maltophilia* and *P. aeruginosa* respectively.

a) Immunised rabbits

The sera from post-immunised MALTO and PA rabbits against CMx proteins from *P. maltophilia* and *P. aeruginosa* were also used in Western blotting to determine the polypeptide profiles in whole cell, IMP, OMV and CMx preparations.

The results of screening all membrane antigens and bacterial whole cells of *P. aeruginosa* against rabbit PA/anti CMx proteins of *P. aeruginosa* antibodies on western blot are shown in figure 5.4a. WC lysate shows major reactions of rabbit PA antibodies against polypeptides with 67, 54, 44, 41, 39, 37, 34, 30 and 14 kDa. Both WC lysate and IMP shared some common polypeptides such as the 41 kDa and 14 kDa that were not revealed in the blots of the CMx and OMV preparations. There were two major proteins that share the common molecular size in WC, OMV and CMx preparations, that is at 39 kDa and 37 kDa. The OMV and CMx had one most obvious common polypeptides at 21 kDa.

Post vaccinated sera from rabbit MALTO/anti *P. maltophilia* did not show a strong binding intensity against IMP on the Western blot so the result is not included in figure 5.4b. Results of screening all other membrane antigens of *P. maltophilia* against rabbit anti CMx of *P. maltophilia* antibodies on Western blot are shown in figure 5.4b. The WC lysate showed only the 43 kDa polypeptide that was associated with both CMx and OMV protein preparations. On the other hand the 40, 35 and 32 kDa in the OMV and CMx fractions were not detectable in WC.
Figure 5.4: Immunoblot analysis of antisera from rabbit immunised with CMx.

Figure 5.4a represents results from rabbit PA immunised with CMx of *P. aeruginosa* and figure 5.4b represents results from rabbit MALTO immunised with CMx of *P. maltophilia*. The lanes contain whole lysate (WC), inner membrane proteins (IMP) and outer membrane proteins (OMV) from sarkosyl extraction. Outer membrane proteins (CMx) from chloroform-methanol extraction. Molecular weight standards (in kilodalton) are indicated on the left. Proteins were separated by 12 % SDS-PAGE, blotted on nitrocellulose and reacted with sera as described in materials and methods.
b) Immunised sheep

Within each group, sheep showed similar results on the immunoblots. To simplify things, the immunoblots in figure 5.5 represent the reactivity of one selected resistant (Figure 5.5a) and one susceptible sheep (Figure 5.5b) antisera against the polypeptide profiles WC, IMP, OMV and CMx from *P. aeruginosa* preparations. Under the same conditions of antigens and antisera preparations, resistant sheep antibodies recognised more polypeptides in the outer membrane protein fractions prepared by Sarkosyl (OMV). The major antigenic proteins in the OMV (Figure 5.5a) recognised by resistant sheep were the 39, 34, 30, 28, 25, 21, 18, 17 and 14 kDa (due to photographic reproducibility the 39 and 17 kDa can not be shown in OMV lane). There are a few bands such as 34, 30, 28, 25, 18, 17 and 14 kDa were missing in the OMV lane when the susceptible sheep sera were used (Figure 5.5b). Similarly when WC, IMP and CMx antigens were used all resistant sheep showed additional distinct bands compared to the susceptible sheep. For WC, both resistant and susceptible sheep antibodies recognised similar polypeptides at molecular weights of 44, 39, 37, 30 and 18 kDa while 87, 67, 42, 28, 25, 21, 17, 15 and 14 kDa were missing in the susceptible sheep. For IMP, both resistant and susceptible sheep antibodies recognised similar polypeptides at molecular weights of 30 and 18 kDa while 25, 21, 17 and 14 kDa were missing in the susceptible sheep. It was also of interest to compare the polypeptide compositions CMx profiles recognised by sheep sera. There is greater reactivity to the CMx when using sera from resistant sheep, particularly against the polypeptide of 37, 30, 28, 25, 17, 15 and 14 kDa. These polypeptides were missing from the patterns for CMx in the susceptible sheep.

In summary, firstly, when the same antigen, that is outer membrane proteins in CMx preparation from *P. aeruginosa* was given to rabbits, the results indicated (1) that there was antibody production found in all tested animals, (2) the recognition
of antibody binding in rabbits against outer membrane proteins in OMV and/or CMx were homogenous, and (3) there is common antigenic determinants between the bacterial whole cell WC, the IMP and OMV/CMx.

Secondly, when the same antigen was given to sheep with two different genetic background, the results were more interesting. Both resistant and susceptible sheep produced antibodies against outer membrane proteins; however, the resistant sheep showed higher antibody titres than the susceptible sheep. The somewhat higher titre for all antigens (WC, IMP, OMV/CMx) may be due to the fact that there were more antigenic recognition in resistant sheep than susceptible sheep.
Figure 5.5: Immunoblot analysis of antisera from sheep immunised with outer membrane proteins from *P. aeruginosa* preparation. Figure 5.5a represents results from resistant sheep and figure 5.5b represents results from susceptible sheep. The lanes contain the same antigens/membrane proteins and standards as described in Figure 5.4.
5.4. Discussion

The basis of structure and biological function of outer membrane proteins have been the subject of many publications. Miura and Mizushima (1968) first described the difference in density between inner and outer membrane fractions of *E. coli*. In their procedure, cells were disrupted by the osmotic lysis of lysozyme-EDTA sphaeroplasts. Bacterial cells can also be disrupted by using a French press (Thornley *et al.*, 1973; Smit *et al.*, 1975), or by using urea as described by Lohia *et al.* (1984).

Attempts have been made to separate the outer membrane proteins from inner membrane proteins in the bacterial membrane mixtures using methods based on differences in density or electric charges. In the first method the fraction with higher density containing outer membrane fragments can be separated from the less dense fraction with inner membrane fragments by sucrose gradient centrifugation (Schnaitman, 1970). In the second method the outer membrane has a relatively higher number of negatively charged groups due to the presence of lipopolysaccharides (White *et al.*, 1972), thus the outer membrane can also be separated from the inner membrane by the use of electrophoresis.

Other methods concerned with the separation of inner membrane proteins from outer membrane proteins have been carried out by the complete solubilisation of the total membrane by non ionic detergent (Schnaitman, 1971) or by ionic detergent (Filip *et al.*, 1973). The treatment of the cell envelope with non ionic detergents using material such as Triton X-100 (Schnaitman, 1971) and Triton X-114 has been reported (Clemetson *et al.*, 1984). This resulted in complete solubilisation of inner membrane proteins, leaving behind the outer membrane proteins. By using the ionic detergent, Sarkosyl, inner membrane proteins are
also solubilised leaving the outer membrane proteins. After non-ionic/ionic detergent treatment the outer membrane proteins are then separated using sucrose gradient centrifugation. However, depending on the bacterial species, it has been reported that this method may yield inner membrane proteins contaminated with proteins from the outer membrane and visa versa. In some instances, sucrose gradient separation can not effectively resolve inner membrane proteins from outer membrane proteins (Chopra and Shales, 1980).

Because the outer membrane proteins are associated with lipopolysaccharides and lipid bilayers in the gram negative bacteria, the hydrophobic domains are always found in their membrane structure. Hence chloroform-methanol could be used to solubilise the lipid which in turn will facilitate the release of hydrophobic proteins. This approach was adopted by Brunden et al. (1987) and adopted in this study. The results presented in figure 5.1a and 5.1b demonstrate that organic phase partitioning apparently extracts outer membrane proteins identical to those obtained by the Filip - Schnaitman method and there is indirect evidence that more outer membrane proteins could be extracted by chloroform-methanol (CMx) than the alternative procedure (OMV). Another advantage associated with the chloroform-methanol extraction procedure is the fact that there is no need for a further sucrose gradient centrifugation step after the total 35 kp preparation (35 kp contains total IMP and OMV/CMx). Sonication and dissolution of 35 kp proteins in acetic acid results in hydrolysis of the contaminating lipopolysaccharides. Dialysis of the mixture therefore eliminates lipid A contamination resulting in a preparation with significantly reduced endotoxin content and gives rise to an outer membrane protein CMx.
The organic phase partitioning method when compared with the Sarkosyl-sucrose gradient is therefore a simpler and less laborious method for separating outer membrane proteins.

Breeding sheep which are resistant to fleece rot and fly strike has been carried out at Trangie research station since the 1970s. By combining direct and indirect selection (Chapter 1), Raadsma and his co-workers (1987a,b) were able to separate sheep into two phenotypes, the genetically resistant sheep with improved resistance to fleece rot and fly strike and the other genetically susceptible sheep. The question is whether outer membrane proteins can elicit an immune response in both types of sheep. Antisera were raised in sheep against outer membrane proteins.
proteins of *P. aeruginosa*. In this experiment the nature of the antibody response of genetically selected sheep was examined. For this purpose, the sera were examined for specificity by ELISA and immunoblots. ELISA revealed that all sheep had antibodies which recognised respective antigens and that reactivity is greater for outer membrane proteins but less for inner membrane proteins (Figure 5.5a and 5.5c). There is a consistently higher serological titre in resistant sheep against outer membrane proteins but a lower titer against inner membrane proteins. Immunoblots revealed that the antibodies from resistant sheep could react more to a larger number of polypeptides in the outer membrane preparation, although many of these are also found in inner membrane preparation. Two major outer membrane proteins were found to be immunogenic and can elicit antibodies in all sheep. They are Opr F and Opr H with the molecular weights of 39 and 21 kDa. These proteins were reported to be protective in mice (Matthews-Geer and Gilleland, 1987; von Specht et al., 1995) and rats (Fox et al., 1994). However, no human trial has been reported yet.

If it is true that the immune response in sheep of different genetic background varies then, in natural conditions, a resistant sheep with several minor episodes of bacterial infections may evoke an immune response, therefore eliminating further invasion from the same organisms.

The failure of antibodies from susceptible sheep to recognise more antigens could be due to impairment of the sheep skin associated immune response. It could be immunosuppression, i.e., lack of the antigen presenting or processing (Streinlein and Grammer, 1989) or even fewer lymphocytes at the skin level (Van Dinther-Janssen et al., 1983; Bishop et al., 1989).

Although the number of sheep used in the experiment from each group were
limited, it is possible to conclude that the antibody response showed some degree of differences in the capacity to elicit antibody response to proteins found in inner or outer membrane proteins.

