

Analysis of the *Mycoplasma hominis* hsp70 gene
and development of a PCR ELISA assay

by

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B.Sc.(Hons) (Natal)

Submitted in partial fulfilment of the
requirements for the degree of
Master of Science
in the
Department of Biochemistry
University of Natal
Pietermaritzburg

May 1998

PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg, from February 1996 to January 1998, under the joint supervision of Professor John Hastings and Dr Romilla Maharaj.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.



Nicollette Shearer

14 May, 1998

ABSTRACT

Mycoplasmas conform most closely with the theoretical concept of 'minimum cells', existing as the smallest, free-living organisms capable of self-replication. They survive as parasites of plants, insects, animals or humans, with the most common human colonising species being *Mycoplasma hominis*. *M. hominis* has been characterised as a human pathogen responsible for a variety of infections, which pose a significant threat particularly to immunocompromised patients and neonates. However little has been elucidated about the cell physiology and molecular structure of this organism. Of interest to this study were the investigation of the heat shock response of *M. hominis* and the diagnostic assays used for its detection.

The heat shock response is a ubiquitous physiological feature of all organisms and displays unprecedented conservation. This phenomenon is particularly evident in the 70 kDa family of heat shock proteins (hsp70) which exhibits a high degree of homology between different species. The hsp70 gene from *M. hominis* was cloned and preliminary partial sequencing indicated the similarity with other hsp70 homologs. The regulation of hsp70 expression at the transcriptional and translational levels was investigated. The level of hsp70 mRNA was found to increase correspondingly in response to heat shock, more visibly than the level of hsp70 protein. However immunochemical studies of the *M. hominis* hsp70 translation product demonstrated further the homology with other species.

To facilitate rapid diagnosis of *M. hominis* infections, a PCR ELISA diagnostic assay was developed and optimised. The amplification of a conserved region of the *M. hominis* 16S rRNA gene was linked to subsequent hybridisation to an appropriate capture probe in a microtiter plate format. The sensitivity of the assay was comparable to other molecular assays although the PCR ELISA produces more rapid results and is less labour intensive.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to the following people for their contribution to this thesis:

Professor John Hastings, for his support and willingness to adopt an orphaned project.

Dr Romilla Maharaj, for initiating this research study.

The biochemistry department academic staff, Professor Clive Dennison, Dr Edith Elliot, Dr Theresa Coetzer, Dr Trevor Anderson and Dr André van der Hoven, for their support and encouragement.

Lesley Brown, for expert efficiency and invaluable contribution to the running of this project; Ron Berry, Jenny Schwartz, Melody Webber and John Geysler, for their assistance throughout this study.

Ramola Chauhan, for her advice and technical assistance with sequencing.

Nic Scholfield, for her buoyant enthusiasm, ingenious suggestions and brain-storming, and Rory Morty, for being a source of constant entertainment and encouragement.

Brendon Price, for his endless advice, patience and support during this study, and Chè Pillay, for my introduction to heat shock studies and his encouragement.

Fellow postgraduate students in the biochemistry and genetics departments, for providing an enjoyable working environment, in particular Frieda Dehrmann, Thembile Dalasile, Phillip Fortgens, Linda Troeberg, Jonathan Edwards, Adam Dawe, Lizette Moolman and Thunicia Moodley.

The Foundation for Research Development and the University of Natal, for financial assistance.

Andrew Winter, Heather Atkinson and Gavin Schafer, for their friendship.

Kerry Taylor, for preserving my sanity throughout my university career and for dappled sunshine and tulips always.

My sister, for her encouragement and support, particularly during the writing of this thesis.

My mother and father, for their unending moral and financial support, understanding and encouragement throughout my university career.

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ABBREVIATIONS

A ₆₀₀	absorbance at 600 nm
ABTS	2,2'-azino-di-(3-ethyl)-benzthiozoline sulfonic acid
ADP	adenosine diphosphate
AIDS	acquired immune deficiency syndrome
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indoyl-phosphate
Bis	N,N'-methylenebisacrylamide
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
CIAP	calf intestinal alkaline phosphatase
2D	two dimensional
ddH ₂ O	distilled deionised water
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
dist. H ₂ O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ddNTP	dideoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
8	relative centrifugal force
hsc	heat shock cognate protein
hsp	heat shock protein
hsp70	70 kDa heat shock protein
hsp60	60 kDa heat shock protein
IgG	immunoglobulin G
IPTG	isopropyl-(3-D-thio-galactopyranoside
kb	kilobase pairs

kDa	kilodalton
MAb	monoclonal antibody
MHC	major histocompatibility complex
min	minute
MOPS	3-(N-morpholine)-propanesulfonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
NBT	nitroblue tetrazolium chloride
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PID	pelvic inflammatory disease
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
S	sedimentation coefficient
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tricine	N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	transfer ribonucleic acid
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indoyl-P-D-thio-galactopyranoside

CHAPTER 1

GENERAL INTRODUCTION

1.1 Mycoplasmas

Mycoplasmas are the smallest and simplest self-replicating micro-organisms, intermediate in size between bacteria and viruses. They conform most closely with the concept of 'minimum cells' as their structural components, metabolic pathways, mechanisms of protein synthesis and encoding genes are present at an essential minimum (Razin, 1992). Members of the *Mycoplasma* genus have the smallest genomes recorded in prokaryotes, ranging from about 600 to 800 kb (Herrmann, 1992). This genome size is approximately one-sixth that of *Escherichia coli* K-12, which has recently been determined as 4639 kb (Blattner *et al*, 1997). The complete sequencing of the *Mycoplasma genitalium* and *Mycoplasma pneumoniae* genomes have revealed coding capacities for 479 and 677 proteins respectively (Himmelreich *et al.*, 1997), compared to the 4288 coding genes of *E. coli* K-12 (Blattner *et al*, 1997).

Mycoplasmal cells may have diameters as small as 0.3 μ m (Bredt *et al*, 1973) and consist only of a cell membrane, ribosomes and prokaryotic chromosomes (Freundt and Razin, 1984). The absence of a cell wall is responsible for most of the unique properties of mycoplasmas, including resistance to β -lactam antibiotics, their 'fried-egg' appearance on agar and pleomorphism among individual cells (Limb, 1989). The mycoplasmas have limited biosynthetic capabilities, which necessitate a parasitic existence, and consequently they require complex media for cultivation (Cassell *et al*, 1994; Razin, 1983). As the tricarboxylic acid cycle is lacking, the hydrolysis of sugars, arginine or urea (depending on the species) provides the major energy-yielding system (Razin, 1992).

With very few exceptions, all mycoplasmas cultivated and identified thus far are parasites of humans, animals, arthropods or plants. Although some mycoplasmas are commensals and are recognised as part of the normal flora, most species are pathogens (Razin, 1983a). Mycoplasmas in humans and animals predominantly colonise the epithelial linings of the oro-respiratory and genitourinary tracts, but may also infect the conjunctivae, ear and mammary glands of certain animals (Taylor-Robinson, 1995). From the initial site of colonisation, mycoplasmas may migrate

to various other locations, especially in immunocompromised hosts, with certain highly virulent species exhibiting invasive characteristics (Meyer and Clough, 1993). The mechanisms of mycoplasma pathogenicity are largely unknown and infections tend to manifest chronically. The unusually close association between the parasite and host cell membranes allows the mycoplasmal metabolic by-products to cause oxidative damage (Tyron and Baseman, 1992). Some of the pathogenic mycoplasmas are capable of variable surface antigen expression, which aids host immune system evasion (Wise *et al*, 1992). In certain mycoplasma infections, the host immune response contributes to the symptoms of the infection. The prolonged presence of mycoplasmal antigens may induce chronic inflammatory responses which produce tissue damage (Clyde, 1983a). Many mycoplasmas display a complex relationship with the immune system, having both stimulatory and suppressive effects. *Mycoplasma fermentans*, *Mycoplasma penetrans* and *Mycoplasma pirum* have been implicated as putative cofactors in the pathogenesis of AIDS; specifically through the activation of the immune system, the production of superantigen or through contribution to oxidative stress, and the possibility exists that urogenital mycoplasmas could facilitate viral transmission (Blanchard and Montagnier, 1994). At least twelve of the seventy established mycoplasma species have been isolated from human sources. *Mycoplasma hominis* is the most frequently isolated of these species, predominantly from the female genital tract (Taylor-Robinson and McCormack, 1980) and has been found to contribute to a variety of infections (Taylor-Robinson, 1996).

1.2 *Mycoplasma hominis*

M. hominis was most likely the mycoplasma first isolated from a human source by Dienes and Edsall (1937). It has since been characterised as a human pathogen involved in infections of the urogenital tract, certain extragenital disorders and diseases in newborns. *M. hominis* displays the unique properties responsible for its classification, such as lack of a cell wall and extremely small genome, as well as extensive antigenic and genomic heterogeneity within the species (Krause and Taylor-Robinson, 1992).

1.2.1 Cell structure

The morphology of *M. hominis* is variable depending on the environmental conditions to which cultures are subjected (Robertson *et al.*, 1975). Under optimal conditions cells assume a coccoid or ovoid shape and replicate by binary fission. However under less favourable conditions and in older cultures, cells appear more degenerate and pleomorphic; filamentous forms, replicating buds and amorphous cells may occur (Robertson *et al.*, 1983).

Due to the lack of a cell wall, the cytoplasmic membrane forms the outer boundary of the *M. hominis* cell. The membrane is typically trilaminar consisting mainly of proteins and lipids with a small amount of carbohydrate. Membrane lipids consist of neutral lipids which cannot be synthesised and are taken up from the growth medium, and polar lipids which are synthesised from fatty acids present in the medium. The dominant lipid component is free cholesterol, a neutral lipid that is an essential growth factor and maintains membrane integrity under osmotic pressure variation (Rottem and Razin, 1973). However the presence of cholesterol renders the membrane vulnerable to damage from chemical components such as saponin and some polyene antibiotics that complex sterols (Taylor-Robinson, 1992). Membrane proteins display distinct and reproducible variation between strains, particularly in the high molecular weight range (Christiansson and Mårdh, 1983). Significant amounts of Mg^{2+} and small amounts of Ca^{2+} ions are associated with the membrane which most likely contribute to the stabilisation of the lipid bilayer and the binding of the membrane to peripheral proteins (McElhaney, 1992). *M. hominis* has been described as capable of capsule expression, although the presence of such an extracellular structure has not been detected (Rosenbusch and Minion, 1992).

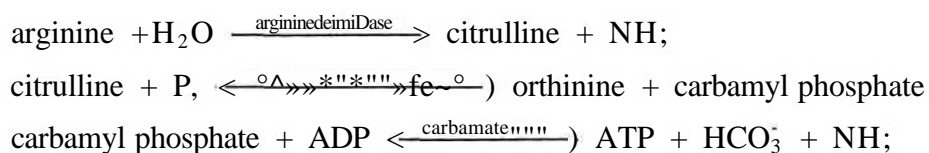
The cytoplasm is filled with nuclear material and typical 70S bacterial ribosomes. The chromosome consists one circular double-stranded DNA molecule of about 4.5×10^8 Daltons with an average length of 270 μm , significantly larger than the average 0.4 μm diameter of a cell (Christiansson and Mårdh, 1983). The *M. hominis* genome displays intraspecies size variation, ranging from 650 to 800 kb and has a characteristically low G+C content, below 30% (Freundt and Razin, 1984). Ribosomes sediment at 71S and can dissociate into two subunits of 33S and 53S with three associated rRNA species, 22S, 16S and 5S (Christiansson and Mårdh, 1983). A plasmid of 18×10^6 Daltons has been reported in a strain of *M. hominis*, however its function is

unknown. Virus-like particles were identified in *M. hominis* strain 14027, but this appears to be an isolated occurrence (Barile *et al.*, 1983).

1.2.2 Metabolism

M. hominis is a non-fermentative organism which utilises arginine as the metabolite in its principle energy-yielding pathway. A possible mechanism for the degradation of arginine is the three-step arginine dihydrolase pathway (Fig. 1.1 A). The arginine deiminase appears to be inducible and is only detected late in the logarithmic growth phase. Its late emergence may indicate that this pathway is not induced until some other energy-yielding metabolite has been exhausted (Vinther, 1983). An alternate pathway for energy production (Fig. 1.1B) involves the generation of ATP through the use of phosphate acetyltransferase and acetate kinase (Kahane *et al.*, 1978).

A:



B:

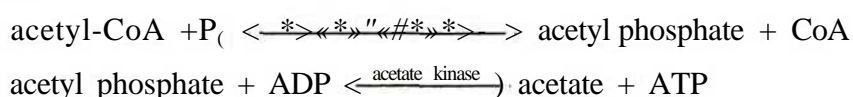


Figure 1.1. The putative metabolic pathways of ATP production in *M. hominis*. A, the arginine dihydrolase pathway (after Schimke and Barile, 1963); B, the alternative pathway (after Kahane *et al.*, 1978).

The first two enzymes of the arginine dihydrolase pathway and those of the alternate pathway have been detected in *M. hominis*. Other enzymes found in *M. hominis* include NADH⁺ oxidase and NADPH⁺ oxidase at relatively low levels; ATPase, RNase, DNase, phospholipase A, and aminopeptidase which are membrane bound enzymes; thymidine kinase and adenosine phosphorylase. The phospholipase and aminopeptidase have the potential for destroying host cells. A highly specific L-methionine transmembrane transport system is present in *M. hominis*, similar to active transport systems of other micro-organisms (Vinther, 1983).

1.2.3 Involvement in disease

M. hominis usually acts as a commensal in the normal flora of the genitourinary tract, particularly in females, which complicates the identification of its pathogenesis. However it has been shown to be a cause of invasive and destructive disease in both adults and neonates (Cassell *et al*, 1995). The most common pathogenic association is found in genitourinary tract infections. *M. hominis* is thought to be responsible for approximately 5% of acute pyelonephritis and is strongly associated with bacterial vaginosis, although its pathological role is unclear (Taylor-Robinson, 1996). Pelvic inflammatory disease (PID) is a disease of polymicrobial aetiology in which organisms from the lower genital tract invade the usually sterile regions of the fallopian tubes and ovaries, resulting in inflammation and possible infertility (Limb, 1989). *M. hominis* has been implicated as a primary pathogen in PID and although its role in causing infertility as a consequence of tubal disease is unknown, it does have this potential (Stacey *et al*, 1992; Taylor-Robinson, 1996). In South Africa, PID is a fairly frequent disease of young women and is one of the most common reasons for admission to the gynaecological wards at King Edward VIII Hospital, Durban (Hoosen *et al*, 1989). *M. hominis* has also been isolated from women with severe chorioamnionitis who subsequently underwent premature labour, and found to cause postabortive septicaemia and postpartum fever (Limb, 1989; Taylor-Robinson, 1996).

M. hominis can cause invasive disease in infants and is a cause of perinatal morbidity and mortality, particularly in premature infants. Neonatal infections may develop either from colonisation during birth or transmission in utero (Cassell *et al*, 1995). *M. hominis* has been reported to cause cerebrospinal fluid infections (Waites *et al.*, 1991), meningoencephalitis (Alonso-Vega *et al*, 1997), septicaemia, respiratory distress (Cunningham, 1990), subcutaneous abscesses and adenitis (Cassell *et al*, 1995). An extensive variety of extragenital infections caused by *M. hominis* have also been reported in adults, particularly under conditions of immunosuppression and/or hypogammaglobulinemia (Meyer and Clough, 1993). These infections develop as mycoplasmaemia, wound infections (particularly following surgery), respiratory tract infections (Madoff and Hooper, 1988), septic arthritis, joint infections (Steuer *et al.*, 1996), and central nervous system infections such as meningitis (Cohen and Kubak, 1997) and brain abscess infection (Zheng *et al*, 1997). Cases of invasive pericarditis have been attributed to *M. hominis* (Kenney *et al*, 1993), and several cases of *M. hominis* infection in transplant recipients have been reported (Gass *et al*, 1996; Vogel *et al*, 1997). As the

pathogenicity of *M. hominis* is not clear and its virulence variable, particularly in immunosuppressed individuals, a need for rapid identification of this organism exists to facilitate appropriate chemotherapeutic intervention (Zheng *et al.*, 1997).

1.2.4 Mechanisms of pathogenesis

The pathogenic properties of *M. hominis* have been difficult to define due to the high antigenic and genomic heterogeneity present among the strains, although potentially important virulence factors are being elucidated (Cassell *et al.*, 1994). Strains isolated from different sources display marked heterogeneity in antigenic properties (Hollingdale and Lemcke, 1970), isozyme expression (O'Brien *et al.*, 1981), polypeptide composition (Christiansen *et al.*, 1987a), DNA homology and DNA restriction profiles (Christiansen and Andersen, 1988).

Initially serological heterogeneity was determined when different *M. hominis* strains produced varied results in agglutination reactions, complement dependent mycoplasmacidal tests and metabolic inhibition studies (Lin and Kass, 1974). These results determined that *M. hominis* consisted of at least seven serogroups and that the variable antigens were probably membrane antigens. Andersen *et al.* (1987) compared the protein profiles of 14 *M. hominis* strains by using SDS-PAGE, immunoblot analysis and two-dimensional (2D) gel electrophoresis. The SDS-PAGE analysis indicated a homology of 76 to 99% between strains, while the more detailed analysis obtained with 2D electrophoresis showed a 41 to 72% similarity. Immunoblot analysis produced similar results, confirming the heterogeneity of the strains. Strains isolated from the same anatomical region were thought to be more homologous than strains isolated from different anatomical sites (Barile *et al.*, 1983). However immunoblot analysis of twelve genital isolates indicated significant heterogeneity among these strains (Christiansen, 1992). Strain variation analysis of single antigens using monoclonal antibodies (MAbs) showed no one MAb reacting with the same protein in all strains and supported the observation that surface antigens are more variable than cytoplasmic antigens (Christiansen, 1992).

Genomic heterogeneity could be visualised by gel electrophoresis of restriction enzyme digested DNA and DNA-DNA hybridisation, which indicated 51 to 91% homology between strains (Christiansen *et al.*, 1987b; Razin, 1983a). To establish a molecular basis for antigenic variation, restriction fragment length polymorphism (RFLP) analysis of the rRNA genes was conducted.

Certain restriction enzyme cleavage sites were found to vary slightly within the rRNA genes and more frequently in the surrounding DNA fragments (Christiansen and Andersen, 1988). Since the *atp* genes are conserved in micro-organisms, RFLP analysis using a probe consisting of part of the *atp* operon, detected distinct polymorphisms between the *M. hominis* strains (Christiansen *et al.*, 1987b).

Since surface antigens are the most variable *M. hominis* proteins, analysis of the genes encoding these proteins would provide information on the mechanism of genetic variability within *M. hominis*. Recombinant DNA studies combined with MAb screening demonstrated that recombinant clones expressing *M. hominis* antigens displayed chromosomal deletions, internal sequence repeats within coding genes and base variations (Christiansen, 1992). Sequence analysis of the gene encoding the 135 kDa surface protein harbouring the epitope of MAb 552, designated Lmpl, has indicated complex genetic organisation involving intragenic and extragenic repeating elements (Ladefoged *et al.*, 1995). Intragenic deletions resulting in antigenic variation have been shown to develop during cultivation of *M. hominis* in the presence of a MAb (Torp Jensen *et al.*, 1995). Mutants of *M. hominis* containing alterations in the size of the repeat-containing gene encoding Lmp 1, were isolated after cultivating *M. hominis* in the presence of the Lmpl-specific MAb 552. The deletion mutants displayed a high degree of spontaneous aggregation, which may provide the micro-organism with a method of host immune system evasion, since some of the antigens may be hidden within the aggregates. The *Imp* repeats have been found throughout the *M. hominis* genome in a range of strains (Ladefoged *et al.*, 1996). A repetition in the DNA sequences encoding proteins has been shown in a wide range of pathogenic bacteria to create an extensive potential genetic variability involved particularly in surface protein expression (DiRita and Mekalanos, 1989). The dominant presence of the *Imp* genes within the small genome of *M. hominis* suggests they have a significant function. They may thus provide the mechanism for variable surface antigen expression in *M. hominis* (Ladefoged *et al.*, 1996).

As the pathogenicity of *M. hominis* is closely associated with attachment, the components that mediate attachment are potential immunogens (Izumikawa *et al.*, 1987). Recent studies have focused on identifying adhesins and surface antigens in pathogenic strains. Alexander (1987a) identified eight proteins from *M. hominis* 49L, ranging from 135 to 37 kDa, as candidate surface antigens by examining antibody staining patterns using the sera of patients with invasive

M. hominis diseases. MAbs prepared against the surface proteins of an arthritogenic strain of *M. hominis* demonstrated extreme variation in the number and size of MAb-reactive proteins from clinical and nonclinical isolates (Olson *et al.*, 1991). A 120 kDa surface exposed membrane protein of *M. hominis*, PI20, was detected using a MAb generated against a clinical isolate of *M. hominis* (Christiansen *et al.*, 1994). The encoding gene was found to be present in all strains and contains a hypervariable region and two semivariable regions. The hypervariable, but not constant, domain of PI20 was recognised by the human humoral response. The variable domain may thus be important in the evasion of the host immune response (Nyvold *et al.*, 1997). The attachment mechanism of *M. hominis* to host cells is not clear, however two surface localised cytoadhesins have been identified as the membrane lipoproteins, P50 and PI00 (Henrich *et al.*, 1993). The P100 also designated the variable adherence-associated antigen (Vaa), gene has been characterised and two aspects of variation defined. Size variation caused by repetitive sequence deletion in the *vaa* gene and sequence divergence in the C-terminal end of the Vaa lipoprotein result in antigenic variation (Zhang and Wise, 1996).

The ability of *M. hominis* to metabolise arginine constitutes an important potential pathogenic factor. Arginine-utilising organisms rapidly deplete their environment of arginine, an essential amino acid, which could alter the metabolism and function of affected cells. The release of ammonia, a metabolic byproduct, may also have cytotoxic effects on the infected host cells (Barilertfl/, 1983).

1.2.5 Treatment

Treatment for *M. hominis* infections is limited to only a few families of antimicrobial compounds. *M. hominis* is resistant to sulfonamides, trimethoprim and all antibiotics acting through inhibition of cell wall synthesis (Cassell *et al.*, 1994). Tetracyclines were the primary drugs used for *M. hominis* infections, however resistance conferred by the *tetM* transposon occurs in as many as 40% of clinical isolates (Waites *et al.*, 1997). Neonatal infections of the central nervous system present a difficult therapeutic situation as several antibiotics are contraindicated in infants. Some of the newer quinolones such as trovafloxacin, are showing promise as alternative treatments (Kenney and Cartwright, 1996). Immunosuppressed patients with systemic mycoplasmal infections may harbour multiple resistant strains and therefore currently require prolonged aggressive treatment (Cassell *et al.*, 1994). Inadequate antibiotic control, resistance and the

potential severity of disease support a vaccine development. However development of a vaccine against all *M. hominis* infections poses difficult technical challenges as a result of the extreme antigenic heterogeneity present within the species and the current lack of a dominant antigen (Ellison *et al.*, 1992).

1.2.6 Evolution

As the closest model of the theoretical minimal cell, the evolution and phylogeny of mycoplasmas have been a source of much interest. The most recent model of mycoplasma evolution describes the mycoplasma phylogenetic tree as monophyletic, having arisen from a branch of the gram-positive bacterial phylogenetic tree. Mycoplasma evolution has thus been characterised by rapid evolution and decreasing genetic and physiological complexity (Maniloff, 1992). The current established basis for phylogenetic study is the comparison of rRNA sequences, more specifically 16S rRNA (Weisburg *et al.*, 1991). Comparison of the available 16S rRNA sequence data indicates that the mycoplasmas arose from a branch of gram-positive bacteria having the lowest G+C content, the *Lactobacillus* group as illustrated in Fig. 1.2. Mycoplasma phylogeny from an organism having a cell wall and genome of the *Lactobacillus* size must have been through degenerate evolution. Therefore mycoplasma evolution had to involve a significant decrease in genome size and capacity (Maniloff, 1992). The *M. hominis* group, possessing the smallest genome size range, is found at the bottom of the phylogenetic tree. Thus having apparently lost the most significant amount of genetic information, it is of interest to examine which gene functions have been retained by *M. hominis*, and which lost through degenerate evolution.

M. hominis has retained a variety of genes for intermediary metabolism and energy production. The gene sequence of the enzyme involved in arginine metabolism, arginine deiminase, suggests the gene is highly conserved among arginine-utilising *Mycoplasma* species (Harasawa *et al.*, 1992). However many of the genes for the enzymatic functions required for macromolecule precursor synthesis are absent, as indicated by the complex nutritional requirements of *M. hominis*. Enzymes involved in biosynthesis appear to be substituted with degradative enzymes such as an aminopeptidase and nucleases, for precursor assimilation (O'Brien *et al.*, 1981). Thus amino acids and nucleotides are not synthesised, rather accumulated through the degradation of exogenous peptides and nucleic acids (Vinther, 1983).

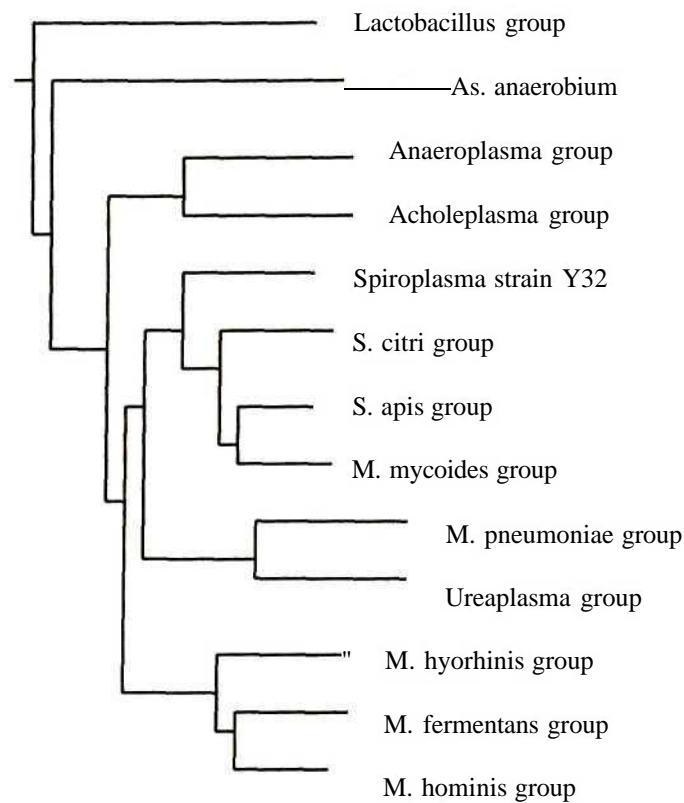


Figure 1.2. Mycoplasma phylogenetic tree reconstructed from 16S rRNA sequence comparisons (after Maniloff, 1992).

Mycoplasmas have low numbers of rRNA genes, either one or two copies, as compared to bacteria which generally have five to ten copies (Amikam *et al.*, 1984). This lack of redundancy is also visible in the mycoplasma tRNAs where the anticodons recognise more than one codon for a particular amino acid. Another interesting feature is the use of the universal stop codon UGA to code for tryptophan (Maniloff, 1992). Mycoplasmas seem to have retained the genes required for macromolecule synthesis. *M. hominis* genes encoding lysyl-tRNA synthetase and the elongation factor Tu, which are involved in protein synthesis, have been characterised (Özkökmen *et al.*, 1994; Ladefoged and Christiansen, 1991). Several bacterial DNA-related systems have been found in mycoplasmas, suggesting that functions as such DNA recombination, repair and modification are still present. The *M. hominis* DNA gyrase gene, which is thought to be essential in these functions, was found to display a unique gene arrangement (Ladefoged and Christiansen, 1994).

A highly conserved function that has been found in every organism studied, is that of a heat shock response. It is characterised by increased synthesis of a group of specific proteins in response to a sudden environmental change, most commonly a temperature increase (Dascher *et al*, 1990). Particularly conserved is the family of heat shock proteins with an approximate size of 70 kDa, the hsp70 family. Due to their large size and high degree of sequence conservation, the hsp70 proteins are well suited for phylogenetic analysis (Falah and Gupta, 1997). A heat shock response has been demonstrated in mycoplasmas, *Mycoplasma capricolum* and *Acholeplasma laidlawii*. The conservation of a heat shock system during degenerate mycoplasma evolution suggests that the system may play an essential role in cell physiology (Dascher *et al.*, 1990).

1.3 Objectives of the present study

The aims of this study were twofold: firstly, to investigate the heat shock response of *M. hominis* and secondly, to optimise and evaluate a rapid diagnostic assay for *M. hominis* infections. As the heat shock response appears to be universally conserved which is an unusual and unique phenomenon, it is of physiological and evolutionary interest, particularly in an organism such as *M. hominis*. The investigation of the heat shock response focused on the cloning of the hsp70 gene from *M. hominis* to establish the existence of the system and elucidate the degree of sequence conservation (chapter 3). The effects of heat shock treatment on the transcription and translation of the hsp70 homolog were also examined (chapter 4). As current diagnostic assays are time-consuming and produce delayed results, a rapid molecular assay using the PCR ELISA technique, was developed for *M. hominis* diagnosis (chapter 5).

