INVESTIGATIONS ON THE TAXONOMY AND THE GENETICS OF CALDOACTIVE BACTERIA

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ABSTRACT

The technique of pyrolysis mass spectrometry was used to attempt to obtain taxonomic groupings for a number of extremely thermophilic bacteria isolated from New Zealand hot springs. The New Zealand isolates were compared with a number of appropriate reference strains obtained from culture collections or as gifts from other laboratories.

Three groups of bacteria were analysed. The *Thermus*-like group was found to consist of three species - level groupings with most of the New Zealand isolates belonging to a single species group. The two reference species *T. aquaticus* and *T. thermophilus* apparently belong to separate species groups.

Bacillus-like strains grown at neutral pH formed three separate species groups which could correspond to the three species of thermophilic *Bacilli* proposed by Wolf and Sharp (1981).

Analysis of a number of archaebacteria confirmed the unique nature of AN1 isolated from a New Zealand hot spring by Morgan and Daniels (1982). The *Thermoproteus*-type strains isolated from New Zealand seem rather different from the type strain *T. tenax* which also has some different physiological properties. An interesting feature of these strains was the contribution of sulphur to the mass spectra.

Taxonomic groupings did not show a close relationship with source temperature and pH but regional groupings were obtained in some cases. The distribution of AN1-like strains seems to be related to the Na^+ content of source pools.

The majority of New Zealand isolates tended to form rather large clusters, probably at the species level, although sub-species level

clusters existed within these. This suggests that although extreme thermophiles may be very variable as reported, this variability is limited. Differences in clustering patterns of different isolates from the same source confirm a considerable level of sub-species level variability does exist.

A method for cloning *Thermus* genes by cosmid packaging was attempted and was apparently successful but gene expression was not demonstrated.

A UV inducible temperate phage was isolated from *Sulfolobus acidocaldarius* strain B-12. It was purified and partially characterised and a method of growing *Sulfolobus* on solid medium obtained.

PREFACE

Aspects of the physiology and enzymology of a number of extremely thermophilic bacteria have been studied at the University of Waikato for several years. It has been found, both here and by other workers in this field, that exact identification of isolates can be difficult and that laboratory cultures often behave unpredictably.

The possibility that life at these temperatures could be associated with genetic instability has been suggested as an explanation of these phenomena (Cometta *et al.*, 1982a). However, at present there is insufficient knowledge of either the genetics or the taxonomy of these bacteria to allow more than speculation about this.

The aim of this study was to investigate the taxonomy and some aspects of the genetics of extremely thermophilic bacteria from both the eubacterial and archaebacterial kingdoms in order to determine whether variability is in fact widespread among extreme thermophiles.

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LIST OF ABBREVIATIONS

PYMS	Pyrolysis Mass Spectrometry

Sampling Sites

Fj or FJ	Fiji
GB	Great Barrier Island
HP	Hipuau
KET or KT	Ketetahi
OK	Orakei Korako
RT or Rt	Rotorua
RK	Rotokawa
SWBS or HWB	Hot Water Beach
TP	Taupo
TK or Tiki	Tikitere
TOK or Tok	Tokaanu
Wai or WN	Waimangu
WP	Waiotapu Tourist Area
WT	Waiotapu Forest Reserve
Wis or WIS	White Island

CHAPTER 1

GENERAL INTRODUCTION

The origin of thermophily is a question of considerable biological interest. In the Group Report (Langworthy *et al.*, 1979) presented to the Dahlem Konferenzen on 'Strategies of Microbial Life in Extreme Environments' it was concluded that thermophily has probably developed gradually (by parallel evolution) within each group of micro-organisms and that life probably began as a single event in a non-thermal sea. Such speculations are always open to question and currently the hypothesis that life began as a single event is being seriously challenged (Woese, 1982) by the concept of a universal ancestral "state".

It has been proposed that the recently described third kingdom of living things, the archaebacteria, (Fox *et al.*, 1980) reflects the level of evolution which had been attained during the period of separation of the three kingdoms in existence today, the eukaroytes, eubacteria and archaebacteria (Zillig *et al.*, in press). Investigations into the origins of archaebacteria have led to the isolation of new species belonging to this kingdom, many of which are extreme thermophiles (Zillig *et al.*, 1981; 1982b; 1983a and 1983b; Stetter *et al.*, 1981; Stetter *et al.*, 1983a). These thermophilic isolates are all obligate anaerobes and include both chemolithotrophs and heterotrophs. The possibility that a thermophilic chemolithotroph was the first fully evolved cell line is certainly conceivable (Fischer *et al.*, 1983).

Thermophilic micro-organisms possess a variety of mechanisms which adapt them to life at high temperatures. There are three general types of mechanisms which make molecules stable (and functional) at

high temperatures

a) Inherent stability

b) Biochemical modification

c) Association with a stabilising co-factor.

When comparing a thermophilic protein with an analogous mesophilic protein it has been found that there may be structural differences which account for the different thermal stabilities e.g. an increase in ionic and hydrogen bonds within the molecule. This can be achieved by changes in a few amino acid residues (Langworthy *et al.*, 1979) with the result that a thermostable protein is produced.

Biochemical modification occurs in tRNA extracted from *Thermus thermophilus* which contains a thiolated base (Oshima, 1979) making the molecule stable at high temperature.

Divalent cations can stabilise proteins, for example Ca^{2+} is required for thermal stability of an extracellular protease produced by *Thermus* strain T351 (D. Cowan, D.Phil. Thesis, 1980). Polyamines seem very important and have been found to stabilise the ribosome-tRNA-mRNA ternary complex (Langworthy *et al.*, 1979). Without the stabilising cofactor the complex cannot function at high temperatures.

Thermostability is therefore a complex property involving both changes in the primary structure of a molecule and modification after synthesis. The genetic control of these changes and therefore of thermophily is not yet known. It seems likely from the variety of methods known to stabilize molecules that thermophily depends on alterations over many genes.

Genetic investigations require "markers" - and auxotrophic mutants of wild-type strains are often used for this purpose. These mutants

have been very difficult to obtain for thermophiles (Langworthy *et al.*, 1979). Although minimal media and conditions for mutagenesis have been developed for some species (mostly *Bacillus*) there have been considerable problems in obtaining reproducible results. It has often been found that thermophiles are naturally extremely variable (Johnson, 1979). For proper genetic investigations it is essential to be able to control the variability of the organism studied.

Genetic investigations also require satisfactory systems for gene transfer. There are some reports of cloning thermophilic DNA into mesophilic bacteria to study thermophile genetics against the background of a well-known genome. The leucine gene from *Thermus thermophilus* has been cloned and expressed in *Escherichia coli* using the plasmid vector pBR322 (Ngahari *et al.*, 1980). The α-amylase gene from an unnamed thermophile *Thermophile V2* has been cloned into *Bacillus subtilis* using a bacteriophage vector (Shinomiya *et al.*, 1981). However gene expression will continue to be a problem using a mesophile as recipient strain. The study of thermophile genetics requires standard methods of producing mutants and the development of suitable systems for gene transfer.

This thesis presents an account of the investigation of the use of a cosmid vector for the transfer of thermophilic DNA and the induction and isolation of a bacteriophage which may be useful both as a vector for transferring DNA and as a system for investigating the mechanisms of genetic regulation of thermophily.

Ecological studies of thermophiles have revealed a large variety of microbial types (Brock, 1978; Castenholz, 1979). However the exact identification of new isolates, at least below genus level can cause difficulties. This can be due to variability of growth (Cometta *et al.*, 1982) and also because the exact criteria to determine a particular

species may not yet have been defined. Recent work (Wolf and Sharp, 1981) suggests that *Bacillus stearothermophilus* may in fact be a collection of organisms which belong to three separate species groups. The taxonomic position of the genus *Thermus* is even less clear. Five separate species have been named but there is not yet general agreement that this status is justified (Degryse *et al.*, 1978).

The problem of variability of growth and characteristics of thermophiles has been widely reported (Johnson, 1979; Cometta *et al.*, 1982) and has been experienced by workers at Waikato University. It was hoped that an investigation of the taxonomic relations of a number of groups of thermophiles would provide some insight into the extent of this variability as well as obtaining satisfactory species groupings for organisms of importance at this University.

The opportunity to work co-operatively with Cadbury Schweppes P.L.C Group Research at Reading, U.K., to use their facilities for pyrolysis mass spectrometry, provided an excellent opportunity for screening a large number of isolates belonging to a variety of microbial families. The results of this work are presented in this thesis.

CHAPTER 2

THE USE OF PYROLYSIS MASS SPECTROMETRY TO CLASSIFY BACTERIA

2.1 Introduction

Classification of micro-organisms into species groups has traditionally been based on morphology and physiological characteristics. However in recent years a number of new and sophisticated techniques have become more widely used. Although the results of classical tests are likely to continue to be the yardstick against which other methods are measured it usually requires a large number of time-consuming tests to obtain results at the species and strain level (Brock, 1974). Under certain circumstances classical methods may actually be less satisfactory than some of the newer techniques. It seems likely that although classifications based on the results of numerical taxonomic analyses of the results of biochemical tests will continue to form the basis of most taxonomic schemes other techniques such as DNA-DNA hybridisation and 16S rRNA homologies will become increasingly important in the future.

In microbial ecology studies it is not uncommon for new species to be isolated which seem very different from any described in the literature, for example those isolated by Zillig (Zillig *et al.*, 1981). In these cases where the physiology is not known classical methods take considerable time to develop and other approaches may be more satisfactory. Classical methods can sometimes fail to distinguish very similar species and are often poor at strain discrimination. In these cases newer and more sophisticated techniques are often advantageous.

Some of the new techniques are very specific and only suitable for use when identification, usually by classical methods, is nearly

complete e.g. serology and radio-antibody methods (Risby and Yergey, 1976). Others which can be used at the species and genus level, but not for more distant relationships include *DNA-DNA hybridisation* (Stephens *et al.*, 1983) and *DNA-RNA hybridisation* (Tu *et al.*, 1982). Hybridisation is very specific, and because it is independent of culture conditions and growth phase is a very powerful tool for determining relationships. However, in practice, results are often ambiguous because some species have extremely variable genomes and there are often experimental difficulties associated with incorporating sufficient radio-isotope.

The technique of *16S rRNA* sequencing (Stackebrandt and Woese, 1981) has proved extremely powerful in determining the relationships between prokaryotes at all levels from kingdom to species. However, it is too expensive and time consuming for general use.

RNA-polymerase cross-matching has been used very successfully by Zillig and co-workers to determine relationships at levels from species to families (Zillig *et al.*, 1982c) but as it requires the use of highly purified enzymes (often extremely oxygen sensitive) it is not practical for rapid, large-scale screening of new isolates.

Immunofluorescence using antibodies prepared against whole cells has been successfully used in some taxonomic studies of thermophiles Bohlool and Brock, 1974) but sometimes the results are difficult to interpret (B. Patel, Ph.D. Thesis, 1984).

Brenda - Bacterial Restriction Endonuclease digestion analysis of total DNA has been successfully used to discriminate closely related bacterial strains. This technique has the advantage of being independent of growth conditions or stage of culture (Hintermann *et al.*, 1981). Total DNA is extracted and purified and restricted with type

II restriction enzymes. This is followed by one-dimensional separation of restriction fragments by agarose gel electrophoresis. A typical banding pattern is obtained for a particular species which is generally stable for both wild-type and mutant strains. It is probable this technique will become more widely used in the future. A small number of *Thermus* strains were analysed using this technique at Massey University, New Zealand with the assistance of Dr R. Marshall, School of Veterinary Science.

Taxonomies based on *SDS-PAGE* (SDS polyacrylamide gel electrophoresis) of soluble proteins derived from whole cells (Jackman, 1982) have been successfully obtained for a number of species. When this method is used in combination with computer matching it has been shown to be very efficient in discriminating at the level of genus or below. Being a measure of gene expression it requires careful standardisation to ensure variables due to differences in culture conditions and growth phase are eliminated. A similar method based on SDS-PAGE of ribosomal proteins has been used successfully particularly with archaebacteria (Schmidt and Boeck, 1982a). These workers are now investigating relationships between archaebacterial species using immuno-electrophoresis techniques with antibodies prepared against ribosomal proteins (Schmidt *et al.*, 1982b).

Pyrolysis (thermal degradation in an inert atmosphere), which yields characteristic volatile fragments for a particular strain grown under standard conditions has been successfully used for identification and classification of many microorganisms. There are two systems of fragment analysis, gas-liquid chromatography and mass spectrometry (or a combination of both). *Pyrolysis gas chromotography* (PGLC) was developed as part of the United States space exploration programme to investigate the possibility of life on Mars (Oyama, 1965). The system

was then further developed for general use in microbiology (Reiner, 1965). PGLC uses relatively simple and generally widely available instrumentation and has been successful in discriminating micro-organisms at the genus, species and strain level. As yet results are only directly comparable when obtained from the same column. Changes in spectra due to slow poisoning of columns and difficulties in obtaining exactly matching columns have probably prevented more widespread use of this technique (Gutteridge and Norris, 1979). A recent study (Magee *et al.*, 1983) has reported considerable success in overcoming these problems by careful quality control, so interest in this method may be increased in future.

Pyrolysis mass spectrometry (PY-MS) was developed because of its potential for overcoming the repeatability problems of PGLC, the suitability of the data for computer analysis and the speed of analyses. It has also been found that PY-MS characterises bacteria better at the genus level than PGLC (Gutteridge and Puckey, 1982). The first use was for the analyses of biopolymers (Zemany, 1952) followed by structural analyses of synthetic polymers in the late 1960's (Meuzelaar *et al.*, 1982). PY-MS continues to be a powerful tool in this type of research and is extensively used for forensic purposes (Irwin 1979b). PY-MS analyses of soil, fuel and water samples (Irwin, 1979b) of biomedical samples (Irwin and Slack, 1978) and food samples (Gutteridge *et al.*, 1983) are increasing in number, a trend which seems likely to continue with improvements in on-line computing systems.

2.2 Pyrolysis Mass Spectrometry (PY-MS)

Several types of PY-MS have been described.

Direct Probe PY-MS

This method uses an accessory which is freely available on most

mass spectrometers with samples being pyrolysed next to the ion source. Fifty strains of Gram-negative bacteria from seven genera were discriminated successfully and unknowns identified with at least 90% success (Gutteridge and Puckey, 1982). However the technique is limited by having a maximum pyrolysis temperature of 300°C and by difficulties in controlling both the starting temperature and the end-point of an analysis (Gutteridge and Puckey, 1982).

Laser PY-MS

This method uses intense laser radiation which can cleave bonds directly as well as fragmentation due to heating. This is undesirable for PY-MS studies as it leads to the formation of ion-molecular complexes which confuse the mass spectrum. The use of infra-red (CO_2) lasers which emit relatively low energy radiation has minimised this effect. In theory this method has a number of advantages - it allows rapid direct heating of the sample only and therefore any desired temperature can be used, also both the final pyrolysis temperature and the temperature rise time can be exactly controlled and reproduced. The resolution is sufficient to allow analysis of 10-12 µg quantities of sample. At present technical problems and high cost are major difficulties and there are no reported analyses of microorganisms by this system (Meuzelaar *et al.*, 1982).

Filament Pyrolysis

There are several types of filament pyrolysers but the one most commonly used for microbial analyses (including this study) involves application of samples to ferromagnetic i.e. Curie-point wires which are heated inductively by a high frequency oscillator. Eddy currents are induced which cause a rise in surface temperature until the Curie-point of a particular wire is reached. At this point the wire becomes

paramagnetic and non-inducible so cooling begins immediately. The temperature of the Curie-point depends on the composition of a particular wire; pure iron having a Curie-point of 770°C and pure nickel of 358°C. A number of intermediate temperatures (e.g. the 510°C wires used in this study) can be obtained using alloys of these two metals. Curie-point wires of the same composition and dimensions are completely inter-changeable and cheap enough to be disposable. Because heating is inductive there is no contact between the heating system and the samples so the system can be automated (Meuzelaar *et al.*, 1978) which considerably increases the potential for rapid analyses.

2.3 Mass Spectrometry

Mass spectrometry involves the production of gas-phase ions from a sample and their separation according to their mass to charge (m/z) ratio. Ions can be produced by electron ionization in which an electron beam intersects the flow of sample molecules. The resulting collisions produce positively charged ions, the aim being to produce singly charged molecular ions. The ions must then be separated according to their masses. There are two main types of separation:

(a) Quadruple Mass Filters

The ion beam is accelerated into the centre of four parallel rods. Alternating radio frequencies are applied which cause the ions to oscillate. Only single charged species pass through the analyser to the detector.

(b) Single-Focusing Magnetic Deflection

In a magnetic field charged particles experience a magnetic force which causes separation of ions according to their m/z ratio.

Separated ion beams are collected by an electron multiplier and

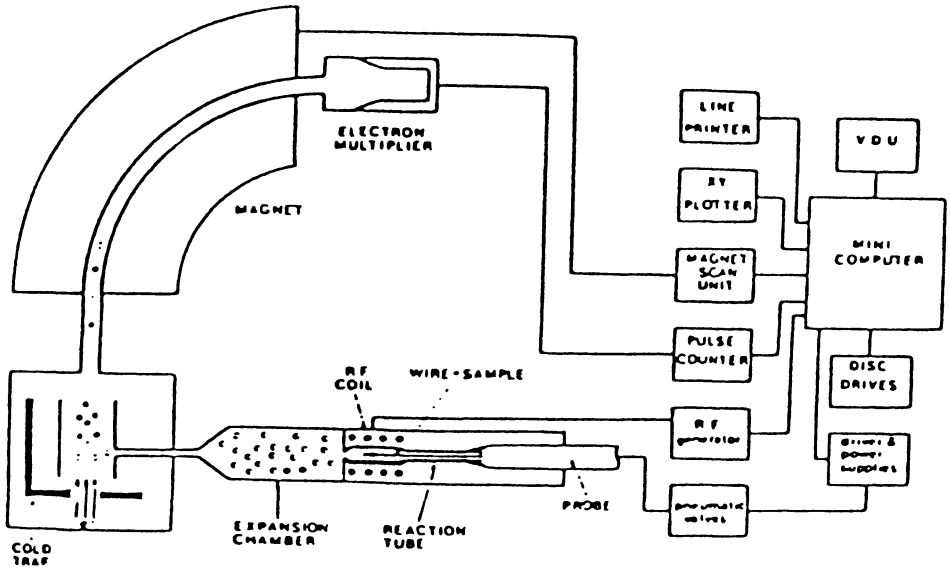


FIGURE 2.1

Schematic representation of the Pyromass 8-80

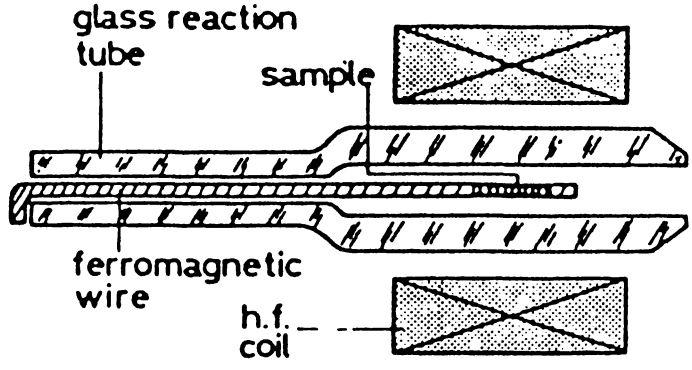


Figure 2.2 Schematic diagram of the Curie-point wire/reaction tube assembly located within the high frequency coil. (from Meuzelaar *et al.*, 1982)

amplified before being recorded (Pecsok *et al.*, 1976).

2.4 The Pyromass 8-80

The instrument used in this study was the Pyromass 8-80 developed in Britain at Cadbury Schweppes Group Research, Reading, in conjunction with VG Gas Analysis, Middlewitch. The Pyromass 8-80 was modelled on a pyrolysis mass spectrometer designed and developed at the F.O.M. Institute for Atomic and Molecular Physics Amsterdam (Meuzelaar and Kistermaker, 1973) and now fully automated (Meuzelaar *et al.*, 1978). A schematic representation of the Pyromass 8-80 can be seen in Fig. 2.1.

Pyrolysis is carried out on a Curie-point filament held in a glass reaction tube (see Figure 2.2) with power supplied by a 1.5KW, 1.1MHz frequency generator. After pyrolysis the pyrolysate passes into a gold-coated expansion chamber and enters the ion-source (via a ceramic tube) as a molecular beam. The entire pyrolysis inlet system is heated to a pre-determined temperature (150^{o}C in this study).

The ion source is a standard electron impace Nier type and was operated at 16eV for all bacterial analyses in this study. High molecular weight organic contaminants are trapped by a liquid nitrogen cooled baffle which surrounds the ion source.

The mass range is scanned exponentially from m/z (mass/charge) 300 to m/z 12 at 1.3s cycle^{-1}. Detection is by an electron multiplier followed by a fast-response amplifier. The pulses of ions generated are counted by a LSI 11/23 minicomputer controlled system, a pre-determined number of scans being averaged and the background spectrum subtracted if required. Raw mass intensity data are stored on floppy diskette. The mass intensities can be normalised and a full or restricted range printed on an X-Y plotter. Normalisation is a

procedure to remove the effect of sample size, and is a necessary part on any mass spectrometric procedure (Hill, 1966).

The Pyromass 8-80 was originally a manual instrument but an automated version is under trial at present. The analysis time is approximately 5 minutes per sample (Gutteridge *et al.*, 1983).

2.5 Pyrolysis of Bacterial Samples

Pyrolysis leads to the production of a series of lower molecular weight substances from complex materials like bacterial cells. Combustion or heating in the presence of oxygen results in production of very small uniform fragments e.g. CO_2, NH_2 and H_2O while pyrolysis is heating under conditions where net oxidation is prevented. In this process molecules are broken at weak points to form smaller more reduced fragments. If conditions are rigorously standardised these products are reproducible and characteristic of the substance pyrolysed.

(1) Pyrolysis Temperature

For reproducible degradation a temperature suitable for the material being analysed must be chosen. A relatively low pyrolysis temperature e.g. <400^{o}C may lead to incomplete degradation of bacterial samples while excessively high temperatures may lead to evaporation or to secondary pyrolysis of condensed products. For bacterial samples it has been reported (Gutteridge and Norris, 1979) that temperatures between 500^{o}C and 1000^{o}C do not affect the products provided the temperature rise time is standardised. The temperature of 510^{o}C used in these experiments was chosen as it has been used successfully at the F.O.M. Institute and suitable Curie-point wires are readily available.

(2) Temperature Rise Time

Although pyrolysis temperature was initially thought to determine the products which formed, it was shown (Farré-Rius and Guiochon, 1968) that heating rate was the factor which had the greatest effect. Long temperature rise times allow high boiling point compounds to escape from the pyrolyser before true pyrolysis (Ericsson *et al.*, 1977) and increase the probability of product reactions occurring. In pyrolysis the primary rupture of bonds can be followed by a complex series of secondary reactions between the products (Levy, 1967), control of which is extremely important in PY-MS. The temperature rise time depends on the power output of the high frequency generator, the care taken to place the sample in the exact centre of the inductive coil and the sample size. It has been found (Meuzelaar *et al.*, 1982) that the amount of non-volatile chars which remain on the filament is inversely proportional to the temperature rise time. To reduce both this problem and also to prevent high boiling tarry substances escaping from the pyrolysis chamber and causing contamination problems in the expansion and ionisation chambers, temperature rise times are adjusted to be as rapid as possible.

2.6 Pyrolysis Transfer

Ideally this should occur without loss, degradation or secondary reactions. Glass reaction tubes are designed to trap relatively involatile substances which could contaminate the ion source and to direct the volatile beam towards it. A beam of pyrolysis fragment molecules enters an expansion chamber which broadens the pressure/time profile and allows time for several mass scans, ensuring a representative sample is obtained. Chamber walls are coated with a chemically inert substance e.g. gold and heated to a temperature which prevents condensation but at the same time does not increase the occurrence of secondary

reactions, usually 150°C for microbial PY-MS. The glass reaction tubes are not directly heated so products with low volatility and large evaporated compounds e.g. lipids tend to remain on the walls which inevitably leads to loss of valuable information. The advantage of this is minimal contamination of the expansion chamber and ion source so that up to a year's use without cleaning is possible.

2.7 Ionisation

Ionisation must be stable enough for long-term repeatability and should cause minimal fragmentation as ideally only ionisation of pyrolysis derived molecules should occur. So-called "soft" ionisation techniques are required to fulfil these conditions. In PY-MS instruments electron impact has been the principal technique used, with energy levels of around 15eV being a good compromise between minimum production of fragment ions and maximum production of molecular ions (Meuzelaar *et al.*, 1982). Deviations of even 0.1eV can cause major spectral changes therefore it is most important to ensure the electron energy is kept constant (Windig *et al.*, 1979).

To prevent contamination by high molecular weight compounds the ion source is surrounded by a liquid nitrogen cooled screen which also acts as a pump due to its large surface area and low temperature. This type of "pumping" is very stable and ensures long-term reproducibility far more efficiently than other systems e.g. diffusion pumps (Meuzelaar *et al.*, 1982). Accumulation of trapped products is prevented by warming the screen to room temperature at the end of the day, which allows for their effective removal by the main vacuum pump of the mass spectrometer.

2.8 Mass Analysis

The instrument used at the F.O.M. Institute was a quadruple mass spectrometer. The Pyromass 8-80 used in this study has a single 8 cm 80^{o} focussing magnetic mass analyser for ion deflection as it was hoped this would have maximum possible stability over long periods of time. Problems of instability due to differences in relative ion transmission are characteristic of quadruple instruments. A problem with magnetic detector instruments is vulnerability to ion source contamination (Meuzelaar *et al.*, 1982).

Ion detection is by pulse counting to allow recording of extremely small signals. As this type of system is usually characterised by slow-speed scanning, workers at the F.O.M. Institute have designed a special high-speed ion counter to ensure maximum possible counts are obtained (Meuzelaar *et al.*, 1982). In the Pyromass 8-80 detection is by an electron multiplier followed by a fast-response amplifier.

The regime used in this study was to scan ions in the m/z 300 to m/z 12 range; 35 scans were averaged, and the raw intensity data stored on floppy diskette, and an X-Y plot printed out for visual comparison.

The operating parameters used on the Pyromass 8-80 in this study were:

Pyrolysis	$510^{o}C$
Inlet temperature	$150^{o}C$
Hold time (prior to pyrolysis within the inlet system	60s
Scan time	1.3 cycles sec^{-1}
Number of scans	35
Electron energy	16eV

TABLE 2.1

MICROBIOLOGICAL APPLICATIONS OF PY-MS

Purpose	Problem	Micro-organism	Reference
Classification	intra-species	*Mycobacterium*	Wieten *et al.*, 1981
	heterogeneity	*Bacillus*	Shute *et al.*, 1984
		Listeria	Eshuis *et al.*, 1977
		Neisseria gonorrhoea	Borst *et al.*, 1978
		Pseudomonas	Risby & Yergey, 1976
		Escherichia coli	Risby & Yergey, 1976
		Arthrobacter	Risby & Yergey, 1976
		Staphylococcus	Anhalt & Fenselau, 1975
		Proteus	Anhalt & Fenselau, 1975
		Streptococcus	Kistemaker *et al.*, 1975
		Mycoplasma	Quinn *et al.*, 1977
		Fungi	Weijman, 1977
	epidemiology	*Klebsiella*	Haverkamp *et al.*, 1979
Identification	pathogenicity	*Escherichia coli*	Haverkamp *et al.*, 1980b
		Mycobacterium	Wieten *et al.*, 1981
	cariogenicity	*Streptococcus*	Kistemaker *et al.*, 1975

Reproduced (with modifications) from Meuzelaar *et al.*, 1982.

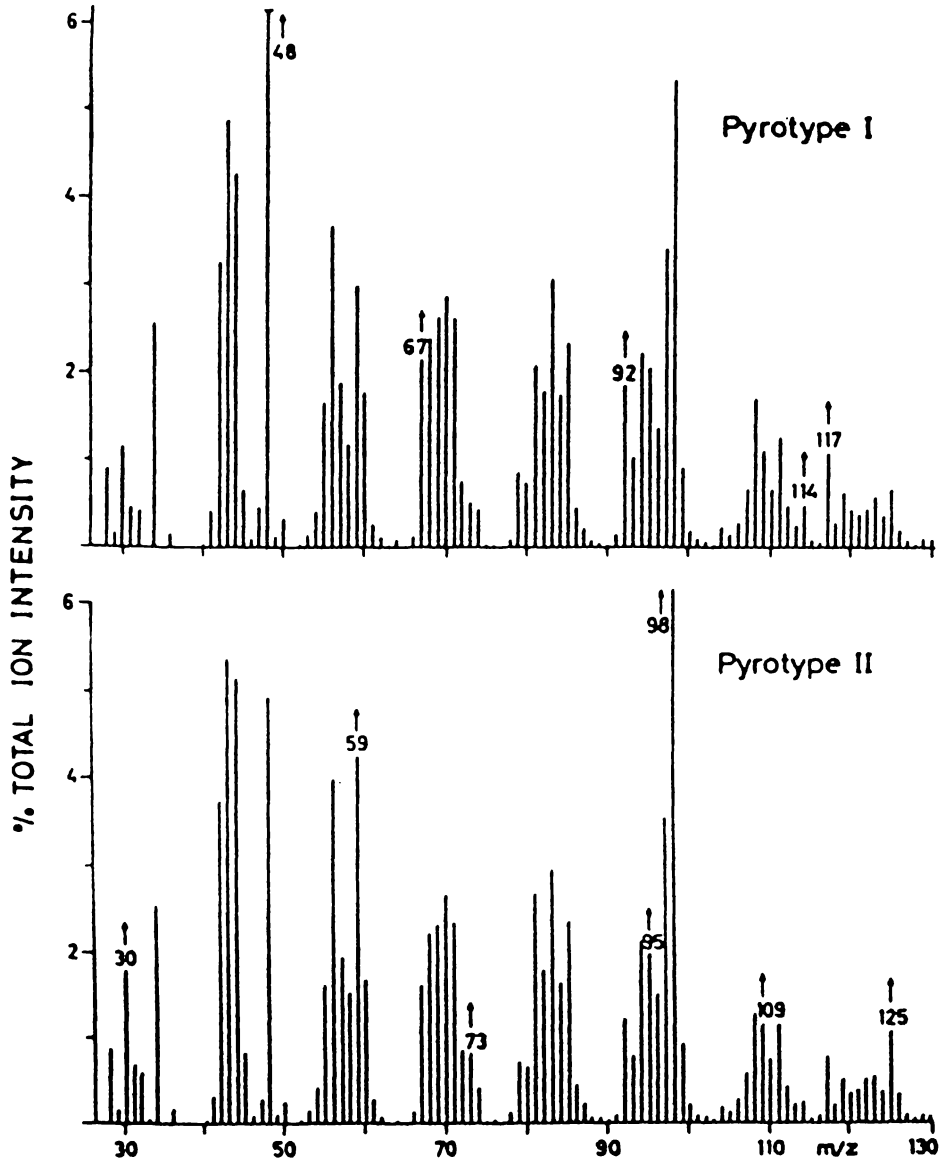


Figure 2.3 (from Meuzelaar *et al.*, 1982)

Examples of pyrolysis mass spectra of two different pyrotypes of *Neisseria gonorrhoea*. Arrows indicate significantly higher peak intensities.

2.9 The Use of PY-MS in Microbial Taxonomy

PY-MS has been used for many microbial investigations since its development by Meuzelaar and Kistermaker (1973) ten years ago. A list of some of the organisms including both bacteria and fungi which have been successfully characterised is given in Table 2.1.

It has been stated (Meuzelaar *et al.*, 1974) that an automated technique for identification of microorganisms which could meet the following requirements would be extremely useful.

a) widespread applicability

b) specificity

c) reproducibility

d) speed

e) sensitivity

Pyrolysis mass spectrometry can currently meet many of these criteria and with continuing improvements in instrumentation and data processing has the potential to meet all five.

The range of micro-organisms listed in Table 2.1 suggests that the technique has widespread applicability to this type of material.

Although biochemical differences between micro-organisms are obviously detected by PY-MS, the complexity of mass pyrograms (X-Y plots of mass intensity data - Irwin, 1979a) can make their interpretation difficult. However there are some examples of changes in pyrograms being related to identifiable cellular components. For example as shown in Figure 2.3 two prototypes of *Neisseria gonorrhoea* were identified by PY-MS and their distinction found to depend on differences in glucose utilization (Meuzelaar *et al.*, 1982). The changes in mass pyrograms were related to an increase in an N-acetyl-amino

sugar component accompanied by a decrease in a protein component rich in methionine (m/z 48), proline (m/z 67) and tryptophan (m/z 117). Similarly identification of a capsular polysaccharide (related to vaccine development) has also been reported for this organism (Haverkamp *et al.*, 1980a) and for antigenic components of *Escherichia coli* (Haverkamp *et al.*, 1980b).

A major problem of microbial identification based on pyrolysis techniques has been that of reproducibility. In the less expensive related technique of PGLC problems of long-term reproducibility (sometimes called repeatability) have limited the potential usefulness of the technique. In part PY-MS was developed in an attempt to overcome these. Both qualitative and quantitative reproducibility are required if results are to be compared on a long-term basis, (usually defined as >1 month in this context) and inter-laboratory reproducibility achieved. As the latter offers the prospect of setting up reference libraries of mass pyrograms it is obviously a very desirable goal. It has been found that qualitative reproducibility is not generally a problem when conditions are carefully controlled, in particular temperature rise time (Meuzelaar *et al.*, 1982).

In this study qualitative and quantitative reproducibility were monitored using glycogen standards. 20 µg of a standard aqueous solution of glycogen was applied to a 510^{o}C Curie-point wire and analysed under the conditions described above. Both the total number of ions produced and the mass pyrogram printed on the X-Y plotter were carefully compared with standards produced when the instrument was set up. If necessary, adjustments to the running conditions of electron energy setting can be made. Gross differences in results usually suggest ion source contamination which mean both this and the expansion chamber surface require cleaning. The reproducibility and repeatability of

TABLE 2.2

RECOMMENDED STANDARD PYROLYSIS CONDITIONS

Wire cleaning method	Reductive
Suspending liquid	Methanol
Sample size	5 - 20 µg
Equilibrium temperature	500 ± 10°C
Temperature rise time	0.1 - 1.5 s
Total heating time	0.3 - 1.2 s
Inlet temperature	150°C

Reproduced from Windig *et al.*, 1979.

glycogen has been thoroughly tested (Windig *et al.*, 1979) and found to be in the order of 6% intensity change in major peaks in short term studies to 8% after 34 days, which is within the range of difference acceptable for biologically variable samples like bacteria. It has been found that although sample preparation and pyrolysis have little effect on quantitative reproducibility when performed under standard conditions, variations in pyrolysate transfer and mass spectrometric analysis can seriously affect results (Windig *et al.*, 1979). A set of recommended standard conditions for pyrolysis has been proposed by these workers as the first step in achieving inter-laboratory reproducibility - see Table 2.2). Because this has not yet been achieved most PY-MS studies (including the one described in this thesis) carried out to date have involved running both reference and unknown samples in the same analysis.

As will be described in Chapter 3 the results of a second analysis of *Thermus* samples were unable to be compared with the results of an earlier analysis possibly because of changes caused by ion source contamination. In an attempt to monitor quantitative differences, glycogen standards were run at intervals as described in Section 1.6. These differences were not detected until the data were processed suggesting the use of standards more similar to the material being analysed might be of more value for future studies.

Although no systematic study on inter-laboratory reproducibility has been undertaken it has been reported that this seems an attainable goal when similar instruments are used (Meuzellar *et al.*, 1982).

Few techniques can compare with PY-MS for speed as untreated microbial samples can be analysed directly and a spectrum obtained in 5 - 6 minutes. As yet, data processing of results requires access to a main-

frame computer but with this facility it is possible to obtain the results of an analysis in a single day. With continuing development and sophistication of small computers the scope of this technique will widen considerably.

Because of the sensitivity of this technique very small samples can be analysed with amounts of 20 - 50 µg only being required. In fact samples which are too large can cause problems as they can be "blown off" filaments during pyrolysis (leading to contamination problems) and also prevent efficient heat transfer.

2.10 Sample Preparation

Curie-point wires must be thoroughly cleaned before use. Wires were soaked in warm 10% HCl for 30 minutes followed by thorough rinsing in tapwater, distilled water and finally acetone. They were then dried overnight at 200°C.

Glass reaction tubes were cleaned by overnight soaking in chromic acid followed by rinsing and drying as for the wires.

For analysis of samples a wire was threaded through a glass reaction tube (See Figure 2.2) with about 4 cms protruding through the wide bore end. Samples were applied to a small section of the wire 3 mm from the tip using a Pasteur pipette for liquid samples and with a bacteriological loop for colonies taken directly from agar plates. The wire was then withdrawn to a point which places the sample in the exact centre of the induction coil (see Figure 2.2). Curie-point wires were inexpensive and were discarded after use.

The bacteria analysed in this study had been freeze dried so it was necessary to resuspend them in methanol (Meuzelaar *et al.*, 1982). An even suspension was obtained by immersing tubes in an ultrasonic bath

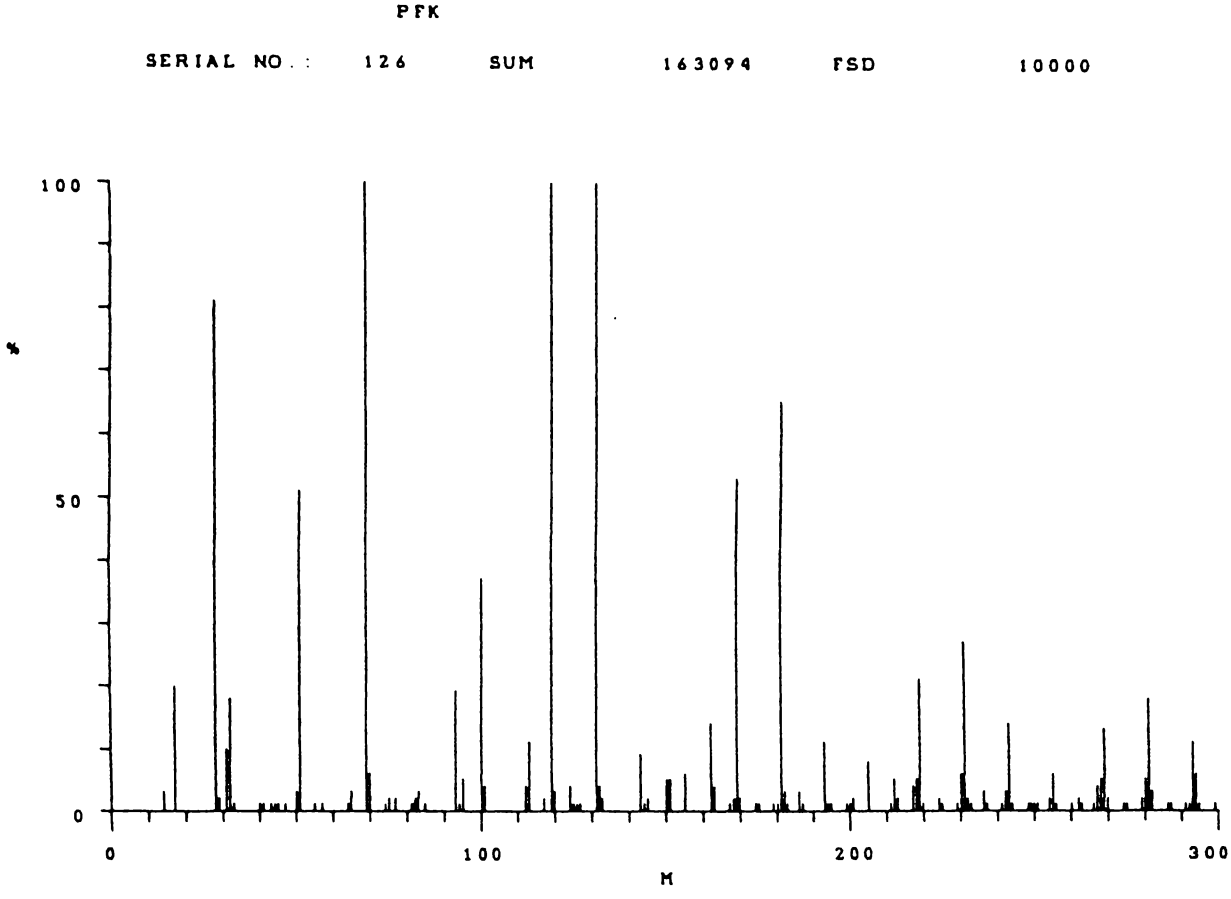


FIGURE 2.4 Mass spectrum (pyrogram) for perfluorokerosene (PFK). A sample of PFK is run to check the correct alignment of masses in the computer memory. Particular attention is paid to the location of a major peak at m/z=69.

for 3 - 5 minutes. It was decided to use only spectra which resulted from samples giving recordings of between 1 x 10^6 and 5 x 10^6 total ion counts, which was usually from the application of 3 - 5 small drops from the tip of a pasteur pipette, with thorough drying between each drop.

2.11 Operating the Pyromass 8-80

The mass scale on the Pyromass 8-80 was divided into 8192 memory locations and before use (and after any change in operating conditions e.g. loss of vacuum) the computer controlled system was reprogrammed to correctly assign masses to their proper location. Alignment of mass scale as well as control of pyrolysis and mass spectrometry conditions was carried out using the computer keyboard.

The mass scale was aligned by allowing a very small quantity of air to be ionised at 70eV. The raw mass data was checked to ensure major peaks were assigned to the correct location and the programmed power supply was adjusted as required. To check alignment a sample of perfluorokerosene (PFK) was analysed. This is a volatile substance which does not require pyrolysing. Pure analytical PFK always gives the same relatively simple spectrum in which changes are easily detected (see Figure 2.4). A 1 - 2 µg sample of PFK was loaded onto a 510^{o} Curie-point wire (but not pyrolysed) and ionised at 70eV; 20 scans being averaged. Raw mass data were compared with standard values for the Pyromass 8-80, in particular for m/z 28. To check that all m/z peaks were recorded at the correct location a second PFK sample was analysed and an X-Y plot obtained for comparison with the standard mass pyrogram shown in figure 2.4. This not only allowed the operator to check the PFK spectrum but also to detect any unusual peaks which could suggest contamination. After the instrument was satisfactorily set up

the electron energy was reduced to 16eV and the pyrolysis conditions set as required. As stated, pre-heating for 60 seconds, 2 seconds pyrolysis and 150^{o}C as sample chamber and transfer-system temperature were used throughout the course of these studies.

Each batch of cleaned Curie-point wires was tested for background by analysing a clean wire (without sample) under normal operating conditions. It was not necessary to subtract background from spectra obtained from samples in this study.

2.12 Analysis of Samples

510^{o}C Curie-point wires were loaded with sample as described in Section 2.9 and analysed under the regime set out in Section 2.7. The mass range m/z 300 to m/z/ 12 was scanned, raw mass intensity data stored on floppy diskette and X-Y plots (pyrograms) of scaled data obtained for visual inspection. The scale was usually 10^{-4} with peaks expressed as percentages.

Four replicates of each strain were analysed to provide pre-formed groups for subsequent statistical analysis and spectra for all samples with total ion counts in the $1 \times 10^{6} - 5 \times 10^{6}$ recorded.

2.13 Data Processing

Initially data was processed on Cadbury Schweppes IBM 3033 main-frame computer but subsequently some of the data was sent to New Zealand and analysed on the VAX computer at Waikato University.

X-Y plots and raw mass data sets were examined to detect any obvious trends and to eliminate extremely large m/z values which have been found to seriously affect the normalisation procedure (MacFie and Gutteridge, 1982). In practise m/z values >1% of the total ion current

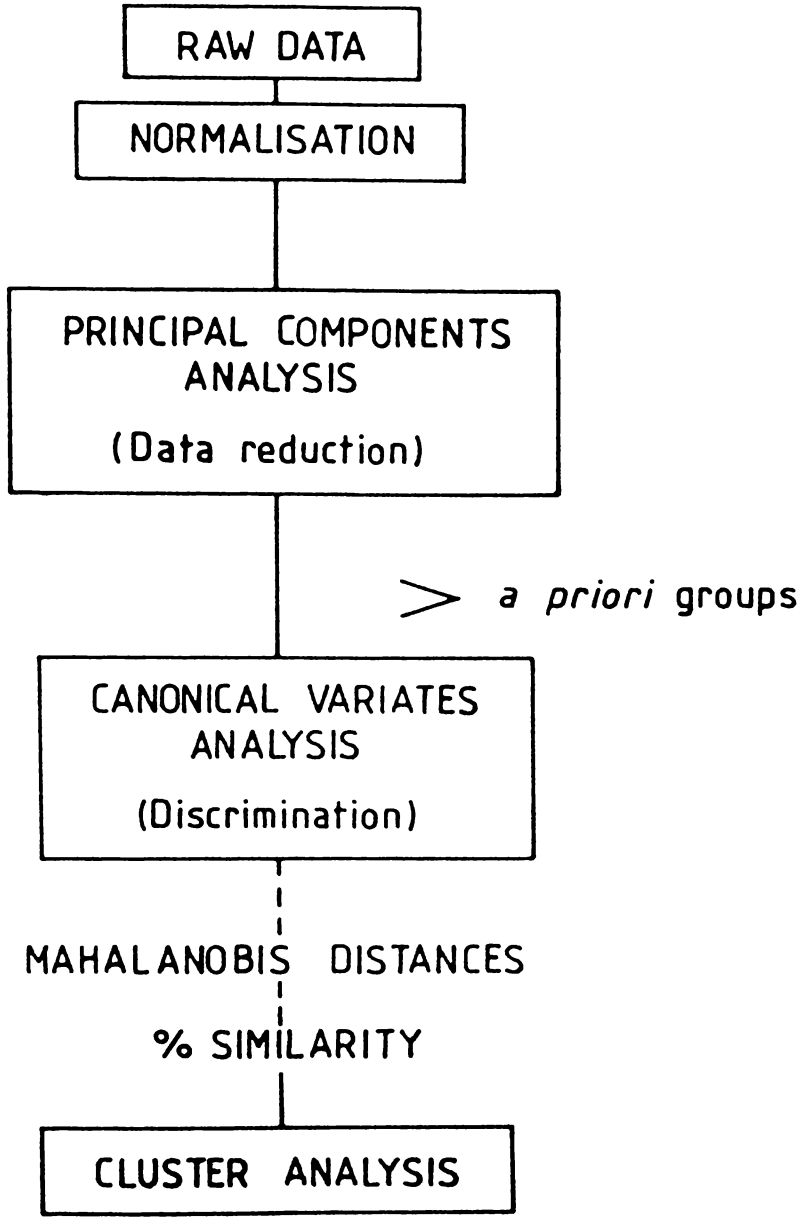


Figure 2.5 Flow diagram of statistical methods used to process the data generated during this study.

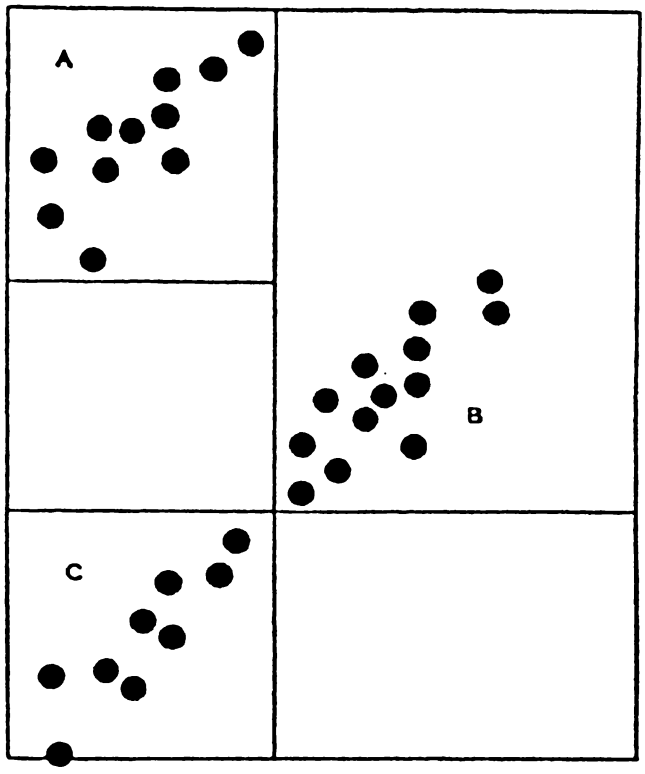


Figure 2.6 The three groups A, B and C have similar dispersion matrices. The Euclidean distance between the means of A and B, and B and C is approximately the same. The generalised distance (Mahalanobis' D^2) calculated by canonical variates analysis takes account of the scatter of the variables and the correlations among them. The generalised distance between A and B is much greater than that between B and C. (from Marriott, 1974)

were eliminated. Peaks with ion counts of less than 10 were also removed as these were considered too small to contain any useful information (Shute *et al.*, 1984). For computer analysis m/z values were normalised by expressing all values as a percentage of the total ion current of the chosen set. This means if 152 ions were chosen for analysis (as for the *Thermus* strains) normalisation was by expressing each peak as a percentage of the sum of the 152 m/z values. Normalisation is necessary to eliminate variations due to sample size (MacFie and Gutteridge, 1982).

Computer analyses were done using the GENSTAT package (Nelder, 1979) and a number of different multivariate statistical techniques which are summarised in Figure 2.5.

2.13.1 Principal Components Analysis

Principal components analysis is a method by which the information contained in a large number of variables can be represented more simply. A set of variables (i.e. the chosen set of m/z values) can be transformed mathematically to a new set of uncorrelated variables (Marriott, 1974). As principal components (P.C's.) are calculated in order of decreasing variance the information contained in the chosen set of m/z values can be represented (without loss) by a very much reduced data set. This is necessary for handling the large amount of data even when using mainframe computers. In these studies the number of principal components which described 99% of the variance between samples was selected (in consultation with Mr L. Vallis, Meat Research Institute, Langford, Briston). For some later analysis the number describing 95% of the variance was found to be more useful.

It has been found that the use of the principal co-ordinates algorithm in which an association matrix is derived from the m/z variables chosen, followed by principal components analysis (Gower, 1966)

is a very suitable method for processing PY-MS data (Gutteridge *et al.*, 1979). Because most of the variance is described by the first few principal components two-dimensional scatter plots relating samples to pairs of the first few P.C's. (pairs of the first three components were used in these analyses) can be drawn for visual inspection. These have been found to be extremely useful for detecting outliers. Outliers are individual analyses, or even strains which fall so far outside the majority of samples they prevent proper discrimination. An example of an outlying sample and strain of *Thermus* can be seen in figure 3.4 which also shows how the other samples are artificially compressed together and cannot be discriminated. Outliers occur in most PY-MS studies and indicate the power of the technique to detect unsuspected variability (MacFie and Gutteridge, 1982). This can be due to an unusual strain which warrants further investigation or some error during growth or preparation of the sample for analysis. Examples of the latter could be a contaminated strain, failure to remove all media or accidentally touching a pyrolysis wire.

Principal components analysis is seldom sufficient on its own to properly discriminate strains especially if they are very similar (MacFie and Gutteridge, 1982). For proper discrimination further data processing is required with all further steps carried out on the P.C. values calculated to describe the required % of the total variance.

2.13.2 Canonical Variates Analysis

Regression analyses are used to find the relationship between a single variate and a set of related variables. In multivariate analyses this involves calculating canonical correlations between pairs of canonical variates (which are linear combinations of the original variables). If one set of variables represents the differences between groups of samples being analysed the method can be used

to determine the variance "between" and "within" the groups of samples. Canonical correlations (like principal components) form a decreasing sequence therefore plots of samples in relation to pairs of canonical axes are very useful for representing relationships between them (Marriott, 1974).

Canonical variates analysis requires pre-formed (*a priori*) groups. In this study an *a priori* group consisted of four replicates of each strain. The GENSTAT package allows graphical plots of the mean position of each strain group in relation to pairs of canonical axes to be drawn. These plots have been found very useful for discriminating between bacteria in PY-MS studies (MacFie *et al.*, 1978).

The dissimilarity (or distance) between each group calculated by this method is defined in terms of Mahalanobis' D^2 values. The advantage of measuring this distance can be seen in Figure 2.6.

It has been found that a D^2 >6 between pairs of samples suggests the difference between them is statistically significant and D^2 >12 suggests highly significant differences (Shute *et al.*, 1984).

If the strains in a study are very similar, canonical variates plots may not distinguish them properly. To include all the information from the canonical variates analysis further data processing is required.

2.13.3 Average Linkage Cluster Analysis

In order to carry out cluster analysis, distances between strains were transformed to % similarities. A % similarity matrix was derived from the Mahalanobis' D^2 values which meant the information contained in all the canonical axes could be used to help discriminate the strains.

In average linkage, clustering points join a cluster, or clusters combine, at the average level of similarity between any two samples

in the new cluster (O'Donnell and Norris, 1981).

A dendrogram giving a pictorial representation of these relationships was computer drawn for visual inspection.

2.13.4 Application of the Data Processing Technique to Thermophile Strains

The method used was as follows:

(1) Raw mass data was inspected to select the appropriate m/z values.

(2) The selected data set was normalised.

(3) Principal components analysis was carried out. Outliers were removed and the required number of P.C's. selected.

(4) Canonical variates analysis calculated D^2 between pairs of strains. Canonical variates plots were inspected for strain discrimination.

(5) A dendrogram was drawn up by applying average linkage cluster analysis to the % similarities which were calculated from the D^2 values.

Canonical variates analysis is susceptible to compression when very dissimilar strains are present (MacFie and Gutteridge, 1982). This affects % similarities and meant that dendrogram patterns, rather than actual % similarity values, were important.

Where reference strains were not discriminated on first analysis this was achieved by removal of highly dissimilar strains from the data set or, if necessary, reducing the number of P.C.'s to that required to describe 95% of the variance.

To date bacteria analysed by PY-MS have had a well known taxonomy. The thermophile samples were characterised to genus level only and few

reference strains were available for any genus. This meant other data processing methods e.g. stepwise discriminant analysis (Shute *et al.*, 1984) could not be used.

All computer programmes used in these analyses were written in GENSTAT language by Dr H.J.H. MacFie and Mr L. Vallis, Meat Research Institute, Langford, Bristol and used with their kind permission.

CHAPTER THREE

THE TAXONOMY OF *THERMUS*

3.1 Introduction

Bacteria belonging to the genus *Thermus* are aerobic thermophiles which are variable in both morphology and physiology. The first published isolate, *Thermus aquaticus*, was from Yellowstone Park, U.S.A. (Brock and Freeze, 1969) and since 1969 a widespread distribution of similar strains has been found in both natural and man-made water systems of mildly alkaline pH and temperatures ranging from 50^{o} - 90^{o}C.

Typically, members of the genus are obligately aerobic, rod-shaped, non-motile bacteria. Strains isolated from natural environments usually have carotenoid pigments, predominately yellow-coloured, which are thought to be photoprotective (Brock, 1978). Growth is chemoheterotrophic and occurs on both complex medium and a limited number of defined substrates in the laboratory. A few strains require the addition of some vitamins or amino acids when grown in defined medium (Saiki *et al.*, 1972; Degryse *et al.*, 1978). In natural environments, dissolved organic carbon, which has been measured as being at least 2ppm in *Thermus* environments is probably used for growth (Brock, 1978). Under unfavourable growth conditions filamentous forms and "rotund bodies" appear; the latter being associations of separate cells connected by fusion of the outer envelope layer (Brock and Edwards, 1970).

Although the general structure is similar to that of mesophilic Gram negative bacteria connections between the outer envelope layer and plasma membrane give a distinctive cross-hatched pattern to thin sections and freeze fractured preparations (Brock and Edwards, 1970; Heinen and Heinen, 1972).

TABLE 3.1 SUMMARY OF THE CHARACTERISTICS OF *THERMUS*

	Pigment	Temperature °C		Moles %	Growth	Formation of filaments and spherical bodies	Generation time (minutes)
		opt.	max.	G & C			
Thermus aquaticus [1]	yellow	70	80	64 - 67	opt. peptone 0.1% nil N.A.	Yes	50 78*
Thermus thermophilus [2]	yellow/ orange	65 - 72	85	69	opt. peptone 2.0% positive N.A.	N.R.	31*
Thermus flavus [3]	yellow	70	81	70	opt. peptone 1 - 3% positive N.A.	N.R.	42
Thermus caldophilus [4]	yellow	75	N.R.	N.R.	opt. peptone 0.8%	N.R.	N.R.
Thermus ruber [5]	red	55 - 60	70	N.R.	opt. peptone 0.1 - 0.5% nil N.A.	Yes	60
'Ramaley's XI' [6]	cream	69 - 71	80	64	opt. peptone 0.1% nil N.A.	Yes	58*
Strain NH [7]	cream	70	80	61.5	opt. peptone 0.1%	N.R.	67
Strain D1	cream	69		62.1	nil N.A.		66
T351 [8]	fawn/ orange	70 - 80	N.R.	N.R.	opt. peptone 0.3% poor N.A.	Yes	120 (estimate)
Strain G [9]	yellow	66 - 68	81	63.3	opt. peptone 0.3%	N.R.	42
K-2 Isolate [10]	pink	60	80	64	opt. peptone 0.1% nil N.A.	N.R.	50

[1] Brock and Freeze, 1969
[2] Oshima and Imahori, 1974
[3] Saiki *et al.*, 1972
[4] Taguchi *et al.*, 1983
[5] Loginova and Egorova, 1975
[6] Ramaley and Hixson, 1970
[7] Pask-Hughes and Williams, 1975
[8] Hickey and Daniel, 1979
[9] Pask-Hughes and Williams, 1977
[10] Ramaley and Bitzinger, 1975

* Values given by Williams, 1975

N.A. = nutrient agar
N.R. = not recorded
opt. = optimum
max. = maximum

In the laboratory most strains grow optimally at 70°C and are therefore classed as caldoactive bacteria (Williams, 1975). Many strains are isolated from pools with temperatures higher than 70°C but as it has been shown that laboratory cultures can gradually be adapted to grow at higher temperatures (Heinen, 1971) *Thermus* species are probably well suited to their natural environment.

To live successfully at high temperature requires stable cellular structures and enzyme systems. The biochemistry of *Thermus* has been studied in some detail and macromolecules found to be stabilised by a combination of small changes in primary structure (compared to their mesophilic equivalents) and by the influence of cations and other molecules (Oshima, 1979; Watanabe *et al.*, 1976; Ray and Brock, 1971c). Changes in culture temperature of a strain can lead to changes in chemical structure, for example increasing the growth temperature causes a correlated increase in the sulphur content of tRNA in *T. thermophilus* (due to thiolation of ribothymidine bases - Oshima *et al.*, 1976). The lipid composition of *Thermus* differs considerably from that of mesophilic Gram negative bacteria (Ray *et al.*, 1971b; Pask-Hughes and Shaw, 1982) and an increase in growth temperature leads to further changes in fatty acid composition (Ray *et al.*, 1971a).

3.2 Physiology of *Thermus*

A summary of *Thermus* properties appears in Table 3.1, which shows that five different species have been characterised and named. The pH optimum of all strains seems similar, being pH 7.0 - 7.5 with growth possible over the pH 6.0 - 9.0 range.

Although all strains of *Thermus* have been found to be extremely sensitive to penicillin, inhibition by other antibiotics is more variable

(Y. Casey, pers. comm.). *Thermus aquaticus* is completely inhibited by 10 μg ml^{-1} streptomycin (Brock and Freeze, 1969), but *T. flavus* is not inhibited until the concentration reaches 100 μg ml^{-1} (Saiki *et al.*, 1979), with similar patterns being found for other aminoglycoside antibiotics.

Table 1 suggests that there are two distinct types of *Thermus* species, one which grows optimally at high (1 - 3%) peptone concentration, and one which can only grow at low concentration (0.1 - 0.5%), the latter group being completely inhibited by 1% peptone and unable to grow on nutrient agar. The high peptone group includes *T. thermophilus*, *T. flavus*, *T. caldophilus* (all Japanese isolates) and 9 Russian isolates (Egorova and Loginova, 1974). The low peptone group includes *T. aquaticus*, *T. ruber*, all the non-pigmeted isolates reported to date and a group of Icelandic isolates (Pask-Hughes and Williams, 1977).