Biological or immunological differences have been extensively examined over the years. Studies by Chin and Watts (1991) indicated that regardless of the sheep origin (resistant or susceptible) and methods of bacterial challenge (epicutaneous with *P. aeruginosa* whole cell or intradermal injection with membrane protein of *P. aeruginosa*), all sheep showed a higher increase in antibody response against outer membrane proteins in resistant sheep than the susceptible sheep.

So far, the O'Meara team (O'Meara *et al.*, 1992, 1995) and the Colditz team (Colditz *et al.*, 1992, 1994) have each published two papers on immune responses related to resistant and susceptible properties in sheep skin. In brief, they suggested that resistant sheep gave a larger response to inflammation than the susceptible sheep in responding to *L. cuprina* implantation. Furthermore, the exudates at the infected sites showed higher levels of serum immunoglobulin (IgG, IgE). Results in this chapter have confirmed their findings.

In conclusion, the results presented in this chapter indicate that immunisation of animals with membrane proteins of *P. aeruginosa* could possibly induce protective immunity to *P. aeruginosa* infection.

Aspects of this chapter were published by James Chin and Yung Dai (1990) in *Veterinary Microbiology*. Volume 22: 69-78, as "Selective Extraction of Outer Membrane Proteins from Membrane Complexes of Pseudomonas maltophilia by Chloroform-Methanol"
Chapter 6

TWO STUDIES IN THE SEROLOGICAL RESPONSE OF SHEEP AGAINST

PSEUDOMONAS AERUGINOSA AND LUCILIA CUPRINA ANTIGENS

6.1. Introduction

Despite the progress made on fleece rot and fly strike studies, there are still many questions to be answered. Firstly, there is the significance of sheep immunity during fleece rot or fleece rot with fly strike development and, secondly, the ability of *P. aeruginosa* and *L. cuprina* to induce an immunity in sheep. *P. aeruginosa* produces many virulent factors such as extracellular enzymes (Chapter 4) that can facilitate skin damage and by overgrowing of *P. aeruginosa* will lead to local inflammation. If the local defense (ie. skin) is effective then the animal should produce some skin-associated-immunity against the infection of *P. aeruginosa* or infestation of *L. cuprina*.

Research by Chin and Watts (1992) in fleece rot has suggested that sheep sera collected after fleece rot outbreaks recognise *P. aeruginosa* to a greater extent than all other fleece bacteria. The antibody activity of the immune system in sheep may be regarded as a significant component of the host defence against *P. aeruginosa* infection. It may also play a crucial role in the clearance of infecting organisms. To support this hypothesis, Burrell (1985) demonstrated that protection can be induced effectively by immunisation of sheep with *P. aeruginosa* vaccines in which the extra-cellular lysate from such bacteria was incorporated with adjuvant. However, he did not evaluate the efficacy of immune activity in those protected sheep. Sandeman *et al.* (1986) and Eisemann *et al.* (1990) have shown sheep can produce antibodies against fly larvae antigen; results of both studies have shown that sheep were infested with *L. cuprina* larvae provided less chance for the larvae to survive challenge. These studies provide a hope that anti *P. aeruginosa* and
anti *L. cuprina* vaccine may be useful for fleece rot and fly strike treatment.

Studies reported in the previous chapter showed that outer membrane proteins from *P. aeruginosa* could induce systemic antibody response in sheep after immunisation. Virtually no information exists on immunity against *P. aeruginosa* in sheep that have fleece rot associated with fly strike. The purpose of this study was (1) to study the role of *P. aeruginosa* in the induction of sheep humoral immunity after experimentally induced fleece rot with or without fly strike; (2) to measure antibody response to bacterial surface antigens of *P. aeruginosa* and the larval antigens of *L. cuprina*; (3) to correlate these antibody levels with *P. aeruginosa* infection and fly larvae infestation and (4) to see if antibody response is a diagnostic marker in sheep with fleece rot and fly strike. The antigenicity of membrane proteins of *P. aeruginosa* and larval protein preparation which may interact with the host immune system during infection were also monitored.

### 6.2. Materials and Methods

#### 6.2.1. Fleece rot trial

All the animal work was conducted by Drs. James Chin and James Watts at the Glenfield Veterinary laboratory. The experiment was conducted on six Merinos, ranging in age from two to three years, housed on a mesh floor during the experiment. Ten millilitre of an overnight culture of *P. aeruginosa* in Luria broth at concentration of $10^9$ - $10^{10}$ cfu per ml was inoculated along the back line of each animal before wetting. This was done by parting the fleece and pipetting 10 ml of *P. aeruginosa* along the back of the sheep. Sheep were kept wet under a simulated rain system consisting of a sprinkler fanning water onto the sheep. The water was sprayed onto the sheep back at a constant rate of 1 mm per minute, lasting for four minutes, for six to seven times per day for 7 days. The sheep were allowed to dry off for a week before the cycle was repeated over a 17 week period. The total
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volume of water sprinkled onto each sheep was approximately 170 mm. Blood samples were collected each week from the jugular veins into plain vacutainer tubes. Serum was separated from the clotted blood and stored at -20 °C. For testing, the samples were diluted to 1 in 200 dilution.

6.2.2. Fleece rot and fly strike formation

The second set of experiments was to set up sheep with a fleece rot/fly strike model. This experiment was carried out in co-operation with Merck Sharp and Dohme at Ingleburn (near Sydney). Sheep were randomly selected for this experiment. There was no sign of fleece rot in these sheep prior to the experiment. Eighteen sheep aged around 2 years of age were artificially infected by epicutaneous challenge with live *P. aeruginosa* using the same technique as the above experiment (Section 6.2.1) and subsequently exposed to the fly species, *L. cuprina*. In this experiment, each sheep was seeded with live *P. aeruginosa* culture (5 x 10⁹ cfu) prior to wetting. Sheep were kept under a simulated rain system consisting of a central dome head sprinkler fanning water down onto the sheep which were housed in a pen with a wire mesh floor. The wetting schedule was carried out in a similar manner to the fleece rot trial but only lasted for 2 days after each episode of bacterial inoculation and the flies were released at day three for two days. This was done by keeping the sheep in a fly-isolator room. The whole process of bacterial inoculation and fly exposure was repeated for 14 weeks at a fortnightly interval. Blood was also collected fortnightly for serology studies. Strikes were detected by the existence of larvae anywhere in the fleece. Struck sheep were monitored by veterinary staff and put down at the end of the experiment.

6.2.3. Larvae antigen preparations

All first, second and third instar *L. cuprina* larvae were grown at the Merck Sharp and Dohme fly breeding laboratory at Ingleburn. A total of 3 g of first, second and third instars (1 g from each group) were washed three times with sterile 50 mM tris
pH 7.4. 10 ml of tris containing 2 mM phenylmethylsulfonyl fluoride (PMSF) was added to the washed larvae and it was kept in an ice bath. The larvae were ruptured by a minimixer until 90% of larvae were ruptured. PMSF was used to inhibit the possible release of protease activity in the larvae homogenate. The homogenate was centrifuged at 15,000 g on a Sorvall RC-5 ultracentrifuge in 60 Ti tubes for 30 min at 4 °C to remove any large fragments. The supernatant was collected between the lipid layer and pellet, and was stored in small aliquots at -20 °C. Protein estimation was carried out by the Markwell et al. (1978) procedure and adjusted for ELISA and immunoblot analysis.

6.2.4. Rabbit anti larval antisera

Rabbit anti-larvae homogenate was raised under standard conditions, as described in Chapter 2. 1 mg of homogenate was used on each occasion for injection. Blood was collected from the ear vein before and one week after the third immunisation. Sera were stored at -20 °C in small aliquots.

6.2.5. ELISA

P. aeruginosa whole cells and membrane proteins were prepared according to the Sarkosyl and chloroform-methanol extraction methods described in Chapter 5. The ELISA plate was coated with P. aeruginosa whole cell and inner membrane proteins (IMP) prepared by Sarkosyl method (Section 5.2.2a). Outer membrane proteins from chloroform-methanol extract (CMx, Section 5.2.2b) were shown to be identical to the outer membrane proteins (OMV) prepared by the Sarkosyl method; outer membrane proteins from chloroform-methanol extraction was used for all ELISA screening. ELISA procedure is described in Chapter 2. Antisera were used at 1 in 200 dilution for the fleece rot trial (without fly strike) and 1 in 75 dilution for the fleece rot/fly strike trial. Sheep antibodies which were bound to test antigens were detected with horse radish peroxide conjugated anti ruminant monoclonal antibodies (Appendix 2). Readings of the peroxidase reaction with O-
phenylenediamine / H₂O₂ substrate were taken at 492nm.

6.2.6. Immunoblots

SDS-PAGE was carried out as described in Chapter 5 by using 12% polyacrylamide separating gel and 4% polyacrylamide stacking gel. Whole cell lysate WC and membrane proteins (IMP, OMV and CMx) were prepared and loaded onto the SDS-PAGE. The antigens were transferred from gels to nitrocellulose membrane. Non specific binding sites were blocked with 1.5% (w/v) gelatine solution (Appendix 2). The nitrocellulose membrane was then incubated with sheep sera at 1 in 200 dilution for the fleece rot trial and at 1 in 75 for the fleece rot/fly strike trial. The antibodies were detected with alkaline phosphate conjugate anti ruminant monoclonal antibodies which gave rise to purple bands after reacting to the reagents p-nitro blue tetrazolium chloride and 5-bromo,4-chloro-3 indolyl phosphate toluidine salt (Appendix 4).

6.3. Results

6.3.1. Fleece rot trial

The photograph below shows sheep after exposure to *P. aeruginosa* on the fleece.
6.3.1.a. ELISA

Evaluation of the immunologic memory of sheep to multiple challenges with the live *P. aeruginosa* against *P. aeruginosa* whole cell WC, IMP and CMx was carried out in ELISA assays. Sheep sera collected before the experiment (prebleed) and weekly after each challenge were used. The detectable antibodies in the blood of sheep is expressed as the mean optical density (ELISA O.D.) of triplicates from each bleed. Figure 6.1 shows the result of ELISA analysis performed on sheep sera before and after each challenge with *P. aeruginosa* over a five month period.

The pattern of antibody levels in each sheep is generally similar in response to different antigens used in ELISA. In all six sheep, the antibody response of each sheep against all antigens, such as WC, IMP and CMx rose after the third infection (bleed 5,6). The temporal responses of each sheep exhibited a rise and fall pattern but overall they maintained this level throughout the 17 week period. Thus, infection with $10^{10}$ to $10^{11}$ *P. aeruginosa* with continuous wetting will give rise to an antibody response after the second or third challenge. The response peaked after week nine. Overall, the ELISA result was less intense with whole *P. aeruginosa* bacteria than with outer membrane proteins as antigen. All sheep sera shared a consistent display of reactivity against all antigens. i.e., sheep sera reactivity were high against bacterial whole cell; there was also high reactivity against the cell membrane proteins. In contrast, sheep sera displayed a low reactivity against WC and also displayed similar results against IMP and CMx.

