BACTERIAL TAXONOMY

-ANALYTICAL AND BIOCHEMICAL METHODS WITH SPECIAL REFERENCE TO MYCOBACTERIA

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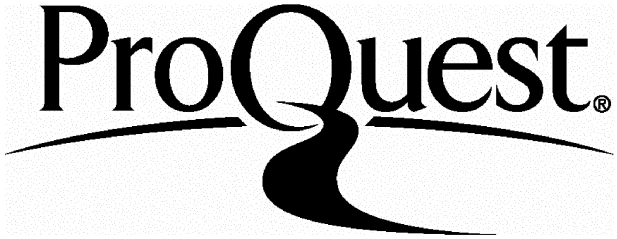

I dedicate this thesis to my Dad, Anton Furst,
I am eternally grateful for his support and guiding light
and for the inspiration he has given me.

And forever cherish his memory with love and pride.

ABSTRACT

Bacterial taxonomy is particularly important in medicine, where the correct identification of an organism enables the optimal treatment to be prescribed. Currently medical microbiology uses a compilation of sequential morphological, staining, biochemical and serological tests to identify an organism. Many of the routine identification tests do not enable sub-speciation of an organism. Sub-speciation can be important in routine circumstances, particularly for infection control; studying the epidemiology of an organism by sub-speciation is vital. Thus there is a need for new microbiological tools and methods to advance microbiological knowledge and this is the basis on which this study was undertaken.

A universal feature of all cells is the uptake and utilisation of certain substances for the synthesis of proteins, the essential tools of cell metabolism. The bacteria were incubated with [^{35}S] methionine and the radiolabelled samples were separated by SDS PAGE. The resulting "bar code" type pattern was then examined by autoradiography and more importantly using a Radioanalytic imaging system that enabled computer analysis of the data. The data were extracted as histograms and normalisation strategies assessed. The data were then grouped and analysed by dendrogram or used in a database for identification. The methods were standardized to allow comparisons of different

species and the use of other sources of [^{35}S] such as inorganic sulphate and thio ATP were investigated. All organisms gave labelled patterns with the methionine (except for *Mycobacterium leprae*) and most did so with the sulphate and ATP and the data were used to investigate speciation and sub-speciation.

Mycobacteria were of particular interest because they are slow growing and there is a need for rapid identification and sub-speciation techniques. Mycobacteria have very thick cell walls with a very high lipid content, this made standardisation of cell break down for analysing the cell content difficult, so the secreted proteins were predominantly analysed. *Mycobacterium leprae* presented particular problems and some studies of the metabolism of this organism were carried out in order to try and apply the labelling methods to this organism. It was not possible to reproducibly label *M. leprae* proteins but some useful metabolic studies were made.

Mycobacteria are unique bacteria in producing iron binding compounds such as mycobactins which are known to be species specific. In this study the methods for extracting, labelling with [^{55}Fe] and separating mycobactins by TLC were improved to increase detection sensitivity and their potential for rapid identification of mycobacteria from clinical specimens was assessed.

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THE AIMS OF THIS THESIS

The aims of this thesis were firstly to continue developing the [^{35}S] methionine labelling techniques of Holland, 1987, and to explore cheaper and potentially useful alternatives to methionine. Secondly to expand the range of organisms that can be investigated rapidly by this method, particularly the slow growing members of the genus *Mycobacterium*. Thirdly to test extensively the computer software as an aid for gel normalisation and thus enable the comparison of large groups of species. Fourthly to build a database that could be used for rapid identification and for the sub-speciation of clinically important species.

The initial stages of this study concentrate on looking at a general and wide range of bacterial genera for assessing the usefulness of the different [^{35}S] compounds and examining the reproducibility of the electrophoresis techniques. The second stage of this study concentrates more closely on a more selective range of bacteria, particularly mycobacteria, and to building and testing the application of a database. There is particular emphasis here on mycobacteria because of their importance as human pathogens and the particular problems associated with their classification and identification. Mycobacteria are particularly slow growing in laboratory culture media requiring 3-12 weeks to cultivate pathogenic strains from patients' samples. Once isolated their identification takes a further 2-8 weeks.

Identification of the species is especially important because of Public Health and therapeutic implications of the species. This is particularly so with the *M.avium, -intracellulare -scrofulaceum* (MAIS) group and the *M.tuberculosis* (tubercle) complex which can cause similar infections but show little similarity in their antibiotic susceptibility. Infection with these two groups of mycobacteria have increased in recent years, particularly in patients with AIDS. Infection with other less common mycobacterial pathogens are also important. These infections should be treated as quickly as possible, but there are few rapid methods to identify the causative organism. There is also a need for new identification and classification techniques for *Mycobacterium leprae*, so the [^{35}S] labelling methods were used to examine this species. The aim was to adapt the methods described by Franzblau, for maintaining *M.leprae in vitro*, for labelling with [^{35}S] compounds in order to obtain [^{35}S] labelled *M.leprae* proteins.

Two approaches to rapid mycobacterial identification have been pursued. Initially using the [^{35}S] labelling method and then using an alternative biochemical method analysing mycobactins, one of the compounds unique to mycobacteria. The mycobactins were labelled with [^{55}Fe] by utilising the natural affinity of these compounds for iron, they were then differentiated using thin layer chromatography (TLC). The method for differentiation of mycobactins by TLC was initially developed by Snow (1965)

and has been used by many other workers. However the methods already in use were not sensitive enough for application to clinical samples. The aim was therefore to improve the sensitivity of the extraction and detection method and to adapt it for use with clinical specimens and isolates.

CHAPTER 1 BACTERIAL TAXONOMY

1.1 INTRODUCTION

All studies of the natural world require an organised method of naming and classifying the items under study. This is so that information can be passed on, conveying the maximum known at that time about the studied organism. Thus whether it be mammals, insects, reptiles, bacteria or other organisms our taxonomic arrangement of these organisms evolves and reflects our view and present understanding of the structure of the natural world. Classification schemes evolve along with our expanding knowledge. This leads to revision of groupings and renaming of taxonomic units. Consequently there is room for discrepancies in opinion between different workers about the classification and particularly the taxonomic arrangement of different organisms.

Shortly after the discovery of bacteria and once distinct organisms were recognized as such the classification of bacteria was attempted. At this time the classification of organisms was dominated by the Linnaean system. In this system the organisms are grouped in a hierarchic manner (Table 1.1) with the lowest taxonomic unit designated as a species. Each of the taxonomic groups in the hierarchy share common features which not only unite them as a group, but differentiate then from other groups. The Linnaean system, however, had been developed to classify

organisms that were composed of eukaryotic cells and the newly discovered bacteria were of a different cellular structure. One of the major differences between the eukaryotic and what came to be known as prokaryotic organisms was the question of the stability of the 'species', even if the concept of a species was valid in classification of prokaryotic organisms. In the classification of animals and plants the direction of the process of classification can be thought of as downwards, from the higher taxa to the species. But in the classification of bacteria it is convenient to think of the direction of classification as upwards from the species. With animals and plants (eukaryotes) the species is clearly defined and maintained so by reproductive and geographical isolation. With bacteria (prokaryotes) because of the lateral transfer of genetic information and the spectrum of characteristics that can be transferred between species the species is less clearly defined as a taxonomic unit and its definition can vary between the major groupings of bacteria (Stanley and Kreig, 1984).

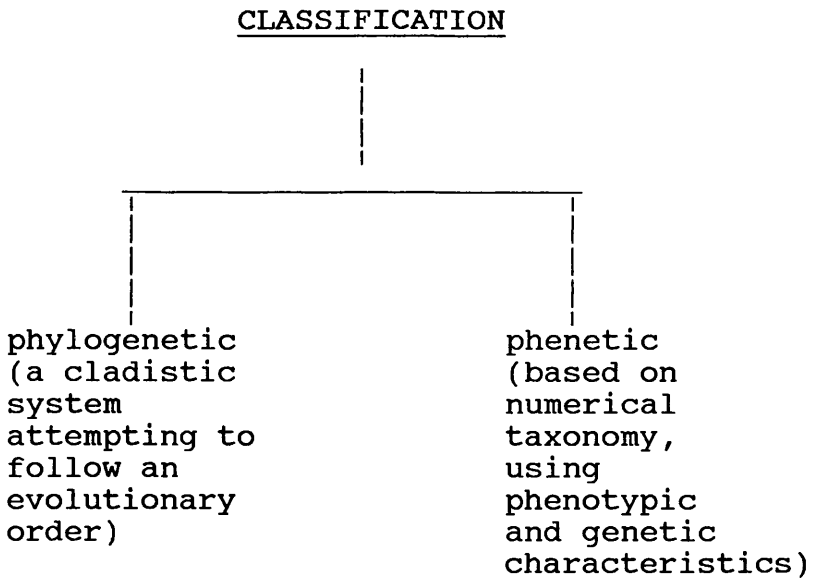
The two major approaches to classification have engendered much debate (Table 1.2). On the one hand is a cladistic or phylogenetic system which tries to demonstrate the progress of evolutionary relationships between organisms and on the other hand, an empiric grouping according to considerations of various characteristics which

Table 1.1. Taxonomic ranks.

Kingdom
Division
Class
Order
Family
Genus
Species

Murray, 1984b; Sneath, 1984a; Holland, 1987.

Table 1.2. Classification strategies.



Norris, 1980; Sneath, 1978; Hill,1983/84; Woese and Fox, 1977; Fox *et al.*, 1980; Cain and Harrison, 1960; Holland, 1987.

are the end product of evaluation (a phenetic system). Which ever taxonomic system is used it should have certain characteristics. It should be stable with respect to newly aquired information. The system should be polythetic including a variety of characteristics from a large number of isolates. Within each grouping the intra-group variation should be less than the inter-group variation. Finally every member of a taxonomic unit need not necessarily possess a given characteristic but the taxonomic unit should be defined by character sets rather than by individual characteristics.

The classification system developed by Linnaeus was a system in which different characters, usually morphologic, had different weighting. An alternative approach to taxonomy is numerical taxonomy (Sneath, 1957a,b) where:

1. each character has equal weighting and is recorded in a binary format as present or absent, '1' or '0'. This sort of data recording clearly lends itself to computerization.
2. a large number of isolates should be used
3. a large number of characteristics should be used
4. the similarity between groups should be recorded as percentage similarity coefficients
5. the isolates should be grouped as clusters according to the shared characters.

Numerical taxonomy gained wide acceptance by bacterial taxonomists because much of the data available could be put

into the biniary forms. However it was criticized by taxonomists of eukaryotic organisms because it was not a cladistic system and rejected the significance of character weighting. However, the application of numerical taxonomic techniques to a database does result in a value of importance for a given character by virtue of a correlation coefficient. Highly correlated characters (with a high similarity coefficient) determine the overall structure of the classification system and thus have more weighting, although this weighting was not assumed a prior.

Despite all the difficulties the clear organisation of bacteria is important as an aid to identifying and studying infectious diseases which are still the highest cause of mortality in underdeveloped countries and continue to cause morbidity in both developed and underdeveloped countries.

1.2 DEFINITIONS AND NOMENCLATURE

The three essential and interdependent components of bacterial taxonomy, as defined by Cowan (1971), are classification, nomenclature and identification. **Classification** places organisms into groups, and different strategies can be used for this classification. **Nomenclature** is the labelling of groups and of individual members within groups according to international rules (Lapage *et al.*, 1975; Sneath, 1992). The validity of nomenclature of bacteria is controlled by the Bacteriological Code and is

periodically reviewed by the International Committee for Systematic Bacteriology which produces Approved Lists of Bacterial Names (Skerman *et al.*, 1980). This list is updated by validation lists that are published in the International Journal of Systematic Bacteriology. The same style of nomenclature is followed as that originally adapted by Linnaeus with a latinized binomial; the genus name is given first, with the first letter capitalized, followed by the species name in lower case letters (Breed, 1948; Sneath, 1984b; Hill, 1983/84). **Identification** is the process of recognizing an unknown organism so that it can be named and classified.

1.3 THE BEGINNING OF BACTERIOLOGY

Micro-organisms were first described by Leeuwenhoek (1632 - 1723) in a series of letters to the Royal Society of London (Dobell, 1932). Leeuwenhoek had constructed a microscope and observed 'animalcules' in water, soil and human material. However, the first recorded attempts at speciation was not until 100 years later by Muller (1786) in his 'Animalcule Infusoria et Marina'.

Over the next 100 years characterisation of bacteria was attempted from descriptions of mixed cultures, which meant that classification was 'often vague and puzzling' and nomenclature was 'chaotic' (Bergey *et al.*, 1925). There was a clear need for developments in isolation of pure

strains. Louis Pasteur, between 1860 to 1890, was the main driving force of this development and thus also of modern microbiology. He experimented with sterilization, an essential process for obtaining a pure culture. He also established that micro-organisms cause disease and founded the principles of immunisation.

Tyndall (1877) used differential heating methods to combat persistent spore forming bacteria, and Joseph Lister (1878) worked on the dilution method to obtain a pure culture. He also applied Pasteur's observations to the prevention of wound sepsis, which revolutionised surgery. The development of a solid medium by mixing gelatin and nutrient broth, by Robert Koch (1884), was a major break through in techniques of bacterial culture. This solid medium was modified by Petri in 1887 by replacing gelatin with agar. This technique is still a fundamental tool in bacteriology today. Koch also discovered the bacterial cause of many diseases, including tuberculosis in 1882, and defined the criteria for attributing an organism as the cause of a specific disease (Table 1.3).

Further progress was made by Gram, in 1884, who developed a bacterial stain that divided bacteria into two classes, those that retained the crystal violet stain and appeared blue in colour under the microscope, called Gram positive, and those organisms in which the crystal violet leached out and could only be seen with a red counter stain-

Table 1.3. 'Koch's postulates', 1884.

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1. The particular infecting microbe must be found in all cases of the disease, preferably in locations and numbers which could explain the lesions and symptoms of the disease.

2. The organism must be grown in pure culture in order to prove that it is an independent, animate article.

3. Inoculation of the pure culture into a healthy host must reproduce the disease, including the presence of the same organism in lesions.

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called Gram negative. This was the first step towards a systematic method of classifying bacteria. Even today it is one of the first tests used in the identification process.

1.4 THE DEVELOPMENT OF DIFFERENT CLASSIFICATION SCHEMES

By the end of the 19th century the field of bacteriology was firmly established with its essential microbiological methods and a number of classification schemes. In Europe the scheme proposed by Lehmann and Neumann (1896 updated in 1927) was in general use. But in America it was the work of Buchanan which dominated bacteriology. Buchanan published a series of studies on the nomenclature and classification of bacteria (1916, 1917, 1918) in the newly established Journal of Bacteriology.

The Committee of the Society of American Bacteriologists (SAB) was established in 1917 by Buchanan and Winslow *et al.* (1917) who saw the need for the formation of a bacterial code. In 1923 the Bergey Committee of the SAB produced the first edition of the 'Bergey's Manual of Determinative Bacteriology' which was somewhat based on Buchanan's classification scheme (Bergey *et al.*, 1925; Breed *et al.*, 1948). Successive editions have provided an up to date account of current bacteriology, although not necessarily the definitive account but a supplement to other manuals available today (such as the 'Manual for the Identification of Medical Bacteria' by Cowan and Steel,

1992).

It was suggested by Murray in 1968 that bacteria be placed in the biological kingdom in the taxon *Procaryotae* (Murray, 1984b). The term was introduced into the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbon, 1975). The term is still controversial as all bacteria may not be procaryotic. Because the ribosome has such an important function in protein synthesis, the structure is highly conserved and has thus resisted rapid evolutionary changes. Woese and Fox (1977) investigated the nucleotide sequence of 16S rRNA from a wide range of different organisms, performing a phylogenetic analysis of the results. They demonstrated that there were 3 clusters which represented primary kingdoms, one containing the typical bacteria (prokaryote), a second represented by the eukaryotic cell and a third (archebacteria) represented by methanobacteria (Woese *et al.*, 1990).

The early grouping of micro-organisms, based on the Linnaen method of classification, relied heavily on the weighting of microbial characteristics according to their relative importance. This introduced subjective interpretations of data which has brought about many variations of taxonomic systems. Thus workers characterized bacteria and arranged them in an order they intuitively thought was the best, but different opinions led to a variety of different 'intuitive' judgments, (Staley and

Krieg, 1984; Hill, 1983/84). Therefore although taxonomic ranks remained constant, some bacteria have been considered to be at genus level by one person but at species level by another (Staley and Kreig, 1984). It was not surprising therefore, when Sneath (1978, 1984a) suggested the term 'species' needed to be qualified and that Fox *et al.* (1980) described bacterial taxonomy as where 'the accepted relationships (are) no more than official sanctioned speculation'.

The current classification scheme was proposed by Gibbons and Murray and is outlined by Murray (1984b). But as 'classification cannot be final ' (Cowan, 1971), Murray (1984a) proposed that 'this one has no more permanence than those that went before'.

1.5 BACTERIAL CLASSIFICATION

Ideally, to classify a bacterial species a large number of isolates, eg. 100 or more, should be examined using many of the available analysis methods. The data are then grouped according to the selected scheme. For higher organisms a hierarchic classification scheme has been adopted and was designed to reflect phylogenic relationship between species. But in bacterial classification this approach has not been so successful. The identification of bacteria is however hierarchic, because identification tests are applied sequentially, using the results of one test to indicate which subsequent tests to apply. However, less emphasis has

been put on the hierarchic classification approach in the most recent Bergey's Manual of Determinative Bacteriology (Kreig and Holt, 1984).

Michael Adanson, who was a contemporary of Linnaeus, published a new classification scheme in 'Familles des Plants', 1763, which was based on equal weight being given to all characteristics (Sneath, 1957a), but it was more difficult to apply and so was not widely used. The principles of the Adansonion method were taken up by Sneath (1957a, b) and became part of the basis for the development of computer aided analysis. This gave a more objective method of classification which still used traditional bacterial characteristics but as mentioned previously gave each characteristic equal weighting so that statistical methods could be applied.

As mentioned previously the significance of the bacterial 'species' is debatable. Cowan and Steel (1974) recognise this problem and went further by not attempting to define the concept of a bacterial 'species' but accepted it as a convenient unit that had different values in different groups of bacteria. Neither do they try to analyse different kinds of characters, the qualities that distinguish one species from another or whether the taxon (taxonomic group) is a species, a variety or a sub-species. Cowan and Steel (1974) also do not believe that the classification they

describe represents the phylogenic groupings and they do not attempt to combine families into even larger groups (orders) which they regard as artificial and highly speculative. They recognize that different kinds of bacteria are not separated by sharp divisions but by slight and subtle differences in the characteristics so that they blend into each other and resemble a spectrum. This makes the definition of a bacterial species more difficult than for eukaryotic organisms where reproduction, and thus recombination of genes, can only occur between organisms of the same species. With micro-organisms gene transfer can occur between organisms of different 'species' which causes the spectrum of micro-organism as described by Cowan and Steel. However, because 'species' is a useful concept to taxonomists, attempts have been made to define the bacterial species. Brenner and Falkow (1971, 1981) employed a phenetic description of species which stated that 'species are groups of strains in which (i) over 70% of their DNA reassociates under moderately restrictive conditions and (ii) the thermal stability of this reassociated DNA is within 4 °C of that of homologous reassociated DNA'. This definition is one of the minimal standards required for the description of new species. The call for minimum standards for the description of a new species was first made in the International Journal of Systematic Bacteriology in 1977. Updates to the International Code of Nomenclature continue to be published in the International Journal of Systematic

Bacteriology and new species are then added to the Approved List of Bacterial Names (Skerman *et al.*, 1980). For example, the minimum standard for assigning a new isolate to the Genus *Mycobacterium* include acid fastness, a DNA G+C content in the range from 61 to 71 mol%, and the prescence of C22-C26 mycolic acid as well as the DNA-DNA hybridisation analysis (Levey-Frebault and Portaels, 1992).

In addition to the traditional characteristics of morphology and biochemistry that have been used to classify bacteria, there have been advances in adapting newer, chemical or molecular biological techniques, as methods of classifying bacteria. For example:

(a) Genetic analysis

New developments in molecular biology have been readily used to investigate the relationship between micro-organisms,

Genetic relatedness can be analysed by calculating the mean nucleotide composition of a DNA sample. This is expressed as the mole percent guanine and cytosine content (mol% G + C) of DNA which can be determined by measuring the melting point of the DNA during thermal denaturing. It can also be measured by determining the density of the DNA in a cesium chloride gradient using ultracentrifugation. The values obtained from this method range from about 25 to 75 percent. This method is best for determining non-relatedness as similarity in G + C content does not necessarily imply

relatedness (Johnson, 1984; Hill, 1983/84; Bradley, 1980; Owen and Pitcher, 1985).

A second strategy that has been used to assess genetic relatedness is called transformation. This involves solubilising DNA extracted from one bacterial strain and transferring it to another strain, causing heritable changes in the second strain. The relatedness of the two strains is calculated from the efficiency of transfer (Jones and Krieg, 1984).

The comparison of nucleotide sequence is the final arbiter of relatedness and has been used in bacterial taxonomy in a number of ways. Firstly the similarity in sequence homology can be evaluated by heat denaturing the double-stranded DNA of two strains being compared and allowing them to reanneal together by slow cooling. The amount of hybrid DNA, measured by ultraviolet spectrophotometry or by means of a labelled DNA probe, will then reflect the degree of sequence homology (Bradley, 1980; Johnson, 1984; Hill, 1983/84). In an alternative methodological approach the denatured DNA can be fixed to a nitrocelulose membrane and then incubated with the denatured radiolabelled DNA or RNA of a second strain. The degree of homology will be reflected in the amount of bound labelled DNA which can be monitored by autoradiography (Johnson, 1984; Bradley, 1980; Hill 1983/84; Owen and Pitcher, 1985). Secondly the neucleotide sequence has been determined

directly by sequencing the actual bases. Most attention has been paid to the 16S rRNA genes because they are highly conserved. Such investigation by Woese and Fox (1977) have led to a fundamental reorganisation of bacterial taxonomic structure and has led, as previously mentioned, to the description of a third line of cellular descent, the archebacterial cell (Woese *et al.*, 1990).

Different strains of bacteria can be discriminated by restriction endonuclease analysis (REA) of chromosomal DNA (Pitt, 1991). The restriction endonucleases, of which more than 500 are known, cleave the DNA into different lengths depending on the position of the individual recognition sequences. The digested DNA is electrophoresed, and the fragments stained with ethidium bromide and viewed under ultra-violet light. The scoring and classification of these fingerprints has been proposed (Stahl *et al.*, 1990).

Gene probes have been used to reduce the number of bands generated by REA and simplify the interpretation of genomic profiles. Variability in the presence of cleavage sites can be detected by the use of DNA probes and this is known as Restriction Fragment Length Polymorphism (RFLP) (Stewart *et al.*, 1993; Small *et al.*, 1993). Probes used for epidemiological typing include: random or specific chromosomal sequences, toxins and antibiotic resistance genes, ribosomal RNA cistrons, and insertion sequences (Einstein, 1990).

(b) Fatty acid analysis by chromatography

There are over 300 known fatty acids in micro-organisms which are now being utilised for bacterial taxonomy (Sasser and Wichman, 1990). Fatty acid analysis is performed by chromatography. Chromatographic separation is dependant on physical partitioning or adsorption of sample components between a mobile and a stationary phase. The mobile phase may be a gas or liquid, and the stationary phase may be a solid or liquid.

Gas chromatography (GC) and high-performance (pressure) liquid chromatography (HPLC) have been developed for taxonomy. Fatty acids in the size range of 9 to 20 carbons in length are easily analysed by GC and are used alone or with biochemical information. Very large fatty acid materials such as the mycolic acids may be analysed by HPLC or thin layer chromatography (TLC) and can be used alone or in conjunction with biochemical tests to identify mycobacteria, nocardiae and rhodocci (Butler and Kilbum, 1988; Butler *et al.*, 1987; Damato *et al.*, 1987; Levy *et al.*, 1986).

Whole-cell fatty acid analysis (9 to 20 carbons in length) for the identification of micro-organisms has been described for a wide range of organisms (Athalye *et al.*, 1985; Brondz *et al.*, 1989; Eerola and Leptonen, 1988;

Lambert and Moss, 1989). In particular fatty acid profiling has been used for identifying Gram-negative nonfermenters (Vays *et al.*, 1989), mycobacteria (Larsson *et al.*, 1989; Maliwan *et al.*, 1988) and yeasts (Brondz *et al.*, 1989).

Quantitation of the various fatty acids and the application of numerical analytic techniques have been used in taxonomic analysis. Thus in a study of coryneform bacteria isolated from bio-films, a numerical analysis of the fatty acids of 79 isolates distiguished corynbacter from mycobacteria and led to the identification of a new corynform bacteria (Bendinger *et al.*, 1992). Fatty acids have also been investigated by fast atom bombardment- mass spectometry. In a study of some Gram negative bacteria the effect of growth conditions upon the stability of the phospholipid content was investigated. The authors could demonstrate some differences in phospholipid content but the variation was very much less than the inter-species variation and those effects could be overcome by standardization of experimental procedure during preparation of the organism under investigation (Aluyi *et al.*,1992).

(c) Pyrolysis mass spectrometry

This method is based on the principle that pyrolysis of pure bacterial cultures, rather than of only some components as mentioned above, gives mass ion spectra which reflect the particular chemical characteristics of the analysed bacteria.

Pyrolysis mass spectrometry (PyMS) is rapid and can be used for inter-strain comparisons for many bacterial species (Freeman *et al.*, 1990). Computer analysis of data is needed to select the mass ions that give the best discrimination between the various spectra. The selected mass ion spectra are then used as unweighted characteristics which are collated in a similarity matrix giving analysis of similarities and differences.

(d) Protein analysis

Bacterial proteins have been analysed by electrophoresis for classification and identification of many different organisms (Table 1.4). Different subpopulations of proteins have been examined such as enzymes (Table 1.5), membrane proteins (Mocca and Frasch, 1982; Heckels, 1977) as well as whole cell proteins (Table 1.4). The most widely used method for studying proteins has been electrophoresis. Originally workers used paper, agar gel or starch gel as a matrix for protein electrophoresis but this was later replaced by polyacrylamide, giving a technique known as polyacrylamide gel electrophoresis (PAGE), which can be used for one or two dimensional separation.

Gel electrophoresis techniques have been based on one of three methods. The first and original method by Davis (1964) was the electrophoresis of cell-free extracts

Table 1.4 Table of some references to whole cell microbial protein profiles analysis by PAGE.

Coomassie blue stained profiles.	
coagulase-negative staphylococci	Druden *et al.*, 1992.
Helicobacter pylori	Perez-Perez *et al.*, 1992.
Serratia marcescens	Gargallo-viola and Lopez, 1990.
Candida spp.	Lehmann *et al.*, 1989.
Clostridium difficile	Pantosti *et al.*, 1988.
	Poxton *et al.*, 1984.
Pseudomonas aeruginosa	Walia *et al.*, 1988.
Clostridium difficile	Ehret *et al.*, 1988.
Staphylococcus spp.	Clink and Pennington, 1987.
Varied species	Kersters and De Ley, 1984.
Campylobacter spp.	Ferguson and Lambe, 1984.
Mycobacterium fortuitum, M.terrae, M.nonchromogencium	Vanden Berghe and Pattyn, 1978.
Zymomonas spp.	Swings *et al.*, 1976.
Enterobacteriaceae	Sacks *et al.*, 1969.
Silver stained profiles	
Aeromonas spp.	Millership and Want, 1989.
[^{35}S] methionine labelled profiles	
Helicobacter pylori	Clayton *et al.*, 1991.
coagulase-negative staphylococci	Brown *et al.*, 1991.
Serratia marcescens	Altwegg *et al.*, 1989.
Aeromonas spp.	Millership and Want, 1989.
Serratia marcescens	Arzese *et al.*, 1988.
BCG	Abou-zeid *et al.*, 1987.
diphtheroids	Asante *et al.*, 1987.
Staphylococcus spp.	Asante *et al.*, 1987.
Clostridium difficile	Holland, 1987
Varied species	Hook *et al.*, 1987.
	Tabaqchali *et al.*, 1987.
	Holland, 1987.

Table 1.5 Table of some references of bacterial enzymes analysed by PAGE.

Candida spp.	malate dehydrogenase superoxide dismutase alkaline phosphastase esterases Lehmann *et al.*, 1989.
Staphylococcus aureus	esterases Branger and Goullet, 1987.
Escherichia coli	carboxylesterase Goullet and Picard, 1986.
Aeromonas spp.	esterases glutamate dehydrogenase malate dehydrogenase lactate dehydrogenase Picard and Goullet, 1985.
Yersinia spp.	esterases Goullet and Picard, 1984.
Clostridium spp.	xylanase Leeuwenhoek, 1992.
Rhodospirillum rubrum	glutathione reductase Libreros-Minotta *et al.*, 1992.

directly in a discontinuous alkaline gel and buffer system. This separated native proteins on the basis of both charge and size and maintained biological activity.

The second method also separated proteins on the basis of size and charge. But in this method proteins were dissociated and denatured by treatment with phenol-acetic acid-water and separated in an acetic acid urea gel and buffer system (Razin & Rottam, 1967).

The third method was described by Laemmali (1970) and separated proteins strictly according to size (Hames, 1981). This was achieved by solubilizing proteins in an ionic dissociating detergent, sodium-dodecyl sulphate (SDS) and electrophoresing the polypeptides in a discontinuous gel and buffer system, which also contained SDS. This method is referred to as SDS-PAGE and is widely used today. The gels were originally analysed only by visual comparison of bands, and by manual measurements to obtain Rf values (Kersters and De Ley, 1975). Vanden Burghe and Pattyn (1979) numerically analysed band positions by manually dividing each lane into a set number of units, using internal standards as reference positions, and scoring bands in each unit. However due to the complexity of bacterial protein banding patterns and the need to normalise data from different gels the software for computer analysis of gels was developed by a number of different workers (Jackman *et al.*, 1983; Schumaker, 1978). Stained proteins are scanned by

densitometry (Swings *et al.*, 1976; Kersters and De Ley, 1984; Ehret *et al.*, 1988; Gargallo-Viola and Lopez, 1990) and radiolabelled bands are scanned by radiometric methods (Arzese *et al.*, 1988; Hook *et al.*, 1987; Tabaqchali *et al.*, 1987).

Initially protein electrophorotypes were always examined by Coomassie blue or silver staining (Table 1.4). However, radiolabelling with [^{35}S] methionine (Table 1.4) now often displaces the original method because it is quicker, and labels only actively formed, new, ie. not waste or degraded, protein and is more sensitive demonstrating differences between isolates which cannot be seen with Coomassie blue staining (Clayton *et al.*, 1991).

[^{35}S] methionine has been used to label bacterial proteins for taxonomy both with (Arzese *et al.*, 1988; Hook *et al.*, 1987; Tabaqchali *et al.*, 1987) and without (Abou-zeid *et al.*, 1987; Asante *et al.*, 1987) the aid of computer analysis. Some examples are the sub-speciation of BCG used for vaccination world wide (Abou-zeid *et al.*, 1987); multiantibiotic resistant skin diphtheroids (Asante *et al.*, 1987); coagulase-negative staphylococci (Asante *et al.*, 1987) and *Clostridium difficile* (Holland, 1987).

[^{35}S] Methionine is chemically unstable and must be immediately aliquoted and stored at -70°C in liquid nitrogen; it also gives off volatile radioactive products (Smith *et al.*, 1989). Thus alternative [^{35}S] labelling

compounds have also been investigated here. As methionine has been shown in some bacteria to be biosynthetically derived from inorganic sulphate (Roberts *et al.*, 1955), [^{35}S] inorganic sulphate was a possible alternative. [^{35}S] Inorganic sulphate is also cheaper than [^{35}S] methionine and stable at room temperature.

Using [^{35}S] inorganic sulphate may result in proteins being labelled by post translational modification to form sulphated proteins and polysaccharides. [^{35}S] Thio ATP was also investigated as an alternative to [^{35}S] methionine as it would also be used in post translational modification, and give phospho proteins. The thio analogue was preferred to using [^{32}P] because [^{35}S] has a much longer half-life and is much safer to handle.

In this study one of the aims was to develop a rapid, general method for mass screening so a standard protocol was needed and thus also a universal growth medium. An enriched medium, based on Eagles MEM, was developed for this purpose (see Table 3.1, 3.2).

In order to maximise the labelling capacity of [^{35}S] methionine, [^{35}S] inorganic sulphate and [^{35}S] thio ATP the standard medium was modified in each case so that it was free of the unlabelled precursor. Thus in the case of [^{35}S] inorganic sulphate, all the sulphate nutrients in the medium were replaced by their equivalent chloride (or other ion)

compounds.

Hitherto much of the work published on bacterial taxonomy has examined the protein fingerprints of the cell components only although it is well known that many bacteria secrete proteins. Patterns derived from these extracellular proteins were likely to be simpler than total cell fingerprints so supernatants and pellets were examined separately in a number of instances after labelling.

All the labelled proteins were examined by SDS-polyacrylamide gel electrophoresis (PAGE) as it is an effective and rapid method of separating proteins. To ensure that the system was rapid enough such that the techniques and data could be developed for use in a routine laboratory, pre-prepared gels were used. The gels prepared on gelbond were preferable for ease of handling and to reduce gel distortion after electrophoresis. One particular problem that has hindered workers using PAGE has been the lack of reproducibility of polyacrylamide gels. As well of the difficulty of comparing many electrophoretic tracks by eye. The development of the AMBIS radioanalytic imaging system (Smith, 1985) together with AMBIS microbiological system software (Tabaqchali *et al.*, 1987) has made it possible to investigate these problems analytically. Thus in this thesis the application of [^{35}S] labelling techniques coupled with the AMBIS rapid imaging and data analysis software have been investigated.

1.6 CURRENT BACTERIAL IDENTIFICATION METHODS

The identification of bacteria is an applied discipline of microbiology. It is an extremely important science and a major tool for the objective diagnosis of infection.

The principles of identification are different to those of classification, although some methods used in classification are also used in identification. The taxonomist will study as many characteristics as possible in classifying a new organism. However, the identification of a clinical isolate must be achieved quickly and with many economic considerations, ideally employing a minimum number of diagnostic tests. This may lead to a compromise between accuracy on the one hand and speed and cost on the other.

Today the accuracy of identification relies somewhat on the thoroughness of the preparatory work such as media making, preparing stains and reagents, and the degree of care taken in carrying out, observing and recording the results of the various tests. Much of this responsibility now rests with the manufacturers of media and identification kits such as API. But for all methods a pure culture is always required.

There are several strategies for identification although all ultimately rely on comparison of an organism with that of known identity. The three main strategies for

identification are as follows. Firstly a strategy where every conceivable test is applied. This method is not generally used as it requires an excessive and unnecessary amount of work. Although all the tests necessary to identify the organism should have been covered, this may not be so and additional tests may still be required.

The second approach is a hierarchic method where a series of tables are consulted sequentially and guide the investigator to the best selection of diagnostic test. This is the most common method. The first step is to establish a few fundamental character by using primary tests such as:

1. Staining reaction eg. by Gram stain of cells and/or spores
2. Morphology of culture and of the cells
3. Cell motility
4. The ability to grow in air
5. The ability to grow under anaerobic conditions
6. The culture media that gave best growth

After assessing the results of the primary tests a selection of secondary tests can be made. An example of a practical and logical hierarchic set of diagnostic tables for medically important bacteria is given by Cowan and Steel (1974).

The results of the primary tests are usually known within 24 hours of obtaining a pure culture. Only once these

results are known can the second stage tests be selected and applied. Sometimes some third stage tests are needed if the selected second stage tests do not identify the species sufficiently.

This process should identify the organism at the species level, but tells the investigator nothing about the sub-speciation and epidemiology of the strain.

A distinct advantage of some of the more recent methods used in bacterial taxonomy, eg PyMS and electrophoretic separation of proteins, is that this produces a 'fingerprint' of the organism which, if reproducible, can be used for Identification and typing by reference to a database of fingerprints from known organism.

The development of a database of [^{35}S] bacterial protein profiles of clinical isolates would thus not only be useful for rapid identification but also for sub-speciation and comparison with strains from previous outbreaks (Tabaqchali and Wilks, 1993).

CHAPTER 2 MYCOBACTERIA AND OTHER SPECIES OF INTEREST

2.1 INTRODUCTION

After the initial pilot studies using a small selection of many species, larger groups of some species were then analysed. The reasons for concentrating on certain species were varied and are discussed below. There is particular emphasis here on the Genus *Mycobacterium*. Mycobacteria are important human pathogens and there is a need for the development of rapid identification methods.

2.2 *STAPHYLOCOCCUS*

Staphylococcus strains are facultative anaerobes, non-motile, nonspore forming, Gram-positive cocci. Coagulase-negative staphylococci (CNS) have become increasingly significant opportunistic pathogens causing a range of diseases (Kolitainen *et al.*, 1990; Raad and Bodey, 1992; Peters, 1988). Disease arises particularly under abnormal circumstances, such as surgical implantation of foreign bodies (Marples, 1986).

Coagulase-negative staphylococci are acquired at birth and along with coryneform bacteria form the major part of the skin flora of man and animals. As staphylococci are such ubiquitous organisms, with most individuals being colonized with more than one bio-type, they often causes hospital outbreaks which have serious consequences for patients, especially if there is an additional problem of

drug resistance. Thus, typing of isolates from a suspected outbreak is extremely important, firstly to establish conclusively that there is indeed an outbreak and secondly epidemiological studies may help identify the source of infection. The combination of typing of isolates and associated epidemiological studies has clearly demonstrated the occurrence of single source outbreaks and of multiple infections (Bialkowska-Hobrzanska *et al.*, 1990; Oppenheim *et al.*, 1989). The results of these studies have undoubtedly had an advantageous effect on hospital practice as well as enhancing understanding of the epidemiology of the organisms.

Studies have shown that 35%-86% of CNS isolates are resistant to penicillinase-stable penicillins (Schwalbe, 1990). Thus treatment of serious cases has become increasingly dependant on vancomyocin and also teicoplanin. Recently, however there have been reports of vancomycin (Schwalbe, 1990) and teicoplanin resistance which may undoubtedly exacerbate the problem of hospital outbreaks. *Staphylococcus haemolyticus* is one of the coagulase negative staphylococci which is multi resistant and also isolated from clinically significant infections (Wynne *et al.*, 1992).

However, typing techniques for CNS such as phage typing and resistance typing are inefficient and methods such as bio-typing are too slow. Thus there is still a definite need for faster and more effective typing methods for CNS.

Twenty four teicoplanin sensitive and four teicoplanin resistant isolates of *Staphylococcus haemolyticus* were analysed to examine their sub speciation. The data were also used to test the reproducibility of the electrophoresis system.

2.3 *PSEUDOMONAS*

Pseudomonads are non-sporing Gram negative motile bacilli. They are strict aerobes although some may grow anaerobically in the presence of nitrate. They are catalase and oxidase positive and attack sugar oxidatively rather than fermentatively. They have a G-C content of 57-70 mol%. The Genus is divided into 5 rRNA homology groups, Group I includes *Pseudomonas aeruginosa* and *P.stutzeri*. Group II is the pseudomallei group and includes *P.pseudomallei, P.mallei, P.cepacia* and *P.picketti* and Group III is the acidovorans group including *P.acidovorars*, Group IV includes *P.diminita* and Group V *P.maltophilia* (Parker and Duerden *et al.*, 1990). A more recent rearrangement of this species has removed some of the species into separate genera eg. *P.maltophilia* is now *Xanthomonas maltophilia* (Van Zyl and Steyn, 1992).

Initially a number of *Pseudomonas* species were studied. *Pseudomonas pseudomallei* was of particular interest because this organism causes a severe dissemination infection called melioidosis (Dance, 1990) in parts of South East Asia and in

Northern Australia were the organism is found in the environment. Melioidosis may present as a chronic tuberculosis-like syndrome, or as an acute septicaemia with high mortality and it is increasingly recognised as an important cause of morbidity and mortality in endemic areas.

An understanding of the epidemiology of *P.pseudomallei* has been hampered by a lack of a suitable typing system. The individual strains are similar both in their biochemical reactions and antimicrobial sensitivity. Protein profiles of this organism have not been examined previously by other workers and it is for this reason that it was studied.

2.4 INTESTINAL SPIROCHETES

A variety of helically or spirally shaped, bacteria have become increasingly recognized in the last couple of decades to be pathogenic (Penn, 1991). One organism, *Serpula (Treponema) hyodysenteriae* (Stanton *et al.*,1991), is a recognized pathogen in pigs causing an illness called swine dysentry (Lysons, 1989), morphologically similar organisms have often been observed in large numbers in the lower intestinal tract in humans (Jeanette-Jones *et al.*, 1986), but their role in disease is still unclear. Study of their role in gastrointestinal disease has been in part hampered by a lack of adequate taxonomic data. It is not clear whether the term intestinal spirochete refers to one species or to several different species of related organisms (Hovind-Hougen *et al.*, 1982). A group of spirochetes,

isolated from the faeces of patients who had diarrhaea, were studied and compared with *Serpula hyodysenteriae* in order to determine their taxonomic relationship.

2.5 *CAMPYLOBACTER* AND *HELICOBACTER*

Campylobacter jejuni and *Helicobacter pylori* are intestinal pathogens they are curved, S- shaped Gram negative bacilli. *Campylobacter* causes gastroenteritis and is found in the lower intestine. *Helicobacter*, a newly recognized organism is found in the stomach of colonized subjects. At the time of this study there was controversy over the taxonomic relationship of *Campylobacter* and *Helicobacter*, in 1989 Goodwin *et al.* placed this organism in a genus separate from *Campylobacter*, called *Helicobacter* (Goodwin *et al.*, 1989). *Helicobacter* is now recognized to cause a chronic gastroenteritis, peptic ulseration and to be a risk factor for the developement of gastric cancer (Rathbone and Heatley, 1992). The epidemiology of this organism is unknown and there is no effective typing system for *Helicobacter*, except REA and RFLP both of which are technically demanding and labour intensive. For this reason both *Helicobacter* and *Capmylobacter* were choosen for study.

2.6 MYCOBACTERIA

(I) Introduction

Mycobacteria are responsible for two important human diseases, namely tuberculosis and leprosy. In the 20th century the incidence of these diseases has dwindled considerably in economically prosperous countries due to increasing Public Health standards. This trend has not, however, been observed in underdeveloped countries which contain the larger part of the world's human population, or in places such as New York were social deprivation, overcrowding and lack of Public Health facilities, has led to an alarming resurgance of tuberculosis, particularly multiple drug resistance strains. This state of affairs has arisen largely because of the underfunding of social provisions such that patients who are diagnosed as having tuberculosis often do not complete a specified course of treatment and thus allow drug resistant bacteria to emerge.

It is difficult to estimate the correct incidence of these disease in third world countries largely due to the lack of registration of the population and migration of individuals and communities. It has been estimated that a third of the worlds population is infected with the tubercule bacilli, and tuberculosis causes more deaths (over 3 million in 1991) than any other specific pathogen (WHO, 1992). The number of leprosy cases for 1991 has been estimated at 5.5 million cases globally (WHO, 1992). Clearly

the human suffering caused by these organisms is enormous, even though tuberculosis and leprosy are both controllable and curable.

In addition to *M.tuberculosis* and *M.leprae*, other mycobacterial species cause human disease eg. *M.fortuitum* and *M.chelonei* can cause abscesses (Plaus and Hermann, 1991; Rappaport *et al.*, 1990) *M.kansassi* and *M.xenopi* can cause tuberculosis (Wolinsky, 1974), *M.ulcerans* causes a necrotizing skin lesion (Wolinsky, 1975) and *M.marinum* causes a granulotus skin condition (Borradori *et al.*, 1991). Recently with the AIDS epidemic, these immunocompromised patients are recognized to be particularly susceptible to infection with MAIS (O'Grady and Fasier, 1992; Nightingale *et al.*, 1992). The patients may present with weight loss, chronic diarrhea, fever and anemia and infection occurs when the CD4 lymphocyte count falls below 200 cell/mm^3. These organisms are resistant to antibiotics usually used for infections with *M.tuberculosis*, but the patients do respond sympomaticaly to different antibiotic regimes. It is therefore particularly important to be able to rapidly differentiate *M.tuberculosis* from MAIS in the patient because this has therapeutic implications.

(II) Diagnosis: current identification methods

The initial diagnosis of mycobacterial infection may be

achieved by examining a Ziehl-Neelsen stained slide prepared from a human specimen such as sputum. The presence of a single acid fast bacillus is sufficient to alert clinicians in the presence of infection. However in many instances many fields of view must be examined to spot a single bacillus in a sputum sample. The absence of bacilli does not mean there is no infection as the sensitivity of microscopy is only about 55% (Savic *et al.*, 1992). Also, acid-fastness is not restricted solely to mycobacteria, but occurs in Nocardia and some Corynbacteria (Dastidar and Chakrabarty, 1990).

The second stage of laboratory diagnosis of mycobacterial infection is culture on Lowenstein Jensen slopes. Laboratory culture often takes many weeks to obtain visible growth sufficient to identify the mycobacterial species and the identification process itself may take several weeks. Cultures are left for up to twelve weeks. Thus, the investigation of rapid speciation and sub-speciation techniques is particularly important with this genus.

(III) Growth and metabolism of mycobacteria

Within the mycobacterial genus there is great variety of growth rate and nutritional needs. For instance *M.smegmatis* gives abundant growth in a few days on simple media, while *M.ulcerans* would need several months incubation on complex media to give the same yield. *M.leprae* has not

so far been grown axenically.

On the whole most mycobacteria grow on a relatively simple media requiring a carbon and nitrogen source and metal ions such as iron and magnesium.

A number of carbon sources can be utilized by mycobacteria such as glucose, glycerol and organic acids, particularly pyruvic acid. Likewise nitrogen can be supplied in several forms such as ammonia, certain amides or amino acids (such as asparagine) and, in some cases, as nitrate ions.

Mycobacteria all require oxygen for growth, although some variation in the oxygen requirement is seen. For instance, the human type of *M.tuberculosis* is a strict aerobe while the bovine type prefers a lower oxygen concentration and thus when cultured in broth the latter grows just below the surface, the former on the surface. This property can be used to distinguish between the two variants. BCG, although derived from a bovine type, resembles the human type in its oxygen preference. Studies on mycobacterial metabolic pathways indicate that in general they do not differ greatly from those in other bacteria and there are several reviews of mycobacterial metabolism (Ramakrishnan *et al.*, 1972; Barksdale and Kim, 1977; Wheeler and Ratledge, 1988). Some mycobacterial enzymes that control DNA replication and repair have been found to be similar to enzymes in *E.coli* (Grange, 1980). This suggests the

processes in mycobacteria are similar to that of *E.coli* and other bacteria.

The process of protein synthesis and enzyme regulation also appear to be similar in mycobacteria to those found in other cells (Grange, 1980).

The genome of mycobacteria have been found to range from 3 to 5.5 X 10^9 daltons (Grange, 1980). This is quite large compared to other bacteria such as *E.coli* which has a genome of 2.5 X 10^9 daltons. As with other bacteria, mycobacterial DNA homology has been found to be of taxonomic interest (Baess, 1979).

(IV) Classification of Mycobacteria

One of the earliest divisions of the Genus mycobacteria was into two major groups, the slow and rapid growers, although these groups do differ in many respects other than their rate of growth. A major contribution to Mycobacterial classification was provided by Runyon (1954), who classified slow growers into groups based on pigment production and growth rates. He divided the slow-growers into photochromogens (Group I), scotochromogens (Group II) and non-photochromogens (Group III); and rapid growers were a separate group.

A serological analysis by Stanford & Grange (1974) did

show distinct differences between the two main groups and it has been postulated that they represent a major evolutionary split in the genus early in existence. This has now been confirmed by rRNA analysis (Rogall *et al.*, 1990; Stahl and Urbance, 1990; Pitulle *et al.*, 1992)

Although there is wide variation within this genus, there are a few characteristics shared by all strains and which are unique to this genus. All mycobacteria are acid fast and Gram positive, even though the latter stain can give poor and apparently irregular results. The mycobacterial cells are not motile and do not form spores. Some species do produce extracellular slime but none produce true capsules. Branching can be induced by altering culture conditions with some species. For instance, in 2 per cent glycine, rapidly growing strains produce distinct mycelia which are weakly acid fast, like those produced by the *Nocardia* to which they are antigenically closely related. As previously mentioned the minimal recommended standards for describing the Genus *Mycobacterium* are a G+C content of 61-71 mol%, C22-C26 mycolic acids and presence of acid fastness. The recommended minimal standards for describing a slow-growing mycobacterial species are based on the results of phenotypic and genetic studies and include the results of the following conventional tests: growth at 25, 30, 33, 37, 42, and 45 °C; pigmentation; resistance to isoniazid, chloride, thiacetazone, picrate, and oleate; catalase activity; Tween hydrolysis; urease activity; niacin

detection; and nitrate reductase, acid phosphatase, arylsulfatase, pyrazinamidase and alpha-esterase activity. DNA-DNA hybridization analysis is also required, where the difference between the denaturation temperature of the homologous and heterologous reactions are determined (Levy-Frebault and Portaels, 1992).

(a) Slow growing mycobacteria

(i) M. tuberculosis

Clearly, identifying features that can be used to subdivide *M. tuberculosis* could be useful clinically if it identifies resistant or particularly virulent strains. This would make it easier for the clinician to correctly treat a patient quickly. At present it is often only after the patient has been treated for many weeks that resistant strains are identified as such and the required treatment then prescribed. Multi-drug resistant *M.tuberculosis* is becoming an increasing Public Health problem (WHO, 1992) as previously mentioned.

The virulence of *M.tuberculosis* has been found to correlate with phage type and lipid composition (Minnikin, 1982). In BCG a correlation between phage types of BCG substrains and the degree of delayed hypersensitivity triggered by these strains has been found (Mankiewicz and Liivak, 1977). This demonstrates that it may be possible to find markers for resistance and virulence of *M.tuberculosis*

in man.

Within the *M.tuberculosis* complex there are a number of variants that have been recognized as separate species status by some workers. These "species" are *M.bovis* (Karlson & Lessel, 1970), *M.africanum* and *M.microti* (Reed, 1957). As the name indicates *M.africanum* was isolated in Africa. The properties so far observed in these strains indicate they lie between the classical human and bovine types. A study by David *et al.* (1978) has shown that strains from West Africa are phenetically similar to the bovine type while those from the eastern regions are closer to the human type. The variant *M.microti* isolated from voles, also has properties intermediate between human and bovine types.

Yates and Collins proposed a simple classification scheme for clinical isolates in 1979 (Grange, 1980). The scheme was based on four properties: resistance to thiophen-2-carboxylic acid hydrazide, nitrase activity, oxygen preference (aerobic or microaerophilic) and resistance to pyrazinamide. Using these criteria Yates and Collins divided this species into human strains, bovine strains and BCG, and further divided the bovine strains into three major variants: European, Afro-Asian, and "Africanum" strains.