Growth on defined media appears limited to relatively few substrates. Glutamic acid supports the growth of most *Thermus* strains as sole source of carbon, nitrogen and energy; similarily, with ammonium added as nitrogen source, acetate can also support growth of most strains (Pask-Hughes and Williams, 1977). Few Krebs cycle intermediates support growth, and as all enzymes of both this and the glyoxylate cycle are present the failure is probably due to lack of the necessary uptake mechanisms (Pask-Hughes and Williams, 1977). The results of some carbohydrate utilisation experiments seemed difficult to interpret and further investigations into these led to the proposal that characterisation of the group should be revised (Degryse *et al.*, 1978).

Glucose supplemented with ammonium did not support growth but glucose supplemented with glutamate gave excellent growth (Degryse *et al.*, 1978). This seemed to suggest an inability to maintain a supply of Krebs cycle intermediates by one or both of the following reactions:

$$\text{pyruvate} + \text{ATP} + CO_2 \rightarrow \text{oxaloacetate} + \text{ADP} + P_i$$

or

$$\text{phosphoenolpyruvate} + CO_2 \rightarrow \text{oxaloacetate} + P_i$$

When a number of strains were tested for the presence of the necessary enzymes it was found all had a constitutive and highly active PEP carboxylase but that pyruvate carboxylase was not present (Degryse *et al.*, 1981). The question of CO_2 availability was then considered and it was found that under the conditions used to grow *Thermus* on glucose this substance became limiting. At high temperature and low pH the solubility of CO_2 decreases and addition of glucose to the medium used (Medium D - Castenholz, 1969) lead to exactly this situation. The addition of sucrose did not cause a drop in pH which explained the observation that sucrose + ammonium could support growth but glucose required the addition of glutamate (Degryse *et al.*, 1978). A medium was designed by these workers which had the buffering capacity at 70°C to prevent a drop in pH on addition of glucose; under these conditions they found that glucose and a limited number of other defined carbon sources could support growth of all but one of the species tested. This species, *T. thermophilus*, seemed to be a lysine auxotroph. These workers also reported that growth on complex medium was identical for all strains tested but they did not state the peptone concentration used (Degryse *et al.*, 1978).

An inefficient utilization of substrate first noted in batch culture (Degryse *et al.*, 1978) was confirmed under continuous culture conditions, a value of only $\sim$0.15 g biomass g^{-1} total carbon used being obtained (Sonnleitner *et al.*, 1982). This inefficiency was proposed by the latter workers to be a survival mechanism to conserve carbon which is often scarce in hot springs.

3.3 Taxonomy of *Thermus*

The well-buffered medium described in Section 3.2 was used to investigate growth and properties of four groups of *Thermus* strains; *T. aquaticus*, *T. thermophilus*, two non-pigmented isolates NH and D1 (see Table 1) and three isolates from a thermally polluted Belgian river. Not only was it found that there was good growth on glucose + ammonium for all strains tested but also that other morphological and physiological differences which had lead to their characterisation as different species seemed no longer tenable. It was suggested therefore, that all strains tested were simply varieties of one species, *Thermus aquaticus* (Degryse *et al.*, 1978).

If all isolates of *Thermus* described to date are in fact simply varieties of one species then the data in Table 1 indicates a high degree of variability. Evidence supporting variability comes from continuous culture experiments and ecological studies (Cometta *et al.*, 1982b). Under continuous culture conditions in defined medium pigments were irreversibly lost from *T. aquaticus*. To exclude the possibility that the white cells were a contaminant, micrographs of freeze-fractured cells were examined and the distinctive "cross-hatched" *Thermus* pattern found (Heinen and Heinen, 1972). When *T. aquaticus* was grown in complex medium in continuous culture, a mixture of white and pigmented colonies resulted from plating of aliquots onto solid medium (Cometta *et al.*, 1982a). It was noted that white cells completely used glucose in chemostat growth on defined medium, but not yellow cells (Sonnleitner *et al.*, 1982) and it has also been reported (Ramaley and Hixson, 1970) that a white strain of *Thermus* had a faster growth rate than a pigmented one. In the laboratory (as in hot water heaters) photoprotective pigments have no selective advantage which could allow faster growing white cells to become dominant in defined medium.

Ecological studies of populations isolated from hot springs and along a thermal gradient in a stream (all located in southern Iceland) indicate a high degree of variability when tested over 33 separate characters. There was no typical population associated with any particular temperature range nor from any sampling area and no homogeneous populations were found even within a single pool (Cometta *et al.*, 1982a). The temperature and pH range of *Thermus* was also studied in an Icelandic stream from the southern region and a wide range of tolerance to both temperature (55^{o} - 85^{o}C) and pH (6.5 - >10) found (Kristjansson and Alfredsson, 1983).

It has been suggested (Cometta *et al.*, 1982b) that as *Thermus* lacks both mobility and the ability to form spores, it can only succeed in nature by being extremely adaptable. This could be the result of having several copies of functionally similar genes on the chromosome and/or some genes coded on plasmids. Plasmids have been reported in association with both *T. thermophilus* (Eberhard *et al.*, 1981) and *T. flavus* (Hishinuma *et al.*, 1978) and from a New Zealand isolate T41A - (Chapter 6).

The taxonomic status of the pink K-2 isolate (Ramaley *et al.*, 1975) is not clear. It appears different from both *Thermomicrobium roseum* - which is considered to belong to a completely different genus of aerobic caldoactive bacteria from *Thermus* (Jackson *et al.*, 1973) - and from other *Thermus* strains, although *T. ruber* (the Russian isolate) has a red coloured pigment. It was not possible to include either the K-2 isolate or a member of the genus *Thermomicrobium* in this study. While in Britain fifty-four uncharacterised *Thermus* strains (the kind gift of Dr R. Sharp, P.H.L.S. Centre, U.K.) were obtained, one of which had a red pigment and which could not be grown at a temperature higher than 65^{o}C on Medium D (Castenholz, 1969). These strains were analysed for

Source of isolated *Thermus* strains

Source	Source Temperature °C	Source pH
ROTORUA - Kuirau Park		
RT 1	70.8	6.5 (papers in lab)
RT 3	70	7.0 (papers in lab)
RT 4(a) Strain T41A	87	7.2
RT 4(b)	72	7.1
RT 6(a)	68.9	9.2
RT 6(b)	81.5	8.6
RT 6(c)	64	9.4
- Whakarewarewa		
RT 14	80	7.1
RT 15 Strain T351	96.4	8.7
RT 34	75.7	6.5
RT 35	80	6.6
RT 100	81	7.3
TAUPO		
TP 10	72	6.8
TOKAANU		
TOK 1	95	7.9
TOK 3	76	6.9
TOK 4	89	5.6
TOK 5	52	5.9
TOK 8	75-80	5.6
TOK 9	86	7.0
TOK 10	75	6.5
TOK 11	98	7.0
ORAKEI KORAKO		
OK 2	57	7.6
OK 4	60	6.9
OK 6	102.5	8.2
OK 10	74	7.9
OK 13	98	7.3
OK 15	97	7.0
KETETAHI		
KT 4	80	6.0
WAIMANGU		
Wai 3	48	5.5
Wai 13	92	6.5
Wai 15	84	6.0
Wai 16	70	N.A.
Wai 17	80.6	8.5
Wai 18	81.6	7.3
Wai 19	79	7.7
Wai 24	97	7.7
HOT WATER BEACH	~60	~6.5
FIJI - Suva Suva Beach		
FJ 3	N.A.	N.A.

N.A. = not available

TABLE 3.2

comparison with New Zealand strains in a separate experiment described in Section 3.6.

3.4 PY-MS of *Thermus* Strains

The taxonomic status of members of this genus is by no means clear so the purpose of this study was to attempt to obtain a pattern using pyrolysis mass spectrometry. To date this technique has been used to reveal underlying taxonomies of well characterised groups (Gutteridge and Puckey, 1982; Shute *et al.*, 1984) or to determine differences at the strain level of a very homogeneous group of organisms (Wieten *et al.*, 1981). *Thermus* species are considered to have a high degree of variability and it was of considerable interest to investigate the use of this technique to detect underlying species groupings. It was extremely important to eliminate variability due to culture conditions so all strains were grown under rigorously standardised conditions as set out in Appendix I. The strains were isolated from a variety of regions as shown in Table 3.2.

Thirty-nine strains isolated from a number of different thermal regions in New Zealand, one from Fiji and three type strains which were obtained from the American Type Culture Collection (Washington U.S.A.) were prepared in New Zealand and taken to Great Britain for analysis. The type strains were:

ATCC 25104 *Thermus aquaticus*

ATCC 27634 *Thermus thermophilus*

ATCC 27978 'Ramaley's XI'

The 43 isolates prepared are set out in Table 3.3 which also lists the computer analysis code number.

Freeze dried samples were prepared for pyrolysis as described in Appendix 1, Section I.6. It was decided to include only samples with total ion counts in the 1×10^6 - 5×10^6 range in the analysis, the

Thermus Strains Analysed

Computer No.	Sample	Source Temp.	Source pH
1	ATCC 25104 *Thermus aquaticus*		
2	ATCC 27634 *Thermus thermophilus*		
3	ATCC 27978 'Ramaley's XI'		
4	T351 (RT 15)	96.4	8.7
5	TOK 8	75 - 80	5.6
6	Wai 19	79	7.7
7	T 41A	87	7.2
8	OK 15	97	7.0
9	OK 6	102.5	8.2
10	RT 6(a)	68.9	9.2
11	TOK 3	76	6.9
12	FJ 3	N.A.	N.A.
13	OK 4	60	6.9
14	OK 2	57	7.6
15	OK 10	74	7.9
16	TOK 5	52	5.9
17	OK 13	98	7.3
18	TOK 1	95	7.9
19	TOK 9	86	7.0
20	TP 10	72	6.8
21	TOK 4	89	5.6
22	TOK 10	75	6.5
23	Wai 3	48	5.5
24	Wai 15	84	6.0
25	Wai 13	92	6.5
26	Wai 18	81.6	7.3
27	Wai 17	80.6	8.5
28	Wai 24	97	7.7
29	Wai 16	70	N.A.
30	RT 6(c)	64	9.4
31	KT a	N.A.	N.A.
32	RT 3	70	7.0
33	RT 1(b)†	70.8	6.5
34	KT 4	80	6.0
35	RT 1 †	70.8	6.5
36	RT 34	75.7	6.5
37	RT 4(b)	72	7.1
**38	RT 14	80	7.1
*39	RT 35	80	6.6
40	RT 6(b)	81.5	8.6
41	RT a	N.A.	N.A.
42	RT 100	81	7.3
43	SWBS	60	6.5
**44	RT 35	80	6.6
*45	RT 14	80	7.1

** and * are duplicate samples

N.A. = not available

† different isolates from the same water sample.

TABLE 3.3 List of strains of *Thermus* analysed by pyrolysis mass spectrometry. Three reference strains were analysed together with forty isolates from natural hot springs. Thirty-nine isolates were obtained from springs located in the central North Island of New Zealand and one, No. 12 (Fj 3) from Suva Suva Beach, Fiji.

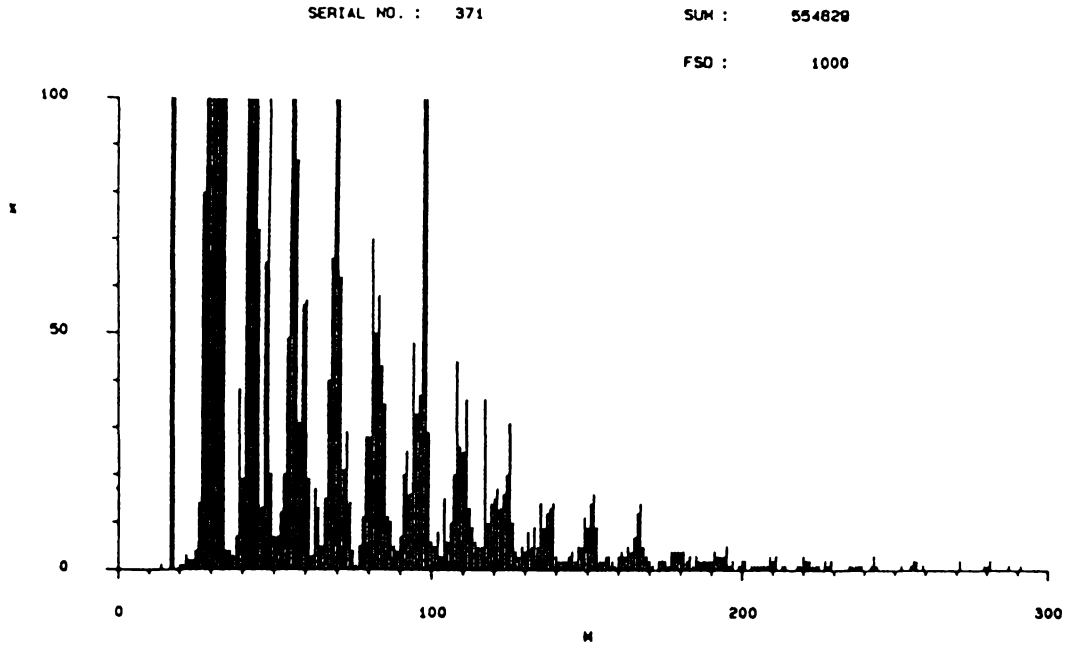


Figure 3.1 Mass spectrum (pyrogram) relating to a replicate of strain Wai 15. Total ion count 554 829 ions. Note that relatively few ions of mass greater than 200 are produced.

```
371
    1     1    11     6     6 110503  4825     5    16    11    11
   25    23    41   141   802   3690  9621  8555 165429 186220 171222
 1053    49    43    38    72    364   194  1231  1666  4253  4744
  726   136   656  1027   202    74    77   127   204   492  1076
 1621   876   316   563   578   195    33   175   136    51   155
  404   662  1050  1137   621   217   296   143    44    19    56
  114   281   286   704   502   587   433   357   119   106    55
   41    74   207   258   168   482   332   378  1180  1534   295
   66    59    82    38   151    60   103   206   447   261   254
  362   139    97    63    51    51   364   100   144   150   177
  137   160   200   316   106    46    30    52    45    84    57
   90    53   142    97   121   131   145    38    27    25    24
   34    47    20    56    54   117    99   140   163    95    24
   21    32    30    22    17    31    47    32    58    41    73
  124   149    58    35    20    15     6    25    21    20    19
   45    49    49    42    46    26    34    17    30    21    22
   21    22    26    41    34    31    39    59    15    20     7
   19    22    23     7    13    12    16    16    10    18    31
   22    30     8    13    10     4     1     4    18    11    32
   27    24    12    18    10     3    26    12    25     7     7
    2     8     8    17    16    13    16    12     8     4    15
   36    14     8     5     4     1     3     9     4    10     8
    1    11    25    20     6    10     5     8     9     2     7
    9     4     7     7     6     7    20     9     8     1     1
    6     8     9    17    12    23     6     6     4     5     4
   13     1     6     6    11     8     6     2     4     3     4
    1     6     2
554629
```

Figure 3.2 Raw mass data relating to a replicate of strain Wai 15 and corresponding to the pyrogram given in Fig. 3.1. Figures refer to m/z = 12 to m/z = 300.

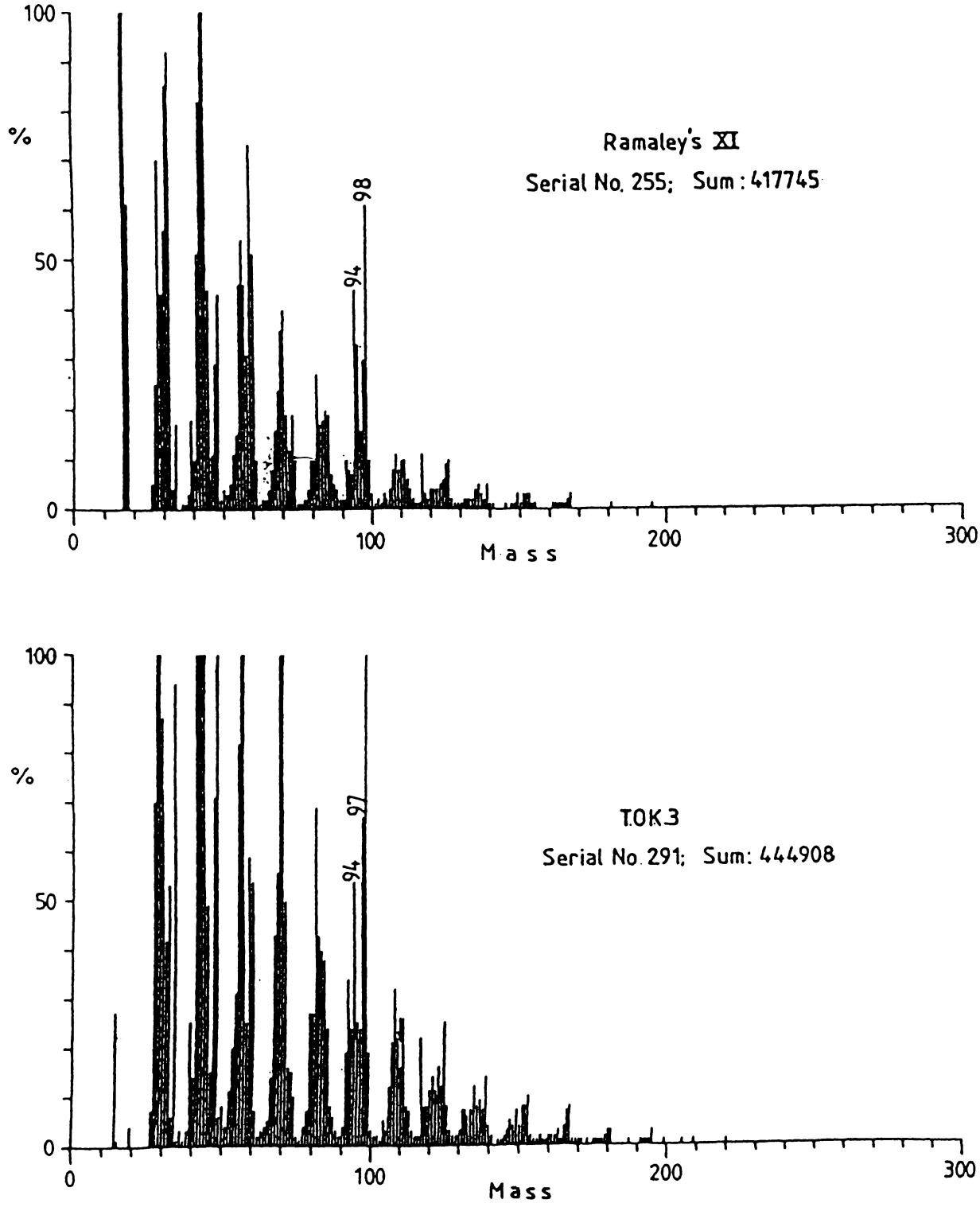


Figure 3.3 X-Y Plots (Pyrograms) for two *Thermus* strains, Tok 3 (Pigmented) and Ramaley's XI (Non-pigmented) which have similar total ion counts (SUM). Note the difference in relative intensity at masses 94 and 97.

number of drops of cell suspension required to achieve this being determined by trial and error. For *Thermus* samples this was generally four small droplets delivered from a Pasteur pipette.

As described in Chapter 2, section 10 samples were inserted into the pyrolysis mass spectrometer and analysed. An X-Y plot was drawn on the line-printer and data for each m/z value in the m/z 12-300 range stored on floppy diskette. An example of an X-Y plot of a *Thermus* sample is given in Figure 3.1 and a table of the corresponding m/z values in Figure 3.2. Four replicates with ion counts in the required range were obtained for each strain.

Data Processing of *Thermus* Strains

The first stage in data processing is the editing of spectra stored on floppy diskette to eliminate replicates with ion counts outside the required range.

X-Y plots were then examined to detect any obvious differences between samples analysed; for *Thermus* samples, differences were noted for the non-pigmented strain, Ramaley's XI (see Figure 3.3). A print-out of m/z values for each sample was obtained and examined to determine which values should be computed using the criteria stated in Chapter 2, Section 13. It was decided to include as many m/z values as possible as it has been proposed (MacFie *et al.*, 1982) this may be a better diagnostic approach than the more commonly used method based on highly discriminatory ions only (O'Donnel and Norris, 1982; Shute *et al.*, 1984). All masses lower than m/z = 35 were excluded as their values were either too high or too low or their derivation was known to be from inorganic ions of no taxonomic importance (Sweatman and Gutteridge, unpublished). It can be seen in Figure 3.2 that rather large intensity values were obtained for m/z = 43 and m/a = 44 (underlined in Figure 2). Not only are these values too large to be included without obscuring information contained in other ions, but as they are probably derived from

a large number of different proteins, nucleic acids and carbohydrates (Sweatman and Gutteridge, unpublished) they are of little taxonomic importance. As very low values were found for m/z = 189 - m/z = 300 these were all excluded from the analysis. The 152 m/z values finally selected were m/z = 35 to m/z = 188 inclusive, but excluding m/z = 43 and 44.

3.5 Data Processing of *Thermus*

3.5.1 Preliminary Analysis of *Thermus*

The complete analysis described in Chapter 2 requires a large amount of computer time. For a rapid preliminary investigation of *Thermus* data a simpler programme code named PCCVPROG also written in GENSTAT by H. MacFie and L. Vallis, was used. This is a principal components analysis programme only. Information is given on:

a) Distribution of loadings for each m/z value.

b) The % discrimination accounted for by each principal component (P.C.O.).

c) 2-D scatter plots relating points to combinations of 2 of the first 3 principal axes.

The information in the print-out of PCCVPROG allows checking that only useful m/z values have been included, selection of a suitable number of principal components and the detection of outliers. Clustering tendencies are rarely indicated by this analysis.

As described in Chapter 2, Section 13.1 outliers are analyses which fall well outside the others in the analysis. Such outliers are common in PY-MS studies and are probably a consequence of the ability of this technique to detect small changes (MacFie and Gutteridge, 1982). A plot

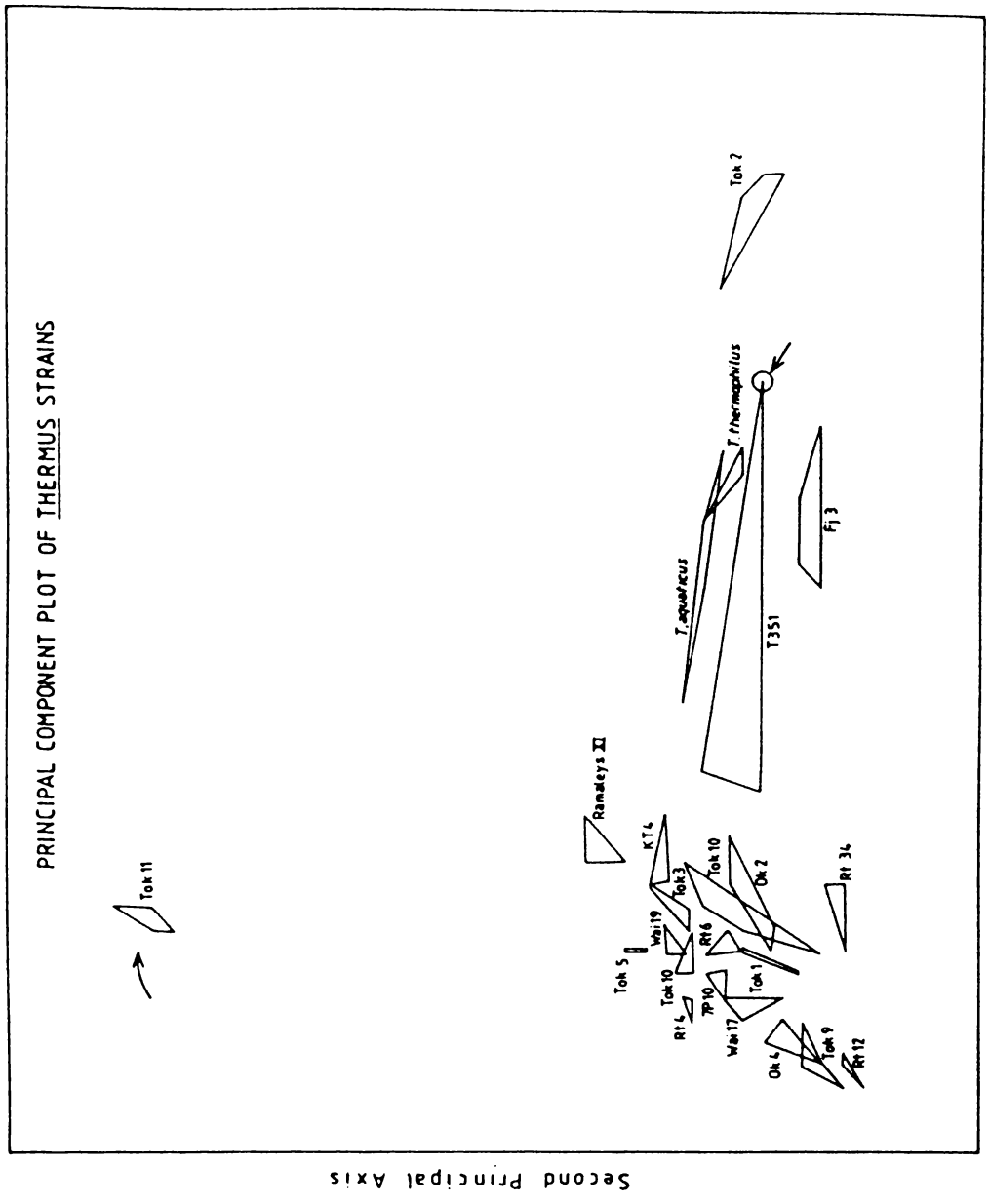


Figure 3.4 Principal Component Plot of *Thermus* strains analysed. The arrows point to an outlying replicate of strain T351 and an outlying sample, strain Tok 11 which were subsequently removed from the data set.
Twenty-one strains which were coincident with the group in the lower left region of the plot were omitted for clarity.

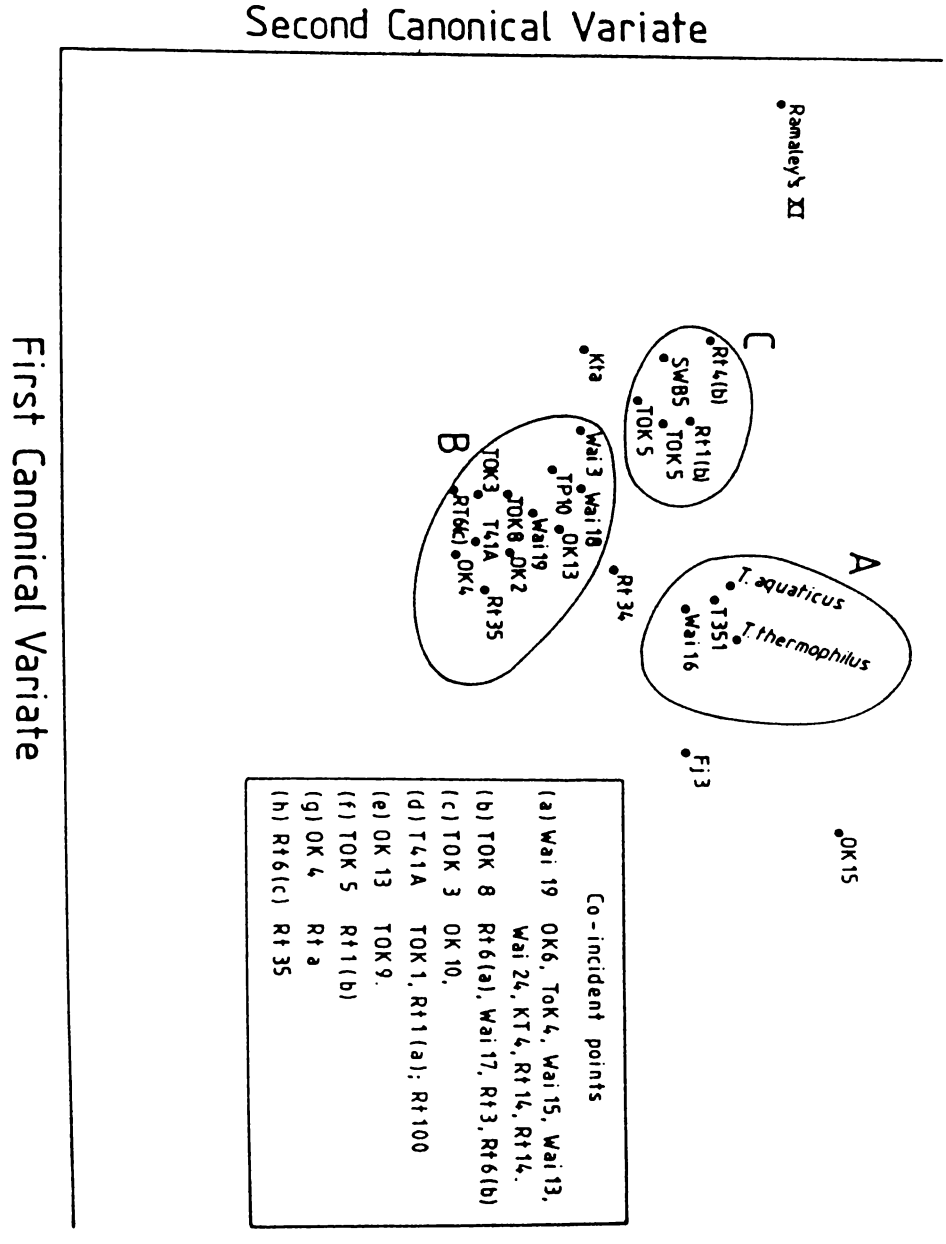


Figure 3.5 Canonical Variates Plot of *Thermus* strains showing clustering into three groups has resulted from this analysis of the data. These two axes display 48% of the variance between the strains.

showing the 2-D relationship of strains with reference to the first 2 principal axes is shown in Figure 3.4. An outlying replicate of T351 is arrowed, and also an outlying strain, TOK 11. To simplify the diagram, 21 strains (see figure), which fell within the lower left-hand cluster have been omitted. Although it is possible that all strains within this cluster are closely related, it is more likely that they have been artificially forced together by the outliers. The five spectra relating to these outliers were removed from the data set to allow proper discrimination of remaining strains.

This analysis indicated the 152 m/z values were satisfactory and also that 26 principal components described 99% of the variation between samples.

3.5.2 Canonical Variates Analyses

The data was then processed by a further GENSTAT programme (code named ROSIE) which carried out principal components analysis and used the data from the stated number of principal components for the calculation of strain position relative to pairs of the first three canonical variates axes. Two-dimensional scatter plots were drawn out. For many data sets these are sufficient for satisfactory strain discrimination (MacFie *et al.*, 1978). As an indication of how well these plots reflect the underlying pattern in the original data, a minimum spanning tree is drawn out. The length of the lines joining strains is related to the differences between them (Dunn and Everitt, 1982).

Figure 3.5 shows a plot relating the strains to the first two canonical variates axes (which describe 48% of the variation). Figure 3.6 gives the minimum spanning tree relating to positions over the first three canonical axes.

The results suggest that most of the New Zealand isolates (31 strains)

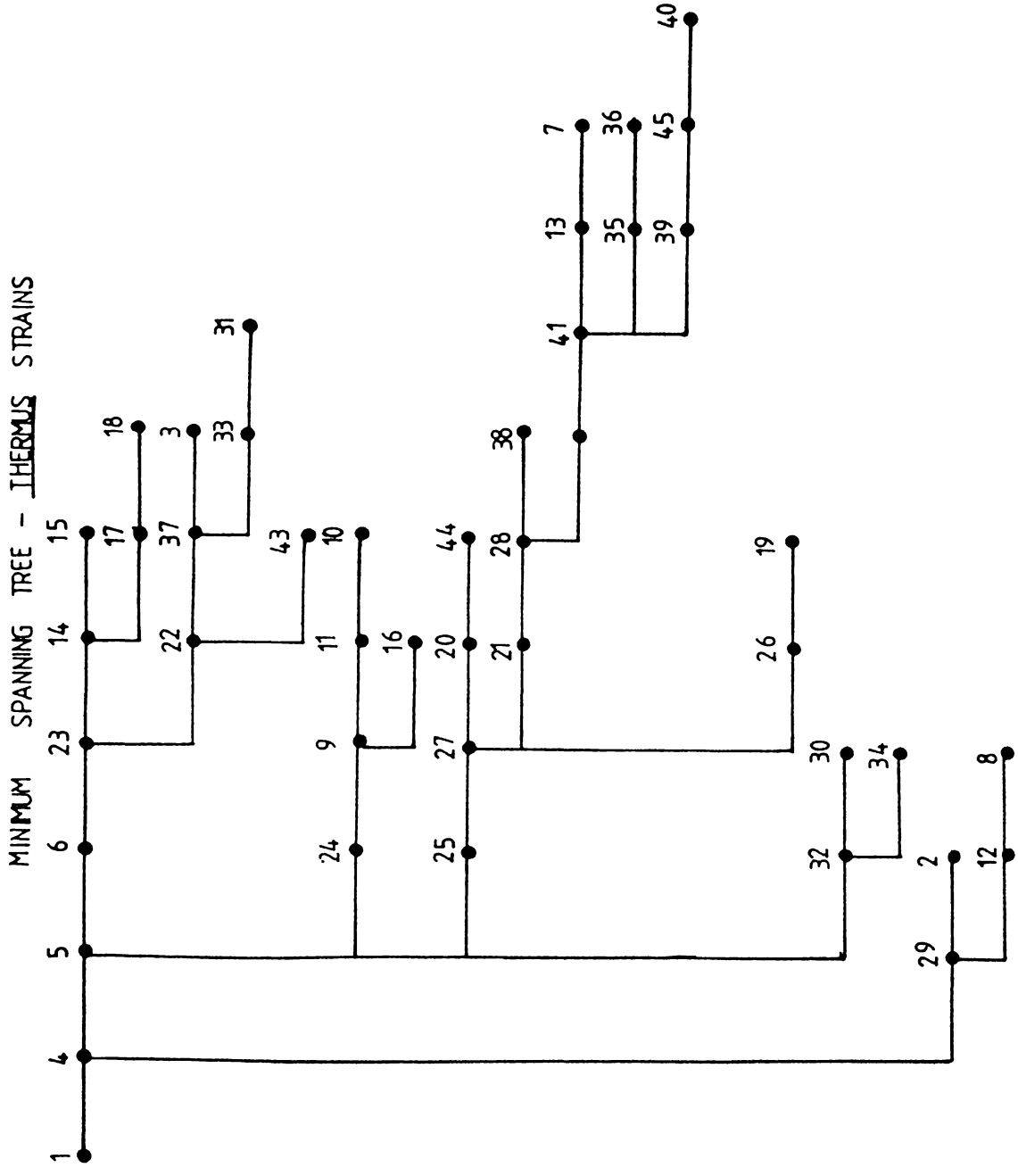


Figure 3.6 Minimum Spanning Tree showing the relationships between strains after canonical variates analysis. For clarity strains are identified by computer numbers as given in Table 3.3

Note: No. 1 = *T. aquaticus*
No. 2 = *T. thermophilus*

fall in one large closely related group. There seem to be two smaller groups; one includes five New Zealand strains, while the third includes two New Zealand strains and two of the reference strains *T. aquaticus* and *T. thermophilus*. Five strains including the third reference strain 'Ramaley's XI' lie outside these groups. Although this plot suggests *T. aquaticus* and *T. thermophilus* (computer numbers 1 and 2) are very similar, the minimum spanning three relating to this data (Figure 3.6) indicates that this is probably not a true indication of their relationship. It seems that canonical variates plots alone are not satisfactory for discriminating these strains.

It was decided that to obtain a more accurate picture of the natural relationsips between strains all possible information should be included. A GENSTAT programme was written which would transform the Mahalanobis' D^2 values generated by canonical variates analysis into % similarity values. These % similarity values were then used to carry out a clustering technique, i.e. average linkage cluster analysis. For the *Thermus* strains, where twenty-six principal components were used, this means that the information in the corresponding twenty-six canonical variates was available to help the discrimination. This programme (code named LLOYD) was used for this and all subsequent sets of data in this study.

3.5.3 Average Linkage Cluster Analysis

The dendrogram (Figure 3.7) can represent the information from all twenty-six dimensions. Essentially the same three groupings as were shown in Figure 3.5 can be seen but there has been further separation of some strains and the appearance of sub-groups within the three major clusters. It can be seen that strains OK 15 and 'Ramaley's XI' are very different from all others in the analysis and that SWBS and KETa also show low similarity. 'Ramaley's XI' is a non-pigmented strain (the only one in this set) and spectral differences between it and a pigmented strain, TOK 3 can be seen in Figure 3.3, although the actual contribution

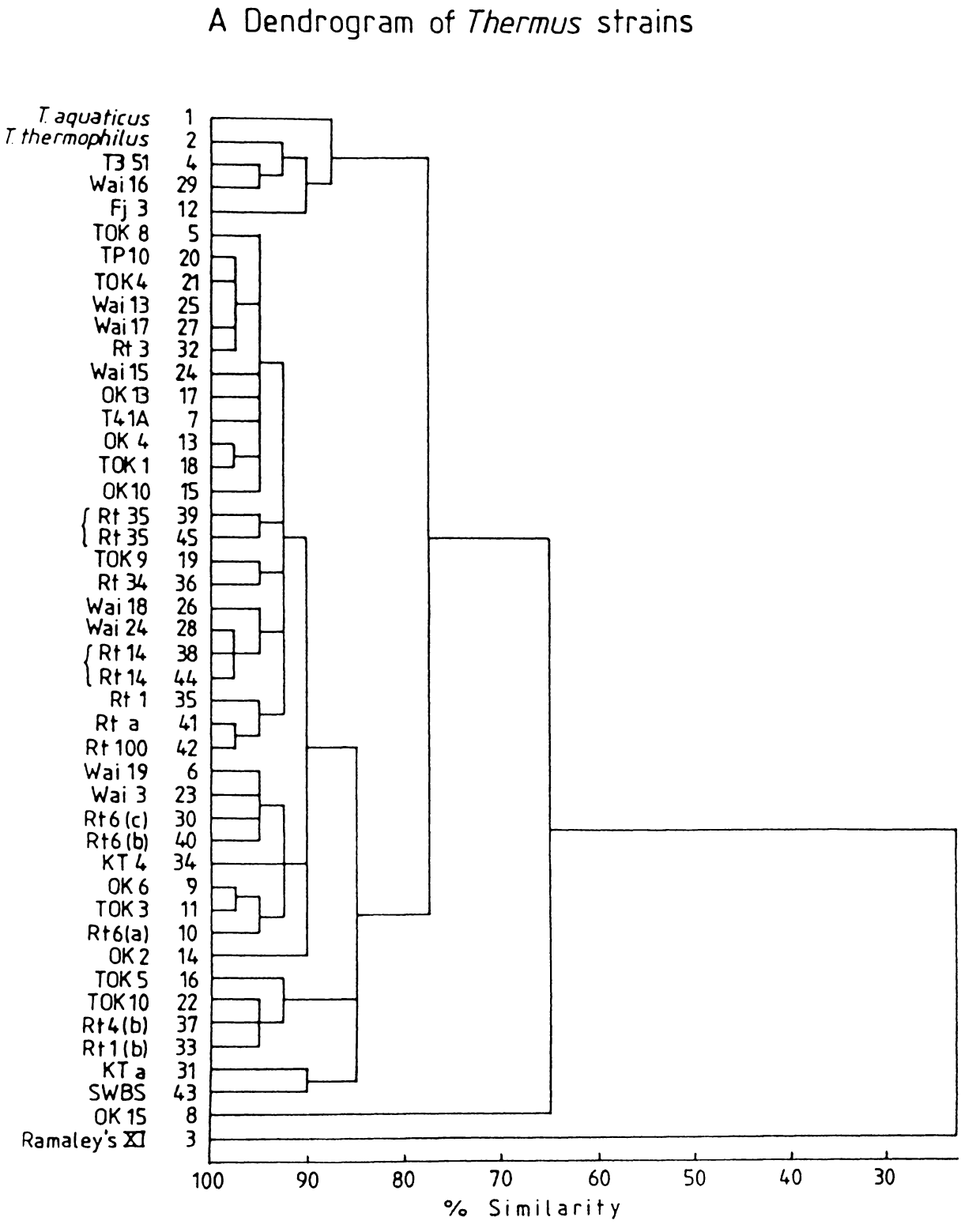


Figure 3.7 Dendrogram derived from average linkage cluster analysis applied to Mahalanobis' D^2 values. Note the three groups at 90% similarity level which probably represent species groupings. The position of the duplicate strains indicates there was no problem with reproducibility during the course of these analyses.

of carotenoid pigments to the spectrum is unknown. The reason for the differences in the three New Zealand strains is unclear and an example of the power of PY-MS to detect strains which warrant further investigation.

A feature of canonical variates analysis which was used to generate the % similarity values is the sensitivity to the presence or absence of individual results (MacFie and Gutteridge, 1982). This means that the numerical value of % similarities between strains is far less important than the patterns of strain groupings in the dendrogram. When the range of % similarity required to include all strains to produce a dendrogram is from 100% to 22%, as it is for the *Thermus* strains, strains which cluster at 95% similarity are probably distinct groups at the sub-species level at least.

It was decided that clusters of three or more strains probably represent meaningful groupings in this analysis.

At 95% similarity six groups representing sub-species i.e. variety level groupings were discriminated. The largest group consists of twelve New Zealand strains (TOK 8, TP 10, TOK 4, Wai 13, Wai 17, RT 3, Wai 15, OK 13, T41A, OK 4, TOK 1 and OK 10). These strains were derived from a number of different sampling regions. The other five groups contain three or four strains and also fail to show regional groupings. The source pH and temperature do not seem to be related to any of these cluster patterns but information relating to organic and mineral content was not available.

Preliminary investigations of antibiotic sensitivity and the ability to grow on either milk agar (Oxoid CM 21) or nutrient agar (Oxoid CM 3) gave no useful information regarding any possible strain groups. A full-scale numerical taxonomy of *Thermus* using a large

number of morphological and physiological and biochemical characters is currently being undertaken by a D.Phil. student at this University.

Biochemical test results suggest that the differences between the cluster-groups are definitely valid. For example, the extracellular proteases produced by strains T351 and T41A differ in properties (D. Cowan, unpublished). Strain T41A produces an inducible β-galactosidase but strain T351 does not (Cowan *et al.*, 1984).

At the 90% similarity level there are three clusters of three or more strains and also clear separation of the two type strains *T. aquaticus* and *T. thermophilus*. It seems reasonable to conclude that these clusters represent species groupings and that the two reference strains are sufficiently different to be considered different species.

The wide separation of the two strains isolated from pool Rt 1 i.e. strains RT 1 and RT 1(b) - similarity 74.3% - is of interest. These two strains were isolated from the same water sample, the second after two years storage at room temperature.

3.5.4 Conclusions on Data Processing of Forty-one *Thermus* Strains.

The results suggest that PY-MS can be used to indicate possible relationships between strains isolated from natural sources. The data processing methods used revealed the major clustering trends in 2-D canonical variates plots, for the *Thermus* strains it appears that there are three distinct, species level groupings. More discrimination resulted from average linkage cluster analysis, which not only confirmed the three "species" groups but also revealed six groupings within this at the sub-species level.

The non-pigmented strain 'Ramaley's XI' occupied an isolated position in the dendrogram,but being the only non-pigmented strain it

was not possible to draw firm conclusions as to its taxonomic status. Three New Zealand strains SWBS, OK 15 and KTa failed to cluster making it possible to speculate that they belong to a separate genus.

Although the fact that the majority of New Zealand strains form a single cluster seems to suggest that the genus may not be as variable as has been suggested, the pattern of clustering within the major groups supports the observed variability. Only two of the three strains isolated from pool 6, Kuirau Park Rotorua, (RT 6(a), (b) and (c)) cluster as a sub-species group. Pools 3 and 4 from this region are sites at either end of a small lake in the Park. Two isolates from pool 4, T41A and RT 4(b) and one from pool 3, RT 3 are all separated in the dendrogram with strain 4(b) lying outside the major group of New Zealand strains. Two strains isolated from the same water sample, collected from pool 1, Kuirau Park RT 1, and RT 1(b) also fail to cluster at a species level.

Similarly there is apparently no simple relationship between the presence of a particular *Thermus* strain and the temperature or pH of the source pool.

It seems that the results of this analysis support the findings of Cometta *et al.* (1982b) that *Thermus* strains do not form homogeneous populations and that these strains are variable, but that this variation is perhaps not as great as these workers suggested.

3.6 Comparison of New Zealand *Thermus* Strains with Isolates from Yellowstone Park U.S.A.

During the course of PY-MS analysis of the New Zealand thermophiles it became possible to obtain forty-nine *Thermus* isolates which had been collected from hot springs in Yellowstone Park, U.S.A. These cultures were the kind gift of Dr R. Sharp (Centre for Applied Mircobiology and Research, Porton Down, Wiltshire, U.K.). The cultures were obtained as single colonies plated onto solid medium (Ramaley and Hixson, 1970) which had been incubated at 65°C. Cultures were coded as series A, B and C without details of specific source of isolation given. It was decided it would be of interest to carry out a comparative study on the two groups of strains.

The New Zealand strains had all been grown on Medium D (Castenholz, 1969) at 70°C so it was decided to attempt to grow the American isolates under the same conditions. One isolate, coded 1X, failed to grow; another isolate, 3D which had a bright red pigment, could only be grown on Medium D at 65°C but all remaining isolates grew satisfactorily under the conditions set out in Appendix I. To keep culture conditions as nearly identical as possible, a portion of the same batch of Coast Biologicals Agar was obtained from New Zealand. Oxoid yeast extract and trypticase peptone had to be used in place of the Merck and BBL products used in New Zealand.

To act as controls, viable cells of five strains were obtained from New Zealand and grown at Reading, together with the American isolates. These were

T. aquaticus (ATCC 27634)
T. thermophilus (ATCC 25104)
T351
TOK3
T41A

The five strains obtained from New Zealand and forty-seven of the American isolates were grown, harvested, freeze dried and autoclaved as

set out in Appendix I. The red American isolate, 3D, was grown at 65°C but all other conditions were identical. To monitor mass spectrometer repeatability further aliquots from the New Zealand grown samples of strains *T. aquaticus*, *T. thermophilus*, T351, TOK3 and T41A were analysed, together with all strains prepared at Reading.

Just prior to this analysis major repairs were carried out on the PYROMASS 8-80 after which there were still problems in maintaining a high vacuum. It was decided to analyse aliquots of New Zealand grown strains together with a limited number of the strains prepared at Reading to check repeatability. The strains analysed were:

T. aquaticus
T. thermophilus
T351
T41A
TOK3

all grown both in New Zealand and at Reading and 15 American strains: 1A, 1B, 1C, 1D, 1E, 1G, 1H, 1I, 1K, 1L, 1M, 1N, 1O, 1P, 1Q.

The sets of spectra relating to the two analyses of the five New Zealand grown control strains should compare with a high degree of similarity to make a comparison possible between the two sets of data, i.e. relating the analysis of all the New Zealand strains presented in Section 3.5 to the American strains grown at Reading. Only if there were no problems with instrument repeatability would it be possible to determine the relationships between the spectra relating to the control strains grown in New Zealand to those of the same strains grown in Reading. As can be seen in Figure 3.8 there was poor instrument repeatability at this time. The problem is even greater than appears in the canonical variates plot as the following Mahalanobis' D^2 values were obtained for aliquots of the same sample analysed at the two different times.

Strain	D^2
T. aquaticus	10.96
T. thermophilus	13.38
T351	12.05
T41A	9.77
TOK3	11.39

Although it was obvious that a full-scale comparative study could not be carried out until the PYROMASS 8-80 was operating at full efficiency (which almost certainly included a thorough cleaning and decontamination of the ion source) it seemed from the C.V.A. plot that the American isolates might form a distinct and separate group from the two reference strains and the three New Zealand strains. It was decided to carry out average linkage cluster analysis on the data obtained in the second PY-MS analysis only.

The results of this can be seen in Figure 3.9. It is clear that the American isolates are well separated from the New Zealand grown strains. Although the slight differences in culture conditions may influence this result to some extent, it does not seem unreasonable to conclude there are probably distinct differences between the American isolates and the New Zealand and reference strains. The American isolates form a reasonably homogeneous group which was sub-divided into three sub-groups. Some of these strains were analysed by SDS-PAGE of whole cell proteins (Section 3.8) and it was also found they formed three sub-groups (see Figure 3.14). In this analysis the group appeared even more closely related and there were differences in the ordering of the sub-groups.

As there is not very good agreement of results between the clustering of the five New Zealand grown strains with that obtained previously (see Figure 3.7) it seems that the problems occurring with the functioning of the PYROMASS 8-80 at this time were interfering with spectral analysis.

It was hoped that it would be possible to complete this analysis but the necessary repairs were of a major nature and time did not permit further work.

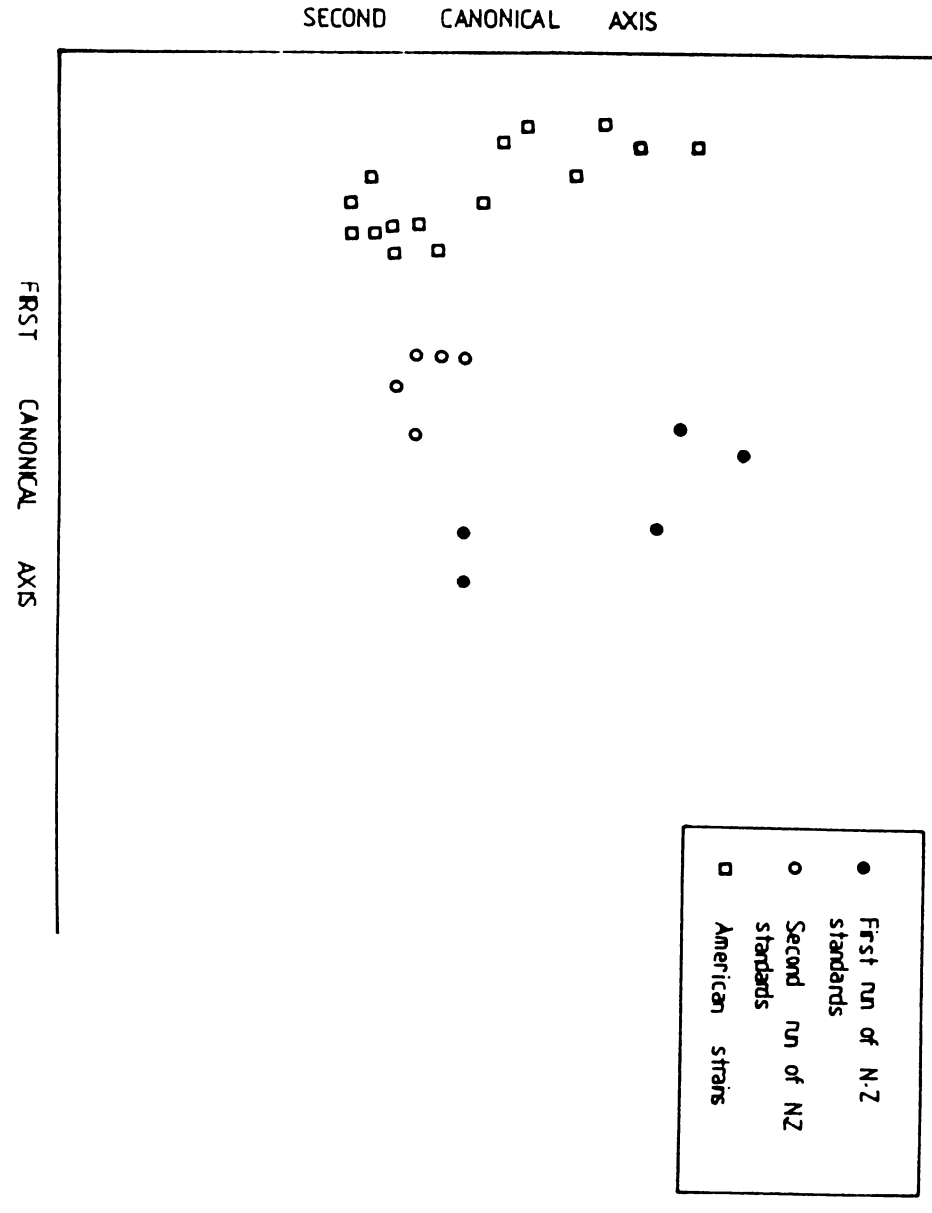


Figure 3.8 *Canonical Variates Plot* of *Thermus* Strains to check repeatability. Note poor replication of standards. Closed and open circles refer to aliquots of the same sample of 5 strains analysed before and after repairs to the PYROMASS 8-80.

Figure 3.9

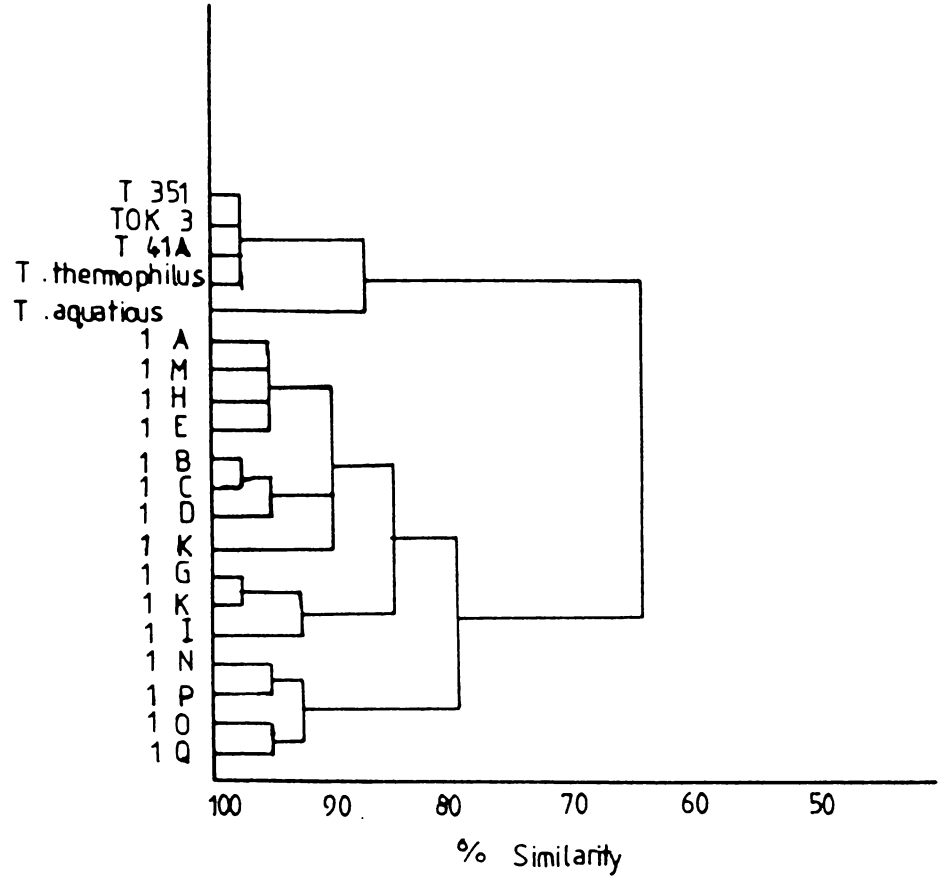


Figure 3.9 *Dendrogram* comparing 15 strains of *Thermus* isolated in Yellowstone Park, U.S.A. to 3 strains isolated in New Zealand, and 2 reference strains.

3.7 A comparison of Some *Thermus* Strains Using the Technique of Restriction Analysis of the Genome by Agarose Gel Electrophoresis

It was decided to investigate the technique of digestion of the cell genome with suitable Type II restriction endonucleases followed by size separation of the resulting fragments by electrophoresis on agarose gels. This technique has been successfully used by R.B. Marshall, Massey University, New Zealand for the identification of *Leptospira* serovars (Marshall *et al.*, 1981). These analyses were carried out with the assistance of Dr Marshall.

The following *Thermus* strains, as analysed by PY-MS were used: *T. aquaticus*, *T. thermophilus*, 'Ramaley's XI' and New Zealand strains T41A, OK 4, TOK 8 and T351. Each strain was cultured in 100 ml of Medium D (Appendix I) in 250 ml Erlenmeyer flasks fitted with cotton bungs. Cultures were incubated overnight at 70°C on a Gallenkamp orbital incubator at 100 revs min^{-1} shaking rate.

3.7.1 Preparation of DNA

Thermus DNA was extracted by the method of Fischer and Lerman (1979). 25 ml of an overnight culture was harvested (3000 g in a Sorvall RC-5B centrifuge), washed twice with phosphate saline buffer (0.1 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 at pH 7.3) and the pellet resuspended in 1 ml TEB buffer (100 mM Tris-HCl, 100 mM EDTA (disodium salt) at pH 7.5. 100 µl of an aqueous solution (3 mg ml^{-1}) lysozyme (Sigma) was added and the mixture incubated at 37°C for 15 minutes. 100 µl of 10% (W/V) solution of sodium lauryl sulphate (BDH) and 100 µl of an aqueous solution (10 mg ml^{-1}) of Pronase (Sigma) was added and the mixture incubated overnight at 50°C. Sodium perchlorate was added from a 5 M solution to a final concentration of 1 M and the mixture incubated for another hour at 50°C and then the volume made up to 5 ml with STE buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA; pH = 8.5).

This mixture was then extracted three times with an equal volume of a mixture of redistilled phenol, chloroform and isoamyl alcohol (25 : 24 : 1) which had been previously saturated with STE buffer. It was noted that most samples still had material at the interface especially 'Ramaley's XI'. Each sample was dialysed (in boiled dialysis tubing) for 24 hours (with three changes of dialysis buffer) against TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8.0) at $4^{o}C$.

The absorbance at 260 nm and 280 nm was measured in a Pye Unicam Spectrophotometer. A ratio of $\frac{A_{260}}{A_{280}} = 2$ is an approximation of "protein free" nucleic acid and samples should have values greater than 1.8.

It was found that most of the *Thermus* samples had ratios of approximately 1.6 - 1.8 except 'Ramaley's XI' which had a ratio of 1.5.

Therefore, all samples were re-extracted twice more with phenol: chloroform:isoamyl alcohol mixture and dialysed again - ratios were now 1.9 - 2.0, except for 'Ramaley's XI'. This sample was heated to boiling point in 1% SDS and then re-extracted.

As RNA had not been removed the DNA concentration was measured fluorometrically (Le Pecq and Paoletti, 1966) as follows:

Approximately 2 µg nucleic acid (estimated from $A_{260} = 1$ being equivalent to 50 µg m^{-1} double-standard DNA) was mixed with 2 ml STE buffer and 800 µl of 12.5 µg ml^{-1} aqueous ethidium bromide. Excitation wavelength was from 365 - 560 nm. A standard curve (Figure 3.10) was drawn up using calf Thymus DNA in the range of 0 - 1 µg ml^{-1}.

