Mycobacteria and Human Disease

Second edition

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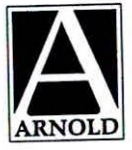

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1 Introduction

From time immemorial tuberculosis and leprosy have ranked amongst the most feared and dreaded of the numerous diseases that afflict mankind. The evangelist John Bunyan dubbed tuberculosis 'the Captain of all of these men of Death', and in India it was known as the King of Diseases. Leprosy may be termed the Disease of Kings, as Robert the Bruce, King of Scotland, and, according to legend, the emperor Constantine are numbered amongst its victims.

The discovery of the causative agent of tuberculosis by Robert Koch in 1882 led to great hopes that this disease would soon be vanquished. In 1908 Leonard Williams wrote: 'The riddle of the white plague, which had so long defied solution, had been read at last; the dreary watches of the night were over; and the dawn, with its promise of victory, peace, and purity, were really at hand'. Who would, therefore, have predicted that, 111 years after Koch's discovery, the World Health Organization, far from celebrating the eventual conquest of tuberculosis, would take the unprecedented step of declaring it a global emergency.

Leprosy had become virtually extinct in the industrially-developed nations by the early twentieth century and, following the introduction of multidrug therapy, the incidence has dropped throughout the world. Fortunately, many caring organizations have maintained their interest in this particularly cruel disease.

Although the classical mycobacterial infections are relatively uncommon in the industrialized nations, opportunist disease due to mycobacteria that normally live harmlessly in the environment is becoming an increasingly serious problem. Once regarded as little more than a curiosity, such disease now frequently complicates the acquired immunodeficiency syndrome (AIDS). The 'little red rods' seem determined to continue to inflict misery on the human race. They are most tenacious and can only be dislodged from individual patients and from society in general with great difficulty. Regrettably, their greatest ally is man's indifference to the sufferings of others.

History

The turning point in the history of tuberculosis occurred at the meeting of the Berlin Physiological Society on the evening of 24 March 1882, when Robert Koch (Fig. 1.1) described the isolation of the causative organism of this disease. This, in fact, was eight years after Armauer Hansen published his

Fig. 1.1 Robert Koch (1843–1910): discoverer of the tubercle bacillus in 1882

not receive the adulation accorded to that of Koch as he was unable to isolate the organism in pure culture.

The work of Hansen and Koch did not occur in scientific isolation. The stage had been set by the clear establishment of the germ theory of communicable disease by Louis Pasteur and, in particular, by the experimental demonstration of the transmissibility of tuberculosis in rabbits by Jean-Antoine Villemin (Fig. 1.2), a French military surgeon, in 1868. It was therefore considered very likely that tuberculosis and leprosy were caused by 'germs' and many workers attempted to isolate them. Koch's critics have remarked that he was only able to discover the tubercle bacillus because he used methods developed by other workers, namely Weigert's stains and Tyndall's inspissated serum medium. On the other hand, Koch's acknowledged industry, patience, tenacity and technical skill must have contributed overwhelmingly to his success. Indeed, Koch's detailed descriptions of his techniques enabled other workers to reproduce his findings and the few antagonists were rapidly silenced.

Koch's discovery heralded the era of hope, and serious research soon took off in three main directions: the isolation and culture of the bacillus for

Fig. 1.2 Jean-Antoine Villemin (1827–92): pioneer of experimental studies on the transmissibility of tuberculosis

diagnostic purposes, the search for an effective cure, and the development of a vaccine. Diagnosis required specific stains and methods for the *in vitro* cultivation of mycobacteria. Koch stained his preparations with an alcoholic solution of methylene blue and used vesuvin as a counterstain. Very shortly afterwards, Paul Ehrlich discovered the 'acid-fastness' of the tubercle bacillus and introduced a staining technique which, with minor modifications by Ziehl and Neelsen whose names the method now bears, is still widely used today. Originally, tubercle bacilli were grown on heat-coagulated serum, then in glycerol-beef broth. Egg-based media were introduced by Dorset in 1902 and were modified by Löwenstein in 1930. Methods for 'decontaminating' clinical specimens were introduced by Petroff and others around 1915. No further significant developments were made for many years so that, in 1954, Dubos remarked that tuberculosis bacteriology was based on 'primitive bacteriological techniques worked out decades ago'. Since that time we have witnessed the introduction of rapid radiometric techniques for the detection of mycobacterial growth and even more rapid techniques for the amplification of

specific DNA in clinical specimens. Nevertheless, the adoption of these new techniques remains a mere dream for tuberculosis workers in most laboratories throughout the developing world.

Koch's discovery coincided with the birth of the discipline of immunology. Koch certainly did not regard himself as an immunologist. Indeed, when Metchnikoff demonstrated the phenomenon of phagocytosis, Koch remarked 'I am a hygienist and it is of no interest to me where the microbes are, whether inside or outside the cells'. Nevertheless he attempted to develop an agglutination test for tuberculosis using the whole bacillus as antigen and also tried to attenuate a human strain for use as a vaccine for tuberculosis in cattle. But his main studies centred on the development of a cure for tuberculosis, and this led to the extraordinary saga of Old Tuberculin. This, as outlined in Chapter 5, followed a meticulous series of experiments on guinea-pigs that led to the description of the tissue necrotizing reaction subsequently named the Koch Phenomenon. Unfortunately Koch was under considerable pressure from his political overlords to announce a cure for the disease and his rather hasty use of Old Tuberculin in patients proved disastrous and almost ruined his reputation. Nevertheless, this was the first attempt at 'immunotherapy' – a form of treatment that was forced into near oblivion by the advent of antibacterial chemotherapy but has returned to offer, perhaps, the only chance of controlling tuberculosis in the face of multidrug resistance. Koch's Old Tuberculin, and the reactions elicited by it, may well have been forgotten if it had not been for Clemens von Pirquet (Fig. 1.3) who developed the tuberculin test – one of the most widely used yet misunderstood of all diagnostic tests.

At the British Congress on Tuberculosis in 1901 Koch made a serious error that was to have far-reaching consequences; namely his statement that bovine tuberculosis was of no danger to man. The veterinary surgeons present were so astounded by this pontification that they persuaded the Minister of Agriculture to convene a Royal Commission to investigate the issue. In a period of ten years the scientists employed by the commissioners, notably Arthur Stanley Griffith and Louis Cobbett, accrued an enormous amount of information on the epidemiology, bacteriology and pathology of bovine tuberculosis and produced irrefutable evidence that humans are susceptible to tuberculosis of bovine origin. The Commission amply demonstrated the benefits of state-sponsored medical research and it was the forerunner of the British Medical Research Council. It also laid the foundations for the bovine tuberculosis eradication programmes which must be hailed as the most effective control measures ever mounted against a bacterial disease.

The general principles of vaccination were well-established by Pasteur, and many workers attempted to attenuate the tubercle bacillus for use as a vaccine. One of the first successful attempts was made by Edward Trudeau who attenuated a human strain by repeated passage on coagulated sheep serum for two years. Although this, the R1 strain, was avirulent in the guinea-pig, no further development was undertaken. Trudeau established a tuberculosis research institute, which bears his name, at Saranac Lake, New York State. Years later, while working at that institute, George Mackaness laid the foundations to the study of the mechanisms of cell-mediated immunity and the role of the

Fig. 1.3 Clemens von Pirquet (1874–1929): the pioneer of the tuberculin test

A vaccine was eventually produced by Calmette and Guérin after passaging a bovine tubercle bacillus 230 times on potato slices soaked in bile and glycerol over a period of 13 years. This vaccine, Bacille Calmette–Guérin (BCG), was first used in 1921 as an oral vaccine for infants. Its early use was delayed by considerable controversy concerning its safety and by the 'Lubeck disaster' in 1930 when many children were accidentally vaccinated with a virulent strain of *Mycobacterium tuberculosis* and 73 died. After a further delay caused by the Second World War, a freeze-dried vaccine was introduced and has been widely used since. Whether it has a future or whether, in the light of advances in our understanding of the immunology of mycobacterial disease, we are ready for a totally different approach to vaccination remains to be seen.