(ii) M.kansasii

M.kansasii is a slow growing photochromogenic species, but some strains occasionally are found to be scotochromogenic or non-chromogenic. Colony growth is usually smooth, but rough and mucoid variants have been found. The species was originally named by Handuroy in 1955 and is recognized as one of two species in Runyon's group I classification. Microscopy shows the *M.kansasii* bacilli to be slender rods which stain unevenly to give a barred or beaded appearance.

M.kansasii causes disease in man but its distribution in the population is variable. This species has been found particularly in the United States of America and England. In England the highest incidence is in North East London where it has been isolated from the water supply (McSwiggen and Collins, 1974). It has been realized for sometime that it is associated with pulmonary disease in humans (Francis *et al.*, 1975; Gross *et al.*, 1976).

A species closely related to *M.kansassi* has been isolated from gastric washings and termed *M.gastri* (Wayne *et al.*, 1978). It has not been associated with disease. *M.gastri* has has been regarded as closely related to *M.kansasii* by comparing the organisms microscopically and by colony morphology, but the two strains have been found to be identical by immunodiffusion analysis (Stanford and Grange, 1974). *M.gastri* differs from *M.kansasii* by being nitrase

negative and by failing to agglutinate with anti-*M.kansasii* typing sera.

(iii) M.xenopi

M.xenopi is slow growing. It generally produces light yellow colonies and microscopically often shows small aeriel hyphae. The cells are elongated and occasionally branching. *M.xenopi* will grow at 45°C and is arylsulphatase positive.

Schwabaker in 1959 (Grange, 1980) was the first to isolate this species from a skin granuloma of the toad *Xenopus laevis*. This species can cause chronic pulmonary disease in humans (Gross *et al.*, 1976).

The distribution of this species is not as widespread as some other opportunistic mycobacterial pathogens. Most isolates have come from London, South East England, Belgium and Northern France. This species has also been isolated from tap and other water supplies.

(iv) *M.avium - intracellulare - scrofulaceum* complex

The *M.avium* complex has a wide distribution in the environment. It contains variants such as *M.intracellulare*, *M.brunense*, *M.lepraemurium* and *M.paratuberculosis*. And there is continued debate as to how distinct these variants are, particularly between *M.avium* and *M.intracellulare*. Thus they are often referred to as *M.avium avium* and *M.avium*

intracellulare. *M.avium-intracellulare* can be serotyped and certain serotypes are regarded as *M.avium* and others as *M.intracellulare*. Recently biochemical differences have been identified (Sato *et al.*, 1992).

M. avium paratuberculosis

This organism causes Johne's disease mainly in cattle. It is usually called *M.paratuberculosis* or *M.johnei* and has been established as a variant of *M.avium* by immunodiffusion analysis. No human cases have ever been reported but this organism has been linked with Crohn's disease (Ciclitira, 1993).

Identification of this variant can be made by confirming dependence on mycobactin as strains when initially cultured cannot synthesise their own mycobactin. Some strains do lose their mycobactin dependency on subculture.

M.avium lepraemurium

This organism causes leprosy in rats but that is as far as it's resemblance to *M.leprae* goes, other than the great difficulty in culturing this organism *in vitro*.

These isolates grow as smooth non-pigmented colonies although rough variants are found, a few strains are scotochromogenic. The subspecies can be distinguished by

immunodiffusion analysis (Stanford, 1973) and the smooth strains of both types have been further divided by agglutination serology (Schaefer, 1965).

The incidence of *M.avium* has gone up over the past decade due to the rising prevalence of AIDS. *M.avium* infection in birds is also a growing problem in high density bird reserves which are becoming increasing prevalent due to destruction of habitats. This poses a devastating reserve of infection, for wild bird populations that invariably make use of such reserves as well as for captive birds being bred for release into the wild to restock endangered bird populations.

(b) Rapid growing mycobacteria

(i) M.fortuitum

The two clinically relevant rapid growing mycobacteria are *M.chelonei* and *M.fortuitum*. The others, with rare exception, are entirely environmental saprophytes and rarely cause clinical illness. Both *M.chelonei* and *M.fortuitum* are non-chromogenic rapid growers and have strong arylsulphatase activity. *M.fortuitum* is quite distinct from *M.chelonei* in its ability to reduce nitrate, and by differences in amidase activity, agglutination, immunodiffusion studies, antibiotic sensitivity and heat stable acylesterase activity. The more pathogenic strains of *M.fortuitum* have been found unable to

produce acid from alcoholic sugars, inositol, mannitol and sorbitol (Grange, 1980).

(ii) M. chelonei

There are two major types of *M.chelonei*, *M.chelonei abscessus* and *M.chelonei chelonei* which have been differentiated on the basis of citrate utilization, salt tolerance and antigenic differences. The former type is mostly found in Africa and America, the latter in Europe.

(c) Non-culturable mycobacteria

(i) M.leprae

This organism has posed a particular problem for microbiologists as it has not been reproducibly cultivated axenically. This has hindered studies of its metabolism, genetics and development of drug therapy.

Hansen was first to describe the leprosy bacillus, in 1873 which is why leprosy is often now called Hansen's disease. However it was not classed as a mycobacterium until 1893 by Lehmann and Neumann, who did so on the basis of its acid fastness. Its exact taxonomic position within the mycobacterium genus was debated and undetermined for some time. Studies of the structure of the mycolic acids by Etemadi and Convit (1974) showed *M.leprae* was distinct from other acid fast organisms such as *Nocardia* and *Corynebacterium*.

Studies of 16S rRNA have shown that *M.leprae* is a true member of the slow growing pathogenic mycobacteria (Smida *et al.*, 1988; Teske *et al.*, 1991)

3.1 THE SOURCE OF BACTERIA

A selection of Gram-negative and Gram-positive organisms were obtained from patients at the Middlesex Hospital, except where specified (Table 3.1).

(I) *Staphylococcus haemolyticus*

The *Staphylococcus haemolyticus* strains were isolated from patients at the Middlesex Hospital. The other coagulase- negative staphylococci (CNS) were obtained from Dr R.Marples at the Public Health Laboratories, Colindale.

(II) *Mycobacterium* spp.

Clinical mycobacterial isolates were obtained from the Dulwich culture collection, from the University College Hospital (UCH) and from Dr J. L. Stanford's collection at the Middlesex hospital, except where stated otherwise.

(III) *Pseudomonas* spp.

The *Pseudomonas* species were clinical isolates obtained from UCH, except for the *Pseudomonas pseudomallei* species which were obtained from Dr S. Gillespie, London School of Hygiene and Tropical medicine.

(IV) Spirochetes

The spirochetes were clinical isolates obtained from UCH and *Serpula* was obtained from Dr Lysons, Institute of

Table 3.1. Source of isolates used in study. NCTC, National Collection of Type Cultures; UCH, University College Hospital; *, clinical isolates from the Dulwich culture colection, see Appendix 3.1 for the laboratory culture numbers.

Staphylococcus haemolyticus	28	clinical isolates UCH
Staphylococcus haemolyticus	1	NCTC 11042
Staphylococcus epidermidis	1	NCTC 7291
Staphylococcus aureus		
Methicillin-resistant	2	clinical isolates UCH
Methicillin-sensitive	2	clinical isolates UCH
Streptococcus agalactiae	1	clinical isolates UCH
Enterococcus faecalis	1	clinical isolates UCH
Bacillus sp.	1	clinical isolates UCH
Corynebacterium jeikeium	1	clinical isolates UCH
Corynebacterium D2	1	clinical isolates UCH
Mycobacterium vaccae	1	NCTC 11916
Mycobacterium tuberculosis	1	NCTC 7016
Mycobacterium tuberculosis	20	clinical isolates *
Mycobacterium avium	1	NCTC 8559
Mycobacterium avium	19	clinical isolates *
Mycobacterium chelonei	1	NCTC 94C
Mycobacterium chelonei	19	clinical isolates *
Mycobacterium kansasii	1	NCTC 10268
Mycobacterium kansasii	19	clinical isolates *
Mycobacterium fortuitum	7	clinical isolates *
Mycobacterium malmoense	2	clinical isolate *
Mycobacterium xenopi	2	clinical isolate *
Nocardia spp.	9	clinical isolates *
Pseudomonas aeruginosa	1	NCTC 10332
Pseudomonas spp.	31	clinical isolates UCH
Escherichia coli sp.	1	clinical isolates UCH
Shigella sp.	1	clinical isolates UCH
Salmonella sp.	1	clinical isolates UCH
Hafnia sp.	1	clinical isolates UCH
Pasteurella sp.	1	clinical isolates UCH
Proteus mirabilis	1	clinical isolates UCH
Citrobacter sp.	1	clinical isolates UCH
Klebsiella sp.	1	clinical isolates UCH
Morganella sp.	1	clinical isolates UCH
Enterobacter sp.	1	clinical isolates UCH
spirochetes	11	clinical isolates UCH
Serpula hyodysenteriae	1	NCTC 11615
Helicobacter pylori	1	NCTC 11638
Helicobacter pylori	6	clinical isolates UCH
Campylobacter jejeni	7	clinical isolates UCH

Animal Health Corporation.

(V) *Campylobacter and Helicobacter* spp.

The *Campylobacter* and *Helicobacter* species were clinical isolates from UCH and Middlesex Hospitals.

3.2 THE CULTIVATION AND STORAGE OF BACTERIA

All the organisms, with a few exceptions were grown on blood agar (BA) plates overnight prior to inoculation into labelling medium (Fig. 3.1). Some organisms such as *Proteus* sp. were grown on CLED plates (Oxoid). The mycobacteria were cultured on Lowenstein-Jensen (LJ) medium.

The mycobacterial cultures were stored on Lowenstein-Jensen slopes at room temperature in the dark. All other cultures were stored on nutrient agar slopes at room temperature.

3.3 [^{35}S] LABELLING OF BACTERIA

(I) Materials

Three compounds were used for labelling microorganisms: [^{35}S] methionine (Amersham International), [^{35}S] inorganic sulphate (NEN-Dupont) and [^{35}S] thio ATP (Amersham International).

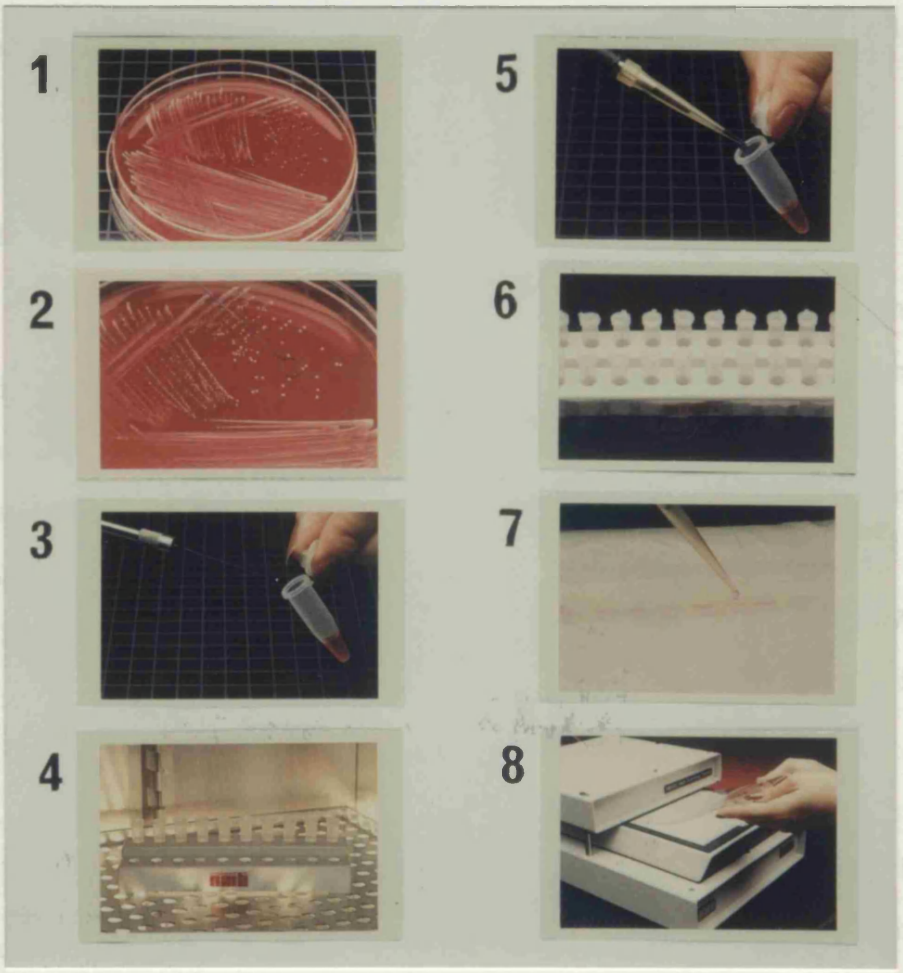


Figure 3.1. Summary of the sample preparation method (AMBIS catalogue): 1 and 2, culture of a pure isolate; 3, inoculation into the incubation medium; 4, incubation of the sample; 5, stopping cell metabolism by adding sample buffer; 6, boiling the sample for 2 minutes in a water bath; 7, loading an aliquot of the sample into the sample applicator ready for electrophoresis; 8, positioning a dried gel on the scanner.

(II) Media composition

Stock solutions were prepared (Table 3.2), mixed and diluted as required (Table 3.3). The stock solutions were stored at -20°C. The prepared sterile medium was stored at 4°C.

(III) Labelling conditions

In all labelling experiments a loopful of an organism was inoculated into 50 microlitres of appropriate incubation medium in sterile screw cap Eppendorf tubes (Fig. 3.1) containing 0.37 MBq of the [^{35}S] compounds. All strains were labelled with [^{35}S] methionine. Most strains were also labelled with [^{35}S] inorganic sulphate and [^{35}S] thio ATP. The majority of organisms were labelled for two hours in air, with the exception of *Campylobacter*, *Helicobacter*, spirochetes and mycobacteria. The *Campylobacter*, *Helicobacter* and spirochetes were all labelled for 18 hours in a micro aerobic or anaerobic environment. Mycobacteria were labelled in air or in a CO_2 incubator for 7 days except where stated otherwise.

All samples were incubated at 37°C.

Table 3.2. Constituents of stock solutions.

Amino Acids (Sigma) mg/50ml (one letter coding):

A: R.HCl, 50; H.HCl.H_2O, 10; I, 26; L, 26; K, 20; F, 16; T, 23; W, 5; Y, 20; V, 23;

A1 = A + C, 12; A2 = A alone; A3 = A + C, 12 + M, 8;
These need a small amount of NaOH 1M and dissolve overnight.

B: P, 10; N, 20; E, 12; D, 12; A, 10; S, 10; G, 10;

H: Q, 292;

D: Bulk salts (BDH) mg/50ml
NaCl, 6800; KCl, 400; $CaCl_2$, 2 H_2O, 265 (prepared from standard 1M soln, BDH); NH_4Cl, 50; phenol red, 17; NaH_2PO_4, 158; glucose, 1000;for D1 and D3, add $MgCl_2.6H_2O$, 150 and for D2 add $MgSO_4.7H_2O$, 200;

E: Trace elements (BDH) mg/50ml
$CuAc_2$, 3.3; KI, 1; $FeCl_3$ 20; $MnCl_2$, 35; NH_4 molybdate, 20; $ZnCl_2$, 33; H_3BO_3, 5;

F: Vitamins (BDH) mg/50ml
biotin, Ca pantothenate, choline.HCl, folic acid, nicotinamide, nicotinic acid, pyridoxal, pyridoxine, pyridoxamine, thyamine.HCl and ascorbic acid all at 10; inositol, 20; riboflavin, 1; PABA, 5;

G: Hepes buffer (BDH) 4.76g/50ml

J: Nucleosides (Sigma) all at 10mg/10ml
A, T, G, C, U, deoxy A, C and G.
Note: readily hydrolysed at room temperature by dilute acid.

Table 3.3. Proportions of stock solutions used to prepare media for [^{35}S] labelling.

Mixture for Incubation with	Met	SO_4	ATP	for 100ml
A1	+			5ml
A2		+		5
A3			+	5
B	+	+	+	5
D1	+			5
D2		+		5
D3			+	5
E	+	+	+	50ul
F	+	+	+	500ul
G	+	+	+	5
H	+	+	+	5
J	+	+	+	1
H_2O				about 70
NaOH, 1M to pH 7.5 @ 15-20° = 7.3 @ 37°C				
H_2O				to 100ml

(IV) Sample treatment and storage prior to electrophoresis

At the end of the appropriate incubation times 50 microlitres of sample buffer was added to the sample which was then boiled for 2-3 minutes. Where supernatants were examined, the sample was centrifuged at 10 000 g (MSE Micro Centaur) for 5 to 10 minutes to pellet bacterial cell debris with removal of the supernatant and addition of sample buffer to the resuspended pellet and supernatant separately.

(V) Filtering supernatants

The supernatants removed at stage (IV) were filtered using a Milex V_4 filter (Millipore). The volumes of the recovered supernatants were estimated and an equal volume of sample buffer added before the supernatants were boiled and stored at -20°C.

3.4 ELECTROPHORESIS

(I) Introduction

The labelled bacterial products were separated according to molecular size using polyacrylamide gel electrophoresis (PAGE) containing the dissociating detergent sodium-dodecyl sulphate (SDS). The principal of electrophoresis is that a charged ion or group will migrate in solution under the influence of an electric field. The solution in this instance was solidified using polyacrylamide but other supports can be used.

Polyacrylamide gels are made from acrylamide and N,N'-methylene bisacrylamide mixtures prepared in a buffered electrolyte solution and polymerised by the addition of chemical catalysts. The SDS is necessary to give a negative charge to polypeptides so that they can migrate and separate on the basis of size only.

Initially pre-prepared 12.5% polyacrylamide gels from AMBIS were used to separate proteins. After the first two and a half years of this study, AMBIS ceased to supply these gel due to financial problems. Gels were then prepared by hand, and LKB and biorad equipment was tested out. However the equipment was not designed for making large batches of gels. The process proved too time consuming, each pair of gels taking more than a day to prepare. Thus a further alternative system was sought. The Pharmacia gradient gel system was selected as the most ideal of the available pre-prepared gel systems. Thus the majority of the non mycobacterial electrophoresis work has been done on the AMBIS gels and the majority of the mycobacterial work has been done on the LKB system with Pharmacia gels. As there are significant differences in the labelling methods used for the mycobacteria compared with most other organisms there was no requirement for comparing data from the two different gel systems.

(II) Materials

(a) Molecular weight markers

(i) Molecular weight markers (NEN DuPont) were purchased separately and mixed to give a total radioactive concentration of 30 kBq/ml which was diluted 1:1 with sample buffer and boiled for two minutes; 10 mcl was loaded for each well. The final mix was stored in 100 mcl aliquots at -20°C. Each aliquot was frozen and thawed as necessary until used up. These molecular weight markers were used with 12.5% polyacrylamide gels (AMBIS).

(ii) Rainbow molecular weight markers (Amersham International) were purchased ready mixed at a total radioactive concentration of 148 kBq/ml in glycerol. An aliquot was removed when needed and diluted 1:1 with sample buffer before being boiled for 1-2 minutes. These molecular weight markers were used with 8-18% gradient polyacrylamide gels (ExcelGel, Pharmacia); 4 mcl was loaded per well.

(b) Sample buffer

The sample buffer was prepared and stored in a light tight bottle at 4° C: 20 mM Dithiothreitol (DTT) (Sigma) in a TRIS buffer pH 7.4 (Tris.HCL (Sigma) 0.888 g, Tris (Sigma) 0.53 g, SDS (Sigma) 4.0 g, bromophenol blue (BDH) 2 mg, bromophenol red (BDH) 1 mg in 100 ml of water).

(c) Electrophoresis buffer

The electrophoresis buffer was supplied as a pre-weighed solid containing glycine, sodium dodecyl sulphate (SDS) and Tris with a total weight of 36.9 g which was made up in 2 litres of distilled water and stored at 4°C (AMBIS).

(d) Fixing solution

Ethanol (BDH), 400 ml, and acetic acid (BDH), 100 ml, were made up to 1000 ml with distilled water.

(e) Destaining solution

Ethanol (BDH), 250 ml, and acetic acid (BDH), 80 ml, were made up to 1000 ml with distilled water.

(f) Preserving solution

Glycerol (BDH), 25 ml of an 87% w/w solution, was made up to 250 ml with destaining solution.

(g) Apparatus

(i) The AMBIS electrophoresis unit

The AMBIS electrophoresis unit (Tabaqchali *et al.*, 1987; Holland, 1987) has two platens that are independently powered and freon cooled. The unit is a large free standing tower that houses the cooling system. The platens sit on top of the unit and have separate lids so that they can be electrophoresed independently.

(ii) The LKB electrophoresis unit

The LKB electrophoresis unit has a 30 x 20 cm platen that lies flat on a base and is cooled by running cold tap water through it. The power was connected to the gels by electrodes that were the same length as the gel and were positioned over and made direct contact with the gel buffer strips that were laid on top of the gel. The power was supplied by a power pack (LKB) that could only supply power to the gel once the lid was in place.

(III) Electrophoresis methods

(a) Gels

For the AMBIS electrophoresis unit 12.5% SDS polyacrylamide gels (AMBIS) were used. They had a stacking gel of 4 cm and a resolving gel of 13 cm. The gels were cast on gelbond and were stored at 4°C.

The pre-prepared gels used with the LKB electrophoresis unit were 8-18% SDS polyacrylamide gradient gels (110 x 245 x 0.5 mm) from Pharmacia.

All gels were used by their expiry date.

(b) Setting up the gel

(i) The AMBIS system (12.5% polyacrylamide gels)

The required platen was cooled to 8°C; 250 millilitres of electrophoresis buffer (AMBIS) was poured into two buffer tanks to submerge the platinum electrodes. The buffer tanks

were placed at the top and bottom of the platen and the contacts made between the electrodes and power supply. The gelbond side of the gel was numbered and three lanes drawn to mark the position of the sample applicator, 1 centimetre from the gel interface, and of the wick contact positions. This aids the correct positioning of the applicator and wicks on the gel. The sample applicator strip (AMBIS) had twenty 2 x 4 millimetre slots that could hold up to 10 microlitres each (Fig. 3.1). The applicator was prepared for each run by rinsing with distilled water and alcohol and then dried by hanging it in the AMBIS warm air gel drier.

The gel was placed on the cooled platen by pipeting *n*-decane (BDH) under the gelbond coated side of the gel and laying the gel down slowly with the exclusion of air bubbles.

The gel was connected top and bottom to the buffer by four wicks, two at each end. The wicks were cut to be the same width as the gel and the two absorbent sides of the plastic backed cotton wool wicks were overlapped so that the combined length of the two wicks would reach between the buffer tank and the gel. Each wick was wetted with buffer before being connected to the gel. The resolving gel was covered with a plastic sheet to prevent the gel drying during electrophoresis.

The dried sample applicator was checked for hairs or

scratches that could allow sample leakage, before being firmly pressed onto the gel.

Sample, 8-10 microlitres, was pipeted into each well of the sample applicator. The end wells were filled with buffer only. Care was taken when loading not to touch the sides of the applicator which might move it and cause sample leakage.

(ii) LKB system (8-18% polyacrylamide gels)

The platen was cooled by cold tap water piped through the platen 15 minutes prior to starting and during electrophoresis. Approximately one millilitre of light paraffin was pipeted on to the platen. The gel was placed carefully and in the correct orientation on to the cooling platen allowing the light paraffin to run underneath the gelbond of the gel without trapping air bubbles.

One cathode and one anode SDS buffer strip (Pharmacia) was opened. The strips were loosened from the package. They were then removed from the packaging with gloved hands, moistened with distilled water. The strips were applied to the cathodic and anodic end of the gel respectively approximately 0.5 cm from the edge of the gel and with the narrow edge of the buffer strip placed on the gel.

The sample applicator (Pharmacia) was washed with distilled water and alcohol and then hung up to air dry.

Once dry the applicator was checked for hairs and damage before being positioned on the gel approximately one centimetre from the cathodic buffer strip and gently pressed into place. The sample applicator contained 26 wells that could each hold a maximum of 40 microlitres.

The samples were pipeted into each well, the electrodes positioned on the electrode holder so that they were centred over the sample buffer strips which were then connected to power plugs in the electrophoresis unit by spring loaded wires. The lid was then placed over the gel and the power cables from the lid were connected to the power pack.

(c) Electrophoresis conditions

(i) AMBIS system

Either the Watts, milliamps or volts could be programmed to be constant. The electrophoresis conditions used in this instance were 20 Watts for 20 minutes then 60 Watts for the remaining time. The power or current were plotted against time and could be monitored during electrophoresis. The plots were filed after electrophoresis for future reference.

The electrophoresis was automatically terminated when the primary ion front reached the 'end of run detector' at a mean resolving gel length of 12.4 centimetres.

(ii) LKB system

The electrophoresis conditions were set at 18 Watts and the gels were electrophoresed until the bromophenol blue front reached the anodic buffer strip which took approximately 1 hour.

(d) Fixing, drying of gels

(i) 12.5% polyacylamide gels (AMBIS)

After electrophoresis the gel was removed from the platen and the gelbond side dried. The pre-prepared AMBIS gels fitted exactly into a cassette that fitted into the AMBIS warm air drier. The gels were dried for 60 to 90 minutes.

(ii) 8-18% polyacrylamide gradient gels (Pharmacia)

The gradient gels were fixed for a minimum of 30 minutes and then soaked in 10% glycerol for 30 minutes to prevent the gels from cracking when dried. The gel was covered with 0.1 mcm mylar to prevent it from sticking to the scanner head or autoradiography film.

Alternatively the gradient gels were fixed in fixing solution for a minimum of 30 minutes, then washed twice in distilled water and dried face down on to paper in a horizontal vacuum drier (BioRad). With this method the gels stuck to the paper and the plastic gel bond had to be carefully peeled off, leaving the dried gel fixed to the backing paper.

(e) Scanning of gels

The dried gels were taped to card mounts 20 x 20 cm and fixed with tape to the scanner base. Gas, 10% methane and 90% argon (BOC), fed continuously into the scanner head and its 78 chambers. The scanner head was cover with a plate containing 78, 0.8 x 0.3 mm slits, such that each slit was positioned over each chamber (Smith, 1985). The plate makes contact with the dry gel and therefore Beta radiations pass up into each detection chamber through each slit. The scanner base moves so that over a period of time, readings are taken over the whole gel. Gels were scanned initially for 15 minutes to check the lanes were straight. Gels were then scanned for three to twenty four hours as necessary. The data were collated at the end of the scanning time to give a computer generated picture of the gel (Fig. 3.2).

(f) Autoradiography

After scanning the gels were removed from the card mounts and sandwiched between two glass plates with autoradiography film (Kodac). The plates were clamped together using bulldog clips. The whole unit was then placed in a light tight bag before being sealed inside a film box. Each film was numbered and the top left hand corner was cut off to enable quick identification of the film orientation after developing.

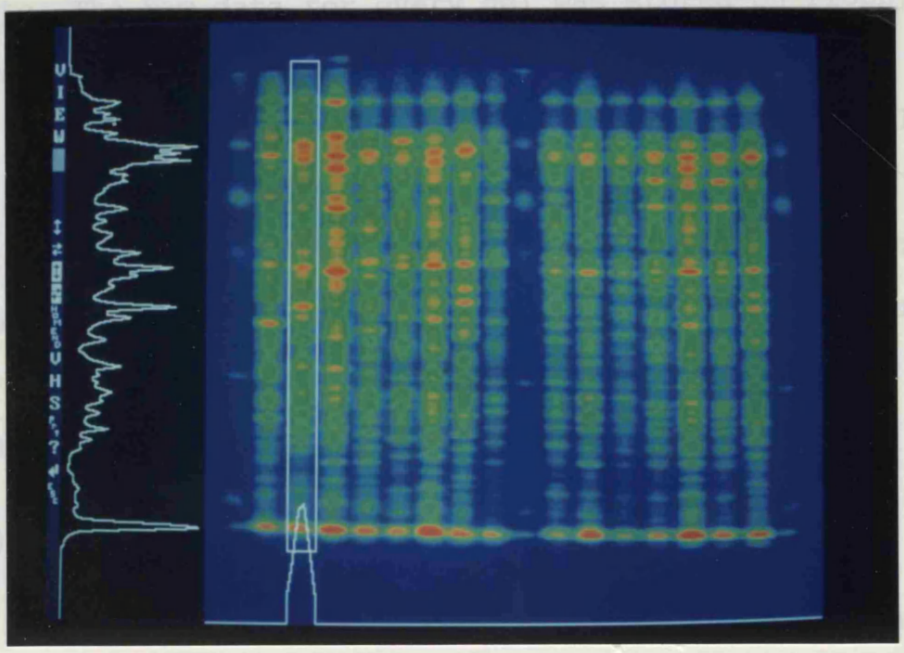


Figure 3.2. Colour enhanced computer image of a gel showing one lane indexed with a box and the corresponding histogram on the left hand side.

3.5 COMPUTER METHODS FOR DATA ANALYSIS

(I) The gel image

The computer generated image of the gel is referred to as the raw data. Gels were numbered consecutively as G1, G2, G3 etc. The raw data for every gel was stored on floppy discs. In order to get the best computer image of each gel the upper and lower contrast limits could be set manually. The AMBIS software versions 1.74, 1.8, 1.84 were black and white only, thus the gel image resembled an autoradiograph of black bands on a white background. Version 2.0 onwards was in colour with a choice of four colour schemes (black/white; hotbody; rainbow; tri-colour).

(II) Extracting lanes and filing

Every lane on every gel was indexed using a box and recorded as a histogram (Fig. 3.2). The histograms were then filed under the gel name followed by the prefix 'L' and the lane number, the lanes were automatically numbered consecutively 1,2,3,4,..... as they were extracted. In later software programs version 2.0 onwards it was possible to number the lanes in any order.

(III) Normalisation

(a) Smoothing

Smoothing of histograms averages data of adjacent peaks to reduce noise.

(b) Clipping

Clipping of lanes cuts off the top and bottom of the histogram at selected points so as to remove data outside the main run length or outside the region of interest (Fig. 3.3 box B and C).

(c) Transformation

Transformation involves shifting points of interest so that specific bands of interest on one lane align with the same bands of another (Fig. 3.3 box B and C). In most instances, this involves shifting marked points, corresponding to the molecular weight marker positions of one gel, from the origin position (marked in Fig. 3.3 box B as >) to the target (marked in Fig. 3.3 box B as -) position. The target positions being the molecular weight marker positions of the gel that is being used as a standard. The histogram is stretched and compressed automatically where necessary in between the marked points. Multiple origin and target positions may be marked, followed by one transformation which will shift all the marked origin points to the marked target position simultaneously.

(d) Alignment

There is also an automatic alignment to allow for minor misalignments. This function stretches and compresses one histogram by a maximum of 5 pixels to get a best fit with a second histogram (Fig. 3.3 box D).

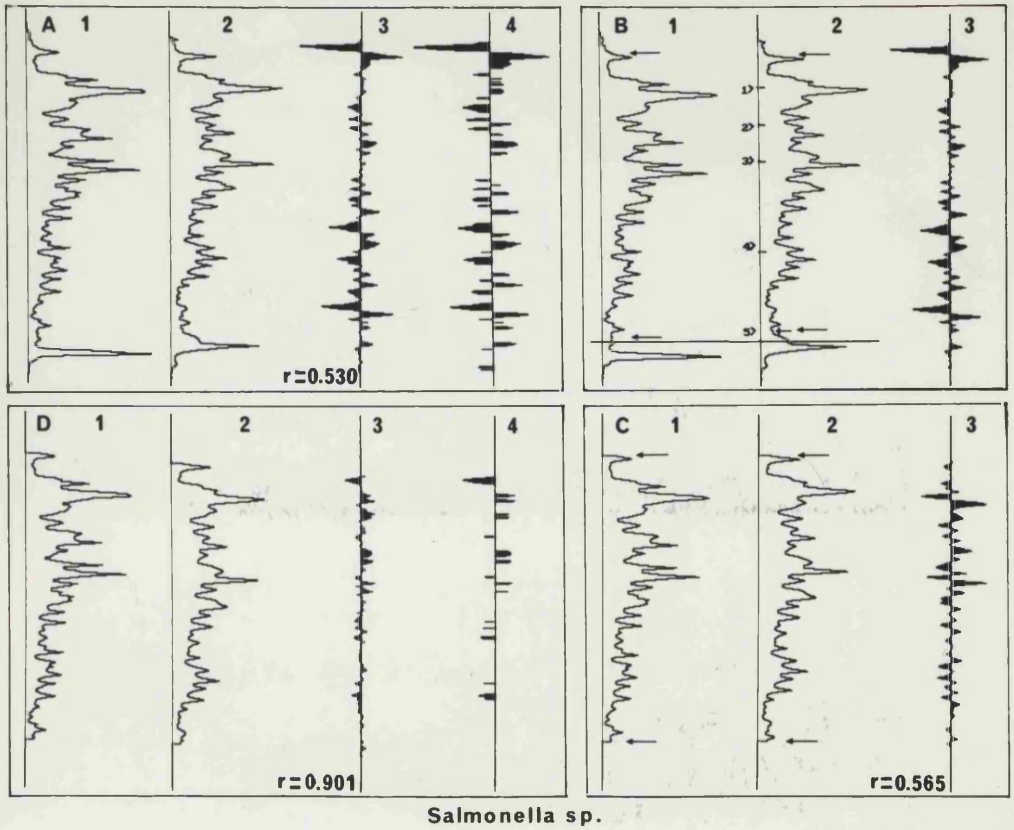


Figure 3.3. Demonstration of the normalisation functions using the protein profile of a *Salmonella* sp. duplicated on two gels (columns 1 and 2); r= coefficient of correlation (column 3). Column 4 shows the differences between the samples in columns 1 and 2, where the observed differences are greater than a standard deviation of 2. Box A: the original histograms of *Salmonella* sp. from two gels and the correlations. Box B: the molecular weight standard positions from each gel are marked (> for gel 1 and - for gel 2) and aligned (this function is called transformation). Box C: the results of transformaton. The top and bottom of the lane are marked with arrows and clipped off. Box D: the results of one auto-alignment, which is a computer controlled adjustment of 5 pixels to give the best fit.

(e) Stretch and compress

This command is generally used to stretch a portion of a histogram to a set maximum length, this function is not essential for normalisation.

(f) Scale adjustment

The histograms are automatically scaled when called up so that the average height of each maximum y-axis value on each lane is the same. The scale can be altered manually.

(g) Data storage

Once the histograms had been adjusted they were filled under their gel name followed by the prefix 'A' and then the lane number.

The data for the gel images were filed on floppy discs. The extracted lanes and adjusted lanes were also filled on floppy discs.

(III) Statistical analysis

(a) Standard curves

The peaks of the molecular weight markers can be plotted using the AMBIS software (Fig. 3.4 and Fig. 3.5) so that the molecular weight of any peak from lanes on corresponding gels can be read off by highlighting the peak using the arrow keys.

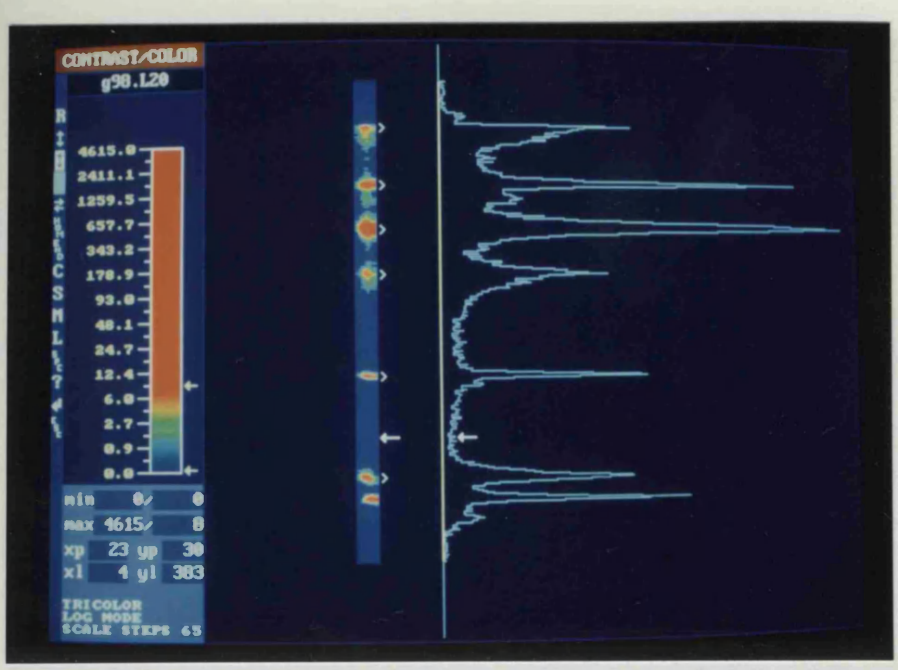
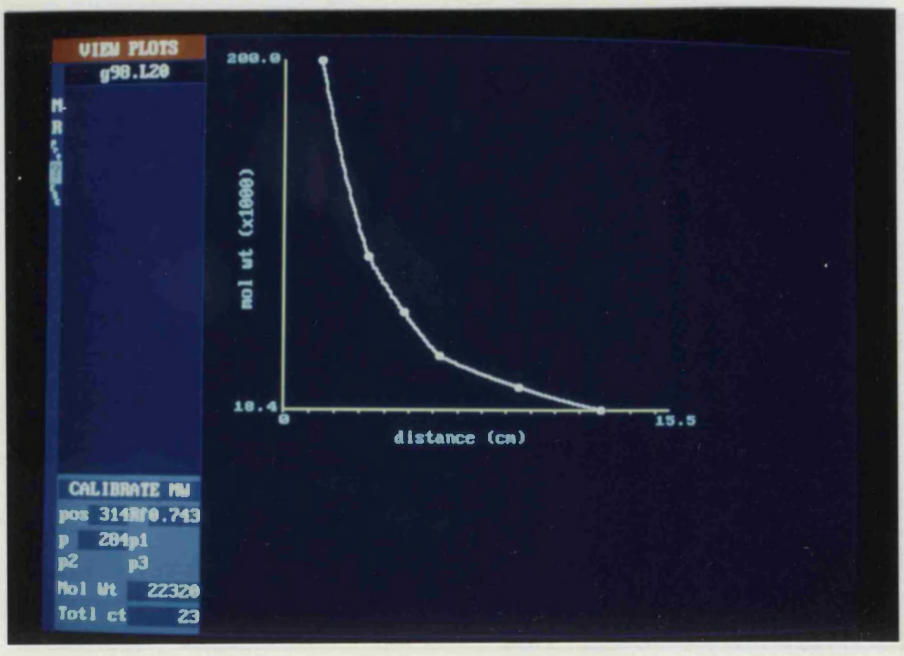


Figure 3.4. Above: the colour enhanced image and the corresponding histogram of ^{14}C molecular weight standards electrophoresed on a 12.5% polyacrylamide gel (AMBIS). Below: the standard curve of the above molecular weight standards (migration distance against the molecular weight)



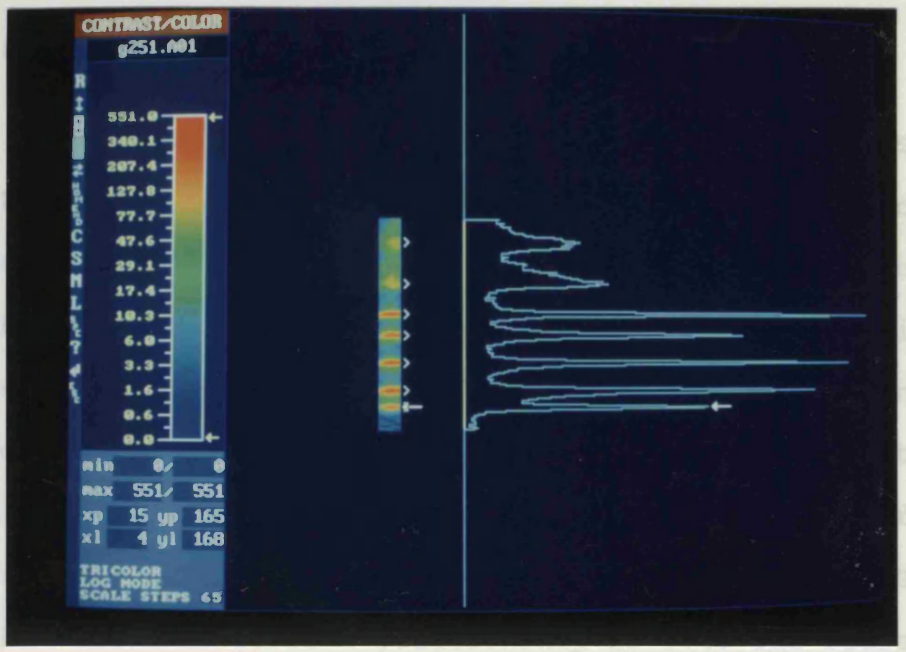
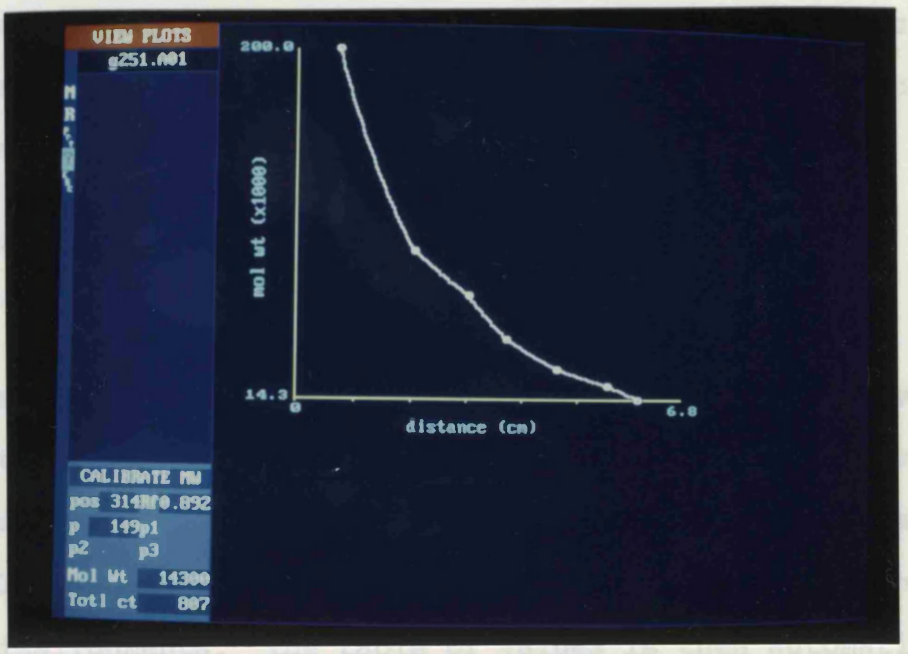


Figure 3.5. Above: the colour enhanced image and the corresponding histogram of [^{14}C] molecular weight standards electrophoresed on a 8 to 18% polyacrylamide gradient gel (Pharmacia). Below: the standard curve of the above molecular weight standards (migration distance against the molecular weight)



(b) Coefficient of correlation

The coefficient of correlation is the factor used in the lane-by-lane comparisons. The Pearson product moment calculation is used to compute the correlation factor (r). The r values are from -1 to +1. +1 is a perfect positive correlation and -1 is a perfect negative correlation. A correlation value of zero means there is no correlation at all. The closeness of the relationship is not linearly proportional to r, for instance a correlation of 0.8 is more than twice as close a relationship as 0.4. The Pearson product moment is extremely position sensitive so that very similar histograms which are not properly aligned can show very poor correlations.

(c) 'Z' factor

The Pearson product moment calculation is also used to calculate the correlation factor but in this instance all data less than a requested standard deviation from the mean is not shown (Fig.3.3 Box A and B, column 4).

(d) Dendrograms

Dendrograms can contain a maximum of 75 lanes, and each lane selected for the dendrogram is compared with every other lane so that a complete table of correlation values is determined (Table 3.3). The lanes can be automatically aligned when preparing a dendrogram to allow for minor misalignments. The table of values is then automatically

Table 3.4. Table of the Pearson product correlation values (r), of five Gram-negative organisms duplicated on two gels, used to produce a dendrogram. Each sample has been correlated with every other sample from both gels: P, *Pasteurella* sp.; E, *Escherichia coli*; S, *Shigella* sp.; C, *Citrobacter* sp.; M, *Proteus mirabilis*.

	P	E	S	C	M
P	0.930 (n=1)	0.521 ± 0.023 (n=4)	0.602 ± 0.038 (n=4)	0.459 ± 0.025 (n=4)	0.598 ± 0.026 (n=4)
E		0.921 (n=1)	0.860 ± 0.026 (n=4)	0.714 ± 0.034 (n=4)	0.662 ± 0.03 (n=4)
S			0.961 (n=1)	0.731 ± 0.025 (n=4)	0.710 ± 0.040 (n=4)
C				0.933 (n=4)	0.713 ± 0.039 (n=4)
M					0.953 (n=1)

analysed using UPGMA (unweighted pair grouping method with averages). The result is a figure that summarises the correlation values (Fig. 4.7).

(e) Database facility

Normalised histograms were filed in a database. The theoretical maximum size of any database was 15000 items. Any database could have up to an eight letter reference name followed by the file code 'ID'. Once created, a database could have additional items added or deleted at any time. Many different databases can be created with the same data.

Each database entry consists of a sine array and a cosine array. There is a sine and a cosine value for the lane at each of the 32 transform frequencies, making a total of 32 coordinates. Matching is done hierarchically, using the simplest transform as a first discriminator. This is like searching through a dictionary, matching 'a', then 'ab', then 'abc', etc. of a word until the whole word has been matched to the dictionary. In the case of the histograms they can be likened to a word with 32 letters which are actually the 32 coordinates.

When matching an histogram with a database the histogram is called up in the challenge position on the left hand side. The lane is matched by pressing 'm' and the name of the required database is typed in. The challenge lane is then compared with every lane in the database using the fast

fourier transform, the spectral correlations of the best matches are then displayed. The maximum number of best matches to be shown can be set, normally ten are selected. The histogram of the closest match is shown along side the challenge histogram on the computer screen. The selected matches can then be further analysed by 'ranking', here the best ten matches are aligned automatically with the challenge lane to give the correlation coefficients ('r' values) and a new order of best match according to the 'r' values. All the data can be printed out.

(i) The spectral match

The fourier transform used in the spectral match produces correlation coefficients from -1 to +1 like the pearson product moment correlation coefficient. But the spectral correlations are based on phase as well as amplitude and are not therefore so position sensitive. The overall pattern is analysed as frequencies from 1 to 32. The different frequencies are used as a means of fast indexing during the database search.

CHAPTER 4 RESULTS

4.1 GEL ANALYSIS

(I) Introduction

When the labelled proteins from bacteria are electrophoresed, a complex array of bands is seen. The eye can quickly and sensitively discern differences in band position and label intensity and, if the patterns have been run on the same gel, can assess if the patterns are identical, very similar or very different. However the more complex the band patterns being analysed, the larger the group of organisms, coupled with the variation due to inherent differences in the gels the more difficult it is to read by eye.

In order to compare banding patterns and compensate for the factors that cannot be fully standardized the banding patterns from bacteria have been converted into histogram graphs using the AMBIS system as described in chapter 3. The histograms represent each band as a peak, the height of the peaks being relative to the intensity of labelling. The histogram is made up of points (pixels) that are then joined to give the graph (histogram). There are 25.2 pixels to every cm of gel. To compare histograms the pixel positions of one histogram are compared with the corresponding pixel positions of a second histogram and a correlation factor (r) is calculated using the Pearson product moment coefficient.

(II) Correlations within a gel

Two isolates of *Staphylococcus haemolyticus* labelled with [^{35}S] methionine were electrophoresed on one gel (Fig. 4.1). From visual inspection of the electrophoretic run it is observed that the within gel variation is minimal.

The histograms for each of the two isolates were 'clipped' (see chapter 3) for maximum length, from the top of the separation gel (at the 200 kDa molecular weight standard position) to, but not including, the free methionine marker at the bottom (at the 18.4 kDa molecular weight standard position), a pixel length of 270 (see Fig. 4.1). The mean correlation for strain 9 was 0.911 $\pm$ 0.083 (n = 21) and 0.912 $\pm$ 0.047 (n = 36) for strain 7. When the same samples were also 'smoothed' (see chapter 3) the mean correlation for strain a was 0.916 $\pm$ 0.079 (n = 21) and 0.923 $\pm$ 0.045 (n = 36) for strain b. When the same samples were 'smoothed' and auto-aligned the mean correlation was 0.969 $\pm$ 0.018 (n = 21) for strain a and 0.968 $\pm$ 0.011 (n = 45) for strain b. Smoothing and auto-alignment both improve correlations even between identical samples.

