CHARACTERISATION OF *PROPIONIBACTERIUM ACNES*

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Characterisation of *Propionibacterium acnes*

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SUMMARY

Propionibacterium acnes forms part of the normal flora of the skin, oral cavity, large intestine and the external ear. Historically, *P. acnes* is considered to be of low virulence; however, in recent years it has been found as the aetiological agent in various pathologies including acne vulgaris, endophthalmitis, endocarditis, osteomyelitis, sarcoidosis, prosthetic hip infections and sciatica. It currently remains unclear why this normally harmless commensal can cause infection and contribute to a number of clinically significant conditions. This thesis has sought to investigate the phenotypic, genetic and antigenic properties of *P. acnes* strains isolated from sciatica patients undergoing microdiscectomy, normal skin, blood cultures, prosthetic hips and acne lesions. Isolates' phenotype was examined by determining their biotype by analytical profile index, antimicrobial susceptibility, virulence factor expression and serotype. A molecular typing method for *P. acnes* was developed using random amplification of polymorphic DNA (RAPD). Patient serum was used to screen *P. acnes* strains for antigens expressed *in vivo* and the chemical composition determined. The serodiagnostic potential and inflammatory properties of identified antigens were assessed. The optimised and reproducible RAPD protocol classified strains into three major clusters and was found to distinguish between the serotypes I and II for a large number of clinical isolates. Molecular typing by RAPD also enabled the identification of a genotype that did not react with the type I or II monoclonal antibodies and these strains may therefore constitute a previously undiscovered subspecies of *P. acnes* with a genetic background different from the type I and II serotypes. A major cell-associated antigen produced by all strains was identified and characterised. A serological assay based on the antigen was used to measure IgG and IgM levels in serum from patients with acne, sciatica and controls. No difference in levels of antibodies was detected. Inflammatory properties of the antigen were measured by exposing murine macrophage-like cells and measuring the release of nitric oxide and tumour necrosis factor-alpha (TNF-α). Only TNF-α was elicited in response to the antigen. The phenotypic, genotypic and antigenic properties of this organism may provide a basis for future studies on *P. acnes* virulence and provide an insight into its mechanisms of pathogenesis.

Keywords: *Propionibacterium acnes*, virulence, molecular typing, immune response

For my family

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ABBREVIATIONS

ANOVA	One-way analysis of variance
API	Analytical profile index
BCG	Bacille Calmette-Guérin
BHI	Brain heart infusion
BSA	Bovine serum albumin
CFU	Colony forming unit
Da	Daltons
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
H_2O_2	Hydrogen peroxide
IFM	Immunofluorescence microscopy
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl-1-thio-ß-D-galactopyranoside
kb	Kilo-base pairs
KCl	Potassium chloride
kDa	Kilodaltons
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
MLS	Macrolide, lincosamide and type B streptogramin
MW	Molecular weight
NCTC	National collection of type cultures
NO	Nitric oxide
NOS	Nitric oxide synthase

ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMN	Polymorphonuclear leukocyte
RAPD	Random amplification of polymorphic DNA
REA	Restriction enzyme analysis
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate
SPSS	Statistical package for social scientists
TAE	Tris acetate EDTA
Taq	DNA polymerase from *Thermus aquaticus*
TBS	Tris buffered saline
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tris	Tris [hydroxymethyl] aminomethane
UPGMA	Unweighted pair group method of arithmetic averages
UV	Ultra violet
X-gal	5-brom-4-chloro-3-indolyl-ß-D-galactopyranoside

CHAPTER 1 INTRODUCTION

1.1 *Propionibacterium acnes*

The genus *Propionibacterium* consists of the classical propionibacteria and the human cutaneous propionibacteria. *P. acnes* belongs to the human cutaneous propionibacteria along with *P. avidum*, *P. granulosum*, *P. innocuum* and *P. propionicum*. Historically, *P. acnes* has been designated *Bacillus acnes* and *Corynebacterium acnes* (Marples and McGinley, 1974) and in papers concerning its role as an adjuvant in tumour therapy the organism is often referred to as *C. parvum* (Eady and Ingham, 1994).

P. acnes is usually regarded as a strict anaerobe but can tolerate oxygen at 100% saturation, growing at reduced rates (Cove *et al.*, 1983). In fact, work by Gribbon *et al.* (1994) has shown that growth of *P. acnes* is promoted at low oxygen concentrations.
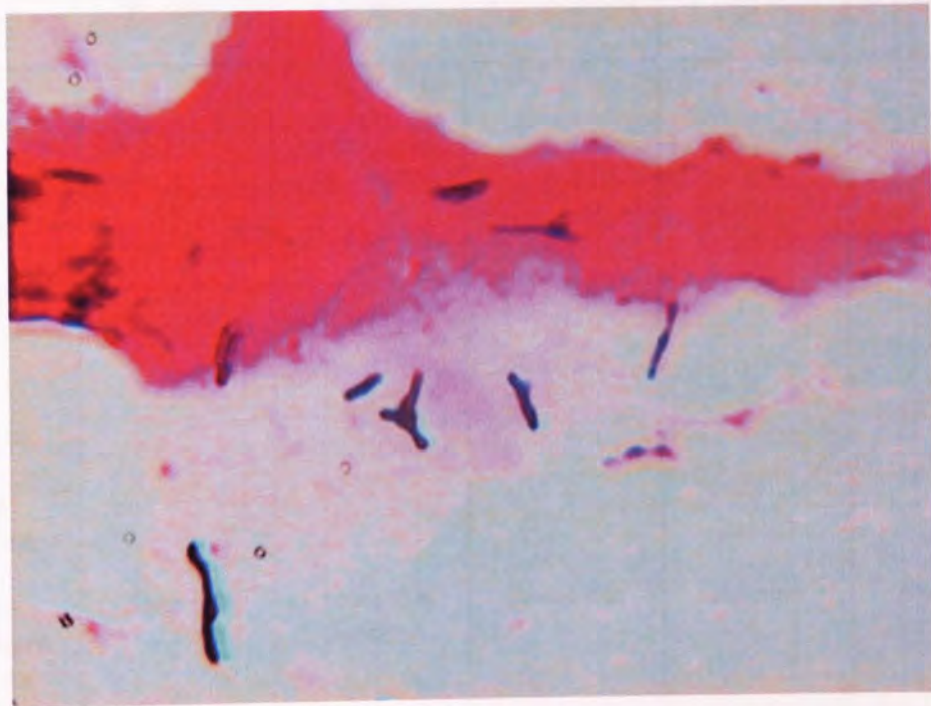
Figure 1.1 Gram stained smears of tissue sample embedded in agarose showing Gram-positive branching rods of *P. acnes* (Stirling *et al.*, 2001)

P. acnes is a non spore-forming, Gram-positive, anaerobic pleomorphic rod (figure 1.1). The cells usually measure $0.3\mu m$-$1.3\mu m$ in diameter and $1\mu m$-$10\mu m$ in length and appear on blood agar as circular cream or yellow opaque colonies of 0.5-2mm in diameter. Colonal morphology is not sufficiently reliable for identification of propionibacteria, but they can be differentiated from other Gram-positive rods by their production of large amounts of propionic and acetic acids which form as fermentation end products. *P. acnes* can ferment glucose, glycerol and fructose but not lactose, sucrose, maltose, xylose or arabinose (Moss *et al.*, 1969). Simple laboratory biochemical tests (table 1.1) can be used to distinguish *P. acnes* from the other commensal propionibacteria (Eady and Ingham, 1994). Attempts have been made to devise strain-specific tests, such as phage typing (Webster and Cummins, 1978), but as yet no reliable routine test exists. Johnson and Cummins (1972) described 2 types of *P. acnes*, type I and II, which differ in cell wall composition, with *P. acnes* type II lacking the sugar galactose. Discrimination between the 2 types can be achieved immunochemically by use of specific antisera or biochemically as type II strains are unable to ferment sorbitol.

Test	*P. acnes*	*P. avidium*	*P. granulosum*	*P. innocuum*	*P. propionicum*
Indole production	+	-	-	-	-
Nitrate reduction	+	-	-	+	+
Aesculin hydrolysis	-	+	-	-	-
Casein hydrolysis	+	+	-	-	-
Catalase production	+	+	+	+	-
Optimum atmospheric conditions	Anaerobic	Anaerobic /aerobic	Anaerobic	Aerobic	Anaerobic

Table 1.1 Tests for the differentiation of commensal propionibacteria (adapted from Eady and Ingham (1994)).

P. acnes possesses a thick cell wall typical of Gram-positive bacteria which is surrounded by a floccular layer of material adherent to the cell surface (Montes and Wilborn, 1970). Chemical analysis of the isolated cell wall shows it to be comprised of peptidoglycan and polysaccharide. The peptidoglycan contains L-diamonopimelic acid in place of the more common *meso*-diaminopimelic acid (Johnson and Cummins, 1972). The polysaccharide component contains mannose, glucose and galactose, as well as an amino sugar diaminohexuronic acid (Azuma *et al.*, 1975; Cummins and White, 1983). No lipoteichoic acids (LTA) have been reported in the cell wall, instead the cells appear to contain a lipoglycan, comparable to that of the lipomannan and lipoarabinomannan found in the related mycobacteria (Sutcliffe and Shaw, 1991; Whale *et al.*, 2004).

P. acnes forms part of the normal flora of the skin, oral cavity, large intestine and the external eye (Brook and Frazier, 1991). In the skin *P. acnes* predominates over other constituents of the normal flora in the pilosebaceous follicles (Funke *et al.*, 1997). The physiology of *P. acnes* reflects their specialist habitat. Compared to other Gram positive organisms they have relatively thick cell walls, providing increased protection to changes in osmotic pressure, ion concentrations, ultraviolet radiation, temperature and mechanical stress (Holland and Bojar, 2001).

1.2 Conditions associated with *P. acnes*

P. acnes is historically considered to be non-pathogenic, however in recent years the bacterium has been implicated as the aetiological agent in various pathologies. It is important to note that compared to other bacterial pathogens, *P. acnes* rarely causes infections and the majority of cases have predisposing conditions (Brook and Frazier, 1991).

The main challenge when *P. acnes* is clinically isolated is to determine whether the organism is a contaminant or represents a true infection. In one survey *P. acnes* proved to be the causative agent in only 7.7% cases (Jakab *et al.*, 1996). The infections that the organism did cause however, were serious involving heart valves, the central nervous system, joints and the eye. Jakab *et al.* also highlighted the

significant role of *P. acnes* in late postoperative infections and the strong association of infections with the presence of foreign body devices.

1.2.1 Acne

Acne vulgaris is a chronic multifactorial condition of the pilosebaceous follicles characterised by the appearance of papules, pustules and nodules on the face and upper trunk (Eady and Bojar, 2001). The disease, which affects 80% of adolescents (Oprica *et al.*, 2002), usually begins when a sharp increase in androgen production occurs during adolescence.

Pilosebaceous follicles are the site of acne and are composed of large multilobulated sebaceous glands, a fine hair and a wide follicular canal lined with a stratified squamous epithelium. The outer part of the canal is termed the infundibulum, it is important in the pathogenesis of acne (Guy *et al.*, 1996) and is the site where skin flora, in particular *P. acnes* reside (figure 1.2). During the normal turnover of skin desquamated cells from the follicular canal are carried towards the infundibulum to the surface of the skin with sebum secreted from the sebaceous glands. If the infundibulum of the pilosebaceous follicle becomes blocked, the mixture of sebum and trapped cells can promote bacterial proliferation and inflammation resulting in the development of acne vulgaris (Gollnick, 2003).

Epidermis

Interfollicular epidermis

Infundibulum

Sebaceous gland

Hair fibre

Inner root sheath

Connective tissue sheath

Arrector pili muscle

Bulge

Dermal papilla

Outer root sheath

Hair matrix

Follicle bulb

Germinative epithelium
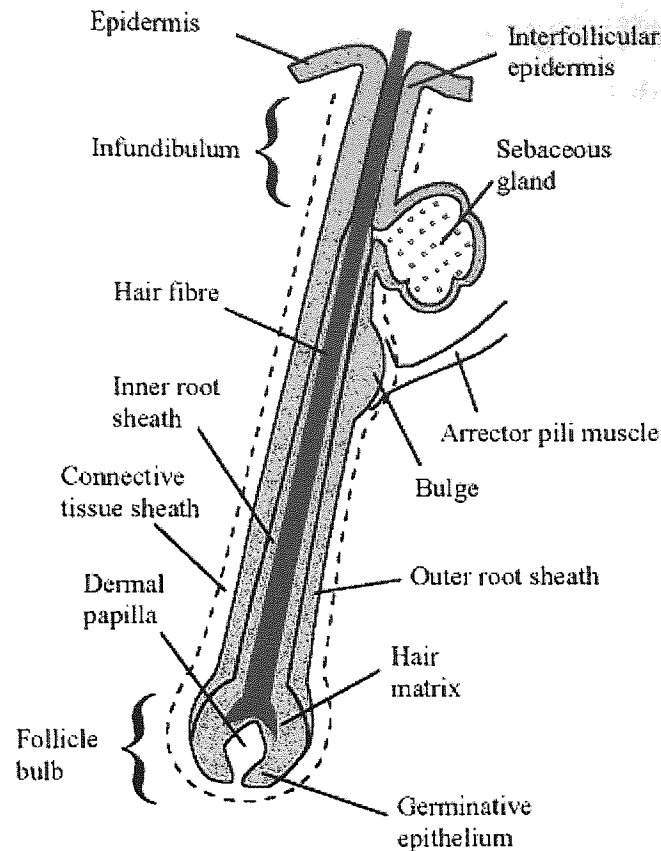
Figure 1.2 Schematic representation of the pilosebaceous follicle adapted from Philpott (2003).

Acne can manifest as non-inflammatory and inflammatory lesions. Non-inflammatory lesions can be open (whitehead) or closed comedones (blackhead) which begin as microcomedones caused by abnormal keratinisation of the infundibular. Visible comedones form due to further blockage of sebum flow and enlargement of microcomedones. Comedones can resolve spontaneously or develop into inflammatory lesions consisting of papules (raised solid lesions), pustules (raised lesions with a visible collection of pus at the surface) and nodules (deep-seated abscesses). The processes behind the formation of acne and the progression from non-inflammatory to inflammatory lesions are not clearly understood. However, the four main aetiological factors are considered to be increased sebum production, abnormal ductal hyperkeratinization, colonisation and proliferation of *P. acnes* within the sebaceous duct and inflammation (Layton, 2000).

Increased sebum production (seborrhoea) plays a key role in the pathogenesis of acne. Sebum is primarily composed of triglycerides, free fatty acids, wax esters and squalene (McGinley *et al.*, 1980) and it is thought that the abnormal composition of lipids in the sebum may also be important in the development of acne (Koreck *et al.*, 2003). In particular, lionoleic acid deficiency is thought to be important in acne aetiology as it impairs the follicular epithelium barrier allowing other free fatty acids to enter the epithelium (Jappe, 2003).

Follicular hyperproliferation and hyperkeratinization are the primary events in the development of an acne lesion. The mechanisms behind these processes have not been fully characterised. It is thought that hyperproliferation may be stimulated by alterations in sebum and lipid levels or the failure of ductal keratinocytes to separate (Koreck *et al.*, 2003). Evidence has also suggested that cytokines may be involved. IL-1α, secreted by keratinocytes in response to changes in sebum, may induce keratinisation (Guy *et al.*, 1996).

The third factor in the development of acne is proliferation of the normal flora, particularly *P. acnes*. The precise role of the organism in acne has never been definitively proven, however there is considerable evidence that *P. acnes* is associated with the condition. *P. acnes* predominate in acne prone areas rich in sebum such as the scalp, forehead, back and ear (McGinley *et al.*, 1978). De Young *et al.*(1984) developed a rat model of inflammation relevant to acne following injection of *P. acnes* intradermally and demonstrated chronic inflammation with the formation of acne-like lesions. Injection of *Escherichia coli*, *Staphylococcus epidermidis* and *Streptococcus lactis* failed to produce a similar response. The importance of *P. acnes* in the development of acne is further supported by the elevation of anti-*P. acnes* cellular and humoral immunity in patients with severe acne (Ashbee *et al.*, 1997; Burkhart *et al.*, 1999b; Holland *et al.*, 1993; Ingham *et al.*, 1987). In addition, antibiotic therapy which suppresses *P. acnes* has been shown to be associated with clinical benefit and *P. acnes* resistance linked to clinical failure (Eady *et al.*, 1989).

During the initial events in the development of acne (non-inflammatory comedones), *P. acnes* is not thought to be involved and therefore not required for comedogenesis

(Lavker *et al.*, 1981). After this process, however *P. acnes* can proliferate within the microenvironment of the comedone as conditions for overgrowth are ideal being that they are anaerobic and provide a lipid rich environment. Lipase secreted by *P. acnes* hydrolyses sebum triglycerides for use as a growth substrate. However, liberated free fatty acids have been shown to have comedogenic properties and promote colonisation by promoting *P. acnes* cell to cell adhesion and anchoring of the cells within the pilosebaceous follicle (Gribbon *et al.*, 1993). Much research on the role of *P. acnes* in acne concerns its pathogenic properties and this is reviewed in detail in section 1.5.

Inflammation is the fourth and final factor involved in acne and the majority of it can be attributed to immunological reaction to *P. acnes*, which is reviewed in section 1.6.

1.2.2 Bone and joint infections

Bone and joint infections caused by *P. acnes* are relatively uncommon (Chia and Nakata, 1996). Cases that have been described and will be discussed in this section include discitis, osteomyelitis, septic arthritis, prosthetic joint infections and most recently, sciatica.

Intervertebral discitis is characterised by lower back pain, disc protrusion and consequently nerve compression and may present with or without fever. *P. acnes* has been reported as an infective agent of intervertebral discitis by Chia and Nakata (1996). In all four reported cases, prior surgery (discectomy in 3 cases) or trauma was noted. The authors suggested that the source of infection was unlikely to be from haematogenous spread and highlighted that inadequate preoperative skin disinfection coupled with the high concentrations of *P. acnes* around hair follicles may increase the chance of postoperative infection.

Spondylodiscitis is an infection of the intervertebral disc and of the adjacent bone. This condition usually occurs through haematogenous spread from a distant site of infection. *P. acnes* however, has been isolated from a case of spondylodiscitis after epidural catheterisation (Halkic *et al.*, 2001; Hernandez-Palazon *et al.*, 2003). Again, these *P. acnes* infections were linked to a predisposing invasive procedure.

Another clear route of infection was apparent in a reported case of vertebral osteomyelitis (Abolnik *et al.*, 1995). The patient had previously undergone a lumbar puncture nine months before presenting with lumbar pain. *P. acnes* was isolated after five days of culture from a biopsy and successfully treated with antibiotics.

A number of infections due to *P. acnes* have been described following spinal instrumentation for the treatment of scoliosis (Richards and Emara, 2001; Richards, 1995; Viola *et al.*, 1997). This method involves straightening and stabilizing the spine after spinal fusion, by surgically attaching hooks, rods, and wire to the spine. Presentation of infection averaged 25 months (Richards, 1995) to 27 months (Richards and Emara, 2001) after initial surgery. All authors suggested that delayed infection resulted from intraoperative inoculation followed by an extended subclinical period.

The National Audit Office reports that the national health service performs over 30,000 total hip replacements each year and some five to ten per cent of hip replacement patients experience complications of varying degrees of severity, including infection and joint loosening (aseptic loosening) (Bourn, 2000). The most frequent route of prosthetic hip infection is surgical contamination but haematogenous infection can also occur (Dupon *et al.*, 2001). Prevention strategies that have decreased the incidence of infection include antibiotic prophylaxis, antibiotic impregnated cement and laminar airflow systems in operating theatres (Tobin, 1999). When infection does occur, however, it is devastating for the patient and can result in permanent joint immobility, amputation and loss of life (Tobin, 1999).

Prosthetic joint infections can be difficult to diagnose on the basis of clinical and microbiological findings. The findings of Tunney *et al.* (1999; 1998) indicate that current methods of detection underestimate the incidence of prosthetic joint infection. In these studies sonication of removed prostheses was used to dislodge bacteria biofilms. *P. acnes* was isolated either alone or with *Staphylococcus* species from 62% of samples (Tunney *et al.*, 1999). It is important to note that 12 culture positive patients had a preoperative diagnosis of aseptic loosening or dislocation (Tunney *et al.*, 1998).

Recently *P. acnes* has been associated with sciatica (Stirling *et al.*, 2001). This is a syndrome characterised by pain radiating from the back into the lower extremities and is most commonly caused by a prolapse of the intervertebral disc. It is usually caused by irritation of a nerve root of the sciatic nerve, often from the compression of a disc or degenerative disease. The pathogenesis of low back pain and sciatica remains poorly understood despite this being one of the most commonest causes for consultation in primary care (OPCS, 1995). While investigating the use of an ELISA (enzyme-linked immunosorbent assay) for Gram positive infection to diagnose pyogenic spondylodiscitis Stirling *et al.* found that sciatica patients, used as negative controls, had raised serum IgG titres. To further investigate this disc and tissue material samples were taken from patients undergoing microdiscectomy to relieve the unremitting pain of sciatica. Of these patients 53% had positive cultures after long term incubation and *P. acnes* was isolated from 84% of these. This raised the possibility that *P. acnes* might contribute to the inflammation and pain associated with sciatica. However, other workers have failed to culture *P. acnes* from microdiscectomy material and suggest that such organisms are derived from the skin as contaminants during the surgical procedure (McLorinan *et al.*, 2003).

Inflammation may be an important component in both sciatica and discogenic back pain. Marshall *et al.* (1977) have demonstrated raised serum immunoglobulins in patients with sciatica. Similarly in herniated disc tissue there is biochemical evidence of inflammation; Kang *et al.* (1996) found that herniated lumbar discs were making spontaneously increased amounts of matrix metalloproteinases, nitric oxide, prostaglandin E2, and interleukin-6. Gronblad *et al.* (1994) investigated inflammatory cell infiltration in disc herniation tissue and found very active inflammation in lumbar disc herniation tissue.

Taken together, the presence of *P. acnes* in disc material and inflammation associated with sciatica raises the question of microbial aetiology, although a direct causal link remains to be established.

1.2.3 Bacterial endophthalmitis

Bacterial endophthalmitis is an ocular infection characterised by inflammation of the interior eye. It is a serious sight-threatening condition as often, despite therapeutic intervention, endophthalmitis results in partial or complete loss of vision (Callegan *et al.*, 2002).

There are three main routes whereby infectious agents can gain access to the eye: postoperative endophthalmitis as a consequence of intraocular surgery; posttraumatic endophthalmitis caused by a penetrating eye injury and haematogenous endophthalmitis due to the spread of bacteria from a distant anatomical site. In terms of incidence, postoperative endophthalmitis is by far the most common, especially following cataract surgery, whereas haematogenous endophthalmitis is relatively rare, comprising 2 to 8% of all cases of endophthalmitis (Okada *et al.*, 1994).

Gram-positive bacteria account for the majority of endophthalmitis isolates, in particular *Staphylococcus epidermidis* (27.8%), viridans group streptococci (12.8%), other coagulase negative staphylococci (9.3%), *Staphylococcus aureus* (7.7%) and *P. acnes* (7%) (Benz *et al.*, 2004). In delayed onset postoperative endophthalmitis *P. acnes* is one of the most common microorganisms isolated (Benz *et al.*, 2004) but the infection is particularly difficult to diagnose and treat (Ciulla, 1999). Patients can present from weeks to years after surgery with low grade uveitis and a white plaque in the capsular bag which is characteristic of infection. Due to the sequestration of *P. acnes* in the capsular bag this kind of endophthalmitis often recurs after standard treatments as it can provide a safe haven for bacteria. Indeed, Aldave *et al.* (1999) and Clark *et al.* (1999) found that *P. acnes* could survive initial treatment with intraocular antibiotics and further therapeutic intervention was required for eradication. Other authors have reported similar findings. Rahman and Holz (2000) reported a case of polymicrobial postoperative endophthalmitis caused by *Alcaligenes xylosoxidans* and *P. acnes*. Initial antibiotic treatment failed and complete vitrectomy with intraocular lens and capsule removal was eventually required for eradication of infection. A case report by Teichmann (2000) highlights the difficulties in diagnosing *P. acnes* endophthalmitis, particularly in the absence of common features such as

capsular plaque or positive intraocular fluid cultures. In this case repeated injections of vancomycin, previously shown to be efficacious, failed to resolve the infection. The author attributes this to sequestration of bacteria in the capsular bag, but also suggests that persistence of organisms in macrophages, biofilms or both may confer resistance to antibiotics. For resolution, the intraocular lens (originally implanted) was removed and ocular inflammation rapidly subsided. Only after nine days was *P. acnes* finally grown in culture from the lens.

1.2.4 Sarcoidosis

Sarcoidosis is a multisystem disorder of unknown cause and is characterised by granulomas primarily affecting the lungs and lymphatic system. It is thought to result from the exposure of a genetically susceptible host to a variety of environmental and infectious agents, including *P. acnes* (table 1.2).

Type of agent	
Infectious	Inorganic/Organic
Viruses (herpes, Epstein-Barr, retrovirus, Coxsackie B virus, cytomegalovirus)	Aluminium
	Pine tree pollen
Borrelia burgdorferi	Zirconium talc
P. acnes	Clay
Mycobacterium tuberculosis and other mycobacteria	
Mycoplasma	

Table 1.2 Environmental agents considered to be involved in sarcoidosis, adapted from Costabel and Hunninghake (1999).

The role of environmental agents is supported by evidence of the inflammatory response in sarcoidosis consistent with the Th-1 type immune response triggered by an antigen and T cell receptor abnormalities in sarcoidosis patients (Costabel and Hunninghake, 1999). *P. acnes* DNA has been detected in the lymph nodes of sarcoidosis patients and if *P. acnes* does cause sarcoidosis, the condition is likely to be

due to a bacterial derived antigen giving rise to a Th-1 immune response (Eishi *et al.*, 2002). This idea is supported by the work of Ebe *et al.* (2000) who found a cellular immune response to a recombinant protein from *P. acnes* in sarcoidosis patients but not in other subjects.

1.2.5 Other infections and conditions associated with *P. acnes*

P. acnes as the causative agent of endocarditis has been reported (Gunthard *et al.*, 1994; Moreira *et al.*, 2000; Vandenbos *et al.*, 2001). These infections characteristically occur on foreign material, for example a teflon ventricular patch in the case reported by Vandenbos *et al.* (2001) and a prosthetic aortic valve reported by Gunthard *et al.* (1994). However, foreign materials are not always involved. Moreira *et al.* (2000) reported on a case of *P. acnes* endocarditis with the only predisposing factor being immunosuppression following liver transplantation.

The abbreviation SAPHO (synovitis, acne, pustulosis, hyperostosis and osteitis) is used to describe a syndrome combining cutaneous lesions with osteoarticular manifestations. Roldan *et al.* (2001) describe a case in a 28 year old male with severe acne who developed arthritis of the left knee and osteomyelitis of the mandible. Other regions of osteomyelitis were also apparent. The patient was given antibiotics and during treatment *P. acnes* was isolated from the mandible on four occasions. *S. aureus* was isolated on one occasion and also from osteomyelitis of the left hip. Continued long term therapy with antibiotics and corticosteroids eventually resulted in the disappearance of osteoarticular symptoms and acne. The authors conclude that there is strong evidence that the disease has an infective origin, however the role of *P. acnes* in the pathogenesis of SAPHO remains unclear and other hypotheses have been presented (Van Doornum *et al.*, 2000).

P. acnes has been described as the cause of glomerulopathy due to chronic infection in a patient with Felty's syndrome (Ohtani *et al.*, 1998). Felty's syndrome presents as a trio of rheumatoid arthritis, splenomegaly and neutropenia. In this case study *P. acnes* was consistently isolated from blood and bone marrow samples and therefore infection-induced nephritis was attributed to the organism. The likely source of the

organism was not cited but the authors emphasize the important role that *P. acnes* plays as an agent in opportunistic infection.

Infections of the central nervous system by *P. acnes* have been increasingly reported, particularly after neurosurgical procedures (Critchley and Strachan, 1996; Ghalayini *et al.*, 2004). Both of these cases illustrate the main features of *P. acnes* intracranial infection, with patients having delayed presentation following surgery and an absence of classical features of such infections, attributed to the organism's low pathogenicity. The source of the contaminating organism is likely to be the scalp due to the high concentrations of *P. acnes* in the sebaceous glands in this area (McGinley *et al.*, 1978). This idea is further supported by the findings of Esteban *et al.* (1995) who reviewed surgical wound infection due to *P. acnes* and found that neurosurgical wound infections predominated, probably for the same reason. Interestingly, Cone *et al.* (2002) reported a case of a cerebral abscess in an otherwise healthy male adult who had been inhaling cocaine for a number of years. Culture of a biopsy from the abscess yielded *P. acnes* and antibiotic therapy commenced. The patient made a complete recovery and it was thought that chronic anaerobic sinusitis may have predisposed the patient to a cerebral abscess caused by *P. acnes* due to inhalation of cocaine.

Noma is a severe gangrenous disease of the oral cavity affecting children in the developing countries and can result in the rapid destruction of both soft and hard tissue. In a study by Paster *et al.* (2002) *P. acnes*, among other organisms, was detected in noma lesions from more than 1 patient using PCR to amplify 16S ribosomal DNA. However, since many different bacterial species were isolated from noma lesions and given that the lesions were open wounds in unsanitary conditions, these findings can only provide information on possible pathogens involved in noma lesions.

In 2000 a case of hepatic granulomatous infiltrates due to *P. acnes* was reported in a patient being treated for leukaemia (Ullmann *et al.*, 2000). As part of the chemotherapy treatment the patient was started on antibiotic prophylaxis, however the patient developed signs of infection and it was noticed that the liver was enlarged. Blood cultures and liver biopsies initially produced negative cultures. A further

biopsy, 76 days after antibiotic therapy was commenced, showed multiple hepatic granulomatous lesions which finally yielded a positive culture of *P. acnes*. The organism was sensitive to the administered penicillin and liver function improved over the next few weeks. This case again serves to demonstrate the spectrum of serious infections *P. acnes* can cause and the difficulties experienced when attempting to diagnose *P. acnes* infection.

1.3 Typing methods

Analysis of bacterial isolates by phenotypic and genotypic methods can provide information on particular characteristics of a species, enable an assessment of the relatedness of strains and associate pathogenic mechanisms with certain strains.

When using a typing method there are several important criteria to consider including typeability, reproducibility, discriminatory power, ease of interpretation and ease of use (Tenover *et al.*, 1994).

1.3.1 Phenotypic methods

The simplest methods of characterising bacteria are those based on observable (phenotypic) differences between organisms. Common methods include colony and cell morphology, biochemical profiling, chemical composition, virulence determinants (exocellular enzyme profile), antibiotic susceptibilities and serological reaction. Studies can be limited, however by the availability of suitable relevant diagnostic tests for identifying and characterising a particular organism.

As phenotypic traits involve gene expression, these properties tend to vary with changes in growth conditions, growth phase and spontaneous mutation (Tenover *et al.*, 1997).

1.3.1.1 Biotyping

Biotyping is based on an organism's reaction to a panel of biochemical tests, for example fermentation of glucose, lactose or other sugars and utilisation of carbon sources. Commercial systems are available which identify organisms by the generation of numerical profiles derived from the biochemical reactions. Each numerical profile can be considered a biotype and can be used to assess the relatedness of strains. However, variations of reactions have been encountered even with a single strain, as microorganisms can alter the expression of many of their cellular products; hence biotyping can have poor reproducibility (Tenover *et al.*, 1997). This could be due to loss of plasmids encoding metabolic functions, variations in incubation conditions, inoculum size and the age of cultures (Tenover *et al.*, 1997).

1.3.1.2 Virulence factors

Virulence determinants can be used in a similar way to biotyping to compare strains but is based on exocellular enzyme production. This method can also provide information on the organism's pathogenic ability. *P. acnes* has been reported to produce a variety of exocellular enzymes and other bioactive exocellular products. These include lipase, hyaluronidase, chondroitin sulphatase, proteinase, haemolysin, neuraminidase and phosphatase (Hoeffler, 1977; Holland *et al.*, 1981; Ingham *et al.*, 1981; Kabongo Muamba, 1982). The liberation of these enzymes by *P. acnes* confers the possibility of direct host tissue damage and inflammation (table 1.3).

Product	Presumed role in health/disease
Lipase	Nutrition. Generation of irritant free fatty acids.
Phospholipase C	Perturbation of membrane function.
Proteinases	Nutrition and proteolysis is the human colon. Complement activation, release of chemotaxins and tissue invasion.
Hyaluronidase	Spreading factor and tissue invasion.
Neuraminidase	Tissue invasion.
Acid phosphatase	Nutrition and scavenging carbon sources.
Histamine and tryptamine	Mediation of acute inflammation

Table 1.3 Role of extracellular enzymes of *P. acnes*, adapted from Eady and Ingham (1994)

1.3.1.3 Antibiotic susceptibility testing

Bacteria can be compared on the basis of their susceptibilities to a range of antibiotics. These can be measured using different methods. Firstly, strains can be exposed to a series of antibiotic concentrations in separate cultures in agar or broth. The minimum inhibitory concentration (MIC) is read at the lowest concentration of antibiotic that inhibits visible growth. Break point sensitivity testing uses fixed concentrations of antibiotic at agreed cut off points giving results simply as resistant or sensitive. The final method uses antibiotic discs which are placed on agar on which the test organism is uniformly seeded. Zones of inhibition can then be measured and used to determine the sensitivity. However these methods are not very discriminatory if all isolates are

sensitive or resistant to the test antibiotics. Furthermore, as resistance to antibiotics is often mediated by unstable extrachromosomal elements it may be subject to frequent and abrupt changes (Towner and Cockayne, 1993). Therefore isolates that are genetically identical may manifest different antibiotic susceptibilities due to the acquisition of new genetic material (Tenover *et al.*, 1997).

1.3.1.4 Serotyping

Specific antibodies can be used to detect antigenic determinants on the surface of bacteria. Serotyping has been used extensively to study the epidemiology of several bacteria for example, *Streptococcus pneumoniae* and *Escherichia coli* 0157:H7 (Busch and Nitschko, 1999).

Two serotypes of *P. acnes* (type I and II) which differ in the composition of their cell wall polysaccharides have been described by Johnson and Cummins (1972). These can also be biochemically distinguished by the inability of type II stains to ferment sorbitol. As a typing system however, two serotypes provide little discriminatory and epidemiological value.

Monoclonal antibodies have been developed to classify *P. acnes* based on cell-associated antigens. Type I and IV were raised against protein antigens and type II and III are carbohydrate containing antigens (personnel communication, Dr Sheila Patrick). It is important to note that these serotypes are different to the classical *P. acnes* serotypes described by Johnson and Cummins (1972), although types I and II in both systems are equivalent.

1.3.1.5 Protein analysis

Whole cell protein patterns can be obtained using polyacrylamide gel electrophoresis (PAGE). Nordstrom (1985) used this method in an attempt to characterise possible protein patterns that may be typical for *P. acnes* strains isolated from acne skin. It was found that the discriminatory power of this technique was very poor as the patterns of *P. acnes* strains isolated from all sources were identical.

1.3.2 Genotypic methods

Advances in molecular techniques have led to the development of new genotyping methods such as plasmid fingerprinting, restriction enzyme analysis (REA), restriction fragment length polymorphism (RFLP), ribotyping and pulsed field gel electrophoresis (PFGE). Furthermore, polymerase chain reaction (PCR) based methods such as random amplification of polymorphic DNA (RAPD), which differentiate organisms at the DNA level have also been developed (Riley, 2004).

1.3.2.1 Plasmid fingerprinting

Plasmids are self-replicating autonomous genetic elements that can be separated from the main chromosome for analysis. The number and size of plasmids present in a strain can be used for typing. However, as many strains of bacteria contain a single plasmid restriction endonucleases can be used to increase the discriminatory power of this method. This is called REA. The enzyme step also makes subsequent analysis easier as uncut plasmid DNA exists in multiple forms (supercoiled, nicked and linear) when analysed by agarose gel electrophoresis.

1.3.2.2 Chromosomal DNA restriction enzyme analysis (REA)

Chromosomal DNA can be analysed by digestion of the DNA using restriction endonucleases and separation of the fragments electrophoretically. Variations in patterns, referred to as restriction fragment length polymorphisms (RFLP), can result from a single base alteration within the restriction site, or by more major changes such as insertions, deletions or sequence rearrangements. Profiles obtained, however, consist of a large number of bands making results difficult to interpret (Busch and Nitschko, 1999).

1.3.2.3 DNA hybridisation and restriction fragment length polymorphisms

To overcome the problems of REA this method can be combined with nucleic acid probes to decrease the number of bands obtained. Following REA, fragments are

transferred to nitrocellulose or nylon by Southern blotting and hybridised with a labelled probe. Probes can be derived from insertion sequences or specific to a particular gene. As only DNA fragments that hybridise to the probe are visible, analysis is simplified.

1.3.2.4 Ribotyping

This technique uses Southern blot analysis of RFLP within the genes encoding ribosomal RNA. The genes for 16S, 23S and 5S ribosomal RNA are highly conserved which makes this method suitable as a universal bacterial probe. Ribotyping can be used to type many bacteria, in particular *Staphylococcus* spp., as they have more than five ribosomal operons and thus produce ribotype patterns of five to 15 bands (Struelens, 1998). However, for bacteria containing only one or two copies of ribosomal genes such as *Mycobacterium* spp., ribotyping is not applicable (Busch and Nitschko, 1999).

1.3.2.5 Pulsed field gel electrophoresis (PFGE)

Large fragments of DNA generated by rare cutting restriction enzymes can be analysed by PFGE. Fragments are electrophoresed in an alternating electric field. Separation of fragments is achieved by the orientation of larger fragments through pores in agarose being slower than that of smaller ones. This method therefore allows the analysis of a small number of fragments generated by restriction enzymes from the entire genome. PFGE is the most commonly used epidemiological tool and is considered the gold standard of molecular typing techniques.

1.3.2.6 Polymerase chain reaction (PCR) and RFLP

PCR using gene-specific primers can be used to amplify specific genetic loci which can then be subjected to RFLP analysis. Common examples include restriction analysis of bacterial 16S rRNA genes and the *recA* gene (Jawad *et al.*, 1998). This method requires only a fraction of the amount of DNA required for Southern blotting and can be completed in a much shorter time (Towner and Cockayne, 1993).

1.3.2.7 Random amplification of polymorphic DNA (RAPD)

RAPD is a PCR-based method which uses short primers (six-ten bp) whose sequences are not directed at any specific sequence in the genome. These short primers will hybridise at random sites on the chromosome to initiate DNA polymerisation. Polymorphisms within the template vary the proximity, number and location of priming sites between strains, giving a DNA fingerprint that can be electrophoresed and visualised. The advantage of this method compared to methods such as RFLP, is that no prior knowledge of the template DNA sequence is required.

The first round of the amplification cycle is carried out under conditions of low stringency achieved by using low annealing temperatures. This allows the primer to anneal randomly to the template where the sequence is not exactly complementary to that of the primer. The amplification products synthesised in the first round become the preferred templates for subsequent amplification cycles, whilst the template DNA is only linearly amplified.

The main criticism of RAPD is lack of reproducibility, however this does not present a problem if critical parameters affecting RAPD are optimised and controlled. These include reaction conditions, primer design and concentration, template concentration, type and make of *Taq* DNA polymerase and PCR buffer components. Providing the conditions of the reaction are optimised, RAPD can give a high level of typeability, discriminatory power and reproducibility. However it has been demonstrated to be slightly less discriminatory than PFGE (Speijer *et al.*, 1999).

RAPD has been used to type a wide range of organisms including Gram positive bacteria such as coagulase negative staphylococci (Raimundo *et al.*, 2002), enterococci (Vancanneyt *et al.*, 2002) and *S. aureus* (Pereira *et al.*, 2002) in addition to Gram-negatives such as *E. coli* (Hopkins and Hilton, 2001; Menichetti *et al.*, 1999) and *Salmonella* spp.(Hilton *et al.*, 1996).

1.3.2.8 Molecular typing of *P. acnes*

Molecular typing of *P. acnes* and other cutaneous propionibacteria has not been widely applied in the clinical situation. Dairy propionibacteria have been analysed previously by RAPD (Matte-Tailliez *et al.*, 2002; Rossi *et al.*, 1998) and REA (Rossi *et al.*, 1998). Furthermore, PFGE has been used to compare strains of *P. acnes* isolated from cases of endophthalmitis (Ting *et al.*, 1999). Progress in molecular typing of *P. acnes* has been hindered by the lack of an effective lysis technique, as *P. acnes* has a relatively thick cell wall. This has been overcome to some extent by the use of penicillin in the lysis method (Tipper *et al.*, 1993).

1.4 Antibiotic resistance

In general, propionibacteria are susceptible to a wide variety of antibiotics and infections caused by *P. acnes* are relatively simple to treat. However, acquired resistance has occurred due to the widespread use of antibiotics to treat acne. Tetracyclines and macrolides in particular have been extensively used to treat the condition. Wang *et al.* (1977) and Denys *et al.* (1983) investigated the susceptibility of *P. acnes* isolates to common antibiotics and both authors reported high sensitivities. Metronidazole was the only agent to which *P. acnes* was consistently resistant and aminoglycosides were found to be weakly active. Since these studies, decreased sensitivities have been reported. In a 15 year retrospective study in Japan Kurokawa *et al.* (1988) demonstrated a progressive increase in antibiotic resistance of *P. acnes* isolates from acne lesions. The highest levels of resistance were seen with clindamycin, erythromycin and tetracycline, reflecting prescribing trends. Similar findings have been found in many other countries (reviewed by Eady *et al.*, 2003). Later work by Kurokawa (1999) again showed resistant strains from acne patients to erythromycin, clindamycin and tetracycline and suggested minocycline as the drug of choice in the treatment of acne due to the high sensitivity of strains to this antibiotic. However, in 2001 a collaborative study between clinics in Europe, USA, Japan and Australia reported high levels of minocycline resistance (Ross *et al.*, 2001).

The molecular basis of resistance has been shown to be caused by 3 point mutations in genes encoding the 23SrRNA for erythromycin (Ross *et al.*, 1997) and a single 16SrRNA base mutation for tetracycline (Ross *et al.*, 1998). The 3 different point mutations involved in resistance to erythromycin have been found to confer different levels of cross resistance to macrolide, lincosamide and type B streptogramin (MLS) antibiotics (Ross *et al.*, 1997). The fact that these are chromosomal mutations allays fears of dissemination of resistance by transmissible genetic elements. However, a further mechanism of MLS resistance has been found to be transposon based (Ross *et al.*, 2002). The transposon, Tn*5432*, from *Corynebacteria* was found to be non-conjugative but the mechanism by which it is transferred has not been elucidated to date.

1.5 Pathogenicity

Most research relating to the pathogenic potential of *P. acnes* is focused around its association with acne, thus most of the work discussed in this section, will relate to this condition.

Several characteristics of *P. acnes* contribute to the organism's success as a pathogen. As already discussed (section 1.3.1.2), it produces a number of exocellular products as well as a number of metabolites including acetate, propionate (Allaker *et al.*, 1987) and indole, hydrogen sulphide and porphyrins (Lee *et al.*, 1978) which can cause direct host damage. Allaker *et al.* (1987) also identified the presence of histamine, tryptamine and short chain fatty acids which, if produced *in vivo*, may contribute to inflammation without mediation of the immune system. Cytotoxic effects of *P. acnes* culture supernatant on monkey kidney cells and fibroblasts have also been reported (Allaker *et al.*, 1985; Csukas *et al.*, 2004).

P. acnes is slow growing and can resist phagocytosis and persist intracellularly within macrophages (Webster *et al.*, 1985b). The organism's resistance to phagocytosis has been attributed to the structure of its cell wall, in particular the complex fibrillar layer (Montes and Wilborn, 1970).

P. acnes may cause inflammation in acne via its interaction with antibody and complement, production of chemotactic factors and by cell mediated immunity (Burkhart *et al.*, 1999a). Elevated levels of antibodies to *P. acnes* have been demonstrated in acne patients and will be examined in more detail in the next section. These higher levels of antibodies might enable activation of complement in acne patients, indeed Webster *et al.* (1978) demonstrated that *P. acnes* could activate the classical pathway and also the alternative pathway. In addition, activation of these pathways generates chemotactic factor, C5a, attracting neutrophils to the site.

Two putative mediators of inflammation, the heat shock proteins GroEL and DnaK, have been cloned and sequenced from *P. acnes* (Farrar *et al.*, 2000). Heat shock proteins are known to be highly immunogenic and Farrar *et al.* are undertaking further studies to investigate whether these proteins are involved in the initiation of inflammation in conditions associated with *P. acnes*.

Cell mediated immunity has been shown with *P. acnes* but its exact role in acne has yet to be proven. Evidence obtained by Norris and Cunliffe (1988) who examined early acne lesions pointed towards a cell mediated immune response, as the predominant cells in the initial cell infiltrate were found to be lymphocytes. Jappe *et al.* (2002) demonstrated that *P. acnes* could activate T-lymphocytes by two mechanisms; antigen and mitogen driven activation. *In vivo* this would then lead to a proinflammatory response and may cause the inflammation associated with acne. *P. acnes* has also been shown to induce cellular infiltration in the cornea (Suzuki *et al.*, 2002). Here the response observed resembled that of delayed hypersensitivity in the skin, with neutrophil infiltration followed by mononuclear cells including macrophages and lymphocytes. Further immunohistochemical investigation revealed that CD4 T cells predominated over CD8 T cells.

