Population biology of *Propionibacterium acnes* and *Pseudomonas aeruginosa* in ophthalmic infections and the development of novel diagnostic tools

by

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DECLARATION

I declare that the work presented in this thesis was conducted by me under the supervision of Professor Chris Dowson, Professor Phillip Murray and Dr. Colin Fink, except where the contribution of others has been acknowledged. The work described in this thesis has not been used in any previous application for a degree at Warwick University or other Universities.

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ABSTRACT

Bacterial keratitis and bacterial endophthalmitis are two of the most devastating sight threatening eye infections. It is currently unclear whether the organisms isolated from these infections represent specialised members of these species or whether all strains are equally likely to cause infection. One method of differentiating strains genotypically is by a typing technique known as multilocus sequence typing (MLST). Using this technique, a better understanding of the molecular epidemiology of eye infections can be achieved.

Propionibacterium acnes (*P. acnes*) is an important anaerobic organism causing several types of eye infections. Although it is now beginning to be recognised as a serious opportunistic pathogen, few studies have been done to investigate the population biology of *P. acnes* at the molecular level. Our continuing inability to distinguish between strains of *P. acnes* means that we still do not fully understand how antibiotic-resistant strains spread, nor whether certain strains, or clonal complexes, of *P. acnes* are associated with certain infections. These are key issues that can now be understood with our development of an MLST system for *P. acnes*. A diverse culture collection of 125 *P. acnes* isolates have been analysed using the MLST scheme developed. Sequence analysis shows that there are phylogenetically distinct groups within *P. acnes* and identified a novel cluster not previously described. Analysis of recombination using several methods suggests that frequent recombination occurs within these subgroups. There appears to be no association between these subgroups and clinical manifestation of *P. acnes* infection or geographical location. The *P. acnes* MLST scheme was validated against 16S rRNA gene and complete *recA* gene typing as well as immunofluorescence microscopy (IFM) and random amplification of polymorphic DNA (RAPD) analysis.

P. acnes is a slow growing organism and is difficult to culture from ocular samples. A real-time PCR assay was developed in order to overcome the low culture positive rate and delay associated with conventional culture methods. Primers targeting one of the seven housekeeping genes used in the MLST scheme (*gmk*), specific for *P. acnes* were selected. The real-time PCR assay was both specific to *P. acnes* and highly sensitive.

Pseudomonas aeruginosa (*P. aeruginosa*) is another important organism in the development of serious eye infections, and is the commonest cause of contact lens related microbial keratitis. Infections with this organism can lead to rapid deterioration of vision and possible blindness. A previously developed MLST scheme has been applied to 117 eye isolates from around the UK and China. This typing data was compared to 166 *P. aeruginosa* isolates from other clinical and environmental sources. Overall, MLST data supports previous findings that *P. aeruginosa* has a non-clonal population with epidemic clones. Sequence analysis showed that eye isolates do not cluster away from isolates from other clinical infection sites.

ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
A. israeli	*Actinomyces israelli*
BHI	Brain heart infusion
bp	Basepair
BPH	Benign prostatic hyperplasia
BURST	Based upon related sequence types
CDS	Protein coding sequence
CET	Central England temperature
CF	Cystic fibrosis
CFU	Colony forming units
CI	Confidence interval
CNS	Central nervous system
DLV	Double-locus variant
DMSO	Dimethylsulphoxide
d_N	Non-synonymous substitutions
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide trisphosphates
d_S	synonymous substitutions
ECF	Extracytoplasmic function
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
et al.	And others
EVS	Endophthalmitis vitrectomy study
FBGT	Fatal bacterial granuloma after trauma
FISH	Fluorescence *in situ* hybridisation
FITC	Fluorescein isothiocyanate
g	Gram
H. influenza	*Haemophilus influenza*
HSV	Herpes simplex virus
I_A	Index of association
IFM	Immunofluorescence microscopy
IgA	Immunoglobulin A

IL	Interleukin
IOL	Intraocular lens
ITS	Internal transcribed spacer
Kb	Kilobase
LASEK	Laser assisted subepithelial keratectomy
LASIK	Laser in situ keratomileusis
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
M	Molar
MAb	Monoclonal antibody
Mb	Megabase
ML	Maximum likelihood
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
mg	Milligram
ml	Millilitre
mM	Millimolar
MW	Molecular weight
NA	Not applicable
NCTC	National collection of type cultures
ND	Not determined
ng	Nanogram
P. acnes	*Propionibacterium acnes*
P. aeruginosa	*Pseudomonas aeruginosa*
PAUP	Phylogenetic analysis using parsimony
P. avidum	*Propionibacterium avidum*
PBS	Phosphate buffered saline
PC	Platelet concentration
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pg	Picogram
P. granulosum	*Propionibacterium granulosum*
P. propionicus	*Propionibacterium propionicus*
RAPD	Random amplification of polymorphic DNA

RBC	Red blood cells
rDNA	Ribosomal DNA
REA	Restriction Endonuclease Analysis
Rep-PCR	Repetitive-sequence-based polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RPE	Retinal pigment epithelium
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAPHO	synovitis, acne, pustulosis, hyperostosis and osteitis
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SH	Shimodaira Hasegawa
SLV	Single-locus variant
S. marcescens	*Serratia marcescens*
S. pneumoniae	*Streptococcus pneumoniae*
spp.	Species
ST	Sequence type
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
Temp.	Temperature
TLV	Triple-locus variant
TNF	Tumour necrosis factor
TTSS	Type III secretion system
UV	Ultraviolet
w/v	Weight to volume ratio
V	Volts
v/v	Volume to volume ratio
WHO	World Health Organisation
µg	Microgram
µl	Microlitre
UPGMA	Unweighted pair group method with arithmetic averages
×*g*	Centrifuge force

TO MY FAMILY

CHAPTER 1 INTRODUCTION

1.1 Infections of the eye

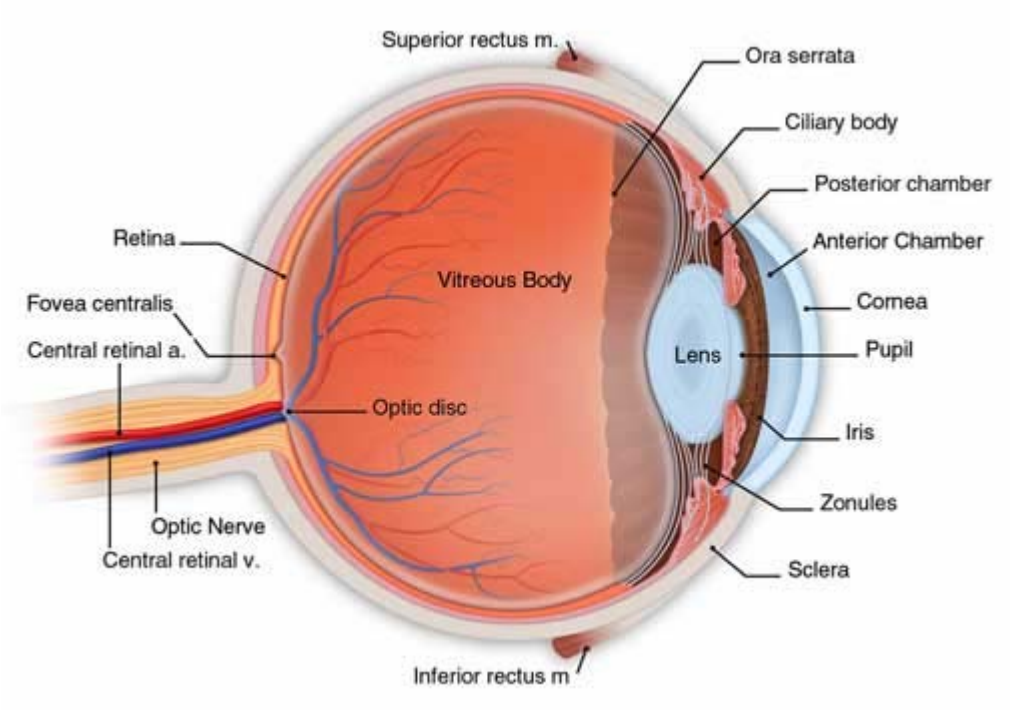
1.1.1 Anatomy of the eye

The eyeball is made up of two main segments; the anterior segment and the posterior segment. The cornea is a transparent structure at the front of the eye (Figure 1.1). Its transparency allows light to enter the eye and it acts as the main refractive structure, focusing light onto the retina (Snell & Lemp, 1998). The cornea also serves as a protective physical barrier against the environment, with the aid of the tear film. Tears contain lysozyme, immunoglobulin A (IgA) and beta-lysin (bactericidal protein), which contribute to protection against infection (Doane, 1981; Ffooks, 1962).

The anterior and posterior chambers of the eye are filled with a clear fluid called aqueous humour (Snell & Lemp, 1998). This fluid contains glucose, amino acids, ascorbic acid and dissolved gases, which help to provide the metabolic needs of the avascular lens and cornea (Snell & Lemp, 1998).

The vitreous body fills the space between the lens and the retina, occupying about four-fifths of the eyeball (Bron *et al.*, 2001). The vitreous fluid is a colourless, transparent, gelatinous substance consisting of 98% water. It also contains hyaluronic acid, amino acids, soluble proteins, salts and ascorbic acid (Snell & Lemp, 1998). Importantly, the vitreous body allows light to be transmitted to the retina. It supports the back of the lens and assists in supporting the neural part of the retina against the pigmented part of the retina (Snell & Lemp, 1998).

Figure 1.1 Anatomy of the eyeball.
Taken from www.matossianeye.com/anatomy.htm



1.1.2 Microbial keratitis

1.1.2.1 Epidemiology

It is estimated that around 38 million people globally are bilaterally blind and about 110 million have severely impaired vision in both eyes (WHO, 1997). Corneal disease is one of the major causes of blindness in the world, second only to cataracts, and includes a wide range of infectious and inflammatory eye diseases (Whitcher *et al.*, 2001). The prevalence of corneal blindness differs in each country and even varies from one population to another (Smith & Taylor, 1991). In parts of Africa and Asia, the incidence of cornea-related visual loss in children is 20-times higher than in developed countries (Whitcher *et al.*, 2001). The epidemiology of corneal blindness is diverse and those of worldwide importance include trachoma, onchocerciasis, leprosy, ophthalmia nenatorum and xerophthalmia (Whitcher *et al.*, 2001). Recent public health programmes to control these causes have resulted in a decrease in cases but has highlighted the significance of ocular trauma and corneal ulceration in corneal blindness (Whitcher *et al.*, 2001).

The incidence of ulcerative keratitis due to infection varies greatly between different countries. In Olmsted County, Minnesota, USA, the incidence was 11 per 100,000 per year (Erie *et al.*, 1993). Applying this incidence rate to the 1990 US population yields an estimate of 27,000 corneal ulcers annually. In contrast, the annual incidence of corneal ulceration in Madurai District, South India was 113 per 100,000 (Gonzales *et al.*, 1996). By applying this rate to all of India, around 840,000 people per year develop an ulcer (Whitcher *et al.*, 2001). Extrapolating the data to the rest of Africa and Asia, the number of corneal ulcers occurring annually in the developing world is estimated to be 1.5-2 million (Whitcher *et al.*, 2001). This is likely to be an underestimate as in Bhaktapur District, Nepal, the annual incidence of corneal ulceration was found to be 799 per 100,000 people (Upadhyay *et al.*, 2001), 70 times the rate in the United States.

1.1.2.2 Clinical findings

Patients with corneal ulceration often complain of pain, photophobia, watering of the eyes, foreign body sensation, redness and decreased vision (Agrawal *et al.*, 1994). The anterior segment of the eye is usually inflamed and congested. The eyelids may be oedematous and the palpebral conjunctivae inflamed. Diffuse conjunctival vessel injection and chemosis are commonly observed. Discharge from the eye may be thick and is often mucoid to purulent in consistency. The infected area of the cornea usually has a focal area of stromal infiltrate with an overlying epithelial defect (Figure 1.2 and 1.3). The cornea may become oedematous and visual acuity is reduced. The severity and degree of visual loss depends on the extent and location of the lesion. Severe cases can lead to profound anterior chamber reaction and hypopyon (accumulation of white blood cells in the anterior chamber). Ciliary body inflammation can cause hypotony (low intraocular pressure). However, the presence of inflammatory cells in the aqueous may also block the trabecular meshwork, leading to an increase in the intraocular pressure (Suwan-Apichon *et al.*, 2007).

Microbial keratitis is relatively rare in the absence of any predisposing factors and only 8% to 15% of patients with microbial keratitis do not have an identifiable risk factor (Bourcier *et al.*, 2003; Keay *et al.*, 2006; Seal *et al.*, 1999; Wong *et al.*, 2003). Contact lens wear is now the most common risk factor for developing a corneal infection in developed nations (Bourcier *et al.*, 2003; Schaefer *et al.*, 2001). This is followed by ocular surface disease, such as herpetic infection, Sjögren's syndrome, severe blepharitis, bullous keratopathy and basement membrane dystrophy (Seal *et al.*, 1999). Ocular trauma and ocular surgery, including corneal grafts, are also predisposing factors to microbial keratitis (Bates *et al.*, 1990; Harris *et al.*, 1988; Keay *et al.*, 2006; Wong *et al.*, 2003).

Figure 1.2 *Pseudomonas* corneal ulcer.
Image from http://mcrcr4.med.nyu.edu/Ophth/dosc/cornea.htm

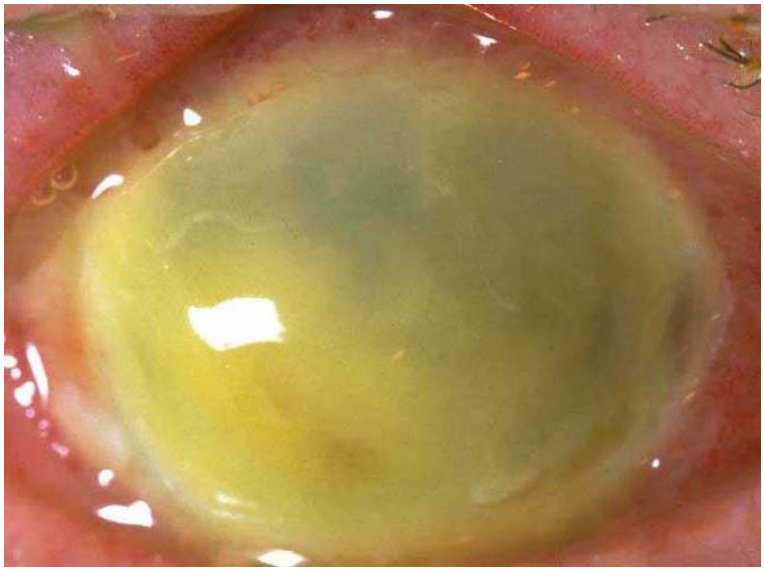
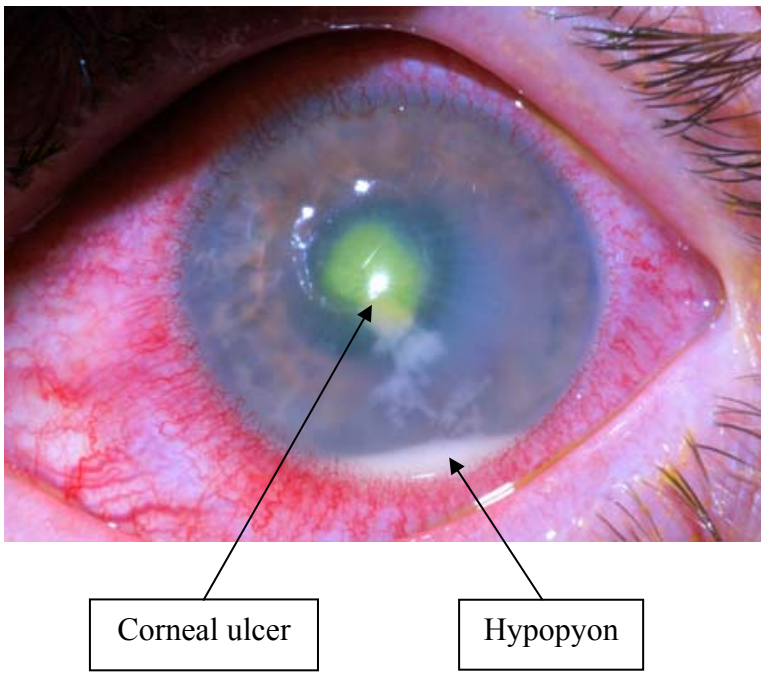


Figure 1.3 Bacterial corneal ulcer with hypopyon.
Image from http://bestpractice.bmj.com/best-practice/monograph/561/resources/images.html



Any laser refractive surgical procedure which breaches the epithelial barrier poses a small risk of infection of the cornea, with an estimated 0.02% to 0.1% occurrence in laser in situ keratomileusis (LASIK) (Alio *et al.*, 2000; Aras *et al.*, 1998; Levartovsky *et al.*, 2001; Lin & Maloney, 1999; Quiros *et al.*, 1999; Reviglio *et al.*, 1998; Watanabe *et al.*, 1997). Bacterial keratitis has also been reported after laser-assisted subepithelial keratectomy (LASEK), another surgical technique used to correct refractive error (Laplace *et al.*, 2004).

1.1.2.3 Causative organisms

The spectrum of microbial organisms is partly dependant on the climatic conditions. Gram-positive bacteria and *Acanthamoeba*, associated with contact lens wear are commonly found in temperate climates such as in the UK (Tuft & Matheson, 2000). In contrast, *P. aeruginosa* and filamentous fungi tend to predominate in tropical and semitropical areas such as South Florida (Liesegang & Forster, 1980), Ghana (Hagan *et al.*, 1995), Bangladesh (Dunlop *et al.*, 1994; Williams *et al.*, 1991) and South India (Bharathi *et al.*, 2007; Srinivasan *et al.*, 1997).

At Moorfields Eye Hospital in London, a study of bacterial keratitis cases demonstrated that of the 1,312 organisms isolated, Gram-positive organisms accounted for 54.7%, and the most common organisms were *Staphylococcus* spp. (33.4%), *Pseudomonas* spp. (24.8%), *Streptococcus* spp. (19.0%), *Moraxella* spp. (5.9%) and *Serratia* spp. (3.0%) (Tuft & Matheson, 2000).

A study in Florida demonstrated that of the 1,338 organisms isolated, the overall numbers of Gram-positive and Gram-negative isolates were similar (48% and 50% respectively) (Alexandrakis *et al.*, 2000a). *P. aeruginosa* and *S. aureus* were the most common isolated organisms (25.7% and 19.4%, respectively). Over the 9-year study period, 108 (8%) *P. acnes* isolates were also recovered.

A recent study in Taiwan showed that of the 272 bacterial pathogens isolated, 32.4% were Gram-positive and 61% were Gram-negative (Fong *et al.*, 2007). The remainder were non-tuberculous *Mycobacterium*. *Pseudomonas* spp. were again demonstrated to be the most commonly isolated organism (46.7%), followed by *Staphylococcus* spp. (11%), *Propionibacterium* spp. (8.1%), *Streptococcus* spp. (7.6%), and non-tuberculous *Mycobacterium* (6.6%).

A study in Aukland, New Zealand looking at 103 cases of presumed infective keratitis found a high proportion of Gram-positive organisms (72%) (Wong *et al.*, 2003). The most common organisms identified were coagulase-negative *Staphylococcus* (16.4%), *P. acnes* (13.9%), *S. epidermidis* (11.5%), *S. pneumoniae* (9%), *S. aureus* (5.7%) and *P. aeruginosa* (5.7%).

Bourcier *et al.* investigated 300 cases of bacterial keratitis in Paris and also observed a high percentage of Gram-positive bacteria (83%) (Bourcier *et al.*, 2003). The most common organisms identified were coagulase-negative *Staphylococcus* (48.5%), *P. acnes* (15%), *P. aeruginosa* (10.2%) and *S. aureus* (7.8%). In the contact lens wear group, *P. aeruginosa* was the most common organism identified.

1.1.2.4 Conventional diagnosis

Microbial keratitis is a sight threatening condition, and if delay in the diagnosis and initiation of appropriate antibiotics occur, then only 50% of eyes are estimated to heal with a good visual prognosis (Jones, 1981). Definitive diagnosis of microbial keratitis requires culture of the causative organism from a corneal scrape sample. This can be obtained using a Kimura spatula or the tip of a sterile disposable needle (Tuft & Matheson, 2000). Smears are routinely examined by Gram staining and also inoculated onto solid and liquid media (e.g. blood agar, Robertson's cooked meat, thioglycolate broth, and brain heart infusion broth) (Tuft & Matheson, 2000).

Culture positive rates from corneal scrapes vary considerably from centre to centre. Studies from around the world have demonstrated culture positive rates ranging from 50% to 86% (Alexandrakis *et al.*, 2000a; Bennett *et al.*, 1998; Bourcier *et al.*, 2003; Fong *et al.*, 2007; Neumann & Sjostrand, 1993; Schaefer *et al.*, 2001; Wong *et al.*, 2003). In cases where corneal scrape cultures are negative and the clinical course worsens despite intensive topical antimicrobial treatment, a cornea biopsy may help in providing material for culture and histopathologic examination (Allan & Dart, 1995). Alexandrakis *et al.* observed a positive culture result in 27 (82%) of 33 corneal biopsies taken for microbial keratitis unresponsive to intense topical treatment (Alexandrakis *et al.*, 2000b).

Confocal microscopy is a non-invasive, high-contrast imaging technique which allows direct visualisation of the organisms *in vivo* and can be helpful in diagnosing fungal and Acanthamoeba keratitis (Brasnu *et al.*, 2007; Matsumoto *et al.*, 2007).

1.1.2.5 Treatment

Treatment for microbial keratitis is commenced once cultures have been obtained from the lesion to enable identification of the organism and test its sensitivity to antibiotics. Gram stain result has been used to determine initial therapy (Jones, 1979; Sharma *et al.*, 2007) but this is not always available and may not be reliable enough to select a specific antibiotic. The mainstay of treatment for microbial keratitis has been a combination treatment (dual therapy) of two fortified antibiotic preparations to cover the whole range of Gram-positive and Gram-negative pathogens (Tuft & Matheson, 2000). Despite its effectiveness, problems with toxicity of the aminoglycosides and the poor stability at room temperature of fortified preparations, has led to the use of alternative antibiotic treatments (Bowe *et al.*, 1991; Davison *et al.*, 1991; Petroutsos *et al.*, 1984). Monotherapy with fluoroquinolones (e.g ofloxacin and ciprofloxacin) became a popular alternative to combination therapy due to their broad spectrum coverage, low toxicity, good corneal penetration and their efficacy at a commercially available strength (Chin & Neu, 1984; Diamond *et al.*, 1995; McDermott *et al.*, 1993; Neu, 1991; Price *et*

al., 1995). Unfortunately, the systemic use of fluoroquinolones resulted in rapid emergence of resistance, especially in *Staphylococcus* spp. and *Pseudomonas* spp. (Ball, 1990; George *et al.*, 1990; Humphreys & Mulvihill, 1985; Raviglione *et al.*, 1990). Fluoroquinolone resistant bacterial keratitis isolates were then reported (Bower *et al.*, 1996; Knauf *et al.*, 1996; Maffett & O'Day, 1993; Snyder & Katz, 1992), with high rates of resistance at some centres (Garg *et al.*, 1999; Goldstein *et al.*, 1999; Kunimoto *et al.*, 1999b).

A study done in London looking at bacterial keratitis cases between 1984 and 1999 did not find an increase in resistance against the combination therapy of cefuroxime and gentamicin or monotherapy with ofloxacin (Tuft & Matheson, 2000). However, it did identify a significant increase in the proportion of Gram-negative organisms resistant to chloramphenicol, the routine prophylactic treatment of corneal epithelial defects at the centre (Tuft & Matheson, 2000).

In Florida, *S. aureus* resistance to fluoroquinolones amongst eye isolates increased from 11% in 1990 to 28% in 1998. *S. aureus* resistance to aminoglycosides (e.g. tobramycin and gentamicin) was 11% over the whole study period, with little fluctuation between the years. In contrast to *S. aureus*, *P. aeruginosa* only showed 1% resistance to fluoroquinolones and 0.6% to aminoglycosides (Alexandrakis *et al.*, 2000a). From their findings, the authors recommended initial empiric intensive treatment using either a fluoroquinolone or fortified cefazolin with a fortified aminoglycoside (Alexandrakis *et al.*, 2000a).

Newer topical fluoroquinolones, including third-generation levofloxacin and fourth-generation gatifloxacin and moxifloxacin, have been introduced. These provide better corneal penetration, greater potency for Gram-positive organisms and reduced likelihood of resistance compared to ciprofloxacin and ofloxacin (Alfonso & Crider, 2005; Blondeau, 2004; Hwang, 2004).

The role of corticosteroids as adjunctive therapy to topical antimicrobials remains controversial. Many practitioners are cautious about using corticosteroids as their immunosuppressive effect may potentiate bacterial replication and delay healing of the corneal ulcer. However, if adequate sterilisation has been achieved, corticosteroids can be beneficial in reducing the inflammatory response and limiting neovascularisation and scarring (Suwan-Apichon *et al.*, 2007). In severe cases of corneal ulceration when antibiotic treatment has failed, penetrating keratoplasty (corneal graft transplant) has been necessary for therapy or visual rehabilitation (Chen *et al.*, 2004; Miedziak *et al.*, 1999).

1.1.3 Endophthalmitis

1.1.3.1 Background

Endophthalmitis is an inflammatory reaction of intraocular fluids or tissues of the eye following introduction of an infectious organism into the posterior segment of the eye. Organisms enter the eye through one of the following routes: (a) as a consequence of intraocular surgery (postoperative), (b) following penetrating injury of the globe (posttraumatic), or (c) from haematogenous spread of infection to the eye from a distant anatomical site (endogenous) (Callegan *et al.*, 2002). 90% of postoperative endophthalmitis occurs after cataract surgery (Verbraeken, 1995), as this is the most frequently performed surgical procedure in the UK and US (http://www.dh.gov.uk). Other procedures that can lead to postoperative endophthalmitis include pars plana vitrectomy (Eifrig *et al.*, 2004), penetrating keratoplasty (Taban *et al.*, 2005a), glaucoma filtering procedures (Busbee *et al.*, 2004), strabismus surgery (Recchia *et al.*, 2000), pterygium excision (Farrell & Smith, 1989) and suture removal (Culbert & Devenyi, 1999).

Over 300,000 cataract operations are carried out in England and Wales each year (http://www.dh.gov.uk) and the incidence of endophthalmitis following cataract surgery has been estimated to range from 0.06% to 0.30% (Aaberg *et al.*, 1998; Kamalarajah *et al.*, 2004; Norregaard *et al.*, 1997; Powe *et al.*, 1994; Somani *et*

al., 1997; Taban *et al.*, 2005b; Wejde *et al.*, 2005; West *et al.*, 2005). This gives an estimated 180 to 900 cases of postoperative endophthalmitis after cataract surgery per year. Alarmingly, endophthalmitis rates have increased over the past decade (Taban *et al.*, 2005a), possibly related to more frequent use of clear corneal incisions (Cooper *et al.*, 2003; Nagaki *et al.*, 2003).

1.1.3.2 Clinical findings

Acute postoperative endophthalmitis

Acute postoperative endophthalmitis is classified as endophthalmitis occurring within six weeks of surgery (Kresloff *et al.*, 1998). Ciliary injection, chemosis and lid oedema may be present. If infection spreads to the orbit, restriction of extraocular movement and proptosis may occur. Signs and symptoms of intraocular inflammation include decreased visual acuity, afferent pupillary defect, pain, hypopyon, corneal oedema, corneal infiltrate, fibrin in the anterior chamber, vitreous inflammation, retinitis, and retinal periphlebitis (Figure 1.4 and 1.5) (Kresloff *et al.*, 1998).

Chronic postoperative endophthalmitis

Postoperative endophthalmitis occurring more than six weeks after surgery is classified as chronic (Kresloff *et al.*, 1998). Patients typically present with a persistent low-grade uveitis that may respond to corticosteroids. However, they may also present with the same signs and symptoms as acute postoperative endophthalmitis. A small hypopyon may be visible. The uveitis is commonly granulomatous, usually with vitritis. Fibrin strands may be present in the anterior chamber and keratic precipitates may be seen on the intraocular lens as well as on the corneal endothelium. A white plaque on the posterior capsule, intraocular lens or retained lens particles suggests the diagnosis (Carlson *et al.*, 1998). This plaque contains a mixture of lens material and adherent organisms and may be observed in both bacterial and fungal infections (Chien *et al.*, 1992).

Figure 1.4 Postoperative endophthalmitis.
Image from www.revophth.com/index.asp?page=1_13822.htm

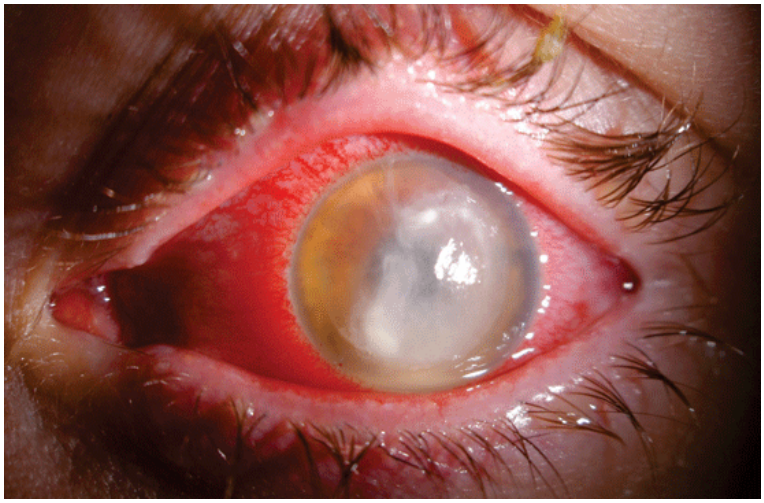
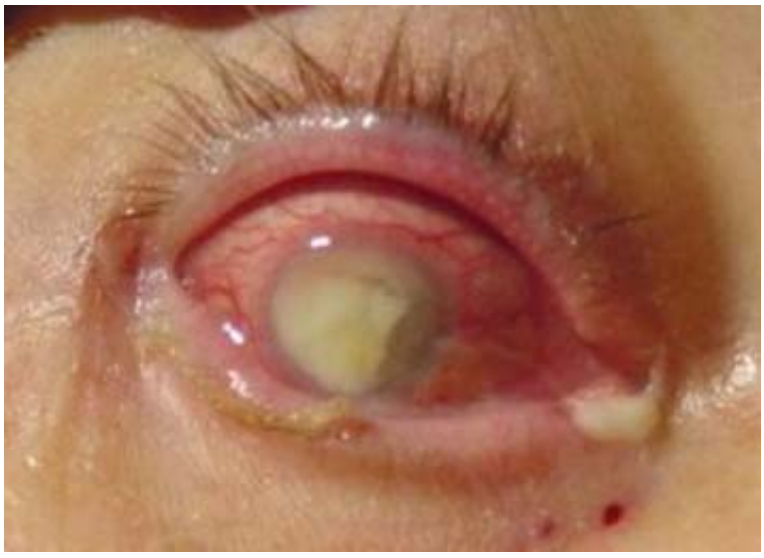


Figure 1.5 *Serratia marcescens* endophthalmitis with scleral injection, corneal oedema and hypopyon (Williams *et al.*, 2006).



1.1.3.3 Causative organisms

The ocular surface and adnexa (e.g. eye lids, lacrimal sac and conjunctiva) are thought to be the main sources of organisms for postoperative endophthalmitis (Bannerman *et al.*, 1997; Kresloff *et al.*, 1998; Speaker *et al.*, 1991). Molecular techniques showed that the external bacterial flora matched the bacteria isolated from vitrectomy specimens in 14 of 17 (82%) cases of postoperative endophthalmitis (Speaker *et al.*, 1991). Outbreaks or clusters of endophthalmitis cases may have originated from external sources, such as contaminated surgical instruments, irrigation solutions, donor corneas, tubing, intraocular lenses, airborne contaminants, and viscoelastics (Arsan *et al.*, 1996; Roy *et al.*, 1997).

Acute postoperative endophthalmitis

The Endophthalmitis Vitrectomy Study (EVS) demonstrated that of the 69% of patients with culture-positive endophthalmitis, 70% were infected with coagulase-negative staphylococci (mostly *S. epidermidis*), 10% with *S. aureus*, 9% with *Streptococcus* spp., 2% with *Enterococcus* spp., 3% with other Gram-positive spp. and 6% with Gram-negative spp. (Doft, 1991; Han *et al.*, 1996). *P. acnes* and fungi, commonly associated with delayed-onset infections, may also cause acute postoperative endophthalmitis (Stern *et al.*, 1989; Winward *et al.*, 1993).

Chronic postoperative endophthalmitis

P. acnes is the most common cause of chronic postoperative endophthalmitis, followed by *S. epidermidis* (and other coagulase-negative *Staphylococcus* spp.), fungi (mainly *Candida* spp.), anaerobic *Streptococcus* spp., *Actinomyces* spp., and *Nocardia asteroids* (Fox *et al.*, 1991; Menikoff *et al.*, 1991; Rao *et al.*, 1991; Roussel *et al.*, 1991; Zimmerman *et al.*, 1993).

1.1.3.4 Risk factors

Preoperative risk factors include blepharitis, conjunctivitis, canaliculitis, dacryocyctitis, lacrimal duct obstruction, contact lens wear, ocular prosthesis in

the fellow orbit (Morris *et al.*, 1993), immunosuppression, diabetes mellitus (Johnson *et al.*, 1997; Phillips & Tasman, 1994), upper respiratory tract infections (Wilson, 1987), atopic dermatitis and keratoconjunctivitis sicca (Sunaric-Megevand & Pournaras, 1997). Intraoperative risk factors include suboptimal lid and conjunctival disinfection, prolonged length of surgery of more than sixty minutes, vitreous loss, use of prolene haptic intraocular lenses, and ocular penetration during ocular surface surgery (Menikoff *et al.*, 1991). Postoperative risk factors include wound leak/dehiscence, inadequately buried sutures, suture removal, vitreous incarceration in the surgical wound, and the presence of a filtering bleb (Katz *et al.*, 1985; Wolner *et al.*, 1991).

1.1.3.5 Prophylaxis

If risk factors for developing postoperative endophthalmitis are identified prior to surgery then it is important to control or treat these beforehand. Some ophthalmologists routinely perform conjunctival cultures preoperatively on patients with risk factors for developing endophthalmitis, although their value is debatable (Hara & Hoshi, 1996; Starr, 1983).

It has been shown that preoperative administration of topical 5% povidone-iodine solution into the conjunctival sac significantly reduced the incidence of endophthalmitis as compared to silver protein solution (Speaker & Menikoff, 1991). Povidone-iodine has been shown to be effective against bacteria, fungi, viruses, protozoa and spores (Boes *et al.*, 1992). Prophylactic preoperative topical antibiotics are commonly used even in the absence of risk factors. This is based on the fact that a reduction in the number of organisms after a 1- or 2-day preoperative antibiotic course have been observed (Burns & Oden, 1972; Shapiro, 1982). More recently, it has been shown that topical moxifloxacin may provide protection against *S. aureus* endophthalmitis in a rabbit model (Kowalski *et al.*, 2004). However, prophylactic use of topical antibiotics is expensive, may lead to antibiotic resistance and can induce allergic reactions.

Subconjunctival antibiotics administered postoperatively may bypass the corneal epithelial barrier, allowing diffusion of high concentrations of the drug into the corneal stroma and aqueous humour. However, vitreous concentrations remain low. Current clinical data remain inconclusive, despite experimental evidence that subconjunctival injections of ceftazidine, gentamicin, and ciprofloxacin prevent endophthalmitis after cataract extraction (Ng *et al.*, 1996).

1.1.3.6 Conventional diagnosis

Once infectious endophthalmitis is suspected, urgent diagnosis and prompt therapeutic management is essential in preventing severe visual loss. Conventional diagnosis relies on obtaining intraocular fluid (aqueous humour and vitreous humour) for culture and Gram stain. Vitreous samples yield positive cultures more often than aqueous samples (Barza *et al.*, 1997; Forster, 1978; Forster *et al.*, 1980). Nonetheless, aqueous samples have been found to be the sole source of positive culture in a minority of cases (Barza *et al.*, 1997; Mollan *et al.*, 2007). Therefore, combination of aqueous and vitreous sample is required to provide the optimal diagnostic yield (Mollan *et al.*, 2007). In chronic postoperative endophthalmitis, intraocular plaques should also be cultured.

Aqueous material can be obtained with a 30-gauge needle attached to a tuberlin syringe through a limbal stab incision into the anterior chamber, and 0.1-0.2ml of fluid aspirated. Vitreous specimen may be obtained by vitreous needle tap using a 27-22 gauge needle attached to a tuberlin syringe or by vitreous biopsy with a vitrectomy probe attached to a tuberlin syringe. 0.1-0.3ml of vitreous is then removed from the anterior vitreous cavity (Kresloff *et al.*, 1998; Lemley & Han, 2007).

Intraocular fluids should be directly inoculated on blood agar, chocolate agar, and thioglycolate broth, and incubated at 37ºC (Speaker & Menikoff, 1993). Samples should also be inoculated on Sabouraud's agar and incubated at 25ºC to isolate fungi. Aerobic and anaerobic blood culture bottles may also be inoculated with

vitreous samples, as they provide results comparable to those with direct solid media inoculation or membrane filtering systems (Joondeph *et al.*, 1989). Chronic endophthalmitis cases should be maintained for a minimum of two weeks to grow anaerobes and fungi. Samples should also be placed on glass slides for Gram and Giemsa stain. Although smears should be used to guide initial therapy, specific treatment should be dictated by culture results, as only approximately two thirds of smears in culture-positive cases are consistent with the organism cultured (Durfee & Smith, 1982; Forster *et al.*, 1980). Results of antibiotic susceptibility testing of any cultured organism is essential for appropriate antimicrobial treatment (Kresloff *et al.*, 1998).

A positive culture is determined by growth of the same organism on two different media, confluent growth on a solid medium, or any growth in an anaerobic medium (Forster *et al.*, 1980; Hibberd *et al.*, 1991; Speaker *et al.*, 1991). Current culture techniques may yield a positive result in approximately 22% to 30% of aqueous humour (Auclin *et al.*, 2001; EVSG, 1995; Fisch *et al.*, 1991; Ng *et al.*, 2005) and 40% to 69% of vitreous humour (Donahue *et al.*, 1993; EVSG, 1996; Han *et al.*, 1999; Kunimoto *et al.*, 1999a; Ng *et al.*, 2005; Sharma *et al.*, 1996). This low detection rate may reflect low microbial count, sterilisation of infection by host defenses (Meredith *et al.*, 1990), sequestration of organisms in phagocytes or retained lens material, infection by fastidious organisms, or sterile inflammation (Speaker & Menikoff, 1993).

Electron microscopy may be used to visualise microorganisms within phagocytes and to identify organisms (Sawusch *et al.*, 1989). Ultrasound visualisation of the globe should be carried out if significant opacification of media prevents adequate view of the fundus. Vitreous opacities secondary to vitritis and chorioretinal thickening are consistent with a diagnosis of endophthalmitis. The presence of retinal or choroidal detachment, dislocated lens material, or retained foreign bodies should be excluded on ultrasound examination (Lemley & Han, 2007).

1.1.3.7 Treatment

Acute postoperative endophthalmitis

Injection of intravitreal antibiotics is the mainstay of treatment for acute postoperative endophthalmitis. The intraocular concentration of antibiotics after intravitreal injection is greater than that achieved by any other modality, such as topical and subconjunctival (Baum *et al.*, 1982; EVSG, 1995). Because rapid initiation of therapy is essential for the successful treatment, antibiotics must be initiated before culture results are available. Vancomycin is considered the drug of choice for the Gram-positive organisms, including methicillin-resistant *Staphylococcus* spp. and *Bacillus cereus* (EVSG, 1995). Aminoglycosides (gentamicin and amikacin) have traditionally been recommended for Gram-negative coverage. However, several clinical and laboratory reports have shown that aminoglycosides are toxic to the retina and retinal pigment epithelium (RPE) at doses close to therapeutic (Bennett & Peyman, 1974; Campochiaro & Conway, 1991; Campochiaro & Lim, 1994; D'Amico *et al.*, 1985; McDonald *et al.*, 1986). In view of this, ceftazidime has now been recommended as an alternative antibiotic to cover Gram-negative organisms because of its broad therapeutic index, lower risk of retinal toxicity, and *in vitro* antimicrobial activity, which is as effective as the aminoglycosides against Gram-negative bacteria (Lemley & Han, 2007; Mochizuki *et al.*, 1992; Roth & Flynn, 1997). Sensitivities of Gram-positive bacteria to vancomycin and Gram-negative bacteria to ceftazidime have been shown to be 99% and 100% respectively in the US (Recchia *et al.*, 2005). In India, however, susceptibilities of Gram-negative bacteria to amikacin and ceftazidime have been observed to be only 68% and 63% respectively (Anand *et al.*, 2000) In suspected cases of vancomycin-resistant enterococcus endophthalmitis, ampicillin or linezolid have been suggested as alternatives (Lemley & Han, 2007; Roth & Flynn, 1997). If no clinical improvement occurs or if worsening is noted within 48 to 72 hours, repeat vitreous tap and injection of antibiotics (plus pars plana vitrectomy) should be considered, as a single intravitreal injection is not always adequate.

In addition to intravitreal antibiotics, subconjunctival and topical antibiotics are often used in the treatment of postoperative endophthalmitis. The aim is to increase the number of routes of antibiotic delivery to increase the likelihood of achieving high concentrations of antibiotics within the eye and also to achieve higher anterior segment concentrations than are obtainable with intravitreal injections alone (Barza, 1989). However, several studies have shown no additional benefit with subconjunctival antibiotics when given in combination with intravitreal antibiotics (Iyer *et al.*, 2004; Smiddy *et al.*, 1989). Topical antibiotics are able to penetrate the anterior chamber much more efficiently than the vitreous (Costello *et al.*, 2006; Solomon *et al.*, 2005).

Intravenous and oral antibiotics have been advocated in the past as adjunct treatments of postoperative infectious endophthalmitis, although controversy exists as to their benefit (Davis, 1996; Engelbert *et al.*, 2004; Hariprasad *et al.*, 2002; Hariprasad *et al.*, 2003; Hariprasad *et al.*, 2006). Despite the fact that the blood-ocular barrier may not be intact in an inflamed eye, it is unclear whether intravitreal antibiotic levels are adequate after intravenous administration (Pflugfelder & Flynn, 1992). If intravenous therapy is considered necessary, vancomycin or cefazolin may be appropriate choice for Gram-positive coverage, and ceftazidime for Gram-negative coverage (Kresloff *et al.*, 1998).

To reduce the destructive effect of the host inflammatory response to the infection in endophthalmitis, many ophthalmologists use systemic, topical, subconjunctival, and intravitreal corticosteroids in combination with antibiotics (Maxwell *et al.*, 1991; Yoshizumi *et al.*, 1998). However, as the evidence for corticosteroid benefit is limited, its use should be carefully considered, especially in patients with contraindications (Das *et al.*, 1999; Shah *et al.*, 2000). Vitrectomy (surgical cutting and aspiration of vitreous contents and replacement with balanced salt solution) has several potential advantages including reduction of infecting organisms and their toxins, removing vitreous membranes that could lead to retinal detachment and improving intraocular antibiotic distribution. However, the absence of vitreous might also predispose to higher levels of drug toxicity as well as drug clearance. Controversy still exists as to whether therapeutic vitrectomy is

necessary in all cases of endophthalmitis and most ophthalmologists tend to reserve therapeutic vitrectomy for severe cases (Kresloff *et al.*, 1998).

Chronic postoperative endophthalmitis

Treatment of chronic postoperative endophthalmitis involves administration of intravitreal antibiotics. If inflammation is not severe, antibiotic therapy can be delayed until smear, culture, and sensitivity data are available from aqueous and vitreous specimens (Kresloff *et al.*, 1998). However, if inflammation is severe, then management is the same as for acute postoperative endophthalmitis. Surgical removal of vitreous infiltrate and partial capsulectomy, including areas of white capsular plaque, is sometimes required (Aldave *et al.*, 1999). Removal of the intraocular lens and total capsulectomy may be necessary to completely eliminate the infection and prevent recurrent inflammation (Aldave *et al.*, 1999; Kresloff *et al.*, 1998; Winward *et al.*, 1993; Zambrano *et al.*, 1989).

1.2 *Propionibacterium acnes*

1.2.1 Background

Propionibacterium acnes (*P. acnes*), *Propionibacterium avidum* (*P. avidum*), *Propionibacterium granulosum* (*P. granulosum*) and *Propionibacterium propionicus* (*P. propionicus*) belong to the human cutaneous propionibacteria (Perry & Lambert, 2006). In the past, *P. acnes* has been designated as *Bacillus acnes*, *Corynebacterium acnes* (Marples & McGinley, 1974) and *Corynebacterium parvum* (Eady & Ingham, 1994).

P. acnes is a Gram-positive, non-sporulating pleomorphic rod that forms part of the normal skin microbiota, but also inhabits the conjunctiva, oral cavity, external ear canal, respiratory tract, genitourinary tract and large intestine (Brook & Frazier, 1991; Funke *et al.*, 1997; Ingham, 1999). *P. acnes* is capable of growing under aerobic conditions but it is treated as an anaerobe in the clinical setting as anaerobic conditions are necessary for optimal isolation from specimens (Cove *et al.*, 1983). *P. acnes* is still frequently dismissed as a contaminant when isolated from clinical samples due to the perception that it has a very low pathogenic potential and, therefore, low capacity to cause infection (Jakab *et al.*, 1996). This view now appears incorrect as increasing numbers of reports are linking the organism to a range of serious conditions including postoperative endophthalmitis (Aldave *et al.*, 1999), keratitis (Jones & Robinson, 1977; Perry *et al.*, 1982; Wong *et al.*, 2003; Zaidman, 1992), endocarditis (Brook, 2002; Pan *et al.*, 2005; Vandenbos *et al.*, 2001), osteomyelitis (Sulkowski *et al.*, 1994), central nervous system infections (Beeler *et al.*, 1976; Ramos *et al.*, 1995; Richards *et al.*, 1989), fatal bacterial granuloma after trauma (FBGT) (Gao *et al.*, 2002), septic arthritis (Yocum *et al.*, 1982), prosthetic hip infections (Tunney *et al.*, 1999), SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis and osteitis) (Kirchhoff *et al.*, 2003), sarcoidosis (Eishi *et al.*, 2002; Moller & Chen, 2002; Yasuhara *et al.*, 2005), prostate cancer (Cohen *et al.*, 2005) and periodontal and dental infections (Goldberg, 1971; Le Goff *et al.*, 1997). *P. acnes* also plays an important role in

the pathogenesis of the common skin condition acne vulgaris (Leyden *et al.*, 1998).

The genome of *P. acnes* strain KPA171202 (GenBank accession no. AE017283, Gottingen Genomics Laboratory, www.g21.bio.uni-goettingen.de) is currently the only completely sequenced species of the genus *Propionibacterium* (Bruggemann *et al.*, 2004). Based on recent *recA* and *tly* phylogenetic analyses, this strain was identified as a type IB strain (see Section 1.2.2) (McDowell *et al.*, 2005). The genome consists of a single circular chromosome of 2,560,265 basepairs, which encodes approximately 2,333 genes (Figure 4.1) (Bruggemann *et al.*, 2004). 1,587 of these genes have been given a predicted function based on significant identity to database entries. Publication of the *P. acnes* genome sequence, as well as more recent studies, have further highlighted the pathogenic potential of the organism by identifying enzymes involved in tissue degradation, putative toxins, immunogenic proteins and phase variable cell surface proteins (Bruggemann *et al.*, 2004; Lodes *et al.*, 2006; Valanne *et al.*, 2005).

1.2.2 Population biology of *P. acnes*

Johnson and Cummins first identified two distinct phenotypes of *P. acnes*, known as types I and II, based on serological agglutination tests and cell wall sugar analysis (Johnson & Cummins, 1972). They demonstrated that the cell walls of the type I strains contained galactose, glucose, and mannose whereas those of type II strains only contained glucose and mannose (Johnson & Cummins, 1972). No difference in the GC content between types I and II, which ranged from 57% to 60% were observed (Johnson & Cummins, 1972). Fermentation typing (Higaki *et al.*, 2000; Kishishita *et al.*, 1979; Pulverer & Ko, 1973), bacteriophage typing (Webster & Cummins, 1978), and immunofluorescence with polyclonal antisera (Holmberg & Forsum, 1973) have all been used to discriminate between *P. acnes* types I and II.

Random amplification of polymorphic DNA (RAPD) carried out on 46 *P. acnes* isolates revealed two distinct genomic profiles (Perry *et al.*, 2003). Although the type status of these isolates was not identified, RAPD profiles from the type I (NCTC 737) and type II (NCTC 10390) reference strains, did match the two RAPD lineages. Amplified-fragment length polymorphism (AFLP) analysis has been used to study the molecular relationship of *Propionibacterium* spp. responsible for contamination of platelet concentrates (PCs) and red blood cell concentrates (RBCs) (Mohammadi *et al.*, 2005). Based on the AFLP profiles, isolates of PCs and the corresponding RBCs were grouped into three main clusters, designated groups 1, 2 and 3 (Mohammadi *et al.*, 2005). It was not determined whether these groups correlated with the different *P. acnes* types.

Analysis of the *P. acnes recA* gene and a more variable haemolysin/cytotoxin (*tly*) gene sequence has demonstrated that types I and II represent distinct phylogenetic lineages, although the 16S rRNA gene sequences of these groups share 99.8-99.9% identity (McDowell *et al.*, 2005). Furthermore, type I strains have been subdivided into groups IA and IB based on *recA* and *tly* sequences, monoclonal antibody (MAb) typing and pulsed-field gel electrophoresis (PFGE) profiles (Cohen *et al.*, 2005). PFGE has also been used to investigate the phylogenetic relationship between *P. acnes* isolates with different antibiotic resistance profiles (Oprica *et al.*, 2004; Oprica *et al.*, 2005; Oprica & Nord, 2005). Recently, a novel lineage of strains, known as type III, has been described (McDowell *et al.*, 2008). Type III isolates display an atypical cellular morphology for *P. acnes*, with long slender filaments more characteristic of *P. propionicus* and *Actinomyces* spp. The 16S rRNA gene sequence of type III organisms was found to have 99.8-99.9% identity with those of types I and II (McDowell *et al.*, 2008).

Using RAPD analysis, *P. acnes* isolates from different clinical sources were distributed amongst the RAPD profile types (Perry *et al.*, 2003). However, McDowell *et al.* showed that using *recA* sequencing, *P. acnes* isolates from bone, tissue samples, acne, dental infections and surgical incision sites, were predominantly of type I and those cultured from prosthetic hip implants revealed similar numbers of types I and II strains (McDowell *et al.*, 2005).

1.2.3 Pathogenesis of *P. acnes* infection

P. acnes has a ubiquitous presence on human skin, where it usually resides within sebaceous follicles as a harmless commensal (Bruggemann, 2005). However, there is evidence to show that *P. acnes* has some features of an opportunistic pathogen (Bojar & Holland, 2004; Brook & Frazier, 1991; Csukas *et al.*, 2004; Holland *et al.*, 1998; Huynh *et al.*, 1995; Ingham, 1999; Jakab *et al.*, 1996; Jappe *et al.*, 2002; Koreck *et al.*, 2003; Merlin *et al.*, 2002). *In vitro*, *P. acnes* is slow growing and is capable of surviving for up to eight months under anaerobic conditions, suggesting that it could also survive in human tissues at low oxidation potentials (Csukas *et al.*, 2004). The organism's complex cell wall structure and its surface fibrillar layer render it resistant to phagocytosis by macrophages, where it persists intracellularly (Montes & Wilborn, 1970; Webster *et al.*, 1985).

In addition, *P. acnes* produces several exocellular enzymes (Hoeffler *et al.*, 1977; Ingham *et al.*, 1981; Kabongo Muamba, 1982) and metabolites that can directly damage host tissue (Allaker *et al.*, 1987). Individuals with acne have been found to have elevated levels of *P. acnes* antibodies (Ashbee *et al.*, 1997; Holland *et al.*, 1986; Ingham *et al.*, 1987). *P. acnes*-derived components possess chemoattractant properties (Webster & Leyden, 1980) and the organism itself can activate complement, leading to the release of C5-dependant chemotactic factors (Webster *et al.*, 1978). *P. acnes* has also been reported to induce the pro-inflammatory cytokines, interleukin (IL)-1α. IL-1β, IL-6, IL-8, IL-12 and tumour necrosis factor (TNF) - α (Smith *et al.*, 1993b; Tsuji *et al.*, 1999; Vowels *et al.*, 1995).

Extensive research on the modulation of the immune system by bacteria or their products has shown that *P. acnes* is one of the most potent adjuvants (Ingham, 1999; Jung *et al.*, 2007; Roszkowski *et al.*, 1990). Pre-treatment with heat-killed cells of *P. acnes* provide protection against infection and anti-tumour activity in a variety of animal models (Eady & Ingham, 1994). For this reason, *P. acnes* has been used in therapeutic trials designed to combat malignant tumours (Bardana, 1985; Davies, 1982; DiSaia *et al.*, 1987).

***P. acnes* Biofilm**

Some microorganisms are capable of producing a biofilm after adherence to a surface. A biofilm consists of an aggregation of bacteria that encase themselves within an extracellular polysaccharide lining (Burkhart & Burkhart, 2007). Multiple microenvironments exist within a biofilm, allowing for the same species of bacteria to live in diverse niches with varying rates of metabolism, replication, and responsiveness to antibiotics (Burkhart & Burkhart, 2007). Microorganisms within biofilms, compared to free-floating (planktonic) bacteria, are 50 to 500 times more resistant to antimicrobial therapies (Burkhart & Burkhart, 2007). Once a biofilm is established, the bacteria undergoes a phenotypic shift in behaviour in which genes are up- and down-regulated (Horswill *et al.*, 2007). The existence of bacterial biofilm is a common cause of persistent infections (Costerton *et al.*, 1999), and increased tolerance to antibiotics is a major problem for the management of infectious diseases (Donlan, 2001).

The recently published genome sequence of *P. acnes* (Bruggemann *et al.*, 2004) shows that the organism possesses three clusters of genes that encode enzymes involved in extracellular polysaccharide biosynthesis, suggesting that it is capable of forming extracellular biofilm matrix. Several proteins potentially involved in adhesion were also identified (Bruggemann *et al.*, 2004; Burkhart & Burkhart, 2006). The existence of the *P. acnes* biofilm has been hypothesized (Burkhart & Burkhart, 2003a; Burkhart & Burkhart, 2003b; Burkhart & Burkhart, 2006), but conflicting data on the biofilm-forming ability of *P. acnes* exists. Several studies have shown that *P. acnes* strains can form highly resistant biofilms on various orthopaedic biomaterials *in vitro* (Bayston *et al.*, 2007; Ramage *et al.*, 2003), and the failure of hip replacement surgery is often attributed to *P. acnes* biofilm on the implanted material (Tunney *et al.*, 1998). *P. acnes* recovered from cases of FBGT were also shown to be capable of biofilm production and the investigators suggested that this led to the failure of antibiotic treatment in these patients (Qi *et al.*, 2008). In contrast, Takemura *et al.* showed the inability of *P. acnes* to form biofilms on dental root filling gutta-percha points (Takemura *et al.*, 2004).

1.2.4 *P. acnes* in postoperative endophthalmitis

Clinical features and pathophysiology

Delayed-onset endophthalmitis secondary to the *P. acnes* organism is a well-recognised complication of cataract surgery (Meisler *et al.*, 1986; Roussel *et al.*, 1987; Sawusch *et al.*, 1989). Features of *P. acnes* infection include the presence of a white plaque on the posterior capsule or intraocular lens (IOL), hypopyon and beaded fibrin strands in the anterior chamber, granulomatous-appearing keratic precipitates and vitritis (Aldave *et al.*, 1999; Clark *et al.*, 1999; Meisler & Mandelbaum, 1989; Zambrano *et al.*, 1989).

P. acnes endophthalmitis is thought to be caused by sequestration of the organism between the IOL optic and the posterior capsule and colonies of the organism may be visualised as a white capsular plaque (Meisler *et al.*, 1987; Sawusch *et al.*, 1989; Winward *et al.*, 1993). In this encapsulated state, the organism is shielded from antimicrobial agents and host defence mechanisms. Despite preference for the lens capsule, infection following intracapsular cataract extraction with anterior chamber IOL placement has also been reported (Chien *et al.*, 1992), and white plaques can be seen on the posterior corneal surface (Abrahams, 1989; Chien *et al.*, 1992). In addition, the IOL itself can support the bacteria (Busin *et al.*, 1995; Elder *et al.*, 1995); thus the capsular bag is not the only reservoir for *P. acnes*.

P. acnes has the ability to cause persistent inflammation within the eye due to several unique characteristics. Meisler *et al.* noted a lack of inflammatory cells in the capsule on histopathology, despite the presence of a large number of organisms (Meisler *et al.*, 1987). *P. acnes* is able to inhibit suppressor T-cell lymphocytes (Whitcup *et al.*, 1991) and its cell wall structure protects against degradation by neutrophils and macrophages (Webster *et al.*, 1985). Additionally, *P. acnes* is thought to exert an adjuvant effect in provoking immune response with residual soft lens material (Meisler *et al.*, 1986; Piest *et al.*, 1987; Semel *et al.*, 1992; Smith, 1986).

Diagnostic techniques

Diagnosis of *P. acnes* endophthalmitis can be challenging for several reasons. The intraocular inflammation may be indistinguishable from sterile postoperative inflammation. The organism can be difficult to culture, as it usually resides within the capsular bag, and also because it has a very slow growth rate. Its generation time for doubling is 5.1 hours (Hall *et al.*, 1994) and as a result, it may take up to 10 days to obtain a positive culture (Aldave *et al.*, 1999; Clark *et al.*, 1999). Aqueous and vitreous specimens should undergo Gram staining and cultures should include anaerobic and fungal media (Carlson *et al.*, 1998). Laboratories should be specifically instructed to incubate samples for at least 14 days before discarding them as negative (Carlson *et al.*, 1998; Meisler & Mandelbaum, 1989). To overcome the low culture positive rate, molecular diagnostic tests have been developed to aid the diagnosis of chronic endophthalmitis. These are discussed in more detail in Section 1.4.3.

Treatment

Unlike the conventional management of endophthalmitis, intraocular antibiotics alone are not sufficient to treat *P. acnes* endophthalmitis (Meisler *et al.*, 1986; Zambrano *et al.*, 1989). Clark *et al.* (Clark *et al.*, 1999) and Aldave *et al.* (Aldave *et al.*, 1999) reported on relatively large numbers of patients in retrospective reviews of culture-positive cases. Treatment strategies included injection of intraocular antibiotics (usually vancomycin), pars plana vitrectomy, vitrectomy with partial capsulectomy, and vitrectomy with total capsulectomy and IOL removal or exchange. Both groups demonstrated that injection of intravitreal antibiotics alone was associated with a very high rate of recurrence. Pars plana vitrectomy with partial capsulectomy was more efficacious than vitrectomy alone. Vitrectomy with total capsulectomy and IOL removal or exchange appeared to be curative (Meisler *et al.*, 1987; Winward *et al.*, 1993).

1.2.5 *P. acnes* in other clinical diseases

As well as being a cause of eye infections, *P. acnes* has the potential to cause a range of other diseases. Some of these are discussed below.

***P. acnes* in acne vulgaris**

Acne vulgaris is the commonest skin disorder amongst the adolescent population and affects around 80% of individuals at some stage in their lives (Dreno & Poli, 2003). It is a multifactorial disorder of the pilosebaceous follicles, and disease mechanisms include sebaceous hyperplasia, follicular hyperkeratinisation, hormone imbalance, bacterial infection and immune hypersensitivity (Gollnick, 2003; Jappe, 2003; Thiboutot, 2000; Webster, 2002). *P. acnes* is considered to play a vital role in the pathogenesis of acne vulgaris where it is thought to contribute to the inflammatory phase of the condition (Leyden, 2001). The organism is able to metabolise sebaceous triglycerides, releasing free fatty acids that irritate the follicular wall and surrounding dermis (Coenye *et al.*, 2007). In addition, *P. acnes* can also produce exoenzymes and neutrophils chemoattractants (Kabongo Muamba, 1982; Thiboutot, 2000; Webster, 2002).

Treatment of acne includes keratinolytic and sebosuppressive agents like retinoids (Thiboutot *et al.*, 2008) as well as antibacterial agents. Topical antimicrobial agents include benzoyl peroxide, clindamycin, erythromycin, tetracycline, azelaic acid and triclosan (Bojar *et al.*, 1994; Dreno, 2004; Krautheim & Gollnick, 2004; Tan, 2004; Taylor & Shalita, 2004; Webster, 2000). Systemic antibiotics such as tetracycline, doxycycline, minocycline and erythromycin may be indicated in cases where topical treatment is not successful or in patients at risk of scarring and pigmentary changes (Ross *et al.*, 2003).

