THE TAXONOMY AND ECOLOGY OF THE GENUS
THERMUS

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ABSTRACT

The phenotype distribution of Thermus isolates was examined by numerical classification using isolates from New Zealand, Iceland, New Mexico and Yellowstone Park. Analysis of the New Zealand isolates showed that there was a relationship between the hot pool temperatures and pHs, and the phenotypes which could be isolated from them. Evidence also emerged which suggested that geographical factors, between distant thermal areas, may also influence the phenotypes isolated from those areas. Analysis of the Icelandic isolates supported these assertions by showing a lack of similarity to the New Zealand isolates. A classification was constructed using all of the available isolates and this showed the same patterns. All of the isolates could be divided into 8 major clusters and it is suggested that these could represent species. In this classification T.ruber and T.aquaticus would be type strains of two of the species as would the invalidly named "T.thermophilus".

Continuous culture experiments and analysis of isolates taken from hot pools over a period of time, did not support the proposal of genetic instability in Thermus. Varying the pH and temperature of continuous culture did not significantly change the phenotype. Analysis of isolates from the same pools over a time course showed there to be resident and transient strains, as well as showing a limitation of the phenotypes which could be recovered from any one pool.

A new isolate is described, which formed trichomes that further coiled to form braiding. Trichomes also exhibited swellings and necridia. Thin-section electronmicroscopy showed a similar cell wall to *Thermus* with the addition of 2 extra layers which appeared to hold the cells together in the form of trichomes. The isolate showed many features in common with *Thermus* and the only significant difference lay in the morphology. It is suggested that this organism be adopted as a new species of *Thermus* and named *Thermus filiformis*, to reflect its filamentous nature.

The aminopeptidase test was negative for *T.ruber*, *T.aquaticus* and *"T.thermophilus"* which is characteristic of Gram type positive organisms, while the KOH lysis test was positive, for these organisms, which is characteristic of Gram type negative organisms. This evidence adds to the contradictory literature derived data on the genus, which although staining Gram-negatively shows many Gram-positive characteristics. It is suggested that these anomolies may represent adaptations to life at high temperatures or may reflect the divergent evolutionary line of descent of the genus.

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1.1 "Extreme" Environments.

1.1.1 A Definition of "Extreme".

In avoiding any anthropocentric implications perhaps the best definition of "extreme" was given by Kogut (1980) as "characterizing the limits of life-supporting ranges of temperature, salinity, pH etc." An extreme environment can be recognised taxonomically (Brock, 1978) as an environment where the species diversity is low. In many extreme conditions, which are usually populated exclusively by micro-organisms, conditions approaching a pure culture may exist.

1.1.2 Diversity of Extreme Environments.

Only a brief summary will be given here of extreme environments other than the thermal environment, which is probably the best studied. It is important to bear in mind that in any one environment more than one extreme factor may occur, for example Antarctica is not only very cold but it also has very little available water (Uydess & Vishniac, 1976). The range of extreme environments given in Kushner (1978), not including thermal environments, include low temperature, high pressure, extreme pH, high salt concentration, low water availability, high heavy metal concentration and high irradiation.

Cold environments include the oceans, the atmosphere, the soil and areas of permanent ice such as Antarctica. A great part of the biosphere is therefore an extreme environment. High pressure environments are also abundant as the oceans have an average pressure of 400 atmospheres and can exceed 1,100 atmospheres (Kushner, 1978). This extreme environment is therefore largely a part of the low temperature extreme.

Acid environments include acidic hot springs, mine drainage waters and bogs while alkaline environments have been less studied and are usually associated with soils containing alkaline minerals, animal excreta or decaying proteins. Some hot springs also exhibit pH values ranging up to 10.5 (Brock, 1971).

High salt concentrations can be found in processed foods and in salterns used to produce solar salt from sea water. The precise ionic composition of a saline lake is determined by the nature of the surrounding rocks so that Na^+, Mg^{2+}, Cl^- and $SO_4{}^{2-}$ concentrations can all vary considerably, as can concentrations of other biologically important ions.

Saline environments and dry environments are similar in that they both have low water activities. Dry environments present greater problems for organisms as there is no potential for ion pumping. Organisms in these environments do not grow when it is dry, but when water does become available they are able to grow rapidly (Brock, 1978).

Heavy metals can be derived from natural sources such as thermal springs and ore lodes, or can be the result of pollution. Radiation can be divided into ionising and non-ionising. Non-ionising radiation can give rise to photochemical reactions and is lethal to mutant organimsms deficient in DNA repair mechanisms. Ionising radiation can be derived from cosmic rays or from the decay of radionuclides on Earth.

1.1.3 Thermal Environments.

There are a number of natural thermal environments which can attain high temperatures which are not associated with geothermal activity including solar heating, combustion processes and heating caused by radioactive decay (Brock, 1978).

Temperatures up to 60°C have been recorded in black anthracite wastes (Brock, 1978) and in shallow water bodies temperatures up to 40°C have been measured (Tansey & Brock, 1978). These temperatures are transitory, depending on the time of day and year. Combustion processes can occur where quantities of organic matter (e.g. composts, hay, grain, wood chips and coal) are stored due to the self heating effect caused by micro-organisms.

There are many man-made sources of heat which may enable thermophiles to grow including hot tap water (Pask-Hughes & Williams, 1975), hot water heaters (Brock & Boylen, 1973), water discharged from steel mills or nuclear power plants (Stramer & Starzyk, 1981) and sugar refineries (Nystrand, 1984).

Tansey & Brock (1978) give an extensive list of environments of varying temperatures which could potentially yield thermophilic micro-organisms.

1.1.4 The Hot Spring Environment.

Four types of hydrothermal activity have been recorded in New Zealand (Suggate, 1978); direct volcanic exhalations, high-intensity fields in volcanic areas, low-intensity fields in decadent volcanic areas and non-volcanic hot springs. Direct volcanic exhalations consist of magmatic steam and are responsible for the strongly acidic hot springs on active volcanoes such as White Island and Tongariro. High intensity fields are found in the Taupo volcanic zone producing geysers, fumaroles and hot springs in many places e.g. Wairakei, Tokaanu, Rotorua, Waiotapu, and Orakeikorako. The high heat flow in these fields is caused by an intrusive zone of magma which heats groundwater in extensive near-surface systems. Low-intensity fields such as the springs at Ngawha in Northland, Hot Water Beach on the Coromandel Peninsula and other features in the Waikato and on Banks Peninsula gain their heat from shallow hot rock formed from, geologically speaking, fairly recent volcanic activity. Non-volcanic hot springs are derived from high temperature rock brought near the surface by rapid tectonic movements occurring along faulted systems.

In a survey of hot springs from New Zealand, the USA and Iceland, Brock (1971) demonstrated a bimodal distribution curve for the pH values of hot pool waters. Most of the pools sampled had a pH in the range of 2-4 or 7-9 and Brock (1971) used this data to reinforce the proposal that there exist two main kinds of thermal spring: acid sulphate and neutral bicarbonate, and that the buffering effect of these anions played a significant part in controlling the pH of the hot springs. Within New Zealand these two types of thermal waters have been noted by Glover (1967). The near neutral or alkaline waters contained sodium and potassium chloride as major constituents and predominate at Tokaanu, Taupo, Wairakei, Orakeikorako, Waikite, Waiotapu, Rotorua and Kawerau while the sulphate type predominate at Te Kopia and Tikitere. Glover (1967) stated that waters from deep drill holes had almost neutral pH and that if deep water is able to reach the surface easily then near neutral chloride water is discharged. Steam containing carbon dioxide and hydrogen sulphide may also reach the surface and upon condensing with cold surface waters produces the acid-sulphate waters.

The chemical constituents in hot pool water can vary due to factors such as time and temperature of water/rock reactions (Mahon & Klyen, 1968) and mixing with other source waters (Glover, 1967). Therefore each pool tends to have unique chemical characteristics (Brock, 1978) although areas supplied by the same main hot water source tend to have similar chemical properties (see Sheppard & Lyon, 1984 for an example from New Zealand springs).

The chemical characters do not appear to change markedly with time (Glover, 1967; Mahon & Klyen, 1968), but some pools e.g. Kuirau Lake in Rotorua, do show marked fluctuations in temperature and water level (Patel et al., 1985a).

1.1.5 The Hot Spring as a Habitat.

A hot spring cannot be considered an homogeneous environment, as not only are conditions different between pools but they can also change markedly within the same pool. An example is in the cooling of water which typically emerges at a source and cools as it flows down a runoff channel. The decrease in temperature is not the only factor which changes as the decreased temperature of the water brings with it the properties of decreased ionisation, solubility of organic and inorganic compounds, volume and diffusion while the properties of oxygen solubility, pH, density, viscosity, surface tension, heat capacity, compressibility and refractive index all increase (Brock, 1978).

Other factors which may be important include the loss of sulphide on exposure of thermal water to the atmosphere, the presence of sediment or rock substrates to allow attachment of bacteria in order to prevent washing out, and the incident sunlight. Hot pools tend not to have a canopy of trees or bushes as their growth is limited by the elevated temperature. This means that sunlight is available for photosynthetic organisms and may also cause toxic photochemical reactions in other organisms. Castenholz (1969) observed that Iceland, with hot springs at a latitude of ~64° N did not receive enough sunlight in Winter to maintain photosynthetic cyanobacteria in flowing water systems and there was barely enough light to maintain a sparse population in still waters. However in hot pools in the USA at a latitude of 40-45° N where there is more Winter sunlight, growth and algal mat accumulation occurred all year round. Conversely strong sunlight may play a selective role by killing organisms lacking photoprotective pigments (Brock & Boylen, 1973). It could therefore be that some organisms living in Icelandic hot springs would not be able to survive in hot springs nearer to the equator.

There are many sources of energy which are available to organisms in hot springs. For heterotrophs low levels of organic compounds exist in pool water (Brock, 1978). In a flowing system there would be enough carbon to support a standing crop of heterotrophs, especially as grazing on the organisms would be non-existent at higher temperatures. Also availabe for the heterotrophs are the metabolites released by autotrophic organisms, mainly photosynthetics accumulated in mats at temperatures below 70°C.

Heterotrophs probably represent the largest range of caldoactive eubacterial organisms. Historically the first isolates were Bacillus (e.g. Marsh & Larsen, 1953), but when incubations at higher temperatures were used Thermus was isolated (Brock & Freeze, 1969). Since then many other isolates have been obtained and characterised (see sections 1.1.6 and 1.1.7 below).

Hot spring mats are usually formed of a mixture of prokaryotic organisms including non-photosynthetic bacteria, photosynthetic bacteria, and cyanobacteria. Doemel & Brock (1977) found that algal mats formed in alkaline springs in Yellowstone Park were predominantly composed of two organisms, Synechococcus lividus, a unicellular cyanobacterium, and Chloroflexus aurantiacus, a filamentous gliding photosynthetic bacterium. The mat had two zones, an aerobic upper zone where there was sufficient light for photosynthesis and a lower anaerobic zone where decomposition was the dominant process. *Synechococcus* was missing from Icelandic algal mats and was replaced by *Mastigocladus laminosus* (Castenholz, 1969). In New Zealand *Mastigocladus* was present where the pH was above 4.8 and *Cyanidium caldarium* present in more acid conditions (Brock & Brock, 1970). Castenholz (1973) suggested that in high sulphide conditions *Chloroflexus* may form mats on its own, using the sulphide as an electron donor. When forming mats with other photosynthetic organisms the other organism is found only in the top layer while *Chloroflexus* is distributed throughout the mat (Brock, 1978).

The anaerobic layers of the algal mat allow other organisms to grow. Wickstrom (1984) showed that nitrogen fixation occurred in algal mats from Yellowstone Park and isolated 14 morphological types of presumptive diazotrophs including sulphate-reducing bacteria. Interestingly mesophilic (Murray & Zinder, 1984) and thermophilic (Belay *et al.*, 1984) methanogens have now also been shown to fix nitrogen and so they may also be present in these mats.

In addition to autotrophs obtaining their energy from sunlight hot pool water contains suitable inorganic ionic species to allow chemolithotrophs to grow. Hot pool water contains a variety of sulphur compounds which may be used by autotrophs as electron donors. Examples of sulphur compound energy yielding oxidations are given in table 1.1

Table 1.1

Examples of Sulphur Compound Transformations by Eubacterial Thermophiles.

ELECTRON DONOR	END PRODUCT	ORGANISM	REFERENCE
Sulphide, sulphur	Sulphate	"*Sulfobacillus thermosulfidooxidans*"	Golovacheva and Karavaiko, 1979
Thiosulphate tetrathionate, sulphite, sulphur	Sulphate	*Thiobacillus* sp.	Williams and Hoare, 1972
Sulphide, thiosulphate, sulphite	Sulphate	"*Thermothrix thioparus*"	Caldwell *et al*., 1976
Sulphide	Sulphur	*Chloroflexus aurantiacus*	Bauld and Brock, 1973

The archaebacteria, considered to be a separate kingdom of organisms (Woese & Fox, 1977), are hot pool organisms which are predominantly autotrophic. Sulphur metabolism is a common feature of many of these organisms in which carbon dioxide acts as a carbon source and sulphur as terminal electron acceptor (Fischer *et al.*, 1983). Methanogenesis is a mode of metabolism exclusive to the archaebacteria and is a process known to occur in thermal environments

(Belay *et al.*, 1984).

Hydrogen is also present and is derived from volcanic gases (Conrad *et al.*, 1985) allowing the growth of hydrogen utilising chemolithotrophs such as *Calderobacterium hydrogenophilum* (Kryukov *et al.*, 1984), *Hydrogenobacter thermophilus* (Shiba *et al.*, 1985) and *Bacillus tusciae* (Bonjour & Aragno, 1984).

Facultative autotrophs able to use hydrogen have also been described (Aragno, 1978). Carbon monoxide has been shown to be a carbon and energy source in *Bacillus* strains resembling *Bacillus schlegelii* (Kruger & Meyer, 1984).

1.1.6 Aerobic Caldoactive Bacteria Other Than Thermus.

Williams (1975) proposed that the term caldoactive be applied to organisms with a maximum growth temperature greater than 70°C, optimum above 65°C, and minimum above 45°C. The term thermophile was to include those with a maximum above 60°C, an optimum above 50°C and a minimum above 30°C. Exceptions to these classes may occur and so here the key temperature taken will be the optimum for each organism.

Other than *Thermus*, comparatively few obligately or facultatively aerobic bacteria have been isolated from the hot spring environment. This is probably due to two main reasons; the decreased solubility of oxygen in water at high temperatures and the presence of sulphide in many hot springs which acts as a reducing agent.

The best studied group of aerobic caldoactive bacteria other than *Thermus* is the genus *Bacillus*. Caldoactive *Bacillus* spp. include strains growing with carbon monoxide as their sole source of carbon and energy (Kruger & Meyer, 1984), hydrogen oxidisers (Bonjour & Aragno, 1984), acidophiles (Darland & Brock, 1971), strains able to grow above 100°C (Heinen & Lauwers, 1981), filamentous forms

(Golovacheva et al., 1975; Heinen et al., 1982) as well as more ordinary heterotrophic forms (Sharp et al., 1980). Although many species have been isolated and described their abundance in an actively metabolising state in nature is an open question as slide immersion studies rarely showed the presence of Gram-positive organisms (Patel, 1984).

"Thermothrix thiopara" was first described by Caldwell et al. (1976). This filamentous facultative aerobe was shown to be capable of autotrophic and heterotrophic growth (Brannan & Caldwell, 1980). Growth occurred between 62 and 77°C, with an optimum at 73°C. Brannan & Caldwell (1983) showed in a continuous culture study that the actual growth yield was higher than for comparable mesophiles and that the specific growth rate was higher than for most autotrophs.

A number of aerobes have been described which can utilise hydrocarbons as sources of carbon and energy. To date some of these organisms remain unnamed and unidentified. One group of organisms was capable of growth solely on hydrocarbons (Merkel et al., 1978b). Another group could grow on hydrocarbons as well as complex media (Merkel et al., 1978a) and included the then incorrectly named "Thermomicrobium fosteri" (Phillips & Perry, 1976) (the one strain of the genus Thermomicrobium is incapable of growth on hydrocarbons).

More recently the group of organisms able to grow only on hydrocarbons has been assigned a new genus and species, Thermoleophilum album (Zarilla & Perry, 1984). Isolates came from thermal and non-thermal environments although none grew at temperatures less than 45°C, with optima at 60°C.

Thermomicrobium roseum (Jackson et al., 1973a) grew optimally at 70 to 75°C and at a pH of 8.2 to 8.5. The cells were Gram-negative pleomorphic rods and contained a pink carotenoid pigment. An unusual feature of this organism is the apparent lack of peptidoglycan in the cell wall (Merkel et al., 1980). The cell wall contained a protein which appeared as a regular mosaic structure on the exterior cell surface.

Hydrogen oxidising autotrophs have been isolated including Hydrogenobacter thermophilus (Kawasumi et al., 1984; Shiba et al., 1985) and Calderobacterium hydrogenophilum (Kryukov & Savel'eva, 1984). Again these organisms were Gram-negative rods with temperature optima in the region of 74-76°C.

A Gram-positive coccus has been isolated from a beet sugar refinery and named Saccharococcus thermophilus (Nystrand, 1984). The main metabolite of sugar degradation was L-lactic acid. This bacterium is the first eubacterial thermophilic coccus to be described and as yet has not been isolated from a natural environment.

"Flavobacterium thermophilum" (Loginova et al., 1978) shared many properties with Thermus but was motile by means of peritrichous flagella and had a lower optimum growth temperature.

The discovery of the genus Sulfolobus arose from attempts by Brock to isolate Thermoplasma from Yellowstone Park hot springs (Brock, 1978). Organisms similar to Thermoplasma were isolated using a medium at pH2 but they had a subunit structure to the cell wall and so could clearly be distinguished from Thermoplasma. Sulfolobus is an archaebacterial chemolithotroph able to oxidise sulphur (Shivvers & Brock, 1973). The temperature optima for isolates varied considerabely from 63 to 80°C. Subsequently it was found widely ditsributed in acidic hot springs in Yellowstone Park, Iceland, The West Indies, Italy and Dominica (Brock, 1978).

1.1.7 Aerobic Thermophiles.

A facultatively anaerobic photosynthetic bacterium has been isolated and named Chloroflexus aurantiacus (Pierson & Castenholz, 1974). Under aerobic conditions the organism grew heterotrophically while photoheterotrophic growth took place under anaerobic conditions. The temperature optimum was between 52 and 60°C and so the species cannot be called caldoactive. A similar organism which was aerotolerant has been isolated and named Heliothrix oregonensis (Pierson et al., 1985). It however lacked chlorobium vesicles and bacteriochlorophyll c as well as being aerobic and so was placed in a new genus. This organism had an even lower temperature optimum (for photosynthesis) of 45-55°C.

A thiobacillus has been isolated from Yellowstone Park which had a temperature optimum of 50°C (Williams & Hoare, 1972). Poor growth was obtained under all conditions used and heterotrophic growth did occur. An organism similar to a thiobacillus isolated in the Soviet Union has been described and named "Sulfobacillus thermosulfidooxidans" (Golovacheva & Karavaiko, 1979) due to the ability of the organism to sporulate. It used various electron donors and may well be responsible for oxidation of sulphide ores and in their heating (the organism was isolated from a spontaneously heated ore deposit).

Isolates from a coal refuse pile growing at pH 2-3 and at a temperature of 55°C were found to resemble mycoplasmas (Darland et al., 1970) and a new genus created and named Thermoplasma. The optimum growth temperature was determined to be around 59°C, with the optimum pH of 1-2. The organism contained lipids which indicated that it was not a mycoplasma and Brock (1978) commented that this was a unique micro-organism. By 16s rRNA sequencing this organism closely resembles the archaebacteria (Brock, 1978).

1.2 The Genus Thermus.

1.2.1 Current Taxonomy of the Genus

To date only two species have been validly named, T.aquaticus (ATCC 25104) YT-1 (Brock & Freeze, 1969) and T.ruber (AUCM 1258) (Loginova & Egorova, 1975; Loginova et al., 1984). The inclusion of the latter expanded the genus description and now enables the K-2 isolate of Ramaley et al. (1975) to be considered a member of the genus. Other species which have been described but not validly named include "T.thermophilus" HB8 (Oshima & Imahori, 1974) and "T.flavus" AT62 (Saiki et al., 1972). Non-pigmented isolates have also been described (Pask-Hughes & Williams, 1975), including Ramaley's strain X-1 (ATCC 27978) (Ramaley & Hixson, 1970). One isolate has been named in association with an enzymological study as "T.caldophilus" (Taguchi et al., 1982) and other strains have been deposited in culture collections as "T.rubens" (ATCC 31556) and "T.lacteus" (ATCC 31557); these last two are patent strains. Strains ATCC 27737 and NCIB 11245, 11246, and 11247, which are all described as being Thermus species, have not been assigned names.

There have been 3 reported attempts in which Thermus isolates have been compared from a taxonomic viewpoint. Donnison et al. (1986) used pyrolysis mass spectroscopy to classify Thermus strains. The 3 reference strains, T.aquaticus, "T.thermophilus" and Ramaley X-1 were separated from one another and from the majority of isolates which were recovered from New Zealand hot pools. The New Zealand isolates clusterd in 3 distinct groups without any relation to the geography, pH or temperature of the source pools. Degryse et al. (1978) performed a number of biochemical and physiological tests on 8

Thermus isolates and concluded that there were no major differences between T.aquaticus, "T.thermophilus" and the other strains tested, which had been isolated from a thermally polluted river in Belgium. They recommended that all the isolates be considered as essentially the same. However Cometta et al. (1982b) showed considerable variation among 35 Icelandic isolates in a numerical taxonomic analysis using only 33 characters, some of which were redundant, and including no replicates to show the reproducibility of the tests. The clustering of the isolates appeared to follow no pattern with regard to the temperature of the source of the isolates and geographical distribution. Cometta et al. (1982b) hypothesised that Thermus isolates are genetically unstable and it is this feature that enables them to survive in an extreme and changing environment.

1.2.2 Phenotypic Characteristics of Thermus.

Members of the genus Thermus can be divided into two groups represented by the two species; T.aquaticus strains (Brock & Freeze, 1969) which have "high" temperature optima of around 70°C, and T.ruber strains (Loginova & Egorova, 1975; Loginova et al, 1984) which have "low" temperature optima of around 50-55°C. The better characterised of the two groups is the "high" temperature group as these were the organisms on which the early interest centred. Both of these groups of organisms have a negative Gram reaction (Brock, 1984) and are pleomorphic rods (see section 1.2.5 below).

All of the T.aquaticus type organisms have temperature optima of around 70°C, with maxima around 80°C and minima around 40°C (Brock & Freeze, 1969; Pask-Hughes & Williams, 1977; Ramaley & Hixson, 1970; Saiki et al., 1972).The optimum pH for growth is given as 7.5-7.8 for T.aquaticus (Brock & Freeze, 1969) and 7.0-7.5 for "T.flavus" strains

(Saiki et al., 1972). For "T.thermophilus" the optimum growth temperature is given as 65-72°C, the minimum 47°C and the maximum 85°C.

For a number of strains of T.ruber the temperature optimum was given as 60°C (Loginova et al., 1984) and so there is a clear distinction between the two species of T.aquaticus and T.ruber.

A general requirement for standard growth medium appears to be a low concentration of yeast extract and peptone. T.aquaticus was enriched on medium containing basal salts (given in the correct form by Ramaley & Hixson (1970) who also isolated the non-pigmented Ramaley X-1 isolate on the same medium) with 0.1% each of tryptone and yeast extract (Brock & Freeze, 1969). Oshima & Imahori (1974) used only 0.8% polypeptone, 0.4 % yeast extract and 0.3% NaCl in their medium to grow "T.thermophilus. For the growth of "T.flavus" Saiki et al. (1972) used 0.4% (w/v) Kyukoto bouillon powder, 0.4% (w/v) polypeptone and phosphate buffer. T.ruber was grown on a potato-peptone agar (Loginova & Egorova, 1975).

Stramer & Starzyk (1979) reported much improved growth of Thermus on Thermus cell lysate indicating that where Thermus grew en masse in nature there would always be a supply of nutrients and growth factors from the lystaes of other cells.

1.2.2.1 Phenotypic Characters of T.aquaticus-like Strains.

Bacterial colony characteristics varied according to the medium composition, temperature of incubation and available moisture (Degryse et al., 1978). The colony types most frequently encountered were circular, raised and either rough or smooth.

Continuous culture experiments have shown a high maintenance requirement for oxygen by T.aquaticus (Cometta et al., 1982a) and many other reports have confirmed that Thermus strains are obligately aerobic (Brock & Freeze, 1969; Pask-Hughes & Williams, 1975 ;Saiki et al., 1972). An attempt has been made to grow isolates anaerobically (Degryse et al., 1978) on a medium containing 0.1% nitrate. There was an onset of growth but the growth yield was low. The presence of catalase was another indicator that Thermus strains are aerobic. Further work by Degryse et al. (1978), using the Hugh and Leifson test (Hugh & Leifson, 1953) which they had modified, showed that their isolates were unable to ferment carbohydrates. Although the isolates tested were capable of growth on carbohydrates as sole carbon sources, evidence could not be obtained to show that they were oxidised.

Growth of Thermus strains on single carbon sources has often been reported as poor. Pask-Hughes & Williams (1975), using a growth medium in which trypticase peptone and yeast extract had been replaced by 0.5 gl^{-1} ammonium sulphate and 2 gl^{-1} carbon source, found growth to be scarce and often limited to the primary inoculum streak on agar plates. Brock and Freeze (1969) had previously reported that growth on single carbon sources had been slower than that on trypticase peptone and yeast extract. A new medium was devised (Degryse et al., 1978) which had increased buffering capacity and proved successful in obtaining good growth of Thermus strains on single carbon sources. However the range of carbon sources which supported growth did not differ radically between the media used and were limited in number.

Carbon sources which supported growth include; acetate, aesculin, alanine, butyrate, citrate, formate, glucose, glutamate, glycerol, β-hydroxybutryate, malate, mannitol, phenyl acetate, pyruvate, starch, succinate, sucrose and xylose. Carbon sources which have never allowed growth include; acetamide, benzoate, creatine, glycine, heptane, hexane, hippurate, p-hydroxybenzoate, itaconate, mesaconate, methylamine, octanol, pentane, petroleum ether, and propionate.

In general hexoses, organic acids and sugar alcohols supported growth whereas aromatic compounds, alcohols, alkenes, alkanes and C1 compounds could not. All of the carbon source testing was performed using ammonium ions as the nitrogen source, but Degryse _et al._ (1978) showed that gelatin, glutamate, urea and nitrate could also supply nitrogen for growth.

Few extracellular enzymes from _Thermus_ strains have been described. Degryse _et al._ (1978) found all of the strains tested produced a gelatinase and an amylase while none of the strains produced a cellulase or an esterase (lipase). Oshima & Imahori (1974) also demonstrated a gelatinase in _"T.thermophilus"_. A detailed study has been carried out on an extracellular exonuclease (Takahashi & Uchida, 1978). The enzyme had a temperature optimum of 85°C and was shown to act in a manner different to _Escherichia coli_, T2 bacteriophage induced and venom exonucleases. It was also proposed that the thermophilic enzyme would be of use in determining 5' terminal and penultimate nucleotide sequences of oligonucleotides when used in combination with HPLC techniques.

Perhaps the best studied extracellular protein from _Thermus_ isolates is the protease derived from _Thermus_ strain T351 (ATCC 31674)(Cowan & Daniel, 1982; Khoo _et al._, 1984). This enzyme has been patented for potential industrial applications. The enzyme has been shown to contain 13% carbohydrate and a single zinc atom (Cowan & Daniel, 1982) as well as binding 6 Ca^{2+} ions per enzyme molecule to confer stability (Khoo _et al._, 1984). The enzyme showed remarkable stability with a half life of approximately 30 h at 80°C.

A heat stable extracellular protease has also been studied in _"T.caldophilus"_ GK24 (Taguchi _et al._, 1983) and two proteases reported in T.aquaticus (Matsuzawa et al., 1983) although the length of incubation (production started at 4 d) makes it possible that one of the enzymes was released by cell lysis.

Although of interest to the biochemist in adding to information on thermostability these enzymes have found no taxonomic utility in the studies to date.

The antiobiotic sensitivity pattern of Thermus strains has been shown to be very consistent amongst the strains studied. The first report (Brock & Freeze, 1969) noted that T.aquaticus was very sensitive to penicillin, actinomycin D, novobiocin, streptomycin and chloramphenicol whereas the organism could grow in the presence of 10 μg/ml of cycloserine or tetracycline. Degryse et al. (1978), Ramaley & Hixson (1970) and Saiki et al. (1972) all showed the same patterns in a range of isolates. Pask-Hughes and Williams (1975,1977) found this trend and in addition demonstrated a high resistance to sulphurazole.

Other substances have been tested for their action against Thermus strains. Sodium dodecyl sulphate inhibited growth at a concentration of 100 μg/ml (Brock & Freeze, 1969; Degryse et al., 1978; Saiki et al., 1972) and sodium chloride at a concentration of 2% (Brock & Freeze, 1969). The concentration of sodium azide required to inhibit growth is not clear as Brock and Freeze (1969) found inhibition at 500 μg/ml whereas Degryse et al (1978) reported growth at that concentration. Brock and Freeze (1969) found no growth of T.aquaticus on medium containing 1% tryptone plus yeast extract. Oshima and Imahori (1974) reported that "T.thermophilus" could grow in the presence of 2% polypeptone plus 2% yeast extract.

The literature shows that standard bacteriological diagnostic tests have been of little use in discriminating between strains.

Degryse et al. (1978) found that all 7 strains tested were oxidase positive, arginine dihydrolase negative and indole production negative. Cometta et al. (1982b) also showed all 33 Thermus strains gave negative results for indole production as well as all negative for H_2S production and all positive for β-galactosidase production. Bergey's Manual of Systematic Bacteriology states that most strains are positive for conversion of nitrate to nitrite (Brock, 1984).

Thermus strains can contain varying amounts of carotenoid pigments which give colonies a yellow or orange colour. Some strains lack these pigments entirely (Brock & Boylen,1973; Pask-Hughes & Williams, 1975; Ramaley & Hixson, 1970). Non-pigmented strains tended to originate from man-made thermal environments (see section 1.2.6). The carotenoid pigments comprised 60% of the cell membrane lipids (Ray et al., 1971b). The two major carotenoids were phytoene and Δ-carotene. Pask-Hughes & Williams (1977) found two types of absorbtion spectra which differed in major absorbtion peak wavelength. One type had an absorbtion peak at ~450nm and the other a peak at ~410 nm. Absorbtion "shoulders" were present at ~410 nm and ~480 nm so the difference between carotenoid types appears to be due to whichever of the three absorbtion peaks is the major peak and which ones are the "shoulders". Other reports have also described these peaks (Degryse et al., 1978; Oshima & Imahori, 1974; Saiki et al., 1972).

Degryse et al. (1978) tested for storage materials in 7 strains and were unable to detect the presence of glycogen or polyhydroxybutryate.

There have been no reports of motility in Thermus strains.

Studies of the cytochromes of Thermus strains have shown typical aerobic type components. McFeters and Ulrich (1972) showed the presence of cytochromes *a+a3*, *b* and *c*, the proportions of which remained relatively constant as the growth temperature was raised. The cytochrome composition of Thermus strain T351 (Hickey & Daniel, 1979) was also comprised of cytochromes *a*, *b* and *c* and in addition there was evidence for the presence of cytochrome *o*. No unequivocal evidence for the presence of cytochrome *a* was found in two non-pigmented strains, but they did contain *b* and *c* type cytochromes (Pask-Hughes & Williams,1975). A study of the cytochrome *c-552* from "T.thermophilus" (Hon-Nami & Oshima,1977) showed the cytochrome to be highly thermostable.

1.2.2.2 Phenotypic Chraracteristics of T.ruber isolates.

Loginova et al. (1984) described T.ruber type strain AUCM 1258 as being obligately aerobic. Carbon sources which supported growth included acetate, cellobiose, fumarate, galactose, glucose, glycerol, malate, maltose, mannose, mannitol, pyruvate, rhamnose, succinate and sucrose. Nitrate was not reduced to nitrite. The main feature which served to distinguish these isolates from T.aquaticus (apart from the temperature optimum, see section 1.2.2 above) was the possession of a red pigment. Methanol, methanol-acetone and hexane extracts all showed absorbtion peaks at 455, 483 and 513 nm, which resembled the pigments neurosporoxanthin and retro-γ-carotene (Loginova & Egorova, 1975). This represents the rather limited amount of information available on T.ruber phenotypic characteristics.

1.2.3 Genetic Characteristics of Thermus isolates.

The range of G+C mol% values found in Thermus strains has been found to be quite large although some of the variation may be due to the different techniques used. Table 1.2 shows reported G+C mol% values for a range of strains.

Table 1.2

G+C mol % values for Thermus Strains.

STRAIN	G+C MOL %	METHOD USED	REFERENCE
Thermus sp. NH	61.4	Thermal denaturation	Pask-Hughes and Williams, 1975
Thermus sp. DI	62.2	Thermal denaturation	Pask-Hughes and Williams, 1975
Thermus sp. X-1	64	Buoyant density centrifugation	Ramaley and Hixson, 1970
T.ruber	66	Chromatography	Aleksandrushkina and Egorova, 1978
T.aquaticus YT1	67.4	Density-gradient centrifugation	Brock and Freeze, 1969
"*T.thermophilus*" HB8	69	Chromatography	Oshima and Imahori, 1974
"*T.flavus*" AT-62	69-71	Thermal denaturation	Saiki *et al.*, 1972

Plasmids were first demonstrated in *Thermus* strains by Hishinuma *et al.* (1978). Of 8 strains tested 4 possessed plasmids; "*T.flavus*" BS1 contained 2 plasmid types with molecular weights of 6.1 x 10^6 and 17.0 x 10^6, "*T.thermophilus*" HB8, "*T.flavus*" AT61 and "*T.flavus*" AT62 each contained 1 plasmid with molecular weights of 6.2 x 10^6, 6.6 x 10^6 and 6.6 x 10^6 respectively. The authors were not able to determine the functions of these plasmids.

More detailed investigations of other plasmids have been made. Eberhard *et al.* (1981) isolated a plasmid (pTT1) from

"T.thermophilus". The plasmid had a molecular weight of about 5.4 x 10^6 and a G plus C content of 68%, which was similar to the host's chromosome. The degree of supercoiling was similar to that of bacteriophage PM2 DNA. A circular restriction endonuclease cleavage map was produced but no functions ascribed to the plasmid.

Vasquez et al. (1984) characterised 3 Thermus plasmids and found that they too had similar G+C% values to the hosts' chromosomes and degrees of supercoiling comparable with those found in the plasmids of mesophilic organisms. They were able to cure isolates of their plasmids (Vasquez at al., 1983) and found no differences between plasmid containing and plasmid-free strains with respect to antibiotic susceptibility, heavy metal salt susceptibility, viral infectivity, ability to produce bacteriocins and the production of endonucleases.

Recently Munster et al. (1985) demonstrated that 62.5% of Thermus isolates recovered from Yellowstone Park possessed plasmids, and again the plasmids were cryptic.

1.2.4 Morphology and Ultrastructure of Thermus.

Bergey's Manual of Systematic Bacteriology (Brock, 1984) describes Thermus as being straight rods of 0.5 to 0.8 μm in diameter and 5-10 μm in length with filaments from 20-200 μm occurring in some cultural conditions. Lengths of cells have been reported to be as low as 2-5μm (Saiki et al. ,1972) although no variation in cell width has been reported. In T.aquaticus, filaments are formed at temperatures of 75°C and above and in stationary phase cultures (Brock & Freeze, 1969) whereas the presence of filaments of T.ruber decrease with culture age (Loginova et al., 1984).

Brock & Edwards (1970) showed, using thin section electron micrographs, that the cell wall resembled those of most other Gram-negative bacteria. The envelope was shown to be in 3 layers consisting of a cytoplasmic membrane, a thin middle layer and a thick irregular outer layer which appeared to be joined to the thin layer by a series of connections. The outer layer appeared to be corrugated and when seen in tangential section consisted of a series of parallel bands. The cell division method resembled that of typical Gram-negative bacteria. Thin sections of *"T.thermophilus"* (Oshima & Imahori, 1974), *"T.flavus"* and *T.ruber* (Loginova & Bogdanova, 1977) and 4 *Thermus* strains (Pask-Hughes & Williams, 1978) showed the same structures. One strain ,strain Il (Pask-Hughes & Williams, 1978), possessed an extra layer exterior to the outer layer.

The production by *Thermus* isolates of rotund bodies, linear cell arrays and cell rosettes was first noted by Brock & Freeze (1969). Rotund bodies were shown by Brock & Edwards (1970) to be formed from a collection of cells contained within a common outer wall layer. More recently Becker & Starzyk (1984) investigated further the structures of all three of these characteristic cell aggregations using scanning electron microscopy. This study revealed rotund body spheres containing hundreds of cells and exhibiting diameters up to 126 μm. The sequence of events involved in the production of rotund bodies was initiated by the fusion of the outer layers of two cells at the apex of a V-shaped pair. Following this there was a "zipping up" of the outer layers resulting in a cell wall layer spanning the area between the two cells. Subsequent cell divisions resulted in the formation of the large rotund bodies observed.

Linear arrays comprised cells joined end to end arranged in a cylindrical manner. Rosettes were shown to be formed from many cells apparently attached at a common point.

The formation of rotund bodies was described as "highly unique" (Becker & Starzyk, 1984), but this is something of an overstatement as other micro-organisms have been shown to have similar structures. For example Fervidobacterium (Patel et al., 1985b) and Dictyoglomus (Saiki et al., 1985), which are extremely thermophilic glycolytic anaerobes and both show these structures.

1.2.5 Thermus, an Anomolous Gram-Negative Bacterium

Although Bergey's Manual of Systematic Bacteriology (Brock, 1984) describes Thermus as staining Gram-negatively, and thin sections show a Gram-negative type wall (Brock & Freeze, 1969; Pask-Hughes & Williams, 1978) there are many reports in the literature which indicate that Thermus is, at least, an anomolous Gram-negative organism.

Within the cell wall itself there are a number of features which are more akin to the Gram-positive organisms. The dibasic amino acid present in the peptidoglycan layer is ornithine (Pask-Hughes & Williams, 1978; Merkel et al., 1978a), while it is typically diaminopimelic acid for Gram-negative organisms. Merkel et al. (1978a) also reported certain similarities of the peptidoglycan to Gram-positive organisms including the possession of high concentrations of glycine and concentrations of glucosamine nearly equimolar with alanine. Pask-Hughes & Williams (1978) reported a lack of heptose and 2-keto-3-deoxyoctonate which implied a lack of basal core structure of the lipopolysaccharide found in the lipopolysaccharides of enterobacteria. The polysaccharide component of the lipopolysaccharide contained neutral and amino sugars like other Gram-negative bacteria.

A review of the respiratory quinone component and taxonomy of bacteria (Collins & Jones, 1981) showed that all Gram-negative aerobic rods contained ubiquinones whereas, the only exceptions, T.aquaticus and "T.thermophilus" contained menaquinones 7 and 8 as their major components. It was this review which also pointed out the anomolous citrate synthase of T.aquaticus.

It has now become established, largely through the work of P.D.J. Weitzman and Dorothy Jones (Weitzman & Jones,1968; Harford et al., 1976; Weitzman, 1980), that the size and control mechanism of bacterial citrate synthase is an important taxonomic tool which has been of practical use in bacterial taxonomy (e.g. Jones & Weitzman, 1974). An analysis of the citrate synthases from a large number of bacteria (Weitzman, 1980) revealed the following patterns; citrate synthases which were not inhibited by the presence of NADH were all from Gram-positive organisms, while those which were inhibited belonged to Gram-negative organisms. Further to this citrate synthases which were inhibited by NADH could be subdivided. Citrate synthases from obligate aerobes showed reactivation by AMP, while those from facultative organisms showed no such reactivation. The size of the citrate synthase also proved to be of taxonomic relevance in that "large" enzymes (of molecular weight approximately 250,000) belonged to Gram-negative organisms while Gram-positive organisms possessed "small" enzymes of molecular weights of approximately 100,000.

The only eubacterium which has been found to differ from these generalisations is T.aquaticus (Weitzman, 1978). NADH had no inhibitory effect, and the enzyme eluted from a sephadex column at precisely the same time as pig heart citrate synthase, a typical "small" enzyme. The citrate synthase of T.aquaticus was therefore shown to be of a typical Gram-positive type.

It should be noted, however, that the succinate thiokinase of T.aquaticus was of the typical Gram-negative type (Weitzman,1980) on the basis of protein size.

Ray et al. (1971a) noted that the fatty acid composition of Thermus resembled that of Gram-positive organisms rather than Gram-negative organisms. This was based on their findings that T.aquaticus contained a majority of fatty acids which were branched, and did not contain cyclopropane fatty acids which are found in some Gram-negative bacteria.

The antibiotic resistance pattern of Thermus isolates is also unusual for Gram-negative bacteria. All of the published analyses of antibiotic sensitivities for Thermus (Brock & Boylen, 1973; Brock & Freeze, 1969; Degryse et al.,1978; Pask-Hughes & Williams, 1977; Ramaley & Hixson, 1970; Saiki et al., 1972) have demonstrated a suprising susceptibility to penicillin, actinomycin D, and novobiocin.

These results provide a useful set of taxonomic characters with which Thermus can be clearly distinguished from other organisms.

It is of interest that 5SrRNA sequence phylogeny studies (Hori & Osawa, 1979; Hori et al., 1982) have shown that Thermus branched off from the other eubacteria at around the same time that the Gram-negative organisms and Gram-positive organisms were diverging and, on a phylogenetic basis, appeared to be more closely related to the Gram-positive organisms although the error ranges at this level make it impossible to state definitely that this is so.

1.2.6 Ecology.

Very little work has been done on the ecology of the genus, and that which has has concentrated exclusively on the higher temperature strains, with strains of T.ruber receiving little attention.

The initial isolations of Thermus strains (Brock & Freeze, 1969) were made from natural hot springs of Yellowstone National Park, U.S.A., and from hot tap water sources in Bloomington, Indiana, U.S.A., which is over 200 miles from the nearest hot spring. It was therefore quickly established that Thermus strains are associated not only with natural hot water features but also with man-made hot water sources.

Thermus strains have now been isolated from natural thermal areas located in distant regions of the world including in addition to Yellowstone Park, Iceland (Pask-Hughes & Williams, 1977), the Soviet Union (Egorova & Loginova, 1974; Egorova & Loginova, 1975; Khraptsova et al., 1984), Japan (Saiki et al., 1972) and New Zealand (Patel, 1984).