CHAPTER 2

CULTURE REQUIREMENTS FOR *MYCOPLASMA HOMINIS* AND MORPHOLOGY OF CELLS AND COLONIES

2.1 Introduction

Due to their extremely small genome and hence limited biosynthetic capabilities, mycoplasmas are highly fastidious organisms and require specifically enriched media (Kenney, 1991). The various mycoplasmas isolated from humans are unique in their metabolic properties and cultivation requirements, with optimal growth at different pH ranges. No single medium formulation will sufficiently provide for the growth of all organisms (Cassell *et al.*, 1994). In lacking a cell wall, mycoplasmas are more sensitive to environmental conditions such as the tonicity of the medium, and the exposed plasma membrane is susceptible to damage by surface-active substances (Rodwell, 1983). Therefore the morphology, growth rate and composition of mycoplasmas are culture media dependent, with variable properties best attributed to their environment. Some of the medium-dependent properties of mycoplasmas that relate to their variable characteristics include antibiotic sensitivity, antigenicity/immunogenicity, pathogenicity, morphology and composition (Clark *et al.*, 1985).

Mycoplasma growth media may contain peptone; beef heart infusion; yeast extract; animal serum; DNA; glucose, arginine or urea; and a non-ionic solute such as sodium chloride. Peptones contain polypeptides, dipeptides and amino acids while yeast extracts supply labile and other growth factors. Animal sera are used as a non-toxic source of lipids and also provide a range of other nutrients. DNA supplies nucleic acid precursors but is not included in all media. Glucose or another appropriate carbohydrate, arginine or urea may be present as biochemical substrates (Miles, 1992; Rodwell, 1983). Antibiotics such as penicillin, are incorporated into the media to inhibit bacterial and fungal contamination (Cassell *et al.*, 1995). While media formulations are important, the quality of the components may be more so. Rigorous quality control of media components is essential as mycoplasmas are highly sensitive to inhibitors present in some batches of serum, yeast extract and even mycoplasma media, and to prevent extraneous mycoplasma contamination (Taylor-Robinson, 1995).

Culture media not suitable for mycoplasma growth will result in aberrant forms and irregular colony formations. The many variations in morphology could be attributed to the wide variety of culture media and preparative techniques used. The characteristic fried-egg colony morphology used for identification is highly dependent on the properties of the culture medium (Clark *et al.*, 1985). Cell and colony morphology is also dependent on the atmospheric conditions, growth density and age of the culture (Robertson and Boatman, 1983). Growth rates for mycoplasmas are relatively slow, with generation times in liquid media varying from one to six hours depending on the species. The yield of organisms is small with liquid cultures showing at most a slight turbidity. Colonies on solid media are visible microscopically after two to twenty days depending on the species (Kenney, 1991; Taylor-Robinson, 1995).

This chapter presents the specific culture media and techniques used to isolate and cultivate strains of *M. hominis* in our laboratory. The resultant colony and cell morphology is examined using light and scanning electron microscopy respectively.

2.2 Materials

Most of the common chemicals used in this study were from BDH (Poole, England), Merck (Darmstadt, Germany) or Boehringer Mannheim (Mannheim, Germany), and were of the highest purity available.

Yeast extract powder, neutralised soya peptone, mycoplasma agar base, AnaeroJar™ anaerobic gas jars and AnaeroGen™ anaerobic atmosphere generation systems were from Oxoid Unipath (Basingstoke, England). Phenol red was from ICN Biomedicals (Aurora, USA). L-Arginine hydrochloride, L-cysteine hydrochloride, CVA enrichment and ureaplasma differential basal agar were from Gibco BRL (Paisley, England). Penicillin and amphotericin were from Sigma (St Louis, USA) and ampicillin was from Boehringer Mannheim (Mannheim, Germany). Horse serum was from Highveld Biologicals (Sandringham, SA). Ultrapure urea was from Stratagene (La Jolla, USA). Disposable sterile pipettes and 55 mm contact plates were from Bibby Sterilin (Stone, England). Membrane filters (0.22 μ m) were from MSI (Westboro, USA).

2.3 Isolation and cultivation of *M. hominis* strains

M. hominis is a facultative anaerobe but has been cultured under aerobic conditions. Optimal growth conditions appear to be achieved through incubation of broth cultures under atmospheric conditions and agar plates under 5% CO₂ (Cassell *et al.*, 1994). *M. hominis*, the most pH tolerant mycoplasma, is able to grow on media with a pH range of 6.0 to 8.0 (Kenney, 1991). It has a growth requirement for cholesterol, which is essential for cell membrane lipid composition (Limb, 1989). Arginine, the major metabolic substrate in the energy-yielding arginine dihydrolase pathway, is also a requirement (Taylor-Robinson, 1995). *M. hominis* has a generation time of approximately 90 minutes (Robertson *et al.*, 1975) and produces characteristic fried-egg colonies on agar medium under suitable environmental conditions (Limb, 1989).

The reference laboratory strain used in this research, *M. hominis* PG21, was obtained from the American Type Culture Collection, Washington DC, USA. Clinical strains were obtained from the Department of Medical Microbiology, University of Natal Medical School, Durban. *M. hominis* from clinical samples was identified using light microscopy and Dienes stain, and filter-cloned to produce stock cultures.

2.3.1 Growth conditions

The culture media used for *M. hominis* in this study were adapted from the media formulations for H Broth (Hominis Broth) and *Mycoplasma* Agar with supplement G (Atlas, 1993). Clinical samples were initially inoculated onto the Ureaplasma Differential Agar Medium (A7) of Shepard and Lunceford (1976). This medium contains specific components to enable in situ detection of urease in colonies. Urea is present as the substrate and the indicator manganous sulfate allows for the detection of urea hydrolysis. The indicator produces the stable reaction product, manganese oxide, which on precipitation turns urease positive colonies brown (Shepard and Lunceford, 1976).

2.3.1.1 Reagents

M. hominis base broth. Neutralised soya peptone (20 g) and NaCl (5 g) were dissolved in approximately 800 ml dist. H₂O, 2% (m/v) phenol red (1 ml) added and the solution adjusted to pH 7.3 with NaOH. The solution was made up to 1 l, dispensed and autoclaved at 121°C for 15 minutes.

M. hominis base agar. Mycoplasma agar base (2.84 g) was dissolved in 80 ml dist. H₂O with gentle heating, 2% (m/v) phenol red (1 ml) added, the solution adjusted to pH 7.3 with NaOH and autoclaved.

Ureaplasma basal agar. Ureaplasma differential basal agar medium (6.6 g) was dissolved in 165 ml dist. H₂O, the pH adjusted to 5.5 with HCl and autoclaved.

Penicillin (10 000U/ml). Penicillin (57.8 mg) was dissolved in 10 ml dist. H₂O and filter sterilised.

Ampicillin (1mg/ml). Ampicillin (10 mg) was dissolved in 10 ml dist. H₂O and filter sterilised.

Amphotericin dmg/ml). Amphotericin (10 mg) was dissolved in 10 ml dist. H₂O and filter sterilised.

1 M L-Arginine-HCl. L-Arginine-HCl (1.74 g) was dissolved in 10 ml dist. H₂O and filter sterilised.

4% (m/v) L-Cysteine-HCl. L-Cysteine-HCl (0.4 g) was dissolved in 10 ml dist. H₂O and filter sterilised.

10% (m/v) Urea. Urea (1 g) was dissolved in 10 ml dist. H₂O and filter sterilised.

25% (m/v) Yeast extract. Yeast extract powder (25 g) was dissolved in 100 ml dist. H₂O and filter sterilised.

M. hominis broth. *M. hominis* base broth (65 ml) was aseptically supplemented with horse serum (20 ml), 25% (m/v) yeast extract (10 ml), penicillin (2 ml) and 1 M arginine (2 ml).

M. hominis agar. *M. hominis* base agar (80 ml), melted and cooled to 55°C, was aseptically supplemented with horse serum (20 ml), 25% (m/v) yeast extract (1 ml), penicillin (2 ml) and 1 M arginine (2 ml). The agar was dispensed into 55 mm contact plates and incubated overnight at RT.

Ureaplasma differential agar (A7). Ureaplasma basal agar (165 ml), melted and cooled to 55°C, was aseptically supplemented with horse serum (40 ml), 25% (m/v) yeast extract (2 ml), 10% (m/v) urea (2 ml), 0.5% (m/v) phenol red (2 ml), CVA enrichment (1 ml), 4% (m/v) cysteine (0.5 ml), ampicillin (0.2 ml) and amphotericin (1 ml). After thorough mixing, the agar was dispensed into sterile plates and incubated overnight at RT.

2.3.1.2 Procedure

Clinical samples were streaked onto A7 agar plates which were incubated at 37°C in 2.5 l AnaeroJars™ with an anaerobic atmosphere of approximately 5% CO₂ generated by AnaeroGen™ anaerobic generation systems. *M. hominis* colonies identified on these plates were subsequently subcultured in *M. hominis* media. *M. hominis* broth was inoculated either with an aliquot of *M. hominis* stock culture or with an agar section containing a *M. hominis* colony. The broth culture was incubated at 37°C under atmospheric conditions for approximately 72 hours. Stock broth cultures were dispensed into 1 ml aliquots and stored in cryovials at -70°C. *M. hominis* agar plates were inoculated with a 50 µl aliquot of broth culture which was evenly spread over the agar surface. The inoculated plates were incubated at 37°C under anaerobic conditions as described above, for at least 72 hours.

2.3.2 Dienes stain

To distinguish true mycoplasmal colonies from pseudocolonies and artefacts, the Dienes stain may be employed. Pseudocolonies may result from magnesium and calcium soap crystals, water droplets, air bubbles or tissue cells (Cassell *et ai*, 1994). Viable colonies stain a vivid blue while extracellular material and debris do not take up the stain. The method used in the present study for this in situ staining technique follows that of Madoff (1960).

2.3.2.1 Reagents

Dienes stain solution. Methylene blue (2.5 g), azure II (1.25 g), maltose (10 g), sodium carbonate (0.25 g) and benzoic acid (0.2 g) were dissolved in 100 ml dist. H₂O.

2.3.2.2 Procedure

A thin film of staining solution was applied to coverslips and allowed to dry. Small blocks of agar containing colonies were removed from the agar plates and placed right side up on a glass slide. The dye-coated coverslips were then placed stain side down directly on the agar blocks and staining allowed to proceed for a few minutes. The slide was then viewed under a light microscope. *M. hominis* colonies developed a deep blue colour while artefacts and agar appeared light blue.

2.3.3 Filter cloning

To obtain pure stock cultures representative of clinical *M. hominis* strains, clinical isolates were cultivated and subcloned by a filtration-cloning technique. The International Committee on Systematic Bacteriology (1979) described standard purification procedures for preliminary cloning of mycoplasmas, which were based on the observation that mixed species or serotypes of various mycoplasmas could exist as cell clumps in broth or agar media. The recommended technique states that the organism should be cloned three times by gentle filtration of broth culture through a membrane filter with the smallest possible pore size (usually 220 to 450 nm). Most mycoplasmas are able to pass through membranes of 300 and 220 nm, excluding other bacterial forms (Tully, 1983).

2.3.3.1 Reagents

~~*M. hominis* broth medium.~~ See section 2.3.1.1

~~*M. hominis* agar medium.~~ See section 2.3.1.1

2.3.3.2 Procedure

Logarithmic-phase broth cultures of clinical isolates were gently passed through a sterile 0.22 μ m pore size membrane filter immediately prior to plating a few μ l of the cultures on *M. hominis* agar plates. Plates were incubated as previously described (section 2.3.1.1) and well separated colonies were randomly picked in agar blocks and propagated in *M. hominis* broth medium. These steps were carried out three times for each subclone. The subcloned cultures were stored at -70°C and used as stocks for subsequent experiments.

2.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a powerful technique for high resolution morphological studies of mycoplasmas. SEM provides the ability to examine individual cell surfaces and their interactions with the surfaces of host cells and tissues, and produces a three-dimensional topographical view of cell populations (Boatman, 1979). The two main advantages of the scanning electron microscope are its ability to produce greater magnification at high resolution (up to 200 000 \times with a resolution of 3 nm), and a superior depth of field capability over the light microscope (Hayat, 1989).

The vacuum exposure and electron bombardment of SEM presents a very hostile environment, which would render untreated biological samples useless. Specimens for SEM require stabilisation prior to examination, usually involving chemical fixation, dehydration, drying and conductivity enhancement (Gabriel, 1982). Preservation of cellular ultrastructure is achieved with non-coagulant fixatives such as aldehydes and osmium tetroxide. The most commonly used aldehydes in SEM are glutaraldehyde and formaldehyde, with the former the most effective for stabilising proteins. Glutaraldehyde forms intra- and intermolecular crosslinks between reactive side chains of proteins. Osmium tetroxide is a heavy metal fixative that effectively stabilises

unsaturated lipids and may be used for post-aldehyde fixation (Gabriel, 1982). Phosphate and cacodylate buffers are compatible with these fixatives, have readily adjustable osmolarity and tonicity, and are thus popular for SEM (Hayat, 1989).

The most commonly used method of specimen dehydration for SEM is critical point drying. This method makes use of the critical point properties of carbon dioxide to ensure complete dehydration and drying. Samples are chemically dehydrated with an organic solvent, which is then replaced by transitional liquid carbon dioxide. Beyond its critical point, the carbon dioxide vaporises, resulting in a dried specimen (Cohen, 1974). In order to obtain a SEM image, specimens require coating with a very thin layer of electrically conductive metal. This is achieved through sputter coating. A partial vacuum in the sputtering chamber results in the bombardment of a gold filament by gaseous argon molecules, causing released gold particles to be available for deposition on the specimen surface (Hayat, 1989).

Due to their small size and absence of a cell wall, mycoplasmas present delicate samples for SEM preparation. However potential problems can be minimised through the use of double fixation and critical point drying. The combination of aldehyde and osmium fixatives stabilises specimens more effectively and minimises thermal damage from the electron beam. Critical point drying minimises the potential for membrane distortion by reducing stress due to surface tension (Carson and CoUier, 1983).

2.4.1 Reagents

0.1 M Cacodylate buffer. Sodium cacodylate (2.14 g) was dissolved in 100 ml dist. H₂O.

0.05 M Cacodylate buffer, pH 7.2. 0.1M Cacodylate buffer (50 ml) was adjusted to pH 7.2 with HCl and made up to 100 ml with dist. H₂O.

Aldehyde fixative [3% (v/v) Gluteraldehyde in 0.05 M cacodylate buffer]. 0.1 M Cacodylate buffer (25 ml) and 25% (v/v) aqueous gluteraldehyde (6 ml) were made up to 50 ml with dist. H₂O.

Osmium fixative \2% (m/v) Osmium tetroxide in 0.05 M cacodylate buffer. 0.1 M Cacodylate buffer (5 ml) was mixed with 4% (m/v) aqueous osmium tetroxide (5 ml).

2.4.2 Procedure

M. hominis colonies grown on agar medium as previously described (section 2.3.1), were excised on small agar blocks. The agar blocks were fixed in aldehyde fixative for 45 min, followed by washing twice in 0.05 M cacodylate buffer for 5 min. The specimens were then postfixed in osmium fixative for 30 min, and washed as before. Alcohol dehydration was done by incubation in 30% (v/v), 70% (v/v), 90% (v/v) and 100% (v/v) ethanol for 5 min respectively, followed by two subsequent washes in 100% (v/v) ethanol for 5 min. Maintaining the specimens under 100% (v/v) ethanol, they were transferred to the critical point drying apparatus. Following the critical point drying procedure, the specimens were removed from the apparatus and mounted on SEM specimen mounts with double sided tape. The mounted specimens were sputter coated, then viewed and photographed on a SEM.

2.5 Results and discussion

As the initial step in the study of *M. hominis*, successful cultivation and propagation of this organism was essential. Due to its fastidious growth requirements and the heterogeneity amongst different strains, standard media formulations often prove insufficient to support *M. hominis* growth in different laboratories (Cassell *et al*, 1994; Rodwell, 1983). Thus optimisation of culture media and environmental conditions is required to ensure optimal growth of the *M. hominis* strains under investigation.

Modifications to established *M. hominis* culture media, both agar and broth (section 2.3.1), enabled propagation of the *M. hominis* PG21 type strain and clinical *M. hominis* isolates. Growth in broth medium, which was found to be adequate under aerobic conditions, was visible after approximately 72 hours as a slight turbidity or fine suspension towards the bottom of the media. *M. hominis* growth was also accompanied by a colour change from pink to deep red, indicative of the release of alkaline metabolic byproducts. Contamination of the media by other micro-organisms was usually accompanied by a colour change to yellow. However to adequately assess the purity of a culture, aliquots were subcultured onto agar medium. Colony growth on

agar medium was visible microscopically after at least 72 hours under anaerobic conditions. Little or no growth was obtained under aerobic conditions. Depending on their density, the morphology and size of the *M. hominis* colonies varied; in less dense areas, colonies had the characteristic fried-egg appearance (Fig. 2.1), while in more dense areas colonies did not develop as distinctly.

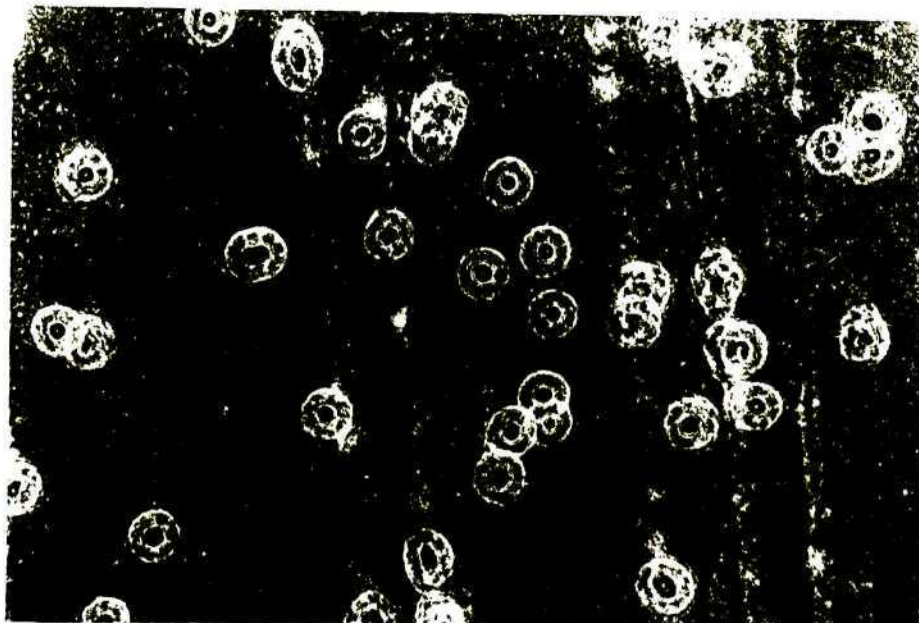


Figure 2.1. *M. hominis* colonies cultivated on *M. hominis* agar under anaerobic conditions. The typical fried-egg morphology is visible under light microscopy (magnification 100x).

Clinical samples inoculated onto A7 agar medium produced multiple aetiological growth, predominantly consisting of yeasts, *M. hominis* and ureaplasmas. Ureaplasma colonies appeared brown or black due to urease production in the presence of the indicator. The small *M. hominis* colonies could be identified by their morphology and confirmed using Dienes stain whereby *M. hominis* colonies stained deep blue, artefacts and the agar light blue and the ureaplasma colonies remained brown. Isolated *M. hominis* colonies were filtered cloned to obtain pure stock cultures for further studies.

SEM examination of *M. hominis* colonies enabled high resolution visualisation of colony and cell morphology. The characteristic fried-egg morphology of *M. hominis* colonies (Fig. 2.2A) is due

to their growth into the agar at the centre of the colony and growth only on the surface at the periphery (Cassell *et al*, 1994). The cell morphology of *M. hominis* has a highly pleomorphic potential dependant on growth conditions (Robertson *et al*, 1983). In both the type strain and the clinical isolate, the dominant morphological form appears to be coccoid (Fig. 2.2B.C). A distinct variation in the size of the cells is present and cellular reproduction by the process of binary fission is visible. Although morphological differences appear to exist between the type strain and the clinical isolate, a definitive conclusion cannot be assumed as the morphology of *M. hominis* is notoriously variable.

The successful cultivation and propagation of the *M. hominis* PG21 type strain and clinical isolates provided a reliable source of material for further investigations.

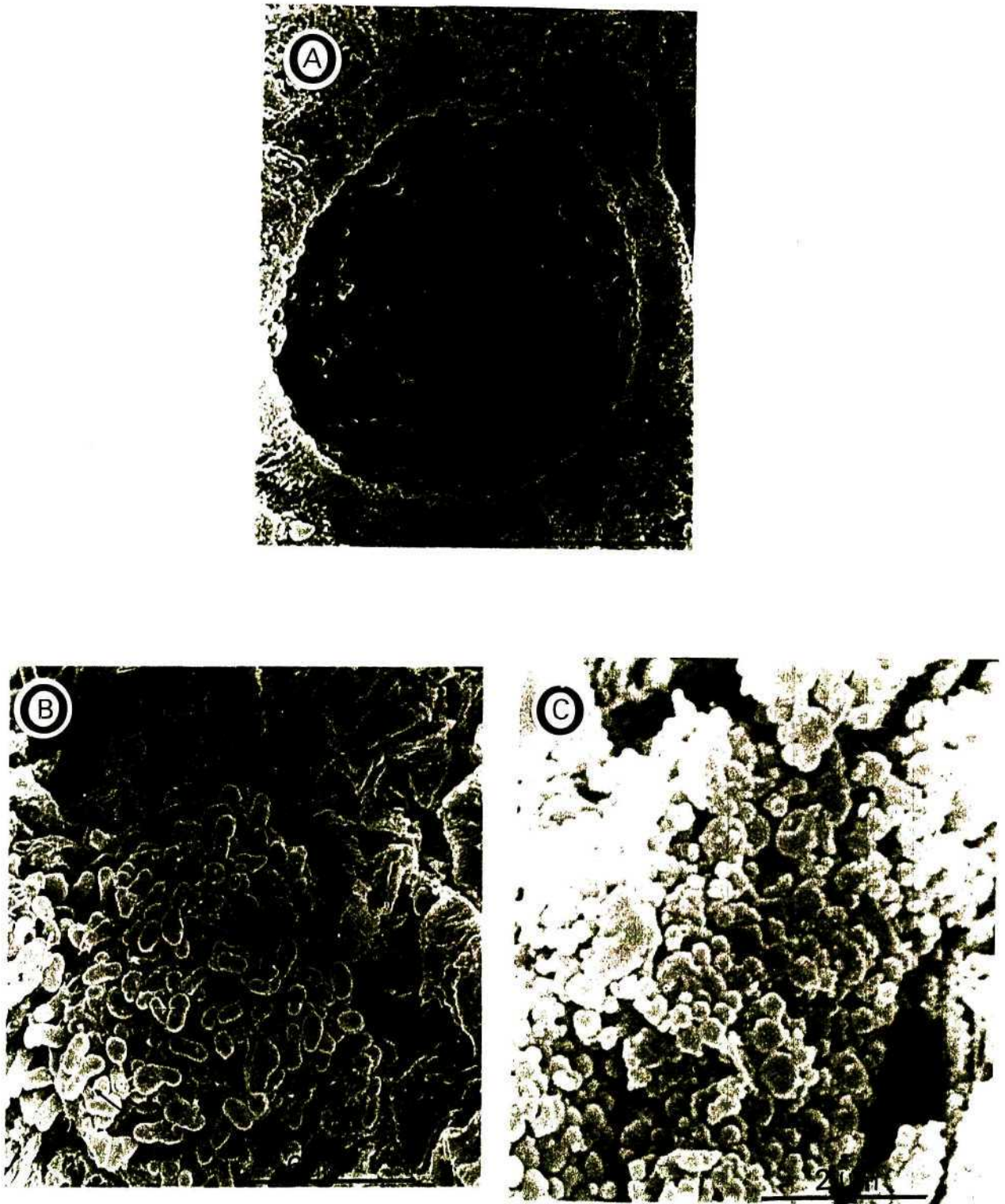


Figure 2.2. SEM examination of *M. liominis* morphology. A, *M. liominis* PG21 colony cultivated on *M. liominis* agar exhibiting features responsible for typical colony morphology; B, *M. liominis* PG21 cells; C, *M. liominis* cells from a clinical isolate. Arrows indicate cells undergoing division by binary fission.

CHAPTER 3

CLONING OF THE HSP70 GENE FROM *MYCOPLASMA HOMINIS***3.1 Introduction**

All organisms share a common response to heat induced stress in the form of the increased synthesis of a group of proteins collectively known as the heat shock proteins or hsp's. This heat shock response is the most highly conserved known genetic system, present in every species studied thus far, from humans to plants to eukaryotes to archaebacteria (Lindquist and Craig, 1988). Molecular analysis of the heat shock phenomenon was initiated in the *Drosophila* species. The heat shock response was viewed as a model system for investigating gene structure and regulation, and investigations proved very successful with *Drosophila* (Lindquist, 1986). While the discovery of heat shock genes preceded discovery of their protein products in *Drosophila*, initial observations on bacterial heat shock were made on proteins with elucidation of their corresponding genes continuing (Neidhardt *et al.*, 1984). These molecular analyses have revealed that some heat shock genes have been highly conserved throughout evolution in both their protein coding sequences and their regulatory sequences (Lindquist, 1986).

Genetic analysis of the heat shock response has revealed many regulatory mechanisms in prokaryotes and eukaryotes, acting transcriptionally and post-transcriptionally (Lindquist and Craig, 1988). Of the prokaryotic heat shock systems, the most characterised is that of *E. coli*. The heat shock response of *E. coli* consists of the induction of the 17 gene hsp regulon and the suppression of the synthesis of non-hsp proteins (Neidhardt *et al.*, 1984). The mechanisms of induction and regulation are complex and have not been fully elucidated. In *E. coli* a regulatory gene, *htpR*, which resembles a DNA binding protein, has indirectly or directly a positive effect on the heat shock response. Some of the hsp's have been implicated as negative modulators of the response and certain signal metabolites such as alaromones, are involved as inducing agents (Niedhardt *et al.*, 1984). Transcriptional regulation is controlled by σ^{32} , a sigma factor that binds to core RNA polymerase and redirects it to heat-shock promoters, which is itself regulated by transcriptional, translational and posttranslational mechanisms (Lindquist and Craig, 1988). The mechanisms regulating the eukaryotic response are more complex, although unusually a general similarity in the molecular mechanism of regulation appears to exist, which together with the strong homology between the hsp's, is unprecedented.

The most universal feature of the heat shock response is the production of proteins encoded by the hsp70 gene family (Lindquist and Craig, 1988). The 70 kDa heat-shock family of proteins (hsp70) constitutes the one of the most conserved classes of proteins found in all organisms characterised thus far, from eukaryotes to prokaryotes to plants to humans, and accounts for the most abundant hsps (Lindquist, 1986; Gupta and Golding, 1993). Although originally identified as inducible proteins, certain hsp70s are also constitutively expressed (Lindquist and Craig, 1988). A single hsp70 homolog is generally found in prokaryotes, while in eukaryotes several distinct hsp70 homologs, encoded by a hsp70 multigene family, have been identified (Gupta and Golding, 1993).

Due to their ubiquitous presence and high degree of sequence conservation, hsp70 homologs provide a useful system for the investigation of molecular evolution (Falah and Gupta, 1997). The phylogenetic relationships between archaeobacteria, eubacteria and eukaryotes are essential to the understanding of evolution (Gupta and Golding, 1993). As discussed in the introduction, current phylogenetic analysis indicates that mycoplasmas arose from a group of gram-positive bacteria through degenerate evolution. However phylogenetic analyses based on hsp70 sequences have revealed several important differences from rRNA-based phylogenies, particularly in eukaryotes where this analysis has been predominantly applied (Boorstein *et al.*, 1994). The elucidation of mycoplasmal hsp70 sequences would thus be useful in establishing their evolutionary relationship to other prokaryotes, and the relative importance of the heat shock system in having been retained in an organism derived through degenerate evolution.

Other than their importance in evolutionary studies, hsps have also generated much interest after being implicated as the dominant antigens of infectious micro-organisms. Heat shock genes are induced very early in parasitic organisms and assume an essential role during adaptation to a new environment, thereby playing an important role during host invasion (Maresca and Kobayashi, 1994). During infection by pathogens, hsps become prominent antigens which induce a significant immune response, with hsp70 and hsp60 representing the major antibody targets. Hsp70 has been identified as an immunodominant antigen in infections caused by *Plasmodium falciparum*, *Trypanosoma cruzi*, *Leishmania* species, *Schistosoma* species, *Mycobacterium* species, *Brugia malayi* and *Onchocerca volvulus* (Kaufmann, 1990). The importance of hsp70 to cellular functioning and immunity is discussed in chapter 4.

A general method to clone the hsp70 gene from any species has been developed using the polymerase chain reaction and specific degenerate primers for conserved regions of the protein (Galley *et al.*, 1992; Gupta and Singh, 1992). This method has recently been used to elucidate the hsp70 gene sequence from *M. capricolum* (Falah and Gupta, 1997) and was used in this study to investigate the hsp70 gene of *M. hominis*.

3.2 Materials

Most of the commonly used chemicals in this study were from Merck (Darmstadt, Germany), BDH (Poole, England) or Boehringer Mannheim (Mannheim, Germany), and were of the highest purity available.