Shortly after the introduction of topical erythromycin and clindamycin in acne treatment over 40 years ago, resistant strains were detected in the US (Leyden *et al.*, 1983). Since then, reports of resistant *P. acnes* strains from acne patients have been described around the world (Kurokawa *et al.*, 1988; Oakley *et al.*, 1995;

Ross *et al.*, 2003; Tan *et al.*, 2001). It has been demonstrated that the proportion of patients carrying resistant bacteria in the UK doubled between 1991 and 1997. Consequently 60% of acne patients were found to carry resistant strains (Eady, 1998). A study carried out in Europe showed that 50% of acne patients were colonised by clindamycin and erythromycin resistant *P. acnes* and 20% of patients by tetracycline resistant *P. acnes* (Ross *et al.*, 2003).

SAPHO and sarcoidosis

In patients suffering from SAPHO syndrome, *P. acnes* has been recovered from bone biopsy, synovial fluid and tissue samples (Schaeverbeke *et al.*, 1998). *P. acnes* has also been isolated from lymph nodes of patients with sarcoidosis, a systemic granulomatous disease of unknown aetiology (Eishi *et al.*, 2002; Ishige *et al.*, 1999; Yamada *et al.*, 2002). This is not conclusive evidence as recent findings have shown that *P. acnes* can normally reside in peripheral lung tissue and mediastinal lymph nodes. Therefore their presence is not exclusive to sarcoidosis (Ishige *et al.*, 1999).

Prostate disease

P. acnes was isolated from the prostate gland in a substantial portion (35%) of patients with prostate cancer and an association was demonstrated between positive culture and prostatic inflammation (Cohen *et al.*, 2005). In addition to this, another study reported *P. acnes* 16S rRNA gene sequences as the most common bacterial DNA extracted from prostatic tissue of patients with benign prostatic hyperplasia (BPH) (Alexeyev *et al.*, 2006). Direct visualisation using multi-colour fluorescence *in situ* hybridisation (FISH) assay of *P. acnes* in prostate tissue has revealed that the organism demonstrated intracellular localisation and stromal biofilm-like aggregates (Alexeyev *et al.*, 2007). It has therefore been proposed that infection of the prostate gland by *P. acnes* might be a contributory factor in developing prostatic inflammation, which in turn might contribute to the development of BPH and prostate cancer through cellular injury and proliferation (Shannon *et al.*, 2006b; Shannon *et al.*, 2008).

Prosthetic joint infections

Prosthetic hip, knee and shoulder-associated infections are common indications for revision arthroplasties (Berthelot *et al.*, 2006; Zeller *et al.*, 2007). *P. acnes* has been implicated in up to 22% of revision operations (Tunney *et al.*, 1998). As *P. acnes* grow predominantly within a biofilm on the surface of the prosthesis, which renders them more resistant to antibiotics, treatment with systemic antibiotics usually fail. Therefore, removal and replacement of the infected implant is often necessary to eliminate infection (Ramage *et al.*, 2003; Tunney *et al.*, 2007).

Spinal surgery and sciatica

P. acnes is described as a cause of wound contamination during spinal surgery and immunofluorescence microscopy (IFM) carried out on surgical specimens suggest that *P. acnes* detected in wounds originates from patient skin (McLorinan *et al.*, 2005). It has also been isolated in cases of spinal osteomyelitis and discitis (Chia & Nakata, 1996; Esteban *et al.*, 1998; Harris *et al.*, 2005; Kowalski *et al.*, 2007; Noble & Overman, 1987). Culture of *P. acnes* from microdiscectomy material removed for the treatment of sciatica has lead to an association between the organism and sciatica (Stirling *et al.*, 2001). However, others have failed to culture *P. acnes* from microdiscectomy material and suggest that these organisms were contaminants from the skin during the surgical procedure (McLorinan *et al.*, 2005).

Central nervous system infections

P. acnes is increasingly recognised as a cause of infection after neurosurgical procedures (Brook, 1988; Brook & Frazier, 1991; Nisbet *et al.*, 2007; Ramos *et al.*, 1995; Skinner *et al.*, 1978). Although it is most frequently isolated from infected neurosurgical shunts, it has also been described as a pathogen in a wide range of central nervous system (CNS) infections including brain abscess (Barazi *et al.*, 2003; Berenson & Bia, 1989; Cohle *et al.*, 1981; Maniatis & Vassilouthis, 1980), subdural and epidural empyema (Critchley & Strachan, 1996; Yoshikawa *et al.*, 1975), and meningitis (Beeler *et al.*, 1976; Everett *et al.*, 1976; Schlesinger & Ross, 1977).

Fatal bacterial granuloma after trauma (FBGT)

P. acnes has recently been identified as the causative agent in FBGT (Gao *et al.*, 2002). The major clinical presentations of FBGT patients were the development of skin lesions after slight trauma to the face, followed by spreading of dark-red plaques on the skin and frequently death from encephalitis within 5 years.

Endocarditis

P. acnes has been shown to cause infection of prosthetic heart valves (Gunthard *et al.*, 1994; Hinestrosa *et al.*, 2007; Horner *et al.*, 1992; Pan *et al.*, 2005), native valves (Mohsen *et al.*, 2001) and annuloplasty rings (Vanagt *et al.*, 2004), leading to endocarditis. Prompt diagnosis and treatment of *P. acnes* endocarditis are essential, since the infection tends to follow a very aggressive clinical course resulting in extensive valvular destruction, congestive heart failure, abscess formation, and systemic embolisation (Delahaye *et al.*, 2005; Horner *et al.*, 1992; Huynh *et al.*, 1995; Lazar & Schulman, 1992; Lewis & Abramson, 1980; Mohsen *et al.*, 2001). Many antibiotics, including penicillin, vancomycin, teicoplanin, and gentamicin, are considered active *in vitro* for the treatment of *P. acnes* infections and have been reported to be effective in eliminating infection in endocarditis (Horner *et al.*, 1992; Huynh *et al.*, 1995).

1.3 *Pseudomonas aeruginosa*

1.3.1 Background

Pseudomonas aeruginosa (*P. aeruginosa*) is considered to be the quintessential opportunistic pathogen, because it can infect a broad range of hosts, from amoeba to humans (Lavenir *et al.*, 2007; Pukatzki *et al.*, 2002; Rahme *et al.*, 2000). It is responsible for infection in cystic fibrosis (CF) patients (Saiman & Siegel, 2004), in burned, mechanically ventilated and immunocompromised individuals (McManus *et al.*, 1985; Richard *et al.*, 1994). It can lead to serious infections including septicaemia, pneumonia, endocarditis, otitis, keratitis and endophthalmitis (Eifrig *et al.*, 2003; Jackson *et al.*, 2003; Kashkouli *et al.*, 2007; Kielhofner *et al.*, 1992). This Gram-negative rod displays a wide range of virulence, from weakly virulent isolates, to highly virulent broad spectrum isolates (Lau *et al.*, 2003; Rahme *et al.*, 1995; Rahme *et al.*, 2000) and is capable of producing numerous virulence factors (Lyczak *et al.*, 2000).

P. aeruginosa is also able to grow in a diverse range of environmental niches including rivers (Pellett *et al.*, 1983), sea water (Kimata *et al.*, 2004), bottled and tap waters (Aoi *et al.*, 2000; Ganguli & Tripathi, 1999; Hunter, 1993; Pellett *et al.*, 1983; Romling *et al.*, 1994), wastewaters (Filali *et al.*, 2000) and soil (Cavalca *et al.*, 2000). It can also colonise plants (Green *et al.*, 1974; Morales *et al.*, 1996) and animals (Lashev & Lasarova, 2001; Marlier *et al.*, 2000; Martin Barrasa *et al.*, 2000). Its ability to survive in different environments allow it to colonise several hospital niches, including taps, drains, water pipes and clinical devices. Genotyping studies have demonstrated the close relatedness of clinical and environmental isolates, and infer an environmental origin of the isolates encountered in several infections (Romling *et al.*, 2005). Therefore, hospital and environmental populations of *P. aeruginosa* represent a serious public health concern.

1.3.2 Population biology of *P. aeruginosa*

P. aeruginosa has been considered to have a panmictic population structure (Denamur *et al.*, 1993; Picard *et al.*, 1994). A panmictic-epidemic population structure with frequent recombination events leading to the evolution of highly successful epidemic clones has been favoured by others (Dinesh *et al.*, 2003; Lomholt *et al.*, 2001; Morales *et al.*, 2004; Pirnay *et al.*, 2002; Romling *et al.*, 1994). Lombolt *et al*. analysed the diversity of 11 metabolic enzymes by multilocus enzyme electrophoresis (MLEE) of 145 *P. aeruginosa* isolates from different origins, and obtained an index of association (I_A) (Smith *et al.*, 1993a) value not significantly different from zero, indicating that *P. aeruginosa* has a non-clonal population structure (Lomholt *et al.*, 2001). From the MLEE data of Martin *et al.* on isolates from cystic fibrosis patients in France, an I_A value of -0.07 was calculated, also suggesting a non-clonal population structure (Martin *et al.*, 1999). The same conclusion was reached by Ruimy *et al.* when they analysed 74 clinical and environmental isolates using RAPD (Ruimy *et al.*, 2001). Pirnay *et al.* compared the sequencing results of three outer membrane genes with the results of one DNA-based and two phenotypic typing methods (Pirnay *et al.*, 2002). They observed no congruence between the results obtained by each method, but showed the existence of clonal complex, further supporting the view that the population structure of *P. aeruginosa* is panmictic-epidemic.

Using multilocus sequence typing (MLST) (Section 1.5.2), Kiewitz *et al.* analysed six sequences of 19 isolates from environmental and disease origins and demonstrated that the frequency of recombination among different *P. aeruginosa* genotypes was high (Kiewitz & Tummler, 2000). Curran *et al.* recently developed an MLST scheme to characterise a large collection of clinical and environmental isolates of *P. aeruginosa*, including epidemic clones from the UK and Europe (Curran *et al.*, 2004). The 143 isolates investigated were assigned to 139 different STs. The I_A value for the 139 individual STs was found to be 0.17, indicating that *P. aeruginosa* has a non-clonal population structure. Both Kiewitz and Curran demonstrated no correlations between habitat and particular clones, in line with previous studies (Pirnay *et al.*, 2002; Romling *et al.*, 1994).

These results are in contrast to a more recent *P. aeruginosa* MLST scheme, where different housekeeping genes to previous MLST schemes were sequenced (Vernez *et al.*, 2005). Of the 34 clinical isolates analysed, 26 different sequence types were identified and the I_A was calculated to be 1.95 (standard error 0.24), indicating linkage disequilibrium in the study population and therefore, a clonal population structure. In support of this, Wiehlmann *et al.* (Wiehlmann *et al.*, 2007) recently developed a microarray method for genotyping of *P. aeruginosa* in both the conserved core and flexible accessory genome of *P. aeruginosa*. They analysed 240 isolates from diverse habitats and geographic origin and showed that the majority of *P. aeruginosa* belonged to a few dominant clones widespread in disease and environmental habitats (Wiehlmann *et al.*, 2007). Majority of the loci were freely recombining with each other, but some loci exist in fixed combinations of genotypes, suggesting that the free flow of genes in the *P. aeruginosa* population occurs at most, but not all, loci of the genome.

1.3.3 *P. aeruginosa* in the eye

P. aeruginosa is an important Gram-negative pathogen in the eye and is capable of inducing keratitis and postoperative endophthalmitis (Eifrig *et al.*, 2003; Irvine *et al.*, 1992; Jackson *et al.*, 2003; Kashkouli *et al.*, 2007). *P. aeruginosa* keratitis is a rapidly developing, devastating disease that may lead to corneal perforation, with a higher incidence in extended wear contact lens users (Mondino *et al.*, 1986; Rattanatam *et al.*, 2001; Wilhelmus, 1996), tropical climates, and in patients that are either debilitated or hospitalised (Laibson, 1990). Studies to identify the source of the infection in contact-lens induced keratitis have shown that *P. aeruginosa* cultured from the cornea, conjunctiva, contact lens case and cleaning solution all had identical PFGE banding patterns (de Melo *et al.*, 2007). Pseudomonal infections often present as a rapidly progressing, suppurative stromal infiltrate with mucopurulent exudates. Yellowish coagulative necrosis surrounded by inflammatory epithelial oedema can be seen and stromal ulceration can lead to significant stromal tissue destruction and loss. A hypopyon is usually present and a ring infiltrate may be seen in the surrounding paracentral cornea. Descemetocele formation or corneal perforation may also result from *P.*

aeruginosa infection (Wilhelmus, 1996). Other sight-threatening complications include development of secondary glaucoma and cataract (Lotti & Dart, 1992). These consequences are due to the hosts' inflammatory response, the influence of bacterial toxins, exoproducts and toxicity from antibiotic treatment, as well as the effects the of corticosteroids (Chusid & Davis, 1979; Hazlett *et al.*, 1977; Hyndiuk, 1981; Kernacki *et al.*, 1995; Moon *et al.*, 1988; Ohman *et al.*, 1980; Steuhl *et al.*, 1987; Thiel *et al.*, 1987). The incidence of antibiotic resistant strains has also started to increase (Chaudhry *et al.*, 1999; Landman *et al.*, 2002).

1.3.4 Pathogenesis of *P. aeruginosa* infection

P. aeruginosa has the ability to adapt to hostile environments and survive on limited nutrients. Because of this, it has a wide environmental and ecological distribution (Lomholt *et al.*, 2001). This versatility is probably due to a substantial collection of enzymes combined with a fit gene regulation (Kiewitz & Tummler, 2000; Stover *et al.*, 2000).

P. aeruginosa has only limited binding affinity to healthy corneal epithelial cells. Binding to and colonisation of the cornea occur predominantly after tissue damage, when exposed stroma uncover receptors for bacterial adhesion (Hazlett *et al.*, 1986; O'Brien, 2003; Stern *et al.*, 1982). Initial adhesion to contact lenses or the cornea may be brought about by lipoplolysaccharide (LPS, endotoxin) and pili (Fletcher *et al.*, 1993; Gupta *et al.*, 1994; Gupta *et al.*, 1997; Zaidi *et al.*, 1996). Type IV pili-mediated twitching motility and flagella-associated motility are important and allow bacteria to move in liquids or along surfaces (Hahn, 1997; Semmler *et al.*, 1999; Zolfaghar *et al.*, 2003). Bacterial flagella also appear to be involved in the invasion of cells by this microorganism (Fleiszig *et al.*, 2001).

The proteases elastase, alkaline protease, and protease IV are associated with virulence, extensive tissue damage, invasiveness, dissemination, and colonisation, and are able to enhance the destruction of corneal tissue (Engel *et al.*, 1998a; Engel *et al.*, 1998b; Hobden, 2002; O'Callaghan *et al.*, 1996; Pillar & Hobden,

2002; Zhu *et al.*, 2001). Protease IV destroys certain host proteins (Engel *et al.*, 1998a) while elastase and alkaline protease mediate penetration through the corneal epithelium (Bejarano *et al.*, 1989; Burns *et al.*, 1990; Heck *et al.*, 1986; Hobden, 2002; Howe & Iglewski, 1984; Twining *et al.*, 1986).

Another determinant of virulence is the type III secretion system (TTSS), present in several Gram-negative bacilli (Galan & Collmer, 1999). Using the TTSS, *P. aeruginosa* is able to produce and secrete virulence factors directly into the cytoplasm of host cells (Ajayi *et al.*, 2003). The system consists of three different protein complexes: the secretion apparatus, the translocation or targeting apparatus, and the secreted toxins (effector proteins) and their cognate chaperones (Hueck, 1998). The effector proteins comprise two ADP-ribosylating enzymes, ExoS and ExoT (Frank, 1997; Frithz-Lindsten *et al.*, 1997; Yahr *et al.*, 1996), an acute cytolytic factor, ExoU (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998), and an adenylate cyclase, ExoY (Yahr *et al.*, 1998).

The genes that encode these effector proteins, which are distributed throughout the *P. aeruginosa* genome, are characterised by variable traits (Stover *et al.*, 2000). The TTSS has been identified in nearly all clinical and environmental isolates but individual isolates and populations of isolates from distinct disease sites appear to differ in their effector genotype (Feltman *et al.*, 2001). Although *exoY* and *exoT* are present in nearly all clinical *P. aeruginosa* isolates, the majority possess only *exoS* or *exoU*, not both (Feltman *et al.*, 2001; Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998). *P. aeruginosa* strains have been classified based on the genotypic expression of these toxins. Invasive strains tend to express ExoS (Fleiszig *et al.*, 1997), whereas non-invasive or cytotoxic strains express ExoU (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998).

1.4 Molecular Diagnostics

1.4.1 Background

The inability of conventional diagnostic protocols to provide rapid and accurate data for identifying pathogens causing microbial keratitis and endophthalmitis leads most ophthalmologists to adopt a conservative management approach by using empiric antibiotic therapy. However, this may potentially lead to added costs, increased rates of antimicrobial resistance and iatrogenic complications associated with unnecessary treatment. A rapid and accurate diagnostic assay is therefore essential to provide early and effective management of infected patients.

Polymerase chain reaction (PCR) technology is perhaps the most important advancement in bacterial molecular diagnostics in recent years. PCR has the ability to amplify a specific target region of DNA exponentialy using oligonucleotide primers, leading to detectable amounts of DNA from one or a few original sequences. Due to their high sensitivity, specificity, and speed of application, PCR-based diagnostic methods can be applied to clinical specimens for identifying organisms that cannot be grown *in vitro* or where existing culture techniques are insensitive or need prolonged incubation times. It also has the advantage of being unaffected by prior treatment with antibiotics unlike culture methods. In these instances, empiric antibiotic therapy that may not be effective can be altered based on PCR results (Song, 2005).

One of the most common approaches to using PCR in rapid diagnostics is the use of broad-range PCR, which is based on the observation that certain genes are universal to many different organisms. Studies have mainly focused on the 16S rRNA genes (Wilson *et al.*, 1990), which are found in all bacteria. These genes have highly variable portions that are considered unique for each bacterium as well as conserved regions that are found in all bacteria. Multiplex PCR (Chamberlain *et al.*, 1988) simultaneously amplifies more than one locus in the same reaction by using multiple sets of primers. Nested PCR (Erlich *et al.*, 1991) uses a second round of PCR with a different primer set internal to the first round,

leading to increased amplification of a specific region within the first PCR-amplified target locus. This is much more specific and sensitive than conventional PCR, allowing the detection of the target DNA at a substantially lower concentration. Reverse transcriptase PCR (RT-PCR) convert RNA to cDNA using the enzyme reverse transcriptase before PCR and has the advantage of determining gene activity (Erlich *et al.*, 1991). Quantitative real-time PCR is a major advancement in PCR diagnostic technology as it eliminates the need for post-amplification processing (i.e. gel electrophoresis) conventionally required for amplicon detection, and allows real-time measurement of DNA synthesis (Heid *et al.*, 1996; Higuchi *et al.*, 1992; Higuchi *et al.*, 1993).

The high sensitivity of PCR-based protocols and their ability to amplify millions of copies of the target sequence come with disadvantages. The principal problems include false-positive results from DNA contamination, the potential for false-negative results, detection sensitivity exceeding clinical significance, and limited ability to detect multiple targets simultaneously (Song, 2005).

1.4.2 Molecular diagnostics in microbial keratitis

Although there have been numerous studies looking at using the PCR technique to detect fungal- (Bagyalakshmi *et al.*, 2008; Ghosh *et al.*, 2007; Kumar & Shukla, 2005) and Acanthamoeba- (Goldschmidt *et al.*, 2008; Lehmann *et al.*, 1998; Mathers *et al.*, 2000) associated keratitis, few exist which look at bacterial keratitis. Available publications have suggested that PCR of 16S rDNA may be a useful diagnostic tool for severe cases of bacterial keratitis (Knox *et al.*, 1998; Lohmann *et al.*, 2000b; Rudolph *et al.*, 2004). Knox *et al.* showed that PCR matched culture results in a series of eleven patients with bacterial keratitis (Knox *et al.*, 1998). However, sensitivity proved to be low as two cases with culture positive results were negative for PCR. Lohman *et al.* reported a series of 16 patients with keratitis that included 10 patients with negative culture results (Lohmann *et al.*, 2000b). PCR was found to be positive for nine of these 10 patients. Rudolph *et al.* investigated a small series of four patients diagnosed with severe infectious keratitis (Rudolph *et al.*, 2004). They used primers to amplify

the 16S rRNA gene, followed by sequencing of the amplified product. Culture results for all cases were negative but PCR detected a causative organism in all four cases.

1.4.3 Molecular diagnostics in endophthalmitis

Several investigators have looked at using PCR to detect the causative organism in endophthalmitis. Therese *et al.* designed primers to target the 16S rRNA gene from aqueous and vitreous samples in culture-negative endophthalmitis cases (Therese *et al.*, 1998). They were able to determine a bacterial cause in 100% of culture-positive and 44% of culture-negative cases. Of the remaining culture-negative cases, one third was found to be fungal. They did not, however, determine the specific organism detected by the PCR. The primary drawback of using PCR directed at the 16S rRNA gene has been the labour and time involved in determining the bacterium responsible for a positive PCR product. To overcome this, the amplicon has been subjected to DNA sequencing (Knox *et al.*, 1999; Lohmann *et al.*, 1998). Although this technique yields a definitive identification of the causative organisms, sequencing of PCR products requires specialised equipment and can be costly.

Carroll *et al.* used nested PCR primers to distinguish between Gram-positive and Gram-negative 16S rRNA gene (Carroll *et al.*, 2000). The test was highly sensitive and could be completed in 3.5 hours. Okhravi *et al.* investigated restriction endonuclease digest fingerprinting of the 16S rRNA gene (Okhravi *et al.*, 2000b). They were able to distinguish the major bacterial causes of postoperative endophthalmitis, but were unable to distinguish between *Escherichia coli* (*E. coli*) and *Serratia marcescens* (*S. marcescens*). DNA sequencing was required to identify these two bacteria. The sensitivity of the assay was a single organism as determined by nested PCR detection of serial tenfold dilutions of genomic DNA and viable organisms (Okhravi *et al.*, 2000b). Application of this protocol to nine eyes with a clinical diagnosis of bacterial endophthalmitis was carried out (Okhravi *et al.*, 2000a). A PCR product was amplified from all 18 intraocular samples, whereas only 55.6% of the vitreous

(n=9) and 11.1% of the aqueous (n=9) samples were culture-positive. Chiquet *et al.* recently carried out a large prospectively study on one hundred patients with clinical diagnosis of acute postoperative endophthalmitis (Chiquet et al., 2008). 16S rDNA amplification and DNA sequencing of aqueous humour and/or vitreous humour from tap or vitrectomy was carried out. The detection rate was not significantly different between cultures and PCR on aqueous and vitreous tap samples (38.2% for cultures versus 34.6% for PCR in aqueous humour samples; 54% versus 57% in vitreous tap samples). However, from vitrectomy samples, after intravitreous injection of antibiotics, PCR detected an organism in 70% of the cases, and only 9% of cases had positive culture results.

Delayed-onset postoperative endophthalmitis is commonly caused by *P. acnes*, *S. epidermidis*, *Actinomyces israelli* (*A. israeli*), and fungi. It presents even further diagnostic challenges as these organisms are frequently present in low numbers and can be difficult to culture. Hykin *et al.* used a nested PCR approach to amplify the 16S rDNA from vitreous samples of patients with clinical delayed-onset postoperative endophthalmitis (Hykin *et al.*, 1994). Seventeen (74%) of 23 cases gave positive results with universal eubacterial primers and eight of 23 samples gave positive results with *P. acnes* primers. However, four (14%) of 29 non-infected cases also gave positive results with the universal eubacterial primers, giving an acceptably high false-positive rate. Lohmann *et al.* used 16S ribosomal primers and fungal PCR primers, in parallel with culture and stain for 25 eyes with delayed-onset endophthalmitis (Lohmann *et al.*, 2000a). They were unable to culture any organisms from aqueous samples and 24% of vitreous samples were positive by culture. In contrast, PCR identified a causative organism in 84% of the aqueous samples and 92% of the vitreous tap samples.

1.4.4 *P. acnes* molecular diagnostics

Dasen *et al.* first published a PCR and sequencing method to identify cutaneous and dairy propionibacteria (Dasen *et al.*, 1998). PCR amplification of *P. acnes* 16S rRNA gene has also been used to identify *P. acnes* in the vitreous humour of sarcoidosis patients (Yasuhara *et al.*, 2005), from an abscess caused by a vascular

prosthesis (Le Page *et al.*, 2003) and from prosthetic hip infections (Tunney *et al.*, 1999). Nakamura *et al.* used primers to target the 16S rRNA and lipase genes to discriminate between *P. acnes*, *P. avidum* and other *Propionibacterium* spp. (Nakamura *et al.*, 2003). Buggage *et al.* used a nested PCR approach to detect *P. acnes* in the vitreous sample of a patient with chronic postoperative endophthalmitis (Buggage *et al.*, 2003). Recently, Lai *et al.* published a case of chronic postoperative endophthalmitis, in which PCR amplification of the 16S rRNA gene fragment of *P. acnes* was used to detect *P. acnes* from the aqueous humour sample and the explanted intraocular lens (Lai *et al.*, 2006). Shannon *et al.* recently developed a PCR-based technique for identifying *P. acnes* types IA, IB and II (Shannon *et al.*, 2006a). Three sets of primer pairs were used to analyse bacterial isolates cultured from urine specimens and facial skin, which showed high sensitivity and specificity to the three *P. acnes* subtypes.

1.4.5 *P. aeruginosa* molecular diagnostics

Several PCR screening tests have been used to identify *P. aeruginosa*. Most significant examples are those targeting 16S rDNA, *toxA, gyrB*, and the ribosomal operon (Kimata *et al.*, 2004; Kurupati *et al.*, 2005; Song *et al.*, 2000; Tyler *et al.*, 1997; Widmer *et al.*, 1998). Some of these protocols were shown to be applicable directly on human samples like skin DNA (De Vos *et al.*, 1997). These gene targets have also been used for FISH detection of *P. aeruginosa* in sputum (Hogardt *et al.*, 2000), in quantitative real-time PCR on blood (Jaffe *et al.*, 2001) and wound biopsy samples (Pirnay *et al.*, 2000). Supression subtraction hybridisation has been used to detect strain-specific sequences. PCR assays were then designed to detect epidemic strains commonly found in CF patients (Panagea *et al.*, 2003; Smart *et al.*, 2006).

Lavenir *et al.* investigated the reliability of the most widely used PCR screenings for *P. aeruginosa* (Lavenir *et al.*, 2007). PCR protocols using the following DNA targets were evaluated: (1) the 16S rDNA, (2) the internally transcribed 16S-23S rDNA spacer (ITS), (3) *gyrB* encoding the DNA gyrase subunit B, (4) *oprI* encoding the lipoprotein I, (5) *oprL* endcoding an outer-membrane peptidoglycan-

associated lipoprotein, (6) *fliC* encoding the C-terminus flagellin, and (7) *toxA* encoding the exotoxin A precursor. With the exception of *toxA* which is *P. aeruginosa*-specific, most of these targets have also been found in other bacterial species (Khan & Cerniglia, 1994). Sensitivity of the PCR assays was determined on a panel of 59 *P. aeruginosa* isolates and their specificity was estimated on a panel of 15 *Pseudomonas* spp. closely related to *P. aeruginosa*. The *P. aeruginosa oprI, oprL,* 16S rDNA and *fliC* PCR assays were found to be highly sensitive, giving the expected PCR products for all *P. aeruginosa* isolates, which is in line with previous findings (De Vos *et al.*, 1997; Qin *et al.*, 2003; Spilker *et al.*, 2004). However, they showed specificity as low as 13%. PCR screening making use of *gyrB* and the 16S-23S rDNA ITS gave the expected PCR products for all *P. aeruginosa* strains of their panel (100% sensitivity), and did not yield any products for the other *Pseudomonas* spp. tested (100% specificity). The *toxA* PCR screening did not yield PCR products for all the *P. aeruginosa* strains tested, a characteristic also observed by Khan and Cerniglia (Khan & Cerniglia, 1994). Lavenir *et al.* went on to design a PCR assay targeting the *ecfX* gene, which encodes an ECF (*e*xtracytoplasmic *f*unction) sigma factor (*ecfX*) and is restricted to *P. aeruginosa* (Lavenir *et al.*, 2007). The sensitivity and specificity testing on 59 *P. aeruginosa* strains and 15 *Pseudomonas* spp. showed 100% sensitivity and specificity. This high sensitivity was further supported by investigating an additional set of 241 clinical *P. aeruginosa* strains of various origins. PCR products of the expected size were obtained for all 241 isolates, suggesting that the *ecfX* gene is highly conserved among *P. aeruginosa*.

DNA microarray for detecting *P. aeruginosa*, using the species-specific 15mer oligonucleotide probes based on the sequences of the 23S rDNA has recently been developed (Keum *et al.*, 2006). Two hundred and ten clinical specimens were examined and *P. aeruginosa* was successfully identified from 25 out of 26 clinical specimens, and the remaining 184 specimens containing other bacterial species were negative, resulting in a sensitivity of 96.2% and a specificity of 100%.

1.5 Bacterial population biology

1.5.1 Methods of strain identification

The characterisation of bacterial pathogens using typing methods allows identification of bacterial isolates and provides information about the evolutionary biology, population biology, taxonomy, ecology, and genetics of bacteria (Wiehlmann *et al.*, 2007). Traditional typing of isolates by serological or other methods (antibiotic resistance, phage typing, etc.) are not necessarily suitable for bacterial epidemiology because it is based on variable phenotypes that may not be related to genetic descent (Achtman, 2002). In addition, phenotypically based typing methods are also limited by their typeability and reproducibility. These shortcomings have led to the development of typing methods based on the microbial genotype or DNA sequences, which try to minimise these problems (Olive & Bean, 1999). Examples of these methods are discussed below and Figure 1.6 compares the laboratory steps involved in some of these techniques.

1.5.1.1 Genotyping methods

Plasmid profile analysis

This technique involves the extraction of plasmid DNA and then separation of this DNA by electrophoresis on agarose gels (Trindade *et al.*, 2003). It is an easy technique to carry out and interpret, but it does have limitations. Plasmids are mobile extrachromosomal elements that can be lost or acquired with relative ease by bacteria. Therefore, epidemiologically related isolates can actually have different plasmid profiles. The reproducibility of the plasmid profiles can also be variable as plasmids exist in different spatial conformations (supercoiled, nicked, and linear), which possess different migration velocities when submitted to gel electrophoresis (Hartstein *et al.*, 1995). Both the reproducibility and discriminatory power of plasmid profile analysis can be improved by carrying out enzymatic restriction of the plasmids, as this procedure identifies the position and frequency of restriction sites between two unrelated plasmids, even though they might have the same molecular mass (Hartstein *et al.*, 1995).

Analysis of chromosomal DNA after restriction endonulease analysis (REA)

This technique involves digestion of chromosomal DNA by restriction endonucleases and then separation of the resulting fragments by agarose gel electrophoresis. Profiles of different isolates can then be compared (Trindade *et al.*, 2003).

Restriction fragment length polymorphisms (RFLP)

In this technique, digestion with REA is followed by separation of chromosomal DNA into a series of fragments of different sizes. Southern blot hybridisation is then carried out, where fragments are separated by electrophoresis or nylon membrane and hybridised using specific nucleic acid probes (Busch & Nitschko, 1999; Maslow *et al.*, 1993). Alternatively, the specific locus to be examined is amplified with gene-specific primers and then subjected to RFLP analysis (PCR-based locus-specific RFLP) (Olive & Bean, 1999).

Pulsed-field gel electrophoresis (PFGE)

PFGE involves the digestion of bacterial DNA with restriction endonucleases that recognise only a few sites along the chromosome, generating large fragments of DNA (Schwartz & Cantor, 1984). During electrophoresis, the orientation of the electric field across the gel is periodically changed, allowing these large DNA fragments to be effectively separated (Maslow *et al.*, 1993; Olive & Bean, 1999). As a result, PFGE allows for the comparison of simpler chromosomal DNA profiles than those produced by high-frequency restriction endonucleases (Weller, 2000).

PFGE has several advantages. It can be used to type most bacterial species and generally has excellent discriminatory power (Bannerman *et al.*, 1995; Olive & Bean, 1999; Tenover *et al.*, 1994; van Belkum *et al.*, 2001). In addition, PFGE results have been correlated with clinical data and standardisation of protocols and interpretation of PFGE has allowed extensive databases to be set up (Blanc *et al.*, 2001; Chung *et al.*, 2000; McDougal *et al.*, 2003; Murchan *et al.*, 2003; Tenover

et al., 1995). There are, however, limitations for the use of PFGE, such as the lengthy time taken to obtain results and the high cost of reagents and specialised equipment required (Ross *et al.*, 2005; Weller, 2000). There are also problems in the interpretation of results, especially between laboratories, as even small differences in electrophoresis conditions can alter the distance travelled by each band (Cookson *et al.*, 1996; van Belkum *et al.*, 1998).

Amplified fragment length polymorphism (AFLP)

This method involves simultaneous restriction-ligation and selective PCR amplification (Savelkoul *et al.*, 1999; Vos *et al.*, 1995). Chromosomal DNA is first digested with two restriction enzymes, and then double-stranded oligonucleotide adapters are ligated to the DNA fragments. Subsequently, adapter-specific primers are used for selective PCR amplification.

Random amplified polymorphic DNA (RAPD)

RAPD assays make use of short random sequence primers, which hybridise with sufficient affinity to chromosomal DNA at low annealing temperatures. If two primers anneal within a few kilobases of each other in the correct orientation, a PCR product is generated. The frequency and position of these random primer sites vary for different strains. Separation of the amplification products by agarose gel electrophoresis produces a pattern of bands, characterised by the particular bacterial strain (Caetano-Anolles *et al.*, 1991; Meunier & Grimont, 1993; Welsh & McClelland, 1990; Williams *et al.*, 1990).

The advantage of RAPD is its relative simplicity and speed. However, the discriminatory power is variable and largely depends on the number and nucleotide sequence of the primers used (Saulnier *et al.*, 1993; Struelens *et al.*, 1993). As the primers are not directed against any specific genetic locus, the priming events can sometimes be the result of imperfect hybridisation between the primer and the target site. Thus, the technique is extremely sensitive to small

changes in the annealing temperature, leading to variability in the banding patterns (Meunier & Grimont, 1993; Welsh & McClelland, 1990).

Repetitive-sequence-based PCR (Rep-PCR)

This is a rapid typing method that amplifies the regions between the non-coding repetitive sequences in bacterial genomes (Versalovic *et al.*, 1991). The sizes of these sequences are unique to each bacterial strain, and therefore the sizes of the amplicon may be different among different strains (Healy *et al.*, 2005; Versalovic *et al.*, 1991). Rep-PCR was found to be much quicker than PFGE for typing MRSA isolates, but it was also less discriminatory than PFGE (Ross *et al.*, 2005).

1.5.2 Multilocus sequence typing

Multilocus sequence typing (MLST) is a nucleotide sequence-based method enabling comparative characterisation of bacterial isolates at the molecular level (Maiden *et al.*, 1998). Since its development in 1998 for *Neisserria meningitidis*, it has been applied to many pathogenic and environmental organisms (Maiden, 2006). MLST is essentially a replacement for MLEE, which identifies variation within multiple core metabolic (housekeeping) genes on the basis of differing electrophoretic mobilities of the gene products (Selander *et al.*, 1986). The major problem with MLEE, and other gel-based methods such as PFGE, is that comparison of results between laboratories it is often difficult and unreliable. This is not a problem with MLST because variation within housekeeping genes is indexed directly by the nucleotide sequencing of internal gene fragments. The resolving power of MLST is determined by the number and the type of genes analysed, the length of the sequenced gene fragment and the level of diversity within the sample being characterised (Cooper & Feil, 2004).

1.5.2.1 The Design of MLST Schemes

Most MLST schemes employ 400 to 600 bp allele fragments, which was originally chosen as the length of nucleotide sequence that could be reliably

produced on a single run of the gel-based automated sequencing machines used in the mid-1990s. This still has advantages in terms of increased speed and reduced costs using current nucleotide sequence determination by the dideoxy chain termination method (Maiden, 2006). The number of genes included in an MLST scheme is usually limited to between 6 to 10. If more resolution is required, such as for outbreak cases which require high-resolution typing, it is more efficient to include genes subject to positive selection and therefore exhibiting more diversity, than to include more housekeeping gnes (Feavers *et al.*, 1999).

The increasing availability of complete genome sequences of bacterial organisms has greatly enhanced the identification of appropriate MLST loci and the design of oligonucleotide primers for PCR amplification and sequencing. Housekeeping genes are chosen because they encode proteins that are under stabilising selection to conserve metabolic function, but at the same time sufficiently diverse to identify multiple variants within the target population (Maiden *et al.*, 1998). Ideally, separate primers should be used for the PCR amplification and the sequencing reaction, the latter nested within the amplified fragment. This approach produces better-quality sequences and therefore more reliable data. In addition, primers should be designed so that the same annealing temperature can be used for all loci for a particular MLST scheme. This emperical step is time-consuming but benefits in the long run when an MLST scheme is used in large-scale epidemiological surveillance or population studies (Maiden, 2006).

Once sequencing data is obtained, it is translated into a universal nomenclature scheme for storing and interpretation (Maiden & Spratt, 1999; Urwin & Maiden, 2003). For each locus, the allele fragment is assigned a unique but arbitrary number in order of discovery (Figure 1.7). For each isolate, therefore, a code made up of numbers for each of the loci is constructed. These codes are referred to as an allelic profile and each individual allelic profile is given a sequence type (ST) number.

1.5.2.2 Databases and analysis tools

An important component of MLST is the availability of databases accessible to public health and research communities (Chan *et al.*, 2001; Jolley *et al.*, 2004). The websites available for the storage and access of MLST data include http://pubmlst.org/, http://www.mlst.net/ and http://web.mpiib-berlin.mpg.de/mlst/. Two approaches are generally used to interpret MLST data: those that determine relationships on the basis of allelic profiles and STs and those that compare nucleotide sequences directly. Both have strengths and weaknesses and the most appropriate method required is determined by the structure of the bacterial population under investigation (Maiden, 2006).

1.5.2.3 Epidemiological and clinical applications

In the clinical setting, MLST has been applied directly to clinical specimens providing both diagnostic value and epidemiological data (Birtles *et al.*, 2005; Diggle *et al.*, 2003; Kriz *et al.*, 2002). It has the advantage of being more rapid and accurate than other diagnostic and characterisation methods that require the isolation of the organism in culture.

MLST data can also provide useful epidemiological information about bacterial organisms. For example, it has been used to confirm that most cases of meningococcal disease are caused by a subset of genetic types, the so-called hyperinvasive lineages (Caugant, 1998; Maiden *et al.*, 1998; Yazdankhah *et al.*, 2004). These studies have also shown that the population of meningococci strains cultured from the nasopharynx is much more diverse than disease-causing strains.

Figure 1.6 Comparison of the steps involved in various molecular typing methods. Adapted from Olive and Bean 1999 (Olive & Bean, 1999).

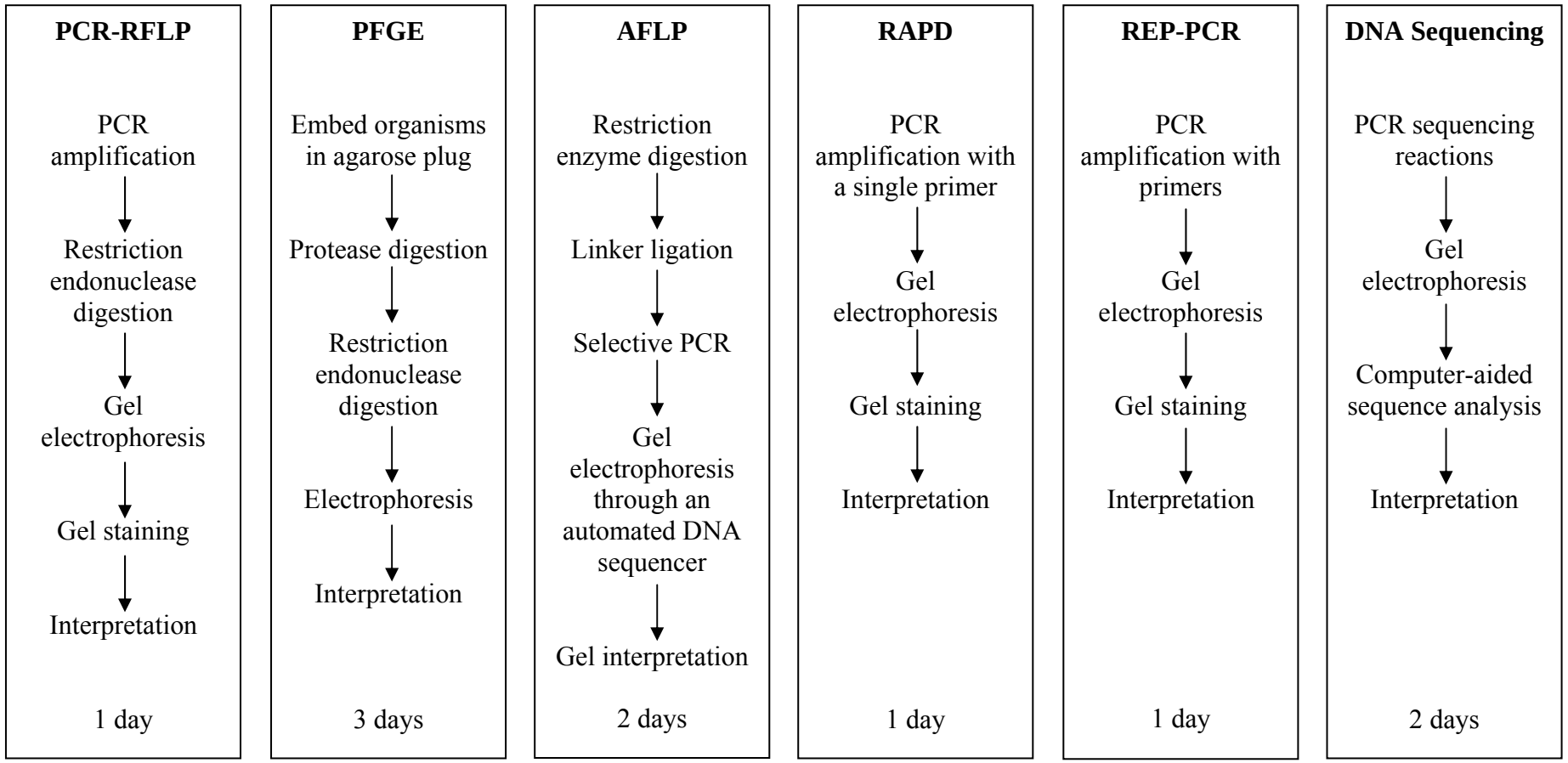
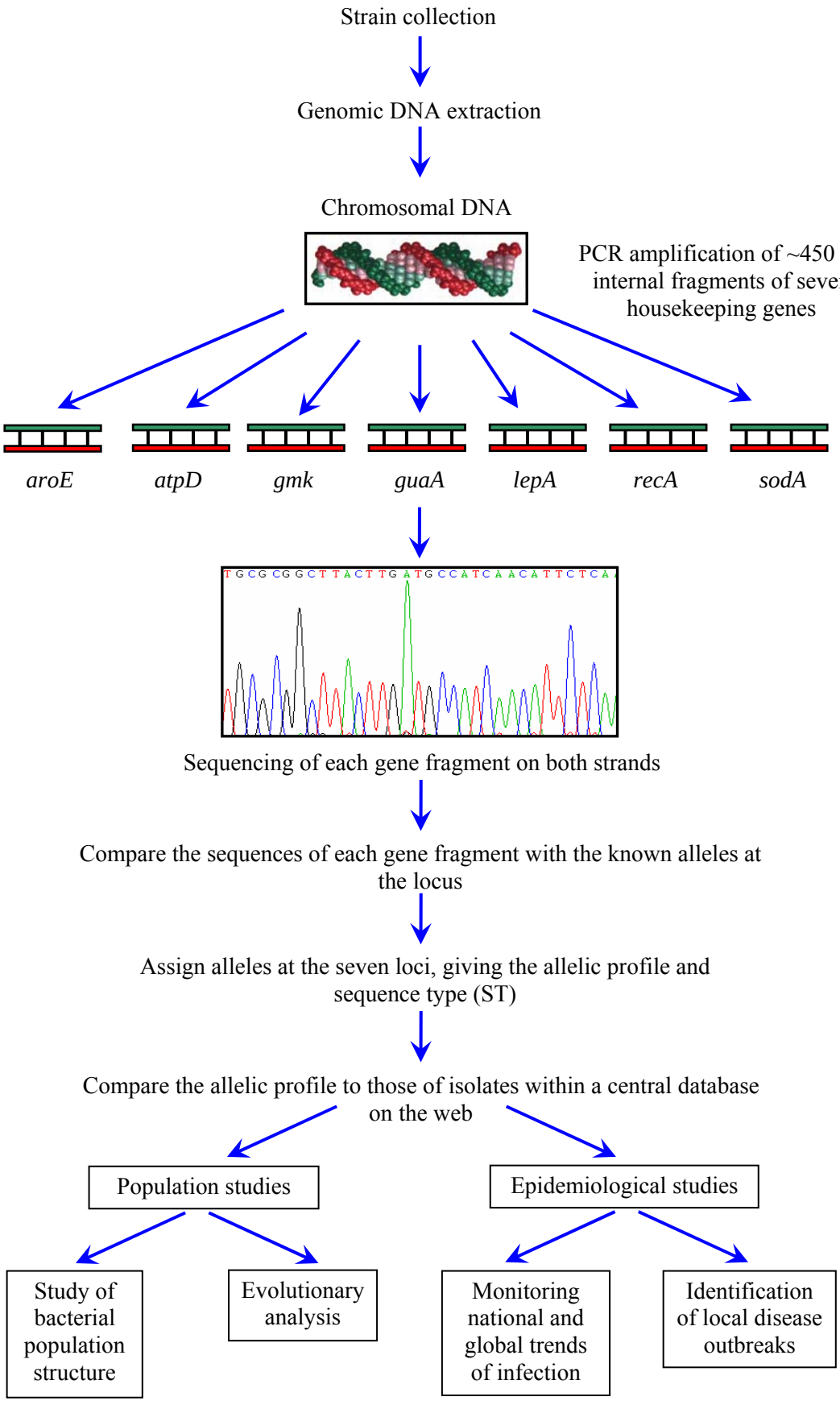


Figure 1.7 Flow diagram of the steps in the multilocus senquence typing (MLST) protocol. Adapted from Spratt 1999 (Spratt, 1999) and Urwin and Maiden 2003 (Urwin & Maiden, 2003).



1.6 Aims and Objectives

Microbial keratitis and endophthalmitis are severe sight threatening eye infections. Prompt diagnosis of the causative organism and treatment with the appropriate topical antimicrobial is essential in providing the best visual outcome. Current culture methods are inadequate in both sensitivity and time taken to obtain culture results. Therefore, a rapid molecular diagnostic test with high sensitivity and specificity is required. In order to tailor the diagnostic assays to eye infections, we need to determine whether microorganisms from eye infections differ from those of other infection sites using a reliable and robust typing system. The main aims of this research project were as follows:

1. To identify the common organisms that cause microbial keratitis in a large tertiary eye centre in the West Midlands, UK.

2. To study the population biology of *P. acnes* and *P. aeruginosa* cultured from eye infections using multilocus sequence typing (MLST). No previous MLST scheme had been developed for *P. acnes,* so a novel scheme had to be set up. This was then used to characterise the population structure of *P. acnes* strain using a diverse collection of strains from eye infections, acne infections and other invasive diseases. The MLST scheme was validated using immunofluorescence microscopy (IFM) and random amplification of polymorphic DNA (RAPD). MLST was also used to type *P. aeruginosa* from eye infections from the UK and China using an existing MLST scheme.

3. To develop rapid polymerase chain reaction (PCR) based diagnostic tests that could be incorporated into routine clinical microbiology to identify the organisms commonly associated with eye infections. These tests would allow faster results and higher pick up rate compared to conventional culture based methods.

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CHAPTER 2 MATERIALS AND METHODS

2.1 Design of *P. acnes* MLST scheme

2.1.1 Chromosomal mapping and selection of genes

Potential housekeeping loci were identified within the *P. acnes* KPA171202 genome sequence (GenBank accession no. AE017283) (Bruggemann *et al.*, 2004) using Artemis genome viewer and annotation software (http://www.sanger.ac.uk/Software/Artemis/). In the selection of candidate loci, a number of criteria were used. Firstly, only genes encoding putative housekeeping products were considered, rather than genes that may come under positive selective pressure such as those encoding putative virulence factors, antibiotic resistance or cell surface antigens. Secondly, if any candidate genes were located near mobile genetic elements they were avoided. Thirdly, genes were at least 500 bp in length so that universal nested primers could be designed to conserved regions that flank a variable central core. The seven housekeeping genes selected, along with their gene products, PCR product sizes and location within the KPA171202 genome are shown in Table 2.1.

2.1.2 Design of primers for gene amplification and nucleotide sequencing

Primer 3 v0.3.0 (http://frodo.wi.mit.edu) was used to design the amplification and sequencing primers for the MLST scheme. The following parameters were set:

Product size range:	1000-2,000 bp	(for amplification)	
	400-600 bp	(for sequencing)	
Primer size:	18 bp		
Primer temp.:	Min. 50ºC	Optimal. 58 ºC	Max. 65ºC
Max. temp. difference:	1ºC		
GC content:	Min. 50%	Optimal. 55%	Max. 60%

Table 2.1 Primer sequences for the amplification and sequencing of the seven housekeeping genes used in the *P. acnes* MLST scheme

Housekeeping Gene	Putative gene product	PPA number[a]	Fragment location[b]	Amplicon size (bp)	Primer sequences (5` → 3`)	
					Amplification (For/Rev)	Sequencing (For/Rev)
aroE	Shikimate 5-dehydrogenase	PPA 1181	1288538-1288961	762	GTGATTGGCCATCCAGTG/ CGCTGTGGACCTCAAAAC	GGGCTATCAGTGACGATG/ GATCTTCAGCACGCCTTA
atpD	ATP synthase beta chain	PPA1239	1346325-1346777	1261	AATTACCCCCGAGACGAA/ CGTGTTCTGGGACAGGAA	TAAGGGTCACGTCTGGAA/ ACATCGCGGAAGTACTCA
gmk	Guanylate kinase	PPA1190	1296456-1296855	444	TAGCCATCCGGAGATCGT/ GCGCAACTGCGTGATCTA	AGATCGTCGTTTCCAGGT/ ACAACGGCGTCAAATTC
guaA	GMP synthase	PPA1764	1926561-1927040	1384	TCGCCTTCATGGAACAAC/ CCATAAGTACGCCCGTCA	GCGTTTGAAGACGTTGAG/ GCTGGTCAGCATTGAGAC
lepA	GTP-binding protein	PPA0901	982280-982731	1218	TCGCGCCCAGTACTTAGA/ CGGATTTCCACTCGATCA	GTCAAGGATGTCCGTCAA/ GCAGGACTGAGAATGGTG
recA	Recombinase A protein	PPA1012	1095604-1096066	877	GCTCTATCATGCGCCTTG/ GCAACATCCGGGTTACCT	GGGGTCGATACAGATTCC/ TGTCAAACTCTGCCTGCT
sodA	Superoxide dismutase	PPA1818	1983249-1983696	488	TGGAACTGCACCATGACA/ GCTAACGACGTTCCACCA	ACAAGCACCACAACACCT/ TAACGTAGTCGGCCTTGA

[a] Locus tag number for KPA171202 genome

[b] Position in KPA171202 genome (base pair)

Table 2.2 Primer sequences for the amplification and sequencing of the seven housekeeping genes used in the *P. aeruginosa* MLST scheme

Housekeeping Gene	Putative gene product	PPA number[a]	Fragment location[b]	Amplicon size (bp)	Primer sequences (5` → 3`)	
					Amplification (For/Rev)	Sequencing (For/Rev)
acsA	Acetyl coenzyme A synthetase	PPA0887	970624-971013	842	ACCTGGTGTACGCCTCGCTGAC/ GACATAGATGCCCTGCCCCTTGAT	GCCACACCTACATCGTCTAT/ AGGTTGCCGAGGTTGTCCAC
aroE	Shikimate dehydrogenase	PPA0025	26883-27380	825	TGGGGCTATGACTGGAAACC/ TAACCCGGTTTTGTGATTCCTACA	ATGTCACCGTGCCGTTCAAG/ TGAAGGCAGTCGGTTCCTTG
guaA	GMP synthase	PPA3769	4226547-4226175	940	CGGCCTCGACGTGTGGATGA/ GAACGCCTGGCTGGTCTTGTGGTA	AGGTCGGTTCCTCCAAGGTC/ GACGTTGTGGTGCGACTTGA
mutL	DNA mismatch repair protein	PPA4946	5551159-5550718	940	CCAGATCGCCGCCGGTGAGGTG/ CAGGGTGCCATAGAGGAAGTC	AGAAGACCGAGTTCGACCAT/ GGTGCCATAGAGGAAGTCAT
nuoD	NADH dehydrogenase I chain C, D	PPA2639	2984590-2984955	1042	ACCGCCACCCGTACTG/ TCTCGCCCATCTTGACCA	ACGGCGAGAACGAGGACTAC/ TGGCGGTCGGTGAAGGTGAA
ppsA	Phosphoenolpyruvate synthase	PPA1770	1915014-1915383	989	GGTCGCTCGGTCAAGGTAGTGG/ GGGTTCTCTTCTTCCGGCTCGTAG	GGTGACGACGGCAAGCTGTA/ GTATCGCCTTCGGCACAGGA
trpE	Anthranilate synthetase component I	PPA0609	671831-672273	811	GCGGCCCAGGGTCGTGAG/ CCCGGCGCTTGTTGATGGTT	TTCAACTTCGGCGACTTCCA/ GGTGTCCATGTTGCCGTTCC

[a] Locus tag number for PAO1 genome

[b] Position in PAO1 genome (base pair)

2.2 Bacterial strains

2.2.1 *P. acnes* strains

A total of 125 *P. acnes* isolates were analysed, including the reference strain NCTC 737 (facial acne isolate) which was obtained from the National Collection of Type Cultures (NCTC; Colindale, UK), and KPA171202 (DSM 16379; contaminated anaerobic culture) which was from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). For the remaining 123 isolates, eight were cultured from brain abscess, bone, lymph node, cadaveric skin and blood samples. A total of 31 isolates were recovered from keratitis (corneal scrape) and endophthalmitis (aqueous and vitreous humour) infections, while 56 isolates were from patients with acne. A further three isolates were recovered from fatal head granulomas, seven from microdiscectomy samples removed during sciatica surgeries and six from dental infections. Five isolates were from surgical skin wounds and seven isolates were recovered from prosthetic hip arthroplasties. The availability of data regarding the time of isolation for all the isolates was limited, but where dates were known they ranged from September 1999 to January 2005.

2.2.2 *P. aeruginosa* strains

A total of 117 *P. aeruginosa* strains from microbial keratitis infections were analysed. 18 isolates were from the Birmingham and Midland Eye Centre, five from Liverpool, ten from Manchester, 30 from Moorfields eye hospital (London), five from Royal Victoria Infirmary (Newcastle), ten from Bristol Royal Infirmary, one from Northwick Park and St. Marks (London) and 38 were from Tong Ren Hospital, Beijing, China. In addition, MLST data on 164 *P. aeruginosa* isolates from other clinical infections and 2 environmental isolates were already available on the *P. aeruginosa* MLST website (http://pubmlst.org/paeruginosa).

2.3 Storage of bacterial strains

A single colony was inoculated onto an appropriate plate and incubated until a patch was produced. The patch was harvested using a sterile swab and resuspended in a 1.5ml centrifuge tube containing 1ml of brain heart infusion (BHI) supplemented with 12% (v/v) glycerol. The cultures were stored at -80ºC until required.

2.4 DNA preparation

2.4.1 *P. acnes* DNA preparation

All bacterial strains were maintained at -80ºC in BHI broth containing 12% (vol/vol) glycerol. Organisms were cultured on BHI and blood agar at 37ºC in an anaerobic cabinet (Mark 3; Don Whitley Scientific, Shipley, United Kingdom) under an atmosphere of 10% H_2, 10% CO_2, 80% N_2. Strains were identified as *P. acnes* by using the API 20A multitest identification system (bioMérieux UK Ltd, Basingstoke, UK) in accordance with the manufacturer's instructions.

For the extraction of chromosomal DNA, *P. acnes* isolates were harvested from agar plates into 500µl of 50mM Tris buffer, pH 8.0 containing 10mM EDTA and 0.8 mg/ml lysozyme (Sigma-Aldrich Company Ltd., Poole, UK) before incubation at 37°C for 30 min. Proteinase K (0.6mg/ml; Invitrogen Life Technologies, Paisley, UK) was then added to the mixture and incubated at 37°C for 30 min before the addition of lauryl sarcosyl (1.6% vol/vol) for 10 min at 30°C. DNA was then obtained following the standard phenol:chloroform extraction and ethanol precipitation protocols previously described and outlined as follows (Sambrook *et al.*, 1989). 500µl of 1:1 of phenol/chloroform was added and mixture inverted and centrifuged at 13,000×*g* for 5 min. The top layer was removed into a clean microcentrifuge tube. Another 500µl of phenol/chloroform was added to the mixture, mixed and centrifuged at 13,000×*g* for 5 min. 400µl of chloroform was then added, mixed well and centrifuged at 13,000×*g* for 5 min. 40µl of 3M sodium acetate (pH 5.2) was added and the solution mixed. 1ml of

ethanol (-20°C) was added and centrifuged at 13,000×*g* for 15 min. The supernatant was removed and 1ml of ethanol (-20°C) was added. The mixture was inverted and then centrifuged at 13,000×*g* for 5 min. The ethanol was then removed with the DNA pellet remaining at the bottom of the 1.5ml tube. The pellet was then air-dried and resuspended in 50µl of sterile H_2O with 1µl of RNase overnight. Extracted DNA was stored at -20°C until needed.

2.4.2 *P. aeruginosa* DNA preparation

Bacterial strains were maintained at -80°C in 12% (v/v) glycerol in BHI broth, streaked to single colonies, and cultured on BHI agar at 37°C under aerobic conditions. Chromosomal DNA was extracted using the Promega Wizard Genomic DNA Purification Kit as follows. A single colony of *P. aeruginosa* was added to 10ml BHI broth and incubated in 37ºC shaker for 16 hours. 1-1.5ml of overnight culture was added to a 1.5ml microcentrifuge tube. This was centrifuged at 16,000×*g* for 2 min. to pellet the cells and the supernatant was removed. 600µl of Nucleic Lysis Solution was added and mixed with a pipette until the cells resuspended. The mixture was incubated at 80ºC for 5 min. to lyse the cells and then cooled to room temperature. 3µl of RNase Solution was added to the cell lysate and inverted 2-5 times to mix. This was incubated at 37ºC for 15-60 min. and then cooled to room temperature. 200µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and vortexed vigorously at high speed for 20 seconds. The sample was incubated on ice for 5 min. and then centrifuged at 16,000×*g* for 3 min. The supernatant containing DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol. The mixture was gently mixed until the thread-like strands of DNA formed a visible mass. The tube was centrifuged at 16,000×*g* for 2 min. The supernatant was carefully poured off and the tube drained on clean absorbent paper. 600µl of room temp 70% ethanol was added and gently inverted to wash the DNA pellet. The tube was centrifuged at 16,000×*g* for 2 min. and the ethanol was aspirated. The tube was drained on clean absorbent paper and air dried for 10-15 min. 50-100µl of DNA Rehydration Solution was added to the tube and incubated overnight at 4ºC. The DNA was stored at -20ºC until required.

2.5 Analysis of DNA by agarose gel electrophoresis

DNA fragments were visualised by gel electrophoresis on agarose gels. The gels were prepared by dissolving agarose (Helena Biosciences Europe, Gateshead, UK) in 1× Tris-acetate-EDTA (TAE) to a final concentration of 1% (w/v). Ethidium bromide (Sigma-Aldrich Ltd, Poole, UK) was added to a final concentration of 0.025µg ml^{-1}. Gels were submerged in 1× TAE and samples containing 20% (v/v) glycerol loading buffer (Sambrook *et al.*, 1989) were loaded alongside 0.5µg of 1 kilobase (Kb) ladder (Fermentas, York, UK). Gels were run at 120V and the DNA was visualised and photographed using a UVI-tec documentation system.