In the study by Norris and Cunliffe (1988) polymorphonuclear leukocytes (PMNs) were also found in acne lesions and it is widely believed that inflammation in acne is contributed to by chemotactic factors produced by *P. acnes*. Material produced by *P. acnes* in the culture medium has been shown to be chemotactic for PMNs and was found to be of low molecular weight ($\leq$2000 Daltons) (Webster and Leyden, 1980).

Pulverer *et al.*(1988) also demonstrated that *P. acnes* released a granulocyte activating factor that was chemotactic. Again the factor was of similar low molecular weight so it could potentially penetrate follicular walls. Webster *et al.* (1980) showed that lysosomal release by PMNs in response to *P. acnes* required the presence of serum whereas *P. acnes* culture supernatant failed to trigger release under any condition. High molecular weight chemotactic factors have also been described. Lipase released by *P. acnes* has been shown to be chemotactic; a response which was inhibited by antibiotics (Lee *et al.*, 1982). This inhibition however, was found not to be chemotactic factor specific as mobility towards zymosan activated serum was also reduced. Jain *et al.* (2002) demonstrated that erythromycin and tetracycline could not only inhibit chemotaxis, but also decrease the release of reactive oxygen species.

Induction of proinflammatory cytokines by *P. acnes* has been reported. Vowels *et al.* (1995) examined the ability of *P. acnes* and *P. acnes* culture supernatant to directly induce expression of cytokines from monocytes. Both *P. acnes* and its culture supernatant induced the production of IL-1β, TNFα and IL-8. IL-8 in particular, along with other *P. acnes* induced chemotactic factors may play an important role in attracting neutrophils (Chen *et al.*, 2002). Kim *et al.* (2002) found that Toll-like receptor (TLR) 2 was expressed on the cell surface of macrophages surrounding pilosebaceous follicles and that *P. acnes* may trigger expression of cytokines by activation of this receptor. TLRs mediate responses to a variety of structures from microbial pathogens and TLR2 has been shown to recognise Gram-positive cell wall components such as peptidoglycan and LTA (Van Amersfoort *et al.*, 2003).

In summary, *P. acnes* has been shown to contribute to pathogenesis by production of exocellular enzymes and metabolites which can directly damage the host. *P. acnes* derived components possess chemoattractant properties and the organism itself can also activate complement by both the classical and alternative pathway giving rise to C5a which is a chemoattractant for neutrophils. Both humoral and cell mediated immunity have been demonstrated. The stimulation of the immune system by *P. acnes* can therefore have a harmful effect by promoting inflammation and tissue damage. These observations however, do not provide direct evidence of the

contribution of *P. acnes* to acne and other *P. acnes* associated conditions; rather they give us an idea as to the pathogenic potential of the organism.

1.6 Immune response

1.6.1 Humoral response

The larger proportion of work on the host's response to *P. acnes* has been centred around the organism's association with acne. The majority of research has shown that antibody levels to *P. acnes* are higher in acne sufferers than in the non-sufferers (Ashbee *et al.*, 1997; Holland *et al.*, 1993; Webster *et al.*, 1985a). The antibodies to *P. acnes* have not been fully characterised but are largely of the IgG class (Ingham *et al.*, 1987). In addition, titres of IgG1, IgG2 and IgG3 against whole cells of *P. acnes* have been demonstrated to be higher in the most severe cases of acne (Ashbee *et al.*, 1997).

The nature of *P. acnes* antigens has been investigated. Dawes *et al.* (1974) described the properties of an antigen from *C. parvum* (*P. acnes* NCTC 10390) which is a type II strain (Johnson and Cummins, 1972). The antigen was found to be released into the growth medium at the end of logarithmic growth and was of high molecular weight, consisting predominately of polysaccharide. Cummins and White (1983) investigated the cell wall polysaccharide further and identified 2,3-diamino-2,3-dideoxyglucuronic acid as one of the components. This component was found in both type I and II strains and to a lesser extent in *P. avidum* and *P. granulosum* but its immunological properties were not investigated. Later work by Iversen *et al.* (1985) on a *P. acnes* antigen resembling the one investigated by Dawes *et al.* (1974) found that it was also released by both type I and II strains. Antibody titres to the antigen from acne patient serum measured by radial immunodiffusion, showed a significantly higher level than was found in serum from healthy blood donors. Holland *et al.* (1993) highlighted specific polypeptide antigens of *P. acnes* using serum from acne patients and normal individuals to probe western blots of cell extracts and culture supernatant fluid of *P. acnes*. The specific properties and role of these antigens remains to be determined.

Burkhart *et al.* (1999b) compared the serological response in acne and normal patients using antigen prepared from the water soluble fraction of a pyridine-insoluble extract of whole cells of *P. acnes*. Using an ELISA to measure IgG no difference in titres in acne patients and blood donors was observed. Preliminary findings concerning the composition of the antigen suggested it to be carbohydrate due to its sensitivity to sodium periodate oxidation. Whether this antigen is related to the one found by Dawes *et al.* remains to be determined since the investigators did not explore secreted antigenic components.

Exoenzyme production by *P. acnes* has been well documented (section 1.3.1.2) and Ingham *et al.* (1987) sought to identify whether antibodies were produced against these secreted factors. Antibodies to acid phosphatase were not found but antibodies to lipase and hyaluronidase were detected. However, titres to these enzymes were no different in acne sufferers than in normal controls. It was concluded that antibodies to exocellular enzymes of *P. acnes* were not involved in the initiation of inflammatory acne.

In one study propionibacterial antigens that evoked a cellular immune response in patients with sarcoidosis were sought (Ebe *et al.*, 2000). A λgt11 genomic DNA expression library of *P. acnes* was screened with patient serum and a recombinant protein identified termed RP35. Further work examining the immune response to the protein showed that serum levels of IgG and IgA antibodies to RP35 were high in patients with sarcoidosis and other lung diseases. An antigen specific proliferative response of peripheral blood mononuclear cells to RP35 was also demonstrated in some patients with sarcoidosis but not in subjects without sarcoidosis.

1.6.2 Immunomodulation

Extensive research exists on the modulation of the immune system by bacteria or their products has shown that *P. acnes* is one of the most potent adjuvants (Roszkowski *et al.*, 1990). Pre-treatment with heat killed cells of *P. acnes* or simultaneous injections with a pathogen have been shown to provide protection against infectious agent and

tumours in a variety of animal models (Eady and Ingham, 1994). Table 1.4 lists the multitude of effects *P. acnes* has been shown to have on experimental animals.

Adjuvant effects	Anti-tumour effects
Splenomegaly	Increased resistance to a range of immunogenic and non immunogenic tumours
Increase in TNFα production by peritoneal macrophages	
Increase in autoantibodies	Increase in tumoricidal activity of macrophages
Augmentation of antibody response to T-dependent and T-independent antigens	
Increase in cytotoxic T lymphocytes	Increase in frequency of tumour specific cytotoxic T lymphocytes
Increased susceptibility to lipopolysaccharide	Increase in antibody dependent cellular cytotoxicity of non adherent spleen cells
Increased susceptibility to indomethacin	Enhancement of thermal resistance to tumours
Increased resistance or total protection in a range of viral infections	
Resistance to bacterial infections	Enhancement of beneficial effects of photodynamic tumour therapy
Resistance to protozoan parasites	

Table 1.4 Immunomodulatory effects of *P. acnes* in experimental animals (Eady and Ingham, 1994).

Wells and Balish (1979) investigated immunomodulation by *P. acnes* using germ free rats. Injections of killed *P. acnes* were found to decrease the immune response to sheep red blood cells but stimulated the response in conventionally reared rats. In addition, the response to bacterial antigens (T cell mitogens were used) was decreased except when an antigen known to be a mixed B and T cell mitogen was administered. These findings led to the conclusion that *P. acnes* has an immunostimulatory effect on B lymphocytes and an inhibitory effect on T lymphocytes. Adlam and Scott (1973) used mice to investigate the lymphoreticular stimulatory properties of *P. acnes* and demonstrated marked increases in spleen, liver and lung weight, adjuvant activity and the ability to sensitise mice to histamine. Pringle and Cummins (1982) suggested that this nonspecific stimulation of the reticuloendothelial system was dependent on cell

wall synthesis. By measuring splenomegaly in animals induced by *P. acnes* treated with different antibiotics they confirmed the association between splenomegaly inducing ability and bacterial cell wall synthesis.

The immunostimulatory effects of *P. acnes* have been utilised in animal and human models of disease, in particular hepatitis. Injection of heat killed *P. acnes* followed by an injection of lipopolysaccharide (LPS) 7 days later has been shown to cause acute liver pathology (Matsui *et al.*, 1997). The stimulatory activity of *P. acnes* has been shown to be caused by its ability to activate cells of the mononuclear phagocyte system and induce cytokine production. A model has been proposed by Eady and Ingham (1994) to explain the potential effects of *P. acnes* on macrophages (figure 1.2). The activation of macrophages by *P. acnes* may boost the proliferation of antigen sensitised lymphocytes and also enhance both the cell mediated and humoral immune response. In the *P. acnes* primed LPS model TNFα, IL-12 and IL-18 have been shown to be produced (Shuto *et al.*, 2004). IL-12 and IL-18, in synergy, induce the generation of T helper type 1 cells which are the main effector cells in LPS induced hepatic injury (Okazaki *et al.*, 2001).

P. acnes has also been used in a murine model of allergic asthma and it was found that it is able to modulate the late phase response of type I hypersensitivity. It was suggested that *P. acnes* treatment before antigen sensitisation induced a cytokine environment allowing modulation of the immune response and promoted a protective response preventing type I hypersensitivity (Braga *et al.*, 2003).

Immunotherapy involves the potentiation of the body's natural antitumour defence mechanisms. During the 1970s there was much work on the use of bacterial agents as adjuvants in particular bacille Calmette-Guérin (BCG) and *P. acnes* (*C. parvum*). BCG has been shown to act as a direct stimulant of the immune system resulting in the non-specific rejection of tumour cells (Molife and Hancock, 2002). *P. acnes* also has similar properties and a number of the organism's characteristics contribute to enhanced immune surveillance of tumour cells. In particular *P. acnes* has been shown to augment antibody production to T cell dependent antigens (Sljivic and Watson, 1977), stimulate the production of macrophages from the bone marrow (Wolmark and

Fisher, 1974) and activate macrophages (Bomford and Christie, 1975). T lymphocyte responses to *P. acnes* may also encourage protection against tumours by macrophages mediated by IFN-γ derived from activated T cells (Keller *et al.*, 1990) (figure 1.2). Although work on *P. acnes* anti-tumour effects is encouraging, results of clinical trails have been disappointing as in human trials the tumours were well established (Eady and Ingham, 1994).
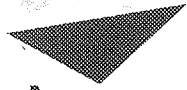
Figure 1.3 Possible mechanisms of adjuvant and tumouricidal effects of *P. acnes*
(Eady and Ingham, 1994)

1.7 Genomic sequence of *P. acnes*

At the start of this project the genomic sequence of *P. acnes* NCTC 737 had been determined by Corixa Corporation (USA) as part of a research programme to identify novel therapeutic targets and candidates for vaccine development. Access to this information was not available without commercial restrictions. In July 2004 the genomic sequence became available for another *P. acnes* strain, DSM 16379, by an academic research group in Germany (Bruggemann *et al.*, 2004). Due to its availability in the final stages of the production of this thesis it was not possible to take full advantage of the detailed sequence information and structural predictions provided by this work. However, a number of important features were identified. The genome was found to be 2,560,265 base pairs and 2,333 putative genes identified. Comparative genomics revealed that the highest number of orthologous proteins were found in *Streptococcus avermitilis*. Only a few regions were found to be linked to mobile genetic elements; four regions harboured complete or fragmented transposase genes. Ten regions containing 'alien' genes were identified by searching for genes with atypical codon usage. Several of these may be linked to pathogenicity such as proteins involved in iron acquisition and the CAMP (Christie Atkins Munch-Petersen) factor, which is analogous to the CAMP factor of *Streptococcus agalactiae* which is a pore-forming toxin capable of causing synergistic lysis of erythrocytes (Lang and Palmer, 2003). A co-haemolytic reaction of sheep erythrocytes with *S. aureus* beta-toxin has been demonstrated in *P. acnes* (Valanne *et al.*, 2003). Numerous genes coding for host degrading molecules were identified including sialidases, neuraminidases, endoglycoceramidases and lipases. A region comprised genes for a system similar to non-ribosomal peptide synthetase (NRPS) found in *Streptomyces* species. This system is involved in the production of secondary metabolites that may act synergistically or contingently against competitors, increasing the fitness of the organism in a particular environment (Challis and Hopwood, 2003). Genes involved in oxygen availability were identified with one cluster showing similarity to *bat*A and *bat*B genes of *Bacteroides fragilis*. These form part of the aerotolerance operon allowing the organism to survive prolonged exposure to oxygen (Tang *et al.*, 1999). Other tentatively identified genes included those involved in quorum sensing, porphyrin biosynthesis and polysaccharide/capsule biosynthesis. Finally, genes

associated with phase variation were found and have been shown to play an important role in bacterial pathogenesis and virulence by generating phenotypic variation allowing bacteria to evade the immune system and adapt to environmental changes (de Vries *et al.*, 2002).

1.8 Aim and objectives

P. acnes is associated with a number of clinical conditions and infections however, the precise role of the organism in these is not clearly understood. The organism is known to produce a number of exocellular enzymes, cytotoxins and immunomodulatory molecules which may be involved in pathogenicity. Whether certain strains of *P. acnes* are associated with particular conditions, and if these strains differ in terms of pathogenicity remains to be determined. To investigate the range of properties expressed by the organism it was necessary to develop a standardised molecular typing system which could be applied to all clinical isolates. The second part of the study was directed towards investigation of the antigenic composition of the organism. The immune response to *P. acnes* was studied in a range of patients to identify any antigens and assess their potential use in diagnosis of infection.

The aims of this study were therefore to:

- Characterise *P. acnes* isolates from a number of clinical sources in terms of their phenotypic traits.
- Develop an effective system for the molecular typing of *P. acnes*
- Determine whether particular phenotypes and/or genotypes are associated with certain conditions.
- Identify *P. acnes* antigens which could be exploited in the serodiagnosis of infection.
- Ascertain the immune response to any detected antigens and investigate their potential inflammatory properties.

CHAPTER 2 BACTERIAL STRAINS, CULTURE, SERA AND REAGENTS

2.1 Bacterial strains

P. acnes were isolated from various clinical sources:

- Disc material removed during microdiscectomy operations on patients suffering severe sciatica at the Royal Orthopaedic Hospital (ROH), Birmingham and processed at the Queen Elizabeth Hospital (QEH), Birmingham (n=33) and BUPA (n=5).

- Strains isolated from blood cultures at the QEH (n=14).

- Clinical material removed from patients undergoing total hip replacements at the ROH and processed at the QEH (n=16).

- Isolates from normal skin (n=17) and acne lesions (n=18) recovered at the QEH.

Ethical approval for the study was obtained from the South Birmingham Local Research Ethics Committee. Informed consent was obtained from all patients and control subjects.

2.1.1 Additional bacterial strains

Propionibacteria: Type strains, *P. acnes* NCTC 737 and NCTC 10390, were included for comparison as well as *P. avidum* and *P. granulosum* (isolates from acne lesions kindly provided by Professor K Holland, University of Leeds).

Other strains: *Staphylococcus aureus* NCTC 11561, *Escherichia coli* NCTC 104118 *Pseudomonas aeruginosa* NCTC 10662 and *Clostridium perfringens* (laboratory strain).

2.2 Storage and culture of bacterial strains

Strains were revived from frozen stocks (Microbank™, Pro-lab diagnostics, UK) maintained at -70°C. *Propionibacteria* were grown on brain heart infusion (BHI) agar under anaerobic conditions at 37°C for 72 hours (MiniMACS anaerobic workstation, Don Whitley Scientific, UK). All other strains were grown on BHI agar and incubated aerobically for 18 hours at 37°C.

2.3 Sera

Serum samples were colleted from sciatica patients prior to microdiscectomy, acne patients and control patients with no clinical evidence of infection in the previous six months.

2.4 Reagents and media

All chemicals were purchased from Sigma Company, UK and media from Oxoid Ltd, UK except where otherwise stated.

CHAPTER 3 PHENOTYPIC CHARACTERISATION OF *P. ACNES*

3.1 Introduction

In this chapter phenotypic properties of *P. acnes* isolates were investigated in order to explore relationships between such properties and clinical source of the strains. Properties investigated included biochemical profiles as determined by the 24 test galleries of commercial analytical profile index (API) strips, generating an eight digit numerical code for each strain. Antibiotic susceptibilities were measured by minimum inhibitory concentration (MIC) determination since breakpoints or zone diameters have not been published for *P. acnes* strains. Virulence factor expression was investigated qualitatively using a range of agar-based tests in which specific substrates were incorporated. Finally serotyping was carried out using a panel of monoclonal antibodies kindly provided by Dr S. Patrick, Department of Microbiology & Immunobiology, Queen's University Belfast, Belfast UK.

3.2 Materials and methods

3.2.1 API biotyping

The API ID32A system (BioMerieux, France) was used to identify and biotype all *P. acnes* strains. The API strips were prepared and read visually according to the manufacturer's instructions.

3.2.2 Minimum inhibitory concentration (MIC) determination by agar dilution

The MIC of each strain against a range of antibiotics was determined using the agar dilution method (Anon, 1991). For each antibiotic eight plates were prepared containing 0.03mg/L to 4mg/L antibiotic, as follows. A stock solution was prepared for each antibiotic (amoxycillin, erythromycin, tetracycline, trimethoprim, fusidic acid, gentamicin, rifampicin, vancomycin and ciprofloxacin; Mast Diagnostics, UK) at 80mg/L in distilled water. This stock solution was filter sterilised and serially diluted two-fold in sterile distilled water to a concentration of 0.6mg/L. Each dilution was

pre-warmed to 50°C and 1ml mixed with 19ml autoclaved molten BHI agar also at 50°C. The agar was poured into a petri dish, allowed to set and dried. Suspensions of each strain equivalent to McFarland 0.5 were prepared and a multi-point inoculator used to deliver 1μl spots of the test strains onto the surface of the agar plates. *P. acnes* NCTC 737 and 10378 were used as controls. Plates were incubated anaerobically for 72 hours at 37°C. The MIC of each antibiotic for each isolate was recorded as the lowest concentration at which there was no visible growth.

3.2.3 Virulence factors

P. acnes strains were examined for the production of exocellular enzymes thought to be involved in virulence by incorporating a number of substrates in the growth media.

3.2.3.1 Haemolysis

Strains were cultured on Columbia agar containing 7% defibrinated horse blood and incubated for 72 hours in anaerobic conditions. Zones of clearance around the colonies indicated β-haemolysis and partial clearance, indicated by a green colouration, α-haemolysis.

3.2.3.2 Non-specific proteinase activity

Sterile cotton swabs were used to streak a suspension of *P. acnes* (McFarland 3) across a skimmed milk agar plate (1% (w/v) agar no. 1, 1% (w/v) skimmed milk powder). *Pseudomonas aeruginosa* NCTC 10662 was used as a positive control and sterile distilled water as a negative control. The plates were incubated for 48 hours in anaerobic conditions and a zone of clearance around the inoculum indicated proteinase activity.

3.2.3.3 Lipase activity

Lipase activity was assessed using olive oil agar (Spare *et al.*, 2003). The basic constituents (1% (w/v) tryptone, 0.5% (w/v) NaCl, 3% (w/v) agar no. 1) were combined and autoclaved. Olive oil (2.5% (v/v) dry heat sterilised olive oil (J

Sainsbury Plc., UK) and rhodamine B (0.001% (w/v) filter sterilised aqueous solution of rhodamine B) were added to the agar which had been pre-cooled to 60°C (Kouker and Jaeger, 1987). Bacterial suspensions (McFarland 3) were streaked across the olive oil agar and incubated for 48 hours anaerobically. *P. aeruginosa* NCTC 10662 was used as a positive control and *E. coli* NCTC 104118 as a negative control. Bright pink colonies and orange fluorescence under ultraviolet (UV) illumination indicated lipase activity.

3.2.3.4 Lecithinase activity

Lecithinase activity was detected using egg yolk agar (nutrient agar, 15% (v/v) sterile egg yolk emulsion) and was indicated by wide zones of opalescence (Cruickshank *et al.*, 1975). Bacterial suspensions (McFarland 3) were streaked across the agar plates and were incubated anaerobically for 21 days at 37°C. *Clostridium perfringens* was included as a positive control and *E. coli* NCTC 104118 as a negative control.

3.2.3.5 DNase activity

Pure cultures of *P. acnes* were stab inoculated into DNase agar plates. *S. aureus* NCTC 11561 was included as a positive control and sterile distilled water as a negative control. Following incubation for seven days anaerobically at 37°C the plates were flooded with 1M HCl and left to stand for two minutes. A zone of clearance around the bacterial colonies confirmed DNase activity.

3.2.3.6 Elastase activity

Bacterial suspensions of *P. acnes* were streaked onto elastin agar plate (brain heart infusion agar supplemented with 0.3% (w/v) bovine neck ligament elastin) (Janda, 1986). *P. aeruginosa* was included as a positive control and sterile distilled water as a negative control. Plates were incubated anaerobically for 21 days at 37°C. Zones of clearance due to elastin degradation indicated elastase activity.

3.2.3.7 Hyaluronidase activity

Suspensions of *P. acnes* (McFarland 3) were inoculated onto hyaluronic acid plates (1.5% (w/v) nutrient agar, 0.1% hyaluronic acid from human umbilical cord) (Balke and Weiss, 1984). *C. perfringens* was included as a positive control and sterile distilled water as a negative control. Plates were incubated anaerobically for 72 hours at 37°C and then flooded with 10% (w/v) cetylpyridium chloride. A translucent area around the colonies was indicative of hyaluronidase activity.

3.2.4 *P. acnes* serotype by immunofluorescence microscopy (IFM)

Monoclonal antibodies raised against *P. acnes* whole cells and specific for four different cell wall antigens were used to serotype *P. acnes*. These were kindly provided by Dr Sheila Patrick, Queens University, Belfast, UK. Samples were prepared using a modification of the procedure described by Lutton *et al.*, (1991). *P. acnes* strains were grown on BHI agar for 72 hours and suspensions prepared in phosphate buffered saline (PBS) to give an absorbance$_{600nm}$ of 0.3. Ten µl volumes of the suspensions were dispensed onto twelve well multi-well slides (C. A. Hendley Ltd, Essex, UK). The slides were air-dried and fixed in 100% methanol for ten min at -20°C. After fixation, slides were air-dried and then stored at -20°C or labelled immediately. To label, 30µl of undiluted monoclonal antibody was applied to each well. The slides were incubated for 45minutes at 37°C in a humidified box. The antibodies were then washed off with PBS, the slide placed in PBS and washed for 30 minutes with gentle agitation. The secondary antibody, goat anti-mouse fluorescein-isothiocyanate conjugate (goat anti-mouse-FITC) was diluted 1:100 in PBS. For visualising all cells, a 1:50 dilution of 1% Evans blue was added into the same solution with the secondary antibody. Thirty µl of the Goat anti-mouse-FITC was applied to each well. The slides were incubated for a further 45 min at 37°C, the washing steps repeated and the slides mounted in Vectashield mounting medium containing an anti-photobleaching agent (Vector Laboratories, USA). Slides were examined using fluorescence microscopy and images were captured using an AxioCam digital microscope camera (Ziess imaging Associates Ltd., UK).

3.2.5 Statistical analyses of phenotypic traits

The significance of the differences between virulence factor production and serotype for the different isolate types were examined using Fisher's exact test (GraphPad Instat version 3.01).

Any relationships between different phenotypic traits was assessed by hierarchical clustering analysis (SPSS version 11.0 computer software, SPSS Inc.).

3.3 Results

3.3.1 API biotyping

All isolates were confirmed as *P. acnes* with 99.9% positive identification. Characteristic reactions were the reduction of nitrates, indole production, alanine arylamidase and glycine arylamidase. Negative reactions observed in all isolates were urease, α-galactosidase, β-galactosidase 6 phosphate, β- glucosidase, α-arabinosidase, raffinose fermentation, alkaline phosphatase and phenylalanine arylamidase, pyroglutamic acid arylamidase and tyrosine arylamidase. The most variable biochemical parameter was α-glucosidase. Within all of the *P. acnes* strains (n=103) 35 different biotypes were identified using the API system. Different distribution profiles of biotypes were seen for each group of isolates (figure 3.1). The most common biotype was different for each group of clinical isolates. The sciatica strains were the most metabolically diverse, as judged by the range of different API numbers in the profile. The two most frequent biotypes found in the sciatica strains were 01033306 and 05033306. These were notably absent from the acne group of strains.

Sciatica isolates

n=38

Skin isolates

n=17

Blood isolates

n=14
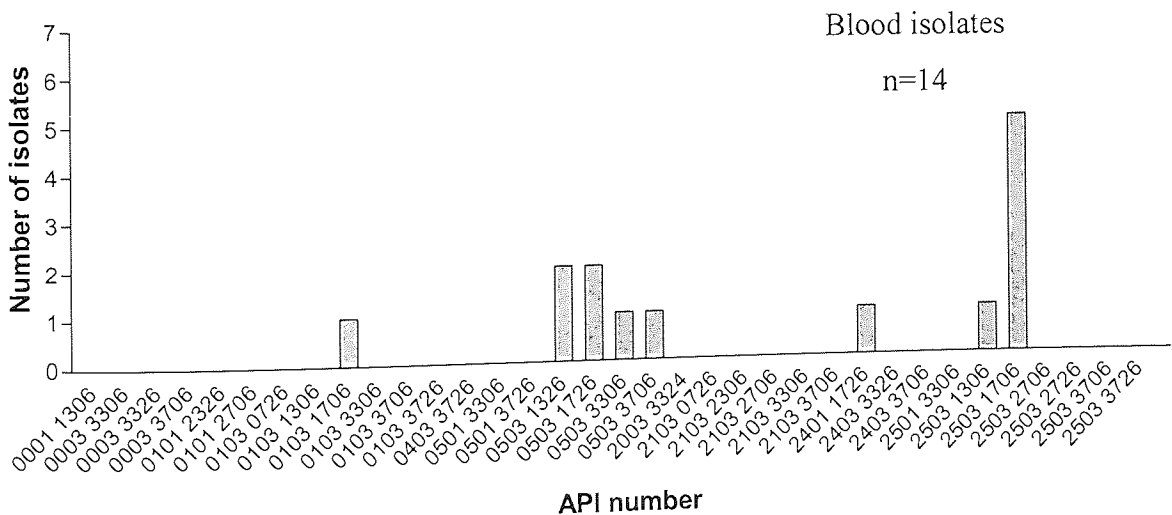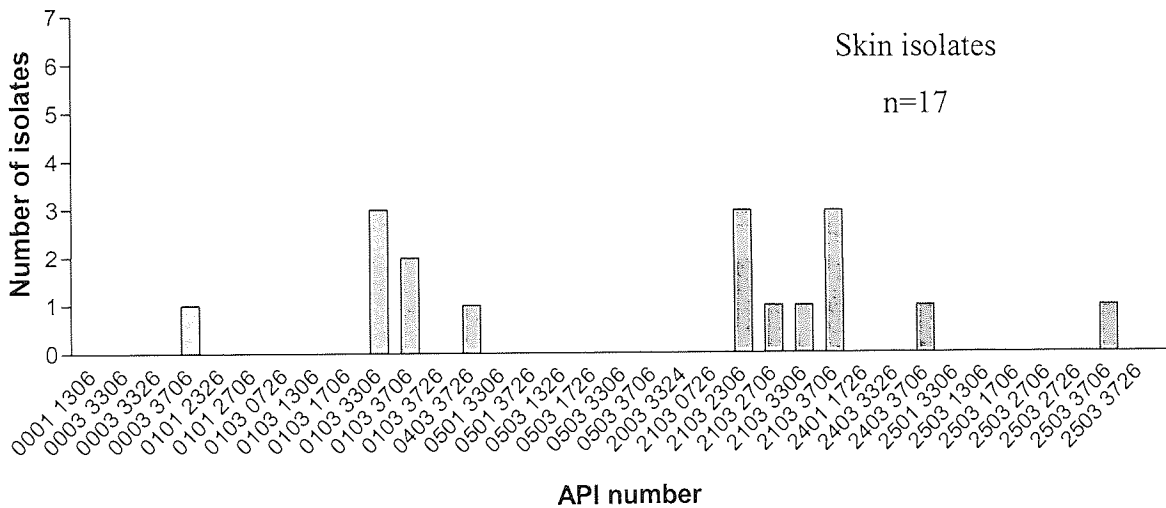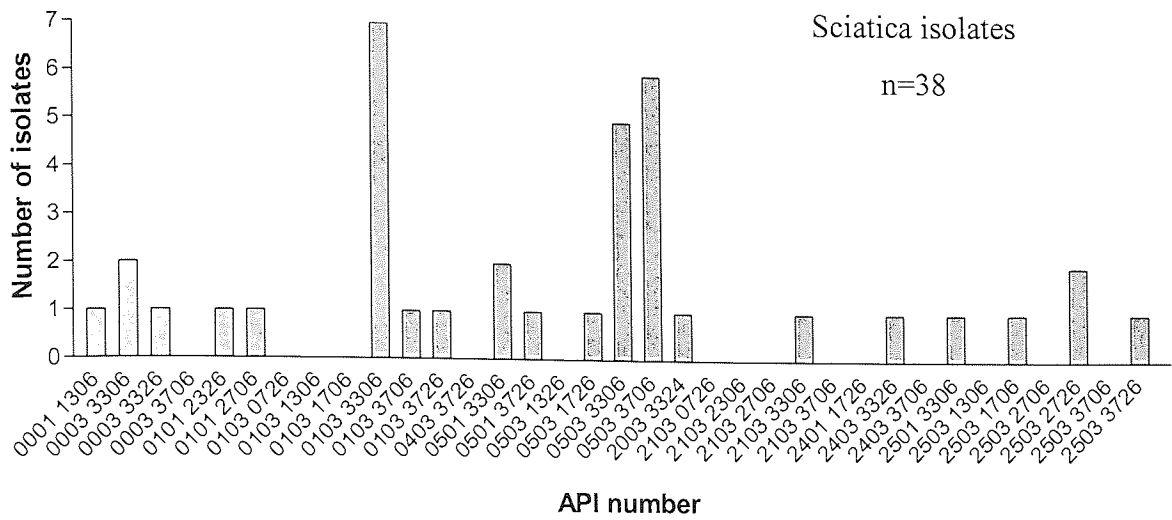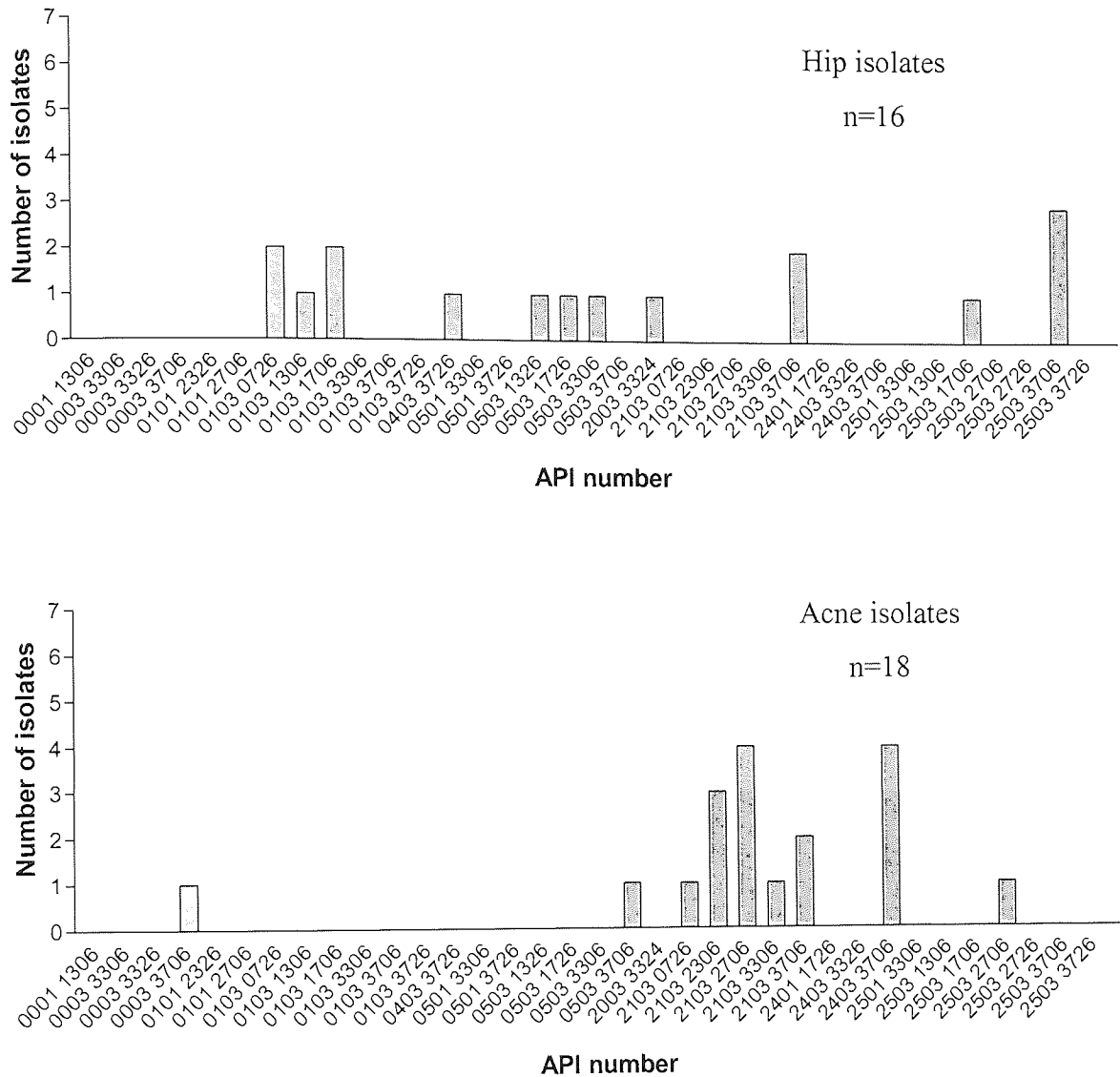
Figure 3.1 Distribution of biochemical profiles among clinical isolates of *P. acnes* generated using API ID32A (BioMerieux, France) .

3.3.2 MIC determination

The MIC for a range of antibiotics of *P. acnes* strains were determined by agar dilution. The MIC_{50} and MIC_{90} values were calculated for each clinical group of strains to give an indication of the sensitivities of these isolates (table 3.1).

As an overall group the *P. acnes* isolates were sensitive to all the major classes of antibiotics. However, tetracycline resistance ($MIC \geq 2mg/L$, Ross *et al.* (2001))

occurred in 13 strains isolated from acne lesions and normal skin. Resistance to erythromycin (MIC $\geq$ 0.5mg/L, Ross *et al.* (2001)) was observed in seven strains of which six were resistant to both antibiotics. These isolates were all taken from acne lesions. Resistance to clindamycin (MIC $\geq$ 0.25mg/L, Oprica *et al.* (2004)) occurred in five acne isolates and all of these were also resistant to erythromycin and tetracycline.

Antibiotic	Clinical source	Number of isolates inhibited at a MIC (mg/L) of								MIC_{50}	MIC_{90}
		≤0.03	0.06	0.12	0.25	0.5	1	2	≥4		
Amoxycillin	Sciatica	25	7[#]	6[*]						0.03	0.12
	Skin	14	3[#]							0.03	0.06
	Blood	11	3[#]							0.03	0.12
	Hip	10	5[#]	1[*]						0.03	0.06
	Acne	12	5[#]	1[*]						0.03	0.06
Ciprofloxacin	Sciatica					13[#]	25[*]			1	1
	Skin					2[#]	15[*]			1	1
	Blood					7[#]	7[*]			0.75	1
	Hip					8[#]	8[*]			0.75	1
	Acne					1[#]	17[*]			1	1
Clindamycin	Sciatica	36[#]	2[*]							≤0.03	≤0.03
	Skin	15[#]	1[*]	1						≤0.03	≤0.03
	Blood	10[#]	3[*]	1						≤0.03	0.06
	Hip	11[#]	3[*]	2						≤0.03	≤0.03
	Acne	12[#]		1	1	2			2	≤0.03	0.5
Erythromycin	Sciatica	37[*#]			1					≤0.03	≤0.03
	Skin	17[*#]								≤0.03	≤0.03
	Blood	13[*#]			1					≤0.03	≤0.03
	Hip	14[*#]	1		1					≤0.03	≤0.03
	Acne	10[*#]	1			2			5	≤0.03	≥4
Fusidic acid	Sciatica		1	10	10	2	13[*#]	2		0.25	1
	Skin					4	10[*#]	3		1	2
	Blood		1	2		1	9[*#]	1		1	1
	Hip					7	9[*#]			1	1
	Acne					6	12[*#]			1	1
Rifampicin	Sciatica	38[*#]								≤0.03	≤0.03
	Skin	17[*#]								≤0.03	≤0.03
	Blood	13[*#]					1			≤0.03	≤0.03
	Hip	16[*#]								≤0.03	≤0.03
	Acne	18[*#]								≤0.03	≤0.03
Tetracycline	Sciatica		5		12	19[*#]	2			0.5	0.5
	Skin				3	10[*#]		3	1	0.5	2
	Blood			7	2	5[*#]				0.185	0.5
	Hip		1	2	9	4[*#]				0.25	0.5
	Acne			1	5	3[*#]		3	6	1.25	≥4
Trimethoprim	Sciatica					1	14[*]	20[#]	3	2	2
	Skin						7[*]	10[#]		2	2
	Blood					1	6[*]	3[#]	4	1.5	≥4
	Hip						6[*]	8[#]	2	2	2
	Acne					2	10[*]	6[#]		1	2
Vancomycin	Sciatica					14	24[*#]			1	1
	Skin					4	12[*#]	1		1	1
	Blood					7	6[*#]	1		0.75	1
	Hip					5	11[*#]			1	1
	Acne					9	9[*#]			0.75	1

Table 3.1 Minimum inhibitory concentration (MIC) of various antibiotics for *P. acnes* strains isolated from sciatica, normal skin, blood, prosthetic hip infections and inflamed acne lesions. MIC of standard strains: *P. acnes* NCTC 737 (*) and NCTC 10390 (#).

3.3.3 Virulence factors

3.3.3.1 Haemolysis

Beta-haemolysis (figure 3.2) was seen in over half of the *P. acnes* strains whilst α-haemolysis was seen in only 5% of strains which consisted solely of isolates from blood cultures and acne lesions (figure 3.8).

Figure 3.2 β-haemolytic colonies of *P. acnes* on a blood agar plate.

3.3.3.2 Non-specific proteinase activity

A zone of clearing around the inoculum on a skimmed milk agar plate indicated proteinase activity (figure 3.3). Isolates from different clinical source possessed similar frequencies in the ability to hydrolyze the casein substrate except those isolated from the skin which had significantly higher frequencies (figure 3.8).
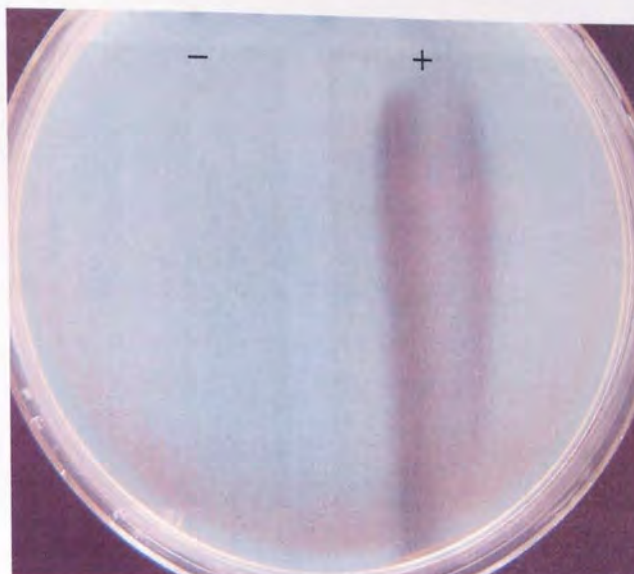
Figure 3.3 Non-specific proteinase activity indicated by zones of clearance surrounding colonies on a skimmed milk agar plate (+). *P. acnes* **proteinase negative strain (-).**

3.3.3.3 Lipase activity

The hydrolysis of lipid on olive oil results in the assimilation of rhodamine B by the colonies thus producing bright pink colonies which display orange fluorescence under UV (figure 3.4). Lipase production was one of the most variable properties, ranging from 94% positivity for skin and acne isolates to 36% for strains from blood cultures. Frequency of lipase production by skin and acne isolates was significantly higher than that of strains isolated from other clinical conditions whilst among blood culture isolates the frequency of lipase production was significantly lower (figure 3.8).
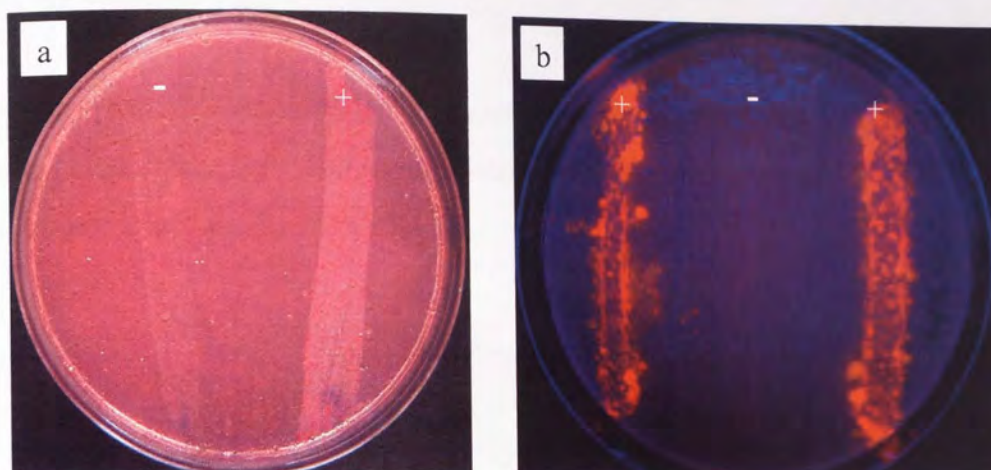
Figure 3.4 Lipase positive (+) and negative strains (-) of *P. acnes* assessed by the olive oil agar method viewed under visible (a) and UV (b) light.

3.3.3.4 Lecithinase activity

Zones of opalescence surrounding the inoculum indicated lecithinase activity (figure 3.5). On average one third of strains produced lecithinase however only 6% of strains isolated from acne lesions were lecithinase positive (figure 3.8).

Figure 3.5 Egg yolk agar inoculated with a lecithinase positive (+) and negative (-) strains.

3.3.3.5 DNase activity

The addition of 1M HCl to the DNase plate caused the precipitation of DNA. A clear zone resulted where DNA was hydrolysed by the activity of DNase (figure 3.6). No strains were found to hydrolyse DNA.

Figure 3.6 DNase activity as indicated by a zone of clearance around the inoculum. Zones of clearance were not observed around *P. acnes* strains (-). *Staphylococcus aureus* NCTC 11561 included as a positive control (+).

3.3.3.6 Elastase activity

A zone of clearance around colonies on an elastin plate indicates elastase activity, no *P. acnes* strains were found to produce elastase.