The correlations within a gel would be effected detrimentally by factors such as temperature differences across a gel during electrophoresis and variation in polymerisation during gel preparation. This could cause a gel front to be distorted in some way after electrophoresis, for instance to give a slope or 'smile' effect. This effect

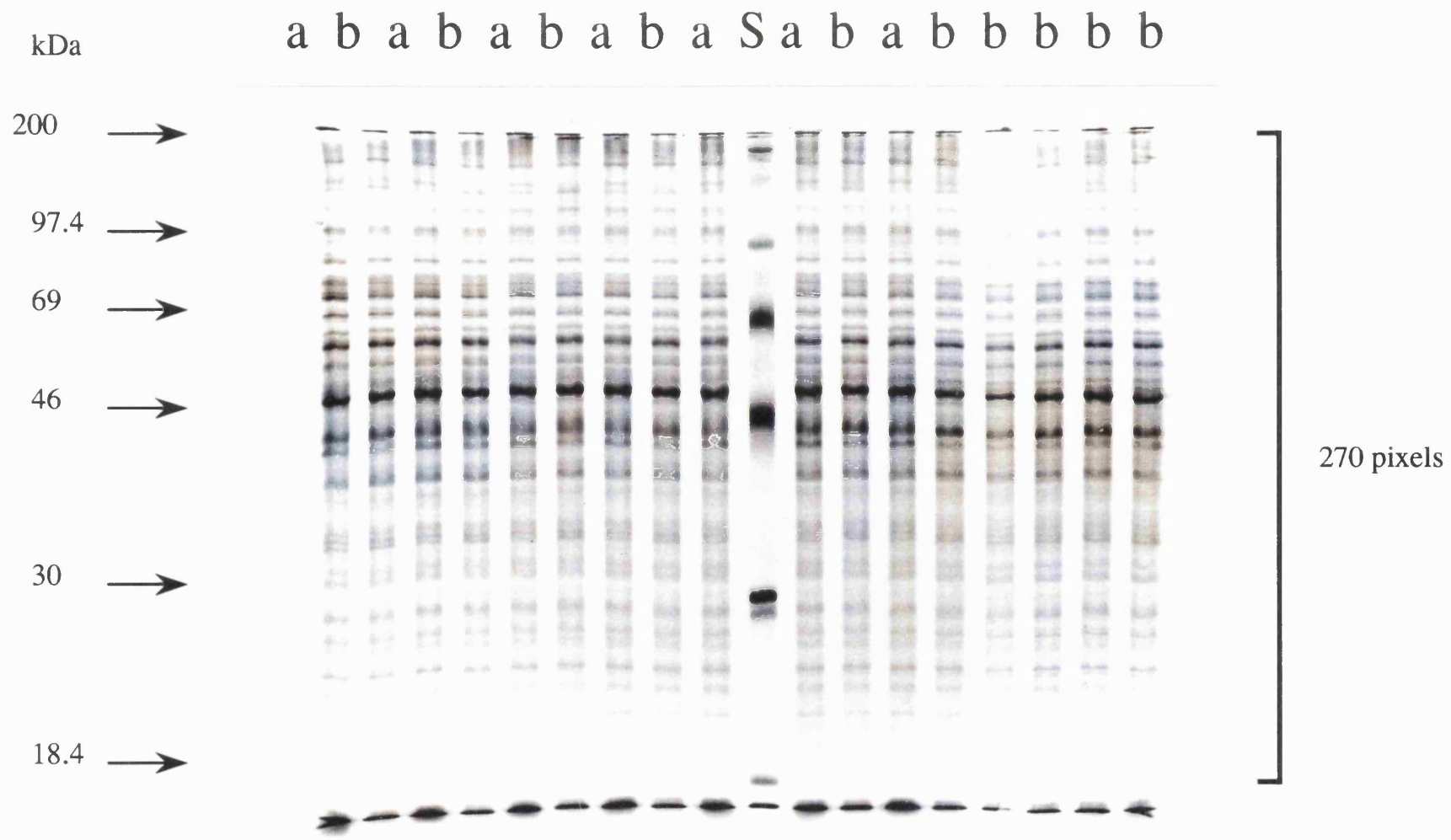


Figure 4.1. [^{35} S] Methionine labelled whole cell proteins of two isolates (a and b) of *Staphylococcus haemolyticus*. S, molecular weight standards.

was artificially recreated by stretching and compressing one of the histograms for strain a by a variety of pixel distances and then comparing the adjusted histogram after one auto-alignment, with its original which would normally give a correlation of 1.00. It was found that if the histogram was stretched or compressed by more than 10 pixels (approximately 0.5 cm) the deviation from 1.00 was more than the standard deviation of the mean correlation for strain a.

(III) The effect of radiolabel intensity on correlations

Two approaches for assessing the effect of radiolabel intensity on correlation values were used, firstly by diluting samples and secondly by scanning a gel for three different times.

A *Pseudomonas* strain was labelled with [^{35}S] methionine and diluted by factors of x2, x4, x6, x8, x10, 12, x16, x20. The dilutions were electrophoresed on the AMBIS gel system and the gel scanned for 900 minutes. The histograms for each dilution were 'smoothed' and 'clipped' for maximum pixel length and then used to prepare a dendrogram with auto-alignment (Fig. 4.2).

In order to assess how critical the scan time was to data processing one gel was scanned for three different scan times: 20, 300 and 900 minutes. All the extracted histograms were 'smoothed' and 'clipped' and a dendrogram prepared of the data without auto-alignment (Fig. 4.3).

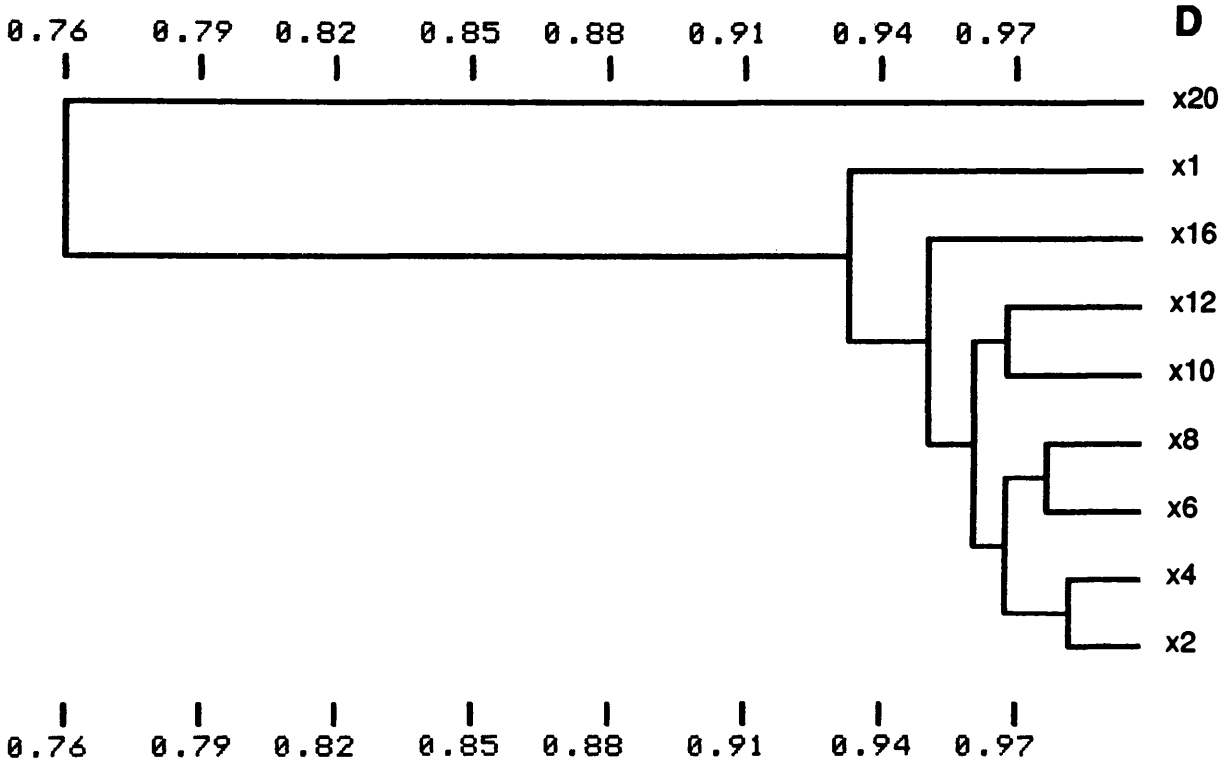


Figure 4.2. Dendrogram of the [^{35} S] methionine labelled whole cell proteins of a *Pseudomonas* sp. diluted (D) x1, x2, x4, x6, x8, x10, x12, x16, x20.

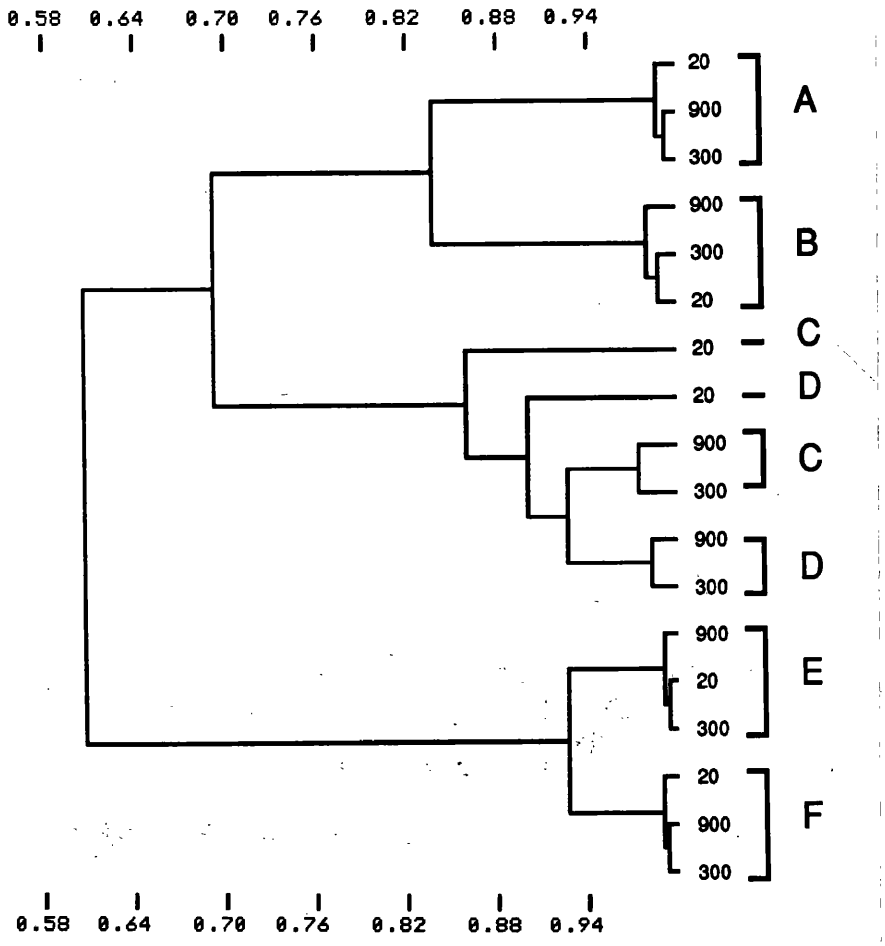


Figure 4.3. Dendrogram of the data from one gel scanned for 20, 300 and 900 minutes: A, *Pseudomonas cepacia* NF144; B, *P.cepacia* NF62; C, *P.aeruginosa* K4; D, *P.aeruginosa* K3; E, *P.stutzeri* Z199; F, *P.stutzeri* NF271.

The intensity of labelling of a band is affected by both the sample dilution and the length of time scanned and it is the intensity that determines the noise to signal ratio, which affects the correlation values. As can be seen with the diluted sample of *Pseudomonas* species, there is a noise to signal threshold point above which the ratio does not affect the correlation significantly, as the mean correlation for all the dilutions from x1 to x16 is 0.970 $\pm$ 0.018 (n = 28) with the lowest total counts for the x16 dilution of 29 000. When the sample was diluted x20 times however, with a total count of 24 296, is below the thresholds and the correlation of this sample compared to the other dilutions is significantly reduced to 0.777 $\pm$ 0.039 (n = 8). Thus the threshold for the diluted sample in Fig. 4.2 is between 24 000 (the counts for the x20 dilution) and 29 000 (the counts for the x16 dilution).

The dendrogram of data scanned for 20, 300 and 900 minutes (Fig. 4.3) shows that the corresponding profiles for the different scan times group together with correlations above 0.988 where the total counts of the histogram are above approximately 24 000 (see samples A, B, E and F in Fig. 4.3). Only the 20 minute scan for samples C and D (Fig. 4.3) gave total counts below 24 000 and in this case the histograms of the 20 minute scans did not give high correlations with the 300 and 900 minute scans. The total count threshold for these data is approximately 24 000, although it is evident that the actual threshold for each

sample will vary according to the distribution of the intensity of radiolabelling between bands in each sample.

The correlations for comparing the corresponding profiles of different scan times show that the lowest correlations and greatest standard deviations are observed when comparing the 20 and 300 minute scans and the highest correlations and lowest standard deviations when comparing the two highest scan times, 300 and 900 minutes. This indicates there is an advantage to longer scan times even when the total counts are above the threshold. In general it was found that the lower the total intensity of labelling of the profile, the lower the correlation values.

The comparison of samples above the threshold level is possible due to the automatic 'scaling' function and shows data of different scan times and concentrations can be compared.

In summary the higher the total counts the lower the noise/signal ratio and the better the correlations, although a significant drop in correlations was only seen for data where the intensity of labelling of a histogram is below 29 000 counts. As the threshold varies with each sample due to the distribution of the radiolabelling within a histogram a threshold of 40 000 counts per histogram was set for general analysis of correlation of subsequent raw data, well above the actual thresholds noted above.

(III) Correlations and the dendrogram

Correlation values are non linear on a scale of -1 to +1. But when used for analysing histograms here the correlation values usually ranged above 0 to below +1. Correlations were always above 0 because negative peaks are not obtained when protein profiles are converted into histograms, and below +1 as no data can be perfectly reproduced.

4.2 GEL NORMALISATION

(I) Introduction

Even with standardised gels and electrophoretic conditions, variation in separation of protein bands occurs. This is easily demonstrated when the molecular weight standards from ten gels are grouped in a dendrogram (Fig. 4.4). Some of the separations are almost identical; for instance where the histograms group together with a high correlation e.g. gel number 1, 2 and 3 on fig. 4.4. Whereas some of the separations are clearly very different and group with a low correlation e.g. gel number 1 and 6. In order to compensate for gel to gel variation the use of normalisation was examined.

(II) Normalisation strategy

A selection of normalisation strategies were investigated using the data from five Gram negative

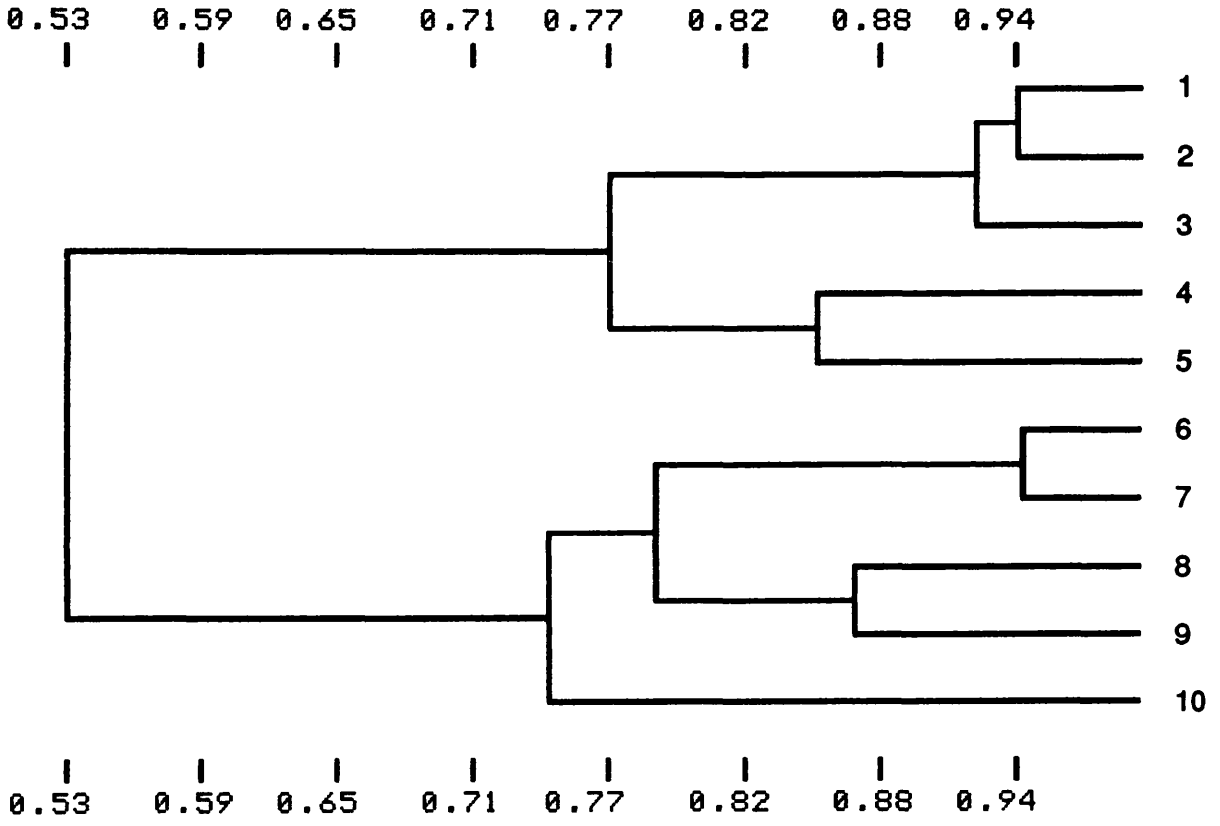


Figure 4.4. Dendrogram of the unnormalised molecular weight standard profiles from ten gels.

organisms that were labelled for 2 hours with [^{35}S] methionine, the pellet of each specimen was processed and electrophoresed on the AMBIS gel system as described in chapter 3. The samples were electrophoresed on two gels. The two sets of five histograms (Fig. 4.5) were analysed (1) by 'moving' the histograms of one gel so that the top of all the histograms were level, and (2) by 'transforming' the histograms of one gel to match the second gel using the molecular weight standard peaks from each gel as reference points. All the lanes were then 'clipped' for the maximum pixel length, from the top of the separation gel to, but not including, the free methionine marker at the bottom of the gel (a pixel length of 270) and 'smoothed'. A dendrogram was prepared from the data with and without auto-alignment.

When no transformation or auto-alignment was used none of the corresponding pairs matched up correctly (Fig. 4.6). When either transformation or auto-alignment was used alone some of the pairs matched correctly. Only when both transformation and auto-alignment were used did all the pairs match correctly (Fig. 4.7). The correlation for each pair was:0.930 for *Pasteurella* sp.

0.921 for *E.coli*

0.961 for *Shigella* sp.

0.933 for *Citrobacter* sp.

0.953 for *Morganella* sp.

a mean correlation of 0.94 $\pm$ 0.017 (n = 5). The different species grouped below a correlation of 0.69,

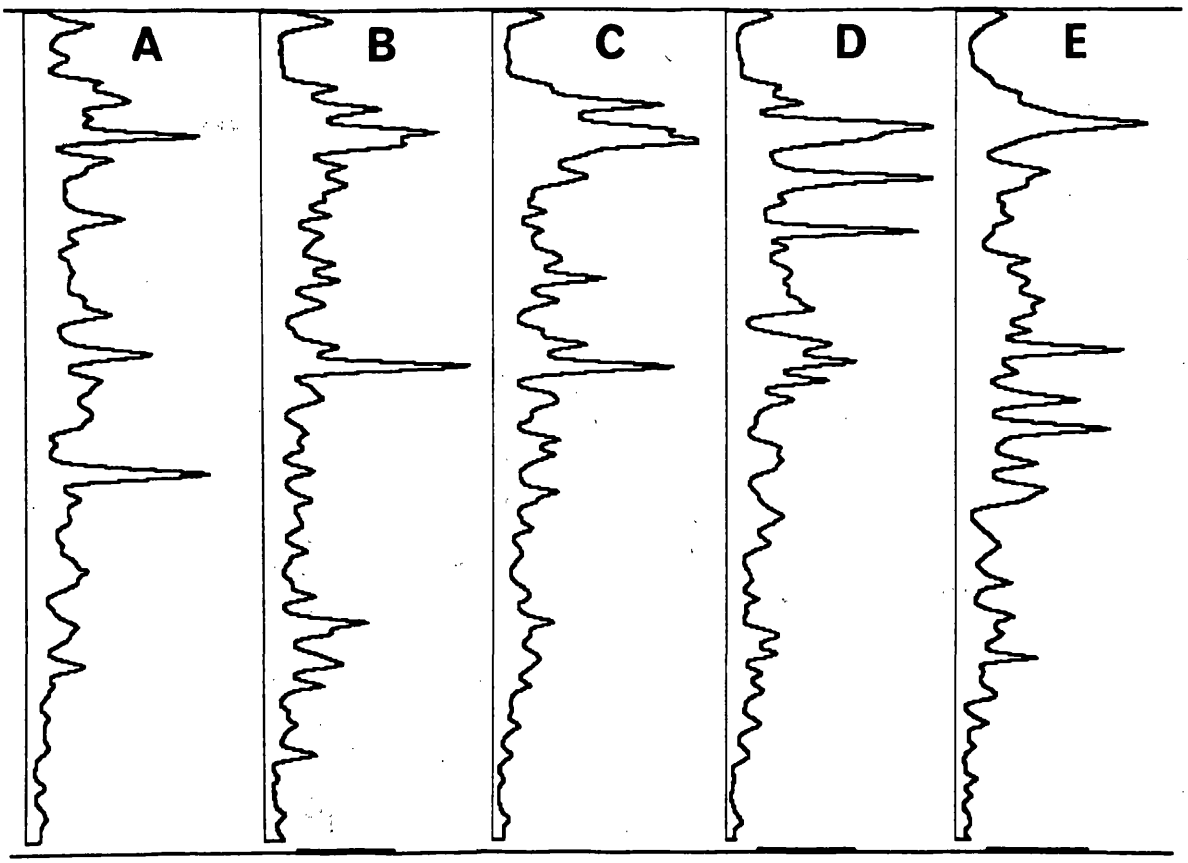


Figure 4.5. Histograms of the whole cell protein profiles of five Gram-negative organisms labelled with [^{35}S] methionine: A, *Pasteurella* sp.; B, *Escherichia coli* ; C, *Shigella* sp.; D, *Citrobacter* sp.; E, *Proteus mirabilis*.

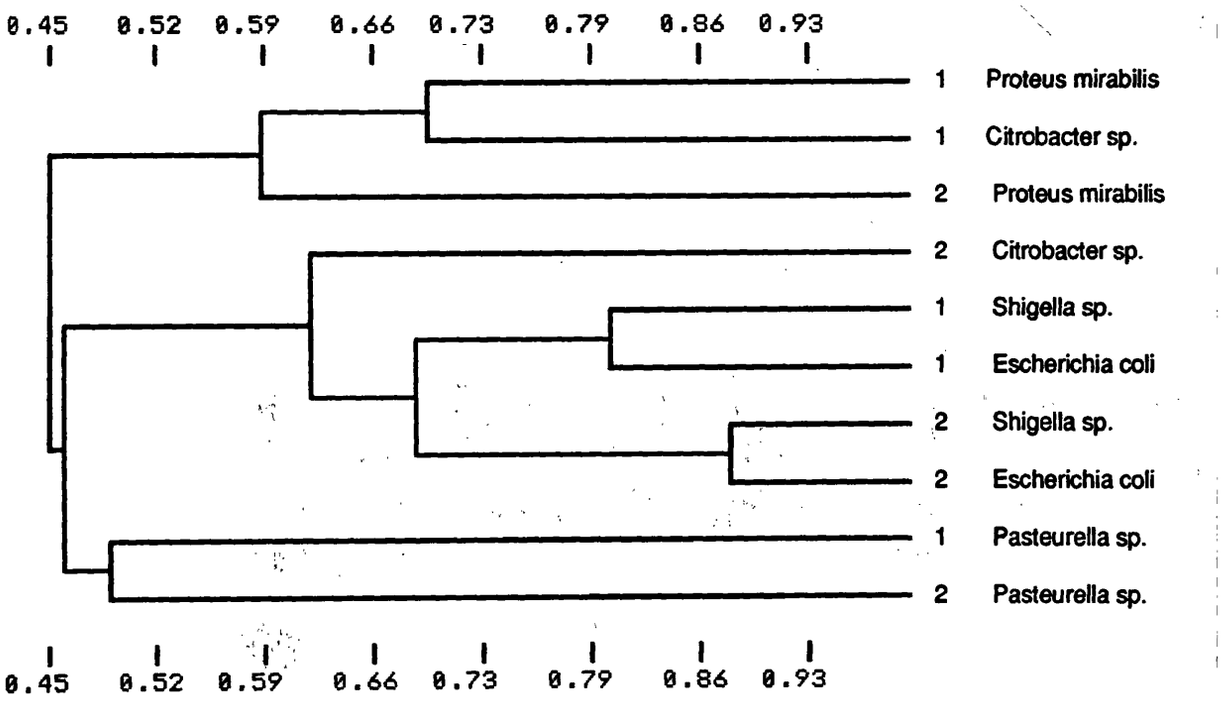


Figure 4.6. Dendrogram of the unnormalised data of the whole cell protein profiles repeated on two gels (1 and 2) of five Gram - negative organisms labelled with [^{35} S] methionine.

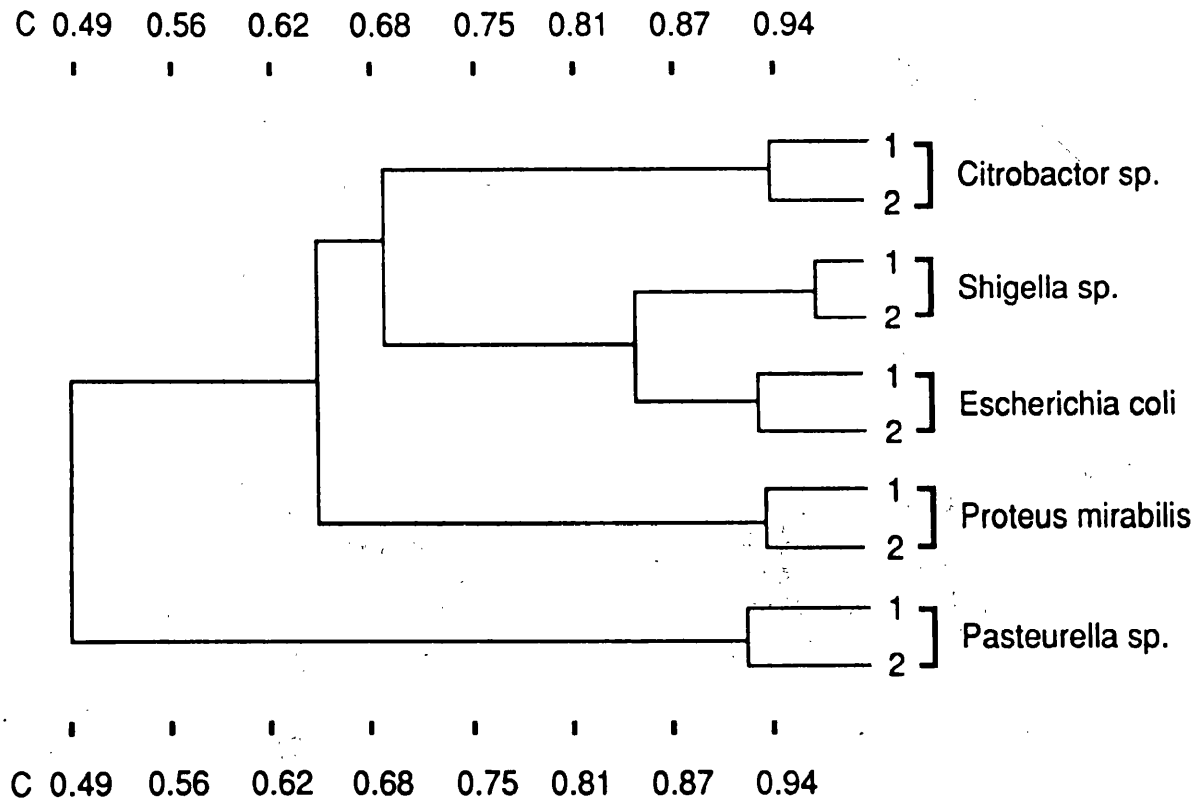


Figure 4.7. Dendrogram of the normalised data of the whole cell protein profiles, repeated on two gels (1 and 2), of five Gram-negative organisms labelled with [^{35}S] methionine.

except for *Shigella* and *E.coli* which clustered at 0.85.

In order to assess the use of clipping and its effect on the dendrogram the five gram negative organisms used above, normalised with the standard method, were cut into three equal parts of 90 pixels each and a dendrogram was prepared. Preparing a dendrogram from only part of a histogram did not effect the correct pairing of the corresponding pairs, although the actual correlations did vary, but did alter the grouping of the species. This was expected as the banding patterns would not vary to the same extent down the lane, in other words some parts of a histogram may be very similar and thus give a high correlation for that region and cluster together, whereas other parts of a histogram may be very different and give a lower correlation. In all three dendrograms, *E.coli* and *Shigella* species grouped together with *Citrobacter* grouping close to them in both the middle and bottom section dendrograms. In the top section dendrogram *Proteus mirabilis* groups closer to *E.coli* and *Shigella* than *Citrobacter*. In all three dendrograms the Pasteurella species was the least related species.

Thus the second normalisation method with smoothing and auto-alignment was selected as the standard normalisation method and the full histogram would be used in all dendrograms particularly when comparing different species. Where different isolates of one species are being

investigated, analysis of specific regions may be appropriate.

When the standard normalisation method was re-applied to the molecular weight standards from ten gels the histograms all grouped above a correlation value of 0.93 (Fig. 4.8).

Five isolates of the same species, *Staphylococcus haemolyticus*, labelled with methionine were electrophoresed on two gels and the data normalised using the standard method. When dendrograms were prepared of the data from the two gels it was found that the samples did not pair correctly but grouped according to which gel they had been run on (Fig. 4.9). The reason for this was that the different isolates were very similar, with correlations between the different isolates being equivalent or higher than the correlations between the normalised data. Thus it was concluded that when large groups of isolates of one species were being analysed it should be done in groups that would always be electrophoresed on the same gel. The amalgamation of the isolates into a larger analysis group would then depend on the correlations observed within each gel.

When looking at a dendrogram it is always important to note the scale. At first glance two dendrograms can look very similar, but if one is on a scale of 0.3 to 1.0 (for instance with different species) and the other is 0.9 to 1.0

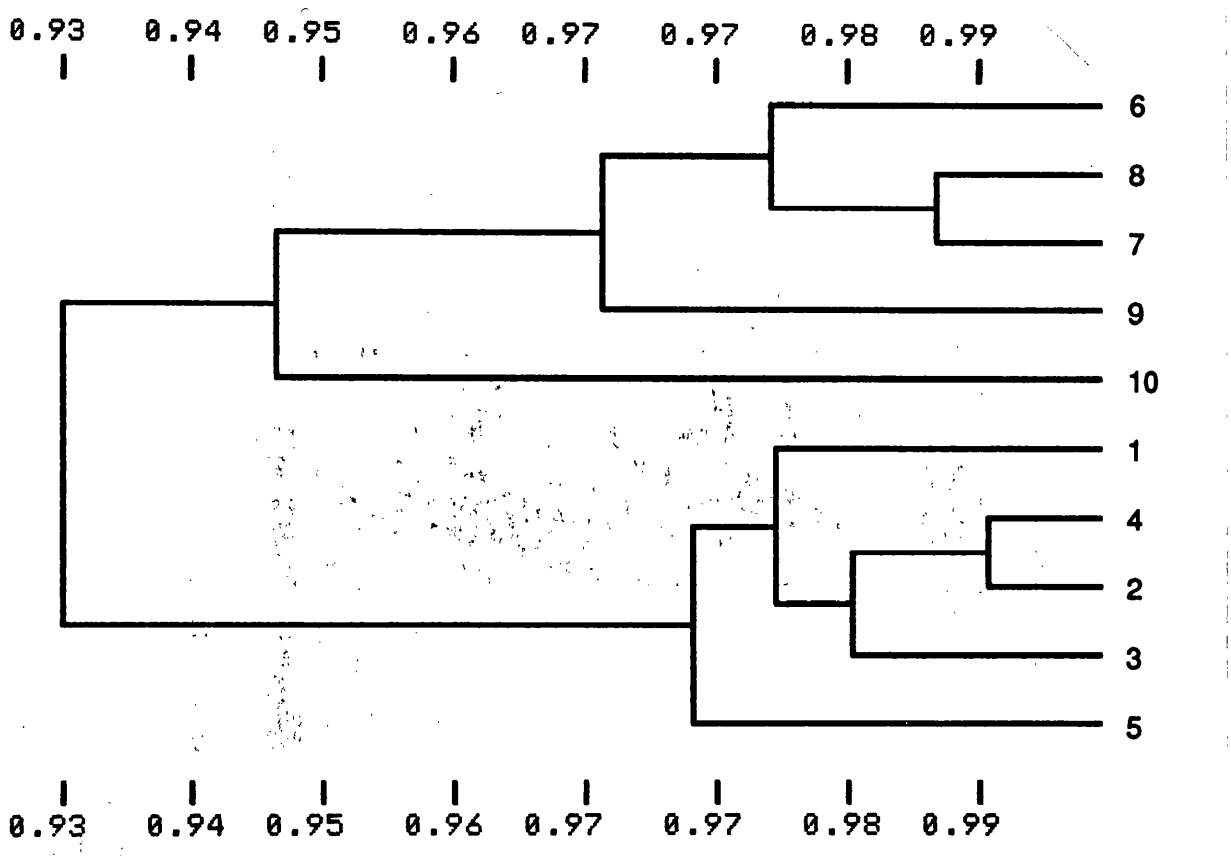


Figure 4.8. Dendrogram of the normalised molecular weight profiles from ten gels (see figure 4.4).

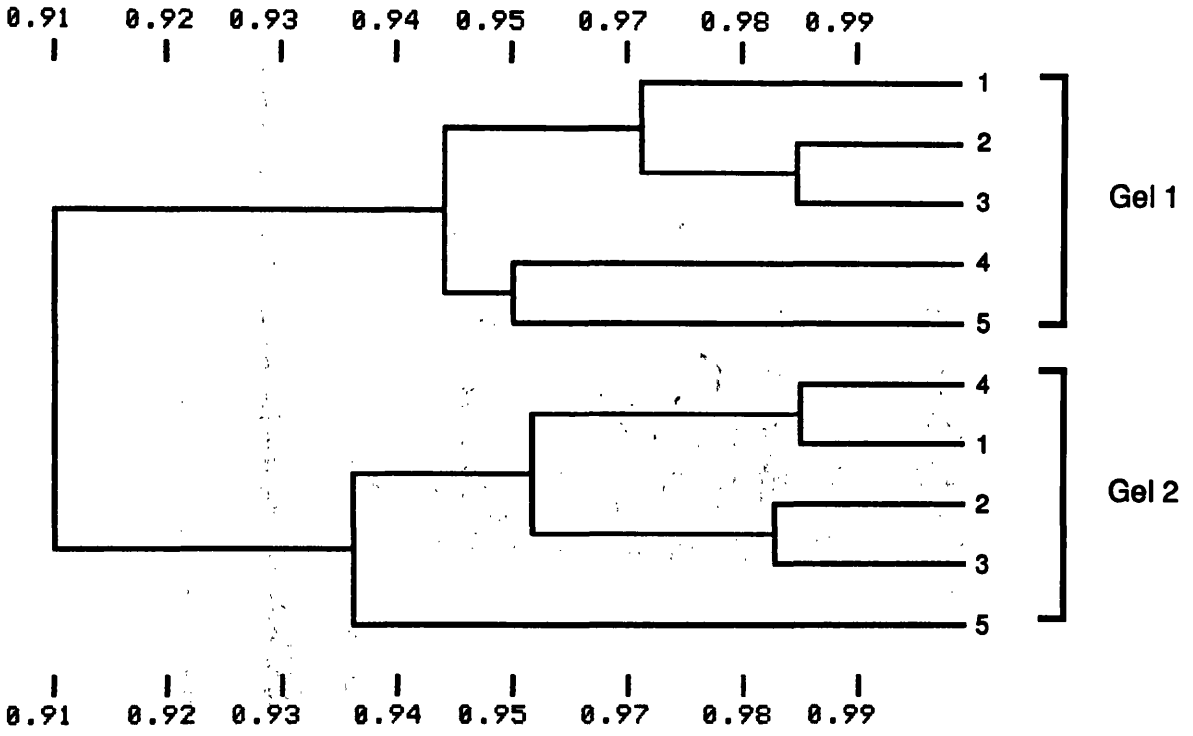


Figure 4.9. Dendrogram of five isolates, duplicated on two gels (1 and 2), of *Staphylococcus haemolyticus* whole cell protein profiles labelled with [^{35} S] methionine.

(with isolates of the same species) then the interpretation is totally different.

4.3 ASSESSING THE USE OF DIFFERENT [^{35}S] COMPOUNDS FOR LABELLING

An isolate from each of 11 Gram negative species and 9 Gram positive species was selected to test labelling with three [^{35}S] compounds: methionine, thio ATP and inorganic sulphate. Each isolate was incubated for 2 hours with each of the [^{35}S] compounds and processed using the methods described in chapter 3 and electrophoresed on the AMBIS gel system. For the [^{35}S] methionine labelled samples only, the supernatants were also electrophoresed.

All the isolates labelled well with the methionine (Fig. 4.10 and 4.11) and some secreted labelled proteins (Fig. 4.12). The Gram negative organisms labelled well with ATP (Appendix 4.1) and weakly with inorganic sulphate. Whereas the Gram positive organisms mostly gave comparatively weak patterns with ATP and no patterns with inorganic sulphate.

The same organisms were then labelled with the three [^{35}S] compounds for three days to give maximum opportunity for [^{35}S] labelled proteins to be synthesized. All of the species labelled with methionine (Appendix 4.2) and ATP (Appendix 4.3) as in the two hour incubations, although the patterns were different. This would almost certainly be due

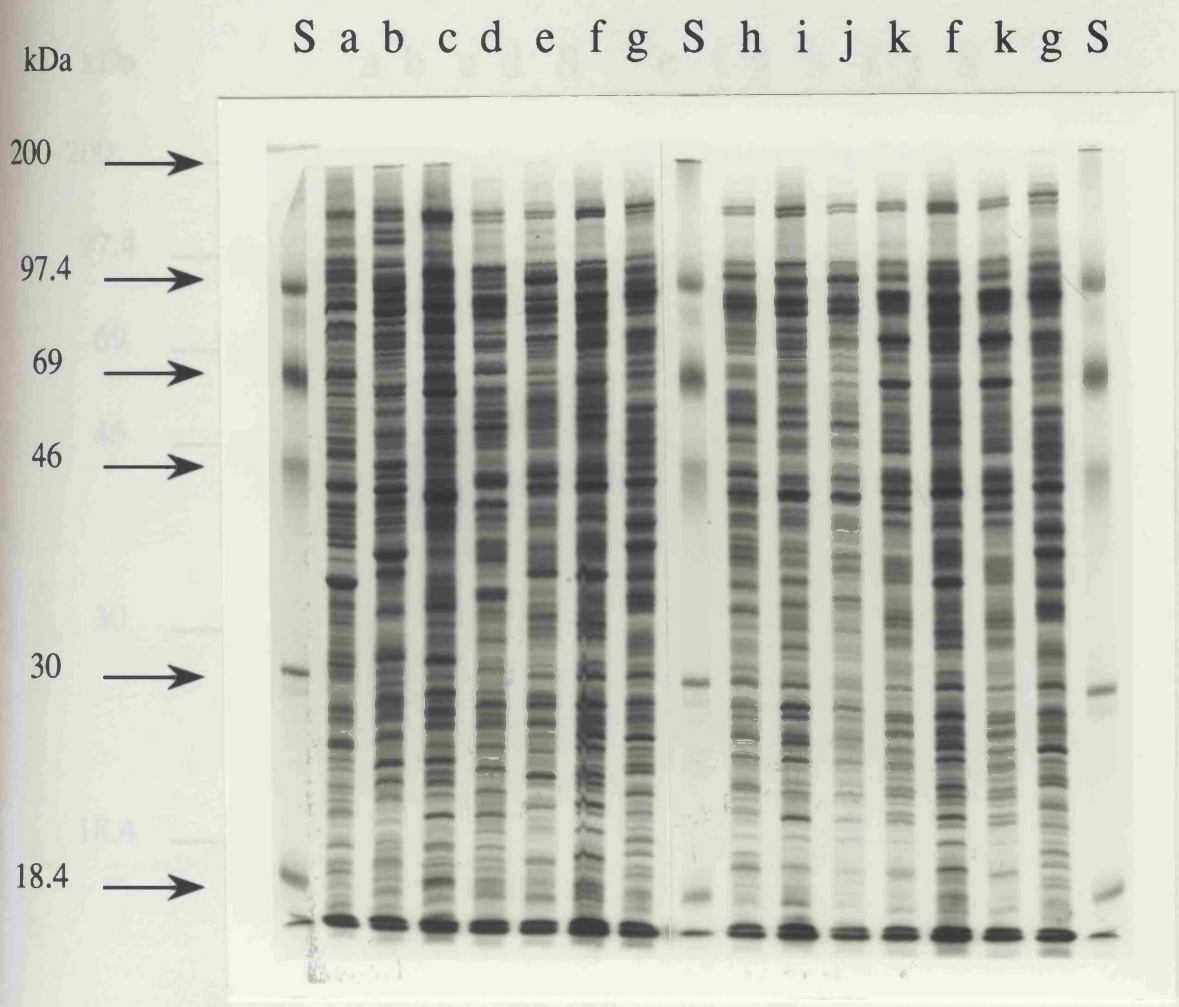


Figure 4.10. Whole cell proteins of Gram-negative organisms labelled with [^{35} S] methionine: S, molecular weight standards: a, *Pasteurella* sp.; b, *Morganella* sp.; c, *Pseudomonas* sp.; d, *Hafnia* sp.; e, *Enterobacter* sp.; f, *Klebsiella* sp.; g, *Proteus mirabilis*; h, *Salmonella* sp.; i, *Escherichia coli*; j, *Shigella* sp.; k, *Citrobacter* sp..

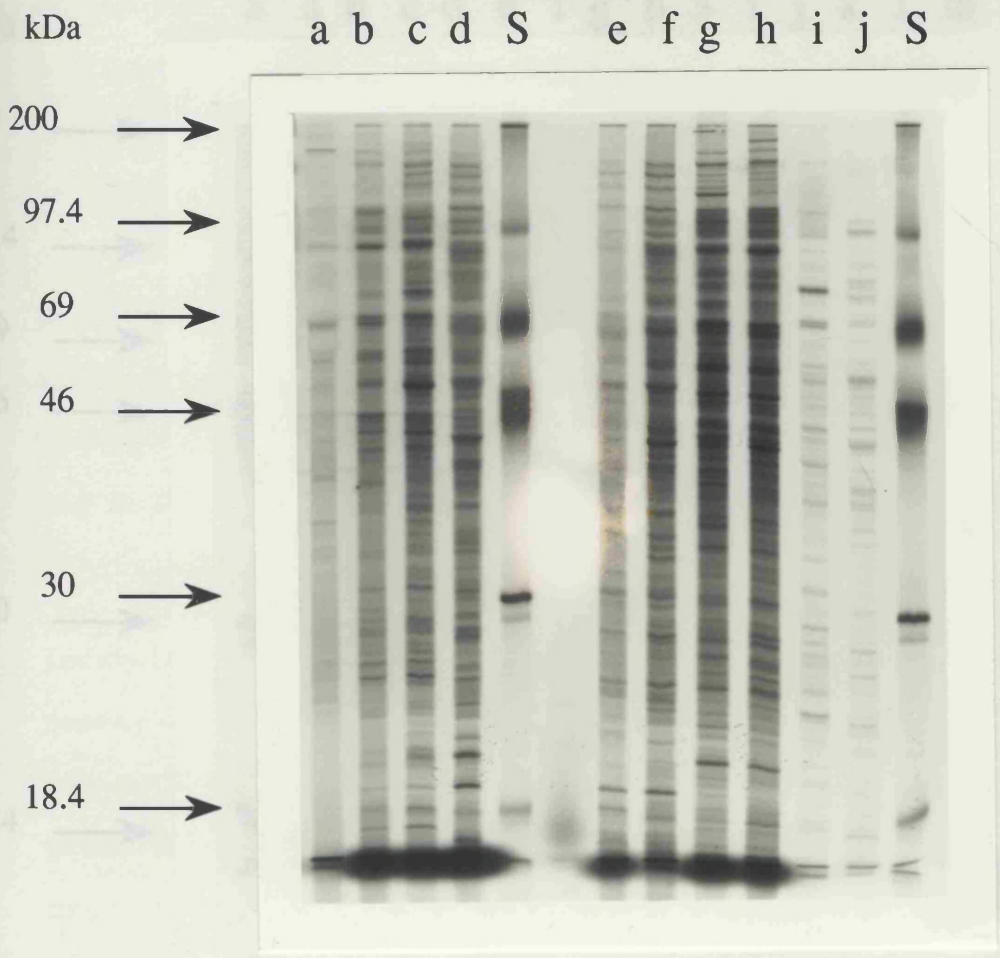


Figure 4.11. Whole cell proteins of Gram-positive organisms labelled with [^{35} S] methionine: S, molecular weight standards; a, *Corynebactium* sp.; b and c, *Staphylococcus epidermidis*; d, *S.aureus*; e and f, *S.aureus*, MRSA; g and h, *S.haemolyticus*; i, *Streptococcus agalacteae*; j, *Enterococcus faecalis*.

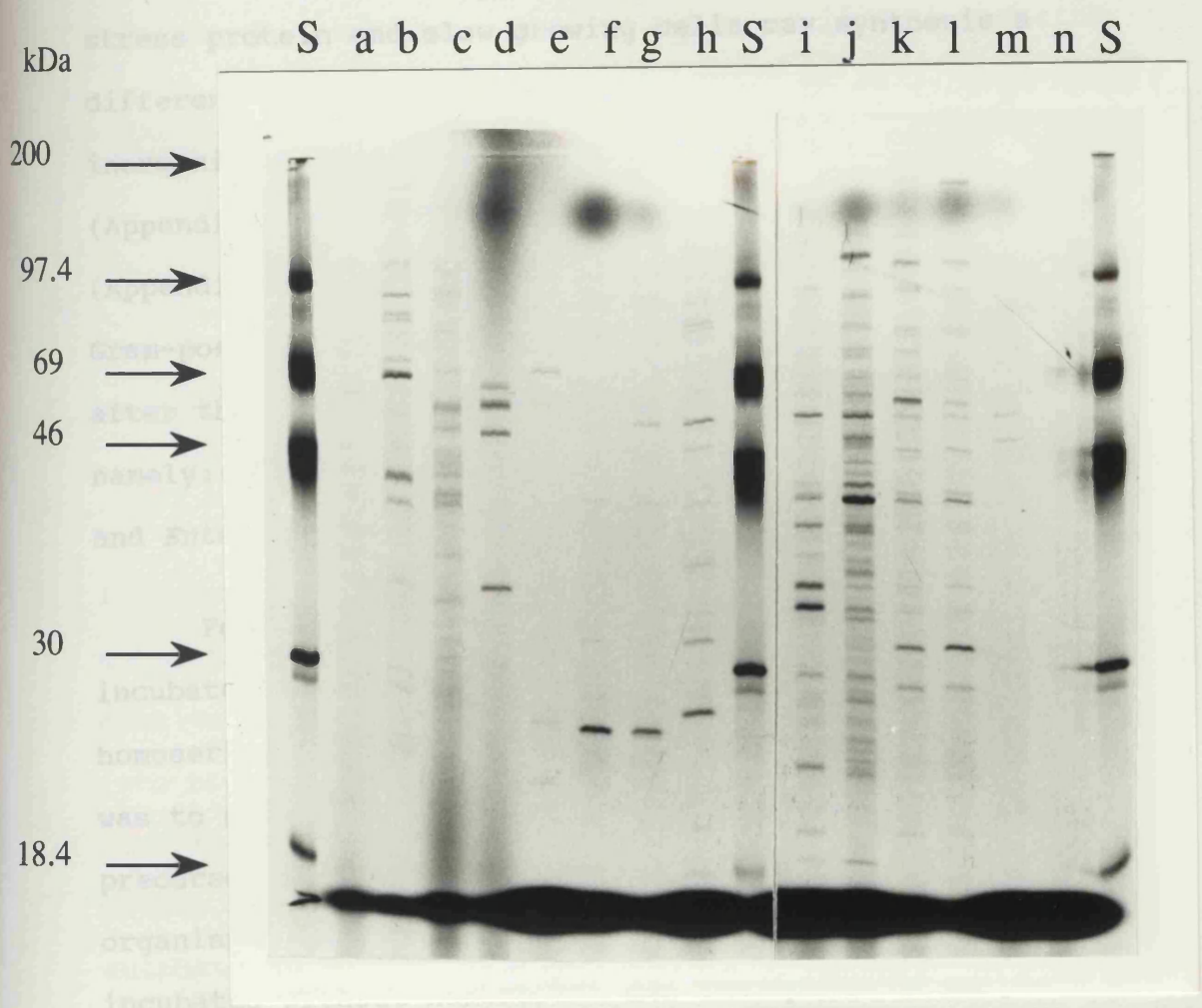


Figure 4.12. Secreted proteins of Gram-negative organisms labelled with [^{35} S] methionine: S, molecular weight standards; a, *Morganella* sp.; b, *Pseudomonas* sp.; c, *Citrobacter* sp.; d, *Salmonella* sp.; e, *Corynebactium* sp.; f and g, *Staphylococcus epidermidis*; h, *S.aureus*, i and j, *S.aureus*, MRSA; k and l, *S.haemolyticus*; m, *Streptococcus agalacteae*; n, *Enterococcus faecalis*.

to cells entering the stationary phase. In the stationary phase cells would become stressed and may begin synthesising stress protein and slow growing cells may synthesis a different set of proteins. More intense bands were seen with inorganic sulphate with the Gram-negative organisms (Appendix 4.4) and some of the Gram-positive organisms (Appendix 4.5) did produce weak bands also. However three Gram-positive organisms did not show any labelled bands even after three days of incubation with inorganic sulphate namely: *Staphylococcus epidermidis*, *Streptococcus agalacteae* and *Enterococcus faecalis*.

Four *Staphylococcus haemolyticus* isolates were incubated overnight with [^{35}S] inorganic sulphate with homoserine (0.06 mg/ml) added to the culture medium. This was to stimulate the uptake of sulphate, as homoserine is a precursor to methionine (Roberts *et al.*, 1955) in some organisms. Very faint bands were seen in one of the isolates incubated without homoserine and the uptake of sulphate was enhanced by the addition of homoserine. But where no trace of uptake was observed no enhancement was found.

The profiles from the sulphate, methionine and ATP labelling were normalised using the standard method and analysed by preparing a dendrogram. The profiles for the different substrates were analysed together and separately. Some species clustered according to the substrate used, this was particularly so with closely related species such as

Escherichia coli, *Shigella* and *Salmonella*. Whereas the different substrate profiles of some species clustered together for instance with *Proteus mirabilis*, *Pasteurella* and the *Pseudomonas* species, the sulphate and methionine profiles clustered together at correlations of 0.8, 0.6 and 0.7 respectively. High correlations between sulphate and methionine profiles would indicate there are bands in common due to inorganic sulphate being converted to methionine. Where the profiles give low correlations it would indicate that these species utilise inorganic sulphate predominantly via a different pathway, for sulpho-proteins and incorporation into cystine and glutathione. Possibly the extra biochemical steps of converting the sulphate to methionine or the other amino acids before incorporating it into proteins accounts for the longer incubation times. It is also possible that cell metabolism is also considerably slower due to the lack of methionine in the inorganic sulphate incubation medium. The complete lack of uptake of sulphate by some Gram-positive organisms may be due to these organisms being methionine dependent. This could be further investigated by adding non labelled methionine into the inorganic sulphate labelling medium, as it has been found with *E.coli* that sulphate uptake is only 48% inhibited (Roberts *et al.*, 1955) in the presence of methionine as an alternative sulphur source. Possibly these species would prefer sulphur in the form of SO_3^{-2}, which was found to be taken up in preference to SO_4^{-2} in the case of *E.coli*

(Roberts *et al.*, 1955).