The next milestone in the history of mycobacterial disease occurred in the [illegible] century with the discovery of the first effective drugs. As in the

case of the discovery of the causative organism, leprosy preceded tuberculosis though with less fanfare and acclaim. Faget and his colleagues found that promin was effective against leprosy in 1943 and streptomycin was discovered, after an extensive search, by the Russian bacteriologist Selman Waksman and his team in 1948. This, and the subsequent discoveries of isoniazid and other drugs, at last removed the fear of tuberculosis. The general public and some physicians were convinced that the disease was conquered and would soon be extinct. Others, including Waksman himself, were not so optimistic and doubted whether antituberculosis drugs alone would solve the problem. Sadly they have been proved correct. Indeed the promises held out by drugs and the Bacille Calmette–Guérin (BCG) vaccine have probably done more to eradicate interest in the mycobacterial diseases than to eradicate the diseases themselves.

The 1950s were a time of great excitement owing not only to the introduction of effective chemotherapy and the early BCG trials, but also to the serious interest being taken in disease due to other mycobacterial species. The first to be described in detail were due to *M. ulcerans* (Buruli ulcer) and *M. marinum* (swimming pool granuloma). In 1954 Ernest Runyon published the first of his studies on the classification of 'anonymous' mycobacteria causing lung disease in man. This, together with the pioneering investigations of Ruth Gordon, led to a renewed interest in the taxonomy of the mycobacteria, culminating in the extensive studies undertaken by the International Working Group on Mycobacterial Taxonomy (IWGMT) established by Dr Larry Wayne. At present there are 41 'approved' mycobacterial species (see Chapter 3), and about 20 others. About half the species are known to cause disease in animals or man.

Before the early 1980s, disease due to environmental mycobacteria was uncommon and few clinicians would have seen more than one case in a lifetime. As with tuberculosis, the human immunodeficiency virus (HIV) pandemic brought about a profound change in interest as these mycobacteria, notably *M. avium* (the avian tubercle bacillus) are common causes of opportunist disease in acquired immune deficiency syndrome (AIDS) patients.

In addition to interest in mycobacteria as pathogens, attention was given to their place in the environment. It is now clear, contrary to earlier views, that the genus *Mycobacterium* is essentially one of environmental saprophytes and that pathogenicity is not their usual behaviour. Thus, the major pathogens *M. tuberculosis* and *M. leprae* are atypical mycobacteria although, paradoxically, this term has been applied to the typical saprophytic species! Ecological studies have proved to be of great relevance to disease as there is little doubt that immunologically effective contact with environmental mycobacteria has a profound influence on the way a person subsequently responds to BCG vaccination or to infection by a pathogenic species.

The period from 1970 until the late 1980s was one of great fascination, interest and confidence. Rifampicin was introduced and made it possible at last to develop short-course curative chemotherapy for both tuberculosis and leprosy. After a decade or so of extensive clinical trials organized by Professors Mitchison and Fox of the British Medical Research Council and their collaborators abroad, 6-month regimens of orally administered drugs

contrasts sharply with the 3000 doses of drugs over a 2-year period used in the early days of chemotherapy. During the same period, short multidrug regimens for leprosy were developed and are now in general use with very great benefit.

Not only was this a period of great advances in chemotherapy, it was also one in which enormous strides forward were made in mycobacterial ecology, taxonomy, structural and biochemical studies (especially on the lipid-rich cell wall) and immunology. This period also saw the beginning of the 'molecular era' of microbiology. It is now possible to clone mycobacterial DNA in alternative hosts and to obtain gene products from the new hosts. This enables pure mycobacterial antigens, even those from the non-cultivable leprosy bacillus, to be obtained in sufficient quantities for diagnostic tests, immunological studies and possible incorporation into new vaccines. The polymerase chain reaction, and related DNA amplification systems and highly specific nucleic acid probes, are set to revolutionize diagnostic mycobacteriology, provided that their high cost can be reduced. Recombinant gene technology and monoclonal antibodies have also facilitated the 'fine structure' analysis of the immune response in mycobacterial disease by permitting the identification and isolation of the various cells and mediators involved.

On the less positive side, this was also a period in which it became even more clear in the collective mind of the medical profession that the days of the mycobacterial diseases were numbered. Professional societies once dedicated solely to tuberculosis switched allegiance to other respiratory diseases: even the International Union Against Tuberculosis appended 'And Lung Disease' to its title. Worse was to come – in what has been termed the greatest blow during the 1980s to the fight against tuberculosis, the British Medical Research Council's Tuberculosis and Chest Diseases Unit was closed. Interest likewise waned west of the Atlantic and in 1986 the US Centers for Disease Control ceased its surveys of drug-resistant tuberculosis. In the research field, funding and interest declined: young scientists and medical practitioners were discouraged from entering the discipline of mycobacteriology and only a few enthusiasts remained. Yet the warning signs were there for those that had eyes to see, not the least of which was the advent of the HIV pandemic.

The future and the challenge

In the early 1990s there occurred what may, in future times, be depicted as one of the greatest shifts of awareness and interest in a disease that has ever occurred in the history of medicine. From being a jaded and exhausted subject, tuberculosis was suddenly the centre of attention! The trigger events for this resurgence of interest were the small but definite upsurge of the disease in the USA, after decades of decline, and the occurrence of outbreaks of HIV-related multidrug-resistant tuberculosis in New York. Thanks to florid newspaper articles and television programmes, the general public was left in no doubt that the 'white plague' had returned. The fact that this plague of yesteryear should strike in the centre of a major metropolis in one of the world's richest nations struck a chord of fear throughout that society and led to calls

The medical profession was soon swept up in this wave of renewed interest and the literature bristled with editorials, of widely varying quality and originality, on the resurgence of the disease. The World Health Organization's Tuberculosis Unit was rapidly expanded and charged with the task of reviewing the magnitude of the problem and developing a new global control strategy. Funds were made available for laboratory studies and there was a renaissance in research activity, particularly in molecular microbiology, in the hope of producing new diagnostic and epidemiological tools, novel vaccines and new therapeutic approaches.

Although the fruits of this scientific renaissance are very exciting intellectually, they have contributed little so far to the practical problems of the control of mycobacterial disease. On the contrary, the fact that the World Health Organization has declared tuberculosis a global emergency eloquently attests to the inadequacy of the control measures at our disposal or, more likely, our abject failure to deploy them responsibly. The high and increasing numbers of persons dually infected with tubercle bacilli and HIV is, as described in Chapter 6, a cause for great anxiety and it must be remembered that dual infection would have been uncommon if tuberculosis had been managed effectively in the past. Sir John Crofton, a greatly respected pioneer of anti-tuberculosis chemotherapy, has remarked that:

> It is a sad reflection on society's incompetence that, more than thirty years after the methods for cure and prevention were evolved, and before the advent of the HIV epidemic, there were already more patients with active tuberculosis in the world than there had been in the 1950s.

With the rapidly increasing and devastating effects of the HIV pandemic on tuberculosis and the emergence of multidrug resistance we need innovative control measures such as immunotherapy as well as the established ones but, more than anything else, we need the vision to realize that the global emergency of tuberculosis is growing daily and that to delay the implementation of adequate control measures is a recipe for disaster. The World Health Organization has drawn attention to the large discrepancy between the incidence of, and mortality due to, tuberculosis relative to other infectious diseases and the funding made available to combat it. Appeals based on the fact that tuberculosis is the most cost-effective of all adult diseases to treat and yet is still the cause of 1 in 4 *preventable* adult deaths appear to have fallen on deaf ears. Let us hope that the global community is granted one more chance to conquer tuberculosis and that this chance will not be lost.

But what of leprosy? Interest in this disease is in danger of being swamped by that currently given to tuberculosis. Certainly, recent surveys show that, thanks to the efficacy of multidrug therapy, leprosy is declining in incidence and there is no evidence that the HIV pandemic will have an adverse effect on this decline. Nevertheless we must hope that we have learnt from the recent upsurge in the incidence of tuberculosis that to permit a decrease in interest and financial investment to mirror the decrease in prevalence of a transmissible disease is most foolhardy. In this context, it is well to remember that, though rarer than many tropical diseases, leprosy is the cause of physical and mental suffering well out of proportion to its prevalence. There is no reason why leprosy

Full scientific, medical and financial cooperation between scientists and field workers and between the developed and developing nations will be required for the eventual conquest of mycobacterial disease. There is no doubt that these are principally afflictions of the socio-economically underprivileged and that the relative freedom of the West from such ills is a direct result of its prosperity. Ironically, our failure to *eradicate* tuberculosis globally is largely the consequence of our success in the *control* of the disease in the developed world and of our parochial indifference to the sufferings of those in distant lands. We are now learning the hard way that 'none are safe until all are safe'. It must be evident by now that until the barriers of race, creed and nationality are broken down, and until mistrust and strife are replaced by brotherly love, compassion and cooperation, the tyrannical reign of the King of Diseases and the Disease of Kings will continue unabated.