The results suggested that sheep with multiple exposure with live *P. aeruginosa* are able to raise serum antibody to an arrays of antigens that could be found in bacterial whole cell, IMP and OMV/CMx.
Figure 6.1: ELISA analysis of fleece rot affected sheep from six animals using different antigen preparations from *P. aeruginosa*. Whole cell (WC), inner membrane proteins (IMP) and outer membrane proteins CMx were prepared as described in Chapter 5. Each panel represents a serological response of each sheep over a 17 week period with the prebleed indicated as the broken line at the beginning of each panel. The results are expressed as the mean of ELISA O.D. of each bleed measured at 492 nm of each serum from each weeks collection.
6.3.1.b. Immunoblots

In order to obtain information about the location of antigens to which sheep antibodies are directed, immunoblots were carried out using *P. aeruginosa* whole cell, IMP, OMV and CMx. Because the antibody response varies in each sheep, only sera collected after 10 weeks were used for immunoblotting. The reactivity profiles for the individual sheep were very similar in terms of the reactive bands. Figure 6.2 reveals the reaction of one representative sheep with fleece rot only (sheep number 2 in Figure 6.1) against various membrane protein preparations and the whole cell preparation. A weaker intensity was found in sheep with low antibody titre detected in the ELISA, such as sheep number 6 (Figure 6.1), however similar positive band patterns can be seen (results not shown).

Immunoblots showed that the antisera specially recognised the 67, 39, 30, 21 and 18 kDa polypeptides, which can be seen in all membrane protein and WC preparations. The major bands of *P. aeruginosa* whole cell polypeptides detected by the sheep sera were 67, 54, 44, 42, 39, 37, 34, 30, 28, 21, 18, 17, 15 and 14 kDa. The antibodies bind strongly to polypeptides in both OMP and CMx with molecular weights of 67, 39, 34, 30, 28, 21, 18 and 14 kDa. There were only six bands found in the IMP fraction, they are 67, 42, 37, 30, 21 and 18 kDa and they all share the common reactivity with the whole cell. There are 4 polypeptides present in all fractions that bind equally well with antibodies, they are 67, 30, 21 and 18 kDa. Thus by exposing sheep to artificial rain and *P. aeruginosa* over a 3 month period, sheep could develop antibodies against whole cell, inner membrane proteins and outer membrane proteins of *P. aeruginosa*. 
Figure 6.2: Representative immunoblots of different \textit{P. aeruginosa} antigens against serum from sheep with experimentally induced fleece rot (bleed 10, sheep number 2). Lanes labelled WC, IMP, OMV and CMx are similar to those described in Chapter 5. Molecular weight standards in kilodalton are indicated on the left.
6.3.2. Fly strike trial

6.3.2.1. ELISA response to \textit{P. aeruginosa}

The combination of \textit{P. aeruginosa} infection and continued wetting encouraged the \textit{L. cuprina} to lay eggs at the infection sites to produce strikes. The strikes were recorded as the result of infestation of larvae in the fleece. The photograph below shows a well developed larvae infestation after 2 days of a successful exposure to a fly wave and \textit{P. aeruginosa} infection.

![Photograph showing a well developed larvae infestation after 2 days of a successful exposure to a fly wave and \textit{P. aeruginosa} infection.]

The same dose of \textit{P. aeruginosa} was given to each sheep with an expectation that each sheep would successfully develop a good serological response against \textit{P. aeruginosa} and would get fly strike after each challenge. This was not the case in all 18 sheep. Table 6.1 shows the total number of strikes monitored after every fly wave. The result indicated that not all eighteen sheep showed evidence of successive strikes. The ELISA response to \textit{P. aeruginosa} whole cell was also variable, that is sheep with higher serological response to \textit{P. aeruginosa} whole cell
antigen did not always suffer a higher number of strikes. In this table, low and high responders were used. The high and low is determined by the ELISA Ratio which is calculated as the ratio of ELISA O.D. of each bleed divided by the ELISA O.D of the pre-bleed. A low responder is defined as having a antibody titre with a ELISA Ratio less than 2 against the whole cell \textit{P. aeruginosa} as antigen. These sheep maintained a low level of antibody throughout the trial. The high responders showed successive increases of antibody as late as after the third challenge with \textit{P. aeruginosa}. As the result shows in this trial, there is a 50 % chance across the board as 9 sheep having fleece rot and fly strike showed high antibody response and 9 had low antibody responses.

Figure 6.3 shows ELISA Ratio analysis of the individual antibody response of sheep before and after the fortnightly challenge with \textit{P. aeruginosa} and \textit{L.cuprina}. Bleed 1 was the sera collected at week 2 after the first challenge and subsequent bleeds were at fortnightly intervals. The value of the ELISA Ratio suggests there is a very slow increase of antibody response across the flock as detected using \textit{P. aeruginosa} whole cell antigen. The response did not peak until after the sixth challenge. Two sheep had barely detectable antibody titre after the sixth challenge; it must be remembered that in this trial the infection procedure used less concentrations of bacteria \((5 \times 10^9 \text{ cfu of } \textit{P. aeruginosa})\) than the fleece rot trial in the previous experiment \((10^{10} - 10^{11} \text{ cfu})\) and this probably was not sufficient to elicit a strong antibody response. The important observation is that antibody activity against \textit{P. aeruginosa} was still induced when sheep are exposed to \textit{P. aeruginosa} and flies (Figure 6.3a). Furthermore, in ELISA studies, antibody activity was not consistent when it was compared between the two membrane proteins, ie. the level of antibody response against outer membrane proteins CMx was not always the same as anti-inner membrane proteins.
Figure 6.3b and 6.3c represent the ELISA Ratio of post infected sera against the pre-infected sera in ELISA using purified membrane proteins as antigens.

ELISA Ratio is calculated by the following formula:

\[
\text{ELISA Ratio} = \frac{\text{ELISA O.D. of bleed 1,2...}}{\text{ELISA O.D. of prebleed}}.
\]

Figure 6.4 is the summaries taken from Figure 6.3a, 6.3b and 6.3c. Each panel represents an average antibody response in 18 sheep detected by ELISA. Results represent the ELISA O.D. at 492 nm for serum antibody in each bleed. By placing all three panels together (a, b, c), it is found that, even though all sheep showed low reactivity to all antigen, the outer membrane proteins were shown to be a favourable antigen to induce antibodies in sheep.
Table 6.1: Frequency of fly strikes and level of anti-\textit{P. aeruginosa} serological responses during the fleece rot and fly strike trials. H is defined as high responder that had average ELISA Ratio > 2 and L is defined as low responder registered average ELISA Ratio < 2 when the sheep sera were screened against \textit{P. aeruginosa} whole cell.

<table>
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<tr>
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<th>No.Strikes</th>
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Figure 6.3: Distribution of the ELISA analysis of individual responses of 18 sheep against *P. aeruginosa* antigen preparations; (3a) for whole cell bacteria (WC), (3b) for inner membrane proteins from sarkosyl method (IMP) and (3c) for outer membrane proteins (OMV/CMx). Results are expressed as a ratio of the ELISA O.D. at each bleed against the pre-bleed. ELISA Ratio of pre-bleed has a value of 1 for all animals. These data represent the mean of triplicate of each bleed tested. Antigens were prepared as described in Chapter 5.
Figure 6.4: Mean of ELISA O.D. of a total of 18 sheep during the course of *P. aeruginosa* infection and *L. cuprina* infestation. These curves are representative of three graphs demonstrated in Figure 6.3 panels. (4a) is average ELISA O.D. with *P. aeruginosa* whole cell as antigen; (4b) is for inner membrane proteins and (4c) is for outer membrane proteins. Data are the average ELISA O.D. from 18 sheep obtained from each bleed collection (Figure 6.3) ± standard error. (4d) is the result obtained from figure 4a, 4b and 4c.
6.3.2.2. Immunoblots to *P. aeruginosa*

Preliminary screening of the titre of the sheep sera against *P. aeruginosa* using ELISA led us to divide the sheep into two groups, the high (H) and the low (L) responders. The high responders were sheep number 2, 4, 5, 6, 7, 10, 13, 15 and 16; the low responders were sheep number 1, 3, 8, 9, 11, 12, 14, 17, and 18. The sera from each group were pooled for immunoblot screening. For immunoblots all *P. aeruginosa* antigens were denatured and loaded onto a 12 % SDS-PAGE and blotted to a nitrocellulose membrane under the conditions described in Chapter 2.

Similar results were obtained with sera from the two groups. Figure 6.5b shows the immunoblot of the low responders against four different *P. aeruginosa* antigen preparations. Figure 6.5a is the results of the blots using sera from the high responders. The intensity of the reactions is greater in the Figure 6.5a but the profiles were similar. For both groups, the results strongly suggest that there are at least 6 major banding reactions against bacterial whole cells in both groups, the polypeptides having a molecular mass of 34, 28, 25, 21, 18 and 15 kDa. When screening against inner membrane protein the greatest intensity were against only two polypeptides, 54 and 30 kDa. In the high responders there are at least four polypeptides with common molecular sizes in both CMx and WC and there are 67, 54, 39, 21, 18 and 15 kDa. The low responders as shown in Figure 6.5b do not have the 39 and 21 kDa polypeptide.
Figure 6.5: Immunoblots of pool antisera from sheep with fleece rot and fly strike against different antigens from *P. aeruginosa* preparations. (5a) represents results from nine high responder sheep and (5b) represents results from the nine low responders. Bleed 7 from each sheep was pooled and used in incubation at 1 in 75 dilution. Lanes labelled WC, IMP, OMV and CMx are described in Chapter 5.
6.3.2.3. ELISA response to *L. cuprina* larval antigen

Although being exposed six times to *L. cuprina*, not all eighteen sheep had the same strike patterns. The strikes were recorded as the incidence of larvae present on the sheep body; the strike was not necessarily always at the *P. aeruginosa* infected sites. Table 1 indicates that sheep were not struck every time they encountered flies; two out of the eighteen were not affected at all. Sera from all eighteen sheep were tested for ability to detect *L. cuprina* larval antigens using ELISA. For the ELISA, larval homogenate (Section 6.2.3) comprised of all the components of the first, second and third instar was used as antigen and adjusted to 20 µg/ml in coating buffer using the procedure as previously described (Chapter 2).