DNA concentrations were estimated as follows:

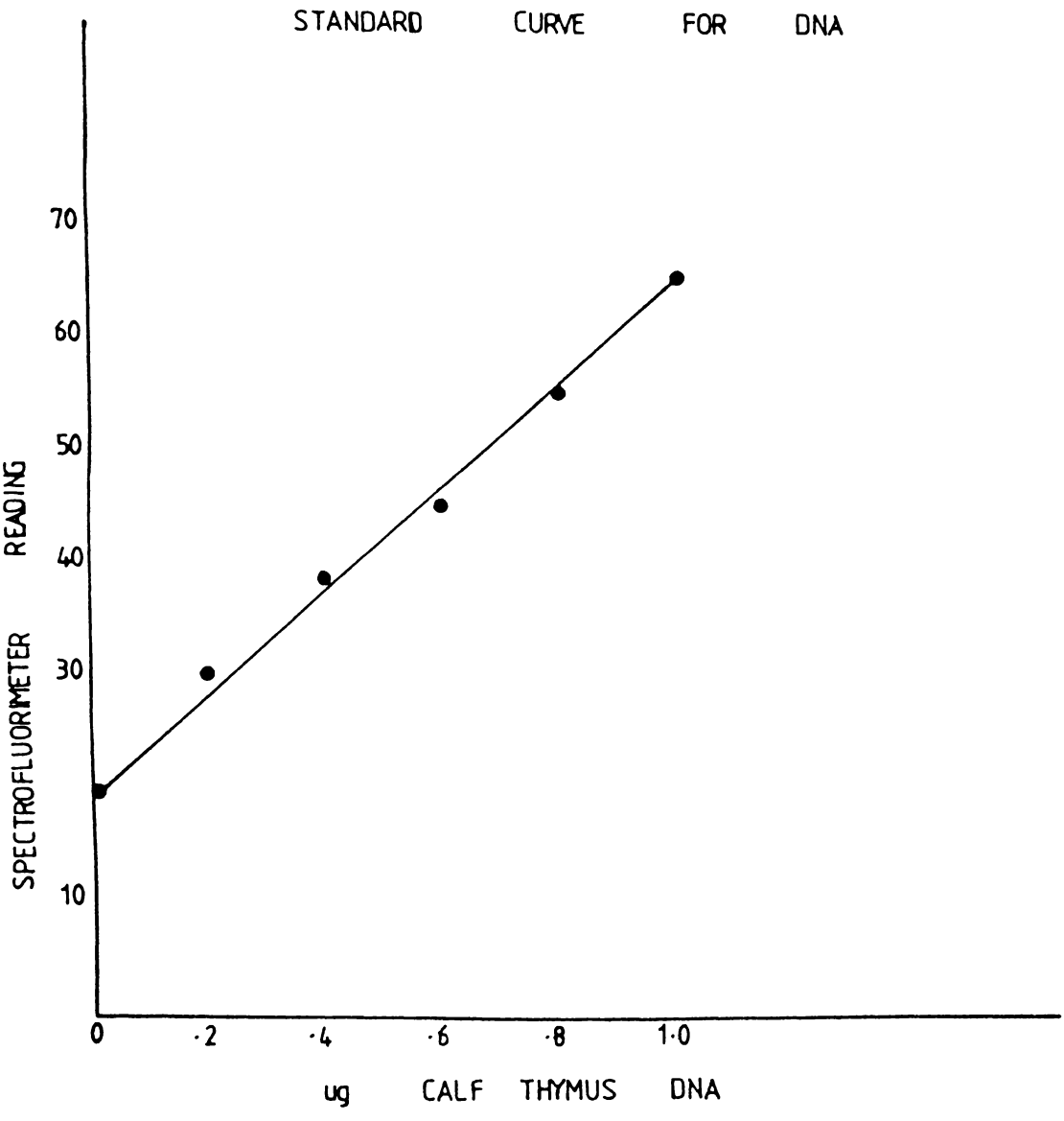


Figure 3.10 Standard Curve for DNA

SAMPLE	(DNA) in $\mu g\ ml^{-1}$
TOK 8	0.87
OK 4	0.74
T. thermophilus	0.45
T. aquaticus	0.36
T 351	0.91
T41A	0.65
'Ramaley's XI'	1.01

3.7.2 Restriction endonuclease Digestion of DNA

λ-phage DNA (Sigma) was used as standard.

2 µg DNA (0.5 µg of λ DNA)

20 µl of enzyme buffer (100 mM Tris-HCl at pH 7.8; 50 mM NaCl, 5 mM $MgCl_2$ and 100 $\mu g\ ml^{-1}$ bovine serum albumin).

4 units of (1) ECo R_1 (Sigma)

(2) Hind III (Sigma)

100 µl of water

were mixed gently and incubated at 37°C for 30 minutes. The reaction was stopped by incubation at 65°C for 10 minutes.

DNA was then precipitated by the addition of 1/20 volume 5 M NaCl and x 2 volume of absolute ethanol and stored at -20°C for 30 minutes. Samples were spun at 12 000 g for 15 minutes in a Sorvall RC-5B centrifuge, the pellet rinsed with cold 70% ethanol and dried in a desiccator.

DNA was resuspended in 45 µl of sample buffer (TE buffer + 20% glycerol + 0.05% SDS) and left overnight at 4°C to redissolve.

3.7.3 Gel Electrophoresis and Photography

The method was that of McDonnell *et al.*, (1977). Each sample was placed in wells of a horizontal slab of 0.7% agarose (Bio-Rad) - Electrophoresis buffer was 40 mM Tris-acetate at pH 7.8, 5 mM sodium acetate, 1 mM EDTA and 0.5 μg ml^{-1} ethidium bromide. Agarose was dissolved in buffer by boiling under reflux. Gels were poured at 40°C. Electrophoresis was for 4 hours at 4 V cm^{-1}.

After electrophoresis, gels were illuminated with UV light (Ultra-violet Products, U.S.A.). Gels were photographed on Kodak Tri-X film by means of a 120 format plate camera through a Wratten 23A gelatin filter.

Figure 3.11 shows the results of electrophoresis of restriction fragments of the 7 *Thermus* strains. Lane 1 contains λ DNA.

The results show that ECo R_1 is not suitable for digestion of *Thermus* DNA - only the bright plasmid bands are present.

Hind III has cut the DNA's satisfactorily with the exception of 'Ramaley's XI' which is just a smear - obviously heating this sample in 1% SDS was too severe.

It is clear from the photograph that all these DNA's are quite different - there is apparently no relationship between any of the strains as measured by this technique.

As the PY-MS results suggest a rather close relationship between T41A, TOK 8 and OK 4 it appears this method is not suitable for such variable wild-type bacteria. It was decided not to proceed with this method for this reason.

3.8 Discrimination of Selected *Thermus* Strains by SDS-PAGE ELECTROPHORESIS of WHOLE CELL PROTEINS

3.8.1 Preparation of Strains

Nine of the *Thermus* strains which were the kind gift of Dr R. Sharp (Porton Downs) were grown on plates on Medium D (Castenholz, 1969) as described in Appendix I (Section I.2) 70°C. These strains were:

Code No.	Strain No.
1	1C
2	1D
3	1G
4	1I
5	1J
6	1K
7	1M
8	1B
9	1L

No further details relating to these strains were available. All strains formed yellow-pigmented colonies. Strains 1B and 1C were slower growing and required 48 hours incubation before harvest. Cells were harvested as described for PY-MS (Appendix I, 5) and were then washed and resuspended in sterile distilled water, and stored at -20°C.

3.8.2 Electrophoresis

The method used was that described by Owen and Jackman (1982) with full details given in Jackman (1984, in press). It was found that boiling in the SDS sample treatment buffer (Laemmli, 1970) was satisfactory for breaking *Thermus* cells. (Sample buffer, 0.625 M Tris-HCl,

pH 6.8; 2% SDS; 5% 2-mercaptoethanol; 10% glycerol). 0.1 g of cells was boiled in 1.0 ml of sample buffer for 10 minutes at 100°C and cell debris removed by centrifugation at 13,000 g for 10 minutes. The supernatant was stored at -20°C.

Electrophoresis was carried out on 1.5 mm thick gels in the vertical multi-gel apparatus (Hoefer, San Francisco, U.S.A.) using the instructions given by the manufacturer and the method of Laemmli (1970). A paper label with the gel number was added to the bottom left corner of each gel.

Gels were loaded with

(a) 15 µl sample

(b) 25 µl sample

Electrophoresis was carried out for 3 hours at 30 mA. Gels were stained overnight in a stirred solution of Coomassi Blue. Destaining was in Fast Destain (1 hour) and then Slow Destain until the background was clear (Hoefer Instruction Manual, U.S.A.) with the patterns being brought back to exactly 100 mm length by brief immersion in Fast Destain. The gels were then dried between cellophane under vacuum on a commercial slab gel drier (Hoefer, San Francisco, U.S.A.).

3.8.3 Analysis of Patterns

Numerical taxonomy of the patterns was carried out on densitometric traces as described by Jackman (1982).

The centre of each gel track was scanned with a densitometer (Joyce Loebl Chromoscan 3) connected to an analogue-to-digital converter. Values were sent to a microcomputer (Commodore PET 3032) for analysis by the programme described by Jackman *et al.* (1983).

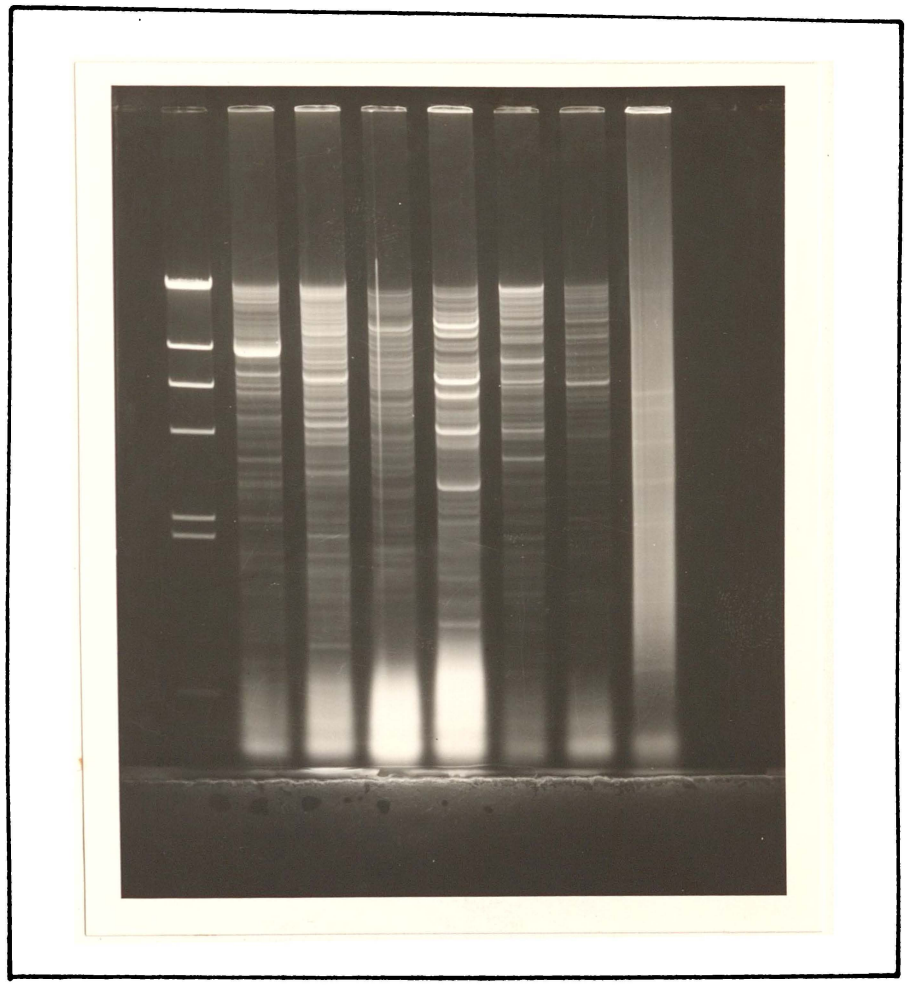


Figure 3.11 Hind III Digestion of 7 *Thermus* strains.
Lane 1 - λ DNA, Lane 2 - TOK 8, Lane 3 - OK 4,
Lane 4 - *T. thermophilus*, Lane 5 - *T. aquaticus*,
Lane 6 - T351, Lane 7 - T41A, Lane 8 - 'Ramaley's XI'

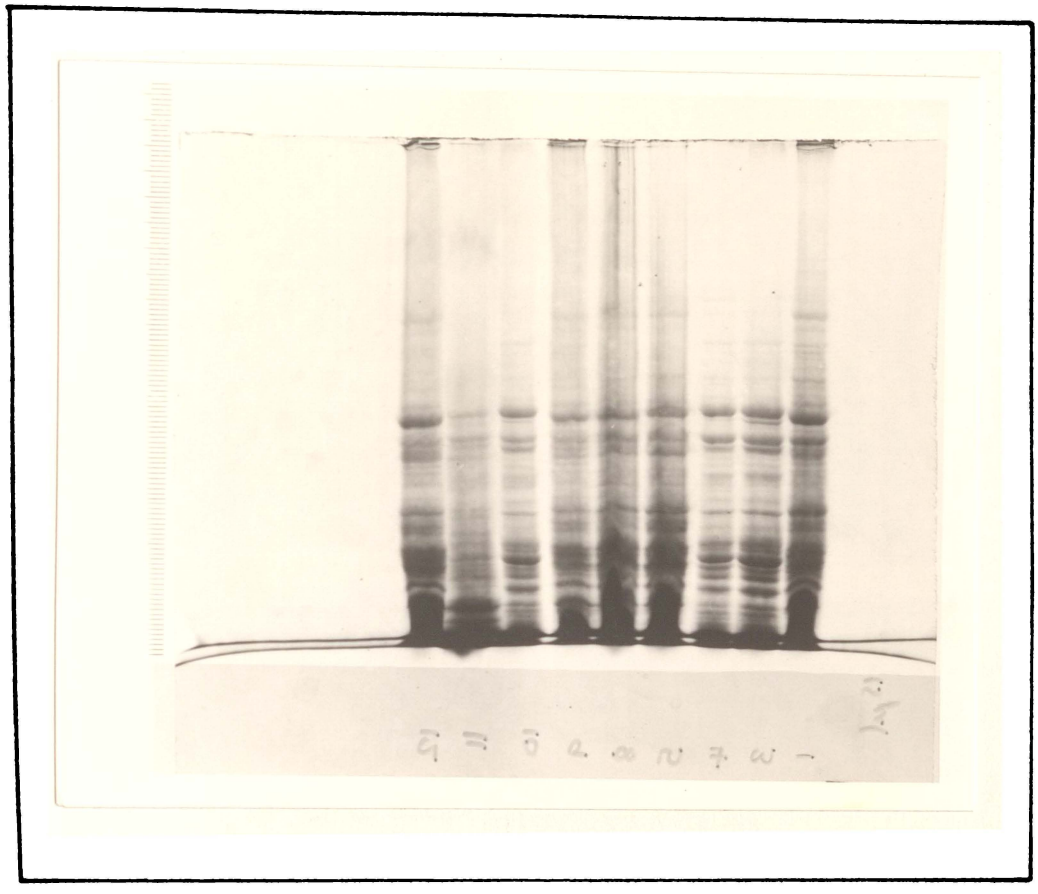


Figure 3.12 SDS-PAGE gel loaded with 15 µl of 9 *Thermus* samples. Samples consist of total cell soluble protein.

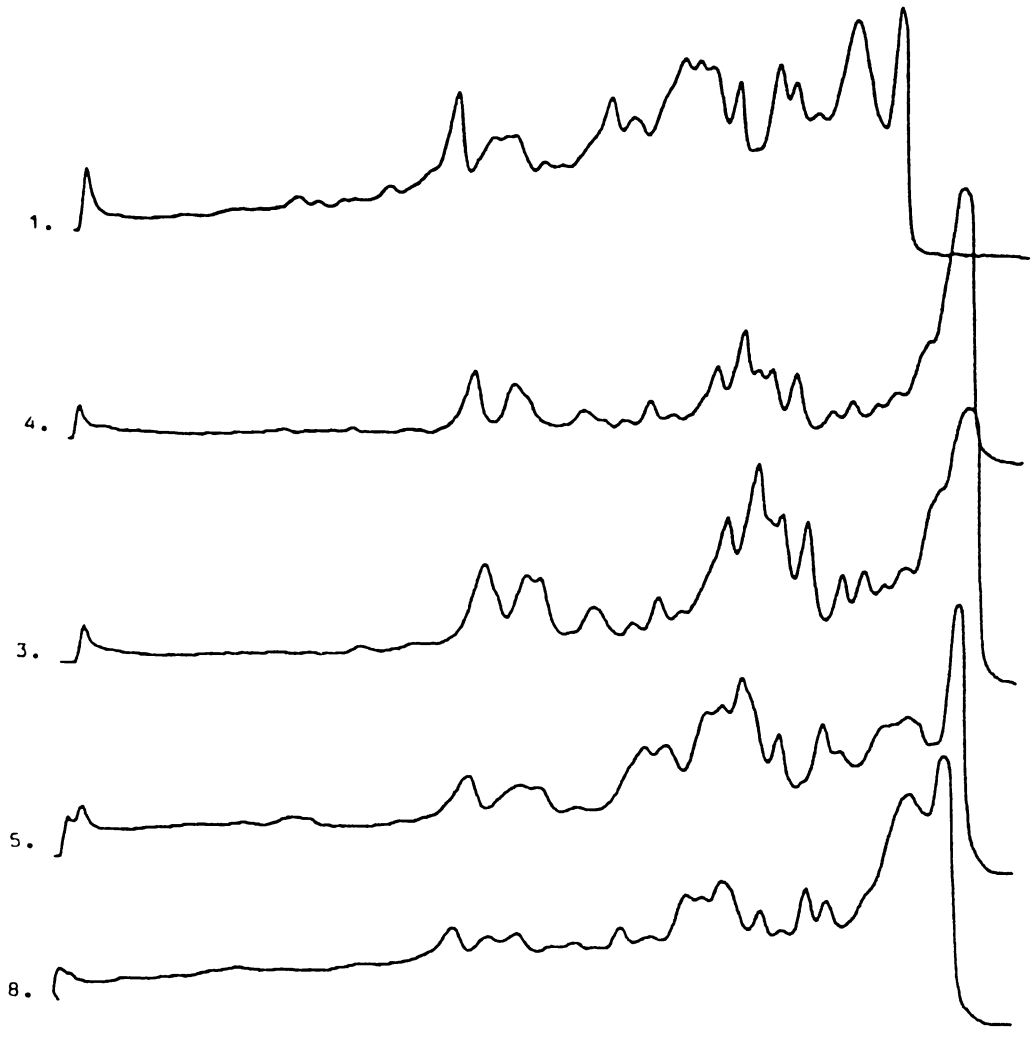


FIGURE 3.13 Densitometric trace of 5 strains of Thermus analysed by SDS-PAGE of whole cell proteins.
Numbers refer to strain numbers (see text).

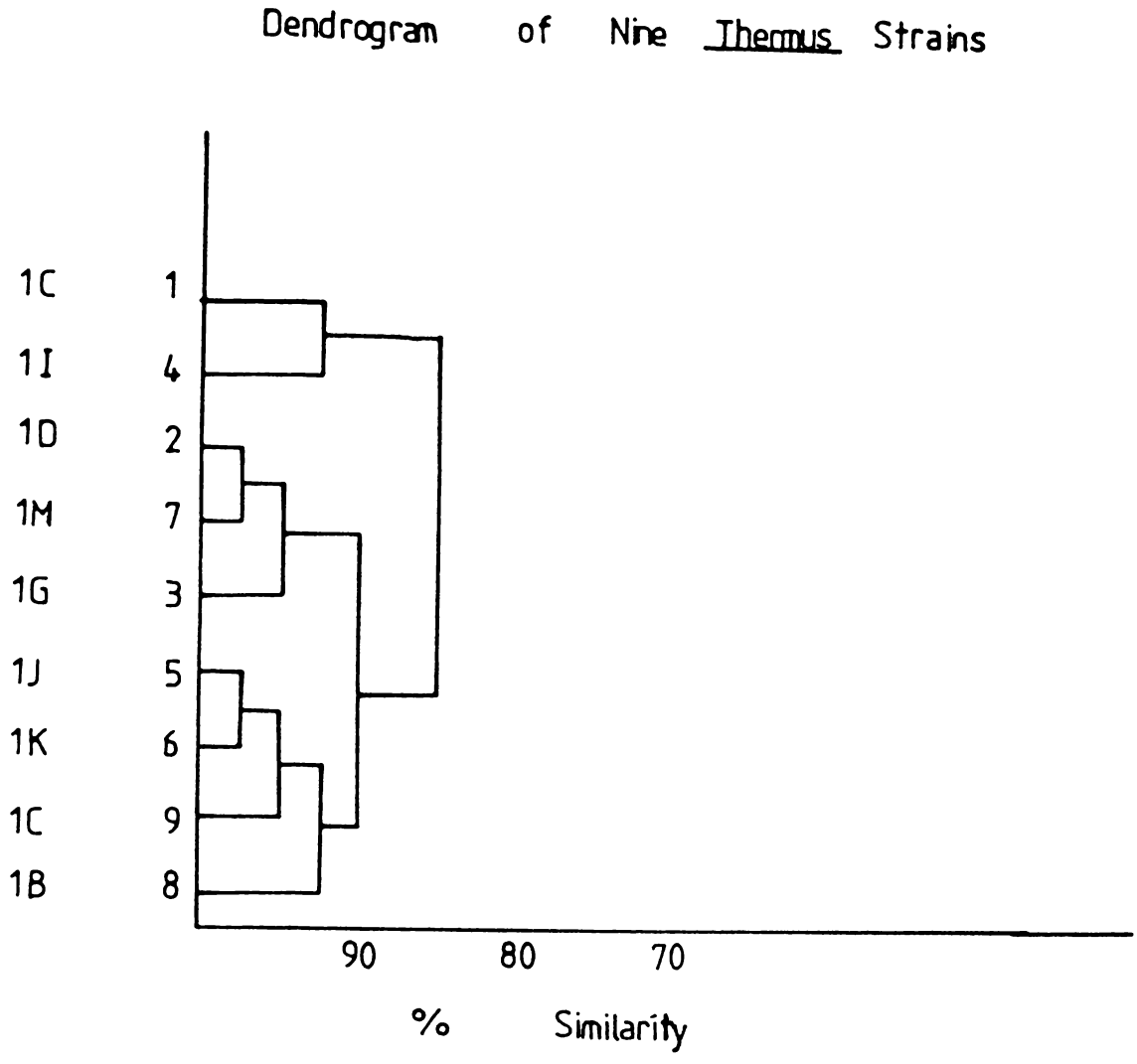


Figure 3.14 Dendrogram of 9 strains of *Thermus* after SDS-PAGE of whole cell proteins, densitometry, digital conversion and unweighted group average clustering applied to a similarity matrix.

Pattern correction and calculation was carried out (Jackman, 1984, in press) and the correlation coefficient of Sneath and Sokal (1973) used to form a similarity matrix. Cluster analysis of this matrix was carried out by unweighted group method using averages (UPGMA) (Sneath and Sokal, 1973).

Figure 3.12 is a photograph of a gel loaded with 15 µl of sample; 25 µl was too much as 'overloaded gels' were obtained.

Figure 3.13 is the densitometer trace of five strains.

Figure 3.14 is the dendrogram relating to these strains by UPGMA sliding best fit ±1 mm using Sneath and Sokal's correlation coefficient.

Seven of the nine strains are very closely related with two strains, 1C and 1I, being rather less similar. However all differences can be considered to be within a single species category.

It appears that this method is very suitable for discrimination of *Thermus* strains and reveals a high degree of similarity between the strains analysed.

These strains were prepared at Cadbury Schweppes (Reading) and analysed at Department of Bacteriology, Institute of Dermatology, London under the guidance of Dr P. Jackman.

No viable New Zealand strains were available for analysis using this method and a suitable densitometer and analogue-to-digital converter are not available at the University of Waikato, so it was not possible to analyse any of the New Zealand strains by this method. Should facilities become available it appears this method would be of considerable value to investigate the taxonomy of thermophiles.

In comparing this grouping with that obtained by PY-MS there does not appear to be any relationship between the two techniques at the "fine" level. This may not be significant, based on the result of only nine strains by SDS-PAGE. A more extensive comparison of the two methods would be of interest, if the opportunity arose.

CHAPTER 4

ARCHAEBACTERIA

4.1 Introduction

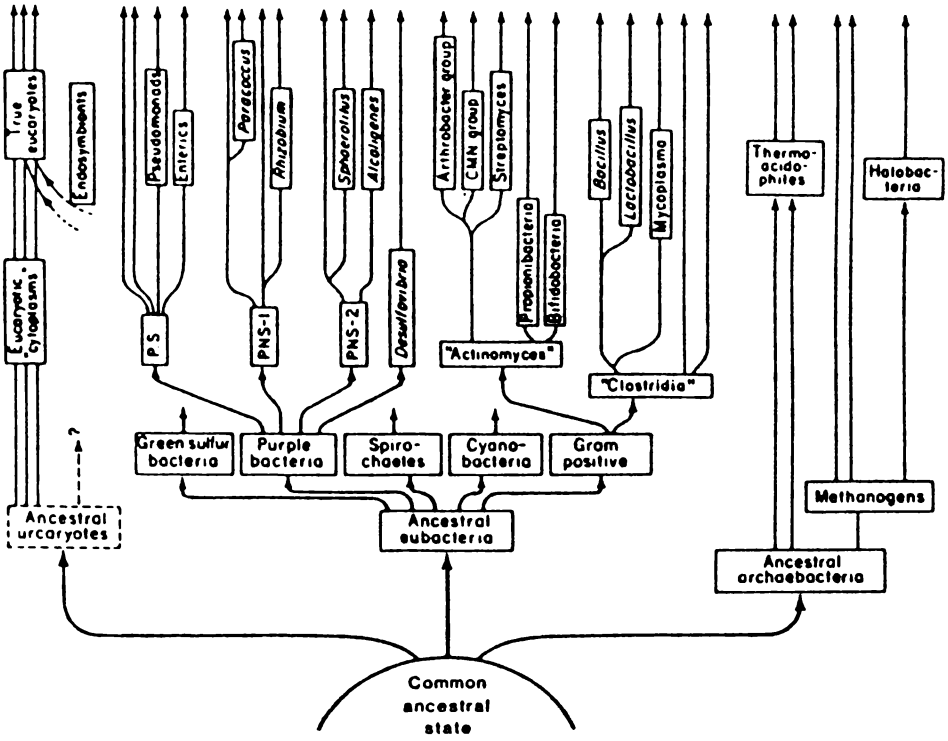
Until recently living organisms were grouped into one of two kingdoms, the Eukaryotes, which included all organisms which had a true nucleus, or the Prokaryotes which did not. However, studies of ribosomal RNA sequence homologies (Woese and Fox, 1977) revealed such wide differences between certain "bacteria" that the Prokaryotes have been regrouped as the eubacteria, or true bacteria (including cyanobacteria and mycoplasmas) and the archaebacteria. As archaebacteria appear to be as dissimilar from eubacteria as they are from eukaryotes it has been suggested that they should be considered as a third primary kingdom (Woese and Fox, 1977).

Members of the archaebacteria are highly diverse, both morphologically and physiologically. The general morphology resembles that of eubacteria with the same range of overall shapes being found. The range of physiological types is wide with examples of aerobic, anaerobic, autotrophic, heterotrophic and photosynthetic metabolism being described (Woese, 1981). The name, which suggests antiquity, was chosen because the best studied group, the *Methanobacteriaceae*, has a metabolism very well suited to conditions thought to have existed on Earth 3-4 billion years ago (Woese and Fox, 1977). At this time archaebacteria may have been the dominant life form and methanogenesis the earliest form of respiration (Woese *et al.*, 1978). The presence of archaebacterial lipids in ancient sediments and petroleum gives further support to the theory of antiquity (Michaelis and Albrecht, 1979).

The archaebacterial kingdom consists of three major sub-groups (Fox *et al.*, 1980): the methanogens, which obtain energy by the

production of methane and require very low redox potentials for growth; the halophiles which require high salt concentrations, and the sulphur-metabolisers (formerly called the thermoacidophiles). Members of the last group which all have the capability of using elemental sulphur during respiration, are found in either hot acid conditions or at temperatures very close to boiling. These are all extreme environments in today's world but may not have been so when archaebacteria evolved. It has been suggested that living in an extreme habitat could be used as a criterion for assigning a newly isolated species to the archaebacterial kingdom (Woese *et al.*, 1978). However, as the concept of the kingdom is so recent it is also possible that species of archaebacteria might be isolated from more moderate environments in future.

Although phenotypically similar to eubacteria, archaebacteria differ considerably at the molecular level. There are the 16S ribosomal RNA differences which were used to characterise the group (Woese and Fox, 1978) (where 16S is an indirect measure of molecular size and refers to the sedimentation rate in an ultracentrifuge). There are also differences in cell wall composition (Brock *et al.*, 1972; Kandler and Hippe, 1977); membrane lipids (Kates, 1972); ribsosomal and transfer RNA's (Fox *et al.*, 1980; Pieler *et al.*, 1982; Schmid *et al.*, 1982 and Gupta, 1982) and in transcription mechanisms (Zillig *et al.*, 1982a). Differences between archaebacteria and eubacteria at a molecular level cause a different pattern of antibiotic resistance which can be exploited in selection and preliminary characterisation of archaebacterial species (Hilpert *et al.*, 1981). Before a newly-isolated species can be classified as an archaebacterium it has been proposed (Woese *et al.*, 1978) that it should have at least the following properties: characteristic ribosomal RNA's, transfer RNA's and DNA-dependent RNA polymerases, ether-linked lipids composed of isoprenoid subunits and cell walls lacking muramic acid.



Schematic representation of the major lines of prokaryotic descent.

FIGURE 4.1 From Fox et al., 1980.

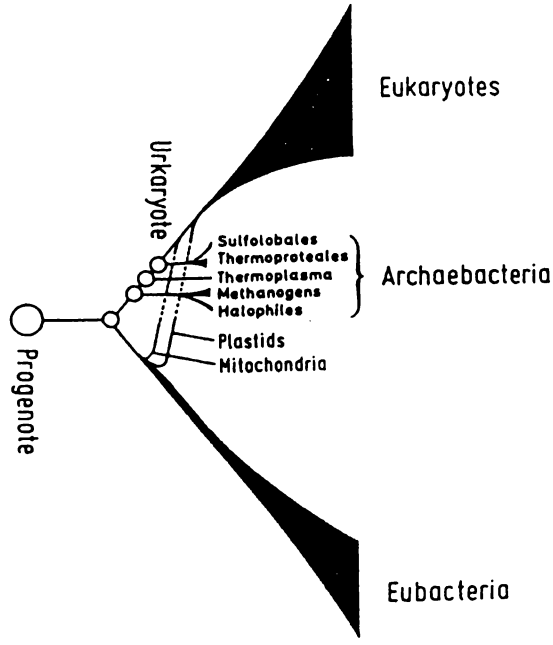


FIGURE 4.2 Proposed phylogeny of the three kingdoms of living things showing the close relationship between the archaebacteria and the eukaryotes (Zillig et al., in press).

4.2 Evolution of Archaebacteria

Analysis of 16S rRNA sequence data led to the realisation that living organisms should be divided into three primary kingdoms (Woese and Fox, 1977), the third kingdom being the Archaebacteria. This concept has led to considerable interest and speculation as to the origin of life. Woese has proposed (Woese, 1982) that the universal ancestor of all living things was a simple rudimentary "cell" he called the progenote. The progenote was probably a collection of ill-defined forms which could readily exchange genetic material and whose biochemical pathways were rather simple and not well regulated (Woese, 1982). A scheme deriving the three kingdoms from the progenote was proposed (Fox *et al.* 1980) and is presented in Figure 4.1. However, there is now a growing body of evidence which suggests a rather close relationship between archaebacteria and eukaryotes. Similarities in molecular detail between archaebacteria and eukaryotes include the following:

i) sequences of ribosomal A proteins (Matheson and Yaguchi, 1982);

ii) reaction of translation elongation factor with diptheria toxin (Kessel and Klink, 1982);

iii) presence of glycoprotein in membranes and envelopes (Yang and Haug, 1979);

iv) structure and function of tRNA's and the genes that encode them (Kuchino *et al.*, 1982; Kaine *et al.*, 1983; Gupta, 1982).

v) resistance to a number of antibiotics which inhibit eubacteria (Hilpert *et al.*, 1981).

vi) subunit structure and immunological cross-reactions between sub-units of DNA-dependent RNA polymerase (Schnabel *et al.*, 1983; Zillig *et al.*, in press).

In view of these similarities it has been proposed (Zillig *et al.*, in press) that archaebacteria may represent the level of evolution during

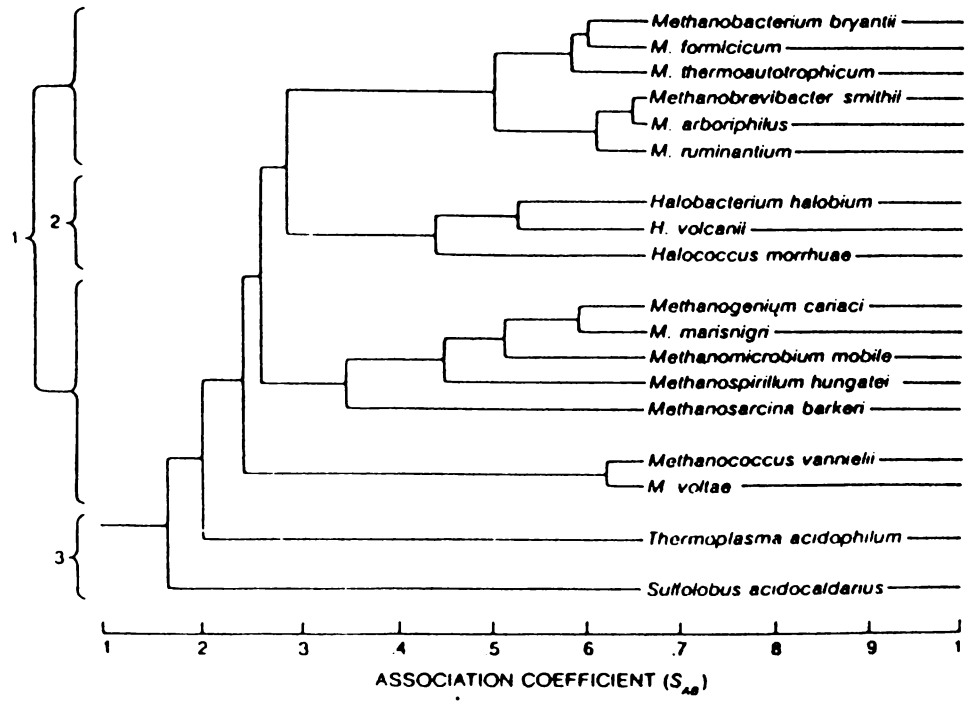


Figure 4.3 Archaebacterial dendrogram showing phylogeny based on 16S rRNA homologies (Woese, 1981).

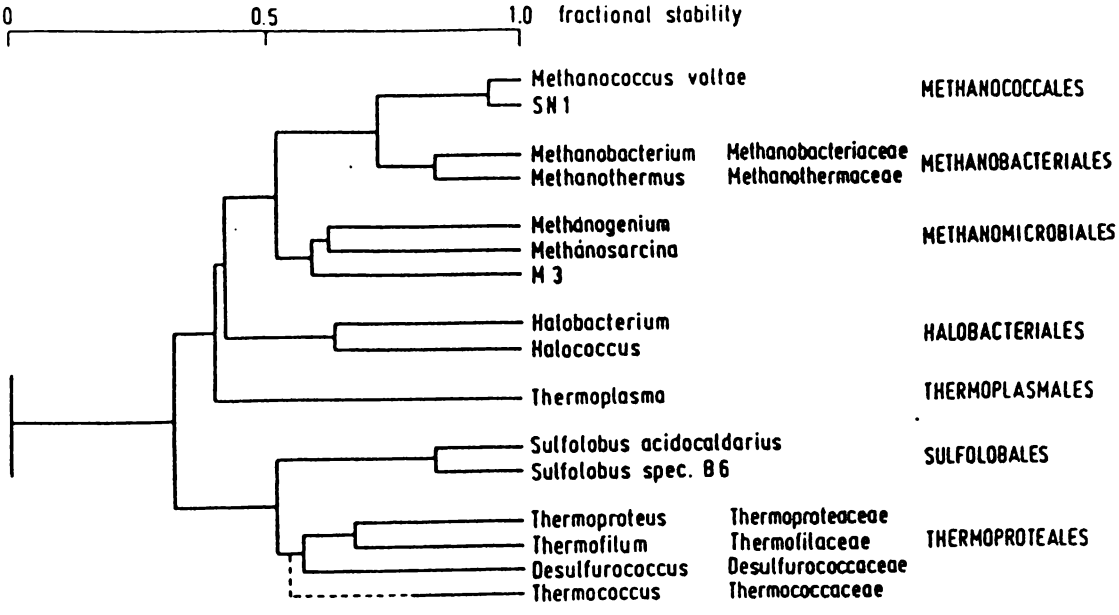


Figure 4.4 Dendrogram depicting archaebacterial phylogenetic relationships based on the stability of DNA-RNA hybrids (Tu *et al.*, 1982).

the period of separation of the three kingdoms. Support for this theory comes from 16S rRNA data which suggests that methanogens are a very ancient group which may in fact be the oldest remaining phenotype (Stackebrandt and Woese, 1981). There seems to be some relationship between methanogens and eubacteria (Zillig *et al.*, in press) which suggests eubacteria separated as a distinct line before the evolution of the sulphur-metabolisers. The eubacterial line has certainly evolved further than the archaebacterial line and possibly more than eukaryotes. The latter appear to have evolved somewhat later and to show rather a close relationship to the sulphur-metabolisers. A diagrammatic representation of this theory is given in Figure 4.2.

Although this picture is still speculative it underlines the importance of archaebacteria in understanding the early evolution of cells (Woese, 1982).

4.3 Phylogeny of Archaebacteria

The technique of 16S rRNA sequence cataloguing has proved a powerful tool for higher order classification (Stackebrandt and Woese, 1981). A dendrogram showing relationships between archaebacteria is shown in Figure 4.3. This study pre-dates the discovery of the anaerobic sulphur-respirers but indicates a close relationship between halophiles and methanogens.

Because of rapid changes in some genes, DNA-DNA hybridisation can only be used to depict relationships below the genus level (Stackebrandt and Woese, 1981) but hybridisation of 16S rRNA with DNA has proved very useful at higher levels (Tu *et al.*, 1982). A phylogeny based on the stability of 16S rRNA-DNA hybrids can be seen in Figure 4.4. This also demonstrates a close relationship between halophiles and methanogens and shows that, with the exception of *Thermoplasma*, the sulphur-

metabolising group is a close knit one. Further evidence for these relationships comes from studies of RNA polymerase structure (Zillig *et al.*, 1982a) and immunological comparison of their components (Schnabel *et al.*, 1983).

At present the Archaebacterial kingdom can be considered to consist of two deep branches:

i) methanogens and halophiles

ii) *Thermoplasma* and the sulphur-metabolisers.

Methanogens form the largest group with three orders and a large number of species. The four remaining orders are the *Halobacteriales* with two genera, the *Thermoplasmales* with only one species described, the *Sulfolobales* and the *Thermoproteales*.

It is not known how successfully PY-MS can be used to discriminate deep relationships as required for a phylogenetic study. However, its ability to discriminate both at the genus level (Gutteridge and Puckey, 1982) and at the sub-species level (Wieten *et al.*, 1981) has been clearly demonstrated. PY-MS analyses of bacteria have been restricted to species which can be grown under identical conditions, however this is not possible when deep relationships are being studied. In spite of this difficulty it was decided to attempt a phylogenic discrimination of as many archaebacterial orders as possible in this study.

4.4 Taxonomy of Archaebacteria

Taxonomic studies of archaebacteria have mainly involved methanogens (Balch *et al.*, 1979) and halophiles (Zwilling *et al.*, 1969). There have also been some studies carried out on *Sulfolobus*, an aerobic member of the sulphur-metabolising group, with some results suggesting an extremely homogeneous group (comparison of ribosomal

proteins (Schmid and Boeck, 1982). Immunological studies, however, indicate different serotypes are found even in the same hot pool (Bohlool and Brock, 1974). Strain differences in temperature optima (Mosser *et al.*, 1974) and in electrophoretic patterns of whole cell proteins (Norris and Marsh, 1983) have also been reported.

It seems probable that there are three distinct species of *Sulfolobus* and a taxonomy supporting this has been proposed (Zillig *et al.*, 1980); unfortunately it was not possible to include the type strains of these proposed species in this study. Detailed taxonomic studies of other members of the sulphur-metabolising group have not yet been reported.

As this study is concerned with the taxonomy of caldoactive bacteria (i.e. bacteria with temperature optima >65^{o}C) known species of these archaebacteria will be described in some detail, including those which were not available for PY-MS analysis. Four species of mesophilic archaebacteria were included in the PY-MS study and a description of these is also given below. With the exception of *Thermoplasma*, a moderate thermophile but a species of considerable evolutionary importance, (Searcy *et al.*, 1978) other moderate thermophiles and mesophiles will not be described. An outline of properties of these species is given in Table 4.1 and a more detailed description follows.

I Sulphur-Metabolising Archaebacteria

(a) *Thermoplasma acidophilum*

This wall-less archaebacterium is found in burning coal-refuse piles (Darland *et al.*, 1970) and has also been isolated from Japanese hot springs (Ohba and Oshima, 1982). Although *Thermoplasma* grows optimally as an aerobic heterotroph it can reduce sulphur to hydrogen

sulphide, but anaerobic growth is very inefficient (Brock, 1978). This property gives it a loose alliance to the sulphur-metabolisers. *Thermoplasma* has a requirement for a factor present in yeast extract and grows well in medium containing this and certain sugars. It has been suggested (Searcy *et al.*, 1978) that *Thermoplasma* may be similar to the theoretical ancestor of the eukaryotes. This theory is based on several features which include the presence of histone-like proteins which conform the DNA into subunits similar to eukaryotic chromatin. *Thermoplasma* lacks cytochromes and has respiratory enzymes not coupled to oxidative phosphorylation, therefore its respiration is similar to that of the microbodies present in all eukaryotic cells. Contractile proteins similar to eukaryotic actin and myosin have been found, which together with the lack of a cell wall, give it the potential to engulf a bacterial endosymbiont. *Thermoplasma* has a tRNA sequence nearly identical to the proposed "ancestral sequence" (Searcy, 1982) which gives further support to the theory of a close relationship to the original eukaryotic cell.

(b) *Sulfolobales*

The distribution of *Sulfolobus* seems to be worldwide with isolates obtained from acidic water and soils over a wide temperature range (Brock, 1978). *Sulfolobus* is the only sulphur-oxidising member of the sulphur-metabolising group. Three species have been described, all with similar general properties.

(i) *Sulfolobus acidocaldarius*

S. acidocaldarius is a facultative autotroph which can obtain energy by the oxidation of elemental sulphur to sulphate. Under certain conditions sulphide can also be oxidised to sulphur but the location of the enzymes responsible prevents complete oxidation from sulphide to sulphate (Brock, 1978). The oxidation of ferrous iron can also be used

to generate energy (Brierley and Brierley, 1973). In the laboratory *S. acidocaldarius* grows heterotrophically on a wide range of substrates (Brock *et al.*, 1972). Growth is generally aerobic but anaerobic (or microaerophilic - Brock, 1978) reduction of ferric iron and molybdenum has been reported (Brierley and Brierley, 1982). Considerable variation has been found in growth requirements for different strains (Norris and Marsh, 1983) and a range of temperature optima has also been reported (Mosser *et al.*, 1974). Cells are lobed cocci which form pili, apparently for attachment to sulphur granules (Weiss, 1973). The cell wall is composed of hexagonally shaped subunits (Weiss, 1974) and the membrane contains cyclic C40 isoprenoid ether-linked lipids (de Rosa *et al.*, 1977). The genetics have been little studied but a plasmid has been isolated and characterised from one strain (Yeats *et al.*, 1982) and a UV-inducible temperate phage from the same strain (Martin *et al.*, 1984).

(ii) *Sulfolobus solfataricus*

An Italian isolate originally named *Caldariella acidophila* (de Rosa *et al.*, 1975) is now considered to be a species of *Sulfolobus* and has been renamed *Sulfolobus solfataricus* (Zillig *et al.*, 1980). This species has similar general properties to *S. acidocaldarius* but temperature optima of strains are generally higher (de Rosa *et al.*, 1975) and there are significant differences both in the molecular weights of the 11 components of its RNA polymerase and in the salt requirements of that enzyme (Zillig *et al.*, 1980).

(iii) *Sulfolobus brierleyi*

This species is an obligate autotroph which differs in several respects from *S. acidocaldarius*: the temperature optimum is rather low (Brierley and Brierley, 1973), cells are much less stable

at neutral pH, the G+C content is significantly lower and the RNA polymerase lacks two components present in the enzymes from the other two species (Zillig *et al.*, 1980).

(c) *Thermoproteales*

This order contains three families of strictly anaerobic organisms most of which can grow heterotrophically by sulphur respiration. Some species can also grow autotrophically using energy obtained from the oxidation of hydrogen by sulphur to assimilate carbon dioxide (Fischer *et al.*, 1983) and a few species are obligate autotrophs. The pathway of carbon dioxide fixation appears to be identical to that described for *Sulfolobus brierleyi* (Kandler and Stetter, 1981) (O. Kandler, pers. comm.).

The family *Thermoproteaceae* contains two genera, *Thermoproteus* and *Thermofilum*.

(i) *Thermoproteus tenax*

Both obligate and facultative autotrophic strains are known (Fischer *et al.*, 1983) and these have been isolated over a wide geographic area. Heterotrophic growth only takes place on complex media (P. Jasperse-Herst, M.Phil. Thesis, 1984). Cells are non-motile, mostly rod-shaped and surrounded by a rigid cell wall. Cells are 0.4 µm wide but the length can vary from 1 µm - 80 µm with shorter cells predominating in unstirred cultures or under favourable growth conditions. Bent cells without septa are sometimes seen and cells with spheric bodies attached (so-called golf clubs) are frequent in actively growing cultures. It is possible that these spheres detach and elongate to form new cells (Zillig *et al.*, 1981). Both cell wall type and lipid composition are similar to *Sulfolobus* (Zillig *et al.*, 1981). Three rod-shaped, temperate phages have been induced from an autotrophic strain following sulphur-depletion in the medium (Janekovic *et al.*, 1983).

(ii) *Thermoproteus neutrophilus*

This species is an obligate autotroph with a higher pH optimum than *T. tenax* (Fischer *et al.*, 1983) which has requirements for both molecular hydrogen and carbon dioxide (Stetter *et al.*, 1983a). Full details have not yet been published.

(iii) *Thermofilum pendens*

First described as "stiff filaments" (Zillig *et al.*, 1981), this organism was found to have an obligate requirement for a component of the polar lipid fraction extracted from *T. tenax* cells (Zillig *et al.*, 1983a) which is thought to be involved in the solubilization of sulphur. Cells are thin rods, 0.2 μm wide and from 1 - >100 μm long with terminal pili; bent forms and "golf clubs" are also found. Swollen sections are seen in some cells and it has been suggested (Zillig *et al.*, 1981) that cells may break at this point to form two daughter cells as well as new cells being formed by the detachment of spheres from "golf clubs". In spite of some similarities to *T. tenax* there is no cross reaction between the RNA polymerases of the two species therefore they represent separate genera.

(d) *Desulfurococcaceae*

Three species have been isolated in this family,all belonging to the genus *Desulfurococcus*. The name describes the coccoid shape of the cells which have a characteristic flexible cell wall. Growth is by sulphur respiration of proteins or peptides and rather inefficient fermentation of peptides is also possible. The growth requirements of all three species are apparently identical.

(i) *Desulfurococcus mucosus*

Cells of this species form a strongly smelling unknown product when growing on sulphur. A slime layer, possibly a mucous polymer, is attached to the cell envelope (Zillig *et al.*, 1982b).

(ii) *Desulfurococcus mobilis*

This species lacks the slime layer of *D. mucosus*. Cells have bundles of flagella attached at one pole but motility is not detectable under the microscope at room temperature (Zillig *et al.*, 1982b). A further species which is surrounded by a dense network of long threads has been isolated but not characterised (Zillig *et al.*, 1982b).

(e) *Thermococcaceae*

One species has been described but it is possible that another as yet unnamed species (Morgan and Daniel, 1982) may represent a second member of this family.

(i) *Thermococcus celer*

A marine organism, *T. celer* has a requirement for sodium chloride with optimum growth occurring at 0.7M concentration. Growth is by sulphur respiration of proteins or peptides or by inefficient fermentation of these substrates. *T. celer* has one of the fastest growth rates among archaebacteria with a generation time of under one hour compared to the 3-4 hours of most species. Cell division is by increasing constriction to form diploforms, daughter cells being linked by narrow bridges before separation. Lysis of cultures sometimes occurs and virus-like particles have been observed in electron micrographs of lysates (Zillig *et al.*, 1983b).

(f) *AN1*

This organism may be closely related to *T. celer* (W. Zillig, pers. comm.), an observation based on preliminary investigations of DNA-polymerase cross-reactions. However, a different relationship was indicated by immunological cross-reactions between ribosomal proteins (G. Schmid, pers. comm.) so it is possible that these two species may not be very closely related. Both organisms share the common

characteristics of sulphur respiration of proteins and peptides, rapid growth rate and formation of diploforms, but AN1 has a higher pH optimum (see Table 1) and a lower salt requirement, 35 mM NaCl optimal (Morgan and Daniel, 1982). The optimal temperature has been reported as 75°C but it has been found (B. Patel, pers. comm.) that if the peptone content of the growth medium is decreased by 90% the temperature optimum increases to 88°C. It is also reported (Morgan and Daniel, 1982) that cystine can replace sulphur as electron acceptor for respiration - hydrogen sulphide is formed as a product as for sulphur respiration.

(g) *Pyrodictium*

This genus contains two species, both obligate autotrophs, which like all anaerobic archaebacteria, including methanogens, can reduce sulphur to hydrogen sulphide (Stetter *et al.*, 1983a). Cells are disc-shaped and if cultures are unstirred a dense network of fibres is produced which enables them to grow up to 110°C. These fibres are composed entirely of protein (K. Stetter pers. comm.) and seem to be involved in ATP production at temperatures >100°C (Stetter *et al.*, 1983a). *Pyrodictium* is a marine organism which requires 0.25M sodium chloride for growth. In laboratory culture precipitates of iron pyrites are formed and it has been suggested (Stetter *et al.*, 1983a) that this species could be responsible for formation of naturally occurring deposits of this mineral. The two species named are *P. occultum* and *P. brockii* which are differentiated by a stimulation of growth shown by the latter species in the presence of 0.2% yeast extract which is thought to be related to solubilization of sulphur (Stetter *et al.*, 1983a).

(h) *Thermodiscus maritimus*

This organism is an obligate autotroph which is moderately halophilic. Cells are flat discs with a thick envelope and long protrusions (Fischer *et al.*, 1983); full details have not yet been published.

The relationship of *Pyrodictium* and *Thermodiscus* to the rest of the *Thermoproteales* has not yet been determined.

II The Methanogens

The methanogens are the largest and most diverse group of archaebacteria; they are found in most natural anaerobic habitats over a wide temperature range and it has been suggested that methane venting from submarine hydrothermal systems could be of microbial origin (Baross *et al.*, 1982). Bacteria isolated from such systems have been reported as growing at 250^{o}C under high pressure (Baross and Deming, 1983) but there has not yet been unequivocal demonstration of growth at this temperature (Trent *et al.*, 1984). Methanogens are a very diverse group with a wide range of morphologies and cell wall types being found (Balch *et al.*, 1979). Most can use molecular hydrogen as the sole source of reducing power and many can synthesize all cellular carbon from carbon dioxide (Zeikus, 1977). There are three orders.

1. *Methanobacteriales*

Two caldoactive species have been described.

(i) *Methanobacterium thermoautotrophicum*

(Formerly known as *Methanobacterium thermoautotrophicus*).

This species is an obligate autotroph (Zeikus and Wolfe, 1972) which grows optimally in the presence of 50mM sodium chloride (Perski *et al.*, 1981). There is an unusual internal membrane system composed of triple membranes which may increase the area of attachment for membrane-

bound enzymes involved in methane production (Zeikus and Wolfe, 1972). Cell walls contain a functionally similar polymer to murein (Kandler, 1982) and give a positive Gram reaction.

(ii) *Methanothermus fervidus*

Only two isolations have been reported and it has been suggested that this species is exceptionally oxygen sensitive (Stetter *et al.*, 1981). Although methane is formed from carbon dioxide and hydrogen, yeast extract is also required for growth in laboratory culture. It is considered probable (Stetter *et al.*, 1981) that biologically formed methane found in Italian Pliocene sediments may have been formed by similar organisms.

2. *Methanococcales*

Two caldoactive species have been described:

(i) *Methanococcus thermolithotrophicus*

This bacterium grows both autotrophically and on formate. A marine organism, it has a requirement for 0.7M sodium chloride and in optimal conditions has a doubling time of only 55 minutes. Unlike mesophilic members of this genus, yeast extract does not stimulate growth. The cell envelope consists of protein subunits and a tuft of flagella is inserted in a defined area on the cell surface.

(ii) *Methanococcus jannaschii*

Growth requirements are very similar to those of *M. thermolithotrophicus* but the doubling rate is exceptionally fast at only 26 minutes under optimal conditions (which include the presence of both 0.5M sodium chloride and of selenium). It has been suggested that this rapid autotrophic growth may make this species an important primary producer in hydrothermal vents. Cells are osmotically fragile and motile with two bundles of flagella inserted near one pole (Jones *et al.*, 1983).

3. *Methanomicrobiales*

One mesophilic species was included in this study.

(i) *Methanosarcina barkeri*

The bacterium has a distinctive microscopic appearance with irregularly shaped cells forming characteristic packets of varying size (Balch *et al.*, 1977). Cell walls strain Gram positive due to the presence of a pseudo-murein type polymer. Methane is produced from acetate in complex media as well as autotrophically from carbon dioxide and hydrogen, and most efficiently of all from methanol in the presence of carbon dioxide and hydrogen (Weimer and Zeikus, 1978).

III The Halophiles

Although some caldoactive archaebacteria have a requirement for sodium chloride, studies of RNA polymerase structure (Zillig *et al.*, 1982a) place these in the sulphur-metabolising group. As yet no caldoactive halophiles have been reported. All extreme halophiles i.e. species which grow best in 20-30% sodium chloride (Larsen, 1977) are archaebacteria. There is only one family, the *Halobacteriaceae* which includes rod-shaped *Halobacterium* species and coccoid-shaped *Halococcus* species, both very similar in physiology. *Halobacteria* are obligate aerobes and also carry out a simple type of photosynthesis. The pigments used are similar to eukaryotic retinal pigments and are used to generate energy and drive ion pumps (Stoeckenius, 1982). Oxygen tensions are often low in high salt environments therefore a supplementary energy generating system is a considerable advantage. The cytoplasmic salt concentration is high which is necessary to prevent dehydration, consequently halobacterial enzymes are adapted to function under these conditions (Dundas, 1977).

TABLE 4.1

GENERAL CHARACTERISTICS OF ARCHAEBACTERIA

Species	Source of first Isolate	Optimal pH	Optimum Temperature	mol % G & C	Reference
I Sulfur Metabolisers					
Thermoplasma acidophilum	Burning coal refuse pile, USA	1.0 - 2.0	59°C	25 - 40	Darland *et al.*, 1970
Sulfolobus acidocaldarius	Hot spring, USA	2 - 3.5	55 - 85°C (strain optima exist)	39 - 44	Brock *et al.*, 1972
Sulfolobus solfataricus	Hot spring, Italy	3.0	75°C and 87°C	36 - 38	de Rosa *et al.*, 1975
Solfolobus brierleyi	Hot spring, USA	2.0	50°C	31	Brierley and Brierley, 1973
Thermoproteus tenax	Hot spring, Iceland	5.0	88°C	55.5	Zillig *et al.*, 1981
Thermoproteus neutrophilus	Hot spring, Iceland	6.8	85°C	N.R.	Fischer *et al.*, 1983
Thermofilum pendens	Hot spring, Iceland	5.0	85 - 90°C	57.4	Zillig *et al.*, 1983,a
Desulfurococcus mucosus	Hot spring, Iceland	6.0	85°C	51.3	Zillig *et al.*, 1982,b
Desulfurococcus mobilis	Hot spring, Iceland	6.0	85°C	50.8	Zillig *et al.*, 1982,b
Thermococcus celer	Submarine solfataric field, Italy	5.8	88°C	56.6	Zillig *et al.*, 1983,b
AN1	Hot spring, New Zealand	7.0	75°C	N.R.	Morgan and Daniel, 1982
Pyrodictium occultum	Submarine solfataric field, Italy	5.5	105°C	62	Stetter *et al.*, 1983,a
Pyrodictium brockii	Submarine solfataric field, Italy	5.5	105°C	52 - 56	Stetter *et al.*, 1983,a
II Methanogens					
Methanobacterium thermo-autotrophicum	Anaerobic sewage digestor	7.2 - 7.6	65 - 70°C	52	Zeikus and Wolfe, 1972
Methanothermus fervidus	Hot spring, Iceland	6.5	83°C	33	Stetter *et al.*, 1981
Methanococcus thermolitho-trophicus	Geothermally heated sediments, Italy	7.0	65°C	31.3	Huber *et al.*, 1982
Methanococcus jannaschii	Submarine vent, East Pacific Rise	6.0	85°C	31	Jones *et al.*, 1983
Methanosarcina barkerii	Sewage digestor	7.0	35 - 37°C	44	Schnellen, 1947
III Halophiles					
Halobacterium halobium	Salt fish	7.2 - 7.4	40°C	66 - 68	Petter, 1931
Halobacterium saccharovorum	Salterns, San Francisco Bay	7.8	40°C	66 - 68	Tomlinson and Hochstein, 1972
Halococcus species	Salt lakes and fish	7.2	30 - 37°C	67	Bergey, 1974

Three species were included in this study -

1. *Halobacterium halobium*

Cells are motile rods with a tuft of flagella. Gas vacuoles are often present and the colour is pinkish red (Bergey's Manual, 1974).

2. *Halobacterium saccharovorum*

This species differs from *H. halobium* in its ability to grow on a range of sugars with the production of acid and gas. It is similar in other respects (Tomlinson and Hochstein, 1972).

3. *Halococcus*

Halococcus species are coccoid in shape with a thick cell wall which makes them more resistant to osmotic damage than *Halobacterium* (Dundas, 1977). The species included in this study was isolated by Dr Lowe (M.I.R.I.N.Z.), but detailed characterisation has not been carried out.

4.5 Isolation and Culture of Caldoactive Archaebacteria

Pyrolysis mass spectrometric analyses are a measure of genome expression, therefore samples must be prepared under rigidly controlled standard conditions predetermined for each group. As some archaebacteria do not grow on solid medium all samples were prepared in liquid culture to eliminate unnecessary differences. Samples analysed in this study were:-

i. type strains obtained either from culture collections or as gifts from other laboratories.

ii. isolates obtained from New Zealand thermal areas.

The standard of purity set for these isolates was three successive serial transfers into media containing a sequence of two different antibiotics known to select for archaebacteria. Phase contrast microscopy was used

to check morphology at each stage in the purification with 20 fields at x1000 magnification routinely examined.

Cells were harvested as described in Appendix II. Subsequently freeze-dried cultures were autoclaved to avoid difficulties in transporting viable material to Great Britain.

4.5.1 Response to Antibiotics

Because archaebacteria and eubacteria differ at the molecular level, their pattern of response to antibiotics is different, making this a useful tool for enrichment and decontamination (Hilpert *et al.*, 1981). Antibiotics used in this study were:

(a) Inhibitors of eubacterial cell wall synthesis

Penicillin G (active at neutral pH) or vancomycin (active at pH<5) inhibit cross-linking during peptidoglycan synthesis in eubacteria and therefore have no target in archaebacteria.

(b) Inhibitors of DNA-dependent RNA polymerase

Rifampicin is a specific inhibitor of eubacterial polymerases but does not affect those of archaebacteria (Zillig *et al.*, 1980). However, inhibition of growth of some archaebacteria was found at concentrations >1000μg ml^{-1} (Zillig *et al.*, 1982b).

(c) Inhibitors of protein biosynthesis

Chloramphenicol binds to the 70S ribosomes of eubacteria and inhibits peptidyltransferase. Some archaebacteria are also inhibited but the target is different, inhibition being due to action on the hydrogenase involved in methane production (McKellar and Sprott, 1979). Streptomycin reduces translation efficiency and causes misreading on the ribosomes of eubacteria; inhibition of archaebacteria has been tested for a few strains (Weisburg and Tanner, 1982) but not reported.

Despite the high incubation temperatures used in this study a rod-shaped, presumably eubacterial contaminant was removed by addition of these antibiotics.

Details of the preparation of antibiotics appears in Appendix II, Section 2.

Two different approaches were used in this study:

I. Isolation of species of caldoactive archaebacteria similar to those already reported in the literature.

II. Isolation of new species of caldoactive archaebacteria.

SECTION I

4.6 Isolation of Known Species of Caldoactive Archaebacteria

4.6.1 AN1-like Strains

4.6.1.1 Isolation of AN1-like Strains

AN1 was isolated in 1978 by Dr H. Morgan from a pool in Kuirau Park, Rotorua, New Zealand; this pool has now dried up and it is of interest that other pools sampled in the Park did not yield this organism.