RNase A and N-laurylsarcosine were from Sigma (St. Louis, USA). Proteinase K, lambda DNA, the DIG labelling and detection kit, T4 DNA ligase, deoxynucleotide triphosphates (dNTPs), isopropyl-(3-D-thio-galactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-fi-D~galactosidase (X-Gal) and restriction enzymes were from Boehringer Mannheim (Mannheim, Germany). The PCR oligonucleotide primers were from Gibco BRL (Paisley, England). *Taq* DNA polymerase was supplied by Bioline (London, UK). The pGEM®-T and pGEM®-7Zf(+) vector systems and M13 universal primers were from Promega (Madison, USA). The T7 Sequenase™ version 2.0 sequencing kit and restriction enzymes were from Amersham (Buckinghamshire, UK). Natural thin-walled PCR tubes (0.2 ml) were from Quality Science Plastics (Petaluma, USA) and the Magnagraph transfer nylon membrane was from MSI (Westboro, USA)

3.3 General methods

The methods described in this section are fundamental molecular biology techniques that were used routinely throughout the subsequent investigations.

3.3.1 DNA extraction

The lack of a cell wall and intracytoplasmic membranes such as a nuclear membrane, allows less stringent conditions to be used for DNA extraction from mycoplasmas. The cell membrane is highly susceptible to lysis, which may be induced by the osmolarity of the buffers, or by a detergent such as SDS. Following the release of the cellular components, the contaminating proteins and RNA can be eliminated through enzymatic treatment and the chromosomal DNA recovered. The DNA isolation method used in this study follows that of Razin *et al.* (1983).

3.3.1.1 Reagents

0.5 M EDTA, pH 8.0. EDTA (18.61 g) was dissolved in 80 ml of dist. H₂O, adjusted to pH 8.0 with NaOH, made up to 100 ml and autoclaved at 121°C for 15 min.

1 M Tris-HCl, pH 8.0. Tris (121.1 g) was dissolved in 800 ml of dist. H₂O, adjusted to pH 8.0 with cone. HCl, made up to 11 and autoclaved.

Tris-EDTA (TE) buffer n mM EDTA, 10 mM Tris-HCl pH 8.01. 1 M Tris-HCl (1 ml) and 0.5 M EDTA (0.2 ml) were made up to 100 ml with dist. H₂O and autoclaved.

10% fm/v) SDS. SDS (10 g) was dissolved in 100 ml sterile dist. H₂O with gentle heating.

RNase A (1 mg/ml). RNase A (10 mg) was dissolved in 10 ml sterile dist. H₂O and stored at -20°C in 1 ml aliquots.

Proteinase K (10 mg/ml). Proteinase K (100 mg) was dissolved in 10 ml sterile dist. H₂O and stored at -20°C.

5 M Potassium acetate. Potassium acetate (49.08 g) was dissolved in 100 ml dist. H₂O and autoclaved.

Tris-buffered phenol, pH 8.0. Phenol (500 g) was melted at 68°C, 8-hydroxyquinoline was added to a final concentration of 0.1% (m/v) and the phenol extracted with an equal volume of 0.5 M Tris-HCl, pH 8.0. The phenol was equilibrated 3 times with 0.1 M Tris-HCl, pH 8.0. Following removal of the final aqueous extraction phase, 0.1 volume of 0.1 M Tris-HCl, pH 8.0 containing 0.2% (v/v) P-mercaptoethanol was added. The phenol was stored in the dark at 4°C.

Phenol:chloroform (1:1, v/v). Equal volumes of Tris-buffered phenol and chloroform were mixed in a dark bottle and left to separate at 4°C. The lower phenol phase was used.

3.3.1.2 Procedure

M. hominis broth cultures were separated into 40 ml aliquots, centrifuged at 10 000 xg for 30 min and washed twice with TE buffer. The washed cell pellet was resuspended in TE buffer (1 ml) and cells lysed by adding 1% (m/v) SDS in TE buffer (2 ml). The lysate was incubated with 50 µg/ml RNase A for 30 min at 37°C, then with 100 µg/ml proteinase K for 60 min at 37°C. The SDS in the lysate was then precipitated with the addition of 5 M potassium acetate (200 µl) and 30 min incubation on ice. Following centrifugation at 15 000 xg for 10 min, the supernatant was transferred to another centrifuge tube and mixed with an equal volume of phenol:chloroform. The aqueous phase was separated by centrifugation at 15 000 xg for 5 min and extracted again with phenol:chloroform. The final recovered aqueous phase was mixed with 2 volumes of cold ethanol and incubated at -70°C for 30 min to precipitate the DNA. The DNA precipitate was collected by centrifugation at 15 000 xg for 15 min and dissolved in a small volume (20 to 100 µl) of TE buffer. The concentration and purity of the preparation was assessed through agarose gel electrophoresis.

3.3.2 Agarose gel electrophoresis

The differently sized components of DNA mixtures require separation, either analytically or preparatively. The technique most widely used for separating DNA molecules, which may vary in size from a few hundred base pairs (bp) to a few hundred kb, is electrophoresis in agarose gels (Walker and Gaastra, 1983). DNA molecules possess, at neutral or alkaline pH, a uniform negative charge which dictates movement towards the anode in an electric field. The electrophoretic mobility of the DNA molecules is inversely proportional to their molecular weight, with larger molecules migrating more slowly due to greater frictional drag and less

efficient movement through the agarose pores (Sambrook *et al*, 1989). Several variables affect the separation of the DNA molecules including the conformation of the DNA, the pore size of the gel, the voltage gradient applied, and the salt concentration of the buffer (Ausubel *et al*, 1991).

The shape and size of DNA molecules influences electrophoretic mobility, demonstrated by the ability of gel electrophoresis to separate different forms of the same DNA, such as superhelical from linear or open circular (Sambrook *et al*, 1989). The gel pore size is determined by the agarose concentration and is linearly proportional to the logarithm of electrophoretic mobility, with common concentrations of 0.5 to 2% used to separate a DNA size range of 0.2 to 50 kb (Walker and Gastra, 1983). The rate of migration is also proportional to the applied voltage, although as the voltage increases, the mobility of the high molecular weight DNA fragments increases differentially. Decreasing the gel thickness or the ionic strength of the buffer will increase the voltage and hence mobility of the DNA (Ausubel *et al*, 1991). Electrical conductance is minimal in the absence of ions, however in buffers of high ionic strength, significant resistance is generated with the risk of DNA denaturation. The most commonly used buffer is tris-acetate but due to its low buffering capacity must be replaced regularly (Sambrook *et al*, 1989).

The common procedure for visualisation of DNA fragments on agarose gels is staining with the fluorescent dye, ethidium bromide. This dye intercalates between the bases of the DNA yielding more intense fluorescence than the free dye (Sambrook *et al*, 1989). The excitation of the dye is most efficient at 254 nm, however nicking and dimerisation of the DNA can occur at this wavelength. The use of 300 nm ultraviolet reduces the photodamage to the DNA without loss in sensitivity, allowing visualisation of as little as 10 ng DNA (Walker and Gastra, 1983).

3.3.2.1 Reagents

50x Tris-acetate-EDTA (TAE) buffer. Tris (242 g), EDTA (37.2 g) and glacial acetic acid (57.1 ml) were made up to 1 l with dist. H₂O and autoclaved. The solution was diluted to 1x for use.

Ethidium bromide (10 mg/ml). Ethidium bromide (0.1 g) was dissolved in 10 ml dist. H₂O and stored in the dark at RT.

DNA size markers. Lambda DNA (0.2 μ g) was restriction digested with *HindIII* (50 U) and restriction buffer (10 μ l) in a total volume of 100 μ l at 37°C overnight.

Loading buffer [0.4% (m/v) Bromophenol blue, 50% (v/v) glycerol and 1 mM EDTA]. Bromophenol blue (0.04 g), glycerol (5 ml) and 0.5 M EDTA (0.2 ml) were made up to 10 ml with dist. H₂O.

3.3.2.2 Procedure

For routine DNA analysis, electrophoresis was carried out using 1% (m/v) agarose gels in the MINNIE submarine agarose gel unit (Hoefer Scientific, San Francisco) at 10 V/cm. When required the concentration of agarose varied from 0.7% for gel purification, to 2% for restriction analysis of small DNA fragments. Large gels (200 ml) were also used for restriction analysis when required, run overnight at 2.5 V/cm. Routine 1% (m/v) agarose gels were made up by melting agarose (0.4 g) in 1x TAE buffer (40 ml), and adding ethidium bromide (1 μ l) when slightly cooled prior to casting. When set gels were submerged in horizontal electrophoresis tanks containing 1x TAE buffer and ethidium bromide (3 μ l). Prior to loading, DNA samples were mixed with loading buffer (2 μ l), and then run towards the anode with DNA size markers (4 μ l). DNA fragments were visualised using a Fotodyne 300 Series Foto UV DNA transilluminator at 300 nm and photographed using a Fotodyne Polaroid camera with Polaroid 667 film.

3.3.3 DNA purification from agarose gels

The recovery and purification of DNA fractionated on agarose gels is an essential procedure for obtaining specific DNA fragments of high purity. Although numerous methods have been developed for this purpose, none have proved completely satisfactory with problems encountered in the recovery of large DNA fragments or small quantities of DNA, and the inability to simultaneously recover several DNA fragments (Sambrook *et al.*, 1989). Several commercial gel purification kits are available and our laboratory assessed the efficiency of different systems. However the most reliable method with consistent yields of at least 50%, was found to be the freeze-squeeze method of gel purification (Benson, 1984).

3.3.3.1 Reagents

3 M Sodium chloride. NaCl (17.532 g) was dissolved in 100 ml dist. H₂O and autoclaved.

3.3.3.2 Procedure

The DNA to be gel purified was separated on 0.7% agarose gels. The agarose gel slice containing the DNA fragment of interest was excised and disrupted in phenol (150 μ l) with a sterile toothpick. The mixture was vortexed and incubated at -70°C for 1 hour. After centrifugation in a microfuge at maximum speed for 15 min, the upper aqueous phase was extracted twice with phenol:chloroform. To the recovered aqueous phase, 1/10 the volume of 3 M NaCl and twice the resultant volume of cold isopropanol were added. The mixture was vortexed briefly and incubated at -20°C for 1 hour. The DNA was pelleted by centrifugation at maximum speed for 15 min and washed with 70% (v/v) ethanol. The DNA pellet was air dried and resuspended in TE buffer. The concentration of the DNA was estimated using agarose gel electrophoresis.

3.3.4 Preparation of competent cells

To improve the efficiency of transformation of bacterial strains by plasmid DNA, the DNA must be introduced into competent bacterial cells. The method used in this research for inducing competency in *E. coli* cells follows that of Alexander (1987b). The particular strain of *E. coli* used must be compatible with the vector of choice and the selection method used for transformants. The pGEM® vectors used in these investigations, use blue/white colour screening for recombinants, therefore the host strains such as JM109, carry the necessary *kcZAM15* and *lac ft* on a F episome. These strains are maintained on minimal plates (M9) with thiamine-HCl, to select for the presence of the F' which carries a nutritional requirement for growth and minimises the number of false positives (Titus, 1991).

3.3.4.1 Reagents

1 M Magnesium sulfate. MgSO₄ (12.324 g) was dissolved in 50 ml dist. H₂O and autoclaved.

1 M Calcium chloride. CaCl₂ (7.35 g) was dissolved in 50 ml dist. H₂O and autoclaved.

1 M Thiamine-HCl. Thiamine-HCl (3.373 g) was dissolved in 10 ml dist. H₂O, filter sterilised and stored at 4°C in the dark.

M9 agar. Na₂HPO₄ (6 g), KH₂PO₄ (3 g), NaCl (0.5 g), NH₄Cl (1 g) and agar (15 g) were dissolved in 900 ml dist. H₂O, the pH adjusted to 7.4 with NaOH, the volume was made up to 1 l and the solution autoclaved. After cooling, 1 M MgSO₄ (2 ml), 1 M CaCl₂ (0.1 ml), 20% (m/v) glucose (10 ml) and 1 M thiamine-HCl (1 ml) were added and the media poured into plates.

Luria broth. Tryptone (2.5 g), yeast extract (1.25 g) and NaCl (1.25 g) were dissolved in 200 ml dist. H₂O and the pH adjusted to 7.0 with NaOH. The volume was made up to 250 ml and the solution autoclaved.

100 mM Sodium acetate, pH 5.5. CH₃COONa.3H₂O (1.36 g) was dissolved in 60 ml dist. H₂O and the pH adjusted to 5.5 with acetic acid. The volume was made up to 100 ml and the solution autoclaved.

Trituration buffer 100 mM CaCl₂ 70 mM MgCl₂ 40 mM Sodium acetate pH 5.5. 1 M CaCl₂ (10 ml), 1 M MgCl₂ (7 ml) and 100 mM CH₃COONa.3H₂O (40 ml) were made up to 100 ml with dist. H₂O.

3.3.4.2 Procedure

JM109 stock bacteria were streaked onto M9 plates and incubated at 37°C overnight. A single colony was inoculated into Luria broth (5 ml) and incubated with shaking at 37°C overnight as a pre-culture. Pre-culture (0.5 ml) was transferred to Luria broth (50 ml) and incubated at 37°C with shaking until the A₆₀₀ was between 0.4 and 0.5. The culture was then incubated on ice for 15 min followed by centrifugation at 5000 xg for 10 min at 4°C. The supernatant was decanted and the cells resuspended in 1 to 2 ml cold trituration buffer. The volume was adjusted to 50 ml with the same buffer and incubated on ice for 45 min. After incubation the cells were spun as before and resuspended in 50 ml cold trituration buffer. The cells were pooled, 80% (v/v) glycerol added to a final concentration of 20% (v/v) and stored in 0.2 ml aliquots at -70°C.

3.3.5 Plasmid preparation from *E. coli* cells

Plasmid DNA was isolated from *E. coli* cells using the alkaline lysis method of Ish-Horowicz and Burke (1981) as modified by Sambrook *et al.* (1989).

3.3.5.1 Reagents

Luria broth. See section 3.3.4.1.

1 N Sodium hydroxide. NaOH (8 g) was dissolved in 200 ml dist. H₂O and autoclaved.

100 mM Glucose. Glucose (1.802 g) was dissolved in 100 ml dist. H₂O and filter sterilised.

Solution I 50 mM Glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA. 100 mM Glucose (5 ml), 1 M Tris-HCl pH 8.0 (0.25 ml) and 100 mM EDTA (1 ml) were made up to 10 ml with sterile dist. H₂O.

Solution II 0.2 N Sodium hydroxide, 1% (m/v) SDS. 1 N NaOH (2 ml) and 10% (m/v) SDS (1 ml) were made up to 10 ml with sterile dist. H₂O.

Solution III 5 M Potassium acetate, pH 4.81. 5 M Potassium acetate (60 ml) and glacial acetic acid (11.5 ml) were made up to 100 ml with dist. H₂O and stored at 4°C. The resultant solution was 3 M with respect to potassium and 5 M with respect to acetate.

3.3.5.2 Procedure

E. coli strains containing plasmids were grown in Luria broth containing 50 µg/ml ampicillin to maintain selective pressure. Depending on the volume of culture, either the mini-, midi-, or maxi-prep method was used with variation only in the volume of solution used (Table 1). The bacterial cells were harvested by centrifugation at 10 000 Xg for 2 min, resuspended in solution I and maintained at RT for 5 min. Solution II was added, the solution mixed gently and maintained on ice for 5 min. Precooled solution III was added, mixed and incubated on ice for 5 min. The precipitated protein, SDS and chromosomal DNA were removed by centrifugation at 10 000 Xg for 5 min. The recovered supernatant was extracted twice with phenol:chloroform.

Two volumes of isopropanol were added to the final supernatant, the mixture incubated on ice for 10 min and the plasmid DNA pelleted by centrifugation at 12 000 Xg for 10 min. The DNA pellet was washed with 70% (v/v) ethanol, air dried and resuspended in TE buffer.

Table 1. Mini-, midi- and maxi-prep extraction of plasmid DNA from *E. coli*.

Solution	Mini-prep	Midi-prep	Maxi-prep
Culture	15 ml	50 ml	200 ml
I	100 μ j	500 ¹	2 ml
II	200 il	1000 \i\	4 ml
III	150 il	750 ¹	3 ml
TE	50 ¹	500 ¹	2 ml

3.3.6 DIG labelling of DNA

The labelling and detection of nucleic acids is an essential feature of molecular biology. One of the most frequently used and successful systems is based on the hapten digoxigenin (DIG), derived from *Digitalis* plants, and anti-digoxigenin antibody conjugates. DIG is an effective nucleic acid label as it is easily chemically manipulated and efficiently incorporated into probes by polymerases. To synthesise probes for use in blots, the preferred labelling method is random primed DNA labelling whereby DIG-dUTP (Fig. 3.1) is incorporated into enzymatically synthesised DNA probes. This method is advantageous in that it is robust, requires small amounts of template DNA and generates an exponential amount of labelled DNA (Höltke *et al*, 1995). The labelling reaction was conducted following the DIG system protocol of Boehringer Mannheim (Mannheim, Germany).

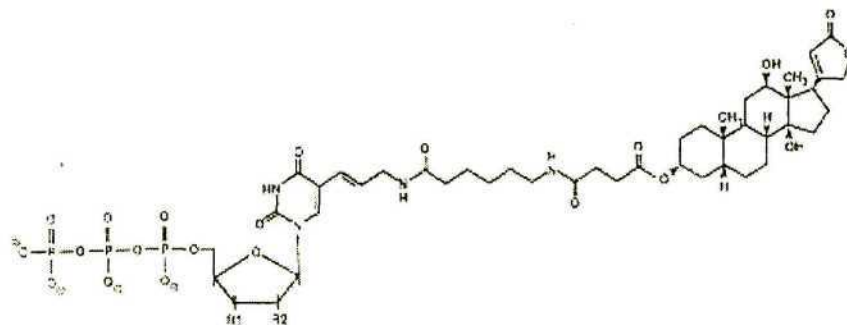


Figure 3.1. The chemical structure of DIG-dUTP (after Höltke *et al.*, 1995). R1 and R2 denote hydroxyl groups.

3.3.6.1 Reagents

0.5 M EDTA. See section 3.3.1.1

4 M Lithium chloride. LiCl (1.696 g) was dissolved in 10 ml dist. H₂O and autoclaved.

Labelling components. Hexanucleotide mix, deoxynucleotide (dNTP) labelling mix and Klenow enzyme were supplied in the DIG labelling and detection kit from Boehringer Mannheim (Mannheim, Germany).

3.3.6.2 Procedure

The DNA template was heat denatured in a boiling water bath for 10 min and snap-cooled on ice before use. The DNA (approximately 1 μ g), hexanucleotide mix (2 μ l), dNTP labelling mix (2 μ l) and Klenow enzyme (1 μ l) were made up to 20 μ l with sterile dist. H₂O and mixed. The reaction was incubated at 37°C for at least 1 hour, generally overnight. The reaction was terminated with the addition of 0.5 M EDTA (1 μ l). The labelled DNA was mixed with 4 M LiCl (2.1 μ l) and cold ethanol (70 μ l), incubated at -70°C for 30 min and centrifuged at 13 000 Xg for 15 min. The pellet was washed with 70% (v/v) ethanol, air dried and resuspended in TE buffer (50 μ l). The labelled probe was stored at -20°C until required. The concentration of the probe was quantified through a dot blot procedure whereby dilutions of supplied DIG-labelled DNA and newly synthesised DIG-labelled DNA were spotted directly onto nylon membrane, detected as for a Southern blot (section 3.3.7) and the relative intensities of the colorimetric reaction compared.

3.3.7 DNA hybridisation and detection

The probing and subsequent visualisation of specific DNA fragments is achieved through Southern blotting in conjunction with a suitable detection system. The transfer of DNA fragments onto nylon membrane and the subsequent hybridisation with a labelled DNA probe follows the method of Southern (1975). The DIG-labelling and detection system provides a convenient, sensitive and non-radioactive method. DIG-labelled hybrids are detected by an enzyme-linked immunoassay producing a colorimetric, fluorometric or chemiluminescent reaction. The most common method of detection uses alkaline phosphatase to catalyse a redox

reaction between 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT) (Fig. 3.2), producing a purple-blue precipitate (Höltke *et al.*, 1995). This was the method of choice for the present study, conducted following the recommendations of Boehringer Mannheim (Mannheim, Germany).

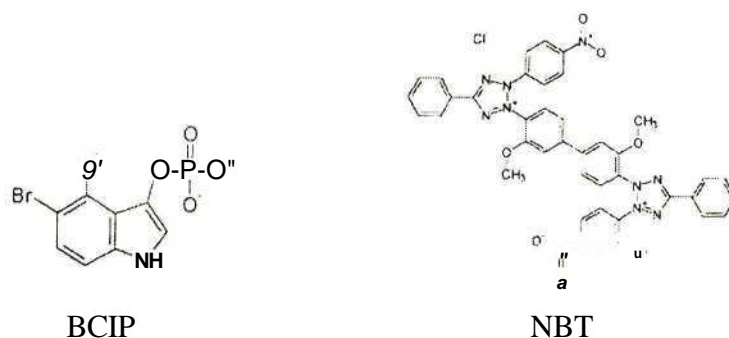


Figure 3.2. Substrates used for alkaline phosphatase colorimetric DIG detection (after Höltke *et al.*, 1995).

3.3.7.1 Reagents

~~1 M Tris-HCl.~~ See section 3.3.5.1.

~~1 N NaOH.~~ See section 3.3.5.1.

~~6 M Sodium chloride.~~ NaCl (175.32 g) was dissolved in 500 ml dist. H₂O and autoclaved.

Denaturation solution ~~0.5 N NaOH.~~ 1.5 M NaCl. 1 N NaOH (125 ml) and 6 M NaCl (62.5 ml) were made up to 250 ml with sterile dist. H₂O.

Neutralisation solution ~~0.5 M Tris-HCl.~~ 3 M NaCl. 1 M Tris-HCl (125 ml) and 6 M NaCl (125 ml) were mixed.

20x SSC stock solution ~~3 M NaCl.~~ 0.3 M sodium citrate. pH 7.01. Sodium citrate (44.115 g) was dissolved in 6 M NaCl (250 ml) and 200 ml dist. H₂O, the pH adjusted to 7.0 with HCl, the solution made up to 500 ml and autoclaved.

Buffer I TO 1 M Tris-HCl, 0.15 M NaCl pH 7.51. 1 M Tris-HCl (50 ml) and 6 M NaCl (12.5 ml) were made up to 450 ml with dist. H₂O, the pH was adjusted to 7.5 with NaOH, the volume made up to 500 ml and the solution autoclaved.

10% (m/v) Block solution. Blocking reagent (10 g) was dissolved in 100 ml buffer I with gentle heating and agitation, and autoclaved.

10% (m/v) N-laurylsarcosine. N-laurylsarcosine (1 g) was dissolved in 10 ml dist. H₂O and filter sterilised.

Standard hybridisation buffer T5x SSC, 0.1% (m/v) N-laurylsarcosine, 0.02% (m/v) SDS, 1% (m/v) block! 20x SSC (50 ml), 10% (m/v) N-laurylsarcosine (2 ml), 10% (m/v) SDS (0.4 ml) and 10% (m/v) block (20 ml) were made up to 200 ml with sterile dist. H₂O.

Buffer II f1% (m/v) Block solution! 10% (m/v) Block solution (10 ml) was made up to 100 ml with buffer I.

Detection buffer HOP mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂1. 1 M Tris-HCl pH 9.5 (25 ml), 1 M NaCl (25 ml) and 1 M MgCl₂ (12.5 ml) were made up to 250 ml with sterile dist. H₂O.

2x Wash solution T2x SSC, 0.1% (m/v) SDS1. 20x SSC (10 ml) and 10% (m/v) SDS (1 ml) were made up to 100 ml with sterile dist. H₂O.

0.1x Wash solution r0.1x SSC, 0.1% (m/v) SDS1. 20x SSC (0.5 ml) and 10% (m/v) SDS (1 ml) were made up to 100 ml with sterile dist. H₂O.

Substrate solution. NBT solution (45 μ d) and BCIP solution (35 μ l) (as supplied by the manufacturer) was mixed with detection buffer (10 ml) just before use.

3.3.7.2 Procedure

The agarose gel containing the DNA fragments of interest, was submerged in denaturation solution twice for 15 min at RT with gentle agitation. After rinsing the gel in dist. H₂O, it was submerged in neutralisation solution twice for 15 min at RT. The gel was then placed on a clean glass surface covered with Whatmann 3MM filter paper in a container with 20x SSC solution. Nylon membrane of the same dimensions as the gel, was soaked in 2x SSC and placed on the gel, followed by two pieces of filter paper also soaked in 2x SSC. A layer of paper towel and a light weight was placed on the assembly and transfer of the DNA by capillary action, allowed to proceed for 4 to 16 hours. The membrane was removed from the assembly, UV crosslinked for 2 min and then air dried.

The blot was prehybridised in standard hybridisation buffer at 68°C for at least 1 hour. The DNA probe was heat denatured in a boiling water bath for 10 min and snap-cooled on ice prior to addition to the hybridisation solution. The DNA probe was used at a working concentration of 30 to 60 ng/ml and the blot hybridised at 68°C overnight. Following hybridisation, the blot was washed twice in 2x wash solution for 5 min at RT, then washed twice in 0.1x wash solution for 15 min at 68°C.

The membrane was equilibrated in buffer I for 1 min at RT with shaking (as for all subsequent incubations). The membrane was blocked by incubation in buffer II for 30 min in a clean container. The anti-DIG-alkaline phosphatase conjugate was diluted in buffer II to a working concentration of 150 mU/ml and the membrane incubated for 30 min. Following antibody incubation, the membrane was washed twice in buffer I for 15 min and then equilibrated in detection buffer for 2 min. The substrate solution was added to the membrane and colour development allowed to proceed in the dark without agitation. After colour development was complete, the membrane was washed in buffer I and air dried at RT.

3.4 PCR amplification of the hsp70 probe

The polymerase chain reaction (PCR) is used for rapid enzymatic amplification of a specific segment of DNA. A DNA polymerase catalyses a series of synthetic reactions between a segment of double-stranded DNA to be amplified and two single-stranded oligonucleotide primers flanking this segment (Ausubel *et al.*, 1991). The template DNA is initially heat denatured in the presence of excess primers and dinucleotides. The reaction is then cooled to an appropriate temperature for annealing of the oligonucleotide primers to their target sequence, followed by DNA polymerase induced extension. The products of preceding amplification cycles act as templates for subsequent cycles, resulting in an exponential accumulation of product (Sambrook *et al.*, 1989).

PCR is a popular technique with a wide range of applications, a common usage being the generation of specific DNA sequences for use as probes. This specific PCR method was required to amplify a segment of the hsp70 gene of *M. hominis* to use as a probe for the entire gene sequence. Galley *et al.* (1992) designed specific degenerate oligonucleotide primers based on highly conserved regions of the hsp70 protein (Fig. 3.3). Although the protein sequence of these regions is almost completely conserved in all species, individual organisms have different codon usage necessitating the use of degenerate primers. Degenerate primers consist of a mixture of oligonucleotides of the same length but varying in base sequence. Ideally degeneracy should be kept to a minimum to ensure maximum specificity. Therefore if possible, the use of peptide regions containing amino acids requiring four or six codons, such as leucine or serine, should be avoided. To ensure primer extension, a non-degenerate base at the terminal 3' position is recommended (Innis *et al.*, 1990).

With the degenerate primers containing 512 different sequence permutations, the concentration of primer in the reaction mix must be higher than that of a homogenous primer (Innis *et al.*, 1990). The other reaction components include magnesium ions for the essential source of divalent ions and thermostable *Taq* DNA polymerase with a 5' to 3' exonuclease activity (Sambrook *et al.*, 1989).

Primer 1 (forward) ¹⁵² Q A T K D A G ¹⁵⁸
 5' - CAR GCN ACN AAR GAY GCN GG - 3'

Primer 2 (reverse) ³⁶⁶ N P D E A V A ³⁷²
 5' - GC NAC NGC YTC RTC NGG RTT - 3'

Figure 3.3. Amino acid sequence of the conserved regions of hsp70 proteins and the corresponding degenerate oligonucleotide primers for PCR. The unusual bases in the nucleotide sequence refer to: Y, either T or C; R, either A or G; D, either T, A or G and N, either A, G, T or C (after Galley *et al.*, 1992).

3.4.1 Reagents

50 mM Magnesium chloride. MgCl₂ (0.102 g) was dissolved in 10 ml dist. H₂O, autoclaved and stored at -20°C in 1 ml aliquots.

10 mM dNTP mix. 100 mM dATP, dCTP, dGTP and dTTP (10 μl) were mixed with 60 μl sterile dist. H₂O and stored at -20°C.

10x PCR buffer. 160 mM (NH₄)₂SO₄, 670 mM Tris-HCl pH 8.8 and 0.1% (v/v) Tween-20 as supplied by the manufacturer.

3.4.2 Procedure

PCR amplification was performed using the Perkin-Elmer GeneAmp PCR system 2400 thermocycler according to the manufacturers instructions. PCR reaction mixtures were set up in 0.2 ml thin-walled PCR tubes as shown in Table 2. Control reactions were prepared by excluding template DNA, primers and *Taq* enzyme from separate mixtures and compensating the volume with sterile dist. H₂O. The amplification procedure consisted of an initial melting cycle at 94°C for 4 min; 30 cycles of 94°C melting for 1 min, 58°C annealing for 30 s, 72°C extension for 1 min; and a final long extension at 72°C for 5 min. After completion of the cycles, the samples were held at 4°C until removed from the thermocycler. Aliquots (5 μl) of the PCR reactions were analysed using agarose gel electrophoresis with ethidium bromide visualisation.