Kristjansson and Alfredsson (1983), in a study of 55 Icelandic hot springs, found that Thermus strains could be isolated from pools with temperatures between 55 and 85°C and a pH range from 6.5 to above 10. Isolates could also be obtained in pools containing up to 1mM dissolved sulphide and having a conductivity up to 2,000 μS/cm. Viable numbers ranged from 10 to 10,000 CFU/100 ml and in a thermal gradient reached highest numbers when the temperature was between 50 and 70°C. These figures agree well with the growth parameters of Thermus isolates (Pask-Hughes & Williams,1977; Saiki et al., 1972).

The types of man-made environments from which isolates have been obtained include hot tap water from two separate systems in the London Hospital (Pask-Hughes & Williams, 1975), American domestic and laundry hot water heaters (Brock & Boylen, 1973), nuclear power plant cooling waters and steel mill process water in the USA (Stramer & Starzyk, 1981). Strains isolated from hot water supplies have been reported to be more frequently non-pigmented than those isolated from natural environments (Brock & Boylen, 1972; Pask-Hughes & Williams, 1975) implying that pigmentation may play some photoprotective role.

Natural aquatic environments subjected to natural or man-made thermal additions are also sources for Thermus isolates. Zeikus and Brock (1972) showed that T.aquaticus could be isolated from river water kilometers downstream from natural hot water sources. They suggested that Thermus could be used as an indicator organism for thermal pollution of waterways.

This idea was further investigated by Stramer and Starzyk (1981) who enumerated Thermus isolates in thermally unpolluted waterways, thermally polluted waterways and in man-made hot water sources. They found very low numbers in unpolluted waterways (0.1 to 1 CFU/100 ml) which was below the detection limit of their standard enumeration technique. Thermally polluted waters, boilers and hot tap water produced counts of of 2.2 to >16 CFU/100 ml. An investigation of the survival of T.aquaticus in filter-sterilised river water showed rapid replication and slow reduction in numbers up to 100 days at 60°C and 70°C, while no cells were viable after 1 day at 100°C. At temperatures from 0 to 20°C viable cells were not found after 6 days at 20°C, 8 days at 10°C and 14 days at 0°C. Therefore it was concluded that Thermus is a good indicator of thermal pollution due to the low "background" count and the ability to grow rapidly and reach high numbers when thermal additions are made. The significance or effect of Thermus cells in the mesophilic environment is unknown.

Ramaley & Bitzinger (1975) demonstrated the appearence of several types of thermophilic aerobe, including Thermus strains, in a stream receiving discharges of hot water from a university. They also showed that the type and distribution of aerobic thermophiles from this source and two natural sources were similar.

Hot pool water has been shown to contain levels of organic matter of the order of 2ppm (Brock, 1978) which, in a continuously flowing system, is sufficient to support a standing crop of Thermus at temperatures where photosynthetic bacteria or algae are unable to grow. Numbers are greater ,however, where photosynthetic organisms are capable of forming mats (Kristjansson & Alfredsson, 1983) and it is likely that Thermus are able to obtain growth substrates from these organisms. At temperatures below about 50°C, where strains similar to T.aquaticus cannot grow, only low numbers are found and these are derived from being washed out from the higher temperature algal mats.

The distribution of different Thermus phenotypes in natural environments has been the subject of only one study (Cometta et al, 1982b). This work was based on a mini-numerical classification of Icelandic isolates. The results showed no typical sampling area specific populations and a diversity of phenotypes. The authors suggested that this variability was due to a genetic instability which enabled organisms to adapt to changing thermal conditions.

1.2.7 Bacteriophages Infectious to Thermus Strains.

A bacteriophage infectious to "T.thermophilus" has been isolated and characterised (Sakaki & Oshima, 1975). The bacteriophage, designated ϕ YS40, had a hexagonal head,a tail, base plate and tail fibres. Thermostability was equally good in hot spring water and in broth used for culturing "T.thermophilus", but a number of other suspending fluids decreased bacteriophage survival. The host range showed that T.aquaticus, 3 from 6 strains of T.flavus, and a number of the authors' own Thermus isolates could not be infected, indicating that there are differences between Thermus strains, at least with respect to bacteriophage receptor sites. The temperature range for replication of the virus was at the higher end of the range of temperatures at which the host grew.

A bacteriophage has also been isolated from a New Zealand hot spring (Patel, 1984) which had a similar morphology to ϕ YS40 and was designated 'phage Wai19-AT2. The two bacteriophages were shown to have different host ranges, Wai19-AT2 being able only to infect a Thermus isolate taken from the same pool as that from which the bacteriophage was isolated. Both bacteriophages showed a build up of defective viral particles in their hosts

1.2.8 Metabolism.

Enzymes from the glycolytic (Embden-Meyerhof-Parnas) pathway have been detected and are shown in table 1.3.

All of the enzymes involved in the TCA cycle were detected by Pask-Hughes & Williams (1977) in T.aquaticus and 4 Icelandic strains. The possession of these enzymes indicates that Thermus metabolism is similar to that of other heterotrophic bacteria.

The mechanisms of anaplerosis have received some attention. When grown on acetate as sole carbon source the glyoxylate cycle served as an anaplerotic pathway (Degryse & Glansdorff, 1981), indicated by the presence of the enzymes isocitrate lyase and malate synthase. A mutant unable to grow on acetate was found to lack isocitrate lyase (Degryse & Glansdorff, 1981).

Also present when the cells were grown on gluconeogenic substrates were the malic enzyme and phosphoenol pyruvate carboxykinase (Degryse & Glansdorff, 1981).

Table 1.3

Enzymes involved in glycolysis detected in Thermus isolates.

ENZYME	SOURCE	REFERENCE
Phosphofructokinase	"T.thermophilus"	Yoshida, 1972
Fructose diphosphate aldolase	T.aquaticus	Freeze and Brock, 1970
G3-P dehydrogenase	T.aquaticus	Hocking and Harris, 1973
Enolase	Ramaley XI	Barnes and Stellwagen, 1973
Enolase	T.aquaticus	Stellwagen et al., 1973
Pyruvate kinase	Strain Z05	Degryse and Glansdorff, 1981

Abbreviation used G3-P dehydrogenase, Glyceraldehyde 3-Phosphate Dehydrogenase.

The enzyme phosphoenol pyruvate carboxylase has been described in strains T.thermophilus" (Bridger & Sundram, 1976) and Z05 (Degryse & Glansdorff, 1976) and is used in anaplerosis when cells are grown on glucose. There was no detectable pyruvate carboxylase even in cells grown on pyruvate (Degryse & Glansdorff, 1976).

Investigations into a key gluconeogenic enzyme, fructose 1-6 bisphosphatase from "T.thermophilus" (Yoshida & Oshima, 1971; Yoshida et al., 1973) have shown that the concentrations of phosphoenol pyruvate and ADP/AMP are responsible for the allosteric control of this enzyme and phosphofructokinase to regulate glycolysis/

gluconeogenesis. The more usual system is controlled by the ATP/AMP balance (Yoshida et al., 1973).

Lactate dehydrogenase has been demonstrated in "T.caldophilus", (Taguchi et al., 1982; Taguchi et al., 1984; Taguchi et al, 1985) and T.aquaticus (Machida at al., 1985), and allosteric control shown dependent on fructose 1,6-bisphosphatase as an effector.

Little attention has been paid to amino acid metabolic pathways in Thermus. Guy & Daniel (1982) described the purification of a specific D-asparaginase from the strain T351, while the enzymes homoserine dehydrogenase (Saiki et al., 1973), threonine deaminase (Higa & Ramaley, 1973) and acetohydroxy acid synthase (Chin & Trela, 1973) have been described from amino acid synthesis pathways.

A variety of other intracellular enzymes have been studied including β-galactosidase from Thermus strains T2 (ATCC 27737) (Ulrich et al., 1972), and 41A (Cowan et al., 1984), NADH dehydrogenase from strain T351 (Walsh et al., 1983), alkaline phosphatase from T.aquaticus (Smile et al., 1977) and T.ruber (Egorova & Loginova, 1984), restriction endonucleases and modification methylases (Vasquez & Vicuna, 1982), DNA polymerase from T.aquaticus (Kaledin et al., 1980) and apparently identical sequence specific endonucleases from T.aquaticus (Sato et al., 1977) and "T.thermophilus" (Sato & Shinomiya, 1978).

When grown on single carbon sources T.aquaticus-like strains (Heinen, 1971) and T.ruber-like strains (Loginova & Khraptsova, 1977) were able to use organic acids at temperatures above their optima while sugars either allowed no growth or reduced growth. Heinen (1971) provided evidence which suggested that the inability to use sugars at higher than optimum growth temperatures was due to the enzymes of glycolysis being less heat stable than those involved in the utilisation of pyruvate. The ability of organisms to grow on sugars was restored when they were returned to their optimum growth temperature.

The major regulatory process known as the stringent response has been shown to be in operation in "T.thermophilus" (Lienert & Richter, 1981). On addition of hydroxylamine, which blocks amino acid acylation, and on lowering the temperature from 75 to 37°C growth and RNA synthesis were restricted while the production of (p)ppGpp increased above the basal level. The (p)ppGpp synthesising activity was localised in the ribosomes, and predominantly pppGpp was formed with only 10% of the total in the form of ppGpp. It was concluded that the (p)ppGpp synthetase from "T.thermophilus had properties similar to those of the enzymes from Bacillus stearothermophilus or Escherichia coli.

1.2.9 Mechanisms of Thermophily as Exemplified by Thermus.

As there is no way for a thermophile to buffer itself against its environment, the cell constituents must all be stable at the growth temperature. Oshima (1979) listed three mechanisms whereby thermostability of cell constituents may be conferred, these being 1) intrinsic mechanisms whereby stability is obtained by altering the internal structure of a molecule or cell component 2) post-translational or post-transcriptional modification and 3) protector mechanisms whereby cell constituents are only stable in the presence of certain protector molecules. More than one of these mechanisms can be operating in any one system at any one time.

The genus Thermus has been the subject of much work on thermostability and hence can be used to furnish examples of the above mechanisms.

1.2.9.1 Protein Stability.

It was postulated by Allen (1953) that survival at high temperatures was due to a high turnover rate of cell components. Bubela & Holdsworth (1966) presented supporting evidence in that they found that cellular proteins in *Bacillus stearothermophilus* had a half life of less than 2 min. at 63°C. However Kenkel & Trela (1979) found in *T.aquaticus* that there was not a high turnover rate, i.e. that *T.aquaticus* enzymes must be stable at the growth temperature. Since then the isolation and characterisation of many *Thermus* proteins has been performed and remarkable thermostability found when compared to the proteins from mesophiles. For just one example Ramaley & Hudock (1973) compared isocitrate dehydrogenase from *T.aquaticus*, *Bacillus subtilis* and *Chlamydomonas reinhardti*. All 3 enzymes showed similar catalytic properties but differed markedly in their thermostabilities. The optimum temperature for catalysis for *B.subtilis* and *C.reinhardti* enzymes was approximately 55°C, while that of *T.aquaticus* was over 80°C. The temperature at which 50% of the activity was lost after 1h incubation was 30°C for the *C.reinhardti* enzyme, 54°C for the *B.subtilis* enzyme and 88°C for the *T.aquaticus* enzyme.

Investigations into the mechanisms whereby thermostability is conferred to protein molecules have shown that there is no structural difference between heat-stable and heat-labile molecules (Grutter *et al.*, 1979) by showing that the three dimensional structure of lysozyme was the same as that of a temperature sensitive mutant. They cited this as evidence for an earlier proposal that the thermostability of proteins is due to subtle changes in hydrophobic interactions, hydrogen bonds etc. and that the net free energy of stabilisation of proteins is small and is derived from a balance between large stabilising forces and destabilising forces. Perutz & Raidt (1975) concluded that thermophile ferredoxins were stabilised by extra salt bridges on the molecular surface. Thus the differnces between heat stable and heat labile proteins have been found to be small and achieved through subtle changes of molecular structure.

1.2.9.2 Fatty Acids, Lipids and Membranes.

Several analyses of the fatty acid composition of Thermus have been carried out to investigate the changes which occur to allow growth at high temperatures. In a comparison of Thermus with 3 other thermophiles of lower temperature optima Oshima & Miyagawa (1974) found that the fatty acids present were qualitatively similar consisting mainly of C_{14}-C_{17} iso and ante-iso branched acids. The predominant acid was iso $C_{17:0}$. Heinen (1970) found a similar composition. Jackson et al. (1973b) showed that the fatty acid composition of T.aquaticus YT-1 and Thermus strain X-1 differed. They both contained a large component of branched chain fatty acids and the kinds of fatty acids present were also similar. Differences lay in the distribution of types of acid, there being a marked difference in the abundance of types of fatty acids between the two organisms. An analysis of T.ruber and "T.flavus" growing at their optimum temperatures (Andreev et al., 1981) showed that the fatty acid composition of these strains did not differ qualitatively but quantities were reported to vary in an unpredictable manner.

Most work has centred on how the fatty acid composition changes with different incubation temperatures. Ray et al. (1971a) found that the proportions of monoenic and branched C_{17} fatty acids and the proportions of iso C_{16} and normal C_{16} fatty acids increased with increasing incubation temperature. Also noted was an increase in the total fatty acid content of cells grown at 75°C compared with those grown at 50°C. The shift in fatty acid type represented a shift to a more thermostable composition as the C_{17} acids have higher melting temperatures than those of the C_{16} acids listed above.

Heinen et al. (1970) analysed the fatty acids of T.aquaticus at 80°C and 50°C and this work showed results at odds with those of other workers in that a major fatty acid component was found to be $n-_{15}$. Ray et al. (1971a) found $n-_{15}$ acids to be trace components.

Thermus does therefore respond to varying temperatures by altering the nature of its fatty acids to accomodate these changes, in that a shift in the fatty acid composition leads to greater stability at increased temperatures.

Ray et al. (1971b) determined the effect of the growth temperature on the lipid composition of T.aquaticus. As the temperature was increased so did the lipid content, which was suggested as a method of permitting thermophily. Over an increase in growth temperature from 50 to 75°C the phospholipids increased 2 fold (approximately) as did the carotenoids while the glycolipids increased 4 fold. It was shown that diacyl phospholipid turnover rate was comparable to that of mesophilic bacteria while the major phospholipid and carotenoids did not turnover. It was suggested that perhaps glycolipids increase the stability of membranes.

A novel glycolipid has been isolated from "T.thermophilus" (Oshima & Yamakawa, 1974) which comprised 70% of the total lipid, and again it was suggested that this molecule contributed to the stability of the membrane. A broader investigation (Pask-Hughes & Shaw, 1982) of several Thermus strains showed that this glycolipid was common to the strains but differed by the sugar component between the strains. A feature of the glycolipid considered to be important with regard to thermophily was the size of the hydrophobic moeity of the molecule, although no definite structural mechanism was advanced.

The presence of polyamines has been shown partially to protect protoplasts from lysis (Ray & Brock, 1971) indicating that they have a role in membrane stability. Subsequently polyamines have been isolated from Thermus strains (Oshima, 1975; Oshima, 1983) which suggests that they also contribute to the thermostability of membranes in this genus. This is an example of the use of one molecule to help stabilise another.

Work on membrane phase transitions has been carried out (Jansen et al., 1982) with strain T351. Three distinct phase transitions were shown to occur between 0 and 85°C, one at 19°C, one at 39°C, and one at 66°C. At temperatures above 66°C the membrane was assumed to be in the liquid crystalline state, thus allowing normal membrane processes to take place.

1.2.9.3 DNA, RNA and Ribosomes.

Although the DNA of Thermus isolates is G+C rich (see Table 1.2) this cannot be taken as a method enabling growth at high temperatures as other caldoactive bacteria have G+C mol% values much lower than Thermus. An example is Fervidobacterium nodosum (Patel et al., 1985b), a glycolytic anaerobe, which has a G+C mol% of 37.

Thermal denaturation profiles of 16s and 23s rRNA from T.aquaticus and E.coli showed that only the 23s rRNA from T.aquaticus showed enhanced thermostability (Zeikus et al., 1970). In the same study tRNA from T.aquaticus was stable up to 68°C and melted at 86°C. The rRNA and tRNA of T.aquaticus also contained a higher G+C mol% value than comparable molecules from E.coli. Extracted tRNA from "T.thermopilus" was shown to have a melting temperature of 87.5°C

(Oshima & Imahori, 1971). Chemical analyses of "T.thermophilus" tRNA has shown the presence of 5-methyl 1-2-thiouridine (Oshima, 1979). It was suggested that this too contributes to the thermostability of tRNA, and this was supported by structural hypotheses. This is an example of a post-transcriptional/translational modification used to enhance thermostability. Proteins involved in tRNA production (Kohda et al., 1984) and aminoacylation (Zeikus & Brock, 1971) have been shown to be thermostable.

Purified ribosomes have been shown to be thermostable when compared to those of mesophilic organisms (Oshima & Imahori, 1971; Zeikus & Brock, 1970), there being a correlation between the temperatures at which ribosomes denatured and the maximum growth temperature of the organism under study.

1.3 Numerical Taxonomy as Applied to Bacteriology.

1.3.1 The Need for Numerical Taxonomy.

While numerical taxonomy has not necessarily been totally accepted by botanists and zoologists even its critics conceed that its use by microbiologists is of great value (e.g. Blackwelder, 1967). The techniques used at the outset still remain relatively unchanged to date although the methods involved in obtaining and processing the data have become more sophisticated.

The need for a new type of taxonomic method was expressed at the end of the 1950's and subsequently by a number of people of whom P.H.A. Sneath, S.T. Cowan and R.R. Sokal were prominant. The book "Principles of Numerical Taxonomy" by Sokal & Sneath (1963) summarised the developments of the previous few years and really marked the start of widespread use of numerical techniques for taxonomy.

Sokal & Sneath (1963) devoted a chapter of their book to a critique of the then commonly used taxonomic methods. A summary of their major criticisms is given below:

1) Self reinforcing circular arguments are used to establish categories.

2) Phenetic taxonomic data is interpreted in a phylogenetic manner in the classificatory process.

3) Classifications are based on homologous characters which is not feasible.

4) Some characters are given more "weight" than others, which although valid in identification is not so in classification.

5) Descriptions and taxonomic work have made no improvement since the turn of the century.

6) The involvement of subjectivity in nomenclature is not helpful.

Numerical taxonomy therefore had the aim of arriving at values of affinity based on unweighted and multiple characters with the maximum degree of objectivity. It was also hoped that numerical taxonomy would produce the most "natural" classification i.e. giving the most information to the "general" user. It was considered that a phylogenetic classification was in fact a special classification.

1.3.2 Some Definitions.

Before a brief discussion of numerical taxonomy is given it is necessary to give some definitions which will be adhered to in the rest of the text.

TAXONOMY: "the scientific study of classifications" (Hedges, 1979).

Taxonomy includes the study of classification, nomenclature and identification.

CLASSIFICATION: "the ordering of organisms into groups (or sets) on the basis of their relationships, that is of their associations by contiguity, similarity or both" (Sokal & Sneath, 1963).

PHENETIC RELATIONSHIP: "the overall similarity as judged by the characters of the organisms without any implication as to their relationship by ancestry" (Sokal & Sneath, 1963).

PHYLETIC (OR PHYLOGENETIC) CLASSIFICATION: "has as one of its aims, the exposition of evolutionary relationships" (Singleton & Sainsbury, 1980).

TAXON; An abbreviation for taxonomic group of any nature or rank. (Sokal & Sneath, 1963).

NUMERICAL TAXONOMY: "the grouping by numerical methods of taxonomic units into taxa on the basis of their character states" (Sneath & Sokal, 1973). This perhaps blurs the distinction between "taxonomy" and "classification" so this description more fittingly describes "numerical classification".

1.3.3 The Principles of Numerical Classification.

Sneath & Sokal (1973) give the following principles which are probably concise enough to warrant direct reproduction;

"1) The greater the content of information in the taxa of a classification and the more characters on which it is based, the better a given classification will be.

2) *A priori*, every character is of equal weight in creating natural taxa.

3) Overall similarity between any two entities is a function of their individual similarities in each of the many characters in which they are being compared.

4) Distinct taxa can be recognised because correlations of characters differ in the groups of organisms under study.

5) Phylogenetic inferences can be made from the taxonomic structures of a group and from character correlations, given certain assumptions about evolutionary pathways and mechanisms.

6)Taxonomy is viewed and practiced as an empirical science.

7)Classifications are based on phenetic similarity."

The general order of going about a numerical classification is discussed by Rohlf & Sokal (1981). The most frequent order is as follows; characters are selected and their states recorded for all of the organisms (or OTUs, Operational Taxonomic Units) involved, measures of resemblance are calculated for each OTU compared with each other OTU, clustering techniques are performed on these values to form taxa, the taxa produced are represented in some manner so that conclusions about the taxa present can be drawn.

1.3.4 A Summary of Steps Involved in Performing a Numerical Classification.

1.3.4.1 Selection of Organisms.

To select organisms for study it is necessary to decide on the criteria used to define the taxon to be studied essentially from the exisiting classification. However care must also be taken to avoid omitting organisms from a study which do not strictly conform to the criteria for inclusion to the taxon to be studied (Sneath & Sokal, 1973). Too few OTUs will lead to sampling error in the classification while too many will be unmanageable. It is desirable to include reference strains and type strains so that comparisons can be made between classifications.

1.3.4.2 Choice of Characters.

A character can be defined as being "...of two or more states, which within the study at hand cannot be subdivided logically except for subdivision brought about by the method of coding" (Sneath & Sokal, 1973). Characters are said to have "states", for example a character may be the possession of β-galactosidase and the states can be either "possessed" or "not possessed". The use of quantitative data may not necessarily conform to this definition as the character states may be continually variable. However it is usual for quantitative data to be regarded as a number of related characters which are broken down from the original data as far as reliability will allow.

Sokal & Sneath (1963) list inadmissable characters including meaningless characters (e.g. culture collection numbers), logically correlated characters (e.g. the length and half-length of a structure), partial logical correlations (e.g. degree of melanisation of the skin and skin colour assessed by some colorimetric method) and invarient characters (e.g. if all bacteria in the same study had the same Gram reaction).

Austin & Colwell (1981) in advice on character selection suggest that tests from a wide variety of biological activity of the organism are chosen including morphological, colonial, biochemical, nutritional and physiological characters. They state that no fewer than 40 tests should be included.

In bacteriology the temperature at which the tests are carried out may be important if the OTUs under study vary significantly in their optimum growth temperature and the choice may need to be made between incubating at a common temperature allowing all the OTUs to grow or to incubate at the optimum temperatures of the organisms. Sneath (1968) presented a mathematical way of overcoming this problem by producing a matching coefficient with a "vigour" and a "pattern" component.

1.3.4.3 Coding of Character Data.

The exact format of coding will depend on the computer program used but it is standard practice to represent a positive result as a 1 and a negative result as a 0. Missing data are represented in some other manner according to the computer program. This method is the one used when coding simple 2 state characters. When a character has more than two states the various states can be assigned numbers if the program allows, or converted to 2 state data. This is also required by some algorithms when continuously varying data is used. Sokal & Sneath (1963) discuss this matter and give 2 methods which have become commonly used; additive and non-additive coding. In additive coding a character with n states is coded into n-1 2 state characters. This method does introduce some weighting to the character in question and involves logical redundancy. Non-additive coding has the advantage of giving the character a weight of 1 and does not suffer from logical redundancy. Sokal & Sneath (1963) state that additive coding is simple and adequate where many characters are used.

Other algorithms which assign similarity values can deal with raw data given as numbers. In this case there is one main method used which was proposed by Gower (1971), and is known as the S_G coefficient. In this, apart from a weighting component (to deal with no comparisons) the similarity for any one character was given by

$$Sijk = 1- \frac{|Xij-Xik|}{R} \qquad [1]$$

where Sijk is the similarity between OTUs j and k for the ith character, Xi is the state of the ith character and R is its range. Thus each comparison has a value between 0 and 1 regardless of the character values in question. Ware & Hedges (1978) suggested that the S_{GA} coefficient would have advantages where equation [1] (a part of the S_G coefficient) was substituted by

$$Sijk = Xij/Xik \qquad [2]$$

and the computer programmmed to ignore with divide by zero errors. It was argued that computer programming would be simplified. Gower (1978) replied to this criticising S_{GA}, as the similarity between two OTUs would not change should more OTUs be brought into the classification. He stated " A duck may not seem very similar to a hawk but it is when compared to a cat".

1.3.4.4 The Calculation of a Similarity Value.

Given the kind of data which would be obtained in a study of this kind a biologist would probably think of using a correlation coefficient to determine the correlations between all of the organisms used. However Eades (1965) pointed out the inappropriateness of using the Pearson product-moment correlation coefficient. He found that there was a chance of fortuitous high correlation when OTUs were not very similar and that there can be unequal weighting of characters under certain circumstances.

The original coefficients are still used today and are based on a table for each pair of organisms as below (Sneath & Sokal, 1973)

		OTU j		
		1	0	
OTU k	1	a	b	a+b
	0	c	d	c+d
		a+c	b+d	n=a+b+c+d

where a,b,c and d represent the number of 1,1 matches, 0,1 mismatches, 1,0 mismatches and 0,0 matches respectively between OTUs j and k. The number of comparisons, n, is given by the total of a+b+c+d.

The simplest coefficient of similarity is that known as S_J (Sneath, 1957) which gives a similarity value as

$$S_J = \frac{a}{a+b+c} \qquad [3]$$

As such it does not count 0,0 matches and it is questionable as to whether they should be ignored or not (Sneath & Sokal, 1973) and in general this coefficient has infrequently been used in microbiologircal work.

Perhaps the most widely used similarity coefficient was introduced by Sokal & Michener (1958) although they did not use it in their study. The coefficient is that of equation [4] below.

$$S_{SM} = \frac{a+d}{a+b+c+d} \qquad [4]$$

Here negative matches are taken into consideration. Outlined above is the S_G coefficient (equation [1]) used in situations where the data has not all been scored in two state characters.

Also commonly used are distance coefficients which calculate the distance between OTUs if they were plotted in n-dimensional hyperspace (where n=number of characters). The main coefficients which have been used calculate the distance between OTUs along the edges of the hypercube (Manhatten metric) or in a straight line across the hypercube (Euclidian metric). Sneath & Sokal (1973) give an account of these metrics and their variants.

Not very frequently used coefficients are those which use probabilistic methods and these are again discussed in Sneath & Sokal (1973).

1.3.4.5 Clustering Methods.

The end product of calculations of similarity values for each pair of organisms is a symmetrical matrix containing these values. This set of data must then be converted into a form for easy assimilation and interpretation. To this end there are a number of methods which may be used each of which have various merits and demerits.

There are many approaches to clustering although one type has become predominant. The two fundamental methods are to have a hierarchical or non-hierarchical system. Non-hierarchical techniques are not frequently used in biology even though they can give good representations of the similarity matrix (Rohlf, 1970). Assuming that a hierarchical technique is to be used clusters may be formed either by gradually adding OTUs to the clusters (agglomerative) or by gradually splitting the OTUs up into clusters (divisive). The ease of programming has meant that agglomerative techniques have become the most commonly used (Sneath & Sokal, 1973). Another major consideration is whether the clusters should be non-overlapping (no OTU may belong to more than one cluster at any one taxonomic rank) or overlapping (where this is allowed). Non-overlapping methods may permit a better representation of the similarity matrix (Rohlf, 1970), but the non-overlapping technique is the more conventional method and allows the formation of the familiar nested clusters.

The most commonly used clustering techniques belong to the SAHN (Sequential Agglomerative Hierarchical Non-overlapping) type, and it is this type which will be briefly reviewed.

Single link clustering was introduced by Sneath (1957) and is also known as the "nearest neighbour" technique. In this method two clusters are joined at the level of the highest similarity value between any member of one cluster and any member of the other cluster. Dunn & Everitt (1982) comment that this method has the disadvantage of "chaining" together of OTUs i.e. groups of OTUs which are distinct may be clustered at a relatively high similarity level by being linked together by a chain of OTUs between the two groups.

Complete linkage is similar except that the two groups only join at the lowest similarity value between all the OTUs in one group and all the OTUs of the other.

Sokal & Michner (1958) introduced average linkage clustering to overcome the extremes of the other 2 methods indicated above. In this method two clusters are joined at the average similarity value for each member of one cluster versus each member of the other cluster. Weighted linkage methods attempt to give candidate clusters equal weight regardless of the number of OTUs in them, so that individual OTUs are given unequal weight, while unweighted average linking (also known as UPGMA) gives each OTU equal weight. Sneath & Sokal (1973) note that the unweighted methods give less distortion when a resulting taxonomic structure is compared with the similarity matrix.

Centroid clustering uses the mean character values for each cluster and links clusters at the similarity values for the two average vectors under consideration.

Other SAHN techniques which are used less frequently are discussed in Sneath & Sokal (1973).

The main question when confronted by these techniques is which is the best to use? There appears to be no definitive answer. Dunn & Everitt (1982) state that no single method is best for every situation, that single linkage while being the most mathematically respectable is the least satisfactory in practice, and that average linking methods are the best overall. This last point is in agreement with Rohlf (1970) when given a choice of the techniques listed above. Farris (1969) came to the same conclusion with the use of the cophenetic correlation coefficient (Sokal & Rohlf, 1962).

The most commonly used strategy used in the bacteriological literature appears to be the use of S_J and/or S_{SM} followed by clustering using average group methods.

1.3.4.6 How Classifications are Displayed.

The most common method of displaying the results of a classification is the dendrogram or phenogram. The term phenogram is to be preferred (Sneath & Sokal, 1973) as the term dendrogram may be taken to include phenograms (displaying phenetic similarity) and cladograms (which represent evolutionary branching sequences). Schnell (1970) makes use of many phenograms. The orientation of phenograms may vary but one of the axes represents taxonomic similarity (or dissimilarity). The points at which various OTUs meet represent their similarity with each other and clusters can be seen. For publication simplified phenograms are being more frequently used (e.g. McCarthy & Cross, 1984), where only the major clusters are shown for ease of interpretation.

Another common method is to use shaded triangles where the organisms are clustered and arranged so that clustered organisms are adjacent. A number of shades from black (high similarity) to white (very low similarity) are used to represent the similarity values between each pair or organisms. The final result is a large triangle with a number of dark triangles along the diagonal edge representing the clusters. McCarthy & Cross (1984) have an example.

Sneath & Sokal (1973) detail other methods. The most commonly used other methods are for representations of non-hierarchical classifications, such as those from principal components analysis. These displays are usually points plotted in the first three components or as stereograms which are able to represent the first three components in a pseudo 3-dimensional manner (Rohlf, 1968).

1.3.4.7 Ordination Methods.

By these methods the OTUs under study are distributed in a large number of dimensions and this distribution is summarised with either orthogonal or non-orthogonal axes depending on the method used. Of these methods principal components analysis (Dunn & Everitt, 1982) is probably the most frequently used, and employs orthogonal axes. This method is good at representing the differences between major clusters but misrepresents the distances between close neighbours (Sneath & Sokal, 1973). Other methods discussed by Sneath & Sokal (1973) include multiple factor analysis, principle co-ordinate analysis, non-metric multi-dimensional scaling and seriation. These methods have not frequently been used in bacteriology and when used have dealt with unusual data sets (e.g. Donnison et al., 1986; Shute et al., 1984, which both interpreted pyrolysis mass spectroscopy peak heights).

1.3.4.8 Identification.

The process of identification has become as statistically orientated as classification and one method of identification is to run the unknowns through a whole series of tests for a numerical classification and to see where they cluster. Keys are a second method of identification. The traditional monothetic key is prone to error with an exceptional organism and while they may be useful in some aspects of bacteriology are not really satisfactory identification schemes (Cowan, 1971). Polythetic keys, where a branch point is determined by more than one character is the most common form of key (Sneath & Sokal, 1973) and here computers can be used to produce the keys and to assign weights to diagnostic tests (e.g. by their discriminatory power (Rypka et al., 1967)). The number of tests should be much reduced from the original classification or the convenience is lost. A simultaneous key compares a vector of character states of an unknown OTU with a table of reference OTUs and the closest match is taken as a correct identification. Another related method is to plot the unknown OTU in multidimensional hyperspace and determine with which hyperspherical cluster it is nearest. Detailed accounts of these methods and others are given in Sneath & Sokal (1973).

The third type of identification method, the application of probabilistic techniques, has now been investigated (e.g Dybowski & Franklin, 1968; Lapage et al., 1973; Wilcox et al., 1973) and promises to improve techniques. In probabilistic methods the organism is identified by calculating which taxon the organism probably belongs to. These techniques allow for variable and error prone characters and also accept that it is impossible to identify an organism with certainty. Identification rates by these methods of 70-90 % were obtained (Lapage et al., 1973), the lower rates being due to the inappropriate tests available for certain groups of bacteria.

1.3.5 Summary.

While section 1.3 above does not cover by any means the full spectrum of numerical taxonomy the basic techniques which have now become standard practice have been briefly discussed. Current work in numerical taxonomy aims to evaluate the classifications produced (e.g. Archie, 1984) and to assess how they are affected by certain factors (e.g. Sneath, 1983). Also still under much discussion are the relative merits of phenetic and phylogenetic classifications under various conditions (e.g Colless, 1981; Sokal et al., 1984). The type of data being analysed has also been changing, reflecting attempts to speed up the classification process by using techniques which are more automated or quicker to run. Examples are shown in pyrolysis mass specroscopy (Donnison et al., 1986; Shute et al., 1984), whole cell electrophoresis (McCormick et al., 1985), enzyme electrophoresis (Chun et al., 1985), pyrolysis gas-liquid chromatography (Macfie et al., 1978), fatty acid methyl ester analysis (Mallory & Sayler, 1984) and others.

The area of nomenclature has not been mentioned as this is now rigidly controlled (Lapage et al., 1975).

1.4 Objectives.

Thermus is a relatively recently described genus (Brock & Freeze, 1969) which had not received any rigorous taxonomic treatment. Only 2 species have been accepted although evidence suggests that there is variety in the genus (Cometta et al., 1982b). A number of other isolates have also been described and given names, which have not been accepted as valid, and tend to confuse the situation because of the incompleteness of some of their descriptions.

This project set out, initially, to devise a suitable number of tests, as too few were then available, so that a numerical classification of the genus could be carried out and some taxonomic conclusions drawn. The classification was to be carried out on isolates from New Zealand hot springs, available in freeze-dried form. Also, to test for phenotype stability in hot springs, isolates were to be taken from hot springs over a period of time and examined for any changes in phenotype which might occur. This work was to be supported by continuous culture studies on a selected strain so that any phenotypic change in response to varying the environmental conditions could be detected, and so demonstrate any genetic instability.

Chapter 2: Development of the Character Set.

2.1 Introduction.

To enable a numerical classification of *Thermus* isolates to be carried out a minimum of 50 tests needed to be devised, and these tests needed to represent a broad spectrum of test types in order that the goal of sampling a random portion of the genome could be achieved. Until this study was undertaken the number of useful tests available was very small as only two phenetic taxonomic investigations (Degryse *et al.*, 1978; Cometta *et al.*, 1982b) had been made. Degryse *et al.* (1978) found that most of their tests were redundant i.e. they were either all positive or all negative for all of the somewhat small number of isolates tested. Cometta *et al.* (1982b) used, a commercially available test system which may not have functioned correctly at 70°C, and again a large number of the tests were redundant if the data from only *Thermus* isolates are compared.

2.2 Materials and Methods.

2.2.1 Strategy.

To devise a series of tests 10 isolates available in the laboratory at the time were selected so that the performance of potential tests could be evaluated with a workable number of organisms. Although some tests might show infrequent result differences if evaluated with a larger number of organisms, and these would be missed where only 10 isolates were used, these tests would provide less discriminatory power than those which could show differences between 10 isolates. Therefore it was felt that any tests which might be missed by the screening procedure would not significantly add to the classification.

2.2.2 Strains Used.

Table 2.1

Isolates Used in Test Evaluation.

ISOLATE	SOURCE	REFERENCE
NCIB 11245	Hot tap water, UK	Pask-Hughes and Williams 1975
NCIB 11246	Hot tap water, UK	Pask-Hughes and Williams 1975
NCIB 11247	Icelandic Hot Pool	Pask-Hughes and Williams 1977
Rt 41A	New Zealand Hot Pool	Cowan *et al.*, 1984
T351	New Zealand Hot Pool	Cowan and Daniel, 1982
"*T.thermophilus*"	Japanese Hot Pool	Oshima and Imahori, 1974
T.aquaticus	US Hot Pool	Brock and Freeze, 1969
Tok 3	New Zealand Hot Pool	Saravani, 1985
Ramaley X-1	US Thermally Polluted Stream	Ramaley and Hixson, 1970
HWBSA1	New Zealand Coastal Hot Pool	-

Table 2.1 shows the strains used for initial screening work. The strains represented a number of geographical sources, pigment types and supposed differences at the species level.

2.2.3 Culture Maintenance.

Inocula used were either grown on Castenholz Medium D (CMD) agar (Ramaley & Hixson, 1970) solidified with 3% (w/v) Oxoid No. 1 agar or in CMD broth for 24 h. CMD contains (gl^{-1}) Nitrilotriacetic acid, 0.1, $CaSO_4.2H_2O$, 0.06, $MgSO_4.7H_2O$, 0.1, NaCl, 0.008, KNO_3, 0.103, $NaNO_3$, 0.689, Na_2HPO_4, 0.111, yeast extract, 1, trypticase peptone, 1, trace element solution 1 ml, $FeCl_3$ solution 1 ml. Trace element solution was made up at 1000 fold strength so that the final concentrations in mg l^{-1} were as follows; $MnSO_4.H_2O$ 2.2, $ZnSO_4.7H_2O$ 0.5, H_3BO_3 0.5, $CuSO_4$ 0.016, $Na_2MoO_4.2H_2O$ 0.025, and $CoCl_2.6H_2O$ 0.046. The stock solution of $FeCl_3$ was also made up at 1000 fold concentration so that the final concentration in the medium was 0.28 mg l^{-1}. All plates were sealed with masking tape to prevent excessive dehydration on incubation. Broth cultures were dispensed in aliquots of 10 ml per 25 ml bottle and were not shaken during incubation. Unless otherwise stated all incubations were at 70°C.

2.2.4. Tests Evaluated.

Unfortunately control organisms which would normally be used to ensure that a known test worked in the usual manner were not available for the temperatures used as there has been little taxonomic work on aerobic thermophiles. The results of tests are compared with those reported in the literature wherever possible. Once a test had been shown to differentiate Thermus isolates appropriate controls could be used.

Abbreviations used in tables are as follows; Ram, Ramaley X1; 45, NCIB 11245; 46, NCIB 11246; 47, NCIB 11247; Taq, T.aquaticus, Tth, "T.thermophilus" and 41A, Rt 41A.

2.2.4.1 Proteolysis on Casein Milk Agar.

METHOD: CMD agar was supplemented with 0.1% (w/v) skim milk powder (Difco) and 0.5% (w/v) Hammarsten Casein (BDH). Organisms were streaked onto each plate so that confluent areas of growth, approximately 2.5 cm x 2.5 cm were obtained and plates were examined after 24 h for the production of a white precipitate around the margin of growth.

RESULTS AND DISCUSSION: Organisms positive were; T.aquaticus, Ramaley X-1, T351, Rt 41A, and Tok 3. As 5 organisms were negative and five positive it was decided that the test was suitable for use in the classifications.

2.2.4.2 Hydrolysis of Other Proteinaceous Materials.

METHOD: An agar overlay technique was used. Approximately 20 ml per plate of CMD agar was poured and allowed to set. An overlay was then poured which contained one of the following; elastin 0.3% (w/v), hide powder azure 0.2% (w/v), fibrin 0.2% (w/v) (all obtained from Sigma Chemical Co.) or keratin 0.1% (w/v) (Merck). Two organisms were streaked per plate as in section 2.2.4.1. Plates were incubated for up to 3 d and examined daily for the presence of clear zones around the margin of growth.

RESULTS AND CONCLUSIONS: The data obtained are shown in table 2.2.

Apart from keratin the other substrates appeared to be suitable for use as test substrates. Additionally plates could be read up to 72 h so that data from all three daily readings might be used if they proved to be sufficiently discriminatory when a larger number of organisms was used. The reading times used in each case are shown in the list of tests for that particular classification.

Table 2.2

Hydrolysis of Proteinaceous Materials by *Thermus* Strains.

ORGANISM	HIDE POWDER AZURE		ELASTIN			FIBRIN			KERATIN		
	24h	48h	24h	48h	72h	24h	48h	72h	24h	48h	72h
T.aquaticus	+	+	+	+	+	+	+	+	-	-	-
"*T.thermophilus*"	-	+	-	-	-	-	-	-	-	-	-
Ramaley X-1	+	+	-	+	+	+	+	+	-	-	-
T351	+	+	-	+	+	+	+	+	-	-	-
Rt 41A	+	+	-	+	+	-	+	+	-	-	-
Tok 3	+	+	-	+	+	+	+	+	-	-	-
11245	-	-	-	-	-	-	-	-	-	-	-
11246	+	+	-	-	-	-	-	-	-	-	-
11247	-	+	-	-	-	-	+	+	-	-	-
HWBS A1	+	+	-	+	+	-	-	-	-	-	-

2.2.4.3 Degradation of Insoluble Materials.

Xanthine decomposition was tested according to the method of Gordon (1966). To CMD agar was added xanthine to a concentration of 0.4% (w/v) before autoclaving. Plates were inoculated with two organisms each as in section 2.2.4.1, incubated for up to 5 d and examined for zones of clearing around the margin of growth.

Chitin was prepared according to the method of Hsu & Lockwood (1975) in the following manner. 6g of chitin (Sigma Chemical Co.) was stirred with 30 ml of 5.25% (v/v) sodium hypochlorite for 10 min. This mixture was added to 500 ml of tap water and filtered. This washing procedure was repeated 5 times and the chitin recovered by air-drying. The bleached chitin was ground in a Waring blender and dissolved in 40 ml of conc. HCl with constant stirring; and colloidal chitin obtained by adding the acidic solution slowly to 200 ml of water which was kept on ice. The chitin was recovered by filtration and washed 3 times in 500 ml of water before finally being filtered and resuspended in water for autoclaving. Chitin was added to CMD agar to a final concentration of 0.3% (w/v). Plates were inoculated with two organisms each as in section 2.2.4.1 and incubated for 3 d before being examined for zones of clearing.

Degradation of carboxymethyl cellulose was tested by the method of Williams (1983). Carboxymethyl cellulose was added to CMD agar to a concentration of 0.1% (w/v) before autoclaving. Incubation was for 48 h. Plates were then flooded with 0.1% (w/v) trypan blue for 15 min., the staining solution was washed from the plates and they were re-flooded with 1 M NaCl, left for a further 15 min. and examined for zones of clearing.