Publications of general and historical interest

Allen, B.W. and Hinkes, W.F. 1982: Koch's stain for tubercle bacilli. *Bulletin of the International Union Against Tuberculosis* **57**, 190–2.

Bishop, P.J. and Neumann, G. 1970: The history of the Ziehl–Neelsen stain. *Tubercle* **51**, 196–206.

Brothwell, D. and Sandison, A.T. (eds.) 1967: *Diseases of antiquity*. Springfield: Charles C. Thomas. (Contains chapters on leprosy, tuberculosis and diseases in the Bible and the Talmud.)

Crawfurd, P. 1911: *The King's Evil*. Oxford: Clarendon Press.

Dubos, R. and Dubos, J. 1952: *The White Plague: Tuberculosis, Man and Society*. Boston: Little, Brown.

Evans, C.C. 1994: Historical background. In Davies, P.D.O. (ed.), *Clinical Tuberculosis*. London: Chapman & Hall, 1–17. (See also the foreword by Sir John Crofton in the same book.)

Francis, J. 1959: The work of the British Royal Commission on Tuberculosis, 1901–1911. *Tubercle* **40**, 124–32.

Grange, J.M. 1982: Koch's tubercle bacillus: a centenary appraisal. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infectionskrankheiten und Hygiene*, Abt. 1. **251**, 297–301.

Grange, J.M. and Bishop, P. 1982: Über Tuberkulose – A tribute to Robert Koch's discovery of the tubercle bacillus, 1882. *Tubercle* **63**, 3–17.

Heifets, L. 1982: Metchnikoff's recollections of Robert Koch. *Tubercle* **63**, 139–41.

Keers, R.Y. 1978: *Pulmonary tuberculosis: a journey down the centuries*. London: Baillière Tindall.

Koch, R. 1882: Die Aetiologie der Tuberculose. *Berliner Klinische Wochenschrift* **19**, 221–38. Translated by Pinner, B. and Pinner, M. 1932: *American Review of Tuberculosis* **25**, 285–323.

Major, R.H. 1945: *Classic descriptions of disease*. 3rd edn. Springfield, Illinois: Charles C. Thomas.

Pallamary, P. 1955: Translation of Gerhard Armauer Hansen: Spedalskheden Aarsager (Causes of Leprosy). *International Journal of Leprosy* **23**, 307–9.

Rosenthal, S.R. 1957: *BCG vaccination against tuberculosis*. London: Churchill. (Includes a historical chapter by Guérin.)

Ryan, F. 1992: *The greatest story never told*. Bromsgrove: Swift Publishers.

Stanford, J.L. and Grange, J.M. 1993: New concepts for the control of tuberculosis in

Villemin, J.A. 1868: *Études experimentales et cliniqies sur tuberculose*. Paris: Baillière et Fils.
Vogelsang, T.M. 1978: Gerhard Henrik Armauer Hansen (1841–1912), the discoverer of the leprosy bacillus. His life and work. *International Journal of Leprosy* **46**, 257–332.
Waksman, S.A. 1964: *The conquest of tuberculosis*. London: Cambridge University Press.
Williams, L. 1908: The worship of Moloch. *British Journal of Tuberculosis* **2**, 56–62.
World Health Organization. 1994: *TB – A Global Emergency*. Geneva: World Health Organization.

2 The genus *Mycobacterium*

The generic name *Mycobacterium* was introduced by Lehmann and Neumann in the first edition of their 'Atlas of Bacteriology' published in 1896. At that time the genus contained only two species, *Mycobacterium tuberculosis* and *M. leprae*. The name *Mycobacterium*, meaning fungus-bacterium, was derived from the way that the tubercle bacillus grows as mould-like pellicles on the surface of liquid media. The name did not, and should not, imply that the mycobacteria are related to the fungi. The non-culturable leprosy bacillus was included in the genus because it shares a staining property with the tubercle bacillus; namely, resistance to decolourization by weak mineral acids after staining with one of the arylmethane dyes. This property, acid-fastness, is the basis of the widely used Ziehl–Neelsen stain, the history of which was reviewed by Bishop and Neumann (1970) and Allen and Hinkes (1982). Acid-fastness, although a useful distinguishing property, is not unique to the mycobacteria: bacterial spores, for example, are often strongly acid-fast and members of the related genus *Nocardia* are weakly acid-fast.

Shortly after the introduction of the generic name, acid-fast bacilli were cultured from birds and cold-blooded animals such as frogs, turtles and fish. Also, at that time, small but constant differences between tubercle bacilli isolated from man and cattle were described. Thus, four 'tubercle bacilli' were recognized; namely, human, bovine, avian and 'cold blooded'. In addition, acid-fast bacilli were isolated from inanimate sources such as hay, compost and butter. As tuberculosis in man and cattle was such a serious problem, these other mycobacteria received scant attention, although there were a few early reports of their involvement in human disease. Despite the lack of clinical interest, numerous supposedly new species were described and the 1966 edition of Index Bergeyana listed 128 validly published species. Paradoxically, this plethora of names made identification of individual isolates so difficult that mycobacteria other than *M. tuberculosis* were often termed 'anonymous mycobacteria'.

Interest in the classification of the genus was awakened in the 1950s by the descriptions of two new mycobacterial diseases of man – swimming pool granuloma and Buruli ulcer (see Chapter 9) – and by the pioneering studies of Ruth Gordon and Ernest Runyon.

Runyon (1959) drew attention to the role of 'anonymous mycobacteria' in human lung disease and placed the responsible strains into four groups according to their speed of growth and pigmentation. These groups are:

I photochromogens (yellow pigment formed in the light)
II scotochromogens (yellow pigment formed in the dark)

III non-chromogens
IV rapid growers

Though now obsolete, this grouping was of great value in that era of taxonomic chaos. Since then, much effort has been devoted to the classification of the mycobacteria and, as a result, many species names have been reduced to synonymity. Indeed, only 16 of the 128 names in the 1966 edition of Index Bergeyana are now in use (Ratledge and Stanford, 1982). Before 1980, the correct name for a species was, by international agreement, the first one to be validly published after 1 May 1753, the publication date of Linne's *Species Planetarum*. It is now only necessary to refer back to the 'Approved lists of bacterial names' (Skerman *et al.*, 1980) published in the *International Journal of Systematic Bacteriology* on 1 January 1980. This list contains 41 species of mycobacteria, but it omits a number of apparently distinct species, and several others have been described subsequently (see Tables 3.1 and 3.2 in Chapter 3). The introduction of techniques for DNA manipulation has provided additional powerful tools for the speciation of bacteria and one such technique known as 'ribotyping' appears to be of value in classifying and identifying mycobacteria, including non-cultivable strains. This technique is described on page 29.

The variation of properties within the genus *Mycobacterium* is extensive and is reflected in the range of virulence, habitat, rate of growth, nutritional requirements and antigenicity. There are, in fact, relatively few properties that are common to all mycobacteria and yet clearly delineate this genus from related ones. Many of the unique characteristics of the mycobacteria are to be found in their very complex lipid-rich cell walls.

The mycobacteria appear to have evolved from the group of Gram-positive aerobic rods which includes the genera *Corynebacterium* and *Nocardia*. Indeed mycobacteria are Gram-positive although they are not easily stainable by this method. Mycobacteria are aerobic (although some such as bovine tubercle bacilli prefer low oxygen tensions), non-sporing and non-motile. They do not form capsules in the strict sense although some strains are very smooth, even slimy, owing to a thick coat composed of lipids termed mycosides (see page 21).

Mycobacteria appear to divide by simple binary fission, although some authors have postulated more complex life cycles, possibly including cell wall-free, or spheroplast, forms. Although such forms may be produced as laboratory artefacts, claims that they occur naturally or indeed that they are the causative agents of certain granulomatous diseases such as sarcoid or Crohn's disease require careful substantiation.

Antigenic structure

Mycobacteria, being complex unicellular organisms, contain many antigenic proteins and sugars. The antigens are conveniently divided into cytoplasmic (soluble) and cell-wall lipid-bound (insoluble) antigens. Both have proved of value for classifying species and typing strains. The soluble antigens are

autolysis or mechanical disruption and those that are actively secreted by living whole cells.