Table 2. Components of the PCR reaction mixture for amplification of the hsp70 probe from *M. hominis*.

Reagent	Initial concentration	Volume (μ l)	Final concentration
PCR buffer	10 x	5	1x
dNTP mix	10 mM	1	200 μ M
MgCl ₂	50 mM	2.5	2.5 mM
Primer 1	100 μ M	5	10 μ M
Primer 2	100 μ M	5	10 μ M
<i>Taq</i>	5U/ μ l	0.5	2.5 U
DNA template	100 ng/ μ l	1	100 ng
Dist. H ₂ O	-	30	-

3.5 Cloning the PCR product into the pGEM®-T vector

In order to sequence the hsp70 probe synthesised by PCR (section 3.4), the PCR product needed to be cloned into a suitable vector. The introduction of T-vectors has greatly improved the efficiency of cloning PCR fragments. Prior to this, cloning was achieved through inefficient blunt-ended ligations, or vector compatible restriction sites were incorporated into the PCR primers. However these methods required several enzymatic manipulations of both PCR product and vector to ensure compatible ends (Walsh, 1996). The T-vectors have terminal 3' thymidine residues which allow for direct cloning of PCR fragments generated by certain DNA polymerases. The pGEM®-T vector used in this case is generated by digesting the pGEM®-5Zf(+) vector with *EcoKV* which yields blunt termini within the multiple cloning site and provide a template for the addition of a terminal 3' thymidine to both ends (Promega, 1997). This system provides a compatible overhang for PCR products generated by particular thermostable polymerases including *Taq*, which through their terminal transferase activity, add a single deoxyadenosine residue to the 3' ends of the PCR product. The addition of the T residues to the 3' termini also prevents religation of the vector and hence the 5' ends have not been dephosphorylated (Mezei and Storts, 1994).

The pGEM®-T vector contains an open reading frame encoding the LacZ α peptide of (3-galactosidase interrupted by a multiple cloning site (Fig. 3.4). Together with the peptide encoded by the F' episome of the host cells, this peptide produces β -galactosidase through α -complementation when induced by isopropyl-P-D-thio-galactopyranoside (IPTG), which allows for blue/white colony selection. Bacterial cells producing this enzyme form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-(3-D-galactosidase (X-Gal). Transformed cells containing the vector with insert produced white colonies as the open reading frame has been disrupted by the insert (Sambrook *et al.*, 1989).

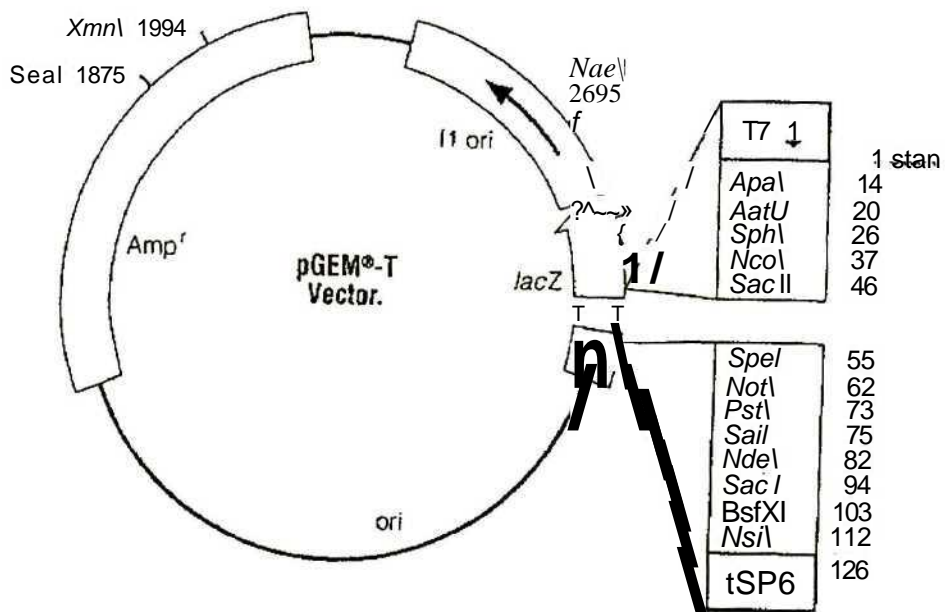


Figure 3.4. Vector map of the pGEM®-T cloning vector (after Promega, 1997).

3.5.1 PCR product preparation

Generally the primers used for PCR are synthetic oligonucleotides with 5'-OH ends, resulting in dephosphorylated PCR products. The cloning of PCR products into linearised phosphorylated vector requires the presence of 5'-phosphate groups on the amplified DNA fragment. This 5'-phosphorylation may be achieved by treating the PCR product with T4 polynucleotide kinase prior to ligation (Walsh, 1996). However the enzyme must be removed after completion of the reaction to prevent any subsequent interference.

3.5.1.1 Reagents

1 mM ATP. ATP (6.05 mg) was dissolved in 10 ml dist. H₂O, filter sterilised and stored at -20°C in 1 ml aliquots.

100 mM EDTA. 0.5 M EDTA (4 ml) was made up to 20 ml with sterile dist. H₂O.

3 M Sodium acetate, pH 5.3. CH₃COONa.3H₂O (4.92 g) was dissolved in 15 ml dist. H₂O, the pH adjusted to 5.3 with acetic acid, the volume was made up to 20 ml and autoclaved.

3.5.1.2 Procedure

A phosphorylation reaction containing PCR product (1 μ g), 10x polynucleotide kinase buffer (3 μ l), polynucleotide kinase (10 U), 1 mM ATP (3 μ l) and dist. H₂O to 30 μ l, was incubated at 27°C for 30 min. The reaction was stopped through the addition of 100 mM EDTA (1.5 μ l) and incubation at 75°C for 10 min. The volume was adjusted to 100 μ l with dist. H₂O and the mixture extracted with phenol:chloroform, followed by chloroform extraction. The aqueous phase was recovered, mixed with 3 M sodium acetate pH 5.3 (10 μ l) and cold ethanol (250 μ l) and incubated at -20°C for 1 hour. The DNA was precipitated by centrifugation at 13 000 Xg for 10 min. The pellet was washed with 70% (v/v) ethanol, spun at 13 000 Xg for 2 min and air dried. The DNA was dissolved in 10 μ l dist. H₂O and the concentration assessed through agarose gel electrophoresis.

3.5.2 Ligation and transformation

Ligation of a DNA fragment to a linearised plasmid vector depends on the formation of phosphodiester bonds between the terminal 5'-phosphate residues and the adjacent 3'-hydroxyl groups (Sambrook *et al.*, 1989). The relative concentrations of the DNA fragments, both insert and vector, are critical in determining the frequencies of specific ligations. A DNA fragment may either join to the end of the same molecule (intramolecular ligation) or to a separate DNA molecule (intermolecular ligation). The intermolecular ligation may then be between molecules of the same type (vector to vector or insert to insert) or between molecules of different types (vector to insert). Typically intermolecular ligation followed by intramolecular ligation is required to generate a final circular plasmid. The optimal cloning efficiency thus occurs at

DNA concentrations that are sufficient to enable intermolecular joining but low enough not to inhibit intramolecular ligation (Struhl, 1987). The following equation is used to calculate the amount of PCR product (insert) to include in the ligation reaction, depending on the molar ratio:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert: vector molar ratio} = \text{ng of insert}$$

The ligase of choice is T4 DNA ligase as it uses ATP as a cofactor rather than NAD which is required by *E. coli* ligase, and it is able to ligate blunt ends under normal reaction conditions (Sambrook *et al.*, 1989). The putative ligated vector-insert plasmids are then transformed into a suitable host cell with an appropriate selection method which may either depend on insertional inactivation or a-complementation (Sambrook *et al.*, 1989). The pGEM®-T vector system uses a combination in the form of blue/white screening as described in section 3.5, to identify colonies containing recombinant plasmids.

3.5.2.1 Reagents

2 M Glucose. Glucose (1.8 g) was dissolved in 5 ml dist. H₂O and filter sterilised.

2 M Mg²⁺. MgCl₂ (1.017 g) and MgSO₄ (1.232 g) were dissolved in 5 ml dist. H₂O and filter sterilised.

1 M Potassium chloride. KCl (0.745 g) was dissolved in 10 ml dist. H₂O and autoclaved.

SOC medium. Tryptone (0.4 g), yeast extract (0.1 g), 1 M NaCl (200 µl)» 1 M KCl (50 µl), 2 M Mg²⁺ (200 µl) and 2 M glucose (200 µl) were made up to 20 ml with sterile dist. H₂O.

0.1 M IPTG. IPTG (0.24 g) was dissolved in 10 ml dist. H₂O, filter sterilised and stored in 1 ml aliquots at -20°C.

X-Gal (80 mg/ml). X-Gal (80 mg) was dissolved in 1 ml N,N'-dimethylformamide and stored in the dark at -20°C.

Luria agar. Agar (15 g) was added to 11 Luria broth (section 3.4.1) and autoclaved.

Luria agar for blue/white screening. Luria agar was cooled after autoclaving, 100 mg/ml ampicillin (1 ml), 0.1 M IPTG (5 ml) and 80 mg/ml X-Gal (1 ml) were added and the medium poured into petri dishes.

3.5.2.2 Procedure

The ligation reactions were composed as in Table 3 and allowed to proceed at 4°C for 60 hours. Ligation efficiency was assessed through agarose gel electrophoresis and ligated fragments transformed into competent JM109 cells. The competent cells were also transformed with negative controls, no DNA and unligated vector. Aliquots of the ligation mixes (2 μ l) and the controls were incubated with 50 μ l competent JM109 cells on ice for 20 min. The transformation reaction was then incubated at 42°C for 50 s, followed by incubation on ice for 2 min. SOC medium (950 μ l) was added to the mix and incubated at 37°C for 2 hours. Aliquots (100 μ l) of the transformation mixes were plated onto Luria agar for blue/white screening, and incubated at 37°C overnight. Colonies were then screened for recombinant plasmids.

Table 3. Reaction components for the ligation of PCR product into pGEM®-T vector.

Reagent	Vector: insert	Vectoninsert
	1:5	1:10
T4 DNA ligase (3U/ μ l)	1 μ l	1H1
10X Ligase buffer	1 μ l	1fH
Vector (50 ng/ μ l)	1 μ l	1JH
PCR product (20ng/ μ l)	2.5 μ l	5 μ l
Dist. H ₂ O	4.5 μ l	2 μ l

3.5.3 Rapid colony screening

To identify putative recombinant plasmids, the plasmids from transformed bacterial colonies must be screened. The cracking procedure, as described by Titus (1991), allows rapid plasmid isolation for examination by agarose gel electrophoresis and was used in the present study.

3.5.3.1 Reagents

10 mM EDTA. 0.5 M EDTA (0.2 ml) was made up to 10 ml with sterile dist. H₂O.

4 M Potassium chloride. KCl (2.98 g) was dissolved in 10 ml dist. H₂O and autoclaved.

2X Cracking buffer. 1 N NaOH (10 ml), 10% (m/v) SDS (2.5 ml) and sucrose (10 g) were made up to 50 ml with sterile dist. H₂O.

3.5.3.2 Procedure

Individual colonies were toothpicked off agar plates and smeared near the bottom of microcentrifuge tubes containing 10 mM EDTA (50 μ l). Freshly made 2X cracking buffer (50 μ l) was added and the cells resuspended by vortexing. The samples were incubated at 70°C for 5 min and cooled to RT. 4 M KCl (1.5 μ l) and 0.4% (m/v) bromophenol blue (0.5 μ l) were added, the mixture incubated on ice for 5 min and then centrifuged for 3 min at 13 000 rpm. Aliquots of the samples were analysed using agarose gel electrophoresis.

3.5.4 Restriction analysis of putative recombinant clones

The pGEM®-T vector contains multiple restriction sites within the multiple cloning site (Fig. 3.4) which enables a double digestion to release the insert from the vector. Two enzymes with unique restriction sites near to and on either side of the point of insertion were selected, *SphI* and *NdeI*, to analyse the recombinant clones.

3.5.4.1 Procedure

Purified plasmid DNA from the putative recombinant clone was restriction digested with *SphI* and *NdeI* in the following reaction : DNA (900 ng), high salt buffer (1 μ l), *SphI* (0.5 μ l), *NdeI* (0.5 μ l) and dist. H₂O to a final volume of 10 μ l. The reaction was incubated at 37°C overnight and the digest examined on an agarose gel.

3.6 Hsp70 gene cloning

For cloning DNA that has simple structure and is smaller than 10 kb, the most reliable and efficient vectors are bacterial plasmids. The pUC vectors are the most versatile and widely used plasmid vectors, offering a number of important features such as direct selection for inserts; high copy number; multiple cloning site and controllable promoter (Sambrook *et al.*, 1989). A typical pUC vector contains an ampicillin resistance gene, part of a β -galactosidase gene with a multiple cloning site and an origin of replication. Combining these features with the M13 bacteriophage replication origin, allows for the construction of M13-plasmid hybrid vectors or phagemids (Howe, 1995). The pGEM® vectors are examples of such recombinant vectors.

The pGEM®-7Zf vector (Fig. 3.5) has the ampicillin resistance gene as a selective marker, a multiple cloning site and the typical plasmid origin of replication enabling standard double-stranded DNA cloning. The vector system also incorporates blue/white screening of recombinants through insertional inactivation of the *lacZ* gene. The presence of the T7 and SP6 *E. coli* bacteriophage promoters allows for transcription of cloned DNA sequences. The vector includes the origin of replication of the filamentous bacteriophage *fl* for the production of single-stranded DNA (Promega, 1996).

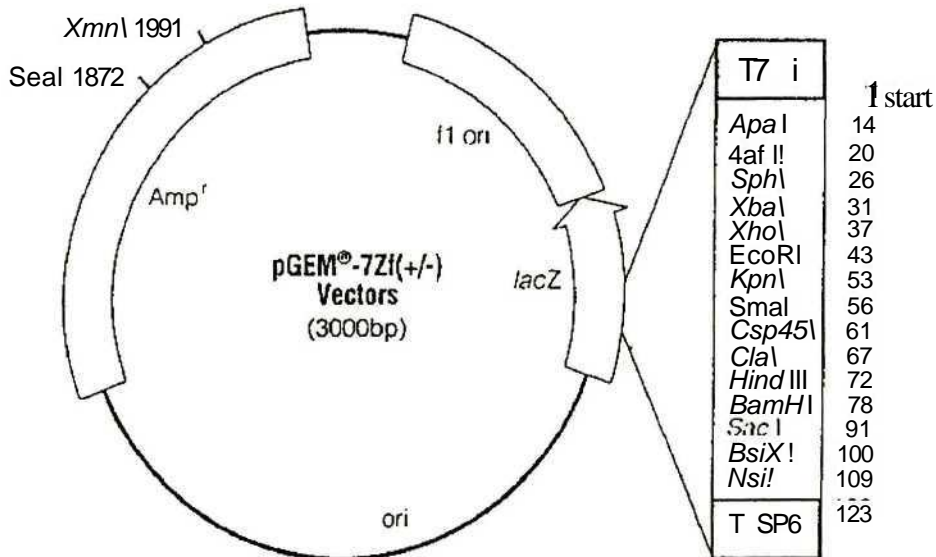


Figure 3.5. Vector map of the pGEM®-7Zf cloning vector (after Promega, 1996).

Using the general cloning method of Gupta and Singh (1992) and the cloning protocols recommended by Promega (Madison, USA), the pGEM®-7Zf(+) vector was used for cloning the fragments of *M. hominis* DNA encoding the hsp70 gene.

3.6.1 Identification of gene segment encoding hsp70

To identify the DNA fragment/s containing the gene encoding for hsp70, *M. hominis* chromosomal DNA was digested with a range of restriction enzymes corresponding to those found in the multiple cloning site of the pGEM®-7Zf vector. The digests were blotted and probed with the labelled PCR product generated using specific degenerate primers (section 3.4).

3.6.1.1 Procedure

Purified *M. hominis* DNA was digested with *Apa*I, *Bam*HI, *Bgl*II, *Eco*KL, *Kpn*I, *Hind*UI, *Sma*I and *Xba*I in separate reactions. The restriction digests contained DNA (1.5 µg), restriction enzyme (50 U), 10X restriction buffer (2 µl) and dist. H₂O to a final volume of 20 µl. All digests were incubated at 37°C overnight, with the exception of *Sma*I digests which were incubated at 30°C. Following incubation, the digests were run on agarose gels and transferred to nylon membrane for Southern blotting (section 3.3.7). The DIG-labelled (section 3.3.6) hsp70 PCR product was allowed to hybridise to the membrane bound DNA. The blot was then

detected using the DIG-detection kit as described in section 3.3.7. The regions containing the restriction fragments of interest were gel purified (section 3.3.3), quantified and used as insert DNA for the subsequent ligation reactions.

3.6.2 pGEM®-7Zf vector preparation

Cloning of DNA fragments with identical ends into plasmid vectors can only be successful if the vector is linearised and has compatible termini. Therefore following linearisation of the vector through restriction digestion, dephosphorylation is essential to prevent self-ligation and recircularisation of the plasmid vector (Sambrook *et al*, 1989). Alkaline phosphatase catalyses the hydrolysis of the 5'-phosphate residues from both termini of linear DNA. Calf intestine alkaline phosphatase (CIAP) is most commonly used as it is readily inactivated by heating (Tabor, 1987).

3.6.2.1 Reagents

7.5 M Ammonium acetate. Ammonium acetate (5.781 g) was dissolved in 10 ml dist. H₂O and filter sterilised.

3.6.2.2 Procedure

The pGEM®-7zf vector (1 μ g) was mixed with restriction enzyme (30 U), either *Apal* or *BamHI*, 10X restriction buffer (2 μ l) and dist. H₂O to a final volume of 20 μ l and incubated at 37°C for 2 hours. The digested vector was dephosphorylated through the addition of CIAP (0.01 U), 10X CIAP buffer (10 μ l) and dist. H₂O to a final volume of 100 μ l, and incubation at 37°C for 30 min. To terminate the reaction, 0.5 M EDTA (2 μ l) was added and the mix incubated at 75°C for 10 min. One volume of phenol:chloroform was added, the reaction mix vortexed and centrifuged at 12 000 rpm for 2 min in a micro fuge. The aqueous phase was extracted again with one volume of chloroform. 7.5 M Ammonium acetate (50 μ l) and 2 volumes of absolute ethanol were added to the recovered aqueous phase and incubated at -70°C for 30 min. The solution was centrifuged at 12 000 rpm for 15 min in a micro fuge and the pellet washed in 70% (v/v) ethanol. After air drying, the pellet was resuspended in 10 μ l dist. H₂O and the DNA concentration determined by agarose gel electrophoresis.

3.6.3 Ligation and transformation

The ligation of restriction fragments into linearised dephosphorylated vector follows the same principles as discussed in section 3.5.2. The pGEM®-7Zf vector system also uses blue/white colour screening for recombinants.

3.6.3.1 Reagents

500 mM Dithiothreitol (DTT). DTT (0.386 g) was dissolved in 5 ml dist. H₂O and filter sterilised.

5X Ligase buffer [150 mM Tris-HCl pH 7.8, 50 mM MgCl₂, 50 mM DTT, 5 mM ATP]. 1 M Tris-HCl pH 7.8 (9 ml), 1 M MgCl₂ (3 ml), 500 mM DTT (6 ml) and 10 mM ATP (30 μ l) were made up to 60 ml with dist. H₂O and stored at -20 °C.

Luria broth. See section 3.3.4.1.

Luria agar for blue/white screening. See section 3.5.2.1.

3.6.3.2 Procedure

The appropriate amount of insert required for the molar ratios of vector to insert was calculated as in section 3.5.2. The ligation reactions were set up as in Table 4 and incubated at 4°C overnight. Ligation efficiency was assessed through agarose gel electrophoresis and the putative recombinant plasmids transformed into competent JM109 cells (section 3.3.4). The competent cells were also transformed with various controls: no DNA; uncut vector; cut linearised vector and cut, dephosphorylated self-ligated vector. Aliquots of the ligation mixes (2 μ l) and the controls were mixed with the competent cells (200 μ l) and incubated on ice for 30 min. The mixtures were heated at 42°C for 50 s and cooled on ice for 2 min. Luria broth (3 ml) was added and the mixtures incubated at 37°C with shaking for 45 min. Aliquots (100 μ l) of the transformation mixes were plated onto Luria agar for blue/white screening and incubated at 37°C overnight. Resultant colonies were screened for recombinant plasmids.

Table 4. Reaction components for the ligation of *M. hominis* restriction fragments into pGEM®-7Zf vector.

Reagent	Vector: insert 1:1	Vector:insert 1:3	Vector:insert 4:1	Self-ligated vector
Vector (50ng/ il)	2 M	2 pi	2 il	1 il
Insert (50ng/ il)	2 il	5 il	0.5 il	-
T4DNALigase(1U/ il)	1 il	1 H	iin	1 il
5X Ligase buffer	2 il	2 il	2 il	2 il
Dist. H ₂ O	3 U	-	4.5 il	5 il

3.6.4 Colony screening

A selection of transformed JM109 colonies were screened using the cracking procedure (section 3.5.3) and examined through agarose gel electrophoresis. The isolated plasmids were transferred onto nylon membrane and probed with DIG-labelled PCR product (section 3.3.6). The blots were hybridised with the probe and then detected with the DIG detection system (section 3.3.7). Colonies that carried positive recombinants were transferred to Luria broth containing 100 |ig/ml ampicillin and cultured at 37°C overnight. The recombinant plasmids were isolated as described in section 3.3.5 and digested with the appropriate restriction enzyme to release the insert. The digests were examined on agarose gels and then blotted and probed as before. Aliquots of transformed cells containing positive recombinants were stored at -70°C.

3.7 Restriction mapping

To obtain a partial restriction map of the hsp70 clone, the recombinant plasmid was digested with a number of restriction enzymes, which either had no, or unique restriction sites within the vector sequence. Enzymes with compatible buffer requirements and incubation temperatures were also used in double digests. Restriction digests were made up of restriction enzyme (10 U), 10X restriction buffer (1 |il), plasmid DNA (500 ng) and dist. H₂O to a final volume of 10 |il, and the digests incubated at suitable temperatures overnight. The digests were analysed through agarose gel electrophoresis.

3.8 DNA sequencing

Currently two different DNA sequencing approaches exist, the enzymatic method of Sanger and the chemical degradation method of Maxam and Gilbert. The enzymatic method uses a DNA polymerase to synthesise a labelled, complementary strand of the DNA template, while the chemical degradation method subjects a labelled DNA strand to a series of base-specific chemical reactions (Sambrook *et al.*, 1989).

The Sanger method of dideoxy-mediated chain termination involves the use of a specific primer for complementary strand extension by DNA polymerase, base-specific chain-terminating dideoxynucleotides (ddNTPs), and polyacrylamide gels to resolve the labelled DNA chains (Sambrook *et al.*, 1989). With the development of Sequenase™ enzyme, a chemically modified form of T7 DNA polymerase, a modified labelling/termination method was established to take full advantage of the high processivity of the enzyme. Initially a limiting amount of the four dNTPs, one of which is radiolabelled, and Sequenase™ are added to the single-stranded DNA template. DNA synthesis proceeds until the dNTPs are exhausted and then the reaction mix is divided into four aliquots and mixed with more dNTPs and a ddNTP. Synthesis continues until specifically terminated by incorporation of a ddNTP (Ausubel *et al.*, 1990).

DNA fragments cloned into an M13-derived vector or phagemid can be sequenced as denatured double-stranded templates using this method. Universal M13 primers which anneal to sequences within the vector, are readily available (Sambrook *et al.*, 1989). Autoradiography enables the detection of bands generated by manual sequencing and commonly the radioactive label used is ³⁵S which is incorporated as [α -³⁵S]dATP at the chain elongation step. As ³⁵S is a weak β emitter producing less scatter, there is relatively little loss in resolution from the gel to the autoradiograph (Ausubel *et al.*, 1990). The DNA fragments generated by these enzymatic reactions are resolved on denaturing polyacrylamide gels, which allows up to 500 nucleotides of a sequence to be read (Sambrook *et al.*, 1989). Automated DNA sequencing incorporates fluorescein labels, such as carbocyanines, into DNA allowing automated detection of sequencing bands. The carbocyanines may either be used as dye-primers or dye-terminators, although the former is the preferred method due to the chemical changes induced in the dideoxy terminators by the bulky dye molecules (Chen, 1994).

3.8.1 Reagents

2 M NaOH/2 mM EDTA pH 8.0. NaOH (0.8 g) was dissolved in 8 ml dist. H₂O, 0.1 M EDTA pH 8.0 (0.2 ml) added and the volume made up to 10 ml with dist. H₂O. The solution was autoclaved and stored at RT in 1 ml aliquots.

2 M Ammonium acetate pH 4.6. Ammonium acetate (1.54 g) was dissolved in 5 ml dist. H₂O and the pH adjusted to 4.6 with glacial acetic acid. The volume was made up to 10 ml with dist. H₂O, filter sterilised and stored at RT in 1 ml aliquots.

Silanising solution \5% (v/v) dimethyldichlorosilane in chloroform. C¹⁸HgCkSi (5 ml) was made up to 100 ml with chloroform in a fume hood.

10X Tris-borate-EDTA (TBE) buffer. Tris-HCl (108 g) and boric acid (55 g) was dissolved in 800 ml dist. H₂O, 0.5 M EDTA (44 ml) added, the volume was made up to 1 l and the solution autoclaved.

6% (m/v) Acrylamide gel mix. Acrylamide (5.7 g), bis-acrylamide (0.3 g), urea (48 g) was dissolved in 10X TBE (10 ml) and 40 ml dist. H₂O, and filter sterilised.

50% (m/v) Ammonium persulfate. Ammonium persulfate (0.5 g) was dissolved in 1 ml dist. H₂O.

3.8.2 Procedure

The double-stranded plasmid DNA template was denatured using alkaline denaturation. Plasmid DNA (4 ng) was made up to 18 μ l with dist. H₂O and 2 M NaOH/2 mM EDTA (2 μ l) added, mixed and incubated at RT for 5 min. The alkaline solution was neutralised with the addition of 2 M ammonium acetate pH 4.6 (2 μ l). The DNA was precipitated with ice cold absolute ethanol (75 μ l) and incubation at -70°C for 15 min. The DNA was pelleted by centrifugation at 15 000Xg for 15 min and the pellet washed with 70% (v/v) ethanol. The supernatant was removed and the pellet dried for approximately 1 min under vacuum and dissolved in 7 μ l sterile dist. H₂O.

To allow template and primer annealing, denatured DNA (7 μ l), SequenaseTM reaction buffer (2 μ l) and M13 sequencing primer (1 μ l) were mixed and incubated at 65°C for 10 min. The reaction mix was then incubated at 37°C for 30 min and allowed to cool to RT. Just before use, labelling mix and diluted SequenaseTM were prepared by adding 5X concentrated labelling mix (5 μ l) to 20 μ l dist. H₂O, and diluting SequenaseTM (1 μ l) in dilution buffer (7 μ l) respectively. To the annealed template-primer mix, 0.1 M DTT (1 μ l), diluted labelling mix (2 μ l), 10 mCi/ml [³⁵S]-dATP (1.5 μ l) and diluted SequenaseTM (2 μ l) were added, mixed and incubated at RT for 5 min. The labelling reaction was terminated with the addition of labelling mix (3.5 μ l) to separate tubes of each ddNTP (2.5 μ l). The mixes were pulse-spun in a microcentrifuge and incubated at 37°C for 5 min. Stop solution (4 μ l) was added to each of the four tubes, mixed thoroughly and stored on ice until loaded onto the sequencing gel.

The glass plates used for the sequencing gel were thoroughly cleaned with suitable detergent followed by 70% (v/v) ethanol. The shorter plate was treated with silanising solution and dried in a fume hood. After a final ethanol rinse, spacers were greased and placed along the sides of the longer plate. The shorter plate was placed on top of the long plate, the spacers aligned and the plates clamped and sealed. The 6% (m/v) acrylamide gel mix (75 ml) was mixed with 50% (m/v) ammonium persulfate (67 μ l) and TEMED (67 μ l). Holding the plate assembly vertical, the gel solution was slowly poured down one side, taking care to gently remove any air bubbles and allowed to set overnight.

The sequencing gel was set up in the S2 Gibco BRL Sequencing system (Gibco BRL, Paisley) with IX TBE buffer. A sharktooth comb was placed at the top of the gel with the teeth penetrating the gel to a depth of about 1 mm. The wells formed in the gel were washed and the gel pre-electrophoresed for 15-30 min at 130 W and 2 kV. The samples were heated at 75-80°C for 3 min and 3 μ l of each sample loaded. The gel was electrophoresed for 1.5 hours at 130 W and 2 kV.