For enrichment 1 ml of water and sediments (see Appendix I, Section I.1 for details of sample collection techniques) was added to 15 ml of AN1 medium (Appendix II, Section II.3) and incubated at 75°C. Penicillin G was added at 50 $\mu g\ ml^{-1}$ to select for growth of archaebacteria. Most positive samples were visibly turbid within three days. Purification was achieved by serial dilution into the same medium with the addition of either 100 $\mu g\ ml^{-1}$ streptomycin or 50 $\mu g\ ml^{-1}$ chloramphenicol. After three successive serial transfers and microscopic examination a culture was considered pure.

Sixty-nine pools from a number of different thermal regions were screened for the presence of this bacterium and thirteen positive isolates obtained (see Table 1, Appendix II).

There seems a definite correlation between region and presence of AN1 with many pools in Tokaanu (TOK) and the Whakarewarewa district of Rotorua (RT) yielding isolates. Other regions sampled gave only occasional positive results. Isolates of AN1 were obtained from pools of pH values ranging from pH = 5 to pH = 7.9. Although there seems to be no absolute correlation between pool temperature and the presence of AN1, this species generally seems to be found in pools with temperatures of less than 90°C. AN1 does have a requirement for 35mM sodium chloride which is apparently a requirement for Na+ (it has been found that Cl- is not required and that Li+ can replace Na+) - (H. Morgan, pers. comm.). Pool data for sodium chloride is available for eight of the pools sampled which is insufficient to make definite conclusions but does indicate a relationship between sodium levels of 400 ppm or higher and the presence of AN1 (see Table 1, Appendix II).

Most successful isolations were made from water samples which had been stored in the laboratory for less than three months.

4.6.1.2 Preparation of AN1-like Strains for PY-MS Analysis

A sample of AN1 was obtained from Dr Morgan and maintained by weekly subculture prior to preparation for analysis. Together with the thirteen isolates, AN1 was grown in 200 ml quantities and harvested as set out in Appendix II, Section 6.

Four strains were also grown using cystine as respiratory electron acceptor, and another two strains were inoculated into medium containing 1/10th concentration of peptones and incubated at 85°C (Appendix II, Section 3).

AN1-LIKE STRAINS ANALYSED BY PY-MS

Strain	Source temperature °C	Source pH	Source Na^+ concentration ppm	Conditions of Growth		
				Sulphur	Cystine	at 85°C
AN 1	85	6.5	N.A.	+	+	-
Rt 12	82	7.0	606	+	-	+
Rt 14	79	7.3	647	+	-	-
Rt 34	75	6.5	411	+	-	-
Rt 44	62	7.8	N.A.	+	-	-
Rt 51	82	7.3	628	+	-	-
Rt 56	90.2	7.9	857	+	-	-
Rt 100	81	7.3	N.A.	+	-	-
Wai 23	95	6.5	N.A.	+	+	-
Tk 14	80	5.9	N.A.	+	+	-
Tok 4	92	5.7	N.A.	+	+	+
Tok 10	75	6.5	N.A.	+	-	-
Tok 11	98	7.0	N.A.	+	-	-
Wis 4	90 - 96	5.0	N.A.	+	-	-

N.A. = data not available

TABLE 4.2

Table 4.2 sets out the strains which were prepared as described in Appendix II, Section 3 and taken to Great Britain for analysis.

4.6.1.3 PY-MS and Data Processing of AN1-like Strains

Freeze dried samples were resuspended in methanol, with sonication, and the resulting suspension applied to 510°C Curie Point wires as described in Chapter 2, Section 2.9. Four replicates of each strain were analysed, each with a total ion count in the 90 000 - 250 000 range.

After inspection of pyrograms and mass data 182 mass values were chosen for analysis i.e. m/z = 28, 30-32, 34-43, 46-170, 174, 176-184, 186, 192-200, 2-3, 206-208, 210-211, 214, 220-225, 229, 231, 243-244, 254-259. The analysis was carried out using 15 Principal components Genstat programme *Lloyd* being used for data analysis. This programme enables clustering of strains by average linkage clustering after principal components and canonical variates analysis have been carried out (Chapter 2, Section 13.4).

The computer print out includes the range of values for each variate (m/z). For AN1 strains, m/z = 31 and m/z = 34 were noted to have extremely large values for most samples. These two variables were therefore removed from the data file before the total analysis of all archaebacterial samples was carried out.

4.6.1.4 PY-MS Taxonomy of AN1

A dendrogram illustrating the similarities between the strains of AN1-like bacteria is given in Figure 4.5. The following trends can be seen.

1. There are three distinct groupings of strains (and one outlier). Two of these groups appear to be due to intrinsic differences between

strains, the third is due to a change in culture conditions.

2. Three of the four strains grown on cystine instead of sulphur (see Section 4.6.2.1) cluster differently - this could be interpreted as evidence supporting the theory of a different mode of utilisation. (Section 4.4, I (f)).

3. Changing the growth temperature also affected cluster position. Temperature-induced changes in cell composition have been reported for *Thermus* strains (Ray *et al.*, 1971a,b) and (Oshima *et al.*, 1976). It has also been reported that different temperature strains of *Sulfolobus* have different lipid compositions with strains of higher temperature optima having a greater proportion of their C40 lipids in the cyclic form (Bu'lock, 1983). Reports of changes for the same strain grown at different temperatures have not appeared in the literature to date. As one of the two strains grown at 85^{o}C was found to be an outlier no definite conclusions can be drawn from the results.

4. One replicate of isolate Wis 4 was an outlier and was removed from the data set before all archaebacterial strains were computed together. (See P.C. plot - Fig. 4.6).

As all archaebacterial strains were to be computed together in an attempt to obtain a phylogenic pattern, the outlying strain was not removed from the data set in order to determine whether such outliers would re-classify with other groups.

The high degree of similarity between the two groups of AN1 strains (linkage at 90% similarity as shown in Fig. 4.5) probably precludes their classification as different species, but the compression effect of the outlier seriously affects the % similarity (MacFie and Gutteridge, 1982).

Figure 4-5

Dendrogram of AN1 – like Strains

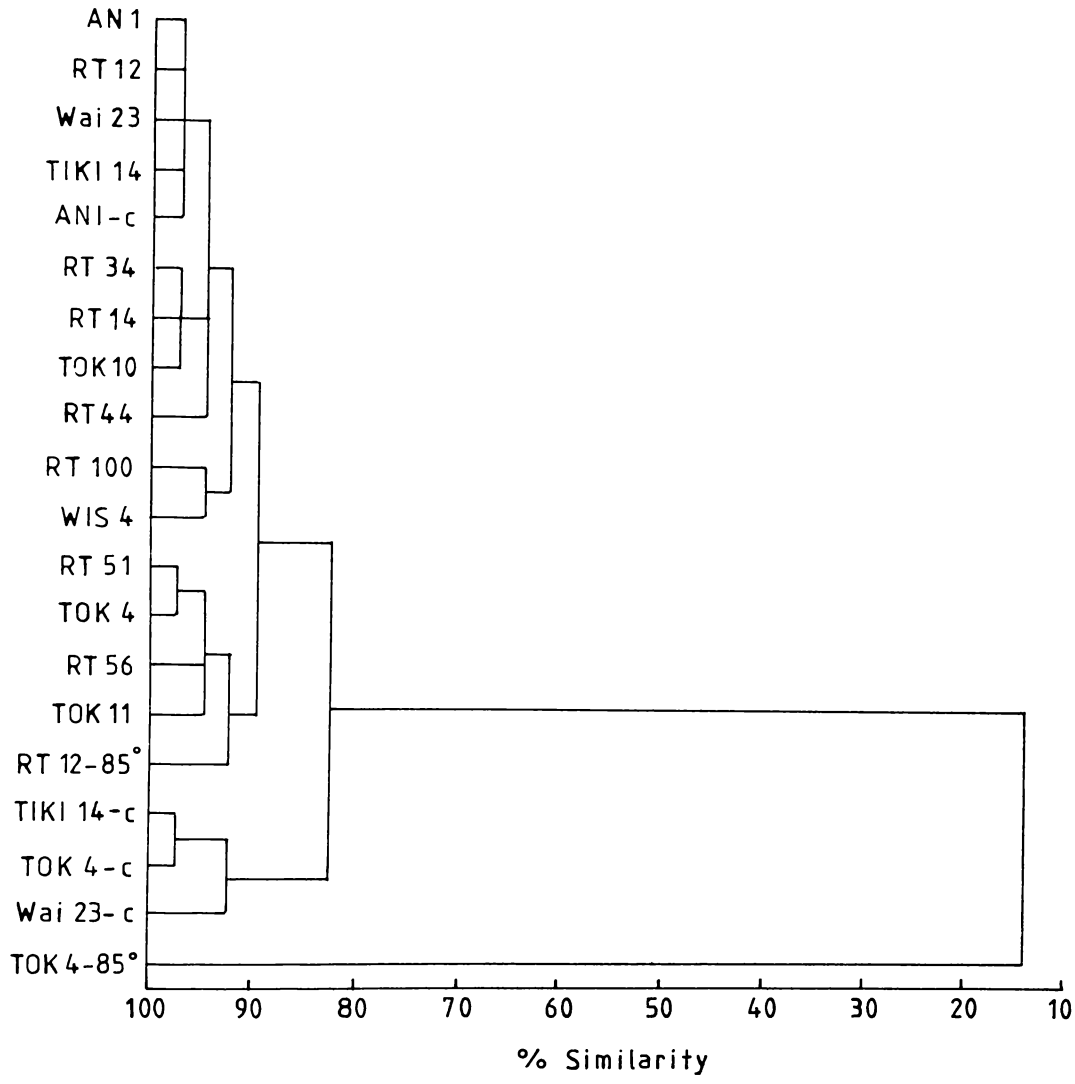


Figure 4.5 Dendrogram of AN1-like strains grown under three different conditions.

-c refers to cultures grown on cystine as terminal electron acceptor.

-85°C refers to cultures grown on low organics at 85°C.

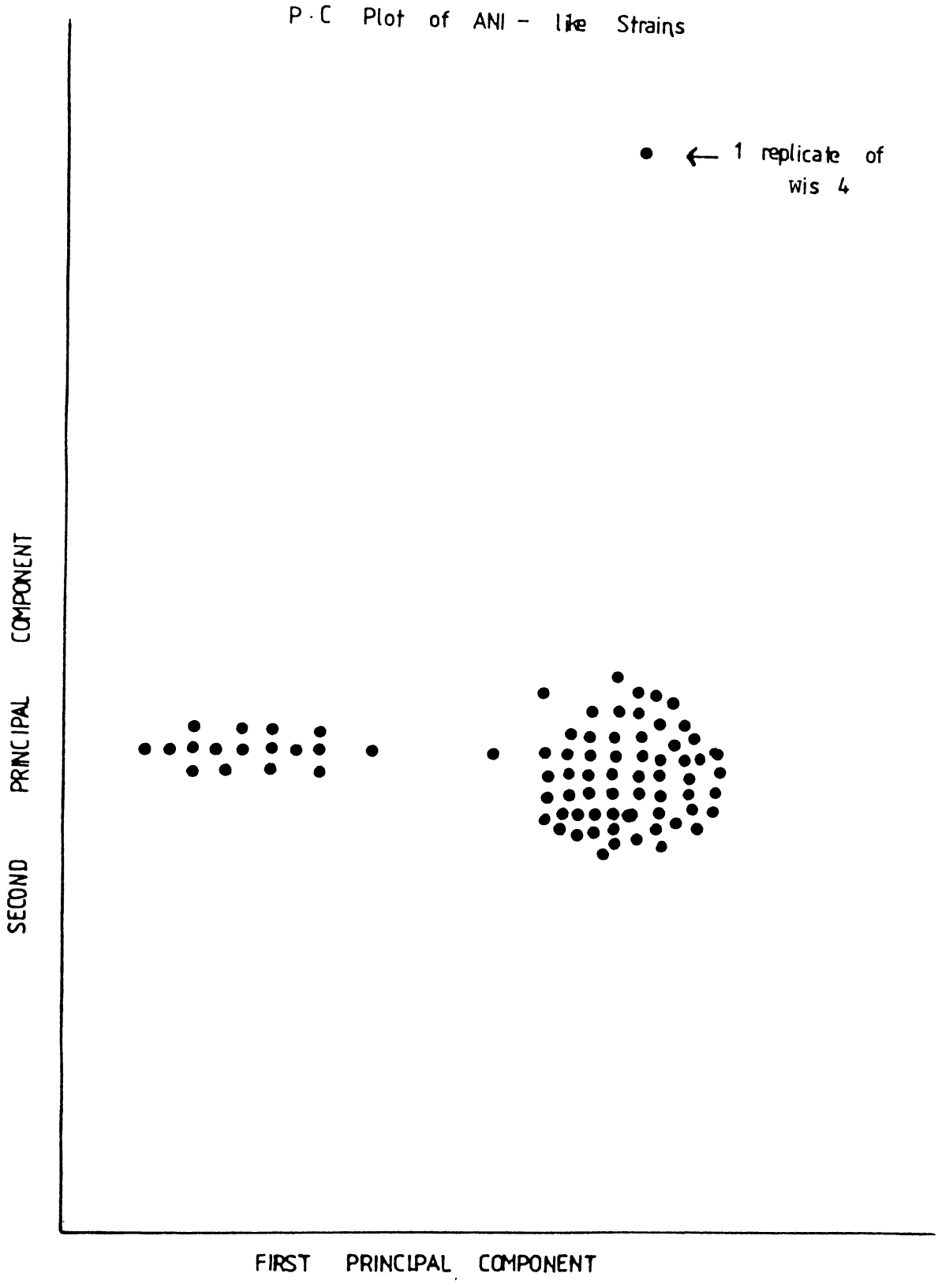


Figure 4.6 Principal Components Plot of ANl-like strains. An outlying replicate of strain Wis 4 is arrowed in the diagram. The spectrum relating to this point was subsequently removed from the data set.

Compression by outliers has a lesser effect on Mahalanobis' D^2 values e.g., strains RT 34 and RT 56 cluster at a 95.5% similarity level which does not seem a significant difference. However, the Mahalanobis' D^2 value for these two strains is 13.6. D^2 values of >6 are considered to indicate a significant difference between pairs of strains in these analyses, with differences >12 considered to be highly significant (Shute *et al.*, 1984). It can be concluded therefore that definite differences, at least at the sub-species level, are revealed by this analysis. Further work using different taxonomic techniques would be of interest to confirm this.

4.6.1.5 The Influence of Sulphur on Mass Spectra

It was noted on examination of pyrograms drawn on the line printer that unusually high counts were recorded for m/z = 256 for all samples, with detectable counts also recorded for satellite ions m/z = 255 and 257. Mass spectra from bacterial samples have not previously shown detectable counts for fragments of such high molecular weight and it was assumed these were due to the cyclic ether-linked lipids of this group of archaebacteria being rather resistant to pyrolysis. On consultation with J. Boon, F.O.M. Institute, Amsterdam - (C. Gutteridge, pers. comm.) it was decided that these were masses derived from polymerisation of sulphur. Inspection of raw mass values revealed high ion counts for the whole sulphur series from sulphur itself, S^O (m/z = 32) to S^8 i.e. (m/z = 256) for all samples grown on sulphur as electron acceptor, far smaller values being recorded for the sample grown on cystine. It seems probable that these ions may be derived from elemental sulphur used in the medium rather than from the bacteria themselves, so it would be desirable to remove the whole sulphur series from the file and recompute the data.

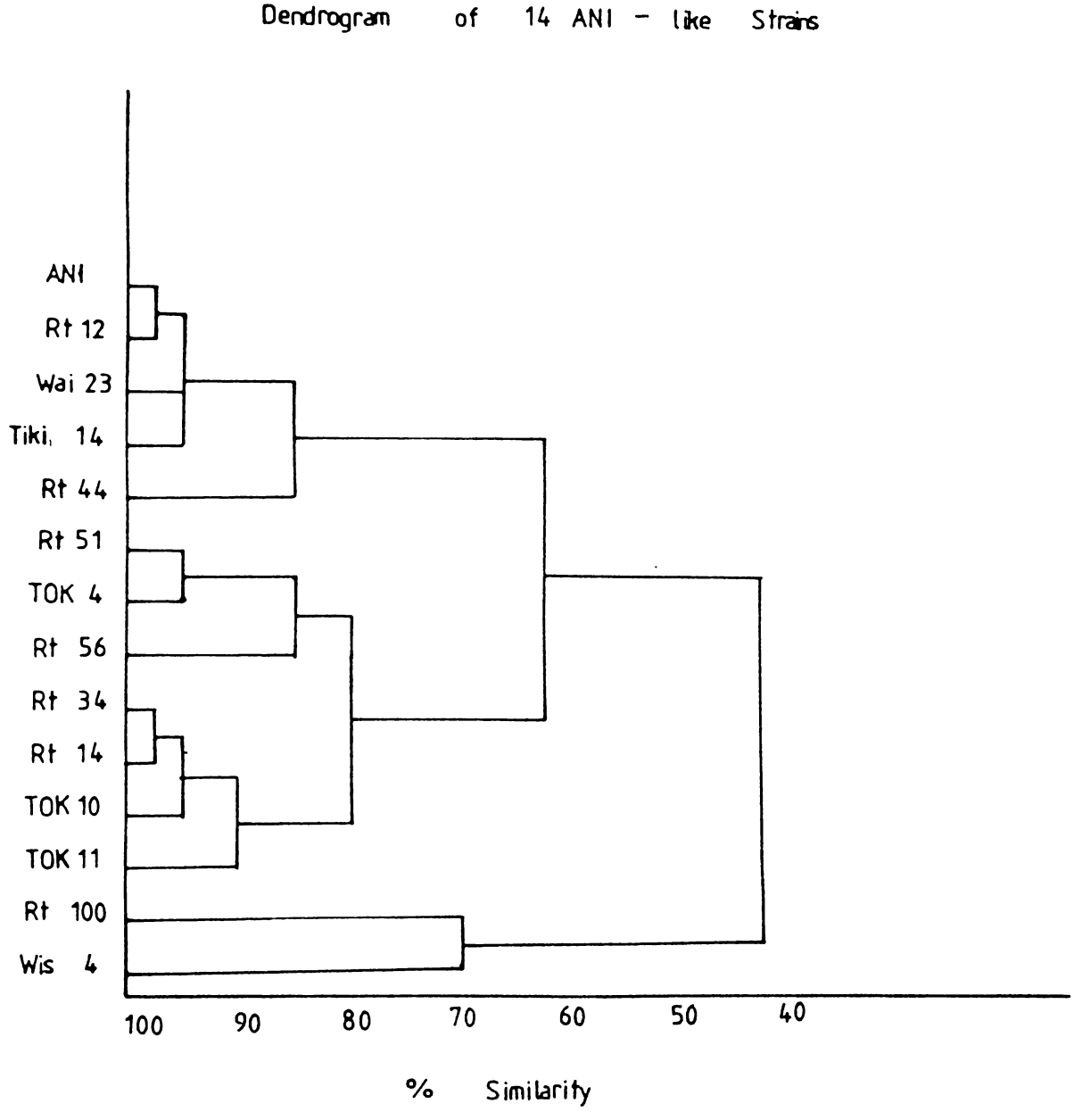


Figure 4.7 Dendrogram showing the relationships between 14 ANI-like strains grown under identical conditions. There appear to be 3 distinct groups, possibly sub-species groupings with 2 strains, Rt 100 and Wis 4 possibly members of a separate species group.

4.6.1.6 Data Processing of AN1 Strains After Removal of the Sulphur Series

As changing the growth conditions by altering the terminal electron acceptor or the temperature had affected the spectra produced, it was decided to carry out further analyses on only those samples grown at 75°C on sulphur.

A new format statement was written to exclude the molecular ions of all the sulphur series i.e. n32 ($1 < n < 9$). 183 m/z values were initially chosen but it was found that m/z 31 and m/z 43 were too large (maximum value 20, 093 and 11,508 respectively) so 181 m/z values were used, which were: m/z 15, 16, 19, 20, 22, 25-30, 33, 35-42, 45-63, 65-95, 97-127, 129-159, 161-173, 175-189, 191, 193-197, 199-201, 203-205, 207-209, 211, 220-223.

Ten P.C.O's were used initially but as 6 P.C.O's. described 95% of the variance it was decided this would be a more satisfactory number as the use of too many P.C.O's emphasises intra-group variance and presents maximum expression of intergroup variance.

The outlying replicate of Wis 4 (arrowed in Figure 4.6) was eliminated leaving 14 groups of 55 samples.

Figure 4.7 is a dendrogram of AN1 clusters formed. Using the criteria determined for *Thermus* strains (Section 3.5.3) i.e. groupings of three or more strains can be considered a significant type-cluster.
There are three distinct types of AN1 strains and two strains which fail to cluster.

Group 1 includes the "type" strain AN1, TK 14, Wai 23, RT 12 and RT 44. Although the dendrogram shows that all three groups are distinctly different, the difference between Groups 1 and 2 is probably

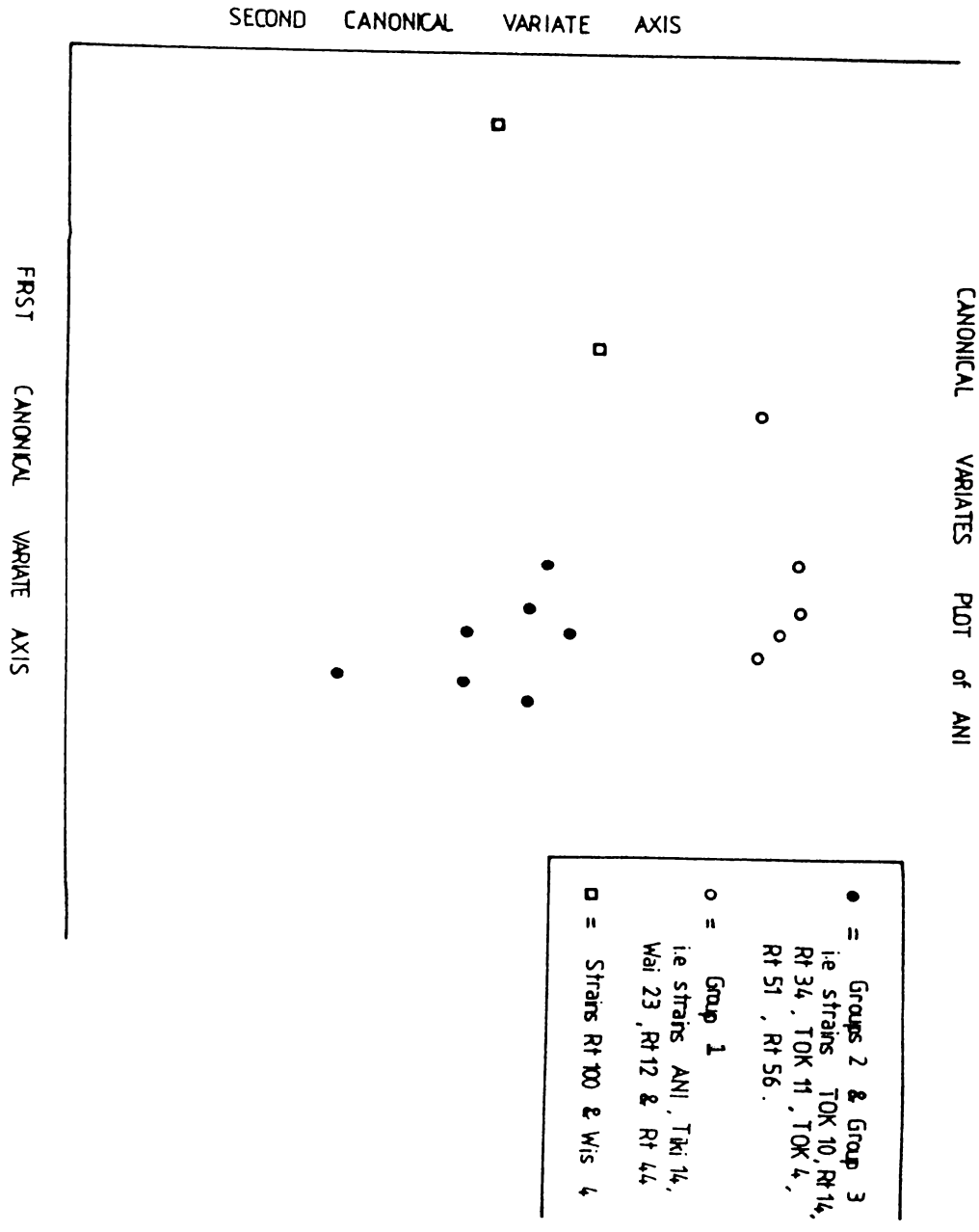


Figure 4.8 Canonical variates plot showing the relationship of 14 ANI-like strains to the first 2 canonical axes. The plot indicates Groups 2 and 3 may be more similar to each other than to Group 1. This could be interpreted as suggesting that Group 1 is also a separate species group.

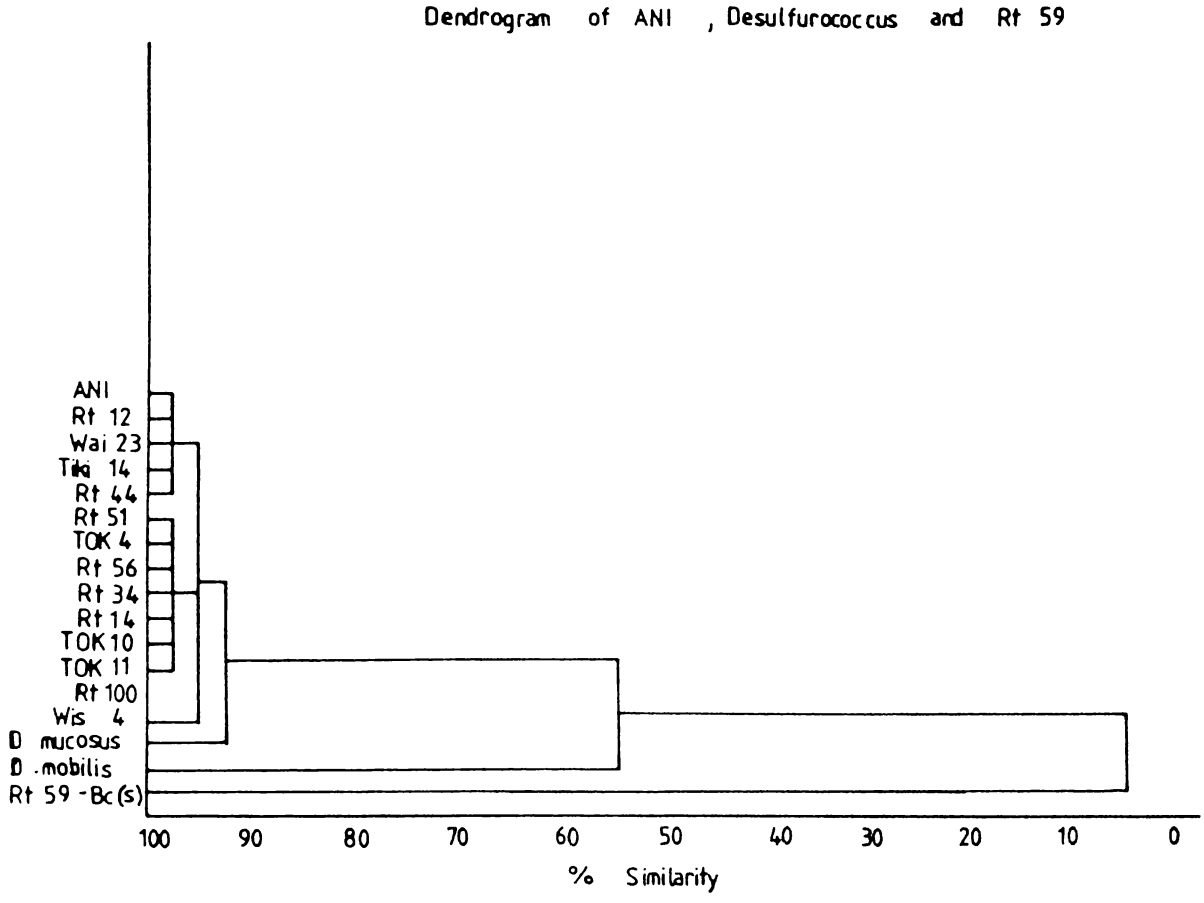


Figure 4.9 Dendrogram showing the relationship between the ANI-like strains, the two *Desulfurococcus* type strains and isolate Rt 59-Bc(s). In spite of the apparent similarity between the ANI-like strains and *D. mucosus* the D^2 value indicates a significant difference. There is no significant relationship between the ANI-like strains and the 3 species analysed.

only at a sub-species level; D^2 between RT 44 and RT 51 is 8.1 and the plot of these strains relative to the first two canonical axes (Figure 4.8) suggests these two groups are varieties of the AN1 species type. However members of Group 3 may belong to a different species (D^2 between RT 56 and RT 34 is 12.1 i.e. highly significant). It is also probable that RT 100 and Wis 4 belong to a separate species grouping. These strains warrant further investigation.

4.6.1.7 Comparison of AN1 with other Cocci

It was decided to compute data relating to AN1 together with that relating to *Thermococcus celer*, the obligate salt-requiring archaebacterium isolated by Zillig (Zillig *et al.*, 1983b). The same conditions i.e. 181 m/z values and 6 P.C.O's were used. There was no relationship between *T. celer* and any of the AN1 strains. *T. celer* joined the dendrogram at 12.5% similarity and D^2 ranged from 72.9 - 80.1.

The AN1 strains were also computed with *Desulfurococcus mobilis*, *D. mucosus* and the coccus isolated by P. Jasperse-Herst (M. Phil. Thesis, 1984), strain RT 59 - Bc(s). There was no significant relationship between AN1 and any of these strains. Although it appears in the dendogram relating to this analysis (Figure 4.9) that *D. mucosus* is similar to AN1, the D^2 values are all >12, and even this is undoubtedly affected by the compression caused by the other two cocci.

It seems that AN1 strains form a unique group which are almost certainly different from all sulphur-metabolising cocci isolated to date. The inter-relationships between the AN1 strains do not seem to be related to site location or parameters measured to date. It is hoped to undertake a thorough investigation of sodium levels of these pools in the future.

4.6.2 *Thermoproteales*

4.6.2.1 Isolation of *Thermoproteus*-like Strains

Samples were collected both as described for *Thermus* (Appendix I) and also anaerobically (Appendix II, Section 1). Enrichments were set up using the modified Hungate technique described in Appendix II, Figure II.1 with either nitrogen or 95% nitrogen plus 5% H_2 or an 80%/20% mixture of H_2/CO_2 as gas phase. 100 µg ml^{-1} Vancomycin (an antibiotic known to be stable under hot acid conditions (Brock, 1978) was added to all enrichments. 1 ml of pool water and sediments were added to 10 mls of 'A' medium (Zillig *et al.*, 1981) designed by Zillig for the isolation of *T. tenax* for Icelandic hot springs; all incubations were at 85^{o} without agitation.

Nineteen enrichments were set up as just described, aliquots were removed daily, using a sterile syringe, and examined by phase contrast microscopy for the presence of bacterial cells. After 7-10 days incubation, a few cells both rod and coccoid in shape appeared in 7 of the 19 enrichments (see Table II.2, Appendix II). Although rod-shaped objects were clearly bacteria, coccoid shapes were more difficult to determine as the presence of both sulphur granules and sediment particles prevented unambiguous identification of "round" bodies. Attempts were made to fix and stain the cells which were successful for rods but not for cocci.

All apparently positive samples were subcultured (using 5% inocula) into the same medium and grown under the same conditions. There were no cells of either type present in any of these subcultures suggesting that medium 'A', based on the mineral composition of Icelandic hot springs and of extremely low phosphate concentration (10^{-4}) was not suited for the growth of New Zealand strains.

It was decided to attempt isolations using the salt base of the

medium used by Brock (Brock *et al.*, 1972) for the culture of *Sulfolobus* species (this has a 3.2 mM phosphate concentration). Other additions were as for medium 'A' and the resulting medium, named medium 'B', (Appendix II, Section 8) was used in a further screening attempt. Fifty-eight samples were set up as described for enrichments using medium 'A', and a few cells, both rods and cocci, were detected in thirteen of these after 7-10 days incubation (Table II.3). All enrichments which appeared to be positive were subcultured into medium 'B' but again no further signs of growth were detected. It seemed probable that glucose, used as the sole carbon source in both medium 'A' and medium 'B', could not support growth of New Zealand strains. This was later confirmed by B. Patel (Ph.D. Thesis, 1984) using ^{14}C labelled glucose.

The presence of a few cells in the primary enrichments just described, strongly indicated the existence of *T. tenax* or a very similar species in New Zealand hot springs, therefore it was essential to attempt further modifications to media to obtain satisfactory cultures. A medium which had been designed for the isolation and growth of *Desulfurococcus* species (Zillig *et al.*, 1982b) was then used (Appendix II, Section 8.c). This was named medium Db and used for a further screening experiment. Fifty samples were added to Db medium at pH = 5.5 using the same conditions of gas phase and incubation as previously described. After 5-10 days there was good growth in most cases (Appendix II, Table 11.4) with 5-10 cells per field being seen (under phase contrast microscopy at x1000). Cultures were not visibly turbid at this stage. In fourteen enrichments both rods and cocci were observed, five containing only rods and seven only cocci.

It was observed during electron microscopic examination of pool flora (B. Patel, D.Phil, Thesis, 1984) that populations of *T. tenax*-

like cells were present in pools of neutral to slightly alkaline pH with temperatures close to boiling. Twenty-eight samples collected from pools with these properties were added to Db medium at pH = 7.0, with 50 μg ml^{-1} of penicillin G as antibiotic, all other conditions being as described for pH = 5.5 enrichments. Eighteen positive cultures were obtained (see Appendix II, Table II.5), all but two containing both cell types.

4.6.2.2 Purification of *Thermoproteus tenax*-like Strains

Having confirmed beyond doubt the presence of both *T. tenax*-like and *Desulfurococcus*-like strains in New Zealand hot springs it was now essential to obtain pure cultures for analysis.

The following methods were used in an attempt to obtain pure cultures using four mixed cultures which consistently grew with a fast growth rate.

1. Dilution series were set up, using Db medium containing 10 μg ml^{-1} rifampicin. Although there was good growth (20 cells/field under phase contrast) when undiluted cultures were used as inocula there was no growth in dilutions of 10^{-4} or less. In most cases both thick and thin rods (thought to be *T. tenax* and *T. pendens*) and cocci were still present therefore other methods of purification were tried.

2. Attempts were made to obtain colonies on plates of solid medium using gelling agents and conditions as set out in Appendix II, Section 9 because agar cannot be used at these temperatures. Oxygen contamination (as detected by resazurin indicator) was a continual problem in these experiments although all manipulations were carried out under a stream of N_2 gas, and later CO_2. (Reported successes of growth of these species on plates have all involved the use of an anaerobic chamber, unavailable for these experiments). After numerous attempts in which no sign of colonies were detected it was decided to try other

methods of purification.

3. The agar "shake" method used for the isolation of oxygen-sensitive photosynthetic bacteria (Pfennig and Trueper, 1981) was tried in an attempt to set up a satisfactory anaerobic gradient. Although agar melts on incubation at 85°C it was found that a solid core remained after 15 hours, which was sufficient for appreciable growth of these four cultures in liquid medium. However, no sign of colonies was detected. To allow a longer period for production of colonies, tubes were incubated at a range of temperatures; 75°C, 77.5°C, 82.5°C and 80°C, but although a solid core of agar remained for several days at 75°C, there was never any sign of colonies in any tube so these attempts were discontinued.

It was later suggested (W. Zillig, pers. comm.) that agar is toxic to these organisms.

4. It has been reported (Zillig *et al.*, 1981) that malate can replace sulphur as an electron acceptor for respiration varying concentrations of malate and yeast extract were added to Db medium but no growth occurred.

Under different conditions, growth on malate has been confirmed (P. Jasperse-Herst, M.Phil. Thesis, 1984).

5. Autotrophic growth has been reported for many strains of *T. tenax* (Fischer *et al.*, 1983). This was attempted using the salt base of medium Db, sulphur and an 80%/20% H_2/CO_2 gas phase at pressures of both 1 atmosphere and 2 atmospheres (achieved by injection of an equal volume of gas). There was no growth, which was probably due to leakage of hydrogen from the Hungate seals after incubation at 85°C as pressures of 2 atmospheres were not maintained, therefore this approach was discontinued.

6. Attempts were made to selectively lyse cocci using either 100 $\mu g\ ml^{-1}$ rifampicin (Zillig *et al.*, 1982b) or 0.05% Triton-X100 in Db medium. In most cases cocci survived two transfers under these conditions so the method was abandoned.

7. Yeast extract at >1% concentration was reported to inhibit growth of *T. tenax* (Zillig *et al.*, 1981). Yeast extract at this concentration was added to Db medium but both rods and cocci grew vigorously and this method was abandoned.

8. It was observed by B. Patel when attempting enrichments of pool water at 98°C that only "thick" rods of the *T. tenax* type were found in cultures obtained. It appeared that both *T. pendens* and *Desulfurococcus* species were eliminated at this temperature. Cultures were set up in Db medium at pH = 5.5 and pH = 7.0 under these conditions. After two successive transfers at 98°C, followed by serial dilution, cultures were deemed sufficiently pure for mass spectrometry.

4.6.2.3. Preparation of *Thermoproteus*-like Strains for PY-MS

For discrimination by PY-MS, conditions of preparation of bacterial samples must be rigorously standardised. It was therefore decided to attempt to find a single pH level which would support the growth of strains currently grown at either pH = 5.5 or pH = 7.0. Two strains from pools of pH <5 isolated at pH = 5.5 and two strains from pools of pH >7 isolated at pH = 7.0 were grown over the pH range pH = 3-9. It was found good growth occurred in all four strains at pH = 6 - 6.5. All strains were subsequently grown at pH = 6.5. Cultures were harvested when cell density was 15-20 cells per field (counted over 10 fields under phase contrast at x1000 magnification).

Time permitted preparation of only a limited number of strains so thirteen strains from a range of thermal areas were grown in 400 ml quantities and harvested as described in Appendix II, Section 12. These

are set out in Table 4.4.

In addition a culture of *T. tenax* (the kind gift of Professor W. Zillig, M.P.I. Martinsried) was prepared under identical conditions.

This same *T. tenax* culture was also grown on medium 'A' (Zillig *et al.*, 1981) and also autotrophically, as described in Section 4.6.4.2 (5). Cells were harvested as for other *T. tenax*-like strains.

4.6.2.4 Isolation of Desulfurococcus-like Strains

Most cultures which were set up for the isolation of *Thermoproteus* also contained cocci, presumably strains of *Desulfurococcus*. In the course of isolating *Thermoproteus* strains at pH = 5.5 and pH = 7 thirteen pure or nearly pure cultures of cocci were obtained (see Tables 4 and 5, Appendix II). A further three strains of cocci were isolated by a modification to Db medium (Zillig *et al*, 1982b) in which 0.2% yeast extract was the sole carbon source; this medium was coded Medium Da. The pH used was 5.5 with other conditions identical to those used for the growth of *Thermoproteus* on medium Db (Appendix II, Section 8).

The use of high temperature had proved very successful in eliminating these cocci from mixed cultures, and as it had been reported that *Desulfurococcus* had a greater tolerance to lower temperatures than *Thermoproteus* (Zillig *et al.*, 1982b) it was decided to incubate cultures at 75^{o}C. This definitely reduced the numbers of rods in mixed cultures. However, there was still the problem of three reported *Desulfurococcus* species with the same requirements for growth which only really effective serial dilution could separate. Time was now very limited so it was decided not to attempt to prepare these strains for PY-MS analysis.

Later consultations with Professor Zillig suggested that the problems encountered in growth efficiency were related to the sterilisation of sulphur. These problems had been overcome in his laboratory

SCREENING FOR THERMOCOCCUS CELER-LIKE STRAINS

Source	Sample Code	Source pH	Source Temperature °C	Results (+ incubation temperature)
HOT WATER	HWB 1	7.0	58	- (at 75°C)
BEACH	HWB 4	6.0	58	- (at 75°C)
GREAT BARRIER ISLAND	GB 1	6.5 - 7.0	75	- (at 75°C)
FIJI	Na SS Fj 1	8.2	96.4	- (at 75°C)
	Na SS Fj 4	7.6	95.5	- (at 85°C)
	SS Fj 5	7.8	98.5	- (at 85°C)

TABLE 4.3

by the use of non-sterile sulphur. This method was then adopted at this University and has proved successful for the isolation of pure cultures of New Zealand strains (Jasperse-Herst, M.Phil. Thesis, 1984).

A culture of *Desulfurococcus*-like cocci isolated and purified by serial dilution in Db medium with non-sterile sulphur was posted to Britain at a later date and included in the PY-MS analysis.

4.6.2.5 Screening for *Thermococcus celer*

A number of samples stored in the laboratory were known to be "salty". Water and sediments from six of these were added to *T. celer* medium (Zillig *et al.*, 1983b). If the pool temperature was known to be more than 90°C, enrichments were incubated at 85°C. Samples from pools of lower temperatures were incubated at 75°C (see Table 4.3).

There was no sign of growth in any of these enrichments after two weeks incubation so attempts to isolate this marine organism were abandoned.

A culture of *Thermococcus celer* (the kind gift of Professor W. Zillig, M.P.I., Martinsried) was grown in the medium published for this species (Zillig *et al.*, 1983b) under an N_2 gas phase at 85°C

T. celer was grown for PY-MS in 2 x 400 ml quantities in 500 ml bottles as described for *T. tenax* (Appendix II, Section 12) and harvested as described for those strains, except that 4% NaCl was added to the rinsing buffer and centrifugation was as described for AN1.

Data processing of *T. celer* was done with the *Thermoproteus* strains, and later with AN1-like strains.

Thermoproteales Strains Grown for PY-MS Analysis

Sample	Source pH	Source Temperature	Comments
(1) *Thermoproteus tenax*-like Strains			
KT 1	4.0	"boiling"	
KT 8	6.0	85	
KT 10	6.0	89	
TK 6	4.7	84	
WT 1	2.1	88	
TOK 11	7.0	98	
TOK 12	6.9	97	
Wis 3	2.9	83.7	
Rt 47	3.2	100.5	Arikapakapa
Rt 50	3.1	N.A.	Arikapakapa
Rt 56	7.9	90.2	Whakarewarewa
Rt 57	7.2	97	Whakarewarewa
Rt 60	7.2	97.7	Kuirau Park
Thermoproteus tenax			grown on Db medium
Thermoproteus tenax			grown on A medium
Thermoproteus tenax			grown autotrophically
Desulfurococcus strains			
Desulfurococcus mucosus			
Desulfurococcus mobilis			
Rt 59	7.6	103	
Thermococcus celer			grown on *T. celer* medium

TABLE 4.4

4.6.2.6 PY-MS Analysis of Thermoproteales

The data processing of the strains in Table 4.4 was carried out using 15 principal components and 182 m/z values (as for the analysis of AN1 strains, Section 4.6.2.2).

Inspection of principal component plots revealed there were 4 outlying samples which were subsequently removed (before the analysis of all archaebacterial strains). These were replicates of TOK 11, RT47, *T. celer* and *T. tenax* on Db medium.

4.6.2.7 PY-MS Taxonomy of Thermoproteales Strains

A dendrogram illustrating cluster groupings of *Thermoproteales* can be seen in Figure 4.10. There appear to be two separate groups of *T. tenax*-like strains. One group, which includes the strain obtained from Professor Zillig, contains 9 strains from a number of different thermal regions. The other group which is deeply separated from the first contains 4 strains, each from a different region. There seems no obvious relationship between the source temperature and pH and these 2 clusters.

One type strain of *Desulfurococcus*, *D. mucosus*, was an outlier and has caused "compression" of the other strains in the cluster analysis. In spite of this, spectra from this strain were left in the data file for analysis of all archaebacterial strains; however they should be removed for any future analyses of the *Thermoproteales* samples.

Two anomalies can be seen in this analysis: a New Zealand isolate of *Desulfurococcus* appears to have clustered with the smaller group of *T. tenax*-like isolates. However, the Mahalanobis' D^2 distance between it and TOK 11, the nearest *T. tenax* isolate is in fact 13.06, which represents a highly significant level of difference.

The second anomaly is the extremely close clustering of the type strains of *T. tenax* grown on medium 'A' and autotrophically, in this

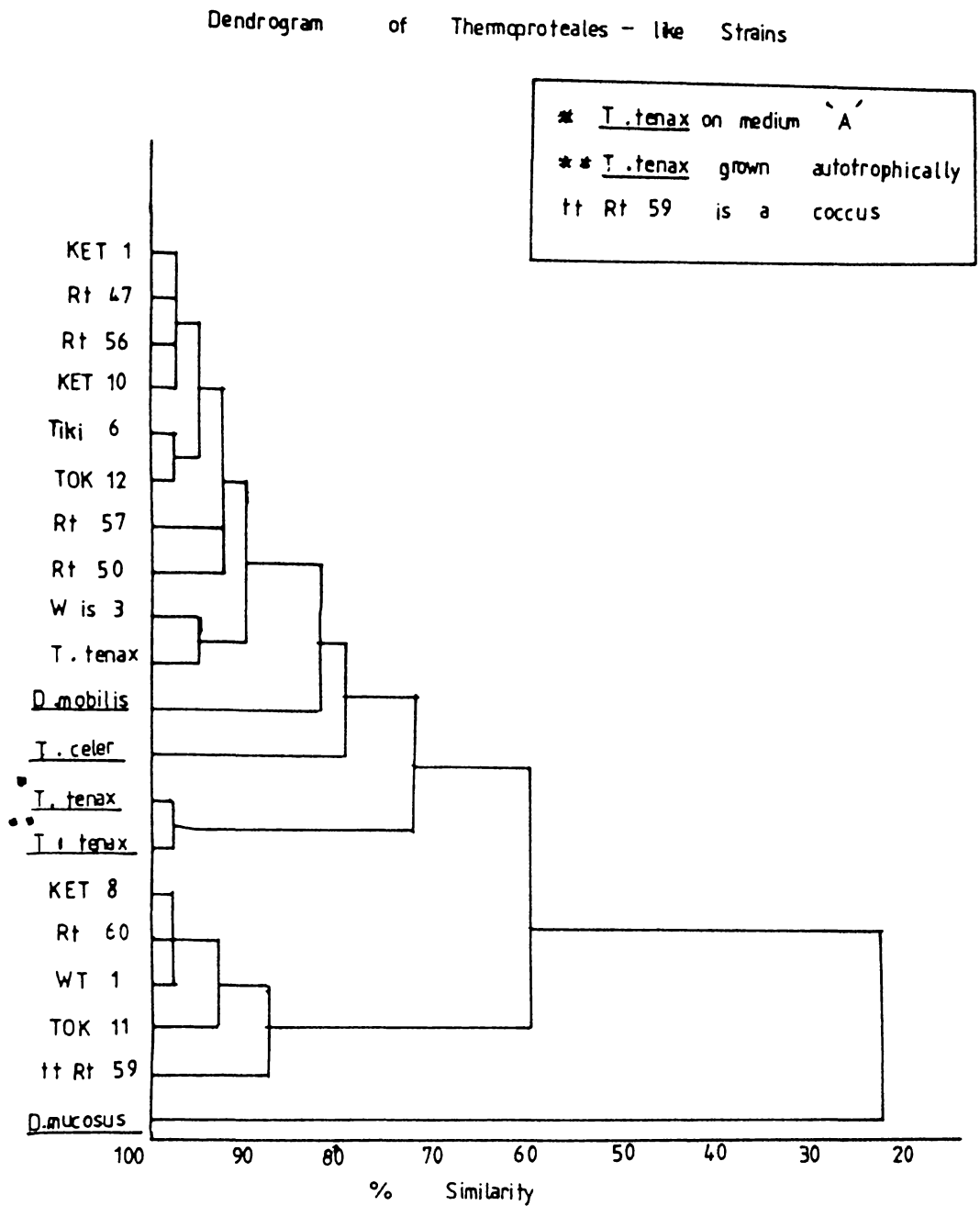


Figure 4.10 Dendrogram showing the relationships among the *Thermoproteales* strains analysed. There is clear separation of *T. celer* and the 2 *Desulfurococcus* strains from the N.Z. isolates. It appears that one group of N.Z. strains could be similar to *T. tenax* grown under the same conditions.

Note: This dendogram was based on an analysis including the sulphur series of m/z peaks.

case D^2 = 3.9. In contrast to this the sample grown on Db medium has D^2 = 16.1 and 15.7 with the other two samples of this strain. Lack of knowledge of the metabolism of these bacteria prevents any conclusions being made with regard to this observation.

4.6.2.8 Data Processing of *Thermoproteales* After Removal of Sulphur Peaks

It was decided to compute the data relating to *Thermoproteus*-like strains after removal of the sulphur series of m/z peaks. The same 181 m/s's as selected for the analysis of AN1 (Section 4.6.1.6) were used initially, but it was found necessary to eliminate m/z = 33 as it was very large (maximum value 10, 696). It was found that 12 P.C.O's were required to describe 95% of the variance. The principal components analysis run under these conditions revealed a sample of both RT 47 and *T. tenax* as outliers, so these were removed from the data set before complete data processing was carried out.

The dendrogram showing the relationship between these strains is given in Figure 4.11. It appears that the New Zealand strains are very different from the type strain *T. tenax* isolated by Zillig (Zillig *et al.*, 1981). It also appears that there are two distinct groups of New Zealand strains. This is clearly shown in Figure 4.12, the plot relating these strains to the first two canonical axes. These groupings do not appear to be related to site location or parameters measured at sampling.

4.6.2.9 Comparison of the *Thermoproteus*-like Strains with other *Thermoproteales*

The *Thermoproteus*-like strains were computed with the two *Desulfurococcus* strains isolated by Zillig (Zillig *et al.*, 1982b). The results are shown in Figure 4.13. Although it appears there is a relationship between *D. mobilis* and the New Zealand *Thermoproteus*-like strains, the D^2 values indicate this relationship is not significant, merely an artifact of the severe compression due to the high dissimilarity between *D. mucosus* and the other strains. It seems that the removal of the

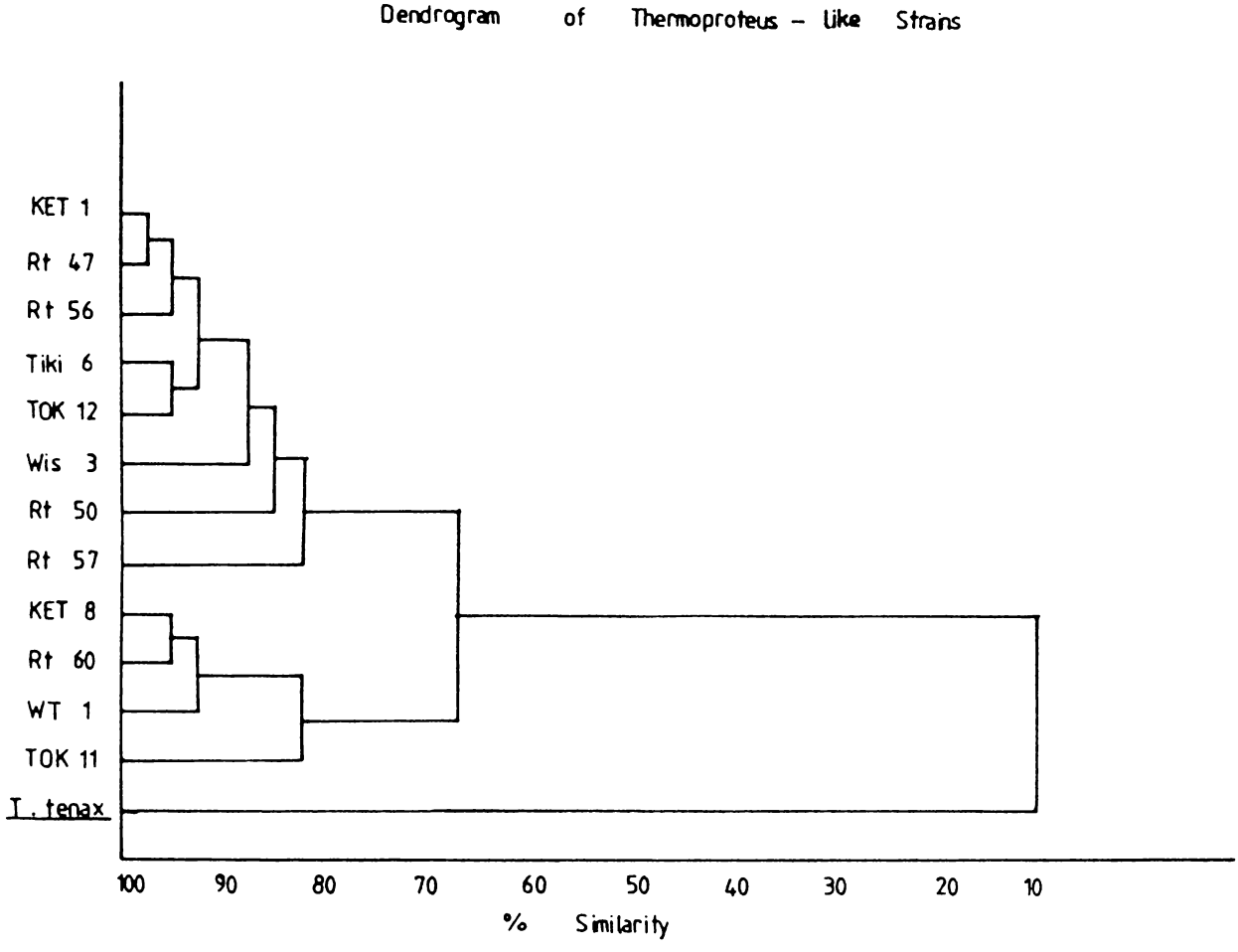


Figure 4.11 Dendrogram of *Thermoproteus*-like strains grown on Db medium. The dendrogram clearly indicates that the New Zealand isolates are different from *T. tenax* at a species level. This analysis was carried out after the sulphur series of m/z peaks were removed from each mass spectrum in the data set.

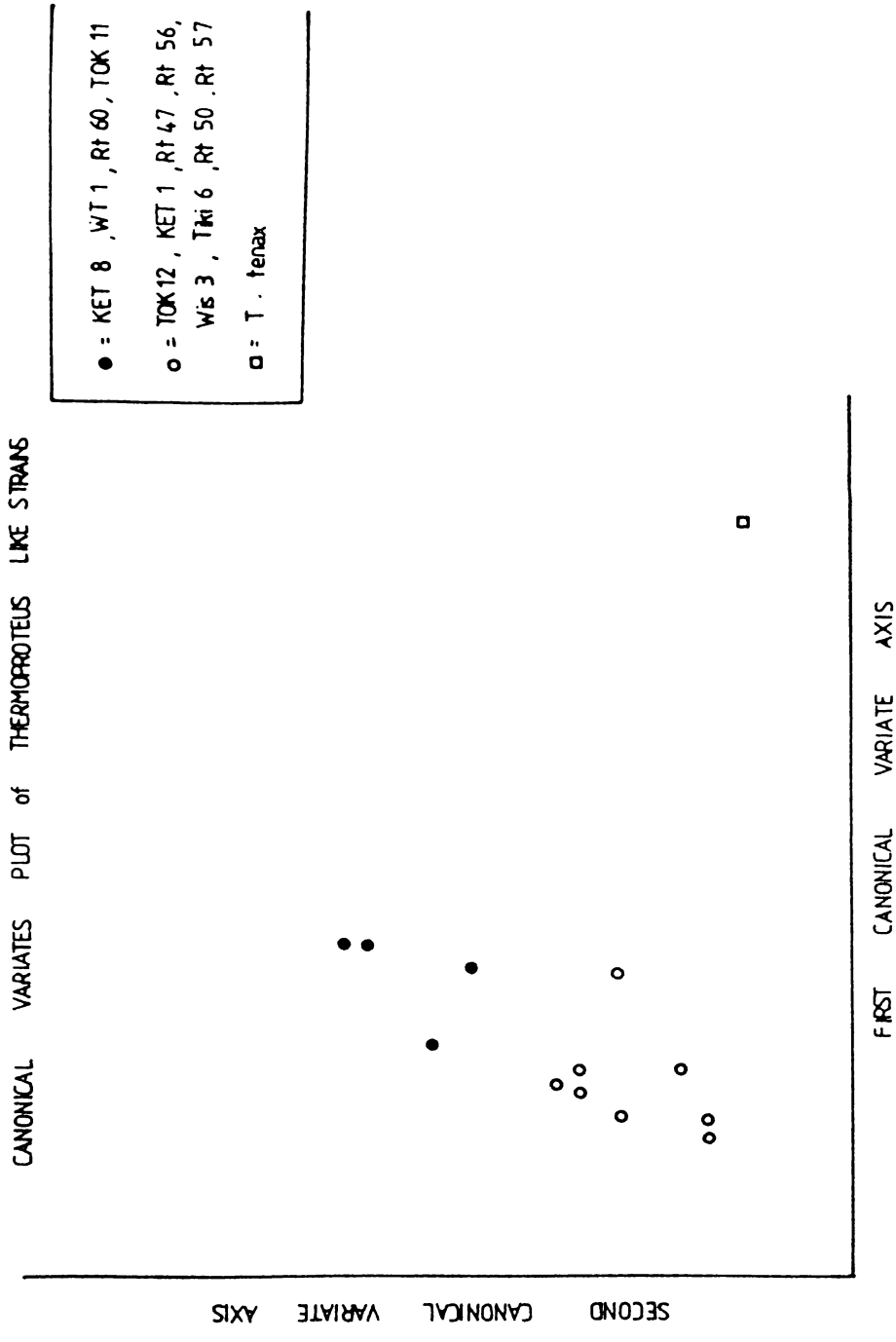


Figure 4.12 Relationship of the *Thermoproteus*-like strains grown on Db medium to the first 2 canonical axes. The diagram suggests that the 2 groups of New Zealand isolates differ from *T. tenax* at a species level but from each other at probably a sub-species level.

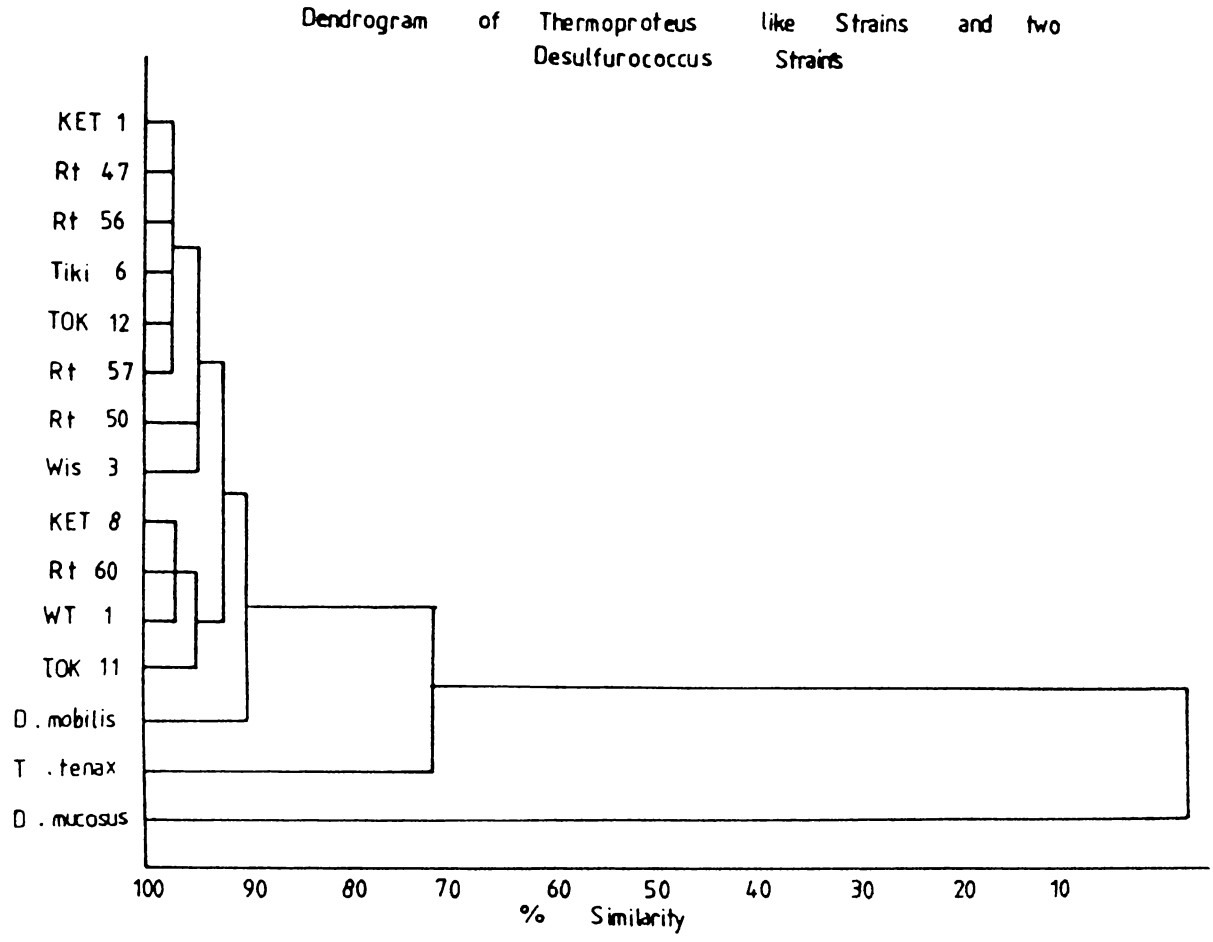


Figure 4.13 Dendrogram of four types of *Thermoprotealeas* grown under identical conditions. The difference between the N.Z. isolates and *D. mobilis* is in fact significant. This confirms the difference between N.Z. strains and the type strains which were isolated from Icelandic hot springs.

sulphur series of m/z peaks has not significantly altered the PY-MS taxonomy of most of the New Zealand isolates. This analysis has successfully discriminated the four reference species *T. tenax*, *T. ceter*, *D. mucosus* and *D. mobilis* and indicates that there may be two species groups among the New Zealand isolates. The New Zealand isolates do not appear to be closely related to any of the reference species.