3.3.3.7 Hyaluronidase activity

Hyaluronidase activity was assessed using agar containing hyaluronic acid. The addition of cetylpyridinium chloride precipitates undigested hyaluronic acid therefore clear zones around the inoculum ensued to substrate degradation (figure 3.7). Hyaluronidase was the most common virulence factor of *P. acnes* strains from all clinical sources. Compared to strains from other sources, the frequency of

hyaluronidase production was significantly lower in those isolated from prosthetic hip infections (figure 3.8).
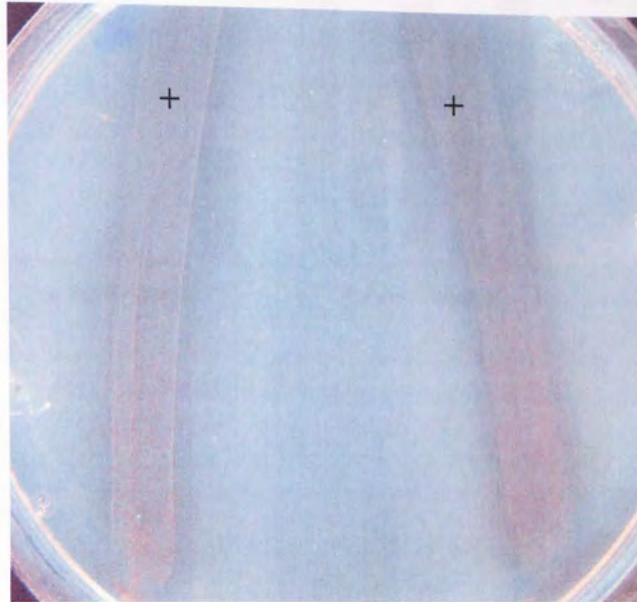
Figure 3.7 A hyaluronic acid agar plate inoculated with two hyaluronidase positive *P. acnes* strains (+).
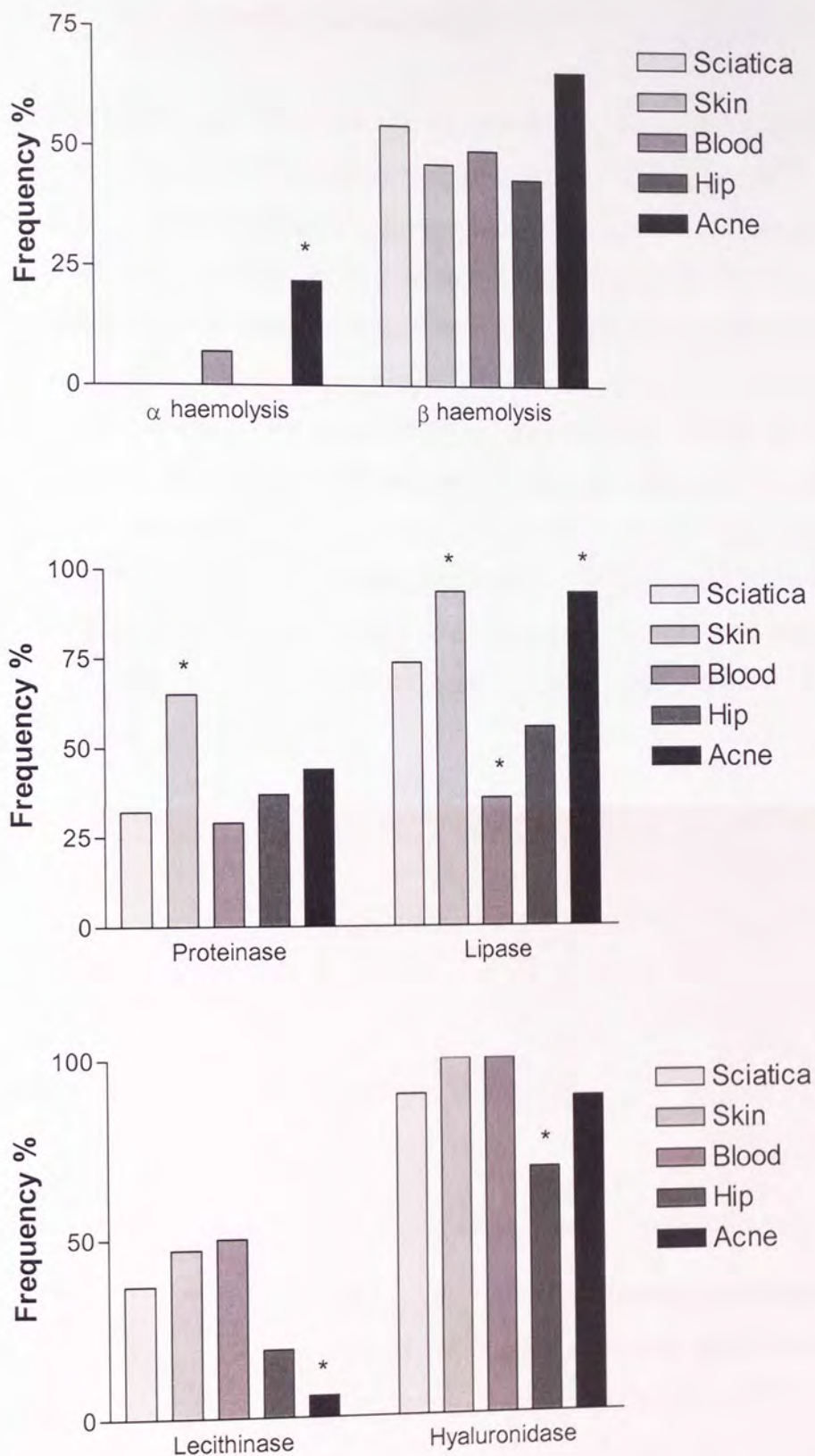
Figure 3.8 Frequency of virulence factor production by *P. acnes* strains isolated from sciatica patients undergoing microdiscectomy (sciatica), the skin (skin), blood cultures (blood), prosthetic hip infections (hip) and acne lesions (acne). * $p < 0.05$ indicates statistical significance using Fisher's exact test.

3.3.4 IFM using *P. acnes* monoclonal antibodies

Strains were classified into serotypes I-IV by determining their ability to bind four different monoclonal antibodies directed against cell wall antigens of *P. acnes*. Typical results are shown in figure 3.9, the reactivity of strains with each monoclonal antibody are described in table 3.2 and are listed according to isolate source. The most discriminatory monoclonal antibodies were found to be those detecting types I and II which, for most isolates, were mutually exclusive. In contrast, reaction with type III and IV antibodies was common to all strains. The type IV monoclonal antibody reacted with both the cells and background material whereas the other antibodies only reacted with the cells. A number of strains showed no reactivity with either the type I or type II monoclonal antibodies. In terms of the relationship between the clinical source of an isolate and its serotype, serotype I was significantly associated with skin isolates (p=0.048) although those recovered from acne lesions were also predominately of serotype I.

| Clinical source | Reactivity of strains with monoclonal antibodies (%) | | | |
	I	II	III	IV
Sciatica (n=38)	63	16	100	79
Skin (n=17)	88*	13	100	82
Blood (n=14)	43	36	100	100
Hip (n=16)	56	19	100	94
Acne (n=18)	83	11	94	94
All (n=103)	67	17	99	87

Table 3.2 Reactivity of *P. acnes* strains with monoclonal antibodies against type I, II, III and IV antigens.   *p=0.0498 indicating statistical significance using Fisher's exact test.
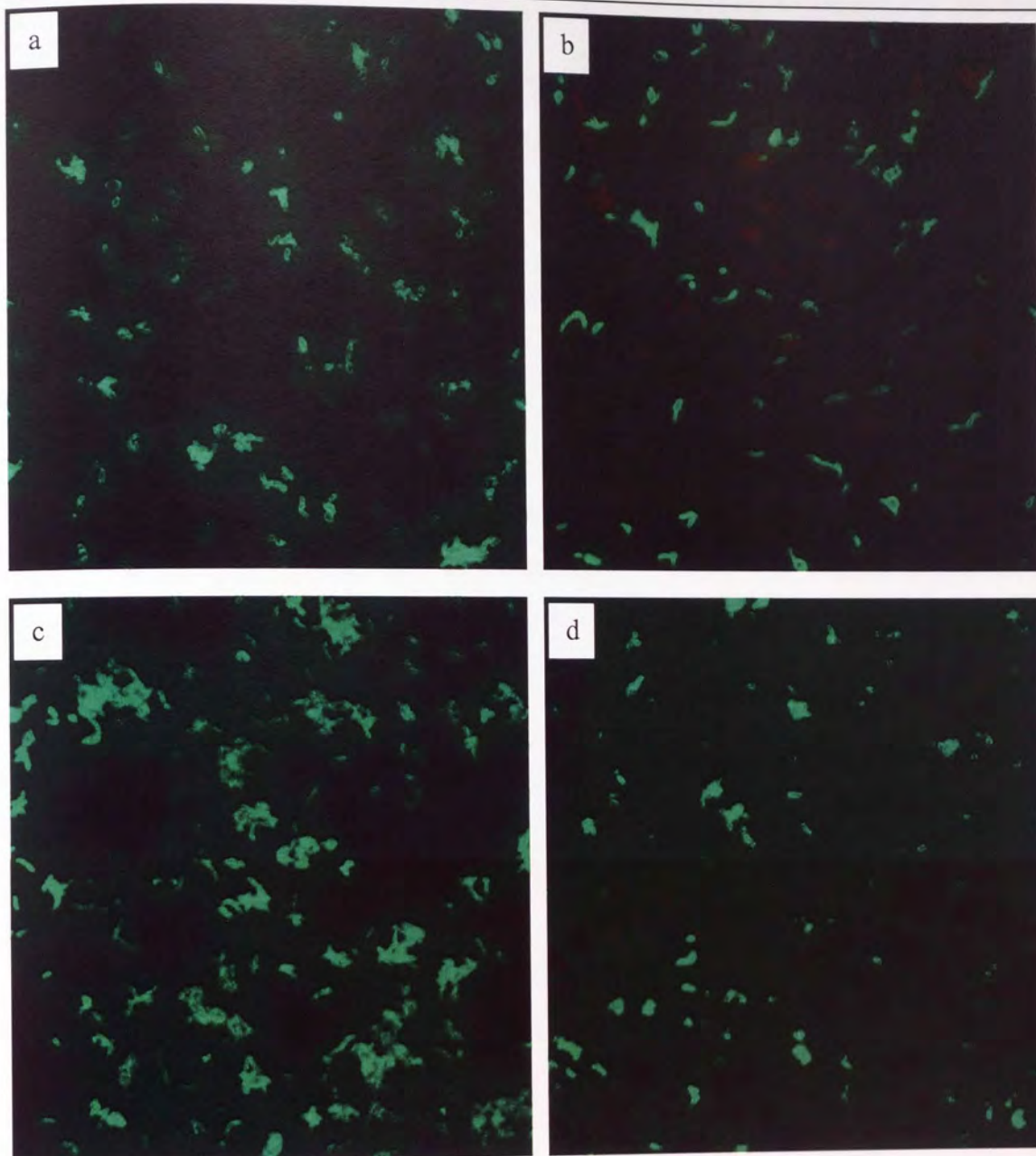
Figure 3.9 IFM of *P. acnes* cells labelled with type I (a), type II (b), type III (c) and type IV (d) FITC monoclonal antibodies.

3.3.5 Phenotypic associations

Relationships between different phenotypic traits were examined by hierarchical clustering analysis (figure 3.10). Portraying the clusters as dendrograms using a between-group linkage cluster method based on squared Euclidean distance revealed that α-haemolysis, lecithinase and proteinase are associated with serotype II whilst lipase, hyaluronidase and ß-haemolysis are associated with serotype I.

```
                          Scale of dissimilarity
              0         5        10        15        20        25
      Num     +---------+---------+---------+---------+---------+

Serotype II   ┐
α haemolysis   ┘
Lecithinase
Proteinase
Serotype I    ┐
Lipase         ┘
Hyaluronidase
β haemolysis
```
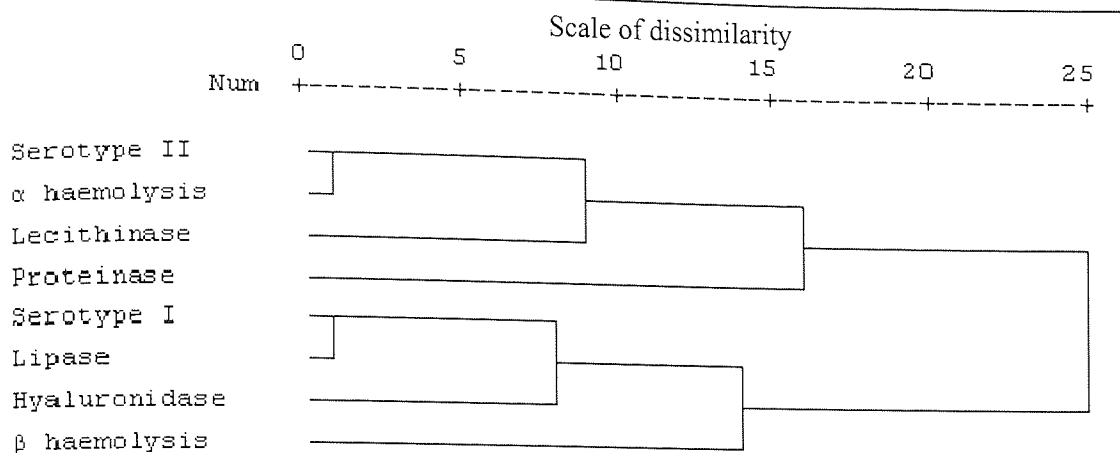
Figure 3.10 Hierarchical cluster analysis for expression of six virulence factors and expression of serotype I or II antigens by *P. acnes*. The scale is an index of dissimilarity and shows the average linkage between groups based on squared Euclidean distance.

3.4 Discussion

All isolates were identified as *P. acnes* by the API ID32A system which generated 35 different biotypes. Overall the isolates were not evenly distributed across the different biotypes as determined by API number. Clustering of some biotypes with clinical source was particularly apparent with the sciatica isolates which were most strongly correlated with API numbers 01033306 and 05033706. By contrast these biotypes occurred rarely or not at all amongst the skin, acne, hip and blood isolates. Although much larger numbers of isolates would be needed to confirm a definite association between API number and isolate source, these results do suggest that certain biochemical properties which determine the API biotype, may influence the association of strains with certain clinical conditions. The most strikingly different API number distribution profiles were found for isolates from sciatica and acne patients. The most frequently occurring biotypes from acne, 21032306 and 24033706, did not occur among the sciatica isolates. Again this suggests that certain biochemical properties of *P. acnes* strains enable these bacteria to inhabit different anatomical niches.

Antimicrobial susceptibility of *P. acnes* isolates was determined using the agar dilution method. This approach was adopted since there are no published guidelines for susceptibility testing of *P. acnes* for break point antibiotic concentrations or zone diameter measurements. However, some workers have defined breakpoint concentrations for the detection of *P. acnes* resistant to erythromycin, clindamycin and tetracycline (Oprica *et al.*, 2004; Ross *et al.*, 2001). With the exception of some skin and acne isolates the *P. acnes* strains were susceptible to a wide range of antibiotics. This concurs with the findings of other workers. Denys *et al.* (1983) studied the susceptibility of 104 clinical isolates by broth micro-dilution and found that *P. acnes* strains were susceptible to a range of penicillins and cephalosporins as well as tetracycline, erythromycin and clindamycin. Hall *et al.* (1995) found that all *P. acnes* strains isolated from cases of endophthalmitis were susceptible to various test antibiotics.

Resistance to tetracycline was observed in acne and skin isolates whilst erythromycin and clindamycin resistance was only exhibited by acne isolates. Of the seven acne isolates resistant to erythromycin six were also resistant to tetracycline. Furthermore, all strains (five) resistant to clindamycin were resistant to erythromycin and tetracycline. *P. acnes* resistance to these antibiotics has been previously reported (Kurokawa *et al.*, 1999; Oprica *et al.*, 2004; Ross *et al.*, 2001; Tan *et al.*, 1999). It appears that the widespread use of antibiotics to treat acne has led to the acquisition of resistance by selective pressure. In contrast to the findings of Ross *et al.* (2001) who stated that resistance to erythromycin has always been much more common than resistance to tetracycline, this work found that *P. acnes* was more sensitive to erythromycin than tetracycline. Tetracyclines are the first line oral antibiotics for the treatment of acne in the UK (Department of Health, 2004) and the difference observed between these findings and those of Ross *et al.* may therefore reflect prescribing trends in different countries as this study incorporated only strains from the UK. The molecular bases of resistance to tetracycline and erythromycin have been identified and are due to point mutations in the genes encoding the 23S rRNA (erythromycin) and the 16S rRNA (tetracycline) (Ross *et al.*, 1998; Ross *et al.*, 1997). Ross *et al.*(1997) identified four phenotypes of erythromycin resistant *P. acnes* based on their patterns of cross-resistance to macrolide-lincosamide-streptogramin B antibiotics. The strains resistant to erythromycin and clindamycin in this study did not fall clearly into any of these described phenotypes.

As a means of distinguishing between strains, antibiotic susceptibility testing is not very discriminatory where the majority of isolates are sensitive (as in this case) or resistant to the test antibiotics. Susceptibility testing however is indispensable for the selection of appropriate therapy. It appears from this study on the sensitivities of *P. acnes* isolates from five clinical sources that infection can be treated without concern of failure due to resistance, as seven different classes of antibiotics being effective.

Virulence factor production by *P. acnes* was investigated to assess pathogenic potential and to determine whether strains isolated from different clinical sources are associated with particular pathogenic traits. To aid in the invasion and colonisation of host tissues bacteria produce constitutive and inducible enzymes that can degrade components of the host including cells and their contents, the extracellular matrix and

macro-molecules present in the body fluids. This may benefit the organism by enabling colonisation and spread through the host whilst taking up any nutrients released and allowing evasion of host defences. *P. acnes* is known to produce a number of exoenzymes (Hoeffler, 1977; Kabongo Muamba, 1982) and in this study the production of haemolysins, non-specific proteinase, lipase, lecithinase, DNase, elastase and hyaluronidase by strains isolated from a number of clinical sources was investigated. *P. acnes* produced a range of exoenzymes, including haemolysin, non-specific proteinase, lecithinase and hyaluronidase, but no strains were able to hydrolyse DNA or elastin. This is in concordance with the findings of Marples and McGinley (1974) and Dick *et al.*(1976).

The ability of *P. acnes* strains to produce haemolysins was detected using agar containing blood cells and observing clear zones around the colonies where the blood has been lysed. Approximately half the strains tested exhibited ß-haemolysis and this haemolytic activity did not vary between isolates from different sources. Relatively few strains displayed α-haemolysis and isolates that did, consisted of blood culture and acnes isolates. These findings agree with those of Hoeffler (1977) who found that 42.5% of *P. acnes* strains demonstrated complete (ß-haemolysis) and 4% incomplete haemolysis (α-haemolysis). Haemolysin production was higher in *P. acnes* serotype I (79%) than serotype II (28%). Valanne *et al.* (2001) also found that haemolysin production among serotype I strains (77%) was greater than among type II strains (6%). Haemolysins are often referred to as cytolysins due to their broad target cell range (Semedo *et al.*, 2003). Elaboration of cytolysins can be of benefit to an organism by providing essential nutrients such as the acquisition of iron (Lebek and Gruenig, 1985), aiding tissue invasion by lysis of host cells and avoidance of the host's defence system by lysis of cells of the immune system (Patrick and Larkin, 1995).

Non-specific proteinase activity was significantly higher in isolates from normal skin (65%) compared to isolates from other sources (29-44%). These results match the findings of Hoffler *et al.* (1985) who found that isolates from the skin of healthy controls were more proteolytic than those from comedones and pustules. Production of proteinases may confer an advantage to organisms in terms of tissue invasion and destruction, evasion of host defences and modulation of the host immune system

during infection and inflammation (Travis *et al.*, 1995). Furthermore, bacterial proteinases can cause deregulation of the kallikrein-kinin pathway and pathogens may exploit this property to ensure a supply of nutrients to the site of infection by increasing vascular permeability. Proteinases have also been shown to inactivate complement factor C5a, reducing neutrophil recruitment (Molla *et al.*, 1986). Extracellular proteinases are produced by *P. acnes* during exponential growth whereas other known extracellular enzymes are produced later in the growth cycle (Ingram *et al.*, 1983). This suggests that proteinases may play essential roles by increasing the availability of amino acids during active growth rather than providing a scavenging function for survival later in the growth cycle.

Lipolytic ability was the most variable virulence factor ranging from 94% frequency in skin and acne isolates to 36% in blood culture strains. Kabongo Muamba (1982) and Marples and McGinley (1974) found 100% and 96% respectively, of *P. acnes* strains investigated produced lipase. The strains investigated in these studies were predominately skin isolates and their results therefore support the findings for the skin and acne isolates investigated in the study. The high level of lipolytic activity seen in skin and acne isolates compared to the other strains may reflect the environment they colonise. *P. acnes* is located deep in the pilosebaceous follicles through which sebum passes to the skin. Therefore *P. acnes* may gain nutritional advantage from the degradation of sebum triglycerides. Lipases are hydrolases acting on carboxyl ester bonds present in triglycerides to liberate glycerol and fatty acids. *P. acnes* lipase can hydrolyse a range of triglycerides (Ingham *et al.*, 1981) and is normally produced at the end of the log phase (Jaeger *et al.*, 1994) but are optimally detected at zero oxygen tension (Cove *et al.*, 1983). *P. acnes* lipase is regarded as a possible colonisation factor because free fatty acids efficiently increase cell to cell adherence thereby promoting colonisation and persistence of *P. acnes* (Gribbon *et al.*, 1993). The gene encoding *P. acnes* lipase, *gehA*, has been cloned and sequenced (Miskin *et al.*, 1997). Further research by the workers is in progress to produce a lipase-deficient mutant of *P. acnes* to test its ability to colonise the skin and to determine the role of lipase in the pathogenesis of acne.

On average, a third of *P. acnes* strains produced lecithinase (phospholipase C), however, when grouped by clinical source, production was significantly lower in acne

isolates (6%). Lecithinase production has not been as widely investigated as other exoenzymes of *P. acnes*. Hoeffler (1977) failed to detect lecithinase in 40 *P. acnes* strains but in later work Hoffler *et al.* (1985) found that 17% of acne isolates produced this enzyme. Nakamura *et al.* (1984) and Bulkacz and Erbland (1997) both investigated single oral strains of *P. acnes* and found that they were able to produce considerable amounts of phospholipase C. The natural function of phospholipase C may be to secure supplies of phosphate but it can also can be cytolytic (Titball, 1993). Phospholipase C hydrolyses two components of the host cell membrane: phosphatidyl choline (lecithin) and sphingomyelin. The enzyme lyses all mammalian cells indiscriminately since its substrate, phosphatidylcholine (lecithin) is ubiquitous in mammalian membranes. It can therefore cause damage to the membranes of leukocytes, fibroblasts and muscle cells as well as causing platelets to aggregate and lyse (Patrick and Larkin, 1995).

Hyaluronidase was found to be the most common exoenzyme produced by *P. acnes*. Hoeffler (1977) similarly found that 73% (n = 40) of *P. acnes* strains isolated from various sources were able to depolymerise hyaluronic acid. In terms of the production of hyaluronidase by strains from different clinical sources, production was significantly lower in those isolated from prosthetic hip infections. A wide variety of microorganisms produce enzymes capable of degrading hyaluronic acid including *Streptococcus*, *Staphylococcus* and *Clostridium* species (Hynes and Walton, 2000). Hyaluronic acid is an unbranched polysaccharide consisting of repeated glucuronic acid/N-acetylglucosamine disaccharide units and is ubiquitously present in the extracellular matrix. Changes in the synthesis and degradation of hyaluronic acid modulate the extracellular packing of collagen and fibrin, fibre size and porosity of extracellular substrates. Hyaluronidase production by an invading microbe may therefore provide a selective advantage by enabling the spread of the focus of infection throughout the tissues (Matsushita and Okabe, 2001). *P. acnes* hyaluronidase has been purified and partially characterised by Ingham *et al.*(1979) and the gene cloned and sequenced by Steiner *et al.* (1997). The hyaluronidase gene encodes an 82kDa protein that shares homology with hyaluronidases from *S. pneumoniae*, *S. agalactiae* and *S. aureus*.

Characterisation of *P. acnes* isolates using four monoclonal antibodies against cell wall antigens revealed that the majority of strains isolated from normal skin and acne lesions were serotype I. For typing purposes the monoclonal antibodies directed towards serotypes I and II were most discriminatory and the majority of strains belonged to either one of these serotypes. By contrast, antibodies to serotypes III and IV reacted with the majority of strains. These findings therefore concur with those of other workers who found that *P. acnes* can be divided into two major serotypes (Johnson and Cummins, 1972; Kishishita *et al.*, 1979)

In a previous study of 38 *P. acnes* strains from acne and normal skin no difference in biochemical or physiological properties between isolates was found (Puhvel, 1968). However, examination of the phenotypic characteristics of the isolates in this thesis revealed some correlation between phenotype and isolate source. The main feature was the predominance of type I strains amongst the acne and normal skin isolates. This suggests a more common occurrence of this serotype on the skin.

Biochemical profiling using API test system revealed a wide range of biotypes among the isolates studied. This information may lead to a better understanding of the combination of biochemical properties that are associated with strains from individual conditions. In turn this might reveal an insight into relevant pathogenic mechanisms involving ability of strains to colonise, survive host defences, proliferate and cause damage. However, although the distribution of API profiles differed between isolates from different clinical sources, the generation of a large number of biotypes made it difficult to effectively cluster strains. Likewise, Tenover *et al.*(1994) found that biotyping recognised too many types for it to be useful for clustering outbreak strains of *S. aureus*. Furthermore, API tests can be difficult to interpret, involving subjective judgement of colour changes in the test strips; other workers have also commented on this problem (Geary *et al.*, 1997).

Antibiograms based on MICs revealed that most isolates were broadly sensitive to a wide range of antibiotics. As most strains were sensitive to the antibiotics tested, the discriminatory value of the antibiograms was low. However, sensitivities to tetracycline were the most discriminatory between isolates from different sources.

The highest MICs were obtained for the acne and skin isolates, possibly reflecting the widespread use of tetracyclines in the treatment of acne.

Analysis of virulence factor production also highlighted differences in acne and skin isolates when compared to other strains, in terms of lipase, proteinase (skin isolates), α-haemolysis (acne strains) and lecithinase (acne strains). Information on the production of specific virulence factors by strains isolated from a particular clinical source may provide an insight into the pathogenic mechanisms that are associated with those conditions.

This chapter has illustrated the wide range of phenotypes expressed by *P. acnes* isolates obtained from different clinical sources. Little is currently known on the molecular mechanisms involved in gene expression of the organism, for example response to environmental stresses such as nutrient limitation. With the recent availability of the genomic sequence of *P. acnes* studies on gene expression using microarrays should provide a deeper insight into the phenotypic properties expressed by the organism during infection.

CHAPTER 4 GENOTYPIC CHARACTERISATION OF *P. ACNES* USING RAPD

4.1 Introduction

Traditional typing techniques based on an organism's phenotypic properties have been increasingly challenged by the development of molecular based typing methods. Phenotyping is limited by the restricted number of characteristics that can be examined and results can be misleading because of variations in gene expression (Busch and Nitschko, 1999). Molecular methods have several advantages over traditional typing methods including higher discriminatory power, application to a broader range of bacterial species and, in some cases, speed (Tenover *et al.*, 1997).

Random amplification of polymorphic DNA (RAPD) is a PCR-based technique that uses a single primer to anneal and prime at numerous locations on the genome producing an array of products that serve as a genetic fingerprint. This method requires no prior knowledge of the target DNA and was therefore particulalry useful for genotyping *P. acnes* at the start of this work when the genomic sequence of the organism was not available in the public domain. Amplification based DNA fingerprinting methods, such as RAPD can be sensitive to changes in reaction conditions resulting in changes in profiles and therefore careful optimisation of typing protocols is required (Tyler *et al.*, 1997). In this chapter an optimised RAPD protocol for the epidemiological typing of *P. acnes* is described. The relationship between genotype and clinical source of *P. acnes* isolates, as well as correlations with phenotypic properties are explored.

4.2 Materials and methods

4.2.1 DNA extraction

P. acnes strains were grown at 37°C in BHI for 72 hours. The cultures were supplemented with 20μl penicillin G (20mg/ml) and incubated for a further four hours to weaken the cell wall and to aid subsequent lysis. DNA was extracted using a modification of the method described by Hilton *et al.* (1996). Cells were harvested from 6ml of culture (12,000g, 5minutes) and the pellet resuspended in 270μl Tris-EDTA (TE) (10mM Tris, 1mM EDTA, pH 8.0). The cell suspension was heated at 75°C to inactivate DNases. The cells were lysed by addition of 30μl sodium dodecyl sulphate (SDS) (100mg/ml) and the lysate treated with 3μl proteinase K (10mg/ml). The suspension was incubated at 65°C for three hours and diluted to 600μl with sterile distilled water. DNA was then extracted by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) in phase lock eppendorf tubes. After brief vortexing and centrifugation (12000g, 1 min) the aqueous phase was recovered. The DNA was precipitated by addition of 0.1 volumes of 3M sodium acetate pH 5.2 and two volumes of ice cold ethanol. The suspension was incubated at -20°C for 20 minutes and the DNA pelleted (12000g, 20 min). Following a 70% (v/v) ethanol wash, the DNA was dried at 40°C for 10 min. The pellet was redissolved in 30μl PCR grade water and left to rehydrate overnight at 4°C. All DNA preparations were stored at –20°C until required.

4.2.2 Determination of DNA quality and quantity

DNA preparations were examined by gel electrophoresis to ensure that the DNA was not excessively sheared. Ten μl of each DNA preparation was mixed with 2μl of 6 x loading buffer (0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol (v/v)) and loaded in wells on a 2% agarose gel (Bioline, UK) containing 1μg/ml ethidium bromide. Five microlitres of HyperLadder DNA standard (Bioline, London, UK) was also added and the gel electrophoresed in Tris acetate EDTA buffer (TAE) (40mM Tris acetate, 2mM EDTA, pH 8.0) at 100 volts for one hour. The

profiles obtained were visualised under UV light transilluminator using Grab-IT software (UVP, CA, USA).

DNA was quantified by measuring the absorbance at 260nm (1 absorbance$_{260nm}$ unit = 50 μg/ml) using a UV spectrophomoter (Camspec, Cambridge, UK). Absorbance measurements were also taken at 280nm and the ratio of 260nm and 280nm readings calculated as an indication of purity. Ratios of 1.8-1.9 indicate highly purified preparations of DNA.

4.2.3 RAPD reaction

PCR was carried out in a 25μl reaction containing 1 x commercial PCR buffer (Promega, UK) until buffer composition was optimised (section 4.2.4.2), 200μM dNTPs (Promega, UK), 100pmol primer (section 4.2.4.2), 1.25U Taq polymerase (Promega, UK) and 2μl of DNA template (section 4.2.4.3). The amplification *procedure comprised* of one cycle for 4.5 min at 94°C followed by five cycles of 30 s at 94°C, 2 min at 20°C, 2 min at 72°C and 35 cycles of 30 s at 94°C, 1 min at 32°C, 2 min at 72°C. The amplification was concluded with a final extension step of 5 min at 72°C and the reactions stored at 4°C until analysis. Amplified fragments were separated by electrophoresis as described in section 4.2.2. Digitally captured RAPD profiles were analysed with GelCompar software (Applied Maths, Belgium) with the band matching coefficient of Dice and un-weighted pair groups using mathematical averages (UPGMA) clustering to determine profile relatedness.

4.2.4 RAPD optimisation

4.2.4.1 Primer

P. acnes genomic DNA was subjected to RAPD analysis (section 4.2.3) using primer detailed in table 4.1. Primers which gave clearly distinguishable RAPD profiles with a suitable number of amplicons were selected for further use.

Primer	Sequence	Reference
GEN	5'-GTTTCGCTCC-3'	Dautle *et al.* (2002)
EP015	5'-AGAACTGCTC-3'	Tambic *et al.* (1997)
KAY1	5'-AGCAGCCTGC-3'	Tambic *et al.* (1997)
1254	5'-CCGCAGCCAA-3'	Hilton *et al.* (1996)

Table 4.1 Primers used for RAPD typing (MWG biotech, Germany).

4.2.4.2 PCR buffer

The optimum buffer composition for PCR reactions was determined using the Opti-Prime™ buffer matrix (Schoettlin *et al.*, 1994). Each buffer was tested with DNA template from three strains. The PCR reaction was carried out as described in section 4.2.3 using each of the 12 buffers detailed in table 4.2 and the primer selected in section 4.2.4.1. The buffer producing profiles with suitable discrimination was selected for further testing.

Tris-HCl 10 mmol/L	$MgCl_2$ mmol/L	KCl 25 mmol/L	KCl 75 mmol/L
pH 8.3	1.5	Buffer 1	Buffer 2
pH 8.3	3.5	Buffer 3	Buffer 4
pH 8.8	1.5	Buffer 5	Buffer 6
pH 8.8	3.5	Buffer 7	Buffer 8
pH 9.2	1.5	Buffer 9	Buffer 10
pH 9.2	3.5	Buffer 11	Buffer 12

Table 4.2 Opti-Prime™ buffer matrix (Schoettlin *et al.*, 1994).

4.2.4.3 DNA concentration

RAPD was carried on DNA preparations from two strains using the following concentrations of template DNA: 40, 20, 10, 7, 5 and 3ng/µl. PCR was carried out as described in section 4.2.3 using 2µl of each concentration of template DNA. The

profiles obtained were visualised under UV and the optimum DNA concentration selected for use in subsequent RAPD typing.

4.2.5 RAPD Intra- and inter-reproducibility

To confirm RAPD intra- and inter- reproducibility using the selected primer, PCR buffer and quantity of DNA, DNA samples underwent RAPD singularly and in duplicate in separate reactions 3 months apart.

4.2.6 RAPD typing of *P. acnes* strains

Using the optimised RAPD method all *P. acnes* strains underwent analysis. *P. acnes* NCTC 737, *P. acnes* NCTC 10390, *P. avidum* and *P. granulosum* were included for comparison.

4.2.6.1 Determination of typeability and discriminatory power of optimised RAPD

The typeability of the RAPD method was calculated as the percentage number of strains which could be assigned to typing groups. The discriminatory power was determined using Simpson's index of diversity (D) (Hunter and Gaston, 1988):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$$

where N is the total number of strains in the sample population, s is the total number of types described, and n_j is the number of strains belonging to the jth profile type.

4.2.7 Relationship between RAPD genotype and phenotypic factors

To investigate any relationship between the RAPD genotype and phenotype of a strain, dendrograms generated from RAPD profiles were annotated with phenotypic data.

4.2.8 Determination of 16S rRNA and *recA* sequence

To confirm the type status of strains categorised by RAPD the 16S rRNA and *recA* sequences of representative strains were elucidated by Dr Andrew McDowell of the department of Microbiology and Immunology, Queen's University, Belfast, UK.

4.2.9 PCR Cloning

The nature of fragments generated from RAPD were investigated by cloning and sequencing.

4.2.9.1 Isolation and purification of a RAPD fragment

P. acnes NCTC 737 genomic DNA underwent optimised RAPD as in section 4.2.3 with the exception of the final extension step of the PCR reaction which was increased to 10 minutes to ensure efficient addition of a single adenine (A) nucleotide overhang at the 3' terminus of each amplified fragment during the PCR procedure. Amplified fragments were separated by gel electrophoresis in 2% Seaplaque low melting point agarose (Biowhittaker molecular applications, USA) for 1 hour at 100 volts. The 200bp band was cut from the gel visualised on a transilluminator (UVP Products) and extracted from the agarose gel using a Gel Extraction Kit, Qiaex II (Qiagen, UK). The agarose containing the RAPD fragment was dissolved in buffer QX1 (Qiagen, 0.3ml per 100mg agarose) and mixed with 30μl Qiaex II silica resin (Qiagen). The mixture was incubated at 50°C and vortex-mixed every two minutes for ten minutes. Following centrifugation (12,000g, 30 seconds) the supernatant was removed and the pellet washed with 500μl of Buffer QX1 (Qiagen). The pellet was resuspended by vortexing and was centrifuged (12000g, 30 seconds) to remove residual agarose. The

supernatant was removed and the pellet washed with 500μl of Buffer PE (Qiagen). The pellet was resuspended by vortexing and centrifuged (12000g, 30 seconds) to remove residual salt contaminants. The supernatant was removed and the pellet was left to air dry for 15 minutes. DNA was eluted by the addition of 20μl of water and was resuspended by vortexing. After the suspension had been incubated at room temperature for 5 minutes it was centrifuged (13000g, 30 seconds) and the supernatant was retained. The DNA concentration was determined by agarose gel analysis by comparing the sample to known amounts of DNA. Different amounts of the DNA sample (4μl, 2μl & 1μl) and 25μl MassRuler™ DNA Ladder, High Range, (Helena Biosciences, UK) were electrophoresed through a 1% agarose gel (Bioline, UK). Quantification of DNA was obtained by densitometric measurement of band intensity and comparison with a standard curve derived from the quantitative DNA ladder using GeneToolsAnalysis software (SynGene, UK).

4.2.9.2 Ligation of the RAPD fragment to the cloning vector

The pDrive pre-digested cloning vector (figure 4.1, Promega, UK) was used in accordance with the manufacturer's instructions. A 5x molar ratio excess of insert was used in the ligation reaction. The reaction consisted of 1μl pDrive Cloning Vector (50ng/μl), 2μl RAPD fragment (32.5ng/μl) and 5μl of 2x ligation master mix. Ligations were incubated overnight at 4°C.
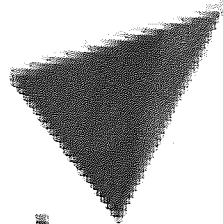
Aston University

Illustration removed for copyright restrictions

Figure 4.1 pDrive cloning vector (Reproduced from Qiagen PCR cloning handbook).

4.2.9.3 Preparation and transformation of competent cells

Competent cells were prepared using the rubidium chloride method. Briefly, a 0.25% (v/v) inoculum of overnight culture of *E. coli* DH5α (SupE44 ΔLacU169 (Ø80 Lac Z ΔM15) hsdR17 recA1 end A1 gyrA96 thi-1 rel A1) was added to sterile SOB media (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, pH 7.0) and incubated at 37°C until the cells entered logarithmic growth phase ($Absorbance_{550nm}$ 0.4). The culture was chilled on ice for 30 minutes and centrifuged (3000g, 15 minutes). The cells were resuspended in ice cold RFB 1 buffer (100mM RbCl, 50mM $MnCl_2$, 30mM potassium acetate, 10mM $CaCl_2·2H_2O$, 15% v/v glycerol) at 33% of the original volume of the culture and incubated on ice for 60 minutes. Cells were then pelleted by centrifugation (3000g, 15 minutes) and resuspended in RFB 2 buffer (10mM MOPS, 10mM RbCl, 75mM $CaCl_2$, 15% (v/v) glycerol) at 8% of the original volume of culture and stored on ice for 15 minutes. Cells were frozen in liquid nitrogen and stored at -80°C until use.

Prior to transformation competent cells were thawed on ice. Once defrosted 2μl of the ligation mixture was added to the cells and mixed by flicking. After a 30 minute incubation on ice the cells were heat shocked for 45 seconds at 40°C and then returned to ice for one minute. Aliquots of the transformed cells (20μl, 50μl and 80μl) were grown overnight at 37°C on Luria Bertani agar (1.5% agar, 1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, pH 7.0) containing ampicillin (50μg/ml), 1.6μl/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal, Promega, UK) in dimethylformamide (50mg/ml) (DMF, Promega, UK) and 0.1mM isopropyl-ß-D-thiogalactopyranoside (IPTG, Promega, UK). Colonies were screened using blue/white selection.

4.2.9.4 Screening of presumptive colonies

Presumptive colonies from blue/white selection were screened by colony PCR. Each 25μl reaction consisted of 1x PCR buffer, 1.5mM $MgCl_2$, 200μM dNTPs (Promega, UK), 0.4μM of both forward and reverse plasmid primers (M13 forward (-20) 5'-GTAAAACGACGGCCAGT-3', M13 reverse 5'-CAGGAAACAGCTATGAC-3'; MWG biotech, Germany), and 1.25 units of *Taq* polymerase (Promega, UK). For the bacterial template presumptive colonies were transferred to an eppendorf tube containing 2μl of PCR grade water using a toothpick. The amplification procedure comprised of two minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, one minute at 55°C and 20 seconds at 72°C cycle followed by a final cycle of seven minutes at 72°C. Products were analysed by agarose gel electrophoresis as in section 4.2.8.1 using HyperLadder IV (Bioloine, UK).

4.2.9.5 DNA insert sequencing

The PCR products of colonies containing the insert were independently sequenced by the Functional Genomics and Proteomics Laboratory, School of Biosciences, University of Birmingham using the ABI PRISM® BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit with AmpliTaq® DNA Polymerase.

4.2.9.5.1 DNA sequence and amino acid analysis

The sequence data from different clones were aligned using Multialin software (http://prodes.toulouse.inra.fr/multalin/multalin.html) to obtain a consensus sequence. This was then used to interrogate nucleic acid sequence databases (GenBank bacterial sequences) using the BLAST program provided by the BiologyWorkbench package (http://workbench.sdsc.edu). The sequence was translated into the six possible open reading frames (ORFs) and these were used to search peptide sequence databases (GeneBank bacterial sequences) for homologies using BLASTP.

4.2.9.6 Detection of the sequenced RAPD fragment in *P. acnes* strains using PCR

Primers were designed to screen *P. acnes* isolates for the sequenced RAPD fragment. The PCR was carried out as described in section 4.2.9.4 using primer pairs P1 and P2 (table 4.4) and the PCR products sequenced (section 4.2.9.5).

4.3 Results

4.3.1 RAPD optimisation

4.3.1.1 Primer

Genomic DNA from *P. acnes* NCTC 737 and 10390 was subject to RAPD using primers GEN, EP015, KAY1 or 1254 giving the profiles shown in figure 4.2. Fragments were only generated using primers KAY1 and 1254 for both DNA templates. Primer KAY1 produced a lower yield of amplicons (2 bands) therefore primer 1254 was selected for use in further optimisation studies.
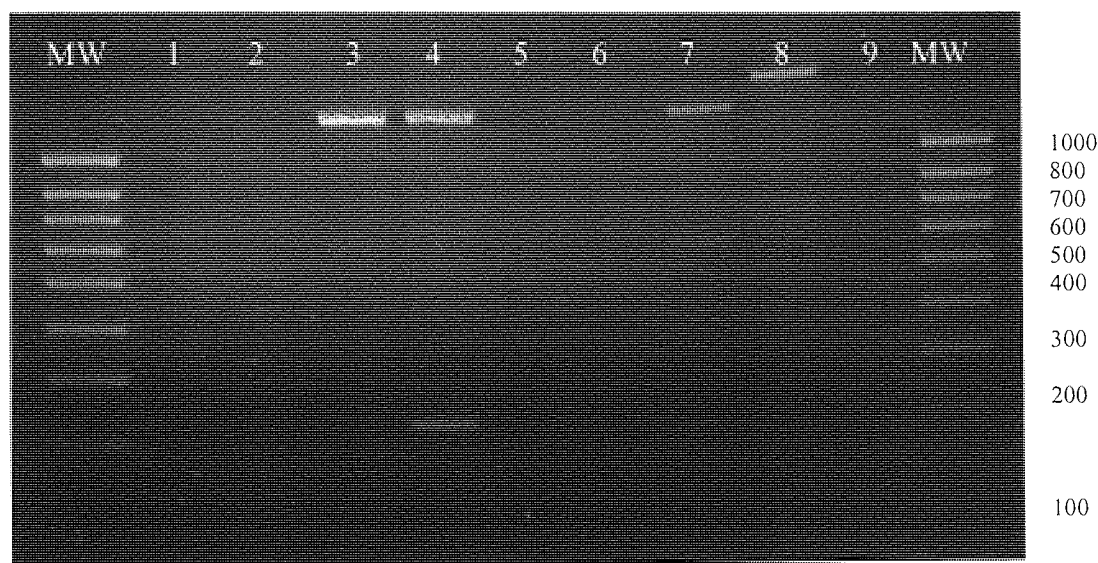
Figure 4.2 RAPD profiles amplified from genomic DNA from *P. acnes* NCTC 737 (lanes 1-4) and NCTC 10390 (lanes 5-8) using primers GEN (lanes 1 and 5), EP015 (lanes 2 and 6), KAY1 (lanes 3 and 7) and 1254 (lanes 4 and 8) (primers detailed in table 4.1). Lane 9: negative control, lane MW: 1 kb DNA molecular weight ladder (Bioline, UK).