After completion of the run, the apparatus was disassembled and the shorter plate removed. A sheet of Whatmann 3 MM filter paper was placed on the gel and the gel lifted from the plate. The gel was covered with a film of clingwrap and dried for 1.5 hours at 80°C on a Hoefer Gel

Drier (Hoefler Scientific, San Francisco). The gel was then exposed to X-ray film in a dark room and left to expose overnight at RT. The autoradiograph was developed in X-ray developer for 4 min and rinsed in H₂O for 30 s. The film was fixed in X-ray fixer for at least 2 min, washed in running water for 10 min and hung up to dry.

3.9 Results

3.9.1 PCR amplification of the hsp70 probe

Purified *M. hominis* DNA was subjected to PCR with specific degenerate primers and produced a PCR product of approximately 600 bp (Fig. 3.6), which compared favourably with the expected size based on the location of the primers in the hsp70 sequence (Galley *et al.*, 1992).

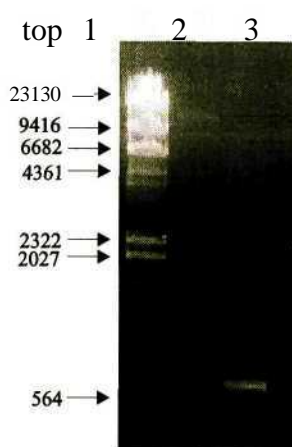


Figure 3.6. **PCR** amplification of the hsp70 conserved fragment from *M. hominis* DNA. (1) *HindIII* DNA size marker; (2) purified *M. hominis* DNA; (3) PCR product.

3.9.2 PCR cloning

Attempts at direct sequencing of the PCR product were unsuccessful, therefore it was necessary to clone it into a suitable vector for sequencing. High concentration ratios of insert to vector DNA (5:1, 10:1) were required to achieve ligation between the PCR product and the pGEM®-T vector (Fig. 3.7). Competent JM109 cells transformed with the ligation mixes produced a mixture of blue and white colonies which were screened for possible recombinants with the cracking procedure (Fig 3.8). The negative control, cells transformed without DNA, produced no growth while cells transformed with only vector, produced only blue colonies.

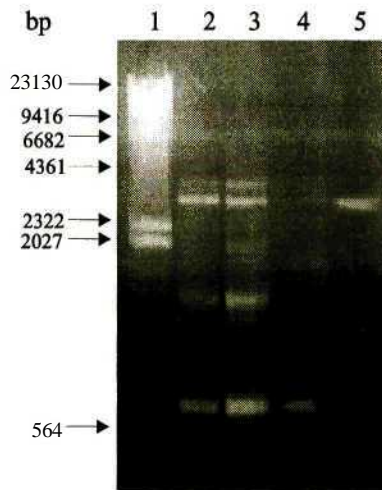


Figure 3.7. Ligation mixes of hsp70 PCR product and pGEM®-T vector. (1) *HindIII* X DNA size markers; (2) 5:1 insert to vector ligation; (3) 10:1 insert to vector ligation; (4) PCR product; (5) pGEM®-T vector.

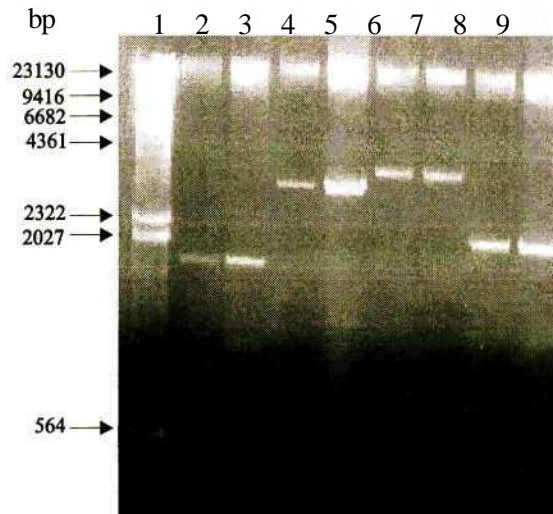


Figure 3.8. Rapid screening of plasmids isolated from transformed JM109 cells. (1) *HindIII* X DNA size markers; (2), (3) plasmid from cells transformed with vector only; (4), (5) plasmid from a white colony of 10:1 insert to vector transformants; (6), (7) plasmid from a blue colony of 10:1 insert to vector transformants; (8), (9) plasmid from a white colony of 5:1 insert to vector transformants. These last two lanes represent the putative recombinant plasmid.

The putative recombinant plasmids were isolated, purified and analysed through Southern blotting and restriction digests. Southern blot analysis (Fig. 3.9A) where the plasmids were probed with labelled PCR product, confirmed the presence of the insert in the putative recombinant. The restriction analysis with enzymes selected to release the insert, produced an insert of the expected size and revealed an internal *NdeI* or *SphI* site within the insert (Fig. 3.9B).

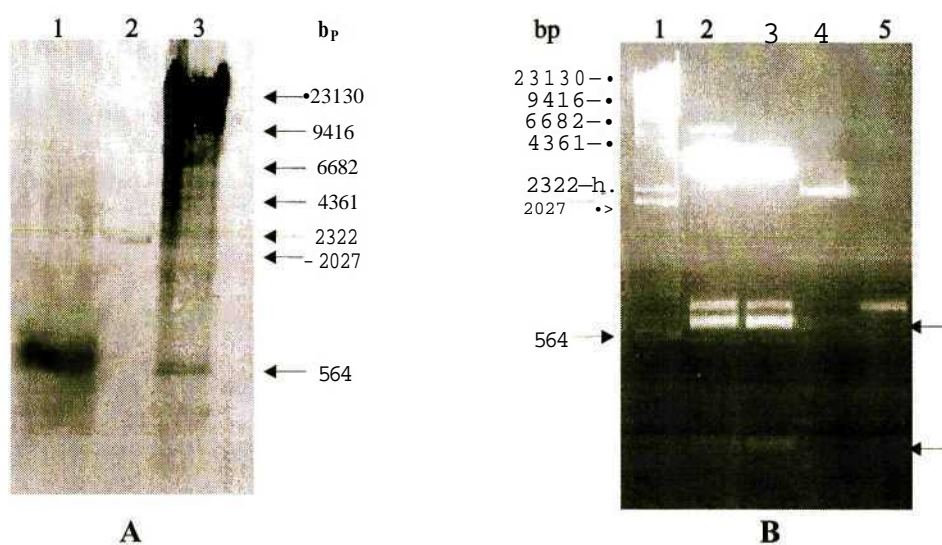


Figure 3.9. Analysis of the putative recombinant plasmid. A, Southern blot analysis of plasmids probed with labelled PCR product. (1) PCR product; (2) recombinant plasmid DNA; (3) *HindIII* X DNA size markers. B, Restriction analysis of the recombinant plasmid. (1) *HindIII* X DNA size markers; (2), (3) *NdeI*, *SphI* digested plasmid; (4) undigested plasmid DNA; (5) PCR product. The arrows indicate fragments generated by digestion of an internal restriction site.

3.9.3 PCR product sequencing

The hsp70 PCR product cloned into pGEM®-T was partially sequenced using M13 universal forward and reverse primers in manual sequencing (Fig. 3.10).

A:

```

5'- TGCCACAGCT  TCGTCTGGAT  TCACGTCCTT  GCGGGCGTCG  CGGCCGAAGA
    ACTCGCGCAC  CTGCTCCTGC  ACCTTCGGCA  TG-iCGCGTCAT  GCCGCCGACC
    AGGATCACGT  CGTCGATGTC  GCTGACCTTG  ACGCCACAGT  CcTTgATCGC
    GATNGgCACG  GTTCGATGGT  GCGGGTGATC  AgCTCCTG . . . GGCGATCTCn
    GATGATCGAG  ATGTCGAAGG  TACCgcNGCC  GAGGTCATAC  ACcGCGATCT
    TGCGGTCCG  CTTCTCGTTC  TTGTCCATGC  CGAAGGCCAg  NGcGGCCGCG
    GTCGGCTCGT  TGATGATGCG  CTTGACCTCC  AGGCCGGCGA  TGggGCCCGC
    GTCTTTAGTA  GCCTG -3'

```

B:

```

QATKDAGPIAGLEVKRIINEPTAAALAFGMDKNEKGDRKIAVYDLGGTFDISII ... IAIKD
VGVKVSDIDDVILVGGMTRMPKVQEQRVREFFGRDARKDVNPDEAVA

```

Figure 3.10. Preliminary partial sequence of the hsp70 probe. A, nucleotide sequence. Lower case letters denote unsure bases, N denotes the presence of a base which could not be determined and the dots indicate an unknown length of sequence. The arrow indicates the position of the internal *SphI* restriction site; B, amino acid sequence displayed in standard single letter code.

The sequence homology of the *M. hominis* hsp70 probe to other sequences was investigated using the BLAST (Basic local alignment search tool) program to search sequence databases (Table 5.) and indicate the alignment of the amino acid sequence to other mycoplasma hsp70s (Fig. 3.11).

Table 5. Representative results of the BLAST search for sequences similar to the partial *M. hominis* hsp70 probe sequence.

Sequences with homologous regions	P-value*
<i>Ralstonia eutropha</i> dnaK gene	6.6e-65
<i>Pseudomonas cepacia</i> hsp70 gene	8.9e-50
<i>Caulobacter crescentus</i> hsp (dnaK) genes	1.3e-35
<i>Rhizobium meliloti</i> hsp70 gene	2.5e-31
<i>Bradyrhizobium japonicum</i> dnaK gene	9.0e-29
<i>Thermomicrobium roseurn</i> hsp70 gene	1.1e-28
<i>Brucella ovis</i> hsp70 gene	4.4e-27
<i>Rhodopseudomonas</i> sp. dnaK gene	5.7e-26
<i>E. coli</i> dnaK gene	5.5e-24
<i>Halobacterium cutirubum</i> hsp70/dnaK gene	9.6e-24
<i>Salmonella typhimurium</i> dnaK gene	1.1e-23
<i>Crithidia fasciculata</i> mitochondrial hsp mRNA	1.5e-23
<i>Plantomyces maris</i> dnaK gene	1.0e-20
<i>Leishmania major</i> hsp70 gene	1.2e-20
<i>Agrobacterium tumefaciens</i> dnaK gene	2.1e-19
<i>Leishmania amazonensis</i> hsp70 gene	5.9e-19
<i>Pirellula marina</i> dnaK gene	1.4e-18
<i>Trypanosoma congolense</i> P69 antigen gene	1.1e-17
<i>Mesocestoides corti</i> hsp70 gene	5.1e-17
<i>Rhodobacter capsulatus</i> hsp70 gene	8.0e-14
<i>Vibrio cholerae</i> dnaK (hsp70) gene	1.2e-12
<i>Trypanosome cruzi</i> mitochondrial hsp70 gene	6.7e-11
<i>Drosophila melanogaster</i> hsp70 gene	6.7e-10
<i>Leptospira interrogans</i> heat shock gene	2.0e-09
<i>Mycobacterium tuberculosis</i> dnaK gene	3.7e-09
<i>Mycobacterium paratuberculosis</i> hsp70 gene	3.7e-09
Mouse heat-shock protein gene	2.1e-08
<i>Trypanosoma brucei</i> hsp70 gene	2.1e-08

Table 5. Continued.

Sequences with homologous regions	P-value*
Human heat shock protein HSPA2 gene	2.1e-08
<i>Neurospora crassa</i> major stress-induced protein gene	1.0e-07
Pig hsp70 gene	1.2e-07
<i>Rattus norvegicus</i> hsp70 gene	1.2e-07
<i>Haemophilus ducreyi</i> hsp70 gene	3.1e-07

* The P-value represents the probability that the sequence homology is a coincidence, with values close to zero indicating true homology. The values are presented in scientific notation i.e. 6.6e-65 is 6.6×10^{65} .

Mycoplasma hominis (mh) vs *Mycoplasma capricolurn* (me) 70% identity

```

mh 1  QATKDAGPIAGLEVKRIINEPTAAALAFGMDKNEKGDRKIAVYDL-GGTFDISII 54
      ATKDAG IAGL V RIINEPTAAALA G DK K I VYDL GGTFD SI
me 128 KATKDAGTIAGLQVERIINEPTAAALAYGLDKQDK-EETDLVYDLGGGTFDVSIL 181

```

Mycoplasma hominis (mh) vs *Mycoplasma genitalium* (mg) 69% identity

```

mh 2  ATKDAGPIAGLEVKRIINEPTAAALAFGMDKNEKGDRKIAVYDL-GGTFDISII 54
      ATK AG IAGL V RIINEPTAAALA G DK K VYDL GGTFD S
mg 136 ATKTAGKIAGLNVERIINEPTAAALAYGIDKASR -EMKVLVYDLGGGTFDVSLL 188

```

Mycoplasma capricolum (me) vs *Mycoplasma genitalium* (mg) 79% identity

```

me 128 KATKDAGTIAGLQVERIINEPTAAALAYGLDKQDKEETILVYDLGGGTFDVSIL 181
      ATK AG IAGL VERIINEPTAAALAYG DK E LVYDLGGGTFDVS L
mg 136 ATKTAGKIAGLNVERIINEPTAAALAYGIDKASREMKVLVYDLGGGTFDVSLL 188

```

Mycoplasma hominis (mh) vs *Ralstonia eutropha* (re) 92% identity

```

mh 1  QATKDAGPIAGLEVKRIINEPTAAALAFGMDKNEKGDRKIAVYDL-GGTFDISII 54
      QATKDAG IAGL VKRIINEPTAAALAFGMDKNEKGDRKIAVYDL GGT DISH
re 152 QATKDAGRIAGLDVKRIINEPTAAALAFGMDKNEKGDRKIAVYDLGGGTLDISII 206

```

Figure 3.11. Amino acid alignment of mycoplasma hsp70 segments.

3.9.4 Hsp70 gene cloning

Probing restriction digests with the labelled PCR product (Fig. 3.12) identified the *M. hominis* DNA fragment encoding the hsp70 gene.

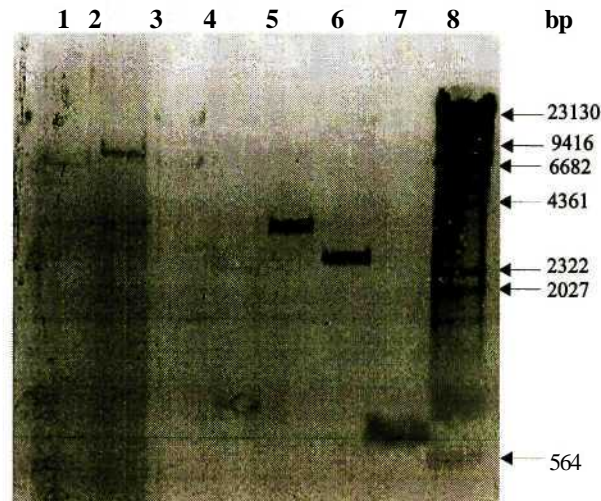


Figure 3.12. Southern blot of restriction digests hybridised to labelled hsp70 probe. (1-6) *KpnI*, *HindIII*, *EcoRI*, *BglI*, *BamHI* and *ApaI* restriction fragments of *M. hominis* DNA; (7) Hsp70 PCR product; (8) *HindIII* A, DNA size marker.

The *ApaI* and *BamHI* fragments were ligated to the pGEM®-7zf(+) vector in 1:1, 1:3 and 4:1 vector to insert concentration ratios (Fig. 3.13) and transformed into competent JM109 cells. The transformants were screened for recombinant plasmids using the rapid screening protocol and Southern blotting (Fig. 3.14). Transformation with the *ApaI* ligation mixes were more successful than the *BamHI* ligation mixes, and the negative controls of cells transformed with no DNA and cut vector did not produce any growth.

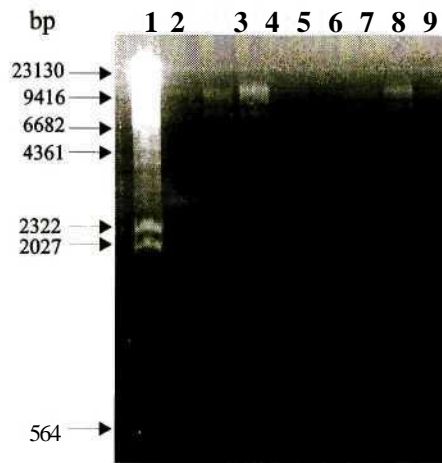


Figure 3.13. Ligation reactions between restriction fragments and pGEM®-7zf vector. (1) *HindIII* X DNA size markers; (2-5) *Apal* fragments and *Apal* cut vector; (5-9) *BamHI* fragments and *BamHI* cut vector; (2), (6) cut dephosphorylated vector; (3), (7) 1:1 vector to insert ratio; (4), (8) 1:3 vector to insert ratio; (5), (9) 4:1 vector to insert ratio.

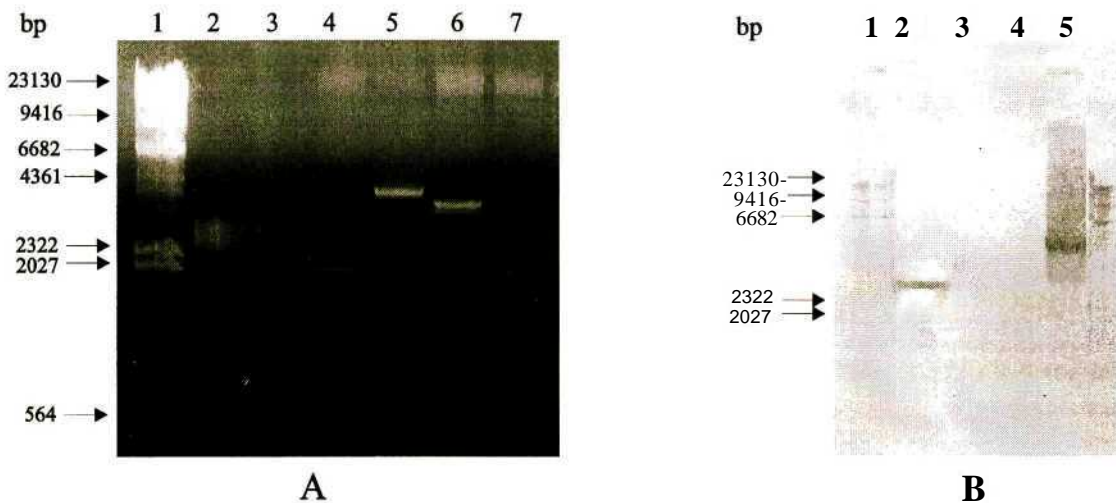


Figure 3.14. Screening of recombinant *Apal*/hsp70 plasmids. A, Agarose gel electrophoresis and B, Southern blotting. (1) *HindIII* X DNA size markers; (2) *Apal* restriction fragment; (3) *Apal* cut pGEM®-7zf vector; (4-7) plasmids isolated from cells transformed with *Apal* insert to vector ratio 1:1.

The recombinant plasmid which hybridised with the probe (Fig. 3.14B) was isolated, purified and analysed further through restriction digestion and Southern blotting (Fig. 3.15). Restriction digestion of the plasmid with *Apal* released a fragment of the expected size that hybridised with the hsp70 probe.

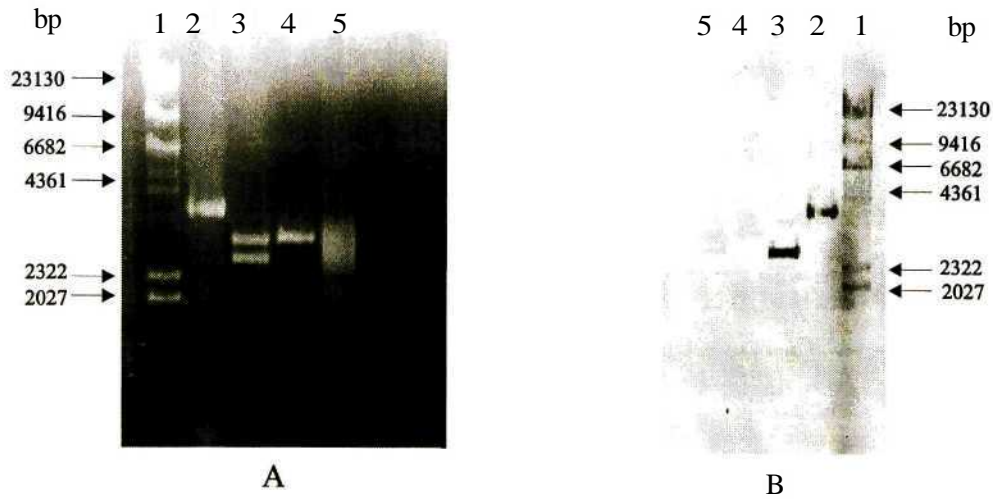


Figure 3.15. Analysis of the putative recombinant hsp70 clone. A, Restriction digestion. B, Southern blot of the plasmid and restriction fragments. (1) *HindIII* X DNA size markers; (2) recombinant plasmid; (3) *Apal* digested plasmid; (4) *Apal* digested vector; (5) *Apal* insert restriction fragments from *M. hominis* DNA.

3.9.5 Recombinant hsp70 plasmid sequencing

The recombinant *Apal* plasmid was sent for automated DNA sequencing using the Pharmacia ALF-Express automated DNA sequencer, which proved unsuccessful. Therefore manual sequencing with M13 universal forward and reverse primers was used for preliminary sequencing of the hsp70 insert DNA (Fig. 3.16).

```

5'-  GGCCCCATAG  ACCCGAAAGT  TCTGTTCCCA  CACACGCTGA  ATNTTCACCA
      GAAGTTGTTC  ATCCCTCTTG  GCTCGTAGGC  TTCgAGTTCC  GGATTCCGCA
      TCGggCAGCA  TATCcCCGGT  ACgtGACgGg  gCGACCTGCA  ACACCTTGCA
      GATCggCTCC  GACCCCATAC  AGGCCCCGAT  GCTGGTCGAT  GTAGGCGTTT
      ATTTCTTCAG  T.....CT  ACNCCTTAGg  CCTTGAgcTT  cNGATGCTc
      tTTCTCCCTA  CTTGTTGAAG  ACCACTTATA  AGTCcCACAC  ACCCTTGTCT
      TGAAAGCCCA  GATACCCCGG  -3'

```

Figure 3.16. Preliminary partial DNA sequence of the *M. hominis* hsp70 clone obtained using manual sequencing. Lower case letters indicate unsure bases, N denotes the presence of a base which could not be determined and the dots indicate an unknown length of sequence.

A partial restriction map of the hsp70 recombinant clone was determined through restriction analysis using single and double restriction digests (**Fig. 3.17**).

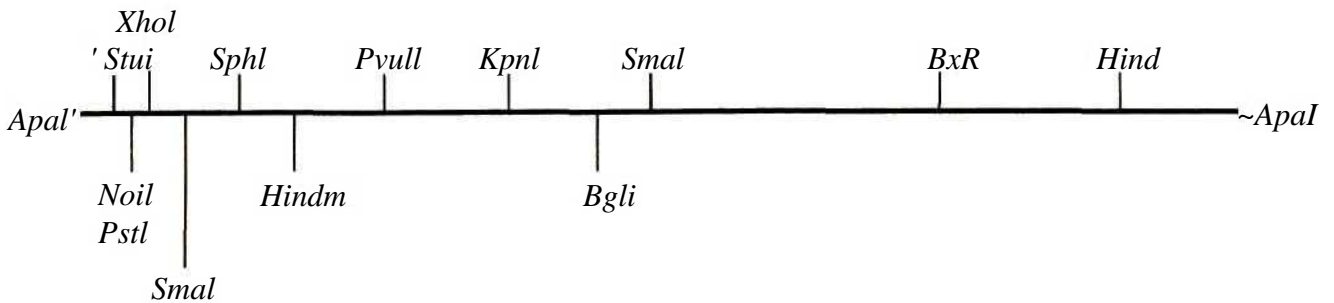


Figure 3.17. Partial restriction map of the *M. hominis* hsp70 recombinant clone.

3.10 Discussion

With the elucidation of the unprecedented degree of conservation among members of the hsp70 family and their significance in cell structure and function and in immunological roles, the cloning and sequencing of hsp70 genes from different species has gained impetus. As mycoplasmas are the smallest free-living organisms, the conservation of the hsp70 gene is also of interest as an evolutionary indicator.

Using PCR and the degenerate primers specific for conserved regions of hsp70, a DNA fragment of approximately 600 bp was amplified from *M. hominis* PG21 (Fig. 3.6). Based on the location of the primers within the hsp70 sequence, this fragment corresponded with the expected size (Galley *et al.*, 1992). Confirmation of the identity of the PCR product required determination of its nucleotide sequence. Although direct sequencing of PCR products is possible, it was unsuccessful in this study. A major problem in sequencing linear double-stranded DNA is the renaturation of the template which affects the annealing of the primer (Casanova, 1996). This problem can be overcome with excess primer but this is difficult to achieve when using degenerate primers that require much higher molar excess to compensate for their degeneracy. Therefore in order to sequence the PCR product, it was necessary to clone it into a plasmid vector. As the PCR product was amplified using *Taq* polymerase, which

incorporates terminal deoxyadenosine residues, it was cloned into the pGEM®-T vector based on T-A cloning. Following ligation (Fig. 3.7) and transformation into appropriate host cells (Fig. 3.8), recombinant plasmids were identified for sequencing using Southern hybridisation and restriction analysis (Fig. 3.9).

Partial nucleotide sequencing of the PCR product determined a unique yet homologous hsp70 partial sequence (Fig. 3.10) and confirmed the presence of an internal *SphI* restriction site as indicated by restriction analysis (Fig. 3.9B). Hybridisation of the PCR product to *M. hominis* restriction digested chromosomal DNA in Southern blot analysis (Fig. 3.12), confirmed the origin of the hsp70 fragment as *M. hominis*. The results of a BLAST similarity search indicated a high degree of homology between the *M. hominis* hsp70 probe and the hsp70 homologs of many other species (Table 5.). The *M. hominis* hsp70 fragment demonstrated a high degree of similarity with the hsp70s of *M. capricolum* and *M. genitalium*, although it appeared to have greater identity with other bacterial hsp70s, particular *Ralstonia eutropha* (Fig. 3.11, Table 5.). Phylogenetic analysis currently assigns *M. capricolum* and *M. genitalium* to the *M. mycoides* and *M. pneumoniae* phylogenetic branches respectively (Weisburg *et al.*, 1989; Maniloff, 1992). These mycoplasma phylogenetic groups are the least closely related to the *M. hominis* group (Fig. 1.2), therefore the extent of identity between their hsp70s correlates to their phylogeny, and compliments the evidence that *M. hominis* has diverged significantly. As the amplified hsp70 probe is derived from the most highly conserved segments of the hsp70 protein, the homology with other bacteria, particularly other gram-negative eubacteria such as *Ralstonia eutropha*, is not unexpected. The range of species that display homology within this hsp70 segment, demonstrates the nature of universal hsp70 conservation, with homology between hsp70s from bacteria, fungi, protozoa, insects, rodents and humans.

Using the labelled hsp70 probe, two restriction fragments that displayed strong hybridisation, were identified for cloning (Fig. 3.12). The 2.4 kb *ApaI* and 3.0 kb *BamEI* fragments were also of an appropriate size: sufficiently small to manipulate into a plasmid vector but larger than the expected size of the gene. The *M. capricolum* hsp70 gene has been determined as 1.8 kb (Falah and Gupta, 1997). Following ligation (Fig. 3.13) and transformation (Fig. 3.14), putative *ApaI* recombinant plasmids were identified and found to release cloned insert fragments which hybridised with the hsp70 probe (Fig. 3.15). Sequencing of the confirmed *ApaUhsplO* cloned insert was then attempted and the insert partially restriction mapped (Fig. 3.17). Automated

sequencing was unsuccessful, therefore sequencing was done manually using the universal M13 primers to elucidate a preliminary partial sequence of the insert (Fig. 3.16). Complete sequencing of the insert is required to fully compare the degree of hsp70 sequence conservation in *M. hominis*.

Using the general cloning method of Galley *et al.* (1992), the hsp70 gene of *M. hominis* was successfully cloned and preliminary sequencing undertaken. Complete sequencing of the insert will require either exonuclease digestion to produce sets of nested deletions or primer walking, which will enable further evolutionary and homology comparisons.

CHAPTER 4

THE EFFECT OF HEAT SHOCK ON THE TRANSCRIPTION AND TRANSLATION OF *MYCOPLASMA HOMINIS* HSP70

4.1 Introduction

The heat shock response is a universal physiological phenomenon and heat shock proteins perform necessary cellular functions as molecular chaperones, such as recognition and stabilisation of partially folded intermediates during translation, translocation across membranes, polypeptide folding, and assembly and disassembly of multimeric structures (Ellis and van der Vies, 1991). These proteins, particularly hsp60 and hsp70 homologs, are also the dominant antigens of many pathogenic micro-organisms and have been associated with both protective and immunopathological responses (Hamel *et al.*, 1997).

Most eukaryotic cells contain both heat-inducible and constitutive (hsc70) forms of hsp70, with different homologs present in different intracellular compartments. Prokaryotic cells carry a single hsp70 homolog, which is constitutively expressed and is inducible under stress conditions. Based on the high degree of sequence conservation between members of the hsp70 family, a consensus model for the hsp70 structure has been proposed. All hsp70 homologs consist of a highly conserved amino terminal ATPase domain and a less conserved carboxyl terminal domain thought to contain the peptide binding site (Hendrick and Hartl, 1993). The ATPase related fragment is a two-lobed domain with a deep cleft in which ATP binds, causing a conformational change related to the binding specificity of the carboxyl terminal (Flaherty *et al.*, 1990). The binding reactions between hsp70 and polypeptides or proteins, therefore require ATP hydrolysis (Lindquist and Craig, 1988).