The removal of the sulphur series of m/z peaks has significantly altered the relationship between *T. tenax* grown on Db medium and the New Zealand grown strains (see dendrograms 4.10 and 4.11). Although considerable care had been taken to remove all visible traces of sulphur it is obvious that traces were still present which interfered with the proper discrimination of these strains.

4.6.3 Isolation of *Sulfolobus* Strains

Seven strains were isolated from a number of different regions with source pH values ranging from 2.0 - 4.5 and temperatures from 48-100°C (Table 4.5).

1 ml of pool water was added to 10 mls of the medium used by Brock (Brock *et al.*, 1972) for the culture of *Sulfolobus*. The pH was adjusted to 2.5 and medium was dispensed as 10 mls into 28 ml McCartney bottles fitted with rubber-lined screw caps. Cultures were incubated at 70°C without agitation.

Time did not permit attempted isolations of different temperature strains or of obligate autotrophs.

4.6.3.1 Preparation of *Sulfolobus* Strains for PY-MS

Cultures were set up as 250 mls of 1 litre Erlenmeyer flasks fitted with cotton bungs and grown as 70°C on a Gallenkamp orbital incubator at 100 rpm shaking rate.

Cells were harvested after 20 hours growth by centrifugation at 4000 g (max) for 15 minutes in a Sorvall RC-5B centrifuge, fitted with a GSA rotor.

Cells were washed in the salt base of the medium at pH = 6 and resuspended in a minimum of this solution.

4.6.4 Isolation of Methanogens

Twenty-five pools of around neutral pH were screened for methane-producing bacteria. Inconclusive results were obtained which seemed to be related to problems in maintaining the necessary 2 atmospheres gas pressure in the Hungate tubes used at the temperature required for incubation.

It seemed probable that a species similar to *Methanobacterium*

TABLE 4.5 Strains of *Sulfolobus*, Methanogens and Halophiles Analysed by PY-MS

Code Name	Strain	Source pool	Source °C	Source pH
(1) *Sulfolobus* strains				
S 1	Tiki 8	Tikitere 8	80.5	3.2
S 3	KET 5	Ketetahi 5	80	3.0
S 4	RT 47	Rotorua 47	100.5	3.2
S 5	Wai 13	Waimangu 13	78	3.5
S 6	Wis 10	White Island 10	65	2.4
S 7	Wis 3	White Island 3	83.7	2.9
S 8	KET	Ketetahi	N.A.	N.A.
(2) Methanogens				
M 12	M 1	Rotorua 1	85	N.A.
M 13	M 3	Rotorua 3	70	7.2
M 14	*Methanosarcina barkeri* 227 (D.S.M. 800)			
(3) Halophiles				
H 1	*Halobacterium halobium* (D.S.M. 670)			
H 2	*Halobacterium saccharovorum* (D.S.M. 1137)			
H 3	*Halococcus* spp. M.I.R.I.N.Z. (Dr Lowe)			

thermoautotrophicum was present in thirteen of the samples tested, but because of the seal leakage problems exact determination was not possible and these experiments were discontinued.

Two isolates apparently similar to *M. thermoautotrophicum* were subsequently grown under these conditions, but using new Hungate seals, and posted to Britain. These were grown autotrophically at 65°C on medium 2 (Balch *et al.*, 1979) under 2 atmospheres of 80%/20% H_2/CO_2. These isolates were both obtained from Rotorua hot pools located in Kuirau Park:

(a) Rt 1 is a pool which has now dried up but which, when sampled, had a temperature of 85°C and a pH of 6.5 (the latter taken in the laboratory after storage for 12 months).

(b) Rt 3 which has a temperature of 70°C and pH = 7.2.

A culture of *Methanosarcina barkeri* - Strain 227 (D.S.M. 800) was grown heterotrophically under a N_2 gas phase in medium 1 (Balch *et al.*, 1979), with an incubation temperature of 37°C.

4.6.4.1 Preparation of Methanogens for PY-MS

All methanogens were grown as 10 x 10 ml cultures in the appropriate medium as described above. 20 ml Hungate tubes were used and the techniques for preparation of media and inoculation of samples were the modified Hungate method described in Appendix II, Figure II.1. Cells were grown to early stationary phase and harvested by centrifugation for 15 minutes at 3000 g (max) in a Sorvall centrifuge, SS34 rotor. Cells were rinsed twice in distilled water and resuspended in a minimum of distilled water prior to freeze-drying.

4.6.5 Preparation of Halophiles for PY-MS

Two species of *Halobacteria* were grown in medium HI (Appendix II, Section 13). These were *Halobacterium halobium*

(D.S.M. 670) and *Halobacterium saccharovorum* (D.S.M. 1137). A culture of an uncharacterised *Halococcus* species (see 4.4 Section III, 3) was grown on the same medium solidified with 2% agar.

Halobacterium species were grown at 37°C without agitation as 500 mls in 1.5 l Erlenmeyer flasks fitted with cotton bungs. Cells were harvested at early stationary phase by centrifugation at 4000 g for 15 minutes in a Sorvall centrifuge, Model GSA rotor.

The *Halococcus* species was grown on agar plates. A single colony was cross-hatched streaked onto solid medium and incubated for several days at 37°C until colonies were clearly visible. Colonies were removed from plates using 0.1M Tris-HCL + 25% NaCl at pH = 7 , and cells rinsed and harvested using this buffer and the method described for *Thermus* strains (Appendix I).

Rinsing and resuspending of *Halobacterial* strains were carried out in 0.1M Tris-HCl + 25% NaCl at pH = 7.

These strains could not be freeze-dried so were left as liquid suspensions, autoclaved, transported and stored at room temperature

4.6.6 PY-MS Analysis of Sulfolobus, Methanogens and Halophiles

These groups were analysed together, both to check for outlying replicates and also to investigate the ability of PY-MS to detect the underlying phylogeny when a representative from each branch of the archaebacterial kingdom was included in the data set. Conditions of analysis were the same as those used for the preliminary analysis of AN1 and *Thermoproteales* strains, i.e. 182 m/z values and 15 principal components.

Although replicates from one strain of *Sulfolobus*, Wis 3, were rather scattered, no point was a real outlier and all samples were retained in the data set.

The phylogenetic groupings were not detected by the principal components analysis but reasonable discrimation occurred after canonical variates analysis was carried out. A two-dimensional plot relating the mean position of each strain to the canonical axes 2 and 3 is given in Figure 4.14.

A dendrogram based on average linkage clustering is given in Figure 4.15. Although this depicts the phylogenetic groupings reasonably well, the *Sulfolobus* strains have been divided into two groups which are separated by the *Halobacteria*. It may be significant that the second group of *Sulfolobus* strains consists of two isolates from pools of pH less than 2.5; all members of the other cluster were isolated from pools of pH higher than 2.9 but numbers of strains are not large enough for statistical validity. There seems no obvious correlation between these groupings and the source temperature.

The five *Sulfolobus* strains which group together have been clustered at more than 97% similarity but are separated by distances of D^2 ranging from 9.5 - 24.9, indicating considerable strain variation. However, values for pairs of strains from each cluster are higher than this, particularly for strain WiS 10, suggesting the two clusters differ at a deeper level.

It is of interest that although none of these strains are grown in the presence of sulphur, values for the "sulphur series" are relatively high for both *Sulfolobus* and methanogen strains. This could be interpreted as suggesting an important role for this element in the metabolism of these groups. As yet little work has been done on the

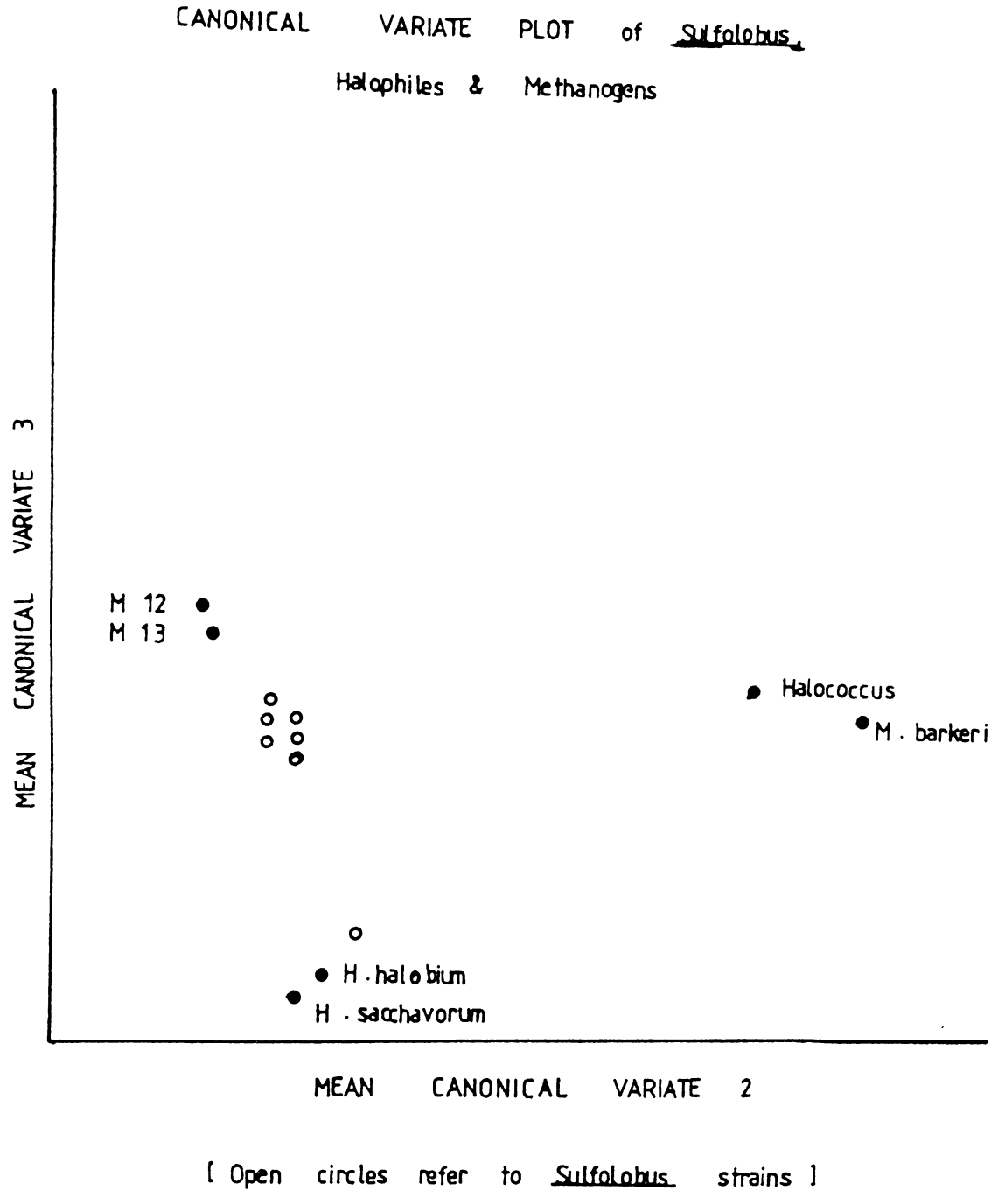


Figure 4.14 Two-dimensional scatter plot showing the relationships among members of 3 different genera of archaebacteria to the 2nd and 3rd canonical axes. There has been reasonably good strain separation at this stage.

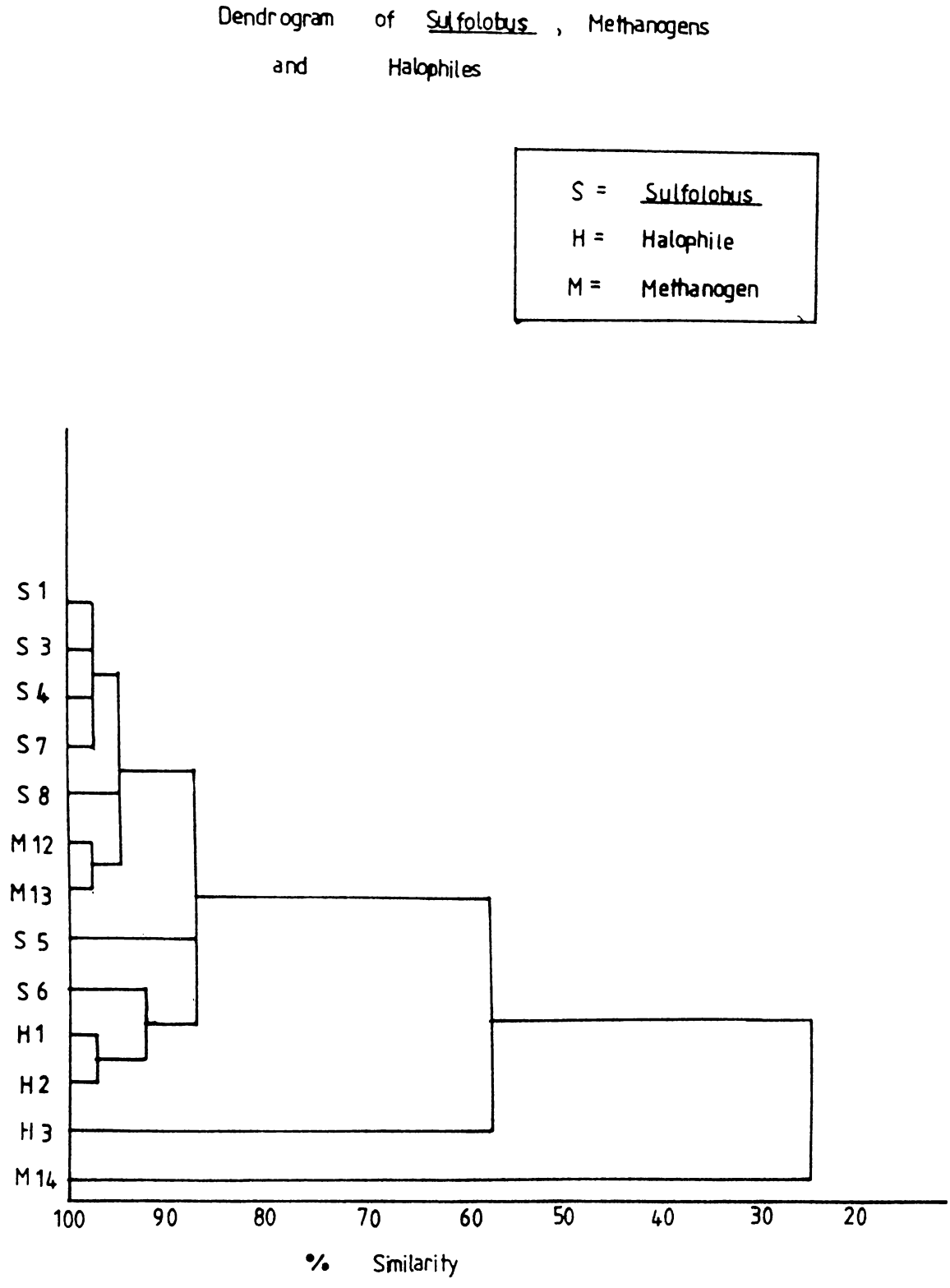


Figure 4.15 Dendrogram of 3 archaebacterial genera. The two thermophilic methanogens have clustered closely as have 4 strains of *Sulfolobus* and the 2 strains of *Halobacterium*. The remaining strains appear somewhat scattered in the dendrogram.

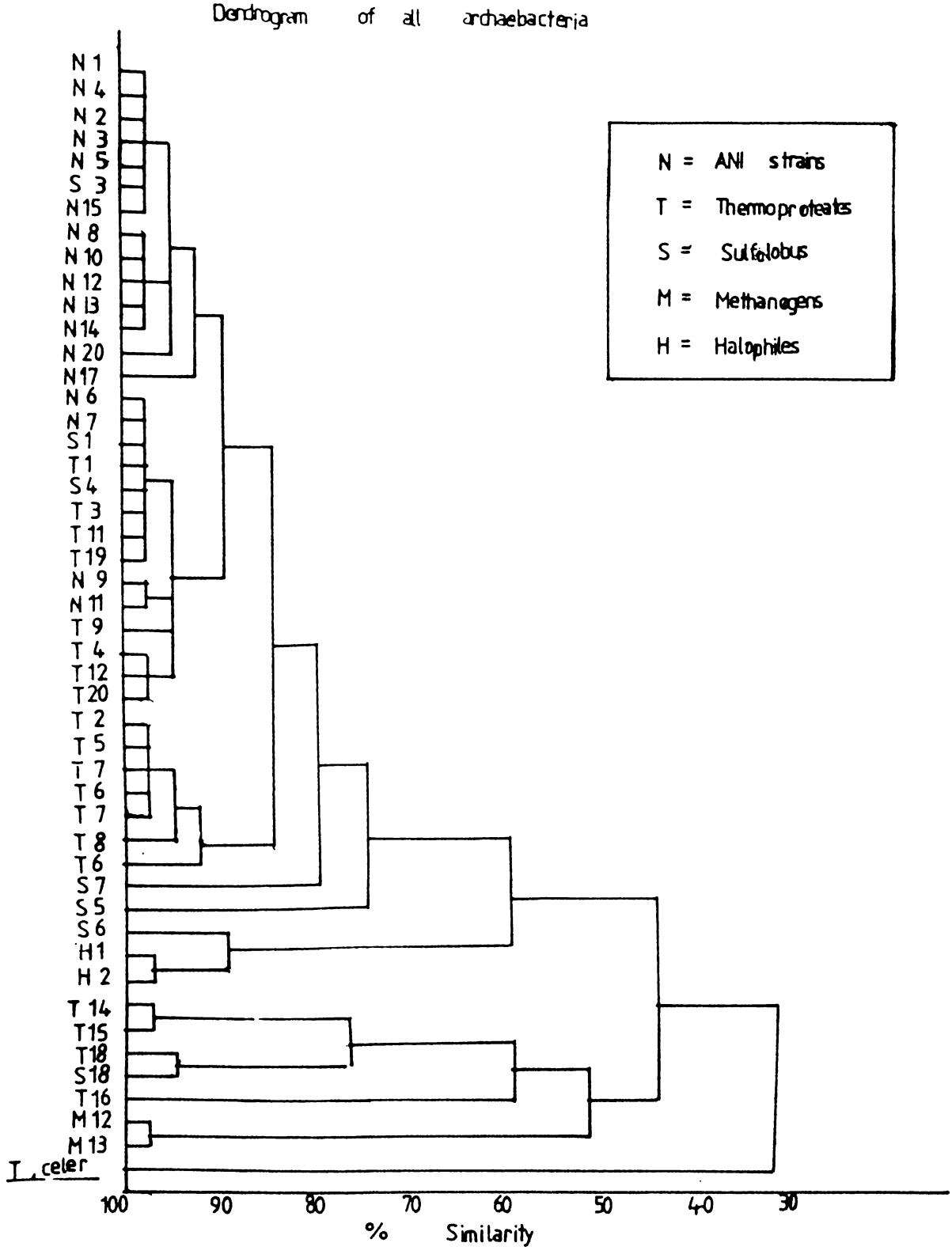


Figure 4.16 Dendrogram showing the relationships among members of 6 archaebacterial genera. There has been reasonably good separation of genera but *Sulfolobus* strains appear somewhat scattered. The results suggest that either these strains are too variable to analyse successfully together or that successful discrimination will have to await further refinements in data processing techniques.

sulphur-methabolism of archaebacteria but it has been reported that the methanogens as well as the sulphur-metabolising group can reduce elemental sulphur to hydrogen sulphide (Stetter *et al.*, 1983b).

4.6.7 PY-MS Analysis of All Archaebacterial Strains

All archaebacterial strains were computed using 180 m/z values (m/z = 31 and m/z = 34 having been removed from all spectra). By accident only 9 principal co-ordinates were used in this analysis which involved 209 samples representing 54 strains.

The plot relating the position of replicates to the first two principal axes showed *M. barkerii* to be a definite outlier while CVA plots depict both this and the *Halococcus* species as outliers. The majority of other strains are seriously compressed in the dendrogram; 32 strains including AN1-like strains, *Thermoproteales* and *Sulfolobus* all cluster at 100% similarity. This compression is so severe that Mahalanobis' D^2 values are all very small e.g. $D^2 = 5.2$ between a strain of AN1 and *Sulfolobus*

The outlying strains were removed and the data recomputed, again using 9 P.C.O's. With the exception of the *Sulfolobus* strains which appeared to be "scattered" throughout the dendrogram, illustrated in Figure 4.16 , other strains were grouped reasonably well. No really useful information is contained in the P.C.O. or C.V.A. plots derived from this analysis. The 9 principal co-ordinates used describes only 86.6% of the variance so it was decided to recompute the data using 13 principal co-ordinates which would describe 93.4%. This resulted in a far greater mixing up of strains from different genera. It seems that intra-strain variability should be minimised in analyses of this type.

Although the attempt to discriminate five genera of archaebacteria was only reasonably successful, it was of interest that there was considerable improvement when fewer P.C.O's. were used. It has been suggested (Woese, 1982) that archaebacterial genomes may not be as strictly controlled as those of eubacteria. If this is so it would be reasonable to assume that a high degree of variability of spectra would result from PY-MS analyses of archaebacterial species.

SECTION II

4.7 Screening for New Species of Archaebacteria

(1) Anaerobic archaebacteria reported to date belong to either the methanogens or the *Thermoproteales*. Members of the latter group all require media low in carbon-containing compounds (< 0.2%). It was therefore decided to attempt isolations in richer media. Variations of the medium used for the culture of caldoactive glycolytic anaerobes (Zeikus *et al.*, 1979) were tried. These variations were:

(i) Increasing yeast extract concentration from 0.3% to 0.5% or even 1.0%

(ii) Omitting glucose

(iii) Reducing trypticase peptone concentration from 1% to 0.5%

(iv) Replacing the reducing agent specified with 0.1% thioglycolic acid

All culture attempts were made with the addition of 0.5% sterile elemental sulphur (prepared as described in Appendix II) and antibiotics to prevent the growth of eubacteria.

The Orakei Korako and tourist area of the Waiotapu series of samples. (see Tables 4.6 and 4.7) were all tested. For samples of source pH of 7 or higher, media of pH 7 - 7.5 were used; for samples of acid pH, media at pH 4 was used. The antibiotics used were 10 $\mu g\ ml^{-1}$ rifampicin for neutral pH media and either 50 $\mu g\ ml^{-1}$ vancomycin or 40 $\mu g\ ml^{-1}$ amoxil for acid pH samples. Enrichments were incubated at $70^{o}C$ - $75^{o}C$, and $80^{o}C$ under a N_2 gas phase.

When vancomycin was unavailable 250 mg amoxil tablets, a form of penicillin which can be taken orally and which is therefore acid stable. were used.

There was no evidence of growth in any of these samples after 5 days, and as media had become extensively caramellised the samples were discarded.

It was also decided to attempt isolations using different gas phases. Three samples (TP9, WP19 and WP18 - see Tables 4.7 and 4.8) were set up in the medium described at pH = 5 under an 80%/20% H_2/CO_2 gas phase and incubated at 80°C and 90°C.

Five samples, the above three plus TK4a and WP1 were also set up in the same medium at both the above temperatures but in a 90%CO/10% H_2/CO_2 gas phase (the H_2/CO_2 from an 80%/20% commercial mixture).

There was no sign of growth in any of these samples after 1 week and the experiments were discontinued.

(2) It has been reported that phosphate is inhibitory for growth of some bacteria (Canale-Parola, 1973). The salt base of the medium described above contains 15mM PO_4^{2-} so it was decided to attempt isolations using a medium which contained no phosphate in the salt base, but which contained silicate (Heinen and Lauwers, 1981). Carbon sources were 0.5% glucose plus 0.5% yeast extract. Sterile sulphur (0.5%) was again added and 10 μg ml^{-1} rifampicin. The pH was 5, the gas phase N_2 and the incubation temperature was 80°C. All samples from the Waiotapu Forest Reserve area (see Table 4.9) were tested but there was no growth observed in any sample.

ORAKEI KORAKO

POOL	TEMPERATURE	pH
OK 1	80.2	4.8
OK 2	57	7.6
OK 3	81	8.6
OK 4	60	6.9
OK 5	66.5	8.6
OK 6	102.5	8.2
OK 7	98.2	6.6
OK 8	83.5	8.5
OK 9	83	6.9
OK 10	74	7.9
OK 11	96	7.4
OK 12	80	7.4
OK 13	98	7.3
OK 14	97	3.9
OK 15	95	7.0
OK 16	78	4.7
OK 17	90	7.2
OK 18	77	3.9
OK 19	80	7.6

TABLE 4.6

WAIOTAPU TOURIST AREA

POOL	TEMPERATURE	pH
WP 1	89.8	2.9
WP 2	89.9	2.9
WP 3	81.0	2.8
WP 4	86.2	2.9
WP 5	98.5	N.D.
WP 6	100.5	N.D.
WP 7	95	N.D.
WP 8	83.6	6.8
WP 9	56	2.9
WP 10	92	3.7
WP 11	76.1	3.4
WP 12	79.4	3.5
WP 13	76.4	4.5
WP 14	76.2	5.7
WP 15	57	4.3
WP 16	75.5	4.0
WP 17	81.7	4.0
WP 18	87.2	3.6
WP 19	78.6	2
WP 20	74.8	5.6
WP 21	67.7	6.6

TABLE 4.7

TAUPO

POOL	TEMPERATURE	pH
TP 4	95	N.D. (Soil sample)
TP 5	89	6.5
TP 6	78	7.3
TP 9	92	7.1
TP 10	72	6.8
TP 11	96	6.6

TABLE 4.8

WAIOTAPU FOREST RESERVE

POOL	TEMPERATURE	pH
WT 1	88	2.1
WT 3	76.8	4.0
WT 4	73.6	N.D.
WT 5	69.8	5.5
WT A	93	1.4
WT B	96	7.0
WT C	78	3.8
WT D	84	4.4
WT E	82	2.9

TABLE 4.9

(3) Autotrophic growth under different conditions was also attempted.

Using the medium designed for the cultivation of hydrogen oxidisers (Aragno and Schlegel, 1981) at pH = 6.8 and with a 5%O_2/75%H_2/20% CO_2 gas phase, sterile sulphur (0.5%) was added and the samples were incubated at 80°C. Three samples TP9, WP18 and WP10 were tested but there was no sign of growth after 1 week's incubation and the samples were discarded.

In retrospect it seems that not only was insufficient time allowed for the growth of autotrophic strains in these primary cultures, but also the leakage problems with Hungate seals at temperatures greater than 70°C precluded the chance of obtaining success with these experiments. It seems probable that a more thorough screening in future may be justified.

4.7.1 Ghostly Cocci

B. Patel had been investigating samples collected from White Island in a screening experiment for autotrophs. The hot pools from this region are extremely acidic and the scarcity of vegetation suggests autotrophs could be very important in the microbial flora. He set up enrichments using the salt base of medium DB (Appendix II, Section 7) at pH = 3.5 with 0.5% sulphur and a H_2/CO_2 (80%/20%) gas phase. These enrichments were set up in medium which had been boiled to drive off oxygen but no reducing agent had been added. Fifteen ml test tubes fitted with rubber bungs were used and the incubation temperature was 70°C. Although there was no visible turbidity there were definitely rather transparent coccoid shaped cells present, which had a "fragile" appearance, lysed on prolonged incubation or standing at room temperature, and were called "ghostly" cocci.

Isolations were set up using water and sediment samples from 21 acidic pools from a number of different regions. The conditions used by B. Patel were modified by the addition of 0.05% of sodium sulphide to the medium, which was now prepared by the modified Hungate technique described in Appendix II, figure II.1. Eighteen positive enrichments were obtained from these experiments (see Table 4.10). There was not a complete correlation with source temperature, but most positive isolates were obtained from pools below 90°C.

Positive enrichments were subcultured in the same medium containing 100 μg ml^{-1} vancomycin. It seemed highly likely that these cells were archaebacteria.

The optimum temperature for most of the strains isolated proved to be 70°C, with one strain, RT39, reaching a higher cell density at 75°C. Growth at 80°C was poor and there was no growth at 90°C.

The optimum pH for most strains was pH = 3.5 with an optimum of pH = 3 for WiSl2 and pH = 4 for WPl0.

It was found that the addition of 0.1% yeast extract led to increased growth to the point where cultures became slightly turbid.

Strains WiS9 and RT39 grown at 70°C and 75°C respectively, were purified by serial dilution in the same medium using three successive serial transfers. Their response to other antibiotics was then tested. An inhibition of 50 - 60% (estimate only) in growth was found on addition of 200 μg ml^{-1} rifampicin and a slight inhibition was noted on addition of 200 μg ml^{-1} chloramphenicol and 200 μg ml^{-1} streptomycin. With the exception of streptomycin, these results are consistent with those found for other caldoactive archaebacteria grown at acidic pH (Zillig *et al.*, 1982b) which seems to confirm the archaebacterial nature of these isolates.

ISOLATION OF "GHOSTLY" COCCI

Source	Sample Code	Source pH	Source Temperature °C	Results
ROTORUA				
Whakarewarewa	RT 30 DSIR No. 414	3.6	97	+
Kuirau Park	RT 38 DSIR No. 607	3.2	72.9	+
	RT 39 DSIR No. 666	4.0	61.8	+
Waiotapu Tourist Area	WP 1	2.9	89.8	+
	WP 2	2.9	89.8	+
	WP 10	3.7	92	+
	WP 12	3.5	79	+
	WP 13	4.5	76	+
White Island	Wis 1	2.5	52.8	+
	Wis 2	3.1	95.2	-
	Wis 3	2.9	83.7	+
	Wis 4	5.0	90-96	-
	Wis 5	3.5	64-88	+
	Wis 6	2.8	74.5	+
	Wis 7	3.2	74.5	+
	Wis 8	N.A.	100.5	-
	Wis 9	2.6	89.7	+
	Wis 10	2.4	64	+
	Wis 11	3.8	56.2	+
	Wis 12	2.9	91.4	+
	Wis 13	3.0	65	+

TABLE 4.10

An unsuccessful attempt was made to grow these cultures on solid media. The gelling agent used was silica gel (Zillig *et al.*, 1980) with both polysulphide and elemental sulphur tried as sulphur source (Appendix II, Section 8) and plates equilibrated overnight in the growth medium.

Later consultations with Professor Zillig suggested that caldoactive archaebacteria do not tolerate high ionic strength, therefore several changes of media are required during the equilibration stage, also this process should be carried out in an anaerobic chamber.

It seemed a possibility that these cells could be strains of *Sulfolobus* growing anaerobically. A culture of RT39 was set up using the salt base, sulphur and 0.1% yeast extract under aerobic conditions. There was good growth, the same cell density being reached as in a parallel culture set up anaerobically. It was therefore decided that these isolates were definitely strains of *Sulfolobus*, and as time was limited no further work was done on them.

Sometime later during the course of electron microscopic studies of archaebacteria B. Patel investigated both "ghostly cocci" and *Sulfolobus* and concluded there were structural differences between the two (Patel, 1984), although the effect of anaerobic growth on the structure of *Sulfolobus* was not determined. However, he found when a culture of *Sulfolobus* was added to medium containing sodium sulphide there was immediate lysis. Clearly these cultures warrant further investigation.

CHAPTER 5

PY-MS CLASSIFICATION OF SOME THERMOPHILIC AEROBIC SPORE-FORMERS

5.1 Introduction

The first extreme thermophile to be cultured in the laboratory was a species of *Bacillus* isolated from the River Seine (Miquel, 1888). Since this date many thermophilic *Bacillus* strains have been isolated and their taxonomy studied in some detail (Wolf and Sharp, 1981).

Early work suggested all strains capable of growing at temperatures of 65°C or higher belonged to the species *Bacillus stearothermophilus* (Smith *et al.*, 1946). However a study based on morphological and physiological characteristics of 230 strains of *B. stearothermophilus* resulted in their sub-division into three major groups (Walker and Wolf, 1971). Supporting evidence for these three groupings has also been obtained from electrophoretic patterns of esterases (Baillie and Walker, 1969), by immunological studies against spores from each group (Wolf and Sharp, 1981) and by the analysis of polar lipids (Minnikin *et al.*, 1977).

Three thermophilic aerobic sporeformers were isolated by Heinen (1971) and Heinen and Heinen (1972). These three strains were differentiated on their temperature optima, sporulation characteristics and the morphology of walls and membranes, and given separate species status as *B. caldovelox*, *B. caldotenax* and *B. caldolyticus*. When these strains were compared to *B. stearothermophilus* (Sharp *et al.*, 1980) it was found that *B. caldotenax* and *B. caldovelox* classified with the Group 1 type (of Walker and Wolf's scheme) and *B. caldolyticus* had some

characteristics from both Group 1 and Group 3. It has been proposed (Wolf and Sharp, 1981) that distinct groups within *B. stearothermophilus* should be given separate species status. These workers suggest that Group 1 organisms be designated as *B. kaustophilus* (Prickett, 1928) Group 2 is not yet named and Group 3, the largest and most variable, as *B. stearothermophilus*.

Bacillus thermocatenulatus (Golovacheva *et al.*, 1975) seems to be a separate species and the first chemolithotroph to be described, *B. schlegelii* (Schenk and Aragno, 1979) has not yet been compared to the other strains.

The *B. stearothermophilus* - type strains grow at around neutral pH but *B. acidocaldarius* (Darland and Brock, 1971) grows over the pH range 2 - 6. Strains have been reported from a number of different countries at temperatures ranging from 40°C - 75°C.

5.2 Isolation and Preparation of Aerobic *Bacillus*-like Strains growing at Neutral pH

These strains were isolated using Oshima's medium (Oshima and Imahori, 1974) at 70°C. The technique was the same as that used to isolate *Thermus* strains (see Appendix I) i.e. 0.5 ml of spring water was added to 15 mls of liquid medium in Universal bottles. Positive enrichments were usually visibly turbid within three days. Cultures were purified over single colonies on Oshima's medium solidified with 1.75% Coast Biologicals Agar (Penrose, Auckland). Three successive plate to plate transfers were carried out with cultures tested for purity by Gram staining and for the presence of spores by phase-contrast microscopy. It has been found that sporulating cultures produce quite different mass spectra and that strain differentiation is poor at this stage (Shute *et al.*, 1984). Harvest and preparation for PY-MS was as

for *Thermus* strains (Appendix I). Twenty-nine strains which were isolated from a number of different regions and two reference strains, *Bacillus caldotenax* (DSM 406) and *Bacillus caldovelox* (DSM 411) which were obtained from the Deutsche Sammlung für Mikroorganismen (Goettingen, West Germany) were analysed. A list of these strains is given in Table 5.1. Eight other reference strains, including three strains of *B. stearothermophilus* had to be omitted from this experiment as the PYROMASS 8-80 required repairs to the pumps before these were analysed. Spectra from aliquots of the same sample run before and after these repairs were not identical so direct comparisons were not possible. This problem was possibly related to ion source contamination, but time did not permit decontamination or a complete re-run of all the *Bacillus*-like strains.

One strain of *Thermus*, TOK 11 was a complete outlier on the *Thermus* principal components analysis. As many of the initial *Thermus* enrichments were contaminated with spore-formers it was decided to analyse the spectrum relating to this strain with those of the neutral *Bacillus*-like strains to check that the reason why this strain was an outlier was not due to it still being contaminated.

5.3 Pyrolysis Mass Spectrometry and Data Processing of Neutral pH *Bacillus*-like strains

The samples were prepared for PY-MS analysis in the same way as the *Thermus* strains (Chapter 3) with four replicates of each strain being analysed.

For the initial comparison of *Thermus* strain TOK 11 with the neutral *Bacillus*-like strains the regime was the same as for the analysis of the *Thermus* strains (Chapter 3). This was using 152 m/z values (m/z 35-188 exempting m/z 43 and 43) and 26 principal components. It was found that there was no significant relationship between *Thermus* TOK 11 and any of the *Bacillus*-like strains. The Mahalanobis' D^2 distances

TABLE 5.1 Strains of Neutral pH Spore-Formers Prepared for PY-MS

Code Number	Source pool	Source temperature °C	Source pH
B1	DSM 406 -	*Bacillus caldotenax*	
B2	DSM 411 -	*Bacillus caldovelox*	
B3	Waimangu 3	96	8.5
B4	Waimangu 7	67	6.5
B5	Erebus 2	N.A.	N.A.
B6	Rotorua 1	N.A.	N.A.
B7	Tokaanu 8	75	5.6
B8	Rotorua 7	N.A.	N.A.
B9	Taupo 10	72	6.8
B10	Fiji 2	N.A.	N.A.
B11	Orakei Korako 3	57	7.6
B12	Tokaanu 4	89	5.6
B13	Tokaanu 6a	97	7.0
B14	Tokaanu 7	53	6.0
B15	Tokaanu 12	97	7.0
B16	Waimangu 9	67	6.5
B17	Ketetahi 2	N.A.	N.A.
B18	Ketetahi 5	80	3.0
B19	Ketetahi 6	87	5.5
B20	Ketetahi 7	N.A.	N.A.
B21	Ketetahi 8	N.A.	N.A.
B22	Rotorua 1a	85	N.A.
B23	Rotorua 7a	91	7.3
B24	Rotorua 8	70	9.1
B25	Rotorua 9	84	8.4
B26	Waimangu 15	84	6.0
B27	Waimangu 17	80.5	8.5
B28	Rotorua 41	N.A.	N.A.
B29	Rotorua 363	N.A.	N.A.

N.A. = not available

ranged from 25.4 - 44.3 all very much greater than the value of 12 which represents a highly significant difference (Shute *et al.*, 1984).

5.3.1 Selection of m/z values and P.C.O's for Analysis

Raw mass spectra were examined to select a suitable set of m/z values for analysis. One hundred and ninety-one peaks were chosen for analysis. These were m/z 21, 26, 27, 33, 35-42, 46-51, 53-78, 80-182, 184-213, 218-224, 228-229, 265-266 and 278-280. Thirteen P.C.O's were required to describe 95% of the variance between samples.

No outliers were detected in the principal components analysis. A modification to the computer programme enabled a print-out of the variate loadings with respect to the first two principal components to be obtained. The plot for these samples is given in Figure 5.1.

Points which lie at the extremes of the graph are those which are important in determining the positions of the samples with respect to the first two principal axes. It can be seen from the plot that most of the discrimination has depended on peaks m/z 48, 56, 59, 60, 61, 75 and 76. Data relating to these peaks is not yet available for these strains but this type of information could be useful for future analyses. Two prototypes of *Neisseria gonorrhoea* were discriminated by PY-MS due to differences in a few m/z peaks. These peaks were found to have been derived from a single amino sugar and one protein peculiar to prototype II (Meuzelaar *et al.*, 1982) - see figure 2.3.

The canonical variates plots of this data suggested the majority of strains were rather similar with five strains: B19, B26, B29, B30 and B31 lying outside the group including the other 26 strains.

The dendrogram which represents the information in all 13 dimesnions revealed the 26 strains could be sub-divided into five groups with four

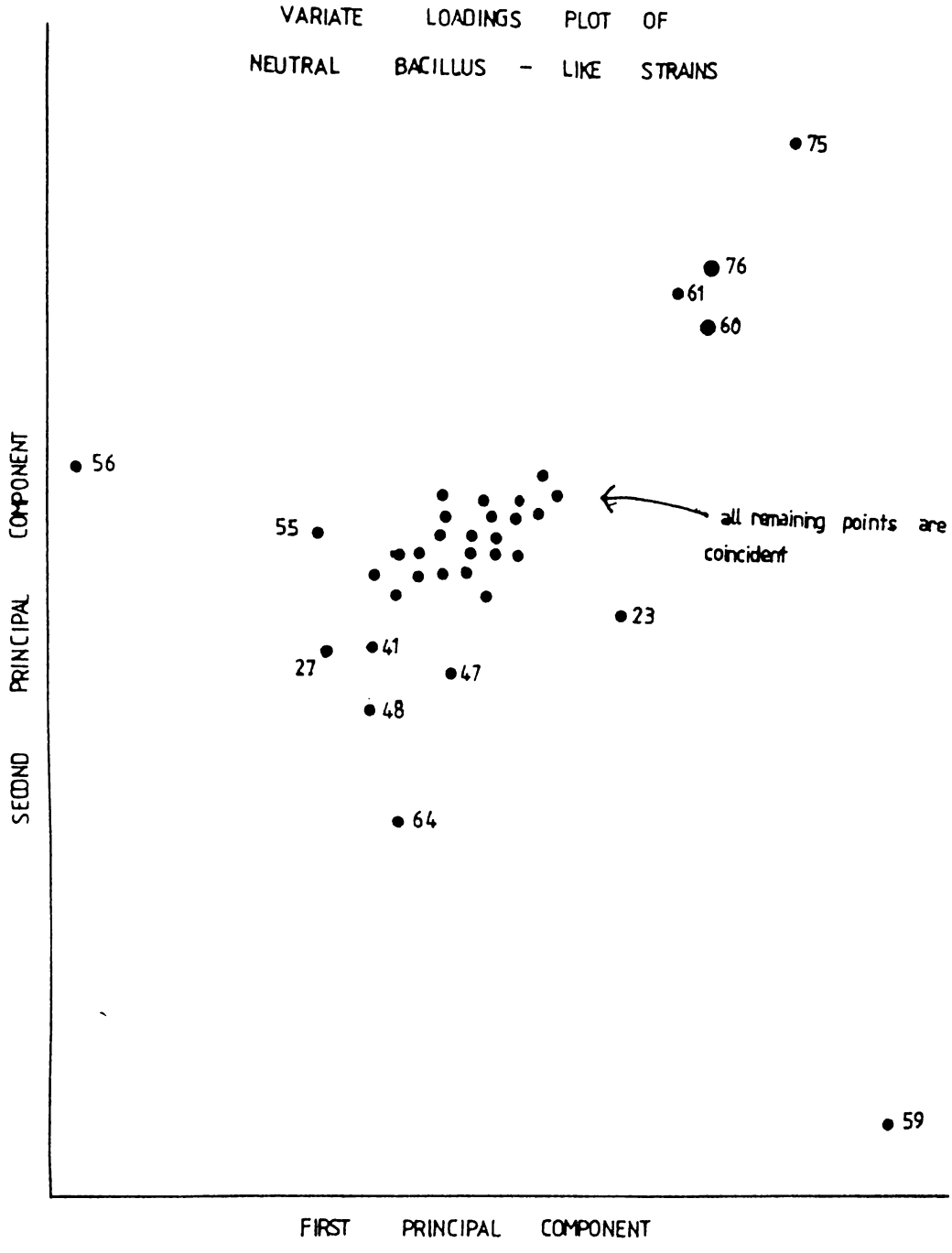


Figure 5.1 Plot relating the m/z peaks used in the data processing to the first two principal axes. Most of the discrimination depends on the peaks which lie outside the main group; these are labelled with the m/z value in the diagram.

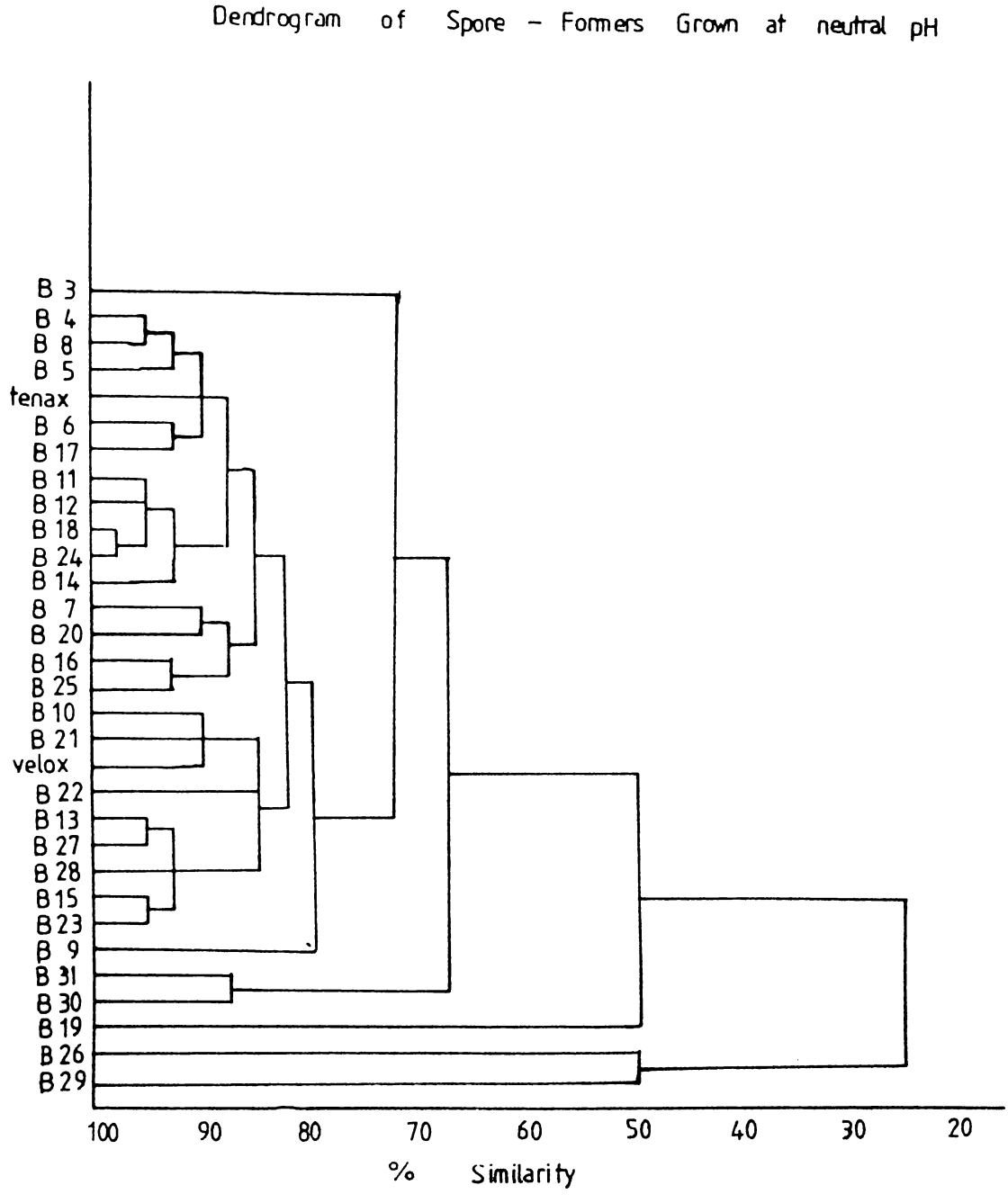


Figure 5.2 Dendrogram of spore-formers grown at neutral pH. Five strain groupings can be seen. Strains B3, B19, B26 and B29 seem rather different from the others in the analysis.

tenax = *Bacillus caldotenax*
velox = *Bacillus caldovelox*

These two type strains fall into separate clusters, probably at a sub-species level ($D^2 = 9$).

strains, B3, B19, B26 and B29 different from all others. Figure 5.2 is the dendrogram relating to these strains. Although both reference strains fall within a single group in the CVA plot, they lie in separate groupings in the dendrogram. The Mahalanobis' D^2 value for these two strains is 8.9, which probably represents a difference at the sub-species level and seems to agree with the finding of Wolf and Sharp (1981) that these two strains belong to a single species group. The differences between most strains within this group are $D^2 < 10$, so it may be that all five groups are at sub-species level.

5.4 Isolation and Preparation of Aerobic, Acidophilic, Spore-Formers.

Thirty-four isolates of acidophilic *Bacillus*-like strains were obtained from a variety of New Zealand thermal springs by enrichment in Brock's medium (Darland and Brock, 1971) at pH = 3.5. The incubation temperature was 70°C. Cultures were purified by three successive plate to plate transfers of single colonies with purity monitored by Gram staining. Phase contrast microscopy was also used to check for the presence of spores. All stages of harvesting the acidophiles were carried out using the same procedure as for *Thermus* strains (Appendix I), with sterile distilled water also used for washing and resuspending these cultures. A tester of isolates is given in Table 5.2.

5.5 PY-MS and Data Processing of Acidophiles

Four replicates of each strain were analysed as for *Thermus* strains (Chapter 3).

It was decided to carry out the data processing procedures under the same conditions as used for the neutral *Bacillus*-like strains to enable a comparative analysis of both strains to be made. These conditions used 191 m/z values (Section 5.3) and 13 principal

TABLE 5.2 Acidophilic Spore-Formers Prepared for PY-MS

Code Number	Source pool	Source temperature °C	Source pH
C1	Orakei Korako 16	78	4.7
C2	Taupo 6	78	7.3
C3	Taupo 26	N.A.	N.A.
C4	Waimangu 21a	82	6.3
C5	Waimangu 21b	82	6.3
C6	Waimangu 31	N.A.	N.A.
C7	Rotorua 29	N.A.	N.A.
C8	Rotorua 30	97	3.7
C9	Waimangu 31a	N.A.	N.A.
C10	White Island 2	95.2	3.1
C11	White Island 3	83.7	2.9
C12	Waimangu 21	67	6.0
C13	Ketetahi 3	N.A.	N.A.
C14	Rotorua 3	70	7.2
C15	Rotorua 7	91	7.3
C16	Rotorua 8	70	9.1
C17	Rotorua 9	84	8.4
C18	Orakei Korako 18	77	3.9
C19	Tikitere 1	45	2.1
C20	Tokaanu 6	97	7.0
C21	Tokaanu 6a	97	7.0
C22	Taupo 3	95	N.A.
C23	Taupo 5	89	6.5
C24	Taupo 6	78	7.3
C25	Taupo 7	72	N.A.
C26	Taupo 8	48	N.A.
C27	Taupo 10	72	6.8
C28	Waiotapu 13	76.4	4.5
C29	Waiotapu 15	57	4.3
C30	Waiotapu D	84	4.4
C31	Waiotapu E	82	2.9
C32	Rotorua 4	87	7.2
C33	Rotorua 37	63	5.0
C34	Rotorua 41	100	7.4

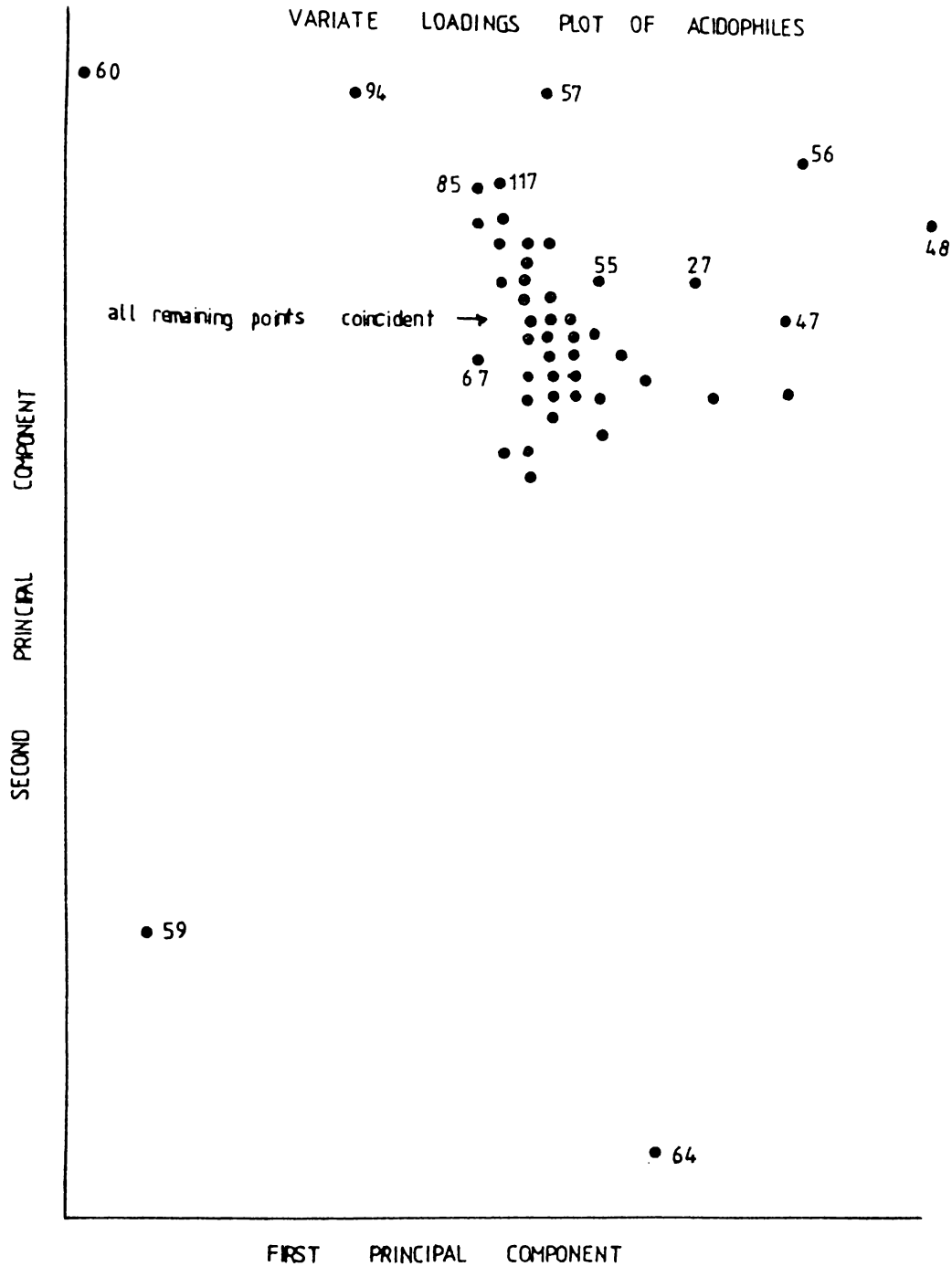


Figure 5.3 Plot relating the m/z values used to discriminate the acidophiles. The important peaks in this analysis are very similar to those used to discriminate the neutral pH strains. See Fig. 5.2.

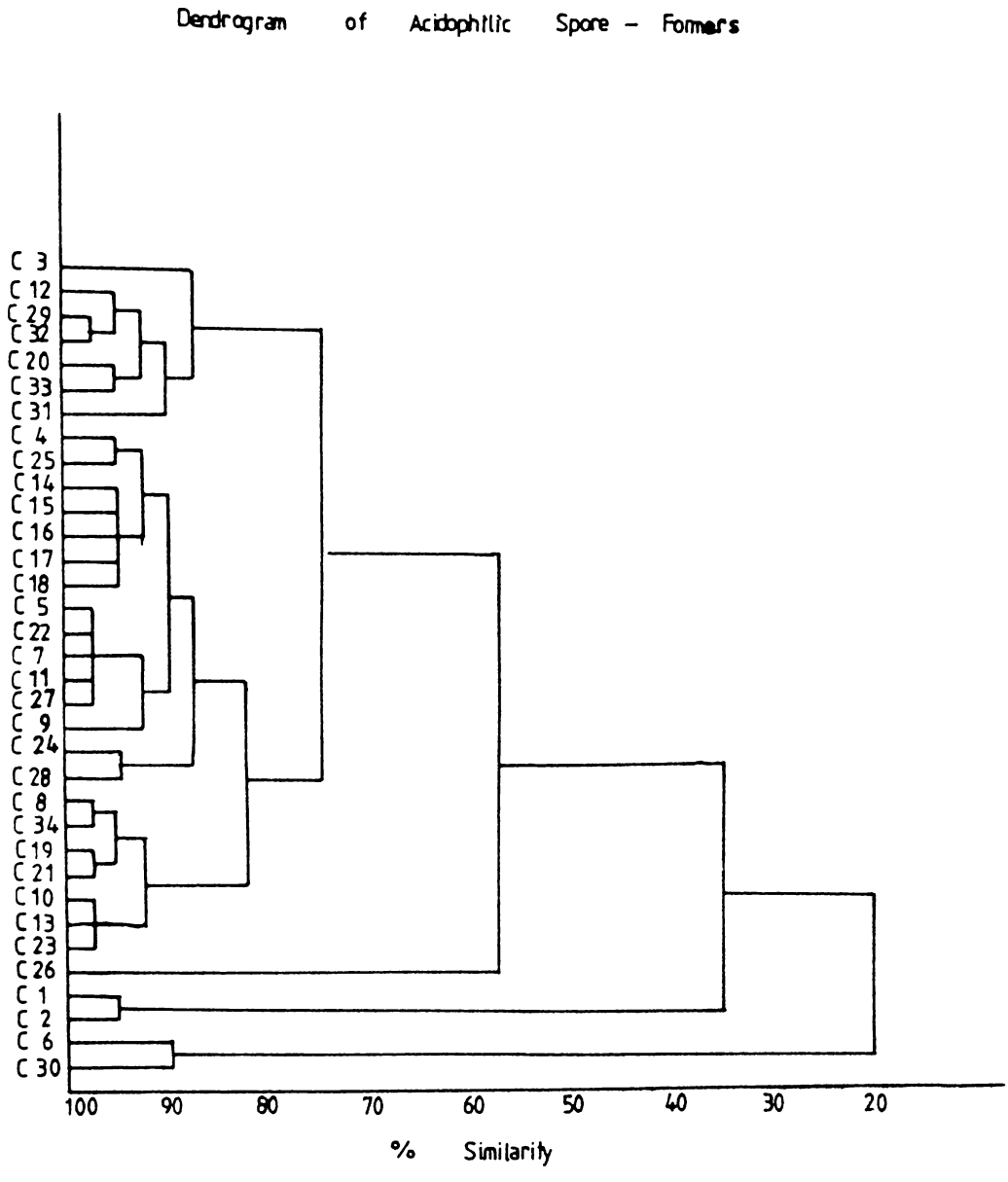


Figure 5.4 Dendrogram of spore-formers grown at pH = 3.5. There are 4 groups of strains falling within clusters of three or more strains at the 90% similarity level. No reference strains were available for this group.

components. For these samples 13 P.C.O's described 93% of the variance.

Four outlying samples (1 each of C2, C3, C5 and C21) were removed from the data set following principal components analysis.

The variate loadings plot (Fig. 5.3) shows that the m/z peaks which were important in discriminating these samples were rather similar to those which discriminated the neutral *Bacillus*-like strains. Three peaks important for these strains, but not the latter group, were m/z = 57, m/z 60 and m/z 64.

Plots of relationships of strains to pairs of the first three canonical axes did not result in unambiguous groupings, but the dendrogram (using the information contained in all 13 P.C.O's) indicates definite strain clusters are present (see Figure 5.4).