4.3.1.2 PCR buffer

The PCR buffer was optimised using primer 1254 to maximise the discriminatory power of RAPD. Profiles obtained should contain a sufficient number of amplicons to allow unrelated strains to be distinguished, but not so many as to make the results difficult to analyse. Profiles from PCR reactions carried out on three strains (*P. acnes* NCTC 737, NCTC 10390 and a clinical strain) using 12 buffers which varied in pH and $MgCl_2$ and KCl concentrations are shown in figure 4.3. At each pH, a high concentration of $MgCl_2$ (3.5 mmol/L) with a low concentration of KCl (25 mmol/L) did not support product amplification (lanes 3, 7 and 11). However, a decrease in $MgCl_2$ concentration to 1.5 mmol/L with a low concentration of KCl improved amplification (lanes 1, 5 and 9). A high concentration of KCl (75 mmol/L) with a low or high concentration of $MgCl_2$ supported product formation but gave smeared profiles with low discrimination (lanes 2, 4, 6, 8, 10 and 12). Increasing the pH appeared to have little effect on the profiles obtained relative to the effect of altered KCl and $MgCl_2$ concentration. Similar effects of buffer composition upon RAPD profile were found for all strains examined, with buffer 9 giving the clearest and most discriminatory profiles. Therefore buffer 9 was selected for further use.
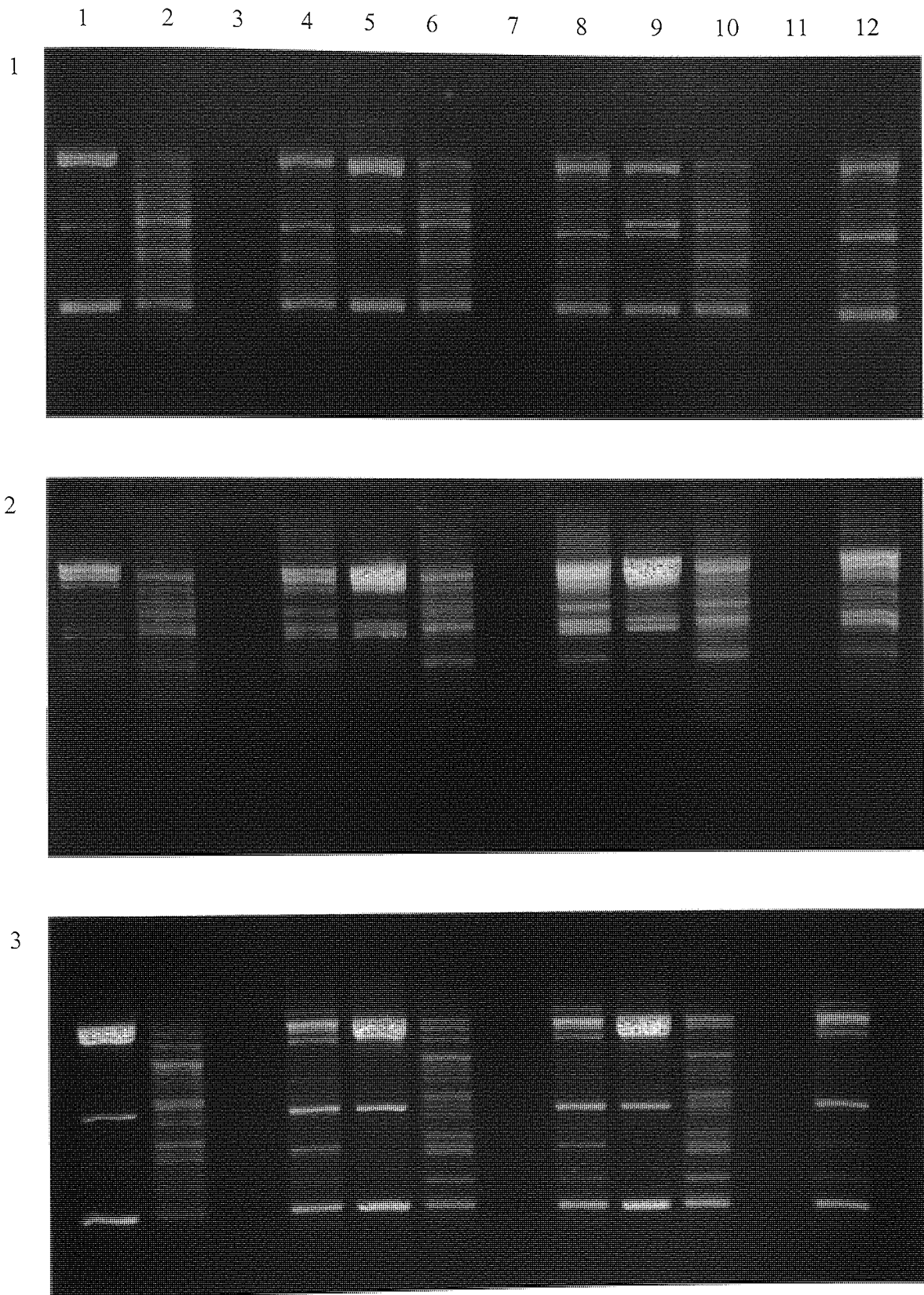
Figure 4.3 RAPD profiles of three strains of *P. acnes* (1. *P. acnes* NCTC 737, 2. NCTC 10390 and 3. clinical strain) using 12 different PCR buffers. Lanes numbered according to the 12 buffers detailed in table 4.2.

4.3.1.3 DNA concentration

Buffer 9 and primer 1254 were used in RAPD reactions with dilutions of DNA from *P. acnes* NCTC 737 and 10390 (figure 4.4). Profiles obtained remained consistent throughout the concentration range. A DNA concentration of 10ng/μl was chosen for subsequent reactions.

DNA concentration 40   20   10   7   5   3 (ng/μl)

DNA concentration 40   20   10   7   5   3 (ng/μl)
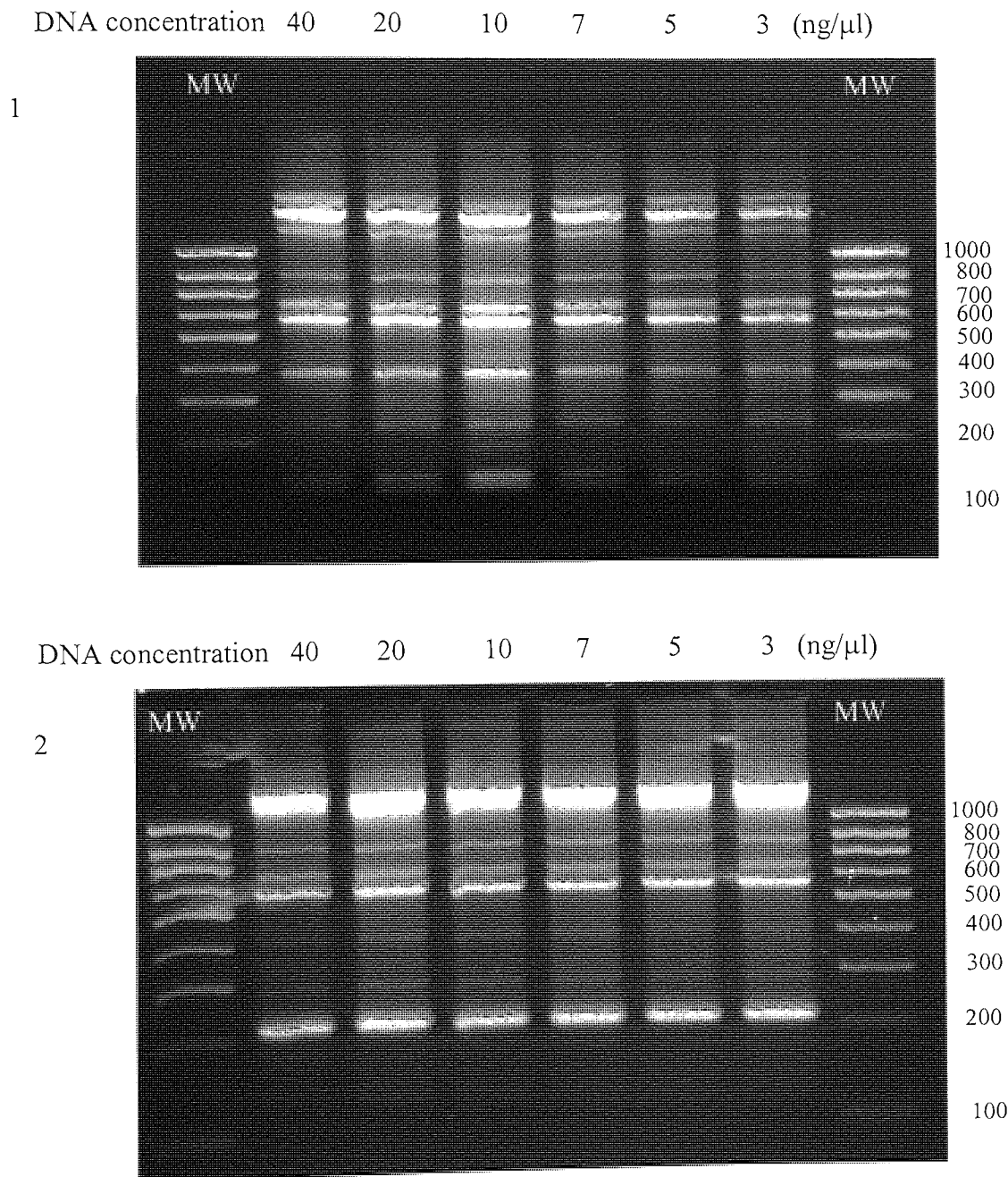
Figure 4.4 RAPD profiles obtained from diluted DNA from *P. acnes* NCTC 10390 (1) and NCTC 737 (2). MW: 1kb DNA molecular weight ladder (Bioline, UK).

4.3.2 RAPD intra- and inter- reproducibility

To investigate reproducibility DNA preparations from *P. acnes* strains (numbered 1-11) underwent RAPD in duplicate (profiles a and b) and were then prepared again three months later (profile c). RAPD profiles obtained using the optimised method were shown to be 100% intra- and inter- reproducible by dendrogram analysis (figure 4.5).
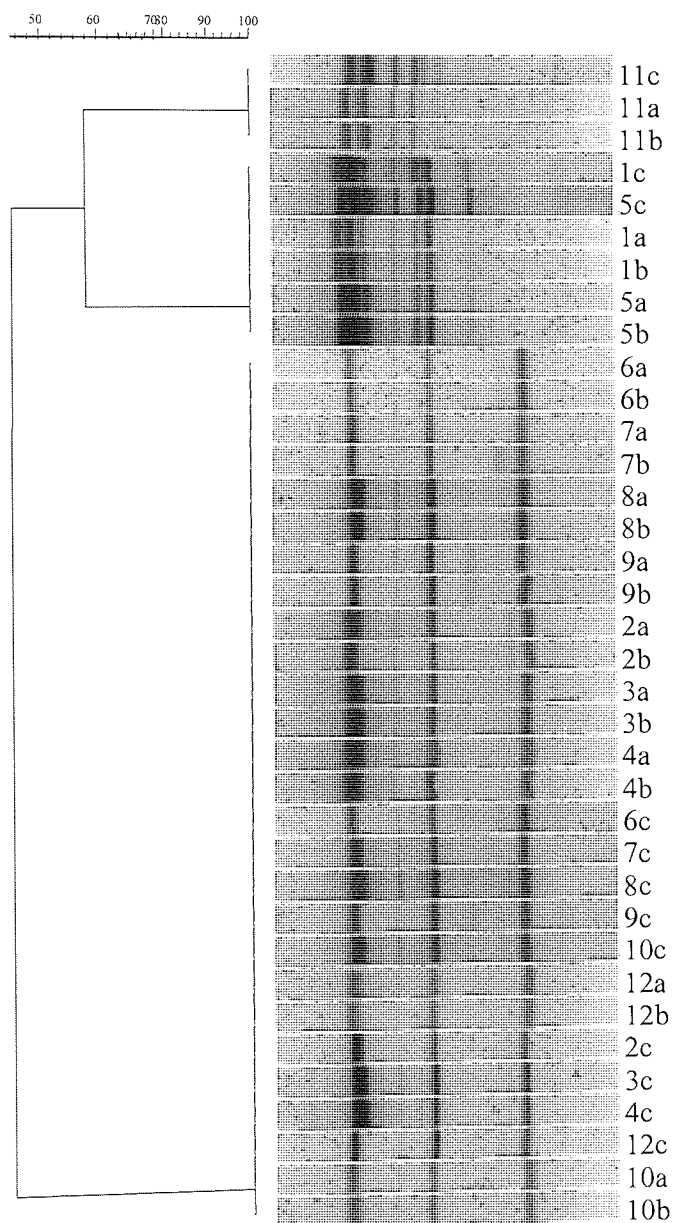
Figure 4.5 UPGMA dendrogram showing comparability of RAPD profiles generated in duplicate (strains a and b) and singularly on a different occasion from a separate DNA preparation (c).

4.3.3 RAPD typing of *P. acnes* strains

One-hundred and three strains of *P. acnes* isolated from sciatica patients, normal skin, blood cultures, prosthetic hip infections and acne lesions underwent RAPD analysis. An example of the electrophoretic profiles generated is shown in figure 4.6. Profiles consisted of five to seven bands ranging from over 1000bp to 200bp. Dendrogram analysis by calculation of the Dice correlation coefficient and construction of a dendrogram by the UPGMA method revealed that clinical isolates of *P. acnes* were represented in a major cluster at 65% similarity, which further subdivided into three distinctive profile types (RAPD type A, B and C). Strains isolated from different clinical sources were distributed amongst the RAPD profile types within the *P. acnes* clusters. The RAPD system differentiated between *P. acnes* and the related *Propionibacterium* species, *P. granulosum* and *P. avidum*, which gave markedly different profiles with less than 40% similarity to those of *P. acnes* (figure 4.7).
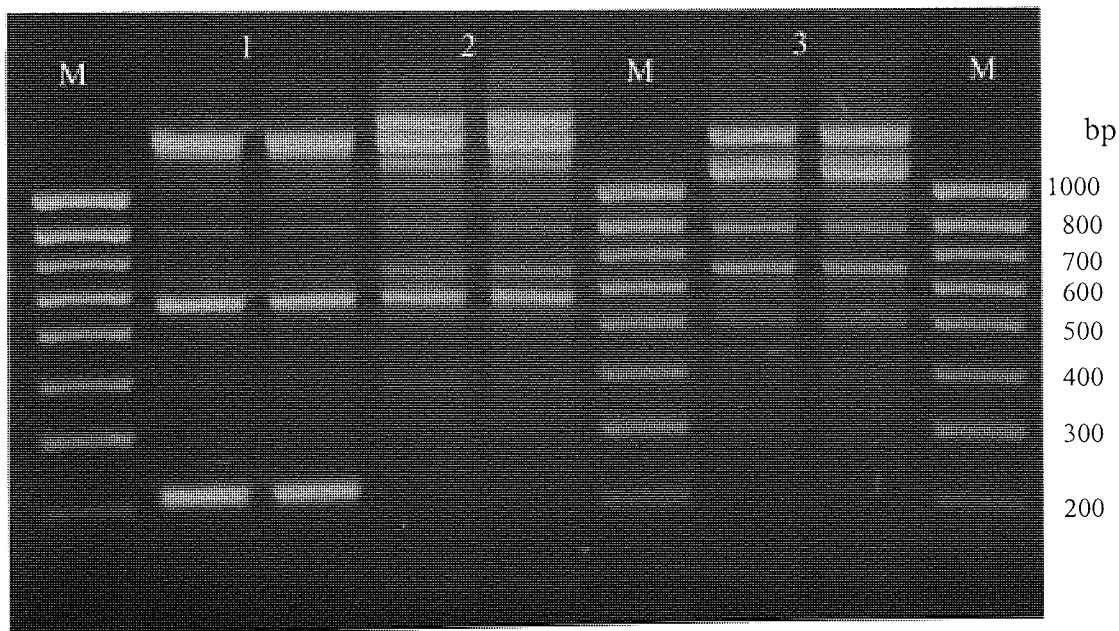
Figure 4.6 Example of RAPD profiles from genomic DNA of three *P. acnes* isolates (1, 2 & 3). Lanes M: 1kbp molecular weight markers.
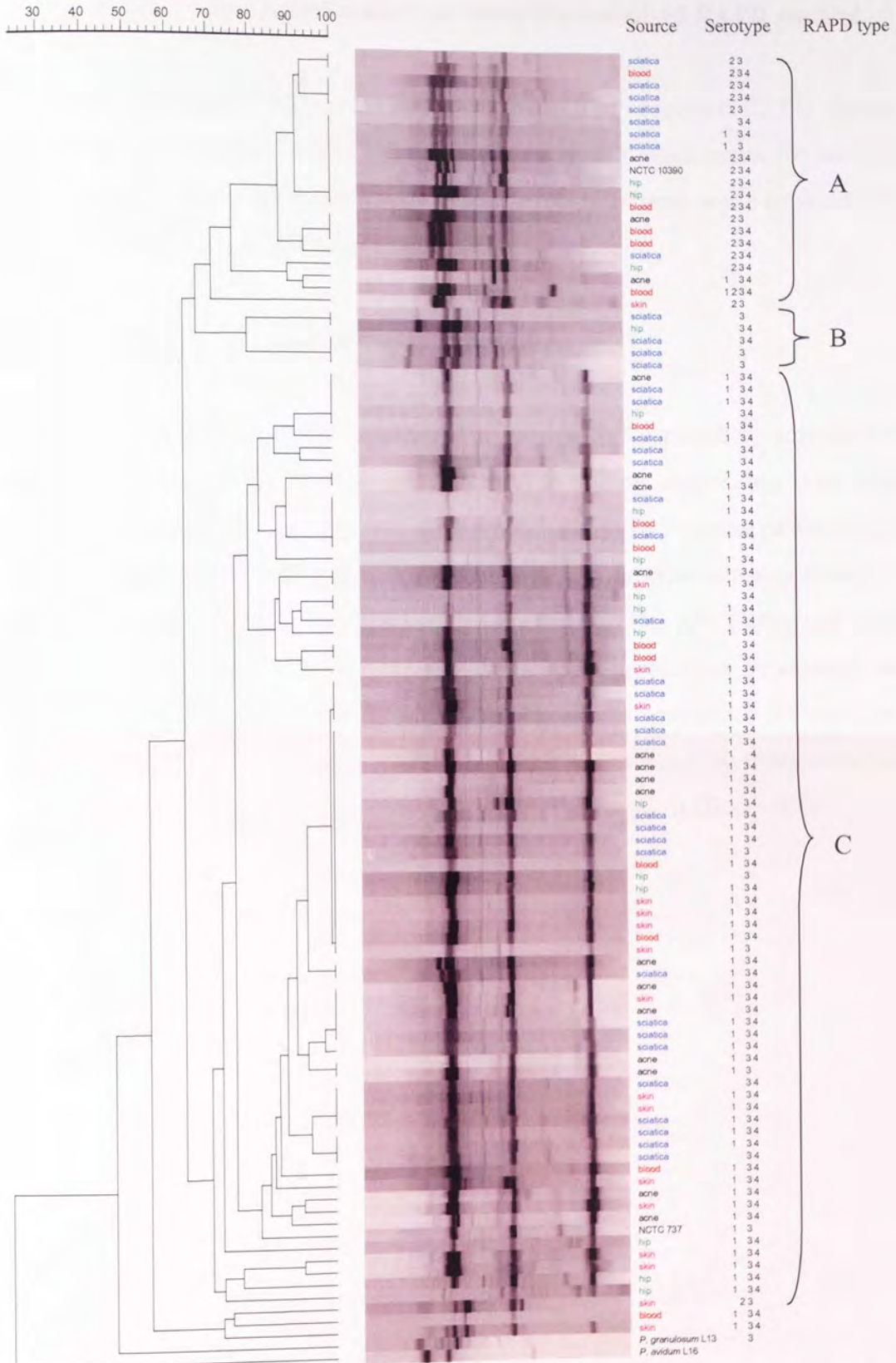
Figure 4.7 UPGMA dendrogram analysis of relatedness (Dice coefficient) of *P. acnes* strains isolated from sciatica patients (blue), blood cultures (red), prosthetic hips (green), normal skin (pink) and acne lesions (black). Dendrogram annotated with serotype (1, 2, 3 or 4) and RAPD type (A, B or C).

4.3.3.1 Typeability and discriminatory power of the optimised RAPD method

All isolates were typeable using the RAPD method. The optimised RAPD protocol was highly discriminatory (DI, 0.95), in keeping with the requirement for an index greater than 0.9 (this is desirable if the results of a typing scheme are to be interpreted with confidence (Hunter and Gaston, 1988)).

4.3.4 Relationship between RAPD type and phenotype

The relationship between RAPD profile and phenotype was explored by annotation of the dendrogram with phenotypic information. A strong association was found between the serotype and RAPD type of a strain. Serotype I strains predominately had RAPD profile (C), serotype II strains had type (A) profiles whereas strains not belonging to serotype I or II had profile (B) (figure 4.7). The API biotype of strains did not relate to the RAPD type (figure 4.8). Beta-haemolysis production was dominant among isolates within the largest RAPD cluster containing the serotype I strains. Furthermore, the number of virulence factors expressed by these serotype I strains appeared to be greater than those expressed by serotype II (figure 4.8).

API
biotype

■	0001 1306
API2	0003 3306
API3	0003 3326
API4	0003 3706
API5	0101 2326
API6	0101 2706
API7	0103 0726
API8	0103 1306
API9	0103 1706
API10	0103 3306
API11	0103 3706
API12	0103 3726
API13	0403 3726
API14	0501 3306
API15	0501 3726
API16	0503 1326
API17	0503 1726
API18	0503 3306
API19	0503 3706
API20	2003 3324
API21	2103 0726
API22	2103 2306
API23	2103 2706
API24	2103 3306
API25	2103 3706
API26	2401 1726
API27	2403 3326
API28	2403 3706
API29	2501 3306
API30	2503 1306
API31	2503 1706
API32	2503 2706
API33	2503 2726
API34	2503 3706
API35	2503 3726

Figure 4.8 UPGMA dendrogram analysis of relatedness (Dice coefficient) of *P. acnes* strains isolated from sciatica patients, blood cultures, prosthetic hips, normal skin and acne lesions. Dendrogram annotated with the API biotype (see figure for key) and virulence factor production of strains.

Virulence factors: α-haemolysis ▌, ß-haemolysis ▌, Lipase ▌, Lecithinase ▌, Proteinase ▌, Hyaluronidase ▌

4.3.5 Investigation of RAPD type using 16S rRNA and *recA* sequence analysis

The 16S rRNA and *recA* sequences were determined for *P. acnes* NCTC 737, NCTC 10390 and representative strains from RAPD types (A), (B) and (C). This work was kindly carried out by Dr Andrew McDowell, Department of Microbiology and Immunology, The Queen's University of Belfast, Belfast, UK. The 16S rRNA sequences of all strains gave 99-100% identity with *P. acnes* (by comparison with GenBank data), confirming all strains as *P. acnes*. The *recA* sequences of strains from RAPD types (C) and (A) matched those of *P. acnes* NCTC 737 (type I) and NCTC 10390 (type II), respectively. However, the *recA* sequences of strains with RAPD type B were found to contain polymorphisms of a mixture of type I and II sequences as well as five additional unique polymorphisms (figure 4.9). These strains could therefore be representatives of a separate group of *P. acnes* distinct from those of types I and II.

```
            1                                                              50
NCTC737     ATGGCAGCGA CCGCTGACCG CGAAAAGGCC CTTGCCACGG CCTTGCAGCA
NCTC10390   ATGGCAGCGA CCGCTGACCG CGAAAAGGCC CTTGCCACGG CCTTGCAGCA
RAPD B      ATGGCAGCGA CCGCTGACCG CGAAAAGGCC CTTGCCACGG CCTTGCAGCA

            51                                                             100
NCTC737     GATCGAGAAG CAGCACGGCA AGGGCTCTAT CATGCGCCTT GGCGAGCAGG
NCTC10390   GATCGAGAAG CAGCACGGCA AAGGCTCTAT CATGCGCCTT GGCGAGCAGG
RAPD B      GATCGAGAAG CAGCACGGCA AGGGCTCTAT CATGCGCCTT GGCGAGCAGG

            101                                                            150
NCTC737     AGACCGTTAA GATCGCCGCT ATCCCCACGG GCTCGGTGGC CCTCGACGTC
NCTC10390   AGACCGTTAA GATCGCCGCT ATCCCCACGG GCTCGGTGGC CCTCGACGTC
RAPD B      AGACCGTTAA GATCGCCGCT ATCCCCACGG GCTCGGTGGC CCTCGACGTC

            151                                                            200
NCTC737     GCGTTGGGTG TCGGAGGCCT CCCACGTGGC AGAATCGTGG AGATCTATGG
NCTC10390   GCGTTGGGTG TCGGAGGCCT CCCACGTGGC AGGATCGTGG AGATCTATGG
RAPD B      GCGTTGGGTG TCGGAGGCCT CCCACGTGGC AGGATCGTGG AGATCTATGG

            201                                                            250
NCTC737     TCCCGAATCC TCCGGCAAGA CGACGGTGGC TTTGCACGCA ATCGCTAATG
NCTC10390   TCCCGAATCC TCTGGCAAGA CGACGGTGGC TTTGCACGCA ATCGCTAATG
RAPD B      TCCCGAATCC TCCGGCAAGA CGACGGTGGC TTTGCACGCA ATCGCTAATG

            251                                                            300
NCTC737     CCCAGGCTGA AGGCGGCATC TGTGCCTTTA TTGACGCCGA GCACGCTCTC
NCTC10390   CCCAGGCTGA AGGCGGCATC TGTGCCTTTA TTGACGCCGA GCACGCTCTC
RAPD B      CCCAGGCTGA AGGCGGCATC TGTGCCTTTA TTGACGCCGA GCACGCTCTC

            301                                                            350
NCTC737     GACCCGGAAT ACGCACGCAA ACTCGGGGTC GATACAGATT CCCTGCTGGT
NCTC10390   GACCCGGAAT ACGCACGCAA ACTCGGGGTC GATACAGATT CCCTGCTGGT
RAPD B      GACCCGGAAT ACGCACGCAA ACTCGGGGTC GATACAGATT CCCTGCTGGT

            351                                                            400
NCTC737     GAGCCAGCCT GACAATGGTG AGCAGGCTCT CGAGATTGCT GACACCCTCG
NCTC10390   GAGCCAGCCT GACAATGGTG AGCAGGCTCT CGAGATTGCT GACACCCTCG
RAPD B      GAGCCAGCCT GACAATGGTG AGCAGGCTCT CGAGATTGCT GACACCCTCG

            401                                                            450
NCTC737     TCCGCTCGGG TGCCTTGGAG CTCATCGTTG TTGACTCGGT GGCCGCTCTG
NCTC10390   TCCGCTCGGG TGCCTTGGAG CTCGTCGTTG TTGACTCGGT GGCCGCTCTG
RAPD B      TCCGCTCGGG TGCCTTGGAG CTCATCGTTG TTGACTCGGT GGCCGCTCTG

            451                                                            500
NCTC737     ACCCCGAAGG CTGAGATCGA GGGCGAGATG GGTGATTCCC ATGTTGGTTT
NCTC10390   ACCCCGAAGG CTGAGATCGA GGGCGAGATG GGTGATTCCC ATGTTGGTTT
RAPD B      ACCCCGAAGG CTGAGATCGA GGGCGAGATG GGTGATTCCC ATGTTGGTTT

            501                                                            550
NCTC737     GCAGGCCCGC CTTATGAGCC AGGCCCTGCG CAAGATGACC GGTGCGTTGA
NCTC10390   GCAGGCCCGC CTTATGAGCC AGGCCCTGCG CAAGATGACC GGTGCGTTAA
RAPD B      GCAGGCCCGC CTTATGAGCC AGGCCCTGCG CAAGATGACC GGTGCGTTGA

            551                                                            600
NCTC737     ATGCGGCCGG TACCACTGCC ATCTTCATTA ATCAGCTGCG CGAGAAGATC
NCTC10390   ATGCGGCCGG TACCACTGCC ATCTTCATTA ATCAGCTGCG CGAGAAGATC
RAPD B      ATGCGGCCGG TACCACTGCC ATCTTCATTA ATCAGCTGCG CGAGAAGATC
```

```
              601                                                  650
NCTC737       GGTGTCATGT TTGGCTCCCC GGAGACAACG ACAGGTGGTC GCGCCCTCAA
NCTC10390     GGTGTCATGT TTGGCTCCCC GGAGACAACG ACAGGTGGTC GCGCCCTCAA
   RAPD B     GGTGTCATGT TTGGCTCCCC GGAGACAACG ACAGGTGGTC GCGCCCTCAA

              651                                                  700
NCTC737       GTTCTACTCG TCCGTGCGCC TCGACGTGCG GCGTGTCGAA ACTCTTAAAG
NCTC10390     GTTCTACTCG TCCGTGCGCC TCGACGTGCG GCGTGTCGAA ACTCTTAAAG
   RAPD B     GTTCTACTCA TCCGTGCGCC TCGACGTGCG GCGTGTCGAA ACTCTTAAAG

              701                                                  750
NCTC737       ACGGATCAGA GATGGTCGGT AACCGCACTC GTGTCAAGGT TGCCAAGAAC
NCTC10390     ACGGATCAGA GATGGTCGGT AACCGCACTC GTGTCAAGGT TGCCAAGAAC
   RAPD B     ACGGATCAGA GATGGTCGGT AACCGCACTC GTGTCAAGGT TGCCAAGAAC

              751                                                  800
NCTC737       AAGGTCGCCC CACCTTTTAA GCAGGCAGAG TTTGACATCC TTTACGGACA
NCTC10390     AAGGTCGCCC CACCTTTTAA GCAGGCAGAG TTTGACATCC TCTACGGACA
   RAPD B     AAGGTCGCCC CACCTTTTAA GCAGGCAGAG TTTGACATCC TCTACGGACA

              801                                                  850
NCTC737       GGGCATTTCT CGCGAGGGAA GCCTCATCGA CATGGGCGTA GATTGCGGCA
NCTC10390     GGGCATTTCT CGCGAGGGAA GCCTCATCGA CATGGGCGTA GATTGCGGCA
   RAPD B     GGGCATTTCT CGCGAGGGAA GCCTCATCGA CATGGGCGTA GATTGTGGCA

              851                                                  900
NCTC737       TCATCACCAA ATCCGGTTCG TGGTTCAGCT ACAACAATGA GCAGTTGGGT
NCTC10390     TCATCACCAA ATCCGGTTCG TGGTTCAGCT ACAACAATGA GCAGTTGGGT
   RAPD B     TTATCACCAA ATCCGGTTCG TGGTTCAGCT ACAACAATGA GCAGTTGGGT

              901                                                  950
NCTC737       CAGGGTAAGG AGAACGTCCG AAAGTTCCTC AGAGGTAACC CGGATGTTGC
NCTC10390     CAGGGTAAGG AGAACGTCCG AAAGTTCCTC AAAGGTAACC CGGATGTTGC
   RAPD B     CAGGGCAAGG AGAACGTCCG AAAGTTCCTC AAAGGTAACC CGGATGTTGC

              951                                                 1000
NCTC737       CAATGAGATT GAGGACAAGA TCCTGACTCA CCTTGGCCTG CGCGAAGCCG
NCTC10390     CAATGAGATT GAGGACAAGA TCCTGACTCA CCTCGGCCTG CGCGAAGCCG
   RAPD B     CAATGAGATT GAGGACAAGA TCCTGACTCA CCTCGGCCTG CGCGAAGCCG

              1001                                                1047
NCTC737       AGGTCCCTGA AGGTGTCGAT CCCCGAACTG GCGAGGTGGA ATTCTGA
NCTC10390     AGGTTCCTGA GGGTGTCGAT CCCCGAACTG GCGAGGTGGA ATTCTGA
   RAPD B     AGGTTCCTGA GGGTGTCGAT CCCCGAACCG GCGAGGTGGA ATTCTGA
```

Figure 4.9 *RecA* sequences of *P. acnes* NCTC 737 (type I), NCTC 10390 (type II) and a representative strain of RAPD type B (RAPD B). The sequence from the RAPD type B contained polymorphisms of both *P. acnes* type I (shown in blue) and II (shown in red) as well as five unique polymorphisms (shown in pink).

4.3.6 PCR cloning

The RAPD cluster containing the serotype I strains (cluster C, figure 4.7) was characterised by the presence of a band of 200bp which was absent from type A and B strains and *P. granulosum* and *P. avidum*. The sequence of this band was determined, after cloning by AU tailing into the pDrive cloning vector, both as a guide to the genetic differences between the two serotypes of *P. acnes* and to explain the absence of this band in the other RAPD types.

4.3.6.1 Screening of presumptive clones

Blue/white screening was used for identification of transformed bacteria that contained recombinant plasmids, however no white colonies were seen on selection plates. This may occur due to in-frame insertions of the PCR fragment into the X-gal gene allowing its expression and accounts for the variation in the intensity of blue colonies observed. Consequently a range of phenotypic colonies were analysed by PCR using plasmid primers. Of the 14 presumptive colonies screened ten appeared to contain plasmid with an insert of approximately 200bp (lanes 2, 4, 5, 6, 7, 8, 9, 12, 13, 14) (figure 4.10). No amplification products were formed from three clones screened (lanes 1, 3, 15). These false positives can occur when the selective antibiotic (ampicillin) becomes depleted from the agar. Lanes 10 and 11 represent self-ligated plasmid.
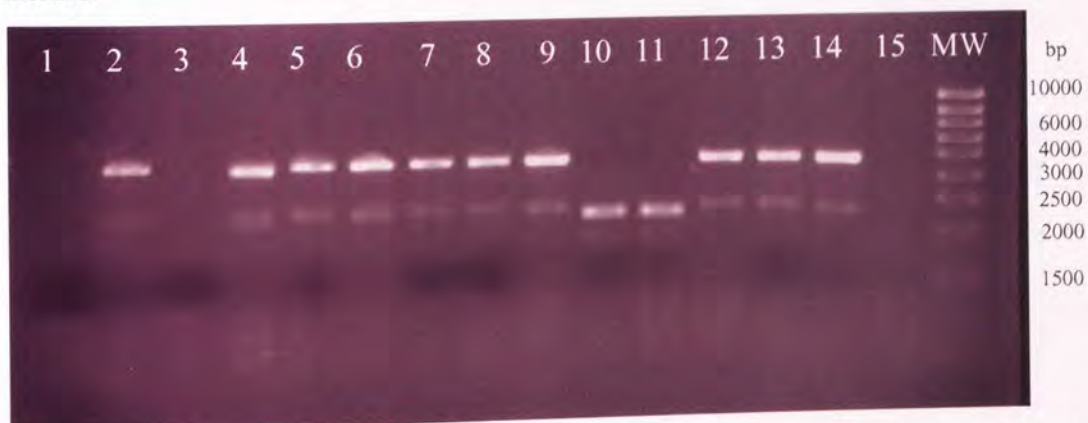
Figure 4.10 PCR products amplified using plasmid primers. Products containing the insert (lanes 2, 4, 5, 6, 7, 8, 9, 12, 13 & 14) were approximately 200bp larger than those without the insert (lanes 10 & 11). Lane MW: 10kb MassRuler™ DNA Ladder, High Range.

4.3.6.2 DNA sequence and amino acid analysis

Both strands of the DNA insert were sequenced from four of the clones screened by PCR. The nucleotide sequences of these were identical when aligned and were 199bp in size. The DNA sequence was translated into the six possible ORFs of which one putative ORF of 66 amino acids was found (figure 4.11).

```
      S   E   G   K   G   S   V   T   L   T   D   L   V   R   Q   C   L   R   M   R
2     tctgagggtaaaggatcggtgactctgaccgacttggtccgtcagtgcttgcgaatgagg   61
      P   D   R   I   V   L   G   E   V   R   G   A   E   L   R   D   L   L   M   A
62    cccgaccggatcgtccttggagaggtgcgaggggcggaactgcgagatctcctcatggcc   121
      L   N   T   G   H   E   G   G   C   G   T   V   H   A   N   G   V   A   E   V
122   ctcaataccggtcatgagggtggttgcgggacggtgcacgccaacggggtggctgaggtg   181
      P   A   R   L   E   A
182   ccggctcgtctggaggct   199
```

Figure 4.11 Nucleotide sequence of the 199bp RAPD band and the translated putative ORF amino acid sequence.

Both the nucleotide sequence and putative ORF were used to interrogate GenBank databases. The nucleotide sequence and ORF gave 100% match with the recently sequenced genome of *P. acnes* strain, DSM 16379 (Bruggemann *et al.*, 2004) and the translated ORF fell within a putative secretory protein ORF. High homology was found with secretory and conjugal transfer proteins from closely related bacteria (table 4.3).

Search	Accession number	Function	Organism	Homology	
				Identities	Positives
DNA	AE017283		*P. acnes* DSM 16379	100%	
	U77780	-	*Chlorobium limicola* strain DSM 249 endogenous plasmid pCL	92%	-
	AF057718	Autoinducer synthase (*tra*I) gene	*Agrobacterium tumefaciens* plasmid pTiC58	90%	-
	AE009422	-	*Agrobacterium tumefaciens* strain C58 Ti plasmid	90%	-
	AP005039	-	*Streptomyces avermitilis*	87%	-
Translated ORF	AE017283	putative secretory protein	*P. acnes* DSM 16379	100%	100%
	AL939117	Putative secretory Protein	*Streptomyces coelicolor*	78%	84%
	AP005215	putative conjugal transfer protein	*Corynebacterium efficiens*	70%	90%
	BX248354	Conserved hypothetical protein	*Corynebacterium diphtheriae*	71%	87%
	AP005039	Hypothetical protein	*Streptomyces avermitilis*	75%	84%

Table 4.3 DNA and amino acid homologies to the nucleotide and translated ORF sequence of the 199bp RAPD band using GenBank bacterial deposited sequences.

4.3.6.3 Detection of 199bp sequence in *P.acnes* strains using PCR

The nucleotide sequence of the cloned 199bp RAPD fragment was used to design primers to use as probes. Strains were screened for the 199bp sequence using primers P1 (table 4.4). All strains examined were PCR positive using primers designed to amplify this sequence (figure 4.12) including strains which were not serotype I. Therefore, primers which would amplify upstream and downstream of the 199bp sequence were sought to try and account for the absence of the 199bp RAPD band in the non-type I strains. The complete ORF, obtained from the published sequence of *P. acnes* DSM 16379 (Bruggemann *et al.*, 2004), within which the 199bp sequence occurred was used to design primers (P2, table 4.4) to amplify regions outside of the 199bp region (figure 4.13).

```
1    CTCTGAGGGTAAAGGATCGGTGACTCTGACCGACTTGGTCCGTCAGTGCTTGCGAATGAG
        >>>>>>>>>>>>>>>>>>>>>

61   GCCCGACCGGATCGTCCTTGGAGAGGTGCGAGGGGCGGAACTGCGAGATCTCCTCATGGC
                                                    <<<<<<<<<<<

121  CCTCAATACCGGTCATGAGGGTGGTTGCGGGACGGTGCACGCCAACGGGGTGGCTGAGGT
        <<<<<<<<<

181  GCCGGCTCGTCTGGAGGCT
```

Figure 4.12 Primers designed to be used as probes for the 199bp sequence. >>>>>> forward primer (P1_F, table 4.4), <<<<<< reverse primer (P1_R, table 4.4).

Name	Sequence	Amplicon size (bp)
P1_F	5'-TGAGGGTAAAGGATCGGTGA-3'	126
P1_R	5'-GTATTGAGGGCCATGAGGAG-3'	
P2_F	5'-CTGCTTTCCGCGATGC-3'	517
P2_R	5'-CGTCGCCAAGAGTCATC-3'	

Table 4.4 Primers (R=reverse, F=forward) MWG biotech, Ebersberg, Germany

```
1     ATGAGCGGAGAAGAACCGGATCTCAAGCTCGTCGAGCAGGTGCGACGCCGACTGGCTGGC

61    CTTGCGCGGGACTGGACACCGATGGACGTCGCCCACGCCATTCTCGACTGTGGCCGGGTG

121   GCTTCAGACAAAACCGTACTGGCGGTCGTCGAAGAGCTACGCCGCACCAGCACTGGGGCC

181   GGGCGATTAGATCCTTTGCTGGCAATGGACGGGGTCACTGACGTCCTCGTCAACGGTCCC

241   AACCAGGTGTTTATCGACCGCGGACAAGGCCTGGAACTGACCGACGTCACCTTCAGCGGA

301   CCTGAGGAAGTAAGGGCTTTAGCAGTGCGGCTGGCTGCAGCGGTGGGGCGTCGGCTCGAT

361   GACGGCAATCCGTGGGTGGATGCCAGGCTGCCAGACGGCACCCGGGTTCACGCTGTCTTG

421   GATGTGCTGGCGAGGCCAGGGACCTGTCTTAGTTTGCGAGTGCCGTCCCGACGCTGCCTC

481   TCCCTCGACGATTGGGTAACCGGAGGATCGATGGCACCGGGGTGCCGACGGATGATCGAC

541   ACAATTTTGAGCCGGAAGTTGGCCTTCTTGGTAACTGGGGGTACCGGGACCGGAAAGACG

601   ACCCTGCTTTCCGCGATGCTGACGAGCTTGCCGTCGGACCAAAGGCTGGTCATTGTGGAG
            >>>>>>>>>>>>>>>
661   GACTCCCGGGAGATCTGGATCGACCATCCGCACTGGGTGTCAATGGAGGCCCGCGCAGCC

721   AACTCTGAGGGTAAAGGATCGGTGACTCTGACCGACTTGGTCCGTCAGTGCTTGCGAATG

781   AGGCCCGACCGGATCGTCCTTGGAGAGGTGCGAGGGGCGGAACTGCGAGATCTCCTCATG

841   GCCCTCAATACCGGTCATGAGGGTGGTTGCGGGACGGTGCACGCCAACGGGGTGGCTGAG

901   GTGCCGGCTCGTCTGGAGGCTTTGGCTGCTTTGGGTGGGTTATCTCGGGAGGCGGCATCG

961   GCTCAGATCACCGCAGCCCTTCACACCGTCATTCATGTGGAGAGGGGAGAGAGGGGACGA

1021  CGGGTGGCGGAGATTGGGGTTTTCGTCGATGTCGAGGGTCGAGCTGTGGTCCTACCCGCC

1081  GTGCGGTGGGATGCCGACGGCGTGATGACTCTTGGCGACGGTGCGGGGAGGTTGTCACAG
                        <<<<<<<<<<<<<<<<
1141  TTGCTCGGAATGGAGGTGGCGCGATGA
```

Figure 4.13 ORF nucleotide sequence (1167bp) within which the 199bp RAPD fragment is located. The 199bp sequence is highlighted (blue) along with the position where the RAPD primers bind (green). Start codon (_), primers designed to amplify either side of the 199bp sequence are designated >>>>>> forward primer (P2_F, table 4.4), <<<<<< reverse primer (P2_R, table 4.4).

All three strains (*P. acnes* NCTC 737 (type I), NCTC 10390 (type II) and a clinical strain) examined by PCR with primers P2 (table 4.4) gave an amplicon of approximately 520bp. Sequencing of the PCR product revealed that the clinical strain was identical to *P. acnes* NCTC 737. Nucleotide differences were seen between *P. acnes* NCTC 737 and *P. acnes* NCTC 10390 in 7 positions. The most notable difference being that where the 3' region of the reverse RAPD primer binds to *P. acnes* NCTC 10390 (figure 4.14). The nucleotide change from T to C prevents the 3' region of the reverse RAPD primer from annealing to *P. acnes* NCTC 10390 DNA. This accounts for the absence of the 199bp band in *P. acnes* NCTC 10390 and other type II strains.

```
NCTC 10390  5'-GAGCTTGCCGTCGGACCAAAGGCTGGTCATTGTGGAGGACTCCCGGGAGATCTGGATCGA
NCTC 737    5'-GAGCTTGCCGTCGGACCAAAGGCTGGTCATTGTGGAGGACTCCCGGGAGATCTGGATCGA

                                              5'-F-3'
NCTC 10390  CCATCCGCACTGGGTGTCAATGGAGGCCCGCGCAGCCAACTCTGAGGGTAAAGGATCGGT
NCTC 737    CCATCCGCACTGGGTGTCAATGGAGGCCCGCGCAGCCAACTCTGAGGGTAAAGGATCGGT


NCTC 10390  GACTTTGACCGACTTGGTCCGTCAGTGCTTGCGAATGAGGCCCGACCGGATCGTCCTTGG
NCTC 737    GACTCTGACCGACTTGGTCCGTCAGTGCTTGCGAATGAGGCCCGACCGGATCGTCCTTGG


NCTC 10390  AGAGGTGCGAGGGGCGGAACTGCGAGATCTCCTCATGGCCCTCAATACCGGTCATGAGGG
NCTC 737    AGAGGTGCGAGGGGCGGAACTGCGAGATCTCCTCATGGCCCTCAATACCGGTCATGAGGG


NCTC 10390  TGGTTGCGGGACGGTGCACGCCAACGGGGTGGCCGAGGTACCAGCTCGTCTGGAGGCTCT
NCTC 737    TGGTTGCGGGACGGTGCACGCCAACGGGGTGGCTGAGGTGCCGGCTCGTCTGGAGGCTTT

            3'-R-5'
NCTC 10390  GGCTGCTTTGGGTGGGTTATCTCGGGAGGCGGCATCGGCTCAGATCACCGCAGCCCTCCA
NCTC 737    GGCTGCTTTGGGTGGGTTATCTCGGGAGGCGGCATCGGCTCAGATCACCGCAGCCCTTCA


NCTC 10390  CACCGTCATTCATGTGGAGAGGGGAGAGAGGGGGCGACGGGTGGCGGAGATTGGGGTTTT
NCTC 737    CACCGTCATTCATGTGGAGAGGGGAGAGAGGGGACGACGGGTGGCGGAGATTGGGGTTTT


NCTC 10390  CGTCGATGTCGAGGGTCGAGCTGTGGTCCTACCCGCCGTGCGG-3'
NCTC 737    CGTCGATGTCGAGGGTCGAGCTGTGGTCCTACCCGCCGTGCGG-3'
```

Figure 4.14 Nucleotide sequence of *P. acnes* NCTC 737 and NCTC 10390 amplified using primers P2 (table 4.4). Complementary (-) and non-complementary ($\searrow$) regions to RAPD primers (forward (F) and reverse (R)), and nucleotide alterations (red) are illustrated.