As constitutively expressed proteins, members of the hsp70 family, in co-operation with other hsp, have crucial physiological functions under normal circumstances. The formation of stable folded tertiary structures inhibits the movement of proteins across membranes, therefore many proteins are translocated between intracellular compartments in an unfolded state. Hsp70 and hsp60 cognates are responsible for unfolding proteins, maintaining the unfolded state during subsequent translocation, then facilitating refolding of the proteins and if required, the subsequent

assembly into multimeric complexes. A well studied example belonging to the hsp70 family, is the heavy chain binding protein, BiP, which participates in immunoglobulin assembly. Hsp70 homologs also seek out unfolded proteins and prepare them for degradation. Under conditions of stress such as heat shock when disassembly and unfolding of proteins occurs, the hsps are required to prevent or reverse these denaturation mechanisms. When refolding becomes impossible, hsps facilitate the removal of denatured proteins (Kaufmann, 1990; Hendrick and Hartl, 1993).

Some hsps are also developmentally induced, particularly during stage differentiation of parasite organisms, conferring thermotolerance. Members of the hsp60 and hsp70 families of bacterial and lower eukaryotic pathogens have been determined as immunodominant antigens, and responses of the immune system to these proteins are implicated in the pathology of several autoimmune diseases and inflammatory mechanisms associated with pathogenic organisms (Maresca and Kobayashi, 1994). Lymphocyte and macrophage activation by interleukins or mitogens, in which a variety of proteins are produced, induces upregulation of hsps at mRNA and protein levels. Induction of the heat shock response has been shown to protect target cells from attack by natural killer cells and lymphokine-activated killer cells. Similarities between MHC class I polypeptide processing and the cellular functions of hsp70 suggest that hsps may be involved in antigen processing. Increased hsp levels are associated with virus-transformed and chemically induced tumour cells and may participate in the recognition and subsequent rejection of such cells; although certain hsp homologs have been identified as tumour antigens which induce protective immunity (Kaufmann, 1990). Human hsp70 homologs have been found to confer resistance to stress-induced apoptosis in thermotolerant cells, which could limit the efficacy of certain cancer therapies (Mosser *et al*, 1997).

The heat shock response of parasites is proposed to play an essential role during host invasion and hsps have been implicated in many parasitic protozoan, pathogenic fungal and bacterial infections. The stress response of parasites, unlike most organisms, is a physiological part of their life cycle whereby the parasitic hsps play a vital role during infection in the process of differentiation, adaptation and protection from the host (Maresca and Kobayashi, 1994). However hsp60 and hsp70 homologs are also immunodominant antigens that are the targets of humoral and cell-mediated immune responses, including T-cell activation which induces both

protective immunity and immunopathology (Kaufmann, 1990). The antigenicity of the hsps may be due to their abundance, the presence of conserved epitopes, preferential processing or an intrinsic role as virulence factors. Despite the extensive homology among hsp70s, a host immune system is generally able to discriminate between its own hsp70 and that of a parasite (Maresca and Kobayashi, 1994). However hsps have been implicated in certain autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus. Under stress conditions some host cells may express self epitopes of hsps which are recognised by previously activated T-cells and antibodies, initiating an autoimmune response (Kaufmann, 1990).

The mycoplasmal heat shock response has been investigated in *M. capricolum* and *Acoleplasma laidlawii* and found to resemble that of other organisms. The hsp70 homologs from these mycoplasmas cross-reacted with antibody to *E. coli* hsp70, DnaK, indicating a significant degree of conservation (Dascher *et al*, 1990). The influence of heat shock on the transcription and translation of the hsp70 homolog from *M. hominis* was investigated in this study.

4.2 Materials

Most of the chemicals commonly used in this study were from Merck (Darmstadt, Germany), BDH (Poole, England) or Boehringer Mannheim (Mannheim, Germany) and were of the highest purity available.

The diethyl pyrocarbonate (DEPC) was from ICN Biomedicals (Aurora, USA). BCIP/NBT substrate tablets and Triton X-114 were from Boehringer Mannheim (Mannheim, Germany). RNase AWAY™ was from Molecular Bio-Products (San Diego, USA). Guanidine thiocyanate, 3-(N-morpholine)-propanesulfonic acid (MOPS), mouse IgG, rat IgG, goat anti-mouse IgG-alkaline phosphatase conjugate and goat anti-rat IgG-alkaline phosphatase conjugate were from Sigma (St. Louis, USA). Mouse monoclonal anti-DnaK antibody was from Stressgen (Victoria, Canada) and rat monoclonal anti-Hsp70 gene family antibody was a gift from Dr. Susan Lindquist (Howard Hughes Medical Institute, University of Chicago, Chicago, USA).

4.3 RNA analysis

As the heat shock response is regulated at both the transcriptional and translational levels (Lindquist, 1986), the initial quantifiable change would occur in mRNA abundance. The upregulation of hsp gene expression which causes the increased prevalence of hsps, typically involves changes in either the rate of synthesis or stability of mRNA, and hence abundance. Northern hybridisation using specific probes homologous to the hsps can be used to examine the induction of mRNA abundance (Cochrane *et al.*, 1994). To examine the effect of heat shock on the level of hsp70 mRNA in *M. hominis*, total RNA was isolated from normal and heat shocked cells, denatured and Northern blotted using the DIG-labelled PCR product (section 3.4) as a probe.

As RNA is highly susceptible to degradation by ribonucleases (RNases), successful isolation of RNA relies on the elimination of these enzymes from all solutions, glassware, pipettes, tips and the working area (Berger, 1987). Autoclaving is not sufficient to completely destroy all RNase activity, therefore many solutions are treated either directly or indirectly with the nonspecific RNase inhibitor, diethyl pyrocarbonate (DEPC). However all traces of DEPC must be removed by autoclaving, where it degrades into carbon dioxide and ethanol, as it can interfere with subsequent reactions. Several other reagents are routinely used during the isolation procedure to eliminate RNase activity, such as the guanidine salts, SDS, N-laurylsarcosine and phenol (Farrell, 1993). In this study, solutions not including any of the former reagents, were treated with DEPC or made up in DEPC-treated H₂O, all glassware, containers and electrophoresis equipment were soaked in DEPC-treated H₂O before use, and all surfaces and pipettes were treated with RNase-AWAY™ to reduce RNase contamination.

4.3.1 RNA isolation

The isolation of undegraded RNA from cells requires the inhibition of endogenous nucleases, deproteinisation of the RNA, and removal of the RNA from the homogenate (MacDonald *et al.*, 1987). The initial two requirements can be achieved simultaneously through the use of guanidine salts. Guanidine thiocyanate and hydrochloride are highly effective protein denaturants and strong inhibitors of ribonucleases, with the former the most efficient (Chomczynski and Sacchi, 1987). Denaturation is further enhanced with the inclusion of a reducing agent such as

dithiothreitol or p-mercaptoethanol. The RNA may then be separated from the remaining components either through selective precipitation or selective sedimentation (MacDonald *et al*, 1987).

This study uses the acid-guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987) for rapid RNA isolation from *M. hominis* cells.

4.3.1.1 Reagents

0.1% (v/v) DEPC-treated H₂O. DEPC (1 ml) was added to 1 l ddH₂O, stirred for at least 30 min in a fume hood and then autoclaved at 121°C for 15 min.

1 M Sodium citrate, pH 7.0. Sodium citrate (5.882 g) was dissolved in 15 ml DEPC-H₂O, the pH adjusted to 7.0 with NaOH, the volume was made up to 20 ml and autoclaved.

10% (m/v) N-laurylsarcosine. N-laurylsarcosine (1 g) was dissolved in 10 ml dist. H₂O and filter sterilised.

Solution D [4 M Guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% (m/v) N-laurylsarcosine, 100 mM P-mercaptoethanol]. Guanidine thiocyanate (9.456 g) was dissolved in 15 ml dist. H₂O, 10% (m/v) N-laurylsarcosine (1 ml), p-mercaptoethanol (140 μ l) and 1 M sodium citrate (0.5 ml) were added, the volume made up to 20 ml with dist. H₂O and the solution filter sterilised.

2 M Sodium acetate, pH 4.0. Sodium acetate (3.281 g) was dissolved in 10 ml dist. H₂O, the pH adjusted to 4.0 with acetic acid, the volume was made up to 20 ml and the solution autoclaved.

Tris-buffered phenol, pH 7.0. The phenol was made up as described in section 3.3.1.1 using Tris-HCl pH 7.0.

4.3.1.2 Procedure

M. hominis cells were either maintained at 37°C or heat-shocked for various times at 47°C, collected by centrifugation at 10 000 xg for 5 min, and resuspended in cold solution D (0.5 ml). To the suspension, 2 M sodium acetate pH 4.0 (50 μ l), phenol (0.5 ml) and chloroform (0.1 ml) were added in order and mixed thoroughly following the addition of each reagent. The mixture was shaken vigorously for 10 s after all the reagents had been added. The sample was incubated on ice for 15 min and then centrifuged at 4°C. The aqueous phase was recovered, mixed with an equal volume of isopropanol and incubated at -20°C for at least 1 hour. The mixture was centrifuged at 10 000 xg for 20 min at 4°C and the supernatant discarded. The RNA pellet was dissolved in solution D (150 μ l) and reprecipitated with the addition of an equal volume of isopropanol and incubation at -20°C for 1 hour. The RNA was collected through centrifugation at maximum speed in a microfuge for 15 min. The supernatant was discarded and the pellet washed twice in 70% (v/v) ethanol. The pellet was recentrifuged and dried to near dryness. The RNA was redissolved in 20 μ l DEPC-H₂O and stored in aliquots at -70°C.

4.3.2 Denaturing agarose gel electrophoresis

The most efficient method for assessing the quality and integrity of purified RNA is to run the RNA sample on a denaturing gel. Intact, competent RNA bands in a reproducible pattern under denaturing conditions. Generally the formation of distinct 28S and 18S ribosomal RNA (rRNA) bands with minimal smearing between them is an indication of intact RNA. The smaller rRNA species and transfer RNA (tRNA) comigrate at the leading edge. Smearing throughout the lane of the gel may be the result of certain isolation procedures or persistent secondary RNA structure (Farrell, 1993). Formaldehyde is an effective denaturant of RNA and agarose/formaldehyde gels allow good electrophoretic separation of RNA molecules (Lehrach *et al.*, 1977). However the formaldehyde must be rinsed from the gel prior to ethidium bromide staining as formaldehyde gels display very high background fluorescence, and prior to Northern blotting to improve the efficiency of transfer (Farrell, 1993; Sambrook *et al.*, 1989).

4.3.2.1 Reagents

10x MOPS buffer [200 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7.0]. MOPS (10.45 g) and sodium acetate (1.025 g) were dissolved in 200 ml DEPC-H₂O and 100 mM EDTA (25 ml) added. The pH was adjusted to 7.0 with NaOH, the volume made up to 250 ml with DEPC-H₂O and the solution autoclaved.

Loading buffer 10 mM EDTA, 0.4% (m/v) bromophenol blue, 50% (v/v) glycerol. 100 mM EDTA (0.1 ml), bromophenol blue (0.04 g) and glycerol (5 ml) were made up to 10 ml with dist. H₂O.

4.3.2.2 Procedure

Agarose (0.4 g) was melted in 34 ml ddH₂O in a microwave oven and when cooled, 10x MOPS (4 ml) and 37% (v/v) formaldehyde (2.2 ml) were added. The gel was cast as a minigel for use with the MINNIE submarine agarose gel unit (Hoefer Scientific, San Francisco). Once set, the gels were submerged in 1x MOPS running buffer. The RNA samples were denatured by adding 10x MOPS (1 μ l), 37% (v/v) formaldehyde (3.3 μ l) and formamide (10 μ l), and incubating the mixture at 55°C for 15 min. Following denaturation, loading buffer (2 μ l) was added to the samples, mixed and the samples loaded on the gel. The gels were run at 10 V/cm until the bromophenol blue had migrated about 80% of the gel length. To visualise RNA with ethidium bromide staining, the gels were soaked in several changes of sterile DEPC-H₂O for at least 15 min to remove the formaldehyde. The gel was then stained in DEPC-H₂O (100 ml) with ethidium bromide (5 μ l) for at least 30 min and then viewed on a UV transilluminator.

4.3.3 Northern blotting

Northern blotting is a convenient and efficient method for the determination of the size and abundance of specific mRNA molecules in total RNA preparations. RNA separated using denaturing agarose gel electrophoresis, is effectively transferred to nitrocellulose or nylon membrane similarly to DNA in Southern blotting. Nylon membranes often produce higher background than nitrocellulose but are preferred as they are easier to handle, bind nucleic acids more strongly and display higher sensitivity for non-radioactive detection (Höltke *et al.*, 1995).

The transferred RNA may be probed with double-stranded DNA fragments, single-stranded DNA, synthetic oligonucleotides, or RNA synthesised *in vitro*, labelled either radioactively or non-radioactively with DIG for example (Sambrook *et al*, 1989). While RNA probes are the most sensitive, DNA probes are effective under more stringent prehybridisation and hybridisation conditions. The present study made use of the non-radioactive DIG labelling and detection system as described in section 3.3.7, with a DIG-labelled DNA probe.

4.3.3.1 Reagents

20X SSC T3 M Sodium chloride. 0.3 M sodium acetate. pH 7.01. Made up as in section 3.3.7.1 but using DEPC-H₂O.

2X Wash solution \2x SSC. 0.1% (m/v) SDS1. Made up as in section 3.3.7.1 using DEPC-H₂O.

0.1X Wash solution TO.1x SSC. 0.1% (m/v) SDS1. Made up as in section 3.3.7.1 using DEPC-H₂O.

Maleic acid buffer IU1 M Maleic acid. 0.15 M NaCl pH 7.51. Maleic acid (11.61 g) and NaCl (8.766 g) were dissolved in 800 ml DEPC-H₂O, the pH adjusted to 7.5 with NaOH, the volume was made up to 1 l and autoclaved.

10% (m/v) Block solution. Blocking reagent (10 g) was dissolved in 100 ml maleic acid buffer with gentle heating and agitation, and autoclaved.

10% (m/v) N-laurylsarcosine. See section 3.3.7.1.

1 M Sodium phosphate pH 7.0. NaH₂PO₄ (7.8 g) was dissolved in 40 ml dist. H₂O, the pH adjusted to 7.0 with NaOH, the volume was made up to 50 ml and autoclaved.

High SDS hybridisation buffer \1% (m/v) SDS, 50 mM sodium phosphate pH 7.0, 50% (v/v) formamide, 2% (m/v) block, 5X SSC, 0.1% (m/v) N-laurylsarcosine. Formamide (50 ml), 20X SSC (25 ml), 1 M sodium phosphate (5 ml), 10% (m/v) blocking solution (20 ml), 10% (m/v) N-laurylsarcosine (1 ml) were mixed. SDS (7 g) was dissolved in the solution with gentle heating.

Northern hybridisation buffer. DIG-labelled DNA probe (section 3.3.6) diluted in high SDS hybridisation buffer.

4.3.3.2 Procedure

Following electrophoresis in a denaturing formaldehyde gel (section 4.3.2), the gel was equilibrated in 20x DEPC-SSC twice for 15 min. Nylon membrane of the same dimensions as the gel, was soaked in 2x DEPC-SSC. The gel was placed on a clean glass surface in a container with 20x DEPC-SSC and covered with the membrane, followed by two pieces of Whatmann filter paper also soaked in 20x DEPC-SSC. A layer of paper towel and a light weight was placed on the assembly and the RNA blotted from the gel by capillary action for 4 hours at RT. The membrane was then removed and fixed by UV exposure for 2 min.

The blot was prehybridised in high SDS hybridisation buffer at 52°C for at least 1 hour. The DIG-labelled PCR product (section 3.4) was denatured in a boiling waterbath for 10 min, snap-cooled on ice before use and diluted to a final concentration of 200 ng/ml in the Northern hybridisation buffer. The blot was hybridised at 52°C overnight. After hybridisation, the blot was washed twice in 2x wash solution for 5 min at RT, then washed twice in 0.1x wash solution for 15 min at 68°C.

The blot was then detected using the DIG detection kit (Boehringer Mannheim, Germany) as described in section 3.3.7 with the following exception: buffer I was substituted with maleic acid buffer.

4.4 Protein analysis

Following the transcriptional changes induced by the heat shock response, corresponding translational changes appear in the form of increased abundance of the hsp70. To investigate the effect of heat shock on the protein abundance of *M. hominis*, total protein fractions were isolated from normal and heat shocked cells, and examined electrophoretically. As a result of the conservation between particularly hsp70 homologs, immunological methods have been used to quantitate the abundance of specific hsp70 during the heat shock response. In this study, antibodies against the *E. coli* hsp70 homolog, DnaK, and the eukaryotic hsp70 gene family, were used to determine the degree of conservation between the *M. hominis* hsp70 and other species, and any translational changes during heat shock.

Protein extracted from normal and heat-shocked *M. hominis* cells was quantified using the Bradford dye-binding assay prior to analysis by Tris-tricine SDS-PAGE and Western blotting.

4.4.1 Protein isolation

The lack of a cell wall makes mycoplasmas susceptible to lysis by gentle methods that are usually ineffective for bacteria with a cell wall. In the present study, total protein fractions were isolated using osmotic lysis (Razin, 1983b).

4.4.1.1 Reagents

0.25 M Sodium chloride. NaCl (0.146 g) was dissolved in 10 ml dist. H₂O.

4.4.1.2 Procedure

M. hominis cells were either maintained at 37°C or heat shocked for various times at 47°C, collected by centrifugation at 10 000 xg for 10 min, washed once in 0.25 M NaCl (300 [41]) and then resuspended in 0.25 M NaCl (40 [41]). ddH₂O preheated to 37°C (1 ml) was added to the suspension and incubated at 37°C for 15 min to obtain a total protein extract. Any unlysed cells and other debris were removed by centrifugation at 10 000 xg for 2 min and the total protein in the supernatant quantified.

4.4.2 Bradford dye-binding assay

The Bradford dye-binding assay is a rapid, reproducible, inexpensive and sensitive protein concentration assay which uses the dye characteristics of Coomassie Brilliant Blue G-250 (Bradford, 1976). The dye exists in a cationic red form and an anionic blue form. The cationic form is present in the acidic dye reagent with an absorption maximum at 465 nm, and is converted to the anionic form on binding to protein with a concurrent shift in absorbance maximum to 595 nm. The extinction coefficient of the protein-dye complex is greater than that of the free dye allowing for sensitive protein quantitation.

An important advantage of the Bradford assay is the lack of interference by chemicals such as metal ions and reducing agents. The most significant disadvantage of the assay is the variation in colour response of different proteins. Read and Northcote (1981) modified the original method substituting Serva Blue G dye for the Coomassie Brilliant Blue G-250 and altering the dye and acid concentrations, which reduced this variation but resulted in less sensitivity at higher protein concentrations.

The micro-assay for the determination of less than 5 μ g of protein (Read and Northcote, 1981) was used in this study.

4.4.2.1 Reagents

Dye reagent. Serva blue G dye (50 mg) was dissolved in 88% (v/v) phosphoric acid (50 ml) and 99.5% (v/v) ethanol (23.5 ml). The solution was made up to 500 ml with dist. H₂O, stirred for 30 min, filtered through Whatmann No. 1 filter paper and stored in an amber bottle. The solution could be stored for up to 6 months at RT, although visual checks for precipitation were made before use.

Standard protein solution. Ovalbumin (10 mg) was dissolved in dist. H₂O and diluted to 0.1 mg/ml for use in the assay.

4.4.2.2 Procedure

Protein standards or samples were diluted to 50 μ l with dist. H₂O, dye reagent (950 μ l) added and the solution mixed by inversion. Colour development was allowed to proceed for 2 min after mixing and the absorbance read at 595 nm against appropriate blanks. Plastic microcuvettes (1 ml) were used, as the dye reagent binds to glass (Bradford, 1976). A standard curve of the standard protein solution (0-5 μ g) was constructed and linear regression analysis of the data generated the following equation that was used to calculate subsequent results:

$$y = 0.0238x + 0.018$$

4.4.3 Gel electrophoresis

In this investigation polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was used to examine the various protein preparations from *M. hominis*. In conjunction with Western blotting, SDS-PAGE was also used to evaluate the reactivity between anti-hsp70 antibodies and *M. hominis* proteins.

The basis of modern PAGE is the discontinuous system developed by Ornstein (1964) and Davis (1964) which makes use of two different gel and buffer systems to resolve protein bands. The gel buffers are composed of Tris-HCl, with the stacking gel at pH 6.8 and the resolving gel at pH 8.8, while the tank buffer contains Tris-glycine. At pH 6.8 the glycinate ions are weakly dissociated with a slight negative charge and hence low mobility; the chloride ions have a high mobility, and the proteins have an intermediate mobility between the two ions. When a current is applied, the proteins stack at a sharp interface generated by the leading chloride and trailing glycinate ions due to the prevailing voltage gradient. Once the conditions of the resolving gel are encountered, the increased pH promotes dissociation, and hence mobility, of the glycinate ions which then overtake the proteins and form a front with the tracking dye and chloride ions. The proteins migrate through the smaller pores of the resolving gel, separating under the influence of size, shape and charge.

In order to separate proteins purely on the basis of MW, SDS-PAGE was introduced by Laemmli (1970). SDS, an anionic detergent, binds to proteins in a constant ratio which imparts a net negative charge to the protein in proportion to its MW. To enable complete disruption of tertiary

structures, proteins may be boiled in the presence of reducing agents such as (3-mercaptoethanol or dithiothreitol).

An alternative to the Tris-glycine SDS-PAGE system has been developed using tricine as the trailing ion (Schägger and von Jagow, 1987). The Tris-tricine SDS-PAGE system enables improved resolution of proteins in the range of 5-100 kDa, particularly those with low MW. The use of the tricine ion allows stacking and separation of proteins to be achieved under ideal conditions i.e. at the same pH in both stacking and resolving gels, and at low acrylamide concentrations (Schägger and von Jagow, 1987).

Thus proteins may be separated on the basis of size by Tris-tricine SDS-PAGE, and the MW of a protein may be determined through comparison against a set of standard proteins with known MW.

4.4.3.1 Reagents

Monomer solution [49.5% (m/v) Acrylamide, 3% (m/v) N,N'-methylene-bisacrylamide]. Acrylamide (48 g) and N,N'-methylene-bisacrylamide (3 g) were dissolved in 100 ml dist. H₂O and stored at RT in an amber bottle.

Gel buffer T3 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris-HCl (36.35 g) and SDS (0.6 g) were dissolved in 80 ml dist. H₂O, the pH adjusted to 8.45 with HCl and the volume was made up to 100 ml.

10% (m/v) Ammonium persulfate. Ammonium persulfate (0.1 g) was dissolved in 1 ml dist. H₂O.

Anode buffer T0.2 M Tris-HCl pH 8.9]. Tris-HCl (24.22 g) was dissolved in 900 ml dist. H₂O, the pH adjusted to 8.9 with HCl and the volume made up to 11.

Cathode buffer r0.1 M Tris-HCl, 0.1 M tricine, 0.1% (m/v) SDS, pH 8.25]. Tris-HCl (12.2 g), tricine (17.9 g) and 10% (m/v) SDS (10 ml) were made up to 11 with dist. H₂O and the pH checked.

Reducing sample treatment buffer 125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) (3-mercaptoethanol, 0.1% (m/v) bromophenol blue. 1 M Tris-HCl (1.25 ml), 10% (m/v) SDS (4 ml), glycerol (2 ml), [3-mercaptoethanol (1 ml) and bromophenol blue (0.01 g) were made up to 10 ml with dist. H₂O.

MW standards [Phosphorylase b, BSA, carbonic anhydrase, ovalbumin, lysozyme (each protein at 2 μ g) in 500 mM Tris-HCl, pH 6.8]. Lyophilised protein (4 mg) was dissolved in 500 mM Tris-HCl pH 6.8 (1 ml), reducing sample treatment buffer (1 ml) added, the mixture boiled for 5 min and stored in aliquots at -20°C.

4.4.3.2 Procedure

Tris-tricine SDS-PAGE was carried using the SE 250 Mighty Small II vertical slab electrophoresis unit (Hoefer Scientific, San Francisco) assembled as described in the manufacturer's manual. Stacking and resolving gels were prepared using 1.5 mm spacers and combs, as described in Table 5. Resolving gels were cast and allowed to polymerise for at least 1 hour, while the stacking gels polymerised within 30 min. The protein samples (containing at least 100 ng per band for silver staining) were mixed in a 1:1 (v/v) ratio with reducing sample treatment buffer and boiled for 2 min. Once cooled, the samples were loaded into the gel wells with a micropipette. The gels were run at unlimiting current and 80 V through the stacking gel, and then the voltage was increased to 100 V. Following electrophoresis, the gels were either silver stained (section 4.4.4) or used for Western blotting (section 4.4.5).

Table 6. Preparation of the resolving and stacking gels for Tris-tricine SDS-PAGE.

Reagent	Resolving gel (10% Acrylamide, 3% Bis)	Stacking gel (4% Acrylamide, 3% Bis)
Monomer solution	3.6 ml	0.5 ml
Gel buffer	6ml	15 ml
Ammonium persulfate	60 μ l	30 μ l
TEMED	6 μ l	12 μ l
Dist. H ₂ O	8.4 ml	4ml

4.4.4 Silver staining of electrophoretic gels

Silver staining utilises the reduction of ionic silver to its insoluble metallic form to detect nanogram amounts of sample (Nielson and Brown, 1983). Under high pH and reducing conditions, silver ions in solution form insoluble coloured complexes with charged side chains. The high background staining which results from non-specific deposition of insoluble silver complexes may be reduced by treating the gel with sodium thiosulfate. By preventing the non-specific precipitation, the level of background staining is reduced without influencing the sensitivity (Blum *et al*, 1987).

4.4.4.1 Reagents

All solutions were made up using deionised distilled H₂O obtained from a MilH-Q Plus Ultra-Pure water system (Millipore, Marlboro, USA).

Fix solution F50% (v/v) methanol, 12% (v/v) acetic acid, 0.2% (v/v) formaldehyde!. Methanol (100 ml), acetic acid (24 ml) and 37% (v/v) formaldehyde (0.1 ml) were made up to 200 ml with H₂O.

Wash solution [50% (v/v) ethanol!]. Ethanol (100 ml) was made up to 200 ml with H₂O.

Pre-treatment solution r0.02% (m/v) Sodium thiosulfate!. Na₂S₂O₃·5H₂O (0.04 g) was dissolved in 200 ml H₂O.

Impregnation solution FO2% (m/v) Silver nitrate. 0.03% (v/v) formaldehyde!. AgNO₃ (0.4 g) and 37% (v/v) formaldehyde (0.15 ml) were made up to 200 ml with H₂O.

Development solution [6% (m/v) Sodium carbonate, 0.0004% (m/v) sodium thiosulfate. 0.02% (v/v) formaldehyde!]. Na₂CO₃ (12 g), pre-treatment solution (4 ml) and 37% (v/v) formaldehyde (0.1 ml) were made up to 200 ml with H₂O.

Stop solution [50% (v/v) methanol. 12% (v/v) acetic acid!]. Methanol (50 ml) and acetic acid (12 ml) were made up to 100 ml with H₂O.

4.4.4.2 Procedure

All steps in the staining procedure were carried out with shaking in clean glass containers at RT. Following the completion of electrophoresis, the gel was removed from the gel assembly, incubated in fix solution for at least one hour and then washed with wash solution (3 x 20 min) to neutralise the gel for subsequent treatment. The gel was then treated with pretreatment solution for 1 min, rinsed in H₂O (3 x 20 s), and soaked in impregnation solution for 25 min. After rinsing the gel with H₂O (2 x 20s) to remove excess silver nitrate, the gel was immersed in development solution until bands were visible. The gel was rinsed in H₂O (2 x 2 min), soaked in stop solution for 10 min and then stored in a sealed plastic bag.

4.4.5 Western blotting

The use of antibodies for the identification and characterisation of proteins or evaluation of antibody specificity is facilitated by Western blotting. Proteins separated by SDS-PAGE are electrophoretically transferred to an insoluble matrix with a high protein binding capacity such as nitrocellulose (Towbin *et al.*, 1979). Once the unoccupied binding sites have been non-specifically blocked to prevent non-specific antibody binding, the membrane is probed with the primary antibody of interest. The resultant antibody-antigen complexes are detected with an enzyme conjugated secondary antibody directed against the primary antibody, which catalyses an appropriate colorimetric substrate reaction.

The method used in this study follows that of Towbin *et al.* (1979) and employed alkaline phosphatase conjugated secondary antibodies with NBT and BCIP (Fig. 3.2) as substrates.

4.4.5.1 Reagents

Blotting buffer T25 mM Tris-HCl, 192mM glycine, 20% (v/v) methanol 0.1% (m/v) SDS.
Tris-HCl (9.08 g) and glycine (43.2 g) were dissolved in 2 l dist. H₂O, methanol (600 ml) and 10% (m/v) SDS (3 ml) added and the volume made up to 3 l.