RESULTS AND DISCUSSON: None of the organisms showed zones of clearing, so none of the tests used were considered to be of use.

2.2.4.4 API-ZYM Reactions.

API-ZYM test strips (API Laboratory Products Ltd) were used according to the manufacturer's instructions. A control of uninoculated medium was included. To prevent excessive dehydration each strip was sealed along its edges with masking tape and strips incubated inside polythene bags also sealed with masking tape.

RESULTS AND DISCUSSION: Results of the tests are shown in table 2.3.

Table 2.3

API-ZYM Results for the Test Organisms.

TEST	Control	Tok3	T351	45	Tth	Taq	Rt41A	RamX1	46	HWBS	47
1	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
3	+/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
4	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
5	-/-	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
6	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
7	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
8	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
9	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
10	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
11	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
12	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
13	-/-	-/-	-/-	-/-	-/-	-/-	-/+	-/-	-/-	-/-	-/-
14	-/-	-/+	-/+	-/-	-/+	-/-	-/+	-/+	-/-	-/-	-/-
15	-/-	-/-	-/+	-/-	-/-	+/-	-/+	-/-	-/-	-/-	-/+
16	-/-	+/+	+/+	+/+	+/+	+/+	+/+	-/+	-/+	+/+	-/+
17	-/-	-/+	-/+	-/-	+/+	+/-	-/+	-/+	-/-	-/+	-/-
18	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
19	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
20	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

Results for two separate trials, - for negative, + positive. The results for the two trials are separated by / symbols. The test numbers stand for the following;1) Control 2) Alkaline phosphatase 3) Esterase (C4) 4) Esterase lipase (C8) 5) Lipase (C14) 6) Leucine arylamidase 7) Valine arylamidase 8) Cystine arylamidase 9) Trypsin 10) Chymotrypsin 11) Acid phosphatase 12) Phoshoamidase 13)α-galactosidase 14) β-galactosidase 15) β-glucuronidase 16) α-glucosidase 17) β-glucosidase 18) N-acetyl-ß-glucosaminidase 19)α-mannosidase 20) α-fucosidase.

The results for the organisms were very similar with a few minor differences in tests 13,14,15,16 and 17. Tests 11 and 12 were not functional at 70°C, and tests 2,3,4,5,6,7,8,9,10,18,19 and 20 were all redundant. Overall the system may have been useful with further standardisation but due to the expense involved in buying test strips for large numbers of organisms and the relatively few tests that were useful per strip it was decided not to use this system. It did however give an idea of which enzyme assays might be useful to test as separate assays. The fact that some of the tests were always positive even in the control wells may mean that such test strip systems are not reliable at elevated temperatures.

2.2.4.5 Arylsulphatase Production.

The method in Kersters & de Ley (1971) was adapted for use with microtitre plates. These plates, supplied by Nunc, Roskilde, Denmark, were of the 96U bottomed well type. Inocula were grown in CMD broth for 24 h and centrifuged to obtain a cell pellet. From each culture 50 μl of cell-free supernatant was added to 2 wells in each of 6 microtitre plates. The cell-free supernatant was discarded and the cell pellet resuspended in distilled water. From this suspension 50 μl was added to another 2 wells in the 6 microtitre plates. To each test well 50 μl of 2·5 mM p-nitrophenyl sulphate dissolved in sodium acetate-acetic acid buffer (pH 5·8) was added, and also to uninoculated medium controls. Plates were sealed with "Gladwrap" plastic film and incubated. Hourly a plate was removed and 50 μl of 0·04 M glycine-NaOH buffer (pH 10·5) was added to each well, and wells examined for the production of a yellow product.

RESULTS AND DISCUSSION: None of the test organisms showed a positive reaction under the conditions used, and so this test was not considered to have any potential use.

2.2.4.6 Phosphatase Production.

Both alkaline and acid phosphatases were tested for by a modification for use with microtitre plates from the method in

Kersters & de Ley (1971). Each test well contained 50 μl of cell suspension or cell-free supernatent and 50 μl of test reagent (0·01 M p-nitrophenyl phosphate in 0·1 M citrate buffer, pH 4·8). Assays were run for 1 to 6 h as in section 2.2.4.5 and the reaction stopped by the addition of 50 μl of 0·04 M glycine-NaOH buffer (pH 10·5) which enabled a yellow colour to form where the enzyme had been active.

Alkaline phosphatase was assayed for in the same manner except that the p-nitrophenyl phosphate was dissolved in 0·04M glycine-NaOH buffer (pH 10·5) and hence no addition of this buffer was needed to visualise the colour production.

RESULTS AND DISCUSSION: No evidence was obtained for the production of acid phosphatase by any of the strains tested. By 2 h of incubation, all of the strains were positive for alkaline phosphatase by resuspended cells. There appeared to be some weak positive reactions by the cell-free supernatant of T.aquaticus, T351, Tok3 and HWBS after 6 h incubation. However the weakness of these positive reactions meant that the test was not easy to score. Since these tests were either redundant or difficult to score they were discarded.

2.2.4.7 β-Galactosidase Production.

Method 1 (5 bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (X-Gal) agar plates were prepared according to the method in Miller (1972) by adding filter-sterilised (0.22 μm pore size-filter) X-gal dissolved in N,N dimethyl formamide to a concentration of 40 mg l^{-1} to cooled (50°C) CMD agar which contained in addition 0.1% (w/v) lactose. Plates were streaked and incubated up to 72 h to allow any colour formation.

Method 2: The method of Gerhardt et al. (1981) was used on cells grown on CMD agar supplemented with 0.1% (w/v) lactose. For each organism a loopful of cells was suspended in 0.25 ml of 0.85% (w/v) NaCl solution. A drop of toluene was added, the tube vortex mixed, and allowed to equilibrate for 5 min. Test reagent was made up as follows; 6.9 g of $NaH_2PO_4.H_2O$ was dissolved in 45 ml of distilled water and then approximately 3 ml of 30% (w/v) NaOH added until the pH was 7.0. The volume was made up to 50 ml. This solution was designated solution A. The actual reagent used in the test was made by dissolving 0.080g of o-nitrophenyl-β-D-galactopyranoside (Sigma Chemical Co.) in 15 ml of distilled water and then adding 5 ml of solution A. This solution was stored at 4°C before use.

0.25 ml of the test reagent was added to the tube and capped tubes incubated for 1 h at 70°C. Tubes showing an orange/yellow colour were scored positive.

RESULTS AND DISCUSSION: Growth on X-gal agar was generally very poor by all of the isolates tested. T.aquaticus and "T.thermophilus" did not grow, the others grew poorly and gave weak positive results. The component of the agar causing inhibition was not investigated further and the test was not considered to be of any use. By method 2 T351, 11247, Tok3, Rt41A, Ramaley X1, T.aquaticus, HWBS, "T.thermophilus", and 11245 were all positive whereas 11246 was negative. This test was considered to be of use in the final classifications.

2.2.4.8 α-galactosidase Production.

METHOD: Method 2 of section 2.2.4.7 was followed with the following modifications; cells were grown on CMD agar containing 0.1% (w/v) melibiose and in the assay p-nitrophenyl-α-D-galactopyranoside was used in the test reagent.

RESULTS AND DISCUSSION: After 1 h incubation T.aquaticus, T351, Tok3, Rt41A, 11245, 11246 and 11247 were positive while "T.thermophilus" and Ramaley X1 were negative. This was considered to be a useful test.

2.2.4.9 DNAase Production.

METHOD 1: The method used followed a method in Gerhardt et al. (1981). To CMD agar was added 2g l^{-1} calf thymus DNA (BDH) and 0.05 g l^{-1} of methyl green before autoclaving. Organisms were streaked onto the agar and incubated for 48 h. DNAase producing organisms should have produced a colourless zone on an otherwise green plate.

METHOD 2: The method of Jeffries et al. (1957) was followed. Calf thymus DNA (BDH) was added to CMD agar to a concentration of 2 g l^{-1} before autoclaving, and plates were poured as soon as the agar had cooled to 50°C. Two organisms were streaked per plate and plates incubated for 48 h. Plates were then flooded with 1 M HCl after growth had been removed from the plate by washing with a jet of water. Where DNA was still present an opaque white precipitate formed and plates were scored as positive where a zone of clearing occurred under or around the area of growth.

RESULTS AND DISCUSSION: Growth on plates prepared by method 1 was very poor, while growth on plates prepared by method 2 was normal, so presumabely methyl green was responsible for the inhibitory effect. Any zones of clearing on the methyl green plates were very hard to discern so this method was not considered further. Organisms grown on the plates described in method 2 did show varying reactions, positive were T.aquaticus, "T.thermophilus", Tok 3, 11245, 11247, HWBS, Rt 41A and T351 while 11246 was negative. As such it was the better of the two tests and the one used in the final classifications.

2.2.4.10 Degradation of p-nitrophenyl Linked Substrates.

METHOD: The method followed was that of Cooper (1982). The assay comprised 0.8 ml of 0.1 M tris HCl buffer, pH 7.5, and 0.1 ml of 8 mM p-nitrophenyl substrate dissolved in 2 ethoxyethanol. Tubes were allowed to equilibrate for 5 min. and 0.1 ml of sample, either whole cell suspension or cell-free supernatant was added and capped tubes incubated at 70°C. Tubes were scored for the production of a yellow colour. Substrates used included p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-β-D-glucanopyranoside, p-nitrophenyl caprylate, p-nitrophenyl laurate and p-nitrophenyl palmitate.

RESULTS AND DISCUSSION: Results for all cell suspension activities on the substrates are given in table 2.4 and those for activities from cell-free supernatant given in table 2.5.

Table 2.4

Degradation of p-nitrophenyl Linked Substrates by Cell Suspensions of the Test Organisms.

Substrate	Incubation time (h)	Cont	Taq	Tth	T351	Ram	41A	45	46	47	Tok3	HWBS
Palmitate	1	-	+	-	+	-	+	-	-	-	-	-
	3	-	+	-	+	-	+	-	-	-	-	-
Laurate	1	-	+	+	+	+	+	+	+	+	+	+
Caprylate	0.5	+	+	+	+	+	+	+	+	+	+	+
β-Glucopyranoside	6	-	+	-	+	-	-	+	-	-	+	-
α-Glucopyranoside	6	-	+	+	+	-	-	+	-	-	+	+

Table 2.5

Degradation of p-nitrophenyl Linked Substrates by Cell-Free Supernatants of the Test Organisms.

Substrate	Incubation time (h)	Cont	Taq	Tth	T351	Ram	41A	45	46	47	Tok3	HWBS
Palmitate	1	-	-	-	+	-	+	-	-	-	-	-
	3	-	-	-	+	-	+	-	-	-	-	-
Laurate	1	-	-	-	+	-	+	-	-	-	-	-
	3	-	-	+	+	+	+	+	-	-	+	-
β-Glucopyranoside	18	-	-	-	-	-	-	-	-	-	-	-
α-Glucopyranoside	18	-	-	-	-	-	-	-	-	-	-	-

Abbreviation used: Cont=control (uninoculated medium).

The use of p-nitrophenyl caprylate was not considered further as a substrate as under these conditions the control turned yellow. The remaining tests showed taxonomically useful reactions. It was decided that for the first classification (see section 3.2) all 4 substrates would be used with both cell-free supernatant and cell suspension with scoring at 1, 3, 6 and 18 h to evaluate the unit characters which could be used on a larger number of organisms. The characters finally selected are shown in Table 3.2 which is the table of tests for the first classification.

2.2.4.11 Phenylalanine Deaminase Production.

METHOD: Phenylalanine deaminase was tested for by the method in Gerhardt *et al.* (1981). Phenylalanine agar contained the following (in gl^{-1}) Yeast extract 1, L-phenylalanine 1, Na_2HPO_4 1, NaCl 0.008, agar (New Zealand) 17.5. The pH was adjusted to 7.6 (the optimum for Thermus), the agar dissolved and the medium dispensed. Bottles were sloped after autoclaving. Tubes were inoculated and incubated for 48 h. A few drops of 10% (w/v) ferric chloride were placed on each slant and the tubes examined for the production of a green colouration caused by the presence of phenylpyruvic acid.

RESULTS AND DISCUSSION: None of the isolates showed the production of phenylalanine deaminase and this test was not considered further.

2.2.4.12 Lipase Production.

METHOD 1: CMD medium was prepared and tributyrin (Sigma) added to a concentration of 1% (v/v). The medium was homogenised in a Waring blender for 30 s before autoclaving and pouring. Inoculated plates were incubated for 48 h and examined for zones of clearing around the growth.

METHOD 2: Medium was prepared according to the method of Degryse et al. (1978) which contained (in gl^{-1}) bactopeptone 5, NaCl 5, agar (Oxoid No.1) 30 and 5 ml of Tween 80 (Sigma). Plates were incubated for 24 h and examined for areas of precipitate around the growth.

RESULTS AND DISCUSSION: At 48 h all of the organisms tested by method 1 had produced zones of clearing to some extent, and so the test was not useful. By method 2 there was very little growth of the organism but zones of precipitate were evident. T.aquaticus, "T.thermophilus" 11245 and 11246 were negative and the other 6 positive. This was therefore regarded as a useful test.

2.2.4.13 Potassium Tellurite Reduction.

METHOD: Tellurite reduction was tested by the method of

Wilkinson & Jones (1977). CMD agar was prepared and autoclaved. While still molten, 10 ml of filter-sterilised (0.22μm pore-size filter) 1% (w/v) potassium tellurite solution was added so that the final concentraion in the medium was 0.01 % (w/v). The agar was mixed well to ensure adequate homogeneity and the plates poured. Two organisms were streaked per plate and growth examined after 24 h for the production of a jet-black colouration due to the formation of metallic tellurium.

RESULTS AND DISCUSSION: Most of the organisms (T.aquaticus, "T.thermophilus", 11245, 11247, Tok3, Ramaley X-1, HWBS and Rt41A) produced an intense black colouration while 11246 produce a light grey colour. Although 11246 reduced the tellurite to some extent there was a clear difference between this and the other strains. The presence of tellurium appeared not to inhibit growth at all and so this was considered to be a useful test.

2.2.4.14 Methylene Blue Reduction.

METHOD: Isolates were grown in CMD broth for 24 h. To each bottle was then added 1 drop from a fifty dropper pasteur pipette of 1% (w/v) methylene blue. Tubes were then re-incubated for up to 6 h and scored at 30 min., 1 h, 4 h and 6 h. Tubes showing reduction had a blue oxidised surface layer and a colourless/turquoise lower layer.

RESULTS AND DISCUSSION: After 30 min. T.aquaticus, T351, Rt41A, 11246 and Tok 3 had all changed from a dark blue to a light blue and after 1 h had completely decolourised the methylene blue, apart from the oxidised surface layer. After 6 h T.aquaticus no longer showed signs of reduction while all the others which had shown reduction at 1h still did. The methylene blue had presumabely killed the T.aquaticus cells causing them to stop reduction. The test was considered to be useful and the time of reading taken to be 1 h.

2.2.4.15 Nitrate Reduction.

METHOD: Nitrate broth (Difco) was rehydrated and dispensed in 20 ml aliquots to 25 ml bottles which contained inverted Durham tubes. Tubes were inoculated with 0.1 ml of 24 h CMD broth culture and incubated up to 7 d. After 24 h 0.1 ml samples were aseptically removed and transferred to tubes containing 1 ml of reagent A (0.02 g of N,N-Dimethyl-1-naphthylamine dihydrochloride (Sigma Chemical Co.) dissolved in 100 ml of 1.5 N HCl), 1 ml of reagent B (1 g of Sulfanilic acid (BDH) dissolved in 100 ml of 1.5 N HCl) and 0.9 ml of water, following the method in Gerhardt et al. (1981). Tubes were examined for the production of a purple colour. The same test was done at 7 d and at both times the presence of gas in the Durham tubes noted.

RESULTS AND DISCUSSION: The results for nitrate reduction are shown in table 2.6.

Table 2.6

Nitrate reduction Test Results for the Test Strains.

PRODUCT	TIME	Taq	Tth	T351	41A	Tok3	RAM	SWBS	45	46	47
GAS	1d	-	-	-	-	-	-	-	-	-	-
NITRITE	1d	+	-	+	+	-	+	+	+	+	+
GAS	7d	-	-	-	-	-	-	-	-	-	-
NITRITE	7d	-	-	-	+	-	+	+	+	-	+

The presence of gas could not be used in the classification, but the presence of nitrite at 1 and 7d was of use. In further tests the Durham tube was omitted.

2.2.4.16 Malonate Utilisation.

METHOD: The method followed was that in Gerhardt et al. (1981). Malonate broth contained (in gl^{-1}) yeast extract 1, $(NH_4)_2SO_4$ 2, K_2HPO_4 0.6, KH_2PO_4 0.4, NaCl 2, sodium malonate 3, glucose 0.25 and bromothymol blue 0.025. The components were combined and the pH adjusted to 7.0 before autoclaving. Tubes were inoculated and incubated up to 72 h and scored for a colour change from green to deep blue.

RESULTS AND DISCUSSION: All of the organisms were able to grow on the medium but none of them produced a colour change and so the test did not prove to be of any use.

2.2.4.17 Triple Sugar Iron reactions.

METHOD: Triple sugar iron slants were prepared from a modified medium of Gerhardt et al. (1981). The agar contained (in gl^{-1}) yeast extract 0.5, trypticase peptone 0.5, glucose 0.1, lactose 0.5, sucrose 0.5, ferrous sulphate 0.2, NaCl 0.008, sodium thiosulphate 0.3, and phenol red 0.024. Also added was 1 ml l^{-1} of CMD trace element solution. The pH was adjusted to 7.7 and the tubes sloped after autoclaving. The medium listed in Gerhardt et al. (1981) contains 41g of organic components and this level was considered likely to inhibit the growth of Thermus (see section 1.2.2.1). The amount of NaCl in the original medium may also have caused inhibition so the quantity was reduced to that of CMD.

Tubes were inoculated with loosely screwed caps for up to 72 h. Tubes were examined daily for yellowing of butt and/or slope, gas production, and hydrogen sulphide production.

RESULTS AND DISCUSSION: Results for daily readings are shown in table 2.7.

TABLE 2.7

Triple Sugar Iron Reactions for the Test Strains.

REACTION	TIME	Taq	Tth	T351	Tok3	45	46	47	Ram	HWBS	41A	Control
Slope	24h	R	R	O	O	R	O	R	R	O	R	O
Butt	"	R	O	O	O	O	O	O	O	O	O	O
Gas Produced	"	-	-	-	-	-	-	-	-	-	-	-
Sulphide produced	"	-	-	-	-	-	-	-	-	-	-	-
Slope	48h	R	R	O	R	R	R	R	R	O	R	O
Butt	"	R	R	O	O	R	O	O	O	O	O	O
Gas produced	"	-	-	-	-	-	-	-	-	-	-	-
Sulphide produced	"	-	-	-	-	-	-	-	-	-	-	-
Slope	72h	R	R	R	R	R	R	R	R	O	R	O
Butt	"	R	R	O	O	R	O	O	R	O	O	O
Gas produced	"	-	-	-	-	-	-	-	-	-	-	-
Sulphide produced	"	-	-	-	-	-	-	-	-	-	-	-

O=Orange, R=Red (compared with control).

The characters of gas production and sulphide production were of no value as they were all negative. The non production of sulphide is in agreement with the data of Cometta <u>et al.</u> (1982b). During the course of the incubation the control tube gradually became yellower, presumably by an effect of the elevated temperature on the medium components (Degryse et al. (1978) reported a drop in pH at 70°C in the presence of monosaccharides). Some of the isolates were able to redden the medium significantly, however there was a gradient of reddening and it was decided that only organisms showing a reddening equivalent to T.aquaticus and "T.thermophilus" would be scored as red, as these showed a very marked reaction. In this case these were the only 2 organisms to show that degree of reddening. Incubation for longer than 24 h made reading the tubes more difficult so the only useful character to be obtained from this test was the reddening of the slope after 24 h incubation. None of the organisms showed signs of anaerobic growth.

2.2.4.18 Growth on Simmon's Citrate Agar.

METHOD: Simmon's citrate ager was prepared according to the method in Gerhardt et al. (1981). The medium contained (in gl^{-1}) sodium citrate 2, NaCl 5, $MgSO_4.7H_2O$ 0.2, $NH_4H_2PO_4$ 1, K_2HPO_4 1, bromothymol blue 0.08 and agar (New Zealand) 15. The pH was adjusted to 7.7. After autoclaving the tubes were allowed to cool in a slanted position. Tubes were streaked with the test organisms and incubated up to 4 d.

RESULTS AND DISCUSSION: None of the isolates were able to grow on the medium and so were not able to use citrate under these conditions. The test was therefore of no use in the final classifications.

2.2.4.19 Methyl Red and Voges-Proskauer Reactions.

METHOD: MRVP broth was prepared as a modification of the medium in Gerhardt et al. (1981). The medium contained (in gl^{-1}) polypeptone 1, K_2HPO_4 5, glucose 1, and NaCl 0.008. Also added were the trace elements and ferric chloride solution as for CMD, and the pH adjusted to 8 before autoclaving. The level of organic compounds in the medium had been reduced from that in Gerhardt et al. (1981) to prevent any inhibition which might occur. For the methyl red test tubes were incubated for 72 h, while tubes for the Voges-Proskauer test were incubated for 48 h.

For the methyl red test 5 drops of methyl red solution were added to each tube. Methyl red was prepared by dissolving 0.1g of methyl red in 300 ml of 95% (v/v) ethanol and bringing the solution to 500 ml with distilled water. A positive test should show a red colour.

For the Voges-Proskauer test 1 ml of each culture was transferred to a test tube. To the tubes was added 0.6 ml of 5% (w/v) α-naphthol (dissolved in 100% ethanol) and the tubes vortex mixed, followed by 0.2 ml of 40% (w/v) KOH solution and tubes again vortex mixed. Tubes were slanted and examined at 15 and 60 min. for the production of a strong red colour at the liquid surface.

RESULTS AND DISCUSSION: None of the organisms showed a positive reaction for either test. The non-production of indole agrees with the results from Cometta et al. (1982b). Neither of the tests was therefore useful for the classification.

2.2.4.20 Aesculin Hydrolysis.

METHOD: The method in Gerhardt et al. (1981) was followed. CMD was supplemented with 0.01% (w/v) aesculin (Sigma) and 0.05% (w/v) ferric citrate. Plates were streaked with two organisms per plate and incubated for 24 h. Plates were examined for the production of a brown colouration around areas of growth.

RESULTS AND DISCUSSION: Organisms Tok 3, Rt41A, Ramaley X-1, 11247, and T351 were able to hydrolyse the aesculin whereas 11245, T.aquaticus, "T.thermophilus and HWBS were not. This test was considered to be of use in the classifications.

2.2.4.21 Arbutin Hydrolysis.

METHOD: The method of Baird-Parker (1960) was modified so that CMD agar contained 0.5% (w/v) arbutin (Sigma Chemical Co.) and 0.5 ml of 1% (w/v) ferric ammonium citrate solution per litre. After incubation for 24h the plates were examined for the production of a brown colouration around the area of growth.

RESULTS AND DISCUSSION: T.aquaticus, "T.thermophilus, 11246 and 11245 were negative and 11247, Tok 3, T351, Rt41A, HWBS and Ramaley X-1 positive. This test was therefore considered to be useful.

2.2.4.22 Litmus Milk Reactions.

METHOD: Litmus milk (BDH) was reconstituted as directed (100 g dissolved per litre of water and autoclaved for 20 min. at 115°C). Tubes were incubated for up to 10 d and checked daily for any reaction (reduction of litmus, acid curd, rennet curd, peptonisation and stormy fermentation).

RESULTS AND DISCUSSION: None of the organisms showed any reaction and the test was not considered further.

2.2.4.23 Colony Characters.

METHOD: Organsisms were streaked out onto CMD agar and incubated for 24 h. Colonies were scored for diameter, form elevation, pigmentation and for a granular appearance using a x10 hand lens.

RESULTS AND DISCUSSION: Colony characters for the ten organisms are shown in table 2.8.

TABLE 2.8

Colony Characteristics for the test organisms.

CHARACTER	41A	Taq	45	46	47	Tok3	Ram	HWBS	T351	Tth
Colony Size (mm)	<1	<1	<1	<1	<1	2	<1	<1	2	1
Colony colour	Y	Y	W	Y	Y	Y	W	W	Y	Y
Form	C	I	C	C	C	C	C	C	I	C
Elevation	C	C	C	C	C	C	C	C	C	C
Granular	-	-	-	-	+	+	-	-	+	-

Abbreviations used: Colony colour Y=yellow, W=white; Form C=circular, I=irregular, Elevation C=convex.

Differences in form, elevation and granularity were not considered at this stage to be of any taxonomic utility due to a grading of the results, while colour was an obvious character to select. As recording colony characters would entail little extra work it was decided to examine organisms for them when full classifications were carried out. The inclusion of more organisms might show useful differences, and characters found to be of use are given in the list of tests for each classification.

2.2.4.24 Growth on Commercially Prepared Media.

METHOD: Attempts were made to grow the organisms on the following media;

Eosin Methylene Blue (BBL): Prepared as directed i.e. 36 g dissolved per litre of water.

Tomato Juice Agar (BBL): Prepared as directed i.e. 51 g dissolved per litre of water.

McConkey Agar (without crystal violet) (BBL): Prepared as directed i.e. 52 g dissolved per litre of water.

Brilliant Green Agar (BBL): Prepared as directed i.e. 58 g dissolved per litre of water.

RESULTS AND DISCUSSION: None of the organisms showed good growth on any of the above media. Reasons for this may include the media being too rich in organic compounds or that agents normally selective against Gram-positive bacteria were selective against Thermus. None of these media could therefore be used as a convenient culture medium or as a taxonomic test medium.

2.2.4.25 Inhibition by Sodium Lauryl Sulphate.

METHOD: CMD broths were prepared containing concentrations of sodium lauryl sulphate (Ajax Chemicals, NSW, Australia) listed below. Broths were inoculated from strains growing on CMD agar plates, and bottles incubated for 48 h.

RESULTS AND DISCUSSION: Control tubes all showed growth. Tubes containing 0.25%, 0.05%, 0.01% and 0.005% (w/v) sodium lauryl sulphate were all negative for growth, while tubes containing 0.0005% (w/v) were positive. Results at these concentrations were therefore redundant and the test was not used further.

2.2.4.26 Inhibition by Phenol.

METHOD: A microtitre plate technique was used. The plate used had 96 U-bottomed wells and was purchased in a sterile pack. To each well was added 0.1 ml of double strength CMD broth and 0.1 ml of the appropriate sterile phenol solution. Two columns were left as uninoculated controls. The inoculum used was 10 μl of a 24 h CMD broth culture. The plate was sealed with plastic film and placed insisde a polythene bag before incubating for 24 h.

RESULTS AND DISCUSSION: No organism showed growth in the 0.5% (w/v) row while all but one grew in the 0.25% row. That organisms, Ramaley X-1, grew in the 0.125% row. The differences in inhibition pattern were not considered great enough to warrant the use of this test in the classifications.

2.2.4.27 Inhibition by Sodium Chloride.

METHOD: The NaCl component of CMD was raised to concentrations of 2% (w/v) and 4% (w/v). Bottles were inoculated and incubated for 3 d before they were examined for growth.

RESULTS AND DISCUSSION: None of the organisms tested was able to grow in a NaCl concentration of 4% (w/v), while at 2% (w/v) "T.thermophilus" and 11247 showed normal growth and the others were completely inhibited. Growth in the presence of 2% (w/v) NaCl was therefore selected as a useful test. The ability of "T.thermophilus" to grow in medium containing this concentration of NaCl was given by Oshima & Imahori (1974) as one of the features distinguishing this organism from T.aquaticus.

2.2.4.28 Inhibition by Trypticase Peptone.

METHOD: The trypticase peptone (BBL) component of CMD was raised to concentrations of 0.5, 1 and 2.5 % (w/v). Tubes were inoculated and incubated for 3 d before they were examined for growth.

RESULTS AND DISCUSSION: All of the organisms were able to grow in the presence of 0.5% (w/v) trypticase peptone. At a concentration of 1% (w/v) and 2.5% (w/v) HWBS was the only organism not able to grow. The concentration of 2.5% (w/v) trypticase peptone as an inhibitor was selected as a test as it would probably be the most discriminatory concentration. According to Brock & Freeze (1969)

T.aquaticus was inhibited by 1% peptone plus yeast extract, whereas in this study it was able to grow at this concentration of organic compounds.

2.2.4.29 Culture pH.

METHOD 1: CMD medium was prepared and supplemented with 0.001% (w/v) phenol red. After incubation bottles were examined for a pH change compared with the control indicated by either a red (alkaline) or yellow (acid) colour.

METHOD 2: After standard CMD bottles had been inoculated and incubated the pH of the cell suspension was measured using a portable pH meter after bottles had cooled to room temperature and the pH meter had been calibrated using pH 7 buffer (BDH).

RESULTS AND DISCUSSION: With phenol red included in the medium Tok 3, T.aquaticus, 11245, HWBS and T351 had all turned redder than the uninoculated control, "T.thermophilus", 11246, Rt 41A and Ramaley X-1 were the same colour as the control while 11247 was more yellow than the control. Apparently therefore different isolates produced different pH changes in the medium. However there was a gradient of colour changes and it was very difficult to decide at what level a tube was significantly different from the control. This difficulty led to the use of method 2, which although requiring more work gave clearcut results. The pH values of cell suspensions were as follows; T351 7.1, Tok 3 7.3, Rt 41A 6.9, Ramaley X-1 7.1, 11245 7.7, 11247 7.1, "T.thermophilus" 7.2, T.aquaticus 6.8 and HWBS 7.1. It was decided to use the character of culture pH > 7.6 scored positive as most of the culture pH values were about the same value (~7) while the value for 11245 was clearly higher. The pH of uninoculated medium was 7.8.

2.2.4.30 Growth at Extreme pH.

METHOD: CMD broth was buffered by the addition of 5 mM boric acid/ NaOH buffer to give a final pH of 9.5 or with 0.01 M sodium phosphate buffer to give a final pH of 6.0. Growth was recorded after 3 d incubation.

RESULTS AND DISCUSSION: At pH 9.5 T351, Tok 3, HWBS, T.aquaticus, Rt 41A and "T.thermophilus" were not able to grow while 11245, 11247 and Ramaley X-1 were. At pH 6.0 T351, Tok 3, HWBS, 11247, T.aquaticus and "T.thermophilus" were able to grow while Ramaley X-1 and 11245 were not. Both of these pH values and buffer systems were therefore considered as being useful in the final classifications.

2.2.4.31 Growth at Extreme Temperature.

METHOD: CMD broths were inoculated and incubated at 45, 75, 80 and 88°C and tubes examined for growth after 3 d incubation.

RESULTS AND DISCUSSION: At 88°C none of the test organisms was able to grow. At 80°C only 11247, "T.thermophilus" and Rt41A grew, at 75°C all grew and at 45°C only T.aquaticus did not grow. It was decided to use growth at 45, 75 and 80°C as characters as they either showed discriminatory power or promised to (in distinguishing T.ruber strains from T.aquaticus strains at 75°C).

2.2.4.32 Oxygen Requirement.

METHOD: CMD agar was prepared and dispensed in 20 ml aliquots to universal bottles before autoclaving. Afterwards the tubes were allowed to cool to 70°C and were then inoculated with 1 ml of 24 h cultures of the test organisms. Each bottle was vortex mixed and allowed to cool until the agar had set. Bottles were then incubated at 70°C for 48 h and the bottles were examined for the depths down to which the organisms were able to grow.

RESULTS AND DISCUSSION: All of the organisms were restricted in growth to the top few mm of the agar and no useful test could be derived from the results. This result is in agreement with the general view that Thermus spp. are strict aerobes.

The following tests were carried out on a larger number of isolates, those listed in table 3.1 i.e. those from the classification of New Zealand isolates.

2.2.4.33 Growth After 28 d Storage at 25°C.

METHOD: The test organisms were streaked onto CMD plates, incubated for 24 h and then plates were stored at 25°C for 28 d in a light-proof box. The plates were still sealed with masking tape. After storage single phase streaks over half plates were made to fresh CMD plates which were then incubated for a further 24 h. Plates were then examined for confluent growth along the streak.

RESULTS AND DISCUSSION: 8 of the 55 isolates did not grow (full data are shown in appendix 2A). The data showed that the test was useful and that plates of Thermus cannot be left for a month if they are required as stock organisms or as sources of inocula.

2.2.4.34 Growth after 14 d Incubation at 70°C.

METHOD: The normal 24 h incubation period was extended to 14 d. At the end of this period 0.1 ml of each culture was transferred to a fresh CMD broth and these incubated at 70°C for 3 d before they were examined for growth.

RESULTS AND DISCUSSION: 9 of the 55 isolates tested did not grow on transfer (full data are shown in appendix 2A). The test was used in the final classification.

2.2.4.35 Tetrazolium Salt Reduction.

METHOD: The method of Wilkinson & Jones (1977) was followed. A 10% (w/v) solution (10 ml) of 2,3,5-triphenyl tetrazolium chloride was prepared and filter-sterilised using a 0.22 μm pore-size filter. Either 1 or 0.1 ml was added to 1 litre of CMD agar to give a final concentration of 0.1 and 0.01 % (w/v) respectively. After incubation plates were examined for tetrazolium salt reduction shown by the production of a red colour by the colonies.

RESULTS AND DISCUSSION: None of the 55 isolates was able to grow in the presence of 0.1% (w/v) tetrazolium salt. With 0.01% (w/v) tetrazolium salt most of the isolates were able to grow but they all showed a reddening of the colonies and hence the test was redundant.

2.2.4.36 Hippurate Hydrolysis.

METHOD: Method 2 in Gerhardt *et al.* (1981) was followed. A 1% (w/v) solution of sodium hippurate was prepared and 0.4 ml aliquots dispensed to screw capped bottles. The tubes were used immediately by supending a large loopfull of isolates grown on CMD plates for 24 h in the solution so that a dense suspension was formed. Bottles were incubated for 2 h at 70°C. After incubation 0.2 ml of ninhydrin reagent (3.5 g of ninhydrin was dissolved in 100 ml of a 1:1 (v/v) mixture of acetone and butanol) added to each bottle and bottles were then further incubated for 10 min. at 70°C. Bottles were examined for the production of a deep purple colour which should be formed by the glycine which is the product of hippurate hydrolysis.

RESULTS AND DISCUSSION: None of the 55 organisms tested showed even the slightest sign of a purple colouration. This test was therefore considered as having no value in the *Thermus* classification.

2.2.4.37 Minimum Inhibitory Concentration Patterns.

METHOD: Minimum inhibitory concentration (MIC) values of antibiotics were determined using a microtitre plate technique. Double strength CMD broth (0.1 ml) was added to each well followed by 0.1 ml of the appropriate antibiotic solution which had been filter-sterilised using a 0.22 μm pore-size filter. The inoculum used was 10 μl of a 1/10 dilution of a 24 h liquid culture in 20 mM sodium phosphate buffer (pH 7.6). The plates were covered with plastic film and incubated in semi-sealed plastic bags for 24 h. Plates were scored using a magnifying lens to examine for the formation of cell pellets at the bottom of the U-shaped wells. Each plate had a row of wells containing no antibiotic solution as a control for growth and two columns of wells with no inoculum as a check for contamination.

RESULTS AND DISCUSSION: The results for the organisms tested are shown in table 2.9.

The results for neomycin, vancomycin, novobiocin, streptomycin and polymixin B were too uniform to provide useful taxonomic characters. The other antibiotics appeared to give useful results. The tests which were extracted from these data were the following; growth in chloramphenicol rows A, B, C or D positive, growth in kanamycin rows A, B, C, or D positive, growth in cycloserine rows A, B or C positive, growth in spectinomycin rows A, B, C or D positive and growth in phosphomycin rows A, B, or C positive.

Table 2.9

Minimum Inhibitory Concentrations for 55 Thermus Isolates.

ISOLATE	Neo	Chm	Str	Cyc	Van	Nov	Phs	Spc	Kan	Pol
T351	H	E	G	E	H	H	D	B	F	D
Tok 8	H	C	G	E	H	H	C	D	F	D
W 28 AT	H	D	F	E	H	H	D	A	E	C
Rt 6A1	E	D	F	E	G	H	D	A	E	C
Tok 3	H	C	F	E	H	H	E	A	E	D
OK 6	H	D	F	E	H	H	D	A	E	D
Rt 41A	H	D	G	E	H	H	C	E	E	D
Ramaley X1	H	D	G	E	H	H	D	E	E	D
NCIB 11245	H	C	F	E	H	H	E	H	F	D
NCIB 11247	D	E	F	E	G	H	C	A	D	C
Wai 33A2	E	D	F	C	G	H	B	A	E	C
Wai 33A3	E	D	G	C	G	H	C	A	E	D
HWBS A1	E	C	E	C	F	H	A	A	E	C
Rt 6A2	F	H	H	F	G	H	F	F	F	D
Oh A1	E	D	E	D	F	H	D	A	E	D
Oh A2	E	E	F	E	F	H	D	A	D	C
ATCC 27737	D	D	E	E	G	H	C	A	E	C
Fiji 3	E	D	F	E	G	H	D	A	C	C
OK 2	E	C	F	E	F	H	C	A	D	C
OK 4	E	D	F	C	F	H	E	A	D	C
OK G	E	C	F	E	H	H	D	A	D	C
OK J	E	D	F	E	F	H	C	A	D	C
Tok 1	E	D	F	D	F	H	F	A	D	C
Tok 20	E	C	F	C	G	H	B	A	E	C
Tok 22	E	D	F	D	F	H	D	B	E	D
Tok 23	E	C	F	D	F	H	C	A	D	D
TP 10	H	D	G	E	F	H	B	A	E	D
WN 2	E	E	H	E	F	H	E	G	F	C
WN 5	E	C	F	E	G	H	D	A	E	C
WN 6	E	D	E	E	F	H	C	A	D	C
WN 8	E	D	E	D	G	H	C	A	D	C
Ket 4	E	D	F	E	F	H	C	B	F	C
B 2	E	B	D	E	F	H	C	A	E	C
B 5	E	E	F	E	F	H	C	C	D	C

CONTINUED

TABLE 2.9 CONTINUED

B 7	E	E	F	E	F	H	D	C	E	C
Rt 1	E	C	E	D	F	H	A	A	E	C
Rt 4	E	D	F	E	F	H	C	C	E	C
Rt 34	E	C	F	D	F	H	C	B	F	C
Rt 35	E	E	F	E	F	H	D	D	E	C
Rt 366	E	E	G	E	F	H	D	F	F	C
Rt 39	E	E	G	E	G	H	F	F	F	D
Rt 355	E	E	F	E	G	H	D	C	E	C
Rt 358A1	E	D	F	E	G	H	D	C	E	C
HWBS A2	E	C	D	C	F	H	B	B	E	C
WKV 2	E	C	F	E	F	H	D	B	E	C
WKV 3	E	D	F	E	F	H	B	B	F	C
Rt 8A1	E	E	F	E	G	H	E	E	F	NC
T.the	E	E	C	D	G	H	D	B	C	C
T.aq	E	E	E	F	F	H	D	A	C	C
Tok 21	E	C	E	D	G	H	D	A	D	C
WN 4	E	D	F	E	F	H	D	A	D	C

Abbreviations used: Neo,Neomycin; Chm, Chloramphenicol; Str, Streptomycin; Cyc, Cycloserine; Van, Vancomycin; Nov, Novobiocin; Phs, Phosphomycin; Spc, Spectinomycin; Kan, Kanamycin; Pol, Polymixin B. NC=No Comparison. All antibiotics underwent doubling dilutions with row A at the highest concentration and row G with the lowest. Row H contained no antibiotic. Antibiotics neomycin, chloramphenicol, streptomycin, kanamycin and polymixin B had gradients with well A= 10μg/ml and well G= 0.15μg/ml. Vancomycin, novobiocin, spectinomycin, phosphomycin and cycloserine had gradients with well A= 100μg/ml and well G= 1.5μg/ml.

2.2.4.38 Growth on Single Carbon Sources.

METHOD 1; Carbon sources were tested using a microtitre plate method. The plates (supplied by Nunc) were of the U-bottomed type, which enabled good cell pellet formation. Each well contained 0.1 ml of double strength medium 162 salts (Degryse et al., 1978), 0.05 ml of 0.02 M sodium phosphate buffer (pH 7.6) and 0.05 ml of 1.6% (w/v) carbon source which had been filter-sterilised using a 0.22 μm pore size filter. Tests were done in duplicate, one well containing in addition L-lysine (20μg/ml) which enables "T.thermophilus" to grow (Degryse et al., 1978). Vitamins were not added. The inoculum used was as for the antibiotic inhibition test as was the method of incubation except that plates were incubated for 48 h. Growth was scored with a magnifying lens by examining for the production of a pellet or turbidity in the wells. Growth in either well was scored positive, although in a majority of cases both wells were identical.

METHOD 2: Method 1 worked well for New Zealand isolates but when isolates from Iceland were tested very little growth could be observed under these conditions. The second medium used was that of Pask-Hughes & Williams (1975). CMD broths were prepared in the normal manner except for the omission of trypticase peptone and yeast extract. To the medium was added 0.5 gl^{-1} of ammonium sulphate and 2 gl^{-1} of the required carbon source. Carbon source test bottles were inoculated with a loopful (0.01 ml) of broth culture and incubation was for 3 d.

For all organisms showing no growth on any carbon source the tests were run again with the addition of 20 μg/ml L-lysine for the reason outlined above.

RESULTS AND DISCUSSION: All of the results are included in the appendices. By method 1 no organism tested was able to grow on either sorbose or arabinose. Generally the pattern followed was consistent with reports in the literature in that all of the carbon sources allowing growth were organic acids, monosaccharides, disaccharides, an oligosaccharide (dextrin), and sugar alcohols. The correspondence between results from the two methods was not exact, and could not be expected to be due primarily to the differences in the buffering of the medium, the compostion of the medium and the concentration of carbon source used. Method 1 was used for the first classification (of New Zealand isolates) while in all other cases, and in the comprehensive classification, the results from method 2 were used.

2.2.5 Concluding Remarks.

The large number of tests which proved to be redundant indicate the general close similarity between the isolates tested and can serve to distinguish Thermus isolates from any other similar organisms which might be isolated. The number of unit characters produced (~65, the exact number varied depending on the isolates being tested, for example all isolates might possess yellow pigments) was sufficient for a classification to be carried out. It is unfortunate that Thermus cannot be shown to ferment or oxidise their carbon sources (Degryse *et al.*, 1978) as this would allow simple carbon source test reading. Also commercial test strips (API-ZYM, API-20E, and Spectrum 10 (Austin Biologicals, Texas)) proved to be of no use; the API-ZYM strip not providing enough data for the cost involved, and the other strips showing all negative results. This too would have greatly simplified the work.