To investigate whether the observed nucleotide differences would be carried through to the amino acid level the nucleotide sequences were translated. The nucleotide and corresponding amino acid sequence of *P. acnes* NCTC 10390 are shown in figure 4.15 and illustrate the differences compared to those of *P. acnes* NCTC 737. All nucleotide changes occurred in the 'wobble position' except one which occurred in the first position (ctg in NCTC 10390 and ttg in NCTC 737) but coded for the same amino acid, leucine. Therefore the differences between type I and type II *P. acnes* revealed by this work are only at the DNA level and would not be apparent in the gene product.

```
      S   L   P   S   D   Q   R   L   V   I   V   E   D   S   R   E   I   W   I   D
 2   agcttgccgtcggaccaaaggctggtcattgtggaggactcccgggagatctggatcgac   61
      H   P   H   W   V   S   M   E   A   R   A   A   N   S   E   G   K   G   S   V
62   catccgcactgggtgtcaatggaggcccgcgcagccaactctgagggtaaaggatcggtg  121
      T   L   T   D   L   V   R   Q   C   L   R   M   R   P   D   R   I   V   L   G
122  actttgaccgacttggtccgtcagtgcttgcgaatgaggcccgaccggatcgtccttgga  181
      E   V   R   G   A   E   L   R   D   L   L   M   A   L   N   T   G   H   E   G
182  gaggtgcgaggggcggaactgcgagatctcctcatggccctcaataccggtcatgagggt  241
      G   C   G   T   V   H   A   N   G   V   A   E   V   P   A   R   L   E   A   L
242  ggttgcgggacggtgcacgccaacggggtggccgaggtaccagctcgtctggaggctctg  301
      A   A   L   G   G   L   S   R   E   A   A   S   A   Q   I   T   A   A   L   H
302  gctgctttgggtgggttatctcgggaggcggcatcggctcagatcaccgcagccctccac  361
      T   V   I   H   V   E   R   G   E   R   G   R   R   V   A   E   I   G   V   F
362  accgtcattcatgtggagagggagagagaggggcgacgggtggcggagattggggttttc  421
      V   D   V   E   G   R   A   V   V   L   P   A   V   R
422  gtcgatgtcgagggtcgagctgtggtcctacccgccgtgcgg   463
```

Figure 4.15 Nucleotide and translated amino acid sequence of region of *P. acnes* NCTC 10390 genomic DNA. Differences in the nucleotide sequence compared to the same region amplified from *P. acnes* NCTC 737 are indicated in red.

4.4 Discussion

The aim of this chapter was to develop a molecular typing method for *P. acnes*, to determine the relatedness of strains and to investigate whether particular isolates are associated with particular clinical conditions. RAPD was the chosen method of choice for typing *P. acnes* as no prior knowledge of the target genome is required. An optimised, robust and reproducible RAPD method was established and all isolates were typeable by this method. The RAPD typing allowed investigation of the nature of a prominent RAPD fragment to provide information about the differences between genotypes.

The RAPD method was optimised to give reproducible and discriminatory profiles using methods described by Hopkins and Hilton (2001). The results demonstrate the importance of optimisation in establishing a reproducible and discriminatory RAPD typing scheme.

Different primers may amplify different parts of the genome so it is important to evaluate the performance of more than one primer in the RAPD methodology (Hopkins and Hilton, 2001). RAPD primers used successfully by other workers (Dautle *et al.*, 2002; Hilton *et al.*, 1996; Tambic *et al.*, 1997) were investigated for their application to the RAPD typing of *P. acnes*. Only one primer (1254) gave promising results and was therefore chosen for its ability to produce distinguishable RAPD profiles. Primer 1254 was therefore selected for further use in optimisation studies.

As demonstrated by other workers (Ellsworth *et al.*, 1993), variation in the magnesium concentration results in marked alterations of RAPD profiles. Magnesium promotes and stabilises primer–template interactions, has an effect on denaturation of the template DNA and is required for *Taq* polymerase activity and fidelity. High concentrations of magnesium may inhibit amplification due to inadequate denaturation of the template DNA and can also lead to the accumulation of non-specific amplification products. By contrast, insufficient magnesium ions will reduce the yield as primers are unable to anneal efficiently to the template DNA (Hopkins

and Hilton, 2001). Potassium can also affect PCR specificity as it facilitates primer annealing (Hopkins and Hilton, 2001) and can directly affect *Taq* polymerase (Hilton *et al.*, 1997a). A lower concentration of KCl in the PCR buffer was optimal for the primer–template combination used in this study. Profiles produced using a buffer with a high concentration of KCl resulted in smeared profiles. This may have occurred due to extension of one primer without extension of a primer on the opposite strand. The pH of the PCR buffer had little effect on the profiles obtained; however, if the pH is too low, non-specific reactions can occur and if too high, yield is reduced. Buffer 9 (1.5 mmol/L $MgCl_2$, 25 mmol/L KCl, pH 9.2) was selected for use in further RAPD reactions.

When optimising RAPD the template concentration can be a critical factor to consider (Tyler *et al.*, 1997). The concentration of DNA can influence the number of products formed resulting in different fingerprints, therefore standardisation of the template concentration is an important factor for reproducibility. Excess template can result in suppression of the amplification process due to competition between template DNA and first-round amplicons and the relative shortage of primers. This phenomenon was not experienced using the DNA concentrations tested in this study.

The optimised RAPD method could type all strains and on repeated testing on two separate occasions, all profiles could be assigned to the same pattern as previously generated giving 100% intra- and inter-reproducibility. The RAPD protocol was also highly discriminatory as the Simpson's diversity index was in keeping with the requirement for an index greater than 0.9, which is desirable if the results of a typing scheme are to be interpreted with confidence (Hunter and Gaston, 1988).

There are no official guidelines for the interpretation of RAPD profiles and problems are often centred around how many differences truly differentiate strains. Many investigators base their interpretations on guidelines published for PFGE (Tenover *et al.*, 1995). Isolates are genetically indistinguishable if profiles are the same. One band difference is likely to arise by one genetic event, i.e. by point mutation, insertion or deletion of DNA; such strains are considered very similar. Strains with two band differences are labelled possibly related and unrelated with three or more differences. These guidelines, however, are based on the creation or loss of restriction sites and

relate to macro-restriction digest profiles. Diversity of RAPD profiles is based on the frequency and location of primer binding sites. Band differences in RAPD can be due to point mutations which may result in the loss or creation of a primer binding site. Similarly, insertions and deletions can create or destroy binding sites. In addition, insertions may increase the fragment length and deletions reduce the length of the fragment. Furthermore, intensity of bands can vary between strains if bands consist of two fragments of the same size.

RAPD profiles obtained in this study were analysed by dendrogram based on un-weighted pair matching of the Dice coefficients calculated for each profile. This compares profiles band by band producing clusters of strains that share a high percentage of similarity. This method is an accepted technique for the interpretation of RAPD profiles and has been used by other investigators (Hilton *et al.*, 1997b; Rossi *et al.*, 1998). *P. acnes* isolates were represented in a major cluster at the 65% level of similarity which further subdivided into three distinctive profile types. The largest cluster contained *P. acnes* NCTC 737 (serotype I) and other clinical strains which were predominately serotype I. Type II strains and NCTC 10390 (serotype II) featured in the second major cluster. The third and smallest cluster contained strains that had unusual serotype results as they were neither type I or II serotypes. The RAPD typing scheme therefore appears to distinguish the two major *P. acnes* serotypes. Other workers have shown that RAPD can cluster isolates within their respective serotypes, for example serotypes of enterotoxigenic *E. coli* (Pacheco *et al.*, 1997), *Salmonella* serotype *typhi* and other *Salmonella* isolates (Shangkuan and Lin, 1998) and serotypes of group B streptococci (Zhang *et al.*, 2002).

The strains featuring in the smallest RAPD cluster which were neither type I or II were further investigated by 16SrRNA and *recA* typing. 16SrRNA typing confirmed that these isolates were *P. acnes* but the *recA* sequences were found to contain polymorphisms of both type I and II serotypes as well as unique ones. This novel *recA* sequence data provides evidence that these strains may constitute a previously undiscovered *P. acnes* subset which is different to the type I and II serotypes (Dr Andrew McDowell, personnel communication).

Cloning and sequencing of the prominent ~200bp RAPD fragment associated with serotype I strains accounted for the lack of this band in the type II serotype strains and identified nucleotide but not amino acid differences between these serotypes. A single nucleotide base change in the 3' region of the primer binding site in serotype II DNA prevented one of the RAPD primers binding adequately. The first three (Sommer and Tautz, 1989) to six nucleotides (Caetano-Anolles, 1993) at the 3' end of the primer must be entirely complementary in order for DNA polymerase binding and extension to occur successfully.

There was no apparent relationship found between RAPD genotype and the source of an isolate. Strains from different sources occurred sporadically throughout the dendrogram generated from RAPD profiles.

The phenotype of an isolate, determined by biotyping, virulence factor expression and antibiotic susceptibility, did not show a relationship with the genotype of the same strain. For example, strains with the same biotype had different RAPD profiles and likewise, the *P. acnes* antibiotic resistant and sensitive populations were found to be polyclonal by RAPD. Oprica *et al* (2004) similarly found that *P. acnes* strains with the same antibiotic susceptibility pattern can be derived from different genotypes. In addition, phenotypic and genotypic characterisation of coagulase negative staphylococci by PFGE, API, antibiogram and slime production showed no correlation (Worthington *et al.*, 2000). The only phenotypic trait of *P. acnes* strains investigated in this thesis which clearly correlated with the RAPD genotype was isolate serotype. This finding enabled the identification of a previously unknown subset of *P. acnes* which were neither serotype I nor II and had a unique RAPD profile.

In summary, a molecular typing technique utilising RAPD was developed for genotyping *P. acnes*. The genotype of a strain did not clearly relate to its clinical source or to its biotype, antimicrobial susceptibility or virulence factor production. The type I and II serotype of *P. acnes* strongly correlated with the RAPD genotype. A small subset of isolates, which did not react with either the type I or II monoclonal antibodies, had a unique RAPD profile and these strains may therefore represent a separate group of *P. acnes* which is distinguishable from type I or II.

CHAPTER 5 ANTIGENIC ANALYSIS OF *P. ACNES*

5.1 Introduction

Many attempts have been made to identify *P. acnes* associated antigens. Previously identified antigens include polypeptides from cell extracts and the culture supernatant of *P. acnes* (Holland *et al.*, 1993), carbohydrate containing antigens (Burkhart *et al.*, 1999b; Dawes *et al.*, 1974; Iversen *et al.*, 1985) and exocellular enzymes produced by the organism (Ingham *et al.*, 1987).

The aim of this study was to investigate and characterise immunodominant *P. acnes* antigens using patient sera from individuals with acne and sciatica patients who had undergone microdiscectomy yielding *P. acnes*. This approach may enable the identification of the major antigens whose expression *in vivo* induces an immune response in the host. Identification of such antigens may provide an insight into the pathogenicity of *P. acnes* and form the basis of a serological test for *P. acnes* infection.

5.2 Materials and methods

5.2.1 SDS-PAGE analysis and Western blotting

The antigenic composition of *P. acnes* was investigated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting of whole cells and culture supernatants.

5.2.1.1 Preparation of whole cell and culture supernatants

P. acnes strains were grown in 25ml BHI in static universals at 37°C for 72 hours. Bacterial cells were harvested by centrifugation (10000g, 10 minutes) and an equal volume of ethanol (100%, -20°C) added to the culture supernatant. After incubation for 18 hours at 4°C the ethanol-precipitated culture supernatant was centrifuged at 12000g for 30 minutes. The pellet was resuspended in 500μl sterile distilled water and

freeze dried. The preparation was adjusted to 100mg/ml using sterile distilled water and stored at −20°C. Cells were resuspended in PBS to absorbance$_{600nm}$ of 1.0. Sterile BHI was ethanol precipitated as a control.

5.2.1.2 SDS-PAGE (Lugtenberg *et al.*, 1975)

An 11% (w/v) separating gel (table 5.1) was poured and allowed to set using the PAGE gel casting system (BioRad, UK). A 5% (w/v) stacking gel (table 5.1) was then poured on top and a fifteen lane comb or a single lane comb inserted 4cm above the stacking gel. Samples for SDS-PAGE were mixed with an equal volume of sample buffer (table 5.1) and denatured at 100°C for 10 minutes. A 15μl aliquot of denatured sample was loaded into an appropriate lane of the 15 lane comb or a 500μl aliquot loaded into the single lane comb. 10μl of prestained SDS-PAGE standard low range (BioRad, UK) or broad range (New England BioLabs, UK) was run in one lane. The polyacrylamide gel was then placed in SDS electrode buffer (table 5.1) and electrophoresis conducted at 200V for 42 minutes using BioRad Mini Protean® II apparatus (BioRad, UK). The gel was then either stained or subject to Western blotting.

	Separating gel 11% (w/v)	Stacking gel 5% (w/v)	Sample buffer	Electrode buffer (pH 8.0)
Acrylamide stock I [*]	5ml	-	-	-
Acrylamide stock II [**]	-	2.5ml	-	-
10% (w/v) SDS	0.5ml	0.15ml	5ml	10ml
1.5M Tris-HCl (pH 6.8)	6ml	-	-	-
0.5M Tris-HCl (pH 6.8)	-	3.8ml	2.5ml	-
Distilled water	8ml	8ml	5ml	1L
TEMED (N,N,N^{1}N^1-tetramethyl-ethylenediamine)	50μl	40μl	-	-
10% (w/v) ammonium persulphate	70μl	50μl	-	-
Glycerol	-	-	2.5ml	-
2-mercaptoethanol			0.25ml	
5% (w/v) bromophenol blue	-	-	0.2ml	-
Tris	-	-	-	3g
Glycine	-	-	-	14.4g

* Acrylamide stock I: 44% (w/v) acrylamide, 0.8% (w/v) bis(N,N^1,-methylene-bis-acrylamide (Severn Biotech Ltd, UK)

**Acrylamide stock II: 30% (w/v) acrylamide, 0.8% (w/v) bis(N,N^1,-methylene-bis-acrylamide (Severn Biotech Ltd, UK)

Table 5.1 Composition of SDS-PAGE gels and reagents.

5.2.1.3 Western blotting

Proteins resolved by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane following a method adapted from Towbin *et al.* (1979). A piece of nitrocellulose membrane (0.45 μm), two pieces of Whatman 3MM paper and two sponges (BioRad, UK) cut to the size of the gel were soaked in transblot buffer [25mM Tris, 192mM glycine, 20% (v/v) methanol, pH 8.3]. The gel was sandwiched onto the nitrocellulose membrane with a piece of Whatman 3MM and a sponge either side. The 'sandwich' was placed in mini transblot apparatus (BioRad, UK) which was cooled internally with an ice. Material resolved by SDS-PAGE was transferred from the gel to the nitrocellulose membrane by electrophoresis in transblot buffer at 100V for 42 minutes. To prevent non-specific binding of proteins the membrane was immersed in 50ml blocking solution consisting of phosphate buffered saline supplemented with 0.05% Tween (PBS-Tween) and 5% (w/v) skimmed milk for one hour with gentle shaking. The blocking solution was discarded and the nitrocellulose membrane washed three times with PBS-Tween. The membrane was cut into strips if a single lane comb was used or otherwise left whole and immersed in the appropriate antibody (section 5.2.1.3.1). Immunoreactive material was visualised by addition of chromogenic substrate containing 10mg of 4-chloronaphthol dissolved in 1ml of methanol diluted in 100ml of 0.01M Tris-HCl pH 7.4 and 40μl of H_2O_2. The reaction was stopped after approximately 15 minutes or until the colour developed by washing the nitrocellulose membrane in distilled water.

5.2.1.3.1 Immunodetection

5.2.1.3.1.1 Patient serum

Sera collected from patients undergoing microdiscectomy for sciatica, those with acne and control patients were diluted 1:400 in Tris-buffered saline Tween 20 (TBS-Tween) 0.01M Tris, 0.85% w/v NaCl, 0.3% v/v Tween 20, pH 7.4) and incubated for 18 hours at 4°C. After washing the membrane three times with TBS-Tween bound antibody was detected by placing the membrane in protein A-horseradish peroxidase

conjugate dissolved in TBS-Tween (0.25µg/ml) for 1.5 hours with gentle shaking. After another washing step the chromogenic substrate was applied (section 5.2.1.3).

5.2.1.3.1.2 Monoclonal antibodies

The relationship between newly identified antigens and previously described cell wall antigens of *P. acnes* was assessed using monoclonal antibodies directed against type I, II, III and IV antigens. Nitrocellulose membranes were incubated with undiluted solutions of monoclonal antibody supernatant for one hour at 37°C with gentle shaking. Membranes were washed 3 times with TBS-Tween and incubated with peroxidase conjugate-goat anti-mouse IgG diluted (1 in 3000) in TBS-Tween overnight at 4°C with gentle shaking. Membranes were the washed three times in TBS-Tween to remove unbound conjugate and reacted with chromogenic substrate (section 5.2.1.3).

5.2.1.4 Gel staining using coomassie blue stain

After electrophoresis each gel was placed in coomassie Blue stain (20% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) coomassie Blue) for 1 hour with agitation. The coomassie Blue stain was discarded and replaced with destaining solution (20% (v/v) methanol, 10% (v/v) acetic acid) for one hour. The destaining solution was replaced repeatedly until the solution remained clear.

5.2.2 Ouchterlony double diffusion immunoprecipitation (Ouchterlony, 1958)

This method was used to investigate the properties of antigen in non-reducing conditions and assess any cross reactivity with BHI media components. A 1% w/v agarose gel was prepared in water, cast onto a glass microscope slide and allowed to set. Wells were punched in the gel to give a central well surrounded by six outer wells equidistance apart. To the centre well 30µl of patient serum was added and an equal volume of *P. acnes* ethanol precipitated antigen or BHI control (section 5.2.1.1) added to the outer wells. After incubation in a moist chamber at 4°C for 18 hours non-precipitated material were removed by pressing and washing the gel according to the

method described by Weeke (1973). Wet filter paper followed by dry absorbent paper tissues were placed on top of the gel which was then pressed evenly for ten minutes to reduce the gel to a thin film. This film was then placed in distilled water to wash and reconstitute the gel. Pressing and washing of the gel was then repeated three times. After the final pressing of the gel it was dried with a hairdryer. Any immuno-precipitins were stained with coomassie blue for ten minutes (1g coomassie brilliant blue R-250, 90ml ethanol, 20ml glacial acetic acid, 90ml distilled water). Excess stain was removed from the gel using destaining solution (90ml ethanol, 20ml glacial acetic acid, 90ml distilled water).

5.2.3 Antigen production during growth phase

To determine the effect of growth phase on antigen production, lag, exponential and stationary phase culture supernatants were assayed for antigen.

5.2.3.1 Calibration curve

An overnight culture of *P. acnes* NCTC 737 in BHI broth was used to establish the relationship between absorbance and colony forming units (CFU). Two-fold dilutions of the culture were made in triplicate using sterile BHI and the absorbance read at 600nm. Colony counts were determined by culture of serial tenfold dilutions using the method of Miles & Misra (1938). Six aliquots of 20µl were inoculated onto BHI agar, incubated anaerobically at 37°C for 72 hours and the colonies counted. A graph of absorbance$_{600nm}$ against CFU/ml was constructed and the equation of the curve used subsequently to obtain CFU/ml of cultures from their absorbance readings.

5.2.3.2 *P. acnes* growth curve

An overnight culture of *P. acnes* NCTC 737 was used to inoculate three conical flasks containing 50ml pre-warmed, reduced BHI. The inoculum was standardised by adjusting the overnight culture to an absorbance$_{600nm}$ of 0.1 and with 500µl used to inoculate cultures. A 1ml sample was taken from each flask immediately and the absorbance read at 600nm using sterile BHI as a blank. Subsequent samples, in triplicate, were taken every three hours until the cells entered the stationary phase.

Growth curves were determined for *P. acnes* NCTC 10390 and a clinical strain (sciatica) using the same method.

5.2.3.3 Antigen detection

Samples of culture supernatant, in triplicate, were collected at 5, 10, 20, 30, 40 and 50 hours of growth and ethanol precipitated as described in section 5.2.1.1. BHI media was ethanol precipitated for use as a control. Antigen was analysed using SDS-PAGE and western blotting as described in section 5.2.1. Quantification of the antigen was achieved using ELISA.

5.2.3.4 Quantification of antigen by ELISA

5.2.3.4.1 Optimisation of antigen coating

Antigen samples collected from the culture supernatant of *P. acnes* NCTC 737 were diluted 1:100 in 0.05M sodium carbonate/bicarbonate buffer (pH 9.6) and 2-fold serially diluted on a microtitre plate (Immunlon 2, Dynex technologies, VA). Ethanol precipitated BHI was included as a control. Plates were incubated for 18 hours at 4°C. Unbound material was then removed and the plates washed three times in TBS-Tween. Unbound sites were blocked by complete filling of the wells with TBS-Tween and incubation at 4°C for four hours. The buffer was then removed and the coated plates stored at -20°C until required. Patient serum was diluted 1:400 with TBS-Tween, 100μl added to each well and the plate incubated at 4°C for 18 hours. The serum was removed from the plate by inversion and the plate washed three times in TBS-Tween. Protein A-peroxidase conjugate was diluted to 0.25μg/ml in TBS-Tween and 100μl dispensed into each well. After an incubation of two hours at 4°C unbound conjugate was removed by rapid inversion and the plate was washed three times in TBS-Tween. A chromogenic substrate was prepared by dissolving 5mg of 3,3',5,5' –tetramethylbenzidine in 500μl of dimethyl sulphoxide. This was then combined with 50ml of 0.1M sodium acetate/citrate buffer (pH 6.0) containing 5μl of 6% (w/v) hydrogen peroxide. Into each well 100μl of chromogenic substrate was dispensed and the plate incubated for ten minutes at room temperature. The reaction was stopped by the addition of 100μl of 0.1M sulphuric acid to each well. The

absorbance$_{450nm}$ was read immediately using a plate reader (Anthos 2001 plate reader, Labtech Instruments). Dilution of the antigen showing good discrimination between samples was subsequently selected for further use.

5.2.3.4.2 Pre treatment of patient serum

To remove antibodies reactive to components in the media, serum (1:400 in TBS-Tween) was incubated with ethanol precipitated BHI for one hour at room temperature. Immune complexes were removed by centrifugation (10 000g, 10 minutes) and the supernatant used for ELISA as described in section 5.2.3.4.1. The results were compared to those obtained without pre-incubation of serum.

5.2.3.4.3 Assay of antigen by ELISA

Antigen samples collected during growth of *P. acnes* NCTC 737, *P. acnes* NCTC 10390 and a clinical strain of *P. acnes* were diluted (as determined in section 5.2.3.4.1) in 0.05M sodium carbonate/bicarbonate buffer and 100μl added to each well in duplicate. ELISA was continued as described in section 5.2.3.4.1 using patient serum pre-incubated with ethanol precipitated BHI (section 5.2.3.4.2).

5.2.4 Chemical and physical destruction of antigen

To elucidate the chemical nature of antigen preparations were treated with periodate to destroy carbohydrate moieties and proteinase K for digestion of proteins.

5.2.4.1 Periodate treatment

The stability of antigen in the presence of periodate was determined by treating 10μl of ethanol-precipitated antigen (100mg/ml) (section 5.2.1.1) with 5μl of varying concentrations of sodium metaperiodate (0, 0.1mM, 1mM, 10mM, 100mM) in sodium acetate buffer (0.1M, pH 7.0). Solutions were incubated overnight at room temperature, after which excess periodate was eliminated by the addition of 1μl butandiol. Additional samples containing only antigen in sodium acetate buffer and

one consisting of reagents without antigen were set up as controls. Samples were then analysed by SDS-PAGE and western blotting as described in section 5.2.1.

5.2.4.2 Proteinase K treatment

A 10μl aliquot of antigen (100mg/ml) was treated with varying concentrations of proteinase K (0, 0.12mg/ml, 0.25mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml) for 4 hours at 55°C. Controls consisting of antigen only and proteinase K alone were also prepared. Samples were then analysed by SDS-PAGE and western blotting as described in section 5.2.1.

5.2.5 Purification of *P. acnes* antigen

5.2.5.1 Fast protein liquid chromatography (FPLC)

5.2.5.1.1 Gel permeation chromatography

Ethanol precipitated antigen (section 5.2.1.1) was injected onto a Superose 12 gel permeation FPLC column (30cm x 1cm, Amersham Pharmacia, UK) at a concentration of 10mg/ml in a volume of 1ml. The column was eluted with distilled water and fifty 0.8ml fractions collected at two minute intervals at a flow rate of 0.4ml/min. Ethanol precipitated BHI media was treated identically as a control. Fractions were analysed by dot blot and ELISA as in described in section in 5.2.5.1.3.

5.2.5.1.2 Hydrophobic interaction chromatography

Column chromatography was performed on an octyl sepharose column (5ml column) using the ÄKTA prime system (Amersham Pharmacia, UK) using a modification of the method described by Fischer (1993). The column was equilibrated with 20ml 0.1M sodium acetate buffer pH 7.4 containing 15% (v/v) propan-1-ol at a flow rate of 1ml/min. A 2ml aliquot of antigen (10mg/ml in equilibrium buffer) was injected onto the column and 2ml fractions collected. After a 10ml was elution with 0.05M sodium acetate pH 7.4 containing a linear propanol gradient from 15% to 65% (v/v) using a total volume of 30ml, a further propanol concentration of 65% was held for 10ml and

then decreased to 15% over another 10ml. Fractions were analysed by dot blot and ELISA as described in section 5.2.5.1.3.

5.2.5.1.3 Analysis of fractions

Eluted fractions were assayed for antigenic content by dot blotting and ELISA using serum from a *P. acnes* culture positive sciatica patient.

5.2.5.1.3.1 Dot blotting

A 1μl spot of each fraction was applied to a nitrocellulose membrane (0.45μm, BioRad, UK) and left to air dry. The membrane was blocked, probed with patient serum and developed as described in section 5.2.1.3.

5.2.5.1.3.2 ELISA

Individual fractions were assayed for antigenic content using an indirect ELISA. Fractions were diluted 1:100 in sodium carbonate/bicarbonate buffer (0.05M, pH 9.6) and 100μl of this dilution dispensed into wells of a 96-well flat-bottomed microtitre plate (Immulon 2, Dynex technologies, Chatilly, VA). Plates were incubated for 18 hours at 4°C and ELISA continued as described in section 5.2.3.4.1

5.2.5.2 Purification by electro-elution from polyacrylamide

Ethanol precipitated antigen, as prepared in section 5.2.1.1, was separated by SDS-PAGE using a wide single lane comb (section 5.2.1.2). Electro-elution was performed according to the manufacturer's instructions (Harrington, 1990) using a model 422 electro-eluter (BioRad, UK). A region of the antigenic bands was excised using the molecular weight marker as a guide. The excised gel was placed in a glass tube to which a dialysis membrane cap filled with elution buffer (25mM Tris, 192mM glycine, 0.1% (w/v) SDS, pH 8) had been fitted. The upper and lower chambers of the electro-eluter in which the glass tube was placed were filled with 100ml and 600ml of elution buffer respectively. Elution was performed at 10mA/glass tube with constant stirring for four hours. The eluted material was recovered from the dialysis membrane

cap and stored at -20°C until use. Effectiveness of elution was determined by SDS-PAGE and western blotting (section 5.2.1).

5.2.6 Modified method for the preparation of antigen using whole cells

Bacterial cells (*P. acnes* sciatica isolate) were inoculated onto BHI agar and incubated anaerobically at 37°C for 72 hours. Cells were harvested from the agar using a sterile loop and placed in an eppendorf tube containing 10mg glass beads (2mm, VWR international Ltd, UK) and 500µl sterile distilled water. Cells were washed by gentle inversion to remove any agar contaminants and centrifuged at 5000g for ten minutes. The supernatant was then removed and replaced with 500µl sterile distilled water. The solution was vortexed for three minutes and centrifuged (5000g,10 minutes) and the supernatant retained. The supernatant was concentrated by freeze drying and resuspended in sterile distilled water to 10mg/ml.

5.2.6.1 Detection of antigen prepared by vortexing from cells

The antigen preparation was separated by SDS-PAGE and detected by coomassie blue stain and western blotting (5.2.1). Gels were also stained using protein silver stain and modified silver stain for carbohydrates. To investigate each stage of the antigen preparation method immunofluorescence using patient serum was employed.

5.2.6.1.1 Protein silver stain

Polyacrylamide gels were stained for proteins using the silver stain plus kit (Bio-Rad, UK). Following electrophoresis, the gel was placed in fixative enhancer solution (10% (v/v) fixative enhancer concentrate, 10% (v/v) acetic acid, 50% (v/v) methanol) for 20 minutes with gentle agitation. The gel was then washed twice in distilled water for 10 minutes. The staining solution (5ml silver complex solution, 5ml reduction moderator solution, 5ml image development reagent, 35ml distilled water and 50ml development accelerator) was prepared immediately prior to use. The distilled water from the final wash step was discarded and replaced with the staining solution. The gel was stained until the desired intensity was obtained and the reaction stopped using 5% acetic acid followed by several rinses in distilled water.

5.2.6.1.2 Modified silver stain for carbohydrates

Material resolved by SDS-PAGE was probed for carbohydrate using a modification of the silver stain method (Fomsgaard *et al.*, 1990). Lipopolysaccharide extracted from *E. coli* 0111:B4 was included as a positive control (5µg per well). After SDS-PAGE the gel was oxidised by immersion in 0.7% periodic acid (v/v) in 40% ethanol-5% acetic acid (v/v) for 20 minutes at room temperature. The gel was washed three times in distilled water for five minutes and then stained with fresh staining solution for ten minutes which was prepared as follows: a 4ml volume of concentrated ammonium hydroxide was added to 56ml 0.1M sodium hydroxide, and 200ml of distilled water, then 10ml of 20% (w/v) silver nitrate was added drop-wise with stirring and the final volume adjusted to 300ml with distilled water. After staining the gel was washed three times with distilled water for five minutes. The water was replaced with 200ml formaldehyde developer containing 10mg citric acid and 0.1ml of 37% formaldehyde. The reaction was stopped when the stain reached the desired intensity by the addition of 10% acetic acid followed by repeated washes in distilled water.

5.2.6.1.3 Immunofluorescence

To visualise each step of the modified antigen method, IFM using patient serum was performed on untreated cells, cells after vortexing with glass beads, cell supernatant from vortexed cells and resuspended cells. After the wash stage of the modified method cells were resuspended to absorbance$_{600nm}$ of 0.3 and a 10µl aliquot applied to a single well of a twelve well multi-well slide (C. A. Hendley Ltd, UK). Subsequently 10µl aliquots were taken at the other stages of antigen preparation. IFM was carried out as described in section 3.2.4 except a 1:100 dilution of fluorescein anti-human IgG conjugate (Vector laboratories, USA) was used.

5.2.7 Chemical analysis

5.2.7.1 Thin layer chromatography

5.2.7.1.1 Application of samples and development of chromatography plate

Spots of 5µl of antigen (10mg/ml) were applied 20mm from the bottom of silica gel 60 coated aluminium chromatography plates (20x20cm, layer thickness 200µm, particle size 2-25µm). The solvent mixture, propanol:water (1:1), was added to a glass chromatography tank and allowed to equilibrate for 2 hours. The dry plate was placed in the chamber and developed until the solvent front reached approximately 10mm below the top of the plate. The plate was then removed, the solvent front marked and the plate fully air dried.

5.2.7.1.2 Visualisation and detection of separated material

Material separated on thin layer chromatography plates was visualised using a number of stains. Immunoreactive material was revealed by immuno-detection. After each detection method plates were immediately photographed.

5.2.7.1.2.1 General detection with iodine vapour

Plates were place in a closed container saturated with iodine vapour until yellow spots developed.

5.2.7.1.2.2 Ninhydrin stain

Plates were sprayed with 0.2% w/v ninhydrin in ethanol, allowed to dry and heated at 100°C for 15 minutes. Material containing amino groups was visualised as purple/pink spots.

5.2.7.1.2.3 α-Naphthol stain

The plate was sprayed with α-naphthol solution (0.5g α-naphthol in 100ml methanol-water (1:1)) until damp and allowed to dry in air. The plate was then sprayed lightly with sulphuric acid solution (H_2SO_4-H_2O (95:5, v/v)) and heated on a hotplate at 120°C until maximum colour developed. Lipids containing sugars give blue-purple spots under these conditions.

5.2.7.1.2.4 Sulphuric acid-acetic acid reagent

The plate was sprayed with concentrated sulphuric acid-glacial acetic acid solution (1:1, v/v) and heated at 90°C for 15 minutes. Non-specific sugars and sterols react with the reagent to give faint pink-brown spots.

5.2.7.1.2.5 Molybdenum blue reagent

Molybdenum blue reagent (1.3% molybdenum oxide in 4.2 M sulphuric acid) was used for the detection of phosphate groups. Plates were sprayed with this reagent and developed at room temperature. Positive spots rapidly appear blue.

5.2.7.1.2.6 Immunostaining

Developed plates were fixed by dipping into 0.05% (w/v) polyisobutyl methacrylate in hexane and air dried. Each plate was then blocked in PBS containing 1% (w/v) bovine serum albumin for 15 minutes at room temperature. After washing three times with PBS plates were incubated with patient serum (1:400 in PBS) for 1.5 hours at room temperature. Conjugate and chromogenic substrate were then applied to the plate as described in section 5.2.1.3.

5.2.7.2 Acid hydrolysis of antigen

A 4mg sample of antigen, prepared as described in section 5.2.6, was dissolved in 300μl of 2M trifluoroacetic acid (TFA) in a glass tube which was then sealed and heated at 100°C for 18 hours. After hydrolysis TFA was removed by repeated drying

under vacuum. The TFA hydrolysate was resuspended in 400μl sterile distilled water to give a concentration equivalent to 10mg/ml of the original antigen. An empty tube was treated with TFA and used as a control.

5.2.7.2.1 Analysis of TFA hydrolysate

5.2.7.2.1.1 Hexose assay

The relative amount of hexose in the TFA hydrolysate was determined using phenol-sulphuric assay for total hexose adapted from the method of Dubois *et al.* (1956). In glass test tubes 15μl of the hydrolysate was added to 185μl of distilled water, in triplicate. Solutions of glucose ranging from 0 to 50μg glucose in a 200μl volume were used as standards and 200μl of distilled water as a control. To each tube 50μl of 8% (w/v) phenol was added followed by 500μl of concentrated sulphuric acid and incubated at 30°C for 20 minutes. Aliquots of 200μl were added to wells of a microtitre plate and the absorbance$_{492nm}$ read (Anthos 2001 plate reader, Labtech Instruments). Hexose content of the antigen was quantified by comparison to a standard curve generated from known glucose concentrations.

5.2.7.2.1.2 Alditol acetates by gas chromatography

Hexoses in the TFA hydrolysate were converted to alditol acetates for identification by GC to determine which sugars were present. 100μl of the TFA hydrolysate was placed in a glass tube with 50μl of 3M NH$_4$OH and 3mg sodium borohydride. The solution was left in the dark at 22°C for 18 hours, after which excess borohydride was removed by adding a drop of glacial acetic acid. The borate was converted to the methyl derivative by addition of 500μl methanol and rotary evaporated at 50°C to dryness. Addition of methanol and evaporation was repeated and 100μl acetic anhydride added. The tube was sealed and heated at 121°C for 3 hours and 400μl of water added. The sample was rotary evaporated to dryness, dissolved in 100μl chloroform and analysed by GC. 1μl of sample was loaded onto a Supelco SP-2380 capillary column (ID 0.25mm, film thickness 0.2μm, length 30m) on a Unicam 610 series system with 1:100 sample splitting and flame ionisation detector (Unicam, UK). The column temperature was maintained at 250°C. The gas phase consisted of helium

(6.5 psi) with nitrogen as the makeup gas (flow rate 25.05 cm/sec) and the flame ionisation detector used hydrogen and air. The injector temperature was 200°C and the detector temperature 280°C. The control TFA hydrolysate and a glycerol standard (1mg in a volume of 100µl) were subject to the same analysis.

5.2.7.2.1.3 Phosphate assay

Phosphate was measured using a colour reagent consisting of 0.5% (v/v) 3M sulphuric acid, 1% (w/v) ascorbic acid and 0.05% (w/v) ammonium molybdate. Standard solutions were prepared using KH_2PO_4 ranging from 0 to 5µg inorganic phosphate. Standards, 25µl antigen hydrolysate and 25µl control hydrolysate were assayed in clean glass test tubes and made up to a volume of 50µl using distilled water. One ml of the colour reagent was added to each tube and the samples incubated at 37°C for 2 hours. After incubation the absorbance was read at 820nm and the standard solutions of inorganic phosphate used to establish a calibration curve.

5.2.7.2.1.4 Glycerol assay

Glycerol was determined colourmetrically using the method described by Hanahan and Olley (1958). Standard solutions of glycerol were prepared ranging between 0 to 20µg in a total volume of 1ml. Solutions of antigen hydrolysate and control hydrolysate were prepared consisting of 10µl, 25µl and 60µl hydrolysate in a total volume of 1ml. To glycerol standards, antigen hydrolysate and control hydrolysate 20µl of 10N H_2SO_4 was added followed by 200µl of 0.1M $NaIO_4$. After 5 minutes 200µl of 10% (w/v) metabisulphite solution and 5ml chromotropic acid solution (10ml 1% (w/v) chromotropic acid, 45ml 24N H_2SO_4) was added. The samples were heated in a boiling water bath for 30 minutes, cooled and 500µl of saturated thiourea solution. The absorbance was read at 570nm and the glycerol standards used to generate a calibration curve.

5.2.7.2.1.5 Determination of *N*-acetylamino sugars

N-acetylamino sugars were measured using the method developed by Ghuysen *et al.* (1966). Standard solutions of *N*-acetylglucosamine were prepared containing 0 to

50µg. Antigen hydrolysate (70µl volume containing 0.2mg antigen) and 70µl of each standard were treated with 50µl potassium borate (5% v/v) and heated in a boiling water bath for 7 minutes. A solution of 16g p-dimethylaminobenzaldehyde was combined with 5ml concentrated HCl and made up to 100ml with glacial acetic acid. For use, the solution (2 volumes) was diluted with glacial acetic acid (5 volumes) and 200µl of this solution added to the samples. After incubation for 20 minutes at 37°C the samples were cooled and the absorbance read at 585nm. A standard curve was generated from the standard solutions and used to determine antigen *N*-acetylamino sugar concentration.

5.2.7.3 Identification of fatty acids by gas chromatography (GC)

Fatty acids present on bacterial cells and antigen were determined by alkaline methanolysis and gas liquid chromatography. Bacterial cells were harvested from 72 hour cultures of *P. acnes* NCTC 737, NCTC 10390 and a clinical strain on BHI agar and each resuspended in sterile distilled water to give a concentration of 400mg/ml. To clean glass tubes 100µl of cells or antigen (10mg/ml) were added, 100µl of sterile distilled water was included as a control. Fatty acids were released from the cell surface by saponification with 1ml a solution containing 45g sodium hydroxide, 150ml methanol and 150ml distilled water. Tubes were sealed and heated in a boiling water bath for 5 minutes, followed by vortexing then a further 30 minutes of boiling. Once cooled, 2ml of a solution consisting of 325ml 6N HCl and 275ml methanol was added. Tubes were vortexed and heated for 10 minutes at 80°C. This process of methylation acts to increase volatility by converting liberated fatty acids to the corresponding fatty acid methyl esters. Extraction was accomplished by adding 1.25ml of a solution of hexane:diethyl ether (1:1 v/v) and rotated for 10 minutes. The aqueous (lower) phase was removed and 3 ml of a solution of 10.8g NaOH in 900ml distilled water added. Following 5 minutes of mixing by rotation the organic phase was removed and retained for analysis. For GC the sample was dissolved in 100µl hexane and 1µl loaded onto capillary column (crosslinked methyl silicone gum, ID 0.32mm, film thickness 0.17µm, length 25m, Hewlett Packard, UK) on a Unicam 610 series system with 1:50 sample splitting and flame ionisation detector (Unicam, UK). The column temperature was maintained at 150°C for 4 minutes, increased at 4°C per minutes to 250°C and finally held at 250°C for 2 minutes. The gas phase consisted up

helium (6.5 psi) with nitrogen as the makeup gas and the flame ionisation detector used hydrogen and air. The injector temperature was 200°C and the detector temperature 280°C. A standard bacterial acid methyl ester mix (Matreya, USA) was included and used to identify and quantitatively estimate fatty acids.

5.2.7.4 Protein assay

Protein content was measured using the folin phenol reagent (Lowry *et al.*, 1951). A solution of alkaline $CuSO_4$ was prepared by combining 1ml of 1% (w/v) $CuSO_4$, 1ml of 2%(w/v) potassium tartrate and 98ml of 2% (w/v) Na_2CO_3 in 0.1N NaOH. Protein standard were prepared using bovine serum albumin containing 0-500µg. To 700µl aliquots of sample and standards 3ml of alkaline $CuSO_4$ added. After incubation at room temperature for 15 minutes 300µl of Folin-Ciocalteu phenol reagent was added and solutions incubated at room temperature for 30 minutes. The absorbance of each sample was measured at 750nm using distilled water as a blank and readings from the standards used to generate a standard curve. The protein content of the antigen was determined by comparison to the standard curve and expressed as a percentage of the original sample.

5.3 Results

5.3.1 Antigenic properties of *P. acnes* by SDS-PAGE and western blotting

In this section the exocellular antigens of *P. acnes* were investigated, initially using ethanol precipitation of culture medium. This method should recover any protein or polysaccharide material released from the cells during growth in BHI. However potential problems could arise from inclusion of BHI-derived material in the ethanol precipitates. Whole cells were also included for comparison purposes.

5.3.1.1 Coomassie blue stain and western blot analysis

Ethanol precipitated supernatant from ten strains (two isolates from each clinical source: sciatica patients, skin, prosthetic hips, blood cultures and acne lesions) were separated by SDS-PAGE on a single gel using a fifteen lane comb. Immunoblots of these strains were probed with sciatica patient serum (figure 5.1(b)) and a corresponding coomassie blue stained gel prepared (figure 5.1(a)). Immunoblotting revealed that the antigenic material was between 34-29 kDa and slight strain diversity occurred. The antigenic material did not appear to be a protein however, the spectrum of proteins in the supernatant varied considerably between strains. Proteins were not detected on the ethanol precipitated BHI control and no material present was antigenic.