2.6 Quantification of DNA

The amount of DNA was determined using the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.7 Amplification of DNA by Polymerase chain reaction (PCR)

2.7.1 Optimisation of PCR conditions (*P. acnes*)

PCR conditions were optimised by varying the following parameters:

- $MgCl_2$ concentration in PCR reaction mix

Final Mg^{2+} concentration in reaction (mM):	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Required volume of 25mM $MgCl_2$ per 50µl reaction (µl)	0	1	2	3	4	5	6	7

- Presence of 10× Q solution (QIAGEN) in PCR reaction mix
- Annealing temperature (12-step temperature gradient varying from 54°C - 60°C)

2.7.2 PCR reactions (*P. acnes*)

Amplification of DNA by PCR using *Taq* polymerase (QIAGEN, Crawley, UK) was used in the amplification of genes for sequencing. Each 50µl amplification reaction mixture comprised the following mixture:

Reagents	**Volume**	**Final concentration**
DNA (~10ng/µl)	1µl	~0.2ng/µl
Forward primer (10pmol/µl)	2µl	0.4pmol/µl
Reverse primer (10pmol/µl)	2µl	0.4pmol/µl
10× PCR buffer (containing Tris-HCl, KCl, $(NH_4)_2SO_4$, 15mM $MgCl_2$; pH 8.7)	5µl	1×
Deoxynucleotide triphosphates (dNTP) mix (10mM each dATP, dCTP, dGTP and dTTP)	1µl	0.8mM
5× Q solution	10µl	1×
$MgCl_2$ (25mM)	3µl	1.5mM
Taq polymerase (5U/µl)	0.25µl	0.025U/µl
H_2O	25.75	-
Total volume	**50µl**	-

Amplification of DNA by PCR was carried out in 0.2ml centrifuge tubes in an automatic thermocycler (DNA Engine Tetrad^{TM} 2 Peltier Theramal Cycler, Bio-Rad Laboratories, Hemel Hempstead, UK). A negative control was prepared with every set of reactions in which DNA was replaced with H_2O.

Amplification primers were designed to have the same melting temperature (T_m) and the thermal cycler conditions were as follows:

Denaturation	94°C	1 min	1 cycle
Denaturation	94°C	1 min	30 cycles
Annealing	58°C	1 min	
Extension	72°C	2 min	
Final extension	72°C	10 min	1 cycle

All PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gels (Helena Biosciences Europe, Gateshead, UK) containing 1× TAE buffer and ethidium bromide (Sigma-Aldrich Ltd, Poole, UK) to a final concentration of 0.025µg/ml. 5µl of product containing 20% (v/v) of glycerol loading buffer (Sambrook *et al.*, 1989) were electrophoresed alongside 0.5µg of 1Kb ladder (Fermentas, York, UK). Gels were run at 120V and the PCR fragments was visualised and photographed using a UVI-tec documentation system.

The forward and reverse amplification primers and product lengths for the *P. acnes* MLST scheme are shown in Table 2.1.

2.7.3 PCR reactions (*P. aeruginosa*)

The method used was as described previously by Curran *et al.* (Curran *et al.*, 2004). Amplification of DNA by PCR using *Taq* polymerase (QIAGEN) was used in the amplification of genes for sequencing. Each 50µl amplification reaction mixture comprised the following mixture:

Reagents	**Volume**	**Final concentration**
DNA (~10ng/µl)	1µl	~0.2ng/µl
Forward primer (10pmol)	2µl	0.4pmol/µl
Reverse primer (10pmol)	2µl	0.4pmol/µl
10× PCR buffer (containing 15mM $MgCl_2$)	5µl	1×
dNTP mix (10mM each)	1µl	0.8mM
Taq polymerase	0.25µl	0.025U/µl
H_2O	38.75	-
Total volume	**50µl**	-

Amplification of DNA by PCR was carried out in 0.2ml centrifuge tubes in an automatic thermocycler (DNA Engine Tetrad^{TM} 2 Peltier Theramal Cycler, Bio-

Rad Laboratories, Hemel Hempstead, UK). A negative control was prepared with every set of reactions in which DNA was replaced with H_2O.

The thermal cycler was programmed as follows:

Denaturation	96°C	1 min	1 cycle
Denaturation	96°C	1 min	35 cycles
Annealing	55°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	10 min	1 cycle

All PCR products were analysed by electrophoresis as described in Section 2.6. The forward and reverse amplification primers and product lengths for the *P. aeruginosa* MLST scheme are shown in Table 2.2.

2.8 Purification of PCR product

Prior to carrying out automated sequencing it was necessary to remove reagents remaining from the PCR reactions. This was carried out using either the MiniElute 96 UF PCR purification kit (QIAGEN) or the QIAquick PCR purification kit (QIAGEN).

2.8.1 QIAquick PCR purification kit

The method used was as described in the manufacturer's protocol. 5 volumes of Buffer PB was added to 1 volume of the PCR sample and mixed. This was placed in a QIAquick spin column with a 2ml collection tube underneath. To bind the DNA, the spin column was centrifuged at 18,000×*g* for 1 min and the flow-through was discarded. To wash, 0.7ml Buffer PE was added to the QIAquick column and centrifuged at 18,000×*g* for 1 min. The flow-through was discarded and the QIAquick column was placed back in the same tube and centrifuged at 18,000 × *g* for an additional 1 min. The QIAquick column was placed in a clean 1.5ml microcentrifuge tube. To elute DNA, 50µl H_2O was added to the centre of

the QIAquick membrane and centrifuged at 18,000×*g* for 1 min. The purified PCR product was stored at -20°C until required.

2.8.2 MiniElute 96 UF PCR purification kit

The amplified product was purified using MiniElute 96 UF PCR purification kit (QIAGEN) following the manufacturer's protocol before being used in a sequencing reaction. A MiniElute purification plate was placed on top of a vacuum manifold and the PCR samples were pipetted onto the purification plate. A 800mbar vacuum as applied for 45-60 min or until the wells were completely dry. The MiniElute purification plate was placed on absorbent paper to remove any liquid that might remain at the bottom of the plate. 20µl of deionized H_2O was added to each well and the plate was placed on a microplate shaker for 2 min at the recommended speed (600rpm). The purified PCR product was recovered by pipetting the elute out of each well into a clean PCR tube. The purified PCR product was stored at -20°C until required.

2.9 *P. acnes recA* gene amplification and sequencing

PCR amplification and sequencing of the *P. acnes* complete *recA* gene using primers PAR-1 and PAR-2 producing a 1,047 bp amplicon was as described previously (McDowell *et al.*, 2005). Sequences for 46 of the *P. acnes* strains had been previously published (McDowell *et al.*, 2005; McDowell *et al.*, 2008). The complete sequences for a further 11 isolates were determined during the current study.

Complete *recA* amplification and sequencing primers

Primer PAR-1	5`-AGCTCGGTGGGGTTCTCTCATC-3`
Primer PAR-2	5`-GCTTCCTCATACCACTGGTCATC-3`

Reagents	**Volume**	**Final concentration**
DNA (~10ng/µl)	1µl	~0.2ng/µl
Primer PAR-1 (10pmol)	2µl	0.4pmol/µl
Primer PAR-2 (10pmol)	2µl	0.4pmol/µl
10× PCR buffer	5µl	1×
dNTP mix	1µl	0.8mM
Taq polymerase	0.25µl	0.025U/µl
H_2O	38.75	-
Total volume	**50µl**	-

The thermal cycler was programmed as follows:

Denaturation	95°C	3 min	1 cycle
Denaturation	95°C	1 min	
Annealing	55°C	30 sec	35 cycles
Extension	72°C	1.5 min	
Final extension	72°C	10 min	1 cycle

All PCR products were analysed by electrophoresis as described in Section 2.6.

2.10 Automated sequencing

2.10.1 DNA sequencing reaction

Internal nested primers were designed using the same method as for the amplification primers (Table 2.1). Nucleotide sequences were determined with BigDye Terminator Ready Reaction Mix v3.1 (Perkin-Elmer Applied Biosystems, Foster City, USA) under standard sequencing conditions according to the manufacturer's protocol.

Primer (3.2pmol/µl)	1µl
10× Buffer	1.8µl
Big Dye 10× CSA Buffer	1.8µl
BigDye (PE Biosystems, California)	0.5µl
PCR product (20ng/µl)	1µl
Total	**10µl**

The thermal cycler was programmed as follows:

Denaturation	96°C	1 min	1 cycle
Denaturation	96°C	10 sec	
Annealing	55°C	5 sec	35 cycles
Extension	60°C	2 min	

2.10.2 Ethanol precipitation of sequencing reaction

Unincorporated dye terminators were removed by precipitation with alcohol as follows. Place samples in a 96-well plate (ABgene, Epsom, UK). To each 10µl of PCR reaction, 10µl of sterile H_2O and 50µl of 100% Ethanol/0.12M Sodium acetate (EtOH/0.12M NaOAc) were added. The mixture was centrifuged at 500×g for 1 min at room temperature. The sample was incubated at room temperature for 60 min and then centrifuged at 2,800×*g* at 4°C for 60 min. The supernatant was removed and the 96-well plate was inverted and centrifuged on 3mm Whatman paper at 500×*g* for 1 min at 4°C. 150µl of 70% (vol/vol) ethanol (-20°C) was added and centrifuged at 2,800×*g* for 10 min at 4°C. The wash step was then repeated and dried samples were stored at -20°C until analysed by an ABI PRISM genetic analyser.

2.10.3 Sequencing

The reaction products were separated and detected on an ABI PRISM genetic analyser (PE Biosystems) using a standard sequencing module with a Performance Optimised Polymer and 5cm array. The sequences from both strands

of a given locus of the same isolate were aligned, trimmed to the desired length and edited using SeqMan II (DNA Star software, Madison, Wison).

2.11 Allele and Sequence Type (ST) assignment

After sequencing, each distinct allele within a locus was assigned an arbitrary number upon discovery. Each isolate was represented by a combination of seven numbers, the allelic profile, which was assigned a sequence type (ST). Subsequent isolates with an identical allelic profile were assigned the same ST identifier and considered to be isogenic as they were indistinguishable at all loci. Each ST was given an arbitrary number in order of analysis. Data for each isolate was uploaded onto the *P. acnes* MLST website (http://pubmlst.org/pacnes/) developed by Keith Jolley and sited at the University of Oxford, United Kingdom (Jolley *et al.*, 2004).

2.12 Immunofluorescence Microscopy (IFM) of *P. acnes*

2.12.1 Production of Monoclonal Antibodies

Monoclonal antibodies (MAbs) were generated by Dr. Andrew McDowell, using the protocol described previously (Tunney *et al.*, 1999). Four BALB/c mice were immunised with killed whole cells (10^8 CFU/ml) of the *P. acnes* strain AT1 (type I; biotype 3), while a further four BALB/c mice were immunised with a combination of killed whole cells of *P. acnes* strains DW1 and ED2 (type II; biotype 2). Hybridoma cell lines producing *P. acnes*-specific MAbs were cloned by limiting dilution (Harlow & Lane, 1988).

2.12.2 Immunofluorescence Microscopy (IFM)

A modification of the IFM procedure described by Patrick *et al.* (Patrick *et al.*, 1995) was performed on pure cultures. Briefly, bacterial cultures were cultured for 18 hours on anaerobic blood agar or BHI, and a suspension of 10^8 CFU/ml in 0.01M phosphate-buffered saline (PBS, consisting of 0.15M NaCl, 0.0075M Na_2HPO_4, and 0.0025M $NaH_2PO_4 \cdot 2H$) was prepared. Samples (10µl) were then

applied to multiwell slides, air dried, and fixed in 100% methanol for 10 min at -20°C. Undiluted human AB serum (30µl) (Sigma-Aldrich Ltd.) was added as a blocking agent, and the slides were incubated at 37°C for 45 min. The slides were then washed in 0.01M PBS containing 0.5% (vol/vol) AB serum for 20 min. The appropriate undiluted MAb-containing supernatant (30µl) was added to each well of the slides and incubated for 45 min at 37°C. After a wash, as before, wells were incubated with a 1:100 dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma-Aldrich Ltd.) in a 0.1% Evans blue (Merck Sharp & Dome, Hoddesdon, England) counterstain (30µl) for 45 min at 37°C. Slides were then washed and mounted in glycerol-PBS containing an antiphotobleaching agent (Citifluor; Agar Scientific Ltd., Stansted, England), and examined by using a Leitz Dialux 20 fluorescence microscope.

2.13 Random amplification of polymorphic DNA (RAPD) analysis of *P. acnes*

RAPD analysis of *P. acnes* isolates were carried out as described previously (Mahenthiralingam *et al.*, 1996).

Primer 208 was used in the PCR reaction 5`-ACGGCCGACC-3`

After optimisation of conditions, the following reaction mixture was used:

Reagents	**Volume**	**Final concentration**
DNA (~40ng/µl)	1µl	~1.6ng/µl
Primer 208 (10pmol)	4µl	1.6pmol/µl
10× PCR buffer	2.5µl	1×
dNTP mix	0.625µl	1mM
10× Q solution	8µl	1.6×
$MgCl_2$ (25mM)	0.5µl	0.25M
Taq polymerase (5U/µl)	0.2µl	0.04U/µl
H_2O	8.175	-
Total volume	**25µl**	-

Amplification of DNA by PCR was carried out in 0.5ml centrifuge tubes in an automatic thermocycler (DNA Engine TetradTM 2 Peltier Theramal Cycler, Bio-Rad Laboratories, Hemel Hempstead, UK). A negative control was prepared with each set of reactions in which DNA was replaced with H_2O.

The thermal cycler conditions were as follows:

94°C	5 min	
40°C	5 min	4 cycles
72°C	5 min	
94°C	1 min	
40°C	1 min	30 cycles
72°C	2 min	
72°C	10 min	1 cycle

All PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gels (Helena Biosciences Europe, Gateshead, UK) containing 1× Tris-borate-EDTA (TBE) buffer and ethidium bromide (Sigma-Aldrich Ltd, Poole, UK) to a final concentration of 0.025µg/ml. 10µl of product containing 20% (v/v) of glycerol loading buffer (Sambrook *et al.*, 1989) were electrophoresed alongside 0.5µg of 1Kb ladder (Fermentas, York, UK). Gels were run at 120V for 3hrs and the PCR fragments was visualised and photographed using a UVI-tec documentation system.

The RAPD fingerprints were analysed both by eye and also by computer with GelCompar II softare (Applied Maths, Sint-Martens-Latem, Belgium).

2.14 *P. aeruginosa exoS* and *exoU* gene amplification

PCR assays were used to determine the distribution of the TTS genes *exoS* and *exoU* (Ajayi *et al.*, 2003) using the primers shown in Table 2.3 and method as previously described (Winstanley *et al.*, 2005).

Table 2.3 Oligonucleotide primers used for *exoU* and *exoS* PCR amplification

Primer	Sequence	Amplicon size (bp)	Target	Annealing temperature (°C)
ExoS forward	5`-GCGAGGTCAGCAGAGTATCG-3`	118	*exoS*	58
ExoS reverse	5`-TTCGGCGTCACTGTGGATGC-3`			
ExoU forward	5`-CCGTTGTGGTGCCGTTGAAG-3`	134	*exoU*	58
ExoU reverse	5`-CCAGATGTTCACCGACTCGC-3`			

Amplification of DNA by PCR was carried out in 0.2ml centrifuge tubes in an automatic thermocycler (DNA Engine Tetrad™ 2 Peltier Theramal Cycler, Bio-Rad Laboratories, Hemel Hempstead, UK). A negative control was prepared with every set of reactions in which DNA was replaced with H_2O.

Reagents	**Volume**	**Final concentration**
DNA (~10ng/µl)	1µl	~0.4ng/µl
Forward primer (10pmol)	1µl	0.4pmol/µl
Reverse primer (10pmol)	1µl	0.4pmol/µl
10× PCR buffer	2.5µl	1×
dNTP mix	1µl	0.8mM
$MgCl_2$ (25mM)	3µl	3.0mM
Taq polymerase (5U/µl)	0.125µl	0.025U/µl
Dimethyl sulphoxide (DMSO)	0.25µl	-
H_2O	15.125	-
Total volume	**25µl**	**-**

The thermal cycler conditions were as follows:

Denaturation	95°C	1 min	1 cycle
Denaturation	95°C	1 min	30 cycles
Annealing	58°C	1 min	
Extension	72°C	2 min	
Final extension	72°C	10 min	1 cycle

All PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gels (Helena Biosciences Europe, Gateshead, UK) containing 1× TAE buffer and ethidium bromide (Sigma-Aldrich Ltd, Poole, UK) to a final concentration of

0.025µg/ml. 5µl of product containing 20% (v/v) of glycerol loading buffer (Sambrook *et al.*, 1989) were electrophoresed alongside 0.5µg of 1Kb ladder (Fermentas, York, UK). Gels were run at 120V and the PCR fragments was visualised and photographed using a UVI-tec documentation system. Presence of *exoU* and/or *exoS* genes were indicated by the presence of gel bands which correspond to the particular primers used.

2.15 *P. acnes* rapid diagnostics

2.15.1 Target gene selection and primer design

Genes from the MLST scheme was selected as targets for the diagnostics assay. The gene most specific for *P. acnes* and which the amplification primers produced the smallest amplicon was selected.

2.15.2 PCR conditions

First round PCR conditions:

Reagents	**volume per reaction (µl)**
Buffer (Labmaster)	5µl
dNTPs (10mM) (Bioline)	5µl
Mg^{2+} (25mM) (Sigma)	3µl
Q solution (QIAGEN)	10µl
Combined primers (5µM each) (Invitrogen)	1µl
H_2O (Sigma)	5.75µl
Taq polymerase (Supertherm)	0.25µl
P. acne107 DNA (0.001pg/µl DNA)	20µl
Total volume	**50µl**

The thermal cycler conditions were as follows:

Denaturation	96°C	1 min	1 cycle
Denaturation	96°C	1 min	30 cycles
Annealing	58°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	10 min	1 cycle

1µl of first round product was then transferred to a second real-time PCR reaction in a LightCycler® capillary.

Second round PCR conditions:

Reagents	**volume per reaction (µl)**
Biogene master mix (with 2mM Mg)	5µl
Combined primers	1µl
Evagreen (Biotium Inc.)	0.5µl
H_2O	2µl
Mg (25mM)	0.5 µl
Template	1µl
Total volume	**10µl**

The thermal cycler conditions were as follows:

Denaturation	96°C	2 sec	30 cycles
Annealing	58°C	5 sec	
Extension	72°C	10 sec	

2.15.3 DNA extraction methods

A suspension of *P. acnes* cells harvested from an agar plate and a negative control was used to compare three DNA extraction different methods:

1. High Pure Viral Nucleic Acid extraction kit (Roche Ltd, Sussex, UK)

2. DNAMITE® kit (Microzone Ltd, Haywards Heath, West Sussex, UK)

3. Chelex (Bio-Rad Laboratories, Hemel Hempstead, UK) extraction method

1. High Pure Viral Nucleic Acid Kit

Method was as manufacturer's protocol. 25µl of *P. acnes* cell suspension was incubated at 37ºC for 30 min with 25µl of 100mg/ml of lysozyme (Sigma-Aldrich Company Ltd., Poole, UK) and 10µl of 1mg/ml lysostaphin (Sigma-Aldrich Company Ltd.). This mixture was then added to a 1.5ml microcentrifuge tube along with 200µl of Binding Buffer supplemented with Poly (A) and 50µl Proteinase K. This suspension was mixed and incubated for 10 min at 72ºC. The sample was then mixed with 100µl Binding Buffer. The High Pure Filter Tube and the Collection Tube were combined and the sample was pipetted into the upper reservoir. The tube was centrifuged at 8,000×*g* for 1 min. The Filter Tube was placed in a new Collection Tube. 500µl of Inhibitor Removal Buffer was added to the upper reservoir of the Filter Tube and centrifuged again at 8,000×*g* for 1 min. The Filter Tube was reinserted into a new Collection Tube and 450µl of Wash Buffer was added. This wash step was repeated again. The Filter Tube was centrifuged at 13,000×*g* for 10 seconds and the Filter Tube was inserted in a new 1.5ml microcentrifuge tube. 50µl of Elution Buffer was added to the upper reservoir of the Filter Tube and centrifuged for 1 min at 8,000×*g*. The eluted nucleic acid was stored at 2-8ºC for later analysis.

2. DNAMITE® DNA extraction kit

Method was as manufacturer's protocol. *P. acnes* cells harvested from an agar plate was added to a 1.5ml microcentrifuge tube. 0.5ml of Solution LA and 10µl of RNase solution was added to the tube. The mixture was incubated at room temperature for 10 min. 20µl of Proteinase K solution was then added and the mixture was incubated at 60ºC for 10 min. After incubation, 30µl of Solution PA was added and the mixture was inverted to mix. The microcentrifuge tube was centrifuged at the manufacturer's recommendation of 10,000rpm for 5 min. 450µl of the supernatant was transferred to a new tube containing 450µl of Solution CA and the mixture was inverted to mix. This was left at room temperature for 5 min and then centrifuged at 13,000rpm for 7 min to pellet the DNA. The supernatant was then removed with a pipette. The DNA pellet was rehydrated with 50µl of TE buffer and left for 30min or overnight.

3. Chelex DNA extraction method

A total of 25µl of *P. acnes* cell suspension was added to 150µl of 10% (w/v) chelex (Bio-Rad Laboratories, Hemel Hempstead, UK) in distilled water. This mixture was incubated at 100ºC on a heating block for 20 min, vortexing half way through the incubation period. At the end of the incubation period, the chelex was pelleted by centrifugation at 8,000×*g* and the supernatant was removed and retained for PCR assay.

2.16 Bioinformatics

2.16.1 Genome sequences

The complete genome sequence of *P. acnes* KPA171202 used for the design of the MLST scheme was located at the following website: http://www.tigr.org

2.16.2 Homology searches

To investigate sequence similarity of sequences identified to those in the databases the following website was used: http://www.ncbi.nlm.nih.gov/BLAST

2.16.3 Sequence analysis tools

Assembly of sequence data, sequence alignments and subsequent cropping of sequences to the desired length were performed using the software SeqMan II (DNA Star).

2.16.4 START v2

Statistical analyses were carried out using the START v2 programme (http://www.pubmlst.org) (Jolley *et al.*, 2001). The number of polymorphic nucleotide sites and the ratio of the number of nonsynonymous-to-synonymous substitutions (d_N/d_S ratio) for all seven loci were calculated. The d_N/d_S ratio

indicates any genes that might be undergoing strong diversifying or directional selection.

2.16.5 LIAN v3.5

Evidence of clonality or recombination among populations was estimated by assessing the level of linkage between alleles at different loci around the chromosome using the index of association (I_a) (Haubold & Hudson, 2000). An I_a not significantly greater than zero after 1,000 computer randomisations would suggest that loci within a monophyletic population is in linkage equilibrium (freely recombining), while a population with an I_a significantly greater than zero ($p<0.001$) is considered to be in linkage disequilibrium (clonal).

2.16.6 MEGA v4.1

Phylogenetic trees of the concatenated sequence of the seven housekeeping genes (3,135 bp) were constructed by the neighbour-joining method using Jukes-Cantor based algorithm (MEGA v4.1; http://www.megasoftware.net). The sequence input order was randomised, and bootstrapping resampling statistics were performed using 1,000 data sets for each analysis. A dendrogram using the unweighted pair group method with arithmetic averages (UPGMA) was also constructed (MEGA v4.1).

2.16.7 SplitsTree v4.1

Phylogenetic relationships were examined by split decomposition analysis using SplitsTree version 4.1 (www.splitstree.org) (Huson & Bryant, 2006). The split decomposition algorithm is a parsimony method that does not impose a branching or tree-like structure on the data set, but permits reticulations or a network structure that may be indicative of past recombination events. As a consequence, a split decomposition graph will look less tree-like and more net-like as the influence of recombination becomes stronger.

2.16.8 eBURST v3

eBURST (based upon related sequence types) analysis was carried out using eBURST v3 programme (http://www.mlst.net) (Feil *et al.*, 2004). Clonal groups were identified using the eBURST v3 clustering algorithm (http://www.mlst.net) (Feil *et al.*, 2004), which divides strains into clonal complexes where each strain has at least six loci in common with at least one other member of the group. The algorithm can also identify the most likely (i.e. parsimonious) ancestral ST within each clonal complex.

2.16.9 ClonalFrame v1.1

ClonalFrame uses MLST data to infer the clonal relationships of bacteria and the position of homologous recombination events (Didelot & Falush, 2007), available at http://www2.warwick.ac.uk/fac/sci/statistics/staff/research/didelot.

Network representation of the output was done using Graphviz (www.graphviz.org).

2.16.10 PAUP v4

Phylogenetic Analysis Using Parsimony (PAUP) assesses the degree of recombination by statistical comparisons between tree topologies for each MLST locus (Wilgenbusch & Swofford, 2003), available at http://paup.csit.fsu.edu.

2.16.11GelCompar II

Programme used to create phylogenetic analysis of Random Amplification of Polymorphic DNA (RAPD) gel banding patterns. (GelCompar II, Applied Maths, Sint-Martens-Latem, Belgium).

2.17 Reagents and Buffers

2.17.1 1 × loading buffer

5 × loading buffer	982.5µl
(0.25M Tris, 1% (w/v) SDS, 50% (v/v) Glycerol, 0.05% Bromophenol Blue pH 6.6)	
β-ME	17.5µl

2.17.2 TE

Tris-HCl (10mM), EDTA (1mM), pH 7.5-8

2.17.3 5 × TAE

Tris-base (2M), acetic acid (1M), EDTA (500mM)

2.17.4 5 × TBE

Tris-base (0.44M), Boric acid (0.44M), EDTA (10mM)

2.17.5 BHI or BHI-blood agar plates

VWR International, Lutterworth, UK

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CHAPTER 3 MICROBIAL KERATITIS STUDY

3.1 Introduction

Microbial keratitis is infection of the cornea (Figure 1.1). It is a common ophthalmic emergency and its clinical outcome depends on prompt diagnosis and initiation of appropriate antibiotics. This potentially blinding infection is relatively rare in the absence of predisposing factors such as contact lens wear, ocular surface disease or trauma (Keay *et al.*, 2006). Patients commonly present with a painful, red, watering eye and decreased visual acuity. A stromal infiltrate with overlying epithelial defect may be present. Deep ulcers may lead to corneal perforation requiring urgent corneal transplantation. The responsible microbial organism varies depending on the geographical location and climatic factors. In the UK, *Staphylococcus* spp., *Pseudomonas* spp. and *Streptococcus* spp. have been identified as common causative organisms (Tuft & Matheson, 2000). Tailored antimicrobial treatment depends on identification of the organism, which is currently dependent on obtaining corneal scrape samples and conventional culture techniques. Reports of culture positive rates vary widely from 50% to 86% (Alexandrakis *et al.*, 2000; Bennett *et al.*, 1998; Bourcier *et al.*, 2003; Fong *et al.*, 2007; Neumann & Sjostrand, 1993; Schaefer *et al.*, 2001; Wong *et al.*, 2003). The consequence of such a low culture positive rate is that the majority of microbial keratitis cases are treated with broad spectrum antibiotics which the infecting organisms may not be sensitive to. This not only leads to poor visual prognosis for the patient but also raises issues of increased antimicrobial resistance.

3.2 Aims

1. To determine the incidence, predisposing risk factors and clinical features of patients with microbial keratitis.
2. To identify the commonly isolated organisms and to determine the culture positive rate of current culture techniques.
3. To determine whether prior use of antibiotics before corneal scrapes lead to reduced culture positive rate.

3.3 Methods

A retrospective, non-comparative case note review of consecutive patients who had corneal scrapes for suspected microbial keratitis at the Birmingham and Midland Eye Centre over a 29-month period between April 2002 and August 2004 was carried out. Patients were first identified through the hospital's microbiology database and their medical records were reviewed. Cases were included if the patients had corneal scrapes taken for suspected microbial keratitis.

The following information was collected for each case:

- Age
- Gender
- Date of presentation
- Whether the patient was admitted into the hospital for treatment
- Length of stay in hospital if treated as an inpatient
- Presenting signs and symptoms
- Duration of symptoms at presentation
- Therapy commenced before presentation
- Past medical and ocular history
- Predisposing risk factors
- Drug history
- Initial treatment
- Gram staining, culture results and antibiotic sensitivities
- Any change of treatment after microbiological results were available
- Surgical procedures carried out as a consequence of the microbial keratitis

Fisher's exact test was performed using GraphPad InStat version 5.1 and GraphPad Prism version 4 (GraphPad Software, San Diego California USA, www.graphpad.com). $P<0.05$ was considered to be statistically significant.

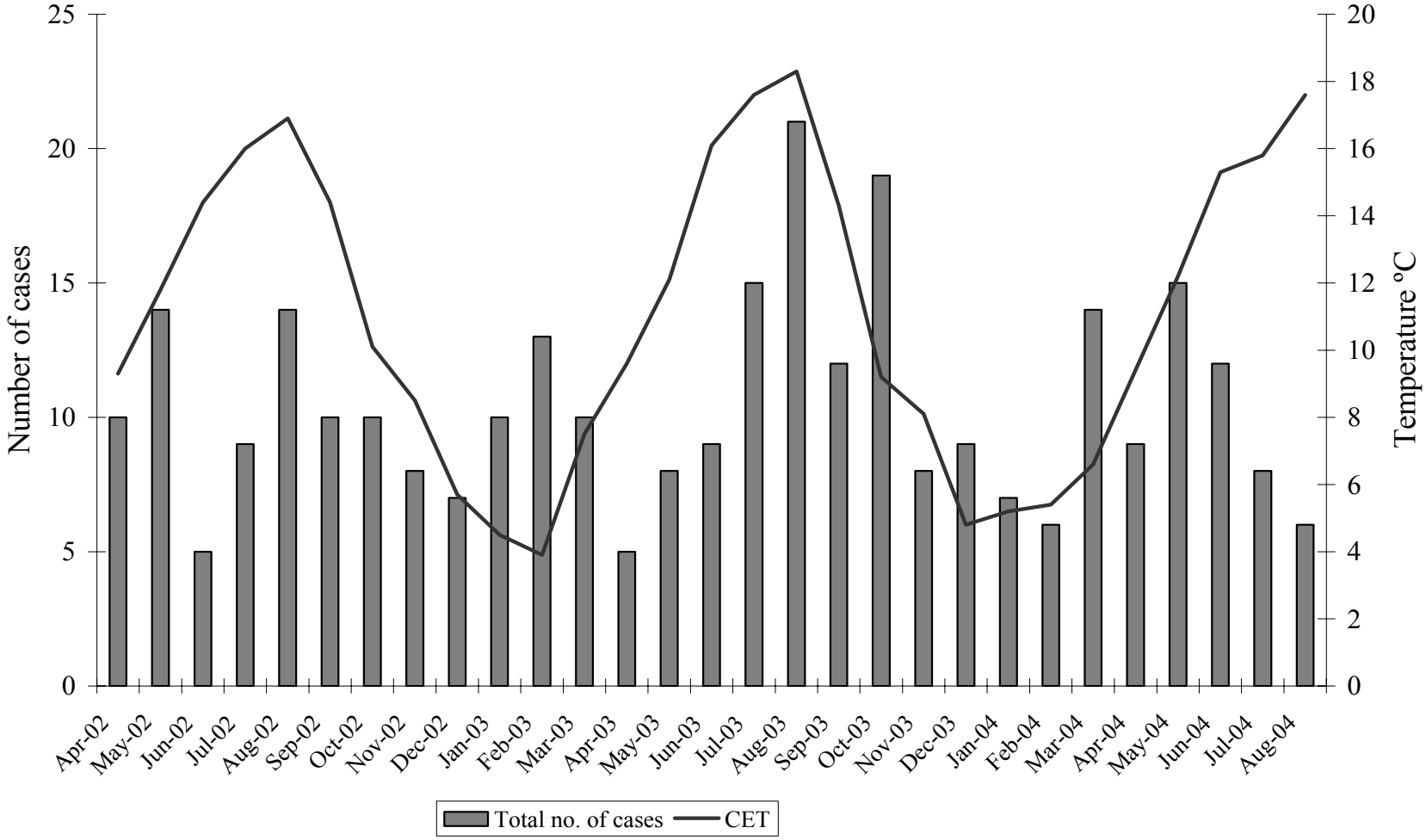
3.4 Results and Discussion

3.4.1 Epidemiological and clinical findings

During the 29 months of the study, 288 patients (303 individual cases) were identified as having suspected microbial keratitis. 149 (52%) were female and 139 (48%) were male with a median age of 43 years (range 5-92 years). 187 (61.7%) of patients self referred to the eye casualty department, 64 (21.1%) were referred by their General Practitioners, 26 (8.6%) were referred by an optician, 21 (6.9%) were referred by another hospital and 5 cases (1.7%) were referred by the hospital's main Accident and Emergency department. 110 (36.3%) episodes required hospital admission for treatment, with a median stay of 7 days (range 1-31 days). 193 (63.7%) were treated in the outpatients clinic.

The incidence of suspected microbial keratitis cases was higher in the summer months (Figure 3.1). In 148 (48.8%) cases, the left eye only was affected, in 152 (50.2%) cases, the right eye only was affected and in 3 (1%) cases, both eyes were affected. Symptoms included pain (79.5%), red eyes (67.7%), photophobia (33.7%), watery eyes (33.3%), blurred vision (18.5%), foreign body sensation (17.2%), discharge from the eyes (13.2%), decreased visual acuity (10.9%) and eyelid swelling (6.3%). The median time interval between the onset of symptoms and presentation to the hospital was 1 day (range 0-56 days). At the time of presentation, 92 (30.4%) had already been treated with topical antibiotics.

Figure 3.1 Number of cases of suspected microbial keratitis and Central England Temperature during each month of the study. (CET data was obtained from the UK Met office)



3.4.2 Predisposing risk factors

The predisposing risk factors were divided into ocular and systemic risk factors. Contact lens wear was by far the commonest predisposing risk factor and was observed in 103 (34.0%) of cases (Table 3.1). As shown in Figure 3.2, the majority of contact lens wearers in the study population were in the 20-29 and 30-39 age groups. The large number of contact lens wearers in these age groups account for the high incidence of contact lens related keratitis cases. Another peak incidence of microbial keratitis is seen in the 60-69 age group and this is more likely to be associated with ocular surface disease and previous ocular surgery. Numerous other studies carried out in developed countries have also identified contact lens wear as the commonest predisposing factor to developing microbial keratitis (Bourcier *et al.*, 2003; Dart, 1988; Keay *et al.*, 2006; Schaefer *et al.*, 2001). Other ocular factors identified in this study include previous ocular surgery in 55 (18.2%) cases, previous microbial keratitis in 47 (15.5%) cases, trauma in 33 (10.9%) cases, and previous herpes simplex virus eye infection in 14 (4.6%) cases. 58 (19.1%) cases had at least one systemic risk factor. Rheumatoid arthritis was the commonest systemic risk factor with 32 (10.6%) of cases with the disease. Diabetes was present in 28 (9.2%) cases, Sjögren's syndrome in 5 (1.7%) cases, facial nerve palsy in 3 (1.0%) cases and Stevens-Johnson syndrome in 2 (0.7%) cases.

Although contact lens wear is the leading risk factor for microbial keratitis in the developed world, other risk factors predominate in the developing world. For example, in Soweto, South Africa, the commonest predisposing factors were found to be blepharitis and ocular trauma (Ormerod, 1987). A study carried out in South India identified ocular injury to be the main risk factor, present in 71% of cases (Bharathi *et al.*, 2007). Contact lens-wear related cases contributed to only 1% of the total microbial keratitis cases in this study (Bharathi *et al.*, 2007).

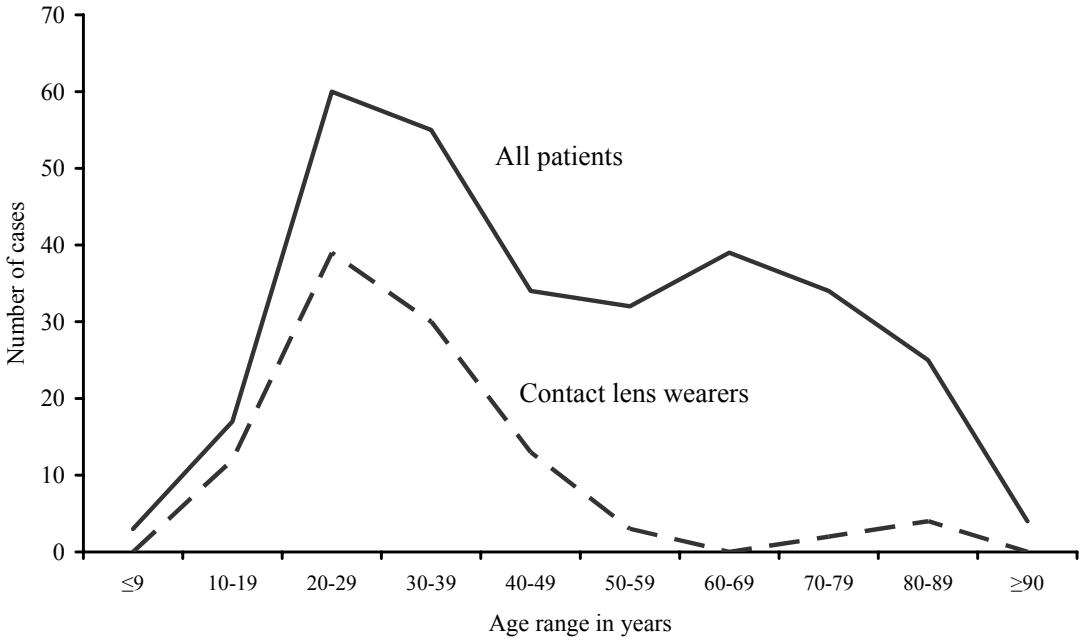
Table 3.1 Ocular predisposing risk factors for developing microbial keratitis.

Predisposing risk factor	Number of cases	%
Contact lens wear	103	34.0
Previous ocular surgery	55	18.2
Previous microbial keratitis	47	15.5
Trauma	33	10.9
Previous herpes simplex virus	14	4.6

Table 3.2 Systemic predisposing risk factors for developing microbial keratitis.

Predisposing risk factor	Number of cases	%
Rheumatoid arthritis	32	10.6
Diabetes	28	9.2
Sjögren's syndrome	5	1.7
Facial nerve palsy	3	1.0
Stevens-Johnson syndrome	2	0.7

Figure 3.2 Microbial keratitis cases by age groups. All cases of microbial keratitis represented by solid line and contact lens related cases represented by broken line.



3.4.3 Microbiology results

Of the 303 cases, at least one organism was identified in 114 (37.6%) cases using conventional culture methods. The culture positive rate in the current study is particularly low compared to previous studies, which have reported pick up rates ranging from 50% to 86% (Alexandrakis *et al.*, 2000; Bennett *et al.*, 1998; Bourcier *et al.*, 2003; Fong *et al.*, 2007; Neumann & Sjostrand, 1993; Schaefer *et al.*, 2001; Wong *et al.*, 2003). Factors thought to contribute to low culture positive rate are the inclusion of sterile ulcers, poor sampling technique, use of topical anaesthetic agents or topical antibiotics prior to sampling (Badenoch & Coster, 1982; Maske *et al.*, 1986; Wong *et al.*, 2003).

In total, 91 (30.0%) of the 303 cases received topical antibiotics treatment without prior cultures. 29 (31.9%) were culture positive and 62 (67.4%) were culture negative. In the group which did not receive antibiotics before cultures were obtained (212 cases), 85 (40.1%) were culture positive and 127 (59.9%) were culture negative. There was no statistical difference in the culture positive rate between the two groups ($P>0.05$, Fisher's exact test). Therefore prior administration of topical antibiotics did not significantly affect the detection rate of the causative organisms. This observation was surprising and data on a larger cohort of patients may identify a difference. Keay *et al.* also observed that there was no correlation between culture positive result and whether or not the patient had topical medication (Keay *et al.*, 2006).

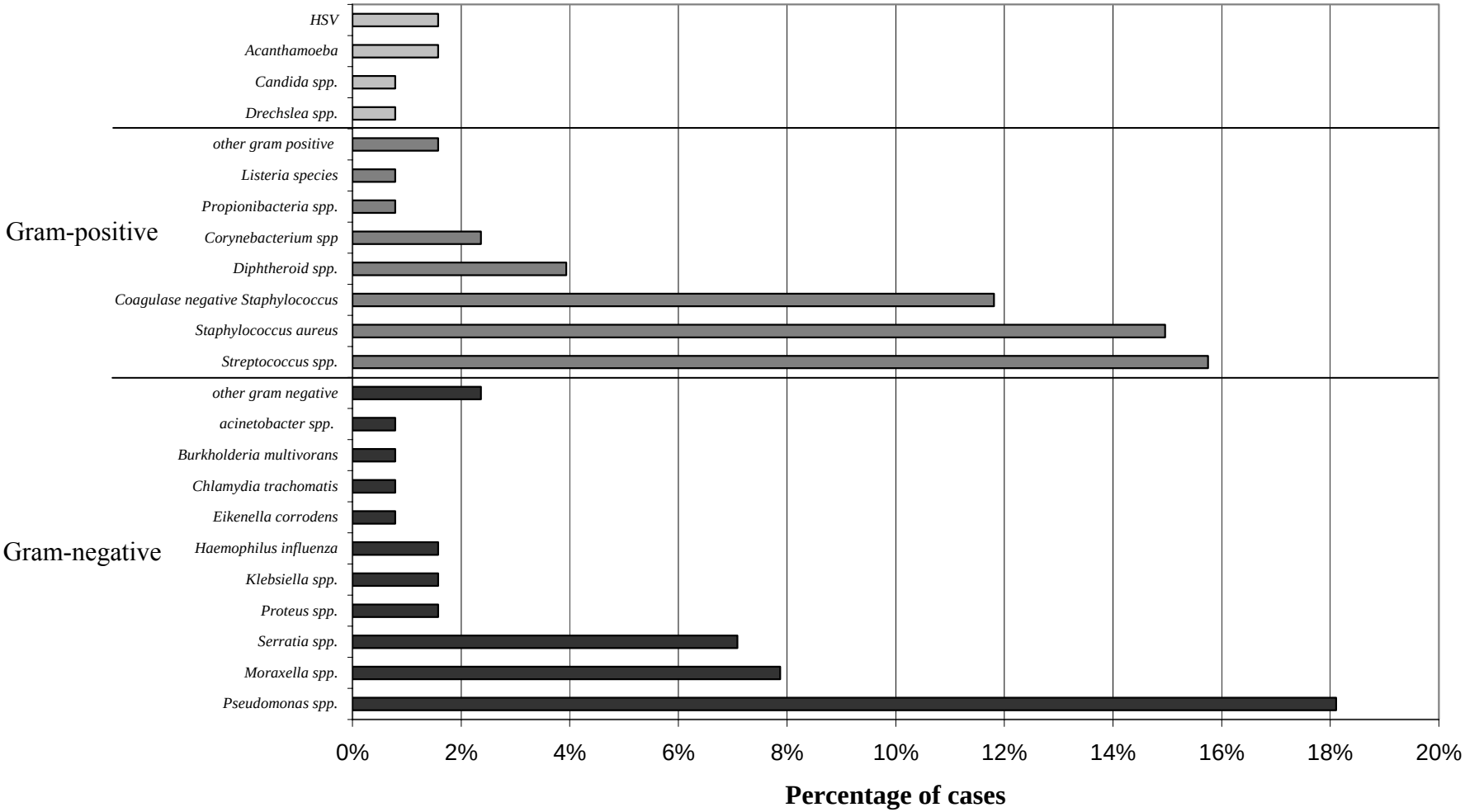
Of the culture positive cases in this study, ten involved mixed bacterial organisms and two cases involved concomitant infection with a viral organism. In total, 127 organisms including bacterial, viral and fungal were identified. 66 (52.0%) were Gram-positive bacteria and 55 (43.3%) were Gram-negative bacteria. The remaining consisted of viral and fungal infections (Figure 3.4). *Pseudomonas* spp. were the most commonly cultured organisms (23 cases, 18.9%) followed by *Streptococcus* spp. (20 cases, 16.4%), *S. aureus* (19 cases, 15.6%), Coagulase negative *Staphylococcus* (15 cases, 12.3%), *Moraxella* spp. (10 cases, 8.2%) and

Serratia spp. (9 cases, 7.4%). Amongst the contact lens related cases, 43.0% of the culture positive cases were caused by *P. aeruginosa*. The mean average time from scrape to a culture-positive result was 5.3 days (range 1-39) with 81.6% of the results available within 7 days. *Pseudomonas* spp. took an average of 4.2 days (range 2-9) to culture. One corneal scrape sample grew a *Propionibacteria* spp. strain, which took 10 days to culture.

Similar distributions of Gram-positive to Gram-negative bacteria to the current study have been reported in South Florida (Alexandrakis *et al.*, 2000). However, others have reported much higher rates of Gram-positive bacteria compared to Gram-negative bacteria in Melbourne, Australia (Keay *et al.*, 2006), New Zealand (Wong *et al.*, 2003), Switzerland (Schaefer *et al.*, 2001) and France (Bourcier *et al.*, 2003). A recent study in Taiwan however, did report higher rates of Gram-negative bacteria (61%) than Gram-positive bacteria (32.4%) (Fong *et al.*, 2007). This variation in the microbial spectrum could be explained by the different climatic conditions, the study population, the relative contribution of different risk factors, e.g. contact lens-wear and the laboratory culture techniques.

Antibiotic sensitivity data was available for 80 (70.2%) of the 114 culture positive cases. Where data was available, the microbes were sensitive to at least one of a standard panel of antibiotics, which included ciprofloxacin, gentamicin, ofloxacin, ampicillin, cefuroxime, chloramphenicol, ceftazidine and methicillin. Antibiotic resistance was detected in 4 cases to cefuroxime, 3 cases each to gentamicin, chloramphenicol and fusidic acid, two cases to ofloxacin and one case to ciprofloxacin. No organism was found to be resistant to ampicillin or ceftazidine. Fluoroquinolone monotherapy has been the treatment of choice for many ophthalmologists in managing microbial keratitis (Allan & Dart, 1995; Hyndiuk *et al.*, 1996; O'Brien *et al.*, 1995). Resistance of systemic and ocular isolates to fluoroquinolones have been increasingly observed, possibly due to their widespread use even in minor infections (Alexandrakis *et al.*, 2000; Garg *et al.*, 1999; Goldstein *et al.*, 1999; Kunimoto *et al.*, 1999).

Figure 3.4 Spectrum of isolated organisms cultured from corneal scrapes in microbial keratitis cases.



3.4.4 Treatment

The visual outcome of patients with microbial keratitis is largely dependant on the successful treatment with topical antibiotics. Empiric treatment with broad-spectrum coverage is generally initiated until culture results confirm the causative organism and its antimicrobial susceptibility (McLeod *et al.*, 1995). Broad-spectrum coverage can be achieved by either using combination therapy with a cephalosporin and an aminoglycoside or monotherapy using a fluoroquinolone. In this study, the majority of episodes (203 cases, 67.0%) were commenced on conventional combination therapy (penicillin or cefuroxime combined with ofloxacin or gentamicin) whereas monotherapy with a fluoroquinolone (ciprofloxacin or ofloxacin) was used in only 31 (10.2%) cases. After culture results and antibiotic sensitivity data were available, the antibiotics regime was altered in 34 (29.8%) cases.

3.4.5 Complications

Despite intensive antimicrobial therapy in the treatment of microbial keratitis, severe cases can lead to devastating complications. In the current study, evisceration (removal of the eye) was required in six cases. Causative organisms included *P. aeruginosa*, *S. aureus*, *Burkholderia multivorans*, *Moraxella nonliquefaciens*, *Serratia* spp. and no growth in one case. One patient underwent penetrating keratoplasty (corneal transplant) for spontaneous perforation caused by *P. aeruginosa* and two cases of smaller perforations were treated with bandage contact lens and glue. One patient developed bacterial endophthalmitis, also caused by *P. aeruginosa* and three patients required tarsorrhaphy (surgical closure of the eye lids) to promote healing.

3.5 Future work

An important observation from the current study is that the culture positive rate of less than 40% is very low compared to previous studies. It is therefore important to ascertain why this difference is so significant and whether culture rates could be improved. Possible causes include lack of training for junior staff in taking

corneal scrape samples, delay in transporting corneal scrape specimens to the laboratory, inadequate quantity of specimens and poor sensitivity of conventional culture methods. One way of overcoming the poor sensitivity and delay in obtaining culture positive results is by using molecular diagnostic techniques such as PCR. Assays can be designed to detect the commonly cultured organisms identified in this study. Real-time PCR protocols require only small amounts of corneal scrape specimen and can detect the causative organism within hours compared to days or weeks using conventional culture methods. 303 cases of microbial keratitis were included in this study. Long term follow up of these patients to investigate their visual outcome, potential complications from the keratitis infection and/or treatment and any recurrent infections would be useful data. They can then be used to identify factors that influence prognosis and effectiveness of different antibiotic treatment regimes.

3.6 Conclusions

The current study looked at clinical and microbiological data for 303 cases of suspected microbial keratitis at the Birmingham and Midland Eye Centre over a 29-month period. Contact lens wear was found to be the most common predisposing factor for the development of microbial keratitis, followed by ocular surgery, previous microbial keratitis and ocular trauma. Using conventional culture methods, the culture positive rate was found to be under 40%, lower than recently reported studies (Alexandrakis *et al.*, 2000; Bennett *et al.*, 1998; Bourcier *et al.*, 2003; Fong *et al.*, 2007; Neumann & Sjostrand, 1993; Schaefer *et al.*, 2001; Wong *et al.*, 2003). This low culture positive rate could be attributed by sampling sterile ulcers, small amounts of sample, poor sampling technique, use of topical anaesthetic agents before sampling and possible delay in transporting the sample to the laboratory. Prior use of antibiotics did not appear to have a significant affect on the culture positive rate. However, inclusion of more keratitis cases may yield more reliable data. *Pseudomonas* spp., *Streptococcus* spp., *S. aureus* and Coagulase negative *Staphylococcus* were the most frequently cultured organisms. Low culture positive rate and prolonged time to culture result, indicates that more rapid and sensitive diagnostic tests are required for optimal management of this sight threatening eye infection.

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CHAPTER 4 MULTILOCUS SEQUENCE TYPING ANALYSIS OF *PROPIONIBACTERIUM ACNES*

4.1 Introduction

P. acnes usually lives on the skin but has also been cultured from the conjunctiva, oral cavity, external ear canal and large intestine (Brook & Frazier, 1991). Although it usually remains a harmless commensal, it has been reported to cause serious clinical infections including sight threatening eye infections such as postoperative endophthalmitis (Aldave *et al.*, 1999) and keratitis (Jones & Robinson, 1977; Perry *et al.*, 1982; Wong *et al.*, 2003; Zaidman, 1992).

Johnson and Cummins first classified *P. acnes* into two distinct phenotypes, known as types I and II, based on serological agglutination tests and cell wall sugar analysis (Johnson & Cummins, 1972). RAPD analysis of *P. acnes* isolates identified two distinct profiles which correlated to type I and II strains (Perry *et al.*, 2003). AFLP was able to identify three main clusters of *P. acnes* although their corresponding phenotypes were undetermined (Mohammadi *et al.*, 2005). Analysis of the *P. acnes recA* gene and *tly* gene sequence has found that types I and II represent distinct phylogenetic lineages (McDowell *et al.*, 2005). Furthermore, type I strains have been subdivided into groups IA and IB based on *recA* and *tly* sequences, monoclonal antibody (MAb) typing (McDowell *et al.*, 2005) and PFGE (Cohen *et al.*, 2005). Very recently, a novel lineage of strains, known as type III, has been described, which appears to have different cellular morphology to type I and II strains (McDowell *et al.*, 2008). Despite developments in our knowledge regarding *P. acnes* phylogeny, the genetic population structure of strains within the different lineages of the organism is poorly understood.

MLST is a nucleotide sequence-based method enabling comparative characterisation of bacterial isolates at the molecular level (Maiden *et al.*, 1998). As it is DNA sequence based, this method has the advantage of being a

reproducible and scalable typing system that is portable between laboratories (Urwin & Maiden, 2003). This chapter describes the development of an MLST scheme to characterise the population structure of a diverse collection of clinical *P. acnes* isolates and to determine whether certain genotypes are associated with particular diseases or geographical locations.

4.2 Aims

1. To develop an MLST scheme to type *P. acnes* isolates.
2. Using this MLST scheme to examine the population biology of *P. acnes* and determine the level of recombination contributing to its diversity.
3. To determine whether strains exhibit disease or geographical associations.
4. Validate the *P. acnes* MLST scheme using 16S rRNA gene analysis, *recA* gene analysis, IFM and RAPD analysis.

4.3 Results and Discussion

4.3.1 *P. acnes* MLST scheme

4.3.1.1 *P. acnes* strains and selection of genes

P. acnes consists of a single circular chromosome (2.5 Mb) containing 2,333 putative genes (Bruggemann *et al.*, 2004). The seven housekeeping genes selected for the MLST scheme and location within the KPA171202 genome are shown in Figure 4.1. To accurately validate the MLST scheme, a collection of *P. acnes* isolates from disparate geographical locations and diverse clinical diseases were selected for analysis (Table 4.1). A total of 125 *P. acnes* isolates were analysed, including the reference strains NCTC 737 (facial acne isolate) and KPA171202 (DSM 16379; contaminated anaerobic culture). For the remaining 123 isolates, eight were cultured from brain abscess, kidney, bone, lymph node, cadaveric skin and blood samples. A total of 31 isolates were recovered from keratitis and endophthalmitis infections, while 56 isolates were from patients with acne. A further three isolates were recovered from fatal head granulomas, seven from microdiscectomy samples removed during sciatica surgeries and six from dental infections. Five isolates were from surgical skin wounds and seven isolates were recovered from prosthetic hip arthroplasties.

4.3.1.2 MLST database

The *P. acnes* MLST database (Figures 4.2a-c), developed by Keith Jolley (Jolley *et al.*, 2004) is publicly available at http://www.pubmlst.org/pacnes. Information on the housekeeping genes, primer sequences for amplification and sequencing, PCR conditions, allelic profiles and sequence types (STs) of the 125 *P. acnes* strains are available on the database. The programme also allows investigators to input sequence data and generate allelic profiles and identify whether strains have STs that correspond to existing strains or represent new STs.

Figure 4.1 Gene map of the *P. acnes* chromosome with locations of the seven housekeeping genes in the MLST scheme (Bruggemann *et al.*, 2004). The protein coding sequence (CDS) is shown in yellow and green, in different strand orientations. 'Alien' gene clusters exhibiting aberrant codon usage are shown in red. The blue circle represents G+C content variation from the mean value, 60% (with lower values pointing inwards) (Bruggemann *et al.*, 2004).

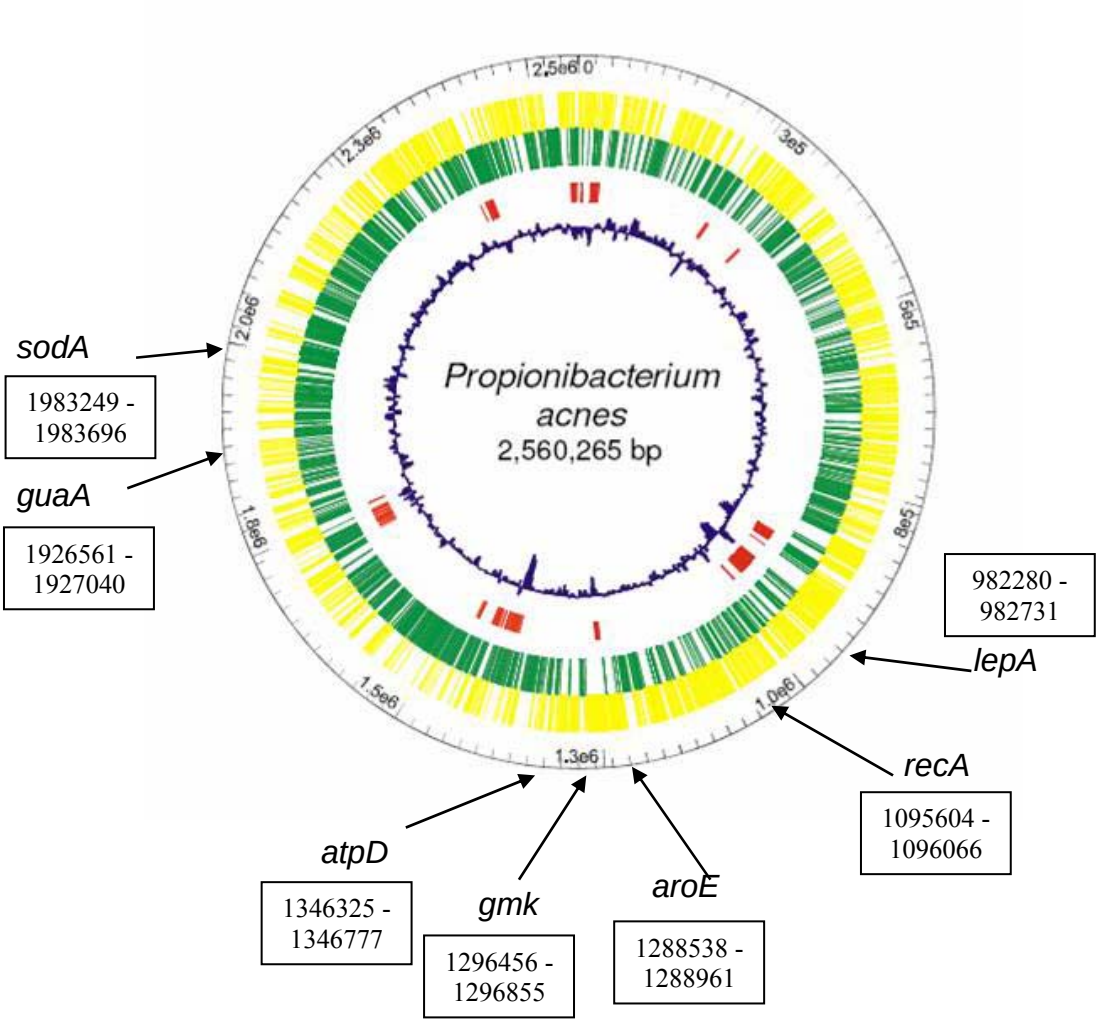
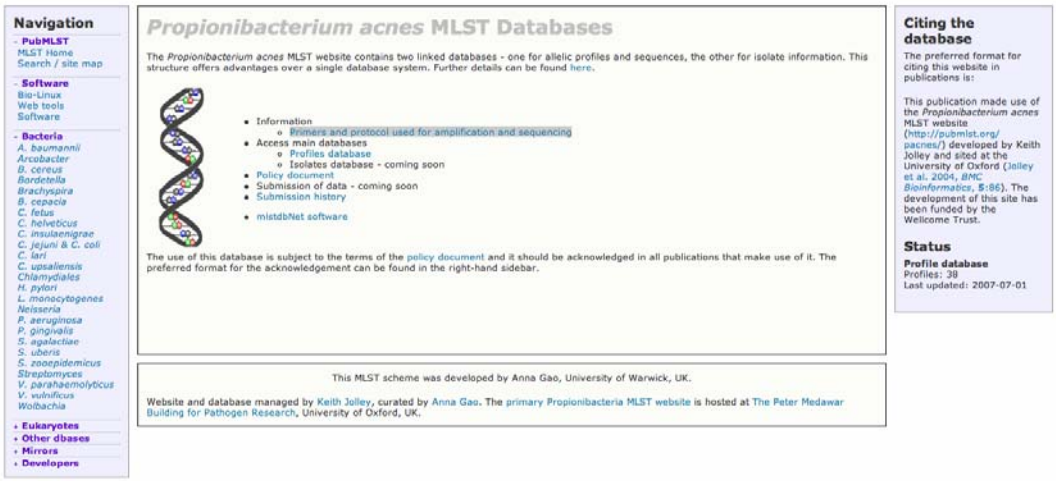
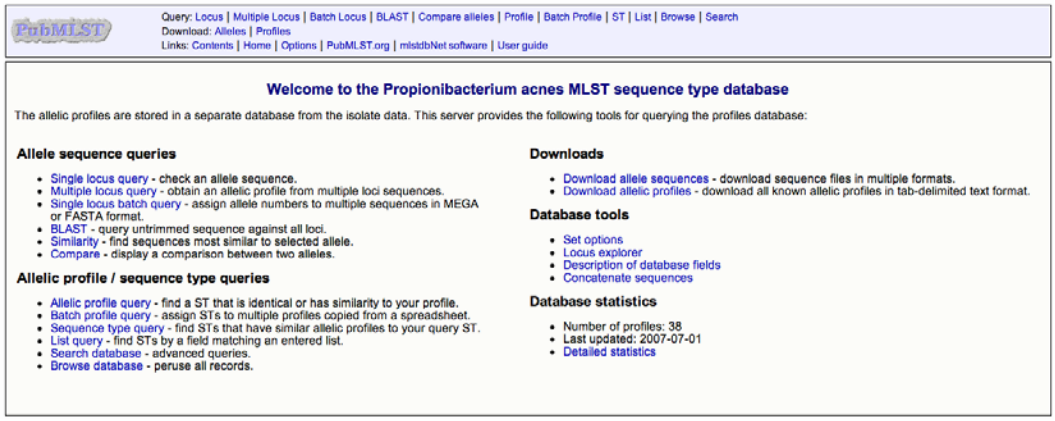


Figure 4.2 Screen shots of online database for *P. acnes* MLST scheme (http://pubmlst.org/pacnes) hosted by Keith Jolley (Jolley *et al.*, 2004). (a) main page for the *P. acnes* MLST scheme (b) profiles database page and (c) primers and protocol used for amplification and sequencing.