Chapter 3. Numerical Classifications of Thermus Isolates.

3.1 Introduction.

It was decided that the classification of the genus Thermus should be carried out using the technique of numerical taxonomy, analysed in a phenetic manner. Several factors were involved in this decision.

Probably the most fundamental division amongst taxonomic philosophy is the division between a phenetic and a phyletic approach to the interpretation of the data (e.g. Hull, 1970). Of course neither system can be described as "correct" and the choice of which to use depends on the objective of the work which is to be undertaken. Phyletic interpretation of the data has been described as a "special" classification (Sneath & Sokal, 1973) as it ignores completely what the organisms are actually like (e.g. Fox et al. (1980) where Escherichia coli is considered to be evolutionarily close to photosynthetic organisms). The state of knowledge of the genus Thermus as regards phenotypic characters and taxonomic structure was very limited at the commencement of this work and so in the first instance it was deemed that the classification should be as "natural" as possible, i.e. that it should convey as much information on the phenetic characters of the organisms under study as possible. Thus the use of a system such as 5s or 16s rRNA analysis was not considered appropriate for initial work. Other taxa in bacteriology have undergone the same sequence of analysis i.e. phenetic followed by phyletic.

The nature of the data which was to be interpreted was also under consideration. Many automated or semi-automated systems of taxonomic data production have been introduced in recent years (see section

1.3.4.9 for a few examples). One feature that all of these systems have in common is that they tell an investigator nothing about the nature of the organisms under study. For example the work of Donnison et al. (1986) using pyrolysis mass spectroscopy to classify Thermus, although of interest in itself, does not help a worker, with a newly isolated Thermus, to identify that organism without access to the appropriate equipment. The clusters produced by this kind of work impart no information on the kind of characters by which they may be routinely recognised. Thus a system based on characters which can be routinely tested in the laboratory is to be preferred for routine work. If the equipment is available, any of the automated systems may be used to identify organisms and to supply information provided that the results from these systems can be correlated with classifications produced by conventional techniques. This will in turn indicate the phenetic nature of the organisms.

It was these factors then which indicated that the use of numerical taxonomy was to be the technique of choice. The results produced below now make it highly desirable that in future some of the other techniques, especially 16s rRNA sequencing, are used and the results compared to the clusters produced in this work.

3.2 Numerical Classification 1: New Zealand Isolates

This section of work (section 3.2) is currently in press (Hudson et al., 1986).

Methods.

3.2.1 Source of strains.

The sources of the strains used in the study are shown in Table 3.1, along with the temperature and pH of the sites from which the isolates originated. Isolates were purified either by enrichment on Castenholz medium D (CMD) broth (Ramaley & Hixson, 1970) followed by repeated streaking on CMD agar plates (CMD solidified with 3% (w/v) Oxoid No. 1 agar) or by direct streaking of pool water onto CMD agar followed by repeated streaking until pure cultures were obtained. Only colonies with cells conforming to the description of the genus Thermus (Brock, 1984) were used in the study.

3.2.2 Characters Tested.

All tests (Table 3.4) had been pre-selected for use, on the basis that they were not redundant, from the tests described in Chapter 2.

Cultures on CMD plates or in CMD broth were routinely used as sources of inocula. All agar plates were sealed with masking tape to prevent excessive dehydration during incubation. Broth cultures were not shaken and used in 10 ml aliquots, unless otherwise stated. All incubations were at 70°C for 24 h unless otherwise stated. Enzyme assays were also performed at 70°C.

3.2.3 Calculation of test error.

At the commencement of isolate testing 14 cultures were each transferred onto 2 plates and each subculture treated as a separate isolate throughout the test procedures. Error was estimated from these replicates by the method of Sneath & Johnson (1972) which computes the probability of obtaining an erroneous result from the proportion of mismatches found between the replicate strains. An error of < 5% was considered by Sneath & Johnson (1972) as being achievable within one laboratory.

3.2.4 Analysis of Results.

Sixty-five unit characters were used, which were scored as one of two mutually exclusive states; plus(+) or minus(-), and missing values encoded so that they were omitted by the computer program.

Data were analysed using the cluster analysis facilities of the GENSTAT program (The Numerical Algorithms Group, Rothamsted Experimental Station, Harpenden, U.K.). Similarity matrices were calculated with the simple matching (S_{SM}; Sokal & Michener, 1958) and Jaccard (S_J; Sneath, 1957) coefficients and clustering was by both single and group pair average (UPGMA) methods, and expressed in the form of dendrograms or minimum spanning trees.

The facilities of GENSTAT were also used for analysis of variance.

TABLE 3.1

Isolates used arranged in order of the clusters shown in Figure 3.1, with their source temperature and pH where known.

CLUSTER	ISOLATE	ORIGIN	SOURCE:pH	TEMPERATURE(°C)
A	ATCC 31674 (T351)	NZ	8.7	96.4
A	OK6	NZ	8.2	102.5
A	Rt1	NZ	NA	85
A	Rt4 A1	NZ	7.2	87
A	OKJ	NZ	7.3	98
A	Wai6	NZ	8.5	80
A	Oh A1	NZ	7	80
A	Wai8	NZ	8.5	80
A	Wai4	NZ	8.2	94
B	Rt6 A1	NZ	9	70
B	Tok21	NZ	NA	NA
B	Tok20	NZ	7	86
-	Wai33 A3	NZ	7.3	73
-	Wai5	NZ	7.7	70
C	Oh A2	NZ	7	80
C	B5 (Rt Pool 1)	NZ	NA	NA
C	B7 (Rt Pool 6)	NZ	NA	NA
-	OK4	NZ	6.9	60
D	Tok8	NZ	5.6	77.5
D	WKV3	NZ	NA	66
D	Wai28 A1	NZ	7.4	75.5
D	Rt358 A1	NZ	7.5	82
D	Rt34	NZ	6.3	77
D	Tok23	NZ	7	97
D	WKV2	NZ	6.8	83
-	ATCC 27737	USA	NA	NA
-	HWBS A1	NZ	6.5	60
E	Tok3	NZ	6.9	76
E	OKG	NZ	7.9	74
E	Tok1	NZ	7.9	96
E	Tok22	NZ	6.5	75
E	OK2	NZ	7.6	57.1
E	TP10	NZ	6.3	70.7
E	Ket4	NZ	6	80
E	Rt4	NZ	8.1	87

CONTINUED

F	ATCC 27978 (Ramaley X-1)	USA	NA	26
F	Rt 39	NZ	4	68.1
F	ATCC 25104 (T.aquaticus Yt-1)	USA	NA	71.5
-	NCIB 11245 (Strain NH)	UK	NA	NA
-	Rt6 A2	NZ	9	48.4
G	Wai2	NZ	3.9	54
G	Rt366	NZ	NA	76
G	Rt8 A1	NZ	9.1	77
G	Rt35	NZ	6.6	80
G	Rt355	NZ	7.3	81
-	B2 (Unrecorded Ket)	NZ	NA	NA
-	HWBS A1	NZ	6.5	60
-	NCIB 11247 (Strain B)	ICELAND	8.9	82
-	Fiji3	FIJI	7.5	85.4
-	ATCC 27634 (T.thermophilus HB8)	JAPAN	6.3	80

Abbreviations used: ATCC, American Type Culture Collection, Rockville, Maryland, USA; NCIB[1] National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland; NA Not available. New Zealand hot pool locations: Tok- Tokaanu, Wai- Waimangu, Rt- Rotorua, OK- Orakeikorako, TP- Taupo, Oh- Ohaaki, HWBS- Hot Water Beach Springs, WKV- Waikite Valley, Ket- Ketatahi. Non-New Zealand location: Savu Savu beach, Fiji. Isolate nomenclature: The general format used represents the thermal region from which the isolate originated, the pool number in that region followed by "A" (aerobe) and a serial number for the isolate. E.g. Wai33 A2 is the second aerobic isolate from pool 33 at Waimangu.

1) NB NCIB references should now be numbered as NCIMB strains

Equivalant Numbering

ATCC	NCIMB	DSM
25104	11243	625
27634	11244	579
-	11245	-
-	11247	-
27978	11248	-

Results.

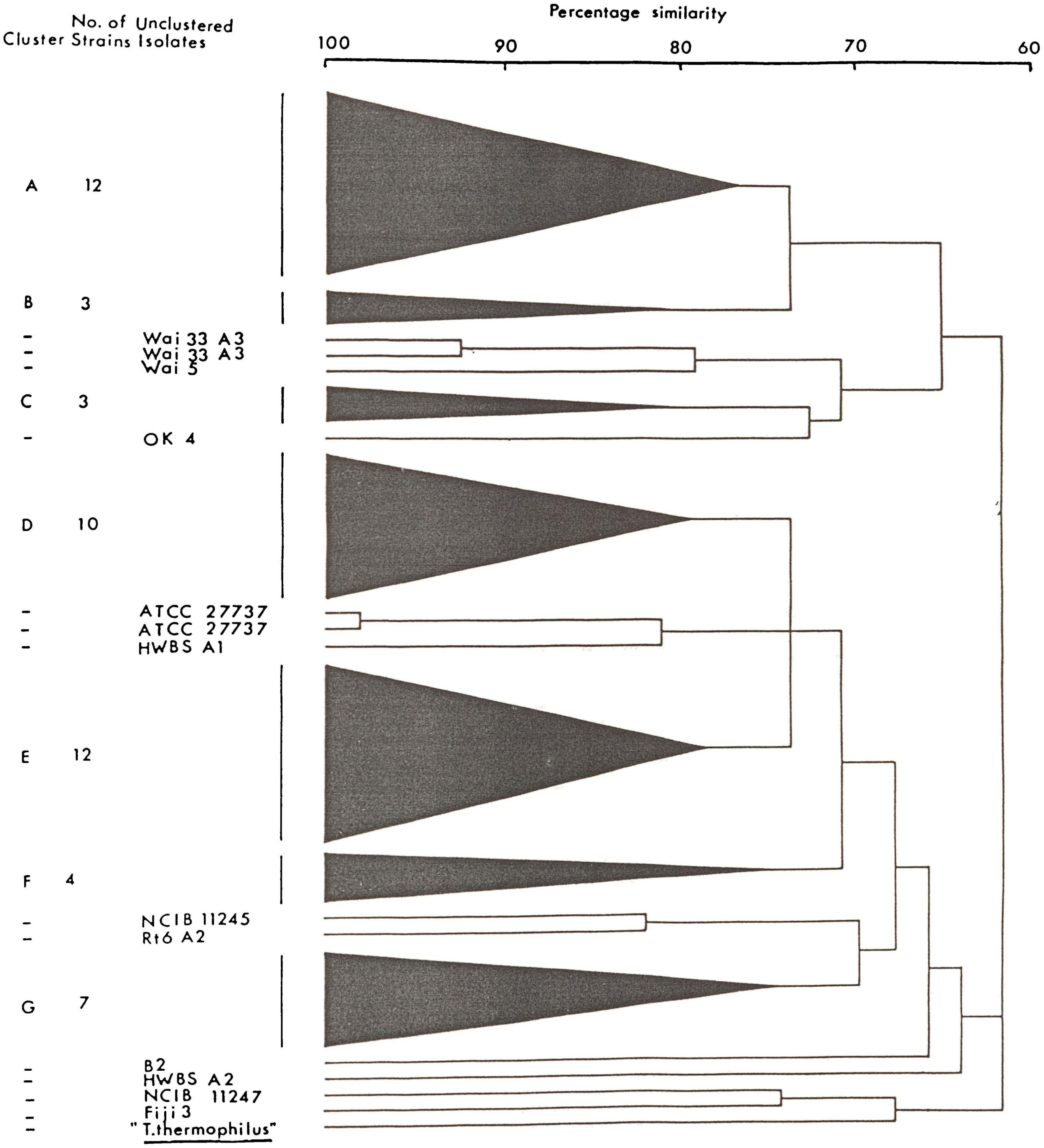
3.2.5 Experimental Error.

Error was estimated at 2.9% which is within the acceptable range given by Sneath & Johnson (1972).

3.2.6. Clustering of the Isolates.

From analysis using the S_{SM} coefficient and UPGMA clustering technique, 7 clusters which each contained 3 or more different isolates were formed at the 73% similarity level (Figure 3.1A and 3.1B; these show a simplified and a full version of the phenogram respectively). These clusters have been labelled A to G. In addition there were 4 two member clusters and 4 single member clusters.

Figure 3.1A. Simplified phenogram of New Zealand isolates based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient.



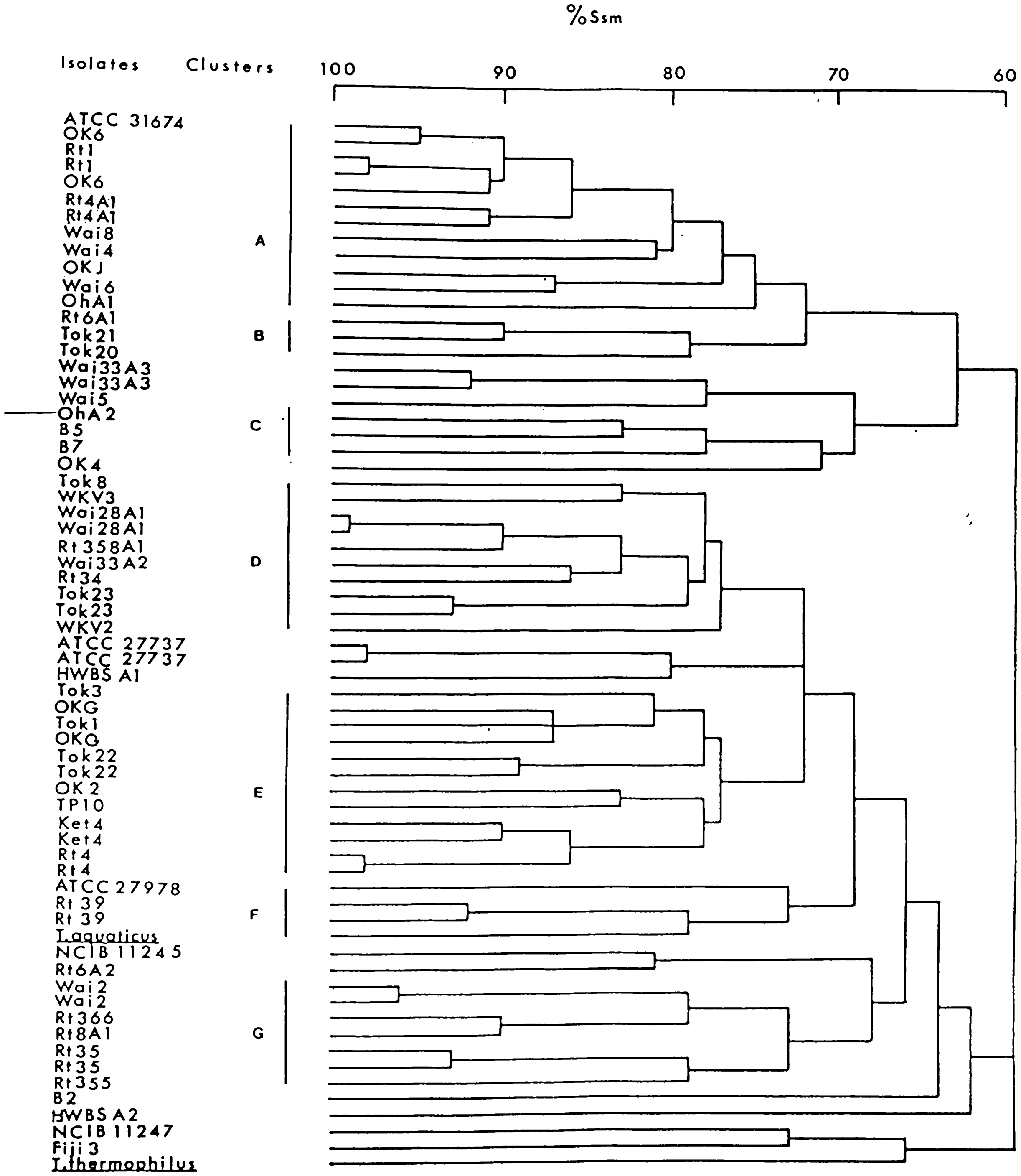


Figure 3.1B. Detailed phenogram of New Zealand isolates based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient.

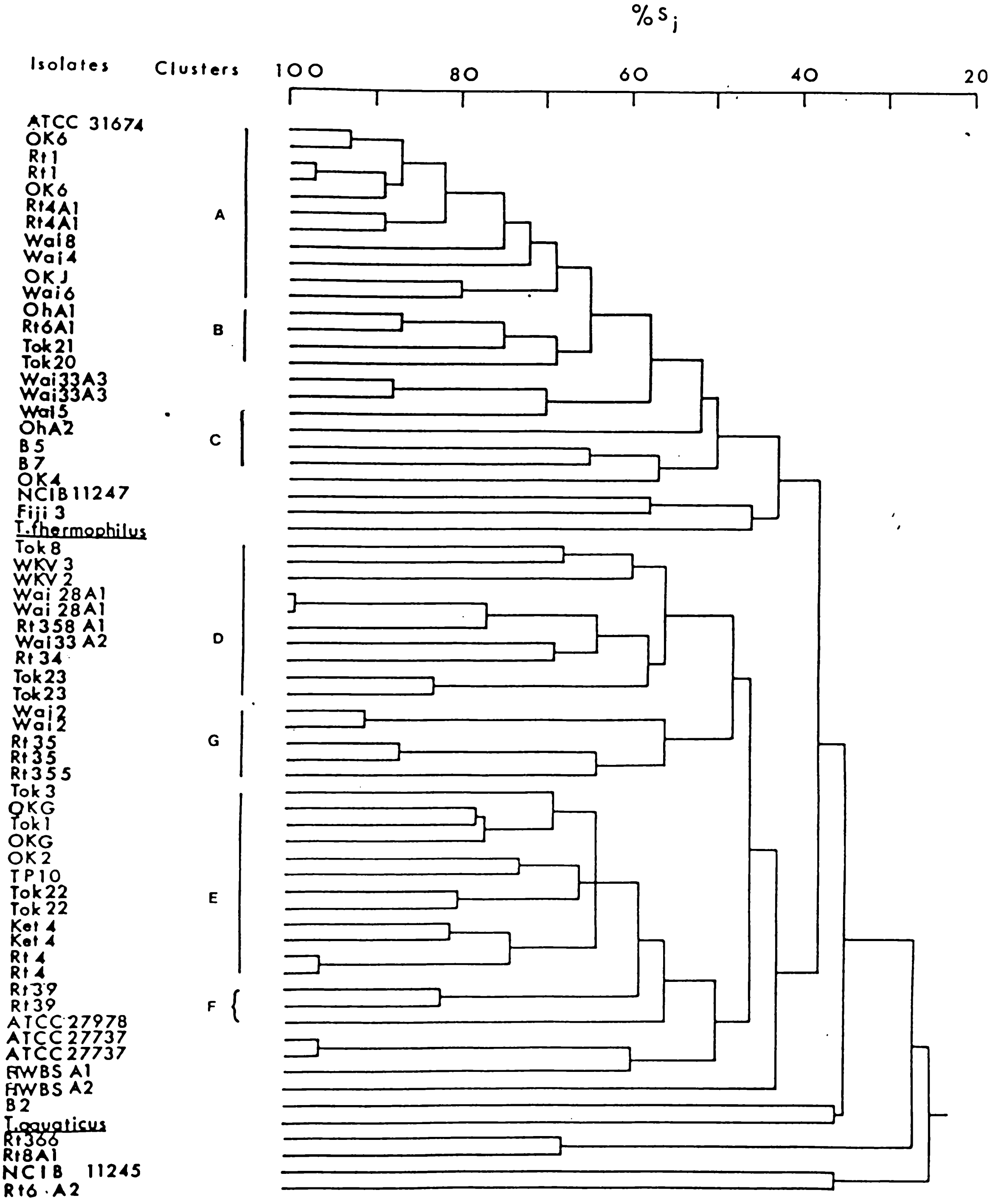


Figure 3.2. Detailed phenogram of New Zealand isloates based on the UPGMA analysis of a similarity matrix derived from the S_J coefficient.

A dendrogram formed when the S_J coefficient was used instead of the S_{SM} (Figure 3.2) coefficient showed some differences from Figure 3.1. These differences indicate that some of the clusters formed in Figure 3.1 were formed from a significant component of negative matches. Irrespective of the similarity coefficient used clusters D and E remained almost identical, apart from minor changes within the clusters. In the S_J analysis one member of cluster A moved to cluster B. Cluster C split into a two member cluster and a single member cluster, cluster F split into two single member clusters and a cluster of a replicate pair, while cluster G lost two members which formed a separate two member cluster.

Single linkage analyses were represented as minimum spanning trees (data not shown) and showed groupings of organisms similar to those obtained by average linking analysis.

In both cluster analyses organisms B2, HWBSA1, HWBSA2, and ATCC 27737 remained in single member clusters. T.aquaticus Yt-1(ATCC 25104) clustered with a minor cluster in the S_{SM} analysis but not with any in the S_{SM} analysis, whereas "T.thermophilus" HB8 (ATCC 27634) did not cluster with any group when either method was employed. Only cluster F contained isolates which were not from New Zealand sources, other non-New Zealand isolates were restricted to single or two member clusters. It would seem therefore that the bulk of the New Zealand isolates do not show a close similarity to strains from other parts of the world.

The four strains lacking a yellow or orange pigment, HWBSA1, HWBSA2, "Ramaley X1", and NCIB 11245, did not cluster together indicating that lack of pigment is not a good character for identification.

In an attempt to discern some pattern to the clusters formed a chi-squared test was performed to compare the distribution of the New Zealand geographical isolate sites within the clusters with that which could be expected by random distribution and it was found that there were no major deviations from the random distribution. Therefore the composition of clusters could not be related to the geographical source of the isolate within New Zealand.

An analysis of variance was carried out, using the appropriate facility of the GENSTAT program, on the two known environmental parameters of isolate sources. For this the pH and temperature values for members of each cluster were compared with the values from each other cluster using a single factor analysis of variance. The distribution of pH and temperature between the clusters was shown to be such that the null hypotheses of each cluster having equal pH means and equal temperature means could be rejected (Tables 3.2 and 3.3). Therefore the members of each cluster tended to be derived from pools with similar pH and temperature values. This was not a rigid rule as isolates from the same pool could be found to cluster separately, for example Wai33 A2 and Wai33 A3, which were isolated from the same sample, clustered in group D and a two member cluster respectively.

TABLE 3.2

Analysis of variance between clusters for isolate source temperatures.

SOURCE OF VARIATION	SUMS OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO
Between clusters	3166.4	6	527.7	4.005**
Residual	3821.7	29	131.8	
Total	6988.1	35		

TABLE 3.3

Analysis of variance between clusters for isolate source pH.

SOURCE OF VARIATION	SUMS OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO
Between clusters	17.86	6	2.98	2.67*
Residual	26.73	24	1.11	
Total	44.59	30		

* denotes significance at the p=0.05 level, ** denotes significance at the p=0.01 level.

3.2.7 Isolate Characterisation.

The distribution of positive characters among clusters and unclustered organisms with the S_{SM}/UPGMA analysis is presented in Table 3.4, and a list of useful tests for identification of the clusters formed given in Table 3.5.

TABLE 3.4

Percentage frequency of positive results for clustered organisms derived from S_{SM} UPGMA analysis.

Cluster No. of isolates	A 12	B 3	C 3	D 10	E 12	F 4	G 7
CHARACTERS							
"pitted" colonies	8	0	0	30	0	0	29
colonies > 3mm	8	0	0	20	17	0	14
orange colonies	100	100	100	100	100	75	100
Growth at 45°C	100	100	100	100	92	0	57
Growth at 75°C	92	100	100	100	100	100	100
Growth at 80°C	0	0	0	0	0	0	0
Growth at pH 9.5	0	0	0	0	8	25	29
Growth at pH 6	92	100	66	100	92	50	71
Carbon sources:							
Acetate	100	100	0	60	42	75	14
Melibiose	92	100	0	0	33	0	0
Glutamate	100	100	33	0	50	0	43
Maltose	100	100	33	0	50	0	43
Succinate	75	33	33	10	42	6	29
Pyruvate	25	67	0	0	25	0	43
Glucose	25	0	0	0	25	0	0
Mannose	83	100	0	10	75	0	29
Rhamnose	42	33	0	10	8	0	0
Mannitol	42	0	0	0	0	0	0
Sucrose	92	100	33	0	17	25	14
Sorbitol	50	0	0	0	0	0	0
Fructose	0	67	0	0	0	0	0
Acetamide	42	0	33	10	0	0	0
Proline	100	100	67	20	30	100	43
Galactose	92	100	0	0	8	0	29
Lactose	100	100	0	0	0	0	0
Casein hydrolysis	75	100	0	30	92	75	57
Elastin hydrolysis							
(24 h)	100	0	0	0	45	100	0
(48 h)	100	67	0	0	100	100	43
(72 h)	100	67	0	30	92	100	71
Hide powder azure hydrolysis							
(24 h)	100	100	0	100	100	100	71
(48 h)	100	100	100	100	100	100	100
Fibrin hydrolysis							
(24 h)	83	100	0	10	100	100	29
(48 h)	100	100	0	30	100	100	43
(72 h)	100	100	0	50	100	100	71
Tween 80 hydrolysis	92	100	67	100	83	75	100

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Antibiotic MIC's (µg/ml)							
Chloramphenicol MIC > 1.25	92	100	0	100	100	25	0
Phosphomycin MIC > 12.5	67	33	33	60	58	0	0
Spectinomycin MIC > 6	92	100	100	100	100	50	57
Kanamycin MIC > 0.6	33	33	67	10	25	25	0
Cycloserine MIC > 12.5	0	33	0	10	0	0	0
Growth in 2% NaCl	8	0	0	0	0	0	0
Growth in 2.5% peptone	75	0	66	40	50	75	0
DNAase under colony	92	33	66	80	83	75	100
p-nitrophenyl substrates:							
β-glucosidase							
cells 6 h	0	100	67	40	33	25	14
cells 18 h	55	100	100	100	100	75	86
α-glucosidase							
cells 6 h	100	100	100	100	100	75	86
CFS 18 h	0	66	0	14	33	0	0
laurate							
CFS 1 h	83	100	100	20	0	0	0
CFS 3 h	100	100	100	82	92	25	100
palmitate							
cells 1 h	92	100	67	60	33	0	15
cells 3 h	92	100	100	70	0	0	86
CFS 1 h	75	100	100	20	0	0	0
β galactosidase	100	100	100	80	83	50	0
α galactosidase	58	67	33	20	8	0	0
Nitrite produced							
1d	83	100	33	40	100	75	0
7d	91	33	66	20	58	75	0
Arbutin degraded	67	100	100	90	100	75	86
Aesculin degraded	67	100	100	100	100	75	100
Triple sugar iron alkaline slant	0	0	0	0	8	25	29
Reduction of:							
Tellurite	100	100	100	0	58	100	100
Methylene blue	100	67	67	100	75	0	86
Viability:							
14d at 70°C	100	100	100	90	75	50	100
28d at 25°C	92	100	100	100	75	100	43
Culture pH > 7.6	0	33	33	10	17	25	0

TABLE 3.5

Diagnostic Tests which Distinguish the Clusters Formed.

+, - and v correspond to ≥80%, ≤20% and 21-79% positive responses respectively.

CHARACTER	CLUSTER						
	A	B	C	D	E	F	G
Potassium tellurite reduction	+	+	+	-	v	+	+
Elastin degradtion (24 h)	+	-	-	-	v	+	-
Growth at 45°C	+	+	+	+	+	-	v
Laurate degradation (CFS 1h)	+	+	+	-	-	-	-
Fibrin degradation (24h)	+	+	-	-	+	+	v

In general the colony characteristics could not be used diagnostically, and neither could growth at extremes of pH. Cluster F had no member able to grow at 45°C. Different clusters showed different carbon source utilisation patterns. Members of clusters A, B and E were able to use a wide range of sources, while members of clusters C, D, F and G were less versatile showing zero or low percentage positives on only a few sources. Members of clusters C and D were generally poorly proteolytic. There appeared to be no pattern to Tween 80 degradation.

Members of clusters C, F and G were sensitive to chloramphenicol while members of clusters F and G were sensitive to phosphomycin and those of cluster G were also sensitive to kanamycin. No member of clusters B or G was able to grow in the presence of 2.5% (w/v) trypticase peptone.

Members of clusters A, B and C showed high percentage positives for *p*-nitrophenyl palmitate degradation, while *p*-nitrophenyl laurate degradation could be achieved by most of the isolates tested. β-Galactosidase was produced by most organisms while very few were able to produce α-galactosidase and cluster B showed the highest perecentage positives able to do this.

No member of cluster D was able to reduce tellurite, and no member of cluster F was able to reduce methylene blue. Nitrate was not reduced by any member of cluster G.

Many other tests such as arbutin and aesculin degradation, triple sugar iron reactions and culture viability did not produce diagnostically useful results.

Discussion.

3.2.8 General Conclusions.

In a numerical classification of Icelandic Thermus isolates Cometta et al. (1982b) were not able to show any correlation between the geographical sources of their isolates and the clusters formed. The data presented here agree with this aspect of their findings, at least with regard to the New Zealand isolates examined. The fact that the bulk of New Zealand isolates did not cluster with isolates from other countries may indicate that geographical variation is important between distant regions. In support of this conclusion the phenogram produced by Cometta et al. (1982b) showed two Japanese isolates, "T.flavus" and "T.thermophilus" in a distinct cluster from their Icelandic isolates.

The data presented here indicate that the phenotype present at any one location may be determined by the environmental conditions prevailing; the clusters formed were shown to be related to the temperature and pH of the sources of the isolates in those clusters. The effect on the distribution of phenotypes by pH was not considered by Cometta et al. (1982b), the phenotype expressed was attributed by them primarily to the temperature of the organism's environment. There may be a number of other factors which also play a part in determining which phenotypes are present; for example the presence of sulphide or toxic metals, knowledge of which could further explain the clusters produced by both this classification and that of Cometta et al. (1982b). The methods used for isolation also determine the phenotype which is found, although in this study direct streaking of pool water onto CMD agar plates was the method used whenever possible so that the most common phenotype was isolated, and not the fastest growing which may be the case with liquid enrichment.

Based on the evidence of their numerical classification and on previous work with Thermus isolates in continuous culture (Sonnleitner et al., 1982; Cometta et al., 1982a), Cometta et al. (1982b) hypothesised that there is a high degree of genetic instability within members of the genus. The results presented here confirmed that there is phenotypic variability, but they do not confirm or refute the hypothesis of genetic instability.

An alternative to the suggestion of genetic instability is that any one pool, or thermal environment of the correct type (neutral or alkaline pH), contains a population of Thermus phenotypes any one of which is numerically dominant (and hence the most likely to be isolated). Conditions in hot pools vary within any one pool both with time (Patel et al., 1985a) and with distance (e.g. the water cools in runoff channels), and they also vary between pools. It may be, therefore, that the dominant phenotype at a particular time and location is determined by the conditions prevailing at that site by selection of a strain, from a population of strains, which is best suited to growth under those conditions.

A study on the population ecology of Sulfolobus acidocaldarius (Mosser et al., 1974; Bohlool & Brock, 1974) showed that a mixture of strains ocurred in the hot pools studied, indicating heterogeneity amongst the isolates. Heterogeneity was shown by optimum temperature, immmunofluorescence and immunodiffusion studies of hot spring populations, so in at least one other group of thermophilic micro-organisms the same heterogeneity of isolates, as these results indicate, has been found.

3.2.9 Taxonomic Conclusions.

Like the study of Cometta et al. (1982b) these results have shown considerable variety within the isolates studied. The validly named species, T.aquaticus Yt-1 does not show a close taxonomic relationship to the New Zealand isolates or to "T.thermophilus" HB8, which was isolated in Japan. If the major clusters formed represent different species then the genus is clearly comprised of many species. However the data are still far from complete, because of the apparent influence of geography on the phenotype isolated and it may be that different thermal areas will be shown to have regionally specific clusters and perhaps other clusters will overlap. These findings prompted the following classifications to be carried out.

3.3 Numerical Classification 2: Icelandic Isolates.

3.3.1 Introduction.

The aim of this section was to investigate further those patterns which emerged from section 3.2, specifically to see if geographical variation on a global scale existed and if the type of hot spring played any part in the distribution of phenotypes. Also more information on the taxonomy of the genus might emerge.

Methods.

3.3.2 Sources of Strains.

Samples were collected in sterile universal bottles from hot springs in Iceland, brought back to New Zealand and processed within 1 week of collection. Site temperatures were recorded with a thermometer and pH values of the samples were determined in New Zealand at room temperature. Great care was taken to ensure that there was no contamination of water samples and all manipulations were done in a laminar air flow cabinet (Microflow, Fleet, Hants, UK). Isolates were obtained in the manner detailed in section 3.2.1, being obtained whenever possible by direct streaking of water samples onto CMD plates and by liquid enrichment if necessary. As there were no historical inconsistencies with regard to isolate numbering the scheme followed was that detailed in the legend to Table 3.1, and strains used listed in Table 3.9, along with the pH , temperature and country where the organism was isolated.

Apart from the Icelandic isolates representative isolates from each of the major clusters A-G of the New Zealand isolate classification (section 3.2) were used as well. "T.lacteus" (ATCC 31557), "T.rubens", T.ruber (DSM 1279), "T.thermophilus" (ATCC 27634), T.aquaticus (ATCC 25104) and "T.flavus" (DSM 674) were also included as reference strains. T.ruber had been validly published as a species since the first classification was carried out and it was considered necessary to include this and the other pink organisms, "T.rubens" and "T.lacteus", in the classification even though they have different temperature optima from the other organisms.

Table 3.6 List of Isolates Used Arranged by Cluster.

CLUSTER	STRAIN	pH	TEMPERATURE(°C)	COUNTRY
-	*T.aquaticus*	NA	71.5	USA
-	ZT1 A1	8	80	ICELAND
A	ZF1 A2	8.7	90	ICELAND
A	ZG5 A3	7.6	55	ICELAND
A	ZG5 A2	7.6	55	ICELAND
A	ZV4B A2	8.3	81	ICELAND
A	ZF2B A2	7.8	75	ICELAND
A	ZV2 A3	7.4	87	ICELAND
A	ZV1E A3	9.2	62	ICELAND
A	ZHG1B A3	9.4	NA	ICELAND
A	ZV5 A1	9.4	80	ICELAND
A	ZG6 A3	8.4	65	ICELAND
A	ZG5 A1	7.6	55	ICELAND
A	ZV1E A1	9.2	62	ICELAND
A	ZV1F A1	9.2	56	ICELAND
A	ZV1C A2	9.2	75	ICELAND
A	ZF1 A3	8.7	90	ICELAND
A	ZV4B A1	8.3	81	ICELAND
A	ZHG1B A1	9.4	NA	ICELAND
A	ZD3 A2	7.8	60	ICELAND
A	ZHG1B A2	9.4	NA	ICELAND
A	ZHG1C A1	9.4	NA	ICELAND
A	ZV1C A1	9.2	75	ICELAND
A	ZD2 A2	8.9	80	ICELAND
A	ZG6 A2	8.4	65	ICELAND
A	ZV4C A2	8.3	71	ICELAND
A	ZV1D A2	9.2	69	ICELAND
-	TOK 22	6.5	75	NZ
-	"*T.lacteus*"	NA	NA	NA
-	"*T.rubens*"	NA	NA	NA
-	"*T.thermophilus*"	6.3	80	JAPAN
-	Fiji 3	7.5	85.4	Fiji
-	Oh A2	7.0	80	NZ
-	ZHG1 A1	9.4	NA	ICELAND

CONTINUED

B	Wai28 A1	7.4	75.5	NZ
B	Rt 366	NA	76	NZ
B	ZA2 A2	6.9	75	ICELAND
B	"*T.flavus*"	NA	NA	JAPAN
B	*T.ruber*	NA	NA	USSR
B	ZV4C A1	8.3	71	ICELAND
B	ZV4D A2	8.3	61	ICELAND
-	Wai 8	8.5	80	NZ
C	Rt39	4	68.1	NZ
C	ZV4A A1	8.3	92	ICELAND
C	ZV1D A1	9.2	69	ICELAND
D	ZF2 A1	9.4	90	ICELAND
D	ZD2 A1	8.9	80	ICELAND
D	ZHG1C A2	9.4	NA	ICELAND
D	ZF1 A1	8.7	90	ICELAND
D	ZV2 A1	7.4	87	ICELAND
D	ZHG1B A4	9.4	NA	ICELAND
D	ZV1C A3	9.2	75	ICELAND
D	ZD3 A1	7.8	60	ICELAND
D	ZG6 A1	8.4	65	ICELAND
D	ZF2B A1	7.8	75	ICELAND
D	ZV4D A1	8.3	61	ICELAND
D	ZV4C A3	8.3	71	ICELAND
D	ZV2 A2	7.4	87	ICELAND
D	ZF2B A3	7.8	75	ICELAND
D	ZT1 A2	8	80	ICELAND
D	ZS1 A1	9.7	68	ICELAND
D	ZA2 A1	6.9	75	ICELAND
-	ZV1D A3	9.2	69	ICELAND
-	ZVIE A3	9.2	62	ICELAND

New abbreviations used: (Z general prefix for Iceland) ZV, Hveragerdi (sites ZV1A-ZV1G are from the gradient sampled by Alfredsson & Kristjansson (1983) and sites ZV4A-ZV4D are also a thermal gradient); ZH, Haegindi (thermal gradient at sites ZH1A-ZH1C); ZT, Aratunga; ZF, Fludir; ZG, Geysir; ZD, Kleppjarnsreykir; ZS, Storias; ZA, Near Hveragerdi.

3.3.3 Characters Tested.

These were basically the same as those in section 3.2 except for a few omissions (where data was redundant for this set of OTUs). Also the carbon source testing was by method 2 (section 2.2.4.38) as Icelandic strains did not grow well under the conditions of method 1. A few extra carbon sources were included and a full list of tests is shown in Table 3.9. All organisms from section 3.2 were also tested for growth on carbon sources by method 2 so that direct comparisons could be made (see Appendix 1 for results). For the three pink organisms, T.ruber, "T.lacteus" and "T.rubens", which have low temperature optima, all incubations were carried out at 55°C, while all other incubations were at 70°C.

3.3.4 Calculation of Test Error.

This was done by the method in section 3.2.3 using 11 pairs of replicate strains. The organisms from section 3.2 were compared with the results from that section so that any changes in phenotypes which may have occurred over the time span between classifications (6 months) could be detected and an inter-classification error rate calculated in a similar manner to that for an intra-classification error value.

3.3.5 Analysis of Results.

This was identical to that in section 3.2.4.

Results and Discussion.

3.3.6 Distribution of Thermus Isolates in Icelandic Hot Springs.

Figure 3.3 shows the distribution of Icelandic water samples which yielded Thermus isolates, along with those that did not, with respect to the temperature and pH of the water sources. The same method was followed for all pools and it is possible that had a greater inoculum of pool water been used where no isolates had initially been obtained, some may have eventually been isolated. However the low numbers which might be present would indicate that those samples were not from environments favourable for the growth of Thermus.

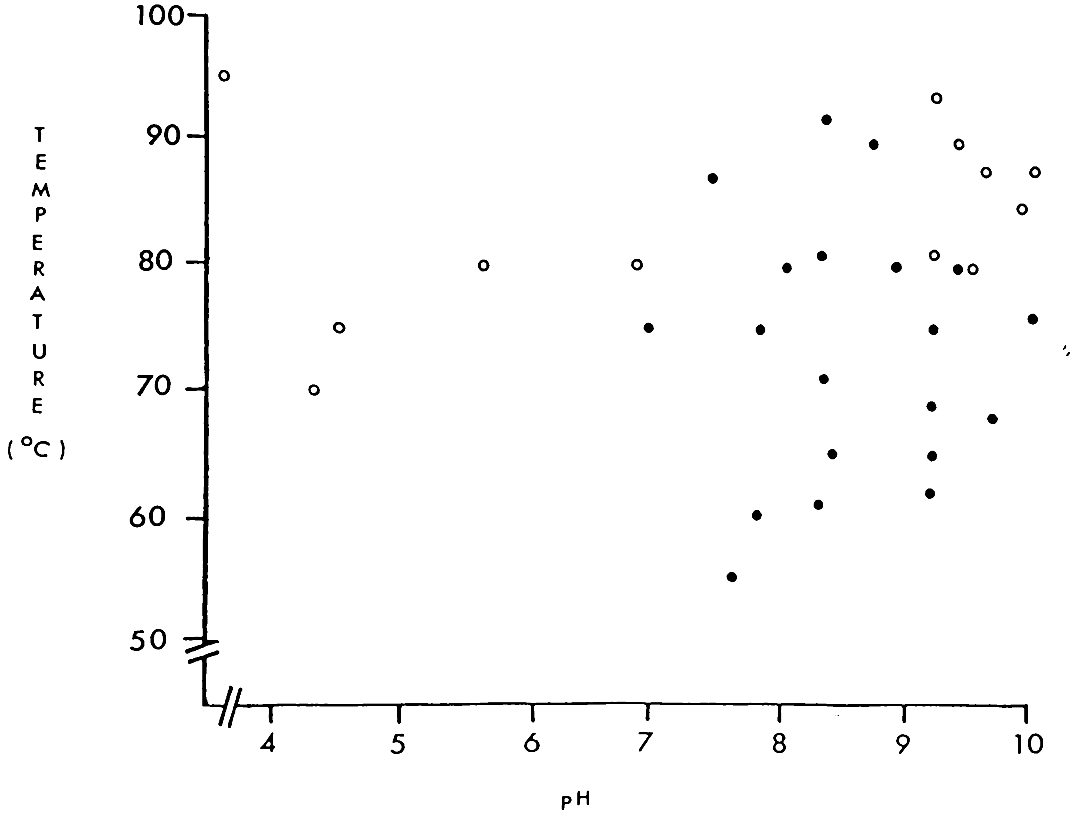


Figure 3.3. Icelandic water samples yielding Thermus isolates in relation to the pH and temperature of the sample sources. Open circles indicate samples yielding no Thermus isolates, closed circles those positive for Thermus isolates.

The results show that Thermus isolates could be obtained from sites which had a pH from just under 7 up to 10 (the highest pH of a water sample tested) and with a temperature from 47°C (the lowest temperature for a source tested) up to just above 90°C. There is an apparent gap where high temperatures and pHs combine, in general samples from temperatures of 80°C or above and of pH 9.5 or above did not yield Thermus isolates. These results agree with those of Kristjansson & Alfredsson (1983).