Mycobacterial antigenic determinants (epitopes) are divisible according to their distribution within the genus. Up to 15 precipitin lines are demonstrable when ultrasonicates of mycobacteria are tested against homologous antisera by double diffusion in agar gel. This technique has been extensively studied for taxonomic purposes by Stanford and his colleagues (see Stanford and Grange, 1974) who described four groups of soluble (diffusible) antigens (Fig. 2.1): those common to all mycobacteria (Group i); those restricted to slowly growing species (Group ii); those occurring in rapidly growing species (Group iii); and those unique to each individual species (Group iv). The species delineated by the Group iv antigens correlates very closely with the speciation obtained by conventional taxonomy and modern molecular techniques.

This antigenic distribution indicates a fundamental difference between the slowly growing and rapidly growing species, and suggests that these groups separated early in the evolution of the genus. Furthermore, some of the Group iii antigens are also found in the genus *Nocardia*, suggesting a close relationship between this genus and the rapidly growing mycobacteria. Many of the common (Group i) antigens are also found in the nocardiae and some are detectable in related genera such as *Corynebacterium* and *Listeria*. This intergeneric sharing of antigens is probably responsible for the notorious lack of specificity of serological tests for tuberculosis.

The more sensitive technique of crossed immunoelectrophoresis (CIE) reveals from 60 to 90 antigens in mycobacterial sonicates and some have been identified as enzymes (Ridell *et al.*, 1987). Data on about 50 mycobacterial antigens, mostly from the *M. tuberculosis* complex and *M. leprae*, have been compiled (Young *et al.*, 1992).

Most of the early work on immune reactivity in tuberculosis was based on the use of crude culture filtrates, notably Koch's Old Tuberculin.

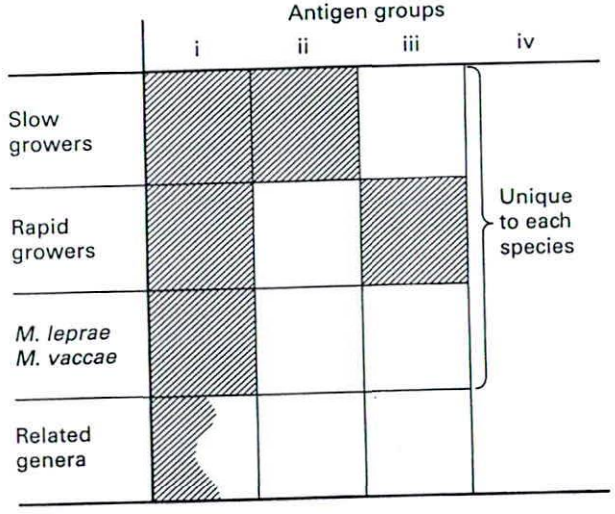


Fig. 2.1 The distribution of soluble antigens in the genus *Mycobacterium*

Subsequently, Seibert (1934) fractionated this filtrate by simple precipitation with acetone and saturated ammonium sulphate solution, thus producing Purified Protein Derivative (PPD) which is still the standard reagent for tuberculin testing.

Many workers have also used chemical and physical fractionation techniques in attempts to isolate the species-specific (Group iv) antigens for use in diagnostic tests, but this task has proved very difficult for two reasons. First, specific epitopes often occur on the same protein molecules as shared epitopes. Even purification methods based on binding to highly specific antibodies (affinity chromatography) cannot separate two determinants if they are on the same molecule. Secondly, a given determinant may be present on a range of molecules of differing physical and chemical properties. Thus, preparative techniques based on such differences (gel filtration and ion-exchange chromatography) have their limitations. Nevertheless, some well-characterized antigens have been prepared in this manner. These include the A60 antigen, the major heat-stable component of PPD, which forms the basis of a commercially available serodiagnostic test for tuberculosis (Charpin *et al.*, 1990).

In recent years, attention has largely turned to the cloning of DNA coding for mycobacterial protein antigens in alternative bacterial hosts and thereby providing a source of such antigens in pure form. This approach has the great advantage that large quantities of proteins coded by DNA from the non-cultivable species *M. leprae* can be produced for research and diagnostic purposes. A limited number of such recombinant antigens are available for research purposes from the World Health Organization (WHO) and gene libraries containing DNA for other antigens have been produced from the major pathogenic species (see page 28).

Insoluble cell-wall bound antigens are usually demonstrated by direct agglutination of whole bacilli by appropriate antisera. This technique is applicable to those species of mycobacteria that form stable, smooth suspensions, and was used extensively by Schaefer and his colleagues (see Wolinsky and Schaefer, 1973) for typing *M. avium*, *M. intracellulare* and *M. scrofulaceum*.

Serotypes are identifiable in several other species but not, unfortunately, in *M. tuberculosis* which is rough and readily auto-agglutinates. The responsible antigens have been identified as the sugar moieties on a group of peptidoglycolipids and phenolic glycolipids collectively termed mycosides (see page 21).

Monoclonal antibodies against some mycobacterial antigens have been produced. In initial studies, many monoclonal antibodies were described but a workshop organized by WHO showed that the range of epitopes recognized by them was rather limited. The third WHO workshop was held in 1992 and the report lists the well-characterized monoclonal antibodies known at that time (Khanolkar-Young *et al.*, 1992).

Heat-shock proteins

Heat-shock proteins (HSPs) are a class of cytoplasmic proteins that are structurally highly conserved and are found in all living cells. Under normal conditions, they are mostly involved in the folding and assembly of newly synthesized, or nascent, proteins and are thus sometimes termed nurse-maid proteins or chaperonins. Under conditions of stress, such as elevated temperature or exposure to toxic agents, their synthesis increases considerably and they are presented on the cell surface where they may elicit immune responses. Their role in immune phenomena is described in Chapter 5.

Being highly conserved structurally, HSPs of mycobacteria have a close homology with well-described ones in *Escherichia coli* (Young and Garbe, 1991). The principal mycobacterial HSPs are listed in Table 2.1. GroEL, the 65 kDa HSP, is the most thoroughly studied of all mycobacterial proteins (see review by Thole and van der Zee, 1990). Superoxide dismutase (SodA) is stress-induced in mycobacteria within living host cells. It is also one of the secreted proteins (see below) and has a function distinct from the others; namely, to protect the bacteria from toxic reactive oxygen intermediates.

Table 2.1 Examples of mycobacterial heat-shock proteins

Protein	*Size* (kDa)	*Function*
DnaK	71	Protein folding and translocation
GroEL	65	Protein folding and translocation
SodA	23–28	Superoxide dismutase
GroES	12–14	Protein folding and translocation

Actively secreted proteins

These proteins have attracted considerable interest as it it likely that they, together with cell surface lipoproteins and HSPs, are the first antigens recognized by the immune system after infection. These proteins have been principally studied in the *M. tuberculosis* complex and include fibronectin binding proteins, superoxide dismutase and some proteins of unknown function. For full details see Abou-Zeid *et al.* (1988) and Andersen and Brennan (1994).

The structure of the mycobacterial cell

The mycobacterial cell consists of cytoplasm bounded by a plasma membrane and enclosed by a complex lipid-rich cell wall. The single chromosome is tightly wrapped into a nuclear body (Fig. 2.2) but is not bounded by a nuclear membrane. Thus, like other bacteria, the mycobacteria are prokaryots (higher unicellular and multicellular forms of life have nuclear membranes and are termed eukaryots). In common with many other bacteria, some mycobacteria contain additional small circles of DNA termed plasmids (see page 30). The cell membrane consists of a bilayer of polar phospholipids with their hydrophobic ends facing inwards and their hydrophilic ends facing outwards. The membrane is closely associated with the enzymes and cofactors involved