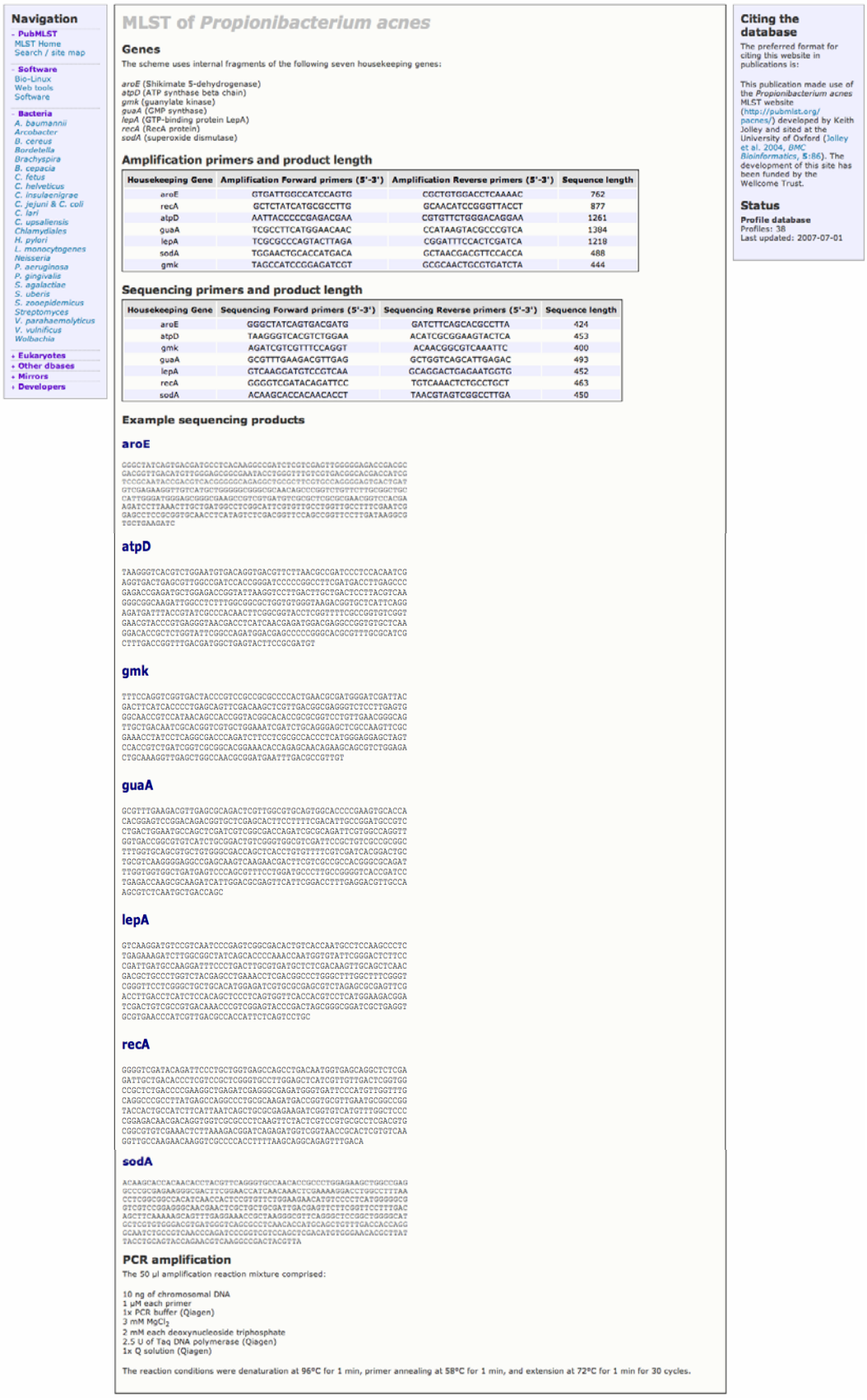
(a)



(b)



(c)



Navigation

- PubMLST
  - MLST Home
  - Search / site map
- Software
  - Bio-Linux
  - Web tools
  - Software
- Bacteria
  - *A. baumannii*
  - *Arcobacter*
  - *B. cereus*
  - *Bordetella*
  - *Brachyspira*
  - *B. cepacia*
  - *C. fetus*
  - *C. helveticus*
  - *C. insulaenigrae*
  - *C. jejuni & C. coli*
  - *C. lari*
  - *C. upsaliensis*
  - *Chlamydiales*
  - *H. pylori*
  - *L. monocytogenes*
  - *Neisseria*
  - *P. aeruginosa*
  - *P. gingivalis*
  - *S. agalactiae*
  - *S. uberis*
  - *S. zooepidemicus*
  - *Streptomyces*
  - *V. parahaemolyticus*
  - *V. vulnificus*
  - *Wolbachia*
- Eukaryotes
- Other dbases
- Mirrors
- Developers

MLST of *Propionibacterium acnes*

Genes

The scheme uses internal fragments of the following seven housekeeping genes:

aroE (Shikimate 5-dehydrogenase)
atpD (ATP synthase beta chain)
gmk (guanylate kinase)
guaA (GMP synthase)
lepA (GTP-binding protein LepA)
recA (RecA protein)
sodA (superoxide dismutase)

Amplification primers and product length

Housekeeping Gene	Amplification Forward primers (5'-3')	Amplification Reverse primers (5'-3')	Sequence length
aroE	GTGATTGGCCATCCAGTG	CGCTGTGGACCTCAAAAC	762
recA	GCTCTATCATGCGCCTTG	GCAACATCCGGGTTACCT	877
atpD	AATTACCCCCGAGACGAA	CGTGTTCTGGGACAGGAA	1261
guaA	TCGCCTTCATGGAACAAC	CCATAAGTACGCCCGTCA	1384
lepA	TCGCGCCCAGTACTTAGA	CGGATTTCCACTCGATCA	1218
sodA	TGGAACTGCACCATGACA	GCTAACGACGTTCCACCA	488
gmk	TAGCCATCCGGAGATCGT	GCGCAACTGCGTGATCTA	444

Sequencing primers and product length

Housekeeping Gene	Sequencing Forward primers (5'-3')	Sequencing Reverse primers (5'-3')	Sequence length
aroE	GGGCTATCAGTGACGATG	GATCTTCAGCACGCCTTA	424
atpD	TAAGGGTCACGTCTGGAA	ACATCGCGGAAGTACTCA	453
gmk	AGATCGTCGTTTCCAGGT	ACAACGGCGTCAAATTC	400
guaA	GCGTTTGAAGACGTTGAG	GCTGGTCAGCATTGAGAC	493
lepA	GTCAAGGATGTCCGTCAA	GCAGGACTGAGAATGGTG	452
recA	GGGGTCGATACAGATTCC	TGTCAAACTCTGCCTGCT	463
sodA	ACAAGCACCACAACACCT	TAACGTAGTCGGCCTTGA	450

Example sequencing products

aroE

```
GGGCTATCAGTGACGATGCCTCACAAGGCCGATCTCGTCGAGTTGGGGGAGACCGACGC
GACGGTTGACATGTTGGGAGCCGGCGAATACCTGGGTTTGTCGTGACGGCACGACCATCG
TCCCCAATACCCACCTCACGGCGGCACAGCCTCCGCCTTCGTCCACGGCGAGTCACTCAT
GTCGAGAAGGTTGTCATGCTGGGGGCGGGCGCAACAGCCCGGTCTGTTCTTGCGGCTGC
CATTGGGATGGGAGCGGGCGAAGCCGTCGTGATGTCGCGCTCGCGCGAACGGTCCACGA
AGATCCTTAAACTTGCTGATGGCCTCGGCATTCGTGTTGCCTGGTTGCCTTTCGAATCG
GAGCCTCCGCGGTCAACCTCATAGTCTCGACGGTTCCAGCCGGTTCCTTGATAAGCCG
TGCTGAAGATC
```

atpD

```
TAAGGGTCACGTCTGGAATGTGACAGGTGACGTTCTTAACGCCGATCCCTCCACAATCG
AGGTGACTGAGCGTTGGCCGATCCACCGGGATCCCCCGGCCTTCGATGACCTTGAGCCC
GAGACCGAGATGCTGGAGACCGGTATTAAGGTCCTTGACTTGCTGACTCCTTACGTCAA
GGGCGGCAAGATTGGCCTCTTTGGCGGCGCTGGTGTGGGTAAGACGGTGCTCATTCAGG
AGATGATTTACCGTATCGCCCACAACTTCGGCGGTACCTCGGTTTCGCCGGTGTCGGT
GAACGTACCCGTGAGGGTAACGACCTCATCAACGAGATGGACGAGGCCGGTGTGCTCAA
GGACACCGCTCTGGTATTCGGCCAGATGGACGAGCCCCCGGGCACGCGTTTGCGCATCG
CTTTGACCGGTTTGACGATGGCTGAGTACTTCCGCGATGT
```

gmk

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TTTCCAGGTCGGTGACTACCCGTCCGCCGCGCCCCACTGAACGCGATGGGATCGATTAC
GACTTCATCACCCCTGAGCAGTTCGACAAGCTCGTTGACGGCGAGGGTCTCCTTGAGTG
GGCAACCGTCCATAACAGCCACCGGTACGGCACACCGGCGGTCCTGTTGAACGGGCAG
TTGCTGACAATCGCACGGTCGTGCTGGAAATCGATCTGCAGGGAGCTCGCCAAGTTCGC
GAAACCTATCCTCAGGCGACCCACGATCTCCTCGCGCCACCCTCATGGGAGGAGCTAGT
CCACCGTCTGATCGGTCGCGGCACGGAAACACCAGAGCAACAGAAGCAGCGTCTGGAGA
CTGCAAAGGTTGAGCTGGCCAACGCGGATGAATTTGACGCCGTTGT
```

guaA

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GCGTTTGAAGACGTTGAGCGCAGACTCGTTGGCGTGCAGTGGCACCCCGAAGTGCACCA
CACGGAGTCCGGACAGACGGTGCTCGAGCACTTCCTTTTCGACATTGCCGGATGCCGTC
CTGACTGGAATGCCAGCTCGATCGTCGGCGACCAGATCGCGCAGATTCGTGGCCAGGTT
GGTGACCGGCGTGTCATCTGCGGACTGTCGGGTGGCGTCGATTCCGCTGTCGCCGCGGC
TTGCGTCGAGCGTGCGTCGTGGGCGACCAGCTCACCGTGTTTTCCTCGATCACGGACTGC
TGCGTCAAGGGGAGGCCGAGCAAGTCAAGAACGACTTCGTCGCCGCCACGGGCGCAGAT
TTGGTGGTGGCTGATGAGTCCCAGCGTTTCCTGGATGCCCTTGCCGGGGTCACCGATCC
TGAGACCAAGCGCAAGATCATTGGACGCGAGTTCATTCGGACCTTTGAGGACGTTGCCA
AGCGTCTCAATGCTGACCAGC
```

lepA

```
GTCAAGGATGTCCGTCAATCCCGAGTCGGCGACACTGTCACCAATGCCTCCAAGCCCTC
TGAGAAAGATCTTGGCGGCTATCAGCACCCAAACCAATGGTGTATTCGGGACTCTTCC
CGATTGATGCCAAGGATTTCCCTGACTTGCGTGATGCTCTCGACAAGTTGCAGCTCAAC
GACGCTGCCCTGGTCTACGAGCCTGAAACCTCGACGGCCCTGGGCTTTGGCTTTCGGGT
CGGGTTCCTCGGGCTGCTGCACATGGAGATCGTGCGCGAGCGTCTAGAGCGCGAGTTCG
ACCTTGACCTCATCTCCACAGCTCCCTCAGTGGTTCACCACGTCCTCATGGAAGACGGA
TCGACTGTCGCCGTGACAAACCCGTCGGAGTACCGACTAGCGGGCGGATCGCTGAGGT
GCGTGAACCCATCGTTGAGCCACCATTCTCAGTCCTGC
```

recA

```
GGGGTCGATACAGATTCCCTGCTGGTGAGCCAGCCTGACAATGGTGAGCAGGCTCTCGA
GATTGCTGACACCCTCGTCCGCTCGGGTGCCTTGGAGCTCATCGTTGTTGACTCGGTGG
CCGCTCTGACCCCGAAGGCTGAGATCGAGGGCGAGATGGGTGATTCCCATGTTGGTTTG
CAGGCCCGCCTTATGAGCCAGGCCCTGCGCAAGATGACCGGTGCGTTGAATGCGGCCGG
TACCACTGCCATCTTCATTAATCAGCTGCGCGAGAAGATCGGTGTCATGTTTGGCTCCC
CGGAGACAACGACAGGTGGTCGCGCCCTCAAGTTCTACTCGTCCGTGCGCCCTCGACGTG
CGGCGTGTCGAAACTCTTAAAGACGGATCAGAGATGGTCGGTAACCGCACTCGTGTCAA
GGTTGCCAAGAACAAGGTCGCCCCACCTTTTAAGCAGGCAGAGTTTGACA
```

sodA

```
ACAAGCACCACAACACCTACGTTCAGGGTGCCAACACCGCCCTGGAGAAGCTGGCCGAG
GCCCGCGAGAAGGGCGACTTCGGAACCATCAACAAACTCGAAAAGGACCTGGCCTTTAA
CCTCGGCGGCCACATCAACCACTCCGTGTTCTGGAAGAACATGTCCCCTCATGGGGGCG
GTCGTCCGGAGGGCAACAACTCGCTGGTCGATTGACGAGTTCTTCGGTTCCTTTGAC
AGCTTCAAAAACGCAGTTTGAGGAAACCGCTAAGGGCGTTCAGGGCTCCGGCTGGGGCAT
GCTCGTGTGGGACGTGATGGGTCAGCGCCCTCAACACCATGCAGCTGTTTGACCACCAGG
GCAATCTCCCGTCAACCCAGATCCCGGTCGTCCAGCTCGACATGTGGGAACACGCTTAT
TACCTCGCAGTACAGAACGTCAAGGCCGACTACGTTA
```

PCR amplification

The 50 µl amplification reaction mixture comprised:

10 ng of chromosomal DNA
1 µM each primer
1x PCR buffer (Qiagen)
3 mM $MgCl_2$
2 mM each deoxynucleoside triphosphate
2.5 U of Taq DNA polymerase (Qiagen)
1x Q solution (Qiagen)

The reaction conditions were denaturation at 96°C for 1 min, primer annealing at 58°C for 1 min, and extension at 72°C for 1 min for 30 cycles.

Citing the database

The preferred format for citing this website in publications is:

This publication made use of the *Propionibacterium acnes* MLST website (http://pubmlst.org/pacnes/) developed by Keith Jolley and sited at the University of Oxford (Jolley et al. 2004, *BMC Bioinformatics*, **5**:86). The development of this site has been funded by the Wellcome Trust.

Status

Profile database
Profiles: 38
Last updated: 2007-07-01

Table 4.1 MLST analysis of *P. acnes* isolates including their sources, geographical locations and *recA* phylotype status.

Isolate name	Source	Date of isolation (mo-yr)	Location	Allelic profile							ST	*recA* phylotype	eBURST group
				aroE	*atpD*	*gmk*	*guaA*	*lepA*	*recA*	*sodA*			
R19606	Brain abscess	NA	UK	1	1	1	1	1	1	1	1	ND	1
R18544	Blood culture	NA	UK	1	1	1	3	1	1	2	4	ND	1
PRP-27	Acne	NA	Australia	1	1	1	3	1	1	1	6	ND	1
PRP-51	Acne	NA	Australia	1	1	1	3	1	1	1	6	ND	1
PRP-1	Acne	NA	Australia	1	1	1	3	1	1	1	6	ND	1
PRP-2	Acne	NA	Australia	1	1	1	3	1	1	1	6	ND	1
NCTC 737	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
R19618	Cadaveric skin	NA	UK	1	1	1	3	1	1	1	6	ND	1
XnL1	Fatal granuloma	NA	China	1	1	1	3	1	1	1	6	IA	1
XnT2	Fatal granuloma	NA	China	1	1	1	3	1	1	1	6	IA	1
XnH	Fatal granuloma	NA	China	1	1	1	3	1	1	1	6	IA	1
JMK9[b]	Surgical skin wound	NA	UK	1	1	1	3	1	1	1	6	IB	1
PRP-10	Acne	Oct-2000	Greece	1	1	1	3	1	1	1	6	ND	1
PRP-17	Acne	Jan-2000	Italy	1	1	1	3	1	1	1	6	ND	1
PRP-26	Acne	Jan-2000	Italy	1	1	1	3	1	1	1	6	ND	1
PRP-30	Acne	Jan-2000	UK	1	1	1	3	1	1	1	6	ND	1
PRP-46	Acne	Jun-2000	UK	1	1	1	3	1	1	1	6	ND	1
PRP-13	Acne	Sep-1999	UK	1	1	1	3	1	1	1	6	ND	1
PRP-69	Acne	Apr-2000	UK	1	1	1	3	1	1	1	6	ND	1
PRP-68	Acne	NA	UK	1	1	1	3	1	1	1	6	ND	1
PRP-109	Acne	NA	UK	1	1	1	3	1	1	1	6	ND	1
PRP-111	Acne	NA	UK	1	1	1	3	1	1	1	6	ND	1
PRP-4	Acne	NA	UK	1	1	1	3	1	1	1	6	ND	1
PRP-52	Acne	NA	UK	1	1	1	3	1	1	1	6	ND	1
PRP-113	Acne	NA	UK	1	1	1	3	1	1	1	6	ND	1
PV10	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1

Isolate name	Source	Date of isolation (mo-yr)	Location	Allelic profile							ST	*recA* phylotype	eBURST group
				aroE	*atpD*	*gmk*	*guaA*	*lepA*	*recA*	*sodA*			
PV13	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV20	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV37	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV41	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV44	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV58	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV84	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV93	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV109	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV113	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
PV138	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
P135	Acne	NA	UK	1	1	1	3	1	1	1	6	IA	1
W891	Dental	NA	UK	1	1	1	3	1	1	1	6	IA	1
W1973	Dental	NA	UK	1	1	1	3	1	1	1	6	IA	1
W3875	Dental	NA	UK	1	1	1	3	1	1	1	6	IA	1
W513	Dental	NA	UK	1	1	1	3	1	1	1	6	IA	1
P.acn11	Aqueous humour	Sep-2003	France	1	1	1	3	1	1	1	6	ND	1
P.acn38	Aqueous humour	Dec-2004	France	1	1	1	3	1	1	1	6	ND	1
P.acn12	Corneal scrape	Oct-2003	France	1	1	1	3	1	1	1	6	ND	1
P.acn13	Corneal scrape	Oct-2003	France	1	1	1	3	1	1	1	6	ND	1
P.acn14	Corneal scrape	Oct-2003	France	1	1	1	3	1	1	1	6	ND	1
P.acn15	Corneal scrape	Oct-2003	France	1	1	1	3	1	1	1	6	ND	1
P.acn25	Corneal scrape	Apr-2004	France	1	1	1	3	1	1	1	6	ND	1
P.acn28	Corneal scrape	Jun-2004	France	1	1	1	3	1	1	1	6	ND	1
P.acn34	Corneal scrape	Aug-2004	France	1	1	1	3	1	1	1	6	ND	1
P.acn26	Eye	May-2004	France	1	1	1	3	1	1	1	6	ND	1

Isolate name	Source	Date of isolation (mo-yr)	Location	Allelic profile							ST	*recA* phylotype	eBURST group
				aroE	*atpD*	*gmk*	*guaA*	*lepA*	*recA*	*sodA*			
P.acn23	Vitreous humour	Mar-2004	France	1	1	1	3	1	1	1	6	ND	1
PRP-29	Acne	Oct-1999	Spain	1	1	1	3	1	1	1	6	ND	1
PRP-23	Acne	Oct-1999	Spain	1	1	1	3	1	1	1	6	ND	1
PRP-7	Acne	Sep-2000	Sweden	1	1	1	3	1	1	1	6	ND	1
PRP-64	Acne	Sep-2000	Sweden	1	1	1	3	1	1	1	6	ND	1
PRP-8	Acne	Sep-2000	Sweden	1	1	1	3	1	1	1	6	ND	1
PRP-9	Acne	Sep-2000	Sweden	1	1	1	3	1	1	1	6	ND	1
PRP-59	Acne	Sep-2000	Sweden	1	1	1	3	1	1	1	6	ND	1
PRP-101	Acne	NA	USA	1	1	1	3	1	1	1	6	ND	1
PRP-105	Acne	NA	USA	1	1	1	3	1	1	1	6	ND	1
PRP-99	Acne	NA	USA	1	1	1	3	1	1	1	6	ND	1
P.acn16	Corneal scrape	Oct-2003	France	1	5	1	3	1	1	1	7	IA	1
P.acn27	Aqueous humour	Jun-2004	France	1	5	1	3	1	1	1	7	ND	1
P.acn21	Vitreous humour	Feb-2004	France	1	1	1	6	1	1	1	8	ND	1
PRP-3	Acne	NA	UK	8	1	1	3	1	1	1	11	ND	1
PV77	Acne	NA	UK	11	1	1	3	1	1	1	13	IA	1
P6	Acne	NA	UK	1	1	6	3	1	1	1	18	IA	1
P9	Acne	NA	UK	1	1	6	3	1	1	1	18	IA	1
R17684	Neck lymph node	NA	UK	1	1	1	14	1	1	1	21	ND	1
P.acn22	Corneal scrape	Feb-2004	France	4	1	1	3	1	1	1	24	ND	1
PRP-60	Acne	Oct-1999	UK	5	1	1	3	1	1	1	25	ND	1
PRP-72	Acne	Jun-2000	UK	5	1	1	3	1	1	1	25	ND	1
P.acn29	Corneal scrape	Jun-2004	France	5	1	1	3	1	1	1	25	ND	1
PRP-78	Acne	NA	Japan	1	1	1	3	7	1	1	32	ND	1
PRP-62	Acne	Jan-2000	Italy	1	1	1	9	1	1	1	33	ND	1
PV139	Acne	NA	UK	12	1	1	3	1	1	1	34	IA	1

Isolate name	Source	Date of isolation (mo-yr)	Location	Allelic profile							ST	*recA* phylotype	eBURST group
				aroE	*atpD*	*gmk*	*guaA*	*lepA*	*recA*	*sodA*			
PRP-107	Acne	NA	UK	1	7	1	3	1	1	1	36	ND	1
Asn7[b]	Microdiscectomy	NA	UK	1	1	1	3	8	1	1	38	IB	1
R18395	Kidney	NA	UK	1	2	1	2	1	1	1	2	ND	2
R19133	Bone (tibia)	NA	UK	1	3	1	2	1	1	1	3	ND	2
P.acn10	Corneal scrape	Sep-2003	France	1	1	1	5	1	1	4	9	IB	3
P.acn24	Aqueous humour	Apr-2004	France	1	1	1	5	1	1	4	9	IB	3
P.acn33	Aqueous humour	Aug-2004	France	1	1	1	5	1	1	4	9	IB	3
P.acn35	Corneal scrape	Sep-2004	France	1	1	1	5	1	1	4	9	IB	3
P.acn39	Aqueous humour	Dec-2004	France	1	1	1	5	1	1	4	9	ND	3
WMK9	Surgical skin wound	NA	UK	1	1	1	4	1	1	4	10	IB	3
MMG9	Surgical skin wound	NA	UK	1	1	1	4	1	1	4	10	IB	3
TON9	Surgical skin wound	NA	UK	1	1	1	4	1	1	4	10	IB	3
Asn2	Microdiscectomy	NA	UK	1	1	1	4	1	1	4	10	IB	3
Asn3	Microdiscectomy	NA	UK	1	1	1	4	1	1	4	10	IB	3
R18466	Neck lymph node	NA	UK	1	1	1	4	1	1	4	10	ND	3
KPA171202[a]	Contaminated culture	NA	DSMZ	1	1	1	4	1	1	4	10	ND	3
PRP-81	Acne	NA	Japan	1	1	1	4	1	1	4	10	ND	3
W1392	Dental	NA	UK	1	1	1	4	1	1	4	10	IB	3
W1998	Dental	NA	UK	1	1	1	4	1	1	4	10	IB	3
P.acn20	Corneal scrape	Jan-2004	France	1	1	1	4	1	1	4	10	IB	3
P.acn32	Aqueous humour	Jul-2004	France	1	1	1	4	1	1	4	10	ND	3
P.acn37	Eye	Nov-2004	France	1	1	1	4	1	1	4	10	ND	3
P.acn40	Corneal scrape	Jan-2005	France	1	1	1	4	1	1	4	10	ND	3
CK17	Prosthetic hip	NA	Sweden	1	1	1	4	1	1	4	10	IB	3
LED2	Prosthetic hip	Mar-1999	UK	1	1	1	4	1	1	4	10	IB	3
RM9	Surgical skin wound	Jan-2000	UK	1	1	1	4	1	1	4	10	IB	3

Isolate name	Source	Date of isolation (mo-yr)	Location	Allelic profile							ST	*recA* phylotype	eBURST group
				aroE	*atpD*	*gmk*	*guaA*	*lepA*	*recA*	*sodA*			
PRP-102	Acne	NA	USA	1	1	1	4	1	1	4	10	ND	3
P.acn31	Aqueous humour	Jul-2004	France	1	1	1	13	1	1	4	27	IB	3
P.acn17	Corneal scrape	Nov-2003	France	1	1	1	5	3	1	5	22	IB	-
P.acn18	Corneal scrape	Nov-2003	France	1	1	1	5	3	1	5	22	IB	-
Asn1	Microdiscectomy	NA	UK	1	1	9	4	1	1	11	37	IB	-
PV66[c]	Acne	NA	UK	9	1	5	8	6	3	8	12	IA	4
PRP-38	Acne	NA	UK	9	1	4	8	6	3	8	29	ND	4
PRP-39	Acne	NA	UK	9	1	4	8	6	3	8	29	ND	4
R18473	Blood culture	NA	UK	2	4	2	4	2	2	3	5	ND	5
RB1B	Prosthetic hip	Jan-1998	UK	13	4	2	4	2	2	3	15	II	5
P.acn19	Corneal scrape	Dec-2003	France	3	4	2	4	2	2	3	23	II	5
PRP-47	Acne	Oct-2000	Greece	1	4	2	4	2	2	3	31	ND	5
JP1B	Prosthetic hip	Jan-1998	UK	15	4	2	4	2	2	3	35	II	5
KC1	Prosthetic hip	Jun-1999	UK	3	4	2	10	4	2	6	14	II	-
ED1	Prosthetic hip	Oct-1996	UK	14	4	2	4	4	2	6	16	II	6
P.acn30	Vitreous humour	Jul-2004	France	6	4	2	4	4	2	6	26	II	6
Asn13	Microdiscectomy	NA	UK	7	6	3	11	5	4	9	17	III	7
Asn11	Microdiscectomy	NA	UK	7	6	3	7	5	4	9	19	III	7
Asn12	Microdiscectomy	NA	UK	7	6	3	12	5	4	9	20	III	7
P.acn36	Corneal scrape	Nov-2004	France	7	6	3	7	5	4	7	28	ND	7
Asn10	Prosthetic hip	NA	UK	7	6	7	7	5	4	9	30	III	7

[a] Strain purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ)
[b] Type IB by *recA* sequencing but eBURST group 1 based on MLST (highlighted in blue)
[c] Type IA by *recA* sequencing but eBURST group 4 based on MLST (highlighted in blue)
NA, not available
ND, not determined

4.3.1.3 Allelic variation

Allelic information for the seven alleles was calculated using START v2 (Section 2.16.4). The total length of the concatenated sequence of all the alleles was 3,135 bp. The mean allele length was 448 bp with sizes ranging from 400 bp (*gmk*) to 493 bp (*guaA*) (Table 4.2). The total number of housekeeping gene alleles ranged from four (*recA*) to 14 (*aroE*), with an average of 9.2 per locus. This provided the potential to distinguish > 5 x 10^6 different genotypes. All alleles for a given locus were found to be of equal lengths for all the isolates examined. The degree of sequence diversity within the housekeeping genes was relatively low with the proportion of polymorphic sites ranging from 0.9% (*recA*) to 8.3% (*aroE*) with an average of 4.2%.

Within this MLST scheme the *recA* gene was the most highly conserved locus investigated with only four distinct alleles and four polymorphic sites in the 463 bp fragment analysed. Previous work, however, focused on the complete 1,047 bp *recA* gene which generated a greater number of alleles and polymorphic regions for phylogenetic study and clustering isolates into distinct lineages (McDowell *et al.*, 2005; McDowell *et al.*, 2008). In the context of the MLST scheme, the *recA* locus was not critical for analysis and the phylogenetic data and clustering produced using the remaining six loci was similar to that produced when *recA* was included. A greater number of isolates will have to be examined, however, before it can be assumed that the gene section of *recA* used in the MLST scheme does not add any additional information to the population study of *P. acnes*.

4.3.1.4 Selective pressure of MLST loci

MLST loci should ideally encode proteins that are under stabilising selection for conservation of metabolic function (Maiden, 2006). The ratio of non-synonymous (d_N) to synonymous (d_S) changes is frequently used to assess the degree of selection operating on a particular locus (Rocha *et al.*, 2006). Synonymous (silent) substitutions are usually considered neutral, or the potential to have a much smaller effect on fitness than non-synonymous (replacement) substitutions. A low

ratio (d_N/d_S <1) signifies strong purifying ("stabilizing") selection and a high ratio (d_N/d_S >1) signifies selection for diversification ("positive selection") (Rocha *et al.*, 2006).

The d_N/d_S ratio was calculated for the seven loci using START v2 (Table 4.2). For all loci the d_N/d_S ratios were less than 1, demonstrating that no strong selective pressure was operating on the genes selected, validating their suitability for inclusion in the *P. acnes* MLST scheme. *recA* and *aroE* were noted to have d_N/d_S values of 0.334 and 0.576, respectively, which were relatively high compared to the other loci. This is not unusual and has been previously observed in MLST schemes developed for other organisms including *Candida albicans* and *Camplylobacter* spp. (Miller *et al.*, 2005). It has been postulated that for closely related strains within a bacterial species, housekeeping genes with a high d_N/d_S value are a consequence of a delay in the removal of slightly deleterious non-synonymous mutations which may remain for a period of time before purifying selection (Rocha *et al.*, 2006). Also, in the case of *recA*, the higher d_N/d_S ratio may reflect the small number of polymorphic sites within the segment analysed (two synonymous and two non-synonymous substitutions) which may generate an artificially high value.

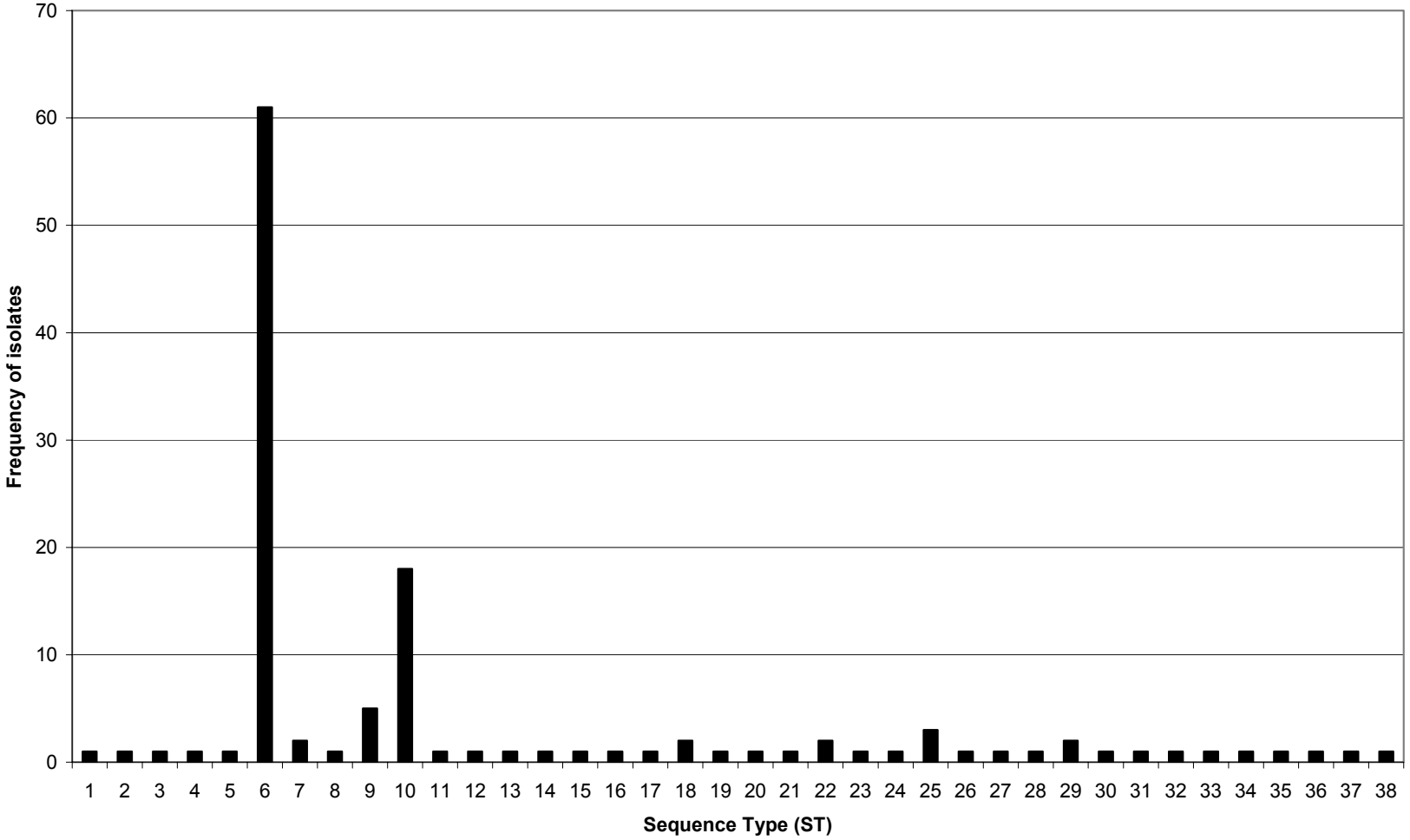
4.3.2 Analysis of *P. acnes* isolates for sequence types (STs)

A total of 38 different STs were assigned to the 125 *P. acnes* isolates under investigation (Table 4.1). All allelic profiles and STs are available on the *P. acnes* MLST database (http://pubmlst.org/pacnes). Upon analysis, ST-6 (n=61; 48.8%), ST-10 (n=18; 14.4%) and ST-9 (n=5; 4.0%) were found to be the predominant allelic profiles (Figure 4.3). All other 35 STs were more evenly spread (at low frequency) across the remaining 41 isolates, with 30 having a unique ST (Figure 4.3). The most well described *P. acnes* strain NCTC 737 (type IA) belonged to ST-6, while KPA171202 (type IB), used for genome sequencing of *P. acnes* (Bruggemann *et al.*, 2004), belonged to ST-10.

Table 4.2 Characteristics of the seven gene loci used in the *P. acnes* MLST scheme

Gene	Size of fragment sequenced (bp)	No. of distinct alleles	No. of polymorphic sites	Proportion of polymorphic sites (%)	Mean G+C content (%)	d_N/d_S
aroE	424	14	35	8.3	61.4	0.5756
atpD	453	7	16	3.5	58.5	0.1510
gmk	400	8	17	4.3	60.1	0.0868
guaA	493	14	29	5.9	61.5	0.0254
lepA	452	8	15	3.3	58.6	0.1180
recA	463	4	4	0.9	58.5	0.3342
sodA	450	10	19	4.2	58.2	0.0605
Mean	448	9	19	4.2	50.8	0.1931

Figure 4.3 Frequency of isolates within the study population of 125 *P. acnes* isolates



4.3.2.1 Recombination occurring across loci

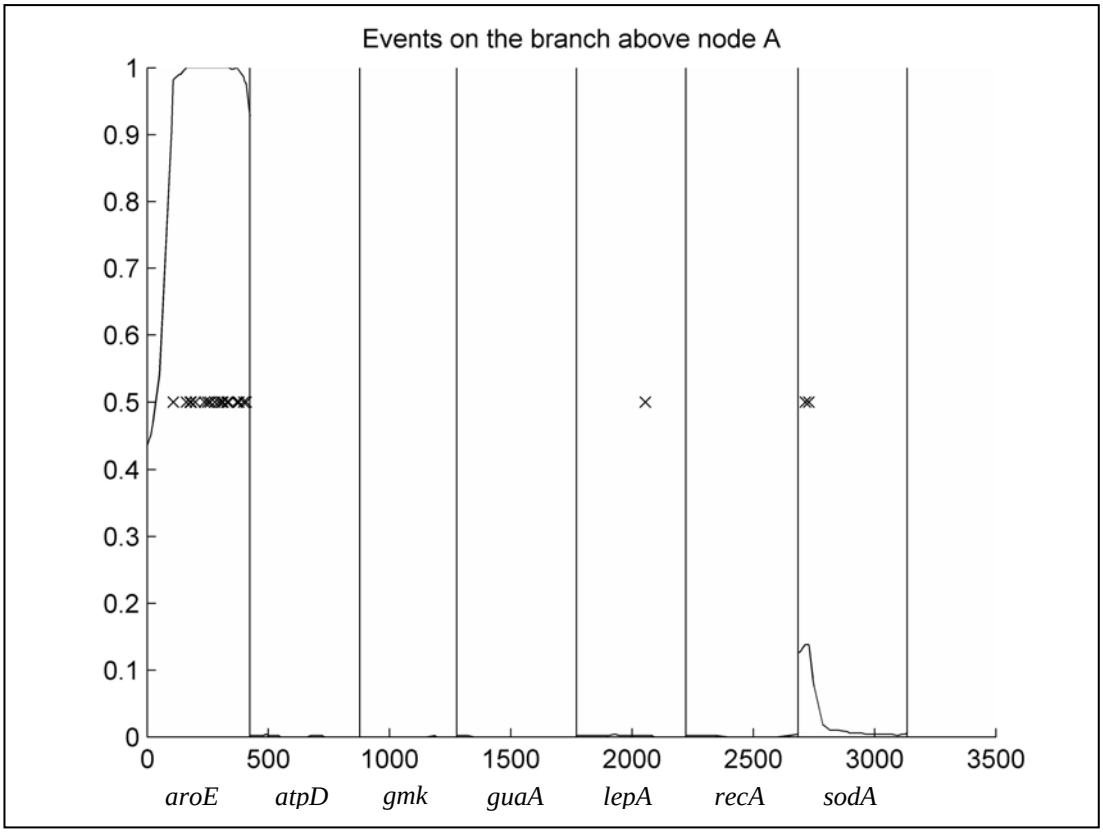
One of the preferred characteristics of housekeeping loci for inclusion in an MLST scheme is that they are equally distributed around the chromosome so that any recombinogenic events do not involve two loci. The *P. acnes* MLST scheme has three housekeeping genes which are relatively close together (Figure 4.1). The *gmk* gene is 7,495 bp downstream of the *aroE* gene and *atpD* is 49,470 bp downstream of the *gmk* gene.

To determine whether a recombinational event has occurred involving two or even three of these genes simultaneously, the allele numbers for each ST are compared between these genes. If two genes are so closely related as to cause a single recombinational event to change both alleles, then the two allelic numbers would usually only be associated with each other. For example, comparing *gmk* and *aroE* allelic numbers (Table 4.3), *gmk* allele 3 is always associated with *aroE* allele 7. The converse is not true as *aroE* allele 7 is also associated with *gmk* allele 7. The close association of these two allelic numbers is also shared with *lepA* and *recA* genes even though they are extremely spread out from each other. The close association in this instance is explained by the fact that these STs belong to the same clonal complex (eBURST group 7, Section 4.3.3.1). It is therefore unlikely that the close position of *aroE* to *gmk* has led to a single recombinogenic event involving both genes. This is further supported by clonal frame analysis of the *P. acnes* MLST data (Figure 4.4) (more details in Section 4.3.3.4). Figure 4.4 shows the inferred events at the node that ST-35 branches off. Frequent substitutions, likely to represent recombination, are shown to occur at *aroE* but not at *gmk*. Now looking at *atpD* and *gmk* genes which are 49,470 bp apart, *atpD* allele 4 and *gmk* allele 2 are the only two allelic numbers that always occur together. It is possible that a single recombinogenic event has led to both genes being genetically altered. However, these allelic numbers are exclusively associated with *recA* allele 2 and all belong to eBURST groups 5 or 6. Again, this suggests that this association is more likely to be due to the genetic relatedness of these genotypes rather than single recombinogenic events leading to their change.

Table 4.3 Allelic profiles and eBURST groups for each ST shown. *aroE*, *atpD* and *gmk* are highlighted to identify any recombinogenic events involving these three genes.

aroE	*atpD*	*gmk*	*guaA*	*lepA*	*recA*	*sodA*	ST	eBURST group
1	1	1	1	1	1	1	**1**	**1**
1	1	1	3	1	1	2	**4**	**1**
1	1	1	3	1	1	1	**6**	**1**
1	5	1	3	1	1	1	**7**	**1**
1	1	1	6	1	1	1	**8**	**1**
8	1	1	3	1	1	1	**11**	**1**
11	1	1	3	1	1	1	**13**	**1**
1	1	6	3	1	1	1	**18**	**1**
1	1	1	14	1	1	1	**21**	**1**
4	1	1	3	1	1	1	**24**	**1**
5	1	1	3	1	1	1	**25**	**1**
1	1	1	3	7	1	1	**32**	**1**
1	1	1	9	1	1	1	**33**	**1**
12	1	1	3	1	1	1	**34**	**1**
1	7	1	3	1	1	1	**36**	**1**
1	1	1	3	8	1	1	**38**	**1**
1	2	1	2	1	1	1	**2**	**2**
1	3	1	2	1	1	1	**3**	**2**
1	1	1	5	1	1	4	**9**	**3**
1	1	1	4	1	1	4	**10**	**3**
1	1	1	13	1	1	4	**27**	**3**
9	1	5	8	6	3	8	**12**	**4**
9	1	4	8	6	3	8	**29**	**4**
2	4	2	4	2	2	3	**5**	**5**
13	4	2	4	2	2	3	**15**	**5**
3	4	2	4	2	2	3	**23**	**5**
1	4	2	4	2	2	3	**31**	**5**
15	4	2	4	2	2	3	**35**	**5**
14	4	2	4	4	2	6	**16**	**6**
6	4	2	4	4	2	6	**26**	**6**
7	6	3	11	5	4	9	**17**	**7**
7	6	3	7	5	4	9	**19**	**7**
7	6	3	12	5	4	9	**20**	**7**
7	6	3	7	5	4	7	**28**	**7**
7	6	7	7	5	4	9	**30**	**7**
3	4	2	10	4	2	6	**14**	-
1	1	1	5	3	1	5	**22**	-
1	1	9	4	1	1	11	**37**	-

Figure 4.4 ST-35 clonal frame shot showing *aroE* recombination but no change in *gmk*. The seven housekeeping gene fragments of the *P. acnes* MLST scheme are represented by blocks on the x axis (numbers represent nucleotides). Each substitution is indicated by a cross. The curved line at each locus indicates the inferred probability for recombination on a scale from 0 to 1 (y axis).



4.3.3 Phylogenetic analysis

Bacterial genomes evolve by a number of different mechanisms, including point mutation, genome rearrangement, deletion, duplication, bacteriophage lysogeny, transposition, slippage mutation in DNA sequence repeats and homologous and non-homologous recombination (Feil *et al.*, 1999; Feil *et al.*, 2000; Lawrence & Hendrickson, 2003; Smith *et al.*, 1993). Recombination can result when bacterial DNA enters the host cell via conjugation, transformation or transduction (Smith *et al.*, 1991; Smith *et al.*, 1993; Spratt, 2004). These numerous different evolutionary mechanisms by which bacteria evolve can present problems when attempting to infer relationship between strains, which often rely on comparing DNA sequence differences.

Point mutations happen approximately randomly and therefore, in the absence of other genetic exchange processes, allow reliable determination of clonal relationships using standard phylogenetic methods (Didelot & Falush, 2007). However, even in housekeeping genes, homologous recombination between bacteria within the same population can change several nucleotides at once (Milkman & Crawford, 1983). Therefore, inaccurate relationships between strains may be inferred as these homologous recombinational events are over-weighted by nucleotide-based phylogenetic methods in comparison to point mutations (Schierup & Hein, 2000). Using these methods, two related isolates which differ at only one locus, can appear unrelated if the variant locus is especially divergent. To take this into account, a variety of methods have been adapted and new ones have been developed to analyse genetic relationships using allelic profiles (Feil *et al.*, 2004; Jolley *et al.*, 2001; Spratt *et al.*, 2004). Other statistical analysis methods have been designed to infer bacterial clonal relationships based on DNA sequences that accounts for both point mutation and homologous recombination (Didelot & Falush, 2007). Here, a variety of phylogenetic methods have been applied to the *P. acnes* MLST data to study its population biology, taking into account of point mutations and homologous recombination.

4.3.3.1 eBURST analysis of STs

MLST analysis of several bacterial species support the view that large proportions of a population belongs to a limited number of clusters of closely related genotypes or clonal complexes (Dingle *et al.*, 2001; Enright & Spratt, 1998; Enright *et al.*, 2000; Enright *et al.*, 2001; Maiden *et al.*, 1998; Meats *et al.*, 2003). One theory for the emergence of clonal complexes is that a founding genotype increases in frequency in the population to become a predominant clone, either as a consequence of a fitness advantage or of random genetic drift (Feil & Spratt, 2001). As the founding genotype increases in frequency, it gradually diversifies, to result in a clonal complex. In terms of MLST, this observation is represented by descendants of the founder initially having identical allelic profile to the founder. Over time, variants in which one of the seven alleles has changed, either by point mutation or recombination, will appear. These genotypes, which differ from the founder at only one of the seven MLST loci, are called single-locus variants (SLVs). SLVs will eventually diversity further, to produce variants that differ at two of the seven loci (double-locus variants [DLVs]), at three of the loci (triple-locus variants [TLVs]), and so on (Feil *et al.*, 2004).

Using MLST data, the eBURST v3 (based upon related sequence types) clustering algorithm (http://www.mlst.net) divides bacterial populations into groups of closely related strains (clonal complexes), predicts the founding (ancestral) genotype of each clonal complex, and displays the patterns of recent evolutionary descent of all other strains from their respective founder (Feil *et al.*, 2004; Turner *et al.*, 2007). The STs within an eBURST group are considered to belong to a single clonal complex if they share identical alleles at six or seven of the seven MLST loci with at least one other ST in the group. For each clonal complex, the primary founder is predicted on the basis of parsimony as the ST that has the largest number of SLVs. In groups that are composed of only a limited number of STs, it may not be possible to assign a primary founder. The frequency of a given ST in the study population is not used to assign founders; however, founders often correspond to the most predominant STs (Feil *et al.*, 2004).

Using the stringent eBURST criteria of assigning STs to the same clonal complex based on having six of seven loci, seven eBURST groups and thus, clonal complexes, encompassing 38 STs, were assigned to the 125 *P. acnes* isolates. Figure 4.5 illustrates the eBURST diagram generated, showing the seven eBURST groups and 3 singletons which did not fall into any eBURST group. Due to the criteria of only designating STs as belonging to the same eBURST group if they have six or more loci in common, the eBURST groups described here correspond to clonal complexes.

ST-6 and ST-19 are assigned the founding genotypes of their corresponding clonal complexes, with bootstrap values of 100% and 88% respectively (Tables 4.4a-g). The predicted founder for eBURST group 3 was ST-10. However, ST-10 had the same number of SLVs and DLVs as the remaining two STs in the group. Therefore, the bootstrap value was only 7%, confirming that it cannot be the ancestral genotype for that clonal complex.

Figure 4.6 is the same eBURST analysis but this time showing DLVs in addition to SLVs. Numerous blue lines connect isolates within eBURST group 1, suggesting that frequent recombinogenic events occur within this clonal complex. eBURST groups 2 and 3 also have numerous DLVs within group 1. Spratt *et al.* suggested that older clonal complexes exhibit extensive diversification with a complicated structure of descending genotypes whilst a clearly apparent founder, with only a few SLV and DLVs, typify young BURST groups (Spratt, 2004). This would suggest that eBURST group 1 is a relatively mature clonal complex whilst groups 5, 6 and 7 are relatively junior clonal complexes. The eBURST diagram displaying the DLV (Figure 4.6) is effectively showing how the groups would cluster if the group definition was less stringent and STs were grouped together if they shared five or more alleles. Groups 1, 2 and 3 would cluster as one BURST group and the remaining four groups stay in the same cluster, clearly showing the diversity between each group. This is interesting because if the previous assumption that groups 5, 6 and 7 were relatively new clonal complexes was true, then why do they not have DLVs within group 1? One possibility is that the

current study population of 125 *P. acnes* isolates does not include ancestral genotypes which all these seven groups descend from.

Singletons ST-22 and ST-37 are DLVs of group 3 genotypes, suggesting that they have diversified from this group or from a common clone. In the same way, ST-14 is a DLV of group 6 strains, providing evidence for a common ancestor between these strains. ST-12 and ST-29 (eBURST group 4) do not have DLVs with any other groups suggesting their distinct nature from the other clusters and the inaccurate assumption that these are type IA strains based on *recA* gene and *tly* gene analyses (Section 4.3.6.2).

As previously mentioned, phylogenetic analysis of MLST data based on concatenated sequences of the seven loci can be misleading. Closely related strains can appear unrelated if considerable amount of recombination has occurred at only one of the loci. This can be observed in the current study population. In Section 4.3.3.2, a phylogenetic dendrogram based on the concatenated sequences of the seven housekeeping genes using the Neighbour-Joining method is shown (Figure 4.7). Based on this dendrogram, isolates which have been clustered together as eBURST group 3 (i.e. ST-9, ST-10 and ST-27), appear to be distantly related. In fact, ST-9 and ST-27 appear to be more closely related to eBURST group 1, 2 and 4 isolates than to ST-10. This inference is most likely incorrect as based on *recA* gene analysis, ST-9, ST-10, ST-27, ST-22 and ST-37 were all classed as type IB isolates and eBURST groups 1, 2 and 4 were classed as type IA isolates. More detailed comparison between MLST analysis and *recA* gene sequencing is discussed in Section 4.3.6.2.

Table 4.4 a-g. The seven clonal complexes derived from eBURST analysis showing the frequency of each ST within the study population and the frequency of SLVs and DLVs. No TLV were identified. Predicted founder for each clonal complex is highlighted in blue. The level of confidence in founding genotypes is indicated by the bootstrap value.

(a) eBURST group 1
(No. of isolates = 80 No. of STs = 16 Predicted founder = ST-6)

ST	Frequency	SLV	DLV	ST Bootstrap
6	61	15	0	100%
25	3	5	10	2%
24	1	5	10	3%
13	1	5	10	4%
11	1	5	10	7%
34	1	5	10	11%
21	1	4	11	1%
8	1	4	11	3%
1	1	4	11	5%
33	1	4	11	9%
7	2	2	13	0%
38	1	2	13	0%
36	1	2	13	0%
32	1	2	13	0%
18	2	1	14	0%
4	1	1	14	0%

(b) eBURST group 2
(No. of isolates = 2 No. of STs = 2 Predicted founder = none)

ST	Frequency	SLV	DLV	ST Bootstrap
3	1	1	0	N/A
2	1	1	0	N/A

(c) eBURST group 3
(No. of isolates = 24 No. of STs = 3 Predicted founder = ST-10)

ST	Frequency	SLV	DLV	ST Bootstrap
10	18	2	0	7%
9	5	2	0	8%
27	1	2	0	14%

(d) eBURST group 4
(No. of isolates = 3 No. of STs = 2 Predicted founder = none)

ST	Frequency	SLV	DLV	ST Bootstrap
29	2	1	0	N/A
12	1	1	0	N/A

(e) eBURST group 5
(No. of isolates = 5 No. of STs = 5 Predicted founder = multiple candidates)

ST	Frequency	SLV	DLV	ST Bootstrap
23	1	4	0	17%
5	1	4	0	16%
15	1	4	0	22%
35	1	4	0	31%
31	1	4	0	46%

(f) eBURST group 6
(No. of isolates = 2 No. of STs = 2 Predicted founder = none)

ST	Frequency	SLV	DLV	ST Bootstrap
26	1	1	0	N/A
16	1	1	0	N/A

(g) eBURST group 7
(No. of isolates = 5 No. of STs = 5 Predicted founder = ST-19)

ST	Frequency	SLV	DLV	ST Bootstrap
19	1	4	0	88%
20	1	2	0	1%
17	1	2	0	3%
28	1	1	0	0%
30	1	1	0	0%

Figure 4.5 eBURST diagram showing the clusters of related STs and individual unlinked STs of 125 *P. acnes* isolates. Seven clonal complexes are identified and labelled. Each ST is represented by a circle with the number beside the circle. The frequency of each ST within the analysed population is indicated by the area of the circle. ST-6 and ST-19 were the founders of their respective clonal complexes. SLVs shown in pink.

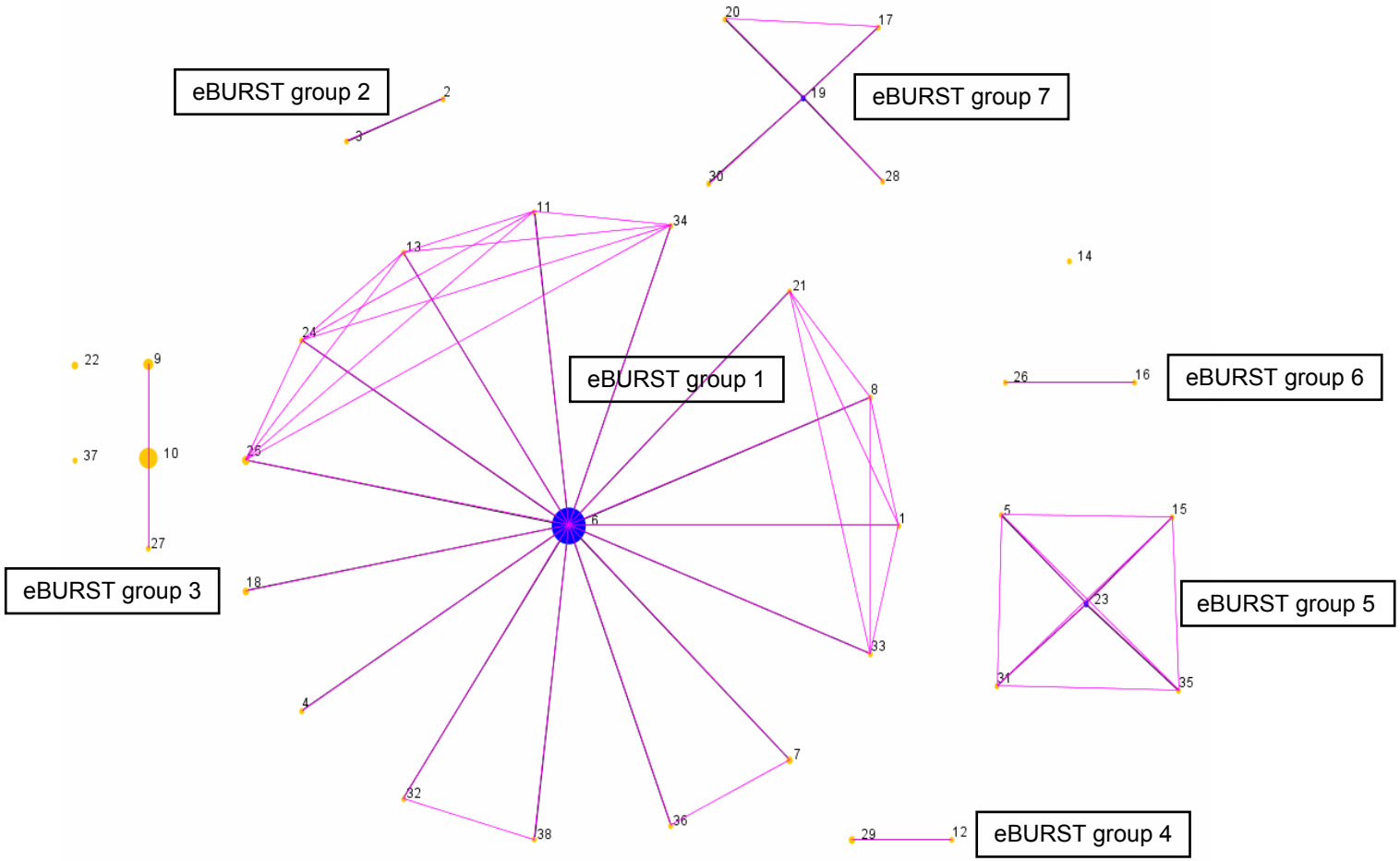
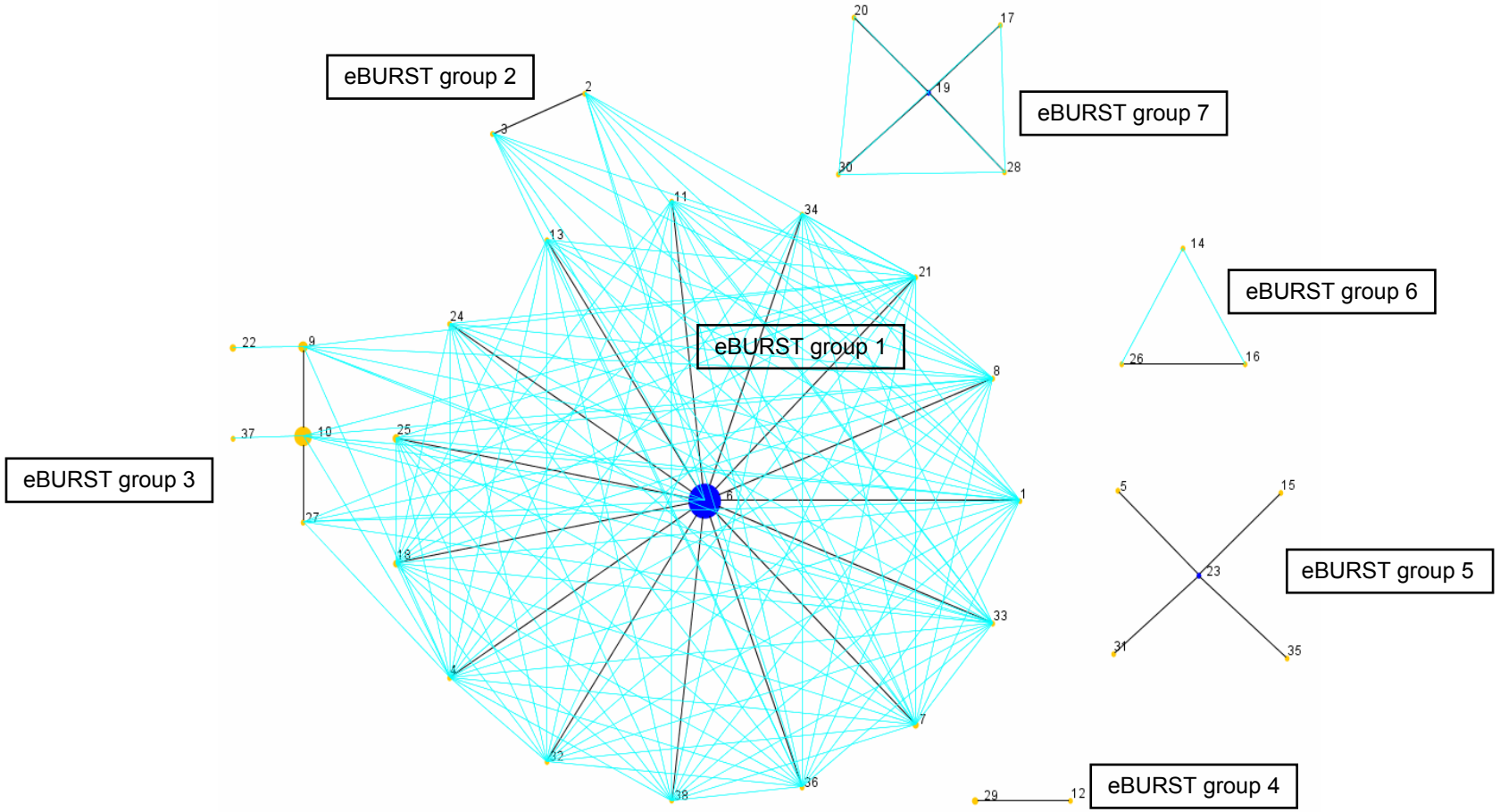


Figure 4.6 eBURST diagram showing the clusters of related STs and individual unlinked STs of 125 *P. acnes* isolates. Seven clonal complexes are identified and labelled. Each ST is represented by a circle with the number beside the circle. The frequency of each ST within the analysed population is indicated by the area of the circle. ST-6 and ST-19 were the founders of their respective clonal complexes. SLVs shown in black and DLVs shown in blue.