Although no reference strains were available for this group it seems reasonable to draw similar conclusions to those of other analyses in this study. Using the same criteria as for the *Thermus* strains, i.e. considering clusters of three or more strains to be relevant groups, it seems there are four distinctly different groups of acidophiles and nine strains which lie outside these. Group 1 (strains C12, C20, C29, C31, C32 and C33), group 2 (strains C4, C14, C15, C16, C17, C18 and C25), and Group 3 (strains C5, C7, C9, C11, C22 and C27) are probably sub-species groupings of one species type. Group 4 (strains C8, C10, C13, C19, C21, C23 and C34) may represent a different species as it clusters at 85% and Mahalanobis' D^2 between members of Groups 3 and 4 are >12. The status of strains C3, C24, C26 and C28 is unclear but two pairs of strains, i.e. C1 and C2 and C6 and C30 seem almost certainly different species from the others in the analysis. This pattern of groupings is taken at the 90% similarity level.

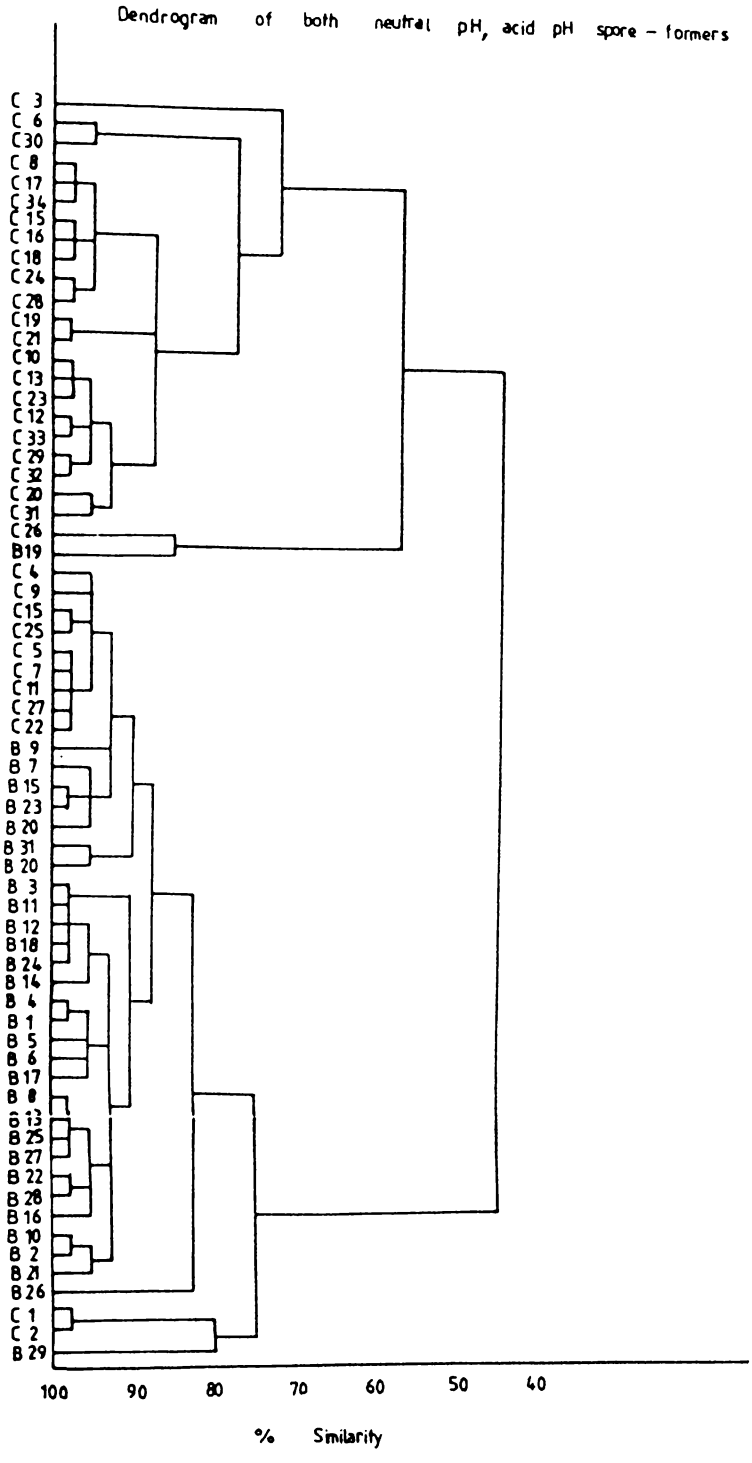


Figure 5.5 Dendrogram showing the results of a combined data processing of all spore-formers. The discrimination of the two types has been good. Note that the misclassified strains, B19, C1 and C2 occupied rather isolated positions in the dendrograms relating to each type. Although the same basic pattern of groupings has been maintained there have been some changes in cluster patterns e.g. strain B8 which originally clustered with B4 and B5 now clusters with B13, B25 and B27. This suggests that analyses of widely different types of micro-organisms grown on different media may require different methods of data processing for accuracy at a fine level of discrimination.

5.6 An Attempted Classification of Neutral and Acidophilic Spore-Formers

It has been found that even small changes in media can alter pyrolysis spectra considerably (Oxborrow *et al.*, 1977) - nevertheless it was felt it would be interesting to investigate the power of PY-MS to discriminate the two groups of spore-formers which were grown on different media. The data was processed using the same criteria, i.e. the same 191 m/z values as for analysing the separate blocks of data relating to the spore-formers, and 13 P.C.O's which were found to describe 93% of the variance. The variates loadings plot with respect to the first two principal axes showed that essentially the same m/z peaks were the most important in discriminating the strains.

The dendrogram produced from this analysis is given in Figure 5.5. It was interesting to see that the two groups of strains were separated quite efficiently. Two strains occupied an anomalous position in the dendrogram, these were C26 and B19 both of which failed to cluster in the individual analysis. A group of acidophiles C4, C5, C7, C9, C11, C15, C22, C25 and C27 were apparently rather similar to the neutral pH spore-formers. Data from the original pools relating to these strains is incomplete but it may be significant that only two are from pools with a pH <5. These strains are mostly from Group 3 in the acidophile classification.

Although there is definite clustering within the acidophile and neutral pH groupings there is not good agreement with the clusters formed in the individual analyses. An extreme example of this is the clustering of strains C4 and C5 together as outliers. In the acidophile analysis, C4 clustered with Group 2 and C5 with Group 3. Therefore this exercise, while demonstrating good pH discrimination, gives ambiguous results as regards classification within the two types of bacteria.

PY-MS is undoubtedly a powerful technique for discrimination of bacteria but new methods of data processing are required for the technique to realise its full potential. The technique of SIMCA pattern recognition (Blomquist *et al.*, 1979) seems very promising in this regard. Problems encountered by the author of the programmes used in this study (MacFie and Gutteridge, 1982) suggest modifications are required to enable SIMCA to be used for these data sets.

As for other thermophiles analysed there seems no obvious taxonomic relationship to either the location of the source pool or to its temperature and pH. Spores from these strains could easily be present over a wide area, therefore the isolation of a strain from a sample pool water does not necessarily mean this is the organism's normal habitat. As some samples from the same pool have yielded both types of strains it is most probable that the acidophilic strains isolated from pools of high pH grew from spores and similarly for the neutral pH strain which was isolated from a Ketetahi pool with pH 3.0.

The proof that *Thermus* strain TOK 11 was not contaminated was a further demonstration of the value of PY-MS in the investigation of new isolates from natural sources. Contaminated cultures of new strains are not always easy to determine in practice.

All strains of spore-formers analysed by PY-MS were kindly isolated and prepared by Y. Casey (Thermophile Research Unit, University of Waikato).

CHAPTER 6

SOME ASPECTS OF THE GENETICS OF *THERMUS*

6.1 Introduction

The *lac* operon of *Escherichia coli* has been studied in detail and considerable information is available regarding the expression and regulation of *lac* genes (Miller and Reznikoff, 1980). Simple and sensitive assays are available to detect β-galactosidase activity (Miller, 1972) which make the study of this operon a suitable topic for laboratory investigation.

It has been reported that an isolate, T2, resembling *Thermus aquaticus*, possesses an inducible β-galactosidase (Ulrich *et al.*, 1972). A New Zealand *Thermus*-like isolate, T41A, has also been found to have an inducible β-galactosidase (Cowan *et al.*, 1984). Therefore it was decided to screen further isolates from New Zealand thermal springs to select an organism with maximum possible β-galactosidase activity. It was proposed to attempt to clone the *lac* genes from the selected organism into *E. coli*. If it were possible to achieve expression of these genes as has been reported for the leucine gene from *T. thermophilus* in *E. coli* (Ngahari *et al.*, 1980), comparisons with the well known *E. coli* system could provide valuable insights into the regulation of *lac* and perhaps general mechanisms of thermostability in extreme thermophiles. Although the *Thermus*-like isolate T41A was finally chosen for the cloning attempt, initial investigations were aimed at isolating any highly active β-galactosidase producer.

6.2 Methods and Materials

Screening for both aerobic and anaerobic β-galactosidase producers was carried out using the following conditions.

(i) Isolation of Aerobes

Water and sediment samples were collected as described in Appendix I, Section I.1 and stored at room temperature for up to six months. 0.5 - 1.0 ml of spring water was added from a sterile Pasteur pipette to 10 ml of medium AS (described below) in 25 ml Universal bottles fitted with rubber lined screw caps. Incubations were carried out in a Clayson ventilated incubator at 70°C (±0.5°C). All turbid cultures were examined for constitutive and inducible β-galactosidase by the method described below.

Positive cultures were purified over single colonies by streaking onto plates of solidified AS medium, with three successive plate-to-plate transfers of well separated single colonies being carried out. Culture purity was monitored by phase contrast microscopy and Gram staining.

'AS' Medium

(a) For constitutive β-galactosidase producers,

Allen Salts (Jackson, 1973)
0.3% Yeast Extract (Merck)
0.3% Trypticase peptone (BBL)
2% Oxoid No. 1 agar was added for solid medium
pH adjusted to 7.2 with 0.1M NaOH.

The medium was dispensed (10 ml into 28 ml Universal bottles) and autoclaved for 15 minutes at 121°C.

(b) For inducible β-galactosidase producers, 0.5% galactose was added from a 10% sterile stock solution either at inoculation, or for primary isolates as soon as cultures appeared turbid, followed by incubation for a further 8 - 12 hours. Initially 0.5% lactose (from

10% filter sterilised stock) was added but this caramellised extensively on prolonged incubation which resulted in cultures of low cell density. Lactose was also found to be a less efficient inducer so its use was discontinued.

(ii) Isolation of Anaerobes

0.5 - 1.0 ml of water and sediments were added to 10 ml of TYEG medium in Hungate tubes. Medium was prepared by the modified Hungate technique described in Appendix II, Figure 1. All stages of preparation and incubation were carried out under N_2.

Screening for both constitutive and inducible β-galactosidase producers was carried out using the assay described below. Positive cultures were purified by serial dilution using the lowest dilution which showed growth as the inoculum for the next transfer. At least three serial transfers were carried out and culture purity monitored by phase contrast microscopy and Gram staining.

TYEG Medium (Zeikus *et al.*, 1979)

The published medium was used for detection of constitutive β-galactosidase producers, but the 0.5% glucose was replaced by 0.5% galactose to screen for inducible enzyme producers. The final pH was 7.2 - 7.4 and all incubations were carried out at 70°C.

Assay for β-galactosidase

0.5 ml cell suspension was pelleted by centrifugation for 10 minutes at 2000 g (max) in an MSE bench top centrifuge.

The cells were resuspended in 0.5 ml McIlvaines citrate-phosphate buffer at pH = 6 (Dawson *et al.*, 1974) and enzyme released by the addition of 0.1 ml of a 2:1 toluene-acetone mixture. The suspension was

incubated at room temperature with frequent agitation for five minutes then immediately assayed. 2.0 mls of ONPG solution (see below) was added to 0.5 ml of toluene-treated cell suspension and the mixture incubated for 10 minutes at 70°C. The reaction was terminated by adding 2.5 mls of 0.5 M Na_2CO_3. The cells were pelleted by centrifugation (10 mins at 2000 g (max) in an MSE benchtop centrifuge) and the absorbance of the supernatant measured at 420 nm on a Beckman model 24 spectrophotometer not later than 1 hour after assay.

All assays were done in duplicate and the mean value calculated.

ONPG Solution

(o-nitrophenyl-β-D-galactopyranoside, Sigma).

5 x 10^{-3} M ONPG was prepared in McIlvaines buffer at pH = 6. Solutions were stored at 4°C in brown glass bottles and discarded after 3 days.

6.3 Isolation of β-galactosidase Producers

Forty-eight samples from thermal springs in the Rotorua, Waimangu, Tikitere, Tokaanu and Taupo regions of the central North Island, New Zealand, were screened for both aerobic and anaerobic β-galactosidase producers. For a simple and quick comparison of enzyme production the following calculation was employed, using the isolate T41A as the standard against which new isolates were measured.

$$\text{Enzyme value for T41A} \quad \frac{A_{420} \text{ for T41A}}{A_{650} \text{ for T41A}} = 100$$

where A_{420} is the absorbance following the ONPG assay and A_{650} is the absorbance of the cell suspension before assay to give an indication of cell density.

TABLE 6.1 AEROBIC β-GALACTOSIDASE PRODUCERS

Source	Source Temperature °C	Source pH	A_{420}	A_{650}	Enzyme Value %	% Inhibition to 1% galactose
TOKAANU						
TOK 4	89	5.6	1.469	0.486	80	-75
TOK 7	53	6.0	1.017	0.394	68	-76
TAUPO						
TP 8	48	not available	0.253	0.608	11	-64
TP 10	92	7.1	0.275	0.668	11	-45
WAIMANGU						
Wai 14	70	7.7	0.416	0.648	17	-42
Wai 19	79	7.7	0.256	0.704	10	-56
ROTORUA						
T41A (reference strain)	-	-	2.715	0.716	100	-69

$$\text{Enzyme value of an unknown} = \frac{A_{420} \text{ of unknown}}{A_{650} \text{ of unknown}} \times \frac{100}{\text{Enzyme value of T41A}}$$

Six aerobic enrichment cultures were carried through to purity and the enzyme value calculated. It was also decided to investigate feedback inhibition due to galactose so these isolates were also assayed in the presence of 1% galactose added with the ONPG. The results are given in Table 6.1

All these isolates were yellow pigmented, Gram negative rods which formed long filaments on prolonged incubation. They were all apparently strains of *Thermus*. As none were superior to strain T41A it was decided that this was the most satisfactory *Thermus*-like β-galactosidase producing isolate.

Nine anaerobic isolates gave positive β-galactosidase assays, as set out in Table 6.2. Cell density was measured on a +++, ++, + scale only.

Table 6.2

ANAEROBIC ISOLATES PRODUCING β-GALACTOSIDASE

Source	Source °C	Source pH	A_{420}	Cell Density	% Inhibition to 1% galactose
TOKAANU					
TOK 2	81	7.1	.346	++	-
TOK 3	76	6.9	.400	++	-
TOK 6a	97	7.0	.650	++	-77
TOK 8	77	5.6	.222	+++	-49
TAUPO					
TP 6	78	7.3	.803	+++	-50
TP 10	72	6.8	.247	+++	-47
WAIMANGU					
Wai 19	79	7.7	.202	++	-
TIKITERE					
Tiki 17	68	6.5	.140	++	-
ROTORUA					
RT 8	70	9.1	.395	+++	-40

A comparison of T41A and the anaerobic isolate TP6, designated ANTP6 was then carried out to determine which isolate to select for the cloning experiments.

A COMPARISON OF T41A and ANTP6 β-GALACTOSIDASES

Isolate	Enzyme Value	% Inhibition to 1% galactose	% Inhibition to 2% glucose
T41A	100	-69	-65
ANTP6	400	-40	+11

Although the β-galactosidase from ANTP6 was apparently superior in terms of amount produced and inhibition properties, it was decided that there could be problems in attempting to use an obligate anaerobe for cloning into *E. coli*.

An investigation of properties of the ANTP6 β-galactosidase have provided the topic for an M.Sc. Thesis in this University.

All attempts at cloning of thermophilic DNA into *E. coli* were made using the aerobic *Thermus*-like strain T41A.

The following work on the molecular genetics of T41A was done at the Cell Biology Department, University of Auckland, New Zealand under the guidance of Professor P.L. Bergquist.

6.4 The use of a Cosmid Vector for Gene Cloning

Very little is known about the genetics of extreme thermophiles including the size and control mechanisms of any particular gene. For successful transfer and expression of β-galactosidase it would be necessary to transfer the entire operon to the recipient, therefore a cloning vector which could transfer large pieces of DNA was required.

The two most commonly used vectors for gene transfer are plasmids (small self-replicating pieces of DNA which are double stranded, circular and exist outside the chromosome) and bacteriophages, particularly the lysogenic phage lambda (λ). These two methods are approved (N.I.H. guidelines) for cloning into F^- strains of *E. coli* (which prevent transmission during conjugation).

Plasmid-donor DNA hybrids have been used in many cloning experiments including the successful transfer of a 3.75 Md fragment which carried the leucine gene from *T. thermophilus* to *E. coli* (Ngahari *et al.*, 1980). Many suitable high copy number plasmids carrying antibiotic resistant markers are available for the purpose. However two practical problems which relate to the use of plasmid vectors are the difficulty of separating hybrid plasmids from parent plasmids and the fact that only small plasmids are taken up efficiently by recipient cells (Collins, 1979).

Only a relatively small section of the λ genome can be replaced by donor DNA, i.e. 26.4 kilobases at most (Hohn, 1979) which is generally too small for whole gene cloning. An important advantage of λ

as a vector is the availability of λ promotors for obtaining high expression rates of cloned genes (Glover, 1980) and the efficient uptake by a suitable recipient.

The inefficiency of inserting large pieces of foreign DNA into recipient cells has been reported to have been overcome by the technique of 'cosmid packaging' (Hohn and Collins, 1980). It therefore decided to use this method to attempt to transfer the *lac* genes from T41A to a *lac* minus strain of *E. coli*.

Cosmids are designed to mimic the *in vivo* production of λ phage particles. A cosmid is a plasmid containing:

(a) The cohesive (*cos*) ends of λ. These are sequences of twelve complementary bases which exist as single stranded "tails" - one on each end of opposite DNA strands when λ DNA is in the linear form (see Fig. 6.1). These *cos* ends are essential for packaging of λ DNA into protein coats (heads) during production of virulent λ phage particles. They also allow efficient circularization (by pairing of the 12 bases) of the λ DNA after infection for insertion into the recipient's chromosome.

(b) Antibiotic resistance markers i.e. genes which enable resistance to one or more antibiotics to be used as a screening assay for recombinants (i.e. recipients carrying cosmid DNA).

(c) Single restriction sites for several restriction endonucleases. This is important as it enables the precise point of insertion of donor DNA to be located. If an endonuclease cleaves through an antibiotic resistant gene, loss of resistance to that antibiotic is a test for hybrid cosmids.

(d) The plasmid origin for independent replication within a recipient bacterial cell. (Hohn and Collins, 1980).

To clarify the action of a cosmid it is necessary to briefly describe the *in vivo* production of λ phage particles. Lambda is a lysogenic phage, which means its DNA is integrated into its host's chromosome but few of its genes are expressed in this state. While integrated, the phage DNA is replicated with the host's and a copy is transferred to each daughter cell at cell division. However certain stimuli (e.g. heat shock) can induce a virulent cycle during which the phage DNA is excised from the host chromosome. All phage genes are now both replicated and expressed to produce many copies of the phage genome and the proteins which provide the outer coat of the mature phage. The phage self-assembles, followed by phage-induced host cell lysis and release of virulent (and infective) phage particles to the environment.

As previously mentioned linear λ DNA has single strained *cos* "tails" of complementary bases. This means that following excision from the host chromosome the phage genome circularises by base pairing. The λ DNA can now be replicated, probably by the method of "rolling circle replication" (Watson, 1977). This method of DNA replication results in long linear double stranded repeats of DNA being formed which are called concatamers.

Lambda phage gene products include the proteins which self-assemble to form the characteristic icosahedral λ head and the corkscrew-like tail of the mature particle. Certain of the head proteins "recognise" the *cos* sequences interspersed along the DNA concatamers. If two adjacent *cos* sequences are separated by a 45 kilobase pair length of DNA a preformed "head" will become attached at the *cos* regions. A phage-coded restriction enzyme then cuts the concatamer adjacent to the *cos* sequence to produce a monomer of λ DNA 45 kilobase pairs long and with 12 base single stranded *cos* tails at each end. This piece

of DNA is folded and packaged into the λ head. The protein tail will now attach, and λ induced host lysis results in the release of mature phage particles.

The development of cosmids arose from a thorough understanding and successful exploitation of λ phage production by Hohn (1979). The essence of the method is the successful production of phage head and tail proteins without the packaging of native λ DNA. The "empty heads" produced can then be filled with required DNA which must have two important properties:

(1) The 12-base single stranded *cos* sequence at each end.

(2) The same sized double stranded centre as native λ DNA i.e., only double stranded DNA pieces within the range 37 - 50 k bases will be packaged with an optimum around 45 k base pairs.

The production of "empty heads" and the tails is achieved by growing up two strains of λ phage (in separate hosts) each with a different head protein mutation. This means that following induction λ DNA replicates and tails assemble but as the "heads" are defective λ particles are not formed.

Growth is stopped before lysis can occur. Both cultures are then mixed but kept cold to prevent further growth leading to lysis (which would release λ components). The mixture is UV irradiated under conditions which have been tested for a particular UV lamp and found to completely destroy all λ DNA. The irradiated cell suspension is concentrated by centrifugation and frozen in liquid nitrogen. When required the cell mixture is rapidly thawed to 37^{o}C which causes cell lysis and release of all the head and tail proteins necessary to form effective λ particles in the presence of suitable (λ mimicking) DNA.

The DNA which will be inserted into the "packaging mix" heads is derived as a result of hybridisation of a high copy plasmid and the *cos* ends of λ to form a small plasmid known as a *cosmid*. The cosmid used in these experiments was derived from the useful plasmid pBR 322 which can form up to 50 copies per cell and has genes for resistance to the antibiotics ampicillin and tetracycline. The portion of pBR 322 containing these genes is ligated to a λ *cos* sequence to form the small cosmid pHc 79. (3.75 M daltons - Hohn and Collins, 1980). The cosmid can be "opened" with a restriction endonuclease and pieces of donor DNA with suitable complementary ends can be inserted and ligated to form linear double stranded DNA molecules with interspersed *cos* sequences, therefore mimicking natural λ concatamers. As the packaging mixture contains all components necessary for forming λ particles except λ DNA, these *cos* sequences will be recognised by phage head proteins, and if the length of DNA between two *cos* sequences are the correct size (~45 k base pairs long) the DNA will be packed into the empty heads; tails will then attach and viable particles form. These can then be adsorbed to a suitable recipient strain of *E. coli*. The infection is highly efficient, resulting in release of hybrid DNA into the recipient cell. Once released the hybrid DNA circularises because of the *cos* ends, but it lacks the genes necessary for integration into the host chromosome. However it has the plasmid (pBR 322) origin and proceeds to replicate to form several copies - up to 15 per cell have been reported (Hohn and Collins, 1980). Yields of up to 9×10^4 colonies containing cloned DNA have been reported per μg of DNA inserted (Hohn and Collins, 1980) but maintainence and copy number depend on the advantage of the inserted genes to the recipient.

The sequence described here is summarised in figure 6.1.

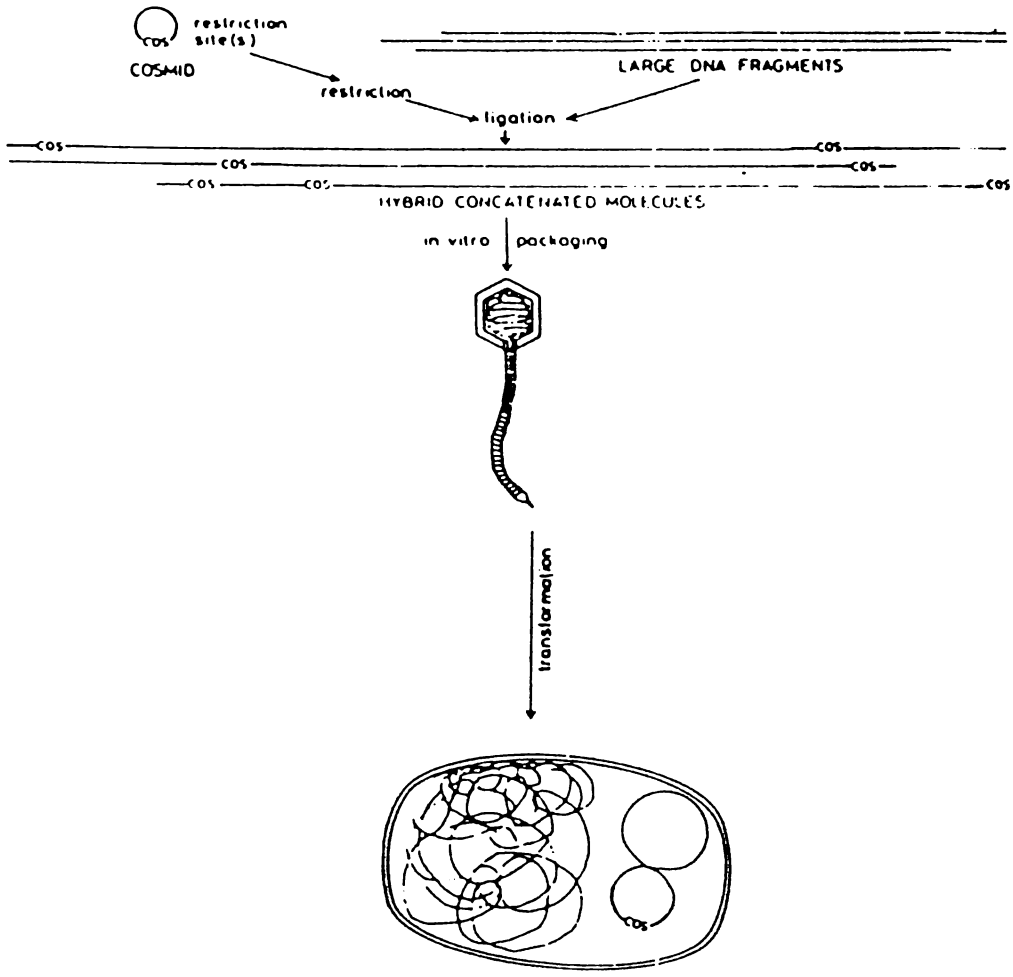


FIGURE 6.1 - COSMID PACKAGING

Reaction sequence

1. A cosmid (e.g. pHc79) is a plasmid which includes the 12 b.p. *cos* sequence from bacteriophage λ.
2. Cosmid and target DNA's are restricted with enzymes producing the same "sticky ends".
3. Ligation yields hybrid concatamers which mimic natural λ DNA.
4. Concatamers are cut at *cos* sequences and packaged into λ phage protein coats.
5. Packaged "phage" can be transduced to a suitable recipient strain.
6. Hybrid DNA is released into the recipient cell, circularises by pairing of the *cos* bases to form a self-replicating plasmid.

6.5 Cosmid Cloning of T41A

6.5.1 Extraction of total DNA from T41A

As a control, total DNA was also extracted from *E. coli* strain PB 2198 which contains functional *lac* genes.

10 ml of an overnight culture of *E. coli* strain PB 2198 was grown in medium L.

Medium L		
	Bactotryptone (Difco)	5 g
	Yeast extract (Merck)	5 g
	NaCl	5 g
	Distilled water	1000 ml
	pH adjusted to 7 with 1 M NaOH	

Cells were pelleted by centrifugation at 3000 g (Sorvall RC-5B) for 5 minutes.

0.5 g (wet weight) of T41A cells which had been grown on AS medium (Section 6.2) were harvested by centrifugation for 10 minutes at 3000 g (max) in a Sorvall RC-58 centrifuge and stored in liquid nitrogen until required.

Both T41A and PB 2198 were then treated in identical fashion.

(1) Cells were washed with 5 ml of TES buffer at pH 8.0 (TES buffer is 500 mM Tris HCl, 5 mM EDTA, 500 mM NaCl) and resuspended in 5 mls of 50 mM Tris HCl/16mM EDTA at pH 8.0. Cells were lysed by the addition of 4 mg of lysozyme (Sigma) and incubated for 10 minutes at 37^{o}C. 2 mg of pronase (Sigma) were added and the mixture incubated for a further 10 minutes at 37^{o}C to digest proteins. This incubation was followed by the addition of 20 mg SDS (sodium lauryl sulphate) with a further incubation for 3 hours at 37^{o}C. The proteins were then

extracted twice with an equal volume of redistilled phenol (saturated with an equal volume of 50 mM Tris HCl at pH 8). The DNA remains in the aqueous phase at this pH, the proteins in the phenol phase - these two phases were separated into two layers by centrifugation at 2000 g (max.) in a benchtop MSE centrifuge with a swinging bucket rotor. The aqueous layer was carefully removed with a Pasteur pipette and DNA precipitated by 2 volumes of absolute ethanol (overnight at -20°C). The DNA was pelleted at 4000g for 10 minutes, drained to remove ethanol and redissolved in 2 mls of 50 mM Tris HCl/5 mM EDTA at pH = 8).

RNA was removed by digestion with 100 µg of (boiled) RNA'ase A (Sigma) and 25 units of RNA'ase T1 (Sigma) at 65°C for 20 minutes. The enzymes were removed by phenol extraction as previously described and the DNA precipitated with ethanol as before. The DNA pellet was redissolved in 500 µl 10 mM Tris HCl/ 1 mM EDTA at pH 8 and dialysed overnight against 1.5 litres of the same buffer.

The DNA concentration was then estimated spectrophometrically at 260 nm on a Carl-Zeiss Spectrophotometer using the formula

$$A_{260} = 1 = 50 \text{ µg ml}^{-1} \text{ DNA}$$

Results

The concentrations obtained were

(1) T41A DNA = 1 µg µl^{-1}

(2) PB 2198 DNA = 0.7 µg µl^{-1}

6.5.2 Preparation of packaging mix

Two strains of *E. coli* pB 2159 and pB 2160 contained lambda phage with separate mutations in genes coding for phage head proteins. Both strains were also rec A mutants, which means they were not able to carry out light induced UV repair. This is important as UV is used to destroy λ DNA.

The two cosmid packaging strains were retrieved from storage at -80°C, purity confirmed over single colonies and plated onto L medium solidified with 2.2% (W/V) Davis agar.

A single colony from each strain was inoculated into 50 ml of NZY medium as follows:

NZY Medium

NZ amine	10 g
$MgCl_2.7H_2O$	2 g
NaCl	5 g
Yeast Extract (Merck)	5 g
distilled water	1000 ml

pH adjusted to 7.0 with 1 M NaOH

The cultures were grown until early log phase (a Klett value of K = 120) and the lysogenic prophage were induced to enter a virulent cycle by heat shock at 45°C for fifteen minutes. The cultures were incubated for a further three hours at 37°C and checked for successful induction by adding a drop of chloroform to an aliquot. Only induced bacteria lyse (i.e. suspension clears) on the addition of $CHCl_3$. The two cultures were centrifuged (at 3000 g for 10 minutes) and resuspended in the original volume of cold M - 9 buffer (Hohn, 1979). The two suspensions were then mixed (kept cold), and transferred to sterile Petri dishes for UV irradiation at 100 μm cm^{-2} (Blak-Ray UV meter) from a Sylvania Germicidal UV Lamp (GTENZ Ltd., Auckland) for 3 minutes (Hohn, 1979). The cells were concentrated x500 by centrifugation and resuspended in packaging buffer (Hohn, 1979), the volume measured and ATP to 1 mM added. 20 μl aliquots were transferred to 1.5 ml Eppendorf centrifuge tubes, frozen in liquid nitrogen then stored at -80°C in a Revco freezer.

6.5.3 Assay for Packaging Mix Efficiency

(a) The recipient strain, pB 2470, a *lac* minus strain which has λ receptors, was grown for 2 - 3 hours (to Klett = 200 i.e. log phase) to induce receptors. The cells were then pelleted and resuspended in 0.01 M $MgSO_4$.

(b) A 20 µl aliquot of packaging mix was thawed and 5 µl of λ DNA added together with 1 µl of 38 mM ATP. The mixture was incubated for 30 minutes at 37°C to "package" the DNA. 0.2 µl of 1 µg ml^{-1} DNA'ase (Sigma) was then added followed by 20 µl chloroform and 200 µl phage buffer (Hohn, 1979).

(c) As a control for packaging of endogenous DNA 5 µl of distilled water was added to a second 20 µl aliquot of packaging mix.

The packaged λ DNA was diluted in series 10^{0} - 10^{-4} in phage buffer (Section 6.8.1). 3 mls of L medium soft agar (0.5% agar) was melted and held at 45°C. To this was added 200 µl of PB 2470 in 0.01 M $MgSO_4$ and 200 µl of diluted "phage". The tubes were mixed on a Vortex mixer and poured over the surface of plates containing ∿25 ml of solid medium L. After 10 minutes plates were inverted, and incubated overnight at 37°C. All assays were done in duplicate. Plaques formed due to phage induced lysis were then counted.

Results

(1) The distilled water control formed no plaques, indicating all native λ DNA in the packaging mix strains had been successfully destroyed during UV irradiation.

(2) The plaque count of packaged λ DNA (means of duplicates)

10^{0} - fully cleared

10^{1} - plaques merged

10^2 - 329

10^3 - 44

10^4 - 7

The result was 3 x 10^4 plaques formed per µl of λ DNA added. This was considered satisfactory to attempt to package cosmid-hybrid DNA's.

Note also

Strain PB 2470, the recipient, is r^- m^+ which means it has no restriction system to remove foreign (including cloned) DNA but it possesses a modification system making it a suitable recipient strain for cloning.

6.5.4 Preparation of Cosmid Vector pHc 79

The cosmid vector is maintained in strain PB 2193.

(a) A 750 ml culture was grown to log phase in L medium. Cells were harvested by centrifugation at 4000 g (Sorvall RC-58) for 10 minutes, then

(b) The cell pellet was washed in 20 mls of buffer (50 mM Tris HCl, 5 mM EDTA, 50 mM NaCl at pH 8.0) respun and resuspended in 2 x 10 mls of lysis buffer (25% sucrose/50 mM Tris HCl) at pH 8.0.

(c) The mixture was placed on ice, 5 mg of lysozyme added and gently shaken (on ice) for 30 minutes.

(d) 16 ml of lysing mix was added slowly while shaking continued.

Lysing Mix	0.3%	Triton X - 100
	0.05 M	Tris HCl pH 8.0
	0.0525 M	EDTA

(e) The mixture was spun at 27,000 g (Sorvall RC-5B) for 30 minutes, the supernatant discarded, and the volume of the pellet measured.

Then 5 M NaCl was added to a final molarity of 0.5 M.

(f) The final volume was noted and 1/10 (W/V) of solid polyethylene glycol (PEG) added very slowly (with stirring on ice). This mixture was left overnight at $4^{o}C$.

(g) The PEG precipitate was pelleted at 3000 g for 5 minutes and gently resuspended in 6 mls of 10 mM Tris HCl/1 mM EDTA at pH 8.0.

(h) The solution was centrifuged at 12,000 g for 10 minutes to remove debris (undissolved PEG) and the supernatant retained.

(i) Caesium chloride was added to a density of 1.56 - 1.59 (determined from $D = \frac{m}{v}$) and $\sim$1 mg ml^{-1} of ethidium bromide added.

(j) Tubes were balanced with paraffin and spun in a Beckmann L265B ultracentrifuge fitted with a swinging bucket titanium 50 rotor for 40 hours at 1 x $10^{6}g$ (average) and $15^{o}C$.

(k) Removal of DNA from the Gradient.

After ultracentrifugation the DNA had banded and could be located by fluorescence of ethidium bromide under UV light. Two DNA bands could be seen. The upper band was DNA from the cell chromosome and open circular plasmid DNA and the lower (narrow) band pure plasmid DNA (closed covalent circular DNA). The lower band was carefully removed with a Pasteur pipette (under UV light).

(l) Ethidium bromide was removed by gently mixing with an equal volume of sec-butanol. The top (butanol) layer was discarded and the extraction repeated until the butanol layer was colourless.

(m) Finally the plasmid DNA was dialysed overnight at $4^{o}C$ against 10 mM Tris HCl/1 mM EDTA at pH = 8.0 to remove caesium chloride.

(n) The concentration of plasmid DNA was estimated by measuring A_{260} and was found to be 0.17 $\mu g\ \mu l^{-1}$.

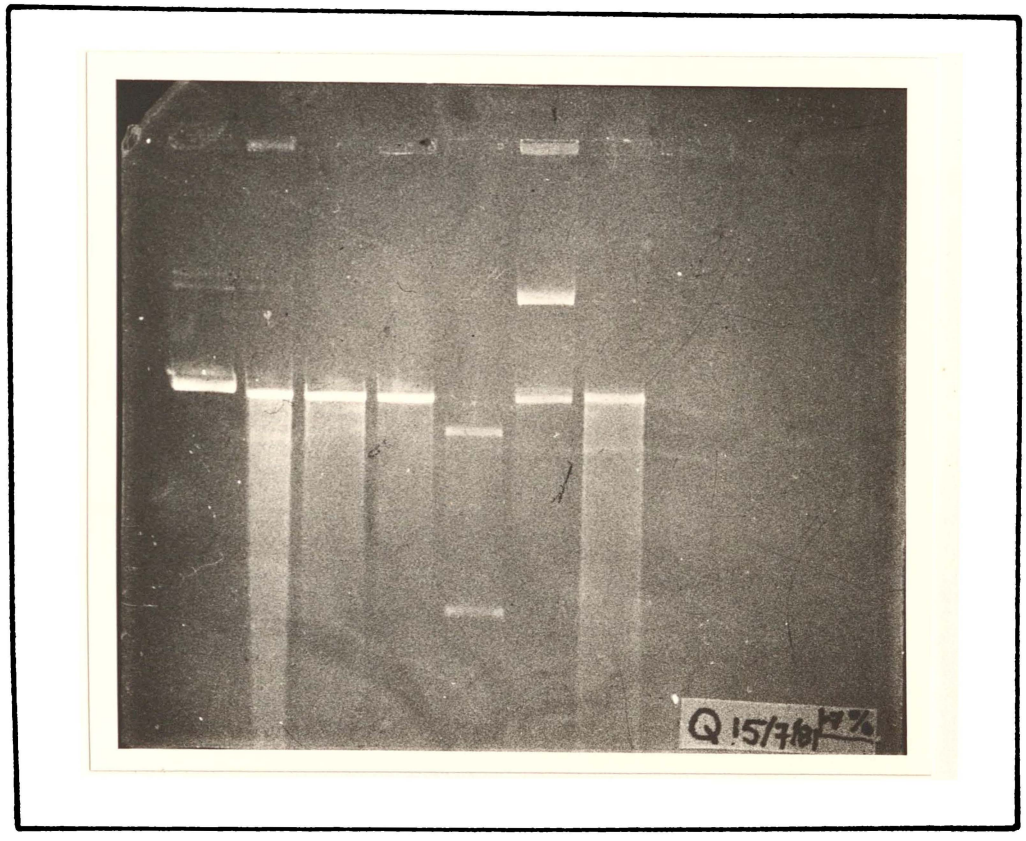


Figure 6.2 The effect of a number of Type II restriction endonucleases on pHC 79 DNA.
Lane 1 uncut; Lane 2 EcoRl; Lane 3 BamHl, the enzyme used in the cloning; Lane 4 Hind III; Lane 5 BGLII; Lane 6 Sal; Lane 7 PST. Electrophoresis in 0.7% agarose at 135V for 3 hours.

(o) The response of the plasmid pHc 79 to a number of restriction endonucleases was determined by electrophoresis in 0.7% agarose and can be seen in figure 6.2.

The enzyme which was used in the cloning attempt was Bam Hl which cuts

5' - G ↓ G A T C C - 3'

3' - C C T A G ↑ G - 5'

In pHc 79 there is a single Bam Hl site at the tetracycline resistance locus, so hybridisation can be checked by replica plating onto medium containing this antibiotic. Any colonies which grow on medium plus tetracycline cannot contain hybrid DNA.

6.5.5 Restriction and Ligation of T41A and pHc 79

The restriction endonuclease Sau 3A produces the same "sticky" ends i.e.

5' - G

3' - C C T A G - 5'

and

5' - G A T C C - 3

G - 5'

as Bam Hl, therefore base pairing can occur between restriction fragments from digestion by either enzyme. Sau 3A was used for the total cell DNA's from T41A (and the lac + *E. coli* strain PB 2198). This enzyme is less stringent in its recognition requirements than Bam Hl and can therefore cleave most genomes into many fragments.

Exact sizing of donor DNA fragments is not essential as only fragments of λ size, i.e. 35 - 50 K base pairs long will be packaged. As Sau 3A usually has many cut sites it was necessary to determine the

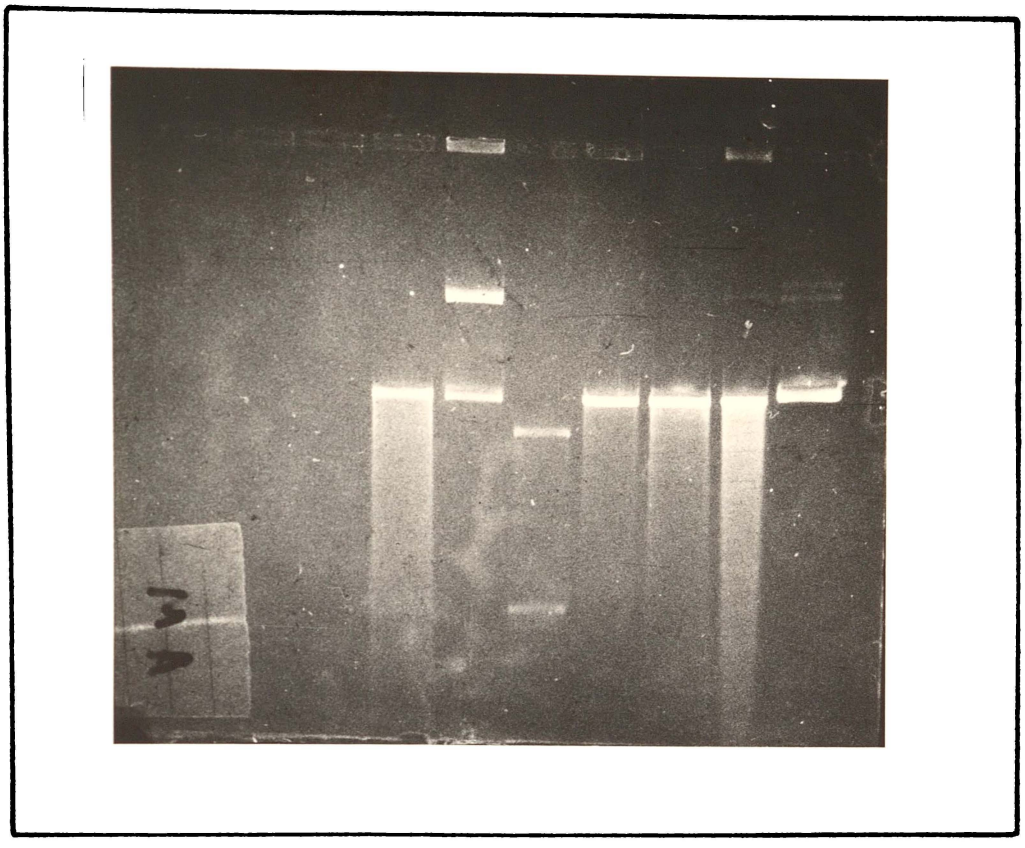


Figure 6.3 Determination of the concentration of Sau3A to just "smear out" the target DNA and produce suitable size fragments for insertion into cosmids. Lane 1 PB 2198 3 µl uncut; Lane 2 + 5 µl Sau3A; Lane 3 + 2 µl Sau 3A; Lane 4 + 1 µl Sau3A; Lane 5 sample (unknown); Lane 6 T41A 3 µl uncut; Lane 7 + 2 µl Sau3A. PB2198 uncut gave a brighter band than T41A although the latter had been calculated to be more concentrated, therefore equal quantities of the two DNA's were used for restriction and insertion into cosmids.
2 µl of enzyme to 3 µl of target DNA were used.
Electrophoresis was in 0.8% agarose at 135V for 3 hours.

enzyme concentrations which would cut the DNA into reasonably large fragments.

The extent of cutting can be monitored by electrophoresis on agarose gels (0.8% agarose in borate buffer at pH 8.35).

Borate Buffer

10.8 g	Tris base
5.56 g	Boric acid
0.46 g	disodium EDTA
1000 ml	distilled water
pH = 8.35	

Bromophenol blue is used as tracking dye. Electrophoresis was usually for 3 hours at 135 v (35 mA).

Gels were stained with 1 μg ml^{-1} ethidium bromide, illuminated on a *Blak Ray* UV transilluminator screen and photographed through red and yellow filters using a Zenit-ΠL camera fitted with an extension tube.

Figure 6.3 shows the effect of different concentrations of Sau 3A on PB 2198 DNA and T41A DNA and also gives a visual comparison of the concentrations of T41A and PB 2198 DNA loaded in equal quantities.

The optimal conditions for hybridisation for cosmid packaging are a 2:1 ratio of vector to target DNA (Hohn and Collins, 1980).

(1) Restriction

(a) Vector

3 μg pHC 79 DNA (0.17 μg ml^{-1}) was restricted with Bam Hl (2 μl/5 μg DNA) for 15 minutes at 37°C in a total volume of 25 μl. Two samples were prepared.

(b) Target

1.5 µg of both PB 2198 (0.7 µg ml^{-1}) and T41A (1 µg ml^{-1}) were restricted with 1.0 µl of Sau 3A for 15 minutes at 37°C in a total volume of 25 µl. All reactions were stopped by heating to 65°C for 15 minutes.

(2) Ligation

One 25 µl aliquot of restricted vector was combined with 25 µl of restricted T41A, the other with 25 µl of restricted PB 2198, and ligated as follows:

50 µl of combined DNA's
20 µl ligation buffer
20 µl 5 mM ATP
3.0 µl DNA ligase (Sigma)
17.0 µl distilled water

then incubated at 14°C for 2 hours. The reaction was stopped by heating to 65°C for 15 minutes.

Ligation Buffer

150 mM Tris HCl at pH 8.1
20 mMCl_2
50 mM $(NH_4)_2SO_4$
6.0 mM EDTA
50 mM dithioethreitol
250 µg ml^{-1} bovine serum albumin

Following ligation, the DNA was concentrated by ethanol precipitation as follows:

to the 100 µl total ligation mixture were added:

10 µl 4 M sodium acetate at pH 5.5 and

200 µl 95% ethanol (at -20°C).

Stand overnight (or at -80°C for 1 hour).

The supernatant was carefully removed and discarded and the pellet resuspended in 50 µl of 70% ethanol at -20°C . At this temperature the salt dissolves but not the DNA.

The DNA was then pelleted for 10 minutes at 12,000 g and dried in a vacuum dessicator over phosphorus pentoxide.

The DNA was then dissolved in 10 µl of 10 mM Tris HCl at pH 8/ 1 mM EDTA.

(3) Packaging

This was carried out as described for λ DNA (Section 6.5.3) followed by infection to *lac* minus strain PB 2470 (prepared as described).

(4) Infection

100 µl packaged DNA

200 µl PB 2470 cells

Incubated 30 minutes at 32°C to adsorb "phage".

2 mls L medium added and incubated 2 hours at 32°C (with agitation).

(5) Plating

The cut site for Bam Hl in the cosmid is through the tetracycline resistance gene, the gene for ampicillin resistance remains. Cells which carry re-ligated cosmids (and hybrid DNA) will be ampicillin resistant and tetracycline sensitive. To confirm this, dilutions of infected PB 2470 were spread to L agar medium + 25 µg ml^{-1} ampicillin as follows: full strength, 10^{-1}, 10^{-2}, 10^{-3}.

Cells which carry functional *lac* genes will form typical red

coloured colonies on McConkeys Agar (BBL). McConkey plates with 1% lactose as an inducer + 25 μg ml^{-1} ampicillin (freshly prepared) were prepared and spread with diluted PB 2470 as just described. Plates were then incubated overnight at 37°C, but no sign of colony formation was detected.

This attempt at cosmid cloning was unsuccessful. Another attempt using a modified method of preparation of the "packaging mix" (Scalenghe *et al.*, 1981) was then tried which was reported to be x5 more efficient.

6.6 Freeze Thaw and Sonic Extract Method for Cosmid Packaging

(a) Freeze Thaw Lysate

The required culture strain PB 2159 was grown in 500 ml L medium at 32°C until cell density was 2 x 10^8 ml^{-1}. The culture was heat shocked (to induce phage) at 43°C for 15 minutes then incubated at 37°C for 2 hours to express phage genes. The culture was chilled and cells pelleted (3000 g for 10 minutes).

The pellet was resuspended in 1 ml of solution F.T. (see below) and 500 μl distributed to 2 x 1.5 ml Eppendorf centrifuge tubes. 30 μl of 2 μg ml^{-1} lysozyme solution was rapidly mixed in and the tubes frozen in liquid nitrogen.

Then the cells were thawed slowly to cause lysis. 125 μl of M buffer were added and the mixture centrifuged at 80,000 g in a Beckman L265B centrifuge for 25 minutes at 4°C to precipitate DNA. The supernatant was distributed as 100 μl aliquots and stored at -80°C.

Solution F.T.

10% sucrose

50 mM Tris HCl at pH 7.0

M Buffer

6 mM Tris HCl pH 7.5

20 mM $MgCl_2$

30 mM mercaptoethanol

30 mM Spermidine

60 mM Putrescine

15 mM ATP

(b) Sonic Extract Mix

500 mls of PB 2160 (the other strain carrying defective λ) was grown as described for the freeze thaw lysate.

The cell pellet was resuspended in 3 ml of S.E. Buffer (see below) and the suspension sonicated, using a Kontes sonicator set at 9 (on a 10 point scale) on ice with several 5 sec pulses without allowing foaming. When the suspension had cleared, debris was removed by centrifugation at 4000 g for 10 minutes.

0.6 ml of M buffer was added to the supernatant which was then distributed in 20 µl aliquots and frozen at -80°C.

S.E. Buffer

20 mM Tris HCl at pH 8.0

1 mM EDTA

3 mM $MgCl_2$

10 mM mercaptoethanol

The efficiency of removal of native λ DNA was assayed as described in Section 6.5.3 and found to be 100%.

6.7 Cosmid Cloning of T41A using the Freeze Thaw Lysate and Sonic Extract Method of Packaging

The procedure was carried out as described in Section 6.5.4 with the following modification to the restriction regime. In the previous attempt, 1.0 µl of Sau 3A was used to restrict the target DNA's - for this attempt the amount was increased to 1.5 µl of Sau 3A.

6.7.1 Packaging

The concentrated ligated hybrid DNA's were added to the two packaging mix preparations as follows:

20 µl freeze thaw lysate

5 µl sonic extract

10 µl hybrid DNA

Incubated 1 hour at room temperature.

200 µl phage buffer and 1 drop of chloroform added.

Phage Buffer

0.1 M KH_2PO_4

0.1 M $MgSO_4.7H_2O$

10 mg Bovine Serum Albumin

1 l distilled water

pH to 7.0 with 3M KOH

The packaged DNA was then infected to PB 2470 as before, and spread to L medium + 25 µg ml^{-1} ampicillin plates and McConkey plates + 1% lactose + 25 µg ml^{-1} ampicillin as follows:

(1) 0.2 ml, 0.1 ml } undiluted PB 2470

(2) 0.2 ml of 10^{-1} dilution of PB 2470 in L medium. All plating was done in duplicate and results are the mean of the two counts.

6.7.2 Results

TABLE 6.3 Colonies resulting from plating out PB 2470.

Plate	Dilution	T41A	PB 2198
		No. of colonies	No. of colonies
L + amp	10^{0} - 0.2 ml	810	435
	10^{0} - 0.1 ml	575	199
	10^{-1}- 0.2 ml	84	0
McConkeys	10^{0} - 0.2 ml	>800	453
+ 1% lactose	10^{0} - 0.1 ml	600	134
+ amp	10^{-1} - 0.2 ml	56	0

Colonies were replica plated onto L medium + 25 µg ml^{-1} tetracycline to confirm the presence of cosmids containing hybrid DNA.

No colonies were found on any of the L + tetracycline plates, suggesting cosmids containing hybrid DNA had been successfully taken up by the recipient strain. The uptake of DNA was calculated as

T41A - 9.6×10^{3} colonies per µg DNA

E coli strain

PB 2198 - 8.2×10^{3} colonies per µg DNA.

It has been reported (Hohn and Collins, 1980) that 1.2×10^{2} cosmid clones would cover the entire *E. coli* genome. No colonies were pink on the McConkeys agar which means there was no successful cloning and

expression of *lac* genes from either the thermophilic donor or the *E. coli* control.

6.8 Attempts to Detect a T41A Hybrid Cosmid Clone with β-galactosidase Activity

A. Colonies from L + ampicillin plates were replica plated onto L + ampicillin + 1% lactose plates and incubated as follows:

(1) 37°C overnight for *E. Coli* clones.

(2) 37°C overnight followed by incubation at

45°C for (i) 1 hour
(ii) 2 hours
(iii) 4 hours

70°C for (i) 1 hour
(ii) 2 hours
(iii) 4 hours

for T41A clones.

Following incubation, a glass filter (Whatman Gf/A - glass fibre) impregnated with 5 mM ONPG in McIlvaines buffer at pH 6 was applied to the surface of the plate and incubated for 30 minutes at 70°C; 2 mls of 0.5 M Na_2CO_3 was added but there was no sign of ONPG hydrolysis.

B. Colonies were replica plated onto L + ampicillin plates and all colonies scraped from the surface with a sterile inoculating loop and added to 0.5% lactose + minimal salts medium (Miller, 1972). There was no growth on this medium.

C. Colonies were replica plated onto L medium + 1% lactose + 25 μg ml^{-1} ampicillin.

The colonies all grew and were assayed for β-galactosidase as follows:

All colonies were scraped from a plate and resuspended in 5 mls of McIlvaines buffer at pH 6 for T41A, and 5 mls of buffer pH 7.0 for PB 2198 clones. 0.5 ml aliquots were assayed as described in Section 6.1. Following the addition of ONPG incubations were as follows:

(1) PB 2198 clones at 25°C

(2) T41A clones at 70°C

After 30 minutes incubation 2.5 ml of 1 M Na_2CO_3 was added but all results were negative.

D. As galactose is a better inducer of T41A β-galactosidase than lactose the procedure described in C. was repeated for T41A clones by adding 0.5% galactose instead of lactose to the medium used for replica plating.

Results were again negative.

6.9 Discussion

The results presented suggest that the Freeze-Thaw/Sonic Extract method of preparation of packaging mix is more efficient than UV irradiation. The native phage DNA was successfully destroyed by this method also, as "phage" were only formed after the addition of DNA.

The transfer of T41A DNA and PB 2198 DNA using the cosmid pHc 79 was successful as determined from expression of the two antibiotic markers, i.e. the colonies were both ampicillin resistant and tetracycline sensitive. However there was no evidence of *lac* gene

expression. There are several possible explanations for this:

(1) the infection was due to religated cosmids only.

(2) The infection was due to cosmids containing religated "tandem" noncontinuous inserts of DNA, i.e. not continuous sequences of whole genes.

(3) The infection was due to a combination of both (1) and (2).

These possibilities have been suggested as reasons for the lack of success often found using cosmid vectors for DNA cloning. (Ish-Horowicz and Burke, 1981).

A method for overcoming these problems by using cosmid pJB8 which is cut with two restriction enzymes, (Hind III and Bam Hl) and then dephosphorylated to prevent self-ligation, has been described (Ish-Horowicz and Burke, 1981). It was proposed to use this vector to attempt to clone T41A DNA. The target DNA is also cut with Sau 3A then dephosphorylated to prevent the formation of "tandem" pieces of DNA.

The enzyme used for dephosphorylation was Bovine alkaline phosphatase (B.A.P.), (Sigma). Unfortunately, it seemed that this preparation was contaminated by DNA-ase, as repeated attempts to dephosphorylate and religate a number of different DNAs and transform competent *E. coli* strain PB 1427 were unsuccessful. The DNA-ase could not be removed by boiling, as B.A.P. is denatured at 70°C, and further attempts were discontinued until a supply of uncontaminated B.A.P. was available.

The only method for unequivocally determining whether target DNA's had been successfully transferred to recipient strain PB 2470 in the absence of *lac* expression would be to use a radioactive probe. At this time no such probe was available for either PB 2198 or T41A.

It seems likely that plasmids containing cosmid target DNA hybrids were transferred, but that random religation prevented the transfer of functional genes.

Although gene expression had not been demonstrated it was felt that there was considerable potential for investigating thermophile genetics using recombinant DNA techniques and these investigations are continuing at Auckland University in association with the research group at Waikato University.

CHAPTER 7

SOME PRELIMINARY INVESTIGATIONS OF THE GENETICS OF *Sulfolobus acidocaldarius*

7.1 Introduction

Members of the archaebacteria seem to occupy an evolutionary position between eubacteria and eukaryotes and it has been suggested that their metabolism is not as well regulated as that of members of the other two kingdoms (Zillig *et al.*, in press). If the archaebacteria have retained a relatively primitive state of organisation, investigations of their genome structure and its control could be extremely valuable in providing insights into the way highly adapted enzyme systems have evolved, including mechanisms of thermophily.

Plasmids and phage have been extremely useful for studying the genetics of eubacteria (Lewin, 1977) and could prove equally valuable for studying those of archaebacteria. To date only a few plasmids and phage have been reported in association with archaebacteria. Phages have been reported from the extreme halophile *Halobacterium halobium*, (Schnabel *et al.*, 1982) and the anaerobic sulphur-metaboliser *Thermoproteus tenax* (Janekovic *et al.*, 1983). Plasmids have been described from a methanogen (Thomm *et al.*, 1983) and from a strain of *Sulfolobus* (Yeats *et al.*, 1982).

In vitro transcription systems which result in synthesis of defined products from defined DNA reading units are a very powerful method of determining gene structure and organisation, and an archaebacterial phage would provide a very satisfactory template for such a system. DNA-dependent RNA polymerases require species specific conditions, i.e.

the enzyme from *H. halobium* requires the presence of 6M NaCl for stability and that from *T. tenax* strictly anaerobic conditions. Such requirements cause considerable difficulties in the practical development of *in vitro* systems.

DNA-dependent RNA polymerase has been isolated from *Sulfolobus acidocaldarius* and found to transcribe *H. halobium* ϕH phage DNA, or poly (d (AT) - d (A - T)) very efficiently at 75°C and pH 8.5 (Zillig *et al.*, 1979). All operations can be done in air in a normal ionic environment. However, the absence of a *Sulfolobus* phage prevented exact determination of *in vivo* conditions.

The following work was carried out at the Max-Planck-Institut für Biochemie, Munich under the guidance of Professor W. Zillig.

During the course of electron microscopic investigations into the properties of a UV-inducible plasmid associated with *S. acidocaldarius* stain Bl2, (Yeats *et al.*, 1982) it was observed that cell-to-cell connections were occasionally found, but only after UV induction. It seemed probable that a bacteriophage was involved in these connections particularly as a copy of the plasmid appeared to be integrated into the host genome. It was decided to attempt to induce phage from this strain as it would be interesting in its own right and also to act as a template for *in vitro* transcriptions.

7.2 Screening for a Bacteriophage associated with *Sulfolobus acidocaldarius* strain Bl2.

It had been observed by previous investigators that "spikes" were occasionally seen projecting from the cell walls of *Sulfolobus* Bl2 after UV induction of the plasmid. Attempts had been made to achieve the maximum number of these spikes with the hopes of obtaining a pure

preparation for investigation, as it was felt they could be virus particles. Previous attempts to produce spikes had involved UV irradiation of cells in early log phase (the best time for maximum amplification of plasmid - Yeats *et al.*, 1982) under a variety of growth conditions.

Sulfolobus B12 was normally grown heterotrophically on a variation of the medium described by Brock (Brock *et al.*, 1972). The basal salts were as described by Brock with 0.1% Yeast Extract (Merck) as carbon source and 0.2% sucrose (Merck) added principally for buffering. The pH was adjusted to pH 3 after autoclaving and the incubation temperature was 80°C. The cells were grown as 5 ml in 50 ml boiling tubes fitted with metal caps in a New Brunswick gyrotory shaker (Model G76) filled with glycerol. Large batches were grown as 400 ml in 1 litre Erlenmeyer flasks under the same conditions.

The variations attempted in previous experiments were principally alterations of carbon source, pH and temperature (S. Yeats, pers. comm.).