3.3.7 Experimental Error.

This was estimated to be 6.4%, which is higher than would be desirable. However Sneath & Johnson (1972) state that only when the error reaches 10% does it become unacceptably high. The error between strains in this and the former classification was 7.8% which is not unexpectedly higher than the intra-classification error value.

3.3.8 Clustering of the Isolates.

From the S_{SM}/UPGMA analysis 4 clusters which each contained more than 2 isolates were formed at the 73% similarity level (Figures 3.4A and 3.4B; these show a simplified and a full phenogram respectively). These have been labelled A to D. In addition there were 5 two membered clusters and 2 unclustered organisms.

The S_J/UPGMA phenogram (Figure 3.5) showed some differences from Figure 3.4. These difference indicate that one of the clusters shown in Figure 3.4 may reflect a significant component of negative matches.

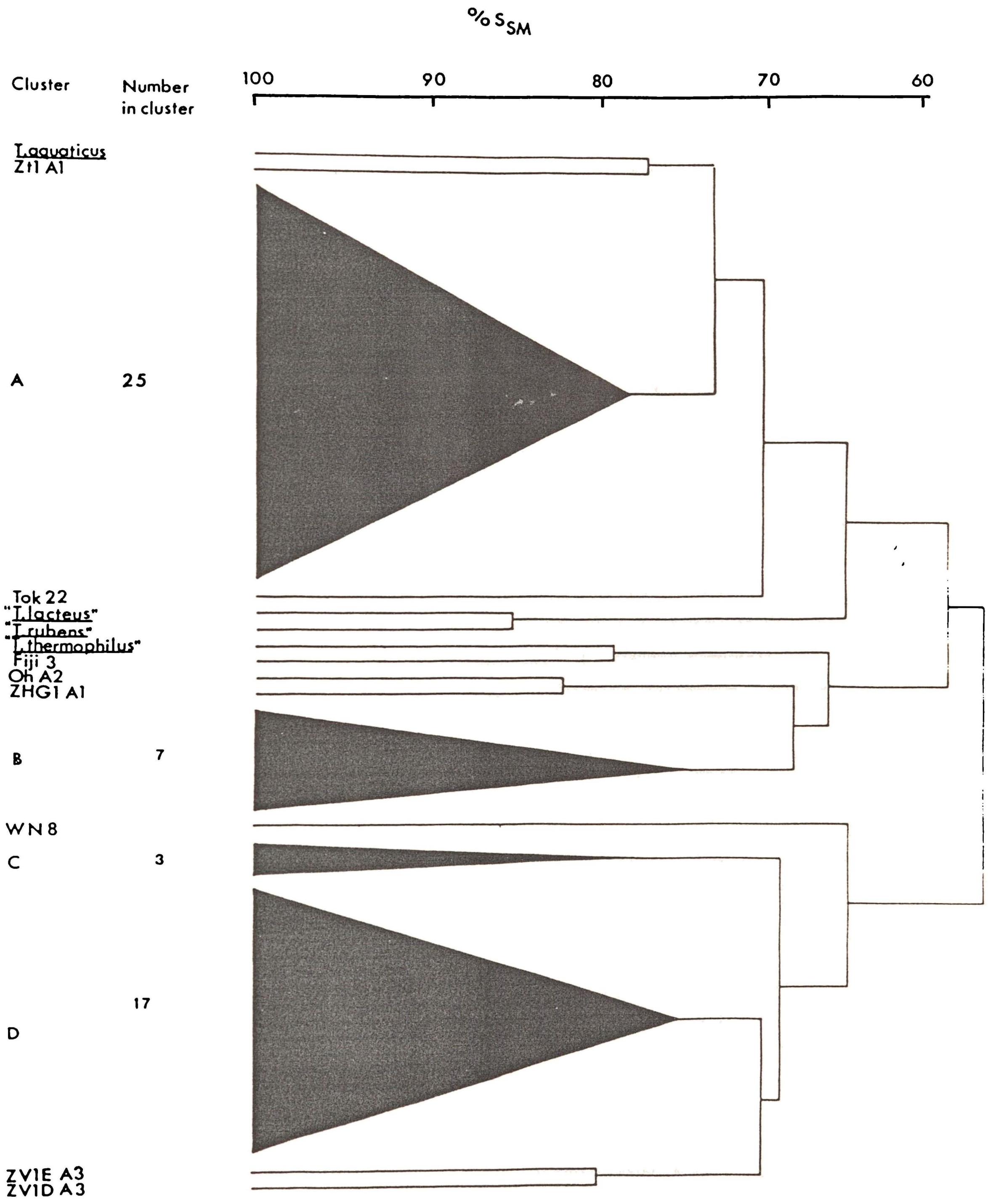
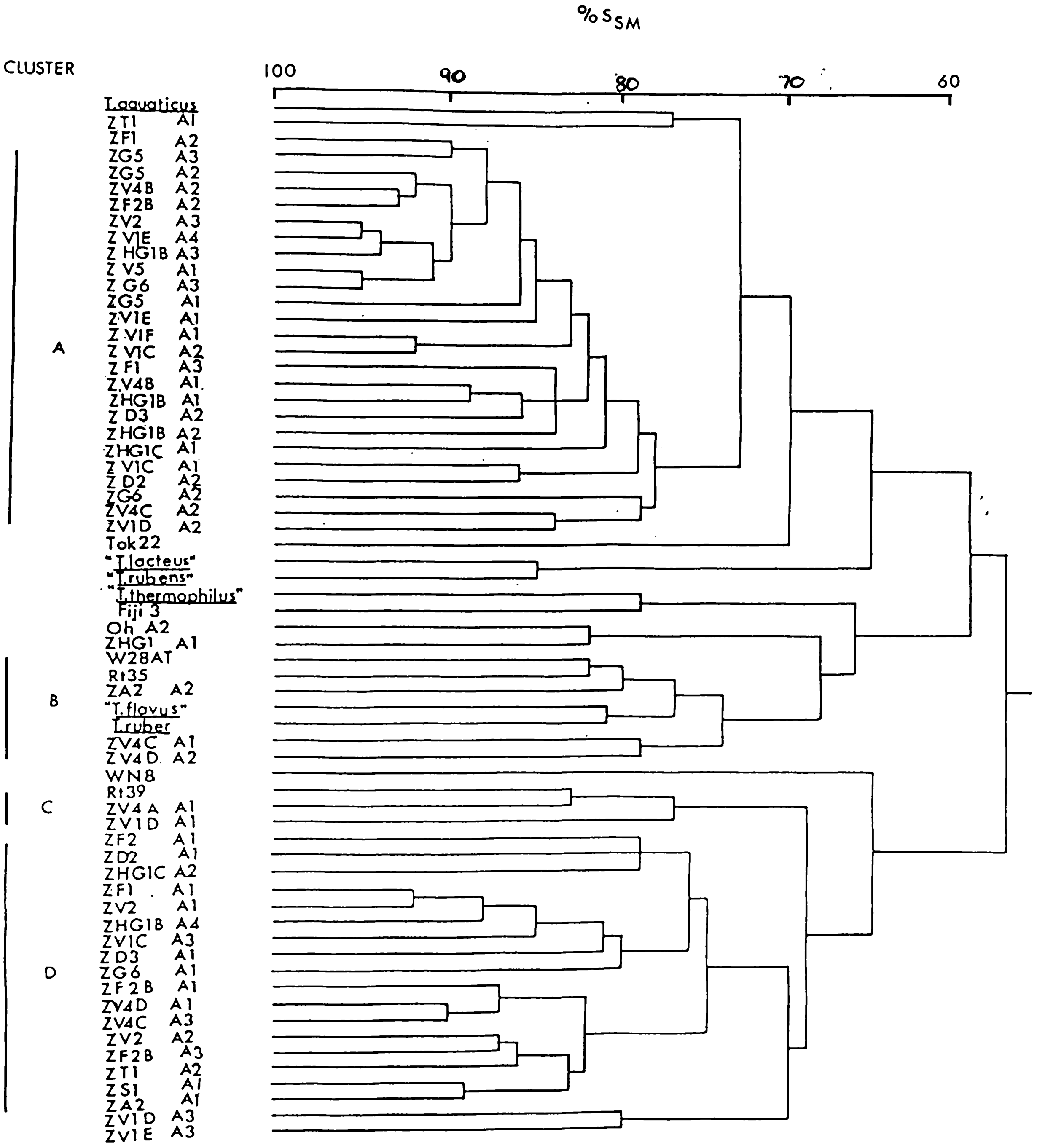


Figure 3.4A. Simplified phenogram of Icelandic isolates based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient.

Figure 3.4B. Detailed phenogram of Icelandic isolates based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient.



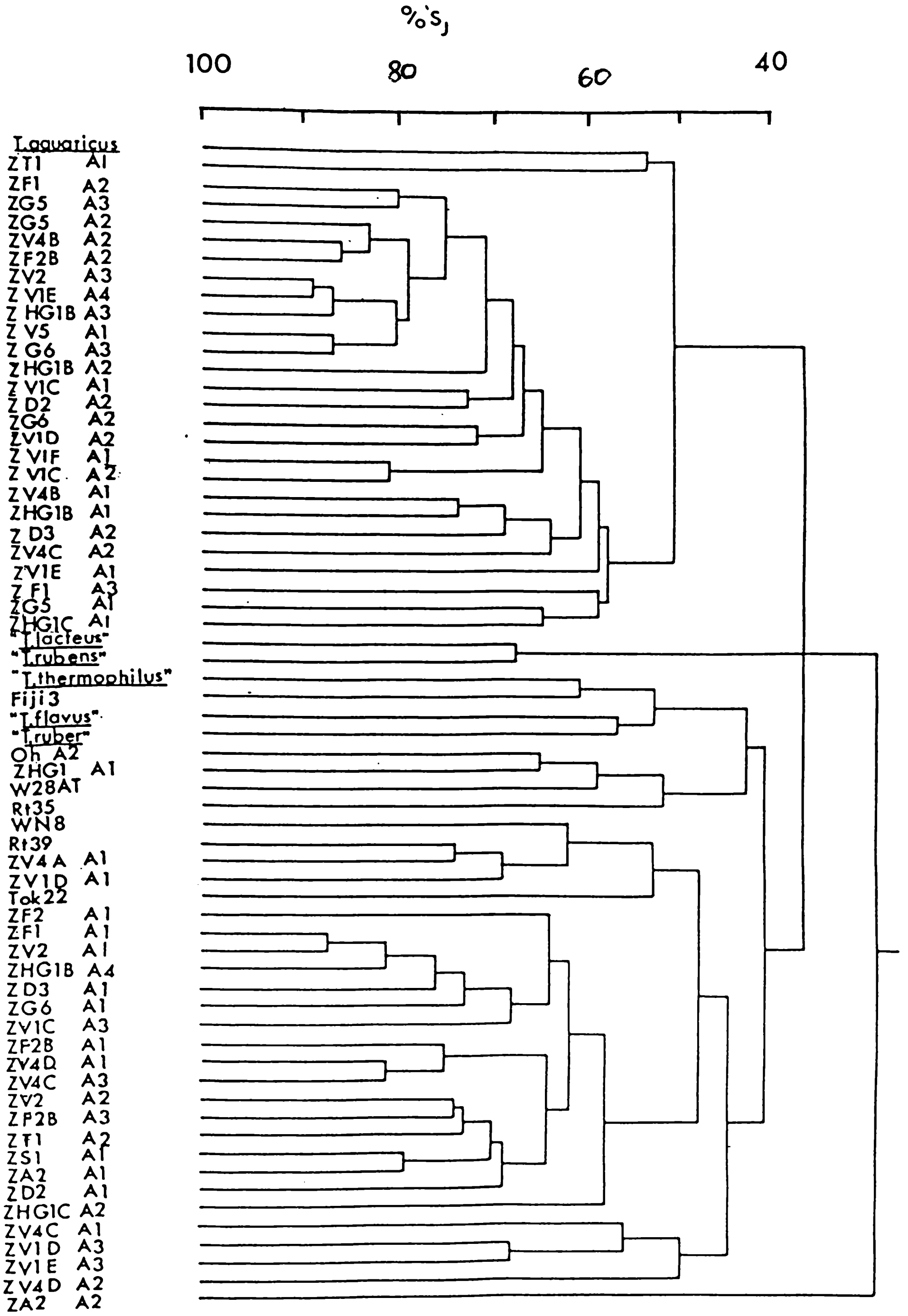


Figure 3.5. Detailed phenogram of the Icelandic isolates based on the UPGMA analysis of a similarity matrix derived from the S$_J$ coefficient.

Irrespective of the similarity coefficient used the 2 largest clusters A and D remained almost identical. In the S_J analysis cluster B had split into 3 two-member clusters and an unclustered organism. Cluster C was joined by two organisms which had been unclustered in the S_{SM} analysis. Single linkage of both S_J and S_{SM} derived similarity values resulted in the same groupings being produced except that T.aquaticus Yt-1 was split away from ZT1 A1.

The two major clusters from the S_{SM} analysis did not contain any of the New Zealand cluster representatives from the clusters produced in section 3.2 or any of the reference strains. In general the representatives of the clusters from the New Zealand classification remained unclustered or in two-member clusters. Cluster B had 2 of these strains. T.aquaticus. Yt-1 clustered at a relatively low similarity level with an Icelandic isolate , ZT1 A1, and "T.thermophilus" clustered with Fiji 3 at a low similarity level. "T.lacteus" and "T.rubens", both pink lower temperature organisms, clustered together at a high similarity level, while T.ruber clustered with "T.flavus" which is a yellow pigmented high temperature organism.

This distribution lends support to the conclusion given in section 3.2.8 in that again the geographical source of the isolates is reflected in the clusters formed. Mixing of geographical sources only occurred in the smaller clusters, and the majority (86%) of the Icelandic isolates did not cluster with isolates from other thermal regions.

Although "T.rubens and "T.lacteus" clustered together, T.ruber clustered with T.flavus, showing that pigmentation is not a useful character for identification. T.ruber also has a low temperature optimum of 60°C (Loginova et al., 1984) while that of "T.flavus" is 70-75°C (Saiki et al., 1972).

Analysis by chi-squared testing of the distribution of the Icelandic sites within the clusters compared with that which would be expected from a random distribution showed there to be no real deviation from the random distribution.

Examination of the pH and temperature values for the isolates of each cluster by analysis of variance in this case showed there to be no significant difference between the temperature values for the 4 clusters, but significance for the distribution of pH values was shown. The results of the analyses are shown in Tables 3.7 and 3.8.

Table 3.7

Analysis of Variance Between Clusters for the Temperature Values of the Clusters.

SOURCE OF VARIATION	SUMS OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO
BETWEEN CLUSTERS	263.4	3	87.8	0.759
RESIDUAL	4626.5	40	115.7	
TOTAL	4890.0	43	113.7	

Table 3.8

Analysis of Variance Between Clusters for the pH Values of the Clusters.

SOURCE OF VARIATION	SUMS OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO
BETWEEN CLUSTERS	7.82	3	2.61	2.965 *
RESIDUAL	39.57	45	0.88	
TOTAL	47.40	48	0.99	

* Denotes significance at the p=0.05 level

There were therefore significant differences between the pH values for the isolates which comprised the clusters. As with the New Zealand isolate classification this was far from a rigid division as, for example, ZF1 A1 and ZF1 A3 cluster in different groups although they came from the same water sample.

3.3.9 Characterisation of the Isolates.

The distribution of positive characters among clusters is presented in Table 3.9, and a list of tests useful for distinguishing the clusters given in Table 3.10. In this classification colony characters were redundant except for colony colour and all clustered organisms were orange/yellow. No diagnostically useful tests could be obtained from growth at extremes of temperature or pH. There was a general trend in the carbon source utilisation pattern. In general members of clusters C and D showed the highest percentage positives for carbon source utilisation while clusters A and B showed low percentage positives for a large number of carbon sources. Few organisms were able to use sorbitol or mannitol as carbon source. Members of clusters B and D were poorly proteolytic while members of clusters A and C showed marked proteolysis after only 24 h incubation. There appeared to be no pattern to Tween 80 degradation. There was little pattern to antibiotic inhibitions except that members of cluster D were kanamycin sensitive. No clustered strain could grow in the presence of 2% (w/v) NaCl while members of clusters A and B were variable for growth in the presence of 2.5% (w/v) trypticase peptone, members of cluster C were completely inhibited under these conditions and members of cluster D were generally able to grow.

Table 3.9

Percentage Positive Results for Clusters of Icelandic Isolates Derived by S_{SM}/UPGMA Analysis.

CHARACTER	CLUSTERS:	A	B	C	D
	No. in cluster	25	7	3	17
Growth at:					
45°C		96	100	100	94
75°C		96	100	100	100
80°C		4	14	33	47
pH 9.5		92	28	33	71
pH 6		68	42	100	100
Utilisation of Carbon Sources:					
Acetate		36	70	100	71
Melibiose		0	0	100	85
Glutamate		16	56	100	76
Maltose		20	14	67	65
Succinate		28	14	33	35
Pyruvate		44	42	100	85
Glucose		12	0	33	6
Mannose		12	0	33	71
Mannitol		4	0	0	0
Sucrose		4	14	100	94
Sorbitol		4	0	0	0
Acetamide		0	0	0	6
Proline		8	42	100	53
Galactose		8	0	100	76
Lactose		4	0	33	88
Dextrin		0	0	100	94
Cellobiose		4	14	100	88
Trehalose		20	28	67	100
Raffinose		4	14	33	65
Salicin		4	14	100	88
D-Asparagine		12	0	100	76
Hydrolysis of					
Casein		80	0	100	12
Elastin 24 h		84	0	33	0
Elastin 48 h		96	0	100	6
Elastin 72 h		96	0	100	6
Fibrin 24 h		100	0	100	0
Fibrin 48 h		100	28	100	18
Hide Powder Azure 24 h		100	42	100	35
Hide Powder Azure 48 h		100	56	100	59
Tween 80		88	28	67	85

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Antibiotic MICs ($\mu g\ ml^{-1}$)				
Chloramphenicol MIC> 1.25	96	100	67	82
Phosphomycin MIC > 12.5	28	28	67	6
Spectinomycin MIC > 6	60	70	67	100
Kanamycin MIC > 0.6	28	28	33	100
Cycloserine MIC > 12.5	0	14	0	0
Growth in:				
2.5% Peptone	24	42	0	82
Presence of DNAase	100	84	100	94
β-Galactosidase	96	84	100	100
α-Galactosidase	4	70	67	100
Degradation of p -nitrophenyl substrates:				
β-Glucopyranoside cells 6 h	0	0	0	6
β-Glucopyranoside cells 18 h	56	28	0	24
α-Glucopyranoside CFS 18 h	0	0	0	6
Laurate CFS 3 h	0	14	33	47
Palmitate cells 1 h	0	0	12	0
Nitrite Production				
1 d	84	0	67	47
7 d	92	14	67	65
Arbutin Degraded	80	100	100	100
Aesculin Degraded	44	56	100	94
Triple Sugar Iron Slant Reddening	0	14	0	0
Reduction of:				
Tellurite	12	42	100	100
Methylene Blue	0	28	0	0
Viability :				
14 d at 70°C	56	84	67	85
28 d at 25°C	96	100	100	42
Culture pH > 7.6	4	28	0	0

All clustered strains were positive for p-nitrophenyl-α-Glucopyranoside degradation at 6 h by cell suspension, and orange colour while they were all negative for p-nitrophenyl laurate degradation by cell-free supernatant after 1 h, p-nitrophenyl palmitate degradation by cell-free supernatant after 1 h or 3 h and growth in 2% NaCl.

Table 3.10

Diagnostic Characters to Distinguish Clusters Produced.

TEST	CLUSTER... A	B	C	D
TELLURITE REDUCTION	-	v	+	+
ELASTIN HYDROLYSIS (24 h)	+	-	+	-
METHYLENE BLUE REDUCTION	-	v	-	-
NITRITE PRODUCTION (7 d)	+	-	v	v

Where - , v and + represent < 20% positive, 21-79% positive and > 80% positive respectively.

Table 3.11

Comparison of Diagnostic Characters Between NZ and Icelandic Isolate Classifications.

TEST	NZ CLUSTERS							ICELANDIC CLUSTERS			
	A	B	C	D	E	F	G	A	B	C	D
TELLURITE REDUCTION	+	+	+	-	v	+	+	-	v	+	+
ELASTIN HYDROLYSIS (24 h)	+	-	-	-	v	+	-	+	-	+	-
GROWTH AT 45°C	+	+	+	+	+	-	v	+	+	+	+
LAURATE DEGRADATION (1 h)	+	+	+	-	-	-	-	-	-	-	-
FIBRIN HYDROLYSIS (24 h)	+	+	-	-	+	+	v	+	-	+	-
METHYLENE BLUE REDUCTION	+	v	v	+	v	-	+	-	v	-	-
NITRITE PRODUCTION (7 d)	+	+	v	v	+	v	-	+	-	v	v

Symbols are those used in Table 3.10.

Most of the organisms were not able to degrade p-nitrophenyl fatty acids. β-Galactosidase was produced by most organisms while only 1 member of cluster A was able to produce α-galactosidase. All members of clusters C and D could reduce tellurite while only members of cluster B showed an ability to reduce methylene blue. Members of cluster B were generally not able to reduce nitrate to nitrite.

Arbutin and aesculin hydrolysis, triple sugar iron reactions and viability did not show diagnostically useful results.

In a comparison of the diagnostic Tables of the New Zealand and Icelandic clusters (Table 3.11) the all Icelandic isolate clusters showed different diagnostic character types, while the Icelandic classification clusters B and C most strongly resembled New Zealand clusters D and E respectively, the differences being between + or - values compared with variable values for percentage positives for some of the tests. This information supports the proposal that thermal areas may have locally restricted phenotypes and other phenotypes which overlap with other thermal areas.

Discussion.

3.3.10 Phenotypic Variation.

The data reinforce earlier conclusions that geography may be important in the phenotypes of Thermus present in thermal areas. It may not be the actual spatial separaration of the thermal regions which is important, but the influence that might be important could be the geology of the region and other factors which could serve to produce a hot pool chemistry characteristic of any one thermal region.

On inspection it appeared that the pH of the Icelandic hot pools which yielded Thermus isolates was generally higher than those of New Zealand Thermus yielding pools. A test to compare the averages of these values by calculating a value for d, the standardised normal deviate, for the pH and temperatures of the two thermal regions was carried out and the results showed that there were significant differences. The value of d obtained for a comparison of the pH data (New Zealand; mean=7.2, N=38: Iceland; mean= 8.54, N=51) was 6.2, indicating significance at the p=0.001 level. For temperature comparisons (New Zealand; mean=78°C, N=41: Iceland; mean= 73°C, N=44) significance was at the p=0.05 level with a value for d of 1.99.

Although these data are interesting there would obviously be a considerable overlap between pool types in the two regions and these differences alone are not sufficient to explain the differences in phenotype. Two possible explanations are 1) that the two regions do have different hot pool chemsitry so selecting for/against certain phenotypes or 2) that Thermus has undergone divergent evolution in the widely separated areas to produce the different phenotypes. This would imply that there is little spread of organisms from one area to another, at least on the scale of distances in this study.

These points indicate that comparisons of phenotypes from more isolated thermal areas should be made.

An examination of the percentage positives for the characters tested between the two classifications, shows the following notable differences in the phenotypes. The Icelandic isolates were more likely to grow at pH 9.5 (which may be connected to the generally higher pHs of the isolate sources), very few could degrade palmitate or laurate, and very few could reduce methylene blue (whereas there was no significant difference in the numbers able to reduce potassium tellurite). These differences alone could not explain the differences in clusters produced, and so the differences must lie in the correlations between characters. The selected tests do however provide useful diagnostic tests to distinguish between New Zealand and Icelandic isolates.

3.3.11 Classification of the Reference Strains.

The classification scheme again shows that T.aquaticus YT-1 and "T.thermophilus" HB8 do not show a very high similarity. The two pink low temperature organisms "T.lacteus" and "T.rubens" clustered close together, indicating that they are similar, but T.ruber did not cluster with them and so pink low temperature organisms cannot be regarded as an homogeneous group. "T.flavus" clustered with T.ruber in one of the smaller clusters and so appears not to represent an unusual phenotype.

3.4 Numerical Classification 3: World-wide Isolates.

3.4.1 Introduction.

Because of the interesting details which emerged from the classifications described above it was clear that a classification involving all of the available organisms should be carried out. It was fortunate that water samples were available from New Mexico (thanks to Prof.H.W.Morgan, University of Waikato), Yellowstone Park (thanks to the provision of samples by Prof. D Ward, Montana State University, Bozeman, Montana, USA), Taiwan (thanks to the provision of samples by Mr. Chihjong Chou), and from the South Island of New Zealand (thanks to Prof.R.M. Daniel, University of Waikato), so that isolates from a diverse range of locations could be obtained. Some of the samples from Yellowstone Park were of particular interest as they were from the same pool from which T.aquaticus Yt-1 was isolated.

METHODS

3.4.2 Sources of Strains.

All of the isolates listed in Tables 3.1 and 3.9 above were included. In addition strains were isolated as indicated in section 3.2.1 from New Mexico, Yellowstone Park and the South Island of New Zealand. Unfortunately water samples from Taiwan did not yield Thermus isolates. These new strains are listed in Table 3.12 along with the pH, temperature and sample location.

Table 3.12

List of New Isolates with pH, Temperature and Location of Sources.

ISOLATE	pH	TEMPERATURE (°C)	LOCATION
YSP1A A1	8	90	Octopus Spring
YSP1B A1	8	90	Octopus Spring
YSP1C A1	8	75	Octopus Spring
YSP1D A1	8	65-73	Octopus Spring
YSP1D A2	8	65-73	Octopus Spring
YSP1D A3	8	65-73	Octopus Spring
YSP1E A1	8	60	Octopus Spring
YSP1E A2	8	60	Octopus Spring
YSP2A A1	8.75	70	Twin Butte Vista Spring
YSP2A A2	8.75	70	Twin Butte Vista Spring
YSP2B A1	8.75	60	Twin Butte Vista Spring
YSP2B A2	8.75	60	Twin Butte Vista Spring
YSP3A A1	7.9	60	Mushroom Spring
NMX2 A1	6	72	Jemez
NMX2 A3	6	72	Jemez
NMX2 A4	6	72	Jemez
HS1 A1	7.6	47	Hanmer Springs
HS2 A1	6.8	37	Hanmer Springs
HS2 A2	6.8	37	Hanmer Springs
HS3 A1	6	47	Hanmer Springs
HS5 A1	6.7	44	Maruia Springs
HS6 A1	6.4	59	Maruia Springs
HS7 A1	6.4	57	Maruia Springs
HS8 A1	7	55	Maruia Springs

Abbreviations Used: YSP= Yellowstone Park, NMX= New Mexico, HS= Hanmer Springs (South Island, New Zealand).

3.4.3 Characters Tested.

The characters tested included all those shown in Table 3.15 and comprised all of the tests devised (i.e. none were redundant). Carbon source testing was by method 2 (see section 2.2.4.38). The New Zealand isolates were re-tested, as method 1 had been used in the previous classification, so as to be consistent with the methods used for the other isolates tested. Data from previous classifications were used where available. The Yellowstone Park, New Mexico and South

Island isolates were tested for all the characters listed in Table 3.15. Full data are given in Appendix 2A.

3.4.4 Test Error.

9 strains were used in duplicate to give an estimate of test error. A significant number of strains from previous classifications was not included for an estimate of error between classifications as values had been obtained twice before and the values were very similar.

3.4.5 Analysis of Results.

Result analysis was identical to that used in both of the previous classifications.

RESULTS

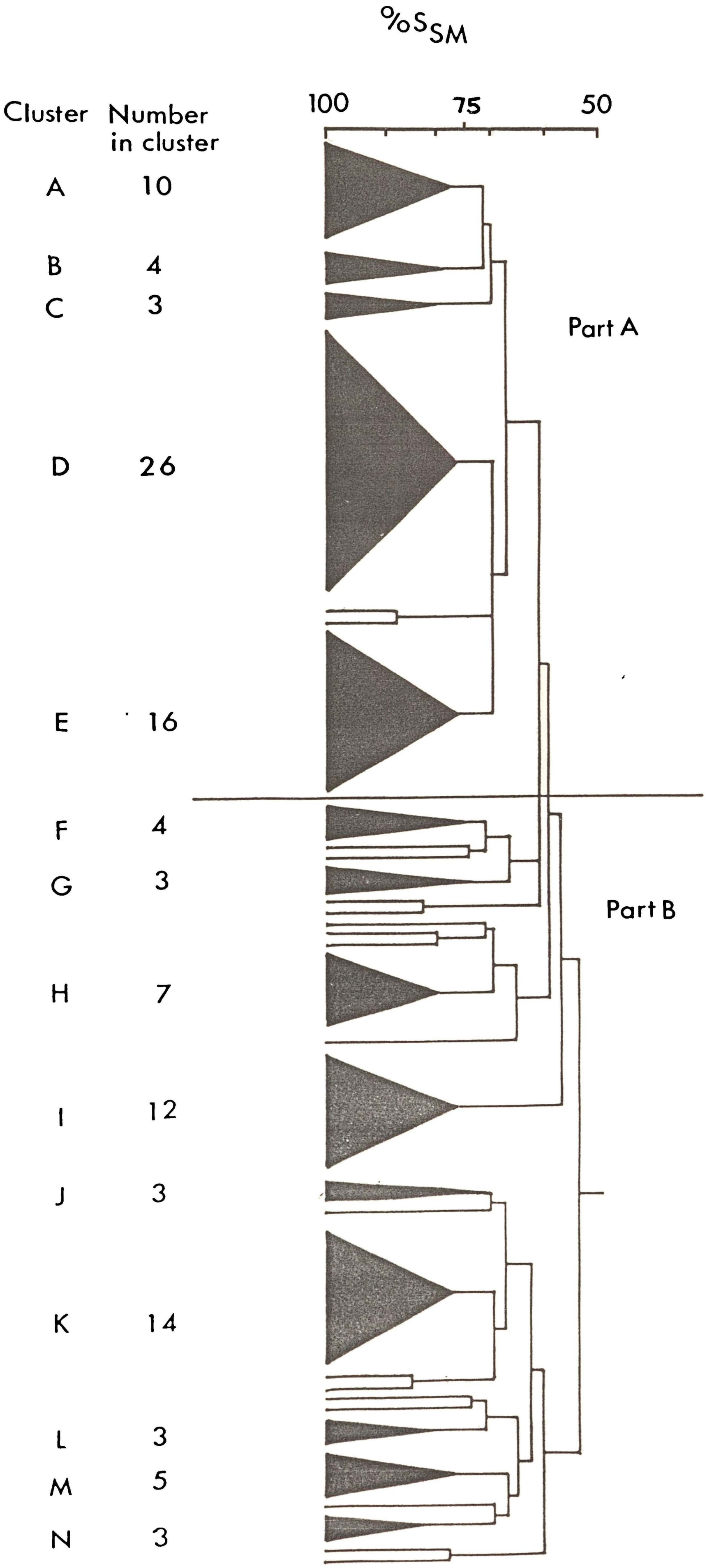
3.4.6 Experimental Error.

This was estimated to be 4.7% by the method of Sneath & Johnson (1972).

3.4.7 Clustering of the Isolates.

Figure 3.6 shows the phenogram produced by S_{SM}/UPGMA analysis. Taking the previously used value of 73% S_{SM} to form the clusters, 14 clusters were formed which each contained 3 or more isolates. In addition there were 7 two-member clusters and 4 unclustered strains.

Figure 3.6. Simplified phenogram of all isolates based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient. Indicated on the phenogram are the two separate parts, A and B, which comprise the detailed phenogram.


%S_SM
100
75
50
Cluster
Number in cluster
A 10
B 4
C 3
D 26
E 16
F 4
G 3
H 7
I 12
J 3
K 14
L 3
M 5
N 3
Part A
Part B

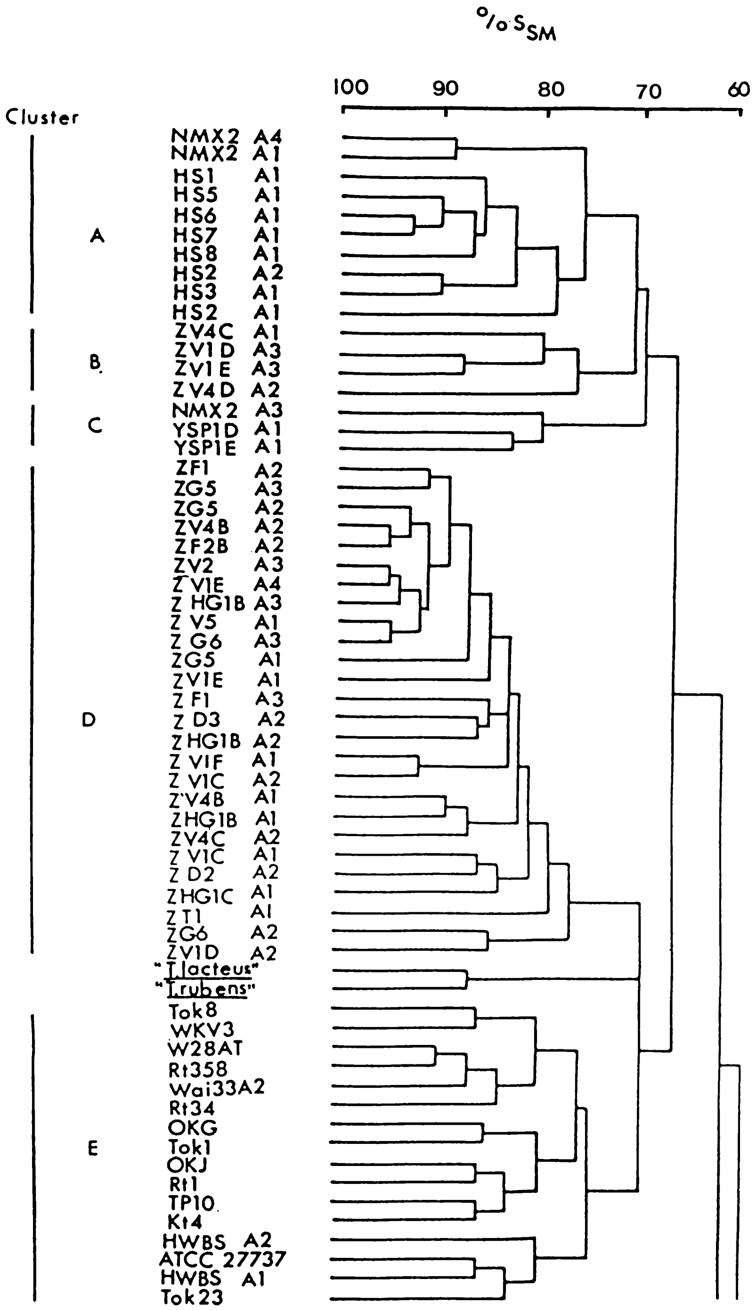
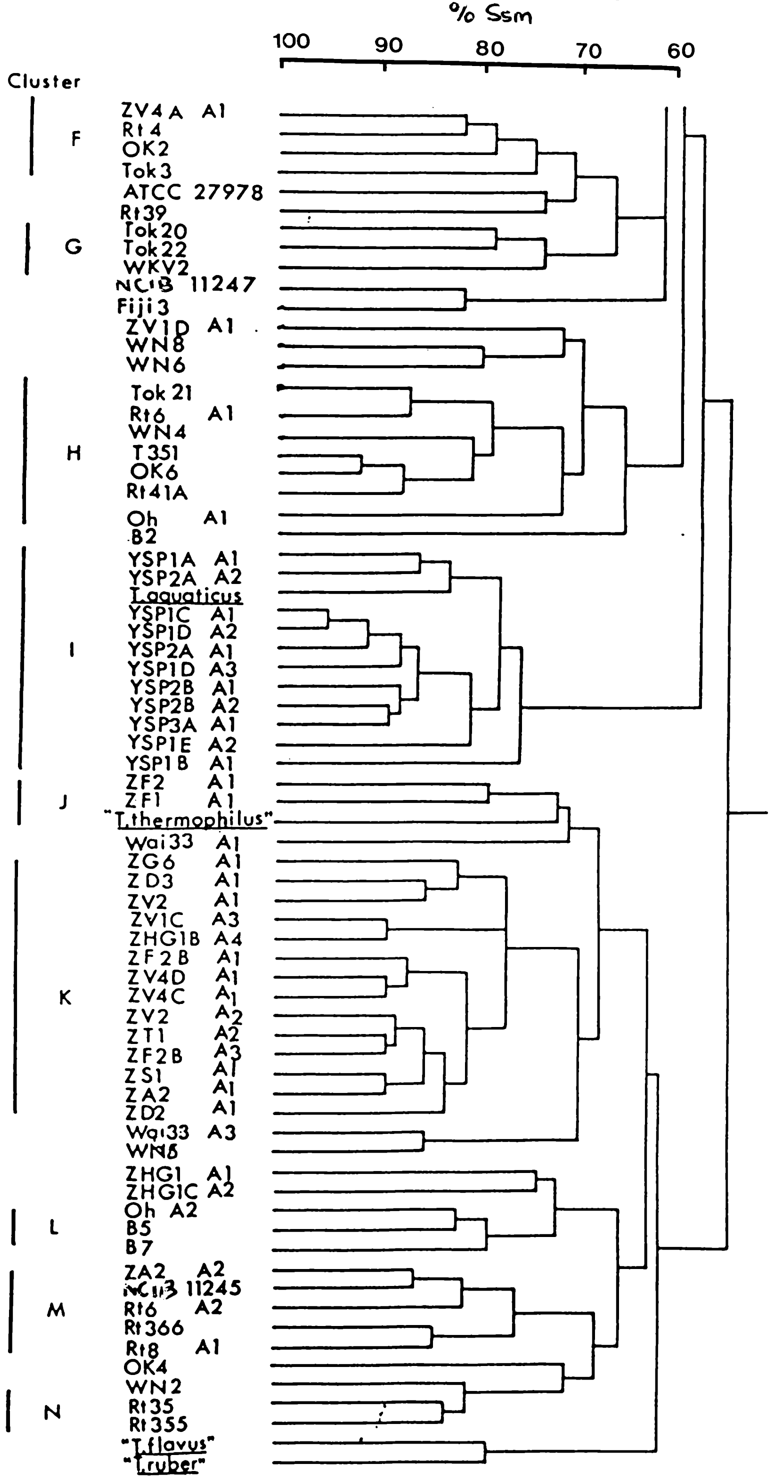


Figure 3.6A. Detailed section of the phenogram shown in Figure 3.6.

Figure 3.6B. Detailed section of the phenogram shown in Figure 3.6.



The S_J/UPGMA analysis (Figure 3.7) shows some differences from Figure 3.6. These differences indicate that some of the clusters shown in Figure 3.6 may reflect a significant component of negative matches. Irrespective of the similarity coefficient used clusters A, B, C, D, I and N remained almost identical. In the S_J analysis compared to the S_{SM} analysis cluster E split into 3 subclusters, the same as those formed in the S_{SM} analysis, cluster F stayed as a discrete sub-cluster and lost one member to another part of the major cluster which contained it. This large cluster incorporated the former clusters F, G, H and K. Cluster J lost "T.thermophilus" which became unclustered, and cluster L split into 2 subclusters as did cluster M.

Analysis by single linkage using minimum spanning trees (simplified minimum spanning trees are shown in appendix 4) showed similar groupings to those described above.

As negative matches are as important as positive ones in this kind of classification further comments will be restricted to the S_{SM}/UPGMA phenogram.

Comparisons with the clusters produced in previous classifications showed that, not unexpectedly, there were some minor differences in the clusters produced. In general the New Zealand isolates clustered as before, even though the carbon source data was different. Some of the New Zealand clusters merged, for example NZ D and NZ E, which formed cluster E in this classification. The Icelandic clusters remained almost completely unchanged, one of the larger clusters losing 4 isolates to others which would be expected where additional, more similar, isolates are introduced into the analysis.

Figre 3.7. Simplified phenogram of all isolates based on the UPGMA analysis of a similarity matrix derived from the S_J coefficient. Indicated on the phenogram are the 3 separate parts, A, B and C which comprise the detailed phenogram.

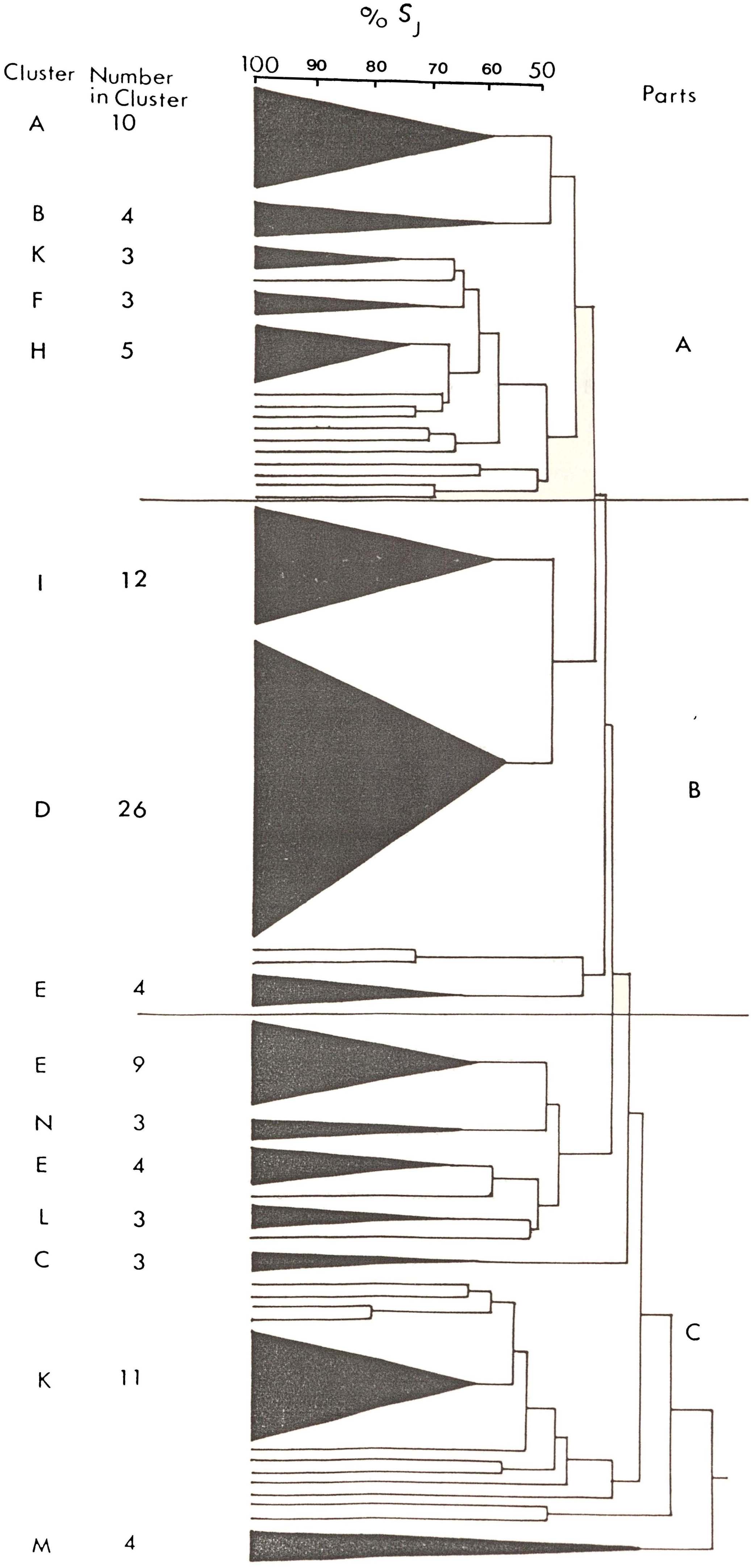

% S_J
100
90
80
70
60
50
Cluster
Number in Cluster
Parts
A 10
B 4
K 3
F 3
H 5
A
I 12
D 26
B
E 4
E 9
N 3
E 4
L 3
C 3
C
K 11
M 4

Figure 3.7A. Detailed section A of the phenogram shown in Figure 3.7.