Figure 6.6 is the ELISA Ratio of sera from sheep experimentally infected with *P. aeruginosa* and fly strike against the mixed larval homogenate. Although the ELISA Ratio shows relatively low values in all sheep, high and low responders could still be detected. However correlating the results of the ELISA against the *L. cuprina* antigens with strike incidence shows that higher incidence of strikes does not result in a higher antibody response to the larval antigen.

A rabbit immunised with larval homogenate was used as a positive control in all ELISA assays. A mixture of larval homogenate could induce a very high serological response in rabbits after three immunisations with Freund's adjuvant. A high antibody response is depicted as ELISA Ratio value of 5 times increase in antibody level after the third immunisation compared with the serum collected before the immunisation. It is clearly demonstrated the larvae homogenate had some antigenicity in the rabbit.
Figure 6.6: ELISA analysis of anti-larval antibodies from rabbits and sheep antisera. The results are expressed as the ELISA Ratio from each bleed relative to the prebleed value when the supernatant of larval homogenate was used as antigen. The values correspond to the mean of a triplicates. Each panel represent the serological response of each sheep after exposure with fleece rot and fly strike. "Post" is result from rabbit serum after larvae immunisation and "Pre" is from rabbit serum before the immunisation.
6.3.2.4. Immunoblots

Following 12% SDS-PAGE analysis of the larval homogenate, immunoblots were undertaken using sera from immunised rabbits and the sheep sera. Lane 1 in figure 6.7 is the Coomassie blue stained SDS-PAGE profile of the larval homogenate prepared by reduction and boiling in β-mercaptoethanol. The major polypeptide bands are 67, 65, 59, 47, 45, 42, 40, 30, 28, 27, 25, 14 and 13 kDa.

Lane 4 in figure 6.7 represents the result of an immunoblot using rabbit antiserum after immunisation with larval antigen. The results showed that the sera reacted strongly with six major polypeptides located at 98, 92, 67, 65, 59, 54, 45 and 25 kDa.

Lane 2 and 3 in figure 6.7 are the two most representative immunoblots when sera from sheep, exposed to both bacteria and flies, were tested against the same larval homogenate. In lane 3 there are five major proteins of 65, 59, 54, 40 and 25 kDa. On the other hand, another sheep showed weaker intensity with 65 and 40 kDa polypeptides and the 25 kDa was completely missing in lane 2. The results indicate that there is a recognition of four common antigens between the sheep, they are 65, 59, 54 and 40 kDa. According to these results the intensity of reactions against larval antigens did not correlate with the strike incidence.
Figure 6.7: Immunoblots of anti-larval antigen probed with serum from rabbit immunised with larval homogenate (lane 4) and sera from two representative sheep with fleece rot and fly strike (lane 2 and 3). Lane (1) is Coomassie blue stained SDS-PAGE of larval antigens on a 12 % polyacrylamide gel under reducing condition (Appendix 4).
6.4. Discussion

Long term research is focused on the potential of biological control for fly infestation such as immunisation rather than insecticide control. It is possible that protective immunity could be developed in sheep against fly strike using immunisation with larval antigen. However, there are doubts that immunisation of crude *L. cuprina* larvae did not protect against the larvae (O'Donnell *et al.*, 1981), and previous studies have suggested that the level of antibodies against larval antigen did not determine the nature of resistance or susceptibility of the sheep to fly strike (Elliott *et al.*, 1980; Sandeman *et al.*, 1986). A study by Chin and Watts (1991) showed that locally administered live *P. aeruginosa* led to a systemic immune response in sheep. These results often were not conclusive because they looked at fleece rot or fly strike, but not both. Thus, this study was developed to pay attention to the antibody responses of sheep with both fleece rot and fly strike problems.

There is debate on the advantages and efficacy of using live or killed whole cell vaccines or of using purified specific components such as membrane proteins or extracellular toxins from *P. aeruginosa* in sheep. Intradermal inoculation of live *P. pyocyanea* in immunised rabbits induced some resistance to infection (Fox and Lowbury, 1953b). It was also demonstrated that patients with cystic fibrosis (Fernandes *et al.*, 1981) and burn wounds (Anwar *et al.*, 1985) produce antibodies which would react with outer membrane proteins of *P. aeruginosa*.

Clearly the route of exposure as well as the nature of antigen can influence the immune response. In this chapter, the exposure of sheep to *P. aeruginosa* infection was assessed. In the first trial, sheep were challenged with live whole cell preparation of *P. aeruginosa* every week with a long duration of wetting after
every challenge. Serological analysis was carried out with ELISA and immunoblot with different antigens such as whole cell proteins and membrane proteins from *P. aeruginosa*. Six sheep exhibited antibody reactivity against various antigens from *P. aeruginosa* soon after the first and second challenge; the ELISA titre increased with the increasing number of challenges of *P. aeruginosa* on the sheep skin. Similar results were obtained in the Western blot analysis in which the intensity of anti-*P. aeruginosa* antibody complexes increased progressively after each challenge with *P. aeruginosa*. The outer membrane protein preparations prepared by two methods indicated polypeptides 67, 39, 34, 30, 28, 21, 18 and 14 kDa reacted with all sheep sera. The inner membrane protein preparations reacted strongly with 67, 42, 37, 30, 21 and 18 kDa only. This is good evidence to show that outer membrane proteins possess antigenic determinants which are recognised by the sheep immune system. When live bacteria are introduced by the dermal route the results indicated that both inner and outer membrane proteins reacted serologically against sheep antibodies. The increased levels of serum antibodies is probably characterised by a constant immunologically stimulation and that the skin associated immune response is present in the sheep.

In the second set of experiments, sheep randomly selected from the field were challenged a number of times with *P. aeruginosa* followed by *L. cuprina* allowing fleece rot and fly strike to become established. ELISA analysis revealed some differences in antibody titre against *P. aeruginosa* antigens. After screening all sheep sera against *P. aeruginosa* whole cell proteins, it was decided that the sheep could be divided into two groups of responders, one high and the other low. Antibodies from the high responder were directed against *P. aeruginosa* whole cell proteins with a molecular mass of 67, 54, 39, 21, 18 and 15 kDa which were also present in the outer membrane proteins.
Data suggested that fly larvae also played a special role in the sheep immune system. However, the antibody response did not correlate with the presence of bacteria and/or number of fly strikes.

The use of larval preparation in ELISA and immunoblot has confirmed the hypothesis of Sandeman et al. (1986) and Eisemann et al. (1990) that the local existence of *L. cuprina* on the skin may trigger the immunological response in the skin, resulting in the presence of circulating antibodies. The data indicated that the antibody titre of struck sheep were similar with non-struck sheep. That means; firstly, the *P. aeruginosa* may not always provide an opportunity for fly strike under experimental conditions; secondly, the difference in the number of strikes did not correspond with the antibody response and; thirdly, the number of times that larvae successfully developed on sheep skin in this experiment did not influence the humoral immune response to larval antigen. Thus, the fly strike-immune response relationship demonstrated by this model did not correlate. This lack of correlation between the strength of immune response and fly strike was demonstrated by Elliott et al. (1980). The result indicates that the antibody response to larvae in sheep with fleece rot and fly strike does not influence the survival of larvae on the skin nor does the antibodies to *P. aeruginosa* influence fly strikes.

However whether preexisting antibodies to any component of *P. aeruginosa* protects against the bacterial penetrations and natural fly strike is still to be determined.

Chapter 7

GENERAL DISCUSSION and CONCLUSION

This thesis has focused on the study of fleece rot and fly strike with particular emphasis on the potential role of *P. aeruginosa*. The aim was to study the following aspects:

1. Investigate the potential role of *P. aeruginosa* during fleece rot development.
2. Investigate the mechanism by which *P. aeruginosa* becomes dominant in fleece rot.
3. Identify the potential roles of *P. aeruginosa* extracellular products in the development of fleece rot.
4. Define the specific antigenic proteins of *P. aeruginosa* outer membranes and their capacity to elicit a serological response.
5. Examine the antibody response of sheep that are experimentally affected by fleece rot and/or fly strike.

It has been well established that *P. aeruginosa* is the predominant bacterial species associated with fleece rot (Burrell et al., 1982; Burrell, 1988; Chin and Watts, 1992). To be the predominant species, it would be expected, firstly, that *P. aeruginosa* is able to manipulate the microenvironment for its successful growth, and secondly, that it has adapted for survival in the fleece. Indeed, in the bacterial inhibition study, whereby a *P. aeruginosa* lawn inhibited or prevented other fleece bacteria species from growing alongside, it was shown that *P. aeruginosa* can not only maintain its existence but can also prevent other bacterial species from growing. Gram positive bacteria were affected more than Gram negative bacteria. Evidence presented in Chapter 3 suggests that pyocyanin which is a blue green extracellular pigment produced by *P. aeruginosa* contributed to the growth advantage of this species. Further, it was found that by adding pyocyanin
(10^{-3} \text{ M}) to a shaking culture of fleece bacteria that the growth of Bacillus cereus, Bacillus coagulans and Staphylococcus epidermidis was inhibited by 60-90 \%. These levels of inhibition were consistent with the results achieved in the lawn experiment. Thus this study has shown the involvement of pyocyanin production by P. aeruginosa as one of the possible mechanisms influencing the bacterial flora during fleece rot development. Whether non pyocyanin-producing strains of P. aeruginosa can inhibit the growth of other bacteria and also cause fleece rot requires further study.

The nutritional requirements for growth of P. aeruginosa are very simple. It is reported to be able to grow in distilled water (Kayser et al., 1975). Simple nutritional requirements and its ability to metabolise a variety of substances allows this organism to survive anywhere sheep can be found. Fleece and skin together with moisture are an ideal environment for P. aeruginosa. Once it is present in the fleece, it proliferates using local nutrients such as sweat proteins and wool fibre. An early increase in the number of bacteria accounts for a discolouration of wool (Mulcock, 1965; Murray, 1979) and local skin inflammation (Merritt and Watts, 1978a; Burrell et al., 1982).