It was decided to investigate UV induction further and then proceed with other types of phage induction (e.g. other mutagens, cold and heat shock treatments).

Liquid nitrogen frozen stocks were used and maintained throughout these investigations. This involved growing 10 ml of culture in a 100 ml Erlenmyer flask (with an extended neck) fitted with a metal cap under the conditions just described. Cells were allowed to reach stationary phase (absorbance $\approx$ 1.3 measured at 600 nm - see Figure 7.1) then cooled to room temperature and the pH adjusted to pH 5.6 (Merck non-bleaching Neutralit pH papers) with solid $CaCO_3$. Cells were harvested by centrifugation for 10 minutes at 2000 g in a bench top centrifuge, then resuspended in fresh medium at x10 concentration. 20% sterile glycerol was added as cryoprotectant and 0.5 ml aliquots stored

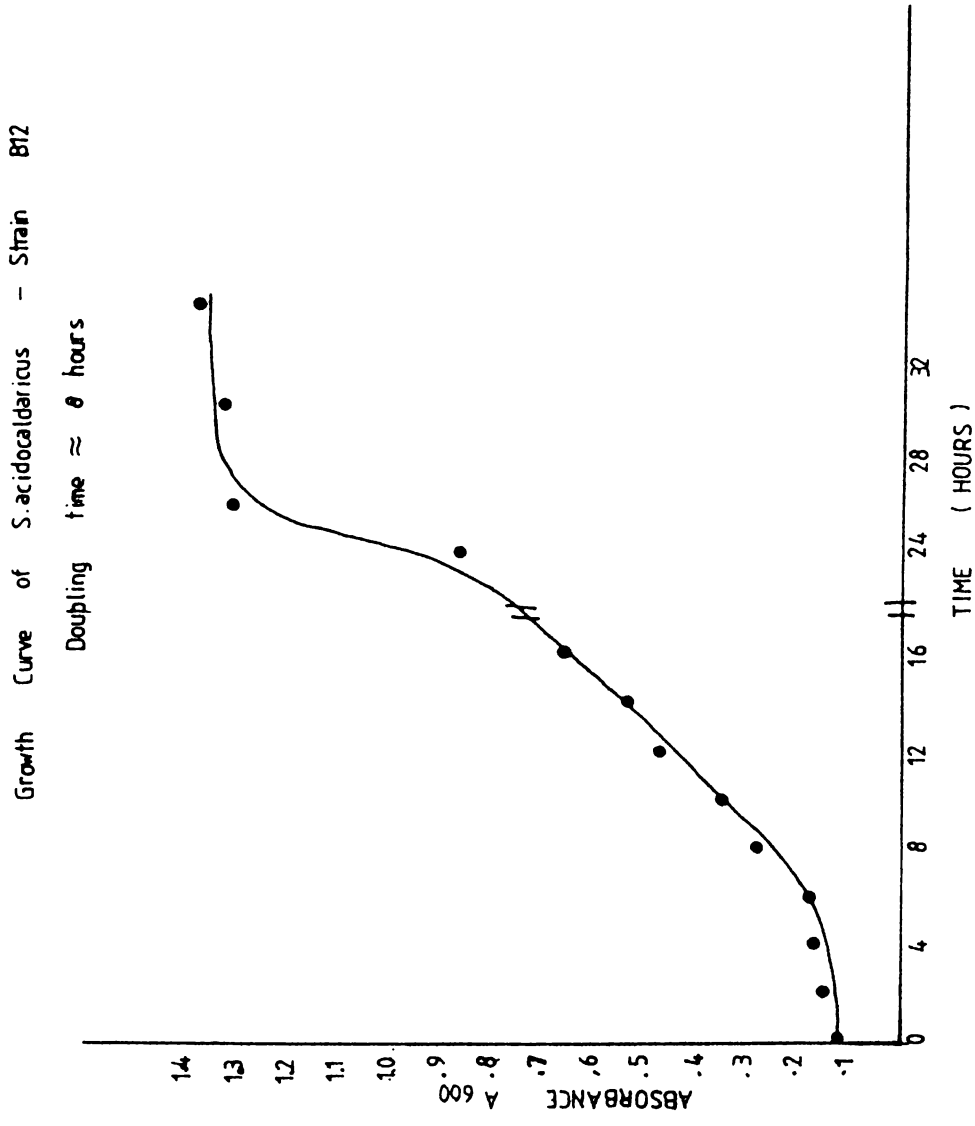


Figure 7.1 Growth Curve of *S. acidocaldarius* - B12 grown at 80°C at pH 3.5.
Generation time was calculated as 8.5 hours.
The formula used being $1/k = \frac{\log_{10} x_t - \log_{10} x_0}{0.301t}$

$x_0 = A_{600}$ at 10 hours

$t = 5$ hours

$x_t = A_{600}$ at 15 hours

in 1.5 ml plastic tubes in liquid nitrogen. Frozen stocks have remained viable for at least a year.

A culture of *Sulfolobus* B12 was grown to determine doubling time which was found to be 8.5 hours (see Figure 7.1) and to investigate the cells at different phases of growth by electron microscopy. All electron microscopy was carried out using an ELM1 1 electron microscope. Cells were negatively stained with uranyl formate on 1 or 2% collodion coated copper grids covered with an ultra-thin carbon film.

There were no spike-like projections seen on non-irradiated cells harvested at any growth stage, the only notable feature was that cells seemed to form large clumps (of up to 20 cells) and also to stain very much darker in stationary phase.

The UV regime which had usually been found to produce a few cells with spikes, was to irradiate 5 ml of early log phase cells in a plastic Petri dish (depth $\sim$0.8 mm) using a Hanua Sterisol f mercury lamp at a setting giving 25 ergs mm^{-2} per irradiation, continually agitating the cells during irradiation. Following irradiation, cells were returned to the culture vessel and grown for a further 6 hours before harvest. Cells were spun down at 2000 g for 10 minutes in a bench top centrifuge and resuspended at x10 concentration in the salt base of the growth medium which had been filtered through a 0.22μm Millipore filter.

7.2.1 Attempts to Induce Phage by varying UV conditions

It was decided to attempt a variety of UV dose intensities. The usual method delivered a dose of 25 ergs mm^{-1} by irradiating at a height of 40 cms for 10 seconds. Cells in early log phase ($A_{600} \approx 0.2$) were irradiated for 10, 20, 30 and 40 seconds, grown for 6 hours, harvested and examined. There were a few spikes on the cells irradiated for 20 seconds and the cells irradiated for 30 and 40 seconds had nearly all lysed.

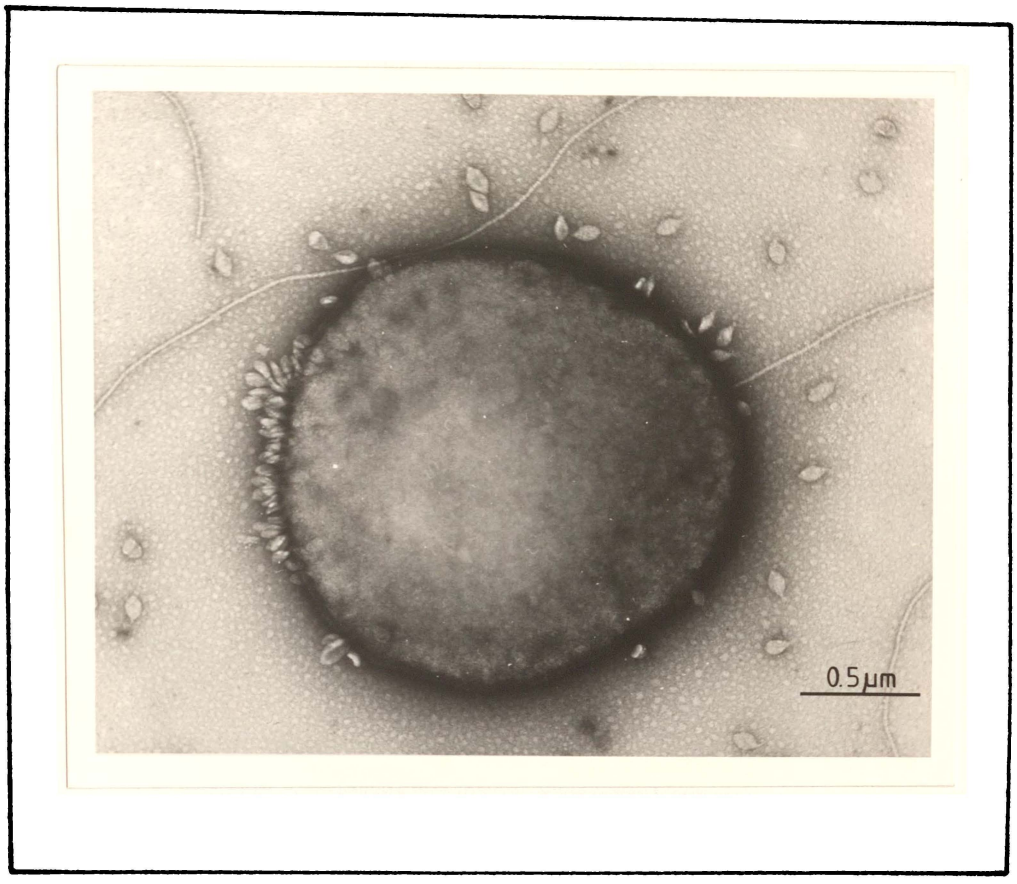


Figure 7.2 *Sulfolobus* -B12 cells surrounded by lemon-shaped particles with a "tail" at one pole. Particles are both clustered around the cell (later determined to being extruded from it) and free outside the cell.
Cells were washed in the filtered salt base of the growth medium and applied to 2% collodian coated 300 mesh copper grids which had been coated with a thin film of vaporised carbon. Contact was for 5 minutes after which grids were washed in filtered double distilled water. Cells were negatively stained in 20 mg ml^{-1} aqueous uranyl formate for 10 minutes, excess stain was removed with filter paper and the speciment coated with a thin carbon film. Grids were examined for the presence of virus under a Siemens Elmiskop 102 electron microscope at 80kv.

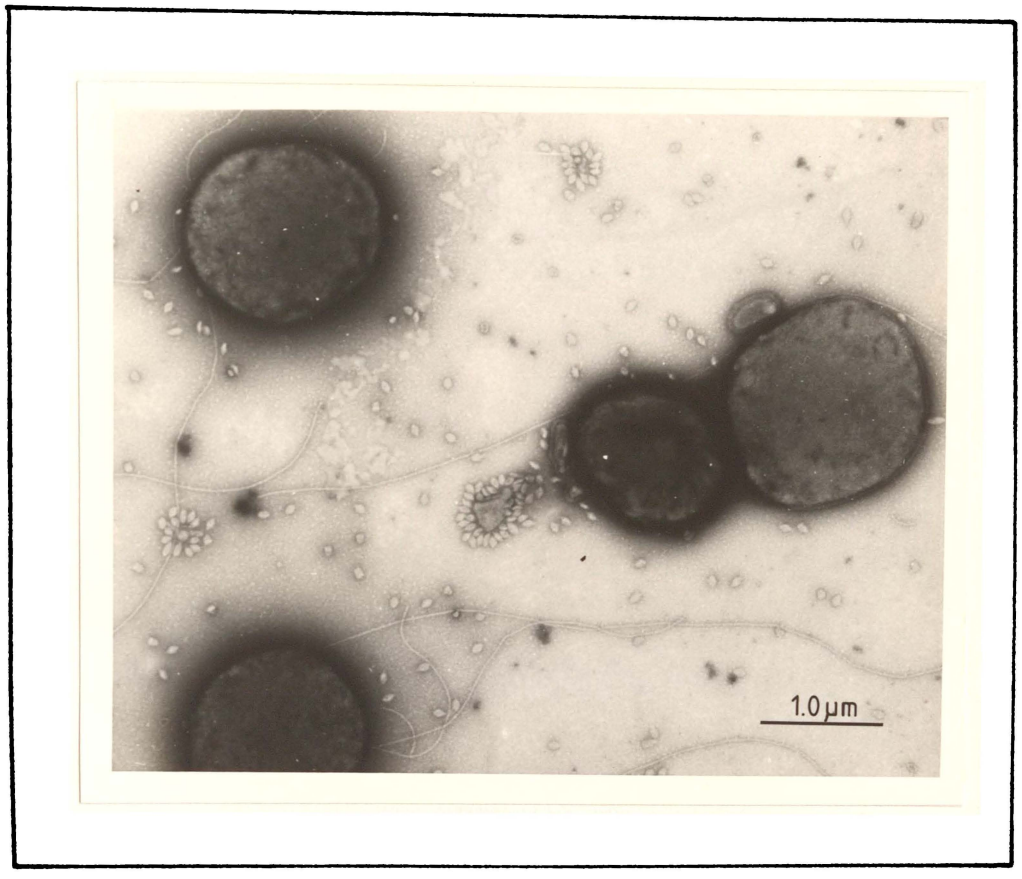


Figure 7.3 Virus SAV 1 can be seen both as free particles and attached to what appear to be cell fragments. These characteristic "sunflowers" appeared in all irradiated cultures in which virus was induced.

It was then decided to give more than one dose of UV at different time intervals. Cells were irradiated,

(1) twice for 10 seconds at 90 minute intervals and grown 6 hours after the second irradiation.

(2) Three x10 second irradiations at 90 minute intervals.

(3) Two x10 second irradiations at 6 hourly intervals then grown 6 hours after the second irradiation.

(4) One 25 second irradiation. (early log phase)

There were no spikes in (1) or (2) but quite a few in (3) and many in (4).

It was decided to repeat (3) and (4) again. On this occasion there were no spikes on the cells given the 2 x10 second irradiation, but the highest number of "spike-cells" yet seen, and also what appeared to be free spikes, were visible in the culture irradiated for 25 seconds (see Figure 7.2). This culture had also been allowed to reach an A_{600} of 0.29 before irradiation, somewhat higher than the usual A_{600} 0.19 - 0.22.

It was then decided to attempt the 25-second irradiation regime on cells at a variety of growth stages. It was also decided to harvest all cells at approximately the same time. This meant the mid-log phase cells were grown for 12 hours after irradiation. These cells had a number of spikes, there were a very large number of free lemon shaped particles and also particles attached to what appeared to be cell fragments (see Figure 7.3).

This regime was repeated and the maximum number of particles was found to be produced after 2.5 seconds irradiation (probably 50 erg mm^{-1} dosage) at A_{600} 0.5 - 0.7 followed by a further 20 hours growth.

E.M. examination of the supernatant after removal of the cells revealed many lemon-shaped particles were present in this fraction. It was decided to purify and concentrate these particles to determine whether they were in fact virus.

7.2.2 Identification of Phage

A large (400 ml) batch of culture was irradiated as 5 ml aliquots under the conditions just described, grown for a further 20 hours and cells removed by 10 minutes centrifugation at 3000 g in a Sorvall RC-5B centrifuge. The supernatant from this spin was layered as 25 ml aliquots onto a 2 ml layer of 30% sucrose (in the salt base of the medium) and spun at 36,000 g in a J-21 centrifuge for 2 hours. 2 ml aliquots of the layer above the sucrose cushion were carefully removed with a pipette and each fraction examined under the E.M. for the presence of virus. One fraction was found to be very enriched with mostly free particles (330 per field at 10,000 x magnification). The other enriched fraction (which spun lower in the gradient) and contained 214 particles per field, consisted largely of particles clustered around what appeared to be cell fragments.

To investigate the nature of these particles it was decided to attempt to extract DNA and recover proteins from the following fractions:

(a) The cells harvested by low speed centrifugation.

(b) The virus-free upper fraction from the high speed gradient spun onto the sucrose cushion.

(c) The free virus fraction (which contained 330 particles per field).

(d) The fraction which contained virus attached to particles.

0.25 ml of cell pellet was resuspended in 1 ml of TEN buffer at pH 7.5 (10 mM Tris HCl, 1 mM EDTA, 100 mM NaCl), and the cells lysed by addition of 50µl sarcosine.

The other three fractions were treated with 12 µl ml^{-1} sarcosine. All four fractions were extracted twice with an equal volume of re-distilled phenol (at pH 7.5), the aqueous layer being separated by 10 minutes centrifugation at 2000 g in a bench top centrifuge. All phenol fractions were kept for subsequent recovery of proteins. Any phenol and remaining proteins were then removed from the aqueous layer by extraction with 9:1 chloroform:octanol followed by two more extractions with chloroform. The DNA was precipitated by the addition of 100 µl 3 M sodium acetate per ml and an equal volume of absolute ethanol and chilled overnight at -20°C. DNA was spun down for 15 minutes at 36,000 g in a J-21 centrifuge at 0°C. The precipitate was dried in a desiccator and resuspended in 100 µg of boiled TE buffer at 7.5 (10 mM Tris HCl, 1 mM EDTA). 20 µl samples were digested with the restriction endonuclease ECO Rl and analysed on 1% agarose gels in Loening's buffer (Loening, 1969). DNA was visualised after electrophoresis by staining with ethidium bromide (5 µg ml^{-1}) and photographed under UV light.

The results showed the cells were plasmid enriched and that fraction (c) appeared to be a partial digest of plasmid DNA. Very faint bands appeared in fraction (d).

Proteins were then recovered from the phenol fraction. Four volumes of absolute ethanol were added to the combined phenol fraction for each of the four samples and left overnight at room temperature to precipitate protein. The precipitate was harvested after 30 minutes centrifugation at 36,000 g in a J-21 centrifuge at 15°C. The precipitate was washed twice with 95% acetone in water. After the final centrifugation the precipitate was resuspended in 25 µl of Laemmli

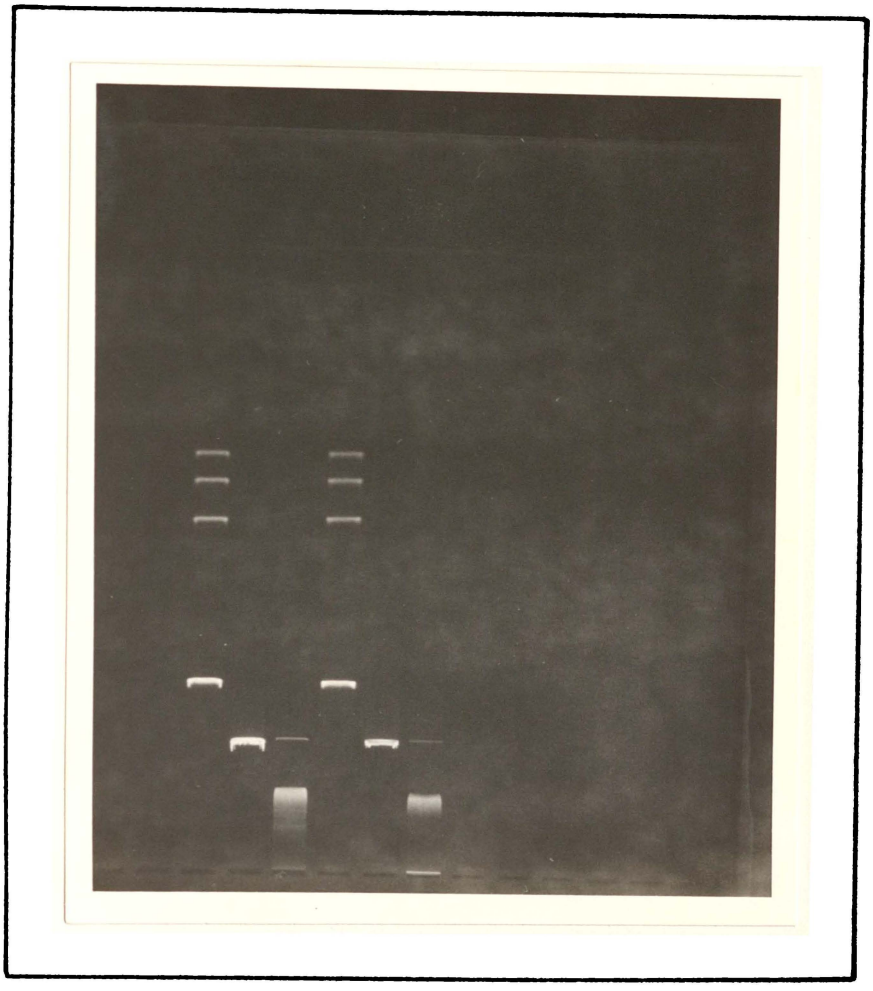


Figure 7.4 A comparison of SAV 1 DNA with plasmid DNA extracted from *S. acidocaldarius*-B12.
Lane 1 plasmid cut with EcoR1, Lane 2 plasmid cut with BamH1; Lane 3 plasmid uncut; Lane 4 virus cut with EcoR1; Lane 5 virus cut with BamH1; Lane 6 virus uncut. The two are clearly identical.
The circular nature of the viral DNA was later confirmed.
Electrophoresis was in 1% agarose.

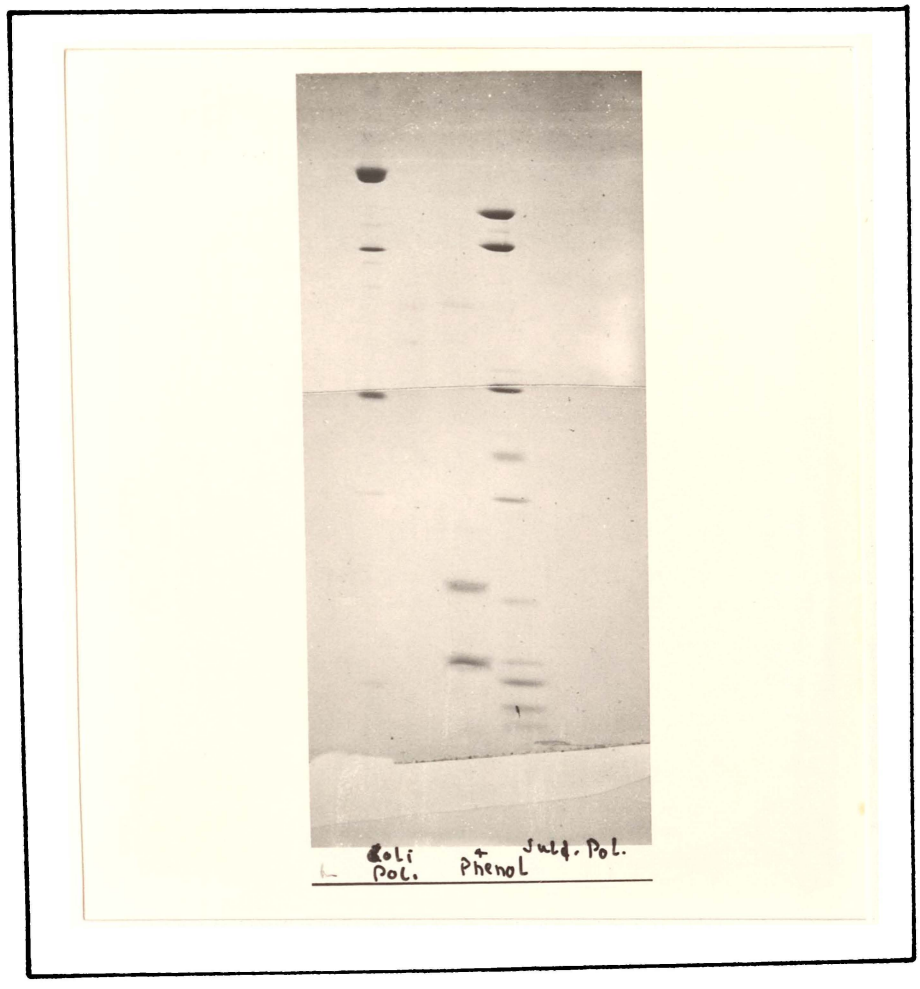


Figure 7.5 Proteins recovered from the phenol phase after extraction of DNA from purified SAV 1 virus. Lane 1 *E. coli* RNA polymerase; Lane 2 SAV 1 proteins; Lane 3 *Sulfolobus* polymerase. The polymerases were run as standards. Two proteins bands appear to have been present in the phenol fraction. Their molecular weights were estimated as being approximately 12 000 and 14 000 daltons. Electrophoresis was carried out overnight in 5 - 25% gradient polyacrylamide gels by the method of Laemmli (Laemmli, 1970).

sample buffer (Laemmli, 1970) at pH 7.0. The proteins were separated on 25% - 5% gradient Laemmli gels at pH 8.8 (Zillig *et al.*, 1978). Although there were many bands present in the cell fraction there was only a suggestion of very faint bands in fraction (c).

It was decided that more efficient concentration of virus was required and a caesium chloride gradient was set up to purify another sample of virus.

After removal of the cells the supernatant was applied in 3 ml aliquots to a stepwise caesium chloride gradient (densities 1.22, 1.38, 1.55 and 1.77) in cellulose nitrate centrifuge tubes. The solution was spun for 12 hours at 80,000 g in an SW 27 rotor in a Beckmann ultracentrifuge. Two clearly visible bands were seen, one, which was very sharp, was found (on EM investigation) to consist of free virus particles, the second lower and wider band was a mixture of free and "attached" particles.

These bands were removed from the gradient using a syringe inserted through the side of the tube - the density of the upper "pure" virus layer was estimated as ∿1.24. DNA extracted from this layer was compared electrophoretically to pure plasmid DNA (prepared by S. Yeats) being digested with both Eco Rl and Bam Hl restriction endonucleases. The results can be seen in Figure 7.4. Lanes 1, 2, and 3 are plasmid DNA uncut, Bam Hl cut and Eco Rl cut. Lanes 3, 4 and 5 are virus DNA subject to the same sequence of treatments. It is clear the two are identical.

Gradient gel electrophoresis of proteins recovered from the phenol phase suggested at least two proteins were present (Figure 7.5). Later work by W.D. Reiter (Martin *et al.*, 1984) using larger volumes has revealed that at least six proteins are present, two of which seem to be hydrophobic and two associated with other constituents, probably carbohydrate (W. Zillig, pers. comm.).

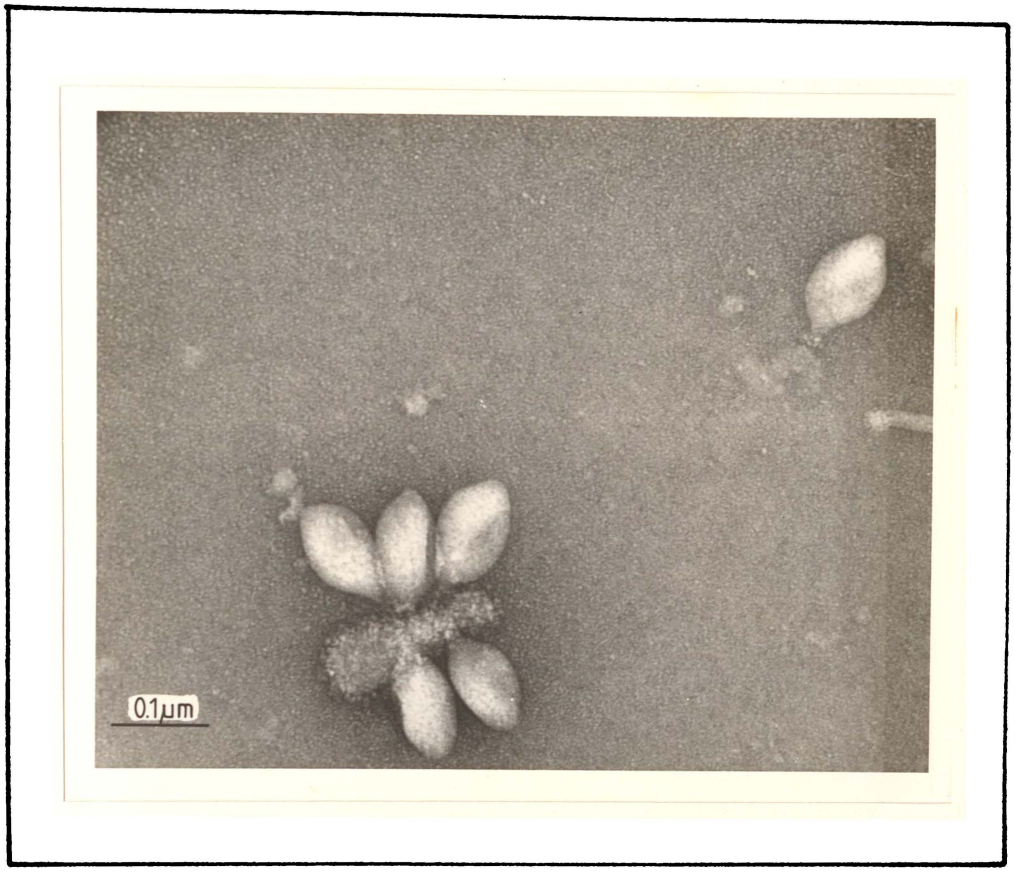


Figure 7.6 Enlargement of SAV 1 virus to examine the "tail" and attachment to particles.
It appears that bundles of fibres extend from the "tail" but this magnification (80 000) is beyond the resolving power of this microscope.
The attachment of particles to the cell wall fragments is clearly by the tail of the virus particle.

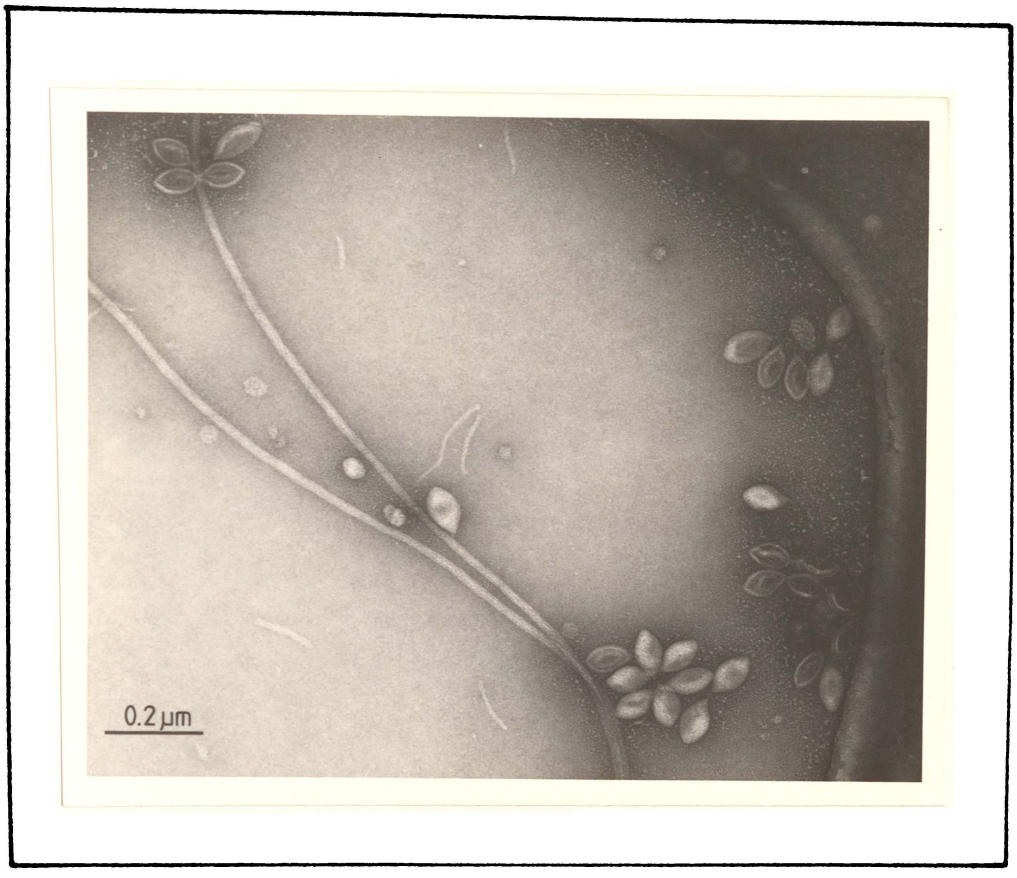


Figure 7.7 SAV 1 virus which appear to have discharged their DNA.

It seemed clear that the particles were definitely virus, with a distinct protein complement and a DNA fraction identical to the plasmid discovered by Yeats *et al.* (1982).

7.3 Structure of Virus

The virus particles were measured at 100 x 60 nm, lemon shaped, and probably carrying tail structures at one pole. It was attempted to determine the fine structure by EM investigation but this proved beyond the resolution capabilities of the instrument. Figure 7.6 shows a x240,000 magnification, where the presence of a tail is clear but its fine structure is not. The tail is obviously the attachment site from the orientation of particles around a particle seen in this figure. Figure 7.7 shows that some attached particles appear to be "empty". It is possible that these have attached to receptors in the wall (and perhaps flagella as shown), and ejected their DNA. This remains purely speculative at present.

The electrophoresis of DNA revealed the virus contained the same DNA as the plasmid induced by Yeats *et al.* (1982). This seemed to indicate that the virus contained circular d.s. DNA. This possibility was investigated by S. Yeats (Martin *et al.*, 1984) and confirmed by heating and chilling DNA samples.

The virus contains at least six proteins, also polysaccharides and lipids (demonstrated by thin layer chromatography). It seems probable the coat proteins are surrounded by a lipid membrane (Martin *et al.*, 1984).

7.4 Production of Virus and Host Infection

A feature of *Sulfolobus* B 12 after UV induction to induce phage, was that under conditions which lead to virus production lysis did not

occur. A time course of virus production was carried out and the optical density seemed to slow down during maximum virus production but to recover and increase after this. This experiment was repeated in detail by W.D. Reiter (Martin *et al.*, 1984) and it was found that virus began to protrude through the cell wall about 4 hours after induction. Liberation of virus continued to a maximum value ($\sim 3 \times 10^{10}$ phage ml^{-1} culture) after about 15 hours. Cell growth was severely retarded during virus extrusion but recovered later. It was also found that virus production can be UV induced again from subculturing, indicating the cells resumed their lysogenic state.

Examination of non-irradiated cells established that a few virus particles are produced, although these were not recognised as such when examining cells for spikes (Section 7.2).

All efforts to infect either *Sulfolobus* B 12 or other strains proved unsuccessful. On one occasion, *Sulfolobus acidocaldarius* strain DSM 639 appeared to lyse 14 hours after the addition of virus. This experiment was not able to be repeated and it has now been decided to attempt to find a host by isolation of more *Sulfolobus* strains from the wild.

Both fine details of virus structure and successful infection systems are the subject of continuing research at the Max-Planck Institute. As yet a completely satisfactory *in vitro* transcription system using this virus as DNA-template has not yet been established but this also is under investigation at present. A publication reporting this work is included in this thesis.

7.5 Attempts to Find Suitable Genetic Markers in *Sulfolobus*

The use of starch to solidify media for growth of *S. acidocaldarius* on plates had been reported (Yeats *et al.*, 1982) but growth on solid medium had ceased to occur. It was decided to carry out further

attempts to grow a number of strains of *S. acidocaldarius* on solid medium and to screen for genetic markers which would be useful for future work. In eubacterial genetics, antibiotic resistance markers have been extremely useful for determining required clones in recombinant DNA experiments. Archaebacteria are resistant to most antibiotics to which eubacteria are susceptible, with the additional problem for *S. acidocaldarius* of growth requirements for high temperature and low pH.

Professor Zillig had achieved success in growing *Thermoproteus tenax* on solid medium at 88°C by the use of a soft overlay technique, which presumably helped prevent dehydration.

The medium used was the same as for growth of liquid cultures with starch (Merck No. 1252) used as gelling agent. For plates, 12% starch (w/v) was added to the basal salts and heated over boiling water until clear and thick. Yeast extract and sucrose were added and the pH adjusted to pH 3 - 3.5 (Merck Acilit non-bleaching papers) with 1 M H_2SO_4. Plates were poured at ∿25 mls and allowed to set overnight at room temperature (plates were stored up to 1 week at 4°C).

The soft starch overlay was made using the same medium with 10% starch. It was found if the overlay was allowed to gel and then remelted before adding the inoculum setting was far quicker. The overlay was made up in 100 ml quantities as described previously and dispensed as 2 ml aliquots into sterile test tubes. When required tubes were melted in boiling water and cooled to 70 - 75°C in a waterbath. Inocula were added (as 0.5 ml from dilution series) mixed by either hand rotation or a rapid vortex, then spread over the surface of plates (which had been dried for a few minutes at 65°C and were still warm). Plates were left upright for approximately fifteen minutes to allow overlay to set then incubated in stacks of at least 6 plates, inverted

and sealed in plastic bags, at $80^{o}C$.

Dilution series of the following strains (from 10^{o} - 10^{6}) were plated out as described:

B12

B 9

B 6

B 6.1

All isolates from Beppu hot springs, Japan and strain DSM 639 (Brock, 1978; Strain 98-3).

After three days there were many colonies on all DSM 639 plates. There were three distinct morphological types:

(a) "fried egg' types

(b) small yellow brown

(c) large yellow brown.

After five days colonies of B12, B6, B6.1 appeared - there were two morphological types of these strains

(a) small yellow brown

(b) large yellow brown

Further attempts eventually produced the same results with strain B9.

Even "large" colonies were only 2 mm in diameter so counting was done under a binocular microscope. Colonies were cut out of the starch (under the microscope) using a fine syringe needle attached to a 1 ml pipette. A single colony innoculated into 5 ml of liquid medium grew to visible turbidity in around 24 hours.

As reported (Yeats *et al.*, 1982) colony size seemed to be heterogeneous and the "fried egg" appearance i.e. due to a clear zone, occurred from time to time in all strains tested.

Virus could be induced from both types of colony of B12 (tested once). Time did not allow extensive screening of a large number of B12 colonies for the presence of virus and it is hoped that further attempts will give rise to a cured cell line. No virus was ever induced from strain DSM 639.

However, on one plate of DSM 639 a few colonies were noted which were surrounded by a zone of clearance. Subculture continued this type of colony which was assumed to be a cell line producing an extracellular amylase able to break down the starch. It is hoped to investigate this further.

Strain B6 is able to grow on lactose as carbon source so it was decided to attempt to find cells with defective lac genes by alternate replica plating onto sucrose and lactose as sole carbon source (i.e. without yeast extract). In all attempts all colonies grew on both substrates - it may be necessary to use mutagenic agents to produce lac minus strains.

Strain B6-2 grows autotrophically on sulphur in 95% air + 5% CO_2 gas phase. It was decided to attempt to grow this strain on solid medium. Plates were made up with the omission of carbon sources as before. The top starch layer had 1.5% sublimed sulphur added and was sonicated for approximately 2 minutes before dispensing the soft starch into tubes. Plating was carried out as previously described and the plates introduced into an anaerobic jar and flushed with a mixture of 95% air + 5% CO_2. After incubation for 1 hour the pressure was checked to ensure it was not too high. It was found that the pressure was not maintained, probably due to a defective 'O' ring. Time did not permit this attempt to be repeated but there seems no reason to doubt that autotrophic strains can be grown successfully on plates.

7.6 Conclusions

Although much work remains to be done on the genetics of *S. acidocaldarius*, the ability to induce virus from strain B12 and to grow all heterotrophic strains tested on plates provides a potentially excellent system for studying archaebacterial genetics and possible adaptations required for life in such an extreme environment.

The unusual properties of SAV 1 virus, i.e. its closed circut ds DNA, its temperate and UV inducible nature with site - specific host intergration (Yeats *et al.*, 1982) and its apparent outer lipid membrane make it a most interesting and unusual virus.

Attempts to grow *Sulfolobus* on solid medium were very successful and it is hoped there will be no further problems with this in future. Using the method of sealing plates in plastic bags (those supplied with empty petri dishes) it was possible to grow all strains of *Sulfolobus* without a soft overlay. However colony counts showed this to be only about 60% efficient. The overlay technique was 90 - 100% efficient in producing colonies as determined by direct cell count.

Colony morphology does not seem to be a useful genetic marker. The only potentially useful marker obtained was the amylase-producing cell line of strain DSM 639. Time did not permit further work on screening for genetic markers.

CHAPTER 8

GENERAL CONCLUSIONS

The aim of this study was to attempt to obtain taxonomic groupings of a number of different genera of extreme thermophiles from both the eubacterial and archaebacterial kingdoms, using the technique of pyrolysis mass spectrometry. Few attempts have been made to classify these bacteria, some of which have been isolated only within the last three years. Although a limited number of type species were available as reference strains, it was hoped that successful PY-MS discrimination of these strains would enable conclusions to be made as to the taxonomic status of New Zealand isolates.

Thermus strains, which have been reported to show considerable variability possibly as a result of unstable genomes (Cometta *et al.*, 1982 a &b) were found to form clusters apparently unrelated to sample source location, pH or temperature. Two isolates from the same water sample did not show a close similarity so it seems that the PY-MS findings also suggest a high degree of variability.

However the *Thermus* strains formed definite and stable groupings, with most of the New Zealand isolates belonging to a single, probably species-level group which was different from the groups containing the three reference strains, *Thermus aquaticus*, *Thermus thermophilus* and 'Ramaley's XI'. The two named *Thermus* species have been reported as being very similar (Degryse *et al.*, 1978) and although the PY-MS taxonomy given in Figure 3.7 suggests that separate species status is probably justified for these two strains, it is clear they are more closely related to each other than either is to the majority of the New Zealand isolates.

A number of *Bacillus*-like strains grown at both neutral and acidic pH were also analysed but only two of the ten reference strains prepared were able to be included in the statistical processing due to technical problems which occurred with the PYROMASS 8-80 during the course of this study. The classification obtained for the neutral pH strains seemed to support the classification obtained by classical numerical taxonomy by Wolf and Sharp (1981).

The pattern of cluster groupings obtained from the analysis of both types of *Bacillus*-like strains did not show any apparent relationship to the source parameters which were measured.

It has been reported that pyrolysis techniques are extremely sensitive to even small changes in culture conditions (Oxborrow *et al.*, 1977) but that under identical conditions a number of different genera and species can be successfully discriminated (MacFie and Gutteridge, 1982). It seems that if bacteria are sufficiently different then discrimination of types can be achieved even when culture conditions are not the same. When the data relating to both *Bacillus*-types was processed there was good discrimination of both types. This analysis, however, revealed one of the inherent problems with currently used methods for processing PY-MS data, that of instability caused by outliers. Outliers are samples which cluster at very low % similarities and can seriously compress more similar samples together. Canonical variates analysis is extremely sensitive to outliers (MacFie and Gutteridge, 1982) which also affect the cluster groupings of those strains with % similarities lower than those of the majority of group members.

The analysis of archaebacteria revealed the difference between *T. tenax* and apparently similar strains isolated in New Zealand.

This is not surprising in view of the higher pH optimum of the New Zealand strains, their ability to colonise pools of higher pH and their inability to use glucose as a carbon source.

The taxonomic status of the New Zealand isolate AN1 remains unclear. Although it is an archaebacterial coccus with a low but obligate requirement for Na^+ it does not appear closely related to any of the sulphur-metabolising cocci described to date.

The presence of relatively large masses associated with the sulphur "series" of m/z peaks was of interest as it was shown by all the sulphur-metabolising groups and the methanogens, including strains which had been grown in media which did not contain sulphur. These bacteria may have an unusual sulphur metabolism. This feature was not shown by the halophiles.

Although the attempt to classify all the archaebacteria analysed (Figure 4.16) had only a limited success, there was sufficiently good discrimination to suggest that with further refinements in data processing this type of exercise will be possible using PY-MS.

The problems with the attempt to compare the N.Z. *Thermus* strains with the isolates from Yellowstone Park, U.S.A. suggest that long term repeatability using PY-MS may still be a problem. It has been reported (Wheals, 1983) that improved results using PY-MS have been obtained for forensic purposes when the instrument was standardised to substances similar to those being analysed. It may be necessary to use reference bacteria to standardise the PYROMASS 8-80, as well as glycogen standards, to obtain long-term repeatability and so make such comparative studies a real possibility.

Thermophile genetics have received comparatively little attention as yet. Attempts were made in this study to carry out some investigations

into the genetics of both *Thermus* and *Sulfolobus*. The difficulties in establishing suitable techniques precluded all but the establishment of a method for gene cloning of *Thermus* and the induction, purification and partial characterisation of a temperate bacteriophage from a strain of *Sulfolobus*. Further work on these two topics is continuing at the University of Auckland and the Max-Planck Institute, Martinsried, Munich.

In conclusion it seems that extremely thermophilic bacteria are variable organisms but that this variability is perhaps not as great as has sometimes been suggested.

APPENDIX I

ISOLATION AND PREPARATION OF *THERMUS* SAMPLES FOR PY-MS

I.1 Collection of Samples

All samples were collected into sterile glass bottles fitted with rubber-lined screw caps. Bottles used were either 28 ml Universals, 100 ml or 250 ml medicine bottles, or 20 ml Hungate tubes (Hungate, 1969) for anaerobic samples.

Source temperatures were taken using a Cole-Palmer Digisense thermistor, or in some cases a 0-200^{o}C mercury thermometer. Source pH was taken using a Cole-Palmer Digisense portable pH meter, calibrated to pH=4 and pH=7 in the field. Sometimes pH papers were used, either Neutralit (pH=5-10) or Acilit (pH=0-6) non-bleaching papers (Merck).

Samples were usually collected by immersing sterile collection bottles (held in long tongs) directly into a hot spring and allowing the bottle to become completely filled with sediment and water.

For highly anaerobic samples the method described by Zillig (Zillig *et al.*, 1981) was used, samples being collected in sterile 10 ml syringes fitted with wide-bore needles and injected into prepared Hungate tubes. If the hot pool could not be safely sampled in this manner, a sterile Universal bottle was lowered into the pool using long tongs, and syringes filled as rapidly as possible from the bottom of the bottle.

All samples were allowed to cool to ambient temperature, transported to the laboratory and stored at room temperature.

Samples were aseptically removed using either sterile Pasteur pipettes or syringes flushed with N_2 gas.

It was found that these samples were able to act as inocula for the isolation of many different strains of bacteria, both aerobes and anaerobes, (when sediments were used for inoculation of enrichment media), for periods of up to two years.

I.2 Medium Used for the Cultivation of *Thermus* Strains

The medium used was Medium D (Castenholz, 1969). There was no change to the published medium, but to ensure standardisation only the following commercially available brands of carbon sources and agar were used.

BBL Trypticase peptone

BBL Yeast extract

Coast Biologicals agar (Coast Biologicals, Penrose, Auckland, N.Z.)

The final pH of both solid and liquid media was adjusted to pH=7.8 (at room temperature), with 1M NaOH.

Agar plates were poured at 25 - 30 ml.

I.3 Isolation of *Thermus* Strains

0.5 - 1.0 ml of shaken water samples were withdrawn aseptically and added to 10 mls of Medium D in 28 ml Universal bottles fitted with rubber-lined screw caps.

Incubations were carried out in a ventilated Clayson incubator set at $70^{o}C$ (+/- $0.5^{o}C$). Positive samples produced heavy growth after 24 - 48 hours.

I.4 Purification of *Thermus* Strains

0.1 ml (2 drops) of shaken culture was 4-phase streaked onto plates of Medium D solidified with 1.75% agar and incubated at 70°C.

Plates were sealed with masking tape and placed directly on the incubator shelves, agar side up.

Single colonies were restreaked in the same manner and checked for purity by Gram staining (Collins and Lyre, 1976) and microscopic examination.

Contamination with spore-formers was a problem with some cultures and was overcome by a second incubation in liquid medium at 75°C, *Thermus* species tolerating this temperature but not spore-formers.

I.5 Harvesting of *Thermus* Strains

Purified strains were 4-phase streaked onto agar plates and grown overnight as above. Colonies were carefully removed, without breaking the agar surface, using a sterile platinum inoculating loop and 10 - 20 ml of sterile distilled water.

Cells were centrifuged at 2500 g (max) in an M.S.E. benchtop centrifuge for 15 minutes.

The cell pellet was washed twice in distilled water, resuspended in a few drops of water and transferred to a 1.5 ml Wheaton sample bottle for freeze-drying.

I.6 Freeze-drying of *Thermus* Strains

Sample bottles were frozen in a slush of liquid N_2 - 95% ethanol and placed on a *Virtis* freeze-drying machine set at -50°C to -60°C under

TABLE I.1 Distribution of *THERMUS* Strains in Some N.Z. Thermal Springs

Site	Pool No.	Temperature °C	pH	Results
ROTORUA				
Whakarewarewa	Rt 14	80	7.0	+
	Rt 15	96.4	8.7	+
	Rt 34	75.7	6.5	+
	Rt 35	80	6.6	-
	Rt 96	94.5	8.7	+
	Rt 97	78	7.1	-
	Rt 98	98	8.5	-
	Rt 100	81	7.3	+
Kuirau Park	Rt 4	87	7.2	+
	Rt 6	64	9.4	+
Government Gardens	Rt 8	90	9.1	-
TOKAANU	TOK 1	96	7.9	+
	TOK 3	76	6.9	+
	TOK 4	89	5.6	+
	TOK 5	52	5.9	+
	TOK 6	97	7.0	-
	TOK 8	80	5.6	+
	TOK 9	86	7.0	+
	TOK 10	75	6.5	+
	TOK 11	98	7.0	+
	TOK 12	97	7.0	+
ORAKEI KORAKO	OK 2	57	7.6	+
	OK 3	87	8.6	+
	OK 4	60	6.9	-
	OK 5	67	8.6	-
	OK 6	102.5	8.2	+
	OK 8	83.5	8.5	-
	OK 10	74	7.9	+
	OK 11	96	7.4	-
	OK 12	80	7.4	-
	OK 13	98	7.3	+
	OK 15	97	7.0	+
	OK 17	90	7.2	-
WAIMANGU	Wai 3	48	5.5	+
	Wai 13	92	6.5	+
	Wai 15	84	6.0	+
	Wai 16	70	N.A.	+
	Wai 17	80.6	8.5	+
	Wai 18	81.6	7.3	+
	Wai 22	92	7.0	-
	Wai 24	97	7.7	+
TAUPO	TP 6	78	7.3	-
	TP 9	92	7.1	-
	TP 10	72	6.8	+
TIKITERE	Tiki 10	75.3	7.3	-
	Tiki 12	78	6.9	-
KETETAHI	KET 4	80	6.0	+
	KET 7	84	6.5	-
HOT WATER BEACH	SWBS	60	6.5	+
FIJI - Suva Suva Beach	Fj 3	N.A.	N.A.	+

N.A. = not available

a vacuum of at least 60 millitorrs. Drying took 6 - 12 hours.

Freeze-dried cells were subsequently autoclaved at 121°C for 15 minutes, dried in a desiccator, caps screwed down and stored at room temperature.

I.7 Preparation of *Thermus* for PY-MS

Freeze-dried strains were resuspended in a few drops of methanol, and sonicated for 5 - 10 minutes to produce an even suspension. The suspension was applied as small drops to 510°C Curie point wires, with thorough drying between application of drops. Initially drops were allowed to air-dry but later a hair-dryer was used.

I.8 The Distribution of *Thermus*

The hot springs listed in Table I.1 were screened for the presence of *Thermus* and thirty-two isolates obtained. The distribution appears to be related to factors other than the location of the site, its temperature and pH. These factors probably include the presence of a suitable carbon-source and the absence of toxic substances e.g. arsenic, which are known to occur in some of these pools (B. Patel, pers. comm.).

These isolates were obtained by Y. Casey (Thermophile Research Unit, University of Waikato).

APPENDIX II

ISOLATION AND PREPARATION OF ARCHAEBACTERIA FOR PY-MS

II.1 Collection of Samples

The majority of the archaebacteria isolated were anaerobes and samples were collected both aerobically and anaerobically. The aerobic samples consisted of both sediments and water with a layer of, at least, 1 cm of sediment at the bottom of sterile glass bottles.

Anaerobic sample bottles were prepared by a modification of the method described by Zillig *et al.* (1981). 0.5 ml of a 1 $\mu g\ ml^{-1}$ aqueous solution of resazurin dye (which is fully reduced at Eh = 110 mV) was added to 23 ml Hungate tubes (Bellco) fitted with butyl rubber stoppers and plastic screw caps. Tubes were autoclaved at 121^{o}C for 15 minutes and filled with 95% N_2 + 5% H_2 gas. Approximately 0.5 g of sterile sulphur was added (under gas) and the tubes sealed. 0.5 ml of H_2S gas (made in the laboratory by the action of dilute HCl on ZnS) was added. from a 1 ml syringe. This fully reduced the dye. Water samples were added as 5 - 7.5 ml from a 10 ml syringe. The syringe was either immersed directly in the hot pool or if this was not possible, water was collected in a sterile Universal bottle held on the end of long tongs and a 10 ml syringe rapidly filled from the bottom of the bottle. Aliquots (0.5 - 1.0 ml) for enrichment cultures were removed with a sterile 1 ml syringe which was flushed with N_2. Tubes were stored at room temperature and remained fully reduced for 3 years.

Sediments were able to act as inocula for enrichments of the anaerobes described in Chapter 4 for 1 - 2 years. One sample KT 1 could not longer act as an inoculum for a *Thermoproteus*-like organism

after 2 years, indicating the value of collecting anaerobic samples for long term storage and possibly for the culture of extremely strict anaerobes which cannot tolerate any exposure to oxygen.

II.2 The Use of Antibiotics for Selective Enrichment of Archaebacteria

All enrichments for archaebacteria were carried out in the presence of antibiotics (Hilpert *et al.*, 1981). The use of antibiotics at these temperatures has been reported by other workers (Brock, 1978; Zillig *et al.*, 1981 and 1982(b)), and it was observed that eubacterial contaminants were completely eliminated by two or three transfers into medium containing a succession of two or three different antibiotics. The species of archaebacteria isolated grew in the presence of the antibiotics listed below. The possibility that prolonged incubation at these temperatures could lead to breakdown of antibiotics with the production of toxic products cannot be ruled out.

The antibiotics used were prepared as set out below and added in concentrations set out in the text. Solutions were prepared fresh weekly and stored at $4^{o}C$ with the exception of penicillin which was stored at $-20^{o}C$. Unless otherwise stated antibiotics were obtained from Sigma.

Penicillin G

Prepared at 20 mg ml^{-1} in distilled water, filter-sterilised and frozen at $-20^{o}C$ in 1 ml aliquots.

Chloramphenicol

Prepared at 20 mg ml^{-1} in 100% ethanol. Stored in brown glass bottles.

Tetracycline

Tetracycline hydrochloride was prepared as a 12.5 mg ml^{-1} solution in ethanol/water (50% V/V); filter sterilised and stored in brown glass bottles.

Streptomycin

Streptomycin sulphate was prepared at 20 mg ml^{-1} in distilled water, and filter sterilised.

Vancomycin

Vancomycin hydrochloride was prepared at 10 mg ml^{-1} in glycine-HCl buffer (data for Biochemical Research, 1974) at pH = 3 and filter sterilised.

Amoxil

At a time when acid-stable vancomycin was not available, 250 mg amoxil tables (an acid-stable form of penicillin) were obtained, crushed and dissolved in water.

II.3 Isolation and Growth of AN1-like Strains

Media used for the Cultivation of AN1-like Strains

AN1-like strains were isolated and grown as follows:

(a) AN1 Medium containing sulphur as electron acceptor.

K_2HPO_4	1.5 g
NaCl	2.5 g
thioglycolic acid	1.0 g
BBL trypticase peptone	10.0 g
distilled water	1000 ml

pH adjusted to 7.3 before autoclaving.

After autoclaving add -

Sterile sulphur	0.05%

All manipulations were done under N_2 gas. The gas phase for incubation was N_2

(b) Using cystine as electron acceptor, 0.05% cystine was added before autoclaving.

All other conditions were the same as for sulphur-containing medium.

All incubations were at 75°C (± 0.5°C) in a Clayson ventilated incubator.

ANl Medium for Growth at 85°C

K_2HPO_4	0.22 g
KH_2PO_4	0.22 g
$(NH_4)_2SO_4$	0.22 g
thioglycolic acid	0.4 g
NaCl	1.0 g
MES buffer	5.0 g
BBL trypticase peptone	0.5 g
BBL yeast extract	0.5 g
trace elements	1.0 ml

(as for Medium D - Castenholz, 1969)

1% $FeSO_4$ solution	120 ml

After autoclaving add -

10% Na_2S solution	30 ml
sterile sulphur	0.05%

Final pH should be 6.8.

. This medium was prepared by the modified Hungate technique as described for *Thermoproteales* strains (see Figure II.1)

The gas phase was N_2.

Preparation of Sterile Sulphur

Elemental sulphur was sterilised by tyndallisation. Universal bottles, fitted with rubber lined screw caps, were half-filled with sublimed sulphur and gently steamed in a water-bath for three hours on three consecutive days.

AN1 Rinsing Buffer

Tris base	6.0 g (0.05 M)
NaCl	3.0 g
$Na_2S.9H_2O$	0.75 g
Distilled water	1000 ml

pH to 7.0 with HCl

II.4 Enrichment of AN1-like Strains

A sterile Pasteur pipette was inserted into the sediment layer at the bottom of the storage bottles and approximately 1 ml of sediment and water withdrawn. This was added to 15 ml of sulphur-containing AN1 medium in 28 ml McCartney bottles (fitted with rubber-lined screw

caps) under a stream of N_2 gas. All enrichments were carried out in the presence of 50 μg ml^{-1} Penicillin G and incubated at 75°C. Positive cultures were visibly turbid within three days.

II.5 Purification of AN1-like Strains

This was carried out as described in Section 4.6.1 with 0.5 ml of the lowest dilution showing positive growth (usually 10^{-6}) used as inoculum. This step was carried out in sulphur-containing medium under the same conditions as described for the isolations with either 10 μg ml^{-1} streptomycin or 50 μg ml^{-1} chloramphenicol added.

II.6 Preparation and Harvest of AN1-like Strains

5 ml of a pure culture of an AN1-like strain was inoculated into 400 ml of medium in a 500 ml medicine bottle fitted with a rubber-lined screw cap. Incubations were at 75°C (±0.5°C) in a ventilated Clayson incubator. Cells were harvested after overnight growth.

All stages of harvesting were carried out under a stream of N_2 gas. The first stage in harvesting was the removal of inorganic sulphur from the medium. It was found that if cultures were allowed to stand for 30 minutes at room temperature most of the sulphur settled to the bottom of the culture vessel. The bacterial suspension was pipetted off, transferred to sterile centrifuge bottles and cells pelleted by centrifugation at 23,400 g (max.) for 15 minutes in a Sorvall RC-5B centrifuge (GSA rotor).

Cells were resuspended in AN1 rinsing buffer, any remaining sulphur allowed to settle out and the bacterial suspension carefully pipetted off. This rinsing procedure was repeated twice with cells pelleted at

TABLE II.1

Attempted Isolation of ANI-like Strains

Region	Sample Code	Source Temperature °C	Source pH	Source Na^+ ppm *	Presence of ANI
ROTORUA					
1. Kuirau Park	ANI (RT 1) DSIR No. 648	85	N.A.		+
	RT 2 DSIR No. 647	82.5	7.5	370	-
	RT 3 DSIR No. 602	70	7.2		-
	RT 4 DSIR No. 601	72.1	7.1		-
	RT 6	81.5	8.6		-
	RT 60 DSIR No. 715	97.7	7.2		-
	RT 66 DSIR No. 656	80	6.1		?
	RT 67 DSIR No. 649	78	6.6		-
2. Ohinemutu	RT 7	91	7.3		-
3. Government Gardens	RT 8	70	9.1		-
4. Arikapakapa	RT 48	98	3.1		?
	RT 50	N.A.	3.1		?
5. Whakarewarewa	RT 12 DSIR No. 428	82	7.0	606	+
	RT 14 DSIR No. 358	79	7.3	647	+
	RT 34 DSIR No. 344	75	6.5	411	+
	RT 36 DSIR No. 346	62	4.0		?
	RT 44	62	7.8		+
	RT 46	90	3.6	636	?
	RT 51 DSIR No. 426	82	7.3	628	+
	RT 56	90.2	7.9	857	+
	RT 71	98.5	3.3		-
	RT 80	>98	7.5		-
	RT 82	>98	6.7		-
	RT 84	>98	7.5		-
	RT 97 DSIR No. 353	78	7.1	617	?
	RT 98 DSIR No. 354	>98	8.5		-
	RT 100 DSIR No. 355	81	7.3		+
6. Bore Samples	RT B1	69.8	N.A.		-
	RT B2	94.2	N.A.		-
	RT B3	84	N.A.		-
	RT B4	89.4	N.A.		-
	RT B5	97	N.A.		-
WAIOTAPU					
Forest Reserve	WT B	96	7.0		-
Tourist Area	WP 8	83	6.8		-
	WP 21	68	6.6		-
TE AROHA	Te A 1	N.A.	N.A.		-
TIKITERE					
Glasshouse Effluent	TG 1	84.1	8.6		-
Tourist Area	TK 4b	90.1	5.8		-
	TK 14	80	5.9		+
	TK 15	84.5	5.3		-
KETETAHI	KT 8	85	6		-
	KT 10	89	6.7		-

Table II.1 cont'd

Region	Sample Code	Source Temperature °C	Source pH	Source Na^+ ppm *	Presence of ANI
WHITE ISLAND	Wis 4	90 - 96	5.0		+
GREAT BARRIER ISLAND	G B I	75	6.5 - 7.0		-
ORAKEI KORAKO	OK 9	83	6.8		-
	OK 10	74	7.9		-
	OK 11	96	7.4		-
	OK 12	80	7.4		-
	OK 13 DSIR No. 119	98	7.3		-
	OK 15 DSIR No. 120	95	7.0		-
WAIMANGU	Wai 13	92	6.5		-
	Wai 18	85	6.5		-
	Wai 23	95	6.5		+
TOKAANU	TOK 4	92	5.7		+
	TOK 9	86	7.3		+
	TOK 10	75	6.5		+
	TOK 11	98	7.0		+
	TOK 12	97	6.9		-
HOT WATER BEACH	HWB 1	58	7.0		-
	HWB 2	48	6.5		-
	HWB 3	58	6.0		-
	HWB 4	58	6.0		-
CHILE	CH 1	"boiling"	N.A.		-
FIJI	Wg Fj 11	95.6	7.9		-
	Wg Fj 14	67.2	6.9		-
	Ta La Fj 3	67.8	8.7		?
	Na SS Fj 3	98.7	8.1		?
	Na SS Fj 7	99.3	8.2		?