Tris-buffered saline (TBS) 20 mM Tris-HCl, 200 mM NaCl, pH 7.4. Tris-HCl (2.42 g) and NaCl (11.69 g) were dissolved in 900 ml dist. H₂O, the pH adjusted to 7.4 with HCl and the volume made up to 1 l.

0.1 % (m/v) Ponceau S stain. Ponceau S (0.1 g) was dissolved in 100 ml 1% (v/v) acetic acid.

Bovine serum albumin (BSA) blocking solution 5% (m/v) BSA in TBS. BSA (0.5 g) was dissolved in 100 ml TBS.

Milk powder blocking solution 5% (m/v) low fat milk powder in TBS. Low fat milk powder (5 g) was dissolved in 80 ml TBS and the volume made up to 100 ml with TBS.

Substrate solution 0.03% (m/v) NBT, 0.015% (m/v) BCIP, 50 mM Tris-HCl pH 9.5, 25 mM MgCl₂. One BCIP/NBT tablet was dissolved in 10 ml dist. H₂O.

4.4.5.2 Procedure

Nitrocellulose membrane, cut to the same dimensions as the gel, was soaked in blotting buffer for 10 min prior to blotting. Following completion of electrophoresis, the gels were removed from the electrophoresis assembly and rinsed in blotting buffer. The Western blot apparatus was assembled with a piece of scotchbrite foam and three pieces of filter paper, soaked in blotting buffer, on either side of the gel with the nitrocellulose membrane on top of it. The sandwich was placed in the blotting tank which was filled with blotting buffer. A low temperature was maintained in the apparatus by attachment to a circulating cooling water bath and stirring within the tank by a magnetic stirrer to ensure even distribution. The gels were blotted either for 2 hours at 200 mA with unlimiting voltage, or overnight at 30 V with unlimiting current. After the completion of blotting, the membrane was removed from the assembly, stained with Ponceau S for 1 min and rinsed in H₂O to determine the position of the molecular weight markers which were marked lightly with a needle point. The blot was then destained with several changes of dist. H₂O.

The membrane was blocked with milk blocking solution for 1 hour, washed in TBS (3 x 5 min) and then incubated with primary antibody in BSA blocking solution for 2 hours. After washing in

TBS (2 x 5 min), the membrane was incubated in alkaline phosphatase-linked secondary antibody in BSA blocking solution for 1 hour and then washed in TBS (3 x 5 min). It was then incubated in substrate solution in the dark until bands were visible. The blot was removed from the substrate solution, washed in H₂O and dried between filter paper.

4.5 Results

4.5.1 RNA analysis

Total RNA was isolated from normal and heat-shocked *M. hominis* cells, quantified on agarose gel electrophoresis (Fig. 4.1) and Northern hybridised with labelled hsp70 probe (Fig. 4.2).

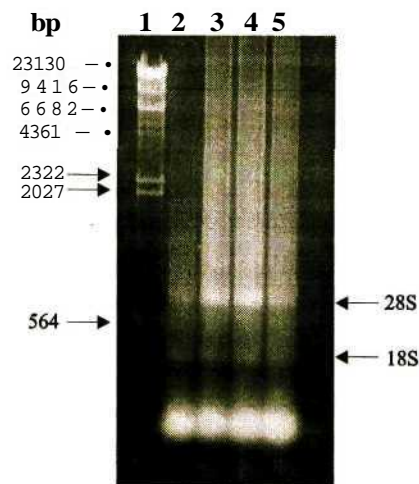


Figure 4.1. Total RNA preparations from *M. hominis*. (1) *HindIII* X DNA markers; RNA isolated from cells heat shocked for (2) 0 min; (3) 10 min; (4) 20 min; (5) 30 min.

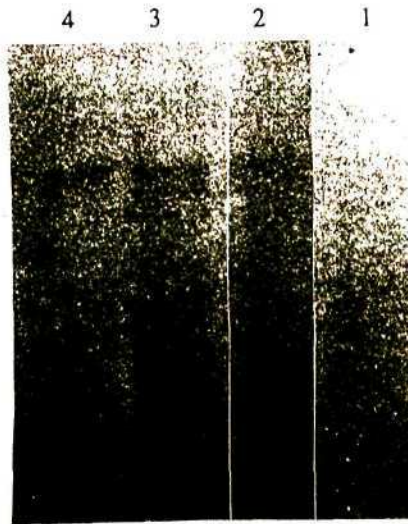


Figure 4.2. Northern blot of denatured total RNA samples hybridised with hsp70 probe. RN A from cells heat shocked for (1) 0 min; (2) 10 min; (3) 20 min; (4) 30 min.

4.5.2 Protein analysis

Total protein was isolated from normal and heat shocked *M. hominis* cells, electrophoresed on Tris-tricine SDS-PAGE and silver stained (Fig. 4.3) or Western blotted (Fig. 4.4).

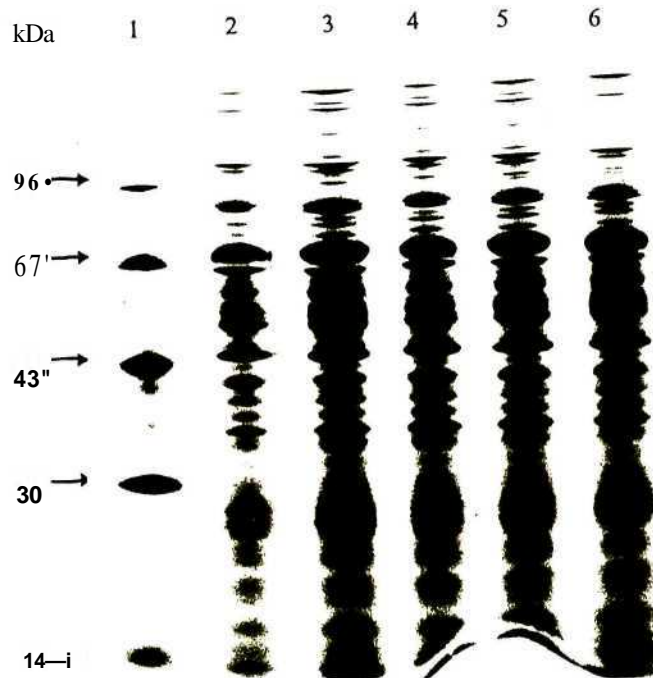


Figure 4.3. Reducing Tris-tricine SDS-PAGE of heat shocked total protein samples isolated from *M. hominis* PG21. Cells heat shocked for (2) 0 min; (3) 5 min; (4) 10 min; (5) 20 min; (6) 30 min, prior to protein extraction. (1), (7) MW markers (phosphorylase b, 96 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; lysozyme, 14 kDa). Visualised through silver staining.

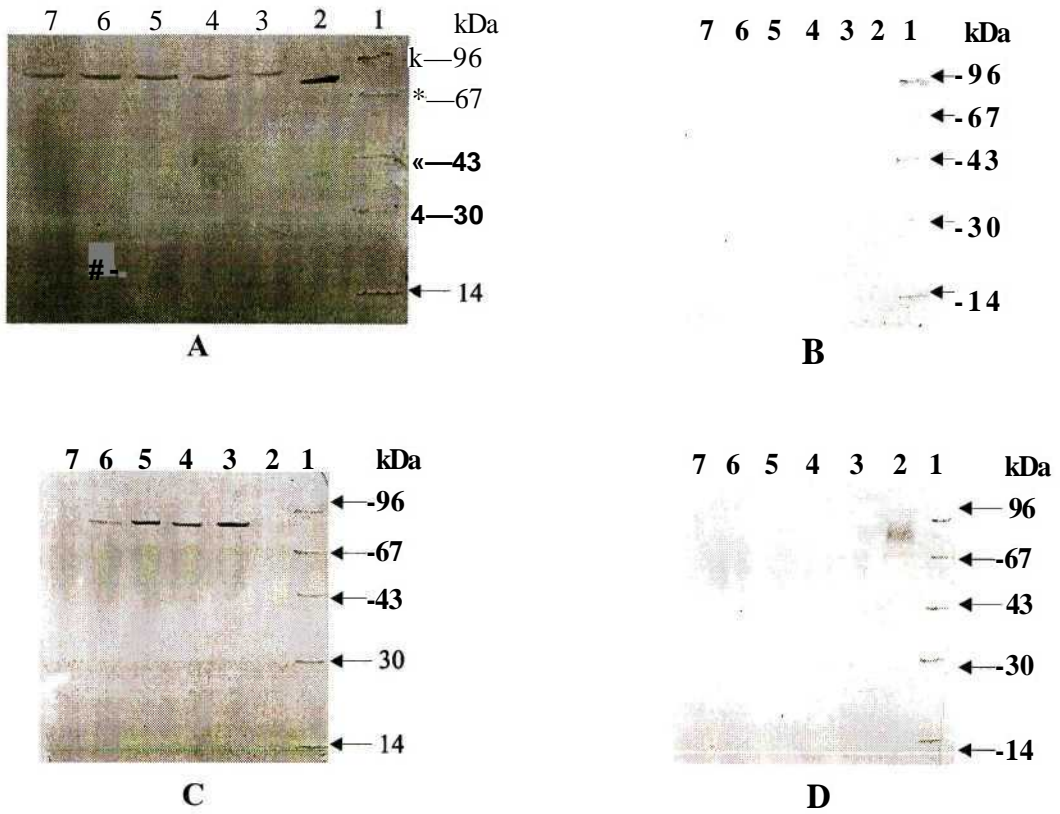


Figure 4.4. Western blots of heat shocked total protein samples isolated from *M. hominis* PG21. Blots were probed with (A), mouse monoclonal anti-dnaK antibody; (B), pre-immune mouse IgG; (C), rat monoclonal anti-hsp70 gene family antibody; (D), pre-immune rat IgG. (1) MW markers (as in Fig. 4.3); (2) *E. coli* total protein extract; total protein from *M. hominis* cells heat shocked for (3) 0 min; (4) 5 min; (5) 10 min; (6) 20 min; (7) 30 min.

4.6 Discussion

The heat shock response is the most universally conserved physiological process and the hsp70 have essential roles in cellular functioning and immunology. Of particular interest are hsp60 and hsp70 which are among the immunodominant antigens of pathogenic organisms (Kaufmann, 1990). In this study, the levels of transcribed and translated *M. hominis* hsp70 products following thermal shock were investigated.

The heat shock response is predominantly transcriptionally regulated with mRNA coding for the hsp70 induced ten to a thousand fold following cellular stress (Lindquist, 1986). Total RNA isolated from normal and heat shocked *M. hominis* cells was of a sufficient quality (Fig. 4.1) to enable the investigation of mRNA induction through Northern blotting. The expected RNA banding pattern of 28S, 18S and smaller molecules comigrating as an indiscreet band in the leading edge (Farrell, 1993) was obtained. The smearing throughout the lane is typical of the acid-guanidine thiocyanate-phenol method of isolation, rather than RNA degradation (Farrell, 1993; Chomczynski and Sacchi, 1987). The presence of mRNA coding for hsp70 in normal and heat shocked cells, was investigated through hybridisation with the DNA hsp70 probe. A low level of hsp70 mRNA was found in normal cells, with a significant increase following exposure to high temperature (Fig. 4.2). The low level of hsp70 mRNA in normal cells indicates the presence of constitutively expressed hsp70, which was confirmed with the protein analysis. While the low level of transcription may also be part of a regulatory mechanism to keep the genes prepared for immediate activation (Velazquez *et al.*, 1983), it must serve a specific purpose within normal cellular functioning.

At the translation level, the enhancement of specific hsp70 on silver-stained polyacrylamide gels is difficult to visualise, although the increased expression of some proteins appears to be induced (Fig. 4.3). In *E. coli*, increased synthesis of at least 17 proteins occurs in response to heat shock, therefore a more sensitive method such as 2D gel electrophoresis or autoradiography would be more appropriate for detection of the hsp70 (Hamel *et al.*, 1997). Immunological methods enable sensitive detection of specific hsp70 such as hsp70. The antibody against the *E. coli* dnaK protein has been shown to cross-react with hsp70 homologs from mycoplasmas, *M. capricolum* and *A. laidlawii* (Dascher *et al.*, 1990). The hsp70 homolog from *M. hominis* was found to cross-react with the anti-dnaK antibody and the antibody against the eukaryotic hsp70 gene family

(Fig. 4.4). The absence of any reaction between the pre-immune IgG and *M. hominis* proteins eliminates the possibility of non-specific interaction (Fig. 4.4 B, D). Hsp70 is present under normal growth conditions, demonstrating a role in cellular functioning other than solely in the heat shock response. While a slight increase in the amount of hsp70 is visible after approximately 10 minutes of heat shock, the translational induction of hsp70 does not appear as significant as the transcriptional induction. This may suggest that the rate of synthesis rather than the steady state level of hsp70 is increased in response to heat shock, as in other bacterial organisms (Hamel *et al.*, 1997). The cross reactivity of the *M. hominis* hsp70 displays the high degree of conservation between hsp70 homologs as was demonstrated in its nucleotide sequence (chapter 3).

The heat shock response of *M. hominis* resembles that of other organisms (Lindquist, 1986; Neidhardt *et al.*, 1984; Dascher *et al.*, 1990; Hamel *et al.*, 1997) with induction of hsp70 at both the transcriptional and translational levels. The conservation of this protein and its constitutive presence within cells suggests an important role in cell physiology and survival.

CHAPTER 5

DETECTION OF *MYCOPLASMA HOMINIS* BY A PCR-ELISA DIAGNOSTIC ASSAY

5.1 Introduction

As discussed in chapter 1, *M. hominis* has a proven pathogenic role in destructive, invasive disease and numerous associated infections (Cassell *et al.*, 1994). While the virulence of this organism is minimal in most normal hosts, *M. hominis* may manifest as potentially fatal infections, particularly those of the central nervous system, in immunocompromised patients and neonates. Due to this pathogenic potential, a need exists for the rapid clinical diagnosis of such infections and the application of appropriate chemotherapeutic agents during the early stages of infection (Zheng *et al.*, 1997).

The gold standard for mycoplasma diagnosis remains isolation by culture. However mycoplasmas including *M. hominis*, are notoriously fastidious organisms requiring highly specialised media (as described in chapter 2), which is generally only available in major medical centres and research laboratories (Cassell *et al.*, 1995). Serological methods whereby identification is achieved through specific antibodies reacting with whole organisms or their antigens, are also widely used, particularly immunofluorescence and immunoblotting assays. The increasing cross-reactivity between species and the multiple serotypes of certain mycoplasma species pose problems for serological identification; *M. hominis* exhibits extreme antigenic heterogeneity (Clyde, 1983b; Cassell *et al.*, 1994). Due to the prolonged time period required for cultural and serological diagnosis of pathogens, interest in the development of rapid molecular diagnostic techniques has increased significantly.

Detection of pathogenic organisms by PCR appears to be a promising technique with regards to rapidity, reproducibility, sensitivity and specificity. PCR identification assays have been developed for a range of pathogens such as *Ureaplasma urealyticum* (Runge *et al.*, 1997), *Trypanosoma cruzi*, the causative agent of Chagas' disease (Wincker *et al.*, 1996), *Mycobacterium tuberculosis* (Wilson *et al.*, 1993) and several mycoplasmas (Cassell *et al.*, 1994). These assays were found to be comparable to culture based detection methods, generally with a higher level of sensitivity. A PCR assay for the detection of *M. hominis* has also been

established (Blanchard *et al.*, 1993). As the primers used for a PCR reaction determine its specificity, primers which amplify species specific DNA fragments are required. The PCR primers used for the identification of *M. hominis*, amplify a 334 bp fragment of the 16S rRNA gene (Fig. 5.1) and were selected from regions conserved between *M. hominis* strains but variable among other mycoplasma species (Blanchard *et al.*, 1993). The PCR detection with the RNAH1 and RNAH2 primers was found to be specific for *M. hominis* when evaluated using a range of mycoplasma species. Nonspecific amplification was obtained with strains of *M. salivarium*, however only *M. hominis* amplified product hybridised with the internal probe RNAH3 (Blanchard *et al.*, 1993). Comparison of PCR with culture for the detection of *M. hominis* in clinical samples indicated that the sensitivity of PCR was higher and PCR reduced the assay time from 3 to 5 days required for culture, to 24 hours (Abele-Horn *et al.*, 1996).

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131 CATTGGAAAC AATGGCTAATGCCGGATACGC ATGG AACCGCATGGTTCCG
      |
181 TTGTGAAANGCGCTGT A AGGCGCN ACT A AAAG ATG AGGGTGCGG AAC ATT
231 AGTTAGTTGGTGAGGTAATGGCCCACCAAGACTATGATGTTTAGCCGGGT
281 CGAGAG ACTGAACGGCCACATTGGGACTGAGATACGGCCNNAACTCCTAC
331 GGGAGGCAGCAGTAGGGAATATTCCACAATGAGCGAAAGCTTGATGGAGC
381 GCACCAGCGTGCACGATGAAGGTCTTCGGATTGTAAAGTGCN GTTATAAG
431 GGAAGAAC ATTTGCAATAGGAAATGATTGC AG ACTGACGGTACC

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Figure 5.1. Nucleotide sequence of the amplified region of the *M. hominis* PG21 16S rRNA gene. The arrows indicate the sequences from which primers for the PCR assay were designed and the internal probe used to confirm the specificity of the amplification is in red (after Blanchard *et al.*, 1993).

While the primers used in PCR assays confer specificity, nucleic acid hybridisation with an internal probe is generally required to exclude nonspecific amplification products, and has the advantage of increasing the sensitivity of detection. Hybridisation of the amplified product is therefore a necessity of routine molecular diagnostic assays (Kessler *et al.*, 1997). This hybridisation usually takes the form of Southern blotting, however this has limitations in the number of samples which can be analysed simultaneously and is time consuming. Recently nonradioactive hybridisation in a microtiter plate has been used in conjunction with PCR, as the basis of a new molecular assay, the PCR enzyme-linked immunosorbent assay (ELISA). The PCR ELISA detects hybrids of DIG or biotin labelled PCR product and probe, in the microtiter

plate format which enables simultaneous processing of large numbers of samples. A commercially available PCR ELISA detection system has been successfully used to establish specific diagnostic techniques for several pathogenic organisms such as *Chlamydia trachomatis* (Royman *et al.*, 1996), sera-specific meningococcal infection (Borrow *et al.*, 1997) and the herpes simplex virus (Scott *et al.*, 1997). PCR ELISA assays have also been developed for *M. pneumoniae* (Kessler *et al.*, 1997), *Mycobacteriwn ulcerans* (Portaels *et al.*, 1997) and *Mycobacterium tuberculosis* (Crawford, 1998), indicating the increasing use of this technique in diagnostic assays.

The PCR ELISA provides high specificity conferred by the internal probe used, high sensitivity, convenience, rapidity compared to other hybridisation techniques, and can be easily adapted. In this study the efficiency and sensitivity of the PCR ELISA for the detection of *M. hominis* was investigated using the previously established primers, RNAH1 and RNAH2, and the internal probe, RNAH3 (Blanchard *et al.*, 1993) and the Boehringer Mannheim PCR ELISA DIG detection system (Mannheim, Germany).

5.2 Materials

Most of the chemicals commonly used in this study were from Merck (Darmstadt, Germany), BDH (Poole, England) or Boehringer Mannheim (Mannheim, Germany) and were of the highest purity available.

The unmodified and biotin labelled oligonucleotide primers were from Gibco BRL (Paisley, England). The DIG labelled oligonucleotide primer, PCR ELISA DIG detection kit, additional streptavidin-coated microtiter plates, restriction enzymes and proteinase K were from Boehringer Mannheim (Mannheim, Germany).

5.3 PCR assay

Using the principles of PCR (as discussed in section 3.4) and the RNAH1, RNAH2 primers of Blanchard *et al.* (1993), the specific assay for *M. hominis* was optimised. The sequences of the oligonucleotide primers were 5'-CAATGGCTAATGCCGGATACGC-3' (RNAH1, forward) and 5'-GGTACCGTCAGTCTGCAAT-3' (RNAH2, reverse). To facilitate ELISA detection of the PCR product, the RNAH2 primer was also synthesised with a 5'-DIG label.

5.3.1 Reagents

50 mM Magnesium chloride. See section 3.4.

10 mM dNTP mix. See section 3.4.

10x PCR buffer. See section 3.4.

5.3.2 Procedure

PCR amplification was performed using the Perkin-Elmer GeneAmp PCR system 2400 thermocycler according to the manufacturers instructions. The reaction mixtures were prepared in 0.2 ml thin-walled PCR tubes as shown in Table 7. For optimisation of the PCR, 100 ng purified *M. hominis* DNA (section 3.3.1) was used as the template, and when the rapid sample preparation method was used, the template was 5 μ l of the lysate. Control reactions were prepared by excluding template DNA, primers and *Taq* enzyme from separate mixtures and compensating the volume with sterile dist. H₂O. The amplification procedure using the unmodified primers consisted of an initial melting cycle at 95°C for 2 min; 25 cycles of 95°C melting for 25 s, 58°C annealing for 30 s, 72°C extension for 30 s; and a final long extension at 72°C for 5 min. When the DIG-labelled RNAH2 primer was used for the PCR ELISA, the annealing temperature was dropped to 48°C. At the completion of the cycles, the samples were held at 4°C until removed from the thermocycler. Aliquots (5 μ l) of the PCR reactions were analysed using agarose gel electrophoresis and ethidium bromide visualisation (section 3.3.2).

Table 7. Components of the reaction mixture for PCR detection of *M. hominis*.

Reagent	Initial concentration	Volume (µl)	Final concentration
PCR buffer	10x	5	1x
dNTP mix	10 mM	1	200 nM
MgCl ₂	50 mM	2.5	2.5 mM
RNAH1	50 µM	2	1 µM
RNAH2	50 µM	%	1 µM
<i>Taq</i>	5U/µl	0.5	2.5 U
Template	-	1-5	-
Dist. H ₂ O	-	to 50 (µl)	-

5.4 Confirmation of PCR product

As the primers used in the PCR assay had previously been shown to be specific for *M. hominis* (Blanchard *et al.*, 1993; Abel-Horn *et al.*, 1996), amplification of the 16S rRNA region was confirmed through restriction analysis. The sequence of the *M. hominis* PG21 16S rRNA gene has been elucidated enabling the identification of unique restriction sites within the region of interest.

5.4.1 Procedure

The PCR product was digested separately with *AM*, *Apal* and *HindIII* in the following reaction: PCR product (5 µl), restriction enzyme (20 U), 10x restriction buffer (2 µl) and dist. H₂O to a final volume of 20 µl. The reactions were incubated at 37°C overnight and the digests examined through agarose gel electrophoresis.

5.5 ELISA detection

Visualisation of PCR products is usually done through agarose gel electrophoresis and ethidium bromide staining, but in diagnostic assays often the small number of target molecules result in faint signals which are beyond the sensitivity limits of ethidium bromide staining. An alternate detection and hybridisation method using ELISA methods is becoming more popular. The ELISA detection of non-radioactive labelled PCR products can be automated for the assay of large numbers of samples, allowing rapid diagnosis (Ritzier and Altweg, 1996).

The method of labelling PCR products in this study, was the incorporation of a DIG label through the use of a DIG-labelled primer (section 5.3). The internal capture probe was biotin-labelled RNAH3 (Blanchard *et al.*, 1993) with the nucleotide sequence 5'-CGCTGTAAGG CGCACTAAA-3'. The biotin-labelled capture probe hybridises to the DIG-labelled PCR product, the hybrid is attached to a streptavidin coated well of a microtiter plate through biotin-streptavidin affinity binding and detected with an anti-DIG peroxidase conjugate and the colorimetric substrate 2,2'-azino-di-(3-ethyl)-benzthiozoline sulfonic acid (ABTS) (Fig. 5.2).

The procedure for the ELISA detection requires the optimisation of several aspects, particularly the temperature and length of hybridisation which is dependant on the characteristics of the capture probe and the concentration of the capture probe. Various parameters of the PCR ELISA were optimised and adapted for the sensitive detection of DIG-labelled PCR products from *M. hominis*.

5.5.1 Reagents

Biotin-labelled capture probe. Lyophilised biotin-RNAH3 was reconstituted in dist. H₂O to a stock concentration of 100 pmol/μl which was diluted to 10 pmol/μl for use.

All other reagents were supplied by the manufacturer and working solutions prepared according to instructions.

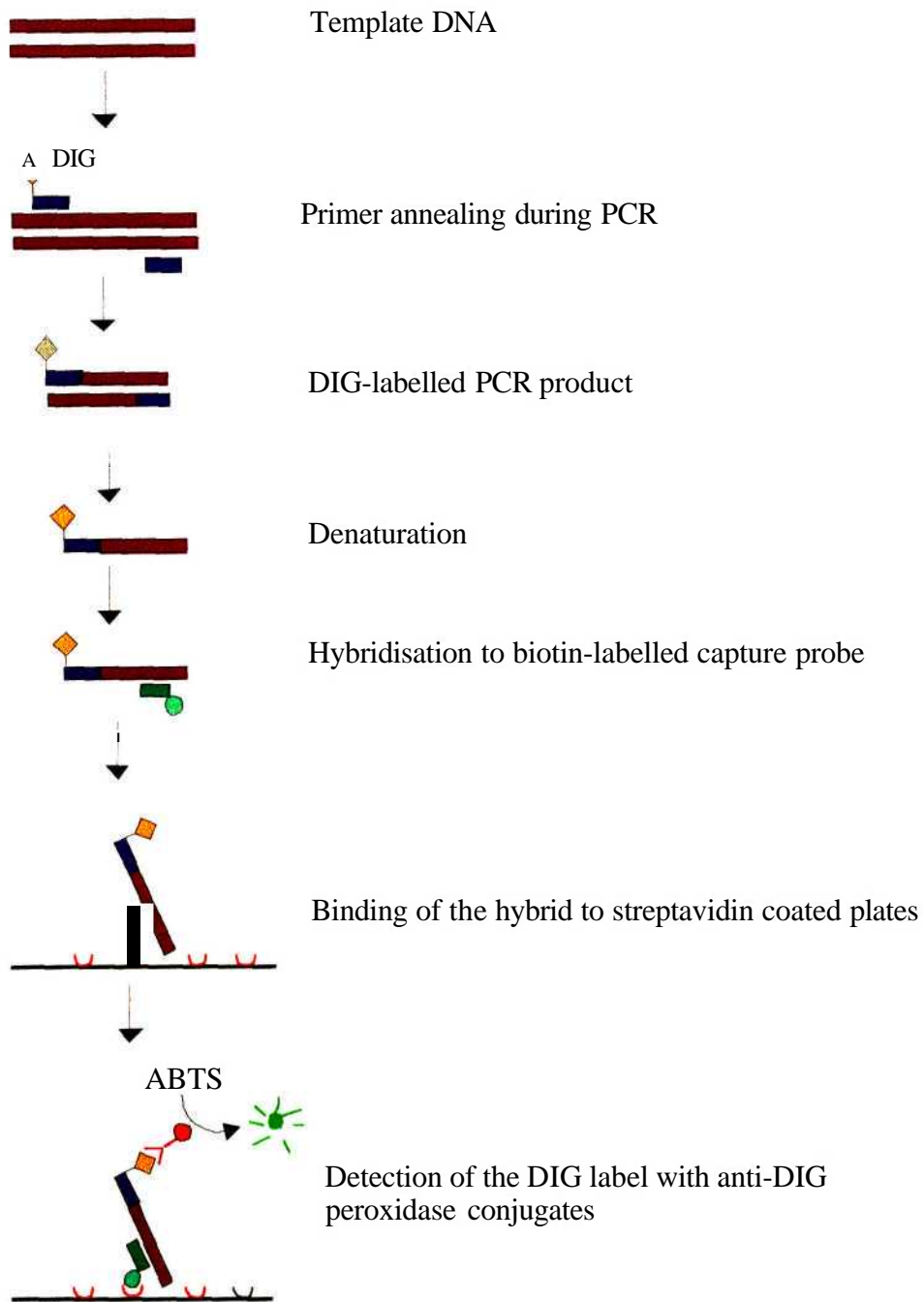


Figure 5.2. The steps involved in PCR ELISA,

5.5.2 Procedure

Aliquots of the undiluted or serially diluted PCR reaction were either denatured by alkali or heat denaturation. For heat denaturation, the samples were boiled for 10 min and snap-cooled on ice and for alkali denaturation, alkali denaturation solution (20 μ l) was added to the sample and incubated at RT for 10 min. The samples were made up to 250 μ l with hybridisation solution containing the biotinylated probe. The samples were either prehybridised before addition to the streptavidin coated microtiter plate or added directly in 200 μ l aliquots. Hybridisation was done using different incubation temperatures and durations. The bound hybrids were incubated with anti-DIG peroxidase conjugate (200 μ l) for 30 min at 37°C, developed with ABTS substrate solution (200 μ l) for 30 min at 37°C in the dark and detected at 405nm. Between each incubation, the plate was washed three times with wash solution.

5.6 Rapid sample preparation for PCR

A time efficient molecular diagnostic technique, such as the PCR ELISA, requires a rapid DNA extraction procedure which is not dependent on complete DNA purification. A simplified sample preparation method that provides an adequate DNA quality for PCR, has been developed (Blanchard *et al*, 1991). This method essentially uses detergents to lyse bacterial cells in suspension and removes contaminating proteins through proteolytic digestion.