4.3.3.2 Phylogenetic Trees

Phylogenetic analysis was carried out as described in Section 2.16.6. Figure 4.7 shows an unrooted phylogenetic tree of the 38 *P. acnes* STs based on concatenated sequences of the seven loci. STs belonging to the same eBURST group have been highlighted for comparison. eBURST groups 1, 2 and 4 formed a distinct lineage based on the concatenated phylogenetic tree and included isolates previously identified as type IA based on *recA* and *tly* gene analysis and IFM (McDowell *et al.*, 2005; McDowell *et al.*, 2008). Although the cluster formed by ST-12 and ST-29 (eBURST group 4) have been designated as type IA, they formed a distinct cluster away from the other type IA isolates found in eBURST groups 1 and 2. The cluster consisting of ST-27, ST-9 and ST-22 were identified to be type IB isolates but clustered away from the other type IB isolates (ST-10 and ST-37). It is possible that this has occurred through exchange of genetic material through recombination at certain loci between type IA and a selection of type IB isolates. However, based on eBURST analysis, all type IB isolates clustered together in group 3. The next lineage correlated with eBURST group 7 and included isolates identified as type III. The final lineage identified consisted of type II isolates and corresponded to eBURST groups 5 and 6. Based on eBURST analysis, ST-14, ST-22 and ST-37 were singletons i.e. they did not belong to any eBURST groups. However, their type designation corresponded to the clustering on the phylogenetic tree.

Another way of determining the phylogenetic relationship between STs is to generate an Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree. Figure 4.8 shows an unrooted UPGMA tree based on the allelic profiles of the 38 STs. Again, the STs that belong to the same eBURST groups are highlighted. As would be expected, this phylogenetic tree correlates with the eBURST groups much more consistently, as both methods are based on the allelic profiles of the STs rather than the concatenated DNA sequences of the seven loci. Interestingly, the clonal complex which includes ST-12 and ST-29 clustered away from all other type IA and IB isolates, even though it has been identified as type IA based on *recA* gene analysis and IFM (McDowell *et al.*, 2005; McDowell *et al.*, 2008). It is possible that this cluster is another subgroup of type I isolates,

different to that of type IA and IB. Unlike the phylogenetic tree based on concatenated sequences, where strains belonging to eBURST groups 5 and 6 were found in the same lineage, the UPGMA tree based on allelic profiles separated these into the two groups.

Phylogenetic analysis of individual genes using the Neighbour-Joining method with 1,000 bootstrapping replications (Figures 4.9a-g) shows that most of the genes give significant resolution forming distinct lineages. r*ecA*, however, appears to provide the least differentiated tree with a mean sequence diversity between all the *recA* alleles of 0.43%, though it is still able to distinguish between the three main lineages, type I, II and III. In contrast, the sequence diversity of the remaining six genes varies from 1.1% to 2.18%, providing sufficient resolution to distinguish between the *P. acnes* lineages I, II and III. The *sodA* gene is the only locus where type IA and IB strains do not cluster together on a phylogenetic tree. Therefore, differentiating between types IA and IB strains appears to be dependent on *sodA*.

Figure 4.7 Unrooted phylogenetic dendogram of the 38 *P. acnes* STs, analysed by Neighbour-Joining method based on concatenated sequences (with Jukes-Cantor-based algorithm and 1000 bootstrapping replications). * denotes STs not belonging to a SLV eBURST group but to a DLV eBURST group.

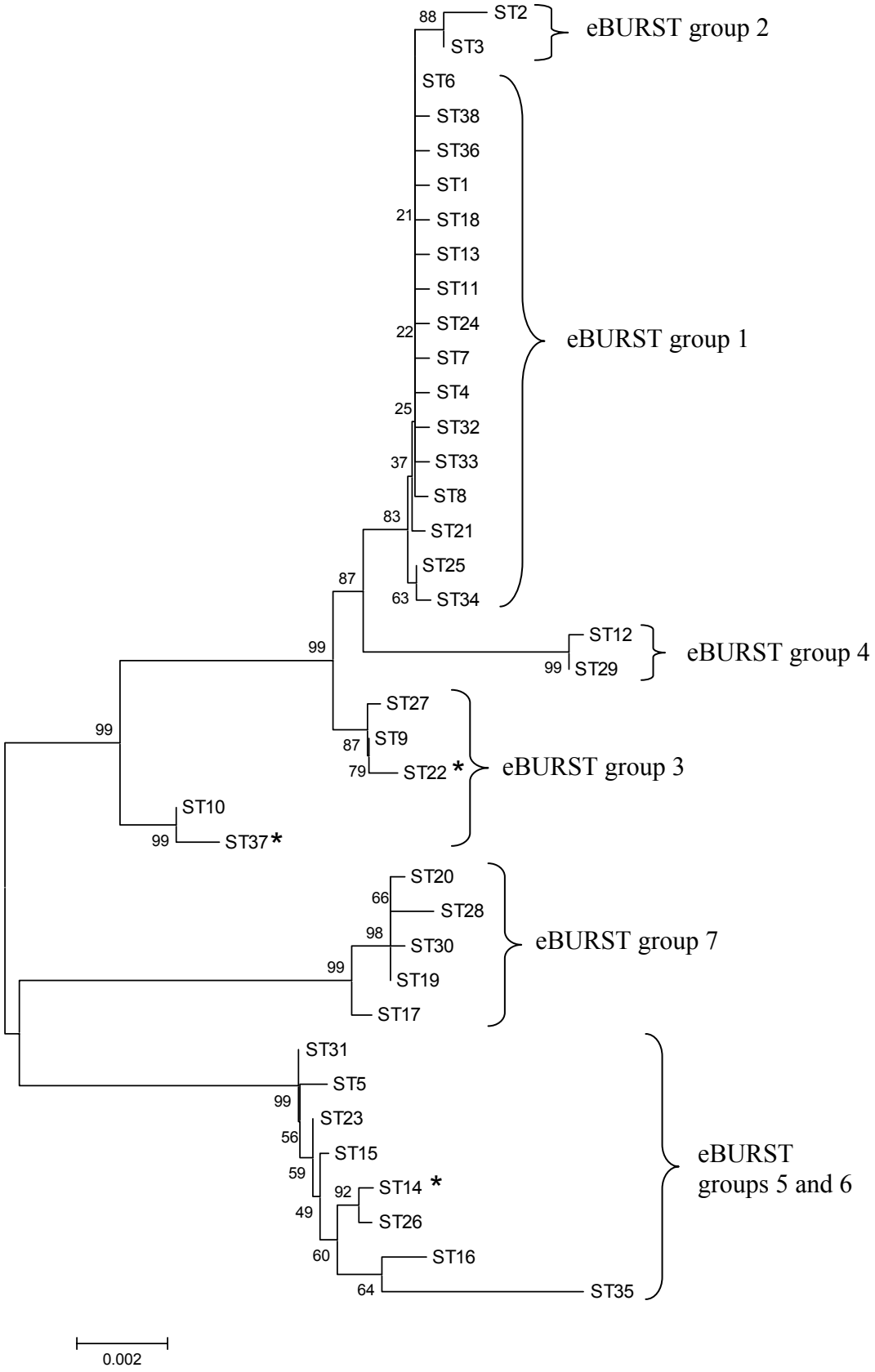


Figure 4.8 Unrooted UPGMA dendrogram, based on allelic profiles of the 38 *P. acnes* STs with Jukes-Cantor-based algorithm. * denotes STs not belonging to a SLV eBURST group but to a DLV eBURST group.

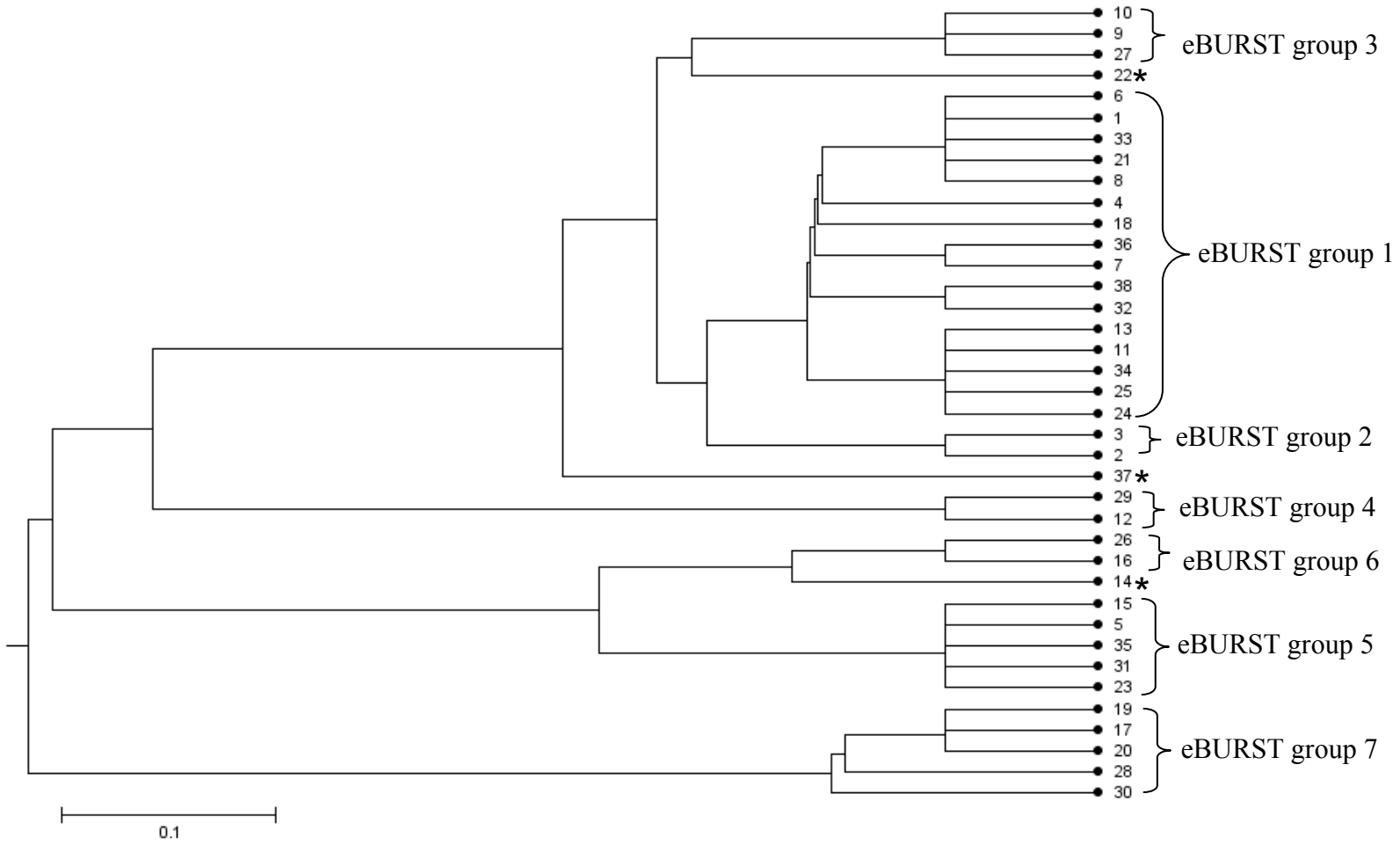
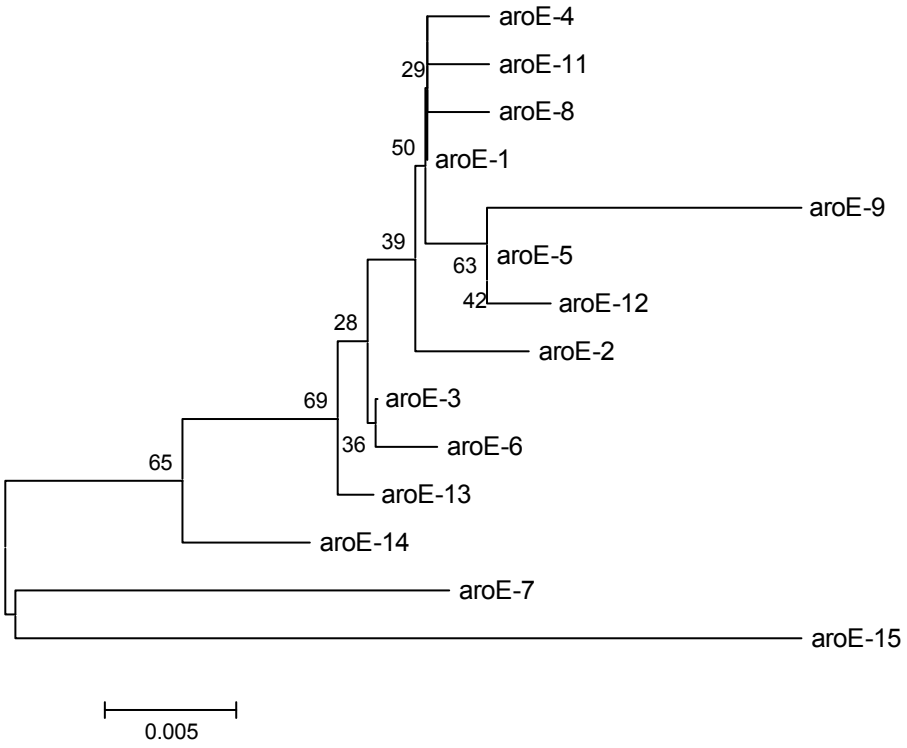
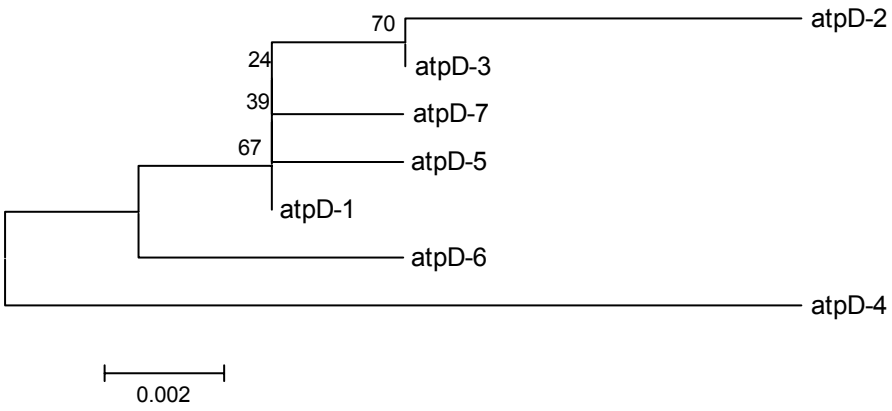


Figure 4.9 (a-g) Unrooted phylogenetic dendrogram, analysed by Neighbour-Joining method based sequences of each of the seven loci (with Jukes-Cantor-based algorithm and 1,000 bootstrapping replications). Allelic numbers are labelled next to the allele name. (a) *aroE*, (b) *atpD*, (c) *gmk*, (d) *guaA*, (e) *lepA*, (f) *recA* and (g) *sodA*

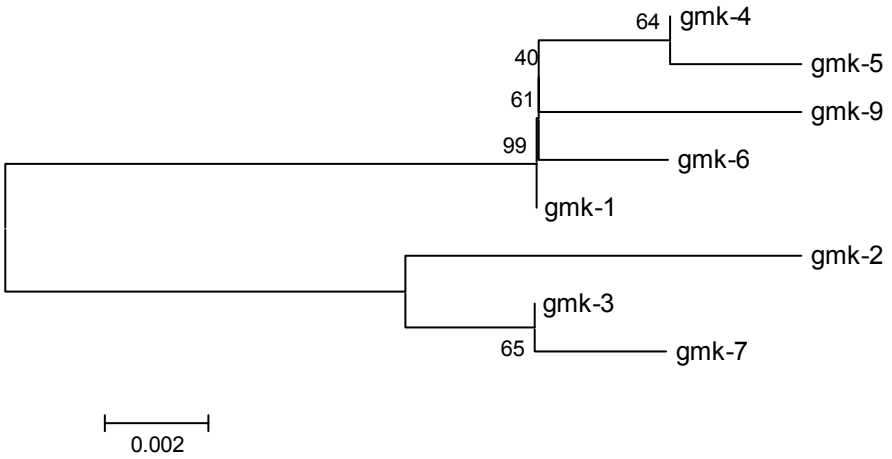
(a) ***aroE***
Overall mean sequence diversity = 1.81%



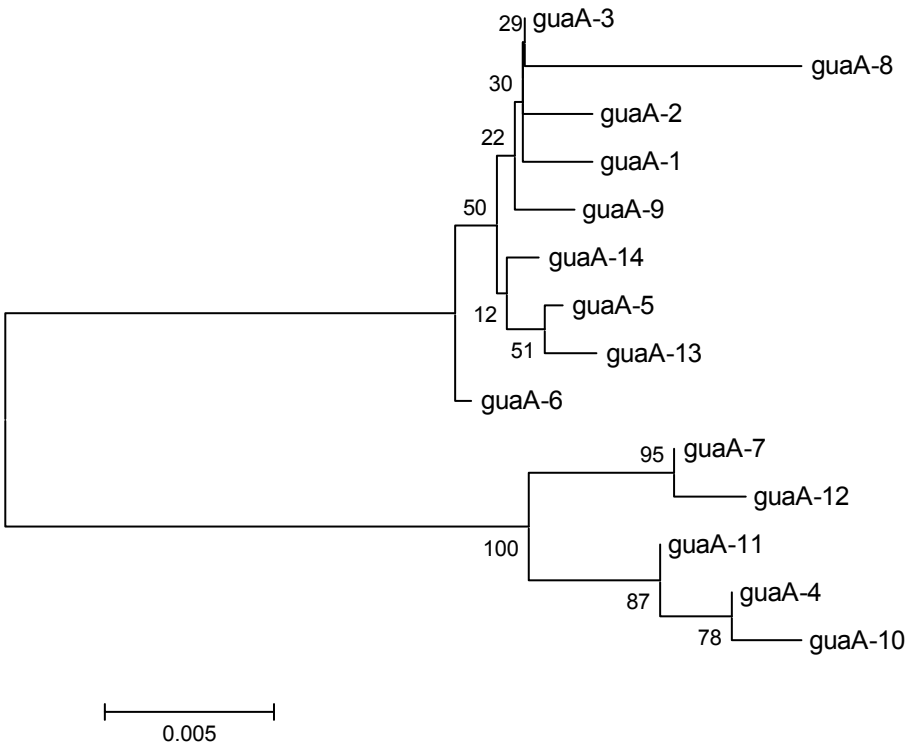
(b) ***atpD***
Overall mean sequence diversity = 1.1%



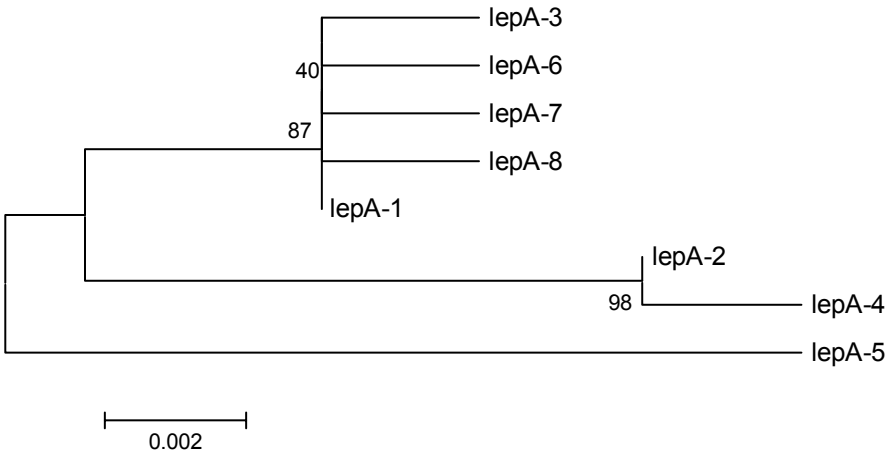
(c) ***gmk***
Overall mean sequence diversity = 1.68%



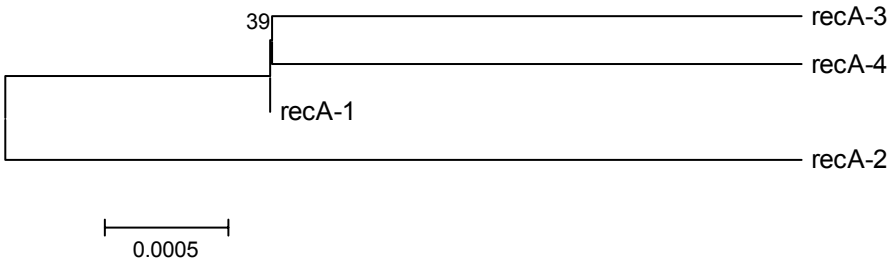
(d) ***guaA***
Overall mean sequence diversity = 2.18%



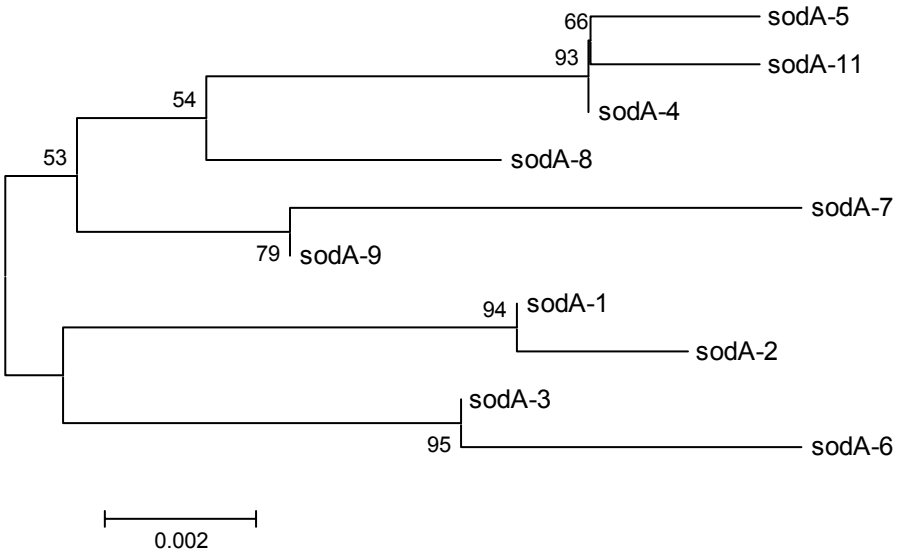
(e) ***lepA***
Overall mean sequence diversity = 1.1%



(f) ***recA***
Overall mean sequence diversity = 0.43%



(g) ***sodA***
Overall mean sequence diversity = 1.36%



4.3.3.3 Splits decomposition analysis

Phylogenetic relationship between STs was also examined by split decomposition analysis using SplitsTree v4.1 (Huson & Bryant, 2006). The split decomposition algorithm is a parsimony method that does not impose a branching or tree-like structure on the data set, but permits reticulations or a network structure that may be indicative of past recombination events. As a consequence, a split decomposition graph will look less tree-like and more net-like as the influence of recombination becomes stronger. As described by Huson, a 'split' is a partition of the taxa into two subsets and in a split network, every edge is associated with a split of the taxa (Huson, 1998). The length of an edge is proportional to the weight of the associated split, which corresponds to the length of a branch in a phylogenetic tree (Huson, 1998).

Figure 4.10 shows splits decomposition analysis of concatenated sequences of the 38 STs and Figure 4.11 shows splits decomposition analysis based on allelic profiles. Strains belonging to the same eBURST group are also shown for comparison. It is evident from both splits trees that there are distinct clusters of STs connected by the network structure, which is fairly consistent with the clonal complexes generated by BURST analysis (Section 4.3.3.1). The splits trees are also very similar to the phylogenetic trees in Section 4.3.3.2.

Figure 4.10 Splits decomposition analysis based on concatenated sequences of the seven housekeeping genes of the 38 STs. ST-14, ST-22 and ST-37 not belonging to an eBURST group.

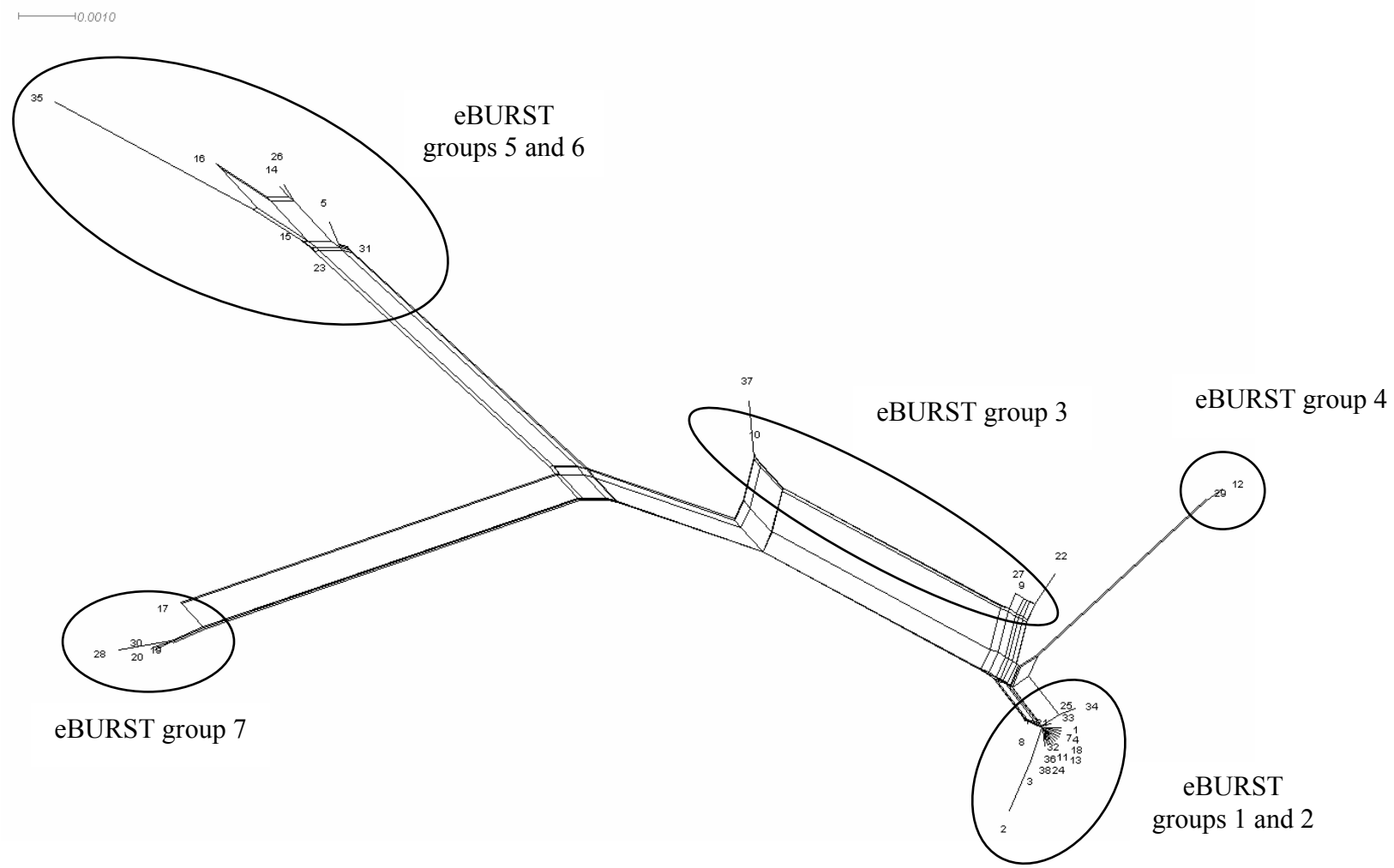
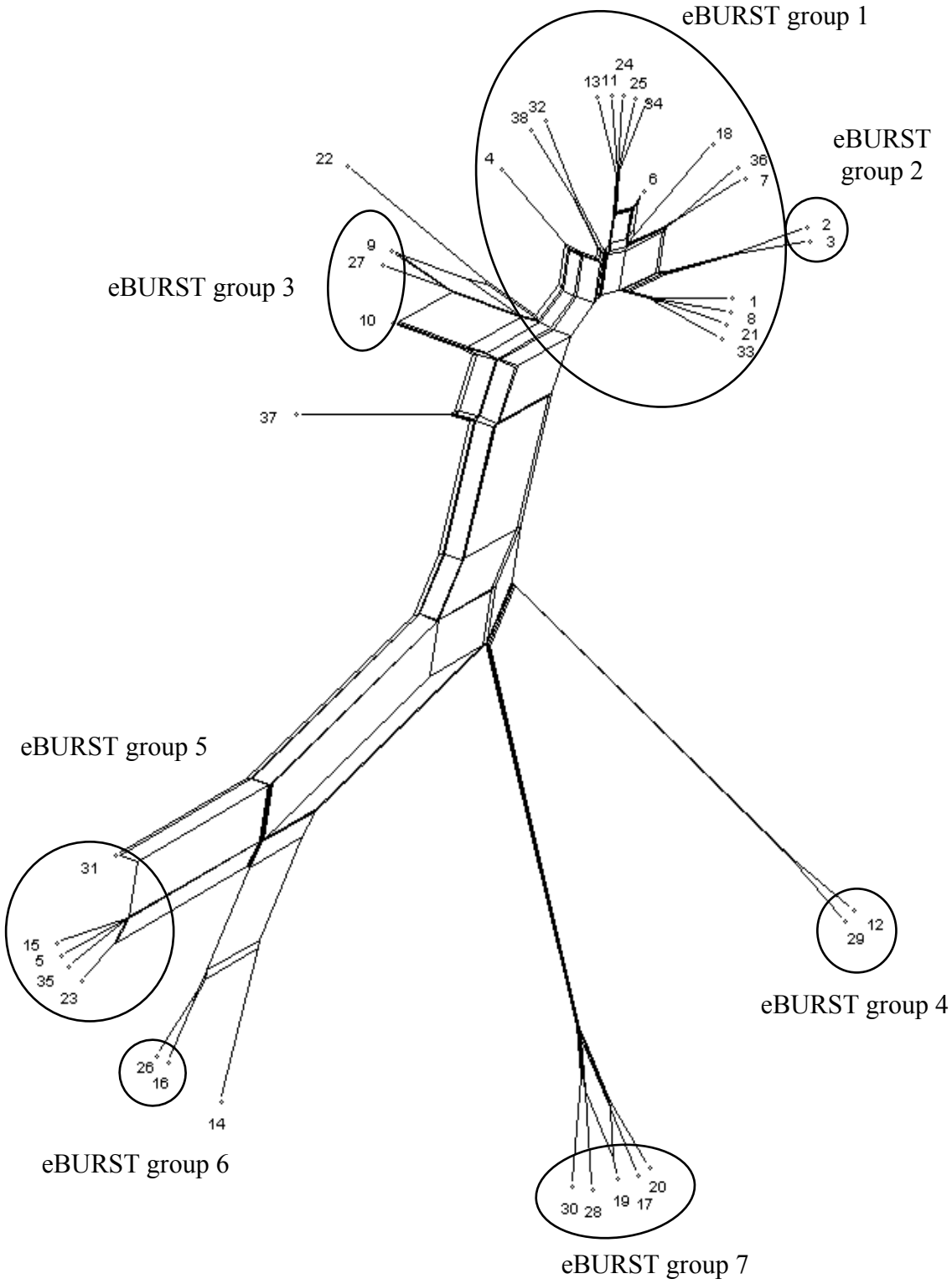


Figure 4.11 Splits decomposition analysis based on allelic profiles of the 38 STs. ST-14, ST-22 and ST-37 not belonging to an eBURST group.



4.3.3.4 Clonal frame analysis

ClonalFrame v1.1 is a model-based method of using MLST data to infer the clonal relationships of bacteria and determine whether a subset of isolates share a common ancestry (Didelot & Falush, 2007). It takes into account of both point mutation and homologous recombination and attempts to infer the parameters and events in the evolutionary process that led to the observed pattern of DNA sequence variation.

P. acnes MLST data was assessed using ClonalFrame and Figure 4.12 is a network representation of the output using Graphviz and Figure 4.13 is a majority-rule consensus tree (i.e. a linear representation of the data). Figure 4.12 shows inferred ancestral nodes in black and the location of isolates in red, with each line indicating a single isolate. Nodes whose ST is not found amongst the study population are shown as an empty circle. The ancestral node of each network component is indicated by a darker circle.

The output diagram suggests that the ancestral node in this network has not yet been identified and that numerous genotypes that have descended from this common ancestor have also not been identified using the current study population. Based on eBURST analysis, ST-6 and ST-19 were the founding genotypes for their respective clonal complexes. Clonal frame analysis, however, does not put particular weight on ST-19 and only regard it as a descendant strain of ST-30 (the ancestral node for that group). ST-6 is inferred as an ancestral node, with two descendent nodes and numerous isolates branching from it. The ClonalFrame-generated network has similarities with both the Neighbour-Joining dendrogram generated from concatenated sequences and also eBURST analysis which is based on allelic profiles. For example, ST-9, ST-22 and ST-27 cluster away from ST-10 and ST-37 (despite the fact that they are all type IB strains based on *recA* gene analysis). This is consistent with the Neighbour-Joining tree but very different to what is implied by eBURST analysis, which clusters ST-9, ST-10 and ST-27 into the same clonal complex (eBURST group 3). Based on clonal frame analysis, ST-35 is the ancestral node to a group which includes ST-31, ST23, ST-5 and ST-15.

ST-35 also gives rise to another separate group consisting of ST-16, ST-14 and ST-26. These two distinct groups are identified as two clonal complexes by eBURST analysis (eBURST groups 5 and 6). However, Neighbour-Joining method combines these two groups and there is no obvious distinction from the phylogenetic dendrogram.

As mentioned in Section 4.3.3.1, eBURST analysis implies that eBURST groups 5, 6 and 7 were relatively new clonal complexes but it was interesting that they did not form any DLVs with any other groups. This can be explained by the current ClonalFrame network, which indicates that there are common ancestors to these groups which have not been identified using the current study population (as shown by the empty circles). Most importantly, the inferred ancestral node for the whole network (darker circle) has not been analysed by MLST.

The ClonalFrame network clearly has advantages over both phylogenetic analysis based on concatenated sequences and eBURST analysis which is solely based on allelic profiles. It is able to clearly represent true relatedness between strains within a group but also infer genetic relatedness and common ancestry between groups. Although eBURST has the ability to correctly identify clonal complexes and cluster together related strains, it does not provide any information as to how theses complexes may be related to each other and therefore how they have evolved over time.

Figure 4.12 A network representation of the ClonalFrame output using Graphviz. The network shows inferred ancestral nodes in black and the location of isolates in red, with each line indicating a single isolate. Each isolate has the genotype of the node it is connected to. Nodes whose ST is not found amongst the study population are shown as an empty circle. The ancestral node of each network component is indicated by a darker circle. Only one isolate representing each ST is shown (i.e. 38 isolates representing the 38 different STs).

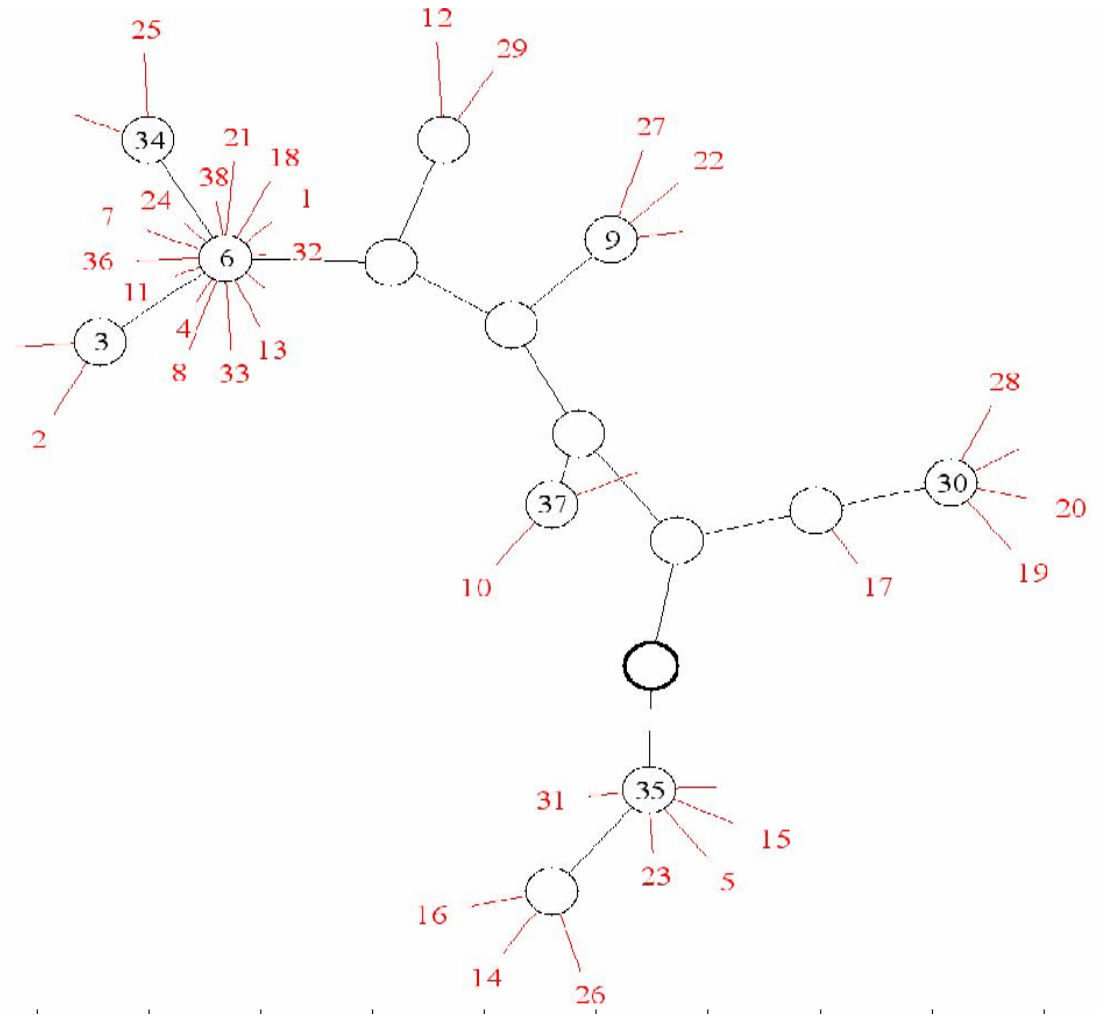
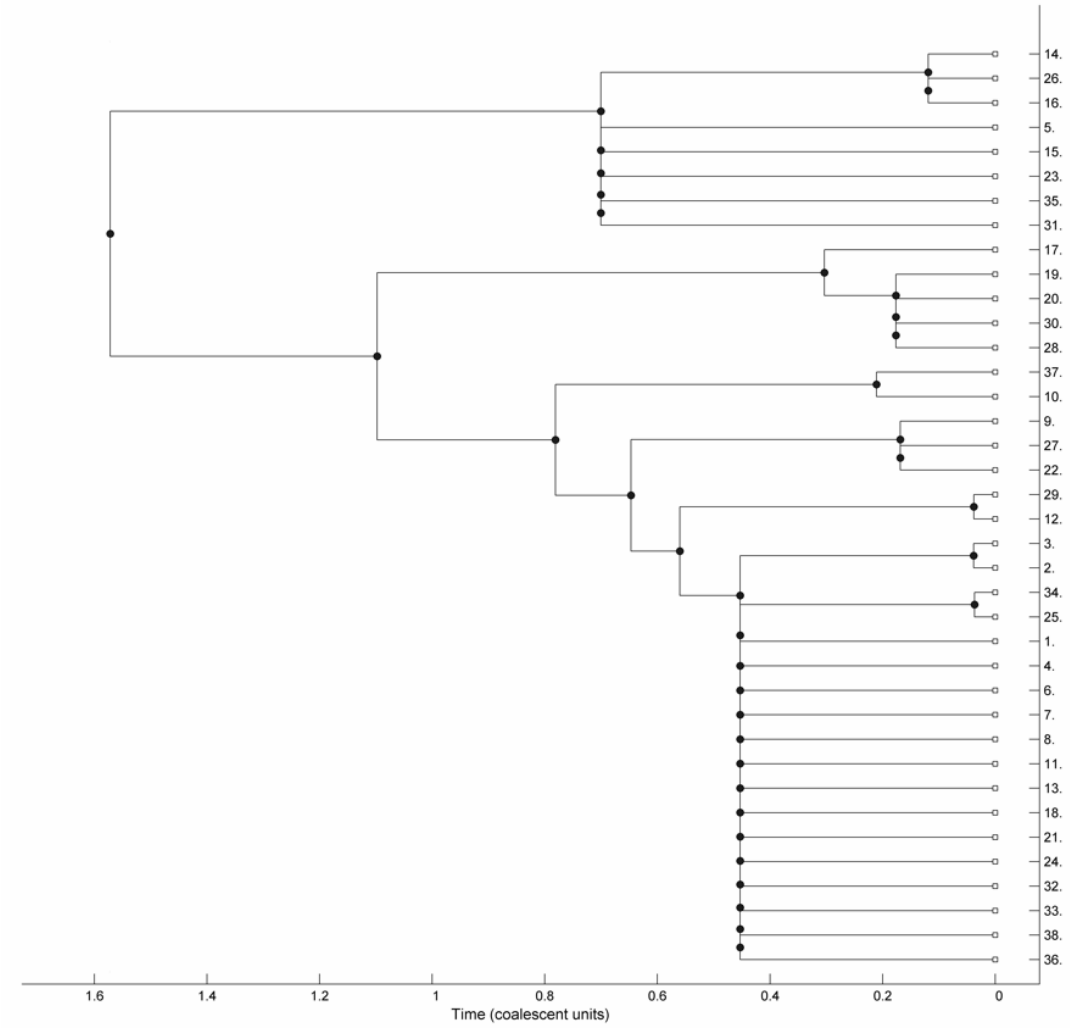


Figure 4.13 Majority-rule consensus tree produced from the ClonalFrame program



4.3.3.5 Sequence diversity

To determine the genetic diversity within populations and between populations, the mean average nucleotide distance within each clonal complex (as determined by eBURST analysis) and between selected clonal complexes were determined using the Jukes-Cantor method in Mega v4. A total of 3,135 nucleotides were compared. The results are shown in Table 4.5. The overall percentage of sequence diversity between all 38 STs is 1.1%.

Sequence diversity within clonal complexes is very small, ranging from 0.03% to 0.41% (Table 4.5a) demonstrating their close genetic relatedness. The diversity between all type I isolates (when eBURST groups 1, 2, 3 and 4 are combined) is also low at 0.26% (Table 4.5b). This is also true for type II isolates (eBURST groups 5 and 6 combined) which only has a mean nucleotide difference of 0.29%. This shows that although these groups are divided into clonal complexes, they actually show very little nucleotide difference. However, there is an obvious increase in the sequence divergence between type I, type II and type III strains (Table 4.6b), verifying their status as distinct lineages. This has also been shown in previous studies looking at complete *recA* gene and *tly* gene sequence analysis. McDowell *et al.* demonstrated that comparison of the 1,047 bp *recA* genes and the 777 bp *tly* genes between two reference strains, NCTC 737 (type I strain) and NCTC 10390 (type II strain), revealed 99% and 98% sequence identity respectively (McDowell *et al.*, 2005). eBURST group 1 strains were identified previously as type IA and groups 5 and 6 were identified as type II strains. r*ecA* gene analysis between a type I strain (NCTC 737) and the recently identified type III strains showed 98.9% identity (McDowell *et al.*, 2008). 99.1% identify were found between type II strain (NCTC 10390) and the same type III strains (McDowell *et al.*, 2008).

Table 4.5 (a) Nucleotide divergence within clonal complexes.

eBURST groups	Strain type	Nucleotide divergence (%)
1	IA	0.06%
2	IA	0.10%
3	IB	0.41%
4	Novel cluster	0.03%
5	II	0.28%
6	II	0.15%
7	III	0.11%

Table 4.5 (b) Nucleotide divergence between clonal complexes.

eBURST groups	Strain type	Nucleotide divergence (%)
1 & 2	IA	0.08%
1, 2 & 3	IA & IB	0.19%
1, 2, 3 & 4	IA, IB & novel cluster	0.26%
5 & 6	II	0.29%
1, 2, 3, 4, 5 & 6	I & II	1.77%
1, 2, 3, 4 & 7	I & III	1.81%
5, 6 & 7	II & III	1.61%
All groups	I, II & III	1.10%

4.3.4 Association between STs, clonal complexes and disease

Amongst the *P. acnes* collection of isolates examined using MLST, ST-6 was found to be the predominant strain (49%). It was also identified as the putative ancestral genotype for eBURST group 1 and was an ancestral node using clonal frame analysis. This particular genotype appears very successful as it was isolated from a variety of sources (acne, skin wounds, fatal granulomas, dental infections) across four continents (Table 4.1). It is currently unclear, however, whether this simply reflects a greater opportunity to cause infection due to an elevated prevalence, or if it relates to increased virulence and capacity to cause disease. The well described and used reference strain NCTC 737, which belongs to ST-6, was isolated from a patient with facial acne in London, 1920. This indicates that the strain is stable and possibly possesses phenotypic characteristics which facilitate its survival in disparate environments. Further studies of the ST-6 genotype in terms of its pathogenicity and antibiotic resistance are therefore warranted. It was interesting to note that significantly more ST-6 isolates were recovered from acne skin than from any other infection sites (odds ratio 6.0219; 95% C.I. 2.76-13.13). Despite this trend, identical or similar STs were also found in isolates from other clinical sources. Table 4.6 lists the clinical infections from which the *P. acnes* isolates were cultured from in order of prevalence. The number of isolates in each eBURST group is shown and the corresponding phylotype based on *recA* gene sequencing is indicated below it. Isolates from acne infection make up a large proportion (46%) of the culture collection. As a large number of these isolates are ST-6, which belong to eBURST group 1, it would explain to a certain extent why there is such a large proportion of ST-6 isolates in the study collection.

There does not appear to be an overall association between ST or clonal complex and clinical manifestation. eBURST group 1 (type IA) strains appear to be responsible for a wide range of infections. eBURST group 3 (type IB) strains are also not restricted to one infection site, however, it does not occur more frequently in acne infections than other infections (unlike type IA strains). Isolates in groups 2, 4, 5, 6 and 7 are too few to make conclusive comments regarding their distribution amongst different clinical diseases. However, four out of six eBURST

group 7 (type III) isolates were recovered from microdiscectomy procedures, with the other two isolated from keratitis and hip infections.

Looking more closely at eye infection, the majority of *P. acnes* cultured from corneal scrape from keratitis infections were from eBURST group 1 and 3 (type IA and type IB, respectively) (Table 4.6). Interestingly, five out of eight aqueous humour isolates from endophthalmitis infections clustered in eBURST group 4 (novel cluster). As mentioned previously, this novel group is phylogenetically distinct from type IA and IB strains. The remaining three aqueous humour isolates clustered in eBURST group 1. Further investigation in aqueous humour samples is warranted to investigate whether this strain is particularly common in this usually sterile environment. There were only two vitreous humour isolates (from endophthalmitis infections) within the study collection. Both belonged to eBURST group 1 (type IA).

Although a few clinical manifestations of *P. acnes* appear to be more related to certain genotypes or clonal clusters, no one infection site is associated with only one clonal complex and vice versa. Any association will require more numbers of isolates from that infection site, ideally from different countries, to be investigated. A previous study using RAPD analysis to distinguish between different clinical *P. acnes* isolates also did not find any clear correlation between clinical source and genotype (Perry *et al.*, 2003).

Table 4.7 lists the different countries the current study *P. acnes* collection was derived from. 54% of the isolates were isolated from different centres around the UK including Cardiff, Belfast, London, Birmingham and Leeds. eBURST 1 (type IA) strains were found in all the ten countries. eBURST group 3 (type IB) strains were found in Europe, USA and Japan. eBURST groups 5 and 6 (type II) and also eBURST group 7 (type III) strains were restricted to European countries although numbers are too few to draw any definite conclusions.

Table 4.6 Clinical disease distribution of clonal complexes and strain type (ST-22 and ST-37 are included in eBURST group 3 and ST-14 is included in eBURST group 7 for ease of comparison). The two type strains are not included in the table. Eye infections (microbial keratitis and endophthalmitis) are highlighted in light grey.

Clinical disease	Clonal complexes and corresponding *P. acnes* strain type							Total no. of isolates	%
	eBURST 1 Type IA	eBURST 2 Type IA	eBURST 3 Type IB	eBURST 4 Novel cluster	eBURST 5 Type II	eBURST 6 Type II	eBURST 7 Type III		
Acne	50	0	2	3	1	0	0	56	45.5%
Corneal scrape (from keratitis)	10	0	6	0	1	0	1	18	14.6%
Aqueous humour (from endophthalmitis)	3	0	0	5	0	0	0	8	6.5%
Microdiscetomy (from sciatica operations)	1	0	3	0	0	0	4	8	6.5%
Hip	0	0	3	0	2	1	1	7	5.7%
Dental	4	0	2	0	0	0	0	6	4.9%
Skin wound	1	0	3	0	0	0	0	4	3.3%
Fatal granuloma	3	0	0	0	0	0	0	3	2.4%
Vitreous humour (from endophthalmitis)	2	0	0	0	0	1	0	3	2.4%
Neck lymph nodes	1	0	1	0	0	0	0	2	1.6%
Eyes	1	0	1	0	0	0	0	2	1.6%
Blood culture	1	0	0	0	1	0	0	2	1.6%
Cadaveric skin	1	0	0	0	0	0	0	1	0.8%
Brain abscess	1	0	0	0	0	0	0	1	0.8%
Kidney	0	1	0	0	0	0	0	1	0.8%
Bone	0	1	0	0	0	0	0	1	0.8%
Total no. of isolates in each group	79	2	21	8	5	2	6	123	
%	64.2%	1.6%	17.1%	6.5%	4.1%	1.6%	4.9%		

Table 4.7 Geographical distribution of clonal complexes and strain type (ST-22 and ST-37 are included in eBURST group 3 and ST-14 is included in eBURST group 7 for ease of comparison). The two type strains are not included in the table.

Country	Clonal complexes and corresponding *P. acnes* strain type							Total no. of isolates	%
	eBURST 1	eBURST 2	eBURST 3	eBURST 4	eBURST 5	eBURST 6	eBURST 7		
	Type IA	Type IA	Type IB	Novel cluster	Type II	Type II	Type III		
UK	41	2	11	3	3	1	5	66	53.7%
Paris	16	0	12	0	1	1	1	31	25.2%
Sweden	5	0	1	0	0	0	0	6	4.9%
Australia	4	0	0	0	0	0	0	4	3.3%
USA	3	0	1	0	0	0	0	4	3.3%
China	3	0	0	0	0	0	0	3	2.4%
Italy	3	0	0	0	0	0	0	3	2.4%
Spain	2	0	0	0	0	0	0	2	1.6%
Greece	1	0	0	0	1	0	0	2	1.6%
Japan	1	0	1	0	0	0	0	2	1.6%
Total no. of isolates in each group	79	2	26	3	5	2	6	123	
%	64.2%	1.6%	21.1%	2.4%	4.1%	1.6%	4.9%		

4.3.5 Analysis of recombination

Bacteria existing as clonal populations evolve diversity by the accumulation of point mutations, while non-clonal populations evolve more through recombination within or between species. There are several ways in which the level of recombination within a population can be measured. Here, the index of association, gene tree congruence, split decomposition and clonal frame analysis, have been applied to determine whether the study population is clonal or recombinogenic.

4.3.5.1 Index of association

The level of linkage between alleles at different loci was estimated by calculating index of association (I_A) values (Haubold & Hudson, 2000) using LIAN 3.5 as described in Section 2.16.5. An I_A value not significantly greater than 0 after 1,000 computer randomisations indicates that loci within a single species population (monophyletic) are in linkage equilibrium (freely recombining), while a population with an I_A value significantly greater than 0 ($P<0.001$) are in linkage disequilibrium (clonal) (Smith *et al.*, 1993).

Table 4.8a shows the I_A values calculated for the different eBURST groups of *P. acnes*. I_A value was 0.538 ($P <0.001$) when all 125 isolates were included and was 0.4768 ($P <0.001$) when a representative of each ST was included. This indicates that the population is in linkage disequilibrium and thus the population structure of *P. acnes* as a whole is clonal. Within eBURST group 1 (type IA strains), the I_A value was found to be -0.1357 ($P=1$) and the analysis programme determined that no significant linkage disequilibrium was detected. Similarly, eBURST group 7 (type III strains) had an I_A value of -0.0996 ($P=1$) also indicating a freely recombining population structure. I_A values for the remaining 5 eBURST groups could not be determined due to too few STs in each group. However, the LIAN program was able to conclude that eBURST groups 3 and 5 did not show significant linkage disequilibrium.

Hence it appears that the population structure of *P. acnes* is composed of subgroups within which there is evidence of frequent recombination occurring but not between subgroups. This is further illustrated by calculating I_A values of different combinations of eBURST groups (Table 4.8b). An I_A value of -0.1167 (P=1) was calculated when eBURST groups 1 and 2 were combined. This suggests that there is frequent recombination occurring within the two groups, which were both identified as type IA strains by *recA* gene sequencing analysis. Combining eBURST groups 1 and 3 (i.e. comparing type IA and IB strains), the I_A value was -0.11 (P=1) demonstrating no significant linkage disequilibrium and therefore frequent recombination between the two lineages. Another explanation for the lack of genetic exchanged between eBURST groups 1, 2 and 3 is that they are descendents of the same ancestor and few evolutionary changes occurred as these subgroups evolved.

When eBURST groups 1 (type IA strains) and 4 (novel cluster) were combined however, the I_A value increased to 0.2179 ($p<0.001$) thus providing additional evidence for eBURST group 4 as a novel cluster. The presence of linkage disequilibrium was also evident between eBURST groups 1 and 5 (I_A value 0.4867, $P<0.01$), between groups 1 and 6 (I_A value 0.3509, $P<0.001$) and between groups 1 and 7 (I_A 0.5094, $P<0.01$). This clearly demonstrates significant phylogenetic distinction between type I, type II and type III strains. Groups 5 and 6, both consisting of type II strains, did not show significant linkage disequilibrium (I_A value -0.074, P=0.991) and therefore frequent recombination occurred between the two groups.

Calculation of I_A values has demonstrated that while recombination was evident amongst the clonal complexes (and thus between the strain types I, II and III) and also between eBURST groups 1 and 2 (i.e. type IA and type IB strains), linkage disequilibrium was detected between all other eBURST groupings, suggesting that *P. acnes* comprise of distinct phylogenetic subgroups with frequent recombination within these subgroups

Table 4.8 (a) Index of Association (I_A) values of all 125 *P. acnes* isolates, for ST representatives and for each eBURST group to measure recombination within groups. An I_A not significantly greater than zero suggests that the population is in linkage equilibrium (LD) and is therefore freely recombining, whilst an I_A value significantly greater than zero is considered to be clonal (Haubold & Hudson, 2000).

Single eBURST groups	No. of isolates	Strain type	I_A value	P value	Linkage disequilibrium
All isolates	125		0.538	P<0.001	LD
ST representatives	38		0.4768	P<0.001	LD
eBURST group 1	16	IA	-0.1357	P=1	No LD
eBURST group 2	2	IA	NAD	-	-
eBURST group 3	3	IB	NAD	-	No LD
eBURST group 4	2	Novel	NAD	-	-
eBURST group 5	5	II	NAD	-	No LD
eBURST group 6	2	II	NAD	-	-
eBURST group 7	5	III	-0.0996	P=1	No LD

Table 4.8 (b) I_A values of combined eBURST to measure recombination between groups.

Combined eBURST groups	No. of isolates	Strain type	I_A value	P value	Linkage disequilibrium
Groups 1 & 2	18	IA	-0.1167	1	No LD
Groups 1 & 3	19	IA & IB	-0.11	1	No LD
Groups 1 & 4	18	IA & IC	0.2179	<0.01	LD
Groups 1 & 5	21	IA & II	0.4867	<0.01	LD
Groups 1 & 6	18	IA & II	0.3509	<0.01	LD
Groups 1 & 7	21	IA & III	0.5094	<0.01	LD
Groups 5 & 6	7	II	-0.1833	0.056	No LD

4.3.5.2 Degree of congruence among loci

The degree of recombination can also be assessed by statistical comparisons between tree topologies for each MLST locus using PAUP v4 (Phylogenetic Analysis Using Parsimony) (Wilgenbusch & Swofford, 2003). The first analysis is the Shimodaira Hasegawa (SH) test which creates maximum likelihood (ML) for each of the genes and compares the topology of one tree to another. For a purely clonal evolving population all the trees should have the same topology regardless of the gene, since with no recombination occurring, their ancestry would be very consistent (congruent). Table 4.9 shows congruence tests for each gene tree compared to other gene tree topologies and random tree data. P value is the probability that the trees are significantly different. $P<0.05$ (indicated by *) shows that there is significant differences in the tree topologies and so the genes compared are not strictly clonal. So although most of the *P. acnes* genes appear clonal, some have significant changes.

The second test, which compares these same trees with random trees, attempts to determine the extent of congruence. The degree of congruence between trees was measured for each of the loci by comparing the differences in log likelihood (Δ-ln *L*) of the ML tree for each locus with those of the ML topologies obtained for the other loci and with 200 randomly generated trees (Figure 4.14a-g). If the Δ-ln *L* values for the comparisons among the different ML trees fall within the 99th percentile of randomly distributed trees, then they are considered significantly different and therefore incongruent. The 99th percentiles in Figures 4.14a-g are indicated by red dotted lines. If the Δ-ln *L* value of the ML tree of the gene is inline with or higher than this line, then it is incongruent and significantly recombinogenic.

Comparison between all seven *P. acnes* MLST genes produced likelihood differences that fell outside the 99th percentile of the random distribution (Figures 4.14a-g), indicating significant congruence between gene trees. Therefore, *P. acnes* as a whole population is not recombinogenic. However, the SH test showed that there was significant incongruence between a few of the gene trees

constructed from different loci, whereas the randomisation test showed that there was still some phylogenetic signal that had not been lost by recombination.

So overall, based on PAUP analysis, *P. acnes* appears to be a mostly clonal population with some significant recombination occurring (as shown by SH test) but the extent of this recombination is not so high as to be freely recombining as the evolutionary relatedness of the STs is still significantly preserved and congruent (as shown by randomisation test). Another explanation for the observed recombination within nucleotide sequences with congruence between gene trees is the existence of ecologically distinct subgroups of the *P. acnes* population. The sequence diversity of *Haemophilus influenza* (*H. influenza*) is considerable, as shown by PAUP analysis (Feil *et al.*, 2001). Like *P. acnes*, all of the *H. influenza* MLST gene trees showed some congruence. The greater level of congruence within this *H. influenza* population has been suggested to reflect barriers to genetic exchange between different subgroups within the population (Feil *et al.*, 2001).