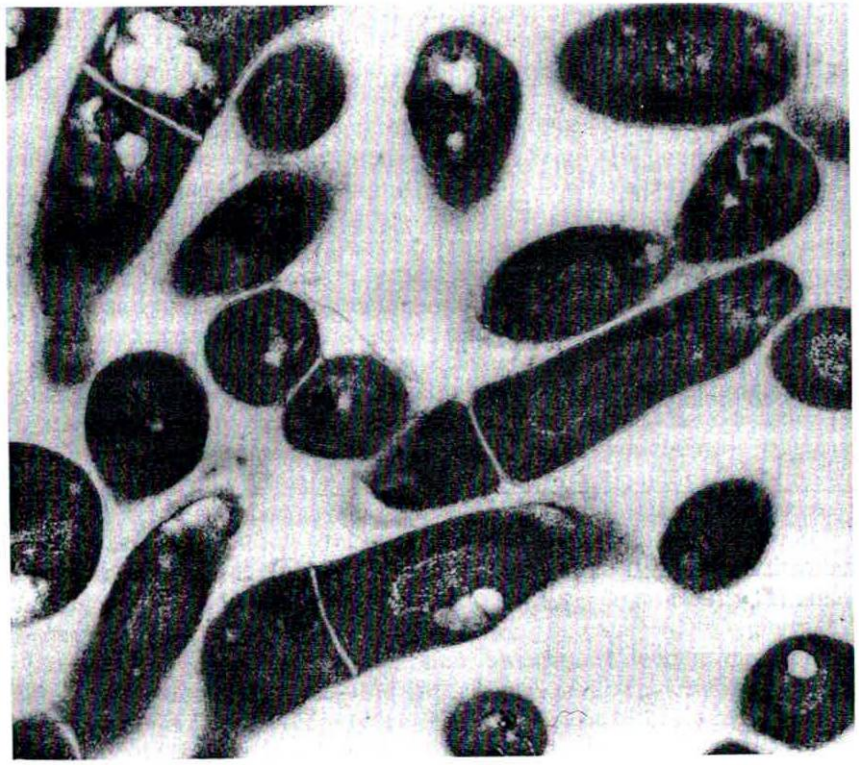


Fig. 2.2 Electron micrograph of a thin section of mycobacteria (Bacille Calmette–Guérin (BCG) vaccine) showing nuclear bodies, cell walls, septa and lipid inclusion bodies (×42 000)

in energy production. For details of the fine structure of mycobacteria and their cell membranes see Paul and Beveridge (1992).

The bacterial cells vary in shape from species to species, and even within an individual strain according to the growth conditions. The cells of the *M. avium* complex may be almost coccoid while those of *M. xenopi* may be filamentous with occasional branching. Cells of *M. kansasii* and *M. marinum* are often elongated and with a distinctive beaded or banded appearance (see page 45). In clinical material, especially corneal scrapings, cells of *M. chelonae* may be long, filamentous and weakly acid-fast and are easily mistaken for *Nocardia* (Khooshabeh *et al.*, 1994).

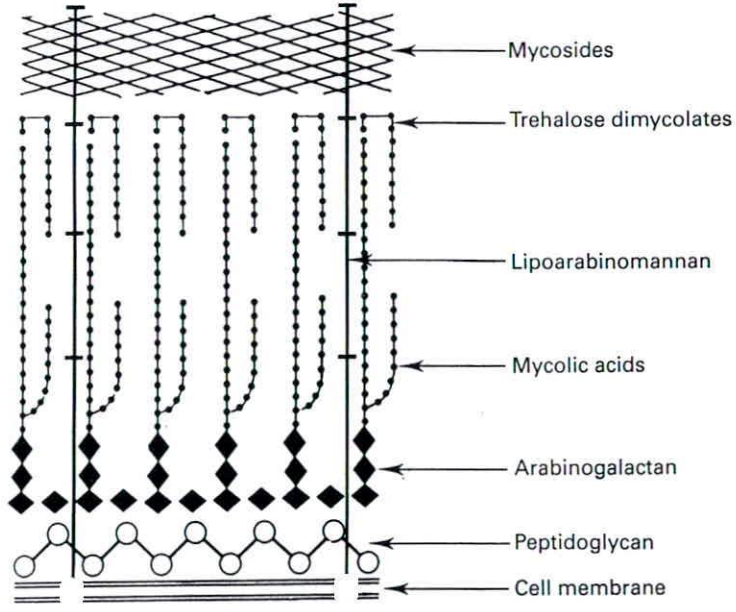
The cell wall

The mycobacterial cell wall is the most complex in all of nature and its major distinguishing characteristic is a very high lipid content. Indeed lipids account for about 60 per cent of the cell wall weight and they consist of a wide range of compounds, some being similar to those found in other organisms and

wall shows three distinct layers: inner and outer electron-dense layers separated by an electron-transparent layer (Paul and Beveridge, 1992). The exact structure of the cell wall has not been fully elucidated but the generally accepted structural model proposed by Minnikin (1982) and elaborated by McNeil and Brennan (1991) is shown in Fig. 2.3. The inner layer, overlying the cell membrane, is composed of peptidoglycan (murein). This, as in other bacteria, consists of long polysaccharide chains cross-linked by short peptide chains, thereby forming a net-like macromolecule that gives the cell its shape and rigidity. The polysaccharide chains contain N-glycolyl muramic acid and N-acetyl glucosamine in alternating positions and the cross-linking peptide chains consist of the four amino acids L-alanine, D-isoglutamine, meso-diamino-pimelic acid and D-alanine. An exception is *M. leprae* which has glycine instead of L-alanine (Draper, 1976). The mycobacterial murein is very similar to that in other genera except that it contains N-glycolyl muramic acid instead of the more usual N-acetyl analogue.

Mycobacteria are powerful adjuvants: Freund's complete adjuvant consists of killed mycobacteria in oil. This activity resides largely in the murein and also in small water-soluble fragments released from murein by digestion with lysosome. One such water-soluble adjuvant, N-acetyl muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP) has been synthesized (Lefrancier *et al.*, 1977).

External to the murein is a layer of arabinogalactan, a branched polysaccharide composed of arabinose and galactose (Lederer, 1971). The terminal



Acid-fastness

Acid-fastness is defined as the ability of the bacterial cell to resist decolourization by weak mineral acids after staining with one of the basic arylmethane dyes. The property is not confined to the mycobacteria: nocardiae, some corynebacteria and related organisms and bacterial spores are weakly acid-fast. Nevertheless, the property is widely used for the microscopic detection of mycobacteria in clinical or environmental specimens.

Despite numerous investigations, the chemical basis of acid-fastness is poorly understood. Mycolic acids are certainly involved and the degree of acid-fastness is related to the size of the acids. Thus the corynomycolic acids, nocardomycolic acids and mycobacterial mycolic acids are progressively larger (see page 19) and are associated with progressively more intense staining. Chemical binding of the dye to the mycolic acid occurs but this is not the whole explanation as disruption of the cell wall by any means reduces its acid-fastness considerably. It has therefore been postulated that the mycolic acids are arranged in certain configurations that cause a trapping of the dye. This view is supported by the finding that acid-fastness is associated with the mycolic acid that is covalently bound to the layer of arabinogalactan rather than that lying free within the cell wall (Goren, 1972).

Pathogenicity and virulence

Pathogenicity is the ability of a micro-organism to cause disease. Clearly this property depends on the susceptibility of the host as well as the aggressiveness of the invading organism. Some micro-organisms are obligate pathogens, having developed a total dependence on a living host for their continued existence. In the case of the mycobacteria this includes the pathogens in the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*) and, possibly, *M. leprae*. Many other mycobacteria are opportunist pathogens, normally existing as harmless saprophytes but becoming pathogens under certain permissive conditions. It is possible that *M. leprae* and other non-cultivable mycobacteria live in certain inanimate environments and the ability to identify species-specific mycobacterial nucleic acids after amplification by the polymerase chain reaction (see page 29) enables this possibility to be investigated.

Virulence is a quantitative measure of pathogenicity and may vary considerably according to the host species. Thus, although virulence may be assayed in a standard, preferably inbred, animal, care must be taken when interpreting such findings in relation to other animals or humans. This is evident within the *M. tuberculosis* complex from the following examples: (a) the vole tubercle bacillus (*M. microti*) is virulent for voles and some other small animals yet attenuated in humans; (b) the bovine tubercle bacillus (*M. bovis*) is much more virulent than the human type for the rabbit; (c) the South Indian variant of, and some isoniazid-resistant mutants of, *M. tuberculosis* are attenuated in the guinea-pig but are virulent in humans; and (d) *M. bovis* is virulent for both cattle and humans yet *M. tuberculosis* rarely causes progressive disease in cattle.