N.A. = no value available

* Na^+ figures by courtsey of Ministry of Works and Development, Hamilton.

NOTE

Samples marked ?

These samples contained a few cells in the primary enrichment but could not be subcultured.
Further isolation attempts also failed.
All these samples were >10 weeks old; successful isolation generally being from fresher samples.
One exception was Wis 4, a 7 month old sample, but this had been stored in liquid N_2 (+ 10% glycerol) since collection date.

17,200 g (SS34 rotor) which resulted in the removal of all visible traces of sulphur. The cell pellet was then resuspended in a minimum of buffer and freeze-drying and autoclaving carried out as described for *Thermus* strains (Appendix I).

11.7 Distribution of AN1-like Strains

Sixty-six pools from a number of different regions were screened for the presence of AN1-like bacteria. The results of this screening are given in Table II.1. Fifteen water samples yielded positive isolates and a further nine samples may have contained AN1 but primary enrichments could not be subcultured. Further attempts to culture bacteria from these water samples gave negative results. As it was not possible to obtain fresh samples from these pools (marked ? in Table II.1) the accurate determination of the presence of AN1 could not be obtained.

The results given in Table II.1 suggest that there is a regional factor involved in the distribution of AN1. Although it was possible to obtain information regarding sodium levels for only a few of the pools screened, it is possible to speculate that >400 ppm Na^+ are required for the presence of this bacterium. An absolute requirement for Na^+ has certainly been established for laboratory culture of AN1 (Morgan, 1982). The apparent absence of AN1-like bacteria from Orakei Korako and Waiotapu may be due to low Na^+ levels in these areas but as yet data relating to these is not available.

It had been expected that the Hot Water Beach samples would yield positive isolates as these pools were situated above the high water level on the sea shore. The absence of AN1 here may be due to Na^+ levels being too high and/or the rather low temperature of these pools.

Temperature and pH alone do not seem to control distribution as isolates were obtained from pools in the 62° - 98°C range and the pH 5.0 - 7.9 range. The only Tokaanu pool which did not yield an isolate was within these ranges.

An investigation into the distribution of ANl and the Na^+ level of source pools is planned as a future research project at this University.

II.8 Culture of *Thermoproteales*-like Strains

Media used in Isolation and Cultivation of *Thermoproteus*

(a) Medium A (Zillig *et al.*, 1981) was used in an isolation attempt. The medium was boiled to drive off oxygen, cooled on ice (under N_2) and 0.1% sodium sulphide (from a 10% stock solution) added as reducing agent. After adjustment of the pH to 5.5,10 ml of medium was dispensed into Hungate tubes (Hungate, 1969) using a modification of the Hungate technique (Hungate, 1969) illustrated in Figure II.1 as designed by B. Patel (D.Phil. Thesis, 1984).

Tubes were autoclaved, cooled, and glucose added from a 20% sterile stock solution to a final concentration of 1%. Ferrous sulphate was added (from a 0.1 M solution which was filter sterilised and stored in 5 ml aliquots at -20°C) at 200 µl/10 ml medium. The pH was checked and 0.05% sterile sulphur added. All manipulations were carried out under a stream of N_2 gas, but cultures were incubated in a final gas phase of 95% N_2 + 5% H_2.

1 ml of water and sediment from sample bottles was added to each tube and incubations carried out in a Clayson ventilated incubator set at 85°C.

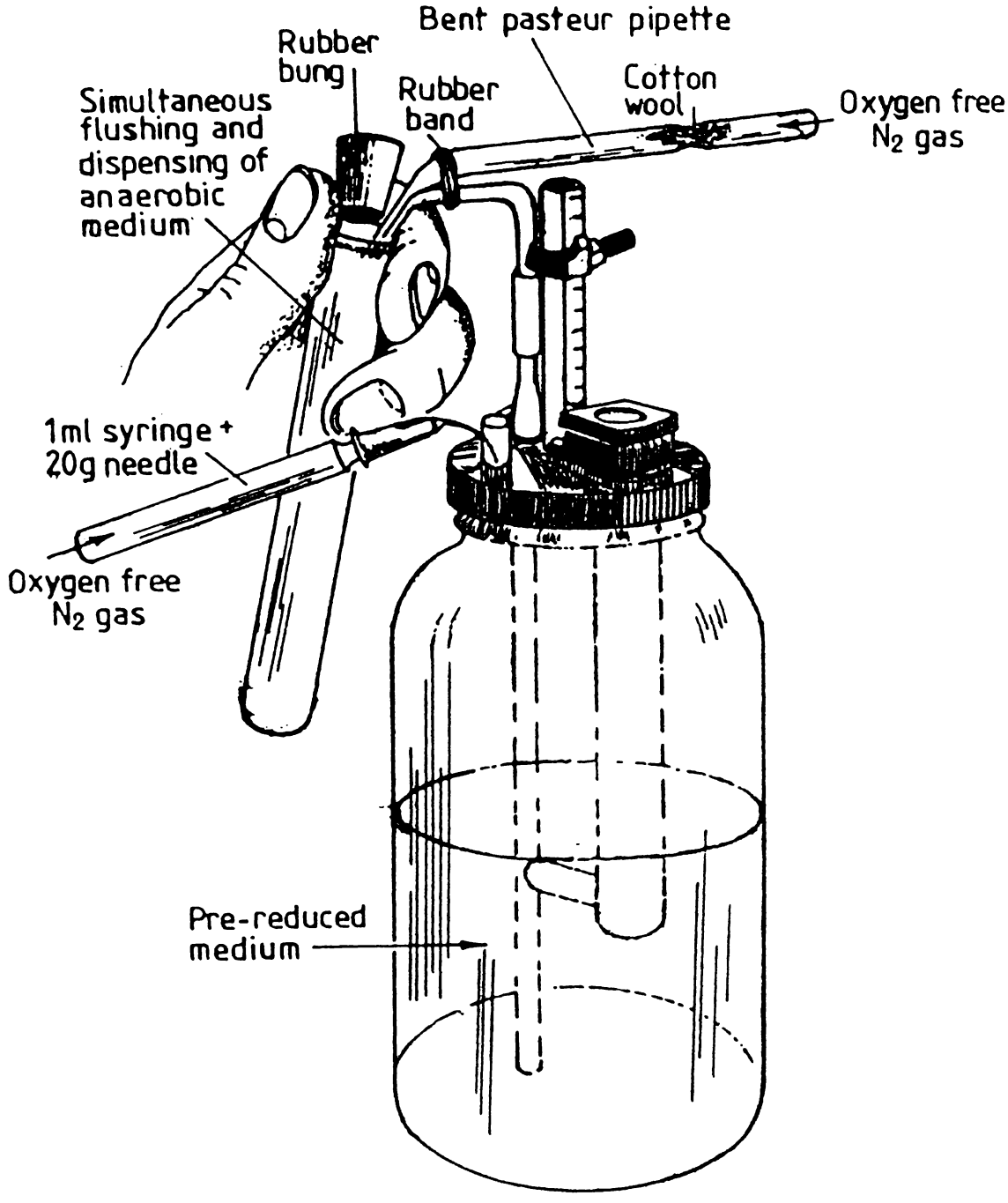


FIGURE II.1 Diagram showing the dispensing of anaerobic medium by a modification of the Hungate technique (B. Patel, D.Phil. Thesis, 1984).

(b) Medium B

The salt base used for the cultivation of *Sulfolobus* (Brock *et al.*, 1972) was used. The medium was prepared as for medium A with 0.05% sodium sulphide added as reducing agent. After autoclaving, glucose, Fe^{2+} and sulphur were added, the pH adjusted to 5.5 and enrichments set up as just described.

(c) Medium Db

This is one of the media described by Zillig for the cultivation of *Desulfurococcus* (Zillig *et al.*, 1982b), the version coded Db contains the following carbon sources:

0.1 g/litre yeast extract (BBL)

2 g/litre bactotryptone (Difco)

The medium was prepared as described for medium A with 0.05% sodium sulphide added as reducing agent and 0.05% sulphur as electron acceptor unless otherwise stated.

The pH was adjusted to either pH = 5.5 or

pH = 7.0

Subsequently pH = 6.5 was used. Adjustments to pH were made using either 1M H_2SO_4 or 1M NH_4OH.

All manipulations and incubations were carried out as for medium A.

II.9 Attempted Growth on Solid Media

1. Sulphur Source

Sulphur is required for respiration, therefore must be evenly distributed throughout solid medium. Several methods were tried to achieve this.

a) Sulphur coated bentonite (Wieringa, 1966);

b) Polysulphide solution (Wieringa, 1966);

c) Sterile S^0 added directly to the gel and stirred until cool enough to pour plates.

Gelling Agent

a) 12% starch - soluble starch (BDH) was added to the basal salts of Db medium and boiled until a gel was formed. Carbon sources were added and the gel autoclaved at 121°C for 15 minutes. After cooling, sulphur source (as required) and reducing agent (0.05% sodium sulphide) were added and the pH adjusted to pH = 5.5.

Later autoclaving of medium was discontinued as it was found that stronger gel formation occurred without autoclaving. Sterile carbon sources and reducing agent were added to the boiled gel and the pH adjusted just before plates were poured.

b) Silica gel - Plates were prepared from both solid sodium silicate (Thatcher and Weaver, 1974) and from sodium silicate solution (Zillig *et al.*, 1980).

Plates were equilibrated overnight with Db medium containing polysulphide as sulphur source, in a desiccator filled with N_2.

C) Polyacrylamide - polyacrylamide plates (Zillig *et al.*, 1981) were polymerised in a desiccator filled with N_2, then equilibrated with medium as for silica gel plates.

II.10 Purification of *Thermoproteus*

This was carried out as described in Section 4.6.4.2(8) - with

TABLE II.2

SCREENING FOR *THERMOPROTEUS*-LIKE STRAINS ON MEDIUM A

Source	Sample Code	Source pH	Source Temperature °C	Results
Craters of the Moon	C.M.	N.D.	98	-
KETETAHI	KT 1	4	"boiling"	long rods + a few cocci
TIKITERE	TK 4	5.8	90	a few cocci
	TK 6	4.7	84	a few cocci
WAIOTAPU TOURIST AREA	WP 8	6.8	83.6	-
	WP 10	3.8	92	-
	WP 13	4.5	76.4	-
	WP 16	4	75.5	a few rods
	WP 20	5.5	74.8	a few long rods + cocci
	WP 21	6.6	67.7	a few rods + a few cocci
ORAKEI KORAKO	OK 1 DSIR No 95	4.8	80.7	-
	OK 7	6.6	98.2	-
	OK 9	6.8	83	-
	OK 14	3.9	97	-
WHITE ISLAND	Wis 2	3.1	95.2	-
	Wis 4	5.0	90 - 96	-
FIJI	FJ 1	N.A.	"boiling"	-
	FJ 2	N.A.	"boiling"	-
CHILE	CH 1	N.A.	"boiling"	chains of short rods

TABLE II.3

Attempted Isolation of *Thermoproteus*-like Strains on Medium B

Source	Sample Code	Source pH	Source Temperature °C	Results
ROTORUA				
Whakarewarewa	Rt 30 DSIR No. 414	3.7	97	-
	Rt 36 DSIR No. 346	6.4	82	rods
	Rt 46	3.6	90	cocci
	Rt 56	7.9	90.2	-
	Rt 89 DSIR No. 388	8.1	94	-
	Rt 99 DSIR No. 363	4.8	94.8	-
Kuirau Park	Rt 38 DSIR No. 607	3.2	72.9	-
Sulfur Flat	Rt 31	2.8	80.4	-
Bores	Rt B1	N.A.	69.8	-
	Rt B2	N.A.	94.2	-
	Rt B3	N.A.	84.0	-
	Rt B4	N.A.	89.2	-
	Rt B5	N.A.	97.0	-
WAIOTAPU				
Forest Reserve	WT 3	4.0	76.8	-
	WT 5	5.5	69.8	-
	WT A	1.4	98	? cocci
	WT B	7.0	93	-
	WT D	4.4	84	-
Tourist Area	WP 2	2.9	89.9	-
	WP 4	2.9	86.2	-
	WP 10	3.7	92	-
	WP 12	3.5	79.4	-
	WP 13	4.5	76.4	-
	WP 16	4	75.5	? cocci
	WP 18	3.6	87.2	-
	WP 20	5.6	74.8	-
	WP 21	6.6	67.7	-
ORAKEI KORAKO	OK 1 DSIR No. 95	4.8	80.7	-
	OK 14	3.9	97	-
TIKITERE	TK 4b	6.1	90.5	cocci
	TK 6	4.7	84	-
	TK 9	3.1	86.2	-
	TK 12	6.9	78	rods & cocci
	TK 14	5.9	80	cocci
	TK 15	5.3	84.5	cocci
TOKAANU	TOK 4	5.7	86	a few rods
	TOK 10	6.5	75	-
	TOK 11	4.0	98	-
	TOK 12	7.0	97	a few rods & cocci
WAIMANGU	Wai 3	5.5	48	-
	Wai 13	6.5	92	-
	Wai 18	6.5	85	-
	Wai 22	6.5	95	a few cocci
KETETAHI	KT 4	6.0	80	a few rods & cocci
	KT 5	3.0	80	-
	KT 6	5.5	88	a few rods
	KT 7	6.5	84	a few rods
	KT 8	6.0	85	a few rods
	KT 9	2.0	85	-
	KT 10	6.0	89	rods

Attempted Isolation of *Thermoproteus*-like Strains on Medium B - Cont'd.

Source	Sample Code	Source pH	Source Temperature °C	Results
ROTOKAWA	RK 1	5.5	91	-
	RK 2	3.5	89	-
	RK 3	2.5	77	-
	RK 4	2.5	93	-
WHITE ISLAND	Wis 2	3.1	95.2	-
	Wis 5	3.5	64-88	-
	Wis 7	3.2	74.5	a few cocci
	Wis 9	2.6	89.7	-
	Wis 12	2.9	91.4	-
CHILE	CH 1	N.A.	"boiling"	-
FIJI	FJ 1	N.A.	"boiling"	-
	FJ 2	N.A.	"boiling"	-
	Ta La Fj 3	8.7	67.8	-
	Wg Fj 10	7.9	92.5	-
	Na SS Fj 3	8.1	98.7	-

Table II.3 cont'd

serial dilutions being set up in medium Db as just described. 0.5 ml inoculum was added to 10 ml medium with 10^{-4} usually the lowest dilution showing growth.

II.11 Screening of Central North Island Hot Pools for *Thermoproteus*-like Strains

A wide variety of thermal regions were screened for the presence of *Thermoproteus* species. Samples were either collected anaerobically (as described in Section 1) or an aliquot from the sediments at the bottom of an aerobic sample bottle used to set up primary enrichments.

As described in Chapter 4 a number of different media and conditions were used in attempts to isolate these bacteria.

Table II.2 lists pools screened using medium 'A' designed by Zillig *et al* (1981) for the isolation of *T. tenax*.

Table II.3 lists the pools screened using medium 'B'. This medium had a different salt base from that of medium 'A'.

Medium 'A' was designed by Zillig (Zillig *et al.*, 1981) to mimic the composition of Icelandic hot pools and had an extremely low phosphate concentration. Medium B contained the salt base used by Brock (Brock *et al.*, 1972) for the isolation and culture of *Sulfolobus*. This had a higher ionic strength including a 3 mM phosphate concentration.

Table II.4 lists the results of isolations using medium Db (Zillig *et al.*, 1982(b)) at pH = 5.5 (see Section II.7).

Table II.5 lists the results of isolations using medium Db at pH 7.0.

Attempted Isolation of *Thermoproteus*-like Strains in Db Medium at pH 5.5

Source	Code	Source pH	Source Temperature °C	Results
ROTORUA				
Kuirau Park	Rt 4 DSIR No. 601	7.2	87	-
	Rt 39 DSIR No. 666	3.3	80.5	cocci & rods
Arikapakapa	Rt 50	3.1	N.A.	cocci & a few rods
Govt. Gardens	Rt 8	9.1	70	-
Bores	Rt B2	N.D.	94.2	-
	Rt B5	N.A.	97	-
Ketetahi	KT 1	4.0	"boiling"	rods & cocci
	KT 4	6.0	80	rods & cocci
	KT 5	3	80+	-
	KT 6	5.5	88	rods & cocci
	KT 7	6.5	84	rods & cocci
	KT 8	6	85	rods & cocci
	KT 9	2	85	-
	KT 10	6	89	rods & cocci
Tokaanu	TOK 4	5.7	86	-
	TOK 9	7.0	92	-
	TOK 10	6.5	75	cocci only
	TOK 11	4.0	98	rods & cocci
	TOK 12	7.0	97	rods & cocci
Waimangu	Wai 1	3.5	78	-
	Wai 3	5.5	48	a few rods
	Wai 8	3.1	85	a few rods
	Wai 10	7.4	91	-
	Wai 13	6.5	92	-
	Wai 15	6.0	84	-
	Wai 17	7.0	75	-
	Wai 18	7.3	81	cocci only
	Wai 21	6.0	62	a few cocci
	Wai 22	7.0	97	a few cocci
Rotokawa	RK 1	5.5	91	-
	RK 2	3.5	89	-
	RK 3	2.5	77	-
	RK 4	2.5	93	-
Tikitere	TK 4b	6.1	90.5	rods
	TK 6	4.7	84	cocci & rods
	TK 8	3.2	80.5	-
	TK 9	3.1	86.2	rods & cocci
	TK 12	6.9	78	-
	TK 14	5.9	80.4	rods
	TK 15	5.3	84.5	rods & cocci
Orakei Korako	OK 7	6.6	98	-
	OK 13 DSIR No. 119	7.3	98	a few rods
Waiotapu Tourist Area	WP 16	4.0	75.5	-
	WP 20	5.6	78.4	a few cocci
	WP 21	6.8	67.7	a few cocci
White Island	Wis 2	3.1	95.2	-
	Wis 3	2.9	83.7	cocci & rods
	Wis 12	2.9	91.4	cocci only
Great Barrier Is./ Kaitoke Springs	GB 1	6.5	75	-
	GB 2	7.0	76	-

TABLE II.4

TABLE II.5

Attempted Isolation of *Thermoproteus*-like Strains in Db Medium at pH = 7.0

Source	Code	Source pH	Source Temperature °C	Results
ROTORUA				
Whakarewarewa	Rt 13 DSIR No. 428	7.4	98	mainly cocci & a few rods
	Rt 42	7.3	97	cocci & some rods
	Rt 56	7.9	90.2	cocci & a few rods
	Rt 70	3.4	98.2	-
	Rt 84	N.A.	"boiling"	cocci & rods
	Rt 98 DSIR No. 354	8.5	"boiling"	-
Kuirau Park	Rt 98 DSIR No. 720	7.6	103	rods & cocci
	Rt 60 DSIR No. 715	7.2	98	rods & cocci
Arikapakapa	Rt 47	3.2	100.5	mainly cocci
	Rt 50	3.1	N.A.	cocci & rods
Bore samples	Rt B2	N.A.	94.2	-
	Rt B5	N.A.	97	-
TOKAANU	TOK 11	4.0	98	mainly rods
	TOK 12	7.0	97	mainly cocci
WAIOTAPU TOURIST AREA	WP 6	N.A.	100.5	-
Forest Reserve	WT 1	2.1	88	rods & cocci
	WT B	7.0	96	-
ORAKEI KORAKO	OK 7	6.6	98.2	-
	OK 11	7.4	96	rods & cocci
	OK 13	7.3	98	-
HIPUAU	HP 1	6.7	>100	-
TIKITERE	TK 4b	6.1	90.5	cocci only
	TK 9	3.1	86.2	rods & cocci
WAIMANGU	Wai 3	5.5	48	rods & a few cocci
	Wai 10	7.4	91	-
FIJI	FJ 2	N.A.	"boiling"	rods & a few cocci
CHILE	CH 1	N.A.	"boiling"	chains of short rods

TABLE II.6

ISOLATION OF *DESULFUROCOCCUS*-LIKE STRAINS

Db MEDIUM AT pH 5.5

Source	Sample Code	Source pH	Source Temperature °C	Results
ROTORUA				
WHAKAREWAREWA	Rt 30 DSIR No. 414	3.6	97	cocci + a few rods
	Rt 34 DSIR No. 344	6.5	75.7	-
	Rt 46	3.6	90	-
	Rt 99 DSIR No. 363	4.8	94.8	a few rods

ISOLATION OF *DESULFUROCOCCUS*-LIKE STRAINS

Da MEDIUM AT pH 5.5

Source	Sample Code	Source pH	Source Temperature °C	Results
ROTORUA WHAKAREWAREWA	Rt 30 DSIR No. 414	3.6	97	-
KUIRAU PARK	Rt 39 DSIR No. 666	3.3	80	a few cocci
TIKITERE	Tk 6	4.7	84	rods and cocci
KETETAHI	Kt 1	4.0	boiling	rods only
WHITE ISLAND	Wis 2	3.1	95.2	a few long rods
	Wis 3	2.9	83.7	cocci + rods
	Wis 12	219	91.4	cocci only

The strains selected for PY-MS analysis from these enrichments are listed in Table 4.3.

It can be seen from Tables II.2 - II.5 that many New Zealand hot pools contain *Thermoproteus*-like species. However, it appears none of the New Zealand strains can grow on glucose as the sole carbon source. Unlike *T. tenax* (Zillig *et al.*, 1981) which is only found in pools in the pH 3 - pH 6 range, the New Zealand strains are also found in pools of neutral to slightly alkaline pH, and in Db medium it seems all strains isolated grew optimally at pH 6.5. As reported by Zillig *et al.*, (1981) *T. tenax* is usually found in association with *Desulfurococcus* (Zillig *et al.*, 1982b). The separation of the two genera is reported in Chapter 4.

Table II.6 lists a limited screening attempt to isolate *Desulfurococcus* without contamination by *Thermoproteus*. Db medium was used for four Rotorua pools where cocci were thought to predominate, (B. Patel, D.Phil. Thesis, 1984) and Da medium which contained 0.2% yeast extract (a concentration reported to be inhibitory to *T. tenax* - Zillig *et al.*, 1981), for screening a small number of pools from a variety of regions. As stated in Chapter 4, not only did these methods fail to selectively isolate cocci but there was also no suitable method available to separate the three reported species of *Desulfurococcus*, (Zillig *et al.*, 1982b), so it was decided not to attempt to prepare *Desulfurococcus* strains for PY-MS.

II.12 Preparation and Harvest of *Thermoproteus*-like Strains

Cultures were grown in 500 ml medicine bottles fitted with rubber-lined screw caps. 400 ml of Db medium (at pH = 5) was added to each bottle which was sterilised, and at the same time pre-reduced, by autoclaving at 121^{o}C for 15 minutes. Bottles were cooled under a stream

of N_2 gas, reducing agent (0.05% Na_2S) added and pH adjusted to pH = 6.5 with 0.1 M NH_4OH. 0.5% sterile sulphur was added then 5 ml of a log phase culture of the required inoculum (1.25% inoculum). Bottles were incubated at 85°C without stirring in a Clayson incubator. Cells were harvested when 20 - 25 cells/field (under x1000 magnification) were present; this was usually after 2 days incubation.

Rinsing and Resuspending Buffer

0.05 M Tris

0.022 M NH_4Cl

1.0 g/litre $Na_2S.9H_2O$

pH to 6.5 with HCl

Cells were harvested in the same way as AN1-like cells except that centrifugation was at 23,400 g (max.).

II.13 Preparation of *Halobacteria*

Both *Halobacterium* species and the *Halococcus* species analysed by PY-MS were grown on the following medium. The *Halobacteria* were grown in liquid medium and the *Halococcus* on solid medium.

Casamino acids	7.5 g
Trisodium citrate	3.0 g
Yeast extract (BBL)	10.0 g
KCl	2.0 g
$MgSO_4.7H_2O$	20.0 g
$FeSO_4.7H_2O$	0.05 g
$MnSO_4.7H_2O$	0.2 g
NaCl	250 g
Distilled water	1000 ml

Agar (Oxoid No 1) 20.0 g

pH to 7.4

Incubated at 37^{o}C without agitation.

All other strains of archaebacteria were grown and harvested as described in the text of Chapter 4.

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SAV 1, a temperate u.v.-inducible DNA virus-like particle from the archaebacterium *Sulfolobus acidocaldarius* isolate B12

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***Sulfolobus acidocaldarius*, strain B12, which harbours a double-stranded DNA species both as a plasmid and in a linear form, which is integrated at a specific site of the chromosome, produces virus-like particles upon u.v. irradiation. These particles contain the same circular DNA and a number of coat proteins and are probably surrounded by a lipid membrane. They are lemon shaped, 100 x 60 nm in size and carry tail structures at one pole. The host cell recovers and remains lysogenic after virus production. Though a large fraction of liberated particles is found attached to structures derived from the cells, neither adsorption nor infection of a number of *Sulfolobus* isolates has so far been observed.**

Key words: archaebacteria/integration/lipoproteins/plasmid/virus

Introduction

The archaebacteria, which form the third urkingdom of organisms (Woese *et al.*, 1977; Fox *et al.*, 1980), are divided into two major branches (Tu *et al.*, 1982; Zillig *et al.*, 1984). In the first branch, comprising the methanogenic and halophilic archaebacteria, a few viruses of *Halobacterium* have been reported (Wais *et al.*, 1975; Torsvik and Dundas, 1980; Schnabel *et al.*, 1982a), of which only the *H. halobium* phage øH has been studied on the molecular level (Schnabel *et al.*, 1982b, 1984a, 1984b). In the other branch, the sulphur-dependent archaebacteria (Tu *et al.*, 1982; Zillig *et al.*, 1984), comprising the *Thermoproteales* and the *Sulfolobales*, only three unusual viruses of the anaerobic extreme thermophile *Thermoproteus tenax* (Zillig *et al.*, 1981) have so far been described (Janekovic *et al.*, 1983). Here we report the isolation of the first virus, SAV 1, of the aerobic thermoacidophilic sulphur-dependent archaebacterium *Sulfolobus acidocaldarius*.

Results

Induction and time course of the production of virus-like particles (SAV 1)

U.v. irradiation of growing cultures of *S. acidocaldarius*, isolate B12, under the conditions described in Materials and methods, induces the production and liberation of virus-like particles. Most of these appear in a free state, but a significant fraction is attached, sometimes in large numbers, to cellular fragments often lacking visible envelopes (Figure 1A).

The first indication of virus production is the protrusion of particles through the cell envelope around 4 h after u.v. induction (Figure 1A). The liberation of virus begins immediately afterwards, followed by a steady increase in the virus titre which reaches its final value, usually ~3 x 10^{10} particles/ml culture, after ~15 h. During the phase of virus extrusion, cell growth is severely retarded. The culture recovers its previous growth rate, however, upon ceasing to liberate virus. Extensive lysis is not observed and the cells are viable when plated or subcultured. Virus production can be induced again from the culture by u.v. irradiation following subculturing, showing that the cells have resumed their original lysogenic state. This suggests that the virus, like the filamentous phages M13, fd and f1 (see review by Denhardt *et al.*, 1978), is largely liberated from cells which remain intact. Thin sections of cells sampled 6 h after u.v. induction showed cell-cell contacts (Figure 1D) which were rarely observed in unirradiated B12 cultures and never in cultures of the strain DSM 639, which does not carry the viral genome. Small amounts of virus (estimated as ~10^7 particles/ml culture) are released spontaneously in unirradiated cultures. Virus is thus also released in nature.

Purification and properties of SAV 1

Usually 20 h after induction the cells were removed from the culture fluid by low speed centrifugation. The virus was then precipitated with ammonium sulphate and further purified by a CsCl buoyant density gradient centrifugation step followed by zonal centrifugation through a pre-formed KBr density gradient, as described in Materials and methods. This method of preparation yields pure virus, as confirmed by electron microscopic inspection.

Electron microscopy

Electron micrographs of negatively stained virus particles are shown in Figure 1. A cell with virus protruding through the envelope, apparently in the process of extrusion, is shown in Figure 1A. Particles of purified virus at higher magnification are shown in Figure 1C. They are shaped like a lemon, ~100 x 60 nm in size, and apparently have several fibres attached to one pole. Sometimes the particles appear elongated, indicating plasticity of form and/or flattened shape. Some particles are of larger, probably double size (Figure 1B, arrowed); others appear as bullet-like structures, possibly representing halves of particles (Figure 1C). In induced cultures, not only free virus particles are seen but also clusters of particles attached to what seem to be cell fragments (Figure 1B). Cell-cell contacts were seen in thin sections of cells from induced cultures (Figure 1D).

Comparison of viral with plasmid DNA

We have previously reported (Yeats *et al.*, 1982) the existence in *S. acidocaldarius* strain B12 of a u.v.-inducible plasmid of length 15.6 kb, a copy of which is also carried on the chromosome, linearised and integrated at a specific site. Our speculation that this element is in fact a viral genome has now been confirmed by comparing the DNA prepared from purified virus particles with plasmid DNA isolated from B12 cells as described previously (Yeats *et al.*, 1982). Agarose gel electro-

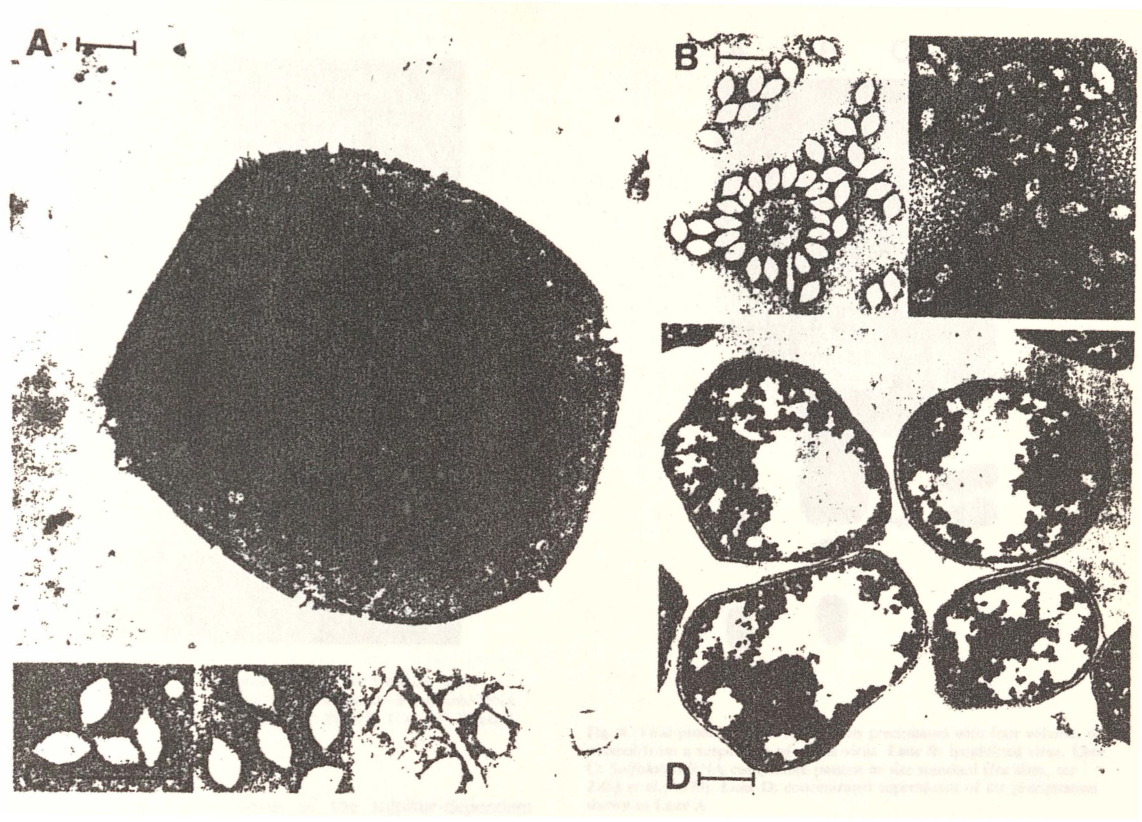

Fig. 1. Electron micrographs of virus particles. **(A)** Cell apparently extruding virus. **(B)** Free virus and virus particles attached to cellular material. Two large particles are arrowed. **(C)** Purified free virus particles exhibiting tail structures. Three bullet-shaped particles are seen on the right. **(D)** Thin sections of cells sampled 6 h after u.v. irridation showing three cell – cell contacts. The bars represent 0.2 μm.

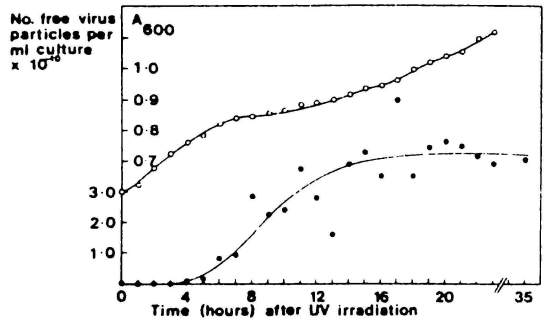

Fig. 2. Time course of virus production. Cultures were grown and irradiated with u.v. as described in Materials and methods and sampled at intervals. The approximate titre of free virus particles was estimated by a standardised electron-microscopic count (Janekovic *et al.*, 1983) together with the corresponding A_{600} of the culture. Closed circles, free virus particles; open circles, A_{600} of culture.

phoresis of uncut DNA and DNA restricted with the restriction endonucleases *Bam*HI and *Eco*RI (Figure 3) proved the two to be identical. To investigate the possibility that the viral DNA is in fact not circular, but rather linear with cohesive ends which cause circularisation, samples were heated at 70°C and chilled on ice before loading on gels. No change in the band pattern was observed (data not shown). We therefore conclude that the DNA is indeed circular.

Other constituents of SAV 1

SDS-polyacrylamide gel electrophoresis of viral proteins revealed defined patterns of at least six proteins depending on the mode of sample preparation (Figure 4). Directly applied lyophilised virus yielded broadened and distorted bands (lane 4B), possibly due to lipids associated with some of the proteins. A simplified pattern was observed when viral proteins were precipitated from whole virus with four volumes of ethanol (lane 4A). Two of the viral proteins were not precipitated by this procedure, but remained in the supernatant (Figure 4D), indicating that these proteins are hydrophobic. Clear blue instead of blue violet tinges after staining with Coomassie Brilliant Blue indicated that two of the peptides are probably associated with other constituents.

The presence of lipids was shown by t.l.c. of chloroform-methanol extracts (data not shown). Though their components appear to correspond to components of the host lipids, the composition is quantitatively very different.

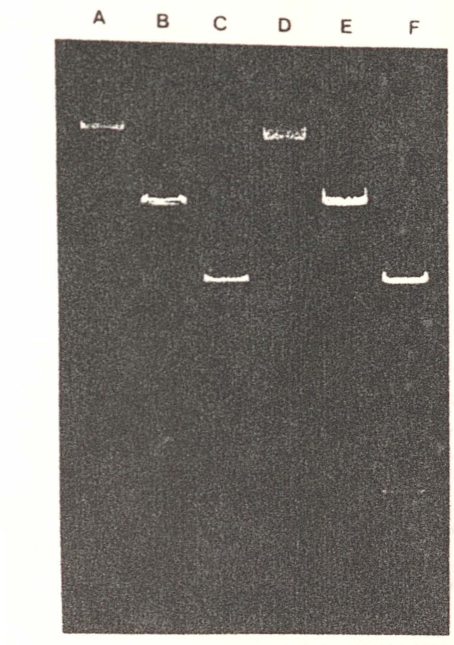

Fig. 3. Comparison of viral with plasmid DNA (for fragment sizes, see Yeats *et al.*, 1982). **Lanes A – C**: viral DNA. **Lanes D – F**: plasmid DNA. **A** and **D**: undigested. **B** and **E**: digested with *Bam*HI. **C** and **F**: digested with *Eco*RI.

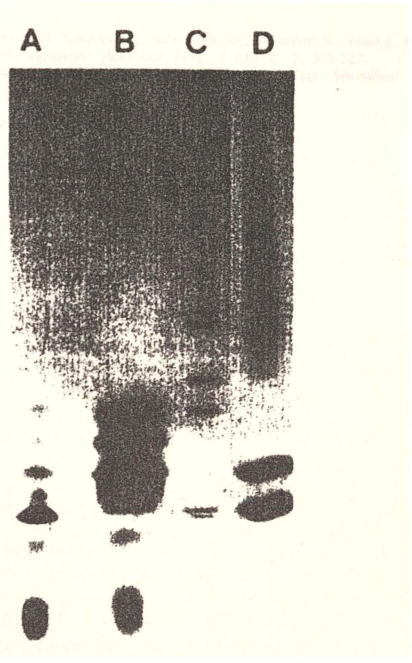

Fig. 4. Viral proteins. **Lane A**: proteins precipitated with four volumes of ethanol from a suspension of whole virus. **Lane B**: lyophilised virus. **Lane C**: *Sulfolobus* RNA component pattern as size standard (for sizes, see Zillig *et al.*, 1979). **Lane D**: concentrated supernatant of the precipitation shown in **Lane A**.

Discussion

SAV 1, the first known virus of the sulphur-dependent thermoacidophilic aerobic archaebacterium *S. acidocaldarius* exhibits some unique properties, such as its structure, and a striking mixture of features known from different virus-host systems. Like two *Pseudomonas* phages PM2 (Espejo *et al.*, 1969) and Rϕ6P (Tucker and Pemberton, 1978), it contains closed circular DNA. Like a number of coliphages, it is temperate and u.v.-inducible and its linearised provirus is site-specifically integrated into the host's genome. The existence in the lysogen of several copies of 'plasmid' besides the linear integrated provirus could be explained by assuming that the 'plasmid' is some viral DNA produced by spontaneous induction. In view of the lack of visible fine structure and the presence of a large amount of lipid in the virus, the envelope is possibly a lipid membrane, like those of the *Pseudomonas* phage PM2 and the *Thermoproteus* virus TTV1. The large difference between the lipid compositions of virus and host (data not shown) points to a contribution of viral structures in the organisation of the envelope.

Given the small size of the DNA (15.6 kb), the virus is surprisingly large, about the size of coliphage T4. Still, it appears to contain only one, or at most a few, copies of DNA per particle as roughly estimated from DNA content, DNA size and virus count.

Both free and integrated viral DNA are stably maintained in the cell. Repeated efforts to cure the cell of viral DNA have been unsuccessful. In view of the co-existence of 'plasmid' and integrated provirus, this is not unexpected. Culture variants occasionally arise in which large-scale virus production is no longer induced by u.v., although both integrated and free viral DNA are present. This hints at a genetic variability at some stage of viral growth.

We have not yet established an infectious system for viral multiplication. Under various conditions tested, the virus does not adsorb to a number of other *Sulfolobus* strains, nor apparently to the B12 strain itself (observed in the electron microscope). Cell-cell contacts are often observed in u.v.-irradiated cultures of the lysogen, strain B12, never in irradiated or unirradiated cultures of strain DSM 639, which does not harbour the virus genome, and rarely in unirradiated cultures of strain B12 (Figure 1D). They are apparently due to a change in the quality of the cell envelope and often lead to a partial removal of the envelope from the membrane. In view of the inability of the virus to adsorb to intact cells, it appears possible that such contacts may be involved in virus transmission and could be exploited to obtain viral infection. Alternatively, mutagenesis to produce virulent mutants or the isolation of more *Sulfolobus* strains from the wild to serve as natural hosts may be the answer. Finally, the virus may be defective. The temperate *Sulfolobus* virus SAV 1 offers a potentially valuable system of studying not only gene expression and its control in sulphur-dependent archaebacteria, but also the structural requirements for the existence of viruses at high temperature and low pH.

Materials and methods

Bacterial strains

Sulfolobus strains and growth conditions were as described previously (Yeats *et al.*, 1982).

U.v. irradiation

Cells were grown in 1 litre Erlenmeyer flasks in 400 ml batches at 80°C in New Brunswick gyratory (model G76) shakers filled with glycerol. After reaching an A_{600} of 0.5 – 0.7, they were irradiated in the culture medium in shallow plastic dishes at a depth of 1.5 mm with manual agitation, using a Hanua Sterisol mercury lamp. The optimal u.v. dose, determined empirically, was 50 ergs/mm^2. For virus preparation, the culture was harvested ~20 h after irradiation.

Purification of virus

20 h after u.v. induction, the cells were removed by centrifugation for 10 min at 8000 r.p.m. in the large rotor of the WKF centrifuge G50K. The virus was precipitated from the supernatant by the addition of ammonium sulphate to 75% saturation at 0°C. The pellet obtained by 1 h centrifugation in the same rotor and centrifuge was dissolved in 20 mM phosphate, pH 3, and 1 mM magnesium sulfate (buffer PM) containing 2 M KCl in addition. After dialysis against buffer PM, caesium chloride was added to give a density of 1.24 and the solution was centrifuged for 2 days in a Beckman Ti 50.2 rotor at 40 000 r.p.m. The virus formed a clearly visible band at a density of ~1.24.

Residual impurities were removed as follows. The virus suspension was dialysed against buffer PM to remove caesium chloride, pelleted by centrifugation for 90 min in the SW27 rotor of the Beckman ultracentrifuge at 27 000 r.p.m. and resuspended in buffer PM containing 2 M KCl in addition. The suspension was layered onto a linear gradient of KBr in buffer PM ranging from 40 to 90% saturation at 20°C and centrifuged in a Beckman SW27 rotor for 1.5 h at 24 000 r.p.m. The virus banded in the middle of the gradient. It was removed, dialysed against buffer PM and kept at 4°C.

Virus count

The approximate virus titre was determined electron-microscopically as previously described for the virus TTV1 (Janekovic *et al.*, 1983).

Preparation and electrophoresis of viral DNA

Viral DNA was prepared and subjected to agarose gel electrophoresis as described previously (Yeats *et al.*, 1982).

Protein gel electrophoresis

This was carried out as described in Zillig *et al.* (1978).

Electron microscopy

Electron micrographs were prepared as described in Zillig *et al.* (1981). Negative staining, however, was done with uranyl formate on 2% collodium grids covered with an ultrathin carbon film.

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Some Properties of a β-Galactosidase from an Extremely Thermophilic Bacterium

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An inducible β-galactosidase from an extremely thermophilic organism, *Thermus* strain 4-1A, has been isolated and partially purified. There were significant dissimilarities to *T. aquaticus* β-galactosidase. It had a pI of 4.5, was inhibited by sulphydryl inhibitors and a number of transition metals, and was activated by EDTA and SH-containing reagents. The β-galactosidase showed strong product inhibition, and weaker inhibition by some other mono- and disaccharides. It was very stable up to 90°C at pH 8. On immobilization by diazonium linkage to porous glass, the pH optimum (6.0), the K_M with ONPG (5m*M*) and the product inhibition were not altered.

INTRODUCTION

The use of β-galactosidases for industrial processing of lactose-containing fluids has become an established technique (see ref. 1). In recent years, the economy and efficiency of these processes have improved as a result of the increased use of immobilized enzyme preparations. Further increases in the economy of lactose hydrolysis can be expected as preparations of greater stability are introduced. While immobilization can often increase enzyme thermostability,[2] the greatest advances in improving the thermostability of β-galactosidases are likely to come from the discovery of new procaryotic sources. This trend has already been exemplified by the successively higher stabilities of β-galactosidases from the thermophile *Bacillus stearothermophilus*,[3] the extreme thermophile *Thermus aquaticus*,[4] and the archaebacterium *Caldariella acidophila*.[5]

METHODS

Bacterial Strain and Culture Medium

The organism used in this study was an extremely thermophilic gram-negative obligate aerobe isolated from the Whakarewarewa thermal region, Rotorua, New Zealand (Patel, Lim and Morgan, unpublished results). It will be referred to as *Thermus* strain 4-1A pending classification.

The bacterium was grown at 75°C in shaking culture on nutrient medium[6] at pH 7.2. Filter-sterilized galactose (0.5%) was added to the nutrient medium after autoclaving.

Partial Purification of β-Galactosidase

A wet cell paste (harvested by centrifugation from 12 hour aerated or 48 hour nonaerated cultures: $A_{650} \sim 1.8$) was suspended in an equal volume of 0.1*M* Tris-acetic acid buffer, pH 8.1. The slurry was sonicated (Dynatech Dismembrator) for about 240 min/L using a continuous flow attachment and a circulation rate of 500 mL/h. Cell fragments were removed by centrifugation for 2 h at 9000*g* (r_{av} = 14 cm) followed by 1 h at $10^5 g$ (r_{av} = 6 cm). The cell-free supernatant was adjusted to 50% saturation with ammonium sulphate (LR grade, Merck, Darmstadt). After standing overnight at 4°C, precipitated protein was removed by centrifugation. The precipitate was redissolved in 0.01*M* Tris-acetic acid, pH 8.1, and dialysed for 24 h against two changes of 0.01*M* Tris-acetic acid, pH 8.1. The dialysate was loaded onto a column (20 × 10 cm) of DEAE-cellulose (Sigma Chemical Co., St. Louis, Missouri, USA) and washed with the same buffer. A simple NaCl step gradient (0.05*M*, 0.1*M*, 0.25*M*, and 1.0*M*) was applied. The major portion of the enzyme was eluted by 0.1*M* NaCl. This fraction was desalted by ultrafiltration (Amicon ultrafiltration cell, UM02 filter) and diluted with 0.04*M* Tris-HCl, pH 9, buffer. This preparation was used for all subsequent experimental work.

Assays

Routine assays were carried out by a modification of the method of Ulrich and co-workers.[4] The *o*-nitrophenol galactoside (ONPG; Sigma Chemical Co., St. Louis, Missouri, USA) was dissolved in 0.1*M* citrate-phosphate buffer,[7] pH 6 (adjusted at 70°C), at a concentration of 5m*M*. Substrate (2 mL) was preincubated at 70°C. The reaction was initiated by addition of 100 μL of enzyme solution. After a measured period, the reaction tubes were removed from the water bath, and the reaction immediately terminated by addition of 2.5 mL cold 0.5*M* Na_2CO_3. Absorbance at 420 nm was converted to molar concentrations

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using $E_M = 4.44 \times 10^3 M^{-1} cm^{-1}$. One unit of β-galactosidase activity is defined as 1 nmol *o*-nitrophenol (ONP) released per minute under the conditions described above.

Whole cells were pretreated with toluene-acetone as previously described.[4] Lactase activity was determined by quantitative analysis of glucose release, using a glucose oxidase reagent (Boehringer Mannheim GmbH Mannheim, West Germany). Protein was estimated either by the Biuret method[8] or by the Bradford method.[9]

Immobilization

For immobilization, 1.5 g of controlled pore glass (CPG-240: Pierce Chemical Co., Rockford, USA) was silanated with γ-aminopropyltriethoxysilane as described previously[10] and then treated with 10% glutaraldehyde. Fifty milliliters of a β-galactosidase solution (2.86 mg/mL in 0.04 Tris-HCl, pH 9) was added, and the suspension incubated with stirring at 40°C for 72 h. The immobilized preparation was washed exhaustively.

Thermal Inactivation

Five-to-ten-milliliter volumes of enzyme were incubated at specified temperatures. At intervals, aliquots were removed and stored on ice for subsequent assay at 70°C.

Molecular-Weight Determination

For the Sepharose gel filtration, a 1.6 × 30 cm column (Sepharose 4B; Sigma Chemical Co., St. Louis, Missouri, USA) was eluted with 0.1*M* phosphate buffer, pH 7.0. For the HPLC gel filtration, a 7.5 × 600 mm TSK G3000SW column (Toyo Soda Ltd., Tokyo, Japan) was used. Proteins were eluted with both 0.01*M* Tris-acetate buffer, pH 7.0, and distilled water, using a Spectra-Physics HPLC solvent delivery system at a flow rate of 1 mL/min. Protein standards (urease tetramer, urease dimer, *E. coli* β-galactosidase, bovine serum albumin dimer, bovine serum albumin monomer, and ovalbumin; Sigma Chemical Co., St. Louis, Missouri, USA) were used in both columns, and the logarithm of molecular weights plotted versus the elution volume and retention time, respectively, to generate standard curves.

Polyacrylamide Gel Electrophoresis

The discontinuous electrophoresis method of Davis,[11] using gels of various acrylamide contents, was used. Conditions of electrophoresis, fixing, and staining were followed as described.[11] The β-galactosidase activity in unstained gels was observed by immersing the gels in 5m*M* ONPG (see the Assays section) for several minutes, followed by incubation at 70°C for 1-2 h. The ONPGase activity was visible as a yellow band.

Isoelectric Point

A pI value was determined using isoelectric focussing on pH 3.5 to pH 9.5 acrylamide gel plates (Pharmacia, Uppsala, Sweden). After electrophoresis for 1.5 h (starting conditions: 500 V and 50 mA), the pH gradient was determined with a Pye surface electrode. The β-galactosidase activity was observed by overlaying the electrophoresis gel with a 1.5% agar gel containing 5m*M* ONPG and 0.5*M* phosphate buffer, pH 7. A region of yellow ONP release was visible after incubation at 75°C for 10 min.

RESULTS AND DISCUSSION

Production

In aerated standard medium containing 0.5% galactose, a maximal level of β-galactosidase activity of approximately 1500 units/mL culture (500 units/mg wet weight of cell mass) was obtained after 12-16 h. The order of effectiveness of inducers was similar to that of *T. aquaticus* β-galactosidase[4] (galactose > lactose > isopropylthiogalactoside).

Purification

The β-galactosidase was partially purified as described in the Methods section. A 43-fold increase in specific activity was obtained after DEAE-cellulose ion exchange chromatography (Table I). DISC electrophoresis showed six weakly staining protein bands and one strongly staining protein band.

Table I. Partial purification of *Thermus* strain 4-1A β-galactosidase.

Fraction	Volume (mL)	Total enzyme × 10^{-3} (units[a])	Total protein (mg)	Specific activity (units mg^{-1})	*x* Purified	Yield (%)
Cell-free supernatant	2,100	5.75	39,270	1464	1	100
Ammonium sulphate precipitate	150	4.19	4155	10,036	6.9	73
Dialyzed precipitate	180	4.30	1048	41,031	28.0	75
DEAE-cellulose eluate	290	1.86	296	62,838	42.9	32

[a]One unit = 1 nmol ONP released per minute at 70°C and pH 6.0.

After immersion in a solution of ONPG, gels incubated at 70°C showed a single region of ONP release, corresponding to the position of the strongly staining protein band. All subsequent characterization was carried out on β-galactosidase of this purity. No α-galactosidase or protease activity was detectible in this preparation.

Elution of this material from both Sepharose 4B and TSK G3000SW columns gave an apparent molecular weight of 4.4×10^5. The β-galactosidase peak was routinely eluted after both the urease dimer ($MW = 4.8 \times 10^5$) and *E. coli* β-galactosidase ($MW = 5.4 \times 10^5$). This molecular weight differs considerably from that of the *T. aquaticus* enzyme, for which a value of ca. 5.7×10^5 from gel filtration on Sepharose 4B is quoted.[4] The isoelectric point was estimated to be 4.5 ± 0.1.

Inhibitors

The chelating agent EDTA, the serine enzyme inhibitor phenylmethyl sulphonyl fluoride, and the sulphydryl reagents dithiothreitol, cysteine and 2-mercaptoethanol, all caused activation of ONPGase activity (Table II). The cysteine enzyme inhibitors, iodoacetic acid and *p*-chloromercuribenzoate, caused substantial inhibition. These results are consistent with the presence of a sulphydryl group in the active site. A variety of metal ions also inhibited ONPGase activity, while Mg^{2+} caused a slight activation. Such metal ion inhibition is typical of cysteine enzymes.

Various mono- and disaccharides acted as inhibitors (Table III). Glucose and galactose in particular, inhibited very strongly at high concentration. Product inhibition by galactose and/or glucose has also been observed with other microbial β-galactosidases.[3,4,12,13]

It is unclear whether the inhibition by lactose is from the disaccharide alone, or from the products of hydrolysis during the period of assay. Inhibition by cellobiose suggests that the specificity of the active site is not absolute. The high level of product inhibition is probably responsible for the incomplete hydrolysis of lactose at high concentration (Table IV). For instance, at a concentration of 20% lactose, no more than 30% of the total available substrate is hydrolyzed. This characteristic alone renders *Thermus* strain 4-1A β-galactosidase an unlikely candidate for industrial application.

Table II. Inhibition and activation of β-galactosidase activity.

Reagent[a,b]	Percent activity (%)
Ethylene diamine tetraacetic acid	187
Iodoacetic acid	0
p-Chloromercuribenzoate	36
Phenylmethyl sulphonyl fluoride	122
Dithiothreitol	157
Cysteine	179
Mercaptoethanol	147
Zn^{2+}	6
Cu^{2+}	9
Cu^{+}	28
Fe^{2+}	52
Fe^{3+}	84
Ni^{2+}	88
Ca^{2+}	100
Mg^{2+}	105
Mn^{2+}	80
Co^{2+}	100

[a]Metal sulphates were used in all cases except CuCl and $CaCl_2$.
[b]Volumes (100 μL) of stock concentrates were added to 2 mL volumes of 5m*M* ONPG in Tris-HCl, pH 6, to give final concentrations of 10m*M* (active-site reagents) and 1.5m*M* (cations).

Table III. Inhibition by carbohydrates.

Carbohydrates[a]	Concentration (m*M*)	Inhibition (%)
Glucose[b]	10	46
	56	63
	278	91
	556	96
Galactose[b]	10	30
	56	52
	278	69
	556	72
Xylose	10	15
Cellobiose	10	15
Lactose	10	22

Arabinose, fructose, mannitol, mannose and sucrose (10m*M*) had no effect on enzyme activity.

[a]This was added to the ONPG reaction mixture at the concentrations specified.
[b]Similar levels of inhibition were obtained in the hydrolysis of ONPG by CPG-immobilized β-galactosidase.

Stability

The enzyme was very stable at pH 8 in dilute buffer solutions at temperatures up to 90°C (Table V). Sodium dodecylsulphate (0.1%) and 10m*M* mercaptoethanol slightly reduced stability at 75°C, while 0.5% NaCl increased thermostability. The substrate (lactose) had no effect at 75°C, but caused more rapid inactivation at 80°C. Enzyme activity was lost very rapidly at 90°C and above. A "protein melting" temperature of ca. 86°C was indicated by inversion of a plot of log *V* vs. $^{1}/_{T}$abs (not shown). The high thermostability of this enzyme (slightly greater than *T. aquaticus* β-galactosidase[4]) is typical of proteins from thermophilic organisms. The limited effects of 0.1% SDS and 10m*M* mercaptoethanol, and its resistance to proteolysis,[14] suggest that the stability of *Thermus* strain 41A β-galactosidase may be a general characteristic. These data, in particular the resistance to proteolysis which implies a more compact conformation,[14] do not seem to be consistent with the hypothesis that thermostable proteins possess a more flexible structure,[5,15] which is responsible for the reduced susceptibility to denaturation.

Immobilization

Immobilization of *Thermus* strain 4-1A β-galactosidase by glutaraldehyde crosslinkage to controlled-pore glass re-

Table IV. Hydrolysis of lactose of high concentrations.

Percent lactose (%)	Glucose released in hydrolysis mixtures at specified times[a] (mg/100 mL)					
	30 min	60 min	150 min	245 min	1380 min	2760 min
5	12.0	24.6	44.4	57.0	137.9	188.0
	(4.8)[b]	(9.8)	(17.8)	(22.8)	(55.2)	(75.2)
10	23.0	33.6	77.8	113.6	193.5	288.8
	(5)	(6.7)	(15.6)	(22.7)	(38.7)	(57.8)
20	26.5	41.8	96.6	143.0	307.4	318.9
	(2.3)	(4.2)	(9.7)	(14.3)	(30.7)	(31.9)
30	31.8	54.7	112.4	167.8	368.8	418.1
	(2.1)	(3.7)	(7.5)	(11.0)	(24.6)	(27.9)

[a]Five milliliters of specified lactose solutions were mixed with 0.5 mL volumes of β-galactosidase solution and incubated at 75°C. (Control solutions were incubated without enzyme.)
[b]Figures in brackets show the percent of total lactose hydrolyzed.

Table V. Thermostability of β-galactosidase.

Temperature (°C)	Incubation conditions[a]	Activity loss
95	—	50% loss in 2 min
90		50% loss in 8 min
85	—	72% loss after 20 h
80	—	17% lost after 20 h
	+ 10m*M* mercaptoethanol	25% lost after 20 h
	+ 5% lactose	92% lost after 20 h
75	—	25% lost after 20 h
	+ 0.5% NaCl	5% lost after 20 h
	+ 5% lactose	26% lost after 20 h
	+ 10m*M* mercaptoethanol	45% lost after 20 h
	+ 0.1% SDS	65% lost after 20 h
65	—	10% lost after 36 days

[a]Enzyme in 0.04*M* Tris-HCl, pH 8, with additions as specified.

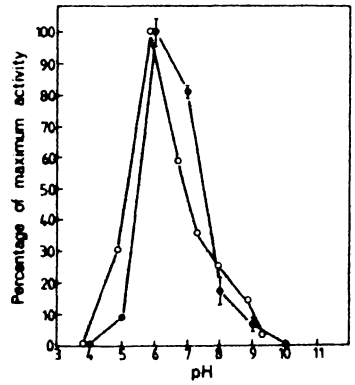

Figure 1. The pH profile for (○) soluble and (●) CPG-immobilized β-galactosidases.

sulted in apparent inhibition of 64% of bound enzyme. Such inhibition is normally attributed to attachment of the enzyme in an orientation in which the active site is sterically hindered.[2] While it is likely that other methods of immobilization would give different levels of inhibition, considerable losses of β-galactosidase activity on immobilization have been observed elsewhere.[16] Since reactions between sulphydryl groups and glutaraldehyde are known to occur,[17] it is possible that the active-site sulphydryl group could be involved in crosslinkage reactions. As SH groups are particularly reactive, a proportion of the activity loss resulting from other immobilization procedures might be accounted for in this way.

Little alteration of the pH-activity profile (Fig. 1), and no change in product inhibition (see Table III) or K_m (ONPG) was observed after immobilization of *Thermus* strain 4-1A β-galactosidase. The K_M value of 5m*M* (for ONPG hydrolysis by the free enzyme, data not shown) is similar to that of *T. aquaticus* β-galactosidase (4m*M*).[4] A K_M of 43m*M* for the hydrolysis of lactose by *Thermus* strain 4-1A β-galactosidase was also unchanged by immobilization. As suggested elsewhere,[5] a low affinity for lactose implies that it is probably not the native substrate *in vivo*.

The product inhibition and narrow pH activity range of *Thermus* strain 4-1A β-galactosidase, and the failure of immobilization to modify either, are likely to render this enzyme of little value for industrial use. However, the evidence presented here and elsewhere[4,5,14] indicates that β-galactosidases from extreme thermophiles possess high stability with respect to various denaturing influences. Other thermostable β-galactosidases with improved catalytic properties are currently under investigation.

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