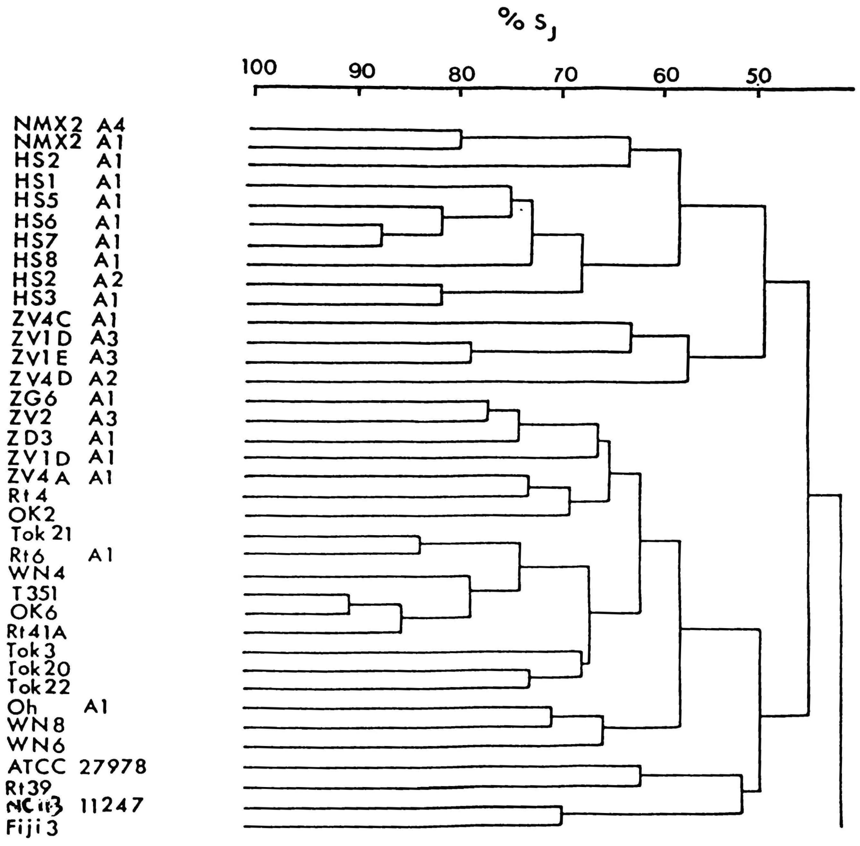


Figure 3.7B. Detailed section B of the phenogram shown in Figure 3.7.

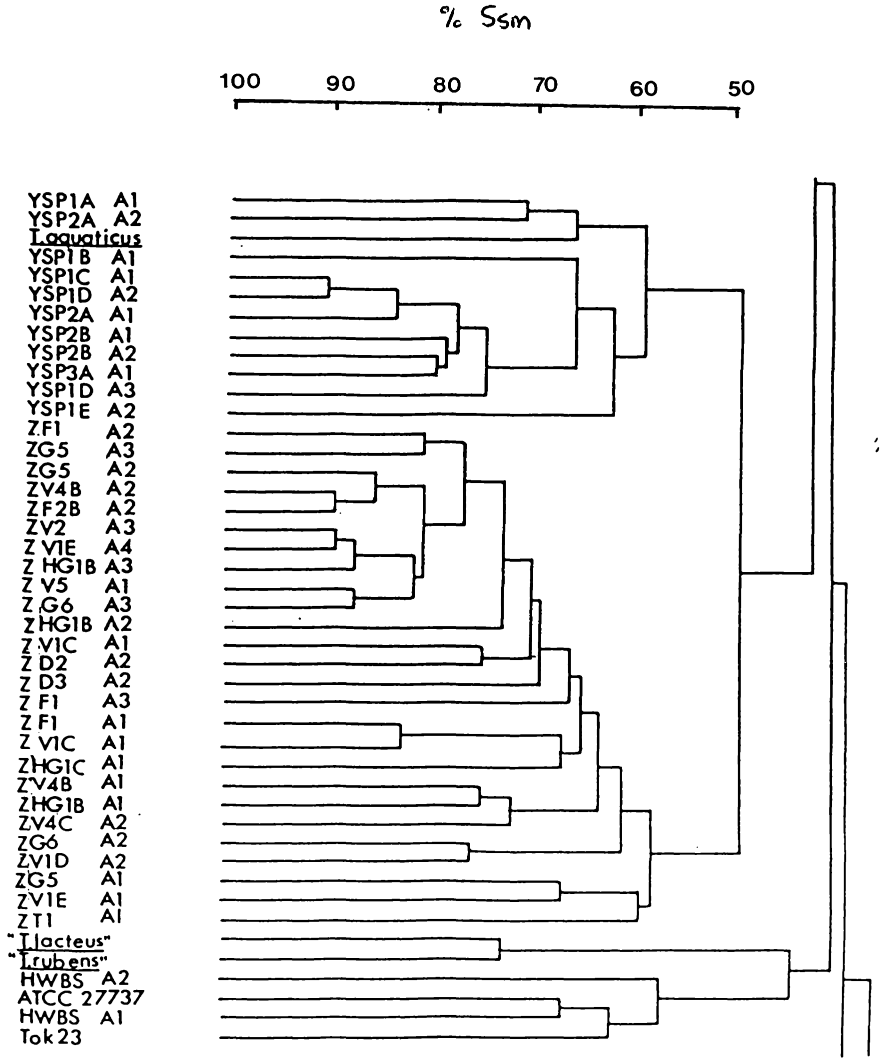
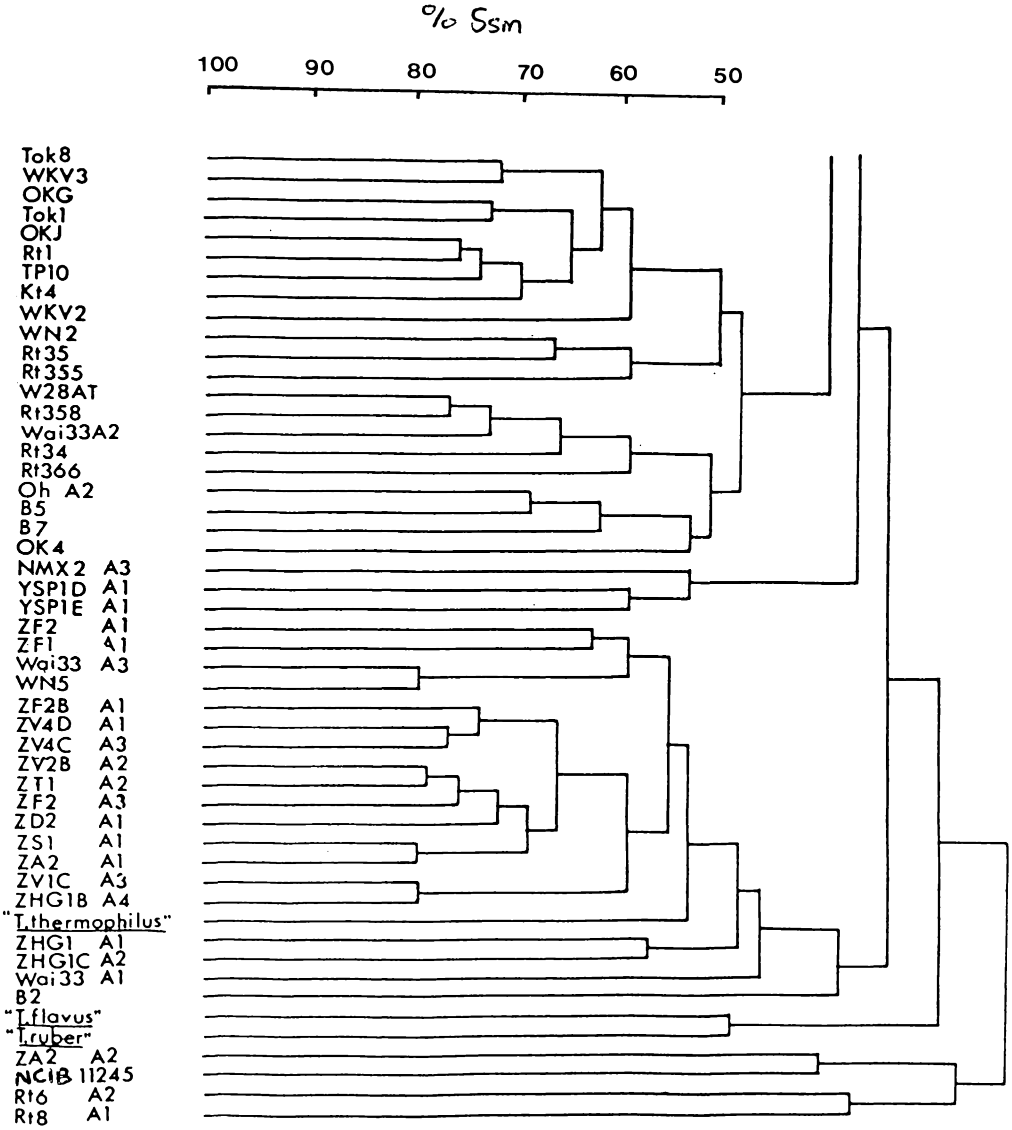


Figure 3.7C. Detailed section C of the phenogram shown in Figure 3.7.



No statistics are required for it to be plain that there is a very strong geographical influence on the cluster formed. Cluster A is comprised of all the South Island isolates plus 2 from New Mexico, cluster B from Icelandic strains, C from US strains, D from Icelandic strains, E from New Zealand strains plus 1 from the USA, G from New Zealand strains, H from New Zealand strains, I from Yellowstone Park strains, K from Icelandic strains and N from New Zealand strains. Clusters F, J, L and M only show real mixes of geographical sources.

Of the reference strains "T.lacteus" and "T.rubens" clustered together as did "T.flavus" and T.ruber, both pairs not being very similar to any other cluster. T.aquaticus clustered in with the Yellowstone Park isolates which is where T.aquaticus Yt-1 was isolated from. The phenotypes of Thermus strains from Yellowstone Park therefore may not have changed over the last 16 years.

NCIB 11247 was isolated from Iceland (strain B, Pask-Hughes & Williams, 1977) although not from a specific area sampled in this study. This organism did not cluster with other Icelandic strains tested here. This therefore did not demonstrate the phenotypic stability shown by T.aquaticus and the Yellowstone isolates. It would have been of interest to have more isolates from known thermal regions which had been recovered some years previously to see if the apparent similarity between T.aquaticus Yt-1 and the other Yellowstone Park isolates is indicative of a general trend.

"T.thermophilus" HB8 remained unclustered while NCIB 11247 and Ramaley X-1 grouped in two-member clusters. NCIB 11245 clustered in a mixed source cluster, cluster M. Wai33 A1 (see chapter 5) remained unclustered.

Analysis of variance of the pH and temperature of the isolate sources showed that both distributions differed significantly from the null hypothesis (Tables 3.13 and 3.14).

Table 3.13

Analysis of Variance Between Clusters for the Temperature Values of the Clusters.

SOURCE OF VARIATION	SUMS OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO
CLUSTERS	7121.5	13	547.8	4.24 **
RESIDUAL	10712.6	83	129.1	
TOTAL	17834.1	185.8		

Table 3.14

Analysis of Variance Between Clusters for the pH Values of Clusters.

SOURCE OF VARIATION	SUMS OF SQUARES	DEGREES OF FREEDOM	MEAN SQUARES	VARIANCE RATIO
CLUSTERS	70.0	13	5.39	8.88 **
RESIDUAL	53.4	88	0.61	
TOTAL	123.4	101	1.22	

** denotes significance at the p=0.01 level.

Table 3.15

CHARACTERS	CLUSTERS													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Number in Cluster	10	4	3	26	16	4	3	7	12	3	14	3	5	3
"pitted" colonies	0	0	0	4	12	0	0	0	0	0	0	0	20	33
Colonies > 3 mm	0	0	0	4	12	0	33	14	0	0	0	0	0	33
Yellow/orange colonies	20	100	100	100	88	100	100	100	100	100	100	100	80	100
Growth at 45°C	100	100	67	96	100	100	100	100	92	100	93	100	100	33
Growth at 75°C	90	100	100	96	100	100	100	86	100	100	100	100	100	100
Growth at 80°C	0	50	0	0	0	0	0	14	33	100	36	33	0	0
Growth at pH 9.5	30	0	67	88	6	0	0	14	67	33	71	0	40	0
Growth at pH 6	60	75	100	69	94	100	100	100	100	100	100	67	60	33
Carbon Sources:														
Acetate	100	100	0	35	6	100	100	71	58	33	79	0	20	33
Melibiose	20	50	0	0	0	50	0	57	0	100	93	0	0	0
Glutamate	100	25	0	19	13	100	0	71	67	67	86	67	20	67
Maltose	20	25	0	19	0	50	67	100	8	100	64	33	0	0
Succinate	60	75	0	27	0	100	100	57	17	100	50	33	0	67
Pyruvate	70	100	67	42	6	100	67	71	0	33	64	0	20	67
Glucose	40	0	67	15	6	75	100	100	0	67	21	0	0	33
Mannose	0	25	0	11	13	50	67	29	17	67	0	0	0	0
Mannitol	50	0	67	4	6	0	0	*	0	33	93	*	*	*
Sucrose	30	75	0	0	*	*	*	29	42	0	0	0	0	0
Sorbitol	70	0	0	8	0	0	0	100	8	100	43	0	0	0
Acetamide	0	0	0	0	0	33	0	0	0	0	7	0	0	0
Proline	90	50	33	8	6	50	67	100	75	100	50	67	0	33
Galactose	0	25	0	8	6	75	0	57	17	100	71	0	0	0
Lactose	10	0	33	4	6	50	67	100	42	67	93	0	0	0
Dextrin	20	50	33	0	0	50	100	100	0	67	93	0	0	0
Cellobiose	0	75	0	4	6	75	0	100	8	0	93	0	0	0
Trehalose	80	100	0	19	0	75	67	86	25	67	100	0	0	33
Raffinose	0	25	0	0	0	25	33	100	8	0	71	0	0	0
Salicin	20	75	0	4	6	50	0	43	0	8	100	0	0	0
Casein Hydrolysis	0	0	0	81	44	100	100	100	100	0	0	0	0	67
Elastin Hydrolysis 24 h	10	0	0	85	25	50	0	71	100	0	0	0	0	0
Elastin Hydrolysis 48 h	20	0	0	96	50	100	33	100	100	0	7	0	0	67
Elastin Hydrolysis 72 h	50	0	0	96	69	75	67	100	100	0	7	0	0	100

Percentage Positive Test Results for World-Wide Clusters

Table 3.15 Continued

	CLUSTERS													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Hide Powder Azure Hydrolysis														
24 h	100	100	67	100	100	100	100	100	100	0	36	0	0	100
48 h	100	100	67	100	100	100	100	100	100	100	100	100	60	100
Fibrin Hydrolysis 24 h	50	0	67	100	50	100	67	71	100	0	0	0	0	33
Fibrin Hydrolysis 48 h	100	100	100	100	62	100	100	100	100	0	7	0	0	67
Fibrin Hydrolysis 72 h	100	100	100	100	75	100	100	100	100	0	7	0	20	100
Tween 80 Hydrolysis	100	75	67	81	94	100	67	100	92	67	64	67	60	100
Antibiotic MICs (μg/ml):														
Chloramphenicol MIC > 1.25	60	100	0	88	100	75	100	86	83	67	79	0	40	0
Phosphomycin MIC > 12.5	0	0	0	31	75	50	33	14	0	33	0	33	0	0
Spectinomycin MIC > 6	90	75	0	58	100	100	100	86	67	100	14	100	20	67
Kanamycin MIC > 0.6	0	0	0	35	25	25	0	29	83	33	0	67	0	0
Cycloserine MIC > 12.5	80	0	0	0	19	0	33	0	58	0	0	0	0	0
								29	0	33	0	0	0	0
Growth in 2% NaCl	0	0	0	0	0	0	0	57	100	100	79	67	0	0
Growth in 2.5% Peptone	90	25	100	27	31	50	33							
DNAase Round Growth	0	0	0	0	0	25	8	0	0	0	0	0	20	0
Under Growth	100	100	100	100	75	100	100	71	100	100	93	67	100	100
α-Galactosidase	100	25	100	4	13	50	0	86	8	100	100	33	0	0
β-Galactosidase	100	8	67	100	100	0	0	100	8	67	100	100	0	0
p-Nitrophenyl Substrates:														
β-Glucoside cells 6 h	*	25	*	0	38	25	33	28	*	0	0	67	20	0
β-Glucoside cells 18 h	100	50	100	54	94	75	67	71	0	33	14	67	20	100
α-Glucoside cells 6 h	*	100	*	100	100	100	100	86	*	100	100	67	20	100
α-Glucoside CFS 18 h	*	0	*	19	25	67	14	14	*	0	7	0	0	0
Laurate CFS 1 h	0	0	0	0	12	0	67	100	8	0	0	100	0	0
Laurate CFS 3 h	30	0	0	0	75	100	100	100	25	67	36	100	80	100
Palmitate cells 1 h	0	0	0	0	56	0	67	100	8	33	0	100	20	0
Palmitate CFS 1 h	0	0	0	0	12	0	0	100	0	0	0	100	0	0
Palmitate CFS 3h	0	0	0	0	44	0	33	100	0	0	0	100	20	100
Nitrate to nitrite 1 d	30	0	0	81	69	100	100	86	8	0	57	33	25	0
Nitrate to nitrite 7 d	40	25	0	92	33	75	100	57	0	33	64	67	50	0
Arbutin Degraded	90	100	33	88	88	100	100	86	0	67	86	100	60	67
Aesculin Degraded	90	50	33	42	81	100	100	86	17	100	79	100	40	100
Triple Sugar Iron Reddening	0	0	0	0	0	25	0	0	83	33	0	0	40	0
Reduction of: tellurite	0	50	0	12	25	75	33	100	100	100	100	100	67	100
methylene blue	0	25	33	0	81	25	100	86	83	33	0	67	40	100
Viability 14 d at 70°C	100	75	67	54	94	75	67	100	83	33	79	100	60	100
28 d at 25°C	100	100	67	96	88	75	100	100	42	67	43	100	60	67
Culture ph > 7.6	30	75	33	8	19	0	0	14	25	0	0	33	40	0

* denotes Not Tested.

TABLE 3.16

Diagnostic Characters for the 73-75% Phena Produced. Symbols Used - < 21% positive, V >20% and < 80% positive, + > 79% positive.

CHARACTER	A	B	C	D	E	F	G	H	I	J	K	L	M	N
TELLURITE REDUCTION	-	V	-	-	V	V	V	+	+	+	+	+	+	+
NITRATE REDUCTION (1 d)	V	-	-	+	V	+	+	+	-	-	V	V	V	-
TRIPLE SUGAR IRON REDDENING	-	-	-	-	-	V	-	-	+	V	-	-	V	-
α-GALACTOSIDASE	+	V	+	-	-	V	-	+	-	+	+	V	-	-
ORANGE COLONIES	-	+	+	+	+	+	+	+	+	+	+	+	+	+
ACETATE AS CARBON SOURCE	+	+	-	V	-	+	+	V	V	V	V	-	-	V
GLUTAMATE AS CARBON SOURCE	+	V	-	-	-	+	-	V	V	V	+	V	-	V
METHYLENE BLUE REDUCTION	-	V	V	-	+	V	+	+	+	V	-	V	V	+
SPECTINOMYCIN RESISTANCE	+	V	-	V	+	+	+	+	V	+	-	+	-	V
CHLORAMPHENICOL RESISTANCE	V	+	-	+	+	V	+	+	+	V	V	-	V	-
LAURATE DEGRADED (1h)	-	-	-	-	-	-	V	+	-	-	-	+	-	-
PALMITATE DEGRADED (3h)	-	-	-	-	V	-	V	+	-	-	-	+	-	+
POWDER AZURE HYDROLYSED (1d)	+	+	V	+	+	+	+	+	+	-	V	-	-	+

3.4.8 Characterisation of the Isolates.

The distribution of positive characters among the clusters with the S_{SM}/UPGMA analysis is presented in Table 3.15, and a list of tests useful for the identification of the clusters given in Table 3.16.

In general the colony characteristics could not be used diagnostically except for the fact that all but 2 members of cluster A were white. Growth at pH 6 was not of diagnostic use but only members of cluster D showed a high percentage able to grow at pH 9.5, and many clusters had very few members able to grow under these conditions. While growth at 45°C and 75°C showed nearly uniform results very few organisms were able to grow well at 80°C, cluster J had all members able to do this. Different clusters showed different carbon source utilisation patterns, but diagnostic value is reduced because of the number of "variable" (between 21 and 79% positive) clusters produced. Clusters C, D, E, I, L, M and N showed low percentage positives on only a few carbon sources, and zero for the rest, while the others showed higher percentage positives on most of the carbon sources. Members of clusters J, K, L and M were poorly proteolytic, while members of clusters A, B and C were able to hydrolyse fibrin but not elastin. Members of clusters A, B, C, J, K, L and M were not able to hydrolyse casein. There was no pattern to Tween 80 degradation.

Sensitivity to chloramphenicol was shown by members of clusters C, L and N. Most organisms were sensitive to phosphomycin, cycloserine and kanamycin, with cluster I showing the greatest percentage positive for the latter. Members of clusters C, K and M were sensitive to spectinomycin. Very few organisms grew in the presence of 2% (w/v) NaCl, and members of clusters M and N generally could not grow in the presence of 2.5% (w/v) trypticase peptone.

The production of α and β glucosidase did not show diagnostically useful results. Members of clusters H and L showed the highest percentage positives for the degradation of p-nitrophenyl fatty acids, and other organisms generally showed no such reaction. β-Galactosidase was produced by most organisms, with clusters I, M and N showing a lack of this enzyme, while only members of clusters A, C, H, K and L showed consistent production of α-galactosidase.

No members of clusters A, C or D were able to reduce tellurite and no members of clusters A, D or K were able to reduce methylene blue. Nitrate reduction was scarce in clusters C, I and N. Only members of clusters I showed consistent reddening of triple sugar iron agar and inability to hydrolyse arbutin or aesculin.

Tests of viability, DNAase production or alkalinisation of growth medium did not show diagnostically useful results.

DISCUSSION

3.4.9 Phenotypic Variation.

The geographical distribution of the isolate sources in Figure 3.6 can readily be seen. Again here the pH and temperature means of the clusters were shown to differ significantly from the null hypothesis. The question to be asked is whether the phenotype distribution is mainly influenced by purely the geography or by the spring types, where of course geography and geology would be important in determining their nature (for example all the pools of the South Island of New Zealand are in general slightly acidic and of lower than average temperature). On their own pH and temperature would not be sufficient to explain the distribution, as there is much overlap between thermal areas of these parameters, which would indicate that there should also be an overlap of phena. Other factors, which have not been measured, could explain the distribution. For example thermal regions may be particularly high or deficient in some biologically important ion, so influencing the types of *Thermus* strains that can grow there. Without full data from the sites sampled no useful comparisons can be made between the areas and so this will have to be left to speculation. However there are only two major types of hot spring (see section 1.1.4), only one of which is inhabited by *Thermus*, that can be defined on a broad chemical basis.

This would indicate that the explanation of the distribution of phenotypes being influenced primarily by environmental parameters is not correct as pools are broadly similar. If there were more groups of springs which could be defined then this would lend more credence to such an explanation as there would be more scope for particular phenotypes to be associated with major divisions of pool types.

Perhaps a more plausible explanation is that it is the geographical remoteness of the thermal regions which has brought about the differences in phenotypes. Great distances between regions suggest that the frequency of cross contamination of strains between regions would be low. Those areas on the same continent (i.e. connected by land) would presumably be more frequently cross contaminated as it is apparent that Thermus can survive in cold water environments for limited periods of time and is found in man-made thermal environments (see section 1.2.6). Thermal areas tend to be on isolated land masses e.g. New Zealand, Fiji, Japan, and Iceland although some are on very large land masses e.g. USA and USSR. The potential for geographic separation is therefore evident.

If these areas are separated from one another then the scope for divergent pathways of evolution exists. Mixed source clusters could represent convergent evolution in distant areas while the distinct clusters may represent discrete lines of descent. Unfortunately the published literature has little to offer on this subject with regard to Thermus. The fact that Thermus diverged from other organisms in the distant past has come from several sources (see chapter 6) but little has been published about relationships within the genus. The only material (Stahl et al., 1985) does show a divergence between T.aquaticus and "T.thermophilus", which supports the suggestion above, based on the evidence of 5s rRNA sequence analysis.

3.4.10 The Taxonomy of the Genus.

Having analysed the data for a large number of Thermus isolates from a variety of geographical sources some conclusion about the taxonomy of the genus should be made. The major difficulty is in where the species division lies. If all clustered and unclustered 73% phena are used then there would be 25 species which could be readily distinguished, but 25 could not be considered to be a reasonable number of species. At values of 60-62% S_{SM} small numbers of clusters are produced but there are inadequate tests to distinguish the different groups. If species are to be defined then they must be distinguishable by a reasonable number of tests so that future identifications can be made, and this level of similarity is too low to allow this.

A suitable compromise would be to take the 65% phena as the species, which would give 8 phena. This level results in T.aquaticus Yt-1, T.ruber and "T.thermophilus" HB8 occupying 3 of the phena and they would therefore give their names to them. Table 3.17 shows the phena, where current species (valid and invalid) are, and the diagnostic tests which may be used in their identification.

Table 3.17

65% Phena for the all Isolate Classification.

TEST	PHENA							
	A	B	C	D	E	F	G	H
NUMBER IN CLUSTER	61	9	2	11	12	20	14	2
TELLURITE REDUCTION	-	V	-	+	+	+	+	+
NITRATE PRODUCTION	V	+	+	V	-	V	-	-
TSI REDDENING	-	-	-	-	+	-	V	-
PALMITATE CFS 3 h	-	-	-	+	-	-	V	-
ß-GALACTOSIDASE	V	+	V	+	-	+	V	+
CASEIN HYDROLYSIS	V	+	-	+	+	-	-	-
GROWTH ON DEXTRIN	-	V	+	+	-	+	-	-

EXISTING NAMED STRAINS A="T.Lacteus", "T.rubens"
E= T.aquaticus YT-1
F= "T.thermophilus" HB8
H= T.ruber, T.flavus

A full table of percentage positives for these phena is given in appendix 3 as in a number of cases the other tests show the same pattern of results as those shown in the Table 3.17.

It would be interesting to see if isolates from other thermal areas, sparsely represented in this scheme, fall in to the categories produced as this would be the true test of the stability of the classification.

Further work to determine G+C mol% values is required before formal species descriptions could be made and an asessment of the usefulness of the classification, as outlined above, needs to be made to validate the scheme.

3.5 Evaluation of Tests Used.

In identification weighting of the tests is perfectly acceptable, and it is therefore useful to know which tests are the more error prone so that the results from this kind of test can be given less weighting than more reproducible tests.

3.5.1 Error Calculation.

Error for each test was calculated from the replicates of all the classifications presented here. Calculations were made by treating each test in a manner identical to that used when finding the error involved between replicates in any particular classification, so that instead of finding the error between a pair of organisms over a number of tests the error for a test was calculated for a number of organisms.

3.5.2 Test Error Results.

Table 3.18 shows the tests arranged in order of error with values of variance and probability of error (calculated according to Sneath & Johnson, 1972)).

TABLE 3.18

Tests Aranged in Order of Reproducibility.

VARIANCE	ERROR (%)	TESTS
0	0	Tellurite reduction; DNAase production around growth; colony size; colony pitting; colony colour; β-glucosidase from cells at 6h, from supernatant at 18 h; palmitate degradation by supernatant at 1 h and 3h; growth at 45°C and 75°C; hide powder azure hydrolysis at 48h; fibrin hydrolysis at 24 and 72 h; arbutin degradation; growth in 2% NaCl, growth on acetamide.
0.014	1.4	Triple sugar iron agar reddening; growth in 2.5% peptone; β-glucosidase from cells at 18 h; β-galactosidase production; fibrin hydrolysis at 48 h, casein hydrolysis, α-galactosidase production.
0.027	2.7	DNAase under growth; viability after 28 d at 25°C; growth at pH 6; methylene blue reduction; growth at 80°C, hide powder azure hydrolysis after 24 h; Tween 80 hydrolysis; growth on mannose and sucrose.

CONTINUED

0.04	4.2	Nitrite production after 1 d; culture pH > 7.6; laurate degraded by supernatant in 1 h; elastin hydrolysed after 24 and 48 h; aesculin degradation; growth on raffinose and sorbitol.
0.054	5.7	Nitrate production after 7d; growth on acetate and salicin; cycloserine MIC.
0.068	7.3	Growth on glutamate and proline.
0.081	8.9	Growth on mannitol and cellobiose; chloramphenicol MIC.
0.095	10.6	Growth on maltose, succinate, pyruvate, glucose and trehalose; phosphomycin and kanamycin MICs; elastin hydrolysis after 72 h.
0.108	12.3	Growth on lactose.
0.122	14.2	Growth on dextrin and galactose; growth at pH 9.5.
0.135	16	Spectinomycin MIC.

It can be seen from this table that most of tests with the worst errors (p>5%) were carbon source utilisation and antibiotic inhibition tests. This would imply that in any future identifications which are made these tests should be given a low weight. A majority of the tests fell below an error of 5%, considered to be the norm (Sneath & Johnson, 1972). Only one test, with an error of 16% was beyond the value of 15% usually taken as the worst permissable. The inclusion of one such error prone test should not have influenced the classifications greatly.

The fact that carbon source utilisation comprised the worst set of tests may imply that optimum conditions for growth on single carbon sources were not used, and indeed are not known. Degryse *et al.* (1978) attributed poor growth to the lack of buffering capacity of the medium. However this explanation cannot be correct, as under continuous culture conditions (Cometta *et al.*, 1982a; this thesis chapter 4) where the pH is strictly controlled, poor growth has also been obtained on single carbon sources. Cometta *et al.* (1982a) went to great lengths to determine what growth factors may be required by *Thermus* but were not able to find any. This question is still at present open.

Sneath & Johnson (1972) considered that it was better to use many less reliable tests than fewer very reproducible tests and this recommendation has been followed here with the inclusion of those tests with errors >5% (although test reliability was not known until after the classifications had been carried out).

3.6 Summary.

Numerical classifications of *Thermus* isolates from New Zealand, Iceland, Yellowstone National Park, and New Mexico have shown that there is a considerable reflection of geography in the isolate sources of the clusters obtained i.e. isolates from individual thermal areas tended to cluster together. In the classification where all isolates were included few of the clusters contained isolates from mixed geographic sources. It was also shown, by analysis of variance, that the pH means and the temperature means of the isolate sources of the clusters differed from the null hypothesis (which states that the pH means and the temperature means of the clusters are equal). Pools from different areas tended to have different ranges of pH and temperature values and so the geographical and pool type influences could not be equivocally separated.

From the phenograms the genus could be divided up into 8 clusters at the 65% S_{SM} level and it was suggested that this should represent the species composition of the genus. Lower S_{SM} values did not allow for clear distinction between the fewer clusters formed and higher values resulted in unreasonable numbers of clusters being formed. Of the 8 suggested species 3 would have T.aquaticus, T.ruber and "T.thermophilus" as type strains. The 8 clusters formed did not reflect the pigmentation of the organisms in them, nor was there a separation of the high and low temperature optimum strains.

CHAPTER 4

Phenotypic Stability in Nature and in Continuous Culture.

4.1 Introduction.

Cometta *et al.* (1982b), from the results of a numerical classification of Icelandic *Thermus* isolates and from observations of colour changes in *Thermus* isolates growing in continuous culture, supported the idea that these organisms are genetically unstable and that this feature helps them to exist in an extreme and changing environment. They suggested that organisms lost genetic information as growth temperature decreased, so that whereas many different types of protein may be found at the upper temperatures for growth, only a few types might be found at points further down the thermal gradient. A similar argument was applied to the response to varying temperature in continuous culture.

As part of the general investigation into the ecology of *Thermus* it was decided to examine this aspect, i.e. the phenotypic stability, of the biology of the genus. Two methods were to be used; firstly an examination of phenotypic stability in hot pools, for if *Thermus* are genetically unstable then presumably this should be manifest by a general instability of phenotypes detected in nature. Secondly a continuous culture run was to be carried out, in which a well characterised organism could be examined for phenotypic changes when environmental parameters were changed.

4.2 Materials and Methods.

4.2.1 Assessment of Phenotypic Stability in Nature.

To investigate the change of phenotypes with time, the following sampling programme was carried out. Samples were taken over a period of approximately 14 months at 2-3 monthly intervals. Five pools were selected because of their ease of access and the knowledge that they were inhabited by Thermus. Samples of hot water and sediment were taken from them on each occasion. Temperature was recorded in situ and pH read at room temperature on returning to the laboratory. Isolation procedure was as previously described (Section 3.2.1) and isolates representing the most common colony morphologies taken. Isolates were freeze-dried and stored until required for use. In addition one pool, Rt350, had samples taken from it at five different locations on one day. A total of 12 isolates were recovered from these samples and included in the analysis.

This collection of isolates was then subjected to the full numerical classification as previously described (chapter 3) and similarly analysed. A random selection of 16 isolates was included to act as indicators of error, and reference isolates from the New Zealand and Icelandic Isolate classifications, also included.

A full list of the isolates used is given in Table 4.1 along with the date of collection (where appropriate), the pH and temperature of the isolate source and the country of origin. Isolates are arranged in the order of the clusters produced and full data are shown in Appendix 2B.

Table 4.2 shows the temperatures and pHs of the sites sampled on the days of sampling and Table 4.3 shows the pHs and temperatures of the sites sampled at Rt 350.

Table 4.1

Isolates Used to Determine Phenotype Stability.

CLUSTER	ISOLATE	DATE COLLECTED	TEMPERATURE (°C)	pH	ORIGIN
A	Rt6 A6	4/84	85	9.9	NZ
A	Rt8 A1	2/83	80	6.6	NZ
A	Rt6 A9	11/84	58	9.3	NZ
A	Rt366 A1	12/83	76	NA	NZ
A	Oh A2	NA	80	7	NZ
A	Rt6 A7	NA	NA	NA	NZ
A	Rt366 A2	11/83	76	NA	NZ
A	Rt6 A8	7/84	73	9.5	NZ
A	Rt4 A11	11/84	36	6.9	NZ
A	Rt358 A1	NA	82	7.5	NZ
B	Rt366 A4	11/84	89	7	NZ
B	ZV5 A1	7/84	80	9.4	ICELAND
B	Rt8 A3	4/84	84	9.6	NZ
B	Rt8 A5	11/84	77	9	NZ
B	Rt8 A4	7/84	80	9.5	NZ
B	Rt4 A4	4/84	44	8.1	NZ
B	Rt6 A5	4/84	85	9.9	NZ
B	Rt358 A7	1/85	83	7.6	NZ
B	*T.aquaticus*	NA	72	NA	USA
B	Rt6 A2	8/83	48.4	9	NZ
C	Rt366 A3	4/84	90	7.3	NZ
C	Rt4 A3	4/84	44	8.1	NZ
C	Rt350 A11	1/85	93	7.3	NZ
D	Rt4 A6	7/84	42	8.7	NZ
D	Rt4 A2	12/83	41	NA	NZ
D	Rt4 A12	11/84	36	6.9	NZ
D	OK2 A1	NA	57.1	7.6	NZ
D	Rt350 A7	1/85	77	7.7	NZ
D	Rt4 A5	7/84	42	8.7	NZ
D	Rt350 A1	1/85	65	8	NZ
D	Rt350 A2	1/85	65	8	NZ
-	ZV4D A2	7/84	61	8.3	ICELAND
-	Rt4 A10	11/84	36	6.9	NZ
-	Rt6 A4	4/84	85	9.5	NZ

CONTINUED

E	Rt35	NA	80	6.6	NZ
E	Rt4 A9	11/84	36	6.9	NZ
E	Wai2 A1	NA	54	3.9	NZ
E	Rt358 A4	4/84	82	7.3	NZ
F	Rt358 A5	4/84	82	7.3	NZ
F	Rt4 A1	NA	87	8.1	NZ
F	Rt8 A2	4/84	84	9.6	NZ
F	Rt41 A	NA	87	7.2	NZ
F	T351	NA	96.4	8.7	NZ
F	Rt358 A6	1/85	83	7.6	NZ
F	Rt350 A6	1/85	77	7.7	NZ
F	Rt6 A1	NA	70	9	NZ
F	ZV1D A1	7/84	69	9.2	ICELAND
F	Rt4 A8	7/84	42	8.7	NZ
G	Rt358 A3	4/84	82	7.3	NZ
G	ZD2 A1	7/84	80	8.9	ICELAND
G	Rt358 A2	11/83	81	7.0	NZ
-	_T.thermophilus_	NA	80	6.3	JAPAN
-	Rt350 A5	1/85	38	8.1	NZ
H	Rt350 A3	1/85	38	8.1	NZ
H	Rt350 A4	1/85	38	8.1	NZ
H	Rt350 A9	1/85	96	7.2	NZ
H	Rt350 A13	1/85	93	7.3	NZ
-	Rt350 A8	1/85	77	7.7	NZ
-	Rt350 A10	1/85	93	7.3	NZ

NA: Not available (dates missing for reference strains only)

Table 4.2

Temperature and pH Values of Pools Sampled.

POOL NUMBER	SAMPLING DATE		pH	TEMPERATURE (°C)
Rt 358	APRIL	'84	7.3	82
	JULY	'84	7.3	79.5
	NOVEMBER	'84	7.0	80.5
	JANUARY	'85	7.6	83
Rt 366	DECEMBER	'83	NR	76
	APRIL	'84	7.3	90
	NOVEMBER	'84	7.0	88.5
	JANUARY	'85	6.8	90
Rt 6	DECEMBER	'83	NR	73
	APRIL	'84	9.9	85
	JULY	'84	9.5	73
	NOVEMBER	'84	9.3	58.3
	JANUARY	'85	9.3	60
Rt 8	DECEMBER	'83	NR	82
	APRIL	'84	9.6	84
	JULY	'84	9.5	80
	NOVEMBER	'84	9.0	77.3
	JANUARY	'85	9.0	77
Rt 4	DECEMBER	'83	NR	41
	APRIL	'84	8.1	44
	JULY	'84	8.7	42
	NOVEMBER	'84	6.9	36
	JANUARY	'85	7.3	43

These results are for samples taken as part of the time course, other previously collected samples are not included. Rt358, Rt366 and Rt4 are specific sites at hot pools whereas Rt6 and Rt8 are runoff channels and the same point sampled would vary in temperature depending on conditions.

Table 4.3

Temperature and pH of Sites Sampled at Rt350.

SITE	pH	TEMPERATURE(°C)	ISOLATE NUMBERS
A	8.0	65	A1, A2
B	8.1	38	A3, A4, A5
C	7.7	77	A6, A7, A8
D	7.2	96	A9
E	7.3	93	A10, A11, A13

4.2.2 Continuous Culture.

The strain of Thermus selected for study was Wai 8, a member of cluster A in the New Zealand isolate classification (see Table 3.1). This organism was chosen in the first instance because of its ability to grow with pyruvate as sole carbon source.

The continuous culture equipment used consisted of the following (all obtained form LH Engineering, Stoke Poges, Buckinghamshire, UK); an LH 502 magnetic drive stirrer, an LH 503 temperature control unit and an LH 505 pH control unit. To supply the medium a Watson Marlow 502S variable speed peristaltic pump was used.

Control of pH was achieved using the pH controller unit connected to an Ingold model 465 steam sterilisable pH electrode. To alter the pH of the medium either sterile 1 M NaOH or 0.5 M H_2SO_4 was pumped by the pH control unit into the continuous culture vessel. Temperature control was via an immersion heater connected to the controller unit. Due to the temperature of operation the cooling finger was not used. At temperatures over 70°C the limit of operation of the existing equipment had been reached and to permit growth at 75°C approximately half of the surface area of the culture vessel was insulated with cotton wool held in place with aluminium foil and masking tape. A condenser was used to stop evaporation.

Compressed air (NZIG) was passed through a sterile filter and into the culture vessel to supply oxygen. Unfortunately a dissolved oxygen controller or meter was not available and so only an estimate of the flow rate, approximately 50 cm^3 $min.^{-1}$, can be given. However later experiments with a different Thermus strain, Wai33 A1, indicated that the flow rate used would not be oxygen limiting at the dilution rates used. Also optical densities obtained from batch culture, on the same medium used in continuous culture with Wai 8, gave the same value as that obtained in continuous culture, indicating again that oxygen was not limiting.

The medium used initially was CMD mineral salts supplemented with 2 g l^{-1} Na pyruvate and 5 g l^{-1} ammonium sulphate (Pask-Hughes & Williams, 1975). This was later abandoned and a medium of CMD with the omission of trypticase peptone but with 1 gl^{-1} yeast extract used instead. To investigate if yeast extract was the limiting nutrient a batch culture experiment was set up as follows.