P. aeruginosa organisms appear to only invade the superficial layer of sheep skin. Burrell et al. (1982) proved this fact by attaching a wool pad saturated with P. aeruginosa to sheep skin for 7 days. While they were able to induce subacute dermatitis which mimics that of fleece rot, the histopathological appearance of skin biopsies from those sheep showed no evidence of invasion of the organisms beyond the epidermis. This led to the conclusion that dermatitis is due to bacterial by-products and/or the immunopathological reaction to these by-product (Burrell et al., 1982). So far, only phospholipase C has been reported to be a virulence factor in fleece rot (Chin and Watts, 1991). However, other
extracellular products, such as proteases, are candidates as they have been virulence factors for diseases induced by *P. aeruginosa* in mice (Diener *et al.*, 1973; Kawaharajo *et al.*, 1975b), in rabbits (Kreger and Griffin, 1974; Gray and Kreger, 1979) and in humans (Margaretten *et al.*, 1961; Kawaharajo *et al.*, 1975a).

Several approaches for the purification of extracellular proteases were employed in the current study. Two isoenzymes were partially investigated for their activity in *vitro* and their pathogenicity in sheep skin (Chapter 4). One enzyme was similar to the previously described elastase (Jensen *et al.*, 1980; Fukushima *et al.*, 1989) and the other appeared to be alkaline proteinase as previously described in the literature (Inoue *et al.*, 1963; Morihara, 1963). The enzymes, in combination, were found to be capable of producing a skin inflammatory response when they were given intradermally but neither enzyme alone could elicit this response, suggesting that the combination of enzymes contributes to the skin damage seen in fleece rot.

Further the current study provides evidence that *P. aeruginosa* replicates to a greater extent with a concomitant increase in extracellular protease activity when sterile sheep wool was added to the bacterial culture. There is little doubt that wool provides an effective nutrient source for growth and that, given the changes in wool consistency observed, the bacteria and potentially the proteases are likely to contribute to the changes of wool consistency that are seen in fleece rot. Researchers such as Nay and Watts (1977) and Burrell *et al.* (1982) reported that *P. aeruginosa*, when living on fleece/fibre proteins, induced narrowed stretches of fibre protein and caused weakened wool fibre. However, this study did not look at the physical properties of the fibre to confirm these observations. A key future experiment could be the impact of purified proteases on the physical and the chemical properties of wool fibre. Further research to elucidate the detailed
effects of proteases on the various layers of skin will also be useful in understanding the pathogenesis of fleece rot caused by *P. aeruginosa*.

Protection from *P. aeruginosa* infection has been difficult to achieve in sheep. No commercial vaccine has been developed for fleece rot. To facilitate studies on the serological response to outer membrane proteins a more effective and less laborious purification process for separating this membrane fraction was desirable. This was achieved using a chloroform-methanol extraction. This procedure enabled outer membrane proteins to be resolved without sucrose gradient centrifugation. Studies reported in Chapter 5 show the successful isolation and purification of outer membrane proteins from both *P. aeruginosa* and *P. maltophila* based on the new method, suggesting that a chloroform-methanol extraction method could be ideal for other Gram negative bacteria.

Once the outer membrane proteins were successfully isolated from *P. aeruginosa* they were then characterised and shown to elicit antibodies in rabbits and sheep (Chapter 5). Examination by Western blotting of the specificity of antibodies generated by fleece rot (Figure 6.2), or fleece rot and fly strike (Figure 6.5) in sheep revealed that all sera recognised the proteins from the outer membrane rather than those from the inner membrane of *P. aeruginosa*. ELISA studies confirmed that sera from these sheep reacted with outer membrane proteins. These experiments support the hypothesis that sheep with fleece rot acquire humoral immunity to *P. aeruginosa* outer membrane proteins. Indeed when sheep were repeatedly exposed to *P. aeruginosa* and wetting, there was an increase in antibody response to outer membrane proteins. Similarly there was a higher antibody response when sheep were given a higher dose of *P. aeruginosa* (10^{10}-10^{11} cfu) than when exposure was low (5 x 10^9) (Chapter 6). While the protective role of the antibody response remains uncertain it can be concluded that there
was a consistent response. In contrast analysis of the antibody response to \textit{L. cuprina} in animals with experimentally induced fleece rot and fly strike (Chapter 6), showed no evidence to suggest that the humoral immune response against \textit{L. cuprina} correlated with signs of fly strike. In fact in an experiment using 18 sheep, no conclusion can be made on whether sheep with more strikes give a higher antibody response against fly larval antigens than those sheep with fewer fly strikes. However, in any subsequent studies looking at sheep antibody responses against larvae, improved quantification of the number of larvae on the sheep is desirable and this may result if larvae are implanted onto the sheep rather than allowing flies to lay their eggs randomly in the fleece of the sheep.

The Western blot studies on sera from six sheep with experimentally induced fleece rot identified the six major polypeptides in the outer membrane fraction recognised by immune sera (Figure 6.2); two of these major outer membrane proteins are identified as Opr F and Opr H with molecular weights of 21 kDa and 39 kDa respectively. These two proteins were first mentioned by Hancock and Nikaido (1978); later by Lam \textit{et al.} (1983) as possible vaccine candidates. The current study supports the immunogenic potential of these proteins. However, the antibodies to other major outer membrane proteins may be as important to protection in fleece rot.

In an attempt to establish the use of antibody response as a diagnostic marker for fleece rot resistant or fleece rot susceptible sheep, the antibody response to \textit{P. aeruginosa} challenge was assessed by ELISA (Chapter 5). When genetically selected resistant and susceptible sheep were intradermally inoculated with outer membrane proteins from \textit{P. aeruginosa}, a positive response to outer membrane proteins was detected by Western blot analysis of sera from both types of sheep, but a lower antibody titre was detected by ELISA in susceptible sheep. The
antisera from both groups of sheep also reacted towards outer membrane protein Opr F and Opr H (Figure 5.5). Having assessed only a few sheep from each group in this study it might be premature to conclude that resistant sheep have a better protective mechanism to *P. aeruginosa* by producing a higher antibody titre to outer membrane proteins than susceptible sheep. Further research, using larger numbers of resistant and susceptible animals is required, with the studies assessing the capacity of resistant sheep to remain resistant with age and to sustain multiple challenges of *P. aeruginosa* and *L. cuprina*.

In conclusion the research reported in this thesis on the microbiological aspects of fleece rot has identified that *P. aeruginosa* inhibits other common fleece bacteria through the production of pyocyanin; that two extracellular proteases together induce dermal damage and that different sheep produce a different serological response against outer membrane protein preparations of *P. aeruginosa*. This knowledge will be beneficial to future studies investigating the potential for a vaccine based on *P. aeruginosa* outer membrane proteins to contribute to the protection against sheep fleece rot as well as the potential of a breeding program selecting for high responders to *P. aeruginosa* outer membrane proteins to enhance resistance in sheep flocks.
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Appendix 1

1. CHEMICALS

Ajax Chemicals, Australia.

- Ammonium sulphate
- AR Butanol
- Glucose
- Di-potassium hydrogen orthophosphate
- Hydrochloric acid
- Potassium chloride
- Potassium dihydrogen orthophosphate
- Sodium carbonate
- Sodium EDTA
- Sodium sulphate
- Sucrose
- Sulphuric acid

Amersham Australia Pty. Ltd., Australia.

- Protein A-HRP

Bacto Laboratories Pty. Ltd., Australia.

- Brain Heart Infusion Broth
- Freund complete Adjuvant
- Freund Incomplete Adjuvant

Bio-Rad Laboratory Pty. Ltd., Australia.

- Alkaline phosphatase
- Amido Black 10B
- 2-mercaptoethanol
- Nitrocellular membrane, 0.2 micron
- SDS-PAGE low molecular weight markers
- TEMED(N,N,N,N'tetramethylethylene diamine), MW = 116.21
- Gelatine powder
- Preweighed Acrylamide and Bis 29:1
Goat anti rabbit Horseradish Peroxidase conjugates
Nitroblue Tetrazolium (NBT)
5-Bromo-4-chloro-3-indolyl-phosphate (BCIP)

Boehringer-Manheim Co., Australia
Aquacide

Calbiochem-Behring Corp., Australia.
Tris (Hydrogen methyl methylamine)

Integrated Science, Australia.
Bovine serum albumin

Merck (BDH) Pty. Ltd., Australia
AR Acetic acid
AR Acetone
Ammonium chloride
Ammonium carbonate
Ammonium hydrogen carbonate
Bromophenol blue
Calcium chloride
Citric acid
AR Chloroform
Disodium hydrogen orthophosphate
Dimethyl formamide
AR Ethanol
Formaldehyde solution 40%(w/v)
AR Glycerol
Glycine
Hepes (n-2-hydroxyethyl piperazine, N-2-ethane sulphonic acid)
AR Isopropanol
Magnesium chloride
Magnesium sulphates
AR Methanol
Potassium hydroxide
pH \text{10.0} buffer
pH \text{7.0} buffer
pH \text{4.0} buffer
Sodium azide
Sodium dihydrogen orthophosphate
Sodium hydrogen carbonate
Sodium hydroxide
Thiomersal merthiolate
Triton X-100

Kodak Pty. Ltd., Australia.

Developer part A
Developer part B
HC 110 Developer
Fixer
F1 Printing paper

Oxoid Pty. Ltd., Australia.

Bacto Tryptone
Yeast Extract
Columbia agar base
Trypticase soy broth

Pharmacia Fine Chemicals, Australia.

SDS-PAGE high molecular weight markers

Sigma Chemical, Australia.

Diaminobenzidine (DAB)
N-Lauryl sarcosine
Tween 20 (polyoxyethylene sorbitan)
2. EQUIPMENT

**Plastic wares**
- Bio freeze vials (Costa)
- Disposable syringes
- 96 Elisa plates, sterile
- Eppendorf tips
- Falcon tubes, 50 ml
- Falcon tubes, 5 ml
- Petridishes 90 mm
- Petridishes 150 mm
- Sterile Transfer pipette

**Sources**
- Stansen, Australia
- Terumo Corporation, USA
- Flow Laboratory.
- Edwards Medical, Australia.
- Crown scientific, Australia.
- Becton Dickinson, Australia.
- Disposable products, Australia.
- Bactolab, Australia.
- Disposable Products, Australia.

**Glass ware**
- Erlenmeyer flasks
- Schott bottles

**Sources**
- Crown Scientific, Australia.
- Newcastle Scientific Supplies, Australia.

**Benchtop centrifuges**
- Eppendorf 5414S
- IEC Centra/ 7R
- Sigma 101M

**Sources**
- Eppendorf, West Germany.
- International Equipment, USA.
- Sigma Lab, West Germany.

**Ultracentrifuges**
- Refrigerated Sorvall RC-5
- Refrigerated L8-M

**Sources**
- Beckman Instruments, Australia.
- Beckman Instruments, Australia.

**Electrophoresis**
1. Minigel
   - Hoefer SE 200

**Sources**
- Australian Chromatography, Australia.

2. Macrogel
   - BioRad Macrogel

**Sources**
- Bio Rad, Australia.

3. Power supply
   - Model 3000/300

**Sources**
- Bio Rad, Australia.
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Appendix 2

PREPARATION OF SOLUTIONS AND MEDIA

**Agarose gel for immunodiffusion test**

1 g Agarose C
100 ml Phosphate buffer saline solution
Dissolve Agarose at 80 - 100 °C. Dispense 10 ml per 90 mm petri dish. Allow to dry at room temperature before punching holes.