In most of the organisms the ATP profiles were found to be totally different from the methionine and sulphate profiles. The exception was *Morganella* where a correlation of 0.9 between the ATP and methionine profiles was obtained and the *Pseudomonas* species where the profiles from all three substrates clustered between 0.7 and 0.8.

When the data from the different substrates were analysed separately (Fig. 4.13 and 4.14), some differences in the arrangement were seen. However there were some general trends, for instance with all three substrates *E.coli*, *Shigella* and *Salmonella* always clustered closely as did *Enterobacter* and *Klebsiella* with *Pasteurella* and *Proteus mirabilis* always clustering at a low correlation with the other Gram negative organisms. this is consistent with current taxonomic arrangements.

From these results it was concluded that [^{35}S] methionine was the most efficient and universally applicable sulphur labelling substrate as all the organisms tested in the above experiments labelled readily in two hours. A standard two hour incubation time was used except for certain species with very long lag phases.

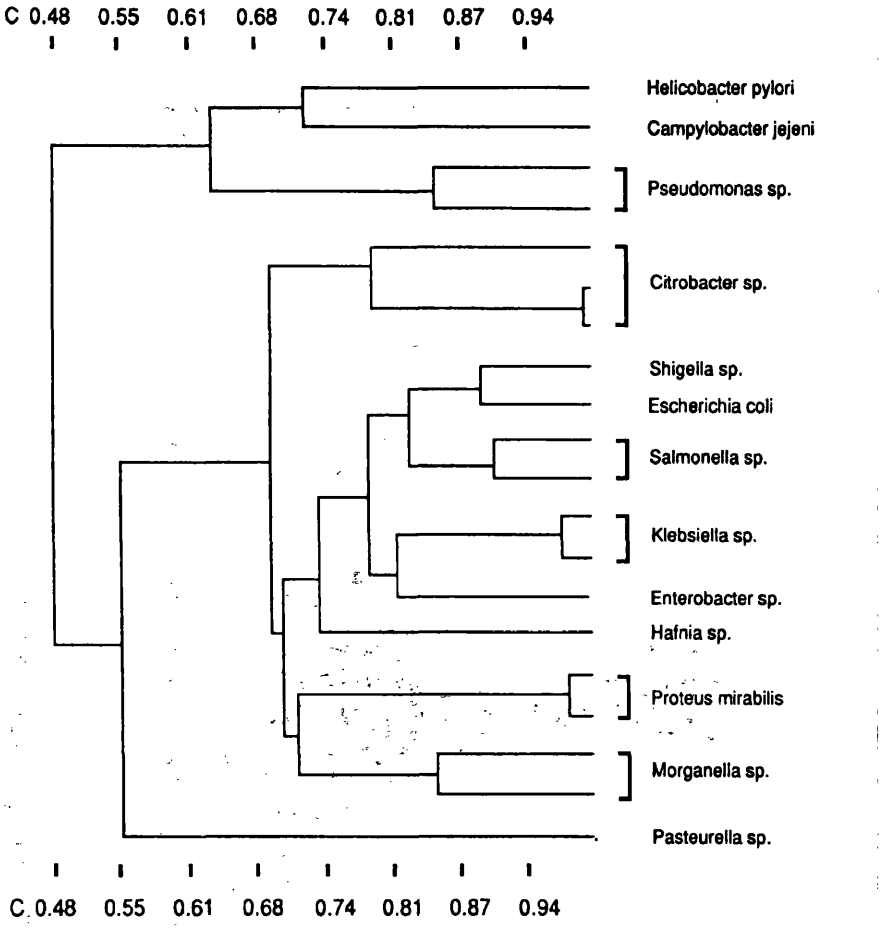


Figure 4.13. Dendrogram of the whole cell protein profiles of Gram-negative organisms labelled with [^{35}S] methionine.

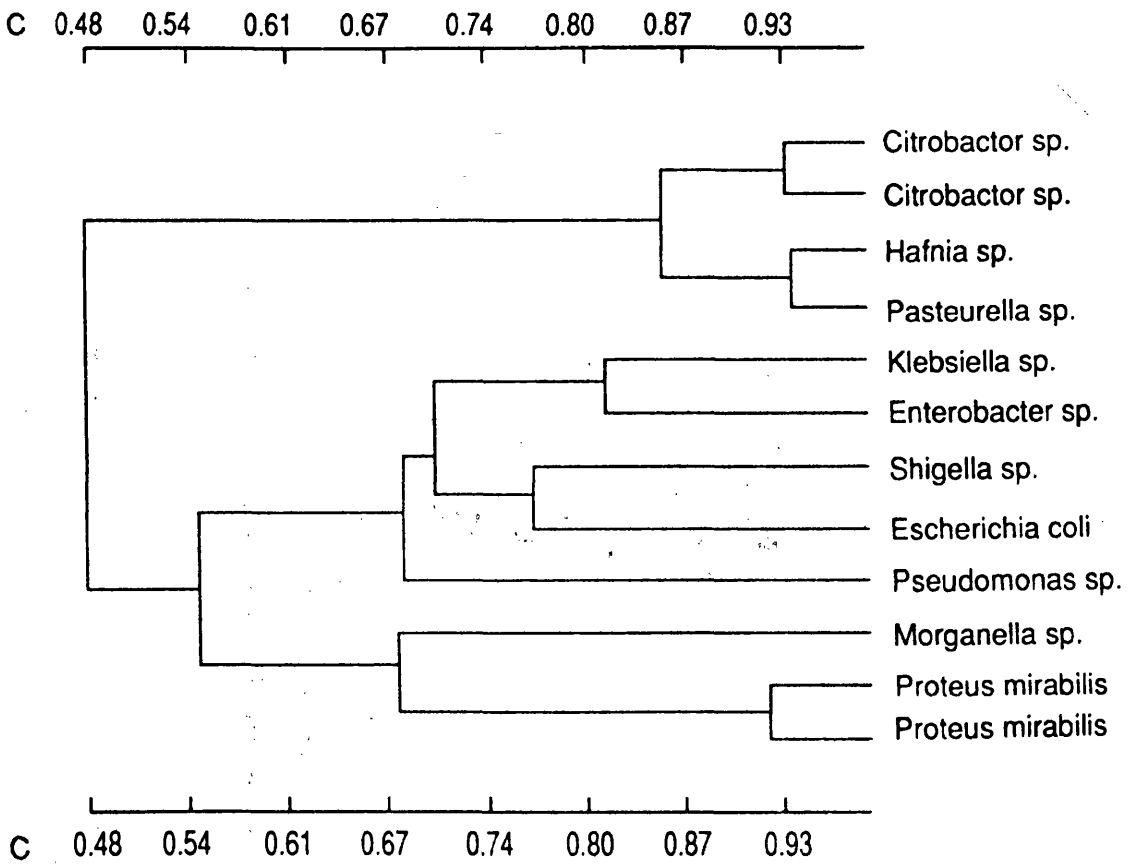


Figure 4.14. Dendrogram of the whole cell protein profiles of Gram-negative organisms labelled with [^{35}S] thio ATP.

4.4 *STAPHYLOCOCCUS HAEMOLYTICUS*

Twenty four tetracycline sensitive isolates and four resistant isolates of *Staphylococcus haemolyticus* were labelled in duplicate with [^{35}S] methionine for 2 hours and the pellets (Appendix 4.6) and supernatants (Fig. 4.15) analysed by the standard method on the AMBIS gel system. Two isolates were incubated in duplicate and electrophoresed on the same gel, When the histograms for each duplicate were normalised and auto-aligned by the standard method the correlations were found to be 0.936 and 0.952. The pellets from the resistant isolates and 18 of the sensitive isolates consistently clustered together above a correlation of 0.9. The 6 remaining isolates did not cluster together or with the main group. The lowest correlation when compared with the main group being 0.7. One of the isolates consistently gave 30% of the counts observed with each of the other isolates. Greater band differences were observed with the supernatant patterns (Fig. 4.15). Nine main bands were observed as being common to most of the isolates with some isolates missing a couple or having extra bands. The resistant and sensitive isolates did not cluster separately. One of the isolates whose pellet patterns grouped with a low correlation with the main group gave no supernatant patterns at all.

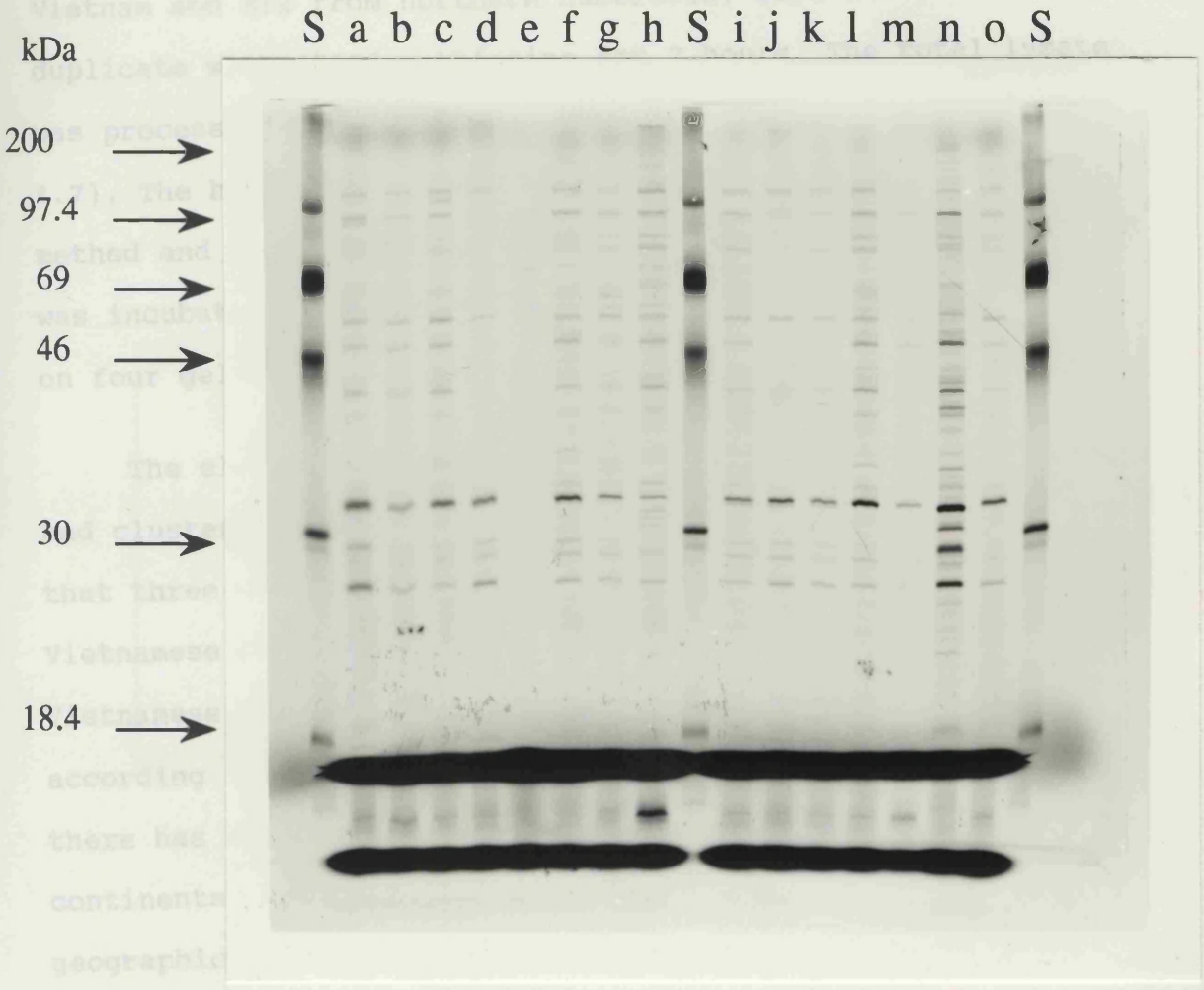


Figure 4.15. Secreted proteins of [^{35} S] methionine labelled *Staphylococcus haemolyticus* : S, molecular weight markers; a to d, teicoplanin resistant isolates of *S.haemolyticus* ; e to o, teicoplanin sensitive isolates of *S.haemolyticus*.

4.5 *PSEUDOMONAS* SP.

Eleven *Pseudomonas pseudomallei* strains, five from Vietnam and six from northern Australia, were labelled in duplicate with [^{35}S] methionine for 2 hours. The total lysate was processed and analysed on the AMBIS gel system (Appendix 4.7). The histograms were normalised using the standard method and a dendrogram prepared (Fig. 4.16). One isolate was incubated in duplicate which was then electrophoresed on four gels.

The eleven strains grouped above a correlation of 0.88 and clustered into three groups above 0.91. It was found that three of the Australian strains and two of the Vietnamese strains clustered together with the remaining Vietnamese strains and Autralian strains clustering according to geographic origin. This may indicates that there has been some spread of isolates between the two continents. More detailed analysis of the isolates geographic origin may enable a route of spread to be determined for this organism.

Within each gel a duplicate incubation of isolate (a) was electrophoresed and after normalisation by the standard method the mean correlation for intra gel comparisons was found to be 0.98 $\pm$ 0.008 (n = 4). The isolates within group 2 and 3 clustered above 0.96, only a correlation of 0.01 less than the standard deviation correlation of the duplicate incubations and thus the isolates within these

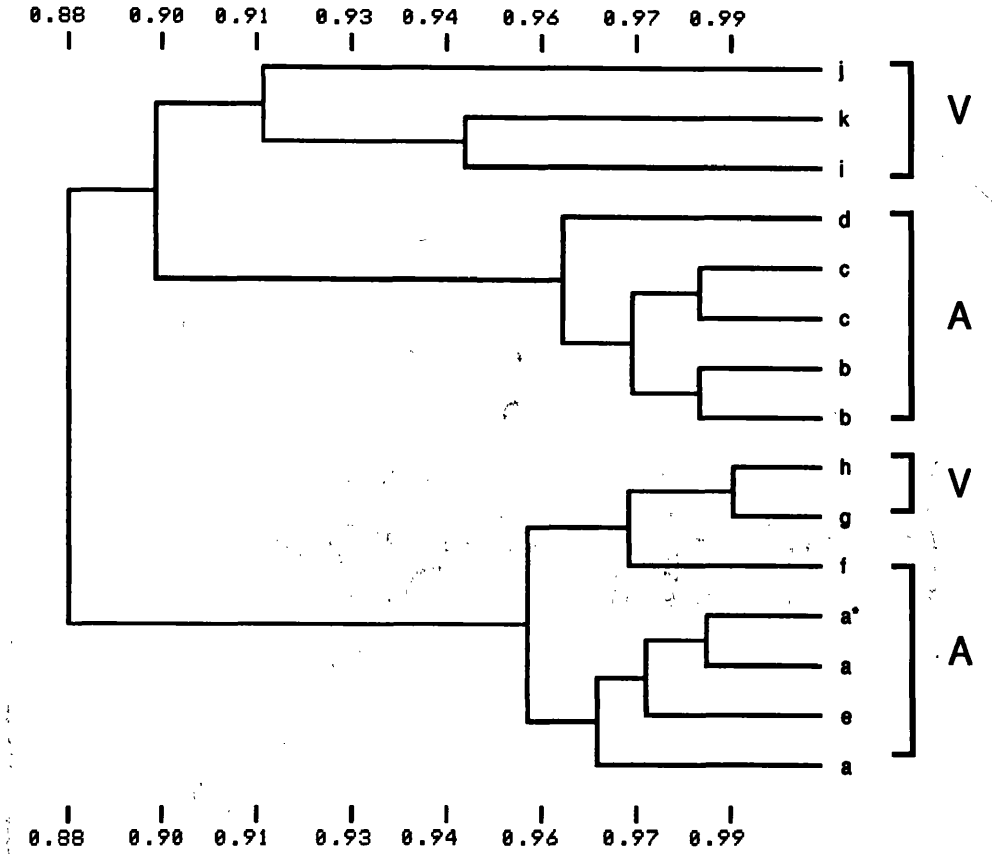


Figure 4.16. Dendrogram of the whole cell protein profiles of eleven *Pseudomonas pseudomallei* isolates, a to k, labelled with [^{35} S] methionine: V, samples from Vietnam; A, samples from Australia. Identical lettering indicates the same sample run in duplicate; * indicates the same isolate labelled separately.

clusters should be considered identical. In group 1 the three isolates cluster up to 0.06 less than the standard deviation which indicates greater isolate variation exists within this cluster.

One to four representatives from eight *Pseudomonas* species were labelled with [^{35}S] methionine for 2 hours and the total lysate electrophoresed on the AMBIS gel system (Appendix 4.8). The histograms were normalised using the standard method and a dendrogram produced (Fig. 4.17). The isolates of each species clustered together, although the clustering of species was in some cases as close as the clustering between isolates of other species. It is clear from this that one cannot set a universal correlation threshold for distinguishing between the species and the sub-species. Although in this instance all the sub-species, with the exception of *P.paucimobilis*, clustered above 0.81 indicating that there is a general trend threshold for distinguishing between the isolates of the same species but that there may always be exceptions to the rule.

4.6 INTESTINAL SPIROCHETES

Eleven spirochete isolates and one control type culture isolate, *Serpula hyodisenteriae*, were labelled in duplicate with [^{35}S] methionine, the pellet was electrophoresed on the AMBIS gel system (Appendix 4.9) and the data analysed. Duplicate incubations of sample i and 1 (see Fig. 4.18) were electrophoresed as controls, a

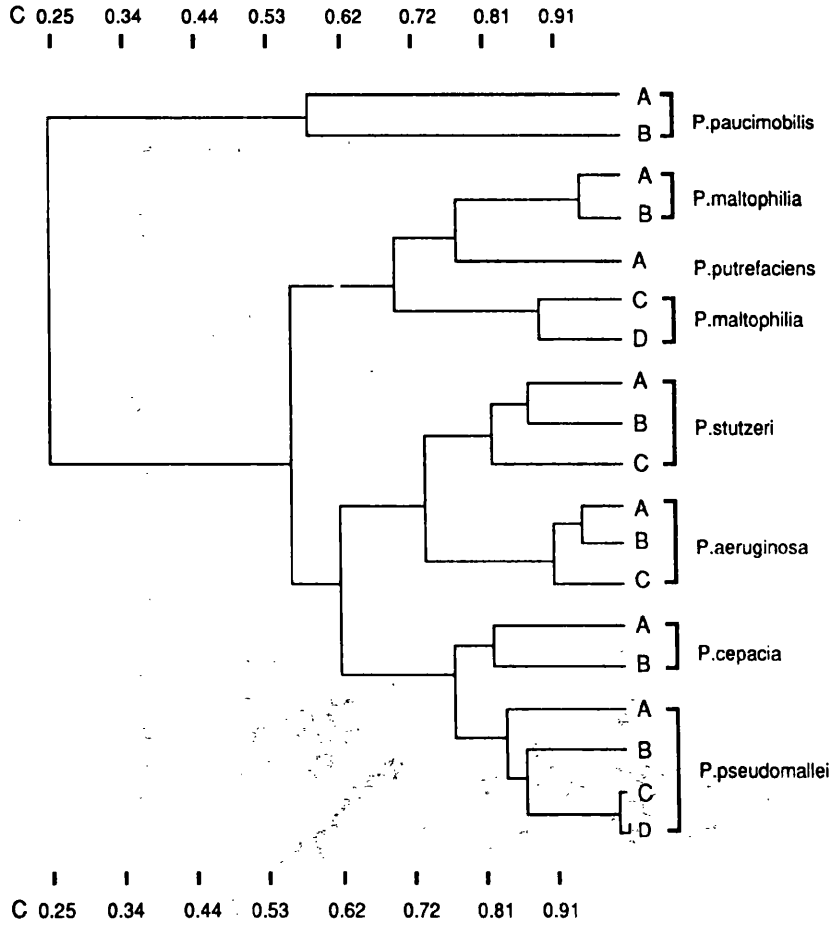


Figure 4.17. Dendrogram of the whole cell protein profiles of [^{35}S] methionine labelled *Pseudomonas* sp.: *P.paucimobilis* (A) NF284 (B) NF60; *P.maltophilia* (A) NF48 (B) NF119 (C) NF24 (D) NF58; *P.putrifaciens* (A) NF270; *P.stutzeri* (A) Z271 (B) Z152 (C) Z199; *P.aeruginosa* (A) K4 (B) K3 (C) K1; *P.cepacia* (A) NF144 (B) NF62; *P.pseudomallei* (A) j (B) i (C) h (D) g (see figure 4.16).

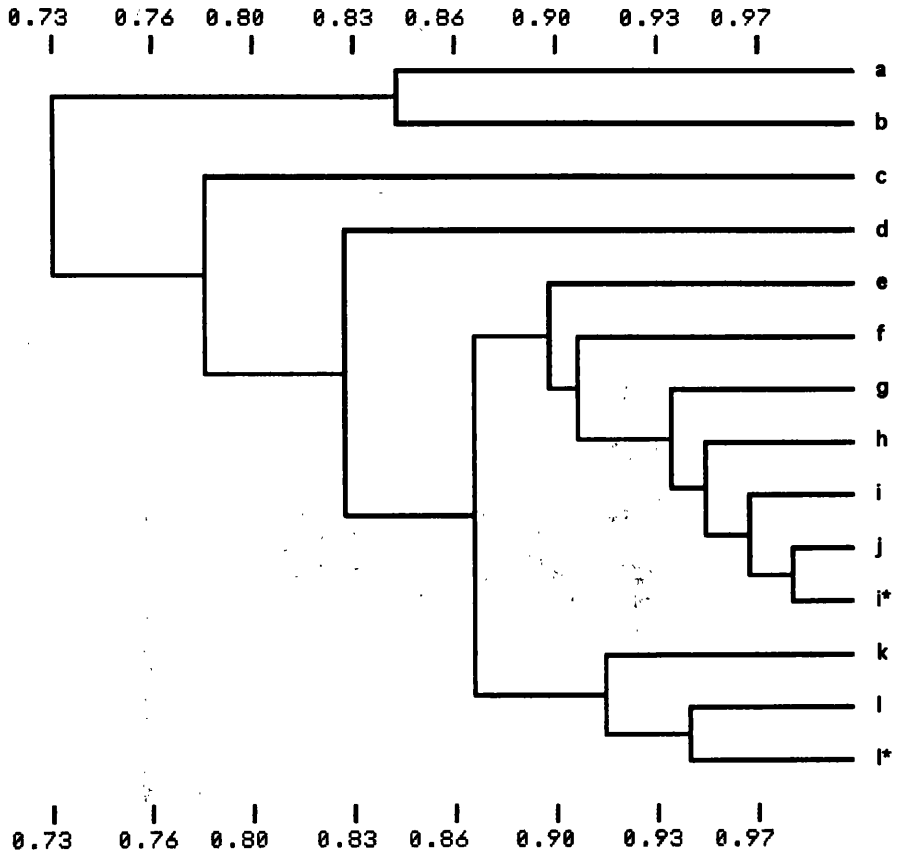


Figure 4.18. Dendrogram of the whole cell protein profiles of twelve spirochete isolates, a to l, labelled with [^{35}S] methionine; * indicates the same isolate labelled separately.

correlation of 0.954 and 0.941 for i and 1 respectively was obtained. By dendrogram analysis (Fig. 4.18) five of the isolates clustered above 0.9 with the the control isolate *Serpula hyodisenteriae*, two other isolates clustered at a correlation of 0.86 with the main cluster. The remaining four isolates clustered at lower correlations with the main group due to one or two band difference.

4.7 *CAMPYLOBACTER* AND *HELICOBACTER* SPECIES

Seven *Campylobacter jejuni* and nine *Helicobacter pylori* isolates were labelled overnight with [^{35}S] methionine in duplicate. The pellets (Appendix 4.10) and supernatants (Fig. 4.19) were electrophoresed separately on AMBIS gels and the data analysed by the standard methods (Fig. 4.20). The profiles of the whole cell preparations of *Campylobacter* and *Helicobacter* clustered in two distinct groups. Duplicate incubations of four isolates of *Helicobacter* were electrophoresed on one gel and the duplicate pairs, normalised by the standard method, were correlated, a mean correlation of 0.890 $\pm$ 0.054 (n = 4) was obtained. Five of the campylobacters clustered above a correlation of 0.9 and thus the profiles could be considered identical, differences between the isolates were evident from the supernatant patterns. Three of the supernatant patterns were identical, one had many more bands and the other had many bands missing but with one extra. The two *Campylobacter* isolates whose cell proteins showed

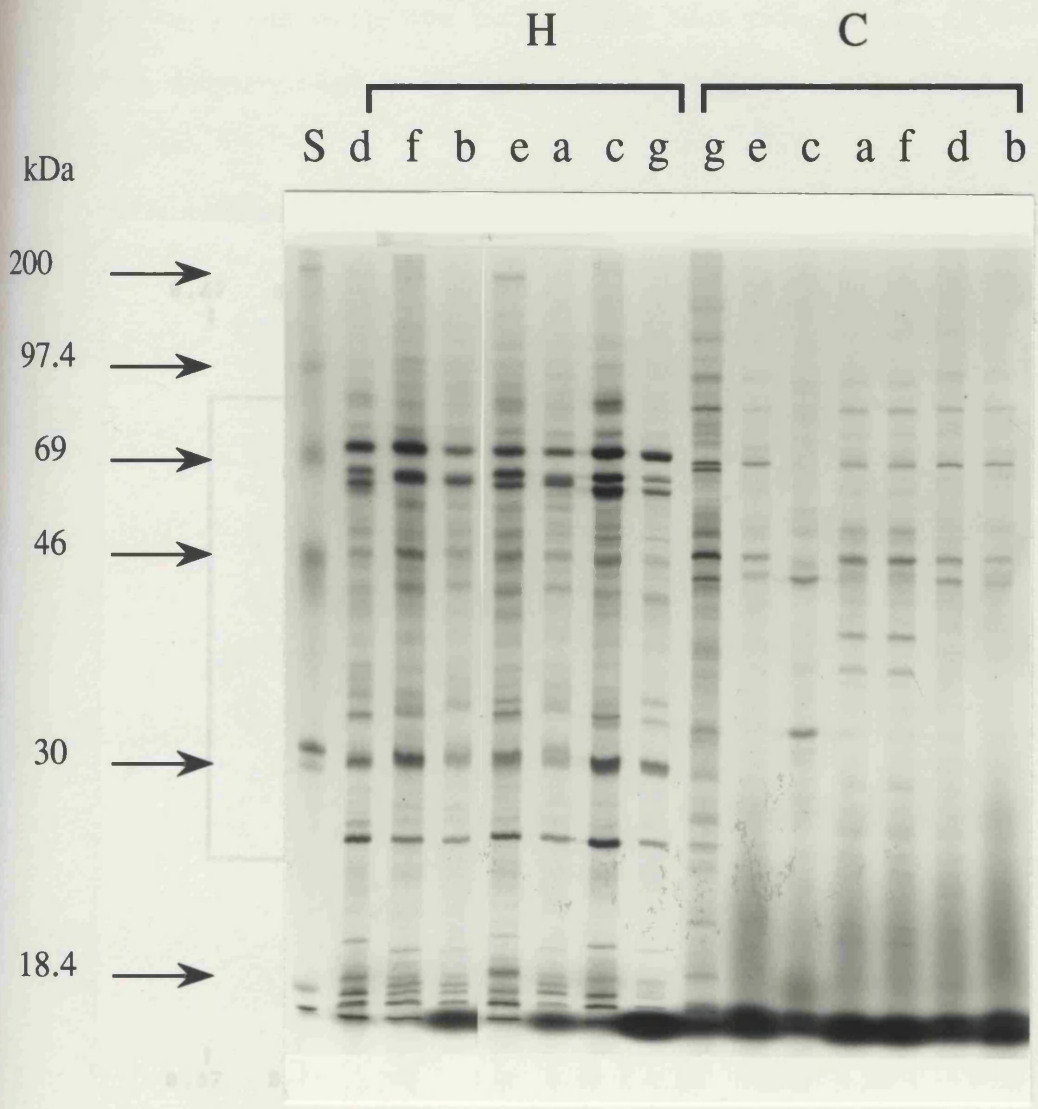


Figure 4.19. Secreted proteins of *Helicobacter pylori* , a to g, (H) and *Campylobacter jejuni* , a to g, (C) labelled with [^{35}S] methionine: S, molecular weight standards.

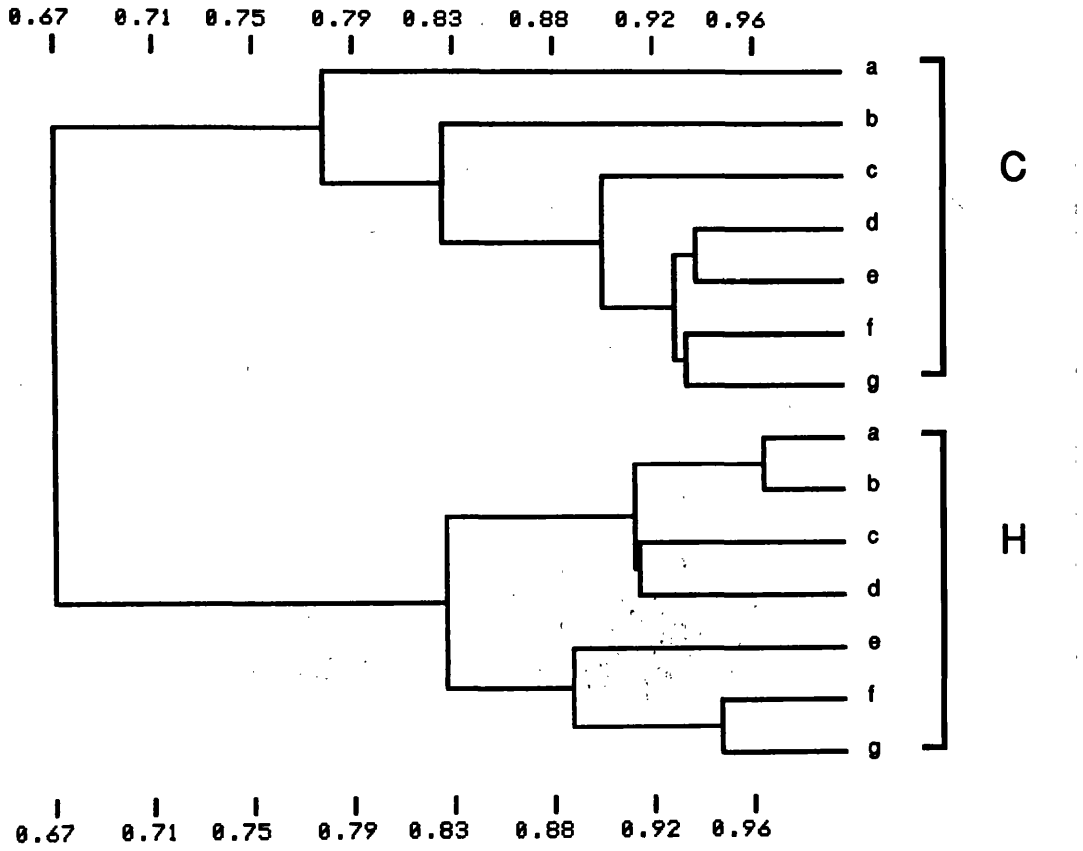


Figure 4.20. Dendrogram of the whole cell protein profiles of *Helicobacter pylori* (H) and *Campylobacter jejuni* (C) isolates labelled with [^{35}S] methionine.

differences with the main group had some bands in common in the supernatant with the other isolates plus extra unique bands.

The *Helicobacter* whole cell profiles clustered above a correlation of 0.8. All the *Helicobacter* isolates shared four prominent bands of 70, 45, 28 and 24 kDa. Some strain differences between the isolates were evident between 67 and 50 kDa. Sub-speciation of the *Helicobacter* isolates was much clearer with the supernatant patterns (Fig. 4.20), the nine isolates sub-divided into two groups on the basis of a doublet of 64 and 62 kDa in five of the isolates and a singlet of 63 kDa in the remaining four. In one of the repeat runs of the supernatants the singlet was just discernable as a very close doublet.

4.8 *MYCOBACTERIA*

(I) AMBIS gels data

A preliminary study using eight pairs of mycobacterial cultures supplied by Dr J. L. Stanford were labelled with [^{35}S] methionine and [^{35}S] inorganic sulphate for one week. The supernatants were electrophoresed on the AMBIS gel system (Fig. 4.21 and Appendix 4.11). Distinct patterns enabled the pairs to be correctly matched by eye. Overall more bands were seen in the methionine supernatants than in the sulphate supernatants except for *M.duvalii* which gave a distinctive laddered effect of bands that were most intensely labelled between the top of the gel and the 96 kDa

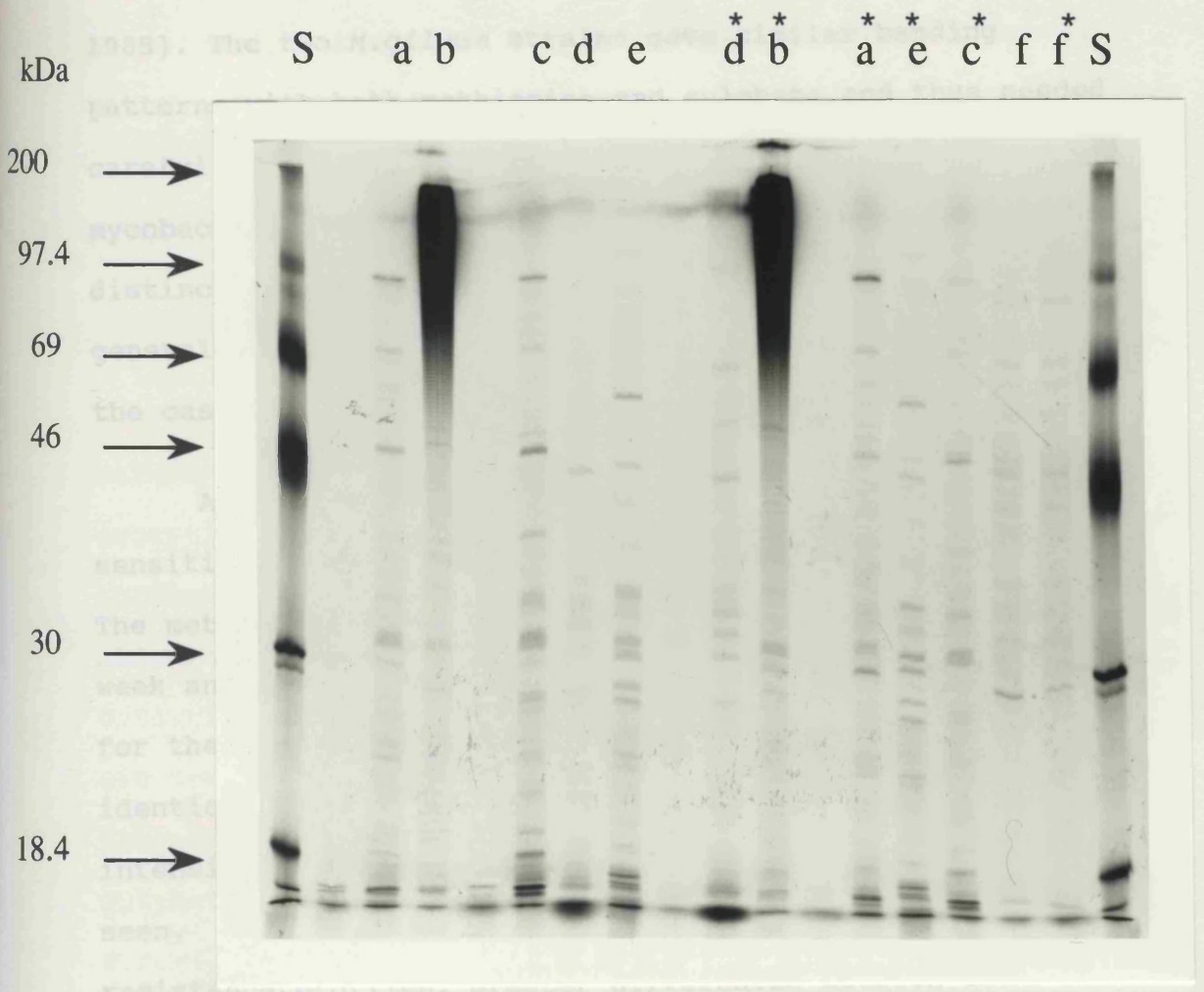


Figure 4.21. Secreted proteins of various Mycobacterial species labelled with [^{35}S] inorganic sulphate: S, molecular weight standards; a and c, *M.gilvum*; b, *M.duvalii*; d and f, *M.vaccae* (d, R877R); e, *M.flavescens*. * , indicates the same organism labelled in duplicate.

molecular weight marker. A similar laddering effect can be seen with lipopolysaccharide (LPS) of *E.coli* (Beck *et al.*, 1985). The two *M.gilvum* strains gave similar banding patterns with both methionine and sulphate and thus needed careful examination to distinguish by eye. The other mycobacterial species included in this set of data gave distinct patterns. The counts of the methionine data were generally greater than the counts using sulphate, except in the case of *M.duvalii*.

A group of *M.tuberculosis* with differing drug sensitivities were analysed using methionine and sulphate. The methionine labelled cultures were incubated for one week and the sulphate for two weeks. The banding patterns for the whole cell preparations within each set were identical (Appendix 4.12). Some differences in the relative intensity of labelled bands within separate patterns were seen, but they could not be correlated with the drug resistance profiles. Greater differences between the isolates were seen with both sulphate and methionine supernatant patterns (Appendix 4.13 and 4.14) but again the clustering did not correlate with the drug resistant profiles.

(II) Pharmacia gels data

As a new gel system was being used a fresh approach was needed, starting with a more extensive range of control labelling experiments.

Two strains of each of the mycobacteria *M.fortuitum*, *M.chelonei*, *M.kansasii*, *M.tuberculosis*, *M.avium*, *M.xenopi* and *M.malmoense* and one strain of *M.vaccae* and *M.neoaurum* were incubated with [^{35}S] methionine and [^{35}S] inorganic sulphate in duplicate. One of the duplicate sets was incubated in air and the other one in the CO_2 incubator (5% CO_2), both at 37 °C (Appendix 4.15, 4.16, 4.17 and 4.18). There was not much difference in the counts of the bands between the air and CO_2 incubated samples. With the exception of *M.avium* all the incubations in the CO_2 incubator and in air with sulphate and some of the methionine incubations clustered above a correlation of 0.93. In these cases pairs incubated in 5% CO_2 and air clustered more closely with each other than with the other isolates of the same species. In the case of *M.avium* sulphate patterns and *M.vaccae*, *M.tuberculosis* and *M.fortuitum* methionine patterns the isolates of the same species clustered most closely than the duplicates incubated in 5% CO_2. This was particularly so for *M.avium* where the separate isolates clustered at a high correlation of 0.96 and at a low correlation of 0.88 with the CO_2 incubated isolates. This phenomenon was even more evident with the *M.vaccae* incubation with methionine where the correlation between the pairs incubated in 5% CO_2 and air was 0.57.

The histograms were normalised using the standard method and a dendrogram prepared and similar grouping

patterns were obtained for both the sulphate (Fig. 4.22) and methionine air incubation data. For instance with both, the different isolates *M.avium*, *M.tuberculosis*, *M.kansasii* and *M.fortuitum* cluster together with one of the two *Nocardia* isolates clustering closely with *M.chelonei*. With both [^{35}S] compounds *M.vaccae* clustered with *M.tuberculosis* which then clustered with *M.fortuitum*. Differences in the clustering of *M.duvalii*, *M.kansasii* and *M.xenopi* with the other mycobacteria were found with the two compounds.

Sulphate was selected as the best source of [^{35}S] for further mycobacterial labelling experiments because with the Pharmacia gel system there was little to choose between the sulphate and methionine data, and sulphate was easier to work with as well as cheaper.

One isolate of five mycobacterial species were incubated with [^{35}S] inorganic sulphate for 4, 7, 10 and 17 days. With *M.kansasii* and *M.chelonei* there was little difference in the counts between the different lengths of time incubated. With the *M.tuberculosis* and *M.avium* there was an increase in counts from 4 to 7 days and 7 to 10 days, with little difference between 10 an 17 days. The labelled bands of *M.xenopi* gave barely visible bands at 4 days which did increase after 17 days, but were still very weak. These results reflect the different growth rates of mycobacterial species. But 7 days is a good compromise incubation time if rapidity and

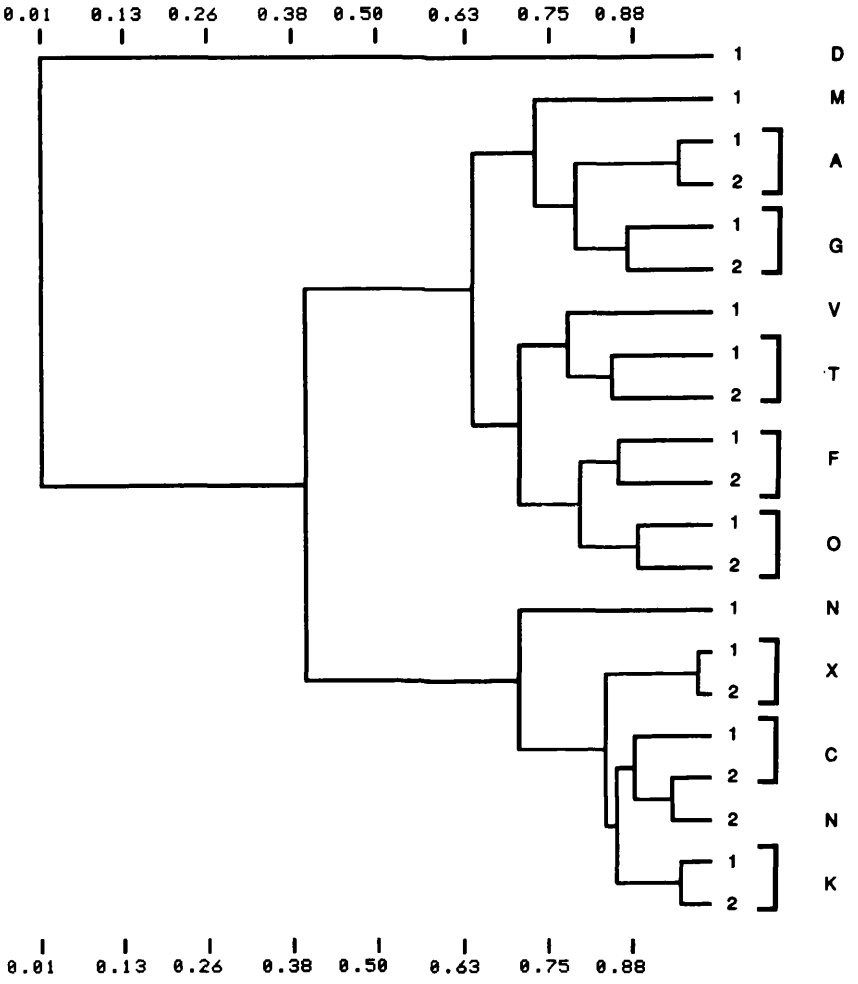


Figure 4.22. Dendrogram of [^{35} S] inorganic sulphate labelled profiles of various mycobacterial species: D, *M.duvalii* ; M, *M.malmoense* ; A, *M.avium*; G, *M.gilvum* ; V, *M.vaccae* R877R; T, *M.tuberculosis* ; F, *M.fortuitum* ; O, *scotochromogen* ; N, *Nocardia* sp.; X, *M.xenopi* ; C, *M.chelonei* ; K, *M.kansasii.*

standardisation is required.

Cultures of different ages from seven mycobacterial species were labelled with sulphate to asses the effect of culture age on labelled protein profiles (Appendix 4.19). The selected cultures were taken from available subculture stored at room temperature. Thus the range of culture ages was determined by what was available. The results show that the length of time a culture was stored could effect the banding patterns, probably due to the stimulation of stress proteins and other cellular changes triggered by the cells entering stationary phase (Siegele and Kolter, 1992). This result demonstrates the importance of using actively growing cultures for inoculation into [^{35}S] incubation media.

Between 7 and 29 isolates of seven species of mycobacteria were labelled with [^{35}S] inorganic sulphate and the pellets electrophoresed on pharmacia gels.

Twenty of the *M.tuberculosis* (Appendix 4.20) strains showed few strain variations, grouping above a correlation of 0.8. One isolate was distinct from the other strains due to the presence of an extra prominent band of 21.5 kDa, this isolate grouped with the other isolates at a low correlation of 0.26.

The *M.avium* (Appendix 4.21) isolates patterns were almost identical, the only band differences were seen at low molecular weight, below a band of 13 kDa common to all the

isolates. Sixteen of the twenty *M.kansasii* (Appendix 4.22) had identical band patterns by visual inspection with a main band of 31 kDa. Of the remaining four isolates, one was identical to the main group but with a particularly prominent band of 15 kDa, two had a unique distinguishing band of 43 kDa and one was almost completely unique.

The band differences between the *M.fortuitum* (Appendix 4.23) profiles were complex and although different isolates had many bands in common it was not easy to group them by eye. Only two isolates appeared to be identical. When analysed two clusters were obtained, each cluster being above a correlation level of 0.8. Distinct patterns were also seen within the *Nocardia* sp. (Appendix 4.23) there were two identical pairs that each paired above a correlation of 0.9, with the remaining three isolates all being distinct from these. The two *M.malmoense* isolates that were labelled gave different patterns but had one prominent band of 44 kDa in common.

Differences between *M.chelonei* (Appendix 4.24) isolates were seen, some isolates were clearly identical. All the isolates had a prominent band of 43 kDa, and the differences between isolates were all seen below that band.

In summary the [^{35}S] inorganic sulphate can be used to sub-speciate mycobacterial species. In some instances the differences between isolates of the same species are minor, one or two band differences such as with *M.avium* and

M.tuberculosis and *M.kansasii*. But with others there major differences between the isolates of the same species, such as with *M.fortuitum* and *M.chelonei*. This may be a reflection of the degree of species analysis and refinement of speciation analysis which would be more prominent in *M.tuberculosis* and *M.avium*. Whereas with less important pathogens such as *M.fortuitum* there has been less emphasis on defining these species as they are not so clinically important.

4.9 DATABASE

A data base containing 84 lanes and consisting of the organisms in table 4.1 was constructed from data analysed on the AMBIS electrophoresis system. All data were normalised by the standard method to one reference gel. Further isolates of some of the organisms making up the database were labelled, electrophoresed and scanned. The extracted data was normalised in the same way as the profiles in the database. A total of 133 histograms were matched with the data base using the fast fourier transform and spectral correlation coefficient to get the best ten matches.

The correct match was observed in 87% of cases. The mean correlation for correct matches was 0.861 $\pm$ 0.084, and the mean correlation for the incorrect matches was 0.720 $\pm$ 0.118. After 'ranking' to give the correlation coefficient the correct match was observed in 92% of the matches. The

Table 4.1 Table of bacterial genera contained within the database: n = number of isolates. See Table 3.1 for stain details.

	n
Staphylococcus	16
Streptococcus	2
Corynebacterium	3
Pasteurella	1
Morganella	1
Pseudomonas	31
Hafnia	1
Enterobacter	2
Klebsiella	2
Proteus	2
Escherichia	2
Shigella	1
Citrobacter	1
Treponema	6
Campylobacter	6
Helicobacter	7

mean correlation coefficient was then 0.869 $\pm$ 0.077, the mean correlation of the incorrect matches was 0.721 $\pm$ 0.119.

When choosing data for the data base, lanes with obvious distortions were not used. Such distortions include extreme streaks down a lane and marks or smears that some times appear on the gels. Lanes that were too weak (<40 000 cpm) were also excluded. Of the gels from which 133 histograms were taken for matching, 7.4% of the data were not used. Even so, 36.4% of this rejected data did still give the correct match with the spectral match, and 54.5% after 'ranking', however the mean correlations for these correct matches were below those for the acceptable data.

The spectral correlation match is much less position sensitive than the correlation coefficient as it compares the overall shape of the histograms, thus it can be used to compare a challenge histogram with the database quickly. It takes approximately 30 seconds to select the ten best matches from a database of 84 histograms. As the correlation coefficient is much more position sensitive, comparing each pixel position of the two histograms, it can only be used in conjunction with the auto-alignment function. It takes approximately 90 seconds to align the one challenge histogram with the ten best matches. Thus although auto-alignment and the correlation coefficient greatly improves the percentage of correct matches it needs to be used in conjunction with the spectral match function.

CHAPTER 5 *MYCOBACTERIUM LEPRAE*

5.1 INTRODUCTION

Mycobacterium leprae has been referred to as the bacteriologist's enigma, as no bacterium showing a close chemical, pathological or immunological similarity to it has ever been cultured *in vitro* (Stewart-Tull, 1982). There have been many attempts and claims of successful culture (Chatterjee, 1983; Chatterjee and Ray 1985; Veeraraghavan, 1983), as well as reports of limited multiplication of the bacilli *in vitro* (Dhople *et al.*, 1988). In order to study *M.leprae*, bacilli have had to be cultured in animal models (Rees, 1988) predominantly in Nude mice (Lancaster *et al.*, 1984, 1986) and armadillos (Kirchheimer and Storrs, 1971). Because of these difficulties relatively little in known about *M.leprae* compared to other, cultivatible micro-organisms. Franzblau (1988) was the first to discover the substrate, palmitic acid, is oxidised by *M.leprae* and that the reaction contributes to ATP formation and synthesis of phenolic glycolipid-1 (PGL-1). Ishaque (1989a) confirmed this result and found direct evidence that *M.leprae* oxidises palmitic acid through the tricarboxylic acid cycle and the electron-transport chain with oxygen as the terminal electron acceptor.

The failure to successfully cultivate the leprosy bacillus *in vitro* has also meant that little has been learnt of the intraspecific variation within the species. Whereas

the tubercle bacillus can be divided into several sub-species, and each sub-species into variants (Collins *et al.*, 1982), some of them with different phage susceptibilities, none of this has been possible with *Mycobacterium leprae*. All that is known of potential variation within the species is that there are strains with slightly different growth characteristics in the mouse, and there are strains differing in their sensitivity to anti-leprosy drugs. The value of sub-speciation and intra sub-speciation of the leprosy bacillus would be a deeper knowledge of the geographical and epidemiological factors in relation to pathogenesis.

Because of the lack of cultivation of *M.leprae* it has been considered separately here from all other bacteria.

5.2 BACKGROUND AND AIMS OF *MYCOBACTERIUM LEPRAE* STUDY

In vitro studies of *M.leprae* cell metabolism have been performed on newly harvested *in vivo* cultured bacilli. Many of these *in vitro* studies have centred around fatty acid oxidation. Wheeler *et al.* (1991) have looked at oxidation of a wide range of fatty acids. Palmitate has been shown to be incorporated by intact *M.leprae* bacilli (Wheeler and Ratledge, 1988) and is also rapidly catabolized to CO_2 (Franzblau, 1988; Wheeler and Ratledge, 1988). Carbon from palmitate also appears in phenolic glycolipid-1 (PGL-1) (Franzblau *et al.*, 1987), the lipid unique to *M.leprae*. Palmitate also stimulates uptake of O_2 by *M.leprae* (Ishaque,

1989a).