It has long been recognized that mycobacteria owe their pathogenicity to

detail in Chapter 5. Although some strains of *M. tuberculosis* certainly liberate toxic compounds, their virulence is not primarily associated with such substances. Thus, claims that certain cell wall lipids with toxic properties, notably cord factor (dimycolyl trehalose) and the sulpholipids are determinants of virulence, have been refuted (Goren *et al.*, 1982). Two other mycobacterial lipids, mycolipenic acid and lipoarabinomannan, may be associated with virulence as described above (see pages 19 and 21).

A further lipid of possible relevance to virulence of *M. tuberculosis* is the attenuation indicator lipid, described on page 21. This is characteristic of the South Indian strains but is not found in isoniazid-resistant classical strains which, in common with the former are often of diminished virulence for the guinea-pig, or in laboratory attenuated strains. Thus its association with attenuation may also be a fortuitous one.

Mycobacterium ulcerans is the only mycobacterium which owes its virulence to a cytotoxic substance (see page 103), while the virulence of *M. avium* appears to be related to colony morphology, suggesting a protective role for surface mycosides (Kuze and Uchihira, 1984). Apart from these examples, the mechanisms of virulence of the opportunist mycobacteria have received scant attention.

In the past, searches for the determinant(s) of virulence of *M. tuberculosis* were based on the comparison of the properties of virulent and attenuated variants of the same strain, such as strains H37Rv and H37Ra, but such searches were singularly unproductive. It is now possible to do much more discriminative investigations by transferring genes for putative virulence determinants from one strain to another (Jacobs and Bloom, 1994).

The idea of finding a single determinant of mycobacterial virulence is an attractive one with great relevance to the possible development of new vaccines. On the other hand, the 'decision' as to whether infection by a mycobacterial pathogen will lead to overt disease may depend more on the nature of the host's immune response than on the invasiveness or other properties of the bacterium. Rook (1991) has suggested that this 'decision' is likely to be based on a complex recognition of the many antigens and adjuvants of the mycobacterium rather than on a single factor. This may explain why searches for specific determinants of virulence have been unsuccessful.

Nutrition and metabolism

Despite the relatively slow growth of mycobacteria and the complexity of their lipid-rich cell walls, most species have very simple nutritional requirements. They do, nevertheless, show an enormous diversity in the substrates that they are able to use as nitrogen and carbon sources – a diversity exploited for purposes of identification. Nutritional requirements include oxygen, carbon, nitrogen, phosphorus, sodium, potassium, sulphur, iron and magnesium. A typical simple medium that supplies all these nutrients is Sauton's medium, composed of asparagine, glucose, glycerol, Na_2HPO_4, K_2HPO_4, $MgSO_4$ and ferric ammonium citrate. A wide range of nutrients, including lipids and nucleic acid precursors are available to mycobacteria within cells and host

antibody. These proteins increase in amount in iron-deprived mycobacteria and may be used to ascertain the iron status of *in vivo* grown organisms such as *M. leprae* (Sritharam and Ratledge, 1990).

Genetics and molecular biology of the mycobacteria

The subject of molecular biology of the mycobacteria has become a huge discipline in recent years. A complete review of the subject is beyond the scope of this book. For more details see McFadden (1990) and Chapters 12–18 in Bloom (1994). In this chapter the aspects of molecular biology of actual and potential clinical significance are outlined.

The mycobacterial genome

In common with all other bacteria, the mycobacteria contain a single circular chromosome (the genome) and some strains also contain additional small circular units of DNA termed plasmids (Crawford *et al.*, 1981). The molecular weights of mycobacterial genomes range from 3×10^9 to 5.5×10^9 (Baess and Mansa, 1978). For comparison the molecular weight of the genome of *Escherichia coli* is 2.5×10^9. The ratio of the base pairs adenine (A) and thymine (T) to guanine (G) and cytosine (C) varies considerably between bacterial genera. The G+C content of the mycobacteria is high, from 66 to 71 per cent of the total base content (Baess and Mansa, 1978).

The degree of relatedness of mycobacterial (or any other) genomes may be determined by the technique of DNA hybridization. This is based on the ability of single strands of DNA to associate into double strands provided that the sequence of the base pairs of the two strands complement each other. The extent of hybridization between fragments of DNA from two different strains or species gives an indication of the similarity of the sequence of the bases. This technique was used as a taxonomic tool to confirm the species boundaries determined by other methods (Baess and Bentzon, 1978). The same technique was used to identify clinical isolates but has been superseded by the use of specific nucleic acid probes produced by recombinant technology. These have the advantage that they give a 'yes or no' result rather than revealing various degrees of homology. Nucleic acid probes for the more commonly encountered mycobacterial species are commercially available.

The development of specific nucleic acid probes was made possible by advances in molecular biology, enabling fragments of DNA representing the entire genome of an organism to be replicated in an alternative organism such as *E. coli*. These self-perpetuating clones of DNA are termed genome libraries and have been produced from *M. leprae* (Clark-Curtiss *et al.*, 1985), *M. tuberculosis* (Young *et al.*, 1985; Eisenach *et al.*, 1986) and BCG (Thole *et al.*, 1985). These are not only the source of DNA for use in diagnostic tests and genetic analysis but the proteins coded by the genes are the source of antigens

DNA amplification – the polymerase chain reaction

The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of DNA. In the living cell, DNA replication occurs during cell division in two stages. First, the double helix is split into its two constituent chains by a gyrase enzyme. Secondly, complementary chains are synthesized by a DNA polymerase, resulting in two new double helices. This process may be induced *in vitro* by heating the DNA to separate the double helix and adding DNA polymerase and the ribonucleotides from which the new chain is synthesized. This synthesis will only occur *in vitro* if a short DNA sequence complementary for part of the single chain is added, thereby forming a short length of double helix. Synthesis of the complementary chain then commences from this site. In practice, the requirement for such 'primers' is of great value as specific primers can be selected so that DNA amplification only occurs if DNA containing base sequences complementary to those of the primers is present in the specimen.

The principles and general methods for conducting PCR are described in detail elsewhere (Brand *et al.*, 1991). In brief, the specimen, ribonucleotides and polymerase are mixed and placed in an automated machine in which the temperature is raised so that the double helix dissociates and then lowered, allowing the complementary DNA chain to be synthesized. (The use of a heat stable polymerase overcomes the problem of heat-inactivation of the enzyme.) The cycle, which only takes a few minutes, is repeated many times so that after two hours there may be a million-fold replication of the target DNA. The product DNA is then detectable as a band on electrophoresis or by hybridization with a chemoluminescent complementary DNA probe. The PCR has been evaluated by many workers for the diagnosis of tuberculosis and infection by other mycobacteria and a number of different primers have been developed. Modifications are continually being introduced, such as isothermal methods and amplification techniques for ribosomal RNA. The latter has the advantage that each bacterial cell contains about 2,000 copies of the target. A test for *M. tuberculosis* based on amplification of 16S ribosomal RNA is marketed by GenProbe (Miller *et al.*, 1994). In view of the rapidity of developments in this field, reference to the latest current literature is essential. The state-of-the-art in 1994 is clearly and succinctly reviewed by Shaw (1994). The advantages and problems encountered in clinical diagnosis are discussed in Chapter 4. In addition to its use for the diagnosis of tuberculosis in the living, PCR has been used to detect mycobacterial DNA in ancient skeletons (Spigelman and Lemma, 1993).

Ribotyping

Ribosomes contain molecules of RNA which are genetically highly conserved. One type of ribosomal RNA, 16S rRNA, has minor variations in its base sequence which appear to correspond closely with the established mycobacterial species. Thus, determination of the sequence of the bases in this RNA may be used to classify mycobacteria and to identify clinical

isolates. This can be achieved rapidly by amplifying the DNA coding for the 16S rRNA by PCR with the appropriate primer and sequencing the amplified product on an electrophoretic gel (Rogall *et al.*, 1990; Kirschner *et al.*, 1993). The method can be used to classify and identify mycobacteria that are non-cultivable or very slowly growing. The species *M. genavense*, a culturally very fastidious pathogen encountered in AIDS patients (see page 56) and some pet birds (Hoop *et al.*, 1993) was identified by this technique. In addition, ribotyping confirms the homology of *M. tuberculosis*, *M. bovis* and *M. africanum* and of *M. kansasii* and *M. gastri* determined by immunodiffusion serology (see page 51). On the other hand, it fails to differentiate between the quite different species *M. marinum* and *M. ulcerans* and it cannot therefore be taken as a gold standard for speciation.