**Agarose- gelatin agar for protease detection**

1 g Agarose C
1 g Gelatin
Dissolve in 100 ml tris- saline pH 7.2. Stir on a hot plate. Add 50 ul 1 M CaCl₂. Allow to cool at 50 - 60 °C. Pour agar onto a glass plate on a level surface (@ 2mm in thickness).

**Alkaline phosphatase substrate**

NBT (Appendix 1): Dissolve 30 mg NBT in 1 ml 70 % Dimethyl formamide
Dissolve 15 mg BCIP (Appendix 1) in 1 ml 100 % Dimethyl phosphate formamide
Add NBT and BCIP solution to 100 ml Carbonate-Magnesium chloride pH 9.8 buffer.

**Amido black staining solution**

0.1 g Amido black
45 ml Distilled water
45 ml Absolute ethanol
10 ml acetic acid
Stir on magnetic stirrer until completely dissolved. Filter solution through Whatman filter paper No. 41
Ammonium carbonate buffer [0.05 M, pH 7.2]

4.2 g Ammonium hydrogen carbonate NaHCO₃
Dissolve in distilled water. Titrate to pH 7.2 with 5 M HCl. Adjust to 1 litre with distilled water.

Ammonium persulphate [10 % (w/v)]

10 g ammonium persulphate
Dissolve in 100 ml distilled water. Store at -20 °C in 50 ul or 450 ul aliquots.

Ammonium sulphate-saturated

100 g ammonium sulphate
Dissolve in 100 ml distilled water on heated-magnetic stirrer. The undissolved salt was allowed to settle in the same container and was shaken prior use.

Blotting buffer [0.25 M Tris; 1.92 M Glycine; 20 % Isopropanol]

6.06 g Tris
28.8 g Glycine
Dissolve in 1.6 litre distilled water. Add 400 ml Isopropanol. Mix gently by inversion.

Bromophenol blue staining solution

0.1 g Bromophenol
45 ml Distilled water
45 ml Absolute Ethanol
10 ml Acetic acid
Dissolve on magnetic stirrer. Filter through Whatman filter paper No. 41

Brain Heart Infusion Broth-Glycerol

3:7 g Brain heart infusion broth
Dissolve in 50 ml distilled water on magnetic stirrer. Add 50 ml AR Glycerol. Autoclave at 121 °C for 15 minutes.
Coating buffer pH 9.6
1.59 g Sodium Carbonate $\text{Na}_2\text{CO}_3$
2.93 g Sodium Hydrogen carbonate $\text{NaHCO}_3$
Dissolve in 1 litre distilled water.

Carbonate - Magnesium Chloride [0.1 M $\text{NaHCO}_3$; 1 mM $\text{MgCl}_2$; pH9.8]
8.4 g Sodium Hydrogen Carbonate $\text{NaHCO}_3$
0.2 g Magnesium chloride $\text{MgCl}_2$
Dissolve in 800 ml distilled water. Titrate to pH 9.8 with 1 M HCl. Adjust to 1 litre with distilled water.

Columbia Blood agar
10.6 g Columbia agar
2 g Bacto agar
Mix well with 225 ml distilled water. Autoclave at 121 $^\circ$C for 15 minutes. Allow to cool at room temperature to 50 $^\circ$C. Add 25 ml sterile sheep blood to give 8-10 % concentration. Mix gently to avoid bubbles. Dispense 20 ml into 90 mm petri dish and allow to set.

Coomassie Brilliant blue G250 [0.1 % (w/v)]
0.1 g Coomassie brilliant blue G250
45 ml Distilled water
45 ml Absolute Ethanol
10 ml Acetic Acid
Dissolve completely on magnetic stirrer. Filter through Whatman filter paper No. 41

Developer HC110 solution for Technical Pan film
30 ml Concentrate HC110
300 ml Distilled water
Make up the solution immediately before use.
Developer solution for Ortho film

Solution A: Dissolve Kodak part A packet with 3.8 l distilled water.
Solution B: Dissolve Kodak part B with 3.8 l distilled water.
Working solution: Combine 1 volume of solution A with 1 volume of solution B.

Diamino Benzidine DAB [1.2 mM]
25 mg 3,3-Diaminobenzidine
Dissolve in 50 ml of Tris [10 mM, pH 7.0]. Add 16 μl Hydrogen peroxide [25 % H₂O₂]. Make up just before use.

Fixer solution
1 packet Kodak fixer
Dissolve in 5 litre distilled water. Store in dark bottles.

Formalin buffer [10%]
4 g NaH₂PO₄
6.5 g Na₂HPO₄
Dissolve in 900 ml distilled water. Add 100 ml formaldehyde.

Gelatine blocking solution
1.5 g Gelatine
100 ml Tris-Saline pH 8.9.
Dissolve gelatine at 80-100 °C.

Gelatine-agar substrate [1 % (w/v)] for radial diffusion gel
1 g Gelatine
1 g Agarose C
Dissolve in 100 ml Tris-Saline pH 7.2. Add 50 ul 1M CaCl₂. Dispense 20 ml into 90 mm petri dish. Allow to cool at room temperature before punching holes.

10 Mm Hepes
2.38 g Hepes (Appendix 1)
Dissolve in 800 ml distilled water. Titrate to pH 7.2 with 5 M Sodium hydroxyde NaOH. Adjust to 1 litre with distilled water.
**Hepes-PMSF solution [2 mM PMSF]**

0.35 g Phenyl Methyl Sulphonyl Fluoride PMSF
Dissolve PMSF in 3.5 ml 70 % Ethanol.
Add PMSF solution to 100 ml 10 Mm Hepes pH 7.2. PMSF is prepared just before use.

**Hepes-Sucrose [10 % (w/v) sucrose]**

10 g Sucrose
Dissolve sucrose in 100 ml Hepes [10 mM]

**Low Nutrient Medium [LNM]**

1.32 g Ammonium sulphate (NH₄)₂ SO₄
0.1 g Magnesium sulphate MgSO₄
0.5 g Dipotassium hydrogen phosphate K₂HPO₄
0.1 g Sodium chloride NaCl
0.1 g Yeast extract
1.5 g Succinic acid
Dissolve in 1 litre distilled water. Titrate to pH 7.0 with Sodium hydroxide.
Autoclave at 121 °C for 15 minutes.

**Luria Bertanie broth [LB]**

10 g Tryptone
5 g Yeast Extract
5 g Sodium Chloride
Dissolve in 1 litre distilled water. Autoclave at 121 °C for 15 minutes.

**Luria Bertanie agar [LA]**

Add 15 g Bacto agar to Luria Bertanie broth, autoclave.

**Merthiolate [10 % (w/v)]**

1 g Merthiolate
Dissolve in 10 ml distilled water. Store in the dark. Working concentration in 0.01 %
**Minimal Nutrient Medium Broth [MNM]**

6 g Disodium hydrogen phosphate $\text{Na}_2\text{HPO}_4$

3 g Potassium hydrogen phosphate $\text{KH}_2\text{PO}_4$

1.3 g Ammonium chloride $\text{NH}_4\text{Cl}$

0.5 g Sodium chloride $\text{NaCl}$

0.25 g Magnesium sulphate $\text{MgSO}_4$

Dissolve in 1 litre of distilled water. Autoclave at 121 °C for 15 minutes. Allow to cool at room temperature. Add 100 ul sterile 1M Calcium chloride $\text{CaCl}_2$ and 10 ml sterile glucose (20% [w/v] stock glucose).

**Nitric acid [0.1M]**

9 ml AR Nitric acid

Dilute to 1 litre with distilled water.

**O-Phenylene Diamine OPD Substrate**

Citric acid [0.1 M]: Dissolve 2.1 g citric acid in 100 ml distilled water

Disodium hydrogen phosphate [0.2 M]: Dissolve 2.84 g Disodium hydrogen phosphate $\text{Na}_2\text{HPO}_4$ in 100 ml distilled water

Citric -Phosphate buffer: Mix 1 volume Citric acid solution; 1 volume Phosphate solution and 2 volumes distilled water.

OPD stock solution: Dissolve 200 mg OPD in 5 ml Absolute Ethanol

OPD Substrate: Add 100 ul stock OPD and 5 ul Hydrogen peroxide [25% $\text{H}_2\text{O}_2$] to 10 ml citric-phosphate buffer. Make up just before use.

**Phosphate Buffer saline pH 7.4**

8 g Sodium chloride $\text{NaCl}$

0.2 g Potassium chloride $\text{KCl}$

0.2 g Potassium dihydrogen phosphate $\text{KH}_2\text{PO}_4$

2.9 g Disodium hydrogen phosphate $\text{Na}_2\text{HPO}_4$

Dissolve in 800 ml distilled water. Titrate to pH 7.4 with 5M HCl. Adjust to 1 litre with distilled water.
Reducing mixture

1.6 g Sodium Dodecyl Sulphate SDS
3 ml Distilled water
5 ml Tris-Cl [1 M; pH 6.8]

Dissolve SDS completely. Add 8 ml AR Glycerol and 4 ml 2-Mercaptoethanol.
Store at -20 °C in 500 ul aliquots.

Running buffer [0.25 M Tris; 1.92 M Glycine]

6.06 g Tris
28.8 g Glycine
2 g SDS

Dissolve in 2 litre distilled water.

Saline [0.15 M NaCl]

0.85 g Sodium chloride NaCl

Dissolve in 100 ml distilled water. Autoclave at 121 °C for 15 minutes.

Sodium Dodecyl Sulphate [10 % (w/v)]

10 g Sodium dodecyl sulphate SDS

Dissolve in 100 ml distilled water. Filter through Whatman filter paper No.41

Sodium hydroxyde [5 M]

40 g Sodium hydroxyde NaOH

Dissolve in 200 ml distilled water.

Tris -Cl; pH 6.8 [1 M]

12.11 g Tris-hydroxymethyl-aminomethane

Dissolve with 80 ml distilled water. Titrate to pH 6.8 with 5 M HCl. Adjust to 100 ml with distilled water.