Measurements of intracellular ATP have also been used to study cell metabolism (Dhople and Green, 1985, 1986; Lee and Colston, 1985, 1986), as has the uptake of radiolabelled thymidine (Sathish and Nath, 1981). The uptake of radiolabelled compounds by *M.leprae* was first demonstrated by Ambrose *et al.* (1974) with the demonstration of uptake of tritiated 3,4-dihydroxyphenylalanine (^{3}H-DOPA).

Studies by Franzblau (1987) have demonstrated metabolic activities, using 1-[^{14}C]palmitic acid assay, by the *M.leprae* bacilli for up to three weeks after harvesting from an infected animal. Franzblau initially developed the 1-[^{14}C]palmitic acid assay (Buddemeyer system) for *M.leprae* as a rapid *in vitro* method for screening anti-leprosy compounds (Franzblau and Hastings, 1987). Since then the method has been used for testing a wide range of compounds for anti-leprosy activity (Franzblau and Hastings, 1988; Franzblau and White, 1990; Franzblau *et al.*, 1989; Franzblau, 1991) and for metabolic studies (Franzblau and Harris, 1988). The commercial BACTEC 460 system was later adapted to quantitate $^{14}CO_2$ evolved from ^{14}C-labelled substrates in an automated system for *M.leprae* studies (Franzblau, 1989).

A large proportion of the metabolic studies on *M.leprae* has been for the purpose of testing anti-leprosy drugs, such as the inhibition of PGL-1 (Harris *et al.*, 1988) and

measurements of hypoxanthine incorporation (Wheeler and Ratledge, 1988).

Although the metabolic activity, observed in *M.leprae* with current *in vitro* methods, may not include the *de novo* synthesis of protein it may include post translational modification of proteins and other molecules. On this premise experiments were undertaken to adapt the methods described in chapter 3 to studying *M.leprae*. Since these labelling methods do not require replication but only cell metabolism, the methods may be valuable for non-cultivatible organism such as *M.leprae*.

5.3 METHODS AND MATERIALS

(I) Media

(a) Dubos medium

Dubos medium without Tween 80 was required because Tween 80 has been found to have a detrimental effect on *M.leprae* at high concentrations (Franzblau, personnel communication). Bacto-Casitone, normally used in Dubos medium, was not available initially thus two alternatives were also tried, casein acid hydrolysate and casein enzymic hydrolysate. The medium constituents per litre were: Bacto-Asparagine (Difco), 2 g; Monopotassium phosphate (Sigma), 1 g; Disodium phosphate (Anhydrose) (BDH), 2.5 g; Ferric ammonium citrate (BDH), 50 mg; Magnesium sulphate (BDH), 10 mg; Calcium chloride (BDH), 0.5 mg; Zinc sulphate (BDH), 0.1 mg; Copper sulphate (BDH), 0.1 mg. To which was added either

(A) Casein acid hydrolysate (Difco), 0.5 g and Trp 25 mg or (B) Casein enzymic hydrolysate (Sigma), 0.5 g or (C) Bacto-Casitone (Difco), 0.5 g. Albumin (Difco) was added at a final concentration of 10 % v/v. These media were adjusted to a pH of 5.5 and/or 6.5, with 1N HCl, depending on the experiment. The media were referred to as Dubos medium A, B and C according to which casein derivative was added.

The above media were subsequently modified by replacing the sulphate compounds with corresponding amounts of their chloride equivalents.

All media were filter sterilized and stored at 4°C, and excess stocks were frozen at -20°C. The antibiotics Ampicillin (Beecham Reseach Laborotories)(2.5 mcg/ml) and Amphotericin (Beecham Reseach Laboratories)(3 mcg/ml) were added prior to use.

(b) Enriched medium

The modified enriched medium described in chapter 3 was used in the initial labelling experiments with the corresponding [^{35}S] compounds.

(II) Origin and transport of tissue and *M.leprae* suspensions.

(a) Mouse and Armadillo derived tissue and M.leprae suspensions

All mouse and armadillo derived tissue and *M.leprae* suspensions were transported on ice. Material from the USA was sent by air on ice in an insulated transport box and

took a minimum of two days to arrive.

Nude mouse tissue and *M.leprae* suspensions were obtained from either St. George's Hospital, London, or The Gillis W. Long Hansen's Disease centre, Carville, Louisianna, USA.

Armadillo tissue or *M.leprae* suspensions were obtained from the Medical Research centre (MRC), Millhill, London.

(b) Human tissue

Biopsy material was sent by post from India and Nepal and took from 6 to 15 days to arrive at the Middlesex hospital by post. Because of the limited ice supply in these countries alternative transport methods were tried such as the use of different transport media. Two media were tried:

(i) 1 ml of cetyl pyridinium chloride (BDH) at 1% was aliquoted into 2 ml screw cap tubes which were packaged in transport holders and padded envelopes and sent to contacts in India and Nepal.

(ii) A 2.5 ml aliquot of 2% warm agar (Sigma) was pipetted into 5 ml screw cap bottles and were left to set (the agar was to be warmed gently to melt it when the biopsy was to be put into it for transport). The bottles were packaged as above and sent out to contacts in India and Nepal.

The aliquoted medium and packaging was sent out to Dr N P Shanker Narayan, V.H.S. Leprosy Project, South India and Dr

M.R.Rajan of the Sacred Heart Leprosy centre, South India.

(III) Preparation of a crude suspension of *M.leprae*

(a) Tissue homogenisation

Homogenisation was used to disrupt tissue cells and release intracellular bacilli.

Dissection and homogenisation of tissue was carried out aseptically. The mouse foot was swabbed with 70% alcohol before removing the foot pad with a sterile blade. Homogenisation was either by hand using a glass grinder or by electrical grinder with a tissue disintergration head (Silverson Machines Ltd.).

(i) Glass grinder method

The mouse foot pad was placed in 5ml of Dubos medium, minced with sterile scissors and poured into a glass grinder (Wheaton tissue grinder). The tissue was ground by raising and lowering the grinder shaft inside the holder. The shear forces generated between the two surfaces caused the tissue to be disintegrated.

(ii) Electric grinder method

The tissue disintegration grinder head consisted of a drive shaft with a blade on the end which was inside a cylindrical shield, the shield had four 1 cm slits on the blade end that allowed tissue shreds to be sucked in and disintegrated by the blade inside (Silverson Machines

Ltd.). A glass universal could then be screwed in place with the blade shaft inside. The drive shaft was sealed so that the whole disintegration head could be autoclaved and the blade inside the universal remained sterile until used.

The tissue was put into a sterile glass universal containing 3-4 mls of Dubos Albumin medium. The sterile grinder head was screwed onto the universal to seal the tissue. Glycerol (BDH) (1%) was poured into the top of the grinder unit to lubricate and cool the grinder drive shaft. The grinder was attached to the electric mixing unit and the tissue was ground

Mouse footpad tissue was ground for 15 seconds at a time until disintegrated.

The human and armadillo tissue was always minced with sterile scissors before being put into the universal for homogenisation. The armadillo and human skin was much tougher than the mouse tissue so that it had to be ground for a much longer time. The universal was cooled with ice. armadillo liver was easily homogenized but was generally more bulky than the other tissues so had to be centrifuged in sections, the homogenates were then pooled.

(b) Double centrifugation of homogenate

The homogenate was centrifuged at 100 x g (Digifuge GL) for 5 minutes to pellet tissue lumps. The supernatant was

removed and centrifuged at 10 000 x g (Beckman) to pellet bacilli. The supernatant from the second centrifugation was removed and discarded. The pellet was resuspended in Dubos medium giving a crude *M.leprae* preparation, 1-2 mls of Dubos medium was used for mouse footpad or 1-5 mls for more bulky human and armadillo pellets. The crude suspensions were transferred to screw cap tubes and stored at 4°C.

(IV) Acid fast bacilli (AFB) estimation and staining

(a) Slide preparation and Acid fast staining

To determine the acid fast bacilli (AFB) count per millilitre of an *M.leprae* preparation, appropriate dilutions were made: 1 in 10, 1 in 100 or 1 in 1000. An aliquot of 10 mcl of several dilutions was spread over a circle of 8 mm in diameter on specially prepared glass slides (C.A.Hendley (Essex) Ltd). The slides were allowed to air dry before being flamed and left in formalin vapour for 15 minutes. The slides were then covered with Carbol Fuchsin (BDH) 10 % and heated until the stain steamed. The slide was left for 10 minutes before being rinsed gently with tap water. Sulphuric acid (BDH) (12.5%) was than applied to the slide for 5 minutes, the slide was rinsed again gently. Methylene blue (Hopkin and williams Ltd) (0.2%) was then added to the slide for 30 seconds and the slide was rinsed for the final time. The slide was dried by blotting it gently on tissue paper.

(b) AFB estimation

The slides were examined first with x40 lens and then under oil emersion with a x100 lens. The number of AFB per 10 ul smear was determined by multiplying the mean number of AFB per field of view by the number of fields of view per smear, from which the number of AFB/ml could be calculated. The area of a field of view was calculated by first measuring the radius of a field of view using a heamocytometer. The radius of bacterial smear was 4 mm and so the area over which bacteria were spread could be calculated. The final calculation is as follows:

$$\text{AFB/ml} = \frac{\text{II} \times \text{radius of smear}^2 \times \text{mean AFB per field of view}}{\text{II} \times \text{radius of field of view}^2 \times \text{mean volume of smear}}$$

A minimum of 20 fields of view were counted on two smears.

(c) Fluorescein Diacetate and Ethidium Bromide stain.

Stock solutions of ethidium bromide (EB) and fluorescein diacetate (FDA) were prepared and stored at -20°C: EB (Sigma), 20 mg in 10 mls of 0.1 M phosphate buffer, pH 7.2 (2 mg/ml); FDA (Sigma), 10 mg in 20 mls acetone (BDH), (500 mc/ml). Prior to staining a working solution was prepared from the stock solutions: 20 mcl of FDA stock solution plus 5 ml of phosphate buffer (0.1 M, pH 7.2) plus 10 mcl of the stock EB solution. The working solution was stable for one day at room temperature if protected from light.

A bacterial smear was prepared on a glass slide which was then air dried. An aliquot of 20 mcl of the working solution was pipetted on to the smear. The smear was then covered with a cover slip and the edges of the cover slip were sealed with nail varnish. The slide was then incubated for 10 minutes in the dark. The bacilli were then observed under incident UV light with either x40 or x100 magnification. The cells that appear uniformly stained green were considered to be alive and 'viable' and the red or dual stained cells were considered dead (Jarnagin and Luchsinger, 1980) (Fig.5.1).

(V) Decontamination of *M.leprae* suspensions

(a) NaOH treatment

M.leprae suspensions were treated with an equal volume of 1N NaOH for a minimum of 15 minutes. To neutralize the suspensions an equal volume of 1N HCl was added and the suspension was diluted with phosphate buffer. The suspension was then centrifuged at 10 000 x g (Beckman) to pellet the bacilli. The supernatant was filtered and kept to check the pH. The pellet was resuspended in an appropriate medium.

(b) Treatment with antibiotics

After resuspending the *M.leprae* in appropriate medium containing the antibiotics ampicillin at 2.5 mcg/ml and amphotericin at 3 mcg/ml the suspensions were incubated at

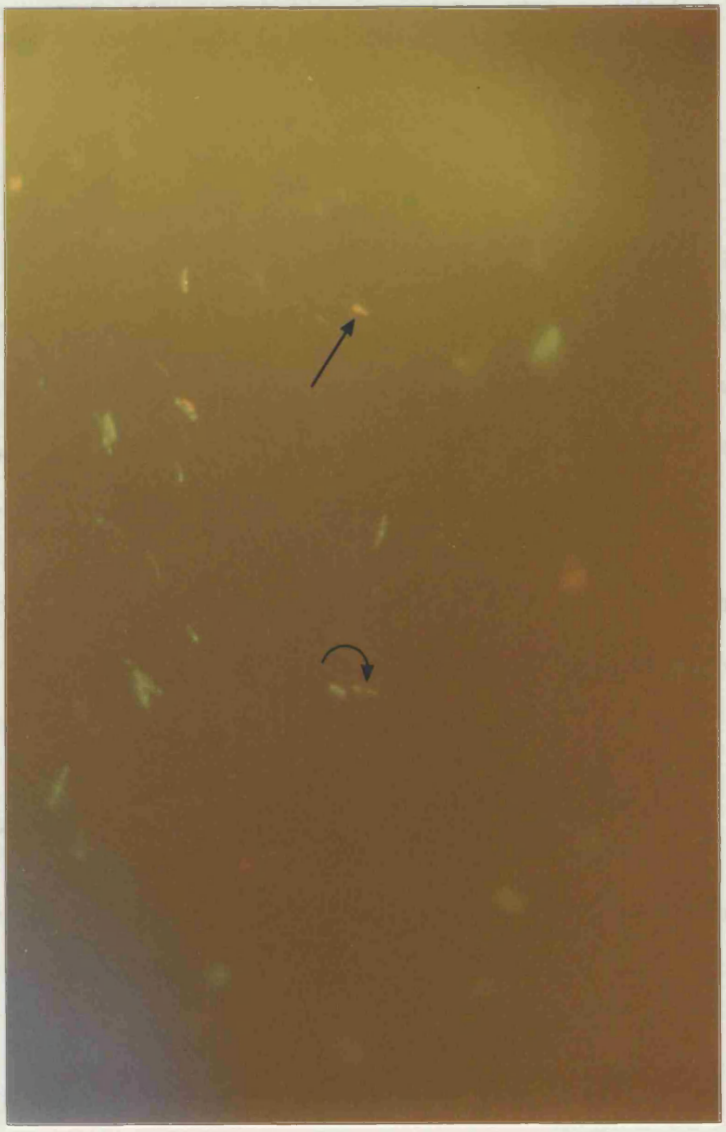


Figure 5.1 Fluorescein diacetate/ethidium bromide viability stain of *M.leprae*. Green stained cells ( ) are considered viable, red or dual stained cells ( ) are considered dead.

37°C for 30 minutes. This allowed any contaminating bacteria to actively metabolize and thus enable the antibiotics to take effect.

(VI) Testing for contaminants.

An aliquot of the crude *M.leprae* suspensions, 10 to 50 mcl, was inoculated on to BA plates and subarose slopes. The aliquot was spread on the plate or slope using a sterile loop. The plates and slopes were incubated at 33°C and checked for contaminants at 24 hours and 1 week.

M.leprae suspensions were tested for contaminants both before and after decontamination and at the end of each incubation with [^{35}S] precursors.

(VII) ^{35}S Labelling

Differentially centrifuged *M.leprae* suspensions were inoculated into Dubos medium A, B or C at pH 6.5 or 5.5 with [^{35}S] precursors at 0.37 MBq per sample. The concentration of bacilli ranged from 2 x 10^6 to 1 x 10^8 AFB per sample depending on the AFB counts and the number of experiments. The samples were incubated for 1 week at 33°C. After incubation a 1 mcl loopful of each sample was tested for contamination by streaking on BA plates and subarose slopes which were then incubated at 33°C and checked after 24 hours and 1 week. An equal volume of sample buffer was then added to each sample and they were boiled in a water bath for 2 minutes. The samples were then stored at -20°C

until electrophoresis on either the AMBIS 12.5% polyacrylamide gels or the 8-18% polyacrylamide gradient gels, see chapter 3 for method details.

(VIII) Percol purification

The differentially centrifuged suspensions were sonicated for one minute in a water bath. 100 mcl each of DNAse (Sigma) 25 mg/10 ml (sterile filtered) and collagenase (Sigma) 20 mg/2 ml (sterile filtered) were added per 10 ml of suspension and the suspension incubated for 1 hour with intermittent agitation. The suspension was then sonicated for a further 1 minute in a water bath before being centrifuged for 5 minutes at 77 x g (Digifuge GL). The supernatant was removed and 0.5 to 1 ml added carefully on top of each Percol gradient. The gradient was made up of 2 mls of Percol (Sigma) at the bottom of a 15 ml tube with 8 mls of of 50/50 Percol/ RPMI medium (Imperial) pipetted carefully on top so as not to mix the two layers. The loaded Percol gradient was centrifuged at 2700 x g (Beckman) for 90 minutes.

The band of bacilli was removed carefully in 2 mls using a Pasteur pipette and put into a 50 ml polypropylene tube. The extracted suspension was then diluted with 45 mls of Dubos Medium and vortexed thoroughly to wash off the Percol. The suspension was then spun at 9200 x g (Beckman) at 10°C for 45 minutes to pellet the bacilli. The

supernatant was discarded and the pellet resuspended in 10 mls of Dubos medium to further wash the cells. The suspension was centrifuged finally at 9200 x g (Beckman) at 10°C for 15 minutes. The pellet was resuspended in 1-5 mls of Dubos medium, 10 mcl was aliquoted on to BA plates and LJ slopes to check for sterility.

(IX) The Buddemeyer system.

Scintillation vials were prepared by dipping 2 x 4 cm strips of Whatman no. 42 filter paper in scintillation fluid (Ecoscint A) and placing them in scintillation vials. 100 mcl of 2 N NaOH was then pipetted on to each strip.

Dubose medium with 37 kBq of 1-[^{14}C]palmitic acid (NEN Dupont) per ml of medium was prepared. An aliquot of bacilli was added to the medium to give between 10^6 and 10^8 bacilli per ml of medium and 1 ml aliquots were pipetted into medium sized glass vials. Each experiment was repeated 2-4 times depending on the available bacilli and the mean scintillation count calculated.

Controls were prepared by aliquoting an equivalent concentration of heat killed bacilli into the appropriate medium and 1ml of this suspension was pipetted into medium sized glass vials. The *M.leprae* had been heat killed by boiling an aliquot in a water bath for 10 minutes.

The lids of the vials were left lose, to allow $^{14}CO_2$ to escape, and each vial was put inside a scintillation vial.

The scintillation vial lid was tightened and the vial incubated at 33°C for 1 to 4 weeks depending on the experiment. The counts per minute were read in the scintillation counter every 2 to 4 days.

5.4 RESULTS

(I) [^{35}S] labelling

An initial pilot study using nude mouse derived *M.leprae* (sample 1: Table 5.1) was carried out in order to test the application of the [^{35}S] labelling method on the *M.leprae* bacilli. Crude double centrifuged *M.leprae* bacilli (sample 1, Table 5.1) were resuspended at 2 x 10^6 bacilli per 50 ul aliquots of Dubos medium A and B and the three [^{35}S] labelling media developed for [^{35}S] labelling with 0.37 MBq per sample of the corresponding [^{35}S] compound. Dubos medium A, containing the casein enzyme hydrolysate, was used for labelling with [^{35}S] methionine, [^{35}S] thio ATP and [^{35}S] inorganic sulphate in triplicate. The *M.leprae* in the Dubos medium B, with the casein acid hydrolysate was only aliquoted for labelling with [^{35}S] thio ATP and [^{35}S] inorganic sulphate in triplicate. The [^{35}S] methionine was not used in this instance as it was presumed that there would be an excess of unlabelled methionine in this medium from the acid hydrolysed Casein. The enriched media described in chapter 3 were used with their corresponding [^{35}S] labelling compounds. Each [^{35}S] labelling experiment was performed in triplicate and incubated at 33°C for 0, 7 and 14 days

Sample no.	Source	Supplied by	AFB per incubation	Incubation medium
1.	NMF	GH	$2x10^6$	Dubos A&B Enriched medium
2.	NMF	GH	$2.4x10^5$	Dubos B pH 6.5
3.	NorMF	MRC	$2x10^6$	Dubos B pH 6.5
4.	NML	GH	-	-
5.	AS frozen	MRC	$6.5x10^6$	Dubos B pH 6.5
6.	ALN	MRC	$2x10^7$	Dubos A,B & C pH 6.5 & 5.5
7.	HT	TDH	$5.6x10^7$	Dubos B pH 6.5
8.	NMF	WGLH	$1x10^7$	Dubos A & B pH 6.5
9.	NMF	WGLH	$1x10^7$	Dubos A & B pH 6.5
14.	HT	I	$1x10^6$	Dubos B pH 6.5
15.	HT	I	$1x10^6$	Dubos B pH 6.5
16.	AS	MRC	$1x10^8$	Dubos B pH 5.5

Codes for the sources of *M.leprae* : NMF= Nude mouse footpad; NorMF= Normal mouse footpad; AS= Armadillo skin; ALN= Armadillo lymph nodes; HT= Human tissue.

Codes for suppliers of *M.leprae* : GH= St. George's Hospital; MRC= Medical Research Council; HTD= The Hospital of Tropical Diseases, King's Cross; WGLH= Gillis W. Long Hansen's Disease Centre, Louisiana, USA. I= V.H.S Leprosy Project, South India.

Table 5.1. List of *M.leprae* samples used in [^{35}S] labelling experiments.

prior to addition of the appropriate [^{35}S] label precursor. The bacilli were then incubated for 7 days at 33 °C. After incubation all samples were treated and stored as previously described in chapter 3. Blank controls were also incubated with their corresponding [^{35}S] precursors.

The *M.leprae* incubated in the enriched medium gave no patterns with any of the labelling compounds. Patterns were obtained with both Dubos media A and B. However controls of the Dubos medium without *M.leprae* showed the patterns with the ATP and with less intensity with methionine. Only the sulphate controls were blank. These patterns were the same as those containing *M.leprae* and could be attributed to the ATP and methionine binding non specifically to the albumin in the culture medium. Thus the only bands that could be attributed to *M.leprae* were those in the [^{35}S] inorganic sulphate labelling experiments with the Dubos medium (Fig. 5.2). The patterns were identical with Dubos medium A and B.

From the initial experiment it was concluded [^{35}S] inorganic sulphate was the only appropriate labelling compound and Dubos medium B was selected for subsequent incubations. Further incubations with mouse, armadillo and human derived *M.leprae* (samples 2 to 12, see Table 5.1) were carried out when samples became available. None of the subsequent samples yielded labelled patterns even when the number of AFB per incubation was increased.

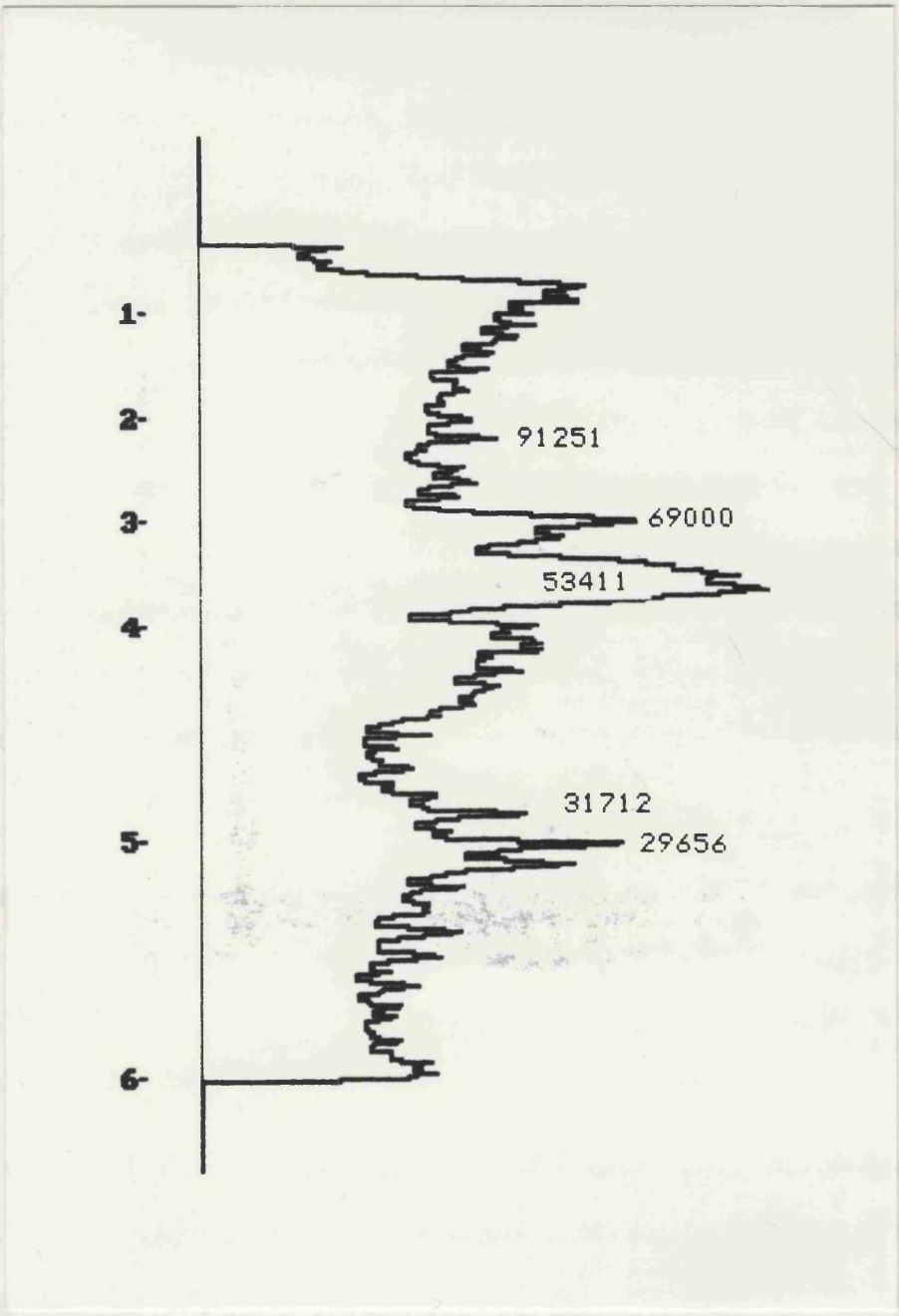


Figure 5.2 Histogram of the [^{35}S] inorganic sulphate labelled protein profile of *M.leprae*. The molecular weight in Daltons has been marked on five of the main peaks; the molecular weight standard positions are numbered: 1, 200 kDa; 2, 97.4 kDa; 3, 69 kDa; 4, 46 kDa; 5, 30 kDa; 6, 18.4 kDa.

Numerous problems were encountered. When the nude mouse liver homogenate (sample 4, Table 5.1) was neutralized with acid a precipitate formed. The sample was centrifuged to pellet the precipitate; the pellet was too large to resuspend in a small enough volume to give > 2 x 10^6 AFB per 50 mcl. Thus the pellet was resuspended in 10 mls of Dubos medium C, pH 6.5, and centrifuged at 100 x g (MSE Centaur) for 3 minutes to pellet the precipitate and leave the bacilli in suspension. However when slides were prepared from the precipitate and supernatant most of the *M.leprae* were still observed in the precipitate, they appeared to be embedded in the precipitate. Thus this sample could not be used for labelling experiments.

M.leprae that have been preserved at -70°C are known to have low viability so one might expect not to obtain labelled bands from the frozen armadillo derived bacilli of sample 5 (Table 5.1).

The trial transport medium that were sent to India were designed to inhibit the growth of contaminants and delay the decomposition of the tissue. On arrival in England the biopsies were homogenized and decontaminated, all the tissue samples were found to be heavily contaminated on arrival and the decontamination treatment was not always successful. The AFB counts were always low which meant that it was not always possible to inoculate enough bacilli into the [^{35}S] medium. The agar that some tissues were embedded in

may have effected the oxygen supply unfavourably. The packages containing the biopsies were found to take a minimum of 9 days to reach England, all these factors would effect the *M.leprae* bacilli viability, and cause cell death.

(II) [^{14}C] palmitic acid study

Subsequent to the problems observed in the [^{35}S] labelling of *M.leprae* proteins an alternative metabolic assay was sought to asses the bacilli metabolism *in vitro*. The 1-[^{14}C]palmitic acid assay described by Franzblau was used with some modifications. The preparation the scintillation tubes was speeded up by dipping the Whatman paper into scintillation fluid and pipetting the NaOH directly onto the paper strips instead of preparing large dried sheets of paper soaked in scintilliation fluid and NaOH and then cutting the paper up into strips.

Crude and Percol purified bacilli suspended in a variety of media at different pH (Table 5.2) values were incubated in the Buddemeyer system (Fig. 3.3). Bacilli treated for different lengths of time with NaOH were also processed (Fig. 5.4) in order to try and assess if the NaOH treatment, routinely used for culture decontamination, had a detrimental effect on the *M.leprae* bacilli.

M.leprae bacilli derived form the human biopsy samples from India were found to have no activity with the 1-[^{14}C] palmitic acid assay and no labelled bands were obtained. However low bacilli counts were obtained with all the human

Sample no.	Source	Supplied by	AFB per incubation	Incubation medium
8.	NMF	GH	$1x10^7$	Dubos pH 5.5 & 6.5
9.	NMF	GH	$1x10^7$ $2x10^6$	Dubos pH 6.5
10.	HT	I	$1.7x10^6$	Dubos pH 6.5
11.	HT	I	$4x10^6$	Dubos pH 6.5
12	HT	I	$2.9x10^6$	Dubos pH 6.5
13.	NMF&NME	GH	$>1.5x10^6$	Dubos pH 5.5
14.	HT	I	$1x10^6$	Dubos pH 6.5
15.	HT	I	$1x10^6$	Dubos pH 6.5
16.	AS	MRC	$1x10^8$	Dubos pH 5.5

Codes for sources of *M.leprae* : NMF= fresh Nude mouse footpad; NorMF= Fresh normal mouse footpad; NME= Nude mouse ear; AS= Armadillo skin; ALN= Armadillo lymph nodes; HT= Human tissue.

Codes for suppliers of *M.leprae* : GH= St. George's Hospital; MRC= Medical Research council; HTD= The Hospital of Tropical diseases; WGLH= Gillis W. Long Hansen's Disease Centre, USA; I= V.H.S Leprosy centre, South India.

Table 5.2. List of the *M.leprae* samples used in the ^{14}C palmitic acid assay.

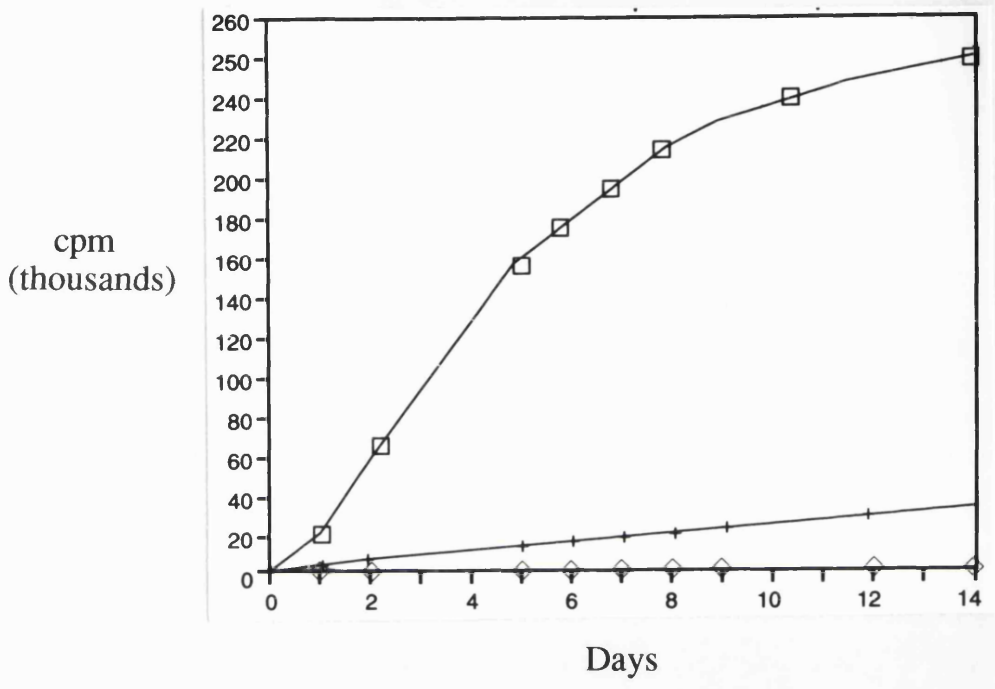


Figure 5.3 Plots of the scintillation counts (cpm) of evolved CO_2 against time (days) from the 1-[^{14}C]palmitic acid assay of crude and Percol purified preparations of *M.leprae*. □ , Percol purified *M.leprae*; +, crude *M.leprae* preparation; ◇ heat killed *M.leprae*.

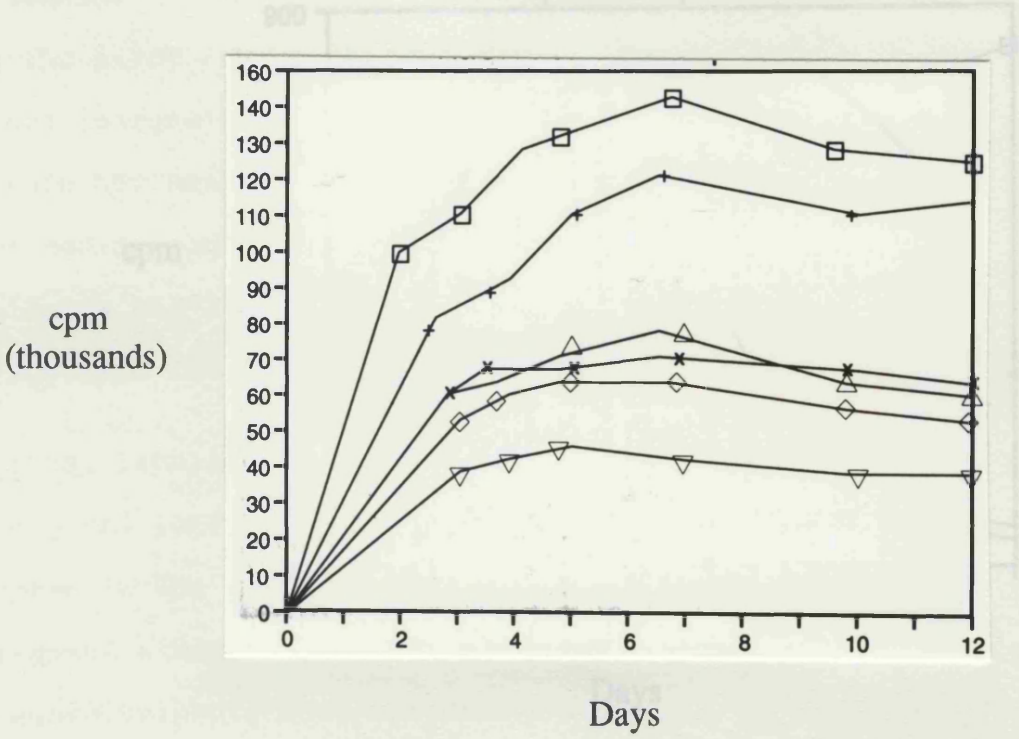


Figure 5.4 Plots of the scintillation counts (cpm) of evolved CO_2 against time (days) from the 1-[^{14}C]palmitic acid assay of NaOH treated *M.leprae* . The *M.leprae* were treated for 10 (□), 12 (+) , 17 (◇), 20 (△), 23 (x) and 25 (▽) minutes.

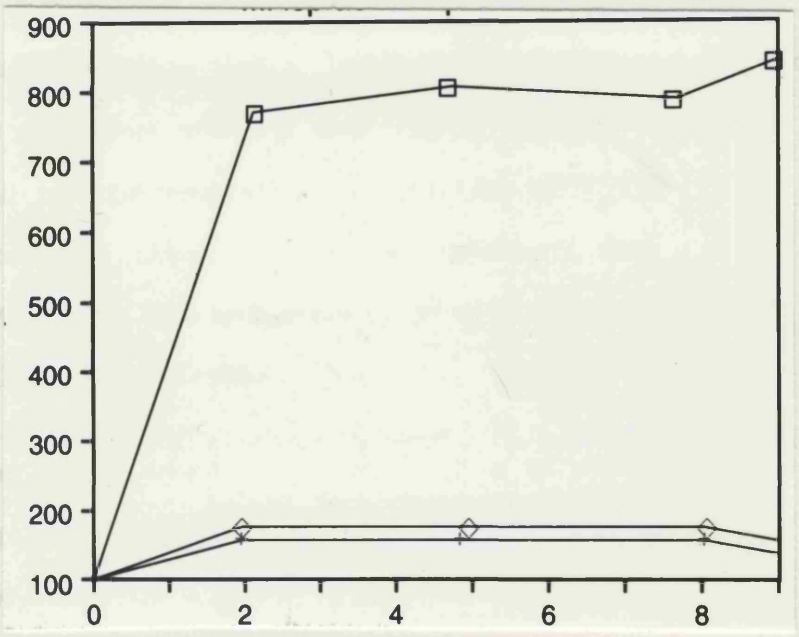


Figure 5.5 Plots of the scintillation counts (cpm) of evolved CO_2 against time (days) from the 1-[^{14}C]palmitic acid assay of human derived *M.leprae* : □, supernatant from homogenised human tissue; +, *M.leprae* extracted from the human tissue; ◇, heat killed *M.leprae*.

samples which may have contributed to the lack of observed activity. A low level of $^{14}CO_2$ was detected in the filtered supernatant of the *M.leprae* homogenate (Fig. 5.5).

5.5 SUMMARY

The pilot study testing the use of [^{35}S] methionine, thio ATP and inorganic sulphate showed that the [^{35}S] methionine and thio ATP could not be used in conjunction with the Dubos medium containing albumin. This was because the methionine and ATP bound non specifically with the albumin and obscured the separating zone.

[^{35}S] labelled bands were observed in the pilot study using [^{35}S] inorganic sulphate and were attributed to *M.leprae* in the absence of any detected contaminant. Subsequent attempts to label *M.leprae* proteins with [^{35}S] inorganic sulphate were unsuccessful.

The Buddemeyer system 1-[^{14}C]palmitic acid assay was used as an alternative method to analyse *M.leprae* bacilli metabolic activity. An increase in activity was observed in Percol purified preparation in comparison with crude preparations using the Buddemeyer system. A decrease in activity was observed between preparations treated for 10 to 25 minutes with NaOH. A reduced level of activity was also observed with media at pH 5.5 in comparison with bacilli in media at pH 6.5.

CHAPTER 6 AN ALTERNATIVE APPROACH TO MYCOBACTERIAL IDENTIFICATION

6.1 INTRODUCTION

There are many biochemical approaches to mycobacterial identification which have not been fully developed, and would utilize the fact that mycobacteria produce a range of unique compounds. However many of these compounds are difficult to isolate and identify, or are unique to specific species and thus would not be appropriate for a general method. One potential range of compounds found in mycobacteria are the cell-associated iron-binding siderophores, termed mycobactins.

Mycobactins have been found to be species specific, (Snow and White, 1969; Hall and Ratledge, 1984). Most species produce up to four molecular variants of mycobactin which differ in the position and length of the five alkyl chain residues (Barclay *et al.*, 1985). They can be differentiated, after extraction with alcohol, by separation using thin layer chromatography (TLC) (Snow and White, 1969; White and Snow, 1968, 1969).

The use of mycobactins for chemotaxonomic identification of mycobacteria has been described by a number of workers (Hall and Ratledge, 1984; Barclay and Ratledge, 1983, 1988). However the methods they described require too many organisms to be of use clinically. The aim

of this study therefore, was to improve the sensitivity of mycobactin detection and investigate its use in clinical situations.

6.2 THE ROLE OF IRON AND IRON BINDING COMPOUNDS

The acquisition of iron is important for nearly all living cells as it is used for many of their biochemical and physiological functions. Ferric iron (Fe^{3+}) is insoluble at physiological pH, existing as the insoluble polymer ($Fe(OH_3)_n$) (Macham *et al.*, 1975). Thus organisms have evolved iron binding proteins that keep iron in a soluble and complex form. These iron binding proteins are not usually saturated with iron so that they scavenge and bind any available free iron leaving little or no iron available for invading organisms. Host animals can further limit iron to microorganisms by actively decreasing the amount of iron absorbed by the intestine and increasing the amount of iron stored as ferritin in the liver (Barclay, 1985).

Bacteria produce their own iron binding compounds in order to scavenge iron from their environment. These compounds, termed siderochromes or siderophores, sequester iron from host iron binding compounds or solubilize ferric iron polymers. They remove iron from the host proteins by having a higher affinity for iron than the host molecules. The ability of mycobacteria to obtain iron may be an important factor in determining their pathogenicity

Table 6.1 Iron complex molecules in mammal.

transport proteins:	transferrin
storage molecules:	ferritin
iron binding proteins:	myoglobin
	haemoglobin
	lactoferrin
iron is important in:	cytochromes
	non-haem iron carriers of the electron transport system

Table 6.2 Effects of iron deficiency on mycobacteria.

DNA content falls	Ratledge, 1987
increase in the activity of DNA-repair enzymes	Winder and Barber, 1973
Iron proteins decrease in activity	Winder and O'Hara, 1964 Ratledge, 1984
Mycobactin and exochelin synthesis is up regulated	Sritharan and Ratledge, 1989

(Sritharan and Ratledge, 1990) as it is in other bacterial pathogens (Griffith, 1983).

Because iron is associated with higher molecular weight compounds most organisms secrete their main siderophore in order to scavenge it from their host. The siderophors that mycobacteria secrete are called exochelins (Ratledge, 1982; Macham and Ratledge, 1975) as well as salicylic acid (Ratledge, 1987). Two types of exochelin are synthesised by mycobacteria, but only one is produced by any particular species. One type is water soluble and the other chloroform soluble. As well as producing two types of secreted siderophore, mycobacteria also produce a cell-associated siderophore which is mycobactin.

It is not clear why mycobacteria produce two distinct types of siderophore and why one is cell-associated. It has been proposed that mycobactin is important for transporting iron from exochelin to the cell interior (Ratledge, 1984). However, exochelin can transport iron across the mycobacterial cell membrane (Stephenson and Ratledge, 1980; Macham and Ratledge, 1975) as well as donate iron to mycobactin (Ratledge, 1984). It is also possible that mycobactin functions as an iron storage molecule during 'feast' times so that iron is available during 'famine' times (Ratledge, 1987). However as mycobactin is only produced in response to iron deficient conditions it is difficult to envisage how any mycobactin is produced to

store extra iron in the first place.

The only other species to produce mycobactin is *Nocardia*, which is related to mycobacteria. *Nocardia* also has a thick lipoidal cell wall and produces a cell-associated mycobactin and an extracellular siderophore (Patel and Ratledge, 1973; Ratledge and Snow, 1974).

6.3 MYCOBACTIN

Mycobactins have various substitutions at position R_1 to R_5 depending on the species (Fig 6.1) (Snow, 1970; Ratledge, 1984; Barclay *et al.*, 1985). The various long alkyl chains particularly at position R_1 do not contribute to the affinity of the molecule for iron, but make it highly lipophilic. The lipophilic tail on the iron binding core permits mycobactin to sit in the lipid cell wall of mycobacteria and thus fulfil its role as an iron ionophore. Differentiation of the chemical types of mycobactins is easily achieved by thin-layer chromatography (TLC) or by high performance liquid chromatography (HPLC) (Ratledge and Ewing, 1978; Hall and Ratledge, 1984; Barclay and Ratledge, 1983, 1988).

Mycobactins are produced in response to deprivation of iron (Ratledge and Marshall, 1972). However not all mycobacterial species, eg *M.leprae* (Ratledge, 1987), have been found to produce mycobactin. Even in those species that are known to produce mycobactin some strains, for instance

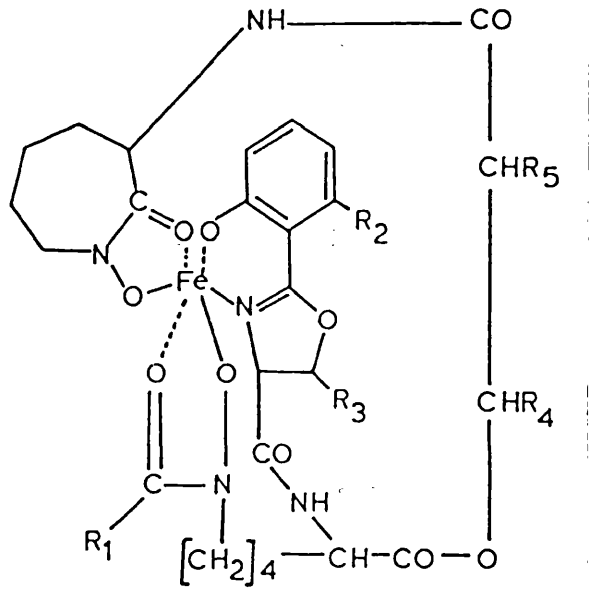


Figure 6.1 Structure of mycobactin, the lipid-soluble, intracellular siderophore of mycobacteria. R_1 - R_5 are various substituents which vary according to the species: R_1 is usually an unsaturated alkyl chain (C_{12} - C_{19}) and R_2 and R_3 are either -H or $-CH_3$. R_4 is normally $-CH_3$ or - C_2H_5 but may be a longer alkyl chain in *M.avium*. R_4 may also be a longer alkyl chain (C_{15} - C_{19}) with *M.marinum* and *Nocardia asteroides* (C_9 - C_{13}) but in these cases R_1 becomes a $-CH_3$ group. R_5 is either -H or $-CH_3$. (Ratledge, 1977).

M.avium do not, and are mycobactin dependant. After successive culture a proportion of these mycobactin-dependent strains will revert to producing mycobactin. This may mean mycobactin production was repressed in these strains (Barclay and Ratledge, 1983) or alternatively affected by moveable genetic elements.

6.4 METHODS AND MATERIALS

(I) Bacterial strains

The following mycobacterial strains were used: *Mycobacterium avium* NCTC 8559, *M.intracellulare* NCTC 10425, *M.intracellulare* M12 (Barclay and Ratledge, 1983), *M.tuberculosis* H37Rv NCTC 7416, *M.bovis* NCTC 10772, *M.bovis* var BCG NCTC 5692, *M.scrofulaceum* NCTC 10803, *M.smegmatis* NCIB 8548, *M.vaccae* NCTC 10916, *M.fortuitum* NCTC 10394, *M.fortuitum* NCTC 10395, *M.thermoresistible* NCTC 10409 and *M.neoaurum* NCTC 10818.

(II) Culture media and growth conditions.

All cultures were stored at room temperature after growth on Lowenstein-Jensen medium at 37°C. To enhance mycobactin synthesis the organisms were grown on low iron defined medium at 37°C prior to extraction. The medium was prepared as follows (/L): glucose (BDH), 10g; glycerol (BDH), 5ml; asparagine (Sigma), 5g; 1.66 mM magnesium sulphate (BDH); 1.8 mcM manganese sulphate (BDH); 7 mcM zinc

sulphate (BDH); 0.18 mcM ferric sulphate (BDH) and purified agar (Sigma), 1.5 g. After autoclaving, solutions were kept warm in a water bath at 60°C and 8ml aliquots were dispensed into sterile bottles. The bottles were left at an angle to give a slope.

The high iron medium was prepared with a final concentration of 18 mcM $FeSO_4$. Where indicated 0.1 mM desferrioxamine mesylate (Sigma), 1 mM 2,2'dipyridyl (Sigma) or B-cyclodextrin 0.5% (w/v) were added to the low iron medium before it cooled.

(III) Extraction and labelling of mycobactin

Mycobacterial colonies were scraped off slopes with a sterile 1 ul disposable loop and inoculated into 0.5 ml of methanol (BDH) or ethanol (BDH) in a screw cap Eppendorf tube. The loop stem was cut, leaving the loop in the alcohol which was then mixed for 15 minutes on a rotator. Cells were centrifuged for 5 minutes at 10 000 x g (Beckman). The methanol was transferred to a fresh Eppendorf tube. The samples were labelled with $^{55}FeCl_3$ (code IES 3, Amersham International) 7.4 kBq in 20 mcl of sterile 20 mcM HCl. After 5 minutes the mycobactin was extracted in 1 ml of chloroform (BDH) by gently mixing for 3 minutes. Distilled water (100 mcl) was added and the tubes centrifuged for 10 s at 10 000 x g (MSE Micro Centaur). The aqueous layer was discarded and the chloroform was removed by heating at 80°C.

(IV) Thin layer chromatography (TLC)

The final chloroform extract (20-50 mcl) was spotted on to the chromatography plates using glass capillary tubes. Initially 20 x 20 cm silica gel TLC plates without a concentration zone (Merck, Germany) were used. They were latter replaced by 10 x 10 cm silica gel 60 high performance thin layer chromatography (HPTLC) plates (Merck, Germany) with a 10 x 2 cm concentration zone on to which the samples were applied. The HPTLC concentrating zone acts like the stacking gel in PAGE and concentrates the samples before separation.

Once prepared the TLC plates were put in glass chromatography jars, with petroleum spirit (BDH): butanol (BDH): ethyl acetate (BDH) 2:3:3 v:v:v to a depth of 0.5 - 1cm, such that the solvent did not cover the sample application site on the plate. The plate was left until the solvent had migrated almost to the top of the plate, then the plate was removed, the solvent front was marked with $^{55}FeCl_3$ or a ^{14}C marker and the plate was left to dry.

(V) Extracting mycobactin from human specimens and preparation for TLC.

(a) Processing of urine

The urine samples (25-50 mls) were treated with half the volume of chloroform (BDH) in 100 ml glass bottles. The samples were mixed overnight on a roller to extract

mycobactin into the chloroform layer. A chloroform/water emulsion was formed due to the constant mixing of the two layers, which were re-separated by centrifugation of the emulsion. The chloroform was removed with a glass Pasteur pipette and transferred to a new glass bottle or polypropylene tube. The chloroform was evaporated off in a water bath and the residue extracted and analysed as previously described.

(b) Processing of sputum

Sputum samples were treated with an equal volume of sputosol (Oxoid) for 20 minutes and then sputa were centrifuged at 10 000 g for 30 minutes. The supernatants were removed and the residues were resuspended in 0.5 mls of 100% ethanol (BDH). The samples were incubated overnight at room temperature. The residues in ethanol were transferred to 1.5 ml screw cap tubes and 1.48 kBq $^{55}FeCl_3$ was added. The samples were left for a further 2-3 hours to extract mycobactin. The sputa were then centrifuged at 10 000 g for 5 minutes. The supernatants were removed and transferred to new tubes. The ethanol was evaporated off in a boiling water bath. The samples were re-extracted in ethanol (150 mcl), chloroform (150 mcl) and finally methanol (BDH) (20-50 mcl). In between each extraction the samples were transferred to fresh tubes and boiled dry in a boiling water bath. The HPTLC was as described above.

(VI) Reading TLC plates and data analysis

The HPTLC and TLC plates were fixed to card mounts and covered with 1.5 micron Mylar. The plates were scanned with the AMBIS radioanalytical imager MK2 (Smith, 1985; Smith and Furst, 1989) for from 15 minutes to 24 hours depending on the radioactivity on the plate.