The results in figure 5.2 show that treating whole cell suspensions with denaturing buffer releases the antigen. However, this was found to be an inconsistent method of antigen release, preparations carried out on different occasions would not show the same amount of antigen release or would show none at all. Furthermore, in some cases antigen would be detected in the culture supernatant but not from whole cells. It was also noted that very little protein was released by boiling cells with denaturing buffer prior to SDS-PAGE.
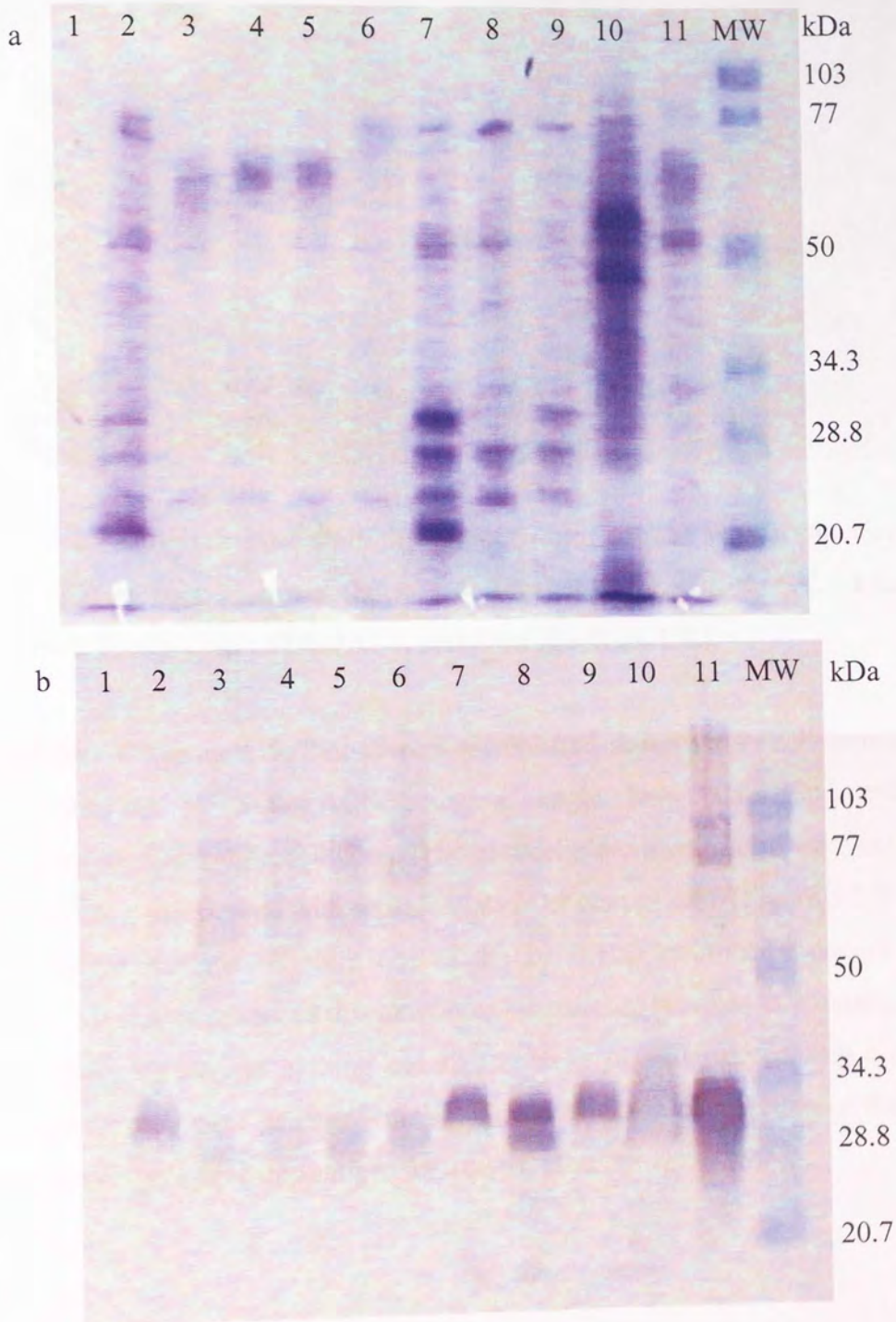
Figure 5.1 Coomassie blue stain (a) and immunoblot probed with sciatica patient serum (b) of ethanol precipitated supernatant from 10 *P. acnes* strains (lanes 2-10). Lane M: prestained low range SDS-PAGE protein markers (BioRad, UK), lane 1: ethanol precipitated BHI control.
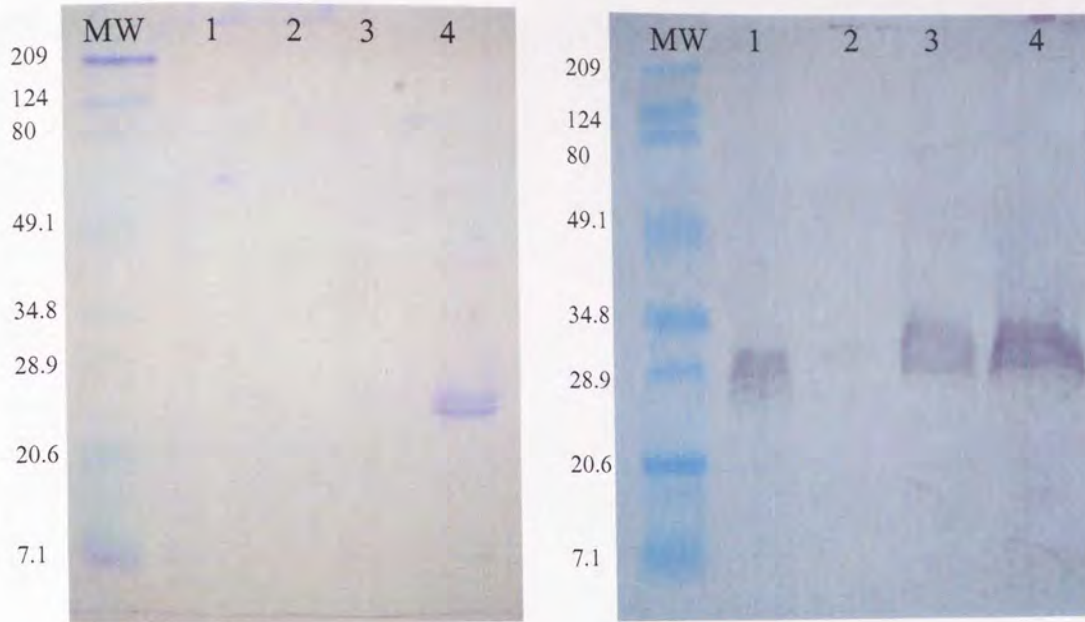
Figure 5.2 Coomassie blue stain and western blot probed with patient serum of whole cells of four *P. acnes* (lanes 1-4) separated by SDS-PAGE. Lane MW prestained broad range SDS-PAGE standard (BioRad, UK).

To examine the antigen further ethanol precipitated supernatant of *P. acnes* strains were separated by SDS-PAGE using a single lane comb and transferred electrophoretically to nitrocellulose. The nitrocellulose membranes were cut into 15 strips and each one probed with separate patient or control sera. Figure 5.3 displays a representative immunoblot from one of the 20 strains examined from all clinical sources. All strains produced this immunogenic material revealed as a cluster of three to four bands by western blotting and all sera from patients reacted with it (lanes 1-10). Sera from control patients with no clinical evidence of infection in the last six months also highlighted this antigen on western blots (lanes 11-15).
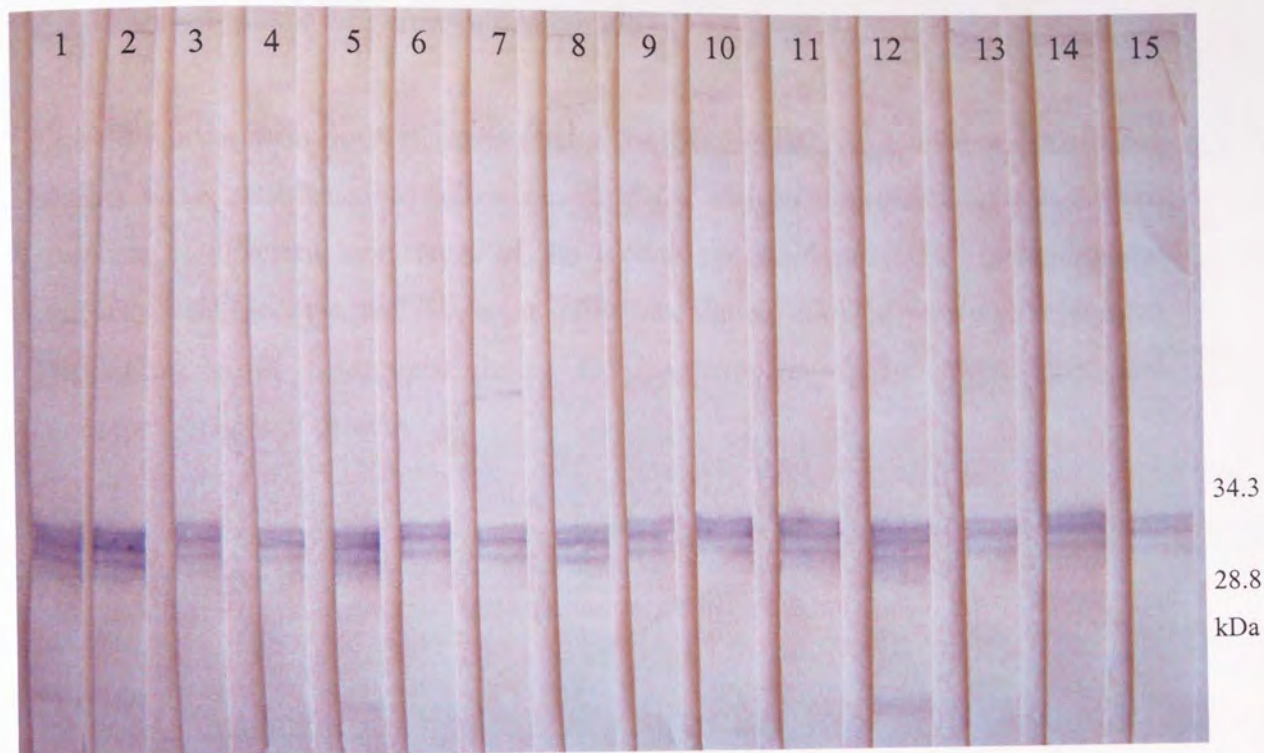
Figure 5.3 Representative immunoblot of ethanol precipitated culture supernatant of a *P. acnes* sciatica isolate probed with different patient (lanes 1-10) and control (lane 11-15) sera.

5.3.2 Ouchterlony double diffusion immunoprecipitation

The *P. acnes* antigen (Figure 5.4 wells marked A) produced a single strong precipitin with antibody from patient serum. The BHI control produced no visible precipitin band (Figure 5.4 wells marked C).
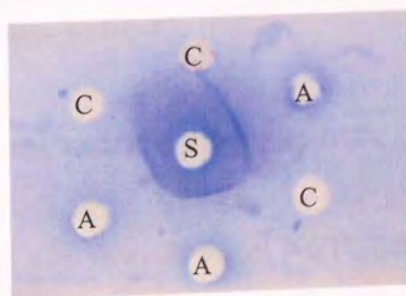
Figure 5.4 Coomassie blue stained double diffusion immunoprecipitation. S: serum, A: ethanol precipitated *P. acnes* antigen and C: ethanol precipitated BHI growth medium.

5.3.3 Growth phase and antigen production

Growth curves with three *P. acnes* strains (NCTC 737, NCTC 10390, and a clinical strain) were established to allow sampling for antigen expression in the culture medium at different key stages of the growth cycle (Figure 5.5). Sampling the cultures between five and 50 hours after inoculation allowed exocellular antigen expression to be determined during the lag, exponential, late exponential and stationary phases of growth.
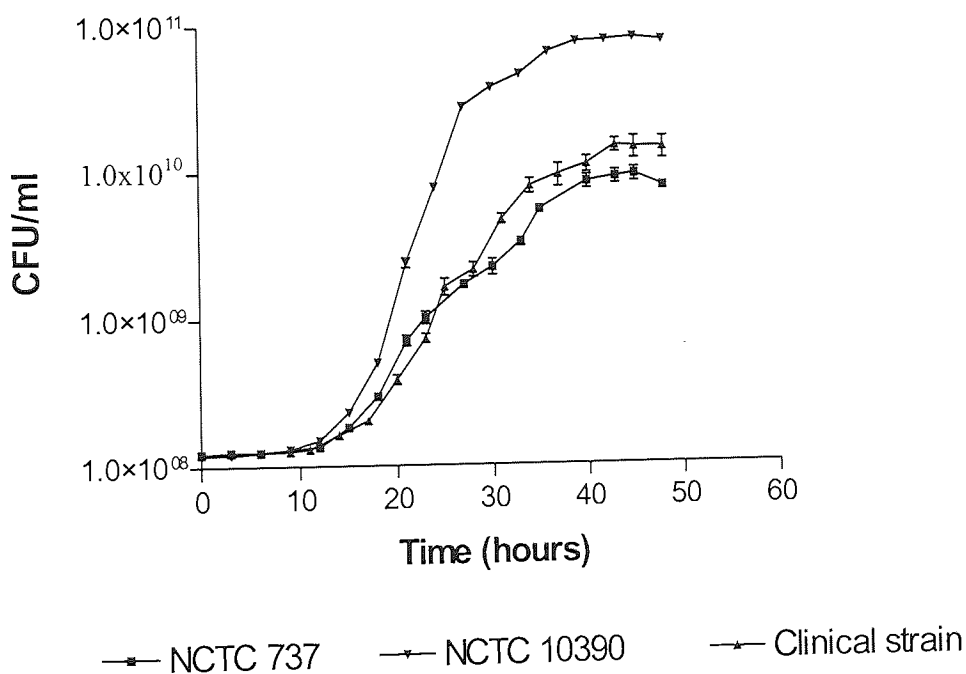
Figure 5.5 Growth curves of *P. acnes* NCTC 737, NCTC 10390 and a clinical strain isolated from a sciatica patient.

5.3.3.1 Detection of antigenic material during growth

The coomassie blue stained gels from *P. acnes* NCTC 737 and NCTC 10390 (Figure 5.6a) showed markedly different patterns of protein present in the ethanol-precipitated culture media. Although not clear in the figure but discernable from the gel itself, the stained material from *P. acnes* NCTC 737 was in the region of 70-80kDa whereas for NCTC 10390 it was around 30 kDa. For *P. acnes* NCTC 737 the stained material ran as a broad band whereas for *P. acnes* NCTC 10390 more discrete individual bands

were visible, suggesting the presence of a number of distinct proteins. The results for the clinical strain were similar to those observed with *P. acnes* NCTC 737 (results not shown). On western blotting of identical profiles (figure 5.6b) antigenic material was only found in the 29-34kDa region and was detected by western blot after 30 hours of growth for both strains. There was a slight difference in the mobility of this material, though it did appear as multiple bands in both cases. The high molecular weight proteins of *P. acnes* NCTC 737 were not antigenic. Close comparison of the coomassie blue stained gels and corresponding blots indicated that the protein bands in the 28-30kDa region for *P. acnes* NCTC 10390 were distinct from the antigenic bands. This is also supported by the absence of protein bands in this region, with a corresponding presence of antigenic bands in *P. acnes* NCTC 737. The likely nature of these antigens as proteins or polysaccharides was investigated later using pre-treatment of the material with proteinase K or periodate.
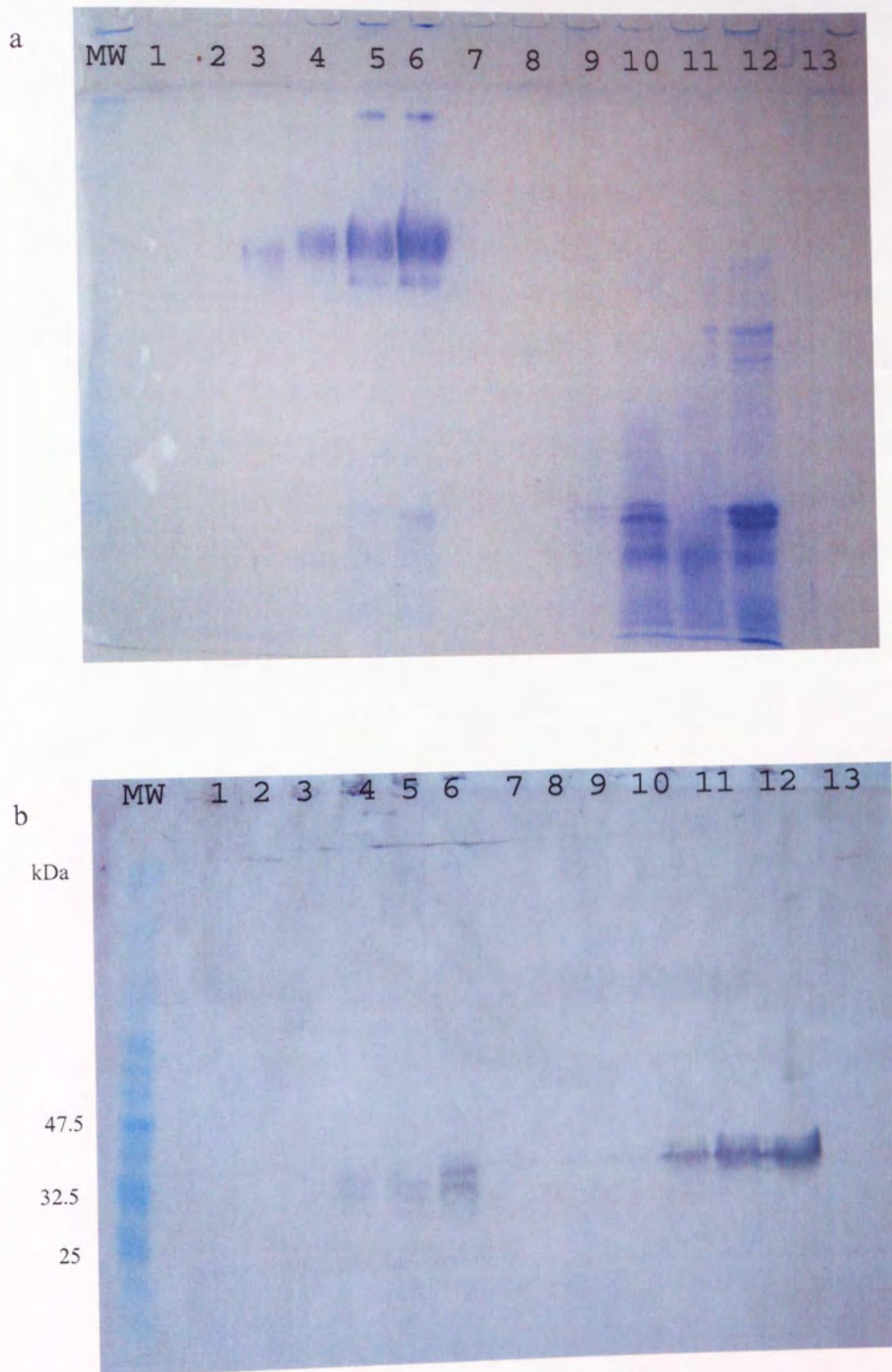
Figure 5.6 Coomassie blue stain (a) and western blot (b) of antigen present in the culture media from *P. acnes* NCTC 737 (lanes 1-6) and *P. acnes* NCTC 10390 (lanes 8-12) sampled after 5, 10, 20, 30, 40 and 50 hours of growth. Lanes 7 and 13 are ethanol precipitated BHI media controls. Lane MW: prestained protein marker, broad range (New England BioLabs, UK)

5.3.3.2 Quantitative assessment of antigen production during growth

5.3.3.2.1 ELISA optimisation

Polystyrene microtitre plates were coated with various dilutions of antigen taken different stages throughout the growth curve of *P. acnes* NCTC 737 and reacted with patient serum followed by protein A-peroxidase conjugate and substrate. The results in figure 5.7 show that there was a considerable reaction with the material present in the BHI control. To avoid this problem patient serum was pre-incubated with the BHI control material for 1hr before reaction with the ELISA plate. The results in figure 5.8 illustrate that this successfully removed the background reaction and that the optimum dilution of sample (1:100) could be selected for quantitative assay of antigenic content.
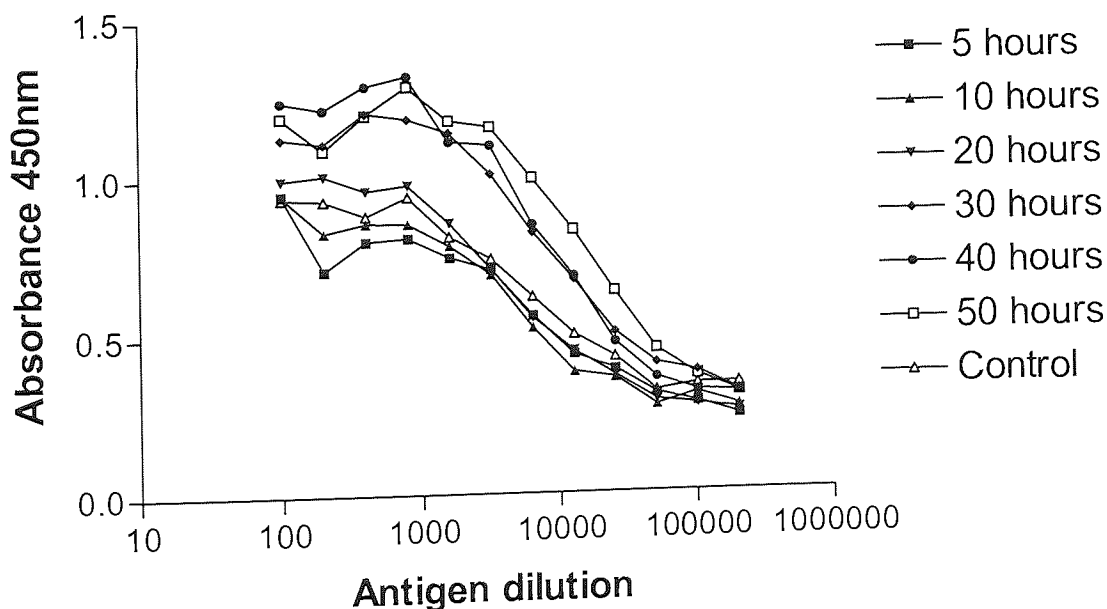
Figure 5.7 Serological response of patient serum to serial dilutions of ethanol precipitated culture supernatant of *P. acnes* NCTC 737 taken at various time points during growth alongside a BHI control.
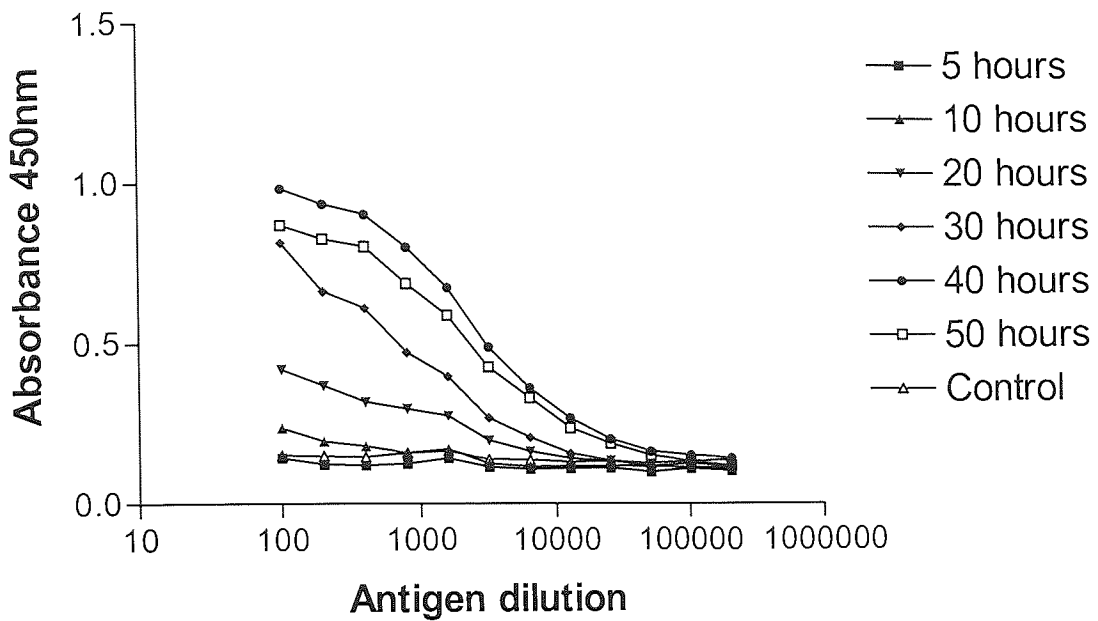
Figure 5.8 Serological response to ethanol precipitated culture supernatant from *P. acnes* NCTC 737 taken during different time points of growth together with a BHI control using patient serum pre-incubated with ethanol precipitated BHI media.

5.3.3.2.2 ELISA to determine antigen production during growth

The results in figure 5.9 show the antigen expression of *P. acnes* NCTC 737 plotted alongside the growth curve. The results indicate that antigen expression increases as the cell density increases but does not correlate with a specific region of the growth curve. This occurred with *P. acnes* NCTC 10390 and the clinical strain (results not shown). The antigen therefore appears to be produced constitutively throughout the growth curve.
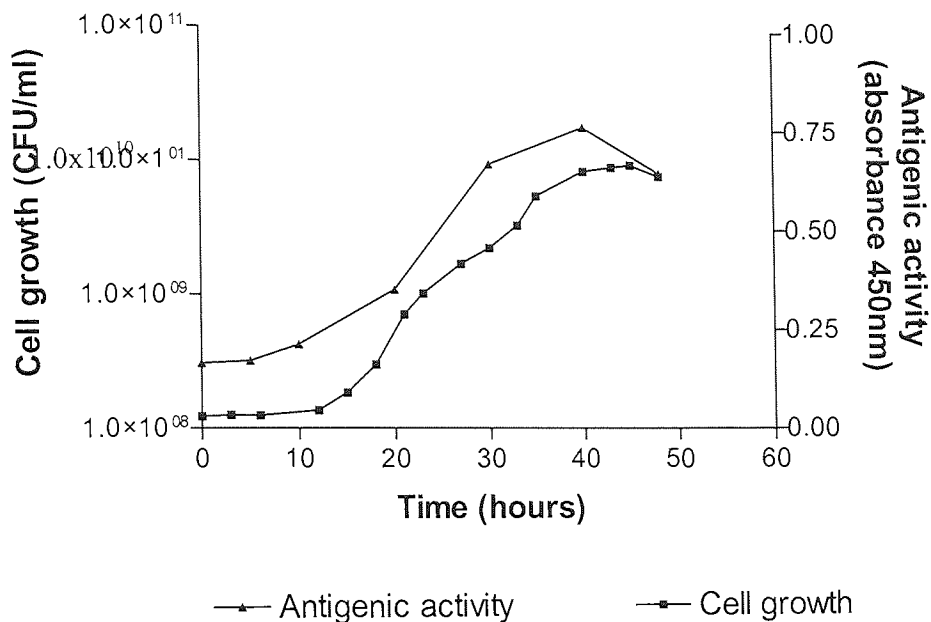
Figure 5.9 A comparison of antigen production and cell growth as exemplified by
***P. acnes* NCTC 737.**

5.3.4 Chemical destruction of antigen

Prior to electrophoresis antigen was treated with periodate or proteinase K to destroy carbohydrate and protein moieties, respectively. Protein silver stain revealed protein bands which ran in the same region as the antigen, however they themselves were not antigenic, as shown by western blotting (figure 5.10). Immunoreactive bands were detected after pre-treatment with proteinase K but were absent after treatment with periodate. The antigenic components in ethanol precipitated supernatant of *P. acnes* were therefore sensitive to destruction by periodate but not proteinase K and must therefore contain carbohydrate moieties.

a

b
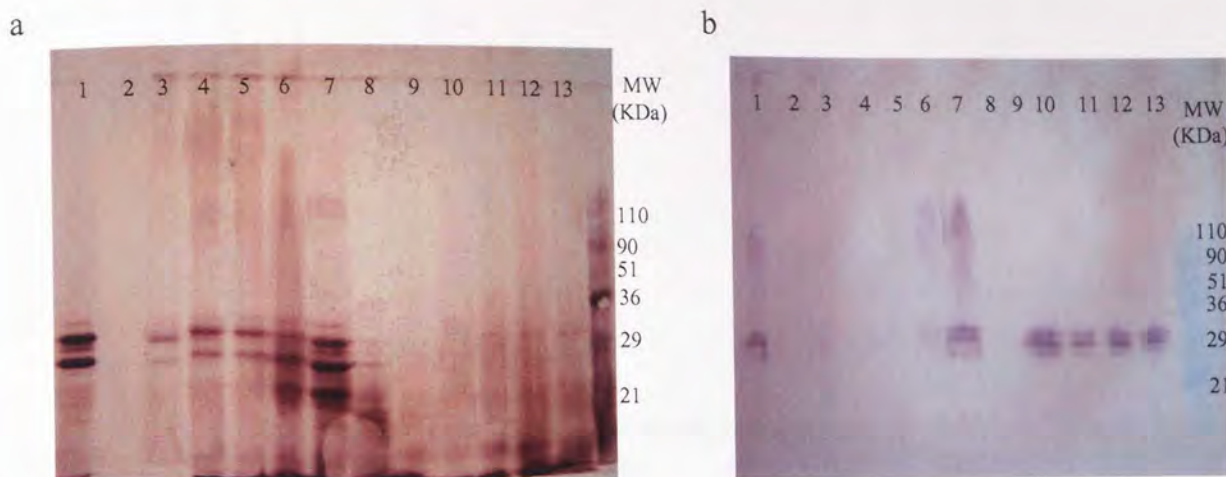
Figure 5.10 Protein silver stain (a) and western blot (b) of ethanol precipitated culture supernatant of *P. acnes* (sciatica isolate) pre-treated with periodate (lanes 3-6, 100mM-0.1mM) or proteinase K (lanes 9-13, 2mg/ml-0.12mg/ml). Lane 1 & 7 untreated antigen, lane 2 100mM periodate, lane 8 2mg/ml proteinase K and lane MW prestained SDS-PAGE standard, low range (BioRad, UK). Western blot probed with sciatica patient serum.

5.3.5 Purification of antigen

5.3.5.1 FPLC

5.3.5.1.1 Gel permeation chromatography

The elution profiles of ethanol precipitated culture medium from *P. acnes* and BHI media control from a Superose 12 column are shown in figure 5.11. The absorbance values at 280nm indicate that the antigen contained two peaks of material: one eluting in fractions 12-16 just after the void volume (fraction 9) and the other in fractions 28-30. Both of these peaks would be of high molecular weight as judged by elution of protein standards (fraction 15 is equivalent to a molecular weight of 150kDa, fraction 17 equivalent to 67kDa). Most notable was the presence of a large quantity of 280nm absorbing material in fractions 10-20 from the BHI control. Presence of this material would make subsequent detailed chemical analysis of the ethanol-precipitated material from the organism difficult.
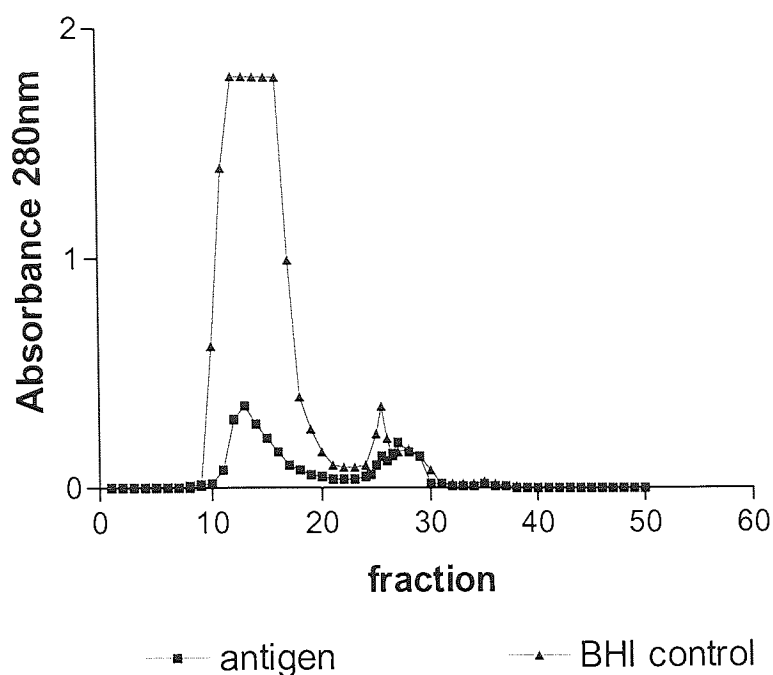
Figure 5.11 Elution profiles of *P. acnes* antigen and BHI media by gel permeation chromatography. Fractions of 0.8ml were collected from a Superose 12 column.

Antigenic detection of fractions by dot blotting gave negative results (not shown) but the more sensitive ELISA method gave a clear representation of the antigenic profiles of the eluted fractions (figure 5.12).
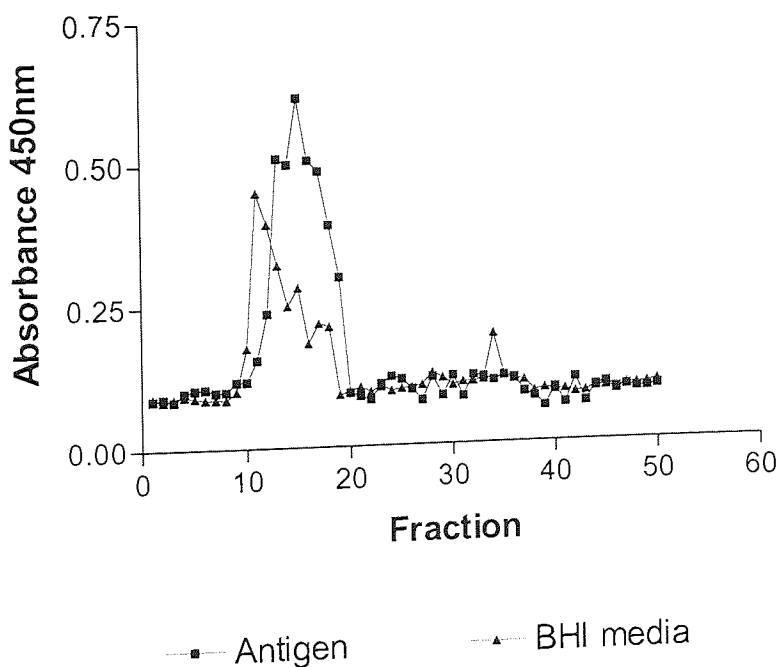
Figure 5.12 Detection by ELISA of antigenic material in fractions of *P. acnes* culture medium and BHI from a Superose 12 column.

The antigenic profile for *P. acnes* ethanol precipitated culture supernatant showed a single major peak between fractions 12 and 20. However, as indicated by the 280nm detection, the BHI control also showed apparently antigenically-reactive material in similar fractions (fractions 10-20). The appearance of this material was not unexpected since the earlier ELISA titrations had shown active material in BHI (figure 5.7). This material was not detected by western blotting or immunodiffusion (figures 5.1 and 5.4) however, serum pre-absorbed with BHI was shown to retain activity in an ELISA using plates coated with *P. acnes* antigen.

5.3.5.1.2 Hydrophobic interaction chromatography

Dot blot analysis of fractions eluted from an octyl sepharose column during loading and elution by an increasing gradient of propanol revealed that the antigenic material did not bind to the octyl sepharose column as it was detected in the initial fractions collected when the column was washed prior to implementation of the elution gradient (figure 5.13). This technique was therefore not pursued further as a purification method.

Figure 5.13 Dot blot of 2ml fractions collected from octyl sepharose hydrophobic interaction chromatography column. Fractions 1-5 loading and washing with start buffer (0.1M sodium acetate containing 15% v/v propanol) fractions 6-20 linear gradient to 65% propanol, 21-25 65% propanol. Blot was probed with patient serum and developed for bound IgG with protein A peroxidase.

5.3.5.2 Purification by electro-elution from polyacrylamide gels

Since purification of antigenic material by FPLC proved inadequate, antigenic bands were excised from a polyacrylamide gel and recovered by electro-elution. The yield of antigen using this method was unsatisfactory as recovery was very low and was almost undetectable by western blot analysis (results not shown). SDS contamination of the eluted product may have also hindered further analysis of the purified antigen.

5.3.6 Modified method for preparation of antigen using whole cells

Due to the contamination of antigen preparations with BHI media components and the poor recovery of antigen by electro-elution an alternative method of preparation was

sought. Vortexing bacterial cells with glass beads was found to release the antigen from cells and antigen could then be recovered from the supernatant.

5.3.6.1 Detection of antigen prepared by vortexing whole cell suspensions

5.3.6.1.1 SDS-PAGE, gel staining and western blotting

Antigen prepared by vortexing was examined by SDS-PAGE followed by gel staining or western blotting and compared to antigen recovered by ethanol precipitation of the culture medium (figures 5.14 and 5.15).

The ethanol-precipitated antigen stained with both coomassie blue and the polysaccharide silver stain suggesting that it contains both protein and carbohydrate (lane 2 fig 5.14a and b). However the antigen prepared by vortexing the cells (lane 3) appeared to contain less coomassie blue stained material with fewer bands. The corresponding western blots (fig 5.15) showed negative staining of the ethanol-precipitated material in the region of the gel (29kDa) which was most heavily stained with coomassie blue. The corresponding region in the vortex-released antigen contained very little coomassie blue stained material but showed strong reaction with antibody. The bands in this material that were stained with silver for carbohydrate matched those which were immunodetected. These results therefore confirm the findings from periodate and proteinase K treatment and suggest that, although the vortex-released antigen contained a small amount of protein, the antigenic material was carbohydrate in nature. The cell vortex method of antigen release was used in preference to the ethanol-precipitation method for further characterisation experiments. The importance of this antigen as the major cellular antigen was confirmed by the inclusion of whole cells on the SDS-PAGE gels and western blot (lane 4). Virtually identical patterns were obtained using the modified silver stain for whole cells and the vortexed released material. The antigenic profiles showed a range of antigens to be present in whole cells including material with a similar mobility to that of the vortex released antigen.
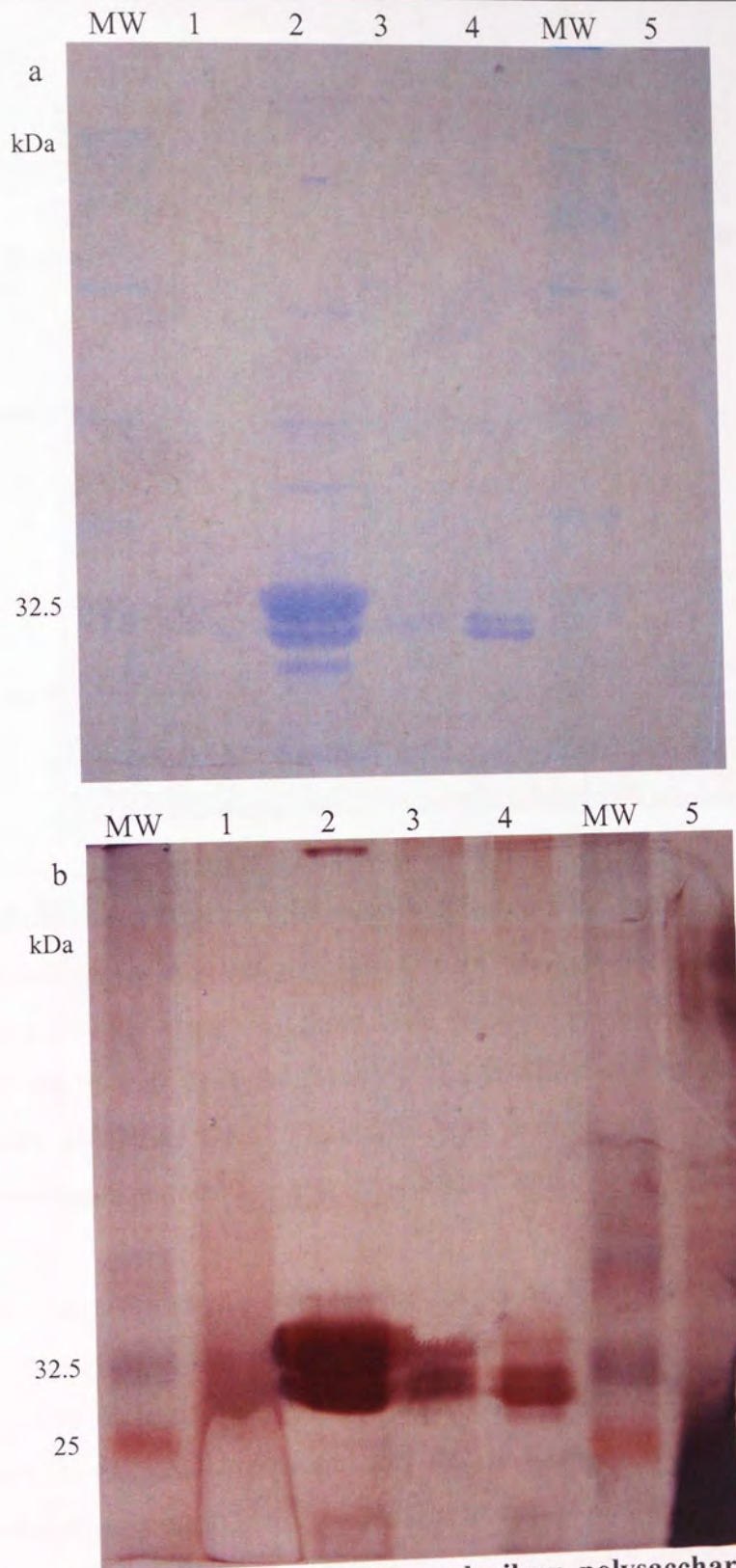
Figure 5.14 Coomassie blue stain (a) and silver polysaccharide stain (b) of ethanol precipitated BHI media (lane 1), *P. acnes* antigen prepared by ethanol precipitation of the culture supernatant (100mg/ml)(lane 2), antigen prepared by vortexing from cells (10mg/ml) (lane 3), *P. acnes* whole cells (lane 4) and LPS from *E. coli* 0111:B4 (lane 5). Lane MW: molecular weight marker, broad range (New England BioLabs, UK).
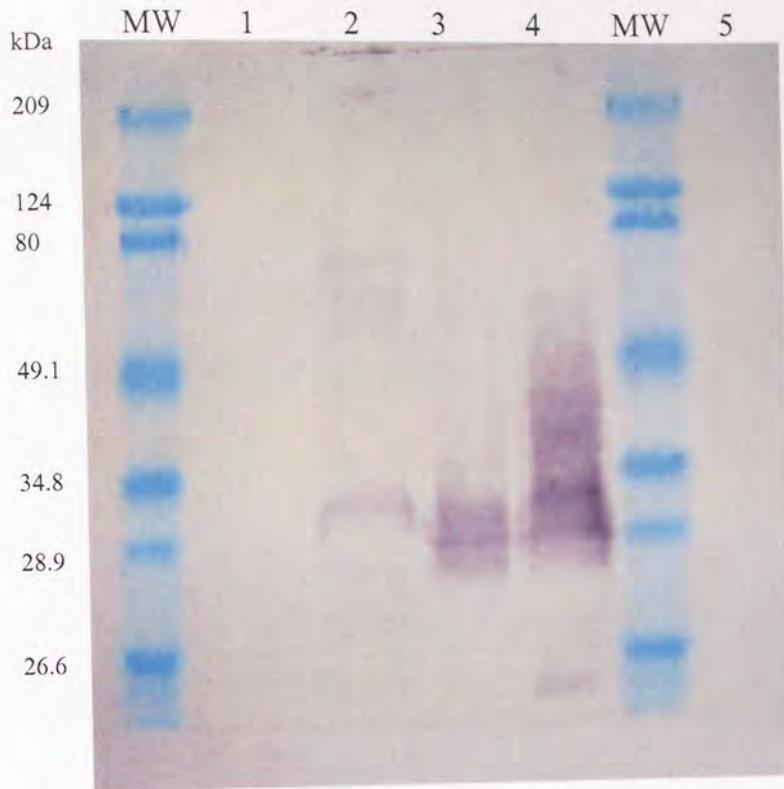
Figure 5.15 Western blot of ethanol precipitated BHI media (lane 1), *P. acnes* antigen prepared by ethanol precipitation of the culture supernatant (lane 2), antigen prepared by vortexing from cells (lane 3), *P. acnes* whole cells (lane 4) and LPS from *E. coli* 0111:B4 (lane 5). Lane MW: molecular weight marker, broad range (BioRad, UK). The blot was probed with patient serum and developed for with protein A-peroxidase bound IgG.

To determine any relationship between the antigen and previously identified *P. acnes* antigens a western blot was probed with *P. acnes* monoclonal antibodies and these bands compared to those seen with patient serum (figure 5.16). Reactivity of the antigen with monoclonal antibodies 1, 2 and 3 was not seen. Lane 4 illustrates distinctive banding in the 20kDa region of the gel and a discrete band of 40kDa which is consistent with published data obtained with the type 4 antigen (Valanne *et al.*, 2001). The characteristic antigenic material reacted with patient serum is shown in lanes 5 and 6. These results demonstrate that there is no association between the antigenic material prepared by vortex-mixing cells and the components classed as type I, II, III and IV antigens.
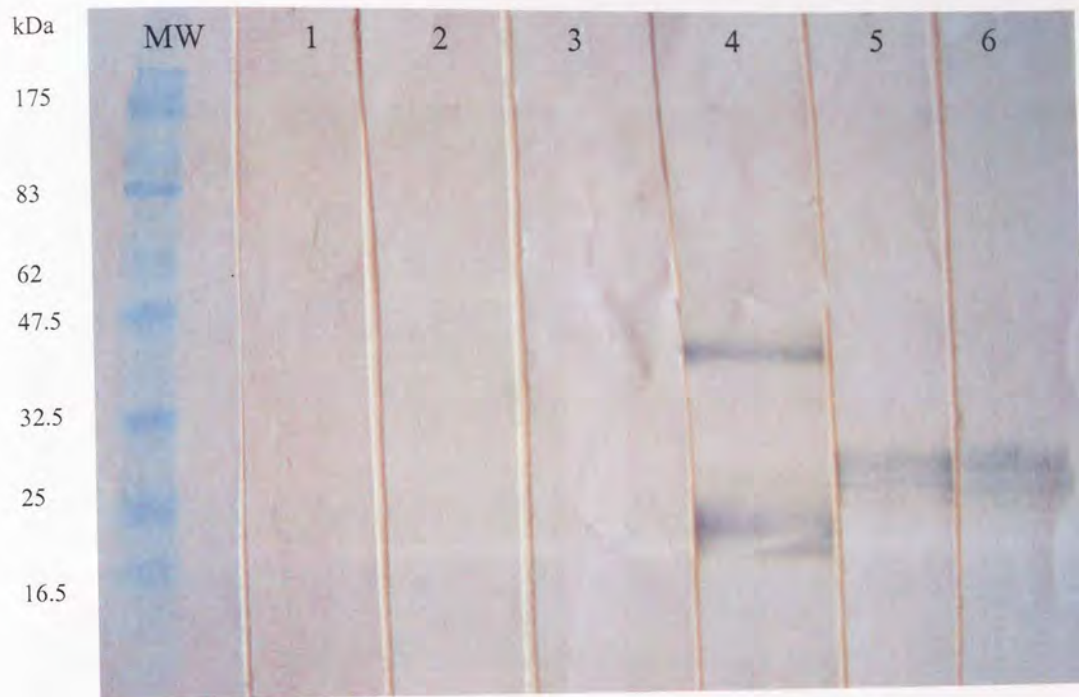
Figure 5.16 Western blot of *P. acnes* antigen prepared by vortex-mixing and probed with *P. acnes* monoclonal antibodies and patient serum. Lane 1: type I *P. acnes* monoclonal, 2: type II *P. acnes* monoclonal, 3: type III *P. acnes* monoclonal, 4: type IV *P. acnes* monoclonal, 5 and 6: sciatica patient serum. Lane MW: molecular weight marker, broad range (New England BioLabs, UK).