Tris Cl; pH 7.5 [10mM]

1.31 g Tris

Dissolve in 800 ml distilled water. Titrate to pH 7.5 with 5 M HCl. Adjust to 1
litre with distilled water.

**Tris - Cl; pH 8.8 [1.5 M]**

18.16 g Tris

Dissolve in 80 ml distilled water. Titrate to pH 8.8 with 5 M HCl. Adjust to 100 ml with distilled water.

**Tris-Glycine solution pH 8.0 [20 Mm Tris; 25 Mm Glycine]**

0.242 g Tris

0.198 g Glycine

Dissolve in 80 ml distilled water. Titrate to pH 8.0 with 5 M HCl. Adjust to 100 ml with distilled water.

**Tris - Saline buffer pH 7.2 [25 Mm Tris; 0.15 M Sodium chloride]**

6 g Tris

17.5 g Sodium chloride NaCl

Dissolve in 1.8 litre distilled water. Titrate to pH 7.2 with 5 M HCl. Adjust to 2 litre with distilled water.

**Tris - Saline - Tween20 [0.5 %]; pH 8.9**

3.02 g Tris

8.75 g Sodium chloride

Dissolve in 800 ml distilled water. Titrate to pH 8.9 with 5 M HCl. Add 5 ml 100% Tween20. Adjust to 1 litre with distilled water.

**Tris - Saline - Tween20 [0.05 %] or Washing buffer; pH 7.2**

Add 1 ml 100 % Tween 20 to 2 litre Tris-Saline

**Trypticase soy broth**

3g Trypticase soy broth

Dissolve in 100 ml in distilled water. Autoclave at 121 °C for 15 minutes.
Appendix 3

BACTERIAL COLLECTION

Maintenance and propagation: bacteria used frequently in the experiments were stored as pure cultures. Stab cultures were used for short term maintenance and freezing and freeze-drying for long term maintenance. Replicate samples were always prepared. One was kept for preservation and the other as working culture.

Stab cultures: A sterile glass vial (10 x 30 mm) was filled with 15 millilitre of hot Luria Bertani agar using a continuous dispenser syringe. The vials were stored at room temperature until use. A single colony was removed from the agar plate using a wire loop. The loop was stabbed into the centre of agar to the bottom of the vial. The lid is slightly loosened to allowed access to air for growth, preferably overnight at room temperature. The lid was then tightened and sealed with paraffin to prevent metabolism and drying.

Freezing: Bacteria were inoculated into a glass tube containing brain heart infusion broth. The culture was incubated at 37 °C in an orbital shaker overnight. An equal volume of 50 % glycerol in brain heart infusion broth was added to the bacterial culture. One millilitre of this culture was transferred into a 1.5 millilitre cryo tube and the tubes were snap frozen in liquid nitrogen to prevent metabolism and contamination.

Freeze-drying (lyophilising): Bacteria were inoculated into a glass tube containing 100% sterile cream milk. The bacterial culture was incubated for 5-6 hours at 37°C in a orbital shaker and two drops of cell suspension (50-100 μl) transferred to sterile ampoules with a Pasteur pipette. Wool plugs were inserted and trimmed off level with the tube. The bacterial suspension was frozen by evaporative freezing under low pressure while centrifuging to prevent frothing. Ampoules were taken out of the centrifuge and cotton wool plugs were pushed halfway down the ampoules. The ampoule was constricted above the cotton wool plug to produce a short capillary of about 2-3 mm in diameter. This was done by using natural gas and oxygen to produce a very hot flame which can melt
A secondary drying process was then employed. The water that remained unfrozen after the primary stage was trapped by refrigerated vapour at low temperature under vacuum, leaving behind about 1% of the moisture content.

After secondary drying, ampoules were sealed in situ on the refrigerated vapour manifold while still under vacuum. This was done by using a portable Bunsen burner to melt the glass at the constricted part.

For locating leaks in the ampoules after secondary drying and sealing, a high frequency spark tester was used. A purple glow or no charge indicated there was a leak. Pale blue/violet shows a satisfactory vacuum.
BACTERIOLOGY IDENTIFICATION

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**PRIMARY TESTS**

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**SECONDARY TESTS**

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Appendix 4

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The SDS-PAGE was carried out under reducing conditions as described by Laemmli (1970).

* Gel casting: Stock solution is needed as follows:
- Sterile water
- Tris-Cl 1.5M, pH 8.8
- Tris-Cl 1M, pH 6.8
- Polyacrylamide/Bis 29:1 (30% w/v)
- SDS (10% w/v)
- Ammonium persulphate (10% w/v)
- TEMED

* Preparation of gel mould: Glass plates, aluminium plates, spacers and template compartments should be cleaned thoroughly prior to setting up the gel mould, using 70% ethanol if necessary. Insert plastic spacers (1.5 mm) between the glass plate (12cm x 8cm) and aluminium plate. Slide the cassette firmly into the compartment of the gel slab casting apparatus so that the glass plate is facing outwards. Repeat the cassette assembling to fit the casting units (2-4 cassettes in a small casting apparatus and 10 cassettes in a large unit). Apply a very thin film of silicone grease to the plastic gasket to avoid leakage. The side of the compartment can be fitted with the aid of clamps.

* Forming of SDS-PAGE: The choice of polyacrylamide concentration is dependent on the experiment and expressed as a percentage of total acrylamide. A gel usually contained a larger pore (stacking gel) and a smaller pore (separating gel). Separating gel was between 8-12% acrylamide frequently has 2-4% acrylamide stacking gel. Low acrylamide gel concentration was useful for separating high molecular weight molecules (e.g. native proteases) and higher concentrations are useful for separation of proteins and low molecular weight peptides.
The gels were made by chemical polymerisation from cross linked bisacrylamide, SDS (0.2 mg/ml polymerisation); ammonium persulphate (APS) catalyst (0.1 mg/ml polymerisation mixture) and a N,N,N,N. tetramethylethlenediamine (TEMED) initiator (approx 30 ml/50ml polymerisation mixture). The polymerisation mixture was always degassed to ensure gas was not present during polymerisation. This polymerisation mixture was gently poured into the gel casting unit and allowed to polymerise at room temperature for 1 hour. To ensure the gel surface was in a straight line, butanol solution (20% v/v in distilled water) was run to seal the top of the gel.

After the gel was set, the butanol was poured off. A scalpel blade was required to separate the individual gel. Gently wash the gel with deionised water before storing in plastic wrapping in a sealed container until use.

* Setting up electrophoresis:
- Running electrophoresis buffer pH 8.6: Running buffer tris-glycine with SDS (25 mm tris, 1.95m glycine, 0.01% SDS) was prepared prior to setting up electrophoresis. Running buffer was placed in both buffer compartments of the Hoefer protein II up to the indicated level.

- Preparing the stacking gel: Insert the ready made separating 12% SDS polyacrylamide gel mould into the slot with clamps. Pour gently the 4% stacking gel onto the separating gels, making sure not to introduce air bubbles. It is advisable to insert the well formed combs into the stacking gel until the bottom of the comb is submerged at least 5 mm below the gel level. Polymerisation of stacking gel is completed after one hour at room temperature. Carefully remove the comb from the stacking gel. Fill the gel to the upper edge of the gel mould with running buffer. Samples can be placed into the wells.

- Preparing the samples: All protein samples are adjusted to 10 mg/ml in 25 mM tris buffer. A mixed liquid protein sample with reducing mixture (denaturing in a ratio of 1:1 and heated for 5 minutes in a waterbottle at 100°C). For solid samples such as bacterial whole cell, a cell pellet from 1 ml of A650 absorbance was resuspended in the reducing mixture. After heating, separate the undissolved component by a brief centrifugation for 5 minutes at 5000 rpm in a
bench top Sigma centrifuge. Molecular weight standard markers were also treated with reducing mixture and boiling but without centrifugation.

A tracking dye indicator (bromophenol blue [0.1% w/v]) was added to all samples. 5-10 μl of samples were loaded into each well slot using a microsyringe.

- Electrophoresis Run: When running a 1.5 mm thick gel at a constant voltage of 150V (4% stacking gel, 12% separating gel), the smallest proteins migrate to the anode in the given system within 2 hours in the presence of an in-built water cooling system.

- Staining and destaining the gel: Upon the completion of the electrophoresis run, i.e. when the bromophenol blue tracking dye reaches the bottom of the gel, voltage was brought back to zero. Gels were taken off the glass plates and placed in the staining solution (0.5% w/v) Coomassie blue R-250 in 27% (v/v) ethanol, 6% (v/v) acetic acid) to stain for 18 hours. To destain, use a solution of 21% ethanol and 6% acetic acid. The destaining process takes 8 hours with several changes of destaining bath.

WESTERN IMMUNOBLOT

With the Western blotting, it is possible to transfer a protein mixture separated in SDS-PAGE entirely into nitrocellular membrane (Towbin et al., 1979) where they can be readily identified by antibodies.

The transfer of proteins from gel onto membrane is done vertically or horizontally in the direction indicated by means of electrophoresis with the aid of blotting buffer (appendix 1) and therefore transfer sandwich.

* Transfer sandwich assembling
   - Blot filter paper sheet and fibre and precut Nitrocellular membrane are soaked in blotting buffer until they are saturated.
   - Fill the electrophoresis tank with blotting buffer. When the bromophenol blue tracking dye reaches the bottom of the gel, voltage was dropped to zero and gels were removed from the glass and aluminium plates. Gels were soaked in blotting buffer for 1-2 minutes.
Meanwhile, a blotting sandwich is assembled. Place the fibre pad on top of a plastic sandwich holder followed by the blotting filter paper. Gel is placed firmly at the centre of blotting filter paper. Precut nitrocellular membrane is gently placed on top of the gel. Blotting filter paper and fibre pad are placed on top of the nitrocellar membrane, followed by a plastic sandwich holder. In this manner, up to 4 gel sandwiches could be transferred at the same time. Put the lid on the unit, connect the leads to the power supply unit so that the electrophoresis would begin from Cathode to Anode.

A complete transfer of gel varies according to the limit of voltage output.

30 volts requires 18 hours
60 volts requires 3.5 hours
90 volts requires 1.5 hours

At the end of the electrophoresis transfer, voltage was brought down to zero before switching it off. As a general rule, molecular weight standard markers could be removed from the nitrocellular blot before the immune reaction is carried out.