Lanes were extracted as previously described in chapter 3 to give histograms and where necessary spots were quantitated using the AMBIS software, to give counts per minute (cpm). Cpm were recorded by indexing the spots to be quantitated using an appropriate shape (rectangle or oval).

6.5 RESULTS

(I) Detection of fast-growing mycobacteria

Three fast growing mycobacteria, *M.smegmatis*, *M.fortuitum* NCTC 10394 and *M.neoaurum*, were used to test the possibility of radiolabelling mycobactin for mycobacterial identification. They were grown on the defined medium slopes at 37°C for 5 days. Sets of one, two, five and ten colonies were picked of the slopes. The mycobactin was extracted with ethanol, labelled with $^{55}FeCl_3$ 0.74 kBq and separated on TLC plates without concentration zones. The plates were scanned.

The method was only just sensitive enough to detect one colony of *M.neoaurum*, but could easily detect single colonies of the other mycobacteria (Fig 6.2), the colonies of which were larger than that of *M.neoaurum*. The overall

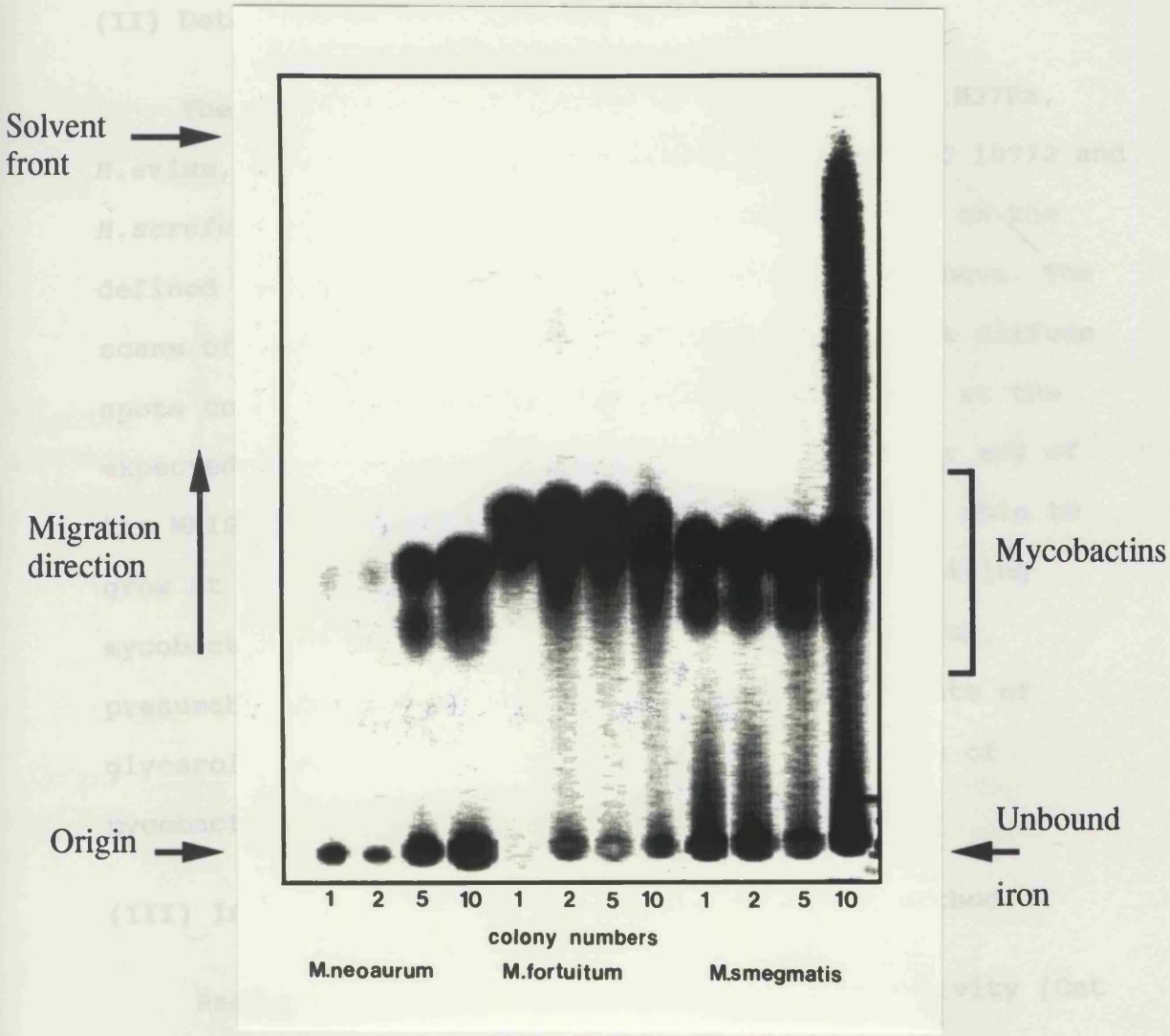


Figure 6.2 Scan of radio-TLC plate of fast-growing mycobacteria: 1, 2, 5 and 10 colonies of *M.neoaurum, M.fortuitum* and *M.smegmatis* grown on iron-limiting medium for 5 days at 37 °C. Mycobactin extracts were labelled with ^{55}Fe and separated by TLC on silica gel 60 with petroleum spirit:butanol:ethyl acetate 2:3:3 v:v:v.

resolution of the plates was poor. As the sample size increased the resolution deteriorated due to lipids that bound the iron and caused streaking on the plates.

(II) Detection of slow-growing mycobacteria

The slow-growing mycobacteria *M.tuberculosis* H37Ra, *M.avium*, *M.intracellulare* NCTC 10425, *M.bovis* NCTC 10772 and *M.scrofulaceum* were incubated at 37°C for 14 days on the defined medium. The mycobactin was extracted as above. The scans of these samples (Fig. 6.3) show that whilst diffuse spots could be detected for the tubercule bacilli at the expected Rf positions, no spots were detected for any of the MAIS group. Thus these organisms appear to be able to grow at lower iron concentrations without synthesising mycobactin. *M.bovis* grew very poorly on this medium, presumably because of the presence of large amounts of glycerol, and failed to produce detectable levels of mycobactin.

(III) Improvement of the mycobactin detection method

Radioactive iron with a higher specific activity (Cat IES 3, Amersham International) was selected to increase sensitivity and thus reduce the growth time. Mycobactin was subsequently detected in fast-growing mycobacteria from overnight cultures and from 4 day cultures with the slow-growers (Fig. 6.4).

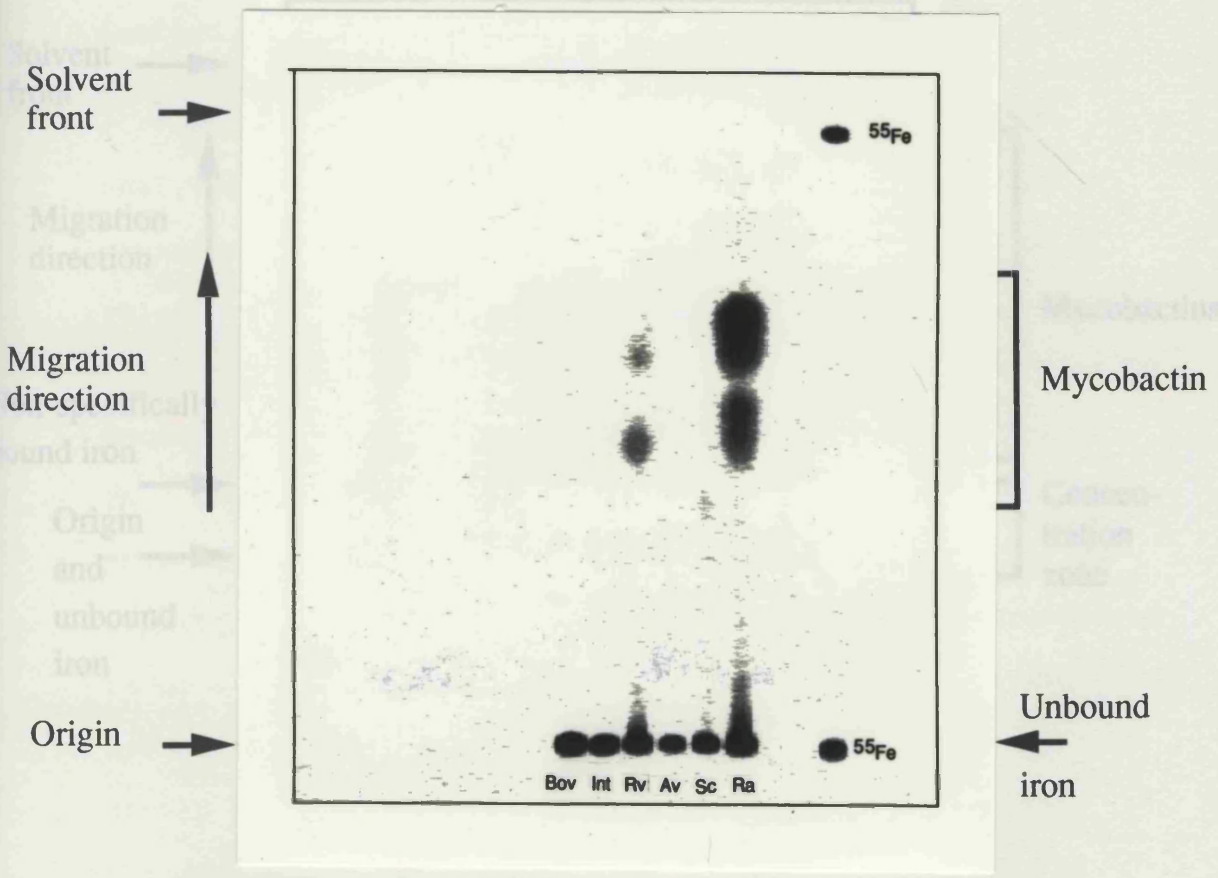


Figure 6.3 Scan of radio-TLC plate of slow-growing mycobacteria by redio-TLC. *M.bovis* (Bov), *M.tuberculosis* H37Rv (Rv), *M.tuberculosis* H37Ra (Ra), *M.avium* (Av), *M.scrofulaceum* (Sc) and *M.intracellulare* (Int) were grown on iron-limited medium for 14 days at 37 °C. Mycobactin extracts from two colonies of these cultures were labelled with ^{55} Fe and separated by TLC on silica gel 60 with petroleum soirit:butanol:ethyl acetate 2:3:3 v:v:v.

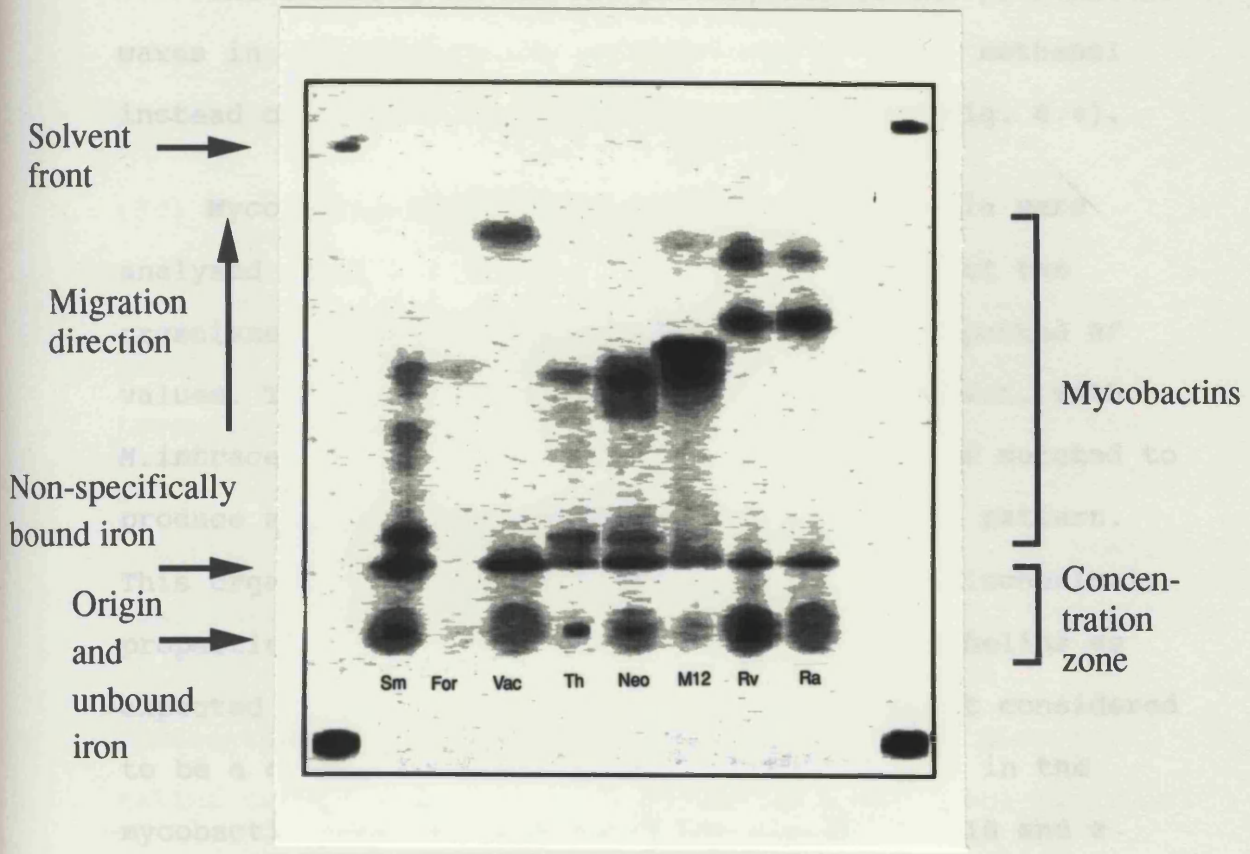


Figure 6.4 Scan of radio-TLC plate showing the improved detection method. *M.neoaurum* (Neo), *M.fortuitum* (For), *M.smegmatis* (Sm), *M.vaccae* (Vac), *M.thermoresistible* (Th) were grown overnight and *M.intracellulare* strain M12 (M12), *M.tuberculosis* H37Rv (Rv) and *M.tuberculosis* H37 Ra (Ra) were grown for 4 days at 37 °C. *M.vaccae* was grown in the presence of of 0.1 mM desferrioxamine mesylate. Mycobactin extracts from a single colony of these cultures were labelled with ^{55} Fe and separated by TLC on silica gel 60 plates with concentration zone using petroleum spirit:butanol:ethyl acetate 2:3:3 v:v:v.

The TLC plates were replaced with the HPTLC which improved the resolution and shortened the separation time. It also increased the cost of the system.

The smearing of the TLC plates, due to the presence of waxes in the extractions, was overcome by using methanol instead of ethanol in the initial extraction (Fig. 6.4).

Mycobactin from a wide range of mycobacteria were analysed using the improved method. Nearly all of the organisms produced mycobactin bands with the expected Rf values. There were abnormalities observed, however, with *M.intracellulare* strain M12 which seemed to have mutated to produce a previously uncharacterised mycobactin pattern. This organism showed its original culture and biochemical properties and produced chloroform-soluble exochelins as expected from *M.intracellulare*, and thus was not considered to be a contaminant. Changes have been observed in the mycobactin profiles of *M.paratuberculosis* NADC 18 and a strain of *M.scofulaceum* (Barclay *et al.*, 1985). These changes, which are stable, have never been observed for mycobacteria other than those belonging to the MAIS group, and have been identified only in the MAIS group organisms that have been grown for many subcultures in iron-deficient media. Thus, for a freshly isolated strain the expected profile would be a spot with an Rf of 0.89 in the solvent system described.

When the solvent system was slightly modified to give

petroleum spirit: ethyl ether: butanol in the ratio of 20:25:30 v:v:v, it was possible to differentiate a single strain of *M.bovis* var BCG from the *M.tuberculosis* (Fig. 6.5). The disadvantage of this method was that although the other separation patterns remained unchanged, their Rf positions differed from those already published.

(IV) Mycobactin detection of MAIS organisms

The MAIS group were inconsistent in the mycobactin produced when grown on either the defined medium or Lowenstein-Jenson medium. To try and stimulate mycobactin production in MAIS the defined medium was made more iron-limiting by the inclusion of the non-utilisable iron binding compounds, desferrioxamine (0.1 mM or 1 mM) or 2,2'dipyridyl (0.1 mM or 1 mM) or by adding B-cyclodextrin. The mycobacteria grew poorly in the presence of 2,2'dipyridyl, taking twice as long to grow as the iron deficient controls. In the presence of 0.1 mM desferrioxamine mesylate or B-cyclodextrin usable microcolonies were produced within 4-6 days. The cells grown in these iron deficient conditions consistently produced detectable levels of mycobactin from a single colony (Fig. 6.5). Using the cpm quantitations evaluated from the AMBIS scans of the HPTLC plates it was calculated that the cells grown in the presence of B-cyclodextrin yielded 31-64% of the mycobactin produced by growth in the presence of desferrioxamine mesylate. In all cases however, culture of

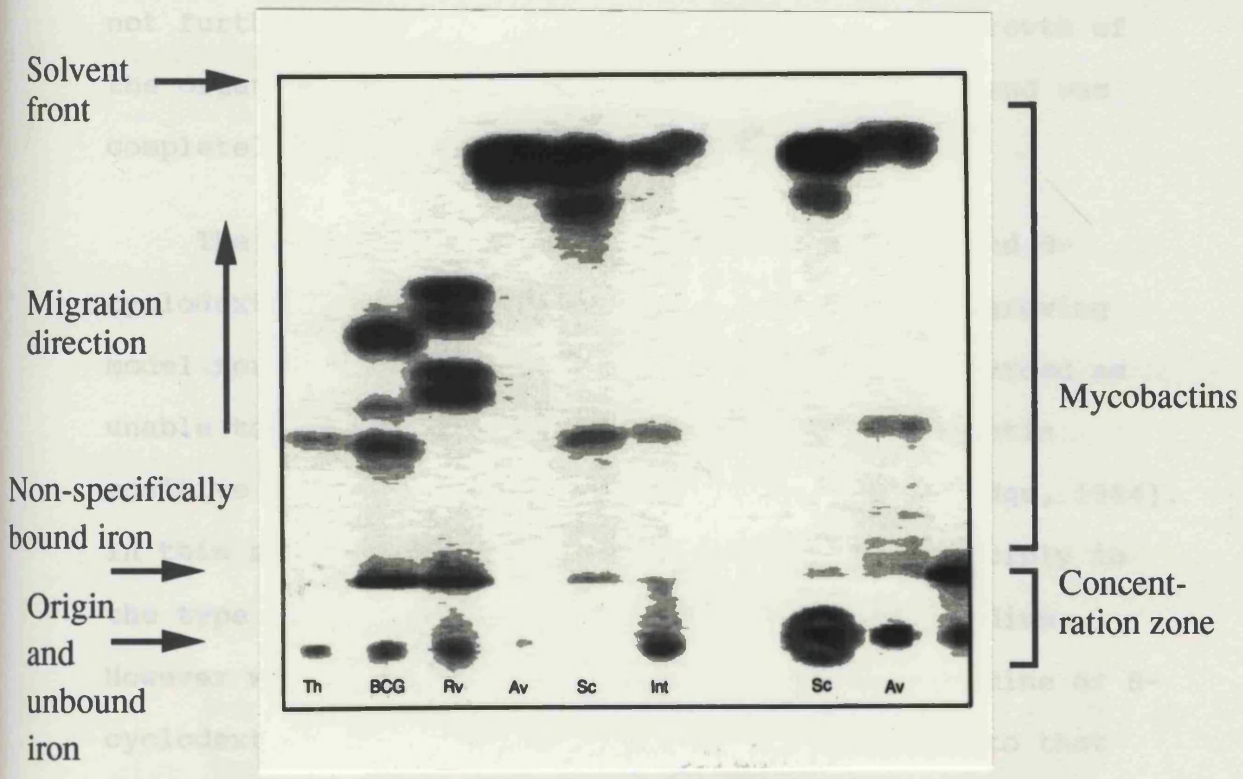


Figure 6.5 Scan of radio-TLC plate showing the mycobactin production by MAIS group organisms using the improved detection method. *M.thermoresistible* (Th) was grown overnight, *M.intracellulare* strain M12 (M12), *M.tuberculosis* H37Rv (Rv) and *M.bovis* var BCG (BCG) were grown for 4 days at 37 °C on iron-limiting medium containing 0.1 mM desferrioxamine mesylate. The *M.avium* (Av) and *M.scrofulaceum* (Sc) samples were grown on iron-limiting medium containing β-cyclodextrin 0.5% w/v. Mycobactin extracts from a single colony of these cultures were labelled with ^{55}Fe and separated by TLC on silica gel 60 with concentration zone using petroleum spirit:butanol:ethyl acetate 20:25:30 v:v:v.

the mycobacteria in the presence of the iron binding additives improved mycobactin detectability despite the fact that the overall growth of cells decreased.The addition of desferrioxamine mesylate in amounts greater than 0.1 mM did not further stimulate mycobactin synthesis. The growth of the organisms, however was considerably reduced, and was completely inhibited at a concentration of 5 mM.

The early experiments with desferrioxamine and B-cyclodextrin were done with *M.vaccae* as a faster growing model for the MAIS group. *M.vaccae* is usually regarded as unable to produce mycobactin, although one mycobactin positive strain has been reported (Hall and Ratledge, 1984). In this study mycobactin was not detected reproducibly in the type strain when grown on the iron-limited medium. However when grown in the presence of desferrioxamine or B-cyclodextrin at 0.1 mM, a band with a similar Rf to that reported previously for strain NCTC 10916, which produced trace amounts, was obtained. This suggests that mycobactin synthesis by *M.vaccae* might be increased or induced under conditions of severe iron limitation.

(V) Sensitivity

M.smegmatis was used to calculate the sensitivity of the method, bacterial numbers were calculated by direct counting using a light microscope. A calibration curve was constructed of cell numbers against radioactive cpm determined with the AMBIS scanner. Desferrioxamine mesylate

was included in the agar, and radioactive iron with a high specific activity was used. The sensitivity of the method was calculated to be 2.87 x 10^3 bacteria. In theory this should be enough for direct detection of bacteria in sputum as most sputum samples contain more than 10^3 cells/ml.

(VI) Testing the potential of pre-applied [^{55}Fe].

High specificity [^{55}Fe] (7.4 kBq in 20 mcl sterile 20 mcM HCL) was applied to five lanes on an HPTLC plate. Five pure mycobactins, prepared by A. Snow and obtained from ICI, were dissolved in methanol at a concentration of 0.1 mg/ml and 5 mcl of each sample was applied directly on to the [^{55}Fe] spots on the HP TLC plate which were then processed as described above.

The mycobactin migrated as expected and bound [^{55}Fe] to give distinct spots that could be detected by the AMBIS Radio Analytical Imager. These results show it is possible to pre-apply [^{55}Fe] to a TLC plate and that unlabelled mycobactin applied on top will bind the labelled iron. This is a potential method that could be developed for speeding up the processing of samples and reduce handling and aliquoting of the isotope for routine laboratory workers.

(VII) Testing mycobactin production under simulated in vivo conditions using macrophages.

Mycobacterium tuberculosis, strain H37R$_v$, cultured on both high and low iron defined medium was used to infect macrophages at an infection ratio of 10 bacteria to one macrophage. The mycobacteria were phagocytosed for 4 hours and then the macrophages were washed to remove unphagocytosed bacilli. After incubation for 0, 3, 5 and 10 days the extracellular medium of the macrophages was collected and mycobactin extracted as described previously. The intracellular bacilli were released by lysing the macrophages with saponin, 5% (w/v) and the mycobactin then extracted. The controls were as follows: (a) extractions of the extracellular medium after phagocytosis and washing of the macrophages at day 0, 3, 5 and 10 (b) extractions of equivalent numbers of bacilli per experimental unit of the original high and low iron cultures of the *M.tuberculosis*, (c) intracellular and extracellular extracts of uninfected macrophages. All the extractions were carried out and analysed according to the improved methods described above.

Mycobactin was detected from the intracellular (phagocytosed) cells after five days incubation (Fig. 6.6). However, the *M.tuberculosis* bacilli collected from the 'extracellular' medium at five days gave much greater counts than the 'intracellular' bacilli. As the macrophages were washed after phagocytosis it must be assumed that the

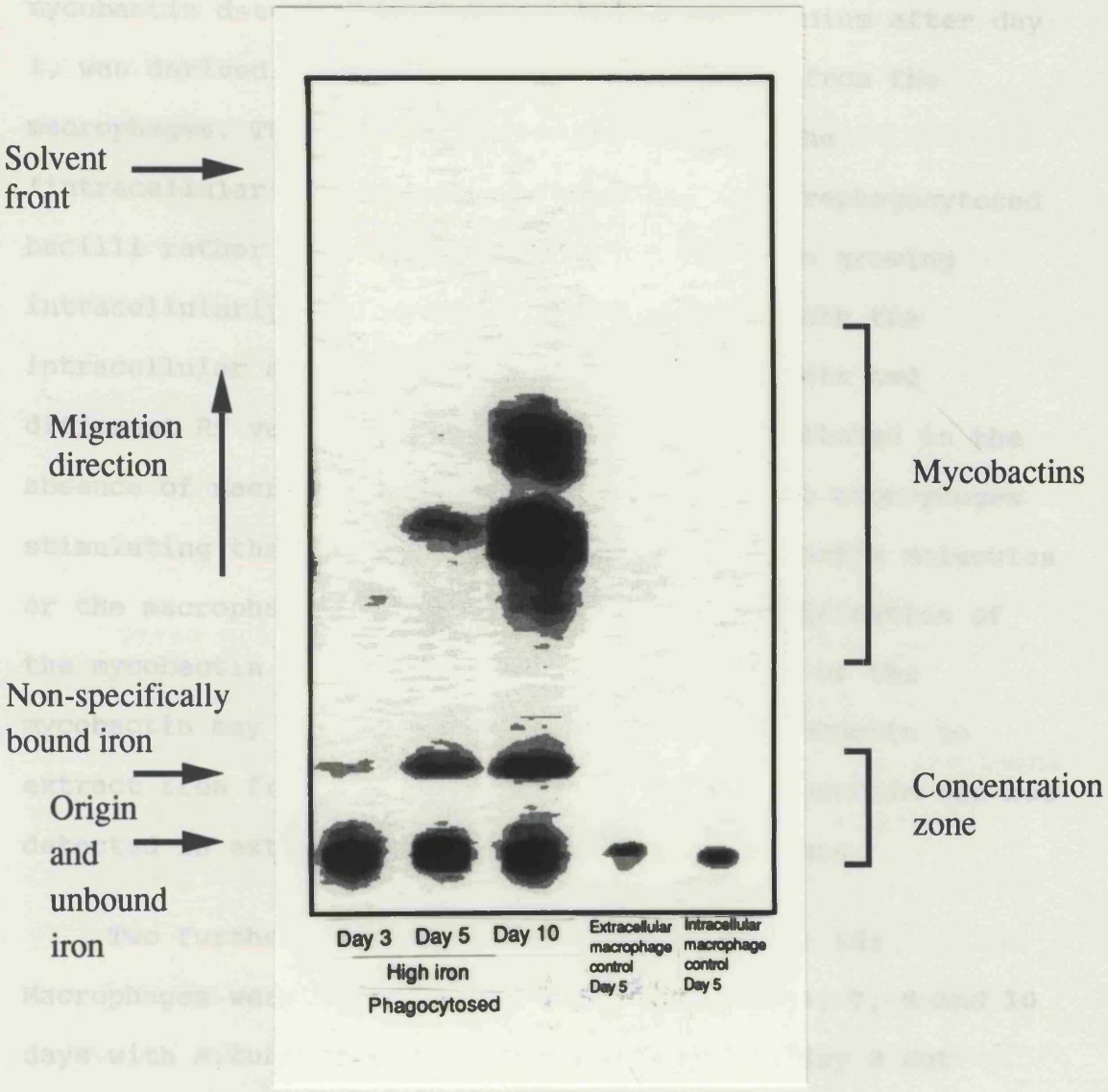


Figure 6.6 Scan of radio-TLC plate of the mycobactins produced by iron-sufficient (high iron) intracellular tubercle bacilli harvested on days 3, 5 and 10 after infection into mouse peritoneal macrophages and non-infected macrophage controls. The macrophages were infected at a ratio of 10 bacteria to one macrophage. The mycobactin extracts were labelled with ^{55}Fe and separated by TLC on silica gel 60 with concentration zone with petroleum spirit:butanol:ethyl acetate 2:3:3 v:v:v.

mycobactin detected in the 'extracellular' medium after day 1, was derived from bacilli that had escaped from the macrophages. Thus it was also possible that the 'intracellular' mycobactin was actually from rephagocytosed bacilli rather than from bacilli that had been growing intracellularly. The mycobactin produced in both the intracellular and extracellular bacilli extracts had different Rf values to the control bacilli cultured in the absence of macrophages. This may be due to the macrophages stimulating the production of different mycobactin molecules or the macrophages may be stimulating the modification of the mycobactin molecules; such a modification of the mycobactin may be necessary to enable the mycobactin to extract iron from the macrophage cytosol. Mycobactin was not detected in extracts from uninfected macrophages.

Two further controls were thus performed: (d) Macrophages were infected for 0, 1, 2, 3, 4, 5, 7, 9 and 10 days with *M.tuberculosis* (day 6 was lost and day 8 not done), the macrophages were washed every day to prevent the possibility of them being rephagocytosed. (e) *M.tuberculosis* bacilli were cultured in the macrophage medium for 0, 1, 2, 3, 4, 5, 6, 7, 9 and 10 days (day 8 was not done).

All the macrophage only controls were negative for mycobactin as expected (Fig. 6.6). The control (d) with extracellular bacilli from 0 to 10 days gave the greatest counts at day 0 and from then onwards the counts varied

randomly for each day. This indicates that a high proportion of bacilli were not phagocytosed at the start of the experiment as the counts in the day 0 extract were greater than on subsequent days, and that bacilli 'escape' continuously over the ten day period. In the control with bacilli only, (e), a gradual increase in counts with time was observed.

(VIII) Testing specimens of clinical origin for mycobactin.

(a) clinical samples

Seven urine samples that were collected from patients with AIDS known to have mycobacterial infections, were tested for the presence of mycobactin. None of the specimens were positive for mycobactin using the sensitive method described. This may have been due to the absence of bacilli or bacilli debris in the urine. Detailed information about the disease status of the patients from whom the urine was taken was not available. However they were all taken from AIDS patients and thus the most likely mycobacterial infection would be MAIS. It was not known how heavily infected these individuals were, or if and how long they had been on treatment. All of the urine samples had blood in them, and were autoclaved prior to extraction, thus it is conceivable that any mycobactin present became saturated with non-labelled iron which would have been freed after the heamoglobin was destroyed by autoclaving. It has been demonstrated that mycobactin is resistant to autoclaving

(Barclay, personnel communication).

The second possible reason for mycobactin not being detected could be that the mycobacterial bacilli were not producing mycobactin *in vivo*. As has been seen from previous data *M.avium*, which is the most likely infecting organism, does not produce mycobactin as readily as many other mycobacteria. Previous workers have also isolated non-mycobactin producing strains, which may be the case in these urine samples. The reason why *M.avium* often does not produce mycobactin is unknown but may be due to the fact that *M.avium* is not an obligate pathogen and is essentially an environmental organism where iron may often be more readily available. It is also possible that *in vivo M.avium* often 'shares' the iron chelating siderophores of other bacteria or needs less iron.

Eleven sputum samples from Nigerian tuberculosis patients were processed as described for sputum samples. Mycobactin was not detected in any of them. It is possible this was due to an ineffective extraction method, rather than an indication that the mycobacteria were not iron deficient enough to produce mycobactin. Although 100% ethanol was used, which is the best solvent for extraction, the sputum residue would have retained a lot of water which would have diluted the ethanol and reduced its ability to extract mycobactin effectively. The method could be modified to overcome this possibility by either drying the sputum

sample before extraction or extracting the sample several times with ethanol and pooling the extractions. This would dilute out the water and thus increase the efficiency of the extraction.

It is also possible that the labelled iron was diluted out and bound by iron binding proteins such as lactoferrin known to be present in sputum (Griffiths, 1983).

Thus it is clear that the extraction method for sputum requires further improvement over that used for cultured bacilli. The method should also be modified further before it can be concluded that bacilli in sputum and urine samples do not produce sufficient mycobactin for identification.

(b) Primary isolates of clinical samples

Over several months 27 isolates from primary culture were obtained and inoculated into 50 mcl of 100% ethanol. The samples were stored at -20°C until processed and run on HPTLC plates.

Of the 27 strains 16 gave positive results (Fig. 6.7), all of the six *M.tuberculosis* strains were easily identified by the presence of two distinct doublet bands. Both scotochromogens, which had not been typed further by the routine laboratory, were readily picked out as being neither of the MAIS or tubercle group The mycobactin banding pattern of both were distinct and uncharacterised. One of the two

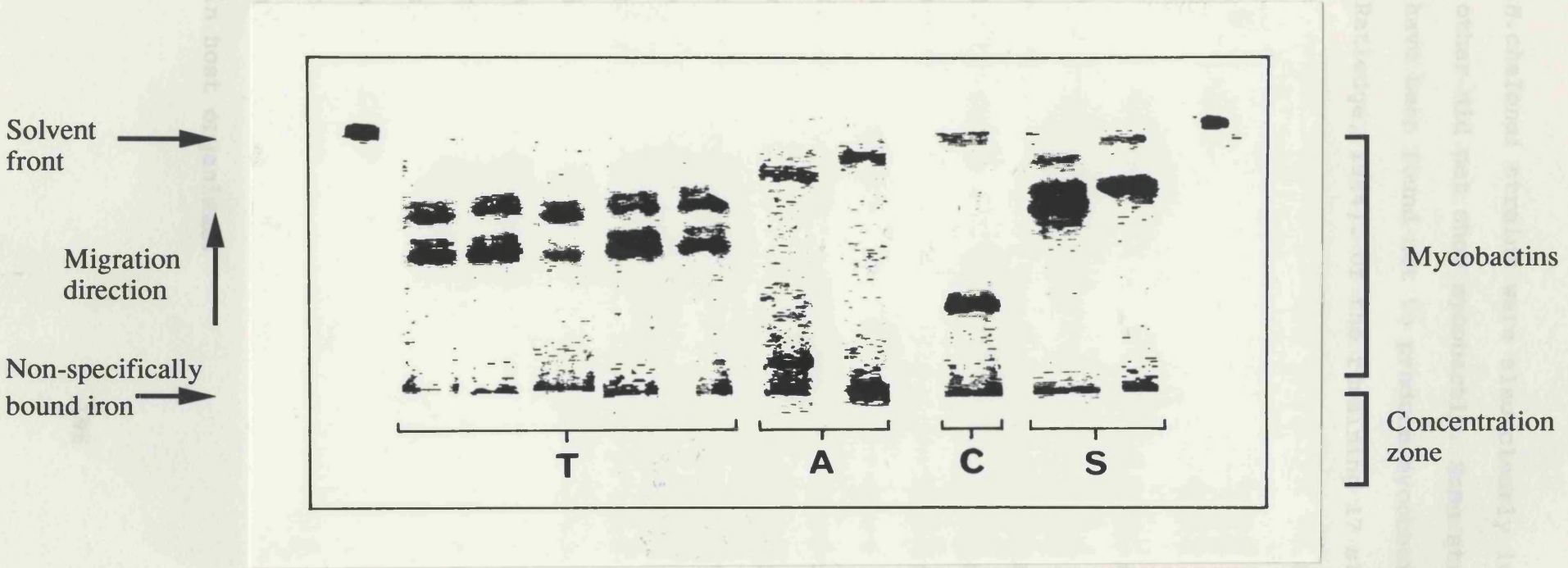


Figure 6.7 Scan of radio-TLC plate of the mycobactins from primary cultures of mycobacteria from clinical specimens:T, *Mycobacterium tuberculosis*; A, *M.avium*; C, *M.chelonei*; S, scotochromogens. The mycobactin extracts were labelled with ^{55} Fe and separated by TLC on silica gel 60 plates with concentration zone using petroleum spirit:butanol:ethyl acetate 2:3:3 v:v:v.

M.chelonei strains were also clearly identified but the other did not show mycobactin. Some strains of *M.chelonei* have been found not to produce mycobactin (Hall and Ratledge, 1984). Of the remaining 17 strains, which were all *M.avium*, 7 were clearly identified as such.

6.6 SUMMARY

The sensitivity of the detection method for mycobactin was greatly improved by making several modifications to the methods used previously. The TLC plates were replaced with HPTLC plates and the results scanned using the AMBIS scanner; [^{55}Fe] of a higher specific activity was used; Ethanol was used for the first extract in the place of methanol and the mycobacteria were cultured on media modified by the addition of non-utilisable iron binding compounds. Using these methods it was calculated for *M.smegmatis* 2 x 10^3 mycobacteria could be detected within 24 hours. The system was found to be suitable for the identification of mycobacteria such as *M.tuberculosis* and *M.avium*. *M.tuberculosis* was found to synthesis mycobactin *de nuvo* after inoculation into macrophages. This may indicate mycobactin is synthesised by mycobacteria *in vivo*. However *M.avium*, *M.intracellulare* and *M.scrofulaceum* were found to have a lower requirement for iron than other mycobacteria and this may influence their growth and mycobactin synthesis in host organisms.

In a preliminary test of this system 27 first isolate cultures were extracted and analysed, 16 gave mycobactin patterns that were characteristic of the species. It is not known why mycobactin was not detected in the other 11, 10 of which were all found later to be *M.avium*, a mycobacterial species known to not always produce mycobactin. Further modification of this method is needed to test its application directly on clinical specimens.

CHAPTER 7 DISCUSSION

7.1 INTRODUCTION

The classification of bacteria originally followed the Linnaean system and was based almost exclusively on morphological and biochemical characteristics. However two major developments have completely changed bacterial systematics. The first was the adaption of numerical systems of classification which differed from the Linnaean system principally in not weighting the characteristics used in the classification system. The second major development was an extension of the type of characteristics used to differentiate bacteria. The characteristics that have become available were the direct result of technological development in chemical-analysis and molecular biology techniques. Analysis of cell wall sugars, fatty acids, respiratory quinones, cell proteins and even complete disintergration of bacterial cells followed by analysis of the fragments by mass-spectrometry, along with analysis of nucleotide sequences in ribosomal RNA have revolutionized bacterial systematics.

There is still clearly a need for advancements in identification techniques and typing systems for many clinically important micro-organisms. Thus the aim of this thesis has been to apply new technologies to some of these old problems in the hope that fresh light may be thrown on

them. Further computerization has enabled the efficient design and use of databases as well as manipulation and analysis of primary data that before would have been extremely difficult and laborious by manual methods.

7.2 [^{35}S] LABELLING AND ANALYSIS

The analysis of whole cell protein patterns has been used in systematics for many years (Kersters and De Ley, 1984; Jackman *et al.*, 1983; Ehret *et al.*, 1988; Gargallo-Viola and Lopez, 1990). The technique gives rise to a fingerprint comprising many separate polypeptide bands of differing molecular weight. Comparisons of the fingerprint from many organisms can be used to derive a taxonomic structure which has been shown to have similar validity to DNA-DNA hybridisation techniques (Picard *et al.*, 1991; Clayton *et al.*, 1991). Because of the large number of bands that can be detected after electrophoretic separation of bacterial cell proteins, comparison of large groups of isolates can only be effectively analysed with the aide of a computer. The original method of PAGE used Coomassie blue staining to detect the separated polypeptides, followed by densitometric analysis of the fingerprint (Jackman *et al.*, 1983). The method used here was originally developed using [^{35}S] methionine to label the proteins and is more sensitive than Coomassie blue staining, detecting a greater number of bands (Clayton *et al.*, 1991) hence there is an even greater need for computer analysis.

Fingerprinting systems such as PAGE and PyMS require standard operating practices to ensure reproducibility of the fingerprint. Part of this study was aimed at developing such standard operating practices both with respect of the labelling of the bacterial cells and the computer analysis.

In order to utilise the fact that bacterial protein profiles are reproducible under a standardised set of conditions there are many aspects of the method that must be strictly controlled. Gel preparation and electrophoresis must be standardised and monitored and the normalisation strategy must be assessed. Under these conditions a high standard of profile analysis is possible.

The first essential step was to develop a standard medium that could be used to label all bacterial cells under investigation. This was achieved by enriching a standard medium with a wide range of nutrients.

Although [^{35}S] methionine was needed originally in this system, other radionucleotides were investigated because of the problems using of [^{35}S] methionine which has volatile break down products which are hazardous (Smith, 1989).

The [^{35}S] compounds, methionine, inorganic sulphate and thio ATP were compared for labelling micro-organisms. Inorganic sulphate was not utilised by many Gram positive organisms, and where it was utilised, lower counts were

obtained in the two hour incubations than for methionine. This could be because sulphate has a different biochemical pathway before being incorporated into proteins than methionine which can be used immediately. ATP was used by all the bacteria examined as was methionine. But methionine was found to be taken up more readily in 2 hours compared with ATP, except for slowly growing organisms such as Campylobacter, Helicobacter, spirochetes and the mycobacteria. These organisms needed longer incubation times of 24 hours or 4 to 7 days for mycobacteria. With these longer incubation times there is a greater risk of a contaminating organism multiplying sufficiently to distort the profiles of the organism being analysed and therefore greater care is needed during sample inoculation and they must be plated after incubation to determine if the culture is still pure. The utilisation of the different [^{35}S] substrates by different organisms is summarised in Table 7.1.

Different patterns were obtained with the different sources of [^{35}S], although with some species, eg. *Proteus mirabilis*, there was a high correlation between the patterns produced by sulphate and methionine labelling indicated there were many polypeptides in common. When the whole cell proteins from larger groups of some species were labelled with methionine and analysed the patterns were found to be species specific. For instance with *Staphylococcus haemolyticus*, *Campylobacter jejuni*,

Table 7.1 Species and genera of microorganisms examined by [^{35}S] labelling: +, labelled proteins; -, no labelled proteins; ND, not done; M, methionine; S, inorganic sulphate; A, thio ATP.

name	no. of strains	M	S	A
coagulase-negative *Staphylococcus*	28	+	-	+
Staphylococcus aureus				
Methicillin-resistant	2	+	-	+
Methicillin-sensitive	2	+	-	+
Streptococcus agaiactiae	1	+	-	+
Enterococcus faecalis	1	+	-	+
Bacillus sp.	1	+	+	+
Corynebacterium jeikeium	1	+	+	+
Corynebacterium D2	1	+	+	+
Mycobacterium vaccae	1	+	+	+
Mycobacterium gilvum	2	+	+	+
Mycobacterium flavescens	1	+	+	+
Mycobacterium duvalii	1	+	+	+
Mycobacterium tuberculosis	36	+	+	ND
Mycobacterium avium	20	+	+	ND
Mycobacterium chelonei	20	+	+	ND
Mycobacterium fortuitum	9	+	+	ND
Mycobacterium kansasii	20	+	+	ND
Mycobacterium malmoense	2	+	+	ND
Mycobacterium xenopi	2	+	+	ND
Nocardia sp.	7	+	+	ND
Escherichia coli sp.	1	+	+	+
Pseudomonas sp.	33	+	+	+
Shigella sp.	1	+	+	+
Salmonella sp.	1	+	+	+
Hafnia sp.	1	+	+	+
Pasteurella sp.	1	+	+	+
Proteus mirabilis	1	+	+	+
Citrobacter sp.	1	+	+	+
Klebsiella sp.	1	+	+	+
Morganella sp.	1	+	+	+
Enterobacter sp.	1	+	+	+
Helicobacter pylori	7	+	+	+
Campylobacter jejuni	7	+	+	ND
Spirochetes	12	+	ND	ND

Helicobacter pylori, many *Pseudomonas* species and mycobacterial species such as *M.tuberculosis*, *M.avium*, *M.kansasii*. With many species eg *Helicobacter pylori*, *Pseudomonas pseudomallei*, *Staphylococcus haemolyticus* and *Mycobacterium chelonei* further sub-divisions were also possible which could prove useful as a means of typing these organisms.

Although methionine was found to be the most readily utilised substrate of the three that were tested, other compounds may be useful for sub-speciation and epidemiological studies in particular circumstances because the different substrates label different sub-sets of bacterial components. Variation between sub-species may not show up with one substrate but may with another. This phenomenon was observed in the *M.tuberculosis* samples labelled with methionine and sulphate. The whole cell patterns for the different isolates were found to be almost identical with both sulphate and methionine but differences between the isolates were found in the secreted protein patterns. With methionine (Appendix 4.13) all the isolates had a characteristic profile of six prominent bands, except for one isolate that was missing one of the prominent bands. With sulphate (Appendix 4.12) there was also a characteristic banding pattern but in this instance there were greater discrepancies between the isolates, several isolates were missing bands or the bands were substantially

fainter.

The next step of the investigation was to asses the usefulness of secreted proteins, compared to whole cell proteins, as a means of differentiating organisms. The secreted protein profiles contained far fewer bands than the whole cell protein profiles, and in many instances the most distinct differences between sub-speciation were observed in the filtered secreted proteins profiles, for instance *Helicobacter pylori* was sub-speciated into two groups and *Campylobacter jejuni* into four groups. Greater strain differences were also seen in the secreted patterns of *Staphylococcus haemolyticus* and *M.tuberculosis* than in their corresponding whole cell patterns.

An important consideration of reproducibility is the variation between gels. Gel to gel normalisation was found to be essential for comparing data from different gels. The best normalisation strategy was smoothing followed by clipping for maximum gel length and transformation prior to clustering with auto-alignment during the preparation of a dendrogram. For gels with less than 0.5 cm discrepancies between the gel front line, and where profiles were not distorted by smearing or overloading, the normalisation gave high correlations. For intra gel comparisons the mean correlation for a repeat sample was found to be 0.98 ± 0.018 and the mean correlation for inter gel comparisons was found to be 0.94 ± 0.017 ($n = 5$).

The software needed to overcome the problem of intra gel variation due to distortion of gels has already been developed for the DNA 'fingerprinting' AMBIS software system. This software has an automatic correction function that 'straightens' out the data using three key molecular weight markers positioned in the middle and at each end of the gel. However this function has not yet been adapted and incorporated into the AMBIS microbiological software described here. If it were added to this software it is likely it would overcome the problem caused by gel distortion.

An alternative method of overcoming the problem of intra gel variation would be to run more molecular weight markers on each gel. If there was a molecular weight marker after every two samples then every sample would be adjacent to a marker which could be used for normalisation. This would reduce the number of spaces for samples on every gel and thus increase the number of gels needed. This tactic has been used by some workers (Millership and Want, 1992). Because gel normalisation cannot always compensate for gel variation a small percentage of data will always be needed to be repeated or discarded.

Other minor technical factors apart from intra gel variation could effect the normalisation process. The molecular weight markers could be distorted either because of degradation of the proteins, which would alter their

relative distribution, or they may not form discrete bands so that the peaks are not clearly defined.

Normalisation of data could also be affected by differences between gels caused by the gel preparation. Differences in the polyacrylamide concentration would alter the separation profile considerably, for instance, separating single bands into many bands. This effect should be minimised by the manufacture of gels in large batches by AMBIS and Pharmacia, with quality control facilities.

At present the normalisation of every profile has to be done manually, this makes the process more time consuming than it need be, and could prohibit the use of this system on a regular or routine basis within a hospital microbiology laboratory. However it would be feasible to develop the software further to include an automatic normalisation function. So that once the vital statistic (molecular weight standard positions and lane positions) of each gel were recorded and the normalisation strategy was selected, normalisation could be completely automated. This coupled with the software to compensate for gel distortion would produce a much improved and effective system.

There is an automatic scaling function in the AMBIS software to enable profiles of different radioactive intensities to be compared. The actual number of counts is dependant on the scan time as well as the sample

concentration and the amount of sample originally loaded on to the gel. The threshold of total count at which samples could be compared with a high correlations was found to be below 40 000 counts per lane. Thus only histograms with counts of 40 000 upward for a full length histogram were used in analysis. The actual threshold for each sample would depend on the distribution of counts within the lane. However in practice, all scan times were over 300 minutes, thus the majority of counts for individual histograms were significantly higher than 40 000 and so well over the threshold level.

There were numerous AMBIS software updates during the period of this study as well as some hardware updates. This improved the speed of analysis and the user friendliness of the software system and the hardware up dates have improved the sensitivity of the detection system. With the development of any new software a close working relationship between developers and potential users is required.

Most samples were incubated in duplicate for standardisation, and one to four duplicates were electrophoresed on the same gel. The correlation of the duplicates were found to be as follows: 0.89 ± 0.054 ($n = 4$) for *Helicobacter pylori*; 0.981 ± 0.008 ($n = 4$) for *Pseudomonas pseudomallei*; 0.937 ± 0.022 ($n = 2$) for *Staphylococcus haemolyticus*; 0.948 ± 0.009 ($n = 2$) for the spirochetes.

The use of the shorter Pharmacia gels caused less band separation than on the 12.5% polyacrylamide gels and therefore produced greater problems in analysis. There were fewer data points on the histograms and as with all statistical analysis the significance of the correlation is greater the greater the set of data points. The bands were also much closer together on the shorter gels which reduced the resolution of bands. Scanner heads with improved resolution are now available which may overcome the problem of crowding on the shorter gels.

A database was constructed from 84 isolates. Further samples of each of the species in the database were labelled and compared to the database. The fast Fourier transform was used to scan the database for the ten best matches for the test profile. A correct match was obtained in 87% of cases and the mean spectral correlation for the correct matches was 0.861 ± 0.084. Where the match was incorrect a much lower correlation of 0.720 ± 0.118 was obtained. When the ten best matches were auto-aligned to the challenge profile the correct match was obtained in 92% of the matches with a mean correlation coefficient of 0.869 ± 0.077, and 0.721 ± 0.119 for the incorrect matches.

The capital cost of the AMBIS system is large (£35 000 currently) and the running costs per identification is approximately £3.25. This compares unfavourable to the cost

of an API, which at current prices costs £1.25 per identification. The method would have advantages where large numbers of organisms were to be identified because of labour saving. However the method is unlikely to gain wide acceptance as a routine identification system because of the cost and the use of radioactivity. It would seem to be much more useful as a means of typing organisms particularly the organisms that cannot be typed readily by other means. The method has a particular advantage for identification when dealing with slowly growing micro-organisms eg. mycobacteria, as it considerably shortens the time needed for identification.

7.3 *MYCOBACTERIUM LEPRAE* STUDY

In the pilot experiment for the [^{35}S] inorganic sulphate labelling of *M.leprae*, labelled bands were obtained after gel electrophoresis. Subsequent attempts to repeat this result were unsuccessful. There is no clear reason for this enigmatic result. The factors that could have been responsible were firstly the methods used or secondly the viability of the *M.leprae* bacilli. It is also possible that the labelled bands were not derived from *M.leprae* but were an artifact, but in the absence of any detectable contaminants this is unlikely.