Table 4.9 Congruence tests for each gene tree compared to other gene tree topologies and random tree data. –ln *L* is the inverse log likelihood of the ML value and Δ-ln *L* is the difference in this log likelihood value from comparing the tree to itself. P value is the probability that the trees are significantly different. P <0.05 (indicated by *) shows that there are significant differences in the tree topologies and so the genes compared are not strictly clonal.

aroE

Tree	-ln *L*	Δ-ln *L*	P
aroE	807.31222	(best)	
atpD	847.44625	40.13402	0.001*
gmk	828.71035	21.39812	0.022*
guaA	865.27895	57.96673	0.001*
lepA	828.71035	21.39812	0.022*
recA	828.71035	21.39812	0.022*
sodA	828.71035	21.39812	0.022*

atpD

Tree	-ln *L*	Δ-ln *L*	P
aroE	769.80655	42.4136	0.001*
atpD	727.39295	(best)	
gmk	732.6117	5.21874	0.426
guaA	769.26288	41.86992	0.000*
lepA	732.6117	5.21874	0.426
recA	733.27356	5.88061	0.411
sodA	733.27356	5.88061	0.411

gmk

Tree	-ln *L*	Δ-ln *L*	P
aroE	691.96979	40.80596	0.000*
atpD	655.92472	4.76089	0.499
gmk	651.16383	0	0.911
guaA	685.70199	34.53816	0.002*
lepA	651.16383	(best)	
recA	660.29054	9.12671	0.262
sodA	660.29054	9.12671	0.262

guaA

Tree	-ln *L*	Δ-ln *L*	P
aroE	1017.44552	157.50512	0.000*
atpD	969.27297	109.33256	0.000*
gmk	962.50152	102.56111	0.001*
guaA	859.94041	(best)	
lepA	962.50152	102.56111	0.001*
recA	973.2177	113.2773	0.001*
sodA	965.13495	105.19455	0.000*

lepA

Tree	-ln *L*	Δ-ln *L*	P
aroE	758.12809	36.80695	0.005*
atpD	734.67598	13.35484	0.132
gmk	729.99302	8.67188	0.33
guaA	760.05409	38.73295	0.005*
lepA	721.32114	(best)	
recA	729.99302	8.67188	0.33
sodA	721.56663	0.24549	0.847

recA

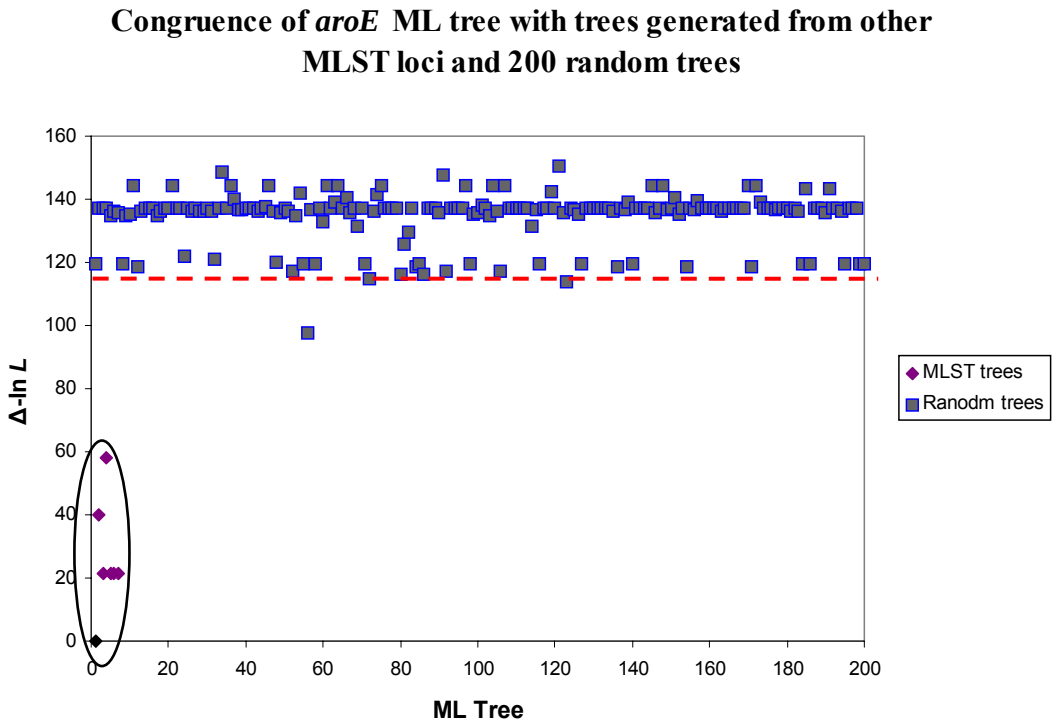
Tree	-ln *L*	Δ-ln *L*	P
aroE	666.29151	7.16423	0.033*
atpD	662.00563	2.87835	0.245
gmk	659.12728	0	0.842
guaA	666.29152	7.16424	0.037*
lepA	659.12728	0	0.976
recA	659.12728	(best)	
sodA	659.12728	0	0.91

sodA

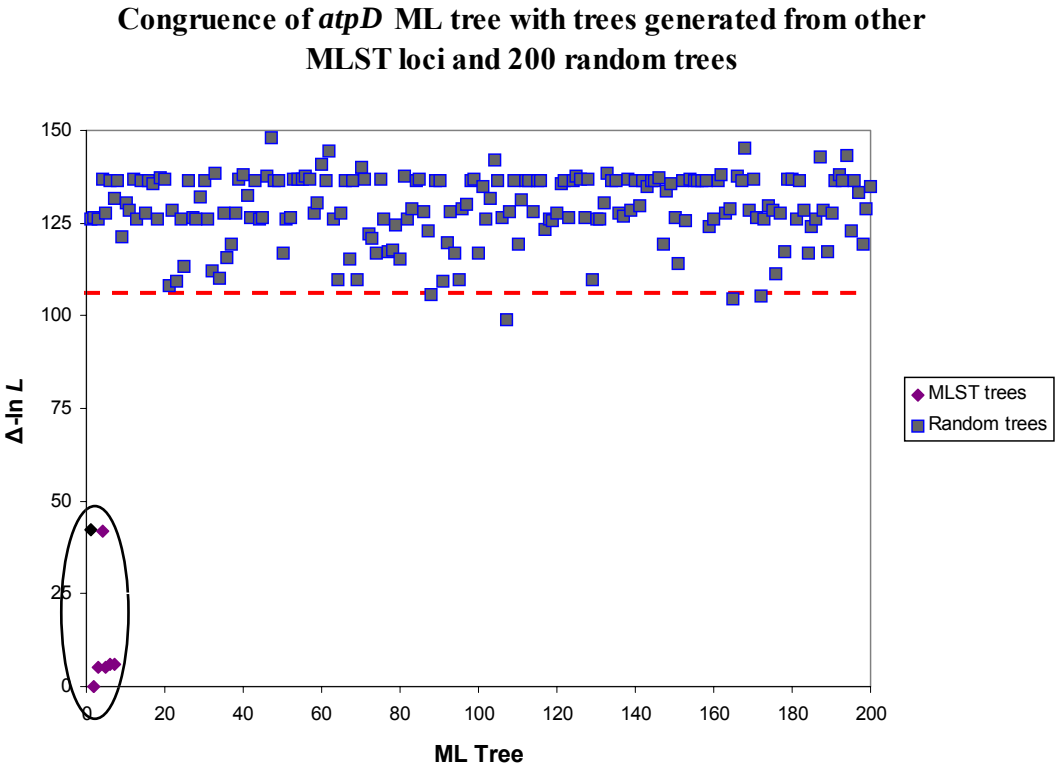
Tree	-ln *L*	Δ-ln *L*	P
aroE	849.02513	96.85518	0.000*
atpD	834.30977	82.13982	0.001*
gmk	824.31159	72.14163	0.002*
guaA	813.11328	60.94333	0.000*
lepA	802.50044	50.33049	0.000*
recA	824.31206	72.14211	0.002*
sodA	752.16995	(best)	

Figure 4.14a-g. Congruence analysis of each *P. acnes* MLST housekeeping gene. 99th percentiles are indicated by the dotted line. (a) *aroE*, (b) *atpD*, (c) *gmk*, (d) *guaA*, (e) *lepA*, (f) *recA* and (g) *sodA*.

(a)

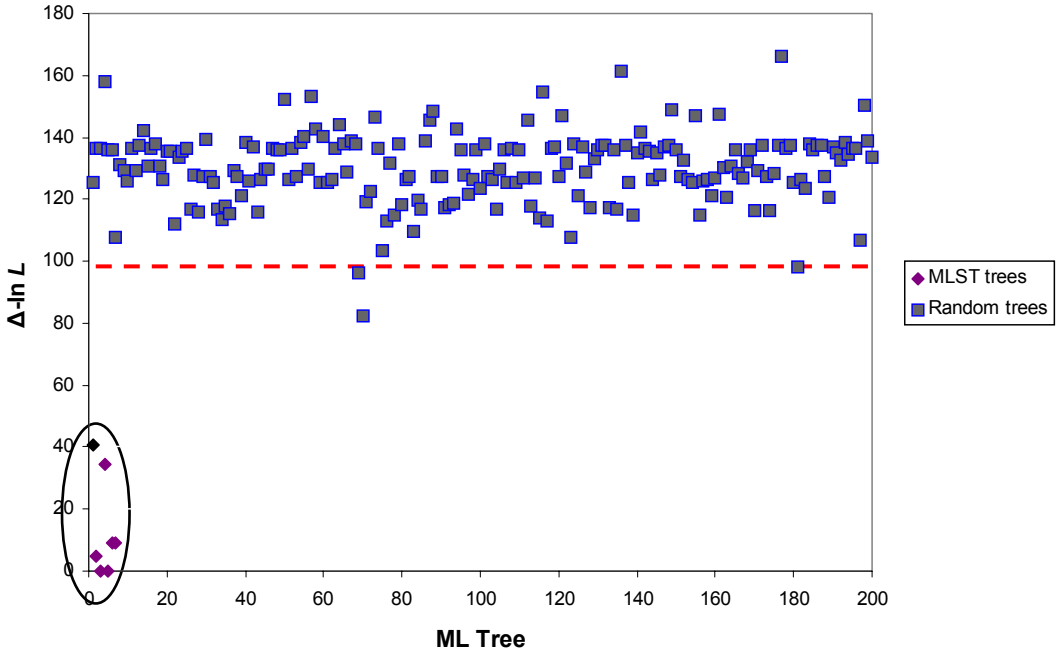


(b)



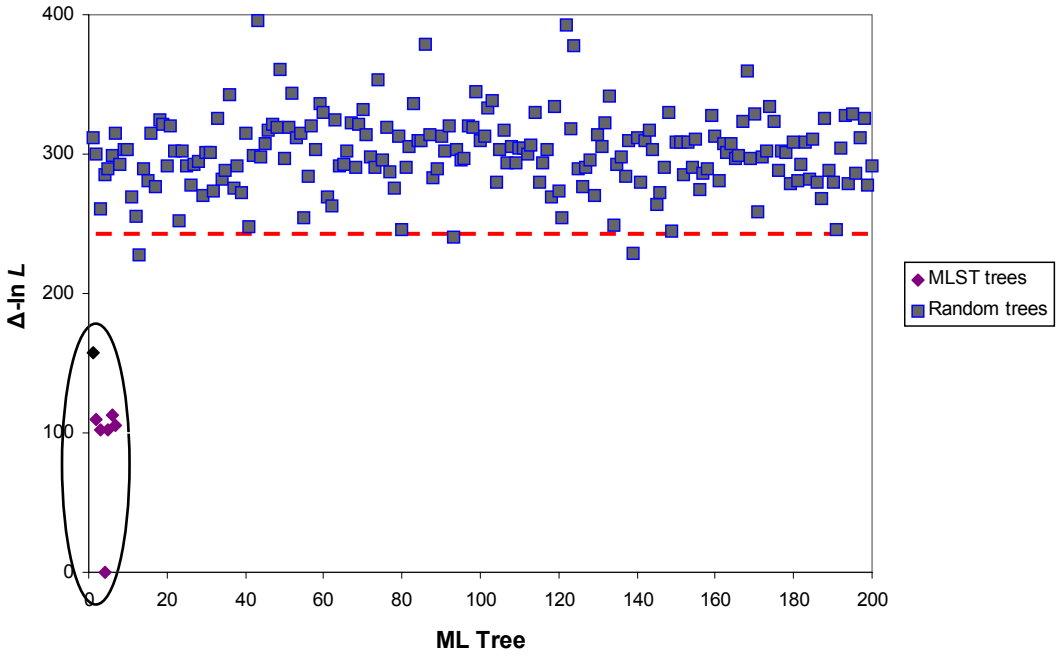
(c)

Congruence of *gmk* ML tree with trees generated from other MLST loci and 200 random trees

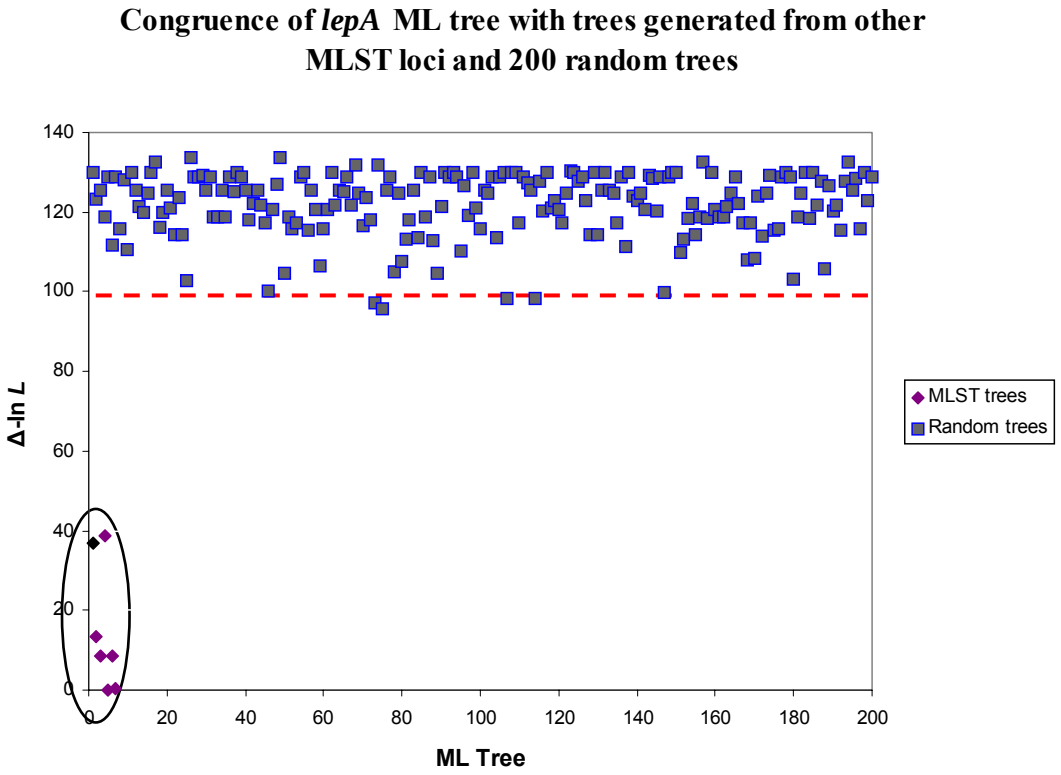


(d)

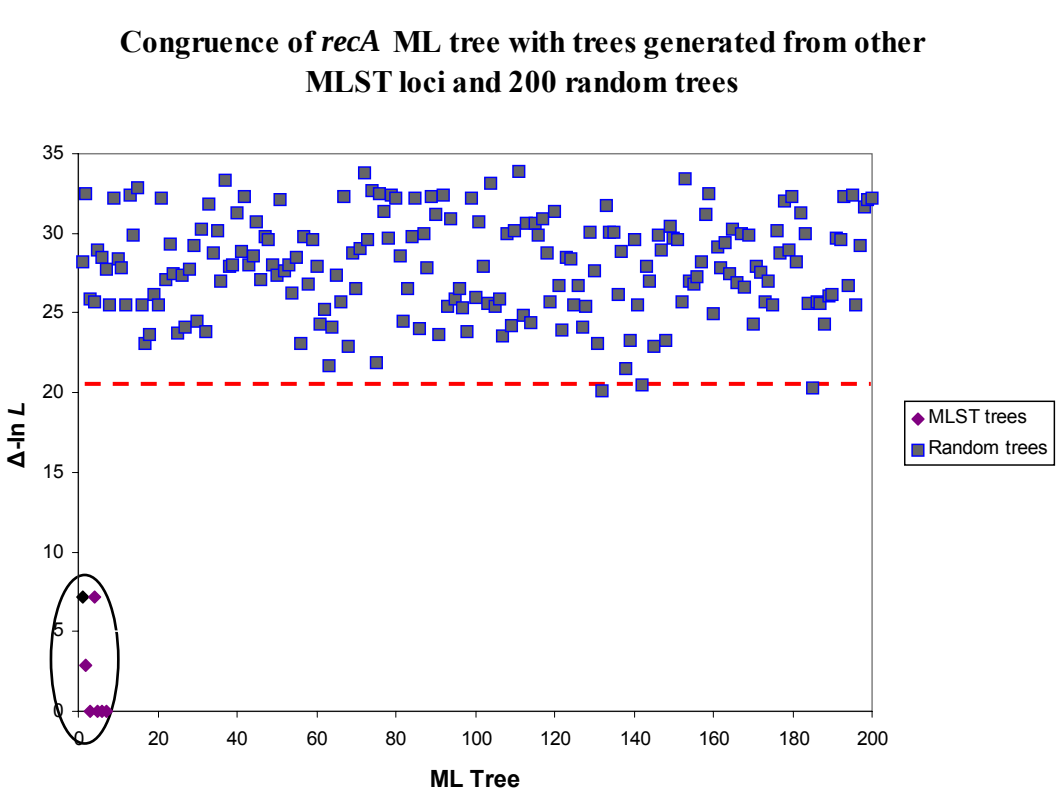
Congruence of *guaA* ML tree with trees generated from other MLST loci and 200 random trees



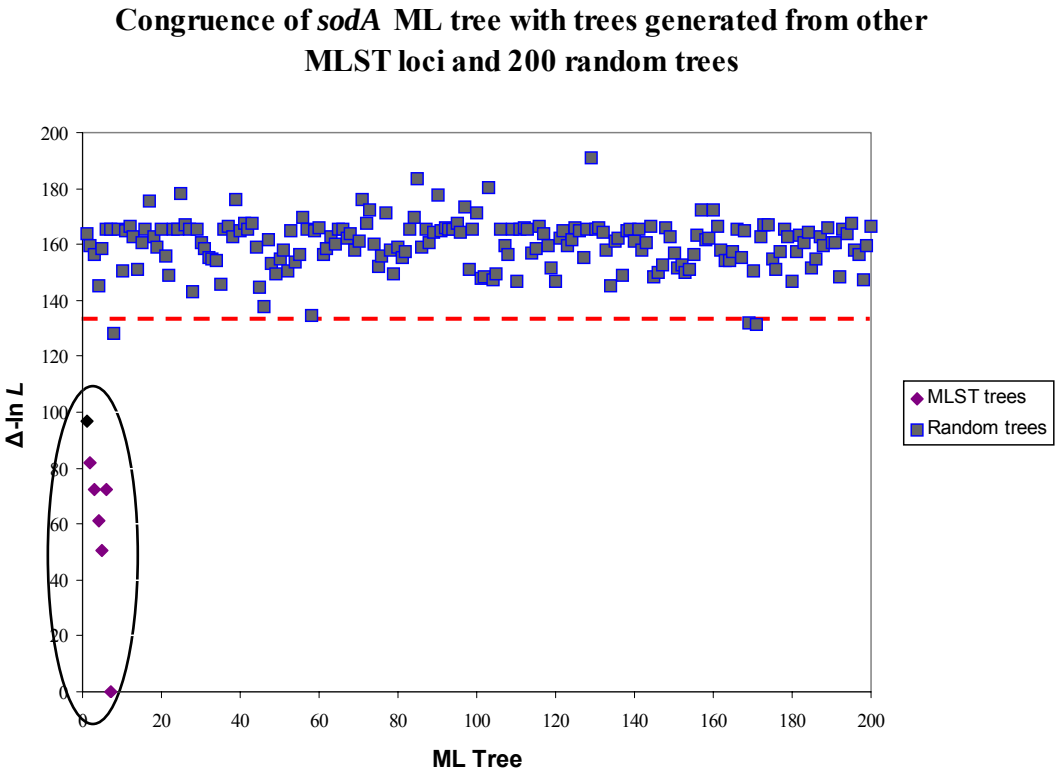
(e)


Congruence of *lepA* ML tree with trees generated from other MLST loci and 200 random trees
Δ-ln *L*
ML Tree
MLST trees
Random trees
0
20
40
60
80
100
120
140
160
180
200

(f)


Congruence of *recA* ML tree with trees generated from other MLST loci and 200 random trees
Δ-ln *L*
ML Tree
MLST trees
Random trees
0
5
10
15
20
25
30
35
0
20
40
60
80
100
120
140
160
180
200

(g)


Congruence of sodA ML tree with trees generated from other MLST loci and 200 random trees
Δ-ln L
ML Tree
MLST trees
Random trees
0
20
40
60
80
100
120
140
160
180
200

4.3.5.3 Splits decomposition analysis

Evidence for recombination having played a role in shaping the genetic diversity within *P. acnes* was displayed by split decomposition analysis of the concatenated data set for each ST (Section 4.3.3.3). The extent of recombination within a population is reflected by the degree of networks computed by the split decomposition programme. As opposed to a clonal tree-like structure, *P. acnes* isolates were interconnected by multiple pathways and formed parallelogram structures indicative of recombinational exchanges (Figures 4.10 and 4.11). eBURST group 4 (ST-12 and ST-29 cluster) formed the least network connections with the other lineages, suggesting infrequent recombination with other strains in this population.

4.3.5.4 Clonal frame analysis

As described in section 4.3.3.4, ClonalFrame v1.1 attempts to infer clonal relationships between strains using MLST data. It is also a useful analysis tool for determining the extent of recombination and mutation on evolution of organisms. Output data generated by ClonalFrame using *P. acnes* MLST are shown in Table 4.10. Rho/Theta is the ratio of recombination versus mutation, and therefore 5.65 mutation events occur for very recombination event in the study population. Inverse delta value is the mean size of the recombination fragment which is 405 bp which suggests that when recombination events occurs, it is more likely to involve the whole allele if not the gene that is recombining rather than small intragenic regions. This is confirmed by examining the site of the polymorphisms that occur at each allele. Alignment of sequences of each allele shows that polymorphisms usually occur across the alleles rather than at specific regions of an allele (data not shown). The r/m value is the impact of recombination versus mutation. If the value is 1, then it implies that the impact of recombination is the same as mutation. r/m value is 0.840 which means that mutation is still the dominant force in evolution than recombination but only marginally. Therefore, based on clonal frame analysis, recombination appears to have an important role in the evolution of *P. acnes* even though it is not the major mechanism. However,

the confidence intervals indicate that the true values could vary across a wide range.

Table 4.10 Output data produced by the ClonalFrame program

Output data	Value	95% confidence interval
Theta	12.373	5.479-19.893
nu	0.0234	0.0154-0.0323
R	1.962	0.655-3.767
delta	0.00247	0.000514-0.000514
R/m	0.840	0.281-1.650
Rho/theta	0.177	0.0451-0.424

4.3.5.5 Point mutation versus recombination

The estimation of recombination rates was carried out by comparisons between STs belonging to the same clonal complex. Clonal complexes were identified where each ST shares at leave six identical alleles with at least one other isolate in the group (Section 4.3.3.3). Comparisons were then made between the sequences of the alleles in each of the SLVs and those in the corresponding putative ancestral genotype. If the variant allele differed from its ancestral genotype at multiple nucleotide sites, it was defined as a recombinational event (Feil *et al.*, 2001). If the difference occurred at only a single nucleotide site, then a point mutation or a recombinational event involving a single site, may have brought about the variant (Feil *et al.*, 2001). These possibilities were distinguished by seeing whether the variant allele appeared elsewhere in the dataset, because an allele introduced by recombination may not be present elsewhere in the dataset, whereas a point mutation is likely to produce a novel allele (Feil *et al.*, 2000).

The seven eBURST groups were analysed separately for the presence of recombinational events and point mutations. As eBURST groups 1 and 7 were the only groups with identifiable founding genotypes, their analyses are presented in Table 4.11 and 4.12. The other groups did not have enough STs within each group for the eBURST program to assign ancestral genotypes or the STs had equal numbers of SLVs. For these groups, the alleles of the ST with the most number of isolates were designated as the typical allele and the other alleles were designated as variant alleles. As shown in Table 4.11, there were a total of 15 allelic variants within eBURST group 1. Of these, 3 differed from the allele typical of the clonal complex by two or more polymorphisms and were therefore considered recombinational imports. The remaining 12 allelic variants differed from the typical of the clonal complex at a single nucleotide site, which is indicative of point mutation. These single nucleotide changes were confirmed likely to be due to point mutations by the fact that these alleles were novel alleles and were not present in any other strains in the study population. In eBURST group 7, there were two recombinational and two point mutational events. Examination of all seven eBURST groups revealed 28 allelic variants in total, with 12

recombinational events and 16 point mutational events. This gives an estimate of the ratio of recombinational events to mutational events per ~450 bp gene fragment of 12:16 (1:1.33), i.e. point mutation has generated new alleles at a frequency 1.33 times as high as recombination (this is similar to the estimation using ClonalFrame of 1.565, Section 4.3.5.4).

Because recombinational imports in relatively diverse species usually introduce multiple polymorphisms, whereas a point mutation only brings about a single polymorphism, the ratio of recombinational events to mutational events does not realistically reflect the impact of these two processes on sequence divergence. Guttman and Dykhuizen suggested that a more accurate method is to estimate the probability that an individual nucleotide site will change by recombination compared with the probability that it will change by mutation (Guttman & Dykhuizen, 1994).

The per-site recombination to mutations (r/m) parameter can be estimated by counting the number of polymorphisms introduced by recombination and mutation. In this analysis, 87 polymorphisms were introduced by the 12 recombinational imports, and a maximum of 16 were introduced by point mutation (r/m is 5.4:1). Therefore, a particular nucleotide site within a *P. acnes* housekeeping gene is at least 5 times as likely to change by recombination as it is to change by mutation. This differs from the conclusion drawn from ClonalFrame analysis (Section 4.3.5.4) where changes are more likely to be brought about by point mutation than by recombination. This could be explained by the fact that the above calculations were done by the detection of polymorphisms between genotypes belonging to the same clonal complex whereas ClonalFrame calculations were carried out on polymorphisms detected between all the genotypes within the study population.

Table 4.11 Polymorphic sites within each of the variant alleles for eBURST group 1. The ST designation and allele numbers are shown. ST-6 is the founder of the group and is shown at the top in red. The polymorphic sites are shown for ST-6 alleles and in the other alleles, only those sites that differ from this sequence are shown. The polymorphic sites are numbered above the sequences in vertical format.

aroE						
			1	1	3	1
		1	1	3	2	4
		4	3	4	1	2
ST-6	Allele 1	C	C	T	T	G
ST-24	Allele 4	.	.	.	C	.
ST-25	Allele 5	.	T	.	.	.
ST-11	Allele 8	T	.	.	C	.
ST-13	Allele 11	.	.	.	C	A
ST-34	Allele 12	.	T	C	C	.

guaA					
			3	3	4
		2	0	8	8
		4	3	1	3
ST-6	Allele 3	G	A	C	C
ST-1	Allele 1	.	G	.	.
ST-8	Allele 6	.	.	T	.
ST-33	Allele 9	A	.	.	.
ST-21	Allele 14	.	.	.	T

atpD			
		2	3
		0	8
		1	8
ST-6	Allele 1	G	G
ST-7	Allele 5	A	.
ST-36	Allele 7	.	T

lepA			
		1	3
		1	7
		0	5
ST-6	Allele 1	G	C
ST-32	Allele 7	A	.
ST-38	Allele 8	.	A

gmk		
		3
		2
		0
ST-6	Allele 1	G
ST-18	Allele 6	A

sodA		
		4
		4
		2
ST-6	Allele 1	A
ST-4	Allele 2	G

Table 4.12 Polymorphic sites within each of the variant alleles for eBURST group 7. The ST designation and allele numbers are shown. ST-19 is the founder of the group and is shown at the top in red. The polymorphic sites are shown for ST-19 alleles and in the other alleles, only those sites that differ from this sequence are shown. The polymorphic sites are numbered above the sequences in vertical format.

gmk		
		4
		4
ST-19	Allele 3	c
ST-30	Allele 7	A

guaA						
				3	3	4
		2	4	3	5	7
		4	9	6	1	7
ST-19	Allele 7	G	G	T	T	C
ST-17	Allele 11	A	.	C	A	T
ST-20	Allele 12	.	T	.	.	.

sodA				
			1	4
		6	6	2
		5	8	8
ST-19	Allele 9	C	C	G
ST-28	Allele 7	T	T	A

4.3.6 Validation of the *P. acnes* MLST scheme

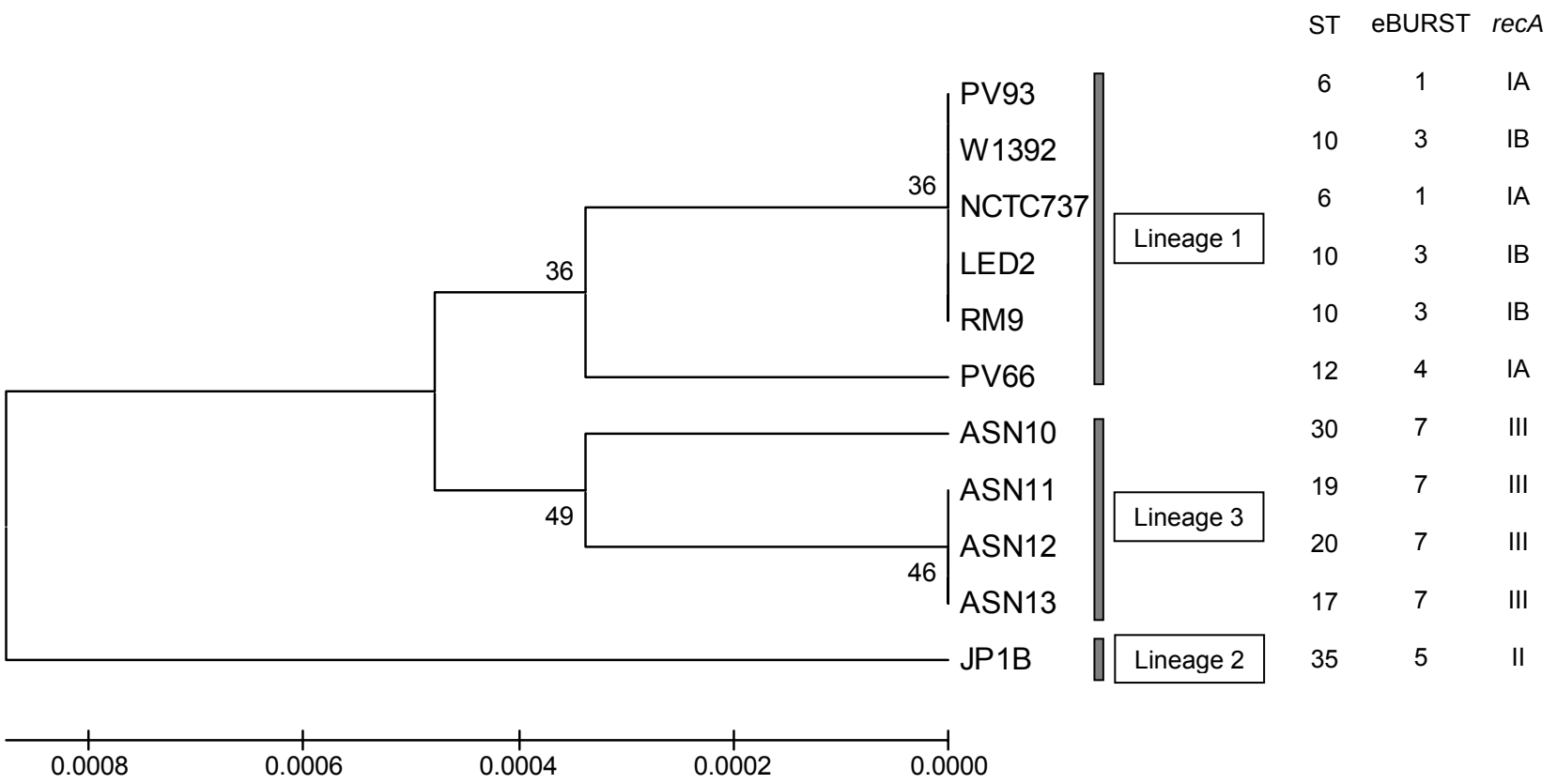
In order to validate the *P. acnes* MLST scheme and its ability to resolve clonal groupings, phylogenetic analysis of the MLST data was compared to several other typing methods including 16S rRNA gene and *recA* gene sequencing, IFM and RAPD.

4.3.6.1 Comparison of MLST with 16S ribosomal RNA gene analysis

16S rRNA gene analysis of eleven of the 125 isolates included in the MLST scheme had previously been carried by McDowell *et al.* (McDowell *et al.*, 2005; McDowell *et al.*, 2008). The selection of isolates included representatives of type IA, IB, II and III isolates. A 1,484 bp fragment was amplified with the universal 16S rRNA-based primers UFPL and URPL. Figure 4.15 is an UPGMA dendrogram of the eleven *P. acne* 16S rRNA sequences. The ST and eBURST groups generated from the MLST data, as well as the phylotype based on the complete *recA* gene analysis, are also shown. The eleven isolates were subdivided into three main lineages based on 16S rRNA gene analysis. Lineage I included eBURST groups 1, 3 and 4 which corresponded to type IA and IB strains by *recA* typing. 16S RNA gene analysis was able to differentiate isolate PV66 (novel cluster) from the other type IA and IB strains, where *recA* gene analysis could not (Section 4.3.6.2). Lineage II was represented by JP1B and corresponded to eBURST group 5 and type II by *recA* analysis. Lineage III represented eBURST group 7 and correlated to type III strains. Overall, it appears that 16S rRNA gene analysis is able to reliably differentiate between types I, II and III strains as well as the novel cluster represented by isolate PV66. However, there was not enough sequence diversity between type IA and IB strains to separate these into distinct groups. The 16S rRNA gene analysis data was in concordance with the eBURST group clustering generated by MLST data and thus validated the MLST scheme. However, MLST provides much more discrimination as shown by the different STs which have identical 16S rRNA gene sequences.

The overall mean sequence identity of the 16S rRNA gene was found to be 99.52% between the five representative sequences of the eleven isolates, confirming their close relationship. In contrast, when a *P. acnes* type III isolate was compared with the sequence from *P. propionicus* strain DSM 43307 (GenBank accession no. AJ003058), much greater differences were observed (95.8-95.9% identity) (McDowell *et al.*, 2008). Although sequence analysis of the 16S rRNA gene is widely recognised as a powerful method for determining the phylogenetic relationships for distinguishing between bacteria, it may not be the most appropriate method of choice for distinguishing between related members of a genus or species (Vandamme *et al.*, 1996). Microbes with 16S rRNAs that are ≤98.7% identical are considered members of different species, because such strong variation in 16S rRNA correlate with <70% DNA-DNA similarity (Achtman & Wagner, 2008). However, the opposite is not necessarily true and distinct species have been found to have 16S rRNAs that are >98.7% identical (Achtman & Wagner, 2008).

Figure 4.15 UPGMA dendrogram of the *P. acnes* 16S rRNA gene sequence (1,000 bootstrap replications). STs and eBURST groups generated by the MLST scheme and the phylogenetic grouping using complete *recA* gene sequencing



4.3.6.2 *recA* gene analysis

Previously, it has been shown that *P. acnes* is composed of a number of distinct clusters or lineages based on phylogenetic analysis of the *recA* gene (McDowell *et al.*, 2005; McDowell *et al.*, 2008). It was therefore important to determine whether these phylogenetic types could be confirmed using MLST data. Of the 125 isolates used in MLST scheme, the *recA* phylotype of 58 isolates was determined based on complete *recA* gene (1,047 bp) sequencing and phylogenetic analysis (Table 4.1). The differentiation of *P. acnes* isolates into distinct groups or lineages based on *recA* sequence analysis (IA, IB, II & III) was mostly congruent with the lineages or clonal complexes resolved from MLST data analysis (Section 4.3.3.1). eBURST analysis revealed that genotypes from *P. acnes* type IA belonged to eBURST groups 1, 2 and 4 (Figure 4.16) and *P. acnes* type IB belonged to eBURST group 3. Strains of *P. acnes* type II were found to form two very closely related clonal complexes or eBURST clusters (groups 5 and 6), while strains belonging to type III, a recently proposed new phylogenetic group within *P. acnes* (McDowell *et al.*, 2008) formed a single eBURST cluster (group 7). To date, only five isolates of this novel cluster, known as type III, have been described suggesting it may represent a minor lineage (McDowell *et al.*, 2005). During this study, however, a further strain was identified (isolate 463040-03; ST-28), isolated from a corneal scrape sample, which clustered with other type III strains.

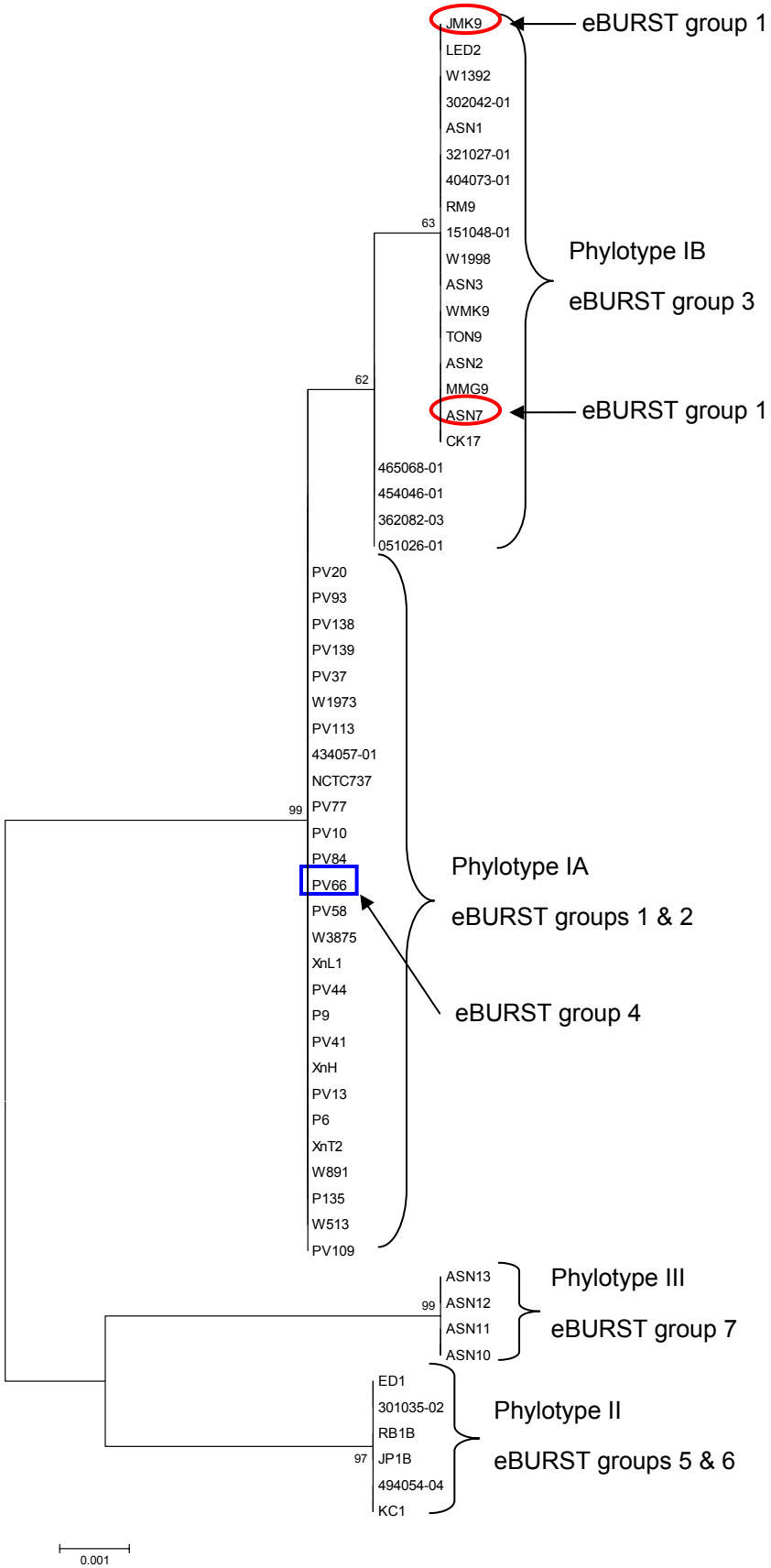
There were a total of five different variants of the complete *recA* sequence. The mean sequence identity between these five sequences is 99.21%. This is comparable to the 99.57% sequence identity of the four *recA* alleles used in the MLST scheme (Figure 4.9f).

While the known phylotype data for *P. acnes* isolates correlated well with their clustering based on analysis of the MLST data, a small number of notable exceptions were observed. Firstly, three acne isolates with ST-12 (n=1) and ST-29 (n=2) formed a highly distinct cluster (eBURST group 4), even though one of these strains (PV66) was previously classified as a type IA organism based on

recA and *tly* gene sequencing (McDowell *et al.*, 2005). Multiple methods of analysing MLST data have shown that ST-12 and ST-29 form a distinct cluster awat from the other *P. acnes* strains. Splits decomposition analysis shows recombination occurring between these two STs but not between these and other *P. acnes* STs. Phylogenetic analysis of the 16S rRNA gene also differentiated PV66 from other type I strains (Section 4.3.6.1). Therefore, this novel cluster does not clearly fall into any of the four previously confirmed phenotypes (IA, IB, II and III).

The second discrepancy between the two methods is that two of the isolates did not show concordance between MLST and *recA* gene analysis data. Isolates Asn7 (ST-38) and JMK9 (ST-6) were found to cluster in eBURST group 1 with other type IA strains. However, based on complete *recA* sequence, they were characteristic of type IB organisms. Both isolates were cultured and sequenced twice in order to eliminate the possibility of labelling or contamination error. The cause of this discrepancy may have come about from a recombinational event occurring at the *recA* gene between type IA and IB strains, resulting in a type IA strain acquiring a type IB *recA* gene segment. These results illustate, therefore, the potential limitations that can arise when using a restricted number of gene loci in the construction of bacterial phylogenies. Therefore, analysis of multiple housekeeping genes is more appropriate and accurate so that sequence data from each gene is buffered against potential recombinogenic events that might occur at other loci.

Figure 4.16 Neighbour-Joining dendrogram of the complete *P. acnes recA* gene. Phylotype based on *recA* gene analysis and corresponding eBURST groups are indicated.



4.3.6.3 Immunofluorescence Microscopy (IFM) analysis

IFM using type-specific based monoclonal antibodies (MAbs) generated by McDowell *et al.* (McDowell *et al.*, 2005; McDowell *et al.*, 2008) was used to validate the *P. acnes* MLST scheme. 76 *P. acnes* isolates were examined with the MAbs QUBPa1 and QUBPa2, which label *P. acnes* type IA and II, respectively, as well as QUBPa3, which reacts with all *P. acnes* strains (method in Section 2.12). Types I and II displayed classical coryneform morphology i.e. clubs, 'tadpole' forms and short bifid forms (Figure 4.17a and b). Previous analysis of reference strains NCTC 737 (type IA strain) and NCTC 10390 (type II strain) revealed lengths that ranged from 0.84 to 2.56μm and 0.89 to 2.80μm, respectively (McDowell *et al.*, 2005). Type III strains, however, consisted of individual cells of variable length and long slender filaments, ranging from 1.20 to 21.8μm in length, that formed very large tangled aggregates (Figures 4.17c and d) (McDowell *et al.*, 2008). In addition, no labelling with QUBPa1 and QUBPa2, which react with proteinaceous and carbohydrate/glycolipid-containing antigen on types IA and II, respectively, was observed for the type III isolates.

A selection of *P. acnes* isolates from eBURST groups 1 and 2 were all shown to be type IA strains based on IFM analysis. All eBURST groups 5 and 6 strains were identified as type II strains and all eBURST group 7 strains were identified as type III strains.

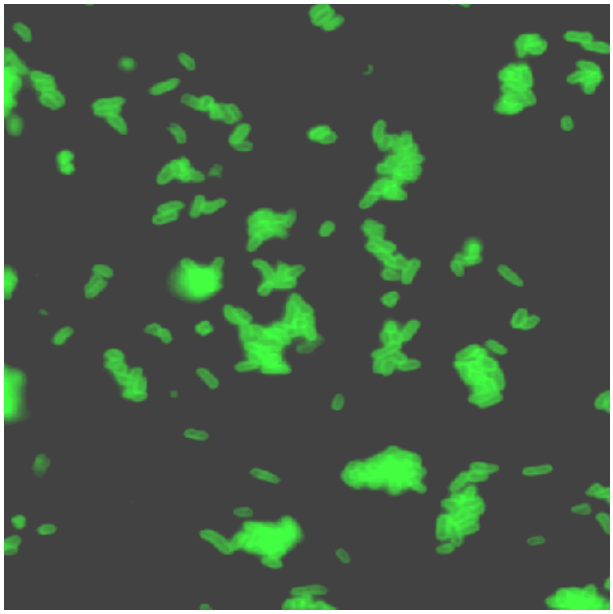
Previous study has shown that a characteristic phenotype of type IB organisms is the absence of reaction with MAb QUBPa1 (labels type IA) and weak–to-no reaction with the MAb QUBPa2 (labels type II), which is believed to reflect differences in the composition of their cell wall compared to type IA strains (McDowell *et al.*, 2005). MLST data shows that the group of type IB isolates upon which this observation was made (W1392; W1998; CK17; LED2; RM9; MMG9; TON9; WMK9) are clonal with an identical allelic profile (ST-10). In fact, the type IB group is represented by five distinct STs (ST-9, ST-10, ST-22, ST-27 & ST-37) which make up eBURST group 3. IFM analysis of other eBURST group 3 genotypes identified in this study (ST-9, ST-22, ST-27 and ST-

37) found that these organisms will react with MAb QUBPa1, but not QUBPa2. Therefore, it would appear that failure to label with QUBPa1 is a phenotypic characteristic of the ST-10 genotype of type IB but not other strains in the type IB lineage. This illustrates that, while useful, immunological typing with MAbs is unpredictable due to the emergence of antigenic variants that do not react.

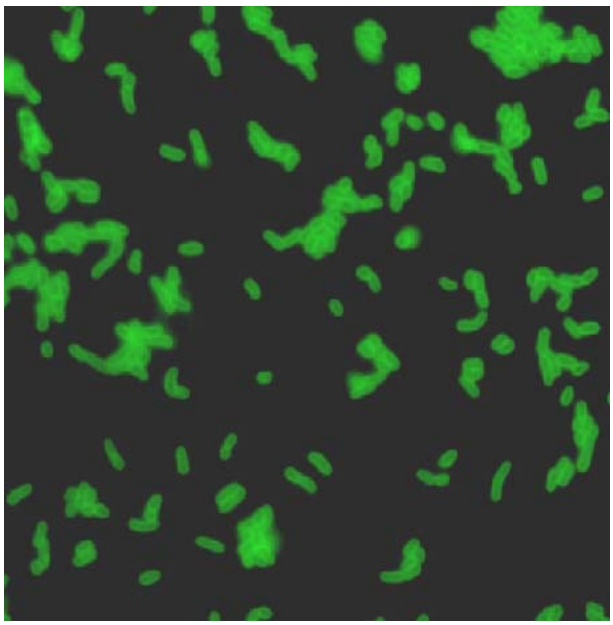
In the study population, a novel cluster comprising three isolates (one ST-12 and two ST-29) was identified, which make up eBUSRT group 4. All isolates within this group displayed dual labelling with MAbs that normally react with either types IA (QUBPa1) or II (QUBPa2), further highlighting their distinct nature (McDowell *et al.*, 2005). It will be important to ascertain if any other phenotypic characteristics that distinguish this group from the other *P. acnes* clusters exist.

Figure 4.17a-d Micrographs of *P. acnes* immunolabelled with a mouse MAb and an FITC-conjugated anti-mouse antibody. Bacteria were counter-stained with Evans blue and viewed with a combined red/green filter (×100 magnification).

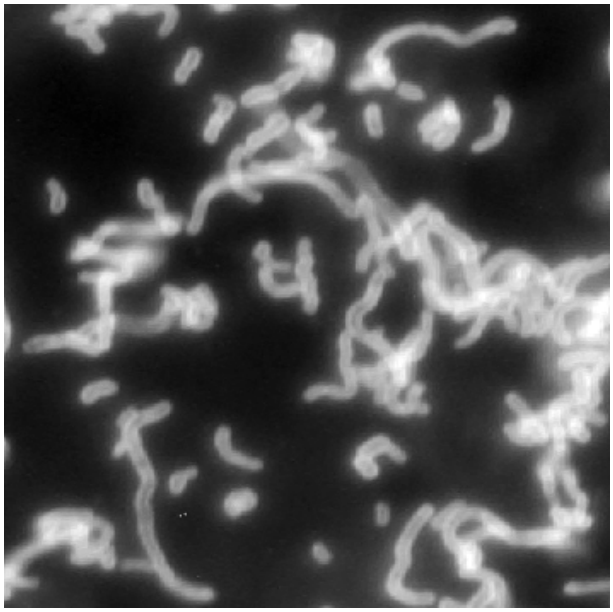
(a) Reference strain NCTC 737 (type IA) immunolabelled with MAb QUBPA1.



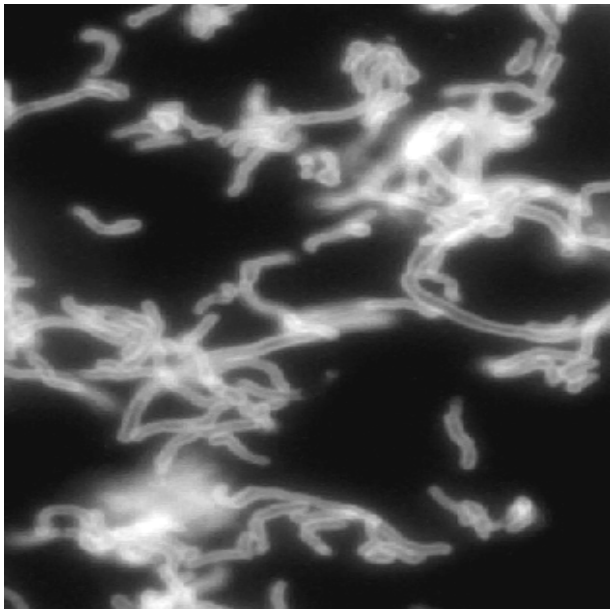
(b) Reference strain NCTC 10390 (type II) immunolabelled with MAb QUBPa2.



(c) Asn10 (type III) immunolabelled with MAb QUBPa3.



(d) Asn12 (type III) immunolabelled with MAb QUBPa3.

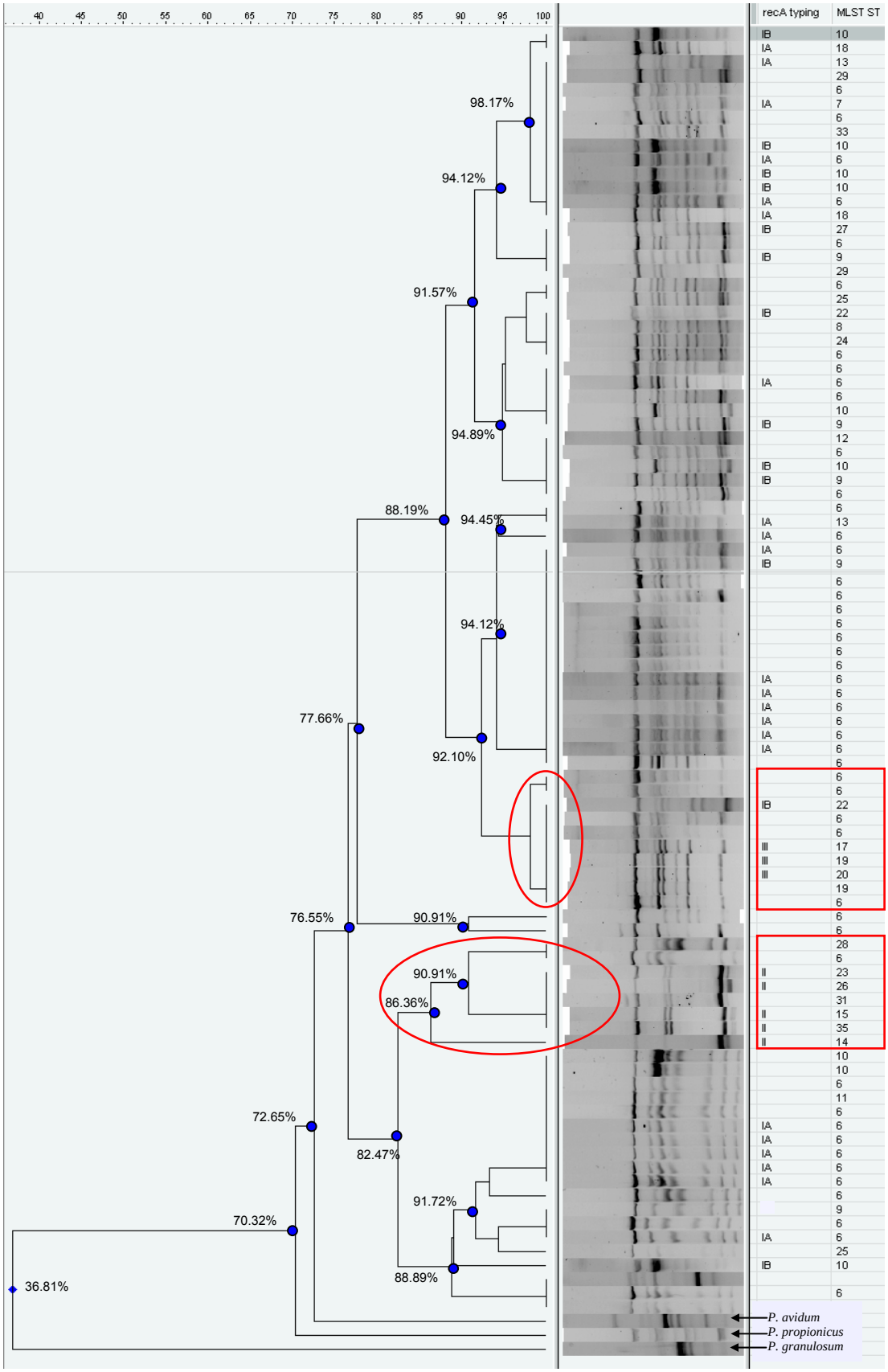


4.3.6.4 Random Amplification of Polymorphic DNA (RAPD) typing

In order to provide comparisons between MLST and a repetitive element PCR typing technique, RAPD typing was carried out on a selection of 92 *P. acnes* isolates as described in Section 2.13. The same RAPD primer (RAPD-208) was applied to *P. avidum*, *P. propionicus* and *P. granulosum* for comparison. Figure 4.18 is an unrooted UPGMA dendrogram using band based Dice correlation coefficient based on RAPD genotyping profiles. The percentage similarity between the bands is displayed next to the dendrogram branch. All the PCR reactions and gels were run on the same occasion to eliminate any differences in laboratory condition which may generate errors in the banding patterns.

RAPD analysis using RAPD-208 primer was able to distinguish *P. avidum*, *P. propionicus* and *P. granulosum* from *P. acnes*. Although *P. granulosum* gave markedly different profiles with less than 40% similarity to those of the other species, *P. propionicus* and *P. avidum* appear to be much more closely related to *P. acnes* at 70% and 73% similarities, respectively. Type II isolates appear to have clustered together (ST-23, ST-26, ST-31, ST-15, ST-35 and ST-14). However, this group also clustered with a ST-28 (type III) strain and a ST-6 (type IA) strain (highlighted in red in Figure 4.18). Similarly, type III strains (ST-17, ST-19 and ST-20) have clustered together but along with a ST-6 (type IA) strain and a ST-22 (type IB) strain. Type IA and IB strains are distributed randomly throughout the dendrogram, indicating that there are too few band differences for the two types to be reliably discerned by RAPD analysis. A previous study using a different RAPD primer (primer 1254) to investigate 46 clinical *P. acnes* strains was able to identify two distinct profile types (Perry *et al.*, 2003). One profile type included the reference strain NCTC 737 which is a type I strain and the other included the reference strain NCTC 10390, a type II strain. The investigators were unable to distinguish between type IA and IB strains using RAPD. They also noted that strains isolated from different clinical sources were distributed amongst the RAPD profiles. It is evident from the current study and from previous work that MLST is a much superior genotyping technique at differentiating between closely related strains than RAPD typing.

Figure 4.18 UPGMA dendrogram analysis of RAPD profiles from *P. avidum*, *P. propionicus*, *P. granulosum* and a selection of 92 *P. acnes* isolates.



4.4 Future work

MLST has reliably identified distinct clonal complexes which encompass closely related strains. ST-6 has been identified as the founder genotype for the clonal complex consisting of the largest number of different STs. ST-6 is also the most frequently found genotype in this study population of 125 *P. acnes* isolates. Is this observation due to the frequent occurrence of ST-6 strains on healthy skin and therefore more likely to be cultured from infection? Or it is a more virulent strain possessing antibiotic resistance traits and other virulent factors that make it "fitter" than the other strains? Perhaps it is a combination of the two factors that allow it to dominate over the other genotypes. One way of answering this question is to culture *P. acnes* from the skin of healthy carriers with no previous history of acne infection. MLST analysis of *P. acnes* strains isolated from the cheek, forehead and back of three healthy carriers with no previous history of acne was carried out in the current study. Two of the carriers harboured only ST-6 strains from the different swab sites and a ST-19 strain was cultured from the cheek of the third carrier (data not shown). Previous ST-6 strains were determined to be type IA and ST-19 strains to be type III. Although this data is limited, it does show that ST-6 is not the only ST found on healthy carrier skin. A much larger collection of *P. acnes* from different parts of the body from healthy carriers of different ethnic origin, of different age groups and from different countries, would provide valuable information about how this successful strain has evolved.

Further studies of different *P. acnes* strains, especially the ST-6 genotype in terms of its pathogenicity and antibiotic resistance would also provide insight into whether certain strains are more likely to cause disease and hence dominate the collection of clinical strains. MLST analysis of virulence genes rather than housekeeping genes may provide further discrimination of lineages within the population structure of *P. acnes*. Although RAPD analysis using a single primer in the current study did not provide accurate and reliable discrimination of *P. acnes* compared to other typing methods, further optimisation and using alternative primers may provide more useful information, allowing for a cheaper alternative to MLST.

4.5 Conclusions

To date, a small number of different genetic fingerprinting schemes for *P. acnes* have been described, including RAPD (Kunishima *et al.*, 2001; Perry *et al.*, 2003) and PFGE (Cohen *et al.*, 2005; Oprica & Nord, 2005; Ting *et al.*, 1999). One common problem with such techniques is the inherent variability that often arises when attempting to match gel banding patterns, which means such methods are not easily transferred between laboratories. Furthermore, due to the nature of its cell wall, *in situ* lysis of *P. acnes* can be problematic during PFGE experiments which may further impact on reproducibility. Typing of strains based on the analysis of unambiguous DNA sequence content rather than genome organisation or restriction fragments is much more robust, reliable and transferable between laboratories. On this basis, MLST schemes provide the opportunity for unequivocal typing and characterisation of bacterial pathogens which is critical for epidemiological studies.

The discriminatory power of the *P. acnes* MLST scheme, and its capacity to estimate levels of homologous recombination, has now enabled the genetic population structure of this organism to be understood in a way previous typing methods have not facilitated. The ability of bacteria to exchange genetic material is of clinical interest, and can confer resistance to antimicrobials, increased virulence and vaccine immunity. All of the lineages within *P. acnes* had a population structure composed of very closely related genotypes. The relatively small degree of sequence diversity observed within the seven core housekeeping genes of these phylotypes, even where allele sharing by recombination was evident (which tends to break up clonal complexes), could be explained by clonal expansion of adapted genotypes (purifying selection) followed by the emergence of minor variants through recombination and/or de novo mutation.

In conclusion, the MLST scheme reported here has proved a valuable tool for high-level strain identification and epidemiological investigation of *P. acnes*. The method has confirmed the previously described phylogenetic groups of the organism and identified an additional novel cluster. There appears to be no

association between these distinct groups and clinical manifestation of the infection, nor the geographical location of these strains. Continued application of the scheme to *P. acnes* isolates from individuals in different localities and asymptomatic carriers will further promote our understanding of the organism's genetic population structure and its role in disease.

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CHAPTER 5 *PROPIONIBACTERIUM ACNES* RAPID DIAGNOSTICS

5.1 Introduction

P. acnes is an anaerobic organism capable of causing a variety of different clinical infections including postoperative endophthalmitis (Aldave *et al.*, 1999) and keratitis (Jones & Robinson, 1977; Perry *et al.*, 1982; Wong *et al.*, 2003; Zaidman, 1992). *P. acnes* endophthalmitis can be difficult to identify as the organism resides within the capsular bag of the eye and is therefore difficult to culture. *P. acnes* also has a very slow growth rate and may take up to 10 days to culture using conventional culture techniques (Aldave *et al.*, 1999; Clark *et al.*, 1999). This delay in diagnosis may lead to inappropriate antibiotic treatment and poor visual outcome.

To overcome the low culture positive rate, molecular diagnostic tests have been developed to aid the diagnosis of postoperative endophthalmitis. Most of these PCR assays use universal primers targeting the bacterial 16S rRNA genes, with subsequent identification of the pathogen using RFLP analysis or DNA sequencing of the amplified products (Knox *et al.*, 1999; Lohmann *et al.*, 2000; Okhravi *et al.*, 2000). A major drawback of this approach is the cost and time involved in the identification of the organism. To overcome this, PCR specifically targeting the *P. acnes* 16S rRNA gene has been developed to identify *P. acnes* directly from vitreous and aqueous humour, and the explanted intraocular lens of patients with chronic postoperative endophthalmitis (Buggage *et al.*, 2003; Hykin *et al.*, 1994; Lai *et al.*, 2006). This approach has also been applied to detect *P. acnes* in vitreous humour of sarcoidosis patients (Yasuhara *et al.*, 2005), from an abscess caused by a vascular prosthesis (Le Page *et al.*, 2003) and from prosthetic hip infections (Tunney *et al.*, 1999).