Volumes of 50 ml of medium were added to 250 ml conical flasks. The medium was CMD with no trypticase peptone, and containing either 0.02%, 0.05%, 0.08%, 0.1% or 0.2% (w/v) yeast extract. Flasks were inoculated with 0.1 ml of a 24 h culture of Wai 8 and incubated using an orbital incubator operating at 150 rpm and 70°C for 48 h. After incubation 3 ml aliquots were removed and their optical densities determined at 650 nm using a Shimadzu UV 200 spectrophotometer. All yeast extract concentration yield determinations were done in triplicate.

Batches of medium were prepared in 20 litre lots and autoclaved for 2 h at a pressure of 15 psi.

The inoculum was prepared by streaking single colonies of Wai 8 on CMD plates twice, to ensure that the inoculum was axenic, followed by growth in CMD broth for 24 h at 70°C. Inoculum was aseptically added to the vessel using a sterile 1 ml syringe to inject through the septum provided. The volume of inoculum used was 1 ml.

It was planned to run the continuous culture for 8 weeks with the changes to the environmental parameters shown in Table 4.4.

Changes every week meant that there were 22 generations between parameter changes (at $\mu=0.13$). During the course of the run compressed air was supplied at the approximate rate indicated above and the impeller speed was at 300 rpm to restrict wall growth.

Table 4.4

Environmental Changes Carried out in Continuous Culture.

SAMPLE (WEEK)	pH	TEMPERATURE(°C)
1	7.6	70
2	7.6	75
3	7.6	70
4	7.6	65
5	7.6	70
6	6.5	70
7	7.6	70
8	8.5	70

After each week a sample was taken and streaked onto two CMD plates. From these plates were randomly selected 10 colonies, which were then streaked onto CMD plates to ensure purity and for storage. Once a collection of 80 isolates had been obtained they were tested according to the diagnostic tests listed in Table 3.5. If they proved to be very similar, one organism from each sample was to be submitted to the full set of taxonomic tests, and if they behaved differently then they were all to be subjected to the full range of tests. All of the taxonomic tests and analyses were as described in chapter 3, the methods for which are in Chapter 2.

4.3 Results.

4.3.1 Numerical Classification.

4.3.1.1 Test Error.

Test error was estimated at 4.4% from the replicates used for error within the classification, and at 8.5% for isolates duplicated from previous classifications. These values are similar to those previously obtained.

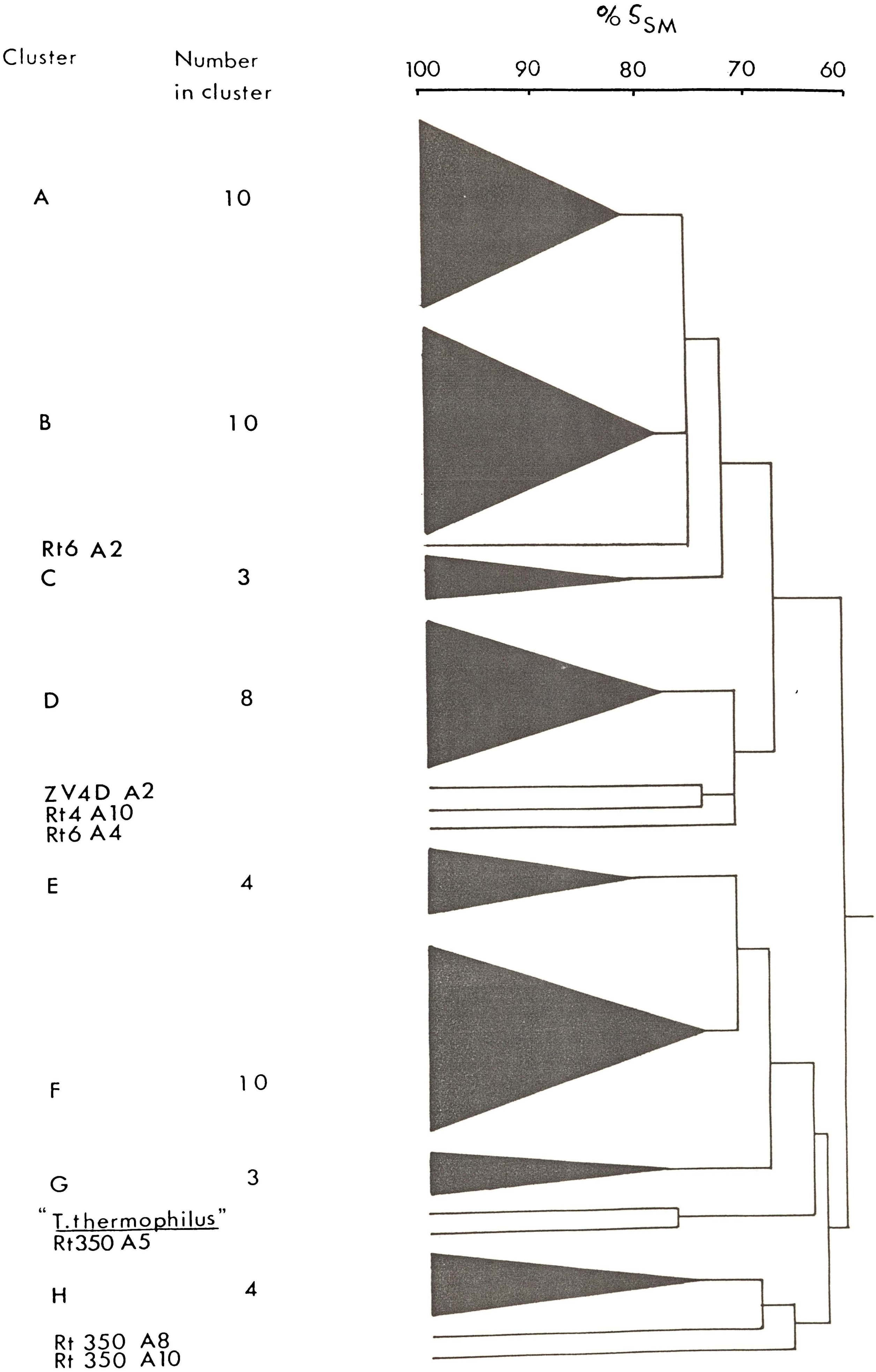
4.3.1.2 Clustering of the Isolates.

From the S_{SM}/UPGMA analysis 8 clusters, which each contained 3 or more isolates, were formed at the 73-75% similarity level (Figures 4.1A and 4.1B show a simplified and a detailed phenogram respectively). These clusters have been labelled A to H. In addition there were 2 two-member clusters and 3 unclustered organisms.

The S_J/UPGMA phenogram (Figure 4.2) showed some differences from Figure 4.1. In the S_J analysis, compared with the S_{SM} analysis, cluster A lost two loosely clustered members to a larger cluster, much expanded over the S_{SM} analysis. This expanded cluster consisted of cluster G with the addition of the two members of cluster A, 2 from cluster D, 1 from cluster F and 3 unclustered organisms. Cluster B lost one member to cluster D. Clusters E and F merged, although they still remained as distinct subclusters. Cluster C split into a pair and an unclustered organism, while cluster G was expanded as reported above. Cluster H remained intact. The overall structure was therefore substantially unchanged except for the expansion of cluster G. As with the previous classifications it can be concluded that some of the organisms in the S_{SM}/UPGMA analysis clustered due to a large component of negative matches. In the context of this work negative matches are probably as important as positive ones and so further discussion is based on the S_{SM}/UPGMA analysis.

Single linkage analysis represented by minimum spanning trees (data not shown) showed groupings like those produced by UPGMA analysis for the similarity coefficients used.

Figure 4.1A. Simplified phenogram of time course isolates based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient.


% S_SM
100
90
80
70
60
Cluster
Number in cluster
A
10
B
10
Rt6 A2
C
3
D
8
ZV4D A2
Rt4 A10
Rt6 A4
E
4
F
10
G
3
"T.thermophilus"
Rt350 A5
H
4
Rt 350 A8
Rt 350 A10

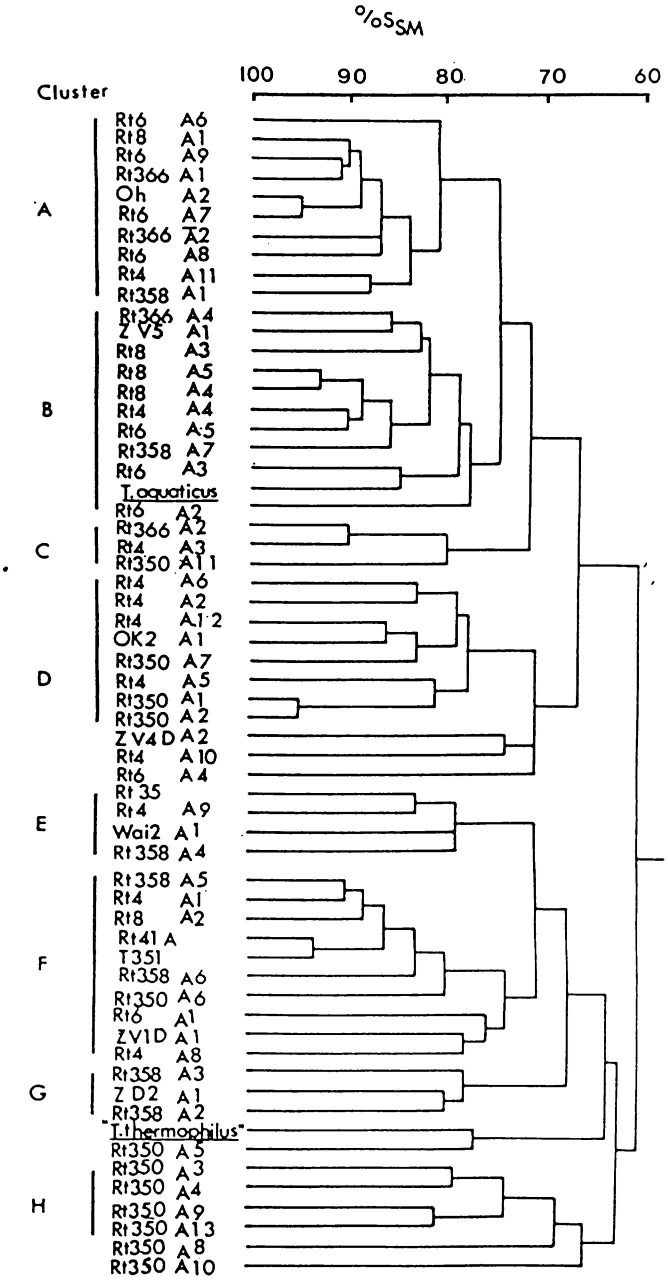


Figure 4.1B. Detailed phenogram of time course isolates based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient.

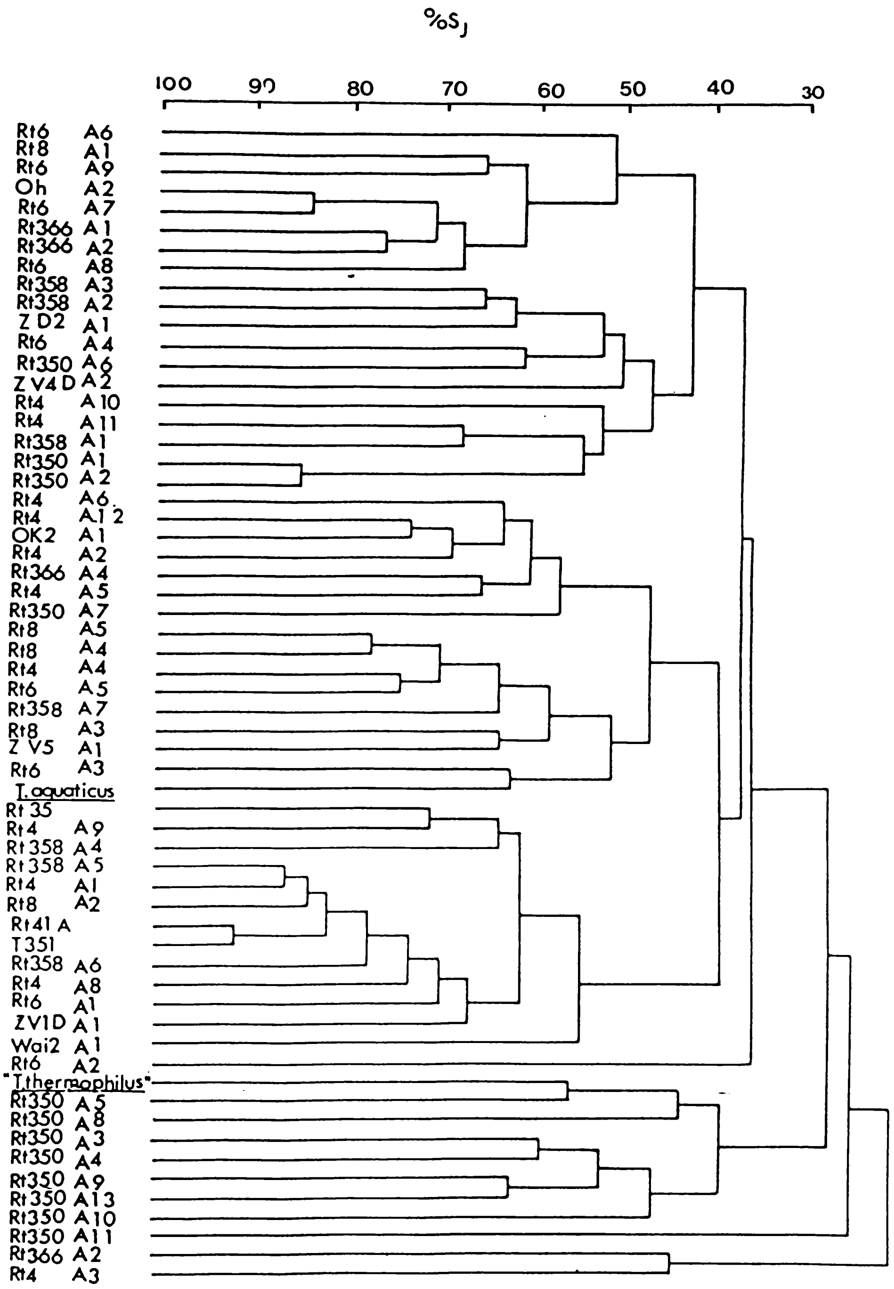


Figure 4.2. Detailed phenogram of time course isolates based on the UPGMA analysis of a similarity matrix derived from the S_J coefficient.

As a comparison with previous classifications the following section will deal with how the reference strains clustered. References to previous classifications will be made to New Zealand (NZ) clusters A to G and Icelandic clusters (ICE) A to D.

Details of clustering of the reference strains are given in Table 4.5.

Table 4.5

Clustering of Reference Strains.

REFERENCE STRAIN	PREVIOUS CLUSTER	TIME-COURSE CLUSTER
Rt41A	NZ A	F
T351	NZ A	F
Rt6 A7 (B 2)	NZ C	A
Oh A2	NZ C	A
Rt4 A2 (Rt4)	NZ E	E
OK2 A1 (OK2)	NZ E	E
Rt35	NZ G	E
Wai2 A1 (WN2)	NZ G	E
Rt358 A1	NZ D	A
Rt6 A1	NZ B	F
T.aquaticus Yt-1	NZ F	B
ZV5 A1	ICE A	B
ZV4D A2	ICE B	Unclustered
ZD2 A1	ICE D	G
ZV1D A1	ICE C	F

Abbreviations Used : NZ= New Zealand Classification Cluster
ICE= Icelandic Classification Cluster

From the New Zealand isolate classification members of NZ A clustered together as did those from NZ C, NZ E and NZ G. The single members fron NZ D, NZ B and NZ F loosely associated with larger clusters. Therefore these reference strains showed consistency with previous results, and indicated the level at which clusters should be formed, which was around the 73-75% S_{SM} level.

Icelandic reference strains did not form unclustered isolates but clustered with the other organisms. The representative from ICE A clustered along with T.aquaticus Yt-1, which it did before. The isolates from ICE B and ICE D clustered with one other organism and two other organisms respectively, and these had previously been in clusters of mixed geographic source. The representative from ICE C clustered in one of the main groups with NZ B and NZ A representatives. This association was however at a fairly low similarity level. Although this organism did not cluster with a New Zealand isolate before, it should be borne in mind that when placed alongside more similar organisms the weighting of the other organisms will tend to reduce the level at which clustering with less similar organisms will occur, as a consequence of the use of the UPGMA clustering algorithm.

References to clusters used from here will be to those produced in this classification of time course isolates. The organisms collected from Rt350 on the same day showed a variety of phenotypes. Two of them, Rt350 A1 and A2, clustered with cluster D, while 4 others, Rt350 A3,4,9 and 13, formed a separate cluster (H), and 1 clustered with cluster C. The others remained unclustered or were members of small clusters. Any one pool then contains a number of phenotypes. The pool in question had a relatively low pH range between sampling sites of 7.3 to 8.1 but a much larger temperature range of 38 to 93°C. It was therefore not unexpected that different phenotypes could be isolated from the same pool.

Table 4.2 lists the temperatures of the sites where the samples were taken. The pools under study, except for Rt6, remained fairly constant over the sampling period, with only minor fluctuations in pH and temperature. However the isolates recovered from these sources showed considerable phenotypic variability.

Cluster A contained 4 Rt6 isolates which were recovered over the period of approximately a year. This pattern was also evident in cluster D, which had 4 Rt4 isolates, and cluster B, which had 3 Rt8 isolates. This indicates that isolates of a similar phenotype could be isolated from the same pool over a long period of time. The final classification of chapter 3 also showed that T.aquaticus YT-1 clustered with isolates from the same source pool which had been isolated at least 16 years later.

All but 2 Rt358 isolates clustered in groups E, F and G, which were three clusters grouped together, showing a loose association between these isolates. All 4 isolates from Rt366 were confined to clusters A, B and C which again indicates the confinement of phenotypes from one source to a restricted set of clusters. Apart from 1 unclustered isolate, all Rt6 isolates clustered in A and B and this was also true for isolates from Rt8. Rt4 isolates were distributed throughout the clusters with a concentration in cluster D as reported above.

There is therefore some evidence that phenotypes do remain constant over time and that isolates from particular pools tend to cluster within a limited range of clusters. A pool with widely varying temperature values and less widely varying pH values yielded a number of phenotypes, which may further indicate that certain phenotypes are dominant under different environmental conditions.

4.3.2 Stability of Phenotype in Continuous Culture.

In attempting to grow Wai 8 on medium with pyruvate as sole carbon source very poor growth was obtained. With aeration and agitation (150 rpm) at a temperature of 70°C and a pH of 7.7 in batch culture there was little growth. When the medium supply was turned on to give a flow rate of 65 ml h^{-1} (equivalent to μ=0.13 with a 510 ml vessel volume) washout appeared to be occurring but in fact the organism was being maintained at a very low optical density. This behaviour is similar to that of "T.thermophilus" in continuous culture (Sonnleitner et al., 1982); and can be partially explained by the high maintenance requirements shown by McKay et al. (1982).

Batch experiments with Wai 8 in CMD with no trypticase peptone showed a linear relationship between the concentration of yeast extract added and optical density up to 0.1 % (w/v) (Figure 4.3). At a concentration of, or above, 0.1 % (w/v) some other component(s) must become growth limiting or are supplied at low levels in the yeast extract. Therefore it was decided to change the medium used to CMD mineral salts plus 0.1% (w/v) yeast extract, which throughout the rest of the experiment proved to maintain satisfactory growth.

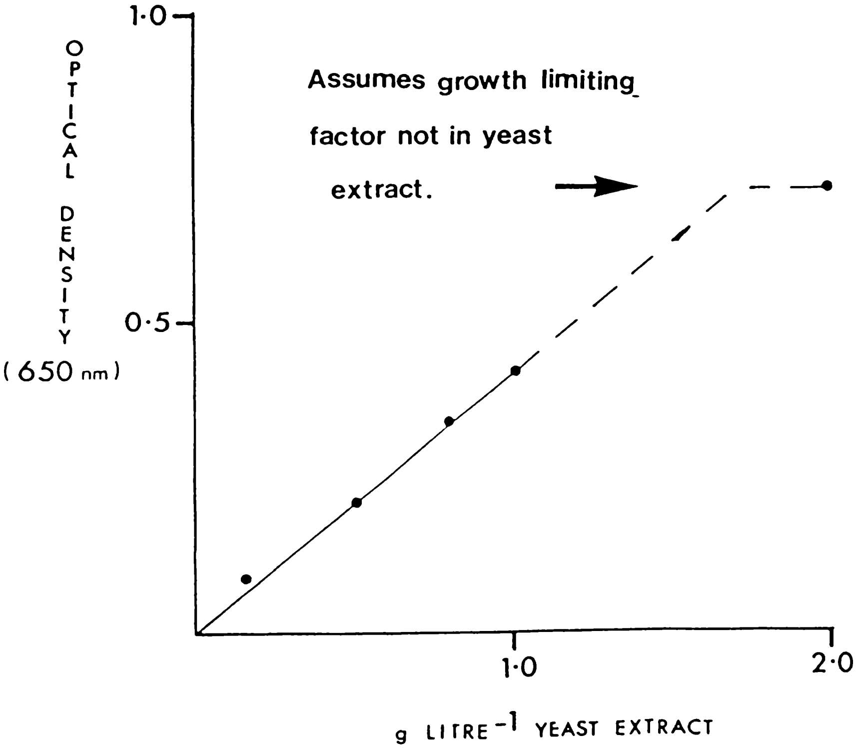


Figure 4.3. Growth yield, assessed by optical density, of Wai33 A1 for increasing concentrations of yeast extract.

The increased impeller speed did not entirely prevent wall growth. This occurred on all metal surfaces (except for the immersion heater), around the inside of the vessel at the medium surface and in the "backwater" created by the outlet weir.

Throughout the run two colony types were frequently encountered, one slightly larger (2 mm), granular with an erose margin and the other non granular with an entire margin. It was usual in sampling therefore to plate out five of each colony type for further taxonomic analysis.

Table 3.6 indicates that Wai 8 (a member of cluster NZ A) should be positive for potassium tellurite reduction, elastin degradation after 24 h, growth at 45°C, laurate degradation after 1 h incubation and fibrin degradation after 24 h incubation. The 80 isolates were passed through these tests as well as the methylene blue reduction test to assess the similarity of the isolates before deciding how many should proceed through the full range of taxonomic tests.

All of the isolates were positive for potassium tellurite reduction, elastin degradation and fibrin degradation. Only 1 isolate gave a negative result for laurate degradation, 4 isolates did not grow well at 45°C and 13 were negative for methylene blue reduction, and most of these (9) were due to the fact that they had not grown well. It was therefore decided that on the basis of these results there had been no dramatic change in phenotypic character over the time of the experiment with the changing conditions used. There was certainly no evidence to show that the two colony types isolated were different for any of the characters tested. One colony from each sample was randomly selected and included in a full run of taxonomic tests routinely used in chapter 3. This was to give a much more sensitve assessment of any phenotypic change which may have taken place.

When the 8 isolates were evaluated against the standard range of tests it was found that of the 65 tests used all 8 isolates showed identical results for 49 of them and of the varying 16 tests 7 of them varied in only one isolate (not the same one). Table 4.6 shows the similarity values between the isolates using the S_{SM} similarity coefficient.

The similarity values are high, and would certainly place these isolates in the same cluster if incorporated into a more extensive phenogram.

Table 4.6

Similarity Matrix of SSM Values of Continuous Culture Isolates.

ISOLATE	1	2	3	4	5	6	7
2	89						
3	88	88					
4	94	92	86				
5	95	89	86	94			
6	95	92	83	92	94		
7	89	88	83	94	95	91	
8	94	92	88	91	94	91	86

ISOLATE

Of the errors which occurred, 78% were carbon source tests and this has been shown to be the least reliable set of tests when error was assessed for the other classifications. The variation which has apppeared can therefore be attributed to error inherant in the tests, rather than to any change in phenotypic charcters.

With the diagnostic tests the organisms behaved identically. It can be concluded that if phenotypic variation did occur then it was at such a level as to be undetected by the tests used.

4.4 Discussion.

Escherichia coli is the most widely studied micro-organism, and the stability of the E.coli population in the gut is one of the aspects of this organism which has come under study. There are some similarities between the human gut and hot springs; they are both flow through systems in which a variety of factors can apply different selective pressures to the organisms present. They are both mixed culture environments and both are environments where adhesion is important for the continued existence of organisms. Obviously there are many differences in the types of parameters found between these two situations, and one of these which may be of great importance is the rate of inoculation of organisms external to the environments under question. With the gut inoculation would be frequent, derived from similar sources and in high numbers (frequency of inoculation is proportional to the amount of canteen food eaten by individuals (Mason & Richardson, 1981)). However in hot springs cross inoculation would usually occur by splashing or aerosol formation and the likelihood of cross inoculation from one pool to another would seem likely to decrease with distance. Pools from geographically distant regions would therefore probably very rarely, if ever, contaminate one another.

Despite these differences some analogies can be drawn between the two environments so that some of the features of the hot pool environment can be paralleled with the ecology of E.coli in the human gut.

In a review of the ecology of E.coli in the human gut (Mason & Richardson, 1981) and of the population genetics of E.coli (Hartl & Dykhuizen, 1984) two types of gut occupant could be described, residents, which could be isolated from volunteers for periods in excess of a year and transients, which ocurred in only single stool samples. Individuals were reported to vary considerably in the rate of turnover of organisms and variation of strains between individuals was also marked. Some resident strains of E.coli have been reported to be displaced by environmental changes but were generally found to be extremely persistent. The large intestine may harbour several strains of E.coli but is, in general, dominated by a single type. However minority populations also occur which, under changing conditions, can rapidly fill new niches.

Is there any evidence for resident and transient strains in hot pools? The data for the numerical classification part of this chapter suggests that there is. Isolates from Rt4, Rt6 and Rt8, which were isolated over a year clustered together as reported in the results section. These organisms may therefore be considered as "resident" strains for those particular pools. Data for Rt358 and Rt366 showed similar but less convincing results. Individual isolates from Rt4 can also be found among many other clusters and these may represent either "transient" strains or perhaps minority strains which were only infrequently isolated. Examples of "transient" strains can be found for all of the other pools. The hot pool environment appears to allow more majority strains, as shown by the results for Rt350 which had the majority of isolates confined to two clusters. Variation between phenotypes of strains isolated from different hot pools has been suggested by the results of previous classifications but the kinds of pool chosen for this study (selected to give a good chance of isolating Thermus strains) probably were not different enough to give a good indication of this here.

Cometta et al. (1982b) suggested that the number of different types of protein (and hence variation in phenotype) would become fewer in a continuous culture run where temperatures were changed. The results from the continuous culture part of this work showed no such trend, as assessed by phenotypic changes, there being minor fluctuations in phenotype (explicable by test error) for the duration of the run. Under the conditions used for continuous cultivation no white strains were recovered, a phenomenon which Cometta et al. (1982b) had reported. However the work of Cometta et al. (1982b) was performed using a defined medium and a sole carbon source, whereas this work used yeast extract to supply carbon and thus the medium was undefined. Optical densities obtained using yeast extract were much better than those obtained by Cometta et al. (1982b) on sole carbon sources and this indicates that conditions suitable for good growth of Thermus on single carbon sources have not yet been found and that this may be a stressful situation for the organism. Growth on single carbon sources was found to be the most error prone type of test used in the classifications (see section 3.5).

Overall there appears to be little evidence from this work to support the idea of genetic instability in Thermus. The rapid turnover of phenotypes of E.coli has been explained by variability, not instability and with Thermus this would also seem to be the case. The size of the genome of E.coli can vary by 23% (Hartl & Dykhuizen, 1984) and there are also differences in the degree of homology between DNA isolated from different E.coli strains. This variation in the genetic information contributes towards the explanation of phenotypic variability and it would be very interesting to examine the genome size of Thermus DNA, to see what variation there is, and also to examine the homology between Thermus strains by DNA/DNA hybridization. If certain isolates of Thermus do have large numbers of gene copies then their DNA would be larger than those obtained from further down temperature in the thermal gradient (as these have less gene copies according to Cometta et al. (1982b)). So far no function has been ascribed to Thermus plasmids (see section 1.2.3), although many characters have been tested for and so at the moment plasmid loss can be ruled out as a source of genetic variation.

Chapter 5: Thermus filiformis nov. sp.

5.1 Introduction.

This section of work arose from the chance isolation of a thermophilic filamentous organism which attracted attention due to its aesthetic qualities when viewed with the microscope. Initially the morphology suggested that the organism was from a previously undescribed genus but as the unusual properties of *Thermus* became clearer (see section 1.2.5) the relationship of this isolate to that genus also became apparent and enabled the identification of the isolate as a *Thermus* sp. to be made with confidence.

Filamentous thermophilic micro-organisms have been observed in hot pools in previous studies (e.g. Brock, 1967; Mann & Schlichting, 1967). Initially it was thought that these organisms were cyanobacteria, but they were too thin to determine microscopically whether they contained chlorophyll or not. Subsequently Brock (1968) suggested that they did not contain chlorophyll A and that they were related to the flexibacteria. Pierson & Castenholz (1974) later found that most of the organisms Brock had observed in algal mats were probably photosynthetic gliding bacteria, and named them *Chloroflexus*. More recently a similar organism with restricted geographical distribution has been isolated and named *Heliothrix* (Pierson *et al.*, 1985).

A non-photosynthetic but morphologically similar organism has been described being given the name *"Thermothrix"* (Caldwell *et al.*, 1976). This organism is a facultative anaerobe capable of autotrophic and heterotrophic growth. Two thermophilic *Bacillus* isolates have also been described which produce filamentous forms; *B.thermocatenulatus* (Golovacheva *et al*, 1975) and *B.flavothermus*

(Lauwers & Heinen, 1983).

This chapter decribes the work which was carried out to determine the taxonomic position of the isolate with respect to the organisms listed above, *Herpetosiphon* (Holt & Lewin, 1968), a mesophile with a similar morphology, and *Thermus*. Preliminary results from this chapter have been published (Hudson *et al.*, 1984).

5.2 Materials and Methods.

5.2.1 Strains Used.

The thermophiles *T.aquaticus* Yt-1 (ATCC 25104), *"T.thermophilus"* HB8 (ATCC 27634), *T.ruber* (DSM 1279), *"Thermothrix thioparus"* (ATCC 29244) and *Chloroflexus aurantiacus* (ATCC 29366) were used for comparative analysis as was the mesophile *Herpetosiphon geysericola* (ATCC 23076). The isolate under study was designated Wai33 A1.

5.2.2 Media and Culture Conditions.

Thermus strains and Wai33 A1 were routinely maintained on CMD[1] agar or broth (see section 2.2.3). Also used were the media Cy and Hp741m (Reichenbach & Golecki, 1975). *"Tx.thioparus"* was maintained on nitrate broth (Difco), *H.geysericola* on K101 medium (ATCC medium 284) and *C.aurantiacus* on ATCC[2] medium 920. *Thermus* and *"Thermothrix"* strains were incubated at 70°C, *H.geysericola* at 30°C and *C.aurantiacus* at 50°C in unshaken cultures unless otherwise stated. Other test media used were TYEG[4] (Zeikus *et al.*, 1979), medium for the growth of *Thiobacillus thioparus* (DSM medium 36[3]) and medium for the autotrophic growth of *"Tx.thioparus"* (Caldwell *et al.*, 1976). Attempts to show oxidative or fermentative catabolism of carbohydrates were made by the methods of Hugh & Leifson (1953) and Degryse *et al.* (1978). Other media such as those used for determining growth on

1) CMD: Castenholtz medium D 2) ATCC: American Type Culture Collection, Rockville, Md, USA 3) DSM: Deutsche Sammlung Von Mikroorganismen, Göttingen, FRG 4) TYEG: Trypticase Yeast Extract Glucose

single carbon sources are given in chapter 2.

To determine colony characteristics the organisms were streaked onto the appropriate medium and incubated at their optimum growth temperature for 24 h.

Attempts were made to grow Wai33 A1 on TYEG, CMD, and nitrate broth under strictly anaerobic conditions using 0.001% (w/v) resazurin as an indicator of reduction and sodium sulphide to a concentration of 0.05% (w/v) to achieve reduction. The inoculum used was 0.1 ml of a 24 h broth culture and incubation was at 70°C. Non-reduced anaerobic/microaerophilic conditions were achieved by filling 125 ml medical flat bottles completely with CMD before inoculation. These bottles were incubated both in the dark and under illumination so that any light requirement or bacteriochlorophyll production could be detected.

To test for autotrophic growth the media for growth of "Tx.thioparus" and Thiobacillus thioparus referenced above were used. With the "Tx.thioparus" medium sulphite, thiosulphate or sulphur were used as an electron donor.

An attempt was made to grow Wai33 A1 in continuous culture to determine temperature and pH optima. The experimental details were as those detailed in chapter 4.

5.2.3 Morphology and Ultrastructure.

Phase microscopy was used to obtain photomicrographs with either an Olympus BH2 microscope equipped with an Olympus PM-10 AF automatic camera system or a Reichert Polyvar microscope. Interference micrographs were obtained using the Reichert Polyvar microscope.

For electron microscopy Wai33 A1 was grown for 48h and washed by centrifugation. Cells were fixed in 2.5% (w/v) gluteraldehyde for 1 h followed by 1% (w/v) osmium tetroxide in veronal acetate buffer for 1 h. Dehydration was achieved using a graded ethanol series and specimens embedded in Spurr resin (Spurr, 1969). Sections were cut with a Riechert ultramicrotome using a glass knife. Sections were stained for 5 minutes in 2% (w/v) uranyl acetate followed by 8 minutes in 0.4% (w/v) lead aspartate. Negatively stained specimens were prepared by staining the cells for 1 min. with 1% (w/v) uranyl acetate on butvar coated copper grids and then blotting dry. A Phillips EM400 electron microscope was used to produce the electron micrographs.

5.2.4 Temperature and pH Optimum Determinations.

As the normal growth type of Wai33 A1 in liquid culture was as a clump it was necessary to devise a method to disperse growth so that optical densities could be measured. To achieve this, ten 4mm glass beads were added per 500ml conical flask along with 50 ml of medium and flasks were placed in an orbital incubator operating at 150 rpm. The inoculum used was 1 ml of a 24 h vortex mixed broth culture and growth was measured by absorbance at 650 nm with a Shimadzu UV 200 spectrophotometer. The temperature optimum was determined in CMD buffered at pH 7.4 with 0.02 M Na_2HPO_4-NaH_2PO_4 buffer, while the pH optimum was determined at 70°C and with varying pH values for the buffer.

5.2.5 G+C mol% Determination.

DNA from Wai33 A1 and T.aquaticus was extracted by the method of Marmur (1961). Wai33 A1 cells were grown in a litre of medium for 48 h and harvested by centrifugation. Cells were washed twice with 0.1 M EDTA/0.15 M NaCl and resuspended in 25 ml of water containing 10 mg of lysozyme and incubated for 45 min. at 37°C. Subsequently 2 ml of 25%

(w/v) sodium dodecyl sulphate was added and incubation continued for a further 3 h until cell lysis was complete as determined by phase microscopy. The suspension was then heated for 10 min. at 60°C. An equal volume of 24:1 (v/v) chloroform:isoamyl alcohol was added and the flask shaken for 30 min. at room temperature. The liquid was transferred to centrifuge tubes and centrifuged at 10,000 g for 5 min. to give 3 layers, the nucleic acid being in the top aqueous layer. This layer was removed, placed in a beaker and twice the volume of cold ethanol layered on. DNA precipitating at the liquid interface was removed by collecting with a glass rod, and redissolved in 1.0 SSC (which contains 0.15 M NaCl and 0.015 M trisodium citrate, pH 8.0).

Purification was by the method of Gibson & Ogden (1979). The DNA solution was treated with 50μg/ml ribonuclease (Sigma) and incubated at 37°C for 30 min. Then 10μg/ml of protease (from Streptomyces griseus, Sigma) was added and incubation continued for another hour. A 2 ml sample of the DNA solution was then applied to a column (1.5 cm x 50 cm) of sepharose 4B (Pharmacia) which had been pre-equilibrated at room temperature. The flow rate through the column of 1.0 SSC was approximately 0.1 ml/min. and the eluent was continuously monitored at 260 nm. Once the ascending limb of the elution curve had been reached samples from this section were pooled to make up a final volume of 3 ml for melting. In general the amount of DNA eluted (as measured by absorbance) was lower than that implied by the reference for the method.

The G+C mol% value was determined by the thermal denaturation method (de Ley, 1970). DNA solution (DNA dissolved in 1.0 SSC buffer) was placed in a quartz cuvette which was in turn placed in a Pye-Unicam SP8-400 spectrophotometer equipped with a flow through cuvette holder. Circulated through the cuvette holder was polyethylene glycol pumped from a circulating water bath heater. The temperature was measured directly using a microthermometer probe connected to a Digi-sense digital thermometer. As the temperature was gradually increased the optical density was recorded at 260 nm. The value for G+C mol% was determined according to the equation in Owen & Hill, (1979) using the melting temperature for T.aquaticus DNA, similarly isolated and determined, as standard.

5.2.6 Diagnostic Tests.

All diagnostic tests were performed according to the methods given in chapter 2.

5.2.7 Aminopeptidase and KOH Lysis Tests.

The aminopeptidase test was carried out according to the method of Cerny (1976), and the KOH lysis test according to the method of Gregersen (1978). Both methods are described in detail in chapter 6.

5.2.8 Respiratory Quinone Analysis.

This was carried out by Dr.M.D.Collins of the Food Research Institute, Reading, England and results supplied as a personal communication.

Wai33 A1 was grown up in 5 litres of CMD broth for 48 h, harvested by centrifugation and washed twice in 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.6). Cells were freeze-dried to provide approximately 600 mg dry weight for analysis.

5.2.9 Pigment Extraction.

Carotenoid extraction was carried out according to the method of Cohen-Bazire et al. (1957) using methanol as the solvent. Growth was removed from plates for organisms growing on the described media at their optimum growth temperatures, and mixed with 3 ml of methanol.

Pigments were allowed to extract for 30 min. Tubes were then centrifuged, the supernatant removed and pigment absorbtion spectra scanned using a Pye-Unicam SP8-400 spectrophotometer.

5.2.10 Antibiotic Inhibiton.

Aliquots (0.1 ml) of vortex mixed 24 h cultures of *T.aquaticus*, Wai33 A1 and *"T.thermophilus"* were used as inocula and added to 50 ml of CMD in 250 ml Erlenmyer flasks which each contained ten 4mm glass beads. 0.1 ml of the appropriate concentration of filter-sterilised antibiotic solution (0.22 μm pore-size filter) was then added to each flask. Incubation was for 24 h in an orbital incubator operating at 150 rpm. Optical densities were measured at 650 nm with a Spectronic 20 (Bausch & Lomb Inc.). Controls with no antibiotic additions were run simultaneously and all tests were run in duplicate.

For each test the mean of the turbidities of the two test cultures was compared with the mean turbidities of the appropriate controls and the result expressed as percentage inhibition with respect to the control.

5.3 Results

5.3.1 Isolation.

The strain, designated Wai33 A1, was isolated by streaking hot pool water from "The Clamshell" (Wai 33), a hot pool in the Waimangu thermal valley near Rotorua, New Zealand, directly onto CMD agar. The plate was incubated for 24 h and a colony of unusual consistency re-streaked until a pure culture was obtained. The site from which the water sample was taken had a pH of 7.3 and a temperature of 81.6°C.

5.3.2 Cultural Characteristics.

Colony characters are shown in Table 5.1 for organisms grown on agar plates.

Table 5.1

Comparisons of Colony Charactersistics of Wai33A1 With Test Organisms.

ORGANISM	MEDIUM	SIZE(mm)	COLOUR	ELEVATION	CONSISTENCY	FORM
Wai33 A1	CMD	1-2	yellow	convex	mucoid	circular
T.aquaticus	CMD	<1	yellow	convex	butyrous	circular
"*T.thermpohilus*"	CMD	1	yellow	convex	butyrous	circular
H.geysericola	K101	4	pink	concave	mucoid	circular
"*Tx.thioparus*"	CMD	2	white	flat	mucoid	lobate

No growth was recorded with media under reduced conditions or with media designed to support autotrophic growth. There was an onset of growth under non-reduced anaerobic/microaerophilic conditions but growth was poor. Analysis of pigments from cells grown in the dark and light showed there to be no difference between the absorbtion spectra produced. It was concluded that no bacteriochlorophyll was produced, that the organism could not grow autotrophically under the conditions tested and that it was an obligate aerobe.

Evidence for the aerobic nature of Wai33 A1 was shown by the results from the continuous culture experiments. It was found that optical densities dropped when dilution rates were increased but that this was not due to the μ max of the organism being exceeded as the optical density stopped falling and resumed a steady state. When the flow rate of compressed air was increased the optical density rose to its former level. Typical data are shown in Table 5.2.

Table 5.2

Typical Data Showing the Oxygen Requirement of Wai33 A1 in Continuous Culture.

DURATION AT PUMP SETTING (h)	PUMP SETTING (ml/h)	OPTICAL DENSITY (650 nm)	AIR FLOW
18	45.5	0.31	LOW
1	97.5	0.29	LOW
7	97.5	0.22	LOW
18	97.5	0.34	HIGH
2.5	162.5	0.31	HIGH
4	162.5	0.29	HIGH

NB Vessel volume=510 ml, temperature 70°C, pH=7.6, impeller speed=300 rpm.

The results showed a decrease in optical density after increasing the flow rate from 45.5 ml h^{-1} to 97.5 ml h^{-1}, until the air flow rate was increased and the optical density returned to its former value. On increasing the pump from 97.5 ml h^{-1} to 162.5 ml h^{-1} the optical density again dropped. The data show that oxygen supply was the limiting factor under some conditions indicating that the organism is an aerobe. It was due to this fact that the continuous culture system could not be used for further investigations as no oxygen control unit was available for use.

By either method of Degreyse et al. (1978) or Hugh & Leifson (1953), neither Wai33 A1 or T.aquaticus showed strong acid production under conditions designed to demonstrate oxidative and fermentative metabolism of carbohydrates. Fructose and mannose led to a yellowing of the medium in the absence of organism, while lactose and sucrose remained blue/green when uninoculated and incubated. Only the latter 2 could therefore be compared under the test conditions. For both Wai33 A1 and T.aquaticus growth was only obtained on the surface of the media (or at the oil/medium interface). T.aquaticus only showed weak acid production on sucrose. Degryse et al. (1978) were also not able to demonstrate oxidative or fermentative metabolism in Thermus isolates.

5.3.3 Temperature and pH Optima.

Figure 5.1 shows the growth curves for Wai33 A1 at various temperatures, and Figure 5.2 shows growth curves for the organism grown at various pH values. Optimum temperature was found to be 73°C (with a doubling time of 1.98 h.) and pH of 7.4 (with a doubling time of 1.8 h) determined at the logarithmic phase of growth. From the curves it is apparent that the logarithmic phase of growth is short and that growth is linear for the majority of the growth phase of the curve. What causes this linear growth is not known but it may be due to the filamentous nature of the organism as filaments of several hundred μm are still present under these conditions.