5.3.6.1.2 FITC immunostaining

Visualisation of cells by UV microscopy with FITC immunostaining gave some indication of the likely location of the antigen. Cells visualised prior to vortex-mixing were strongly stained with patient serum followed by anti-IgG-FITC, with weak background staining (Fig 5.17a). After vortexing, the cells were still stained but the background level appeared to have increased (Fig 5.17b). The supernatant remaining after centrifugation of vortexed cells showed a uniform background staining (Fig 5.17c). The pelleted cells after vortexing were strongly stained as before but the background was clear of immuno-reactive material (Fig 5.17d). Taken together, these results suggest that a portion of the antigen is released from cells during normal growth (accounting for the low level background stain) but that more is released

following vortex mixing. This implies that the antigen is loosely-associated with the cell surface.
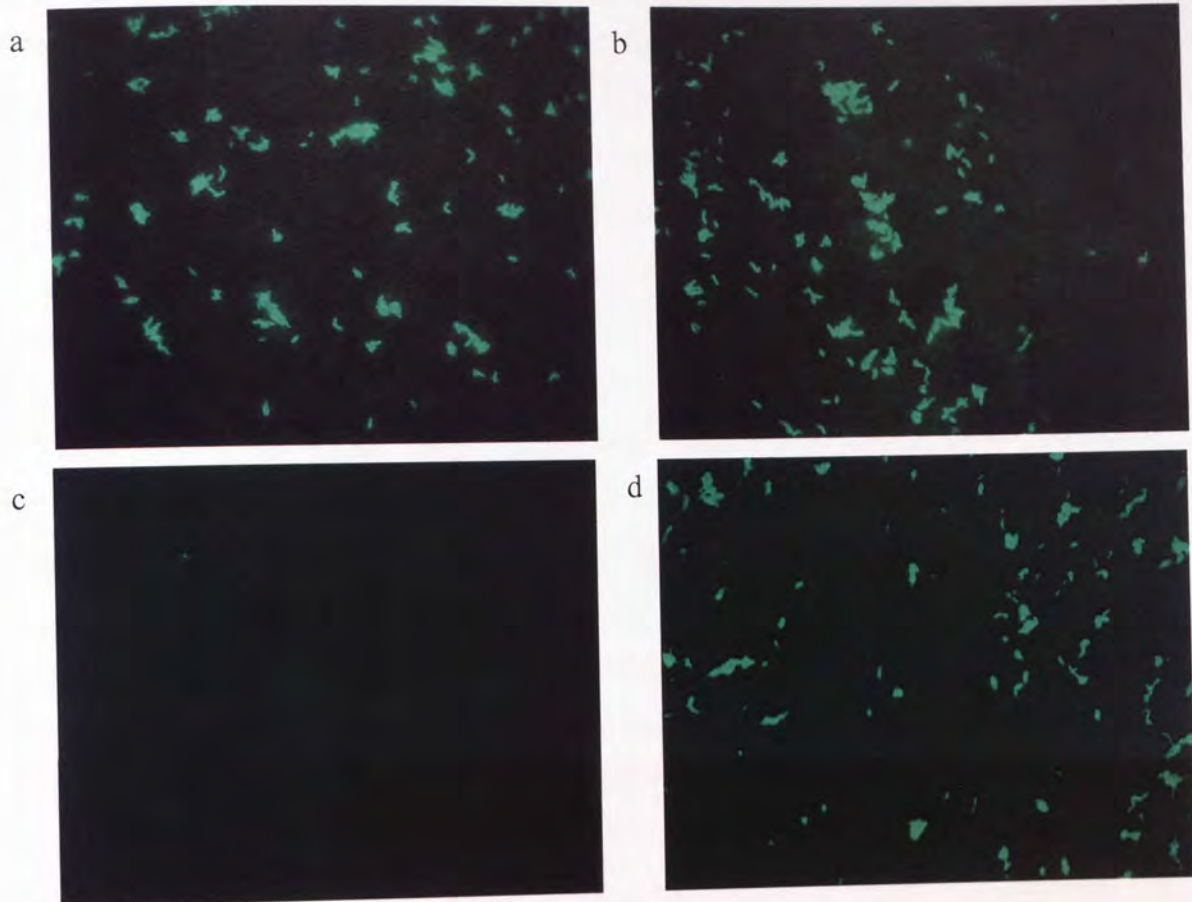
Figure 5.17 Immunofluorescent microscopy of *P. acnes* preparations probed with patient serum and FITC labelled anti-human IgG. Slide a: untreated cells, slide b: cells after vortexing with glass beads, slide c: cell supernatant from vortexed cells and slide d: resuspended cells.

5.3.7 Chemical analysis of *P. acnes* antigen

5.3.7.1 Thin layer chromatography

Thin layer chromatography with a range of chemical detection sprays was employed to give some indication of the chemical composition of the antigen. Silica gel G plates were used with a solvent system of 50% v/v aqueous propanol. This system was selected after numerous trial separations using a range of polar and non-polar solvents including ethyl acetate, ethanol, methanol, chloroform:methanol:water,

(65:25:4). Iodine vapour, used as a non-specific detection reagent, demonstrated that most of the material migrated near to the solvent front (Rf 0.95) (figure 5.18a). Ninhydrin reagent (for detection of amino groups) showed a different pattern of spots with both slow and fast migrating material (figure 5.18b). By contrast, use of sulphuric/acetic acid (detection of non-specific sugars and sterols) revealed strongly-reacting material on the origin and more weakly-staining bands in the Rf 0.95 region (figure 5.18c). Alpha-naphthol spray reagent (which detects glycolipids) revealed material at the origin together with the fast migrating material observed previously (figure 5.18 d). The molybdenum blue spray reagent (for detection of phosphate groups) gave a very weak reaction with the fast-migrating material (figure 5.18f). Finally, immunodetection using patient serum and protein A-peroxidase conjugate showed that the fast-migrating material (or a component migrating in that region of the TLC plate) was antigenic (figure 5.18 e). These TLC results suggest that the antigenic material migrates rapidly using TLC in propanol-water; it possesses the staining properties of a glycolipid (naphthol-reactive), contains amino groups (ninhydrin-positive) and possibly a small amount of phosphate. However, it was noted that the material revealed by immunodetection gave a small, sharp band within the broad band of fast-migrating material, whereas both iodine vapour and ninhydrin detected a much more diffuse band in this region of the TLC plate. Consequently the antigen itself may be part of a number of molecules released from the cells by vortexing, some of which remain at the origin during TLC separation.
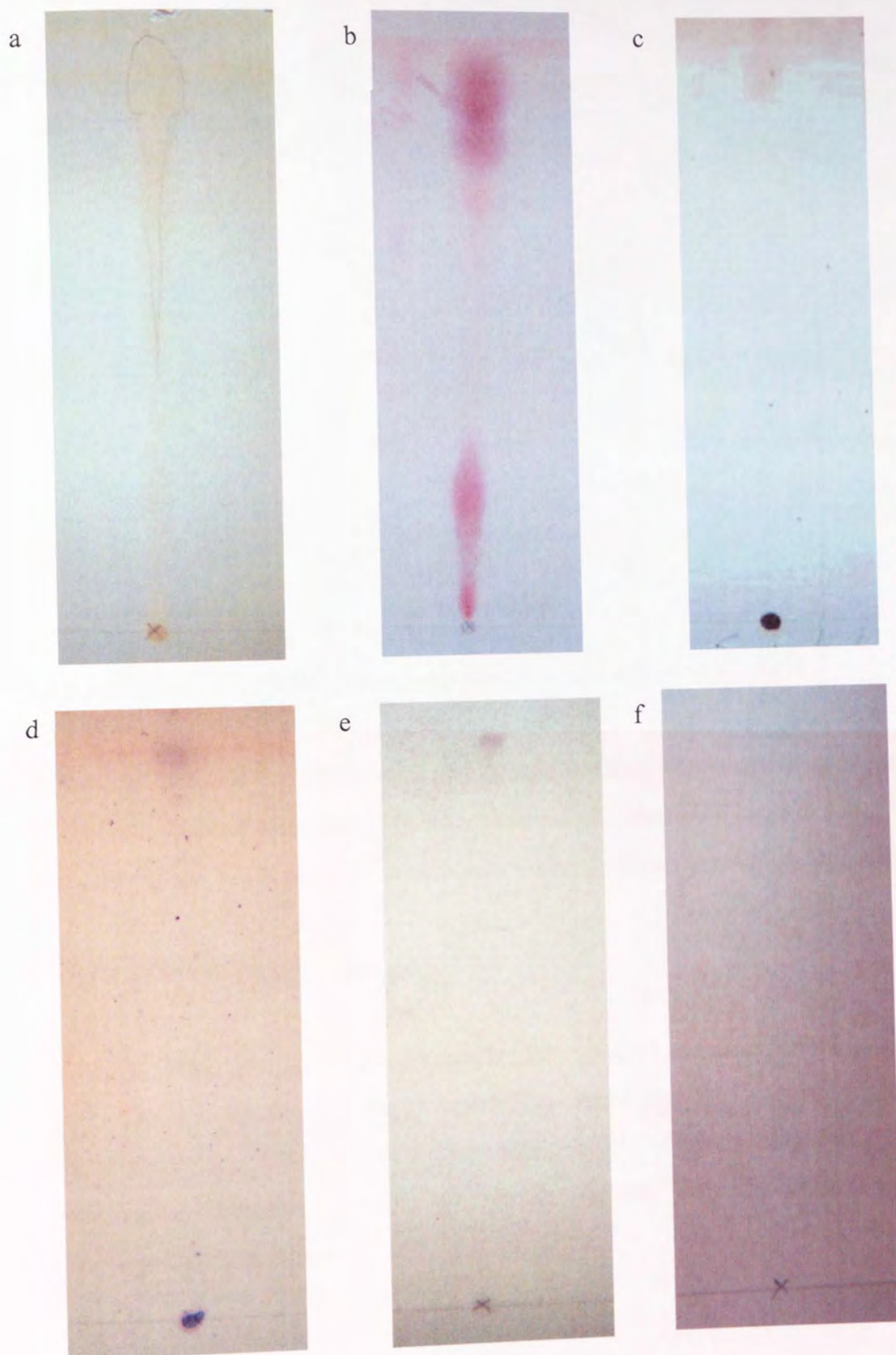
Figure 5.18 *P. acnes* antigen separated by thin layer chromatography detected using iodine vapour (a), ninhydrin (b), sulphuric/acetic acid (c), α-naphthol (d), immunostain (e) and molybdenum blue (f).

5.3.7.2 Chemical analysis of antigen by acid hydrolysis

5.3.7.2.1 Hexose assay
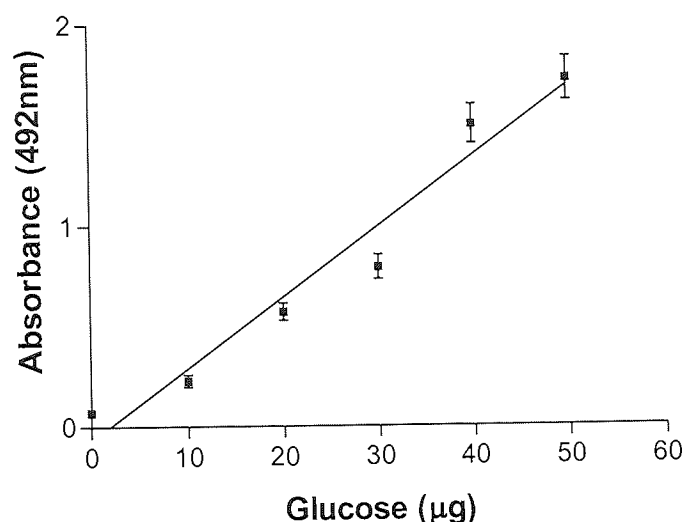
Figure 5.19 Glucose standard curve for phenol-sulphuric acid assay.

The relative amount of hexose in the acid hydrolysis of the antigen was determined by colourimetric assay using standard concentrations of glucose (figure 5.19). The antigen was found to contain 1.26% hexose by weight (12.6µg hexose/mg antigen).

5.3.7.2.2 Alditol acetates by GC

Analysis of alditol acetates of the antigen by GC revealed 4 peaks, ribitol, glucitol, rhamnitol and fucitol, as detailed in table 5.2. Assuming that the alditols were generated from the corresponding sugars this analysis indicates that the antigen contained ribose, glucose, rhamnose and fucose. In total, the sugar content of the antigen was 10.14% by weight.

Alditol acetate	Amount in antigen (% by weight)
Ribitol	5.44
Glucitol	1.90
Rhamnitol	1.88
Fucitol	0.92

Table 5.2 Alditol acetate composition of *P. acnes* antigen

5.3.7.2.3 Phosphate assay

The phosphate content of the antigen was determined by the ammonium molybdate assay using a standard calibration curve of inorganic phosphate (figure 5.20). The antigen was found to contain 0.99% inorganic phosphate by weight.
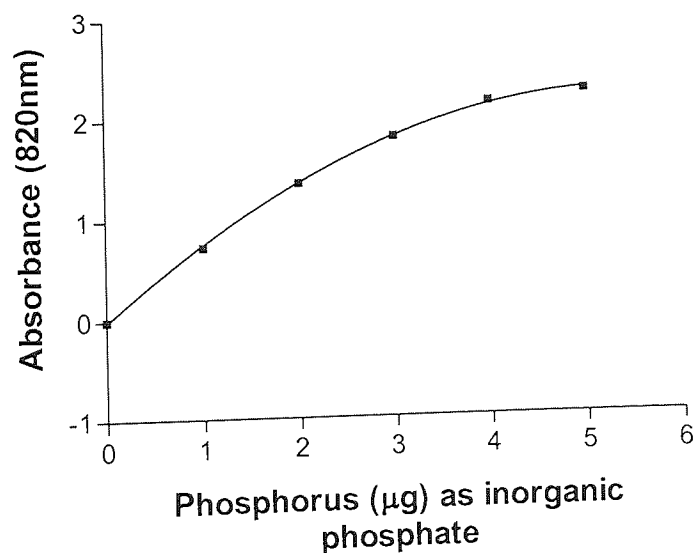
Figure 5.20 Standard curve for the ammonium molybdate assay of phosphate using inorganic phosphate

5.3.7.2.4 Glycerol assay

By use of the chromotropic acid assay (figure 5.21) the antigen hydrolysate was found to contain 0.22% glycerol by weight.
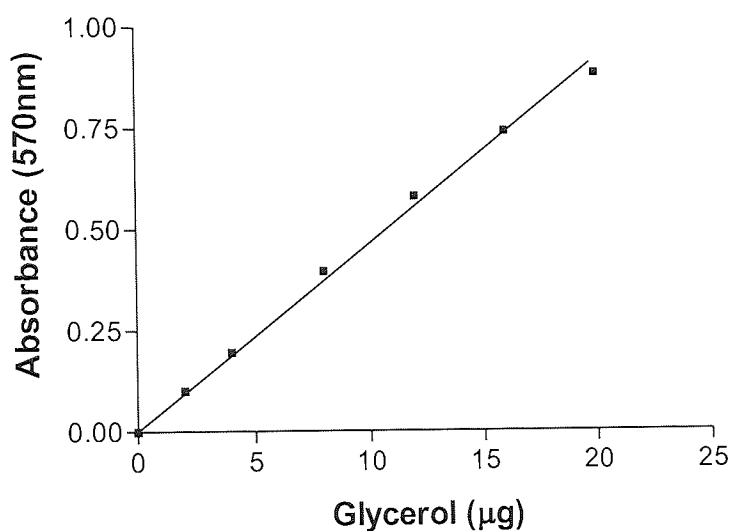
Figure 5.21 Standard curve for the chromotropic acid assay of glycerol.

5.3.7.2.5 Determination of *N*-acetylamino sugars

The standard curve generated by dilutions of *N*-acetylglucosamine was used to determine the amount of *N*-acetylhexosamine in TFA hydrolysed antigen (figure 5.22). The antigen hydrolysate contained 2.16% *N*-acetylamino sugar by weight.
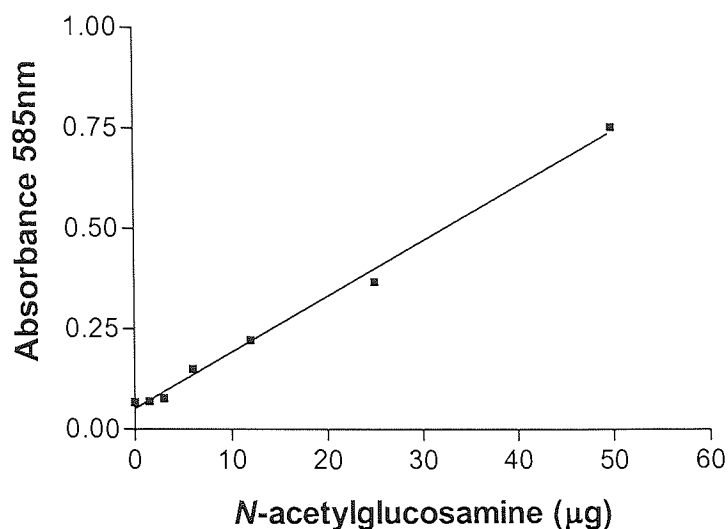
Figure 5.22 Standard curve for *N*-acetylglucosamine assay using *N*-acetylglucosamine.

5.3.7.3 Fatty acids

The profile of fatty acids present in whole cells of *P. acnes* NCTC 737 was comprised of six different fatty acids, the major of which was the branched chain saturated fatty acid i-15:0 (57.4%). The clinical strain also contained this fatty acid as the major component (63.6%) but also contained the unbranched saturated fatty acid C15:0 (table 5.3). When the antigen was treated in the same way as whole cells no fatty acid methyl esters were detected (results not shown). The sensitivity of the GC technique was such that if fatty acids were present as part of the antigen they would have been detected (4mg of dry weight antigen was derivatised and a similar dry weight of whole cells yielded these results). The lower level of detection was estimated as 0.1% by weight of antigen using this method.

Strain	Fatty acid						
	Un	i-15:0	a-15:0	15:0	Un	i-17:0	Un
P. acnes NCTC 737	3.5	57.4	8.3	-	15.5	10.5	4.8
Clinical strain	-	63.6	8.3	6.3	5.0	16.8	-

Table 5.3 Identity and relative amounts of cellular fatty acids of *P. acnes* NCTC 737 and a clinical strain, amounts expressed as % of total fatty acids. Un = unidentified.

5.3.7.4 Protein assay

A standard curve generated from known concentrations of bovine serum albumin using the Lowry (Folin-Ciocalteu reagent) assay (figure 5.23) was used to determine the protein content of the antigen. This was found to be 30.1% by weight.
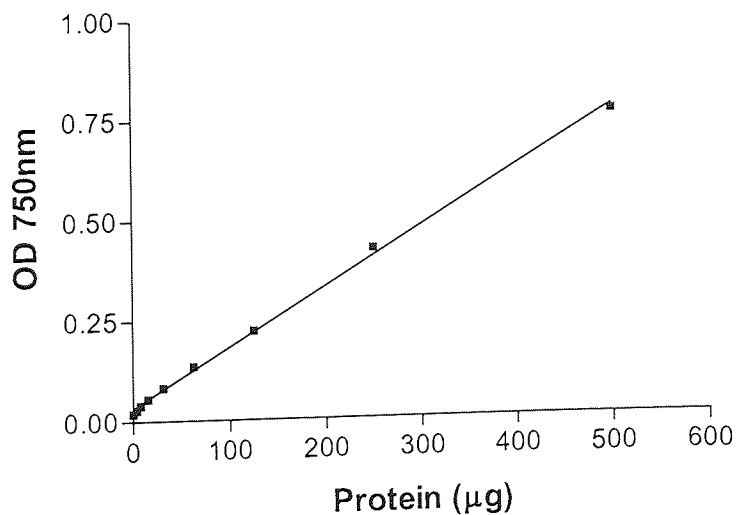
Figure 5.23 Standard curve for protein.

5.3.7.5 Chemical composition of *P. acnes* antigen

The results from chemical analysis of the antigen are summarised in table 5.4.

Component	Relative amount (% by weight)
Protein	30.1
Sugars	10.1
N-acetylhexosamine	2.16
Phosphate	0.99
Glycerol	0.22
Fatty acids	None detected (<0.1)

Table 5.4 Chemical composition of the *P. acnes* antigen.

Chemical analysis accounted for only 45% of the weight of the antigen released by vortex-mixing cells. The remaining material was unidentified. Some may be accounted for by losses through oxidation or polymerisation during hydrolysis. It is unlikely that the antigen contains cellular nucleic acids since a low phosphate content was measured.

5.4 Discussion

SDS-PAGE and western blotting of ethanol precipitated supernatant of *P. acnes* revealed immunogenic material of approximately 29-34kDa which reacts with IgG in both sciatica patients and control subjects. The antigen was produced by all strains investigated regardless of their origin or classical *P. acnes* serotype. To investigate the nature of the antigen it was necessary to recover and separate it from contaminating media components. However, this proved to be difficult due to the co-purification of media components and insufficient yield of antigen from the purification methods and alternative methods were therefore sought. It was found that the antigen present in the culture supernatant was also present on the surface of the cells, some of which could be released by vortexing with glass beads, thus eliminating problems of contaminating media components. The material recovered in this way was investigated for composition, properties and possible utilisation in serological testing (chapter 6).

Evidence for the nature of the vortexed released antigen using SDS-PAGE and western blotting indicated that it contained three closely-migrating bands in a region spanning 29-34kDa. Material corresponding in mobility could be detected by staining with silver using a protocol optimised for carbohydrate detection. Coomassie blue staining detected two bands of protein which migrated similarly, however these protein bands were shown not to be necessary for the presence of antigen by SDS-PAGE. When the antigen was pre-treated with proteinase K, these coomassie blue-stained bands were destroyed but the three antigenic bands, as detected by western blotting were retained. Conversely, when the antigen was pre-treated with periodate, the three antigenic bands were destroyed but the coomassie blue bands remained intact. Taken together, these observations suggest that the antigenic component was carbohydrate in nature but the material extracted from whole cells by vortex mixing contained protein.

Investigation of the antigen using TLC followed by immunodetection or use of selective sprays for chemical detection also provided valuable information on the likely nature of the antigen. Using silica gel plates with a mobile phase of

propanol:water 50:50, the antigenic material migrated as a single spot with an Rf value of 0.95. The high mobility in this system indicates a polar compound, which is likely to be of low molecular weight. The antigenic material gave a positive colour reaction with stains for sugars, amino groups and phosphate. Although no other antigenic spots were detected, material was detected using a number of chemical detection sprays on other regions of the TLC plate. This suggests that the antigen was not pure and contained a number of different non-antigenic components.

Chemical analysis of the material generally supported this view. Approximately 30% of the vortex-released material was protein in nature as determined by the Lowry Folin-Ciocalteu assay. Using the phenol-sulphuric acid reagent, the sugar content was found to be 10% by weight and individual sugars identified by GC included ribose, rhamnose, glucose and fucose. An assay for N-acetylhexosamine gave a content of 2.16% for this sugar. A small amount of phosphate was detected but no fatty acids were present. Despite recognition that the antigen was not pure, these analytical results suggest that the antigenic component released from the cells by vortexing is a water soluble carbohydrate containing phosphate and amino groups. Although the material ran as three closely migrating bands in the 29-34kDa region on SDS-PAGE, their molecular weights were determined for denatured proteins and do not necessarily reflect the size of a polysaccharide. It is also interesting to note that no antigen could be recovered from the SDS-PAGE gel by electro-elution. This may have been due to failure of the dialysis membrane to retain the material, suggesting a molecular weight of less than 10,000 Daltons.

Previous studies have described a range of different materials which could be related to this antigen. The polysaccharide antigen reported by Dawes *et al.* (1974) was found in the supernatant of *C. parvum* 10390 (now *P. acnes* NCTC 10390) and could be released from cells after treatment with hydrochloric acid. This antigen was released into the culture medium after logarithmic growth and the authors concluded that its release at the end of the growth cycle is probably as a result of partial autolysis of the cells. The antigen described in this thesis however, was detected in the culture medium throughout growth and its release was not growth phase related. These findings could reflect the more sensitive ELISA method used for detection of antigen in this study compared to measurement of antigenicity by

counterimmunoelectroosmophoresis (CIEOP) employed in the study by Dawes *et al.* (1974). Chemical analysis of the Dawes' antigen revealed that galactose and N-acetylglucosamine were the major sugar components and amino acids constituted less than 10% of the antigen. However their method of purification of the antigen prior to analysis was by ethanol precipitation of growth culture medium (beef digest broth) which, from the findings in this thesis, will precipitate with the antigen media components. Holland *et al.* (1979) comment on the disadvantages of non-defined complex media such as brain heart infusion as the amino acids and lipids present are qualitatively and quantitatively unknown. Furthermore, peptides and proteins in the media may be themselves antigenic and similar in both molecular weight and charge to some bacterial proteins. In accordance with these comments, BHI was shown in this study to be antigenic by ELISA, resulting in the need for pre-incubation of patient serum with BHI to remove BHI-reactive antibodies.

Another antigenic component was described by Iversen *et al* (1985). These workers demonstrated a single precipitin line by immunodiffusion using antigen prepared by ethanol precipitation of the growth culture supernatant and antibody present in normal human serum. Iversen *et al.* also recovered antigen from whole cells by treatment with urea, a process which releases non-covalently bound material. Antigen released in this way was found to be immunologically identical to the material recovered from the culture medium. Using gel permeation chromatography material was detected (presumably by measuring absorbance) shortly after the void volume. All of these properties are in agreement with those of the antigen described in this thesis.

As in this thesis, the dominant antigen investigated by Burkhart *et al.* (1999b) appeared to contain a carbohydrate component as treatment with sodium periodate modified the antigen, though not all antigenicity was destroyed.

Whale *et al.* (2004) described a lipoglycan macroamphiphile recovered by phenol-water extraction of *P. acnes* whole cells and purified by hydrophobic interaction chromatography and preparative electrophoresis. Although no antigenic properties were documented, the properties of this material suggest that it is related to the lipidated macroamphiphiles such as LTA of Gram-positive bacteria and mycobacterial lipoarabinomannans. The major difference between the lipoglycan described by

Whale *et al.* and the antigen investigated in this thesis is the lack of fatty acids in the latter.

Other exocellular antigens from *P. acnes* include polypeptides from the culture supernatant (Holland *et al.*, 1993) and exocellular enzymes (Ingham *et al.*, 1987).

Probing of an immunoblot containing the antigenic material with monoclonal antibodies to the type I-IV *P. acnes* antigens revealed that the antigen identified in this thesis is not related to any of these previously characterised antigens of *P. acnes*.

Vortexing with glass beads to release surface exposed material has been used in studies on the outermost layer of the cell wall of closely related bacteria. Puech *et al.*(2001) used glass beads to isolate material from Corynebacteria and found it to be largely composed of carbohydrate (glucans and arabinomannans) and protein. Similarly the outermost capsular material of *Mycobacterium tuberculosis* has been isolated in this way and this material also was composed predominately of protein and carbohydrates (Ortalo-Magne *et al.*, 1995). A 120kDa glycogen-type glucan, a 13kDa arabinomannan and a 4kDa mannan were identified as the major surface expressed polysaccharides. Only small amounts of lipid were measured suggesting that most of the non-covalently linked lipids are not exposed in *M. tuberculosis*. The polysaccharides were also shown to be identical to those isolated from the culture medium of *M. tuberculosis*. Lemassu *et al.* (1996) later demonstrated that production of the extracellular polysaccharide of *M. tuberculosis*, measured by ethanol precipitation of the culture media, followed the growth curve of the organism. The findings in the present study that a major antigen of *P. acnes* is produced throughout the growth curve, is loosely associated with the cell wall and can be released by shaking with glass beads therefore match previous observations in related corynebacteria and mycobacteria. Although relatively little work has been done on the ultrastructure of the *P. acnes* cell surface it is interesting to note that early transmission electron microscopy of the organism revealed floccular material attached to the cell surface (Montes and Wilborn, 1970). This material may indeed prove to be the antigen described in this thesis but further immunoelectron microscopy would be needed to confirm the identity.

Extracellular polysaccharide production has been investigated in dairy propionibacteria (Racine *et al.*, 1991). These workers reported the production of an extracellular polysaccharide by *P. acidi-propionici* when grown on whey-based and conventional media. Two fractions of polysaccharide were described, a water-soluble polymer of molecular weight <5,800 Daltons composed of rhamnose (22%), mannose (10%), galactose (34%) and glucose (34%). The water-insoluble polymer (molecular weight not reported) contained fucose (7%), mannose (22%), galactose (40%) and glucose (41%). The analysis of the *P. acnes* antigen showed it to contain rhamnose, fucose, ribose and glucose. This suggests that related polysaccharides could occur in dairy strains of propionibacteria and commensal isolates of *P. acnes*.

In summary, the aim of this chapter was to identify and characterise the dominant antigens of *P. acnes*. The major antigen was found to be carbohydrate in nature, it also contained protein which was found not to be essential for antigenicity. The antigen was cell associated, easily removed from cells by vortex mixing with glass beads and was also released into the culture medium during growth. The next chapter will investigate the potential use of this antigen in the serodiagnosis of *P. acnes* infection and explore whether it can account for the inflammatory properties of *P. acnes* exhibited in many conditions associated with the organism.

CHAPTER 6 SEROLOGICAL AND INFLAMMATORY RESPONSE TO *P. ACNES* ANTIGEN

6.1 Introduction

This chapter investigates the serological response and inflammatory properties of *P. acnes* and the antigen described in chapter five.

Antibody responses to *P. acnes* have been previously investigated by a number of workers. Elevated antibody responses in individuals suffering from acne (Ashbee *et al.*, 1997; Iversen *et al.*, 1985) and in patients with sarcoidosis (Ebe *et al.*, 2000) have been described. However, other workers have observed no difference in the serological response of patients infected with *P. acnes* to those of normal controls (Burkhart *et al.*, 1999b; Ingham *et al.*, 1987). The first part of this chapter therefore explores the antibody response of sciatica patients, individuals with acne and the response of normal human serum to *P. acnes* and the antigen investigated in chapter five.

Bacterial endotoxin or LPS is a well known mediator of inflammation and has been studied in great detail. Less information is available regarding the stimulatory properties of Gram-positive bacteria and their components. Lipoteichoic acid shares many inflammatory properties with LPS and has been shown to trigger the release of reactive oxygen and nitrogen species, hydrolytic enzymes and cytokines from macrophages (Bhakdi *et al.*, 1991; Ginsburg, 2002; Morath *et al.*, 2001). Other components of the Gram-positive cell wall such as peptidoglycan (Heumann *et al.*, 1994; Weidemann *et al.*, 1994) and teichoic acid (Heumann *et al.*, 1994) have also been shown to act as inflammatory cytokine inducers.

In this chapter the inflammatory properties of whole cells of *P. acnes* and the antigen, characterised in the previous chapter, were assessed through measurement of nitric oxide (NO) release and tumour necrosis factor alpha (TNF-α) from J774.2 macrophages stimulated with these components.

Nitric oxide is an important physiological messenger and effector molecule. It is generated by three different isoforms of nitric oxide synthase (NOS). Neuronal NOS and endothelial NOS are constitutively expressed and are calcium dependent. The third isoform, inducible NOS, can be induced in macrophages independently of calcium by bacterial products or cytokines. When generated during immune and inflammatory responses it acts as a toxic agent towards infectious agents. It can affect microbial proliferation and energy flow by inhibition of key enzymes in the mitochondrial respiratory chain and can also cause irreparable damage to DNA. Furthermore, NO can react with other molecules produced during the respiratory burst to form reactive nitrogen intermediates such as peroxynitrite and peroxynitrous acid. It is suggested that these are the major mediators of tissue injury seen during inflammation (Miles *et al.*, 1996).

The biological activity of TNF-α is diverse and the effects of this cytokine can be beneficial or detrimental to the host in different infections. TNF-α is secreted predominately by monocytes and macrophages in response to inflammatory stimuli where it can stimulate cell proliferation and apoptosis required for the normal development and function of the immune system (Curfs *et al.*, 1997). However, excessive signalling can have deleterious effects on the host due to a severe inflammatory response, tissue injury and even septic shock (Read, 1998).

6.2 Materials and methods

6.2.1 Serological response to *P. acnes* antigen by ELISA

6.2.1.1 Optimisation of antigen and serum concentration

Antigen, prepared as in section 5.2.6 from a sciatica isolate, was used to coat a microtitre plate (Immunlon 2, Dynex technologies, VA) from 10µg/ml to 0.009µg/ml by serial 2-fold dilutions across the plate to a final volume of 100µl per well using 0.05M sodium carbonate/bicarbonate buffer (pH 9.6) as the diluent. To the final column, containing no antigen, 100µl of TBS-Tween was added to each well. Plates were incubated for 18 hours at 4°C. Unbound material was then removed and the

plates washed three times in TBS-Tween. Unbound sites were blocked by completely filling the wells with TBS-Tween and maintaining at 4°C for four hours. The blocking solution was discarded and 200µl of patient serum diluted 1 in 100 in TBS-Tween added to each well of the first row. 100µl TBS-Tween was added each empty well and the serum 2-fold serially diluted down the plate. The last row of wells contained TBS-Tween only. ELISA was then continued as in section 5.2.3.4.1.

6.2.1.2 Screening of patient and control serum for IgG antibody binding to *P. acnes* antigen

Microtitre plates were coated with antigen diluted in 0.05M sodium carbonate/bicarbonate buffer (pH 9.6) at the concentration determined in section 6.2.1.1. Plates were incubated for 18 hours at 4°C. Unbound material was then removed and the plates washed three times in TBS-Tween. Unbound sites were blocked by completely filling the wells with TBS-Tween and maintaining at 4°C for four hours. Patient and control serum diluted in TBS-Tween to the concentration established in section 6.2.1.1 were added to each well in 100µl volumes. ELISA was continued as in section 5.2.3.4.1.

6.2.1.3 Screening of patient and control serum for IgG antibody binding to *P. acnes* whole cells

Freeze dried whole cells of *P. acnes* (section 5.2.1.1) were used to coat microtitre plates at the same concentration as for antigen in section 6.2.1.2 and ELISA continued as described this section.

6.2.1.4 Screening of patient and control serum for IgM antibody binding to *P. acnes* antigen and whole cells

ELISA proceeded as described in section 6.2.1.2 and 6.2.1.3 except a different conjugate was utilised. To detect IgM antibody 100µl of anti-human IgM peroxidase conjugate (0.25µg/ml in TBS-Tween) was added to each well.

6.2.1.5 Statistical analysis

Serological data was analysed by ANOVA (Instat, GraphPad Software Inc.) and calculation of Pearson's correlation coefficient (Prism, GaphPad Software).

6.2.2 Inflammatory response to *P. acnes*

6.2.2.1 Murine macrophage cell line

J774.2 macrophages, obtained from the European Collection of Cell Cultures, are a semi-adherent cell line originally obtained from adult female mice BALB/c. Cell cultures were maintained between 3-9 x 10^6 cells/ml in supplemented RPMI 1640 (10% fetal bovine serum, 1% glutamine, penicillin 100U/ml and streptomycin 100µg/ml) and incubated at 37°C in 5% CO_2. Cultures were passaged at confluence by washing with 3ml medium to discard non-adherent cells. Adherent cells were then removed in 3ml of fresh medium using a cell scraper and centrifuged for 10 minutes at 150g. The cell pellet was resuspended in 5ml of medium and 1ml added to 29ml of medium in a 80 cm^2 tissue culture flask (Nunc Brand Products, USA).

6.2.2.2 Macrophage assays

A 1ml aliquot of macrophage cells adjusted to 1×10^6 cells/ml was added to each well of a 24 well plate (Nunc Brand Products, USA). *P. acnes* exocellular antigen (section 5.6.2), *P. acnes* freeze dried whole cells (section 5.2.1.1) and lipopolysaccharide from *E. coli*, Serotype 055:B5 were used as challenging components. *P. acnes* exocellular antigen and *P. acnes* freeze dried whole cells were prepared by 10-fold serial dilution in culture medium to give a range of concentrations from 10-0.01mg/ml. LPS was similarly prepared to give concentrations of 1-0.001mg/ml. A 10µl aliquot of each component was added to wells in triplicate. Wells containing only culture medium, macrophages in culture medium and *P. acnes* whole cells in culture medium were included as controls. Plates were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 for 24 hours. Supernatants were collected and stored at -20°C until analysis. Duplicate plates were set up and incubated for 48 and 72 hours.

6.2.2.3 Measurement of nitric oxide in cell supernatants

Nitric oxide formation was determined by measuring nitrite, which is one of two primary, stable and non-volatile breakdown products of NO. Nitrite was quantified by spectrophotometry using the Griess reagent system (Griess, 1879). Sodium nitrite was diluted from $200\mu M$ to $3.125\mu M$ in culture medium and used to establish a nitrite reference curve. Culture supernatants and sodium nitrite standards were added to a 96 well microtitre plate (Immunlon 2, Dynex technologies, VA) in $50\mu l$ volumes. An equal quantity of 1% w/v sulphanilamide in 5% v/v phosphoric acid was added to each well and the plate incubated at room temperature, protected from light, for 10 minutes. After incubation $50\mu l$ of 0.1% w/v N-1-naphthylethylenediamine dihydrochloride was added to all wells and the plate incubated as previously described. The absorbance was measured immediately at 550nm with an Anthos 2001 plate reader (Labtech Instruments).

6.2.2.4 Measurement of TNF-α in cell supernatants

TNF-α was measured using a mouse TNF-α immunoassay kit (Quantikine, R&D Systems, UK) according to the manufacturer's instructions. Briefly, $50\mu l$ of assay diluent was added to each well of a microtitre plate which had been pre-coated with an affinity purified polyclonal antibody specific for mouse TNF-α. An equal volume of mouse TNF-α standard (1500-23.4pg/ml), control or sample was added to the wells, the plate sealed and incubated for two hours at room temperature. Each well was aspirated and washed using wash buffer five times. After the final wash any remaining wash buffer was removed and $100\mu l$ of mouse TNF-α conjugate added to each well. After an incubation period of two hours at room temperature the conjugate was removed and the plate washed as before. Next, $100\mu l$ of substrate solution was added to each well and the plate incubated for 30 minutes at room temperature protected from light. An equal volume of stop solution was added to each well and the absorbance of each well measured at 450nm with a correction wavelength of 570nm (Anthos 2001 plate reader, Labtech Instruments).

6.3 Results

6.3.1 Serological response

The antigenic properties of whole cells, exocellular antigen and material released from whole cells by vortexing as determined by western blotting were reported in chapter 5. These results illustrated that the major antigen of *P. acnes* runs on western blots as a tight cluster of bands in the region 29-34kD on SDS-PAGE. These antigens were detectable in the culture supernatant but were also present on the cell surface and could be released by vortexing. Studies to characterise the nature of the antigen concluded that they are most likely polysaccharide in nature, but are associated with some proteins undetected with patient serum, which run in the same region of the SDS-PAGE gel. To investigate patient serological response to these antigens two approaches were used. The first used a whole cell ELISA presenting the antigen in its native conformation associated on the cell surface for reaction with antibody. The second approach was to use an ELISA based on the antigen released from whole cells by vortexing. Ethanol-precipitated culture supernatant was not used because of the presence of media components which interfered with the ELISA responses (reported in chapter 5).

6.3.1.1 Optimisation of ELISA conditions; serum concentration and antigen coating

A range of extracted antigen concentrations from 0.009 to 10µg/ml in carbonate buffer, pH 9.6 was used to coat polystyrene microtitre plates. After blocking with TBS-Tween wells were reacted with patient serum applied at an initial dilution of 1:100 and serially diluted 2-fold down the plate. Detection of bound IgG or IgM was by reaction with protein A-HRP or anti-human IgM-HRP diluted according to the manufacturer's instructions. Results in figure 6.1 show the titration curves for antigen reacted with patient serum and developed for bound IgG. From these results an antigen concentration of 5µg/ml and a serum concentration of 1:100 were selected to compare reactivity of sera from a range of patients and controls. To allow direct comparison between immunoreaction of serum to the isolated antigen and whole cells,

the ELISA based on whole cells was carried under similar conditions, using 5μg/ml of freeze dried whole cells in place of the extracted antigen.
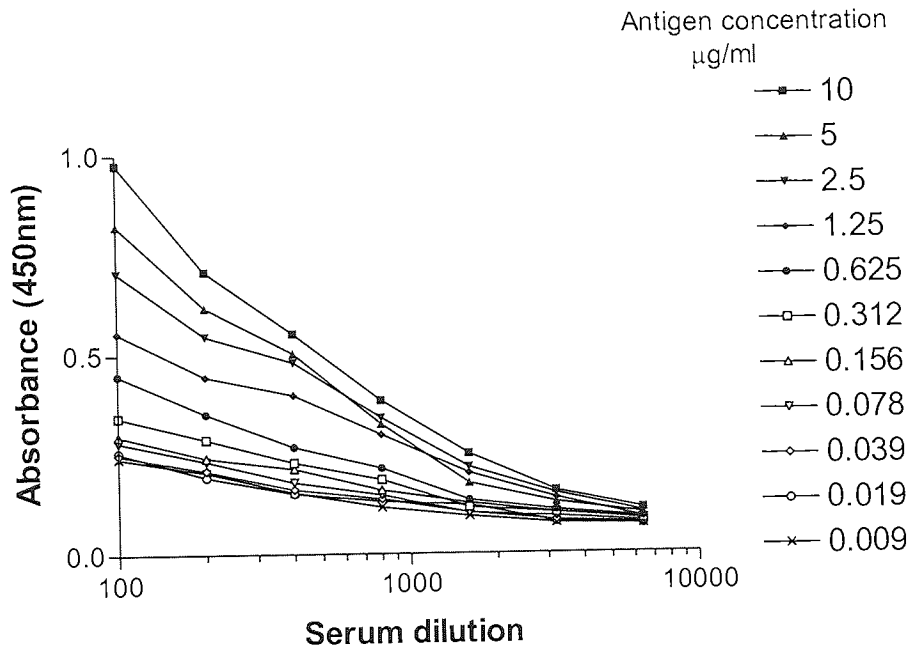
Figure 6.1 Optimisation of serum dilution and antigen coating ELISA plates. Plates were coated with antigen at the concentrations shown, reacted with patient sera at doubling dilutions and developed for bound IgG using protein A-peroxidase.