5.6.1 Reagents

Solution A 100 mM Tris-HCl pH 8.3. 100 mM KCl. 2.5 mM MgCl₂. 1 M Tris-HCl pH 8.3 (0.1 ml), 1 M KCl (1 ml) and 2.5 M MgCl₂ (25 μ l) were made up to 10 ml with sterile dist. H₂O.

Solution B 100 mM Tris-HCl pH 8.3. 2.5 mM MgCl₂. 1% (v/v) Tween 20. 1% (v/v) Triton X-100. 120 μ g/ml proteinase K. 1 M Tris-HCl pH 8.3 (0.1 ml), 2.5 M MgCl₂ (25 μ l), Tween 20 (0.1 ml), Triton X-100 (0.1 ml) and proteinase K (120 mg) were made up to 10 ml with dist. H₂O.

5.6.2 Procedure

Exponential cultures (1 ml) were centrifuged at 12 000 xg, 4°C for 20 min, the bacterial pellet resuspended in a mixture of solution A (250 μ l) and solution B (250 μ l) and incubated at 60°C for 1 hour. The proteinase K was inactivated by heating at 95°C for 10 min. The samples were cooled to room temperature and 5 μ l taken from each sample for PCR.

5.7 Results

Using the RNAH1, RNAH2 primers and an optimised PCR protocol, an approximately 300 bp DNA fragment was amplified from *M. hominis* DNA (Fig. 5.3). This corresponded to the expected size (Blanchard *et al*, 1993) and digests with several restriction enzymes produced the expected fragments based on the sequence of the 16S rRNA gene fragment.

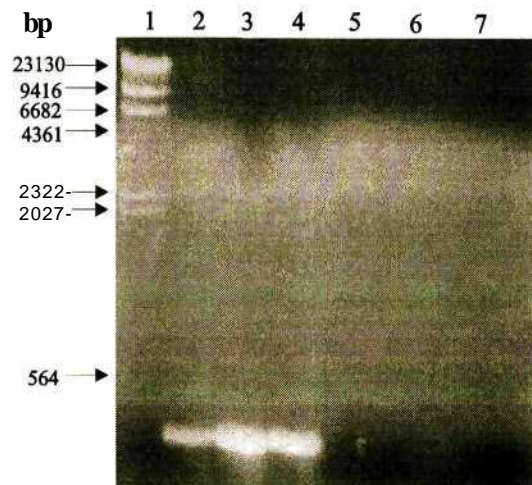


Figure 5.3. DNA amplified from *M. hominis* using RNAH1 and RNAH2 primers. (1) *HindIII* X DNA size marker; (2) *M. hominis* PG21; (3), (4) *M. hominis* isolated from clinical samples; (5) No DNA control; (6) No primer control; (7) No *Taq* control.

The initial parameters to be optimised for the PCR ELISA were the hybridisation conditions, whereby different temperatures (37°C, 47°C and 55°C) and durations (1 and 3 hours) were investigated (Fig. 5.4). Two different methods of DNA denaturation, alkali and heat, were compared in conjunction with varying conditions for prehybridisation of the denatured PCR product and the capture probe (Fig. 5.5). The optimal concentration of capture probe (Fig. 5.6) and the efficiency of the number of PCR cycles (Fig. 5.7) were determined.

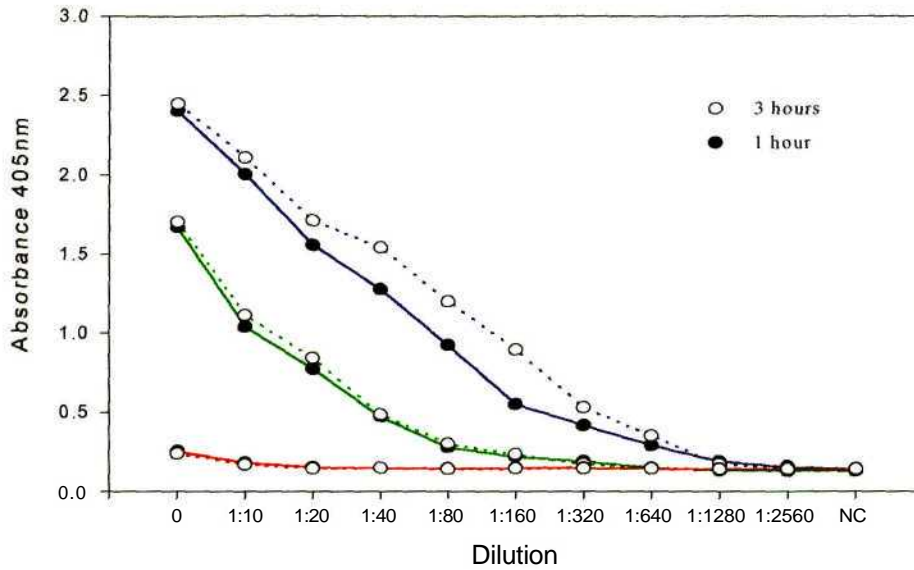


Figure 5.4. The effect of hybridisation conditions on the sensitivity of the PCR ELISA. Serial dilutions of *M. hominis* amplified DNA were hybridised at 37°C, 47°C and 55°C for 1 and 3 hours. NC, negative control without any DNA.

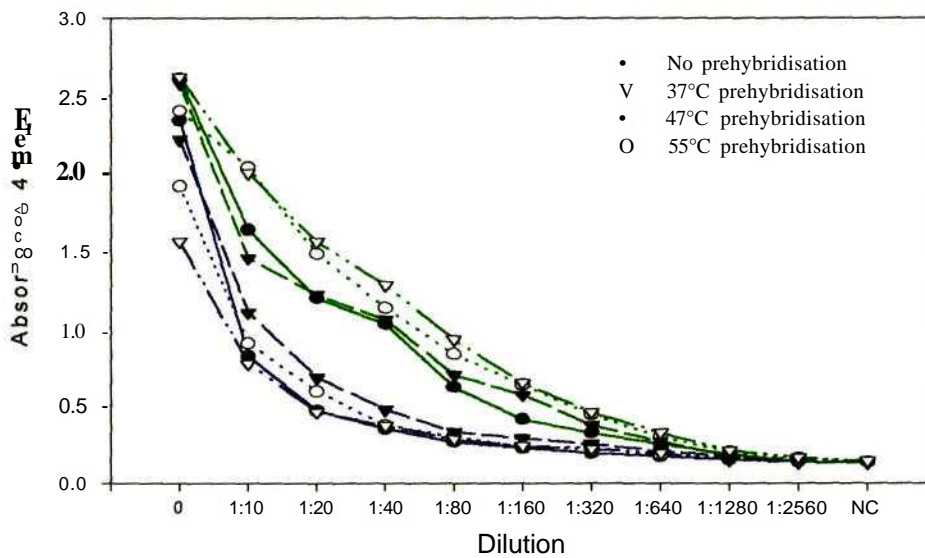


Figure 5.5. Comparison of DNA denaturation and prehybridisation conditions. Serial dilutions of *M. hominis* amplified DNA were denatured using heat or alkali, and either prehybridised with the capture probe at 37°C, 47°C or 55°C for 15 min, or mixed with the capture probe and added directly to the streptavidin coated wells.

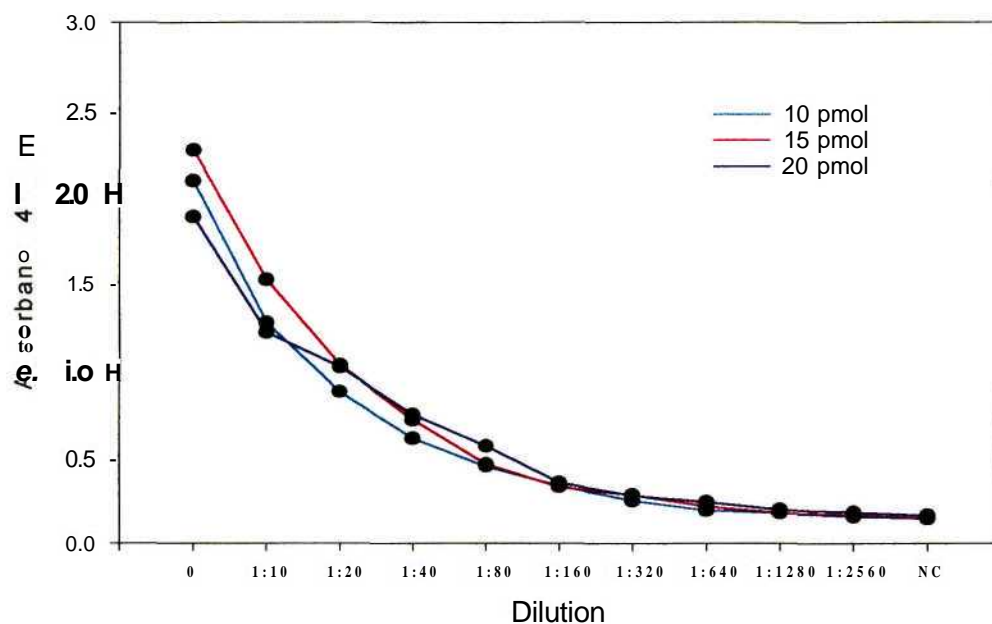


Figure 5.6. Determination of optimal capture probe concentration. Serial dilutions of *M. hominis* amplified DNA were prehybridised with 10 pmol/ml, 15 pmol/ml or 20 pmol/ml capture probe respectively.

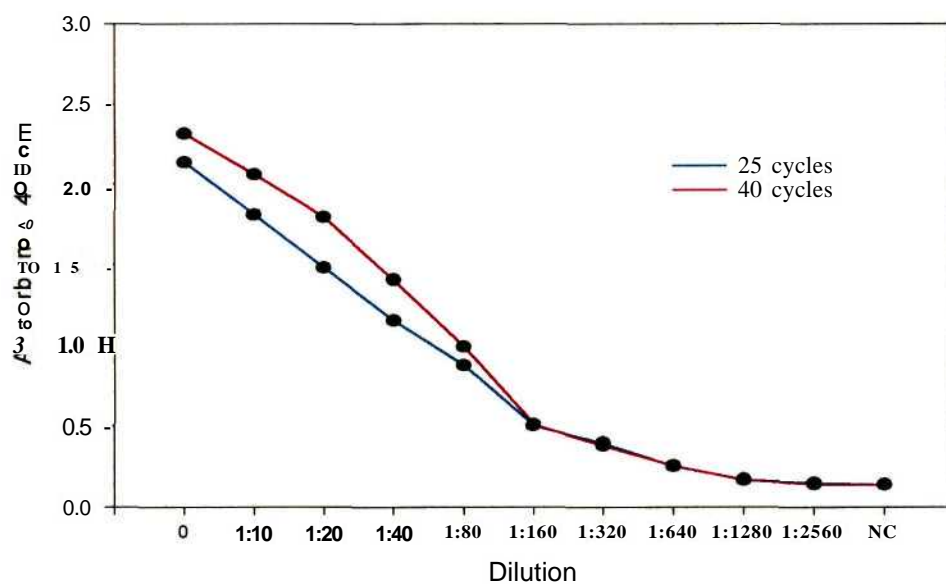


Figure 5.7. The effect of the number of PCR cycles used for amplification. *M. hominis* DNA was subjected to either 25 or 40 cycles of amplification and serial dilutions of the respective amplified DNA detected in the ELISA.

The sensitivity of agarose gel electrophoresis and ethidium bromide staining for the detection of amplified *M. hominis* DNA (Fig. 5.8) was compared to that of the ELISA detection (Fig. 5.9).

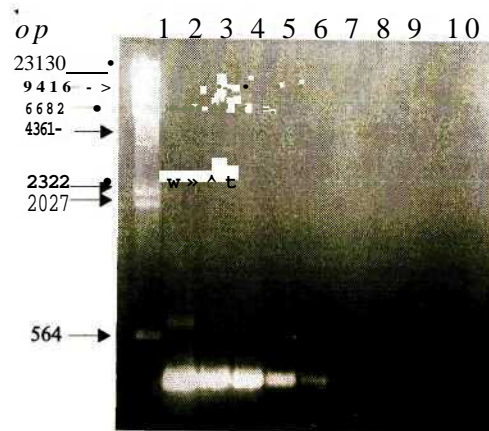


Figure 5.8. Detection sensitivity of ethidium bromide stained agarose gels. Different amounts of *M. hominis* template DNA were used for PCR amplification with RNAH1 and unlabelled RNAH2 primers. (1) *HindIII* X DNA size marker; (2) 10 ng template DNA; (3) 1 ng; (4) 100 pg; (5) 10 pg; (6) 1 pg; (7) 100 fg; (8) 10 fg; (9) no DNA control; (10) no primer control.

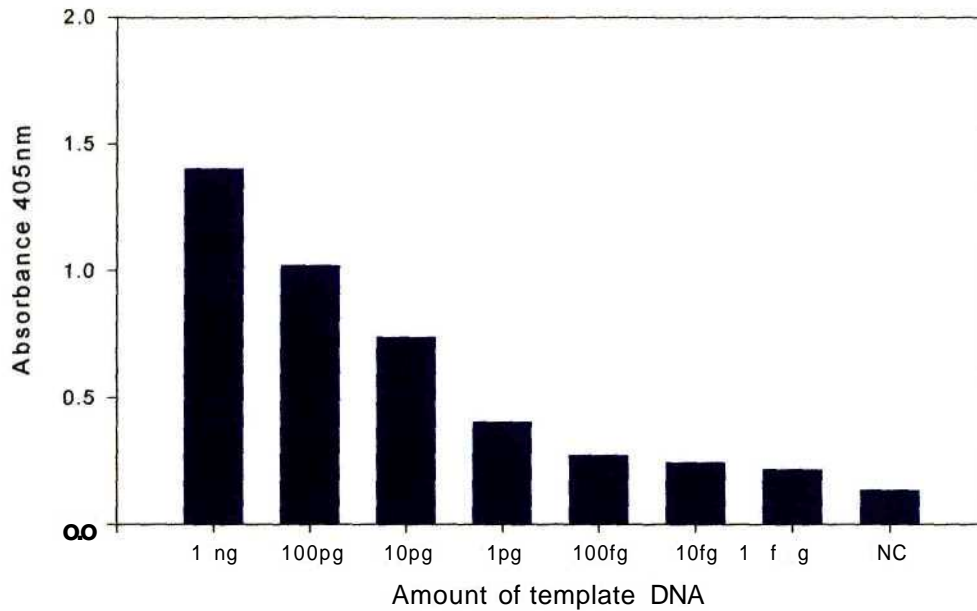


Figure 5.9. Detection sensitivity of the PCR ELISA. Different amounts of *M. hominis* template DNA were used for PCR amplification with RNAH1 and DIG-labelled RNAH2 primers.

The PCR ELISA was tested using clinical culture samples prepared for PCR as described in section 5.6 (Fig. 5.10).

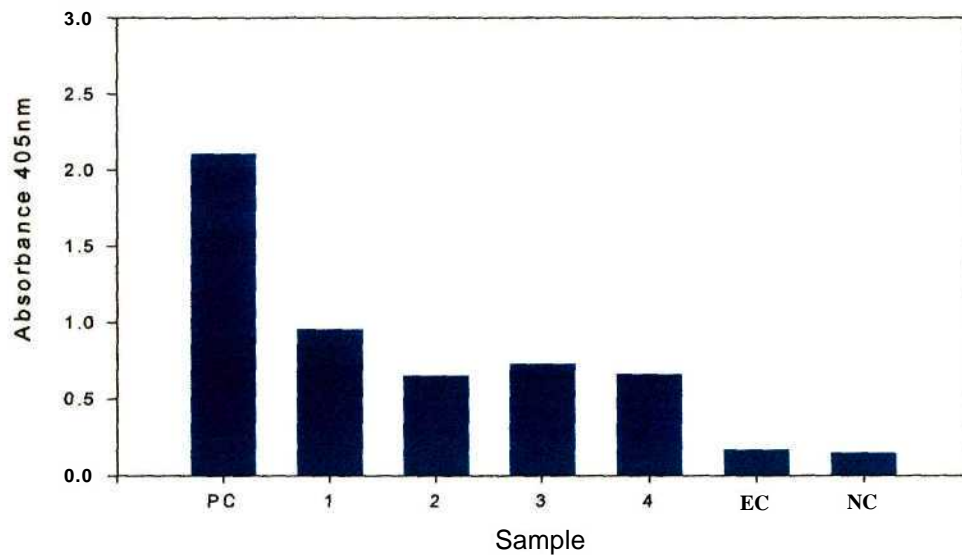


Figure 5.10. PCR ELISA detection of cultured clinical *M. hominis* strains. PC, positive control with 10 ng *M. hominis* DNA as PCR template; 1-4, clinical *M. hominis* isolates prepared by the rapid sample preparation method; EC, *E. coli* prepared by the same method.

5.8 Discussion

The use of the PCR ELISA has wide applications and has shown its potential as an effective diagnostic assay. Due to the current time delay in conventional serological and culture diagnosis of *M. hominis* infections, the PCR ELISA is an attractive alternative. Other proposed molecular diagnostic assays for *M. hominis* require confirmation of the amplified DNA by either Southern hybridisation (Blanchard *et al.*, 1993; Abele-Horn *et al.*, 1997) or sequence determination (Vogel *et al.*, 1997). These additional hybridisation steps are time consuming but can be effectively substituted with ELISA detection using a suitable capture probe.

The efficiency of hybridisation depends predominantly on the nature of the capture probe, and is also influenced by the hybridisation conditions (Ritzier and Altwegg, 1996). The highest incubation temperature stipulated by the PCR ELISA manufacturer is 55°C. This may be due to the potential denaturation of the streptavidin or inhibition of the biotin-streptavidin non-covalent interaction at higher temperatures. While higher temperatures closer to the melting point of the amplified DNA may favour the maintenance of the denatured single-stranded state, they may not favour the formation of capture probe-amplicon hybrids and subsequent immobilisation on the streptavidin coated microtiter plate. For the hybridisation of *M. hominis* amplified DNA, a temperature of 37°C was found to be optimal (Fig. 5.4). At higher temperatures, the sensitivity decreased significantly, with minimal hybridisation at 55°C. The longer duration did increase the absorbance at 37°C although not substantially, while little difference was apparent at the higher temperatures. The optimal hybridisation conditions for the *M. hominis* PCR ELISA were thus determined as 1 hour at 37°C.

The manufacturer's protocol follows an alkali denaturation of the amplified DNA, followed by mixing with the capture probe and direct application to the streptavidin coated wells. In an attempt to simplify the required components and improve the sensitivity of detection, heat denaturation and prehybridisation of the denatured amplified DNA and capture probe, were investigated (Fig. 5.5). Generally the sensitivity of the heat denaturation was higher than that obtained with alkali denaturation. As the exact composition of the alkali denaturation solution is not provided, the stringencies of the methods cannot be compared; however heat denaturation appeared more effective and would minimise the reagents required for the assay.

Prehybridisation increased sensitivity, allowing the capture probe-amplicon hybrid to form prior to the immobilisation of the capture probe to the streptavidin coat. For consistency and simplification, prehybridisation at 37°C was selected as optimal.

The concentration of capture probe should be sufficient to maximise the efficiency of hybridisation and biotin-streptavidin immobilisation. The optimal concentration of biotin-labelled RNAH3 was determined as 15 pmol/ml, which produced slightly higher absorbance than 10 pmol/ml but was comparable to 20 pmol/ml (Fig. 5.6). Although most PCR assays use thirty to forty cycles to generate large amounts of amplified DNA, this was found to be unnecessary in this study. Slightly higher absorbance was generated by DNA amplified from 40 cycles as compared to 25 cycles (Fig. 5.7), however the detection sensitivity after 25 cycles was sufficient. Increasing the volume of amplified DNA analysed in the ELISA, did increase the absorbance obtained, but not in an exponential manner and aliquots of 10 μ l produced high levels of detection. Permanent shaking of the microtiter plates during all incubations also increased the absorbance.

The limit of detection of the PCR assay was previously determined to be between 10 and 15 fg of *M. hominis* DNA (Blanchard *et al*, 1993). In this study, the detection limit using agarose gel electrophoresis and ethidium bromide staining, was between 100 and 10 fg DNA (Fig. 5.8), with the amplified DNA from 100 fg template fairly visible and that from 10 fg beyond clear visualisation. If an absorbance more than twice the absorbance of the negative control is considered positive (Ritzier and Altwegg, 1996), the detection limit of the PCR ELISA is between 100 and 10 fg (Fig. 5.9). The sensitivity of the PCR ELISA is thus comparable to ethidium bromide staining of agarose gels. However as Southern blotting is more sensitive than the latter technique, the ELISA detection would probably be less sensitive than conventional hybridisation. The PCR ELISA produced positive results for the clinical *M. hominis* strains tested from culture (Fig. 5.10), although to comprehensively assess the efficiency of the PCR ELISA as a diagnostic assay, direct testing of many clinical samples would be required.

These initial investigations into the application of the PCR ELISA as a diagnostic assay for *M. hominis* have demonstrated the potential of the technique. Although the procedure has been optimised, testing of clinical samples and comparison with conventional detection methods are essential to demonstrate the specificity of the assay. The PCR ELISA has several advantages

over conventional diagnostic assays: rapid generation of results, the ability to process large numbers of samples, reproducibility, less labour intensive and could be easily automated. With these features, the PCR ELISA can provide a basis for efficient, accessible and rapid diagnosis.

CHAPTER 6

GENERAL DISCUSSION

Mycoplasmas are unique and intriguing micro-organisms, representing the smallest prokaryotes capable of self replication. They exhibit unique characteristics which differ from all other major groups of human pathogens and are responsible for their classification as a separate class, the most distinguishing feature being the lack of a cell wall. Mycoplasmas are a ubiquitous group widely distributed in nature and found in humans, animals, plants, insects, soil and sewage. Most species survive through parasitic or saprophytic relationships with their hosts as their biosynthetic capabilities are limited by the extremely small size of the mycoplasmal genome (Limb, 1989; Cassette *et al.*, 1994, 1995; Taylor-Robinson, 1995).

With a genome that may be as small as 600 kb, mycoplasmas are the closest examples of the theoretical concept of 'minimum cells'. These organisms are composed of the essential minimum number of organelles for growth and reproduction with only DNA, ribosomes and a plasma membrane. Mycoplasmas also lack the major energy production pathways found in most organisms and carry only one or two copies of certain genes that occur in multiples in other bacteria. Having been reduced to the simplest self-replicating organisms through degenerate evolution, mycoplasmas serve as useful models for cell biology studies (Razin, 1992; Maniloff, 1992). They have also been proposed to serve as the paradigm organisms for the complete deciphering of the machinery of a living cell (Morowitz, 1984). The complete nucleotide sequences of the genomes of *M. genitalium* (Fraser *et al.*, 1995) and *M. pneumoniae* (Himmelreich *et al.*, 1996) have been elucidated and found to code for 479 and 677 proteins respectively. One of the identified genes found in both organisms, displaying more than 90% amino acid identity, is that coding for the dnaK/hsp70 homolog (Himmelreich *et al.*, 1997).

The heat shock response is uniquely universally conserved, having been found in every organism studied thus far, with the hsp70 family of proteins displaying unprecedented sequence homology. The hsps function as molecular chaperones and are also the immunodominant antigens of many pathogenic micro-organisms (Kaufmann, 1990). A typical heat shock response has been demonstrated in the mycoplasmas, *A. laidlawii* and *M. capricolum*, with increased synthesis of some specific proteins accompanied by decreased synthesis of others, and cross-reactivity of a

mycoplasmal hsp70 homolog with anti-dnaK antibodies (Dascher *et al*, 1990). The conservation of a heat shock system in mycoplasmas derived through degenerate evolution, implies the system has a significant physiological role.

In the present study, the heat shock system of *M. hominis* was investigated, focusing on the hsp70 homolog. The presence of the hsp70 gene in *M. hominis* was determined using an established PCR method (Galley *et al*, 1992) and sequence analysis, which also demonstrated the high degree of interspecies conservation. The DNA segment containing the hsp70 gene was successfully cloned but only preliminary nucleotide sequence data obtained. Complete sequencing of the cloned fragment will determine the size of the *M. hominis* hsp70 gene and provide the basis for further analysis. Hsp70 sequences are being applied to phylogenetic and evolutionary studies as they are among the most highly conserved proteins known (Gupta and Golding, 1993; Boorstein *et al*, 1993). As mycoplasmas have lost many gene functions through degenerate evolution, their evolutionary relationship to other prokaryotes is of interest. Recently the hsp70 gene of *M. capricolum* was cloned (Falah and Gupta, 1997) and its sequence used to confirm the established phylogenetic model which indicates that mycoplasmas branch from the gram-positive *Lactobacillus* group (Maniloff, 1992). The determination of the *M. hominis* hsp70 sequence may prove useful in analysing the phylogeny within the mycoplasma group.

The heat shock response is regulated at both the levels of transcription and translation, with a variety of mechanisms controlling the response either directly or indirectly. The mechanisms responsible for the induction process are not clear as several external factors such as ethanol, heavy metals, extreme pH and thermal change, may be responsible for activation of the response. However the basic mechanism of transcriptional regulation, involving heat shock transcription factors, seems to be similar in different organisms and autoregulation of the response appears to be a conserved feature (Burdon, 1986; Lindquist and Craig, 1988). The upregulation of hsp expression in response to stress, is initiated either by activation of mRNA synthesis or increased synthesis if the hsp is constitutively expressed (Cochrane *et al*, 1994). The hsp70 mRNA in *M. hominis* demonstrated a significant increase in abundance following heat shock. Low levels of hsp70 mRNA appeared to be present under normal conditions, suggesting constitutive expression. This was confirmed through analysis of the translated products, although the methods employed were not sufficiently sensitive to indicate the expected increased synthesis of

the hsps and decreased synthesis of other proteins. The use of ^{35}S -methionine labelling and autoradiography, or 2D gel electrophoresis would prove more useful in identifying the hsps present in *M. hominis* (Dascher *et al.*, 1990; Hamel *et al.*, 1997). However immunocytochemical assays demonstrated the presence of the hsp70 protein, both constitutively and following heat shock. The cross-reactivity between the *M. hominis* hsp70 and the antibodies against the *E. coli* and *Drosophila* hsp70 homologs, is indicative of the unusually high degree of conservation.

The observations on the hsp70 transcriptional and translational changes in *M. hominis* appear to correlate with those of other investigators. Hsp70 mRNA is maintained at a low basal level with a rapid increase following induction and maintenance of high levels for a prolonged period (Velazquez *et al.*, 1983). It has been suggested that the constitutive low level of transcription may play a regulatory role in keeping the gene ready for immediate activation (Velazquez *et al.*, 1983), although for *M. hominis*, this is more likely to be the physiological consequence of maintaining fairly high levels of constitutive hsp70. The upregulation of hsp70 in response to heat shock was more readily detected at the level of transcription rather than translation, as proposed by Cochrane *et al.* (1994). As determined for *A. laidlawii* and *M. capricolum* using immunoblotting (Dascher *et al.*, 1990), an increase in the level of hsp70 protein between normal and heat shocked cells, is not distinct. This may be the result of an increased rate of synthesis rather than the steady state level of hsp70 (Hamel *et al.*, 1997). *M. hominis* does however appear to produce a high basal level of hsp70 which may obscure the relatively low increase in newly synthesised hsp70. This high level of constitutive hsp70 suggests an essential role in normal cell physiology. The hsp70 homolog of *M. hypopneumoniae*, the causative agent of mycoplasmal pneumonia in swine, has been implicated as an immunodominant antigen. The hsp70 protein may thus act as a major antigenic determinant in mycoplasmas, corresponding to the suggestion that hsps may elicit the host immune response (Dascher and Maniloff, 1992).

Although the mechanisms of its pathogenesis still remain largely unknown, the pathogenic potential of *M. hominis* has been widely demonstrated and is a threat particularly to newborns and immunocompromised patients. In order to facilitate rapid appropriate chemotherapeutic treatment of *M. hominis* infections, rapid clinical diagnosis is essential. Current diagnosis is based on isolation by culture and confirmation through serological methods (Cassell *et al.*, 1995). These assays however require several days to produce results and are complicated by the extreme

heterogeneity the different *M. hominis* strains display (Clyde, 1983b). Due to their time efficiency, reliability, reproducibility and potential for automation, the development of molecular diagnostic assays has increased. Molecular assays for *M. hominis* involving PCR and either Southern hybridisation or sequence determination (Blanchard *et al*, 1993; Vogel *et al*, 1997), have been suggested. However the methods used for confirmation of the amplified product in these techniques are also time consuming and still require several days. The development of a PCR ELISA technique enables same day results and the ability to process many samples simultaneously. This technique has been applied to the diagnosis of several pathogens and is gaining popularity as a rapid diagnostic assay. In the present study, a PCR ELISA was developed and optimised for the detection of *M. hominis*. The appropriate conditions were designed to maximise sensitivity and specificity, while also simplifying the technique and enabling ease of use. The results determined through these investigations represent the initial steps in the development of a diagnostic assay. Comprehensive testing of clinical samples and comparison to conventional diagnostic methods are necessary to fully assess the potential of this technique.

The unique characteristics of mycoplasmas as the smallest free-living organisms, and the heat shock response as the most highly conserved known genetic system, present an intriguing combination. The elucidation of the mechanisms responsible for the functioning of both is a continuing process. The results of the investigations conducted during the present study represent the preliminary foundation for further research.

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