* Immunoreaction: Before immobilised proteins could be detected or bound to antibodies, the free or non specific binding sites were saturated with gelatine at 1.5 % (w/v) in a high pH tris-saline-tween 20 (0.5% w/v) for one hour. The membrane was washed thoroughly with wash buffer tris-saline-tween 20 (0.05% v/v) with three changes before being exposed to antibodies. Antibodies were raised in particular donors such as sheep or rabbits antisera against specific antigens to form a antigen-antibody complex. After 2 hours incubation, excess antibodies or unbound antibodies were washed off thoroughly with tris-saline-tween 20 buffer. These antigen-antibody complexes could be detected by specific second antibodies which were conjugated with enzymes that cause a localised colour reaction which are revealed by colour bands as detailed below.

* Second antibody conjugates
  - Against rabbit antibodies: Bio-rad goat antirabbit IgG or protein A were conjugated either with alkaline phosphatase or horse radish peroxidase
  - Against sheep antibodies: monoclonal antibody against ruminant Ig were
conjugated either with alkaline phosphatase or horse radish peroxidase (kindly donated by Ms B. Pang of EMAI).

* Substrate: Substrates were made immediately prior to use since there is a natural precipitation or color change which may give a false positive reaction. For alkaline phosphate colour development reagents, two reagents must be used in combination to produce purple bands on the nitrocellulose membrane. nitroblue tetrazolium (NBT) and 5 bromo-4 chloro-3 indolyl phosphate (BCIP). For horse radish peroxidase colour development reagents, it is only required to prepare diaminobenzidine (DAB). This produces brown bands on the nitrocellulose membrane.

Nitrocellulose membranes with bound antigen-antibody conjugate complexes are washed in tris-saline-tween 20 to remove all the excess conjugates. Immerse the nitrocellular membranes in the colour development solution. Agitation might be required to aid the colour development. Positive reaction would become visible as purple bands (AP-) or brown bands (HPR-). The incubation in colour development should not proceed further if the membrane produces some visible coloured background.

* Stopper: To stop the reaction, nitrocellular membranes were transferred to a distilled water bath for 5-10 minutes and dried on filter paper. Photographs may be taken using black and white film.

* Storage: Nitrocellulose membrane should be stored between plastic film and protected from light.
Appendix 5

SKIN SECTIONING AND HEMATOXYLIN/EOSIN STAINING

a. **Fixation:** The purpose is to preserve a permanent form of tissue in the living state and the fixation was carried out as soon as possible after the skin biopsy took place. A 10% formalin buffer was used as fixer and the biopsies were stored for a week in this buffer.

b. **Infiltration:** Infiltration is the process of removing formalin buffer, hardening and cleaning the tissues. By passing specimens through ascending grades of alcohol, the formalin buffer is removed and the tissues become harder. Xylol is used to remove any alcohol and make tissue become translucent. The clear tissue was then placed in activated carbon (activated charcoal) and melted paraffin and allowed to remain until the embedding takes place. The infiltration process was carried out in the tissue Tek VIP infiltration processor which requires 18 hours to complete.

c. **Embedding:** Embedding is the process of enclosing the specimen in a convenient block of solid paraffin. The size of the block is determined by the size of the specimens. Hot paraffin (56°C) is poured into small stainless steel dishes and tissues are arranged such that the plane desired for sectioning faces the table surface. Care was taken to ensure that paraffin is above the specimens. A tissue holder was placed on top of the dish followed by a thin film of paraffin over the holder. The dish was transferred to an ice block which harden the paraffin. The paraffin block was lifted of a small spatula or forceps.

d. **Trimming:** Excess paraffin was trimmed as close to the specimen surface as possible before cutting. At this stage the microtome was adjusted as thick as 20 micron sections.

e. **Cutting:** The microtome was adjusted to cut sections as thin as five microns. The paraffin block was placed so that its surface is parallel with the cutting edge of the knife. Successive cutting sections appear in a continuous series.

f. **Spreading:** The ribbon was transferred to a warm water bath and spread on
the slide by placing slide underneath the section. The section was then lifted and allowed to adhere to the glass slide surface. The slide is allowed to dry at room temperature before subjected to staining.

g. Staining: This process was carried out in an automated stainer (Sakura DRS 60). According to the standard method of staining with Harris Haemotoxylin and Eosin.

(i) Dewaxing: the slide was placed in Histoclear which dissolves paraffin.

(ii) Hydrating: Remove histoclear from the section by absolute alcohol then run them through a descending graded alcohol (100%, 95%, 60%) to water.

(iii) Staining: In this process, Harris Haemotoxylin is used. This stain is specific for nucleus and chromatin. Slides are dipped in Haematoxylin solution (10 minutes) then rinsed with water to remove excess Haematotoxylin before it is further stained in counterstain Eosin. The excess Eosin is rinsed off, in absolute alcohol and complete in 60% alcohol.

(iv) Clearing: The stained section was passed through histoclear to remove all excess water and alcohol.

(v) Mounting: The stained section was removed from histoclear and allowed to dry. Mounting medium (DPX) was added to the section between the slide and the coverslip.

(vi) Drying: Any remaining mounting medium is wiped off with paper tissues before slides are allowed to dry at room temperature.

(vii) Slides: All slides are stored horizontally in a cardboard box.
Appendix 6

RECORD OF CALCULATIONS

The following calculations are only applicable to Figure 3.2 and Figure 3.4 of Chapter 3.

Using Student t-test the percentage inhibition, with level of significant difference are shown.

SE : Standard Error

\[
\% \text{Control} = \left( \frac{\text{Mean colony diameter on lawn}}{\text{Mean colony diameter of control}} \right) \times 100
\]

\[
\% \text{Inhibition} = 100 - \% \text{Control}
\]

p: level of significance

NS: Not significant, i.e. p > 0.05

Appendix 6.1: % Inhibition of fleece bacteria by P. aeruginosa lawn

<table>
<thead>
<tr>
<th>Species</th>
<th>%Control ± SE (n=4)</th>
<th>%Inhibition</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas hydrophila</td>
<td>62.4 ± 3.6</td>
<td>37.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alkaligenes faecalis</td>
<td>72.4 ± 3.2</td>
<td>27.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>31.3 ± 1.6</td>
<td>68.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bacillus coagulans</td>
<td>41.7 ± 1.7</td>
<td>58.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>65.4 ± 3.0</td>
<td>34.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>26.7 ± 2.0</td>
<td>72.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pseudomonas alkaligenes</td>
<td>64.8 ± 6.3</td>
<td>35.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>94.2 ± 4.0</td>
<td>5.8</td>
<td>NS</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>57.6 ± 2.9</td>
<td>42.4</td>
<td>&lt;0.005</td>
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</table>
Appendix 6.2: % Inhibition of fleece bacteria by *P. alkaligenes* lawn.

<table>
<thead>
<tr>
<th>Species</th>
<th>%Control ± SE (n=4)</th>
<th>%Inhibition</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>85.3 ± 1.4</td>
<td>15.0</td>
<td>NS</td>
</tr>
<tr>
<td><em>Alkaligenes faecalis</em></td>
<td>82.8 ± 1.4</td>
<td>17.2</td>
<td>NS</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>99.1 ± 1.5</td>
<td>21.0</td>
<td>NS</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em></td>
<td>80.2 ± 0.3</td>
<td>20.0</td>
<td>NS</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>83.6 ± 2.0</td>
<td>16.0</td>
<td>NS</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>84.2 ± 0.8</td>
<td>16.0</td>
<td>NS</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
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<td>12.0</td>
<td>NS</td>
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<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>82.0 ± 3.2</td>
<td>18.0</td>
<td>NS</td>
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</tbody>
</table>

Appendix 6.3: % Inhibition of fleece bacteria by *A. hydrophila* lawn.

<table>
<thead>
<tr>
<th>Species</th>
<th>%Control ± SE (n=4)</th>
<th>%Inhibition</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alkaligenes faecalis</em></td>
<td>83.7 ± 2.0</td>
<td>17.3</td>
<td>NS</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>87.0 ± 1.0</td>
<td>13.0</td>
<td>NS</td>
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<tr>
<td><em>Bacillus coagulans</em></td>
<td>96.5 ± 3.0</td>
<td>4.0</td>
<td>NS</td>
</tr>
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<td><em>Enterobacter aerogenes</em></td>
<td>91.9 ± 4.0</td>
<td>8.0</td>
<td>NS</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>95.0 ± 4.0</td>
<td>5.0</td>
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</tr>
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<td><em>Pseudomonas aeruginosa</em></td>
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<td><em>Pseudomonas alkaligenes</em></td>
<td>82.5 ± 3.0</td>
<td>7.5</td>
<td>NS</td>
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<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>91.6 ± 2.0</td>
<td>8.4</td>
<td>NS</td>
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</table>
Appendix 6.4: % Inhibition of fleece bacteria by *A. faecalis* lawn.

<table>
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<th>%Control ± SE (n=4)</th>
<th>%Inhibition</th>
<th>p</th>
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</thead>
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<td>93.9 ± 0.4</td>
<td>6.1</td>
<td>NS</td>
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<tr>
<td><em>Bacillus cereus</em></td>
<td>85.6 ± 0.5</td>
<td>14.4</td>
<td>NS</td>
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<tr>
<td><em>Bacillus coagulans</em></td>
<td>91.2 ± 2.0</td>
<td>8.8</td>
<td>NS</td>
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<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>96.4 ± 1.0</td>
<td>3.8</td>
<td>NS</td>
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<td><em>Proteus mirabilis</em></td>
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<td>12.7</td>
<td>NS</td>
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<td><em>Pseudomonas aeruginosa</em></td>
<td>92.3 ± 2.0</td>
<td>7.8</td>
<td>NS</td>
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<td><em>Pseudomonas alkaligenes</em></td>
<td>85.1 ± 1.0</td>
<td>14.9</td>
<td>NS</td>
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<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>80.4 ± 1.0</td>
<td>19.6</td>
<td>NS</td>
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</table>
The following tables represent results for Table 3.2 of Chapter 3.

**Appendix 6.5: The average of cfu (n=3) counted (+ SE) on agar plates at each time point after each bacterial species was grown in two different concentration of pyocyanin (0.1 and 1 mM). Control had no pyocyanin.**

### Aeromonas hydrophila

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### Alkaligenes faecalis

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### Bacillus cereus

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### Bacillus coagulans

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<td>4 hr</td>
<td>128.6±7.4</td>
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<td>149.2±10.3</td>
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### Enterobacter aerogenes

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### Pseudomonas aeruginosa

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<td>Pseudomonas alkaligenes</td>
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<td>Proteus mirabilis</td>
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<table>
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