Chapter 4 explored the population biology of *P. acnes* using housekeeping genes and identified distinct clonal complexes. However, there was no obvious

correlation found between a particular clone or clonal complex and the clinical manifestation. Therefore, a PCR assay for the diagnosis of *P. acnes* endophthalmitis infection does not need to target a specific genotype. All seven housekeeping genes used in the MLST scheme were amplified from all the *P. acnes* isolates in the study population. Therefore, any of these genes are potential targets for PCR assays for the detection of *P. acnes* from clinical samples. Molecular identification of organisms based on specific gene targets, such as the MLST housekeeping genes, enables a higher degree of specificity than methods which rely on the use of universal or broad-range primers.

Higuchi *et al.* developed a system whereby PCR products could be detected as they accumulate during a standard PCR reaction (Higuchi *et al.*, 1992; Higuchi *et al.*, 1993). This real-time PCR has the advantage of detecting microbial DNA in a much shorter time than standard PCR protocols as it does not require gel electrophoresis and allows quantification of copy numbers. An additional advantage of the real-time PCR system is that analysis can be carried out without opening the reaction tubes, thus reducing the risk of contamination of the laboratory with the amplified PCR products (Woodford & Johnson, 2004). Current real-time PCR technology relies on two methods of obtaining fluorescent signal following the synthesis of PCR products. One approach utilises fluorescent dyes to bind to double-stranded DNA which once bound, increase their intensity of fluorescence. The second method uses fluorescent reporter probes which are sequence specific and quantify only the DNA containing the probe sequence (Woodford & Johnson, 2004).

5.2 Aims

1. To develop a PCR assay for the detection of *P. acnes* using one of the seven housekeeping genes from the MLST scheme.
2. To determine the PCR assay's sensitivity and specificity using real-time PCR technology.
3. To determine the optimal DNA extraction method for *P. acnes*.

5.3 Results and Discussion

5.3.1 Specificity of PCR assay

PCR reactions using primers targeting the seven *P. acnes* housekeeping genes were carried out as described in Section 2.15.2. To determine the specificity of each housekeeping gene to *P. acnes*, PCR reactions were carried out on the four cutaneous *Propionibacterium* spp. (*P. acnes, P. avidum, P. granulosum* and *P. propionicus*) and four other organisms commonly cultured from eye infections (*P. aeruginosa, S. aureus,* coagulase negative *Staphylococcus* and *S. pneumoniae*). Each PCR mixture contained 20ng of genomic DNA and a negative control without any bacterial DNA was also included. The presence of PCR products was determined by gel electrophoresis on agarose gels. Table 5.1 shows the specificity of each gene to *P. acnes*.

Three genes (*gmk, guaA* and *recA*) were found to be specific for *P. acnes* and were not amplified from any of the other *P. acnes* species or the other bacterial organisms tested. Of the three genes, the smallest PCR product generated by the nested primers used in the MLST scheme was chosen (Table 4.2) because this would allow more rapid PCR cycling. Therefore, the *gmk* gene was chosen to be used in the rapid diagnostics assay for the detection of *P. acnes*.

Real-time PCR technique was applied to different organisms using methods described in Section 2.15 to detect the presence of the *gmk* gene. It was detected in three different *P. acnes* strains (including the *P. acnes* type strain NCTC 737) and could not be detected in *P. avidum, P. granulosum, P. propionicus, S. aureus, P. aeruginosa, S. pneumoniae* or coagulase negative *Staphylococcus*. 1ng/µL of bacterial DNA was used in each sample.

Table 5.1 Amplification of housekeeping genes using primers from the *P. acnes* MLST scheme. g*mk*, *guaA* and *recA* are the only genes found to be specific for *P. acnes*.

	aroE	***atpD***	***gmk***	***guaA***	***lepA***	***recA***	***sodA***
P. acnes	+	+	+	+	+	+	+
P. avidum	+	+	-	-	+	-	+
P. granulosum	-	+	-	-	-	-	+
P. propionicus	-	+	-	-	+	-	+
S. aureus	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-
Coagulase negative *Staphylococcus*	-	-	-	-	-	-	-
S. pneumoniae	-	-	-	-	-	-	-
Negative control	-	-	-	-	-	-	-

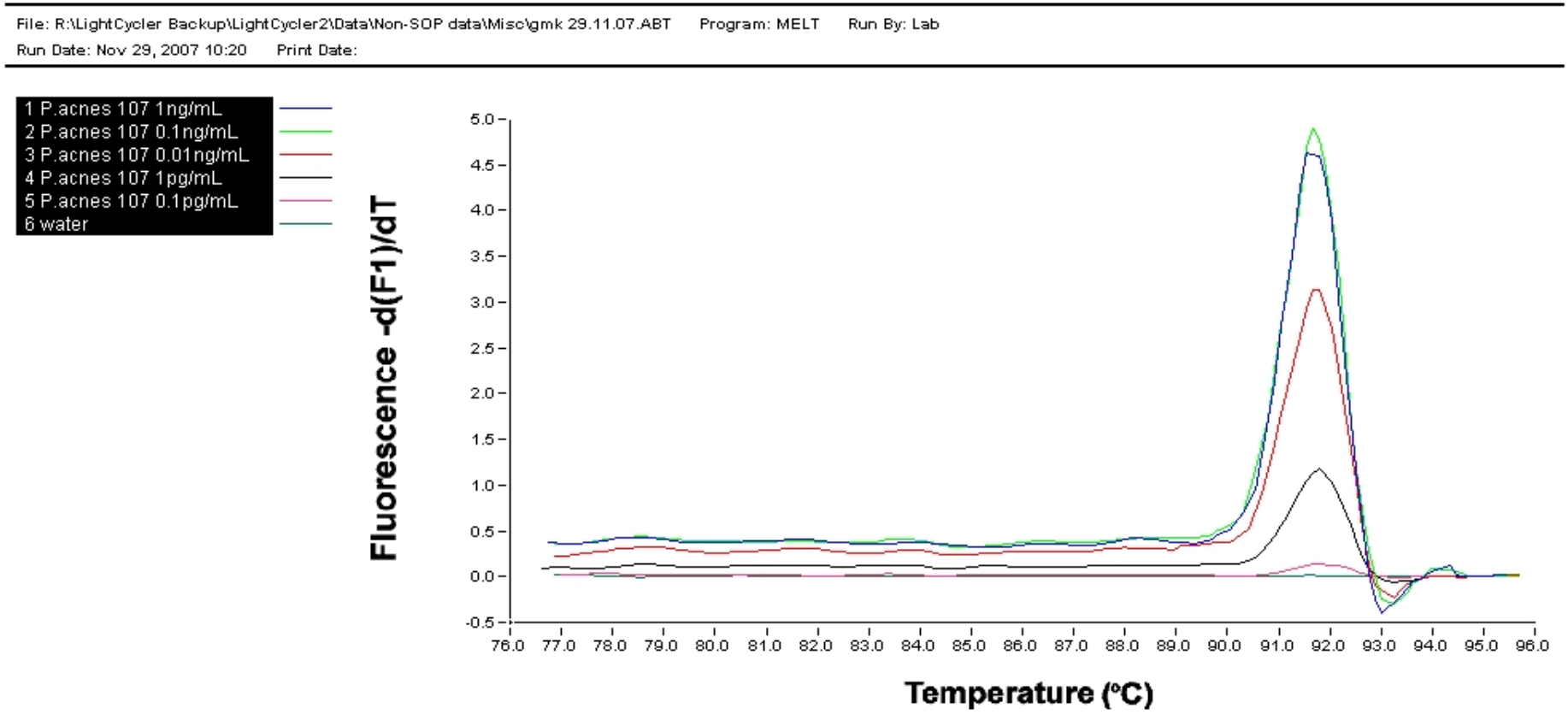
5.3.2 Melting-curve analysis

The real-time PCR method used in the current study relied on the green fluorescent nucleic acid dye, Evagreen (Biotium Inc.) to produce the fluorescent signal. Evagreen exhibits very low levels of fluorescence when free in solution but this level increases by up to 1000-fold when the dye is bound to DNA. The dye has the ability to bind to any double-stranded DNA generated by the PCR. Although this has the advantage that the same dye can be used to detect any amplified product, the disadvantage is that non-specific PCR products such as "primer dimers" also generate a signal.

Melt-curve analysis can be applied when fluorescent dyes are used as reporters in real-time PCR (Ririe *et al.*, 1997). The temperature is gradually increased from one at which the PCR product is known to be double-stranded to one at which all DNA strands are fully dissociated. As the temperature is increased, the amount of fluorescence is measured, and the temperature at which the maximum decrease in fluorescence (or "melting peak") occurs is noted. Melting peaks are visualised on plots of $-dF/dT$ against T (F = fluorescence and T = temperature). If there are multiple PCR products, then more than one melting peak may appear, depending on their physical properties. The temperature of the melting peak corresponds to the point at which half of the DNA is associated. At this point, the population of molecules will consist of fully dissociated, partially associated and fully associated DNA strands. Primer artefacts, such as primer dimers can usually be identified from the target DNA, since they generally have melting-peak temperature ranges quite different from each other (Woodford & Johnson, 2004).

Real-time PCR reactions using primers targeting the *P. acnes gmk* gene were carried out on serial dilutions of *P. acnes* genomic DNA (0.1pg/ml to 1ng/ml). Melt-curve analysis of the real-time PCR reaction using Evagreen carried out on the LightCycler® system (Roche) is shown in Figure 5.1. It shows that the limit of detection of the assay (i.e. the sensitivity) was 0.1pg/ml although a reliable level of detection would be 1pg/ml. There is only one melting-peak at 92ºC confirming that there was a single PCR product and no primer dimers.

Figure 5.1 Real-time PCR melt curves of the PCR products of *P. acnes gmk* gene. 5 different concentrations of *P. acnes* type strain NCTC 737 and a negative control containing water were analysed. The reporter was Evagreen. There is a single peak at 92ºC.



5.3.3 Quantitative real-time PCR

Standard PCR relies on end-point analysis and is therefore non-quantitative in nature. Real-time PCR overcomes this problem by detecting the amount of amplification of a PCR product at a set point in time during each cycle rather than detecting the amount of PCR product accumulated after a fixed number of cycles. An amplification curve can be generated which is the plot of fluorescence signal versus cycle number. Quantification in real-time PCR is calculated by measurement of the number of cycles (threshold cycle, C_T) required for the fluorescent signal to cross a threshold level. This threshold is usually set at three standard deviations above the baseline level. Crossing threshold values are inversely proportional to $\log_{10}$ of the initial target DNA concentration. Quantification values for unknown samples can then be calculated from standard curves constructed using the data obtained from known dilutions of the target DNA (Woodford & Johnson, 2004).

Figure 5.2 shows the amplification plot generated for the real-time PCR amplification of the *gmk* gene using five serial dilutions of genomic DNA from *P. acnes* type strain NCTC 737. The log of the change in fluorescence is plotted against the number of PCR cycles. The plot shows that the higher the starting copy number of target DNA, the sooner a significant increase in fluorescence is observed. From this amplification plot, C_T values for each starting DNA concentration are plotted against the log concentration of the initial target DNA forming a standard curve (Figure 5.3). Quantification of unknown amounts of DNA can be estimated using this standard curve.

Figure 5.2 Real-time PCR amplification plot of the *gmk* gene carried out on *P. acnes* type strain NCTC 737 using Evagreen as the reporter. Five serial dilutions of the genomic DNA are shown. The log of the change in fluorescence is plotted against cycle number.

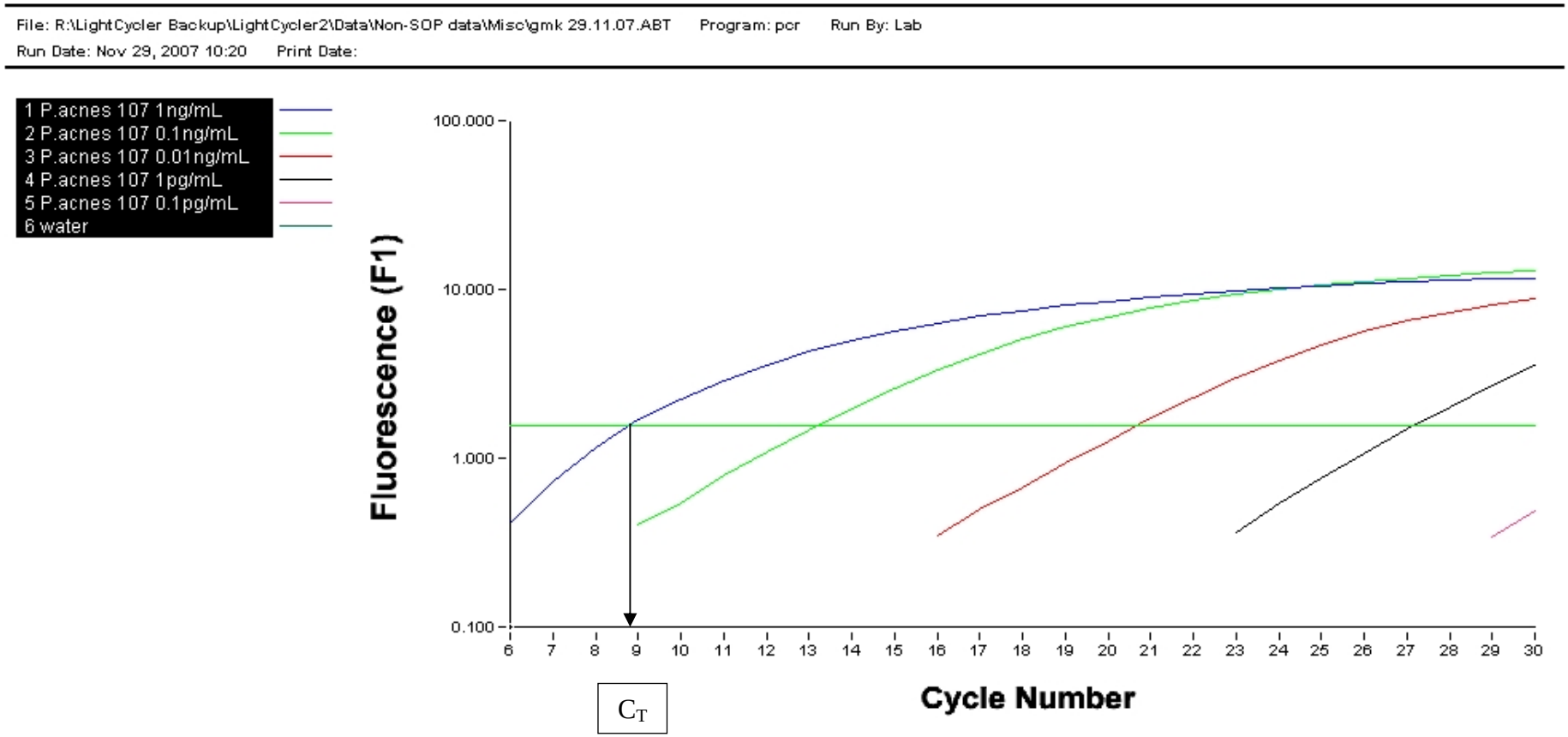
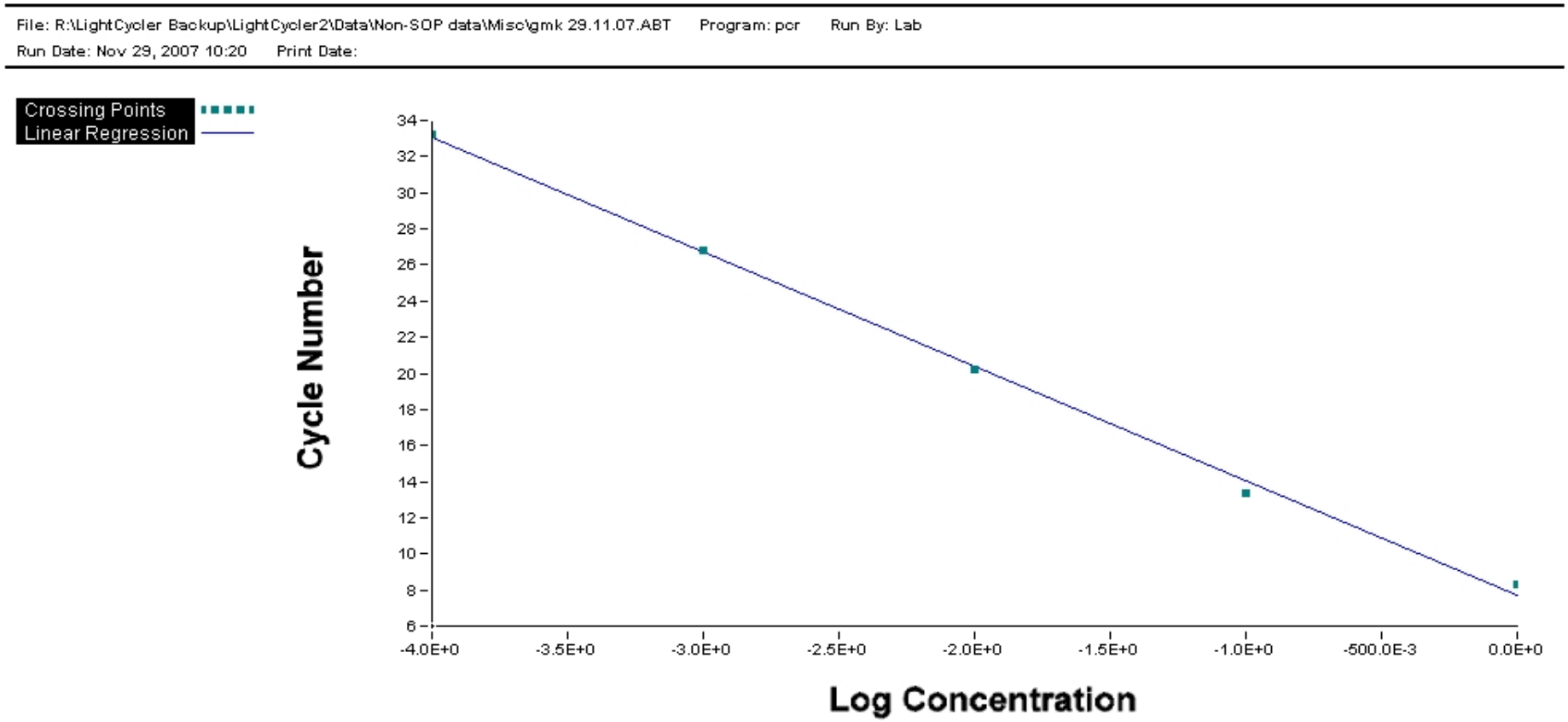


Figure 5.3 Real-time PCR standard curve showing C_T values (threshold cycle) plotted against the log concentration of genomic DNA.



5.3.4 Rapid real-time PCR

Some real-time PCR instruments (e.g. LightCycler®) enable fast and accurate temperature transitions and are therefore commonly used for rapid diagnostic purposes (Woodford & Johnson, 2004). In the current assay to detect *gmk* in the *P. acnes* sample, the first round conventional thermal cycler takes about 1 hour 45 minutes to complete. A single cycle in the second round real-time assay takes around 20 seconds, allowing the second round assay to be completed within 18 minutes. Therefore the total assay time including handling can be completed within about 2 hours 30 minutes, excluding the DNA extraction step. Rapid cycling is achieved because standard heating blocks and PCR tubes are not used in these systems. In the LightCycler, turbulent air is used to control the temperature and glass capillaries act as the reaction vessels. Rapid temperature transitions and short holds also produce better annealing accuracy due to less mispriming, as the sample spends less time at the lower temperatures (Woodford & Johnson, 2004). The real-time PCR method described here to detect *P. acnes* is therefore much more sensitive and rapid than the standard culture method employed in most clinical laboratories, which can take up to ten days.

5.3.5 Comparison of extraction methods

Extraction of genomic DNA from *P. acnes* can be problematic. The phenol/chloroform method has been proven to be very effective at extracting large amounts of clean, genomic DNA from *P. acnes* and this was the method used for obtaining DNA for the MLST study. However, for the purpose of developing a rapid diagnostics protocol for detecting *P. acnes* DNA from small amounts of clinical sample, a more efficient and less hazardous method needs to be established. Therefore, three different DNA extraction protocols were compared 1) HiPure Viral Nucleic Acid extraction kit, 2) DNAmite DNA extraction kit and 3) Chelex extraction method (methods described in Section 2.15.3).

A suspension of *P. acnes* cells harvested from an agar plate was subjected to each DNA extraction protocol. The extracted nucleic acids were then diluted in ten-fold

series and assayed for the presence of *gmk*. The target was detected in neat, 10^{-1}, and 10^{-2} dilutions extracted using the Chelex extraction method and DNAmite kit but only in neat and 10^{-1} dilutions of DNA's extracted by the HiPure Viral Nucleic Acid extraction kit. Therefore, the Chelex extraction method and the DNAmite kit appear to yield the best DNA for use with the *gmk* PCR assay. These methods have the advantage of being less time consuming and involve less hazardous chemicals than the phenol/chloroform DNA extraction method.

5.4 Future work

The real-time PCR assay developed here for the detection of the *P. acnes gmk* gene has been found to be specific for *P. acnes*. This assay needs to be applied to a much larger number of *P. acnes* strains and non-*P. acnes* strains to consolidate this finding. To further test the sensitivity of the assay in clinical samples, known numbers of *P. acnes* organisms can be spiked into sterile aqueous or vitreous samples, the genomic DNA extracted using the Chelex extraction protocol or the DNAmite kit and then subjected to real-time PCR to detect the *gmk* gene. This will allow the different DNA extraction protocols to be compared and also enable the sensitivity of the PCR assay on clinical samples to be determined. Once the optimal conditions are determined, the assay can be applied to aqueous and vitreous samples from suspected endophthalmitis cases. Half of the obtained sample could undergo conventional culture techniques and the remaining tested using the real-time PCR assay, allowing comparison between the two diagnostic techniques. False positive results from contamination of PCR samples are always possible and precautions must be in place to reduce the likelihood of this.

Multiplex PCR allows the simultaneous detection of several organisms using multiple sets of primers specific for each organism. A multiplex PCR assay could be designed to detect the common ophthalmic infections such as *P. acnes. S. aureus, P. aeruginosa, S. pneumoniae* and coagulase negative *Staphylococcus*. Once optimised, this could be applied to aqueous and vitreous samples to diagnose endophthalmitis and corneal scrape specimens from microbial keratitis.

5.5 Conclusions

MLST analysis of a diverse collection of *P. acnes* from differing clinical sites and geographical locations strongly suggest that particular strains or clonal complexes are not associated with disease type. Therefore, diagnostic assays designed to detect *P. acnes* from endophthalmitis infections do not have to differ from those targeting *P. acnes* from other infections. Real-time PCR technology employed here to detect the *gmk* housekeeping gene has proven to be specific for *P. acnes,* as it did not detect any of the other cutaneous *Propionibacterium* spp. (*P. avidum, P. granulosum* and *P. propionicus*) or other common eye infection organisms (*S. aureus, P. aeruginosa, S. pneumoniae* and coagulase negative *Staphylococcus*). The real-time PCR assay was also extremely sensitive, detecting levels of *P. acnes* genomic DNA at concentrations as low as 0.1pg/ml. Another important advantage of the real-time system is its ability to quantify the amount of amplified DNA at each PCR cycle. The standard first round of PCR and the real-time second round PCR can both be completed within two and a half hours. This means that the detection of *P. acnes* from a clinical sample can be achieved well within a day. This is an enormous reduction in diagnostic time compared to conventional culture methods which can take up to ten days.

Compared to previously described PCR assays which use universal bacterial 16S rRNA gene (Knox *et al.,* 1999; Lohmann *et al.,* 2000; Okhravi *et al.,* 2000), the current real-time assay to detect a *P. acnes* specific gene (*gmk*) does not require the need to identify the PCR product using DNA sequencing. This reduces both the cost and time required to complete the diagnostic process.

PCR diagnostics not only has the advantage of being much more rapid and sensitive than conventional culture methods, but it is also unaffected by prior antibiotic use. Further study comparing different DNA extraction methods on clinical samples spiked with *P. acnes* is warranted. The development of a multiplex PCR assay to detect organisms that commonly cause ophthalmic infections would further improve the management of these conditions.

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CHAPTER 6 MULTILOCUS SEQUENCE TYPING ANALYSIS OF *PSEUDOMONAS AERUGINOSA*

6.1 Introduction

P. aeruginosa is a Gram-negative rod and considered an opportunistic pathogen capable of infecting a variety of hosts (Lavenir *et al.*, 2007; Pukatzki *et al.*, 2002; Rahme *et al.*, 2000). It is particularly problematic in cystic fibrosis (CF) patients (Saiman & Siegel, 2004), in burned, mechanically ventilated and immunocompromised individuals (McManus *et al.*, 1985; Richard *et al.*, 1994). It can cause numerous serious infections including septicaemia, pneumonia, endocarditis, otitis, keratitis and endophthalmitis (Eifrig *et al.*, 2003; Jackson *et al.*, 2003; Kashkouli *et al.*, 2007; Kielhofner *et al.*, 1992). *P. aeruginosa* keratitis is much more common in contact lens wearers and can lead to permanent damage of the cornea if appropriate treatment is not instigated immediately (Mondino *et al.*, 1986; Rattanatam *et al.*, 2001; Wilhelmus, 1996). Other sight-threatening complications include development of secondary glaucoma and cataract (Lotti & Dart, 1992).

P. aeruginosa has been considered by some to have a panmictic population structure (Denamur *et al.*, 1993; Picard *et al.*, 1994). A panmictic-epidemic population structure has been suggested by others (Dinesh *et al.*, 2003; Lomholt *et al.*, 2001; Morales *et al.*, 2004; Pirnay *et al.*, 2002; Romling *et al.*, 1994). Kiewitz *et al.* proposed a net-like structure with high frequencies of recombination among different *P. aeruginosa* genotypes (Kiewitz & Tummler, 2000). Curran *et al.* developed an MLST scheme to characterise *P. aeruginosa* strains and demonstrated that *P. aeruginosa* has a non-clonal population structure (Curran *et al.*, 2004). These results are in contrast to another *P. aeruginosa* MLST scheme which supported a clonal population structure (Vernez *et al.*, 2005). This might be explained by the small sample size (n=34) and the possibility of over-representation of epidemic clones. Genotyping of *P. aeruginosa* using microarray showed that the majority of strains belonged to a few dominant clones widespread in disease and environmental habitats (Wiehlmann *et al.*, 2007).

The type III secretion system (TTSS) is present in several Gram-negative bacilli (Galan & Collmer, 1999). Using the TTSS, *P. aeruginosa* is able to produce and secrete virulence factors directly into the cytoplasm of host cells (Ajayi *et al.*, 2003). The effector proteins (secreted toxins) comprise of ExoS, ExoT, ExoU and ExoY (Finck-Barbancon *et al.*, 1997; Frank, 1997; Frithz-Lindsten *et al.*, 1997; Hauser *et al.*, 1998; Yahr *et al.*, 1996; Yahr *et al.*, 1998). The genes that encode these effector proteins are distributed throughout the *P. aeruginosa* genome and are characterised by variable traits (Stover *et al.*, 2000). The TTSS has been identified in nearly all clinical and environmental isolates but individual isolates and populations of isolates from distinct disease sites appear to differ in their effector genotype (Feltman *et al.*, 2001). Although *exoY* and *exoT* are present in nearly all clinical *P. aeruginosa* isolates, the majority possess only *exoS* or *exoU*, not both (Feltman *et al.*, 2001; Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998). *P. aeruginosa* strains have been classified based on the genotypic expression of these toxins. Invasive strains tend to express ExoS (Fleiszig *et al.*, 1997), whereas non-invasive or cytotoxic strains express ExoU (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998).

6.2 Aims

1. To characterise the population structure of a collection of clinical and environmental *P. aeruginosa* isolates using MLST.
2. To evaluate whether *P. aeruginosa* strains from eye infections represent a particular subgroup.
3. To determine whether *P. aeruginosa* eye infection strains between two geographical locations (UK and China) could be distinguished using MLST.
4. To determine the TTSS genotype of *P. aeruginosa* from eye infections.

6.3 Results and Discussion

6.3.1 *P. aeruginosa* strains and MLST scheme

MLST analysis of 117 *P. aeruginosa* isolates from eye infections (115 corneal scrapes from keratitis and 2 vitreous humour from endophthalmitis) was carried out using methods previously described (Curran *et al.*, 2004). Sequences for each allele were uploaded onto the *P. aeruginosa* MLST website (http://pubmlst.org/paeruginosa), developed by Keith Jolley (Jolley *et al.*, 2004). Allelic profiles and STs were also assigned using the database. MLST data on a selection of 166 *P. aeruginosa* isolates from other clinical infections were available on the website. MLST and clinical data for all 283 isolates are shown in Table 6.1.

Allelic information based on the study population, for the seven alleles, was calculated using START v2 (Section 2.16.4). The total length of the concatenated sequence of the seven alleles was 2,882 bp. The mean allele length was 412 bp with sizes ranging from 366 bp (*nuoD*) to 498 bp (*aroE*) (Table 6.2). The number of alleles for each housekeeping gene ranged from 30 (*nuoD*) to 59 (*acsA*) with a mean average of 42. All alleles for a particular locus were found to be of equal lengths for all the study population isolates. The degree of sequence diversity within the housekeeping genes were relatively high, with the proportions of polymorphic sites ranging from 8.6% (guaA) to 17.5% (aroE) with a mean average of 11.8%.

As discussed in Section 4.3.1.4, the ratio of non-synonymous (d_N) to synonymous (d_S) changes is used to assess the degree of selection operating on a gene (Rocha *et al.*, 2006). A low ratio ($d_N/d_S<1$) indicates strong purifying ("stabilising") selection. For all the loci used in the *P. aeruginosa* MLST scheme, the $d_N/d_S<1$ were less than 1, confirming their suitability as housekeeping genes for the scheme (Table 6.2).

Table 6.1 Summary of 283 *P. aeruginosa* isolates with MLST data, eBURST groups, *exoU* and *exoS* gene genotype and serotype. Isolates are grouped together based on eBURST groups. Those isolates not belonging to an eBURST are listed in order of their ST. (Abbreviations: N/A, not available, PME, agglutination with PME serum only)

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
8277	N/A	Sputum	Durham, UK	11	5	1	7	9	8	7	14	1	N/A	N/A	N/A
PSA054	N/A	Corneal scrape	Manchester, UK	11	5	1	7	9	4	7	17	1	+	-	O:1
PS-7	N/A	Sputum	Birmingham, UK	11	5	1	7	9	4	7	17	1	N/A	N/A	N/A
E4SIB2	Sep-99	Sputum	BC, Canada	11	5	1	7	9	4	7	17	1	N/A	N/A	N/A
10478	N/A	Sputum	Birmingham, UK	11	5	1	7	9	4	7	17	1	N/A	N/A	N/A
P10118	N/A	Sputum	Birmingham, UK	11	5	1	7	9	4	7	17	1	N/A	N/A	N/A
8808	N/A	Sputum	Cardiff, UK	11	5	1	7	9	4	7	17	1	N/A	N/A	N/A
9922	N/A	Sputum	London, UK	11	5	1	7	9	4	7	17	1	N/A	N/A	N/A
10099	N/A	Sputum	Manchester, UK	11	5	1	7	9	4	7	17	1	N/A	N/A	N/A
9273	N/A	Sputum	Portsmouth, UK	11	5	1	7	9	4	7	17	1	N/A	N/A	N/A
M2	Apr-94	Sputum	BC, Canada	6	5	1	7	9	4	7	187	1	N/A	N/A	N/A
PSA029	N/A	Corneal scrape	Liverpool, UK	6	5	1	7	9	4	7	187	1	+	-	O:1
8970	N/A	Sputum	Sheffield, UK	6	5	1	7	9	4	7	187	1	N/A	N/A	N/A
B8SIB1	Jul-92	Sputum	BC, Canada	11	5	1	3	9	4	7	202	1	N/A	N/A	N/A
PSA039	N/A	Corneal scrape	Liverpool, UK	11	5	1	7	46	4	7	318	1	+	-	O:1
PSA047	N/A	Corneal scrape	Bristol, UK	6	5	1	7	4	4	7	321	1	+	-	O:1
PSA048	N/A	Corneal scrape	Bristol, UK	11	5	1	7	9	25	7	322	1	+	-	O:1
PSA076	N/A	Corneal scrape	Newcastle, UK	9	5	1	7	9	4	7	339	1	+	-	O:1
PSA110	N/A	Corneal scrape	Beijing, China	112	5	1	7	4	4	7	380	1	+	-	N/A

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
7627	N/A	Sputum	Croydon, UK	6	5	6	7	4	6	3	11	2	N/A	N/A	N/A
8281	N/A	Sputum	N/A	12	5	6	7	4	6	7	15	2	N/A	N/A	N/A
F2	Mar-95	Sputum	BC, Canada	6	5	6	7	4	6	7	27	2	N/A	N/A	N/A
PSA028	N/A	Corneal scrape	Manchester, UK	6	5	6	7	4	6	7	27	2	+	-	O:1
4587	N/A	Sputum	Boston, UK	6	5	6	7	4	6	7	27	2	N/A	N/A	N/A
6954	N/A	Sputum	Glasgow, UK	6	5	6	7	4	6	7	27	2	N/A	N/A	N/A
8184	N/A	Sputum	London, UK	6	5	6	7	4	6	7	27	2	N/A	N/A	N/A
6338	N/A	Sputum	N/A	6	5	6	7	4	6	7	27	2	N/A	N/A	N/A
8101	N/A	Sputum	Stourbridge, UK	6	5	6	7	4	6	7	27	2	N/A	N/A	N/A
8386	N/A	Sputum	Wrexham, UK	6	5	6	8	2	6	7	122	2	N/A	N/A	N/A
8122	N/A	Sputum	Glamorgan, UK	6	5	6	8	21	6	7	128	2	N/A	N/A	N/A
7874	N/A	Sputum	Leeds, UK	6	5	6	7	2	6	7	129	2	N/A	N/A	N/A
M4	Oct-96	Sputum	BC, Canada	6	5	37	7	4	6	7	161	2	N/A	N/A	N/A
PSA069	N/A	Corneal scrape	Manchester, UK	99	5	6	7	4	6	7	334	2	+	-	O:1
D278	N/A	Sputum	N/A	36	27	35	3	4	13	7	144	3	N/A	N/A	N/A
B4	Oct-94	Sputum	BC, Canada	36	27	28	3	4	13	7	179	3	N/A	N/A	N/A
PSA055	N/A	Corneal scrape	Bristol, UK	36	27	28	3	4	13	7	179	3	+	-	O:6
A1	Jul-83	Sputum	BC, Canada	36	27	28	31	4	13	7	180	3	N/A	N/A	N/A
H3	Sep-82	Sputum	BC, Canada	36	27	28	31	4	15	7	188	3	N/A	N/A	N/A
P1	Jul-97	Sputum	BC, Canada	36	27	35	31	4	13	7	200	3	N/A	N/A	N/A
PS-96	N/A	Sputum	Birmingham, UK	88	27	28	3	4	13	7	353	3	N/A	N/A	N/A

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
4785	N/A	Sputum	Boston, UK	7	22	5	3	3	14	19	101	4	N/A	N/A	N/A
S2	Dec-97	Sputum	BC, Canada	28	38	5	3	3	14	19	159	4	N/A	N/A	N/A
9028	N/A	Sputum	Poole, UK	28	5	5	3	3	14	19	171	4	N/A	N/A	N/A
G1	1987	Sputum	BC, Canada	28	22	5	3	3	14	19	175	4	N/A	N/A	N/A
NIL:2427/06	2006	Urine	Poland	28	22	5	3	3	14	19	175	4	N/A	N/A	N/A
PSA046	N/A	Corneal scrape	London, UK	28	22	5	3	3	14	19	175	4	+	-	O:4
9956	N/A	Sputum	Trent, UK	28	22	5	3	3	14	19	175	4	N/A	N/A	N/A
S1	May-88	Sputum	BC, Canada	28	22	5	21	3	14	19	201	4	N/A	N/A	N/A
10258	N/A	Sputum	London, UK	6	5	11	3	4	23	1	146	5	N/A	N/A	N/A
RN64944	Jan-03	Sputum	Hungary	11	20	1	65	45	4	10	343	5	N/A	N/A	N/A
PS-227	N/A	Sputum	Birmingham, UK	6	5	11	81	4	23	1	374	5	N/A	N/A	N/A
10105	N/A	Sputum	Manchester, UK	6	5	11	3	4	6	1	680	5	N/A	N/A	N/A
10108	N/A	Sputum	Birmingham, UK	6	5	40	3	4	23	1	681	5	N/A	N/A	N/A
8711	N/A	Sputum	Cardiff, UK	6	5	11	3	4	4	1	683	5	N/A	N/A	N/A
565	2002	Bronchial lavage	Russia	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11
P3303	2002	N/A	Brazil	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
06-116	2002	N/A	Hungary	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11
3389	2003	N/A	Russia	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11
1913	2003	N/A	Russia	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11
2074	2003	N/A	Russia	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11
05-176	2004	Soft tissue infection	Serbia	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11
3107	2004	Sputum	Russia	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11
MB197	2005	Blood culture	Hungary	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
49010	2005	Bronchial lavage	Hungary	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
NIL:3220/05	2005	Bronchial lavage	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
05-380	2005	N/A	Hungary	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11
NIL:3816/05	2005	N/A	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
NIL:1900/06	2006	Bronchial lavage	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
06-150	2006	Soft tissue infection	Hungary	38	11	3	13	1	2	4	235	6	N/A	N/A	O:11
NIL:1186/06	2006	Urine	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
NIL:155/06	2006	Urine	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
NIL:4586/07	2007	Blood culture	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
NIL:2997/07	2007	Soft tissue infection	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
NIK:2913/07	2007	Urine	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
NIL:3459/07	2007	Urine	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
NIL:652/07	2007	Urine	Poland	38	11	3	13	1	2	4	235	6	N/A	N/A	N/A
PSA010	Mar-04	Corneal scrape	Birmingham, UK	38	11	3	13	1	2	4	235	6	-	+	N/A
PSA098	N/A	Corneal scrape	Beijing, China	38	11	3	13	1	2	4	235	6	-	+	N/A
PSA117	N/A	Corneal scrape	Beijing, China	38	11	3	13	1	2	4	235	6	-	+	N/A
PSA033	N/A	Corneal scrape	Bristol, UK	38	11	3	13	1	2	4	235	6	-	+	O:11
PSA050	N/A	Corneal scrape	Bristol, UK	38	11	3	13	1	2	4	235	6	-	+	O:11
PSA031	N/A	Corneal scrape	London, UK	38	11	3	13	1	2	4	235	6	-	+	O:11
PSA078	N/A	Corneal scrape	London, UK	38	11	3	13	1	2	4	235	6	-	+	O:11
PSA025	N/A	Corneal scrape	Manchester, UK	38	11	3	13	1	2	4	235	6	-	+	O:11
PSA016	Sep-04	Corneal scrape	Birmingham, UK	82	11	3	13	1	2	4	304	6	-	+	N/A
PSA053	N/A	Corneal scrape	London, UK	95	11	3	13	1	2	4	323	6	-	+	O:11

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
PSA061	N/A	Corneal scrape	London, UK	4	11	3	13	1	2	4	342	6	-	+	O:11
8382	N/A	Sputum	London, UK	4	4	16	24	1	6	3	109	7	N/A	N/A	N/A
95149	2004	Bronchial lavage	Hungary	4	4	16	12	1	6	3	253	7	N/A	N/A	N/A
NIL:1185/2006	2006	Urine	Poland	4	4	16	12	1	6	3	253	7	N/A	N/A	N/A
PSA006	N/A	Corneal scrape	Birmingham, UK	4	4	16	12	1	6	3	253	7	-	+	N/A
PSA045	N/A	Corneal scrape	London, UK	4	4	16	12	1	6	3	253	7	-	+	O:10
PSA044	N/A	Corneal scrape	Newcastle, UK	4	4	16	12	1	6	3	253	7	-	+	O:10
PSA038	N/A	Corneal scrape	London, UK	93	4	16	12	1	6	3	317	7	-	+	O:11
5757	N/A	Sputum	France	6	6	4	3	3	4	*7*	9	8	N/A	N/A	N/A
8420	N/A	Sputum	N/A	6	6	4	3	20	4	7	118	8	N/A	N/A	N/A
PSA005	Jul-04	Corneal scrape	Birmingham, UK	6	28	4	3	3	4	7	252	8	+	-	N/A
PS-39	N/A	Sputum	Birmingham, UK	6	5	36	63	4	30	2	231	9	N/A	N/A	N/A
PS-70	N/A	Sputum	Birmingham, UK	6	5	36	63	4	49	2	370	9	N/A	N/A	N/A
PS-257	N/A	Sputum	Birmingham, UK	6	5	36	63	4	30	59	376	9	N/A	N/A	N/A
10106	N/A	Sputum	Manchester, UK	36	5	29	7	4	10	7	170	10	N/A	N/A	N/A
PS-16	N/A	Sputum	Birmingham, UK	36	53	29	7	4	10	7	367	10	N/A	N/A	N/A
PS-169	N/A	Sputum	Birmingham, UK	36	53	29	7	4	10	58	373	10	N/A	N/A	N/A
PS-2	N/A	Sputum	Birmingham, UK	87	70	79	80	53	48	1	366	11	N/A	N/A	N/A
PS-36	N/A	Sputum	Birmingham, UK	90	70	79	80	53	48	1	368	11	N/A	N/A	N/A
PS-95	N/A	Sputum	Birmingham, UK	90	70	29	80	53	48	1	372	11	N/A	N/A	N/A

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
PA0062	Jun-02	Hospital sink	Leeds, UK	3	5	5	4	4	4	3	54	12	N/A	N/A	N/A
P12-Q	1993	N/A	France	17	5	5	4	4	4	3	111	12	N/A	N/A	O:12
06-117	2002	N/A	Hungary	17	5	5	4	4	4	3	111	12	N/A	N/A	O:12
NIL:1010/06	2006	Urine	Poland	17	5	5	4	4	4	3	111	12	N/A	N/A	N/A
8722	N/A	Sputum	Belfast, UK	17	5	5	4	4	4	3	111	12	N/A	N/A	N/A
8756	N/A	Sputum	France	17	5	5	4	19	4	3	113	12	N/A	N/A	N/A
PSA020	N/A	Corneal scrape	London, UK	13	4	5	5	12	7	15	308	13	-	+	O:11
PSA072	N/A	Corneal scrape	Liverpool, UK	6	4	5	5	12	7	15	345	13	-	+	O:11
PSA018	N/A	Corneal scrape	Manchester, UK	33	59	60	3	1	6	4	306	14	-	+	O:11
PSA022	N/A	Corneal scrape	Manchester, UK	5	59	60	3	1	6	4	310	14	-	+	O:11
PSA049	N/A	Corneal scrape	Manchester, UK	14	5	10	7	4	13	7	260	15	+	-	O:6
PSA085	N/A	Corneal scrape	Beijing, China	14	63	10	7	4	13	7	264	15	N/A	N/A	N/A
2620	2003	N/A	Poland	17	5	12	3	14	4	7	244	16	N/A	N/A	N/A
NIL:1659/06	2006	N/A	Poland	17	5	12	3	14	4	7	244	16	N/A	N/A	N/A
NIL:261/06	2006	Urine	Poland	17	5	12	3	14	4	7	244	16	N/A	N/A	N/A
NIK:981/06	2006	Urine	Poland	17	5	12	3	14	4	7	244	16	N/A	N/A	N/A
NIL:2498/07	2007	Urine	Poland	17	5	12	3	14	4	7	244	16	N/A	N/A	N/A
PSA107	N/A	Corneal scrape	Beijing, China	17	5	12	3	14	4	7	244	16	+	-	N/A
PSA120	N/A	Corneal scrape	Beijing, China	17	5	12	3	14	4	7	244	16	+	-	N/A
PSA122	N/A	Corneal scrape	Beijing, China	17	5	12	3	14	4	7	244	16	+	-	N/A
PSA043	N/A	Corneal scrape	London, UK	17	5	12	3	14	4	7	244	16	+	-	O:2
PSA051	N/A	Corneal scrape	London, UK	17	5	12	3	14	4	7	244	16	+	-	O:2
PSA073	N/A	Corneal scrape	London, UK	13	5	12	3	14	4	7	336	16	+	-	O:2

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
PS-175	N/A	Sputum	Birmingham, UK	85	1	59	6	1	33	42	237	17	N/A	N/A	N/A
PS-82	N/A	Sputum	Birmingham, UK	5	1	59	6	1	33	42	238	17	N/A	N/A	N/A
PSA012	Sep-04	Corneal scrape	Birmingham, UK	11	20	1	65	45	4	10	343	18	+	-	N/A
PSA111	N/A	Corneal scrape	Beijing, China	11	20	1	65	4	4	10	381	18	+	-	N/A
PA0052	Aug-02	Sputum	Lincoln, UK	11	5	12	12	1	6	3	45	19	N/A	N/A	N/A
PA0060	Jun-02	Sputum	Wycombe, UK	11	19	12	12	1	6	3	52	19	N/A	N/A	N/A
PSA081	N/A	Corneal scrape	Beijing, China	3	5	72	13	2	4	26	355	20	+	-	N/A
PSA083	N/A	Corneal scrape	Beijing, China	3	5	6	13	2	4	26	356	20	+	-	N/A
PS-4	N/A	Sputum	Birmingham, UK	16	5	5	74	2	10	10	350	21	N/A	N/A	N/A
PS-98	N/A	Sputum	Birmingham, UK	16	5	5	79	2	10	10	354	21	N/A	N/A	N/A
PA0045	Oct-02	Blood culture	Wolverhampton, UK	4	5	16	3	1	4	13	38	22	N/A	N/A	N/A
PA0053	Aug-02	Urine	Lincoln, UK	4	5	16	3	1	17	13	46	22	N/A	N/A	N/A
PSA066	N/A	Corneal scrape	London, UK	97	20	11	3	3	3	7	331	23	+	-	PME
PS-6	N/A	Sputum	Birmingham, UK	22	20	11	3	3	3	7	348	23	N/A	N/A	N/A
9572	N/A	Sputum	Poole, UK	17	5	1	3	13	6	7	148	24	N/A	N/A	N/A
9185	N/A	Sputum	London, UK	11	5	1	3	13	6	7	682	24	N/A	N/A	N/A
9946	N/A	Sputum	Shrewsbury, UK	11	5	1	3	13	6	7	682	24	N/A	N/A	N/A
PSA067	N/A	Corneal scrape	London, UK	98	5	57	13	1	40	3	332	25	-	+	O:8
PSA077	N/A	Corneal scrape	London, UK	11	5	57	13	1	40	3	340	25	-	+	O:8
PSA042	N/A	Corneal scrape	London, UK	94	7	63	13	8	7	8	320	26	-	+	O:11
PSA075	N/A	Corneal scrape	London, UK	33	7	63	13	8	7	8	338	26	-	+	O:11
PSA070	N/A	Corneal scrape	London, UK	6	20	1	3	4	4	2	132	27	+	-	O:6
8440	N/A	Sputum	Carmarthen, UK	6	20	1	3	4	4	2	132	27	N/A	N/A	N/A

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
PSA064	N/A	Corneal scrape	Bristol, UK	5	20	1	3	4	4	2	329	27	+	-	O:6
PSA058	N/A	Corneal scrape	London, UK	35	1	25	6	6	7	5	325	28	-	-	PME
PSA062	N/A	Corneal scrape	London, UK	38	1	25	6	6	7	5	327	28	-	-	PME
8297	N/A	Sputum	Portsmouth, UK	13	8	9	3	1	9	9	16	29	N/A	N/A	N/A
PSA105	N/A	Corneal scrape	Beijing, China	13	8	9	3	1	6	9	316	29	-	+	N/A
PSA109	N/A	Corneal scrape	Beijing, China	13	8	9	3	1	6	9	316	29	-	+	N/A
PSA037	N/A	Corneal scrape	London, UK	13	8	9	3	1	6	9	316	29	-	+	O:11
PSA032	N/A	Corneal scrape	Manchester, UK	13	8	9	3	1	6	9	316	29	-	+	O:11
PSA021	N/A	Corneal scrape	Bristol, UK	13	8	9	3	1	17	15	309	30	-	+	O:1
PSA024	N/A	Corneal scrape	Bristol, UK	13	8	9	3	1	17	15	309	30	-	+	O:11
PSA023	N/A	Corneal scrape	Manchester, UK	13	8	9	3	1	17	4	311	30	-	+	O:11
8237	N/A	Sputum	London, UK	10	4	5	10	1	6	3	13		N/A	N/A	N/A
PA0020	Feb-03	Sputum	Stafford, UK	6	4	1	11	4	7	10	18		N/A	N/A	N/A
PA0021	Feb-03	Sputum	Dudley, UK	14	9	10	6	6	10	5	19		N/A	N/A	N/A
PA0022	Feb-03	Sputum	Cheshire, UK	15	5	11	3	1	7	11	20		N/A	N/A	N/A
PA0024	Jan-03	Sputum	Bedford, UK	16	5	12	11	1	2	1	21		N/A	N/A	N/A
PA0025	Jan-03	Tissue Sample	Northampton, UK	6	4	4	3	3	11	2	22		N/A	N/A	N/A
PA0027	Jan-03	Urine	Tyne and Wear, UK	18	4	5	5	5	4	4	23		N/A	N/A	N/A
PA0034	Dec-02	Blood culture	London, UK	15	10	11	3	2	7	12	28		N/A	N/A	N/A
PA0039	Nov-02	Intravenous Line	London, UK	18	13	13	11	11	4	14	32		N/A	N/A	N/A
PA0041	Nov-02	Sputum	Luton, UK	4	5	16	3	3	7	3	34		N/A	N/A	N/A
PA0042	Oct-02	Sputum	Glasgow, UK	22	14	17	3	3	6	7	35		N/A	N/A	N/A
PA0044	Oct-02	Blood culture	Leeds, UK	23	16	11	4	4	6	3	37		N/A	N/A	N/A

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
PA0046	Oct-02	Blood culture	Folkestone, UK	18	5	5	3	1	6	13	39		N/A	N/A	N/A
PA0047	Sep-02	Tissue Sample	Chelsea, UK	4	5	16	11	11	7	14	40		N/A	N/A	N/A
PA0055	Jul-02	Blood culture	Newcastle, UK	6	5	4	13	1	4	4	48		N/A	N/A	N/A
PA0061	Jun-02	Sputum	Brighton, UK	11	5	19	4	4	4	3	53		N/A	N/A	N/A
PA0063	Jun-02	Blood culture	Bedford, UK	27	5	20	13	1	2	8	55		N/A	N/A	N/A
PA0064	Jun-02	Tissue Sample	Telford, UK	13	17	5	14	3	6	19	56		N/A	N/A	N/A
PA0068	May-02	Tissue Sample	London, UK	28	4	17	15	4	4	3	60		N/A	N/A	N/A
PA0069	May-02	Sputum	Gloucester, UK	13	4	9	3	1	6	11	61		N/A	N/A	N/A
PA0071	Apr-02	Urine	Ormskirk, UK	5	4	21	5	12	10	15	63		N/A	N/A	N/A
PA0074	Mar-02	Blood culture	Dorset, UK	30	10	23	5	4	2	7	66		N/A	N/A	N/A
PA0080	Feb-02	Sputum	York, UK	4	4	16	19	13	10	23	72		N/A	N/A	N/A
PA0082	Jan-02	Blood culture	Wycombe, UK	4	5	1	19	13	7	23	74		N/A	N/A	N/A
PA0084	Jan-02	Skin swab	London, UK	17	21	12	5	1	17	1	76		N/A	N/A	N/A
PA0085	Jan-02	Hospital floor	London, UK	17	22	12	12	1	18	3	77		N/A	N/A	N/A
PA0086	Dec-01	Catheter	Stoke, UK	11	11	11	3	3	19	19	78		N/A	N/A	N/A
PA0088	Dec-01	Trachea	London, UK	35	23	27	6	6	4	5	80		N/A	N/A	N/A
PA0093	Nov-01	Tissue Sample	Folkestone, UK	11	5	1	3	14	17	7	83		N/A	N/A	N/A
PA0101	Sep-01	Blood culture	Windsor, UK	17	5	5	1	1	21	1	91		N/A	N/A	N/A
PA0103	Sep-01	Blood culture	Leeds, UK	22	5	11	13	1	15	4	93		N/A	N/A	N/A
PA0104	Aug-01	Hospital floor	Leicester, UK	24	5	3	5	16	6	28	94		N/A	N/A	N/A
PA0107	Aug-01	Sputum	Leicester, UK	6	5	1	11	17	7	2	96		N/A	N/A	N/A
PA0109	Jul-01	Blood culture	Cheshire, UK	41	12	4	3	3	4	14	98		N/A	N/A	N/A
6950	N/A	Sputum	Southport, UK	15	5	5	3	4	4	3	102		N/A	N/A	N/A

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
7186	N/A	Sputum	Truro, UK	17	5	12	22	14	22	29	103		N/A	N/A	N/A
7215	N/A	Sputum	Bath, UK	4	4	16	3	1	6	30	104		N/A	N/A	N/A
8113	N/A	Sputum	N/A	22	20	11	23	1	3	3	106		N/A	N/A	N/A
8202	N/A	Sputum	London, UK	4	4	16	21	1	6	1	107		N/A	N/A	N/A
8318	N/A	Sputum	Sutton, UK	39	5	20	5	1	6	31	108		N/A	N/A	N/A
PSA103	N/A	Corneal scrape	Beijing, China	15	5	1	3	2	12	7	110		+	-	N/A
8623	N/A	Sputum	UK	15	5	1	3	2	12	7	110		N/A	N/A	N/A
8734	N/A	Sputum	Stoke, UK	6	5	1	25	1	12	1	112		N/A	N/A	N/A
7916	N/A	Sputum	Bristol, UK	17	22	5	3	4	14	3	120		N/A	N/A	N/A
7193	N/A	Sputum	N/A	11	5	7	27	1	7	33	121		N/A	N/A	N/A
5798	Jan-03	Sputum	Sheffield, UK	17	5	5	1	10	12	2	123		N/A	N/A	N/A
PA0111	Jul-01	Blood Culture	London, UK	5	27	5	13	1	13	4	126		N/A	N/A	N/A
8405	N/A	Sputum	Bangor, UK	43	30	31	26	4	24	32	127		N/A	N/A	N/A
8103	N/A	Sputum	London, UK	1	20	32	3	4	25	3	133		N/A	N/A	N/A
7306	N/A	Sputum	Cumbria, UK	6	20	11	7	1	12	19	134		N/A	N/A	N/A
K1SIB1	Feb-90	Sputum	BC, Canada	1	5	7	5	4	4	2	192		N/A	N/A	N/A
8715	N/A	Sputum	Birmingham, UK	28	5	11	18	4	13	3	217		N/A	N/A	N/A
WID283-D06	2005	Sputum	UK	16	5	30	11	4	31	41	233		N/A	N/A	N/A
NIL:2026/05	2005	Urine	Poland	16	5	30	11	4	31	41	233		N/A	N/A	N/A
PSA112	N/A	Corneal scrape	Beijing, China	16	5	30	11	4	31	41	233		+	-	N/A
PS-19	N/A	Sputum	Birmingham, UK	28	5	1	5	4	32	10	236		N/A	N/A	N/A
245	2005	Urine	Poland	39	6	12	11	3	15	2	245		N/A	N/A	N/A
PSA056	N/A	Corneal scrape	London, UK	39	6	12	11	3	15	2	245		+	-	PME

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
PSA082	N/A	Corneal scrape	Beijing, China	5	62	67	3	1	16	50	246		N/A	N/A	N/A
PSA084	N/A	Corneal scrape	Beijing, China	101	5	11	5	4	4	7	247		N/A	N/A	N/A
PSA090	N/A	Corneal scrape	Beijing, China	38	64	69	13	1	2	51	248		N/A	N/A	N/A
PSA001	Aug-03	Corneal scrape	Birmingham, UK	18	54	5	5	1	6	8	249		-	+	N/A
PSA079	N/A	Corneal scrape	Birmingham, UK	18	54	5	5	1	6	8	249		-	+	O:11
PSA015	Sep-04	Corneal scrape	Birmingham, UK	28	5	36	3	1	13	7	250		+	-	N/A
PSA080	N/A	Corneal scrape	Beijing, China	28	61	66	6	1	33	55	263		N/A	N/A	N/A
PSA088	N/A	Corneal scrape	Beijing, China	36	5	68	3	4	10	7	265		N/A	N/A	N/A
PSA093	N/A	Corneal scrape	Beijing, China	16	5	11	72	44	7	52	266		N/A	N/A	N/A
PSA094	N/A	Corneal scrape	Beijing, China	19	5	12	11	11	4	14	267		N/A	N/A	N/A
PSA095	N/A	Corneal scrape	Beijing, China	23	5	70	7	1	12	7	268		N/A	N/A	N/A
PSA097	N/A	Corneal scrape	Beijing, China	6	5	11	3	4	3	7	269		N/A	N/A	N/A
PSA096	N/A	Corneal scrape	Beijing, China	22	3	17	5	2	10	7	270		N/A	N/A	N/A
PSA099	N/A	Corneal scrape	Beijing, China	102	24	37	5	4	7	7	271		N/A	N/A	N/A
PSA002	May-04	Corneal scrape	Birmingham, UK	44	8	5	3	15	6	26	296		-	+	N/A
PSA026	N/A	Corneal scrape	Newcastle, UK	44	8	5	3	15	6	26	296		-	+	O:1
PSA003	Feb-04	Corneal scrape	Birmingham, UK	91	4	5	5	5	20	4	297		-	+	N/A
PSA004	Jun-04	Corneal scrape	Birmingham, UK	18	4	13	3	1	17	13	298		-	+	N/A
PSA007	Aug-04	Corneal scrape	Birmingham, UK	17	5	36	3	3	7	3	299		+	-	N/A
PSA008	Aug-04	Corneal scrape	Birmingham, UK	5	55	7	61	1	4	45	300		+	-	N/A
PSA009	Jun-03	Corneal scrape	Birmingham, UK	38	4	57	62	1	1	26	301		-	+	N/A
PSA011	Aug-00	Vitreous humour	Birmingham, UK	17	5	5	13	45	4	3	302		+	-	N/A
PSA013	Oct-04	Corneal scrape	Birmingham, UK	16	5	12	11	3	4	18	303		+	-	N/A

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
PSA017	N/A	Corneal scrape	Liverpool, UK	92	8	11	11	4	43	7	305		+	+	O:4
PSA019	N/A	Corneal scrape	London, UK	32	11	61	13	1	6	54	307		-	+	O:11
PSA027	N/A	Corneal scrape	Bristol, UK	5	3	57	6	1	33	47	312		-	+	O:1
05-97	2005	Soft tissue infection	Hungary	47	8	7	6	8	11	40	313		N/A	N/A	O:1
PSA030	N/A	Corneal scrape	Liverpool, UK	47	8	7	6	8	11	40	313		-	+	O:1
PSA035	N/A	Corneal scrape	London, UK	6	4	62	66	3	6	26	314		+	-	O:11
PSA052	N/A	Corneal scrape	London, UK	6	4	62	66	3	6	26	314		+	-	O:11
PSA036	N/A	Corneal scrape	Birmingham, UK	5	4	57	62	1	18	26	315		-	+	O:11
PSA034	N/A	Corneal scrape	London, UK	5	4	3	3	1	11	8	319		-	+	O:11
PSA040	N/A	Corneal scrape	London, UK	5	4	3	3	1	11	8	319		-	+	O:11
PSA057	N/A	Corneal scrape	Newcastle, UK	18	8	27	68	15	7	3	324		-	-	O:8
PSA060	N/A	Corneal scrape	London, UK	96	4	16	12	1	7	3	326		-	+	O:10
PSA063	N/A	Corneal scrape	London, UK	33	56	65	5	1	10	49	328		-	+	O:1
PSA065	N/A	Corneal scrape	London, UK	6	20	36	67	1	6	3	330		-	+	O:10
PSA068	N/A	Corneal scrape	Newcastle, UK	33	5	1	13	4	4	7	333		+	-	O:1
PSA071	N/A	Corneal scrape	Bristol, UK	99	57	1	5	47	24	2	335		+	-	O:6
PSA074	N/A	Corneal scrape	London, UK	17	58	21	17	10	16	22	337		-	+	O:4
PSA014	Nov-01	Vitreous humour	Birmingham, UK	5	14	25	3	1	16	46	341		-	+	N/A
PSA041	N/A	Corneal scrape	London, UK	81	8	5	67	1	40	13	344		-	+	O:1
PSA059	N/A	Corneal scrape	Manchester, UK	33	60	64	69	48	24	48	346		+	-	O:3
PS-3	N/A	Sputum	Birmingham, UK	40	5	17	5	4	15	7	347		N/A	N/A	N/A
PS-58	N/A	Sputum	Birmingham, UK	89	30	64	78	48	24	32	349		N/A	N/A	N/A
PS-130	N/A	Sputum	Birmingham, UK	106	66	11	3	29	4	9	351		N/A	N/A	N/A

Isolate name	Date of isolation	Source of isolate	Location	Allelic profile							ST	eBURST group	*exoS*	*exoU*	serotype
				acsA	*aroE*	*guaA*	*mutL*	*nuoD*	*ppsA*	*trpE*					
PS-202	N/A	Sputum	Birmingham, UK	107	3	20	62	4	7	3	352		N/A	N/A	N/A
NIL:2646/06	2006	Urine	Poland	2	4	5	3	1	6	11	357		N/A	N/A	N/A
PSA087	N/A	Corneal scrape	Beijing, China	2	4	5	3	1	6	11	357		-	+	N/A
PSA101	N/A	Corneal scrape	Beijing, China	41	5	26	3	1	15	18	358		+	+	N/A
PSA114	N/A	Corneal scrape	Beijing, China	41	5	26	3	1	15	18	358		+	+	N/A
PSA104	N/A	Corneal scrape	Beijing, China	111	30	80	26	54	44	32	359		-	-	N/A
PSA113	N/A	Corneal scrape	Beijing, China	15	5	36	11	27	4	2	360		+	-	N/A
PSA121	N/A	Corneal scrape	Beijing, China	35	8	82	3	15	7	3	361		-	+	N/A
PS-47	N/A	Sputum	Birmingham, UK	1	5	20	16	3	4	7	369		N/A	N/A	N/A
PSA092	N/A	Corneal scrape	Beijing, China	103	8	5	5	1	6	4	377		-	+	N/A
PSA106	N/A	Corneal scrape	Beijing, China	1	14	3	11	1	15	1	378		+	-	N/A
PSA108	N/A	Corneal scrape	Beijing, China	39	5	11	28	4	4	63	379		+	-	N/A
PSA115	N/A	Corneal scrape	Beijing, China	113	5	81	3	1	6	25	382		-	+	N/A
PSA116	N/A	Corneal scrape	Beijing, China	35	8	27	3	15	45	3	383		-	-	N/A
PSA118	N/A	Corneal scrape	Beijing, China	17	5	11	3	52	4	2	384		+	-	N/A
PSA119	N/A	Corneal scrape	Beijing, China	15	5	5	5	50	4	14	385		+	-	N/A

Table 6.2 Characteristics of the genes used in the *P. aeruginosa* MLST scheme (based on the study population)

Gene	Size of fragment sequenced (bp)	No. of distinct alleles	No. of polymorphic sites	Proportion of polymorphic sites (%)	Mean G+C content (%)	d_N/d_S
acsA	390	59	53	13.6	66.8	0.0725
aroE	498	39	87	17.5	70.5	0.1320
guaA	373	47	32	8.6	65.7	0.0357
mutL	442	40	48	10.9	67.3	0.0440
nuoD	366	30	30	8.2	63.3	0.0090
ppsA	370	34	53	14.3	66.8	0.0435
trpE	443	42	41	9.3	66.2	0.0250
Mean	412	42	49	11.8	66.9	0.0517

6.3.2 Analysis of *P. aeruginosa* isolates by ST

From the 283 *P. aeruginosa* isolates analysed in the study population, 200 STs were generated. ST-235 was found to be the most common ST (n=30; 15%). This genotype was found in a variety of geographical locations (Russia, Brazil, Hungary, Poland, Serbia, China and UK) and from a wide range of infections (keratitis, soft tissue infection, sputum from CF patients, blood culture and urinary tract infections). Ten isolates were assigned ST-244, nine isolates were assigned ST-17, seven isolates were assigned ST-27 and five isolates were assigned ST-253. Again, these genotypes were associated with different geographical locations and clinical diseases. ST-111, ST-175 and ST-316 were assigned to four isolates each. ST-187 and ST-233 had three isolates each. 14 of the STs had 2 isolates each and the remaining 176 isolates had a unique ST. The relatively high number of STs generated indicates that there is frequent alteration in the genetic structure of *P. aeruginosa*, either by point mutation or recombination.