6.3.1.2 Serological response of patient and control serum to *P. acnes* antigen and whole cells

The IgG and IgM response of patient and control serum to *P. acnes* antigen and whole cells are shown in figures 6.2 and 6.3. All sera were tested at a dilution of 1:100 and were developed for bound IgG or IgM. No differences (p>0.05) were observed in serum IgG and IgM titres between patient and control sera to either *P. acnes* antigen or whole cells. The response of patients in all categories to the released antigen was more variable than that towards whole cells.

a
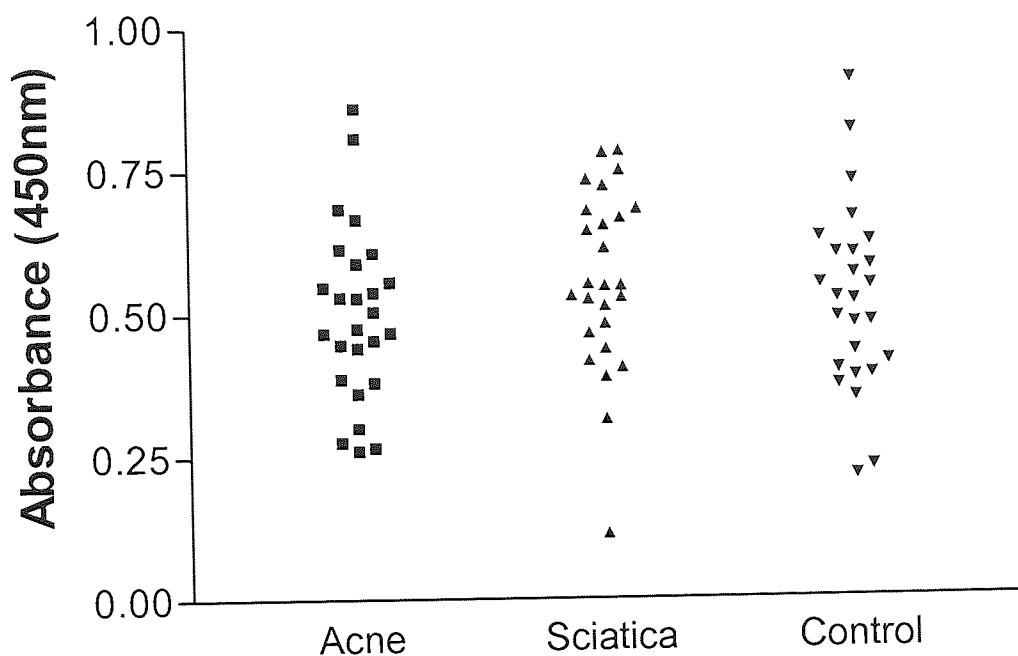
b
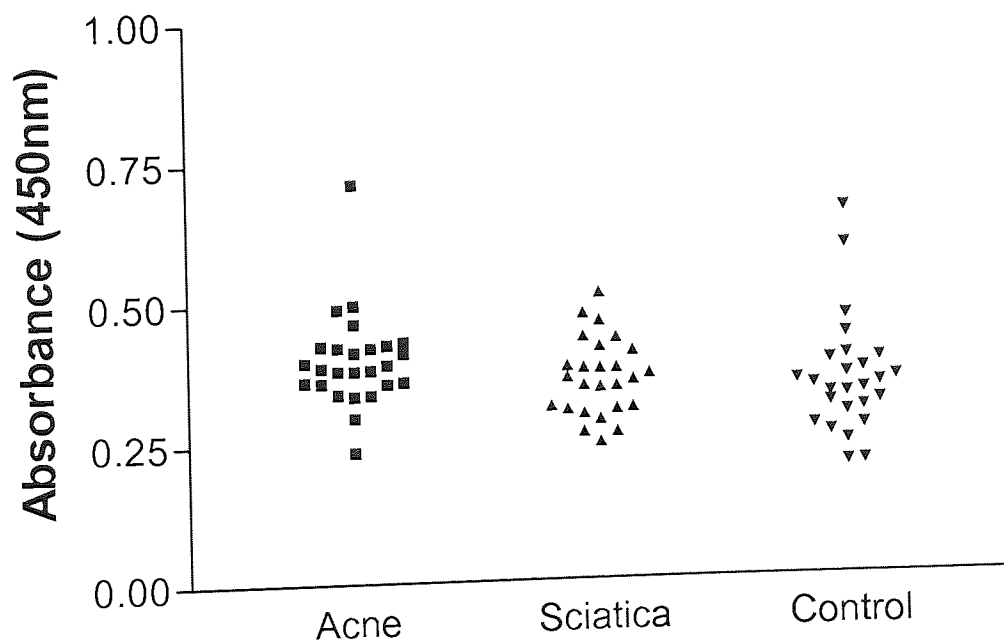
Figure 6.2 IgG response of acne, sciatica and control patient sera to *P. acnes* antigen (a) (p = 0.3837) and whole cells (b) (p = 0.0865). All sera was tested at 1:100 dilution.

a
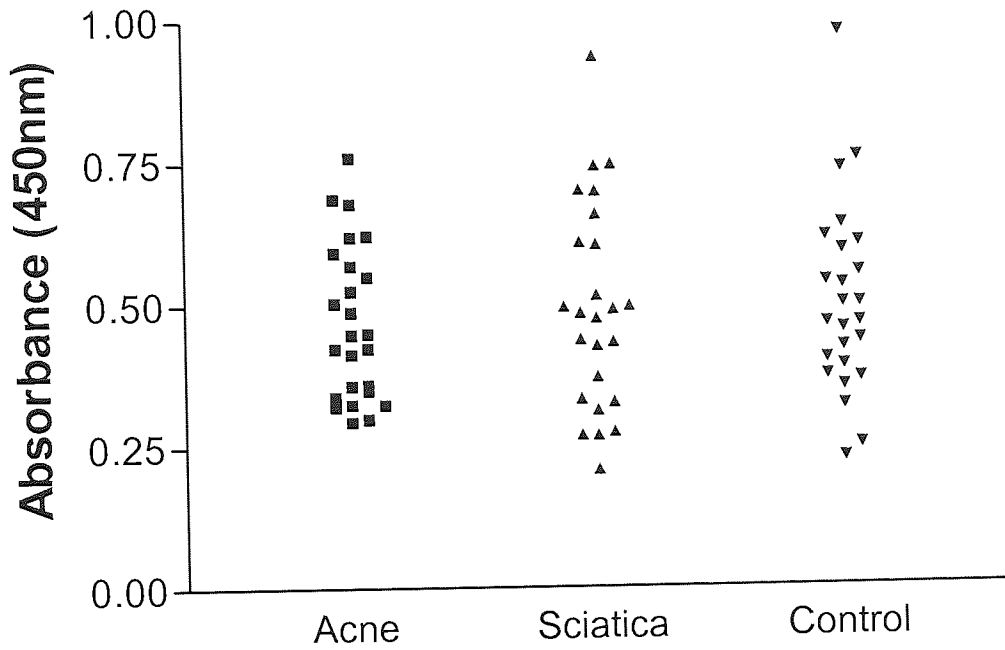
b
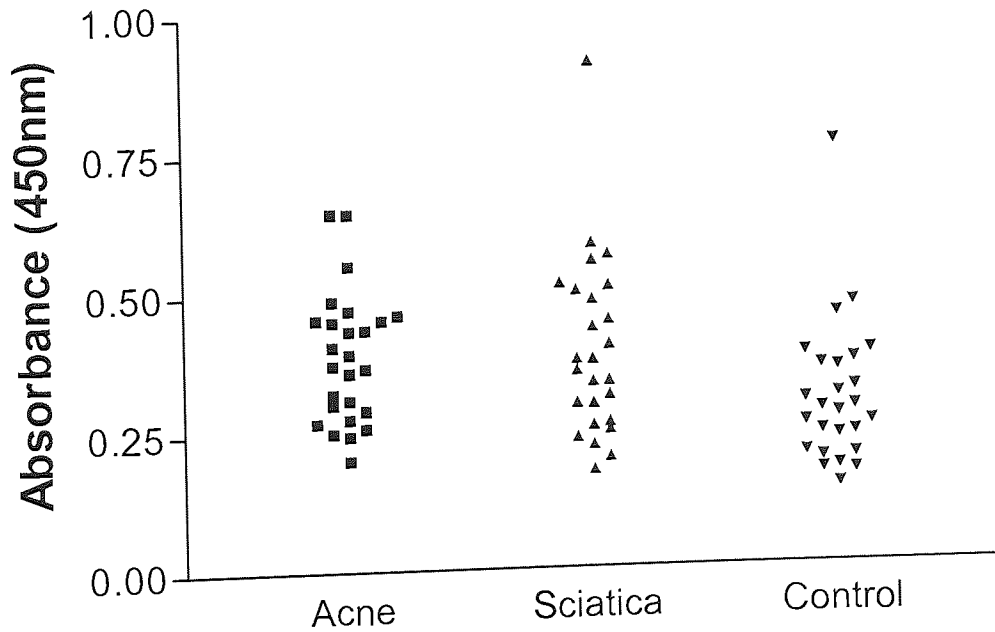
Figure 6.3 IgM response of acne, sciatica and control patient serum to *P. acnes* antigen (a) (p = 0.7372) and whole cells (b) (p = 0.2764). All sera was tested at 1:100 dilution.

Although there were no differences in antibody response between patient groups strong correlations were seen between serum IgG responses to whole cells and the antigen (Pearson r squared value 0.6542, P<0.0001), as well as serum IgM responses to whole cells and the antigen (Pearson r squared value 0.6601, P<0.0001) (figure 6.4). This suggests that the isolated antigen is the major cellular antigen recognised by all patients studied. Some correlation was also found between serum IgG and serum IgM responses to whole cells (Pearson r squared value 0.1806, p=0.0001) and to the antigen (Pearson r squared value 0.05598, p=0.0410) (results not shown).

a
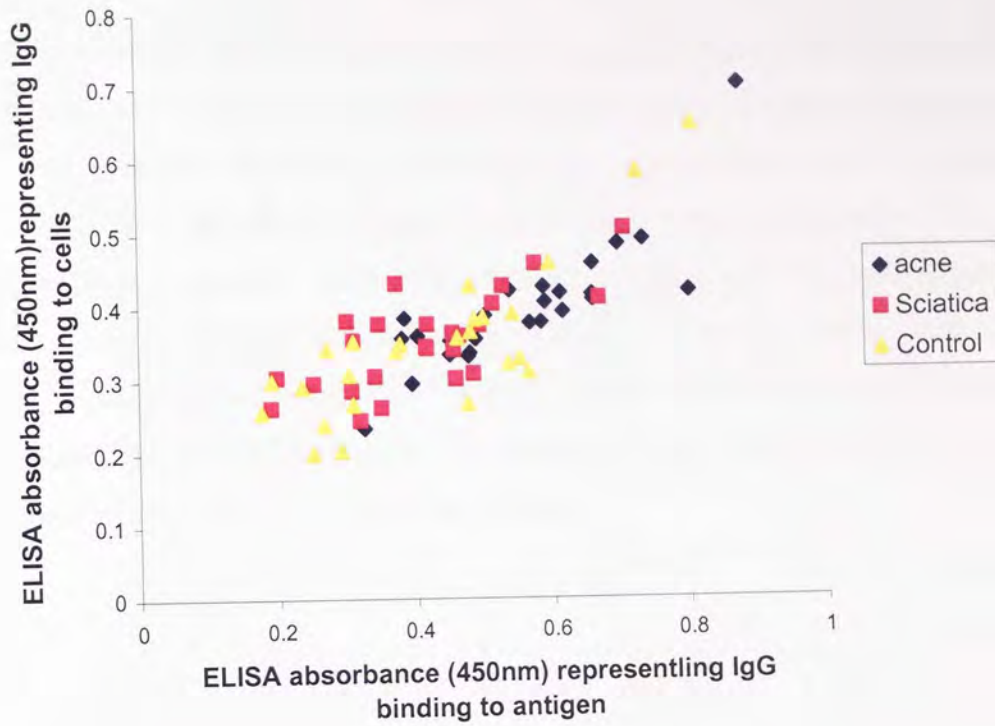
b
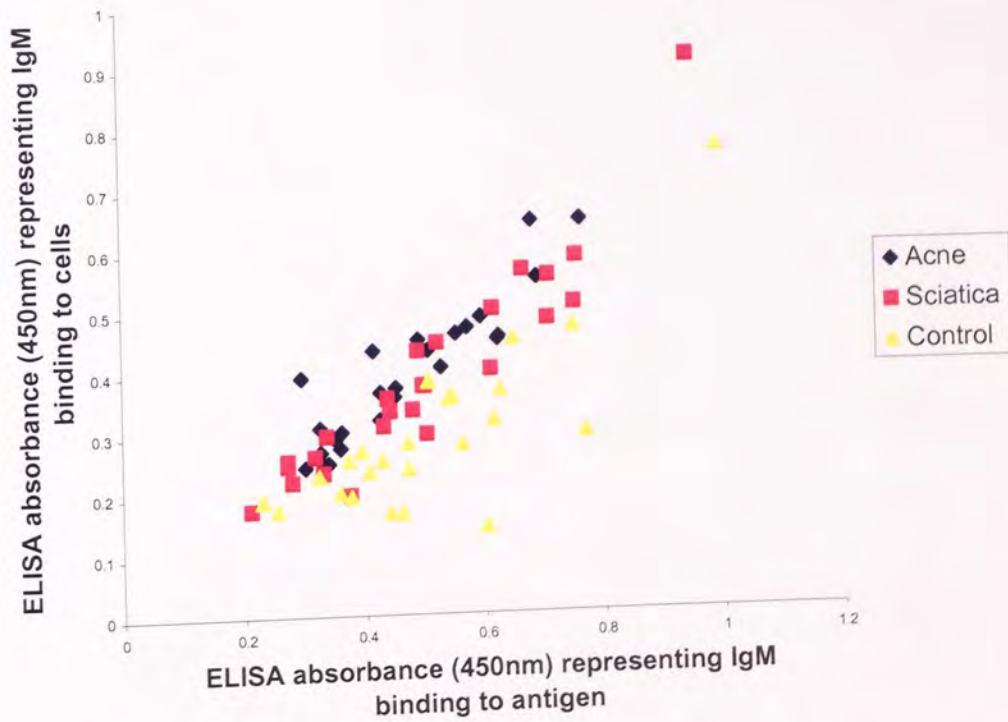
Figure 6.4 Serum IgG response to *P. acnes* whole cells and antigen (a) and serum IgM response to *P. acnes* whole cells and antigen (b) using sera from acne patients, those undergoing microdiscectomy for sciatica and control patients.

6.3.2 Inflammatory response to *P. acnes*

J774.2 macrophages were incubated with *P. acnes* antigen and whole cells and culture supernatants were sampled at 24, 28 and 72 hours. Figure 6.5 shows the appearance of macrophages after 48 hours incubation with the various components. Two images of *P. acnes* cells are shown (b and c), as at the highest concentration there was extensive bacterial growth. Activation of the macrophage cell was evident due to a change in cell morphology and formation of vesicles in the cytoplasm. Particularly evident in panel (e) are pinocytosis vesicles, ruffled cell membrane and extending pseudopodia induced by LPS from *E. coli* ($1\mu g/ml$). These features were also evident in cells stimulated with *P. acnes* cells and antigen.
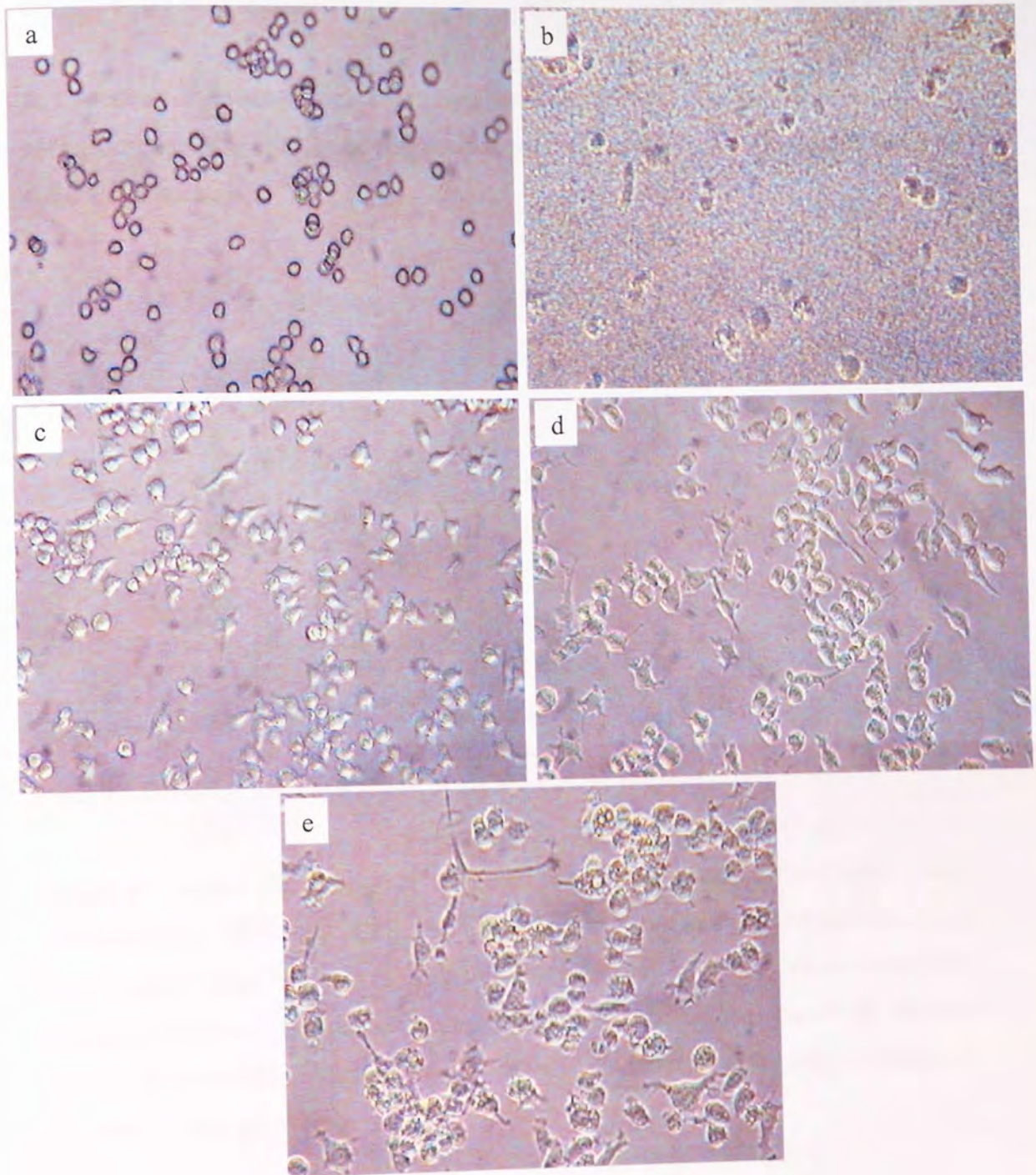
Figure 6.5 J774 macrophage cells viewed after 48 hours incubation (a) and after 48 hours exposure to (b) *P. acnes* cells (10µg/ml), (c) *P. acnes* cells (1µg/ml), (d) *P. acnes* antigen (10µg/ml) and (e) LPS (1µg/ml).

6.3.2.1 Nitric oxide production

A standard nitrite curve (figure 6.6) was established from known concentrations of sodium nitrite using the Griess reagent and used to determine nitrite concentration in culture supernatants.
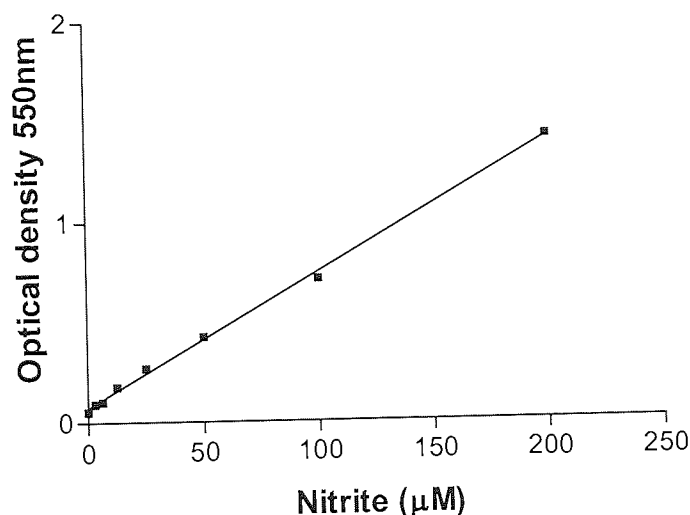
Figure 6.6 Nitrite standard curve using the Griess reagent.

Figure 6.7 shows the effects of *P. acnes* whole cells and antigen upon nitrite production by J774.2 cells. LPS, included in experiments as a positive control, was a potent inducer of nitric oxide synthesis in macrophages. Neither *P. acnes* exocellular antigen nor whole cells induced a response above control levels (figure 6.7). A small amount of nitrite was measured from *P. acnes* cells themselves during incubation in the tissue culture medium.
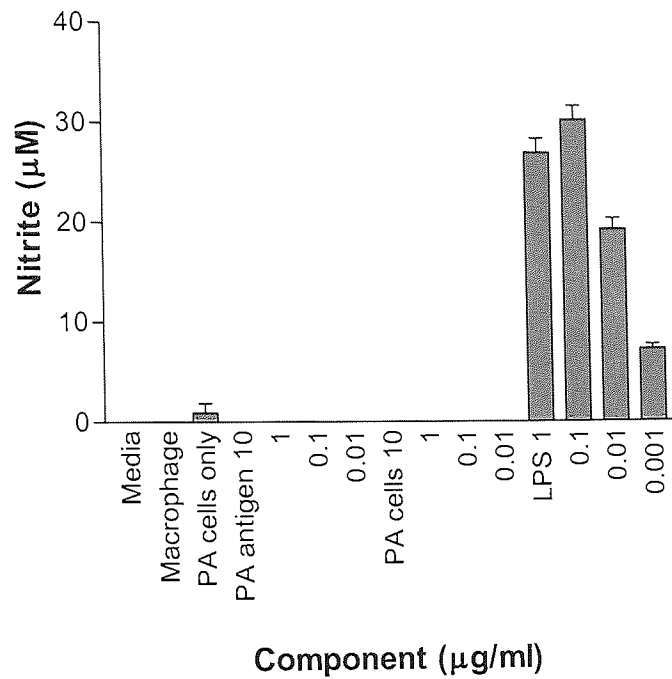
Figure 6.7 Nitrite production by murine macrophages stimulated for 48 hours with *P. acnes* antigen (PA antigen), whole cells of *P. acnes* (PA cells) and *E. coli* LPS.

6.3.2.2 Production of TNF-α

A standard curve using known concentrations of mouse TNF-α was generated using four parameter logistic curve fit (GraphPad Prism, GraphPad Softward Inc.) (figure 6.8) and used to determine TNF-α levels in culture supernatants.
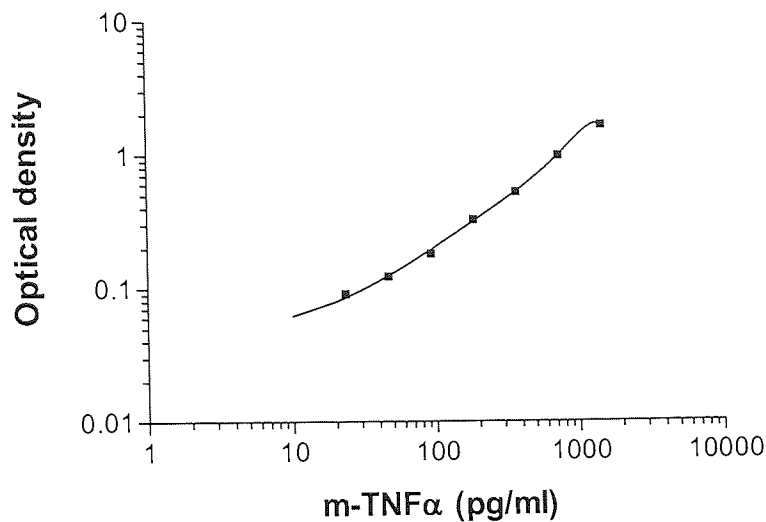
Figure 6.8 Standard curve for mouse TNFα (m-TNF-α) using the quantikine ELISA kit (R & D Systems).

P. acnes whole cells were around 1000 times less potent stimulants of TNF-α than LPS from *E. coli*. The antigen was less active than whole cells but did stimulate production of a measurable amount of TNF-α. The dose dependence of the response to both the whole cells and the purified antigen suggests that the stimulation of TNF-α was genuinely produced by these components rather than by contaminating levels of LPS in the solutions (figure 6.9).
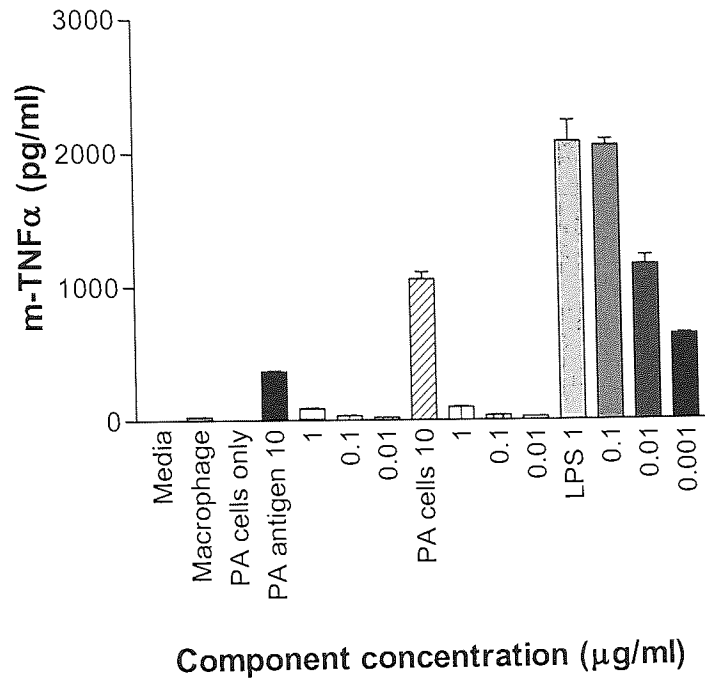
Figure 6.9 TNF-α production by murine macrophages stimulated for 48 hours with *P. acnes* antigen (PA antigen), whole cells of *P. acnes* (PA cells) and *E. coli* LPS measured by quantikine ELISA.

6.4 Discussion

In this chapter a serological response comprising both IgM and IgG to *P. acnes* whole cells and to vortex-released antigen was demonstrated. Antibody levels to whole cells correlated closely with levels directed towards the vortex-released antigen. This suggests that the released antigen is representative of the pattern of whole cell antigens exposed by the organism both in healthy humans and in patients with acne or sciatica and indeed may be the major antigen expressed *in vivo*. However, the antibody levels measured from acne and sciatica patients were no different to those of normal patient sera.

Antibody to *P. acnes* in normal human serum has been previously reported (Wolberg *et al.*, 1977). Antibody titres to whole cells of *P. acnes* were measured by agglutination and widely differing levels of titres ranging from 1:8-1:8192 were observed. Work of Iversen *et al.*(1985) also verified the high prevalence of antibodies against *P. acnes* in healthy humans. These workers did however, find that acne patients had a significantly higher level of antibodies measured by bacterial agglutination and radial immunodiffusion using antigen from the growth culture (BHI) supernatant of *P. acnes*. As identified in the previous chapter, antigen isolated from BHI media may be contaminated with antigenic media components and this may affect the validity of these findings. By use of an ELISA to measure IgG antibody against the water soluble fraction of a pyridine extract of *P. acnes* whole cells Burkhart *et al.*(1999b) found no significant difference in titres in sera from blood donors or acne patients. These findings and those of this thesis are not wholly unexpected since antibody titres to other common skin organisms are found in normal individuals. Previous literature has demonstrated that normal adult human sera contain high levels of antibody reactive with *Candida albicans* mannan (Kozel *et al.*, 2004). Another example where antibody levels to bacterial cell wall antigens have been investigated and found not to differ significantly between infected patients and normal individuals concerns *S. aureus* (Wergeland *et al.*, 1989). In this study antibody levels to the major cell wall antigens, peptidoglycan, wall teichoic acid and LTA exhibited considerable overlap between uninfected controls and patients with active *S. aureus* infection, restricting their exploitation for serodiagnosis of infection.

Although specific subclasses of IgG were not investigated in this study other workers have shown that patients with moderate to severe acne have significantly higher titres of IgG2 compared to controls (Ashbee *et al.*, 1997). The IgG subclass response has been found to vary with structure of the antigen and stimulation by certain antigens may result in a selective increase in certain subclasses. Polysaccharide antigens, in particular have been found to predominantly induce production of IgG2 antibodies (Siber *et al.*, 1980). A common example is IgG2 restriction to pneumococcal polysaccharides (Barrett and Ayoub, 1986) and IgG2 antibodies have been shown to predominate in response to mycobacterial lipoarabinomannan (Da Costa *et al.*, 1993).

Both an IgM and IgG antibody response to *P. acnes* whole cells and antigen has been demonstrated in this work. It has been shown in humans between four months and 14 years old that the majority of antibodies to *P. acnes* are IgM. This is then followed in subsequent years by a switch over from IgM to IgG production (Ingham *et al.*, 1987). The long period of IgM production is characteristic of chronic stimulation of the immune system with levels of antigen too low to stimulate the switch to production of IgG. Ingham *et al.* postulate that the switch to IgG production at adolescence may be stimulated by increased colonisation with *P. acnes* that occurs during puberty. A full quantitative investigation of the IgG subclasses and IgM response would require the use of specific tests in which patient sera are treated prior to application to the ELISA plate with reagent designed to remove other potential competing antibodies. This approach would ensure that only the antibody type to be measured remained in the absorbed serum to react with the antigen on the plate.

Other *P. acnes* antigens not related to the surface antigen identified in this thesis have been shown to stimulate an immune response. The study by Holland *et al.* (1993) found that acne patient sera but not normal sera binds to polypeptides on western blots of cell extracts and the culture supernatant of *P. acnes*. In another study IgG and IgA antibodies to a recombinant protein from *P. acnes* were shown to be elevated in patients with sarcoidosis and other lung diseases (Ebe *et al.*, 2000).

P. acnes has long been recognised to possess immunostimulatory capabilities particularly in relation to adjuvant properties (Roszkowski *et al.*, 1990). The existence of measurable antibody to the organism and to one of its isolated antigens in

normal individuals as well as in patients with various *P. acnes*-related conditions indicates that the immune system recognises and responds to the presence of the organism, regardless of disease state. It is therefore relevant to consider the inflammatory effects that this organism may exert.

In this chapter whole cells of *P. acnes* were shown to stimulate production of the inflammatory cytokine TNF-α in the macrophage-like J774.2 cells. The isolated antigen also stimulated release of TNF-α, but the activity was less than that of the whole cells and less than that of LPS from *E. coli*. Stimulation of murine macrophages with 10µg/ml *P. acnes* cells induced the production of TNF-α at a concentration similar to that produced by treatment with 0.01µg/ml LPS. However, assuming that LPS constitutes 2% (w/w) of the Gram-negative bacterial cell (Ward, 1987), the concentration of LPS used (0.01µg/ml) is equivalent to 5µg/ml whole cells; just half the amount of *P. acnes* cells. Viewed in this way, the activity of the *P. acnes* cells appears to be significant. This is in contrast to previous studies on the comparative inflammatory activities of Gram-positive and Gram-negative cell walls. For example, stimulation with *S. aureus* cell wall components requires 100 times more material than Gram-negative LPS to trigger the release of TNF-α (Majcherczyk *et al.*, 2003).

Vowels *et al.* (1995) found that *P. acnes* and culture supernatants could stimulate the production of pro-inflammatory cytokines (IL-8 and TNF-α) in human monocytic cell lines and peripheral blood mononuclear cells. In agreement with the findings of this thesis, the level of cytokine production was more potent with whole cells of *P. acnes* than with *P. acnes* supernatant. Treatment of *P. acnes* whole cells with heat or paraformaldehyde did not destroy the stimulatory ability (Vowels *et al.*, 1995) and the authors suggested that the active component may be part of the cell wall and not an actively formed product such as an exocellular enzyme. Dialysis and ultrafiltration of the culture supernatant suggested that the cytokine activity was generated by component(s) approximately 3 to 30kDa in size. Furthermore, blocking of the CD14 receptor for LPS and peptidoglycan reduced cytokine production, thus providing evidence that the stimulating factor may be a secreted form of peptidoglycan-

polysaccharide. The antigen described by Vowels *et al.* (1995) shows striking similarities with the *P. acnes* antigen reported in this thesis.

Polysaccharides from cell walls of other Gram-positive organisms can stimulate cytokine production. Polysaccharide from two serotypes of *S. aureus* have been shown to stimulate the production of Il-8, IL-6, IL-1β and TNF- α (Soell *et al.*, 1995a) and streptococcal cell wall polysaccharides can stimulate the release of TNF-α from human monocytes (Soell *et al.*, 1995b). One study has shown that polysaccharides are able to induce cytokines, including TNF-α, *in vivo* (Mancuso *et al.*, 1994).

Other experimental systems have been used to investigate the inflammatory properties of *P. acnes*. Whole cells of *P. acnes* have been reported to enhance TNF-α production in mice when injected with LPS (Matsui *et al.*, 1997) and have been shown to be capable of inducing TNF-α directly. Rossol *et al.* (1990), using peripheral blood mononuclear cells and Park *et al.* (2004), using a human monocytic cell line found that *P. acnes* cells directly induced TNF-α production. Inoculation of mice with heat killed *P. acnes* has been shown to induce acute TNF-α mediated apoptosis of hepatocytes (Chen *et al.*, 1999). Activation of toll-like receptor 2 by *P. acnes* has been found to trigger the release of inflammatory cytokines in monocytes (Kim *et al.*, 2002).

In contrast to the stimulation of TNF-α by *P. acnes* and the isolated antigen studied in this thesis no stimulation of nitric oxide was detected. This result was unexpected since many cells can be induced to produce nitric oxide in response to microbial infection and inflammatory cytokines, including TNF-α (Moncada *et al.*, 1991).

The highest concentration of LPS inhibited the formation of nitrite. This may have been a result of LPS micelle formation preventing macrophage receptor binding. Alternatively, receptors may be saturated at high doses of LPS or LPS may be toxic to macrophages at the higher concentration.

Studies have shown that Gram-positive bacteria and their components are not as potent when compared to Gram-negative organisms at inducing nitric oxide synthesis (Jungi *et al.*, 1999). It could therefore be the case that the concentration of *P. acnes*

components used in this study were too low to stimulate the production of NO or that the levels produced were below the detection limit of the assay. *P. acnes* has been shown to induce NOS in different organs of the mouse (Rees *et al.*, 1995). NOS levels in macrophages were found to be maximal after eight days of incubation. The time required for NO oxide induction by *P. acnes* may therefore be greater than that required for induction by LPS. A period of incubation longer than that possible with the J774.2 cell culture was employed in the study by Rees *et al.* (1995) since an *in vivo* model was used.

In murine macrophage cell lines CD14 has been shown to be involved in LPS (Fujihara *et al.*, 2003) and LTA (Hattor *et al.*, 1997) mediated cell activation and almost certainly acts in concert with additional cellular components such as toll-like receptors to initiate a transmembrane signalling cascade. Differences in activation of NO and TNF-α may be a result of either engagement of a different set of receptors or differences in post-receptor binding events. Failure to induce NO may have been through lack of binding to a receptor or failure to complete the signal transduction cascade.

Various bacteria have evolved mechanisms that block different stages of phagocytosis. The surface components of some bacteria inhibit phagocytosis, for example *S. aureus* protein A and M proteins of group A streptococci (reviewed by Celli and Finlay, 2002). The ability of *P. acnes* to resist phagocytosis has also been reported. *P. acnes* cells can persist for long periods *in vivo* (Scott and Milas, 1977) and *in vitro* (Pringle *et al.*, 1982) which may be associated with the indigestibility of the *P. acnes* cell wall. Scott and Milas (1977) whilst investigating the distribution and persistence of *P. acnes in vivo* found that uptake of *P. acnes* by macrophages was not necessarily a prerequisite for their activation. Furthermore, *P. acnes* has been shown to be ingested by PMNs whilst lysosomal granules remain intracellular and intact (Webster *et al.*, 1980). Lysosomal enzyme release from PMNs has been shown not to be stimulated with supernatants of *P. acnes* and that release stimulated by whole cells of *P. acnes* requires the presence of serum (Webster *et al.*, 1980). The removal of anti-*P. acnes* antibody from patient serum was shown to ablate the ability of serum to promote lysosomal enzyme release in response to *P. acnes*. Further work by Webster *et al.* (1985b) studied the susceptibility of *P. acnes* to killing and degradation by

neutrophils and monocytes. *P. acnes* was found not to be killed by PMNs under any conditions and monocytes reduced the viability *P. acnes* only in the presence of serum. Whether this survival is due to the inability of *P. acnes* to fully activate phagocytic cells or their resistance to degradative enzymes was inconclusive.

CHAPTER 7 GENERAL DISCUSSION

P. acnes normally inhabits the skin, oral cavity, large intestine and the external eye (Brook and Frazier, 1991). On the skin the organism resides in the pilosebaceous follicles and is the prevalent bacterium of the skin glands. Ordinarily a harmless commensal of the skin, *P. acnes* has been recognised as a contributory factor in the development of acne. In addition, the organism has long been documented for its adjuvant properties and consequently it has been investigated for use in anti-tumour therapy and used in experimental models of inflammation. Other infections and conditions attributable to *P. acnes* are increasingly reported in the literature. Like acne, many conditions associated with *P. acnes* are multifactorial, with the organism not solely responsible but contributing to pathologies. Infections caused by *P. acnes* frequently have a predisposing factor, such as trauma, as may be the case in sciatica, or are foreign device-related infections, for example prosthetic joint infections. The characteristics of *P. acnes* infection points towards the organism being a pathogen of low virulence as it does not cause massive pathology associated with highly virulent organisms such as *S. aureus* and group A streptococci. However, it remains unclear why a normally harmless commensal, such as *P. acnes* can cause infection and contribute to a number of clinically significant conditions. The virulence, genetic and antigenic properties of *P. acnes* have not been studied in great detail and this thesis has sought to obtain information which addresses these points.

Firstly, the phenotypic properties of *P. acnes* were investigated by use of API tests, a number of agar based methods for the detection of virulence factors, antibiotic susceptibility testing and four monoclonal antibodies for cell associated antigens. Biotyping using the API system identified a large number of biotypes demonstrating the biochemical diversity of strains. The distribution of biochemical profiles varied between the isolate groups indicating that biochemically distinct strains may exist in different niches.

Antimicrobial susceptibilities of *P. acnes* strains in this study indicated that antibiotic resistance does not pose a problem and infections by the organism can be easily treated. However, there are clear indications from the acne and skin isolates studied

here that resistance to commonly employed antibiotics in the treatment of acne, e.g. tetracycline, erythromycin and clindamycin, is increasing.

Clinical strains were characterised in terms of their ability to produce a range of virulence factors. The pathogenic potential of *P. acnes* was illustrated by the detection of a number of virulence factors produced by the organism, particularly haemolysins, lipase, proteinase and hyaluronidase. The range of virulence factors produced by an isolate was not strongly associated with the clinical source of the strain. It is possible that some strains may express unidentified virulence factors or that all strains may be equally capable of causing a condition. Expression of virulence factors *in vivo* may aid the organism in invasion and colonisation by degradation of host macromolecules, however no direct information regarding regulation or *in vivo* expression was gained. It would therefore be most valuable for future studies to investigate the effect of *in vivo* growth conditions on the regulation of virulence factors. This approach has proved particularly useful for studying the effect of iron restriction upon expression of virulence related genes in a wide range of bacteria. This has been most thoroughly studied in *E. coli* and the regulatory gene, *fur*, identified (reviewed by Litwin and Calderwood, 1993). However, very little work exists to date on *P. acnes* in this respect. However, the approach employed in this thesis to study the antigenic profile of *P. acnes* using sera from patients with *P. acnes* infection, would reflect antigens exposed *in vivo*. The biofilm mode of growth is another important factor which influences expression of virulence factors as well as antibiotic sensitivity (Costerton, 1999). Few studies have been done in this respect for *P. acnes* apart from an investigation of the effects upon antibiotic susceptibility (Ramage *et al.*, 2003).

Recent progress in serotyping the organism following the development of monoclonal antibodies that distinguish four type specific antigens (type I, II, III and IV) allowed the exploration of the isolates' serotype. Isolates belonging to serotype I appeared to express more virulence factors than isolates belonging to serotype II. Notably, type I strains were associated with β-haemolysis and type II strains with α-haemolysis.

P. acnes isolates were genotyped using an optimised RAPD protocol. The advantages of using this molecular typing technique include the fact that it requires no prior knowledge of the target genome, it is cheap, rapid and if optimised, reproducible. In addition, techniques which examine the genome are not affected by environmental conditions unlike phenotypic analysis. RAPD was not specifically designed to distinguish between serotype I and II but it was found to distinguish between these two serotypes for a large number of clinical isolates. RAPD also enabled the identification of a genotype that did not react with the type I or II monoclonal antibodies and these strains therefore may constitute a previously undiscovered serotype. Recently a further phylogenetic subdivision within type I *P. acnes* has been identified (McDowell *et al.*, in press). The RAPD type of an organism was not found to be related to the isolate source. Furthermore, phenotypic traits, except the serotype, varied among strains of the same genotype.

This study identified the major *P. acnes* antigen that induces an antibody response *in vivo* using patient sera. It was found to be released into culture media during growth but was also found in association with the cell wall, some of which could be removed by vortexing with glass beads. Chemical analysis revealed that this antigen consisted mainly of carbohydrate and protein; the latter being non-antigenic. A serodiagnostic test for *P. acnes* infection would be advantageous for conditions such as sciatica or prosthetic hip infection, since both require invasive surgery and culture to confirm infection. However, investigation of the serodiagnostic potential of the antigen demonstrated that both normal human sera and sera from acne and sciatic patients contained antibodies to *P. acnes*. The ubiquitous nature of *P. acnes* antibodies among the population is not unexpected since the organism resides as a commensal in the pilosebaceous follicles of the skin at 10^5 CFU/follicle (Funke *et al.*, 1997). Antibodies in normal human serum therefore reflect prior exposure by commensal strains.

The inflammatory response to *P. acnes* and the isolated antigen was assessed by measuring the release of NO and TNF-α from J774.2 macrophage-like cells. Neither whole cells nor the antigen could induce the release of NO. This work highlighted the resistance of *P. acnes* to phagocytosis and/or failure of the organism to fully activate macrophages. However, TNF-α was produced when J774.2 cells were stimulated with both *P. acnes* cells and antigen. *In vivo* the production of pro-inflammatory

cytokines may contribute to clinical manifestations of disease. Since inflammation and pain are the major features of *P. acnes*-associated conditions (e.g. endophthalmitis) more work needs to be carried out to determine the interaction of the organism and its products with the host immune and nervous system. Early work showed that prostaglandin-like molecules are produced by *P. acnes* (Abrahamsson *et al.*, 1978; Hellgren and Vincent, 1983), these merit further study as possible mediators of inflammation. Chemotactic factors produced by the organism might also be important (Webster and Leyden, 1980), these exocellular products could be released from organisms residing in the pilosebaceous follicles to initiate recruitment of inflammatory cells. Future work on the chemotactic activity of the *P. acnes* antigen identified in this thesis would also be of interest in this respect.

The recently available sequence of *P. acnes* strain DSM 16379 (Bruggemann *et al.*, 2004) provides invaluable information upon which the design of novel protective vaccines and therapeutics for *P. acnes* infection may be based. Accessibility to the sequence opens up the possibility of studies utilising genomic microarrays to investigate gene expression.

The location of *P. acnes* deep in the skin makes its confirmation as the causative organism in conditions such as sciatica difficult, due to issues of contamination. To establish a definite causal relationship between the organism and a specific disease would require the fulfilment of Koch's postulates. The organism should be isolated from patients suffering from the disease and, when inoculated into a susceptible host should produce the characteristic disease symptoms. This quite clearly is difficult to perform if an organism is difficult to cultivate and would also require a suitable animal model. Due to the limitations of Koch's postulates and with the emergence of technological advances, molecular criteria have been suggested for establishment of causal relationships between microbes and disease (Fredericks and Relman, 1996). The use of molecular technology such as PCR has established an association of *P. acnes* with sarcoidosis, where the organism's DNA has been detected in the lymph nodes of sufferers (Eishi *et al.*, 2002). However, this only confirms the presence of the organism and not the extent of the organism's involvement in the disease process.

The growing use of medical devices is likely to lead to an increased incidence of infections caused by skin organisms. This is currently noticeable in postoperative endophthalmitis following cataract surgery and lens implantation where organisms like *P. acnes* and coagulase-negative staphylococci are frequently isolated (Leong *et al.*, 2002). The incidence of prosthetic joint infection caused by bacteria including *P. acnes* may be underestimated (Tunney *et al.*, 1999; Tunney *et al.*, 1998). The incidence of infection may therefore be underestimated in other devices used in the body.

At the start of this study published data proposed that *P. acnes* may be associated with sciatica (Stirling *et al.*, 2001). Other workers have since investigated this association with variable results. McLorinan *et al.* (2003) found *P. acnes* among other skin organisms in disc and muscle material taken during microdiscectomy but suggested that the source of the organism may be from contamination during the surgical procedure. Another group have confirmed the incidence of positive cultures but were still unable to establish a causal link (Vautrin *et al.*, 2004). The current position regarding the association of *P. acnes* with sciatica therefore appears to be neither confirmed nor refuted. The possible association of *P. acnes* with clinical conditions should not be overlooked considering the number of conditions which have subsequently found to have a microbial aetiology. The best example of this is the involvement of *Helicobacter pylori* in peptic ulcer disease (Marshall and Warren, 1984).

How does *P. acnes* cause disease in some individuals whilst remaining a harmless commensal in most? Its role in the pathogenesis of disease appears similar to that of other skin commensals, including the coagulase-negative staphylococci. As low virulence organisms, they are capable of causing significant infection particularly where medical devices are introduced into the body and when host defences are compromised by other disease or cytotoxic therapy. A particular characteristic of *P. acnes* infection is its late onset, for example following surgical procedures on the eye, infection may not be apparent for several months.

The work presented in this thesis provides background information on the properties of *P. acnes* strains isolated from clinical samples. It could promote a greater

understanding of the nature of *P. acnes*, particularly in terms of virulence factors and antigenicity, and provides information regarding the organisms contribution to disease states.

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CONFERENCES ATTENDED

September 2003   Society for General Microbiology 153rd Meeting, Manchester, UK.

April 2003   Society for General Microbiology 152nd Meeting, Edinburgh, UK.

January 2003   *SfAM* January Meeting – Lab on a Chip, Birmingham, UK.

November 2002   Federation of Infection Societies, 9th Annual Meeting, Manchester, UK.

September 2002   Society for General Microbiology 151st Meeting, Loughborough, UK.

April 2002   Society for General Microbiology 150th Meeting, Warwick, UK.

LIST OF PUBLICATIONS

Full Papers

Perry A. L., T. Worthington, A. C. Hilton, P. A. Lambert, A. J. Stirling & T. S. J. Elliot. (2003). Analysis of clinical isolates of *Propionibacterium acnes* by optimised RAPD. *FEMS Microbiol. Letters*. 228: 51-55.

Non-peer reviewed articles

Perry, A. L. (2003) When bugs strike back. *Microbiologist:* March 40-41.

Abstracts

Perry A. L., T. Worthington, A. Hilton, P. Lambert, A. Stirling & T. Elliot. (2002). RAPD typing for *Propionibacterium acnes*.
(FIS Manchester Meeting; published in the proceedings)

Perry A. L., T. Worthington, A. Hilton, P. Lambert, A. Stirling & T. Elliot. (2003). Optimised random amplification of polymorphic DNA (RAPD) typing for genotyping clinical strains of *Propionibacterium acnes*.
(SGM Edinburgh Meeting; published in the proceedings)

When bugs strike BACK

Alexandra Perry asks if **sciatica** can now be added to the list of diseases thought to be caused by an infectious agent?

Aston University

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Analysis of clinical isolates of *Propionibacterium acnes* by optimised RAPD

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