Very closely related mycobacteria, such as *M. avium* and *M. paratuberculosis* are more clearly differentiated by cloning and sequencing the DNA coding for the 23S rRNA and the spacer region between the 16S and 23S rRNA genes (McFadden *et al.*, 1994).

Genetics of drug resistance

Drug resistance is acquired by mutation affecting cell wall permeability, enzymes involved in transport or activation of the drug or the susceptibility of the target molecule. Detailed studies have been conducted on two of the principal antituberculosis drugs, isoniazid and rifampicin.

Two genes are involved in susceptibility to isoniazid: *katG* and *inhA*. The former codes for the catalase–peroxidase enzyme and is either deleted or inactivated by mutation in most isoniazid-resistant strains of *M. tuberculosis* (Zhang *et al.*, 1992). The *inhA* gene codes for a target of isoniazid and ethionamide and is involved in fatty acid biosynthesis (Banerjee and Dubnau, 1994). Mutations in this gene have been found in some, but not all, isoniazid-resistant strains. The discovery of these genes should help to clarify the mode of action of isoniazid.

Resistance to rifampicin is due to mutations in the *rpoB* gene which codes for one of the subunits of the enzyme DNA-dependent RNA polymerase. Sequencing of PCR-amplified *rpoB* gene DNA reveals several different mutations and a technique for the rapid detection of such mutations in clinical practice (PCR–heteroduplex formation assay) has been developed (Williams *et al.*, 1994).

Mycobacterial plasmids

Many environmental mycobacteria contain one or more small extrachromosomal elements of DNA known as plasmids. The evidence for their occurrence in *M. tuberculosis* is less strong: one report indicates that this species may contain an extrachromosomal element composed of single stranded DNA (Zainuddin and Dale, 1990). Plasmids are detected by lysing mycobacteria in ways that do not break DNA chains, removing the heavy principal

electrophoresis. Plasmids of specific types may also be detected by hybridization with cloned plasmid-specific DNA probes.

The effect of plasmids on the properties of mycobacteria is poorly understood owing to the great difficulty experienced in 'curing' these bacteria of plasmids. Some supposedly cured strains have been shown, by DNA fingerprinting, to be unrelated to the wild strain, stressing the importance of avoiding mixed cultures. The only conclusive reported direct plasmid-induced effect is resistance to heavy metal salts in a strain of *M. scrofulaceum* (Meissner and Falkinham, 1986). There is indirect evidence that plasmids of *M. avium* may facilitate growth at 43°C and in the absence of oleic acid as only plasmid-containing strains have these properties.

Plasmids have been most thoroughly studied in the *M. avium* complex (MAC) since their discovery by Crawford and Bates (1979). Meissner and Falkinham (1986) found them in 55 per cent of MAC from people, in 75 per cent of isolates from aerosols but in only 21 per cent of strains from water. This suggests that plasmids might increase the hydrophobic property of the cell wall, facilitating entry into aerosols and, possibly, establishment in the human host. These plasmids weighed between 8.8 and 160 MDa and there were from one to six in each strain. Crawford and Bates (1986) showed that all of 26 strains of *M. avium* from HIV-positive patients contained plasmids. These strains were either serotype 4 or 8 (see page 47); the former usually contained two small plasmids and the latter just one. All strains contained a plasmid that hybridized with one termed pLRT, suggesting that this plasmid might be a determinant of virulence of *M. avium* in HIV-positive patients. On the other hand, plasmids were detected in only 34 of 71 strains from such patients in London (Hellyer *et al.*, 1991), shedding doubt on a causative role for plasmids in the pathogenicity of AIDS-related strains of *M. avium*.

Plasmids have proved useful as vectors for introducing foreign DNA into bacterial cells. Such studies on mycobacteria are rather limited as free plasmids do not readily enter mycobacterial cells. The entry rate is improved by incubating the target cells in agents such as cycloserine or glycine that weaken the cell wall or by a technique called electroporation in which short electrical pulses of high voltage transiently open pores in the cell envelope through which DNA may pass (Snapper *et al.*, 1988). A further problem is that, once within the cell, plasmids do not stably integrate with the host genome although rare mutants permitting such integration have been isolated. It is likely that phage/plasmid hybrids (phasmids) will, as described below, prove to be more useful shuttle vectors for genetic studies on mycobacteria.

Mycobacteriophages

Since the first description of a phage lytic for a mycobacterium by Gardner and Weiser (1947), many have been isolated, mostly from environmental sources though a few were found in naturally occurring lysogenic strains. Such strains often differ from their non-lysogenic counterparts: in particular, their colonies on solid media are often very sticky, and not easily dislodged from the medium, owing to the accumulation of extracellular DNA and are [illegible] in solutions of DNase (Grange and Bird, 1978). In

contrast to the closely related genus *Corynebacterium*, there is no evidence that mycobacterial virulence is associated with lysogeny.

Mycobacteriophages usually have hexagonal or oval heads and long, non-contractile tails (Fig. 2.8(A)), an exception being phage I3 which has a contractile tail (Fig. 2.8(B)). Many of the phages have very wide host ranges that appear to ignore the usual species boundaries. Thus, for example, some bacteriophages isolated and propagated in strains of *M. smegmatis* may also be propagated in certain strains of *M. tuberculosis*.

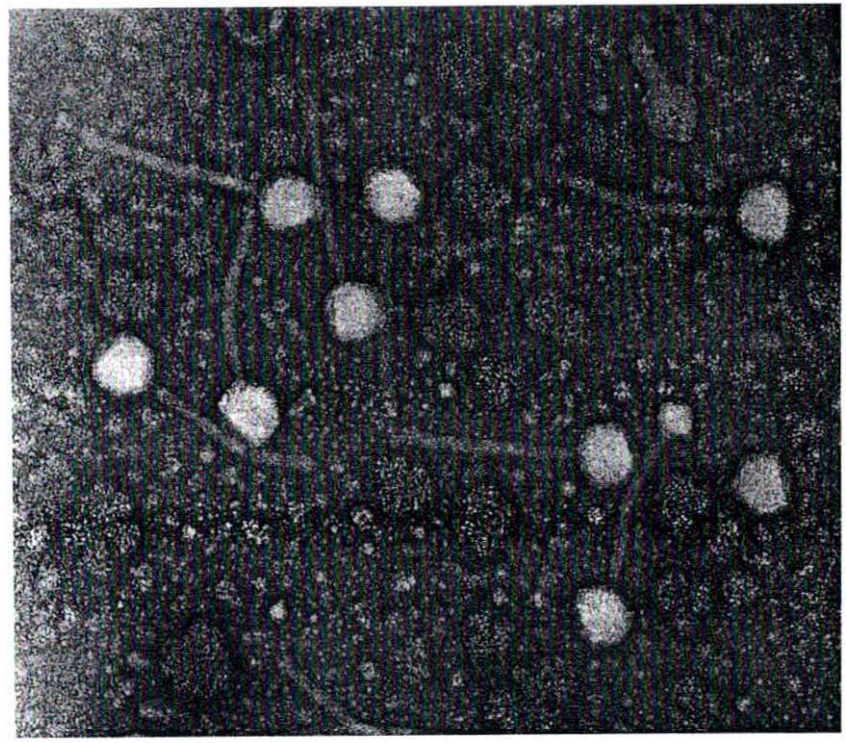
Some mycobacteriophages are virulent, leading to lysis of the cells that they infect and some are temperate. In the latter state, the phage DNA inserts fairly stably into the host genome and persists for many bacterial generations. Several mycobacteriophages, though not virulent, do not stably integrate into the host cell genome but exist as extrachromosomal plasmids: a state termed pseudolysogeny (Baess, 1971).

Initially, interest in the mycobacteriophages centred on their use in the typing of mycobacteria, particularly tubercle bacilli, for epidemiological purposes (reviewed by Grange and Redmond, 1978). In recent years, bacteriophage typing has been superseded by the technically easier and much more discriminative technique of 'DNA fingerprinting' (see below). Interest in the mycobacteriophages has thus moved to the possibility of harnessing their ability to shuttle genes from one bacterial strain to another. This phenomenon, termed transduction, was described in *M. smegmatis* by use of phage I3 (Sundar Raj and Ramakrishnan, 1970). Two other transducing mycobacteriophages, L1 (Snapper *et al.*, 1988) and TM4 (Jacobs *et al.*, 1987) have been described.