The nude mouse tissue homogenation techniques were based initially on personal communications with Dr D. McDermot, St George's Hospital. Advice was also taken

from Dr S. Franzblau, Gillis W. Long Hansen's disease centre, Louisiana and from Dr C. Low, MRC. The initial set of experiments were with tissue containing high counts of bacilli. Contacts were sought in order to obtain specimens for further experiments but there was great difficulty in obtaining large aliquots of fresh *M.leprae* containing specimens.

Nude mice are thought best for *M.leprae* culture because they are bred in hygienic and sterile environments and thus free from other bacterial flora and can yield up to 10^{10} bacilli per footpad (Lancaster *et al.*, 1983) compared with a maximum of 10^{6} bacilli from normal mice. Armadillos yield 100 times more bacilli than nude mice, however armadillos have not yet been bred in captivity and therefore must be take from the wild. Armadillos would therefore be colonized by a wide range of bacteria.

Dr S.Franzblau was able to supply bacilli from nude mice but the material had to be sent from Lousiana by air. The first batch of bacilli supplied from Dr.S.Franzblau was brought to England in the hand luggage of the author and used in trials with the Buddemeyer system. The [^{14}C]palmitic acid oxidising capacity of subsequent batches of bacilli sent by air were found to be much lower than the batch brought by hand. Potentially the sub zero environmental conditions in the airplane hold of a transatlantic flight may have had a detrimental effect on the bacilli; certainly

the viability of *M.leprae* bacilli is considerably reduced by repeated freeze thawing (Portaels *et al.*, 1988).

The human biopsy material did not oxidize the 1-[^{14}C] palmitic acid, which indicates the *M.leprae* did not survive in biopsy tissue sent from India. Pattyn *et al.* (1992) found a 10-fold decrease in the detection efficiency of PCR techniques on boiled skin biopsies kept at room temperature for 3 weeks; indicating the bacilli are not preserved effectively in biopsy tissue at room temperature. A low level of oxidation was observed from the filtered supernatant from the homogenised tissue. This may be an indication that the oxidising enzymes are secreted and remain active after the bacilli have died. Cho *et al.* (1992) found indications that *M.leprae* may actively secrete phenolic glycolipid 1 into the surrounding tissue for a long time. Alternatively the bacilli may be breaking open and releasing active enzymes.

From the 1-[^{14}C]palmitic acid studies it was found that there was a higher level of 1-[^{14}C]palmitic acid oxidation in the Percol purified bacilli. The reason for this observation is not clear. Possibly the Percol purification selects for live/metabolically active bacilli. Alternatively the purification may remove components that are released when the tissue is homogenised and which are detrimental to the bacilli. Further studies would have to be carried out to establish why the oxidation of palmitic

acid is improved with Percol purification. Percol purified bacilli could be used in other metabolic studies as well as in further [^{35}S] labelling experiments.

One problem with the Percol purification is that in most instances only 10-40% of the bacilli are recovered after the purification process. The proportion of recovered bacilli may equate with the proportion of live bacilli if this process does select for live bacilli. But this is as yet unproven and considerably limits the material available for experiments. Alternatively it may be due to polymerisation of fresh mouse foot pad tissue with the Percol as suggested by Mori *et al.* (1984).

Rodde *et al.* (1992) have recently described an improved method of purifying *M.leprae* from armadillo tissue, this method is an improvement on the standard purification method of Draper (1980) for armadillo derived *M.leprae*, which is very labour intensive. The Rodde method uses Percol in a similar way to the methods described here (Franzblau, personal communication) for purifying bacilli derived from mouse tissue, and would be useful for obtaining large batches of *M.leprae* bacilli from armadillos for further metabolic studies.

The activity observed from the 1-[^{14}C]palmitic acid study varied considerably from sample to sample. This may be an indication that the metabolic activity of different

batches of *M.leprae* were variable. The viability of *M.leprae* could have been effected by the methods employed by different suppliers, such as the time of harvest of bacilli. There is the possibility, particularly in nude mice where the immune system is deficient and thus does not inhibit bacilli growth, that as the bacilli load increases some bacilli will be deprived of nutrients and die, thus affecting the overall viability of the batch. Differences between different batches of cell free extracts of armadillo derived *M.leprae* when analysed by SDS-PAGE and immunoblotting with monoclonal antibodies have previously been reported (Ibrahim *et al.*, 1990).

The key factors affecting the cultivation of *M.leprae* have eluded researchers for decades. But it is still an important topic as demonstrated by the advancements that have been made in understanding the previously uncultivated *M.paratuberculosis* after it had been cultivated (Herman-Taylor *et al.*, 1990). An important analytical approach in attempting to cultivate *M.leprae* is metabolic studies and finding ways of assessing the metabolism of *M.leprae in vitro* with different substrates and under different conditions. Relatively little is known about the environmental conditions that are favoured by *M.leprae*, for instance which nutrients does it require, which pH is best? Metabolic studies are an important way of gauging the most favourably conditions for *M.leprae*. It is quite likely that a complex combination of conditions is

required by *M.leprae* for maintenance and ultimately *in vitro* culture and thus the right permutation of temperature, nutrients and pH will not easily be resolved.

7.4 DETECTION AND IDENTIFICATION OF MYCOBACTERIA USING MYCOBACTIN

The method originally proposed by Snow and White, 1969, for extracting and separating mycobactin and developed by Hall and Ratledge (1984) for taxonomic purposes has been refined here for rapid routine identification. Identification is based on the published Rf values of these organisms (Barclay and Ratledge, 1983, 1988; Hall and Ratledge, 1984, 1985). Rapidly growing mycobacteria can now be identified within 24 hours, and slow growing mycobacteria within 5 days. Most mycobacterial species can be identified within a few hours if sufficient organisms are available.

The limitation of this method is that mycobacteria which do not produce mycobactin cannot be identified. Three species do not produce mycobactins in substantial quantities *M.paratuberculosis, M.vaccae* and *M.leprae*. The first two have the potential to produce mycobactins (Hall and Ratledge, 1984; Barclay and Ratledge, 1983; Merkal and McCullough, 1982) and the small amounts produced can normally be increased by growing the organisms under iron-limiting conditions with desferrioxamine mesylate. However,

M.paratuberculosis grows so slowly under these conditions (some strains taking 18 months to grow) that the technique is probably impractical for this organism. Although *M.leprae* has not been shown to produce mycobactin, it has not yet been tested by this highly sensitive method.

The analysis of mycobactin profiles of the MAIS group, using the methods developed here, indicates atypical properties of mycobactin production, and thus iron-acquisition of this group. This is evident by the growth of this group, without mycobactin synthesis, in conditions that would be iron limiting to other mycobacteria and thus stimulate mycobactin synthesis. This may mean the MAIS group have a lower requirement for iron than other mycobacteria or that MAIS organisms utilise the exochelin uptake system more effectively. This difference in mycobactin synthesis could hamper the use of mycobactin profiles for identification.

Growing mycobacteria under iron limiting conditions with the iron chelator, desferrioxamine, improves mycobactin production. It was determined that desferrioxamine at 0.1 mM is suitable for rapid growers but the slow growers need 1 mM of desferrioxamine to promote mycobactin production.

We have demonstrated here that *M.tuberculosis* will produce mycobactin after it has been phagocytosed by macrophages, a situation similar to *in vivo* conditions. Thus although mycobactin has as yet not been detected directly

from clinical specimens it still remains feasible that mycobacteria do produce mycobactin *in vivo*. As the body tissues are recognized to be deficient in free ferric iron and as mycobacteria produce iron binding molecules in response to iron deficient conditions it seems quite reasonable to suggest that mycobacteria would produce mycobactins *in vivo*. This has been found to be the case for other bacteria (Czirok *et al.*, 1990). In the case of mycobacteria the hypothesis that mycobacteria would produce mycobactin *in vivo* is supported by the macrophage data. The lack of detection from clinical samples could be due to the presence of host iron binding compounds, further work is required to investigate this. It would also be beneficial to repeat the macrophage experiment with *M.avium* to see if it produced mycobactin as readily as *M.tuberculosis* in the *in vivo* simulated environment.

In a preliminary test of this system, 27 primary cultures were extracted and analysed, 16 gave mycobactin patterns that were characteristic of the species. It is not known why mycobactin was not detected in the other 11, 10 of which were all found later to be *M.avium*, a mycobacterial species known to demonstrate atypical production of mycobacactin. Further development of this method is needed to test its application directly on specimens, and increase its sensitivity for the clinically important MAIS-group of mycobacteria.

The methods discussed clearly have potential for rapid identification of mycobacterial infection from primary culture and may also prove to be useful directly with clinical specimens. Other techniques of rapid identification are also being developed using PCR and gene probing (Lebrun *et al.*, 1992; Evans *et al.*, 1992). These methods are well on the way to being applied and used routinely in developed countries, but may never be applicable in more under developed countries. This is because of the often expensive and labile nature of many of the reagents needed for these molecular biology techniques. Many of the enzymes have short half lives and require storage at -20°C, and would need good delivery services as well as storage facilities, that are at present impossible in rural areas of the world.

An identification technique based on the mycobactin methods could be developed for application in rural areas. The chemicals are relatively cheap and stable without refrigeration. [^{55}Fe] has a long half life and is a low energy isotope making it easy to handle. There are not the same problems of contamination as is the case with DNA analysis. The HPTLC plates could be pre-scored and sample application sites marked. Reference mycobactin could be pre-spotted and the [^{55}Fe] may also be pre-applied to the plate to reduce the need for workers to handle the isotope. Possibly the amount of [^{55}Fe] used could be reduced as could the number

of extraction/transfer/evaporation steps, and autoradiography film used to visualise plates where the rapid AMBIS Imaging system was not available. The development of chromogenic assay for the bound iron would also be a great advantage by removing the need for radioactivity. These possible modifications may speed up the technique still further without loss of sensitivity.

7.5 SUMMARY AND CONCLUSIONS

(I) The [^{35}S] labelling study

1. A medium that supported the growth of all the bacteria under test was developed for [^{35}S] labelling studies.

2. Of the three [^{35}S] compounds tested,[^{35}S] methionine was found to be the most readily utilised substrate within a two hour incubation period. Some slowly growing organisms did need longer incubation times in the radioactive substrate. All the Gram negatives organisms labelled with [^{35}S] methionine, [^{35}S] thio ATP and [^{35}S] inorganic sulphate. However not all the Gram-positive organisms labelled with [^{35}S] inorganic sulphate.

3. Gel to gel normalisation was essential for comparing lanes from different gels. The best normalisation strategy for histograms was smoothing, clipping and transformation with auto-alignment during analysis to give a dendrogram.

4. The method was found useful for differentiating the species within a genus, with several organisms eg. *Pseudomonas pspeudomallei* and *Helicobacter pylori* the method could also be used to type these species.

5. The AMBIS system has potential as a rapid microbiological Identification system compared to other methods used to identify bacteria.

(II) The *Mycobacterium leprae* study

1. [^{35}S] labelled proteins were obtained from *M.leprae* incubated with [^{35}S] inorganic sulphate in the initial pilot study but attempts to repeat the result were unsuccessful. This was due to a number of complications which resulted in poor test specimens.

2. [^{35}S] thio ATP and [^{35}S] methionine could not be used for labelling experiments with *M.leprae* suspended in Dubos medium because the label bound non specifically to albumin in the medium.

3. The Buddemeyer system was used as an alternative metabolic assay for monitoring the activity of *M.leprae*. Using the Buddemeyer system it was found that Percol purification of the bacilli resulted in a dramatically increased the oxidation of 1-[^{14}C]palmitic acid.

(III) The mycobactin study

1. The sensitivity of mycobactin detection has been dramatically improved by modifying the extraction methods. The detection time has also been rapidly reduced by using the AMBIS scanner to visualise TLC plates. Rapidly growing mycobacteria can now be identified within 24 hours, and slow growing mycobacteria within 5 days. Most mycobacterial species can be identified within a few hours if sufficient organisms are available.

2. Identification is based on the published Rf values of these organisms (Barclay and Ratledge, 1983, 1988; Hall and Ratledge, 1984, 1985)

3. It has been demonstrated that *M.tuberculosis* will produce mycobactin after it has been phagocytosed by macrophages, a situation similar to *in vivo* conditions. Thus although mycobactin has as yet not been detected directly from clinical specimens it still remains feasible that mycobacteria do produce mycobactin *in vivo*.

4. Growing mycobacteria under iron limiting conditions with the iron chelating agent desferrioxamine improves mycobactin production. It can be concluded that desferrioxamine at 0.1 mM is suitable for rapid growers but the slow growers need 1 mM of desferrioxamine to promote mycobactin production.

5. The methods discussed clearly have potential for rapid identification of mycobacterial infection after primary culture and may also prove to be useful for clinical specimens.

7.6 RECOMMENDATIONS FOR FUTURE STUDY.

1. Data analysis of gels using the AMBIS system software could be improved by automation of the lane extraction and normalisation process.

2. Further development of the standardisation of gel manufacture would aid the normalisation process.

3. In order for this system to be used as a rapid Identification system isolates of more species would need to be analysed and added to the database.

4. A database for rapidly identifying mycobacteria could be constructed and would be a great advancement on present slow identification systems for this species.

5. *M.leprae* could be tested with the sensitive mycobactin detection method developed here.

6. Further primary culture isolates could be analysed with the sensitive mycobactin detection method.

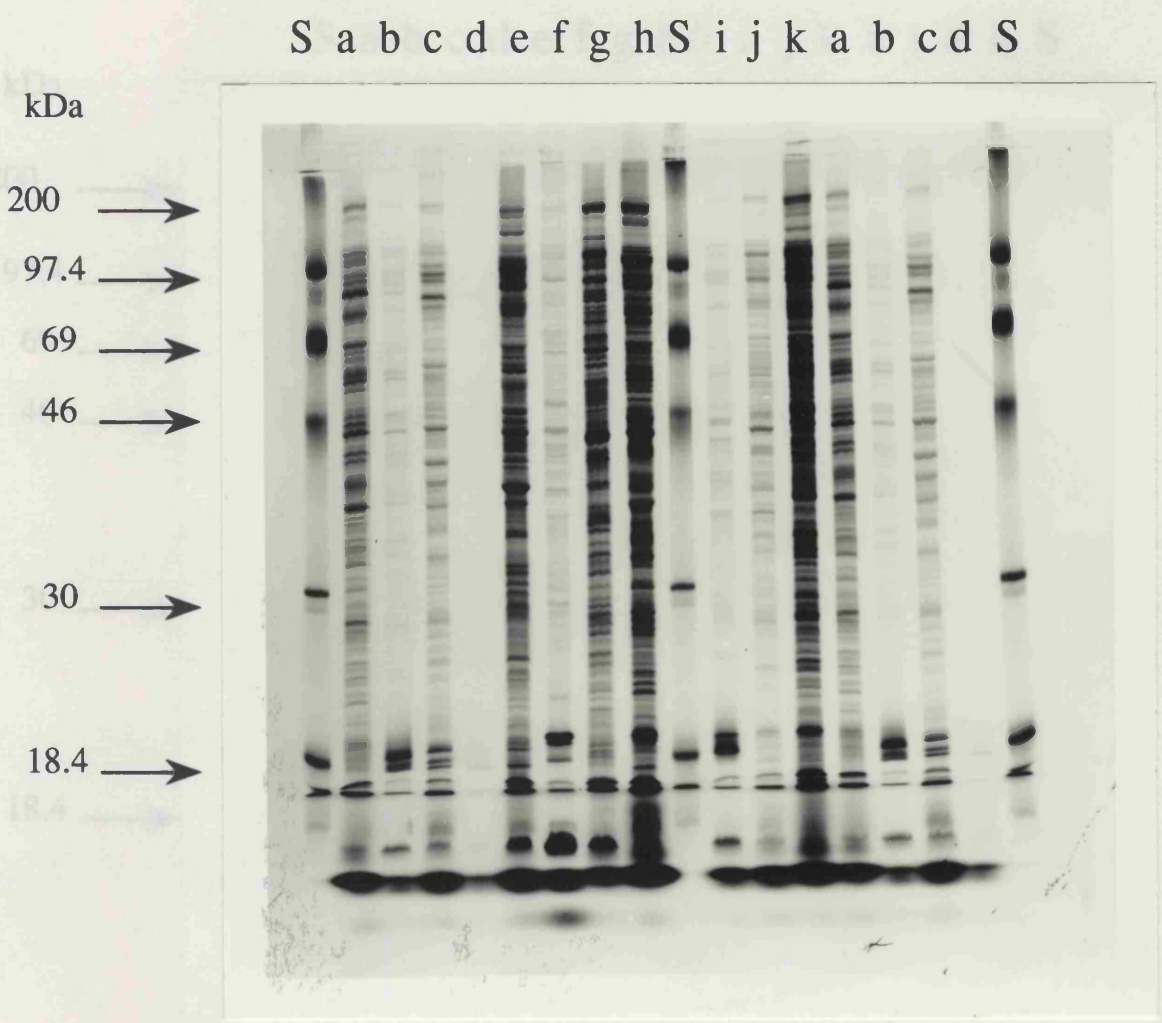
7. The extraction method for clinical specimens needs further development to test for mycobactin detection directly from the clinical specimen.

Appendix 3.1. List of the clinical isolate numbers of mycobacteria from the Dulwich culture colection.

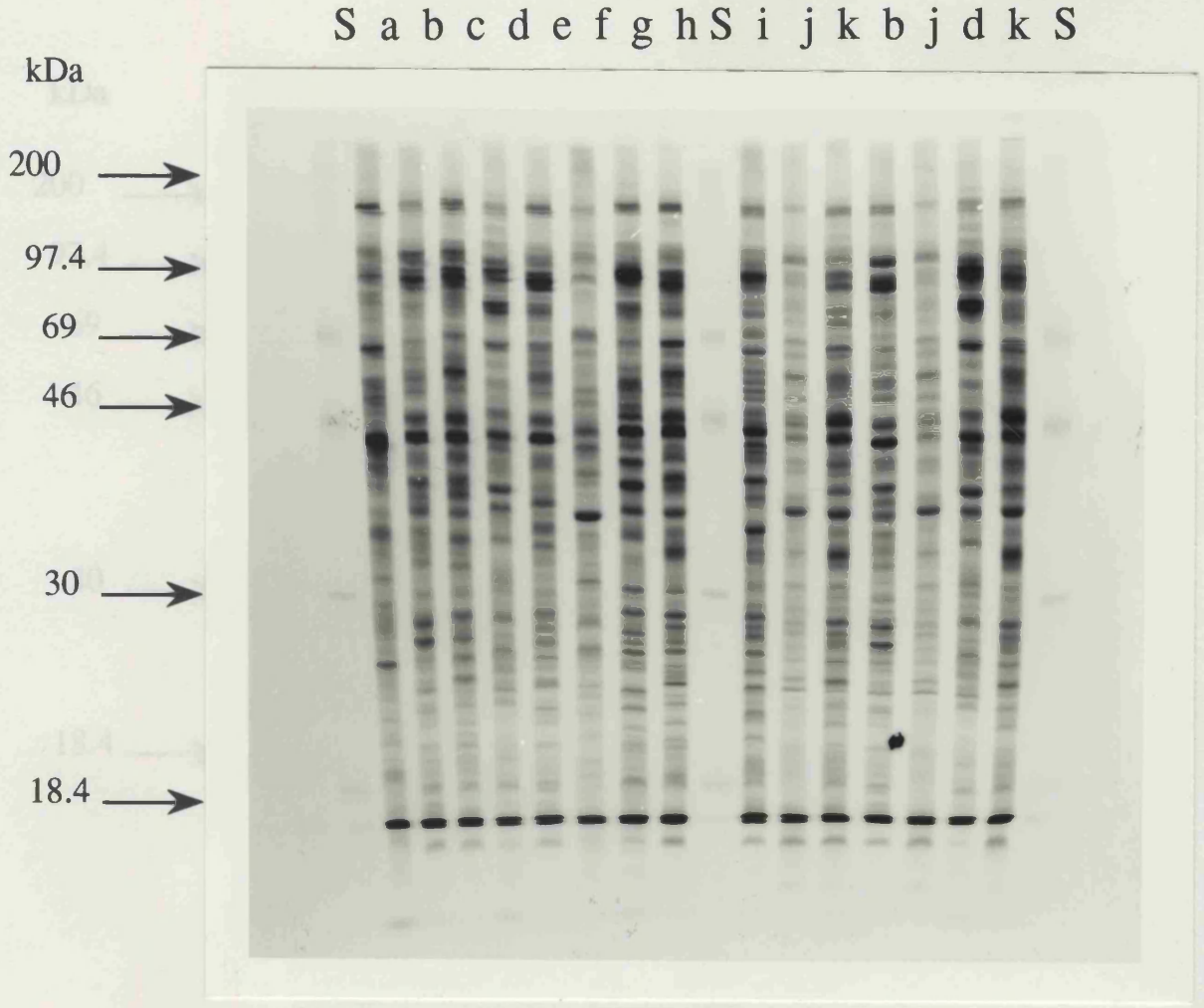
Letter code, isolate number.

M. tuberculosis Appendix 4.20	*M.avium* Appendix 4.21	*M.chelonei* Appendix 4.24	*M.kansasii* Appendix 4.22
a, 00513	a, 00488	a, 00656	a, 00690
b, 01093	b, 00499	b, 00560	b, 00740
c, 00500	c, 00502	c, 00681	c, 00786
d, 01069	d, 00503	d, 01317	d, 00505
e, 01100	e, 00609	e, 01429	e, 00584
f, 01112	f, 00233	f, 02700	f, 00480
g, 01312	g, 00270	g, 02691	g, 00432
h, 00373	h, 00274	h, 02884	h, 00333
i, 00203	i, 00753	i, 02699	i, 01112
j, 00213	j, 01057	j, 02690	j, 00898
k, 00283	k, 01308	k, 02829	k, 00053
l, 00373	l, 01327	l, 02690	l, 00462
m, 01158	m, 01678	m, 02672	m, 01488
n, 00955	n, 01937	n, 01411	n, 00615
o, 00509	o, 01827	o, 01482	o, 00613
p, 00858	p, 01834	p, 01413	p, 00676
q, 01048	q, 01947	q, 01424	q, 00651
r, 01159	r, 00442	r, 01330	r, 00677
t, HRv37	t, 00432	t, 01229	t, 00448
u, 0112o	u, 00443	u, 00707	u, 00480
v, HRa37			

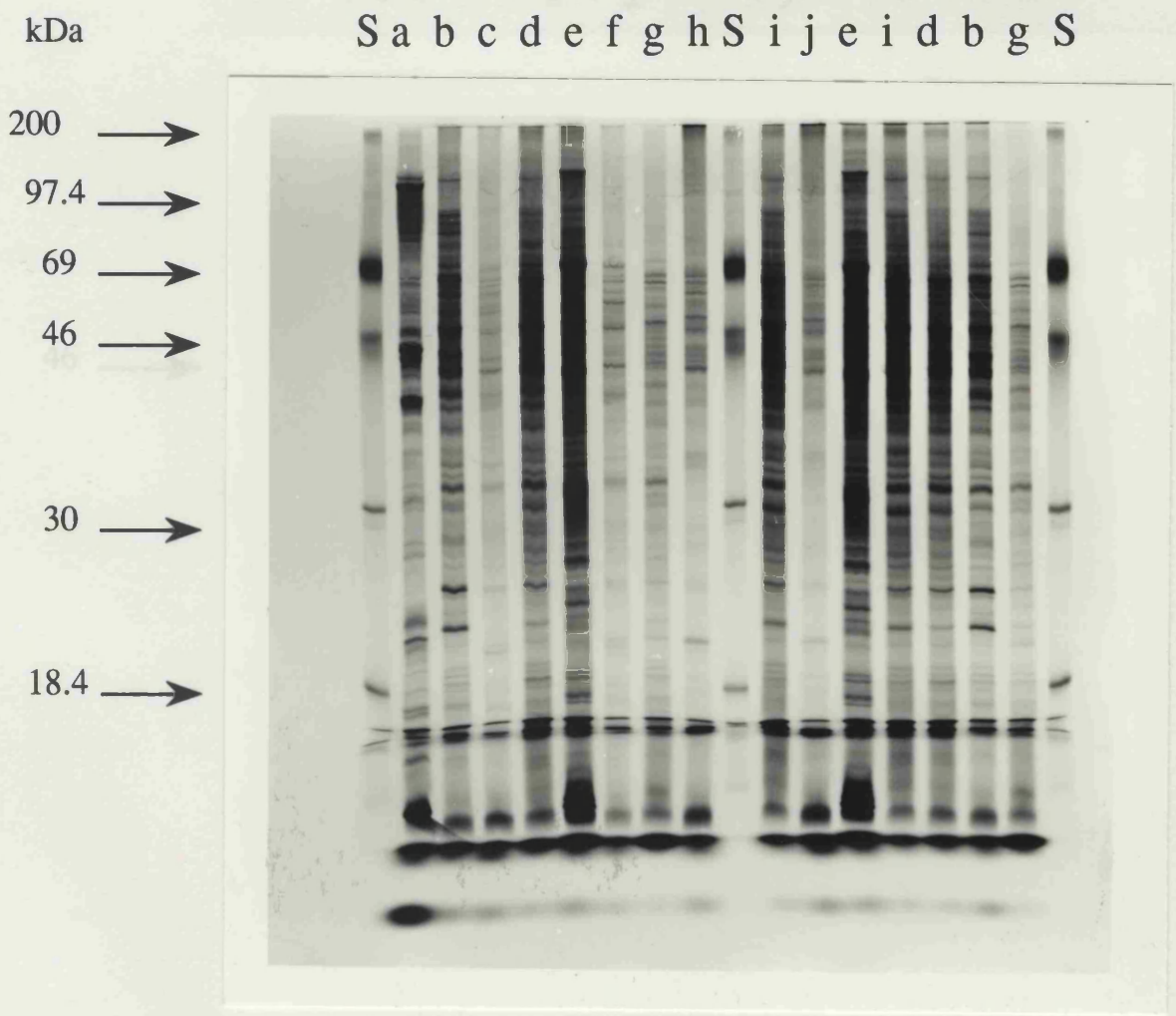
M.fortuitum Appendix 4.23	*Nocardia* spp. Appendix 4.23	*M.tuberculosis* Appendicies 4.12, 4.13 & 4.14
a, 00785	j, 00600	a, 80148
b, 00677	k, 00679	b, 80157
c, 01348	l, 00543	c, 80226
d, 00187	m, 00501	d, 80313
e, 01483	n, 01463	e, 80493
f, 01423	o, 00023	f, 80798
g, 00132	p, 01553	g, 01063
h, 00508		h, 80085
i, 00602		i, 80119
		j, 80709
M.malmoense	*M.xenopi*	k, 80873
Appendix 4.18	Appendix 4.18	l, 00396
		m, 80006
Ma, 11298	X, 00457	n, 00699
Ma',00301	X',00396	o, 00823



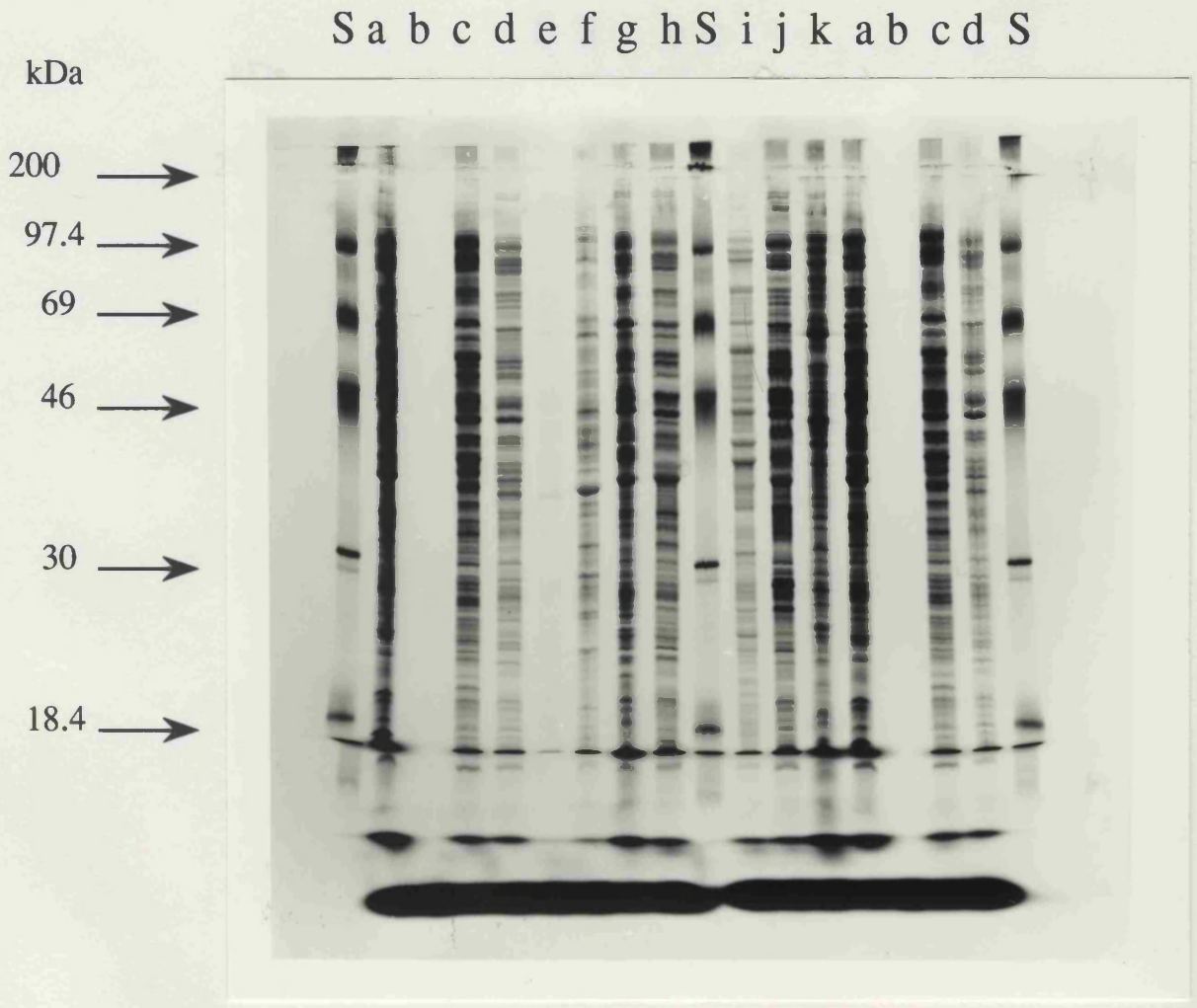
Appendix 4.1. Whole cell proteins of Gram-negative organisms labelled with [^{35}S] thio ATP for two hours: S, molecular weight standards; a, *Klebsiella* sp.; b, *Citrobacter* sp.; c, *Proteus* sp.; d, *Salmonella* sp.; e, *Morganella* sp.; f, *Pasteurella* sp.; g, *Pseudomonas* sp.; h, *Escherichia coli*; i, *Hafnia* sp.; j, *Shigella* sp.; k, *Enterobacter* sp..



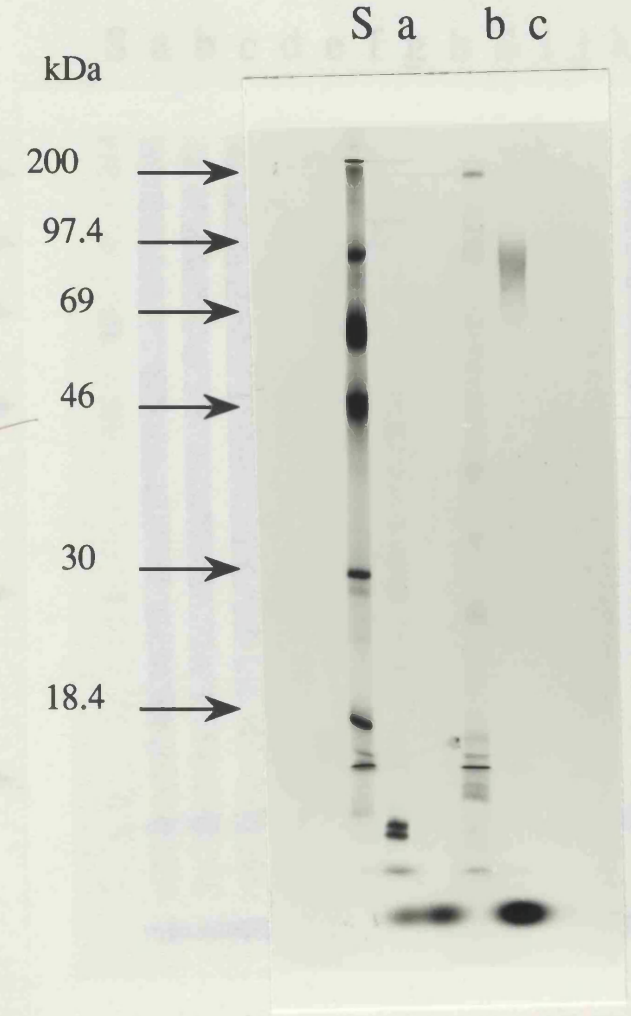
Appendix 4.2. Whole cell proteins of Gram negative organisms labelled with [^{35}S] methionine for three days: S, molecular weight standards; a, *Pseudomonas* sp.; b,*Escherichia coli*; c, *Salmonella* sp.; d, *Morganella* sp.; e, *Shigella* sp.; f, *Pasteurella* sp.; g, *Proteus mirabillis*; h, *Klebsiella* sp.; i, *Hafnia* sp.; j, *Citrobacter* sp.; k,*Enterobacter* sp..



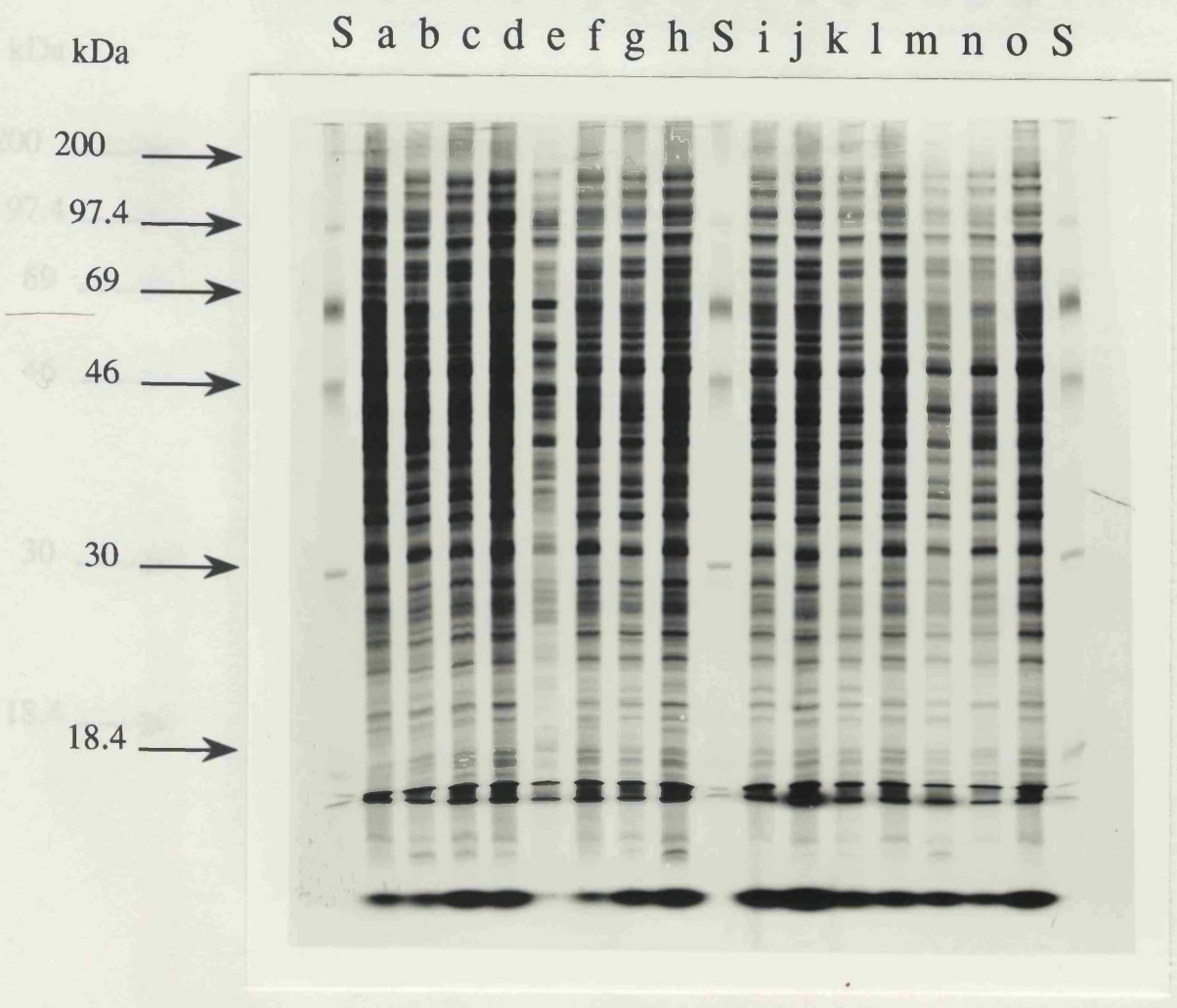
Appendix 4.3. Whole cell proteins of Gram-positive organisms labelled with [^{35}S] thio ATP for three days: S, molecular weight standards; a and e, *Corynebactium* sp.; b and i, *Staphylococcus aureus*; c and f, *S.epidermidis*; d and g, *S.aureus* MRSA; h and j, *S.haemolyticus*.



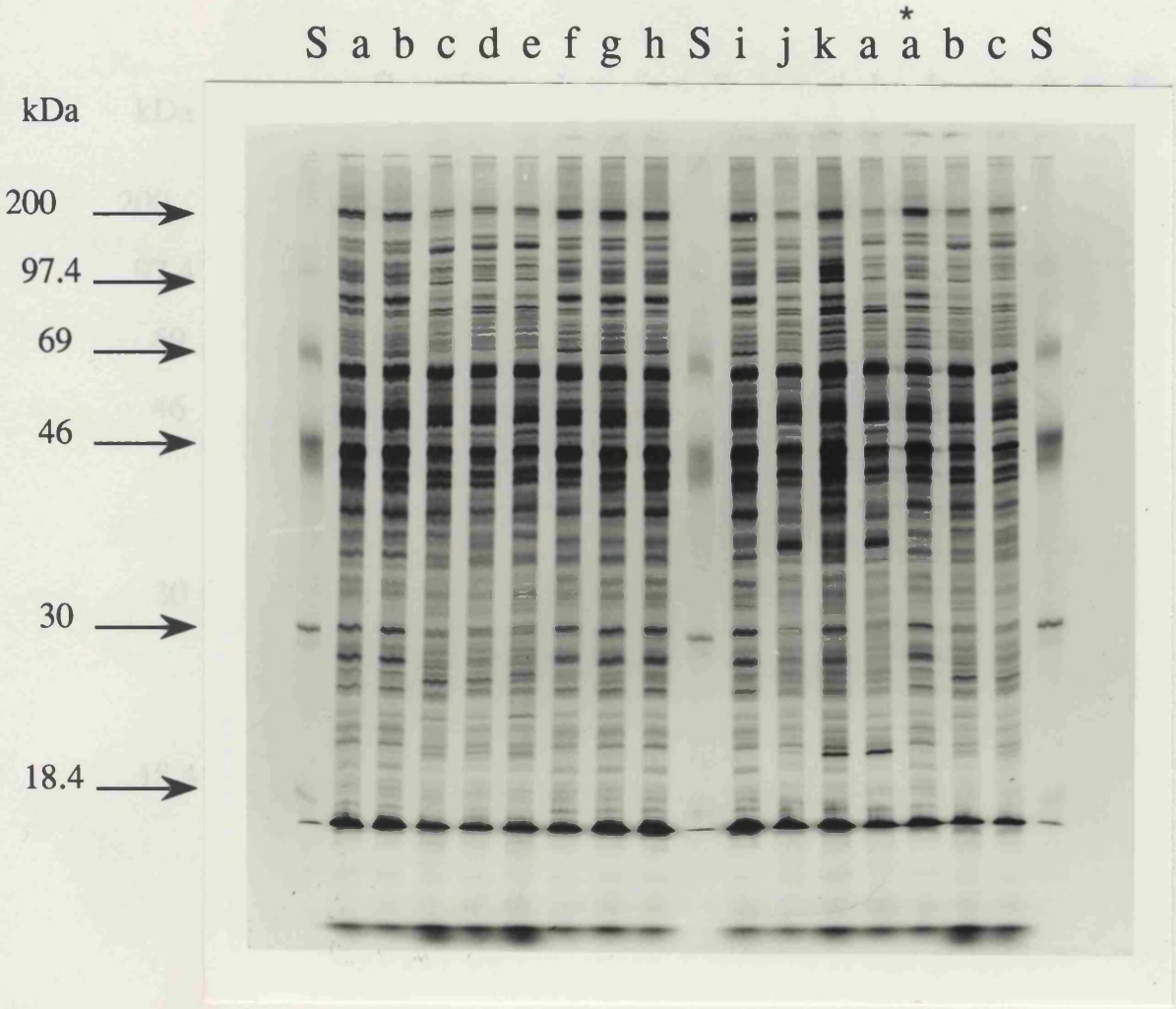
Appendix 4.4. Whole cell proteins of Gram-negative organisms labelled with [^{35}S] inorganic sulphate for three days: S, molecular weight standards; a, *Enterobacter* sp.; b, *Morganella* sp.; c, *Salmonella* sp.; d, *Shigella* sp.; e, *Hafnia* sp.; f, *Pasteurella* sp.; g, *Klebsiella* sp.; h, *Citrobacter* sp.; i, *Proteus mirabilis*; j, *Escherichia coli*; k, *Pseudomonas* sp..



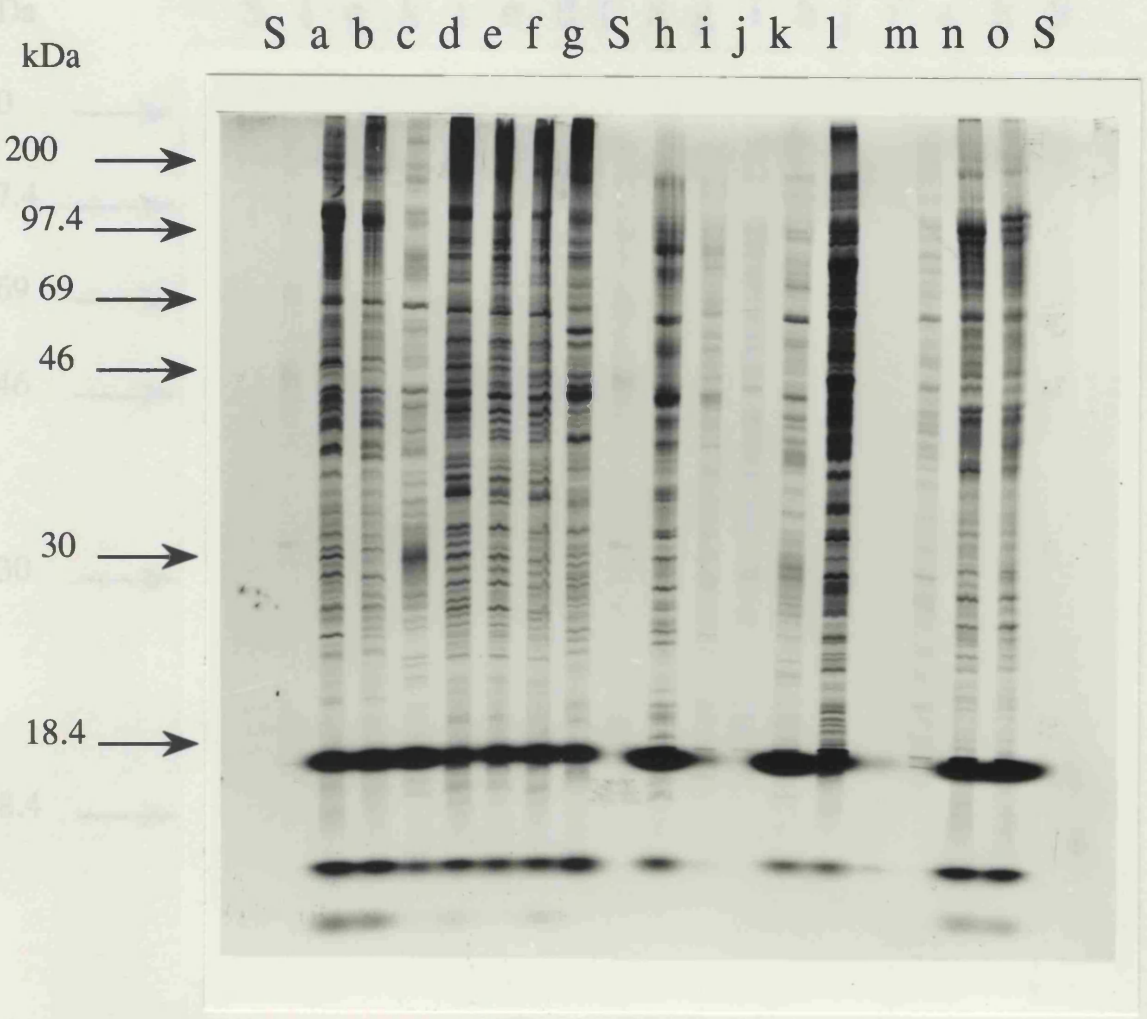
Appendix 4.5. Whole cell proteins of Gram-positive organisms labelled with [^{35}S] inorganic sulphate for three days: S, molecular weight standards; a and c *Corynebactium* sp.; b, *Enterobacter* sp..



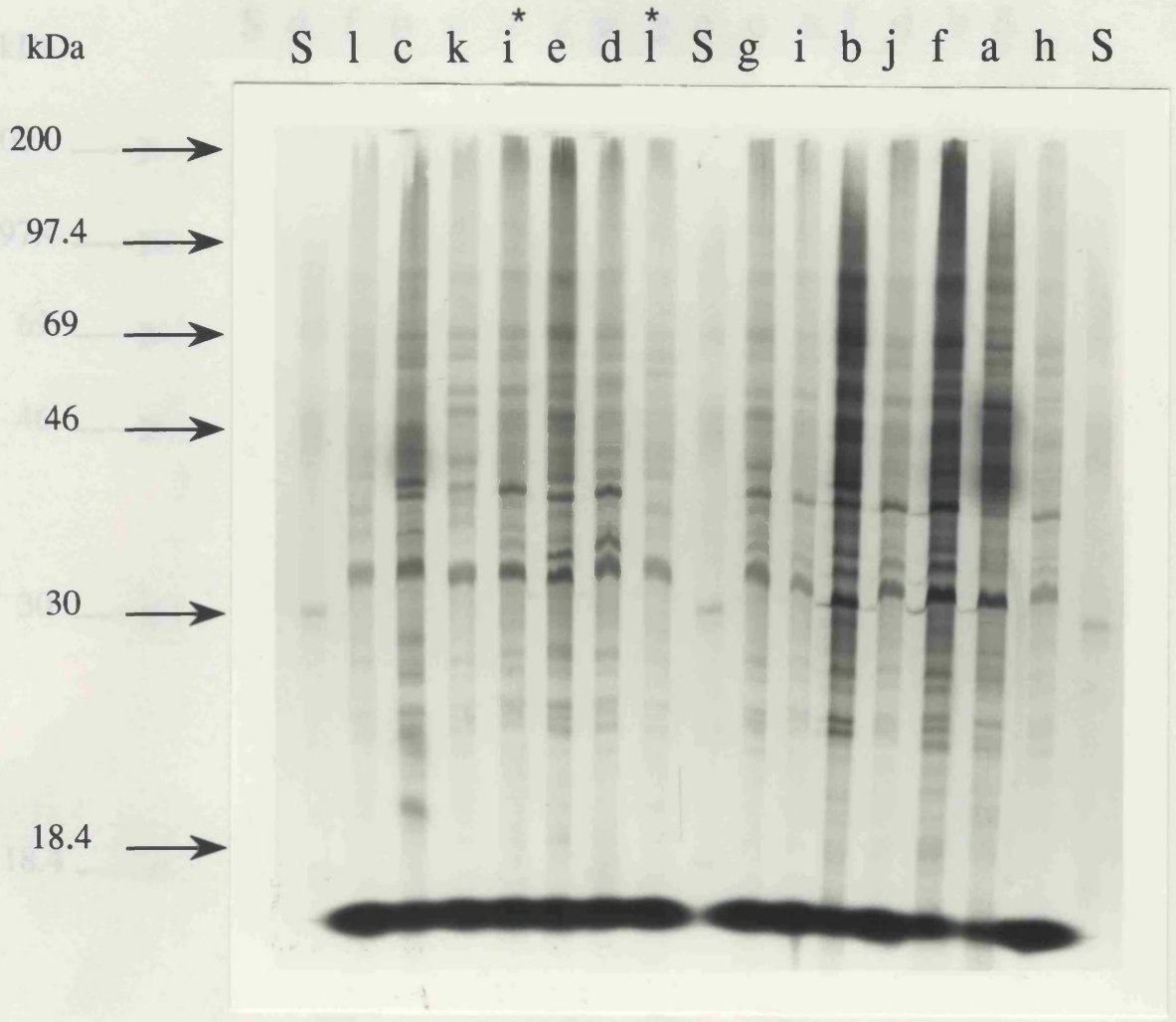
Appendix 4.6. Whole cell proteins of [^{35}S] methionine labelled *Staphylococcus haemolyticus*: S, molecular weight markers; a to d, teicoplanin resistant isolates of *S.haemolyticus* ; e to o, teicoplanin sensitive isolates of *S.haemolyticus*.