6.3.3 Phylogenetic analysis

6.3.3.1 Phylogenetic trees

Phylogenetic analysis was carried out on the 200 *P. aeruginosa* STs as described in section 2.13.6. Figure 6.1 is a Neighbour-Joining dendrogram based on allelic profiles of the 200 STs. eBURST groups 1-6 (Section 6.3.3.2) are highlighted. All 30 clonal complexes identified by the BURST analysis are marked by a red dot. Each clonal complex consists of STs that have 6 or more alleles in common (SLVs). The STs did not sub-divide into distinct lineages as was the case in *P. acnes*. This data suggests that there is frequent point mutation or recombinational events leading to *P. aeruginosa* having a non-clonal population structure.

Neighbour-Joining dendrogram of concatenated sequences divides the STs into numerous clusters (Figure 6.2). One particular cluster, consisting of three isolates represented by three different STs (ST-366, ST-368 and ST-372), is significantly different to the remaining isolates. These isolates were all cultures from sputum

samples from CF patients in Birmingham. Comparison of concatenated sequences of ST-366 and a randomly selected ST from the main cluster (ST-9) showed 4.6% sequence diversity, compared to 1.02% overall sequence diversity of all STs. More detailed analysis of individual loci is described in Section 6.3.4.2. The existence of this smaller cluster suggests that although *P. aeruginosa* has a non-clonal structure, occasional epidemic clones or clonal complexes emerge. This cluster was not identified from the dendrogram of allelic profiles because the frequency of polymorphisms is not weighted and so one point mutation at a locus will change the allelic profile in the same way that multiple polymorphisms would. The existence of this epidemic clone is also demonstrated using split decomposition analysis (Section 6.3.4.2).

Figure 6.1 Unrooted Neighbour-Joining dendrogram based on allelic profiles of the 200 *P. aeruginosa* STs (Jukes-Cantor-based algorithm, 1,000 bootstrap replications).

Figure 6.2 Unrooted Neighbour-Joining dendrogram of the 200 *P. aeruginosa* STs based on concatenated sequences of the seven housekeeping genes (with Jukes-Cantor-based algorithm, 1,000 bootstrap replications).

6.3.3.2 eBURST analysis

eBURST analysis was carried out on the *P. aeruginosa* MLST data, which divides genotypes into eBURST groups. Genotypes within an eBURST group have at least six or more loci in common (SLV) (Feil *et al.*, 2004). 91 of the 200 STs generated from 283 isolates were divided into thirty eBURST groups (or clonal complexes) (Figure 6.3). The remaining 109 STs were singletons, i.e. they did not have any SLVs. eBURST group 1 was made up of 9 individual STs, eBURST group 2 had 8 STs and eBURST group 3 had 6 STs. Both eBURST groups 4 and 5 had 5 STs each. eBURST group 6 had only 4 STs but made up from 33 isolates. This is due to the large number of ST-235 isolates (n=30). The remaining eBURST groups were made up of 2 or 3 STs each.

Figure 6.3 show SLV connected by a pink line and Figure 6.4 has DLV shown in addition to the SLV. These clusters suggest the existence of epidemic clonal complexes within the study population, although none of the eBURST groups were associated with a particular geographical location or clinical disease. Figure 6.5 looks more closely at strains from eye infections. STs associated with keratitis or endophthalmitis infections are circled in red and those associated with both eye infections and other clinical infections are shaded in red. The eBURST diagram shows that there were no clonal complexes made up of three or more STs that were associated exclusively with eye infections. There were eight clonal complexes made up of two different STs that were only found in eye infections. However, there were too few isolates within each of these clonal complexes to conclude if they were solely associated with eye infections.

Figure 6.3 eBURST diagram showing the clusters of related *P. aeruginosa* STs and individual unlinked STs. Thirty clonal complexes are identified. SLVs are shown as pink lines.

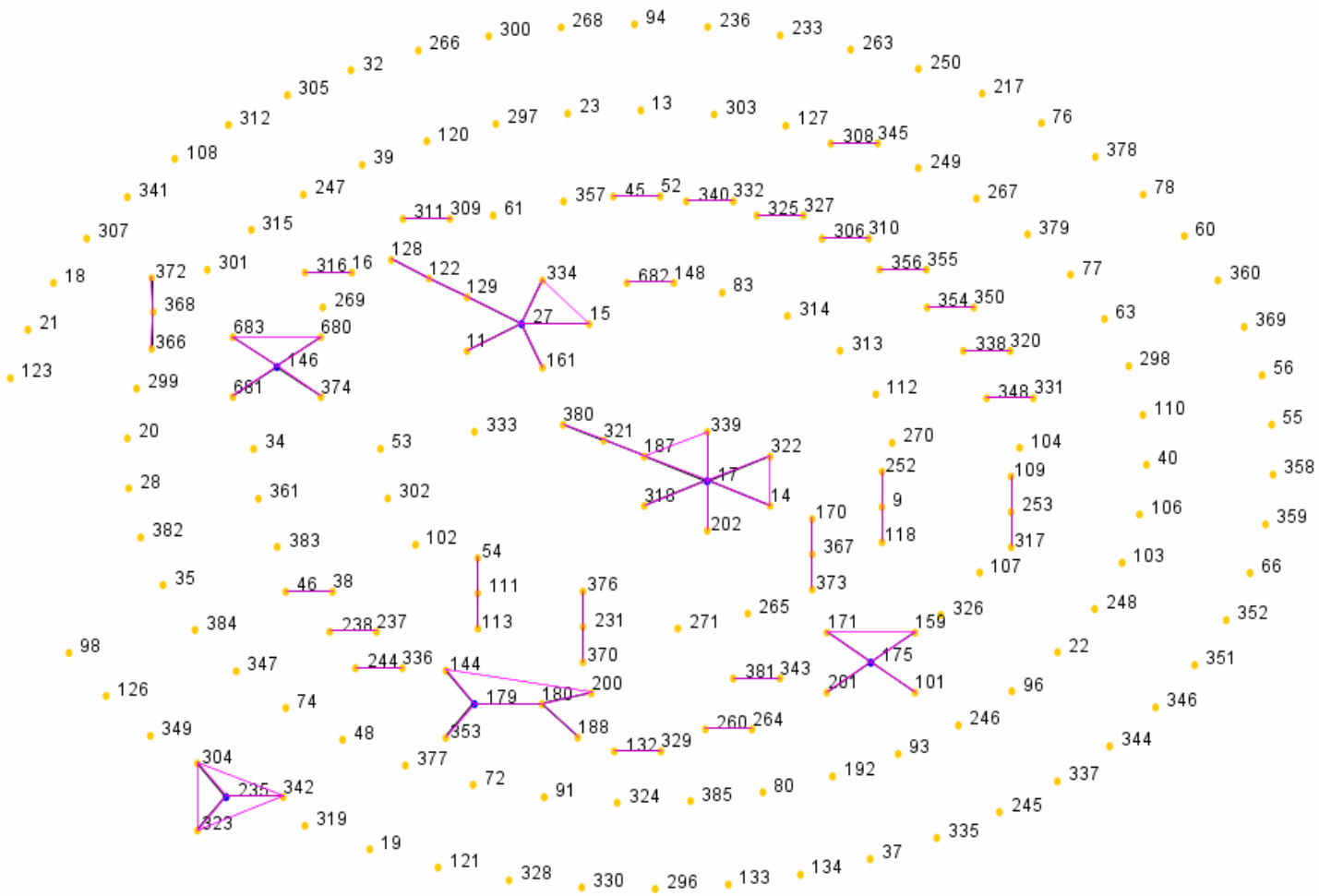


Figure 6.4 eBURST diagram showing the clusters of related *P. aeruginosa* STs and individual unlinked STs. Thirty clonal complexes are identified. SLVs are shown as pink lines and DLVs are shown in blue

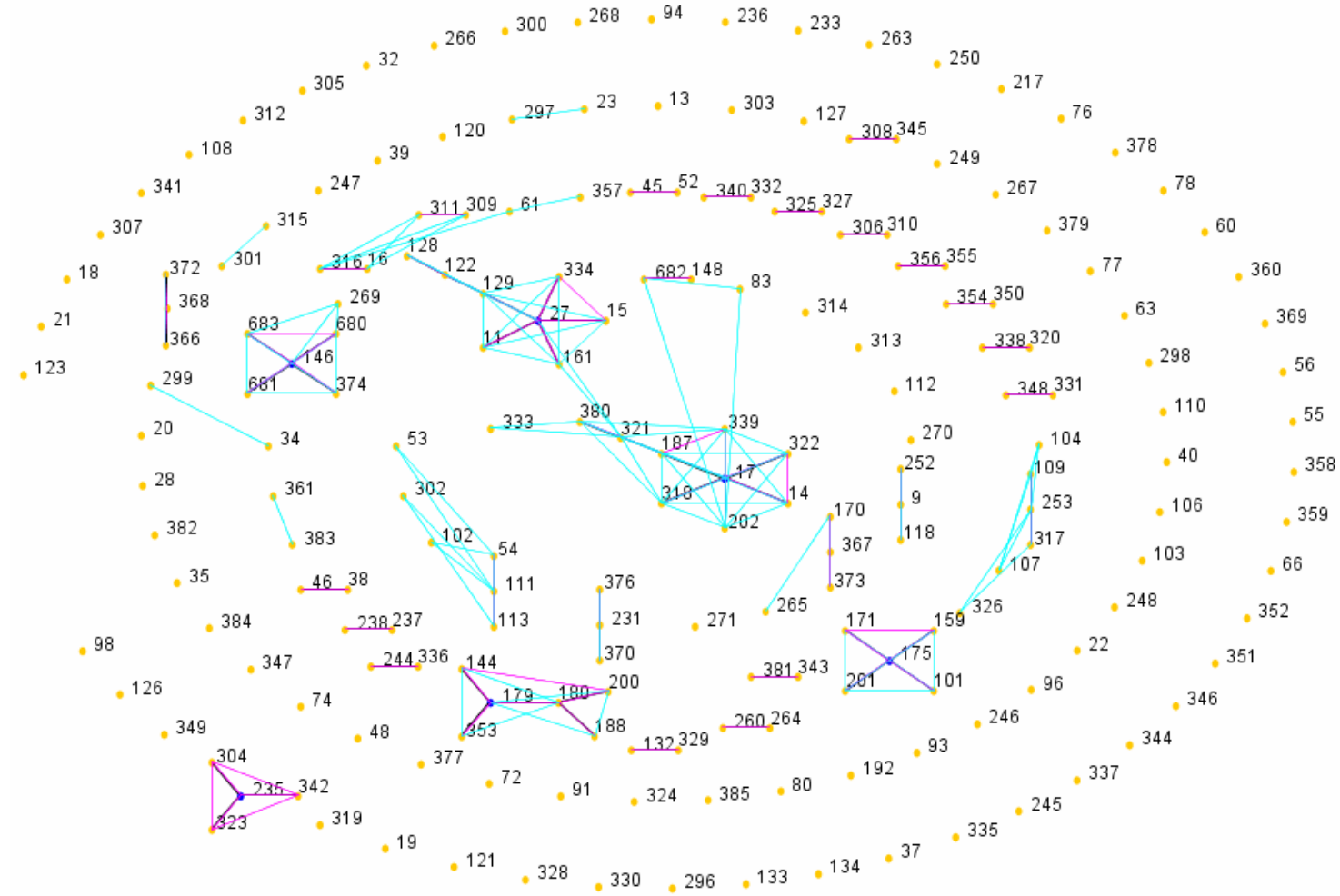
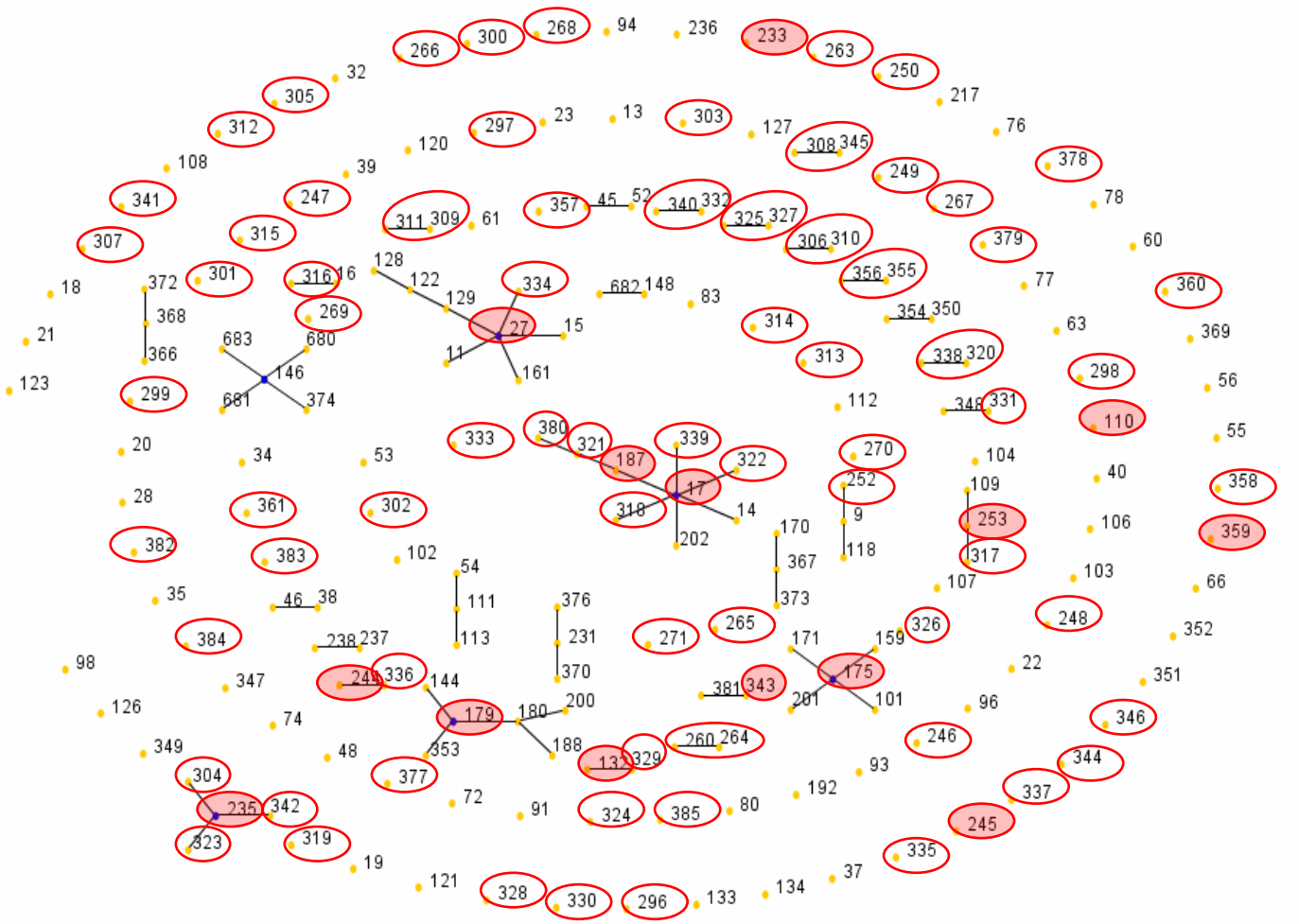


Figure 6.5 Association between sources of *P. aeruginosa* infection and eBURST groups. STs associated with eye infections are circled in red and those associated with eye infections and other clinical infections are in shaded circles.



6.3.4 Analysis of recombination

6.3.4.1 Index of Association

I_A values provides an estimate of the extent of linkage equilibrium within a population (Haubold & Hudson, 2000). The I_A value calculated using LIAN 3.5 (Section 2.16.5) was 0.1835 (P<0.001) for all 283 isolates and 0.0636 (P<0.001) when a representative of each ST was included (n=200). An I_A value not significantly greater than 0 after 1,000 computer randomisations indicates that the loci within the study population are in linkage equilibrium and therefore are freely recombining. The low I_A value supports the theory that overall, the population structure of *P. aeruginosa* is non-clonal.

6.3.4.2 Splits decomposition analysis

As discussed in section 4.3.3.3, the split decomposition algorithm generates a network structure from the MLST data which allows the degree of recombination to be estimated. Split decomposition analysis of the *P. aeruginosa* MLST data was carried out using SplitsTree v4.1 (Huson & Bryant, 2006). Figure 6.6 shows the splits dendrogram based on allelic profiles of the 200 STs. The 'splits' indicate the partition of the taxa and the length of the edge is proportional to the weight of the associated split (Huson, 1998). There appears to be no distinct clusters of strains and the abundance of networks and splits demonstrates that recombination has had a huge contribution in shaping the genetic diversity within *P. aeruginosa*.

Splits decomposition based on concatenated sequences (Figure 6.7) however, gives a slightly different picture. Although there are still numerous parallelogram structures formed between the majority of strains, three STs (ST-366, ST-368 and ST-372) did cluster away significantly from the other strains. This cluster of strains form eBURST group 11 (Section 6.3.3.2) and were also identified as a distinct cluster from the Neighbour-Joining dendrogram based on concatenated sequences (Figure 6.2). Comparisons were made between sequences of the allele from ST-366 and ST-9 (a randomly selected genotype from the main cluster).

This value was compared to the overall sequence divergence of each allele for all STs (Table 6.3). The sequence divergence of *aroE* is significantly higher between ST-9 and ST-366 (13.86% vs 1.71%) indicating that there is significant recombination at this locus. This is also the case for alleles *ppsA* and *mutL* (Table 6.3). The sequence divergence of *acsA* between ST-9 and ST-366 is also slightly higher than the overall sequence divergence (2.34% vs 1.71%). The concatenated sequence divergence between ST-9 and ST-366 was 4.6%, compared to the overall concatenated sequence divergence of 1.02%. Therefore, this group of isolates (eBURST group 11) have evolved from the main cluster of *P. aeruginosa* strains through extensive recombination. These strains were all from sputum samples taken from different CF patients in Birmingham. This observation further confirms that *P. aeruginosa* has a non-clonal population structure with emergence of epidemic clones or clonal complexes.

Figure6.6 Splits decomposition analysis based on allelic profiles of the 200 *P. aeruginosa* STs.

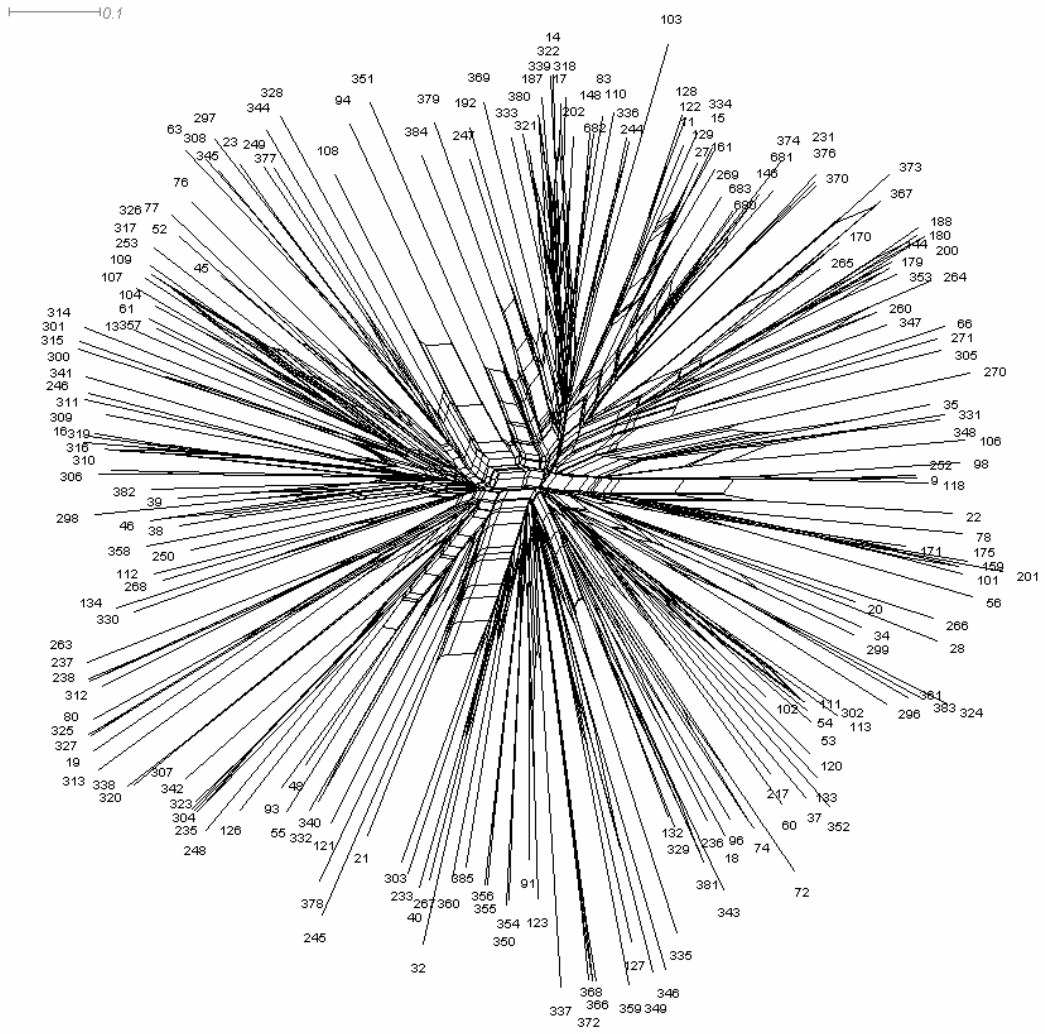


Figure 6.7 Splits decomposition analysis based on concatenated sequences of the seven housekeeping genes of the 200 *P. aeruginosa* STs.

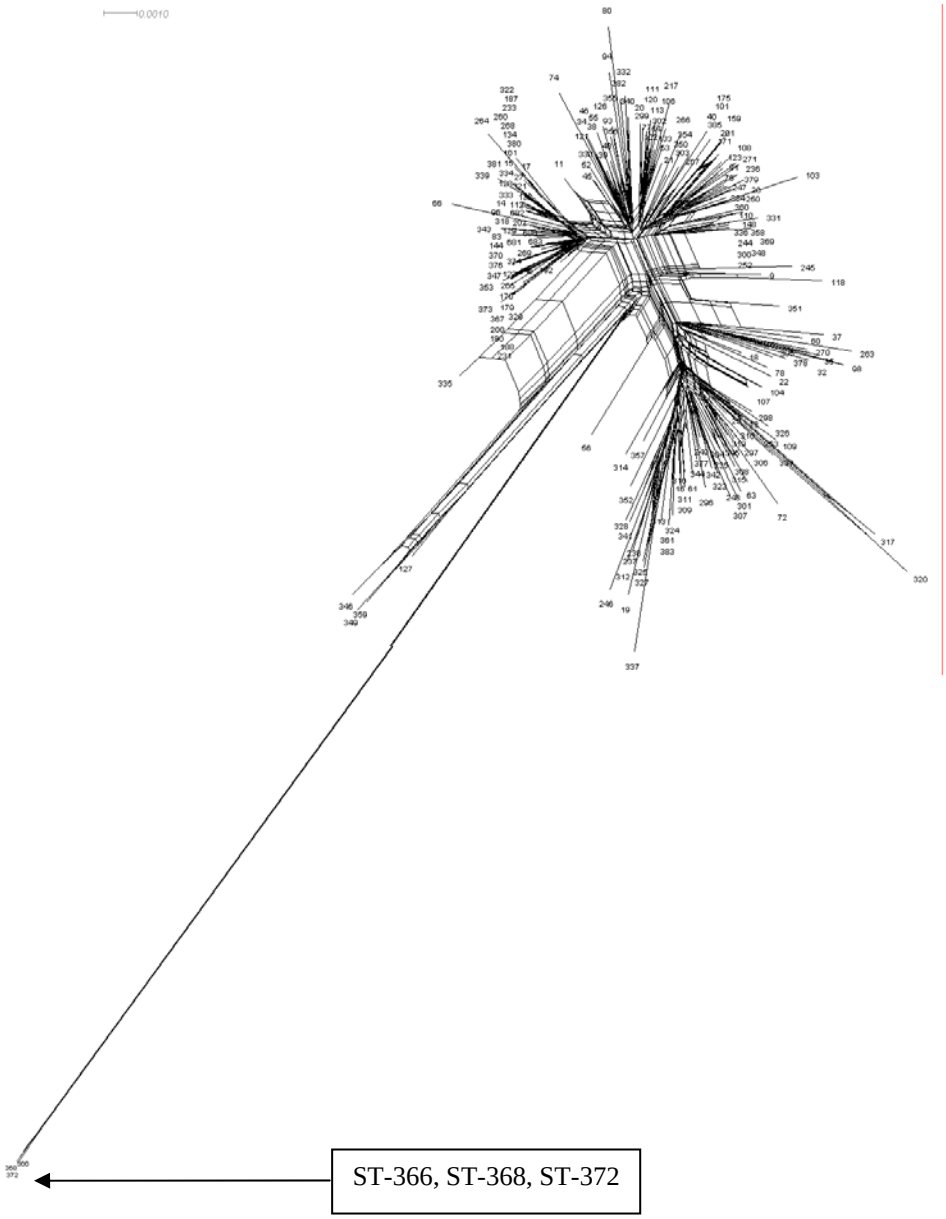


Table 6.3 Sequence divergence between *P. aeruginosa* MLST alleles. The overall percentage divergence of all 200 *P. aeruginosa* STs and the percentage divergence between ST-9 and ST-366 are shown.

Housekeeping gene	Overall sequence divergence (%)	Sequence divergence between ST-9 and ST-366 (%)
acsA	1.71	2.34
aroE	2.30	13.86
guaA	1.14	1.08
mutL	1.02	3.47
nuoD	1.28	1.94
ppsA	1.59	8.23
trpE	1.64	0.23
Concatenated	1.02	4.60

6.3.5 Type III secretion system

6.3.5.1 Determination of TTSS effector genotypes

An important characteristic of *P. aeruginosa*, which allow it to cause a diverse range of clinical infections, is its ability to disrupt the functions of epithelial surfaces. This is partly achieved via a type III secretion system (TTSS). Four toxins (ExoS, ExoT, ExoU and ExoY) are known to be produced and secreted by *P. aeruginosa*, leading to the disruption of vital eukaryotic cellular signalling pathways (Lyczak *et al.*, 2000). Studies have shown that *P. aeruginosa* isolates generally exhibit either cytotoxicity or invasion phenotypes, and these correspond to the secretion of ExoU and ExoS, respectively. Strains secreting ExoU remain extracellular promoting cells death via an acute cytotoxic process and those secreting ExoS become internalised and bring about apoptosis (Ha & Jin, 2001).

In order to determine the TTSS effector genotype of *P. aeruginosa* strains from eye infections, PCR reactions to detect *exoU* and *exoS* were carried out on 105 *P. aeruginosa* eye isolates using methods described in Section 2.14 (Table 6.4). Although this data was already available for 42 of the *P. aeruginosa* isolates from a previous study (Winstanley *et al.*, 2005), the PCR assay was repeated for these isolates, which generated the same results. Overall, more isolates possessed the *exoS*$^{-}$/*exoU*$^{+}$ genotype (n=53, 50.5%) than the *exoS*$^{+}$/*exoU*$^{-}$ genotype (n=44, 41.9%), i.e. more cytotoxic strains than invasive strains (although this is not statistically significant (p=0.27, Fisher's exact test). Three isolates possessed both *exoS* and *exoU* genes (One was a corneal scrape specimen from Liverpool [ST-305], and two were corneal scrape specimens from Beijing, both being ST-358). In five of the isolates, neither gene was detected (two ST-28 strains from London, one ST-324 from Newcastle, one ST-359 and one ST-383 both from Beijing). When a representative of each ST was used instead (to exclude bias due to presence of clones), the number of *exoS*$^{+}$/*exoU*$^{-}$ genotype (n=39, 41.1%) was marginally more than *exoS*$^{-}$/*exoU*$^{+}$ genotype (n=37, 38.9%) (p=0.88).

Analysis of only the UK isolates showed that more isolates possessed the *exoS*$^{-}$/*exoU*$^{+}$ genotype (n=45, 57%) than the *exoS*$^{+}$/*exoU*$^{-}$ genotype (n=30, 38%) ($p<0.05$). In contrast, the Chinese isolates showed more *exoS*$^{+}$/*exoU*$^{-}$ genotype (n=14, 53.8%) than *exoS*$^{-}$/*exoU*$^{+}$ genotype (n=8, 30.8%) although this was not statistically significant (p=0.16).

The current study data confirms that the *exoU* and *exoS* genes are almost mutually exclusive (Berthelot *et al.*, 2003; Feltman *et al.*, 2001; Fleiszig *et al.*, 1997; Lomholt *et al.*, 2001). When a representative of each ST was included in the analysis, the proportion of strains possessing the *exoU* genotype was similar to the number possessing the *exoS* genotype. This is comparable to a previous study by Zhu *et al.* who also found no significant difference between the number of strains carrying the *exoU* gene (n=20, 43%) and those carrying the *exoS* (n=23, 50%) gene amongst 46 *P. aeruginosa* strains cultured from eye infections (Zhu *et al.*, 2006). Lombolt *et al*. showed that amongst 61 keratitis cases, *exoU* and *exoS* genotypes were evenly distributed (Lomholt *et al.*, 2001). Interestingly, when all 145 isolates were analysed, including isolates from a variety of clinical infections and environmental sources, the strains were found to possess predominantly *exoS* genotype (n=95, 66%). Similarly, a study of 115 clinical and environmental isolates which did not include any eye infection isolates, found that the majority (n=83, 72%) possessed the *exoS* gene and only 32 (28%) possessed the *exoU* gene (Feltman *et al.*, 2001). Berthelot *et al.* also demonstrated a higher proportion of *exoS* genotype strains (n=63, 68%) than *exoU* genotype strains (n=27, 29%) amongst 92 bacteraemia isolates from six different French hospitals (Berthelot *et al.*, 2003). They also reported a statistically significant correlation between the presence or absence of the *exoU* and *exoS* genes and TTSS protein secretion (Feltman *et al.*, 2001). Therefore, PCR screening of *exoU* and *exoS* genes can give an indication of the probable secretion phenotype.

P. aeruginosa strains resistant to contact lens-disinfecting solutions have been associated with the cytotoxic phenotype (Lakkis & Fleiszig, 2001). Zhu *et al.* also identified a significant correlation between multidrug resistance, the cytotoxic phenotype and serotype O:11 (Zhu *et al.*, 2006). 23 (39%) of the 59 *P. aeruginosa*

eye isolates in the current study population were observed to harbour the *exoU* gene and belong to serotype O:11. Therefore, a significant proportion may be multidrug resistant and the management of these infections would differ from standard regimens.

6.3.5.2 Correlation between MLST, TTSS genotyping and serotyping

Where TTSS genotype data was available, isolates with the same ST were found to have the same TTSS genotype. Isolates belonging to the same eBURST group were found to have the same TTSS genotype (Table 6.1). Therefore, MLST analysis corresponded well with TTSS genotyping data. Lomholt *et al.* also found a strong correlation between *exoU* genotype and enzymes types based on MLEE data of 145 *P. aeruginosa* strains (Lomholt *et al.*, 2001).

Of the 283 *P. aeruginosa* isolates in the study population, 76 isolates had serotype data available, either from the *P. aeruginosa* MLST website or from previous studies (http://pubmlst.org/paeruginosa) (Curran *et al.*, 2004; Winstanley *et al.*, 2005). Serotype data for 63 of the eye isolates were available from a previous study (Winstanley *et al.*, 2005). 59 of the 63 isolates were assigned to seven different O-serotype groups: O:1, O:2, O:4, O:6, O:8, O:10 and O:11. The remaining four isolates gave agglutination with the polyvalent PME serum but failed to agglutinate with monovalent sera (Winstanley *et al.*, 2005). Serotypes O:11 and O:1 were the most commonly found serotypes (42.4% and 25.4%, respectively) (Table 6.5). There was good correlation between MLST data and serotype data. Of the 59 isolates which were serotyped, isolates with same ST also had the same serotype, with one exception (Table 6.1). Isolate PSA021 and PSA024 were both ST-309 strains and belong to eBURST group 30. However, isolate PSA021 was serotype O:1 whereas isolate PSA024 was serotype O:11.

There was also good correlation between eBURST groups and serotype data. The majority of strains belonging to the same eBURST group also had the same serotype. The only exception was eBURST group 7 where two isolates from this group were serotype O:10 and one isolate was serotype O:11. Strains with the

same serotype were not exclusively found in one eBURST group. Serotype O:1 strains were found in eBURST groups 1, 2 and 30. Serotype O:6 strains were found in eBURST groups 3 and 27. Serotype O:11 strains were found in eBURST groups 6, 7, 13, 14, 26 and 30. This suggests that although MLST data generally correlates well with serotyping data, MLST analysis is more discriminative than serotyping for *P. aeruginosa*.

Previously published serotype data available for 59 of the *P. aeruginosa* eye infection isolates within the current study population (Winstanley *et al.*, 2005) showed that strains having the *exoU* genotype were associated with serotypes O:8, O:10 and O:11 (Table 6.5). *ExoS* genotype strains were associated with serotypes O:2, O:4 and O:6. Serotype O:1 strains were associate with both genotypes although more so with the *exoS* than the *exoU* genotype.

Berthelot *et al.* observed a statistically significant correlation between O serotype and TTSS genotype (Berthelot *et al.*, 2003). In their study of *P. aeruginosa* bacteraemic isolates, all strains belonging to serotypes O:1, O:10 or O:11 (n=24), except for one, harboured the *exoU* gene and all strains belonging to serotypes O:3, O:4, O:6, O:12, or O:16 (n=64), except one, harboured the *exoS* gene (Berthelot *et al.*, 2003). In contrast, lung infections strains belonging to serotype O:1 more frequently harbour the *exoS* gene (Faure *et al.*, 2003). Zhu *et al.* observed that eye infection strains carrying the *exoS* gene were more frequently associated with serotypes O:1, O:3, O:6 and O:16; and those carrying the *exoU* gene were associated with serotypes O:7, O:8 and O:11 (Zhu *et al.*, 2006). It has been postulated that the relationship between serotype and TTSS may be brought about by genomic linkages (Zhu *et al.*, 2006). The *exoU* gene is located adjacent to an insertion-like element that demonstrates homology with an insertion-like sequence that determines the serotype of *P. aeruginosa* (Raymond *et al.*, 2002).

Table 6.4 TTSS genotype of 105 *P. aeruginosa* isolates from eye infections. Isolates from China are compared to those from the UK

exoS	*exoU*	No. of UK isolates (%)	No. of Chinese isolates (%)	Total no. of isolates (%)
+	+	1 (1.3%)	2 (7.7%)	3 (2.9%)
-	-	3 (3.8%)	2 (7.7%)	5 (4.8%)
+	-	30 (38%)	14 (53.8%)	44 (41.9%)
-	+	45 (57%)	8 (30.8%)	53 (50.5%)
	Total	79	26	105

Table 6.5 TTSS genotype and serotype of 59 *P. aeruginosa* isolates from eye infections. Serotype data from Winstanley *et al.* (Winstanley *et al.*, 2005).

exoS	*exoU*	Serotype						
		O:1	O:2	O:4	O:6	O:8	O:10	O:11
+	+	0	0	1	0	0	0	0
-	-	0	0	0	0	1	0	0
+	-	9	3	2	5	0	0	2
-	+	6	0	1	0	2	4	23
	Total	15 (25.4%)	3 (5.1%)	4 (6.8%)	5 (8.5%)	3 (5.1%)	4 (6.8%)	25 (42.4%)

6.4 Future work

Serotype and TTSS data available for a selection of *P. aeruginosa* eye infection isolates has generally correlated well with MLST data. However, this association would need to be verified by serotyping and determining the TTSS genotype of the remaining *P. aeruginosa* infections isolates. The presence of the *exoU* or *exoS* gene does not necessarily equate to protein expression and therefore the production of these exotoxins should be determined to confirm the virulence of these strains. As previously suggested by Zhu *et al.*, *P. aeruginosa* strains possessing the cytotoxic phenotype may be associated with multidrug resistance (Zhu *et al.*, 2006). It would therefore be valuable to determine whether this observation is also seen in our collection of *P. aeruginosa* strains, especially those from eye infections. If this correlation is observed, then it would be useful to determine the TTSS genotype of *P. aeruginosa* cultured from infections and instigate the appropriate management.

6.5 Conclusions

A selection of 283 *P. aeruginosa* isolates including 117 from eye infections were analysed by MLST. 200 individual STs were designated to the 283 isolates, with ST-235 being the most frequently observed ST (n=30). Phylogenetic analysis based on allelic profiles demonstrated that *P. aeruginosa* has a non-clonal population structure. In addition, phylogenetic analysis of the concatenated sequences of the seven housekeeping genes identified the existence of an epidemic clonal complex. Analysis of recombination using the splits decomposition program and the I_A value supports the finding that the population structure of *P. aeruginosa* is non-clonal and that recombination has had an important role in determining its evolution.

eBURST analysis of the MLST data identified 30 eBURST groups. However, 109 of the 283 isolates did not belong to an eBURST group. There appears to be no association between individual clones or eBURST groups and the clinical source of infection or geographical distribution of these strains.

The presence or absence of *exoU* and *exoS* genes in 105 *P. aeruginosa* isolates from eye infections was determined by PCR amplification. Data confirmed that the *exoU* and *exoS* genes are generally mutually exclusive (three isolates harboured both genes and five isolates possessed neither genes). There was no statistically significant difference between the proportion of isolates possessing the *exoU* gene and those possessing the *exoS* gene. The distribution of TTSS genotypes of the eye infection strains in the current study appear to confirm previous studies of eye infection isolates but differ from isolates from other infection sites.

MLST data corresponded well with TTSS genotype. Isolates with the same ST were found to have the same TTSS genotype and isolates belonging to the same eBURST group were also found to possess the same TTSS genotype. With one exception, MLST data and serotype data for 63 of the isolates were found to correlate i.e. isolates from the same eBURST group had the same serotype. The reverse was not true, i.e. isolates with the same serotype could be found in several different eBURST groups. This further validates the higher discriminatory power of MLST compared to phenotyping methods.

In conclusion, MLST analysis of a selection of 283 *P. aeruginosa* shows that this organism has a non-clonal epidemic population structure. Clones or clonal complexes were not found to be associated with particular clinical manifestation or geographical distribution. Further study to determine any association between antibiotic resistance and MLST data would be useful for clinical management of *P. aeruginosa* infection.

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CHAPTER 7 GENERAL DISCUSSION

7.1 Main objectives

The main objectives of the project were (1) to identify the common microorganisms that cause microbial keratitis and endophthalmitis in a large tertiary eye centre in the West Midlands, UK (2) to develop an MLST scheme for *P. acnes*, (3) to study the population biology of *P. acnes* and *P. aeruginosa* cultured from eye infections using MLST, determine whether specific clones were associated with eye infections and (4) to develop a PCR based rapid diagnostic test to detect *P. acnes* from ocular samples.

7.2 Microbial keratitis study

Microbial keratitis is infection of the cornea, the clear transparent structure at the front of the eye. It is an ophthalmic emergency and the visual prognosis of the patient depends on prompt identification of the causative organism and initiation of appropriate topical antibiotic therapy. The causative microorganism is partly dependent on the geographical location and climatic factors. Identification of the organism in most hospitals is dependent on culturing the organism from corneal scrape specimens using conventional techniques. Culture positive rates using these techniques vary widely from 50% to 86% (Alexandrakis *et al.*, 2000; Bennett *et al.*, 1998; Bourcier *et al.*, 2003; Fong *et al.*, 2007; Neumann & Sjostrand, 1993; Schaefer *et al.*, 2001; Wong *et al.*, 2003). The low culture positive rates lead to most cases being treated with broad spectrum antibiotics which the infecting organisms may not be sensitive to. Poor visual outcome and increased antimicrobial resistance are unfortunately the potential consequences.

The current retrospective study looked at patient records for 303 cases of suspected microbial keratitis at the Birmingham and Midland Eye Centre over a 29-month period. Contact lens wear was found to be the most common predisposing factor for the development of microbial keratitis, followed by ocular surgery, previous microbial keratitis and ocular trauma. The culture positive rate

was found to be under 40% and the mean average time taken to obtain a culture positive result was 5.3 days. This low culture positive rate could be a consequence of sampling sterile ulcers, small amounts of sample, poor sampling technique, use of topical anaesthetic agents before sampling, possible delay in transporting the sample to the laboratory and poor sensitivity of conventional culture methods. Prior use of antibiotics before obtaining the specimen was not found to significantly reduce the culture positive rate. *Pseudomonas* spp. were the most frequently cultured organisms and the proportion of *Pseudomonas* spp. infection was even higher in contact lens-related cases. Low culture positive rate and prolonged time to culture result, indicates a more rapid and sensitive molecular diagnostic test, such as PCR, is required for optimal management of this sight threatening eye infection.

7.3 *P. acnes* MLST

P. acnes belongs to the human cutaneous propionibacteria and usually lives on the skin as a harmless commensal (Brook & Frazier, 1991). The potential for this organism to cause serious clinical infections is being increasingly reported. It is the most common organism found in chronic postoperative endophthalmitis (Aldave *et al.*, 1999), a dreaded complication of cataract surgery and has also been reported to cause keratitis (Jones & Robinson, 1977; Perry *et al.*, 1982; Wong *et al.*, 2003; Zaidman, 1992). *P. acnes* was originally classified into two phenotypes, known as types I and II, based on serological agglutination tests and cell wall sugar analysis (Johnson & Cummins, 1972). Analysis of the *P. acnes recA* gene and *tly* gene sequences found that types I and II represent distinct phylogenetic lineages and that type I strains could be subdivided into groups IA and IB (Cohen *et al.*, 2005; McDowell *et al.*, 2005). Recently, a novel lineage of strains, known as type III, has been described (McDowell *et al.*, 2008).

Previously described genetic fingerprinting methods to type *P. acnes*, including RAPD (Kunishima *et al.*, 2001; Perry *et al.*, 2003) and PFGE (Cohen *et al.*, 2005; Oprica & Nord, 2005; Ting *et al.*, 1999), suffer from variability, poor reproducibility and lack of inter-laboratory transferability. Typing methods based

on the analysis of DNA sequences rather than genome organisation or restriction fragments is much more robust, reliable and transferable between laboratories. MLST, developed in 1998, is a nucleotide sequence-based method enabling comparative characterisation of bacterial isolates usually based on seven housekeeping genes (Maiden *et al.*, 1998). MLST has the advantage of being a reproducible and scalable typing system that is portable between laboratories (Urwin & Maiden, 2003). An MLST scheme was set up for *P. acnes* to characterise the population structure of a diverse collection of clinical *P. acnes* isolates and to determine the level of recombination contributing to its diversity. MLST analysis was also done to ascertain whether certain genotypes were associated with particular clinical diseases or geographical locations.

Seven *P. acnes* housekeeping genes: *aroE, atpD, gmk, guaA, lepA, recA* and *sod A* were selected to be examined in the MLST scheme. Although *atpD, gmk* and *aroE* were located relatively close together around the *P. acnes* chromosome, analysis of recombination at these genes demonstrated that a single recombinogenic event was unlikely to have involved two or even three of these genes simultaneously. The ratio of non-synonymous (d_N) to synonymous (d_S) changes calculated for each of the seven genes showed that there was no strong selective pressure operating on these genes and therefore validated their suitability for inclusion in the MLST scheme.

A total of 125 *P. acnes* isolates, including two reference strains and isolates cultured from a variety of clinical infections were analysed. 38 different STs were assigned to these isolates, with nearly half of the isolates being represented by ST-6. The frequent occurrence of ST-6 raises the question as to whether this genotype is just more common, even in healthy individuals or whether it is a more virulent strain with greater capacity to cause disease. Further work using MLST to type *P. acnes* isolates from healthy, carrier skin and analysis of virulence genes would be useful to answer this question.

Phylogenetic analyses of the MLST data, either based on concatenated sequences or allelic profiles, were carried out using a variety of methods. eBURST analysis grouped the 38 STs into seven clonal complexes based on isolates having identical alleles at six or more of the loci. 3 STs did not fall into any eBURST group. ST-6 was identified as the founding genotype, having the largest number of SLVs. Phylogenetic dendrogram representation of the concatenated sequences of the 38 STs correlated with eBURST analysis to some extent although there were differences in designation of isolates into groups. This is not unexpected as analysis based on allelic profiles does not take into consideration of the number of nucleotide substitutions occurring at a gene. Comparison of *P. acnes* isolates does not show an association between a particular ST or clonal complex with certain clinical infections or geographical locations. Some STs were found in numerous different countries, cultured from a variety of clinical sites. A variety of methods used to determine the level of recombination occurring within the study population suggest that frequent recombinogenic events occur within, but not between the *P. acnes* phylogenetic subgroups.

7.4 Comparisons of *P. acnes* MLST with 16S rRNA gene and *recA* gene analyses

Validation of the *P. acnes* MLST scheme was achieved by comparing 16S rRNA gene and the complete *recA* gene sequencing data, IFM analysis and RAPD analysis. 16S rRNA gene analysis of eleven of the 125 isolates included in the MLST scheme had previously been published (McDowell *et al.*, 2005; McDowell *et al.*, 2008). The selection of isolates included representatives of type IA, IB, II and III isolates. The eleven isolates were subdivided into three main lineages corresponding to types I, II and III strains, but could not distinguish between types IA and IB. The 16S rRNA gene analysis data was in concordance with the eBURST group clustering generated by MLST data and thus validated the MLST scheme. However, MLST provides much better discrimination as shown by isolates with the same 16S rRNA gene sequences belonging to different STs.

P. acnes has previously been shown to be composed of phylogenetically distinct clusters or lineages based on sequence analysis of the complete *recA* gene (McDowell *et al.*, 2005; McDowell *et al.*, 2008). It was therefore important to determine whether these phylogenetic types corresponded to MLST clusters. Of the 125 isolates used in the MLST scheme, the *recA* phylotype of 58 isolates was determined based on complete *recA* gene sequencing. The distinct groups or lineages based on *recA* sequence analysis (IA, IB, II & III) were mostly congruent with the clonal complexes resolved using MLST. eBURST analysis revealed that groups 1, 2 and 4 consisted of type IA strains and group 3 consisted of type IB strains. Type II strains were found to form two closely related clonal complexes (eBURST groups 5 and 6), while strains belonging to type III formed a single clonal complex (eBURST group 7).

While phylotype data for *P. acnes* isolates based on complete *recA* sequences correlated well with MLST data, there were a few exceptions. Three isolates formed a highly distinct cluster (group 4) based on eBURST analysis, but one of these strains (PV66) was classified as a type IA organism based on *recA* and *tly* gene sequencing (McDowell *et al.*, 2005). Multiple methods of analysing MLST data have demonstrated that these strains form a distinct cluster away from the other *P. acnes* strains. Therefore, this novel cluster does not belong to any of the four previously confirmed phenotypes (IA, IB, II and III). Two further isolates were found to have conflicting MLST and *recA* gene analysis data. Isolates Asn7 and JMK9 clustered in eBURST group 1 with other type IA strains. However, based on complete *recA* sequence, they were characteristic of type IB organisms. This discrepancy may have come about from a recombinational event occurring at the *recA* gene between type IA and IB strains, resulting in a type IA strain acquiring the segment distinguishing it as a type IB strain. Therefore, analysis of multiple housekeeping genes is more reliable in determining phylogenetic relationships, as sequence data from each gene is buffered against potential recombinogenic events that might occur at other loci.

7.5 Comparison of *P. acnes* MLST with IFM analysis

IFM using type-specific based MAbs generated by Andrew McDowell was carried out on 76 *P. acnes* isolates. Previous IFM analysis have found that MAbs QUBPa1 and QUBPa2 labelled *P. acnes* type IA and II, respectively, and QUBPa3 reacted with all *P. acnes* strains. No labelling with QUBPa1 or QUBPa2 was observed for the type III isolates.

A selection of *P. acnes* isolates from eBURST groups 1 and 2 showed that they were all type IA strains based on IFM analysis. All eBURST groups 5 and 6 strains were identified as type II strains and all eBURST group 7 strains were identified as type III strains. IFM analysis of type IB (eBURST group 3) strains showed variable reaction to QUBPa1 and QUBPa2. Some strains showed absence of reaction with QUBPa1 and weak–to-no reaction with the MAb QUBPa2, whilst other strains were found to react with QUBPa1, but not with QUBPa2. This illustrates that, while useful, immunological typing with MAbs is unpredictable due to the emergence of antigenic variants that do not react.

In the study population, a novel cluster (eBUSRT group 4) comprising three isolates was identified. All isolates within this group displayed dual labelling with MAbs that normally react with either types IA (QUBPa1) or II (QUBPa2), further highlighting their distinct nature.

7.6 Comparison of *P. acnes* MLST with RAPD analysis

RAPD typing using RAPD-208 primer was carried out on 92 *P. acnes* as well as one *P. avidum*, one *P. propionicus* and one *P. granulosum* isolate. Although *P. granulosum* gave markedly different profiles with less than 40% similarity to those of the other species, *P. propionicus* and *P. avidum* appear to be more closely related to *P. acnes* at 70% and 73% similarities, respectively. Type II isolates appear to have clustered together. However, this group also clustered together with a type III and a type IA strain. Similarly, type III strains have clustered together but along with a type IA and a type IB strain. Type IA and IB strains

were distributed throughout the dendrogram, as there are too few band differences for the two types to be distinguished by RAPD analysis. MLST is a far superior genotyping technique at differentiating closely related strains than RAPD typing.

7.7 *P. acnes* molecular diagnostics

Study of the population biology of *P. acnes* using MLST identified distinct clonal complexes but found no distinction between strains from different clinical diseases or geographical locations. Therefore, a PCR assay for the detection of *P. acnes* from ocular samples does not need to target a specific subgroup, so any of the seven housekeeping genes used in the MLST scheme were potential targets for PCR assays. Real-time PCR, developed by Higuchi *et al.* (Higuchi *et al.*, 1992; Higuchi *et al.*, 1993) has the advantage of being more rapid than standard PCR protocols and enables real-time quantification of copy numbers.

The real-time PCR assay developed here for the detection of the *P. acnes gmk* gene was applied to the four cutaneous *Propionibacterium* spp. (*P. acnes, P. avidum, P. granulosum* and *P. propionicum*) as well as other common eye infection organisms ((*P. aeruginosa, S. aureus*, coagulase negative *Staphylococcus* and *S. pneumoniae*) and was found to be specific for *P. acnes*. The assay was repeated on serial dilutions of the *P. acnes* type strain NCTC 737 (0.1pg/ml to 1ng/ml). Melt-curve analysis of the real-time PCR reactions showed that the limit of detection of the assay was 0.1pg/ml of DNA, although a reliable level of detection would be 1pg/ml. The total assay time, excluding DNA extraction, was completed within 2 hours 30 minutes. In order to avoid the routine use of the phenol/chloroform DNA extraction method in the diagnostic setting, three different DNA extraction protocols were compared. The Chelex DNA extraction method and the DNAmite kit appeared to yield better genomic DNA from *P. acnes* than the HiPure Viral Nucleic Acid extraction kit.

The current *P. acnes* real-time PCR assay needs to be applied to sterile aqueous or vitreous samples spiked with known concentrations of *P. acnes* organisms to further test its sensitivity and to optimise the various DNA extraction methods.

The assay can then be applied to aqueous and vitreous samples from suspected endophthalmitis and keratitis cases in the clinical setting.

7.8 *P. aeruginosa* MLST

P. aeruginosa is an opportunistic pathogen capable of infecting a variety of hosts (Lavenir *et al.*, 2007; Pukatzki *et al.*, 2002; Rahme *et al.*, 2000). It is capable of causing severe keratitis, especially in contact lens wearers, and endophthalmitis (Mondino *et al.*, 1986; Rattanatam *et al.*, 2001; Wilhelmus, 1996). It can also lead to numerous other serious infections such as septicaemia, pneumonia, endocarditis and otitis (Eifrig *et al.*, 2003; Jackson *et al.*, 2003; Kashkouli *et al.*, 2007; Kielhofner *et al.*, 1992).

Previous typing studies have reported that *P. aeruginosa* has a non-clonal population structure (Denamur *et al.*, 1993; Dinesh *et al.*, 2003; Lomholt *et al.*, 2001; Martin *et al.*, 1999; Morales *et al.*, 2004; Picard *et al.*, 1994; Pirnay *et al.*, 2002; Romling *et al.*, 1994; Ruimy *et al.*, 2001). Kiewitz *et al.* analysed six sequences of 19 environmental and clinical isolates and demonstrated that the frequency of recombination among different *P. aeruginosa* genotypes was high (Kiewitz & Tummler, 2000). Curran *et al.* developed a *P. aeruginosa* MLST scheme to characterise a large collection of clinical and environmental isolates (Curran *et al.*, 2004). The 143 isolates investigated were assigned to 139 different STs. The I_A value for the individual STs was 0.17, supporting the theory that *P. aeruginosa* has a non-clonal population structure.

In this study, 117 *P. aeruginosa* isolates from eye infections in the UK and China were analysed by MLST. MLST data on a further 166 *P. aeruginosa* isolates from other clinical infections, available on the *P. aeruginosa* MLST website, were also included in the analyses. 200 individual STs were designated to all 283 isolates, with ST-235 being the most frequently observed ST (n=30, 15%). eBURST analysis of the MLST data identified 30 eBURST groups. However, 109 of the 283 isolates did not belong to an eBURST group. No association was found

between STs or eBURST groups and the clinical source of infection or geographical distribution. Phylogenetic analysis of the concatenated sequences of the seven housekeeping genes identified the existence of an epidemic clonal complex. Analysis of recombination using the splits decomposition program and the I_A value supports the finding that the population structure of *P. aeruginosa* is non-clonal and that recombination has had an important role in determining its evolution.

7.9 *P. aeruginosa exoU* and *exoS* genotyping

The *P. aeruginosa* TTSS enables production and secretion of virulence factors directly into the cytoplasm of host cells (Ajayi *et al.*, 2003). The effector proteins (secreted toxins) comprise of ExoS, ExoT, ExoU and ExoY (Finck-Barbancon *et al.*, 1997; Frank, 1997; Frithz-Lindsten *et al.*, 1997; Hauser *et al.*, 1998; Yahr *et al.*, 1996; Yahr *et al.*, 1998). Although *exoY* and *exoT* are present in nearly all *P. aeruginosa* isolates, the majority possess either *exoS* or *exoU*, not both (Feltman *et al.*, 2001; Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998). Invasive *P. aeruginosa* strains tend to express ExoS (Fleiszig *et al.*, 1997), whereas non-invasive or cytotoxic strains express ExoU (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998). The TTSS has been identified in nearly all clinical and environmental isolates but isolates from distinct disease sites appear to differ in their effector genotype (Feltman *et al.*, 2001).

In order to determine the TTSS effector genotype of *P. aeruginosa* strains from eye infections, PCR reactions to detect *exoU* and *exoS* were carried out on 105 *P. aeruginosa* eye isolates. The findings confirm that the *exoU* and *exoS* genes are almost mutually exclusive (Berthelot *et al.*, 2003; Feltman *et al.*, 2001; Fleiszig *et al.*, 1997; Lomholt *et al.*, 2001). When a representative of each ST was included in the analysis, the number of *exoU* genotype strains was similar to the number the *exoS* genotype strains. This correlates with previous studies which found no significant difference between the number of strains carrying the *exoU* gene and those carrying the *exoS* gene amongst *P. aeruginosa* strains from eye infections (Lomholt *et al.*, 2001; Zhu *et al.*, 2006). In contrast, studies of clinical and

environmental isolates which did not include eye infection isolates found that the majority of strains possessed the *exoS* gene (Berthelot *et al.*, 2003; Feltman *et al.*, 2001). MLST analysis corresponded well with TTSS genotyping data as isolates with the same ST were found to have the same TTSS genotype.

Serotype data of the eye isolates were available from a previous study (Winstanley *et al.*, 2005). 59 isolates were assigned to seven different O-serotype groups: O:1, O:2, O:4, O:6, O:8, O:10 and O:11 (Winstanley *et al.*, 2005). O:11 and O:1 were the most commonly found serotypes (42.4% and 25.4%, respectively). There was good correlation between MLST data and serotype data as isolates with same ST also had the same serotype, with one exception: two isolates with the same ST were found to have different serotypes. Strains with the same serotype were not exclusively found in one eBURST group. This suggests that although MLST data generally correlates well with serotyping data, MLST analysis is more discriminative than serotyping for *P. aeruginosa*.

7.10 Conclusions

In conclusion, MLST has been used to study the population biology of *P. acnes* and *P. aeruginos*a, two organisms with potential to cause sight threatening ocular infections. The novel *P. acnes* MLST scheme reported here has proved a valuable tool for high-level strain identification and epidemiological investigation of *P. acnes*. The method has confirmed the previously described phylogenetic groups of the organism and identified an additional novel cluster. There appears to be no association between these distinct groups and clinical manifestation of the infection, nor the geographical location of these strains. These findings have allowed the development of a real-time diagnostic PCR assay targeting the *gmk* gene. The assay has been proven to be specific for *P. acnes* and also highly sensitive. MLST analysis confirmed that *P. aeruginosa* has a non-clonal population structure. As in the case of *P. acnes*, specific clones or clonal complexes were not associated with eye infection isolates or geographical origin of the isolates.

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Chapter 1: Introduction

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