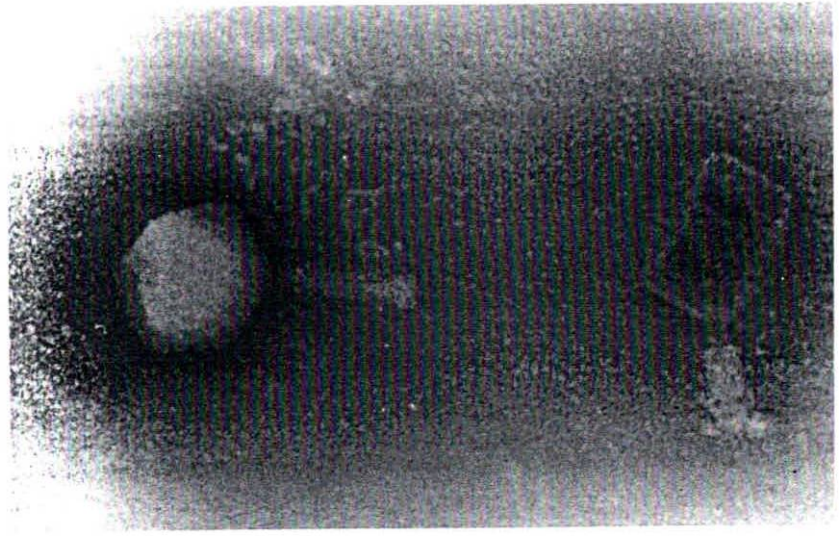
One of these phages, TM4, was used to construct an ingenious hybrid shuttle vector (Jacobs *et al.*, 1987). One of the standard techniques used to clone DNA is to insert the required DNA into a plasmid which is then packaged in a bacteriophage, such as phage lambda, which infects *E. coli*. Numerous copies of the plasmid, and the inserted DNA, are thus produced in cultures of this host bacterium. The problem was how to get this plasmid to replicate in a mycobacterium in a way that it could be transferred to other mycobacteria. This was solved by making a hybrid of the above plasmid with phage TM4. This phage-plus-plasmid (phasmid) functions as a plasmid in *E. coli* and as a phage in mycobacteria. Thus the plasmid, containing the required 'foreign' DNA, is extracted from *E. coli* and introduced into *M. smegmatis* by electroporation (see above) where it replicates and forms phages which may be used to infect various other species, such as *M. tuberculosis*, thereby introducing the required foreign DNA.

Transposons and insertion sequences

Most living cells contain short sequences of DNA that are able to change their position within the genome. In some cases there is just one such mobile element or 'jumping gene' in the genome but in others there are many copies at different sites. There are several different types. The simplest forms only contain the genes responsible for their movement and insertion and are termed



A



B

Fig. 2.8 Mycobacteriophages: A: *Mycobacterium kansasii* phage (×120 000); B: *Myco-*

functional genes, including those determining antibiotic resistance. Some temperate bacteriophages also function as transposons.

Mobile elements are able to disrupt the function of genes by inserting in them and disrupting their continuity. Thus they behave as mutagens, a property termed *transposon mutagenesis* and utilized in genetic analysis. Transposons are used to insert foreign genes into bacterial cells, particularly when many copies of the gene within the cell are required.

Insertion sequences are common in the genus *Mycobacterium*. Some are unique to a species, some to a group of strains with related properties within a species and some are 'private' to just one strain. Some examples are listed in Table 2.2. The number and sites of mobile elements vary greatly within a bacterial species, providing a very useful means of subdividing that species for epidemiological purposes. The technique used is termed restriction fragment length polymorphism (RFLP) analysis or, more popularly, DNA fingerprinting. The technique is described below.

Table 2.2 Examples of mycobacterial insertion sequences (IS)

Sequence	*Origin*	*Number of copies*
IS6110 (IS986)	*M. tuberculosis* complex	0–>20
IS1081	*M. tuberculosis* complex	5–6
IS1245	*M. avium*	2–27
IS901	*M. avium*, bird pathogens only	2–8
IS900	*M. paratuberculosis*	15–20
IS6100	*M. fortuitum*, single strain	4

Within the *M. tuberculosis* complex, the number and position of insertion sequences is very stable over many cell divisions (Fomukong *et al.*, 1992; Cave *et al.*, 1994).

Species-specific insertion sequences can be used as targets for DNA amplification by PCR for diagnostic purposes. They are particularly useful if there are multiple copies within the cell as the sensitivity of the test is thereby greater than when there is only one copy of the specific target sequence.

Some insertion sequences can be transferred experimentally from one species to another and it is probable that interchange of these genetic elements occurs between species and even between genera in nature. Thus IS6100 (Table 2.2) found in just one strain of *M. fortuitum* is identical to an insertion sequence found in the genus *Flavobacterium*.

DNA fingerprinting

The DNA fingerprinting technique relies on the availability of enzymes termed restriction endonucleases that are only able to digest a DNA chain at points where they recognize particular short sequences of bases, usually 4 to 6 in number. Such digestion results in DNA fragments of varying lengths which can be separated according to their size on electrophoretic gels. Changes in the genome by mutation or transposition alter the numbers and sizes of the digestion fragments, resulting in strain-to-strain differences. In early studies, mycobacterial DNA was simply extracted, digested and applied to an electrophoresis gel but this led to a huge number of bands and detection of differences was very difficult.

A much clearer resolution was obtained by performing the digestion and running the gel and then applying a chemoluminescent-labelled probe consisting of one of the insertion sequences described above. Thus IS6110 is usually used to type *M. tuberculosis*, after digestion of the bacterial DNA with the endonuclease *PvuII*. For comprehensive reviews of techniques, interpretation and applications see van Embden *et al.* (1993) and Godfrey-Faussett (1994).

References

Abou-Zeid, C., Smith, I., Grange, J.M. *et al.* 1988: The secreted antigens of *Mycobacterium tuberculosis* and their relationship to those recognized by the available antibodies. *Journal of General Microbiology* **134**, 531–5.

Allen, B.W. and Hinkes, W.F. 1982: Koch's stain for tubercle bacilli. *Bulletin of the International Union Against Tuberculosis* **57**, 190–2.

Andersen, A.B. and Brennan, P. 1994: Proteins and antigens of *Mycobacterium tuberculosis*. In Bloom, B.R. (ed.), *Tuberculosis: pathogenesis, protection, and control*. Washington DC: American Society for Microbiology, 307–32.

Azadian, B.S., Beck, A., Curtis, J.R. *et al.* 1981: Disseminated infection with *Mycobacterium chelonei* in a haemodialysis patient. *Tubercle* **62**, 281–4.

Baess, I. 1971: Report on a pseudolysogenic mycobacterium and a review of the literature concerning pseudolysogeny. *Acta Pathologica et Microbiologica Scandinavica* **79B**, 428–34.

Baess, I. and Bentzon, M.W. 1978: Deoxyribonucleic acid hybridization between different species of mycobacteria. *Acta Pathologica et Microbiologica Scandinavica* **86B**, 71–6.

Baess, I. and Mansa, B. 1978: Determination of genome size and base ratio of deoxyribonucleic acid from mycobacteria. *Acta Pathologica et Microbiologica Scandinavica* **86B**, 309–12.

Banerjee, A. and Dubnau, E. 1994: *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* **263**, 227–30.

Barclay, R. and Wheeler, P.R. 1989: Metabolism of mycobacteria in tissues. In Ratledge, C., Stanford, J.L., Grange, J.M. (eds.), *The biology of the mycobacteria*, vol. 3. London: Academic Press. 37–106.

Barrow, P.A. 1986: Physiological characteristics of the *Mycobacterium tuberculosis–M. bovis* group of organisms with particular reference to heterogeneity within *M. bovis*. *Journal of General Microbiology* **132**, 427–30.

Besra, G.S. and Chatterjee, D. 1994: Lipids and carbohydrates of *Mycobacterium tuberculosis*. In Bloom, B.R. (ed.), *Tuberculosis: pathogenesis, protection, and control*. Washington DC, American Society for Microbiology. 285–306.

Bishop, P.J. and Neumann, G. 1970: The history of the Ziehl–Neelsen stain. *Tubercle* **51**, 196–206.

Bloom, B.R. (ed.) 1994: *Tuberculosis: pathogenesis, protection, and control*. Washington DC, American Society for Microbiology.

Bönicke, R. 1962: Report on identification of mycobacteria by biochemical methods. *Bulletin of the International Union Against Tuberculosis* **32**, 13–68.