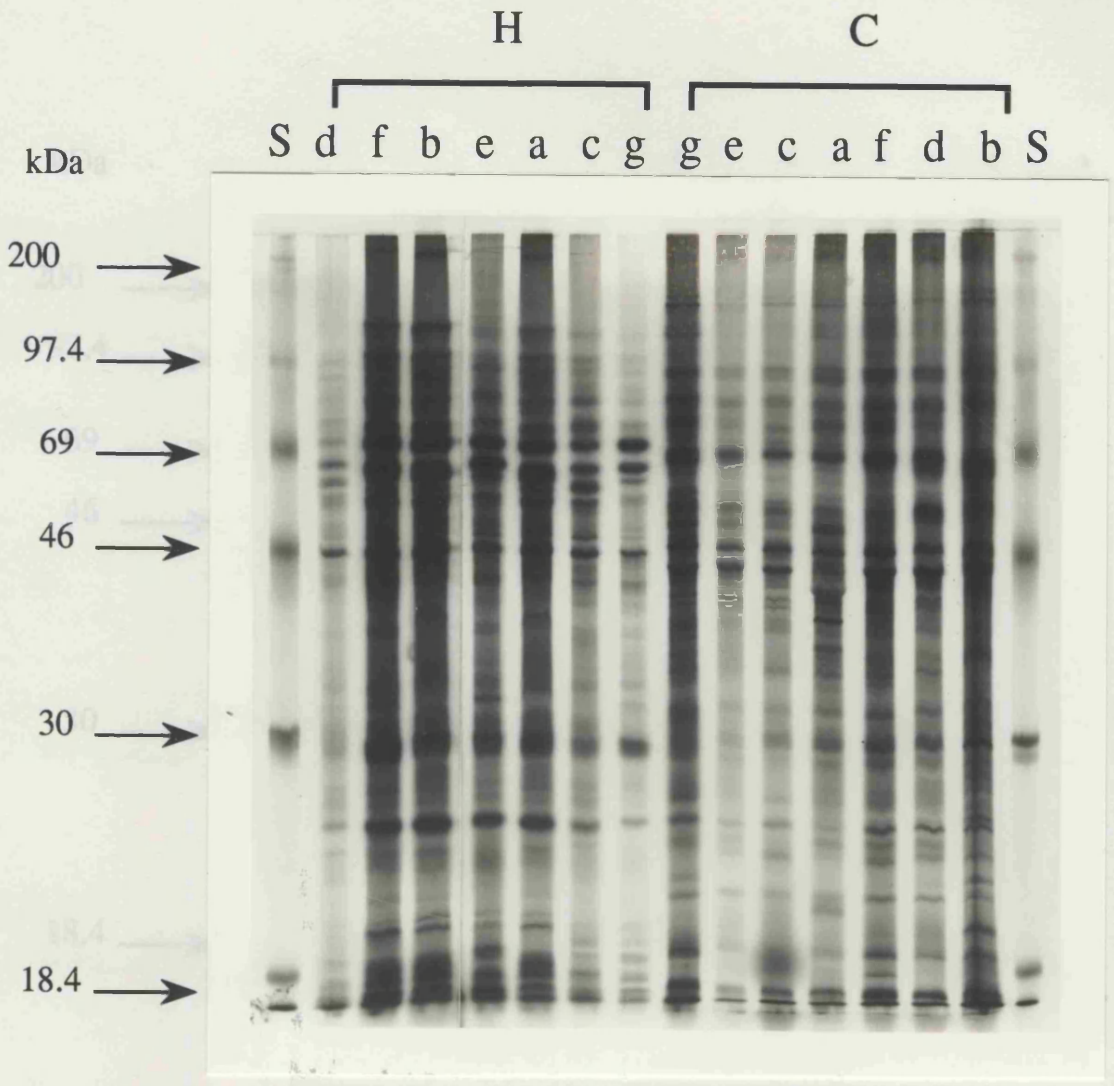
Appendix 4.7. Whole cell proteins of *Pseudomonas pseudomallei* isolates labelled with [^{35}S] methionine: S, molecular weight standards; a to f, Australian isolates; g to k, Vietnamese isolates. Identical lettering indicates the same sample run in duplicate; * indicates the same isolate labelled separately.



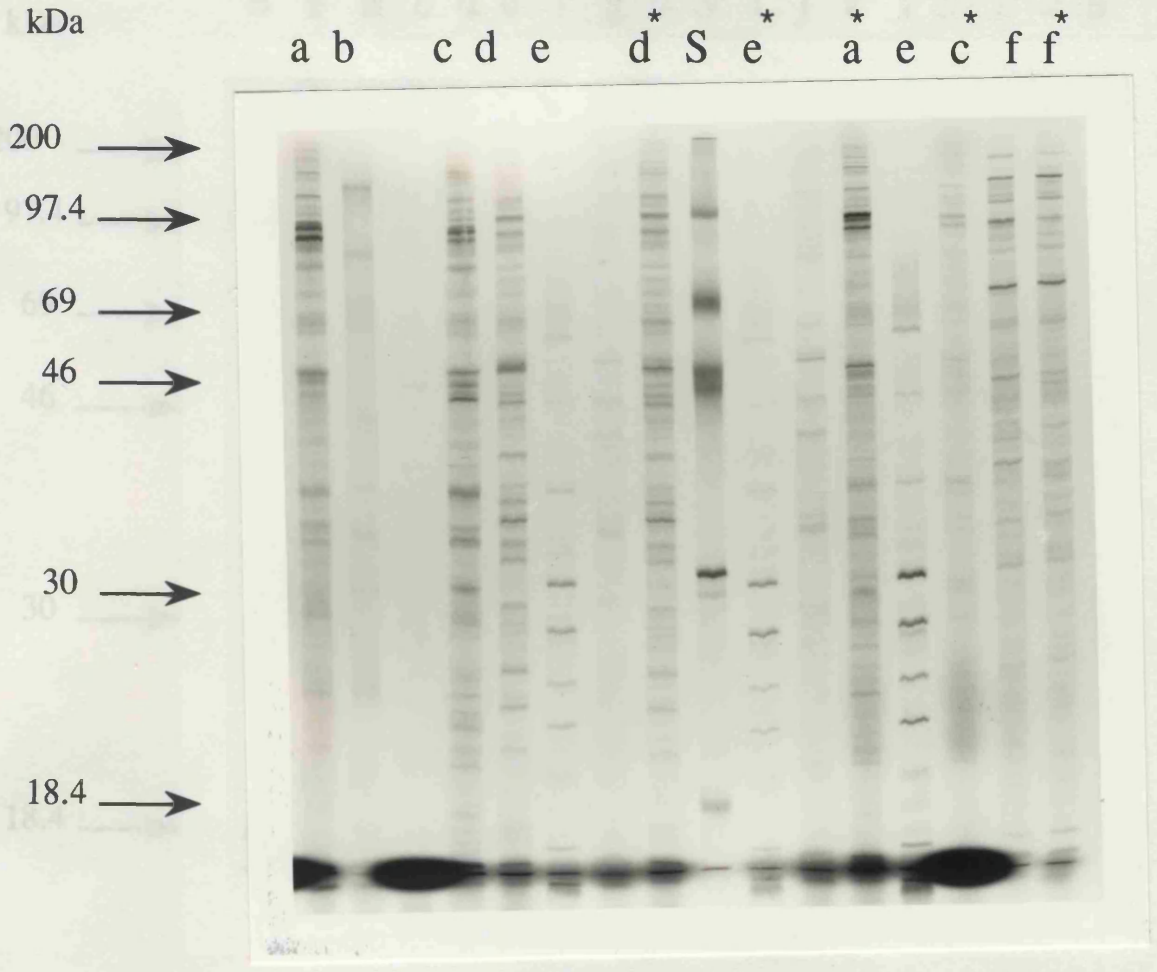
Appendix 4.8. Whole cell proteins of various *Pseudomonas* species labelled with [^{35}S] methionine: S, molecular weight standard; a, b, n and o, *P.maltophilia*, NF58, NF24, NF119 and NF48; c and k, *P.paucimobilis*, NF60 and NF284; d, e and f, *P.aeruginosa*, K1, K3 and K4; g, h and i, *P.stutzeri* Z199, Z152 and NF271; j, *P.putrifaciens*, NF270; l and m, *P.cepacia*, NF62 and NF144.



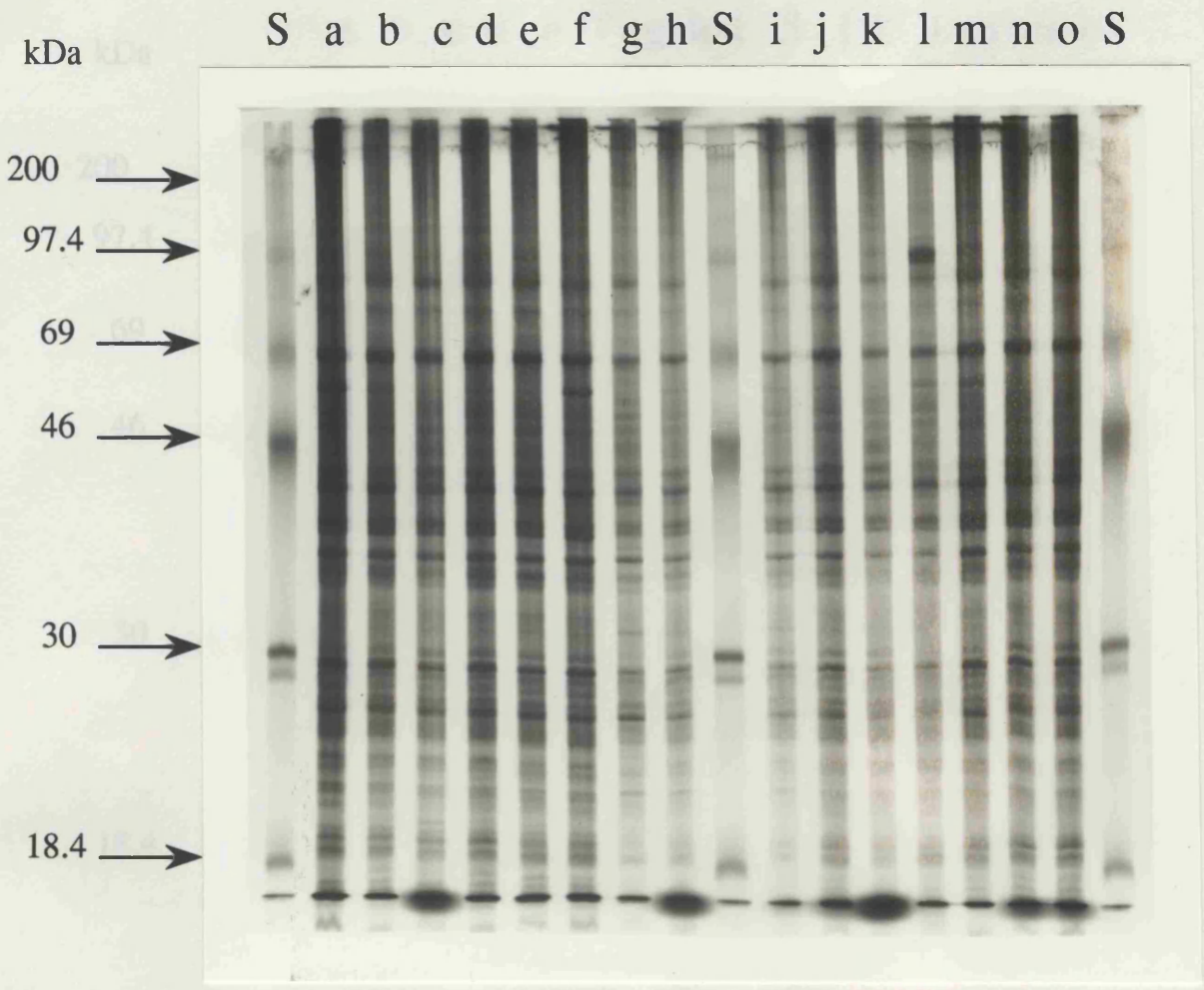
Appendix 4.9. Whole cell proteins of spirochete isolates labelled with [^{35} S] methionine: a to e and g to l, spirochete isolates; k, *Serpula hyodisenteriae* ; S, molecular weight standard; * indicates the same isolate labelled separately.



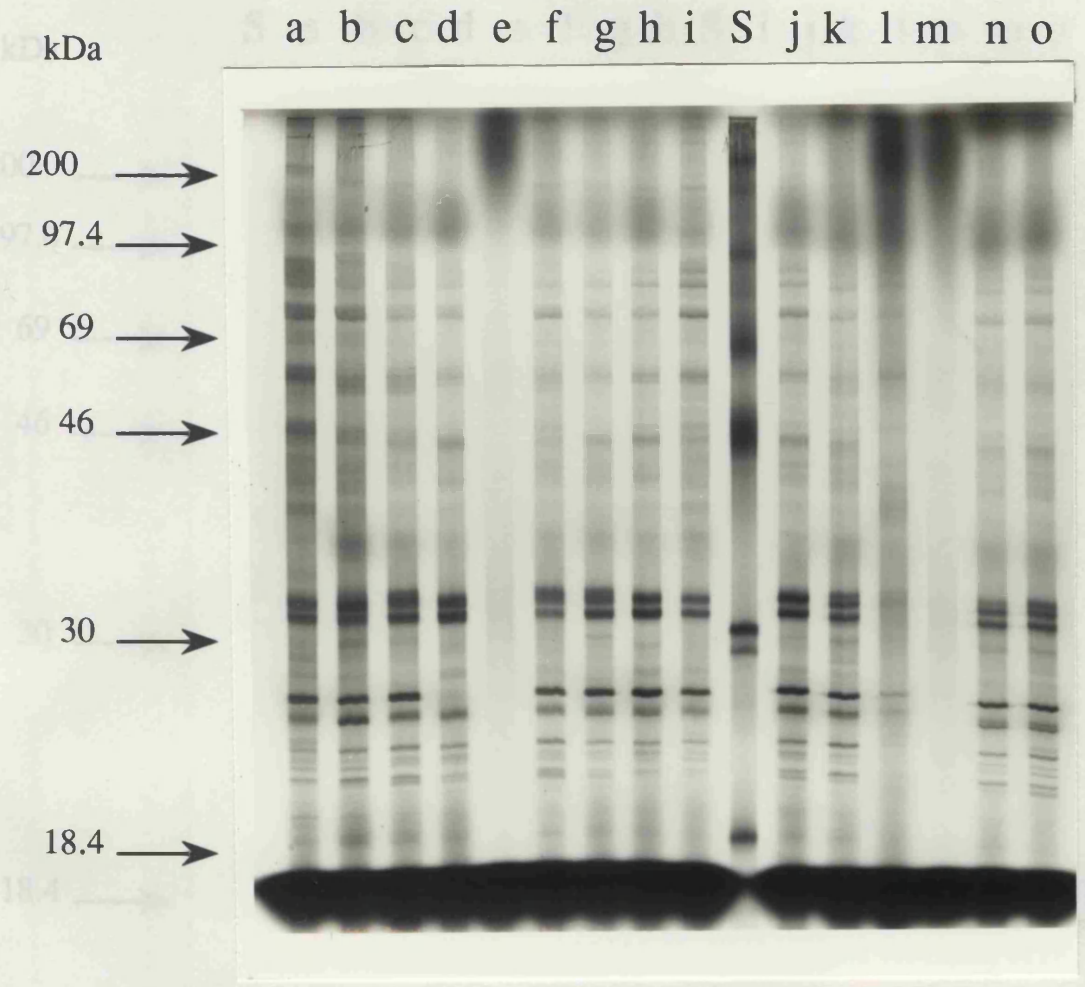
Appendix 4.10. Whole cell proteins of *Helicobacter pylori* (H), a to g, and *Campylobacter jejuni* (C), a to g, labelled with [^{35} S] methionine: S, molecular weight standards.



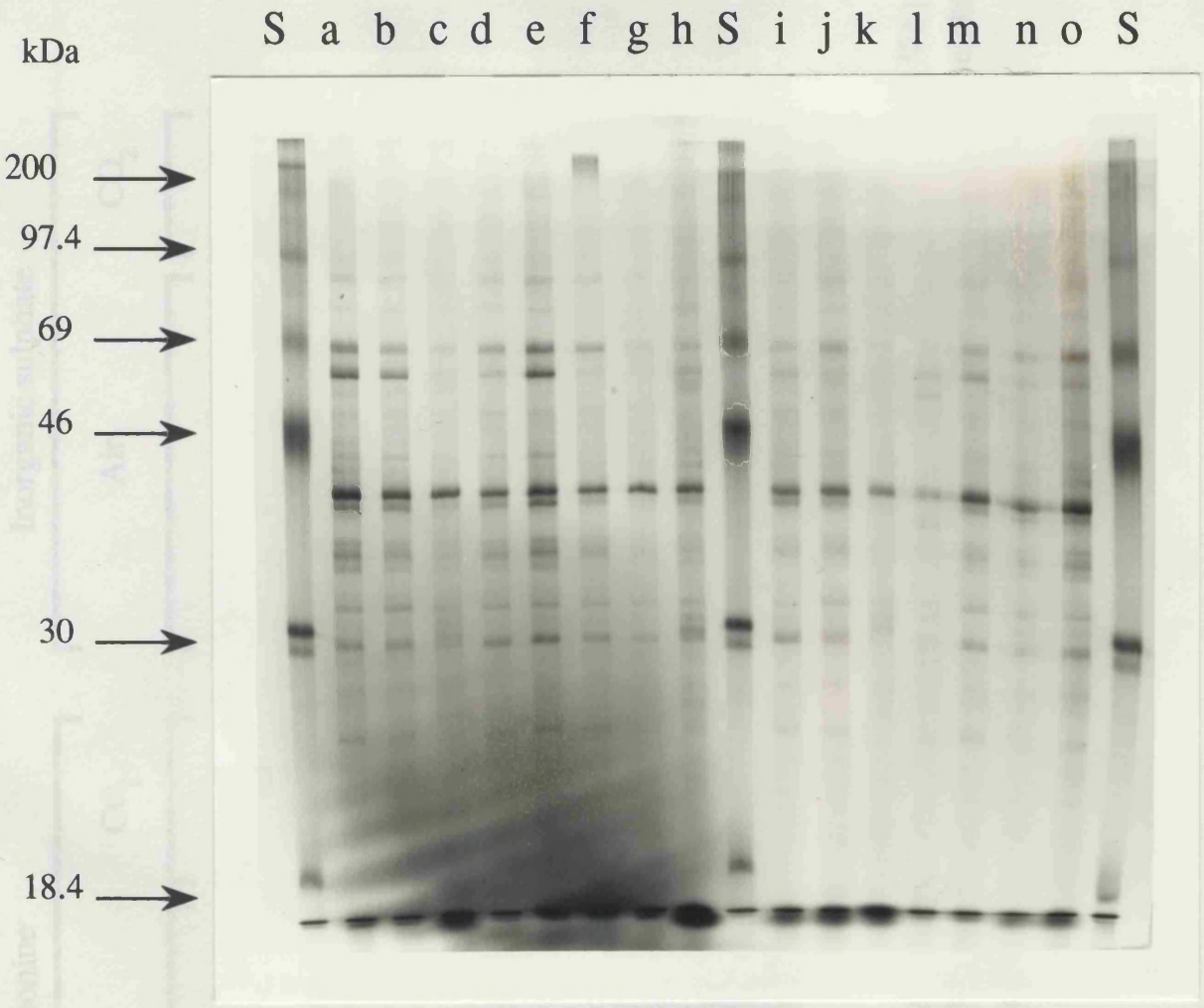
Appendix 4.11. Secreted proteins of various mycobacterial species labelled with [^{35} S] methionine: S, molecular weight standards; a and c, *M.gilvum*; b, *M.duvalii*; d and f, *M.vaccae* (d, R877R NCTC 11659); e, *M.flavescens*; * indicates the same isolate labelled separately.



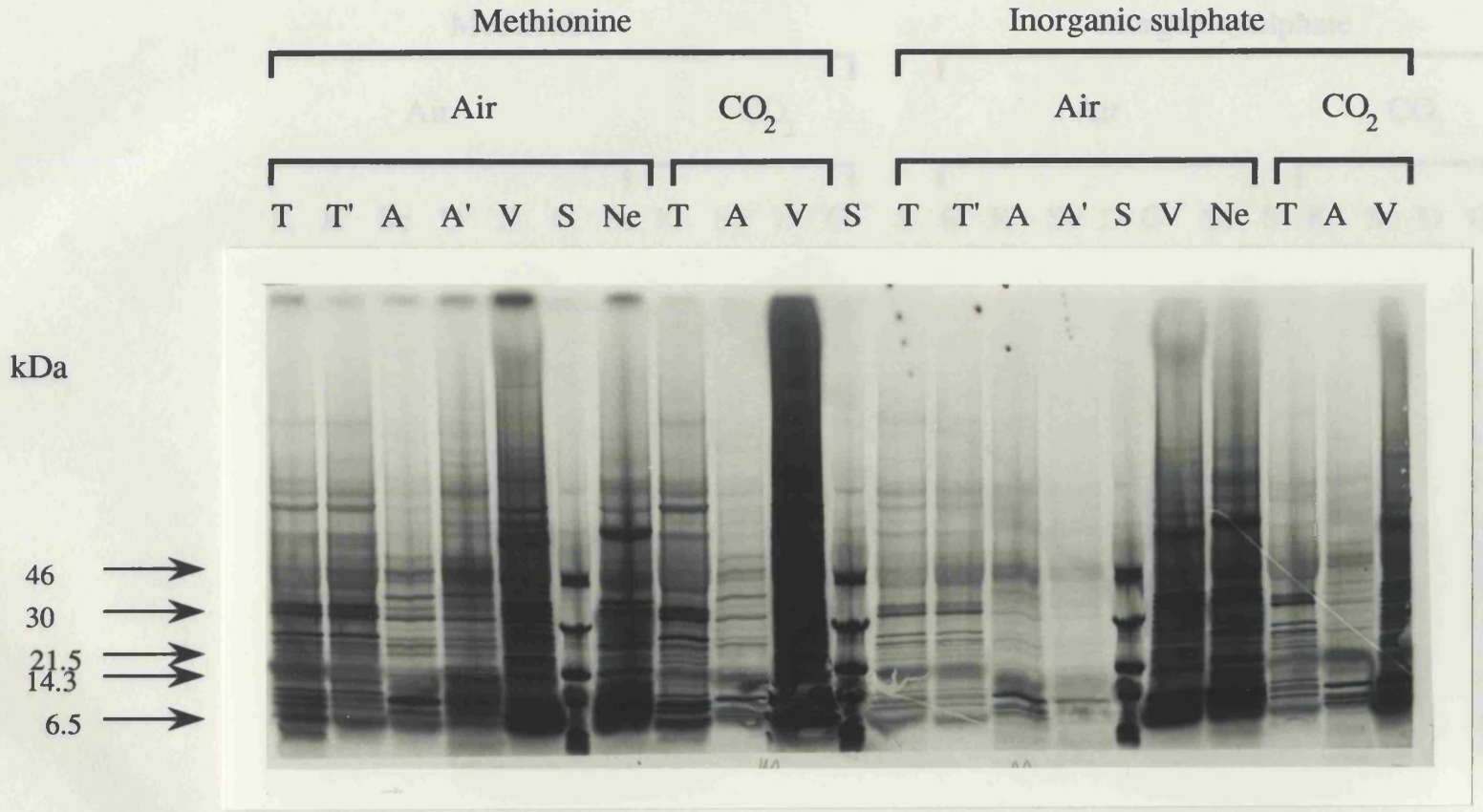
Appendix 4.12. Whole cell proteins of *Mycobacterium tuberculosis* labelled with [^{35} S] inorganic sulphate: S, molecular weight standards; a to f, isoniazid resistant isolates; g to k, streptomyocin resistant isolates; l and m, pyrozinamide resistant isolates; n and o, drug sensitive isolates.



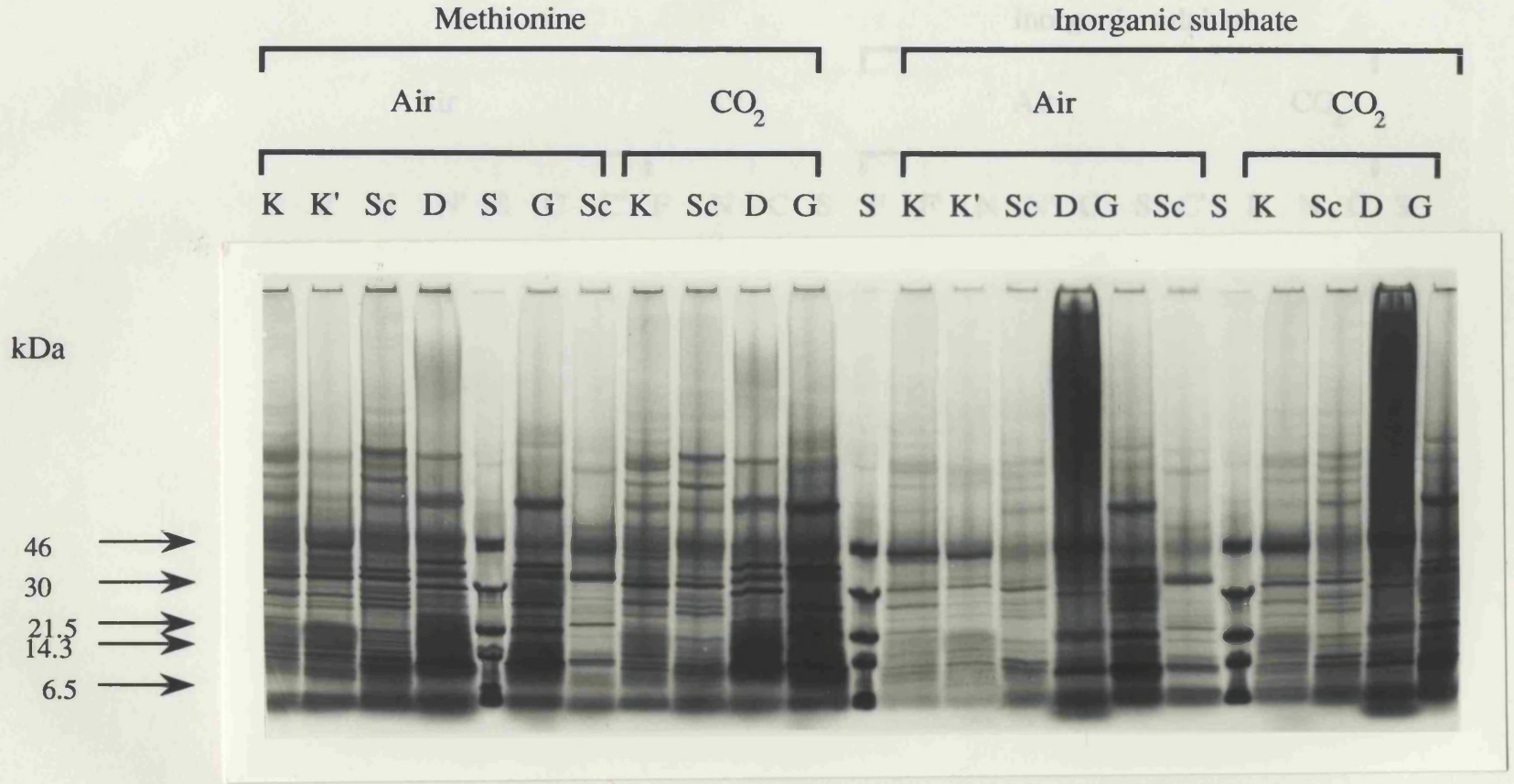
Appendix 4.13. Secreted proteins of *Mycobacterium tuberculosis* labelled with [^{35} S] methionine: S, molecular weight standards; a to f, isoniazid resistant isolates; g to k, streptomyocin resistant isolates; i and m, pyrozinamide resistant isolates; n and o, drug sensitive isolates.



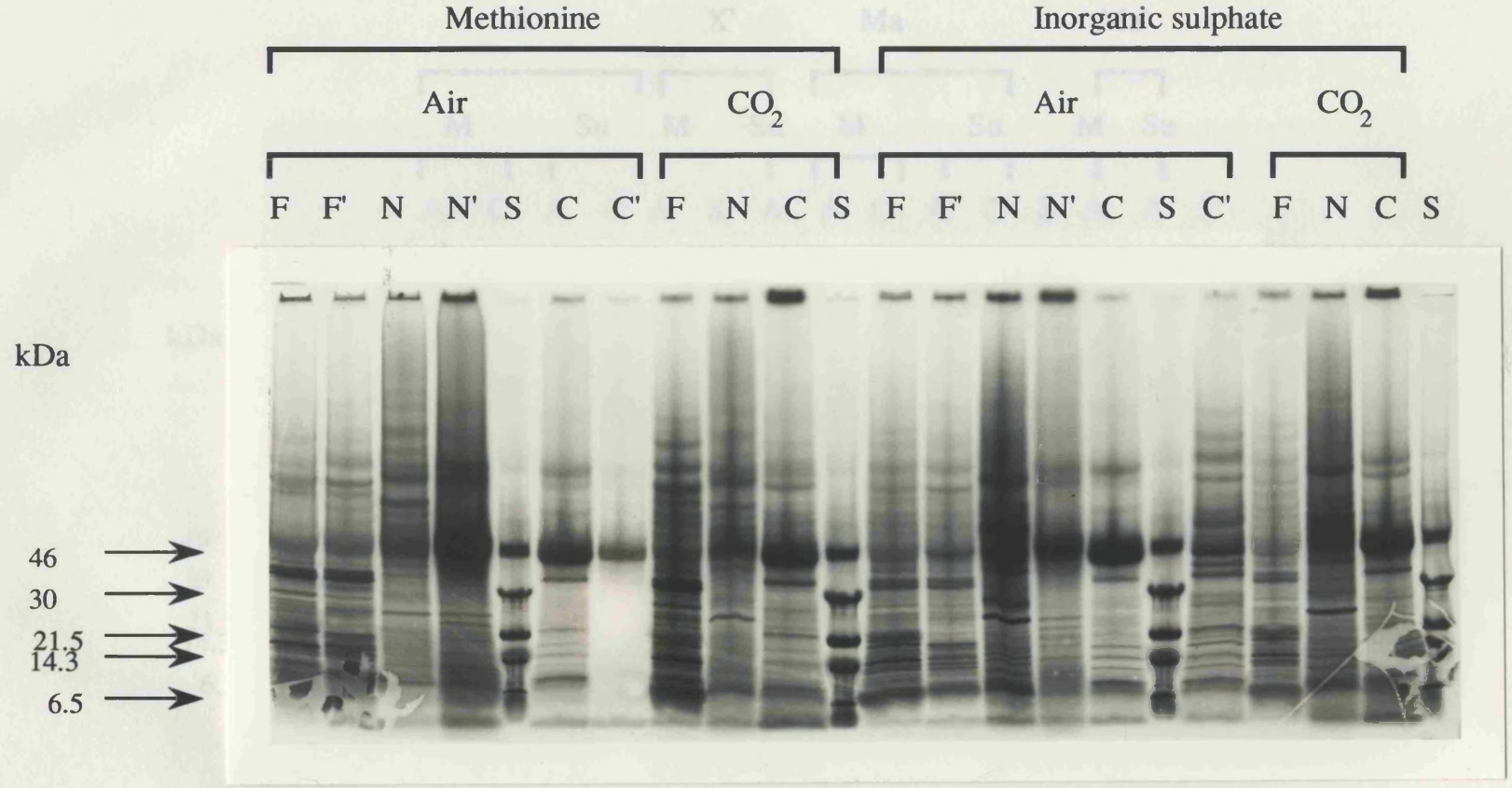
Appendix 4.14. Secreted proteins of *Mycobacterium tuberculosis* labelled with [^{35} S] inorganic sulphate: S, molecular weight standards; a to f, isoniazid resistant isolates; g to k, streptomyocin resistant isolates; i and m, pyrozinamide resistant isolates; n and o, drug sensitive isolates.



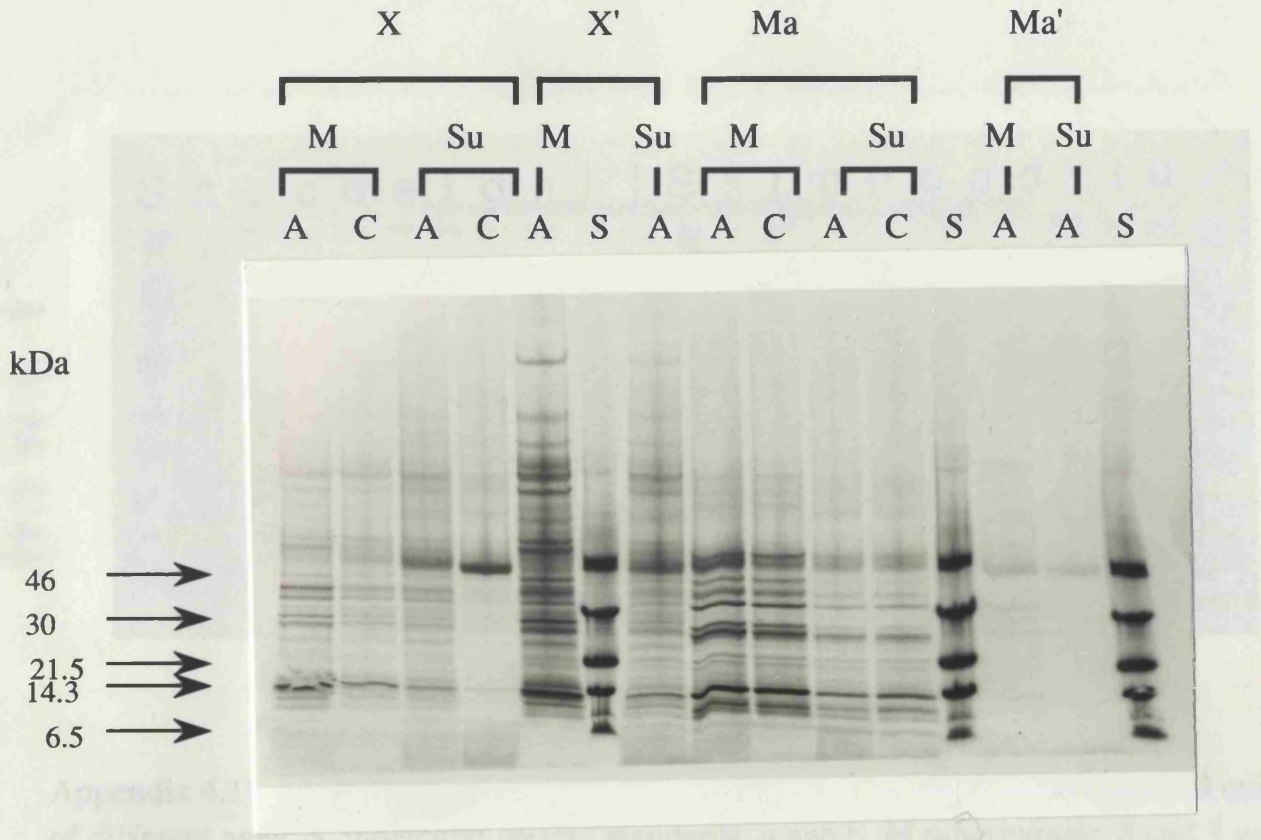
Appendix 4.15. Whole cell proteins of various mycobacterial species labelled with both [^{35}S] inorganic sulphate and [^{35} S] methionine in both air and a CO_2 incubator: S, molecular weight standards; T and T', *M.tuberculosis*; A and A', *M.avium*; V, *M.vaccae*; Ne, *M.neoaurum*..



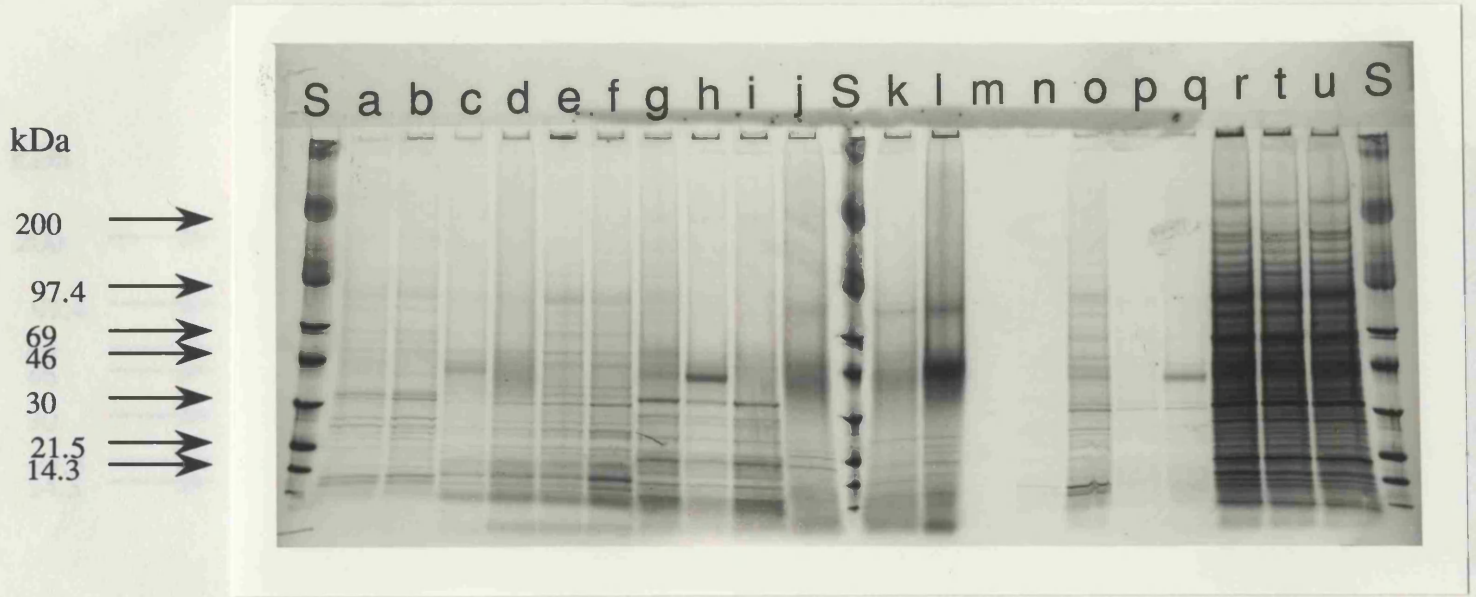
Appendix 4.16. Whole cell proteins of various mycobacterial species labelled with both [^{35}S] inorganic sulphate and [^{35} S] methionine in both air and a CO_2 incubator: S, molecular weight standards; K and K', *M.kansasii* isolates; Sc, a scotochromogenic mycobacterial isolate; G, *M.gilvum*; D, *M.duvalii*.



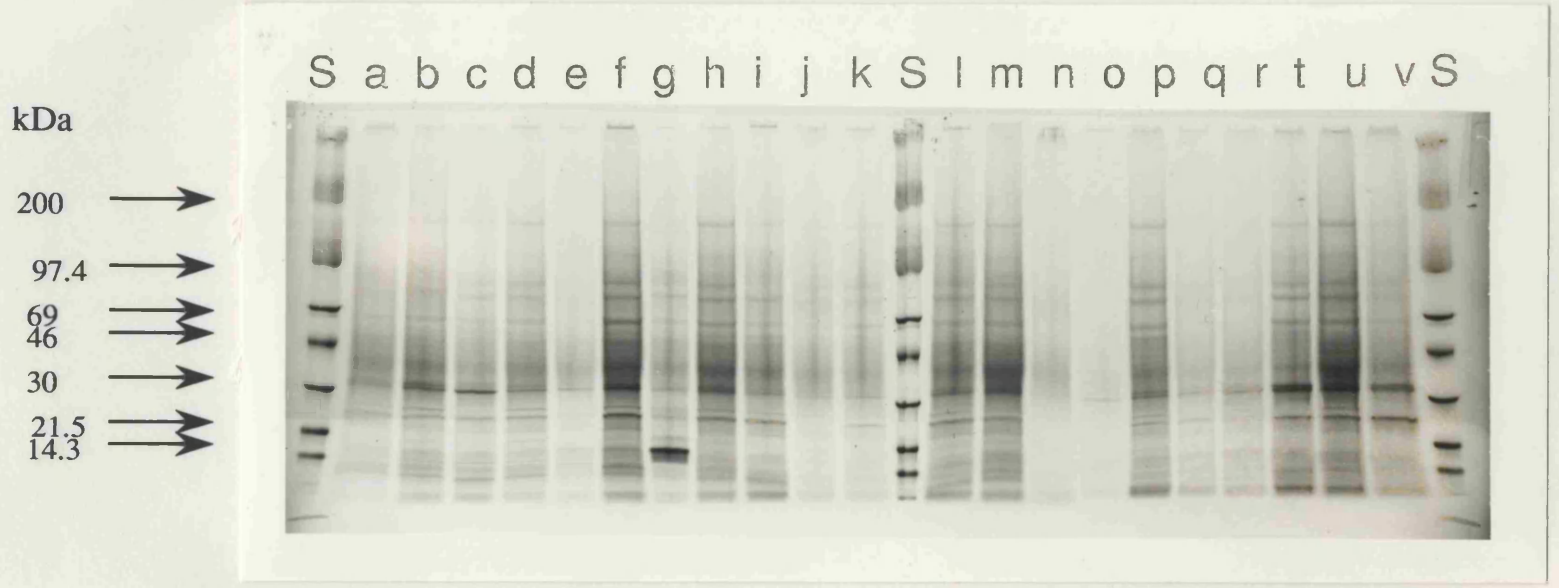
Appendix 4.17. Whole cell proteins of various mycobacterial species labelled with both [^{35} S] inorganic sulphate and [^{35} S] methionine in both air and a CO_2 incubator: S, molecular weight standards; F and F', *M.fortuitum* isolates; N and N', *Nocardia* sp.; C and C', *M.chelonei*.



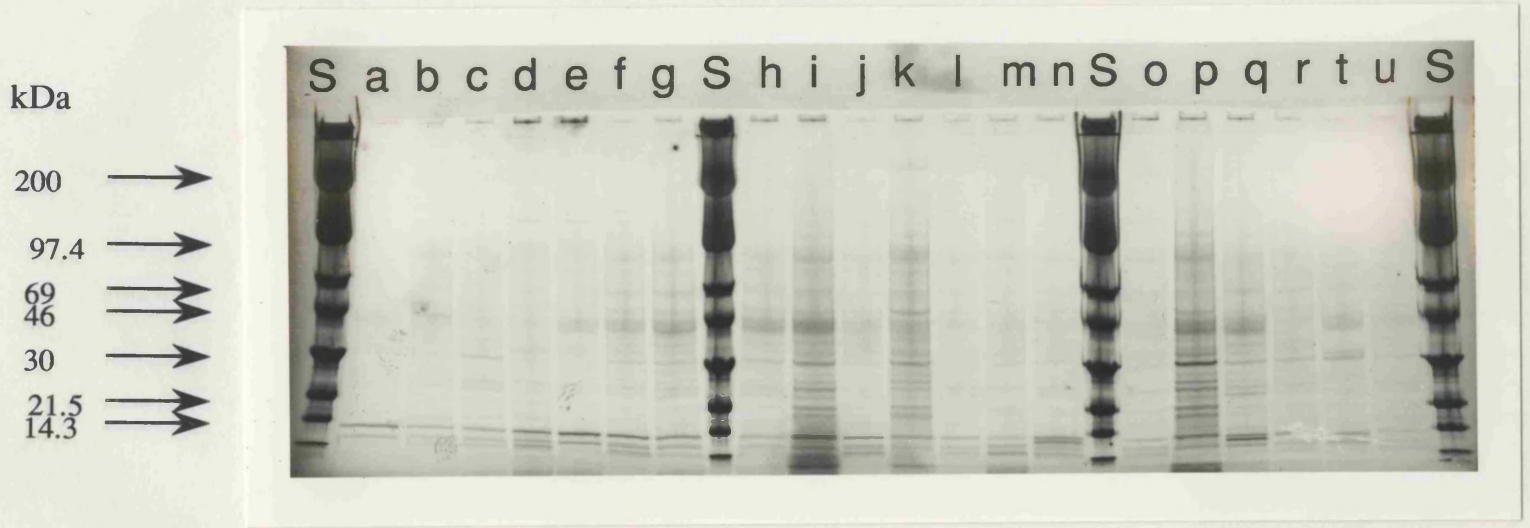
Appendix 4.18. Whole cell proteins of various mycobacterial species labelled with both [^{35} S] inorganic sulphate (Su) and [^{35} S] methionine (M) in both air (A) and a CO_2 (C) incubator: S, molecular weight standards; X and X', *M.xenopi isolates*; Ma and Ma', *M.malmoense* isolates.



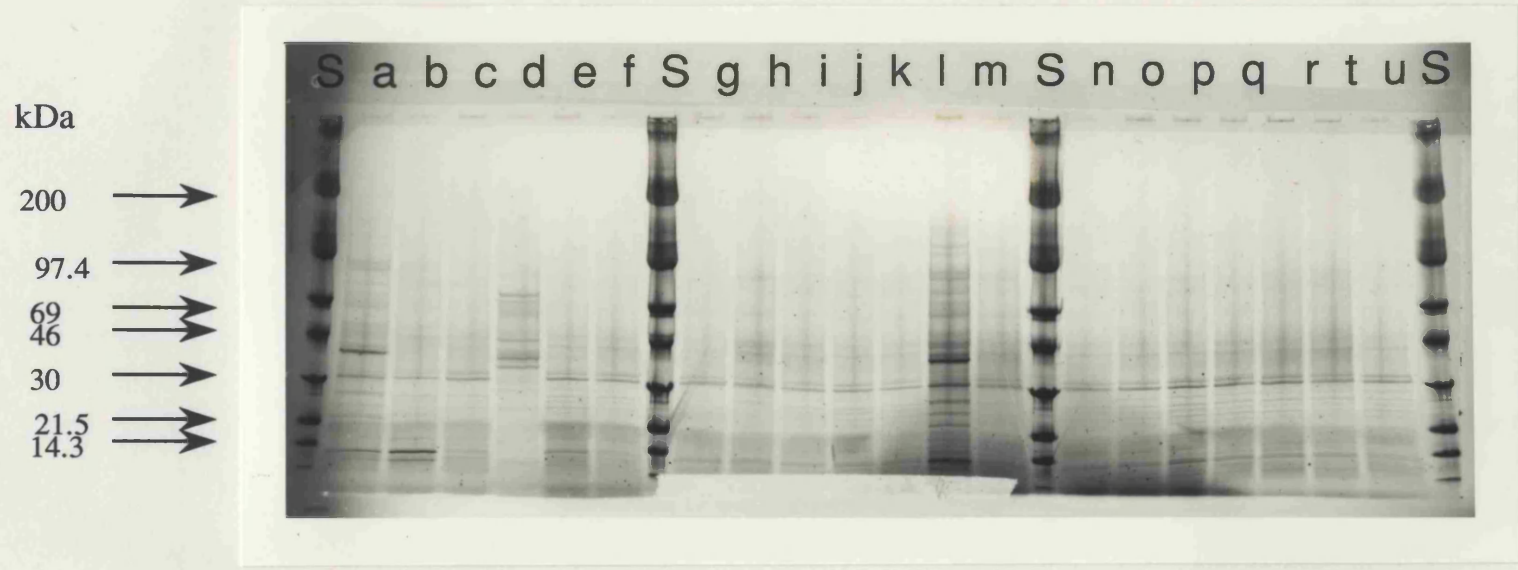
Appendix 4.19. Whole cell proteins of [^{35}S] methionine labelled mycobacterial cultures of different ages: S, molecular weight standards; a and b, *M.tuberculosis,* 8 and 3 weeks respectively; c, d, e and f, *M.avium* , 9 months, 11, 8 and 3 weeks respectively; g, h and i, *M.chelonei*, 8, 3 and 1 week respectively; j, k and l, *Nocardia* sp., 11, 8 and 3 weeks respectively; m and n, *M.xenopi*, 4 and 2 months respectively; o, p and q, *M.kansasii*, 15, 8 and 3 weeks respectively; r, t and u, *M.fortuitum,* 15, 8 and 3 weeks respectively.



Appendix 4.20. Whole cell proteins of [^{35} S] inorganic sulphate labelled *M.tuberculosis* isolates, a to v: S, molecular weight standards.

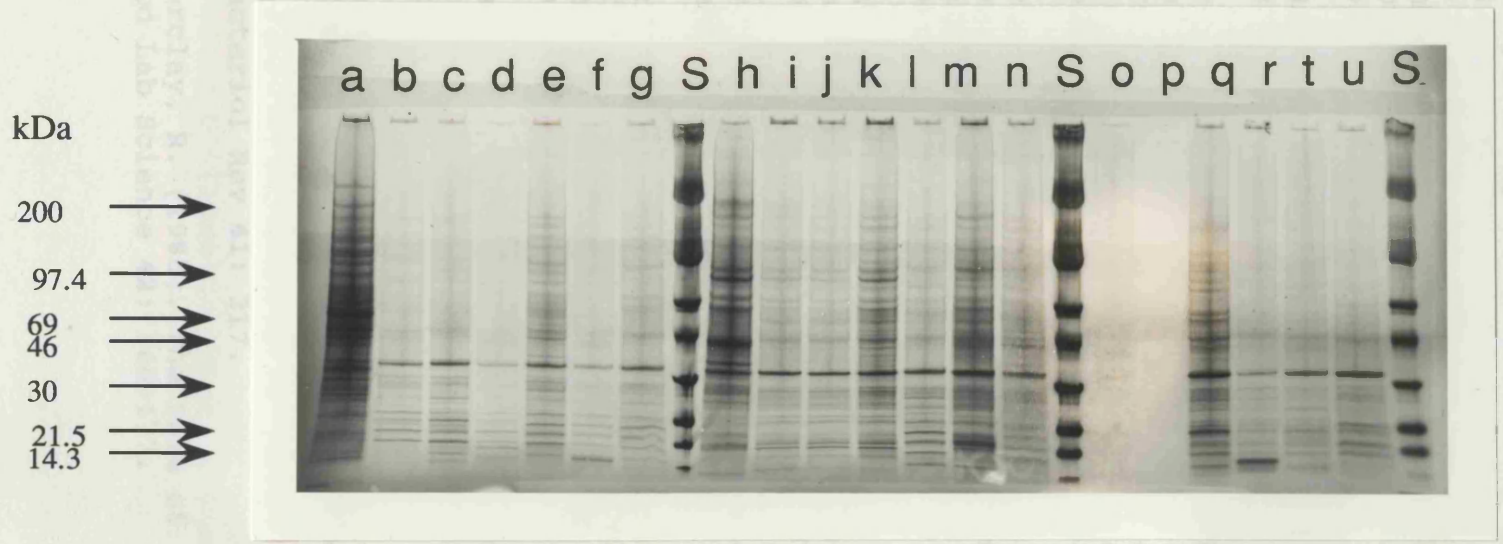


Appendix 4.21. Whole cell proteins of [^{35} S] inorganic sulphate labelled *M.avium* isolates, a to p: S, molecular weight standards.



Appendix 4.22. Whole cell proteins of [^{35} S] inorganic sulphate labelled *M.kansasii* isolates, a to u: S, molecular weight standards.

Appendix 4.23. Whole cell proteins of [^{35} S] inorganic sulphate labelled *M.fortuitum* isolates, a to i, and *Nocardia* sp., j to p: S, molecular weight standards.



Appendix 4.24. Whole cell proteins of [^{35} S] inorganic sulphate labelled *M.chelonei* isolates, a to u: S, molecular weight standards.

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