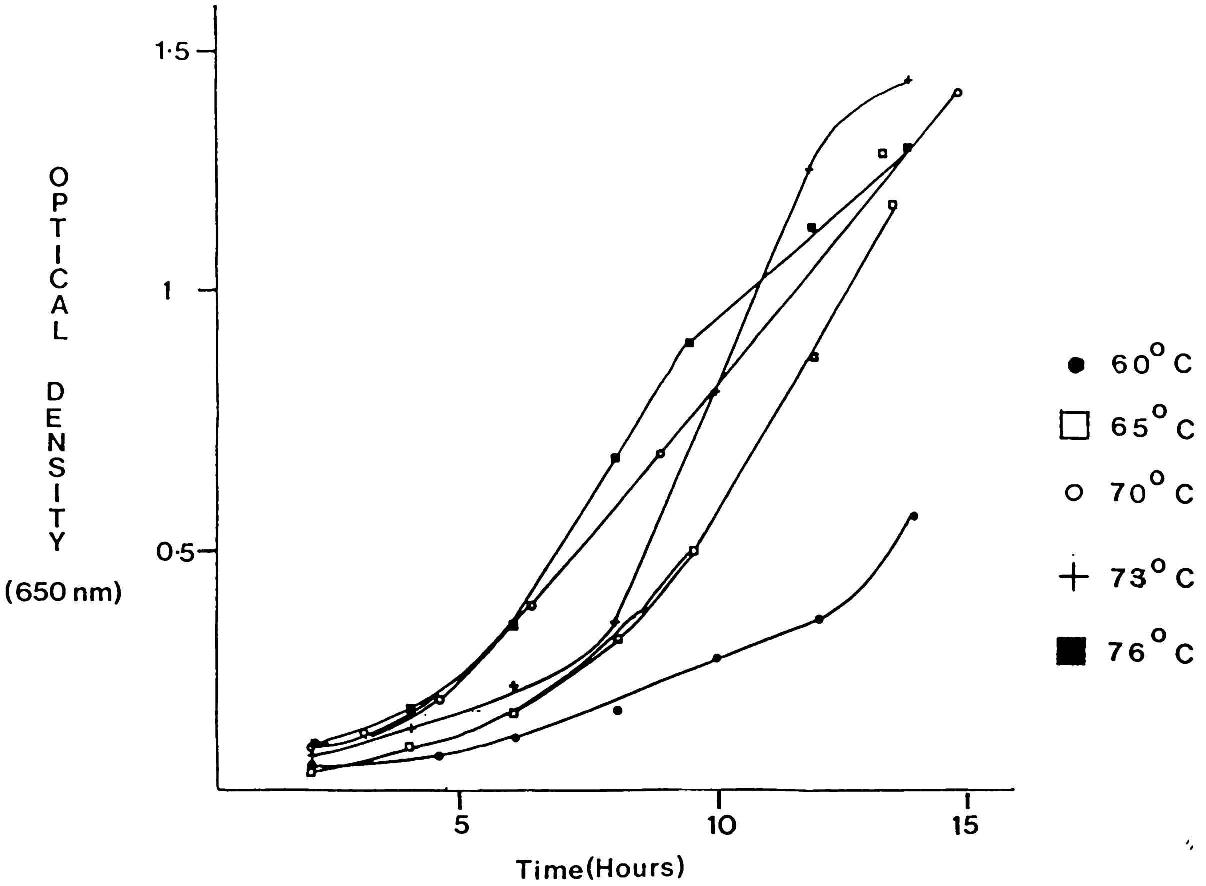


Figure 5.1. Growth Curves for Wai33 A1 Incubated at Various Temperatures at pH 7.4.

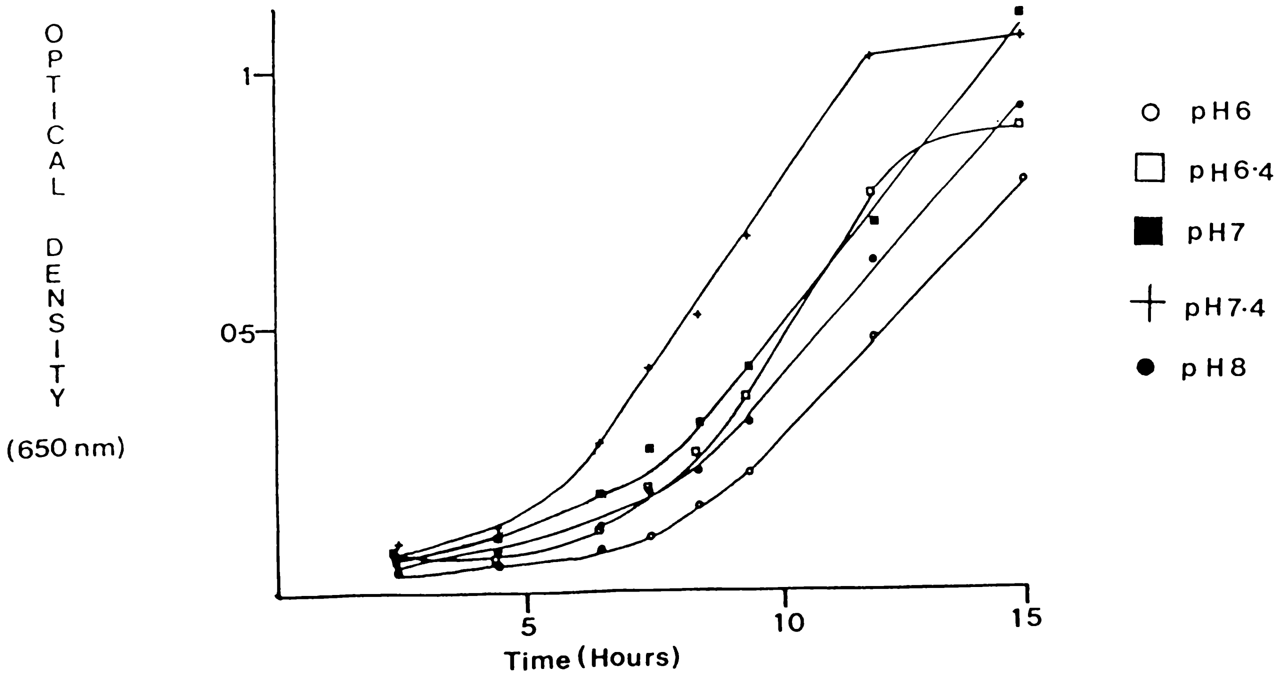


Figure 5.2. Growth Curves for Wai33 A1 Incubated at Various pHs at 70°C.

5.3.4 Morphology and Ultrastructure.

Wai33 A1 showed a very characteristic morphology. In liquid and solid culture the organism formed long filaments consisting of chains of cells (Figure 5.3). Filaments exhibited a characteristic coiling (Figure 5.4) and also the formation of bullae along their length (Figure 5.5). At filament termini two structures were observed; necridia (Figure 5.6) and spherical swellings (Figure 5.7) which could occasionally be seen free in the mounting fluid.

Cell width varied from 0.8 to 1.7 µm within the same filament. The modal cell length was 4-5 µm and ranged up to 11 µm. Overall filament length was indefinite, usually being up to several hundred µm. Unlike the "bulbs" of Herpetosiphon (Reichenbach & Golecki, 1975) the bullae of Wai33 A1 were not refractile under phase microscopy. Necridia were of the same dimensions as single cells.

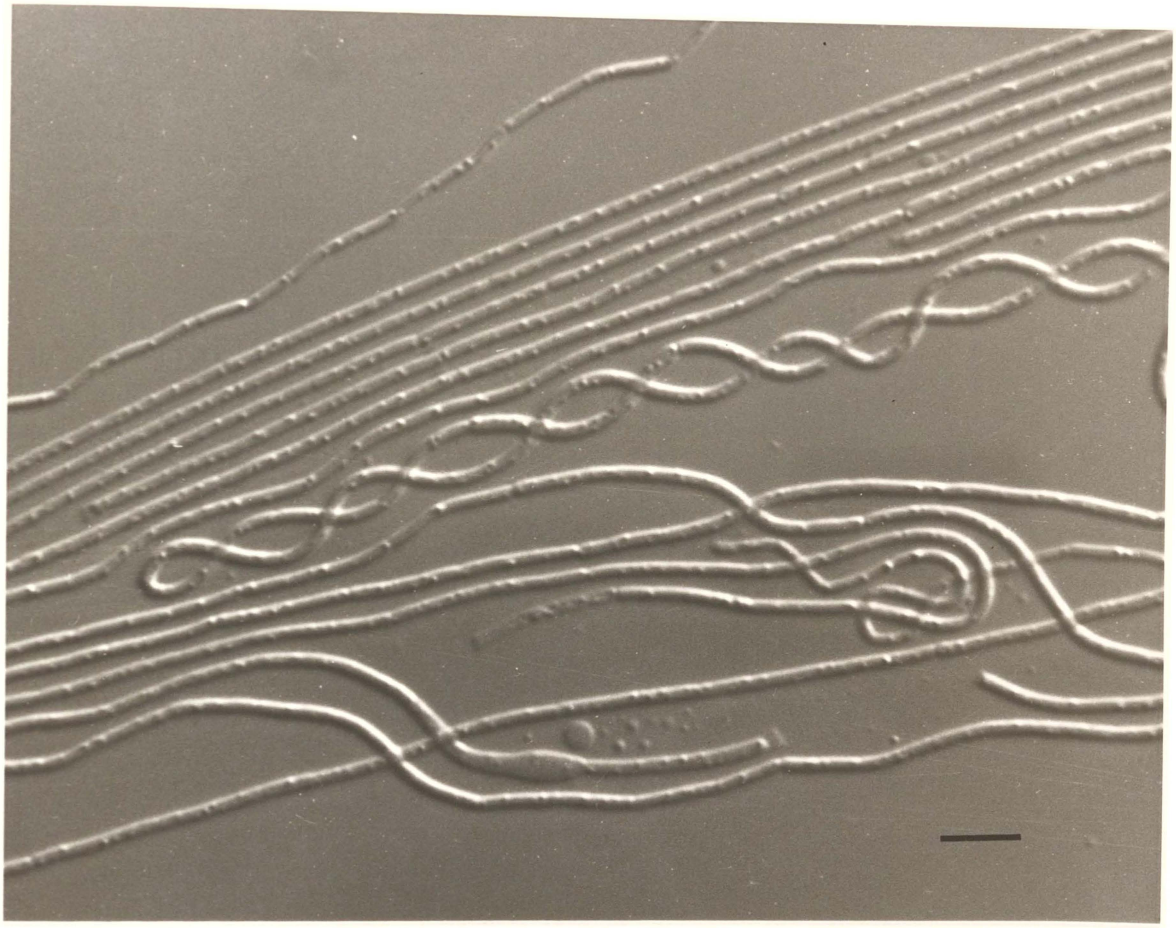
Electron microscopy of thin sections showed the wall to be of the typical multi-layered Gram-negative appearance (Figures 5.8 and 5.9). Adjacent to the cytoplasmic membrane was a thin electron dense layer, outside of which was a thick layer which formed a corrugated appearance in some of the micrographs. External to the corrugated layer there were two more layers which ran uninterrupted along the filaments, apparently playing no part in septum formation (Figure 5.8).

Thin sections of bullae showed there to be no differentiation between these structures and normal cells (Figure 5.10), although the cell wall appeared to be thinner than in other parts of the filament, but of the same overall structure.

When examined using negatively stained preparations small spheres could occasionally be seen between the cells (Figure 5.11). The origin and function of these spheres is not known.

Figure 5.3. Filament Consisting of a Chain of Cells. Bar=4μm.

Figure 5.4. Characteristic Coiling of Wai33 A1 Filaments. Bar=4μm.



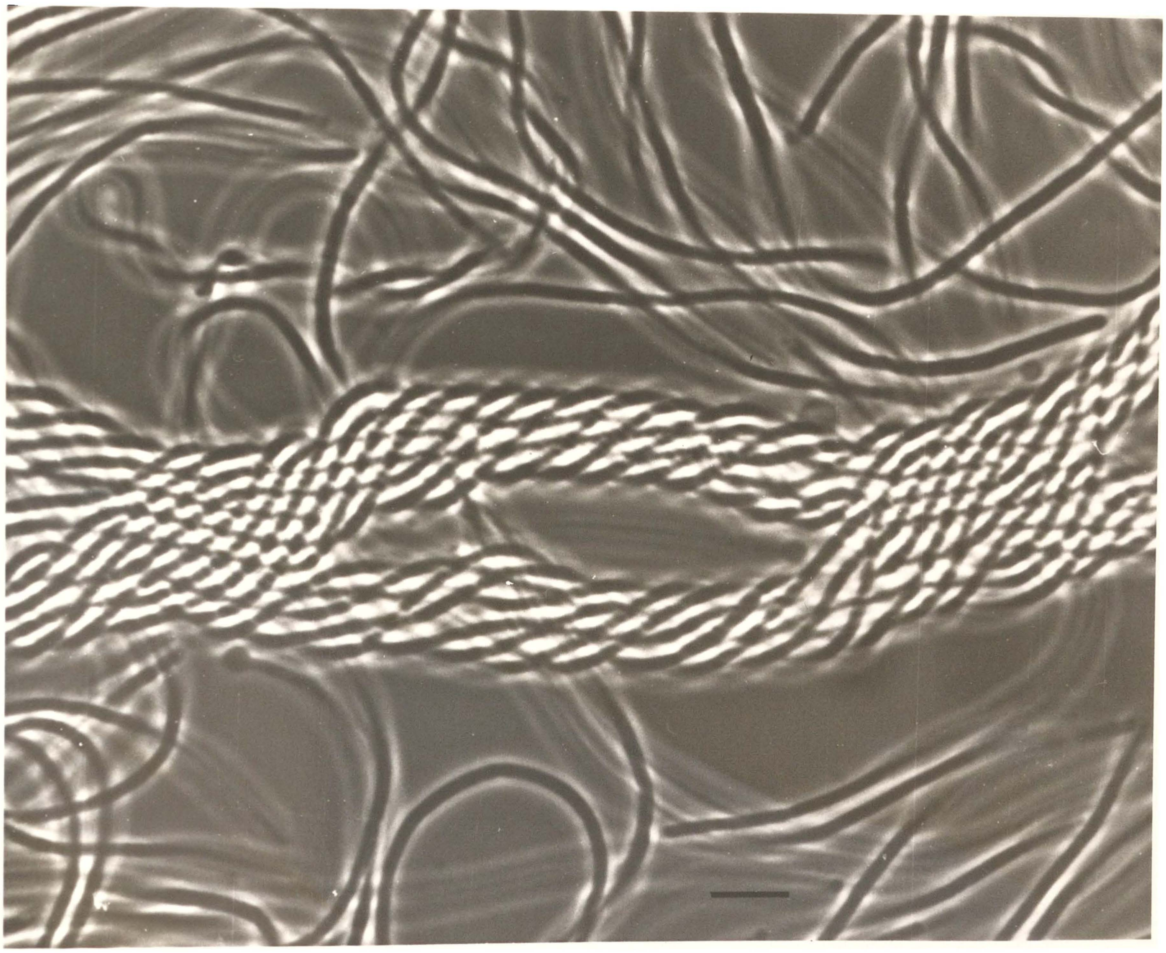
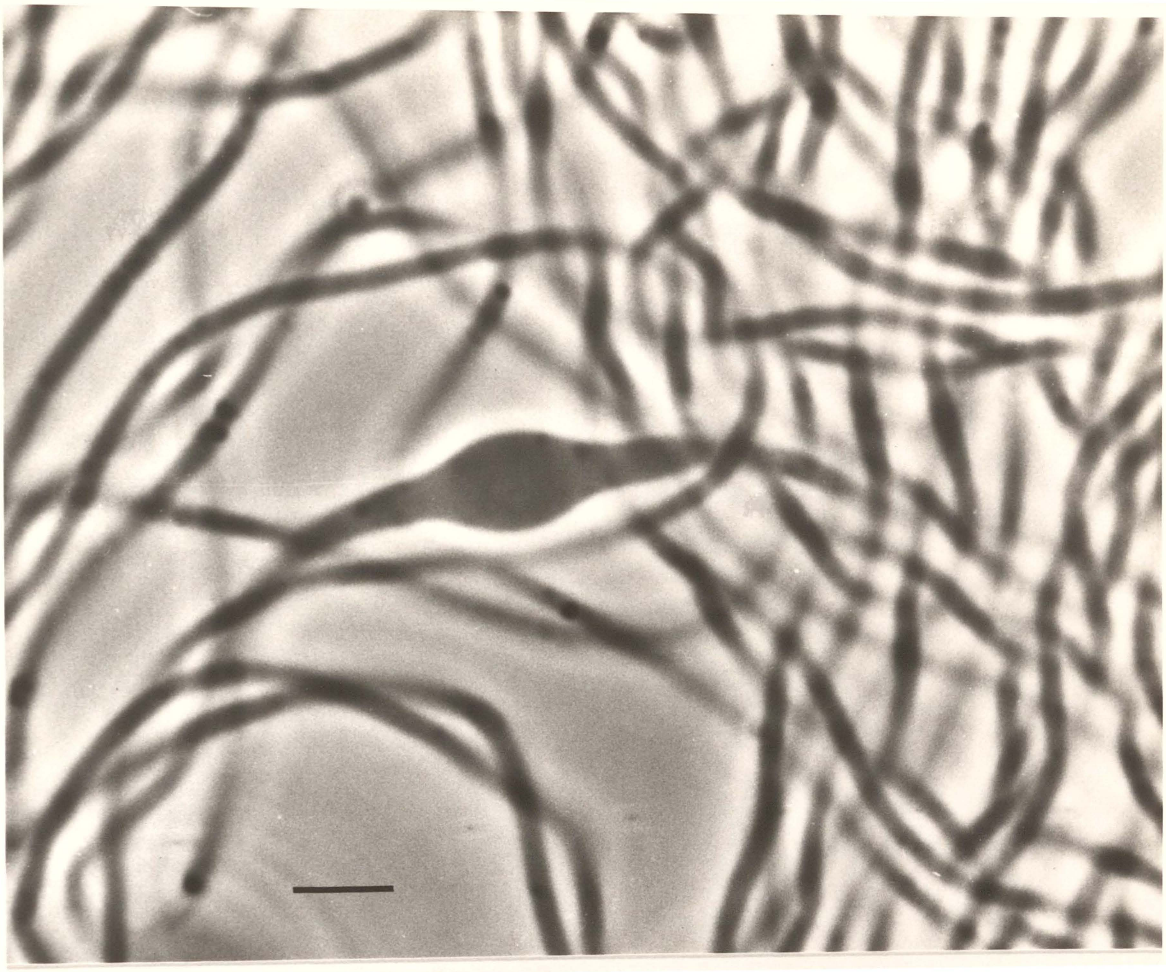


Figure 5.5. Mid Filament Swelling in Wai33 A1 Filament. Bar=4μm.

Figure 5.6. Necridium (Arrow) at Filament Terminus. Bar=4μm.



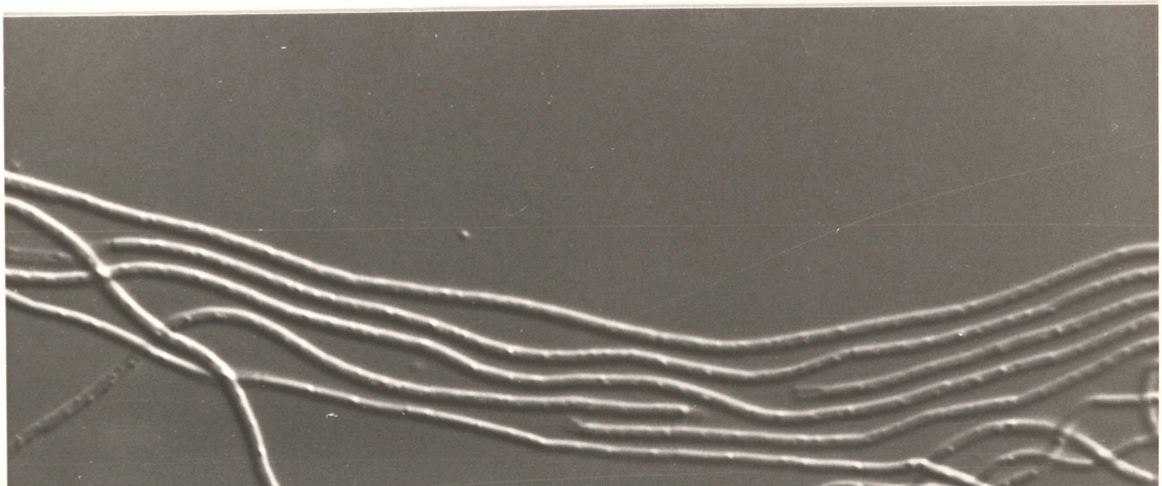
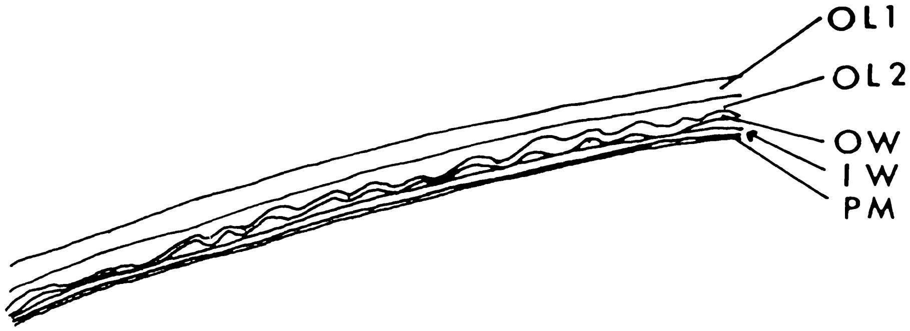
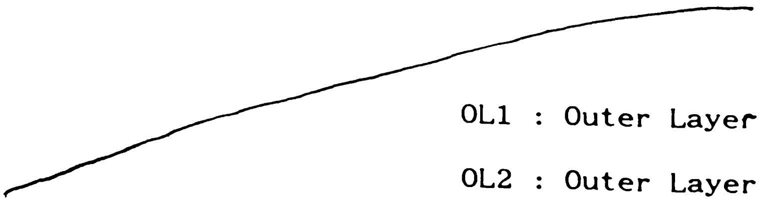


Figure 5.7. Swelling at Filament Terminus (Arrow). Bar=4μm.

Figure 5.8. Thin Section Electron Micrograph of Wai33 A1 Filament Showing Septum. Bar=0.2μm. (Bottom left)

Figure 5.9. Thin Section Electron Micrograph of Wai33 A1 Filament Showing Cell Wall Structure. Interpretation is Shown Below. Bar= 0.2μm. (Bottom right)





OL1 : Outer Layer 1

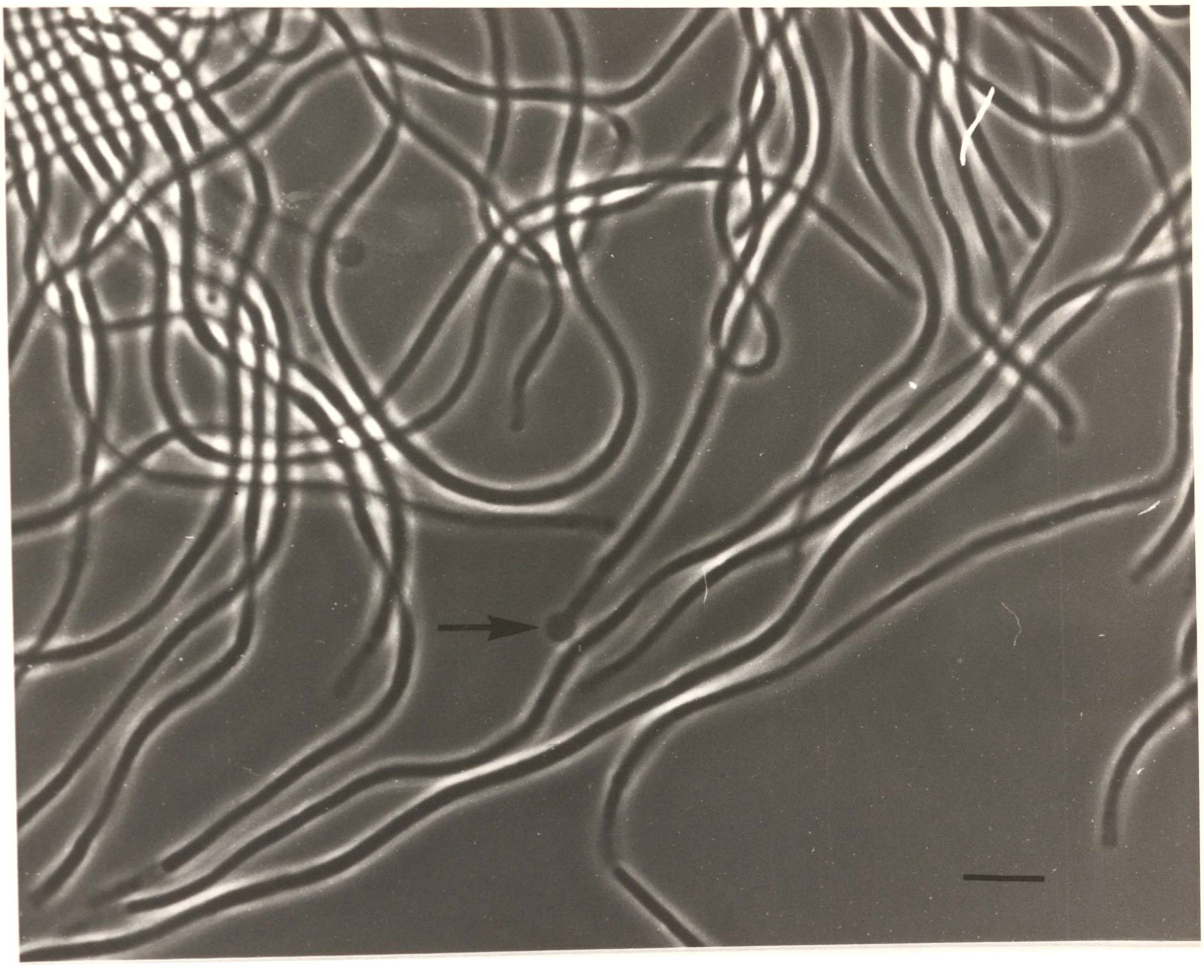
OL2 : Outer Layer 2

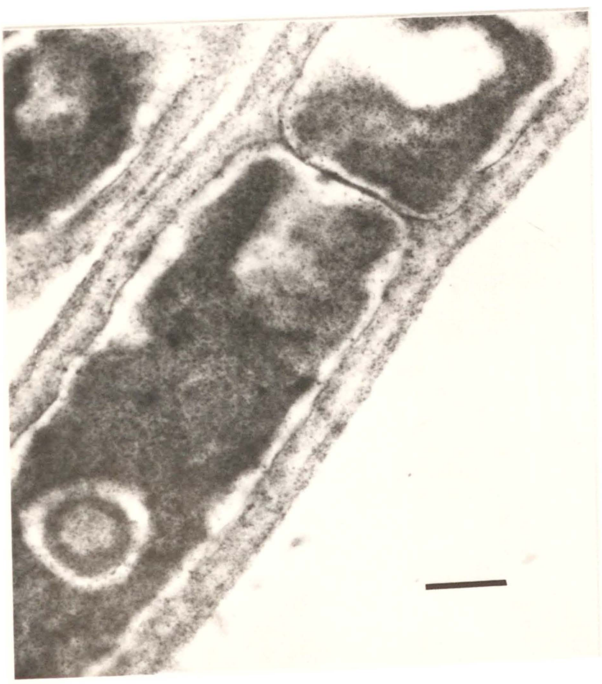
OW : Outer Dense Layer

MW : Middle Light Zone

IW : Inner Dense Layer

PM : Plasma Membrane





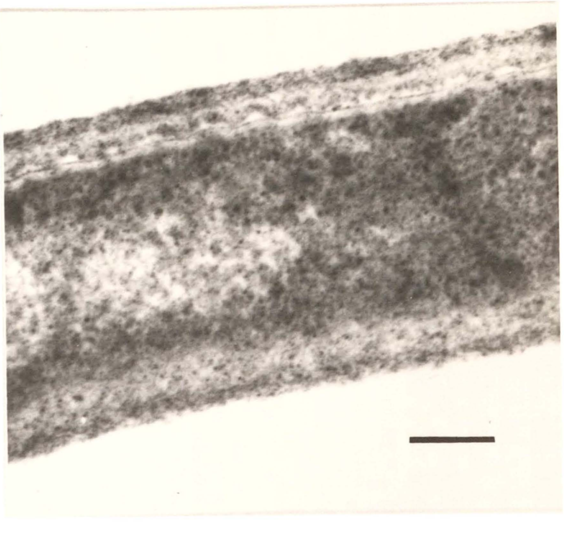
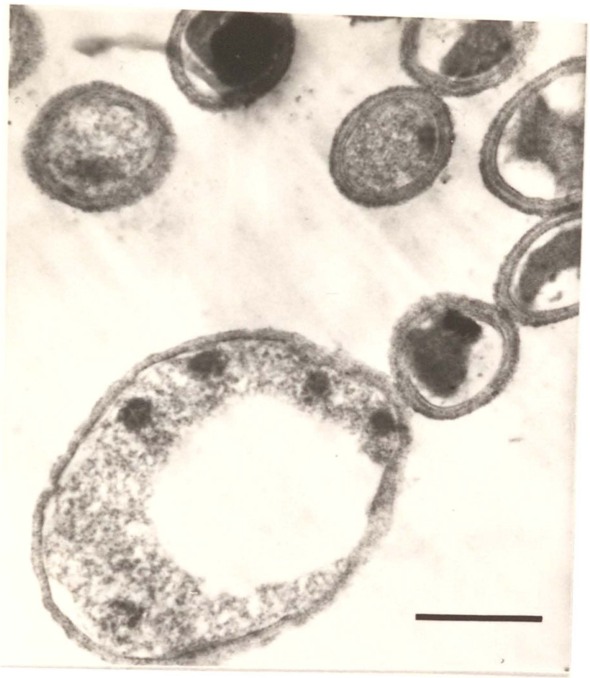


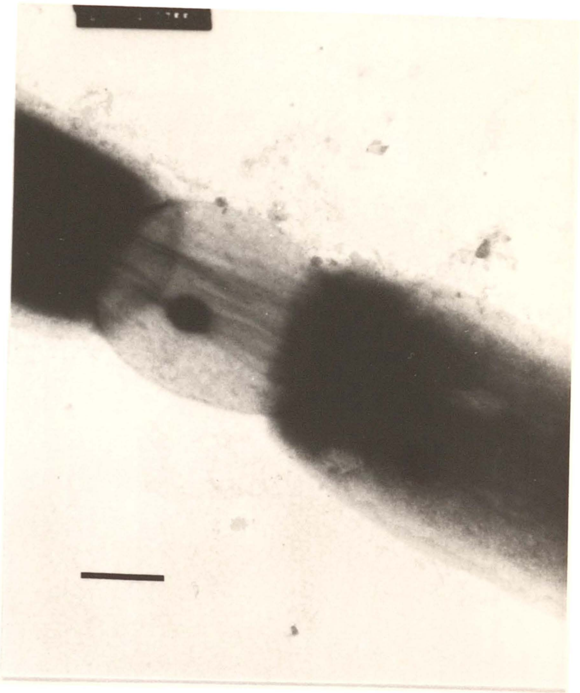
Figure 5.10. Thin Section Electron Micrograph of a Mid Filament Swelling. Bar=1μm.(Top left)

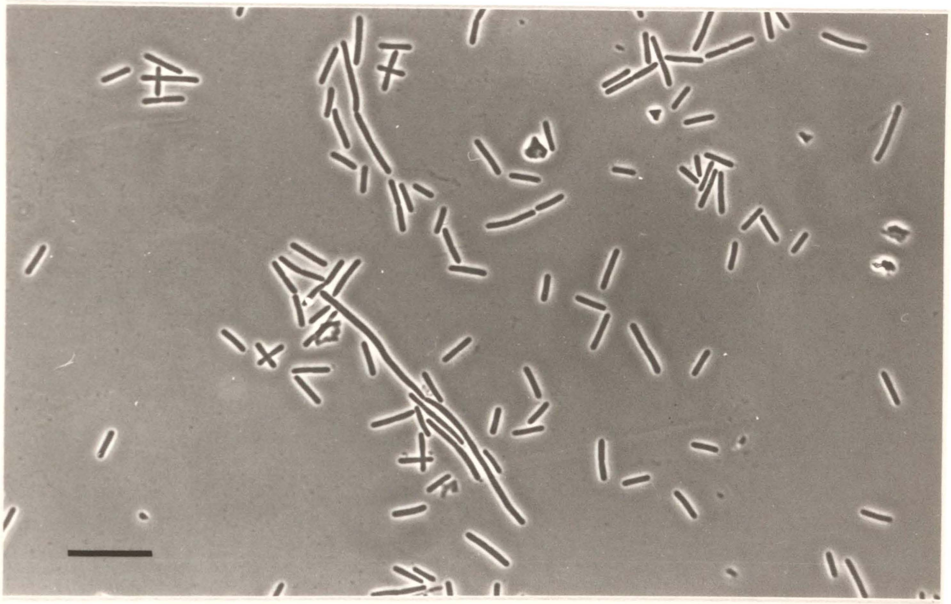
Figure 5.11. Negatively Stained Electron Micrograph of a Sphere Seen in Filaments. Bar=0.28μm. (Top right)

Figure 5.12. T.aquaticus Cell Morphology. Bar=8μm.

Figure 5.13. "T.thermophilus" Cell Morphology. Bar=8μm.







The morphology of T.aquaticus and "T.thermophilus" is that of a pleomorphic rod. T.aquaticus (Figure 5.12) formed rods 5-10μm long by 0.5-0.8 μm wide (Brock & Freeze, 1969) while "T.thermophilus" (Figure 5.13) had average dimensions of 0.5 by 3μm (Oshima & Imahori, 1974). Both organisms showed the ability to form chains (Figure 5.13) but these chains have not been observed to achieve the lengths of those formed by Wai33 A1, and also the septa were clearly visible using phase microscopy, which were rare with Wai33 A1.

Phase microscopy of H.geysericola showed that no coiling was formed, the filaments were very phase dark and septa were clearly visible. The filaments were of even width when grown on solid media but varied considerabely when grown in liquid culture. The cells were of greater diameter than those of Wai33 A1, being 1-1.5μm wide while they were of the same general length (5-10μm). In the mounting fluid there were many free cells and necridia, which were rare with Wai33 A1.

Examination of "Tx.thiopara" showed that most of the cells were either single or in pairs. Septa were clearly visible and the ends of the cells were rounded. Cell width was 0.6μm. Unlike Wai33 A1 the broth culture formed an even turbidity.

C.aurantiacus showed cells of even width (0.6 μm). The length of the cells could not be determined by phase microscopy as the septa were not visible. Filaments were of a length of 150μm or greater and did not show any coiling or bullae.

5.3.5. Mol% G+C Values.

The melting temperature for T.aquaticus DNA was 95.5°C, and that for Wai33 A1 DNA was 94.25°C. The G+C mol% value for T.aquaticus is known to be 67.4% (Brock & Freeze, 1969). The mol% G+C for Wai33 A1 from the equation:

$$\text{mol\% G+C (x)} = \text{mol G+C \% (ref)} + 2.44\,(Tm(x)-Tm(ref))$$

is:

$$x = 67.4 + 2.44\,(94.25-95.5)$$

$$x = 64.4\%$$

5.3.6 Diagnostic Tests.

The results for diagnostic tests are shown in Table 5.3.

Table 5.3

Results of Diagnostic Tests on Wai33 A1.

TEST	RESULT	TEST	RESULT
NITRATE REDUCTION	-	MALONATE UTILISATION	-
AESCULIN HYDROLYSIS	+	ARBUTIN HYDROLYSIS	+
HIDE POWDER AZURE HYDROLYSIS	-	ELASTIN HYDROLYSIS	-
FIBRIN HYDROLYSIS	-	CASEIN HYDROLYSIS	-
TWEEN 80 DEGRADATION	-	DNAase PRODUCTION	-
α-GALACTOSIDASE	+	β-GALACTOSIDASE	+
TELLURITE REDUCTION	+		
p-NITROPHENYL:			
PALMITATE HYDROLYSIS	-	LAURATE HYDROLYSIS	-

+ indicates a positive reaction, - indicates a negative reaction.

Results for carbon source utilisation determined by method 2 of chapter 2 are shown in table 5.4.

TABLE 5.4

Carbon Sources Utilised by Wai33 A1.

CARBON SOURCE	RESULT	CARBON SOURCE	RESULT
ACETATE	+	MELIBIOSE	+
GLUTAMATE	+	MALTOSE	-
SUCCINATE	+	PYRUVATE	-
GLUCOSE	+	DEXTRIN	-
MANNITOL	+	SUCROSE	+
SORBITOL	-	MANNOSE	-
PROLINE	+	GALACTOSE	+
LACTOSE	-	TREHALOSE	+
RAFFINOSE	-	SALICIN	-

+ indicates growth, - indicates no growth.

These results closely parallel the carbon sources used by members of the genus Thermus.

5.3.7 Aminopeptidase and KOH Lysis Tests.

Wai33 A1 was negative for the aminopeptidase test (a Gram type positive characteristic) and positive for the KOH lysis test (a Gram type negative characteristic). See chapter 6 for detailed results. Although the organism stained Gram negatively these results imply that with this organism there is something anomolous about the Gram type as reported with Thermus (see section 1.2.5).

5.3.8 Respiratory Quinone Composition.

The major respiratory quinone of Wai33 A1 was menaquinone 8 (M.D.

Collins Pers. Comm.) which is the same as that for Thermus (Collins & Jones, 1981).

5.3.9 Carotenoid Analysis.

The absorbtion spectra for the organsisms tested are shown in figure 5.14 The absorbtion peaks for Wai33 A1 closely paralleled those of the yellow Thermus species used, while the peaks for T.ruber and H.geysericola were quite distinctively different.

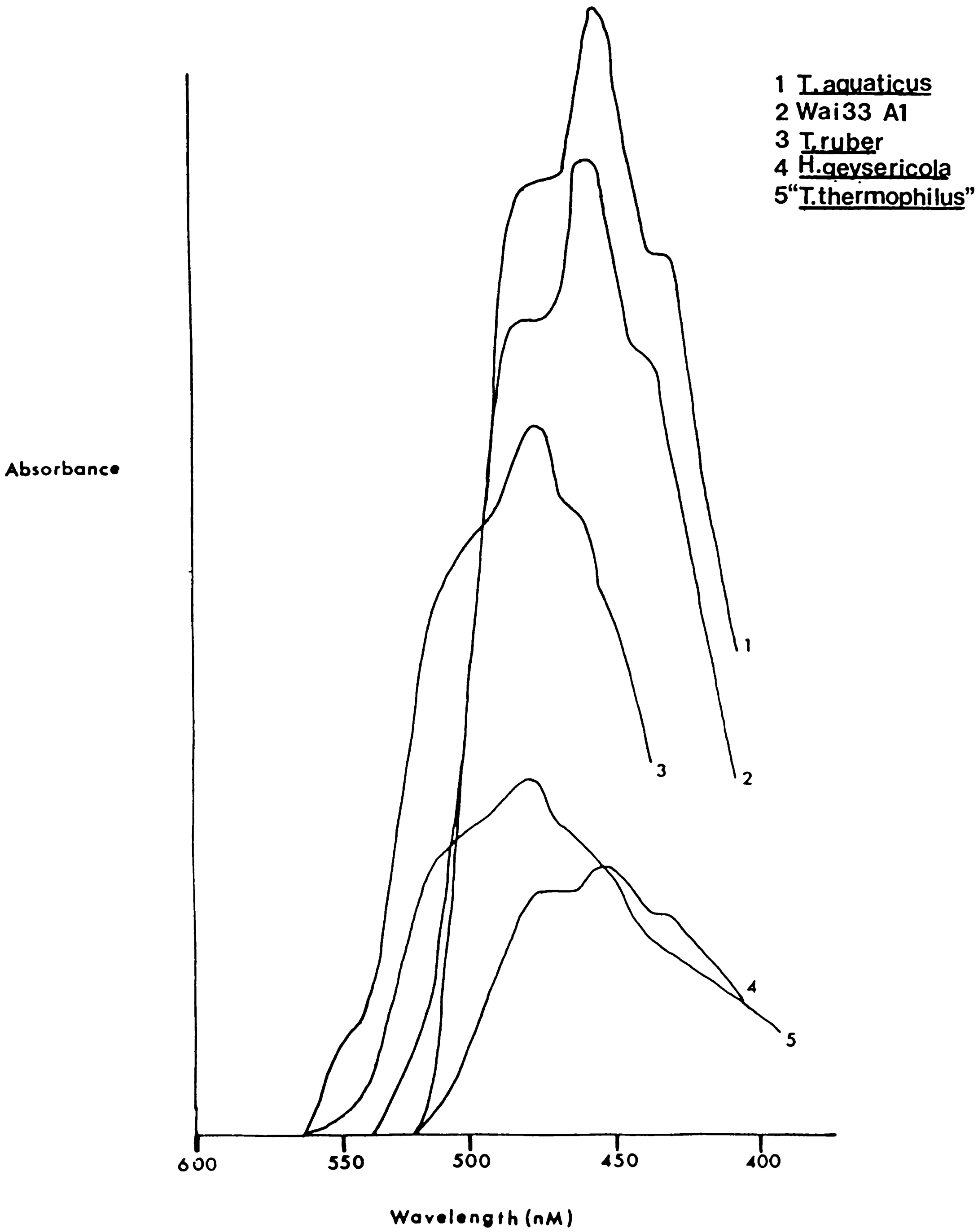


Figure 5.14. Acetone Extract Absorbtion Spectra of Carotenoids of Wai33 A1 and other Organisms for Comparison.

5.3.10 Antibiotic Inhibition Patterns.

Table 5.5

Antibiotic Sensitivities for Wai33 A1, *T.aquaticus* and "*T.thermophilus*" Expressed as Percent Inhibition Compared to the Control.

ANTIBIOTIC	STRAIN								
	Wai33 A1			T.aquaticus			"T.thermophilus"		
	CONCENTRATION (μg/ml)								
	0.2	2	20	0.2	2	20	0.2	2	20
PHOSPHOMYCIN	ND	1	81	ND	0	5	ND	11	11
VANCOMYCIN	ND	0	87	ND	7	94	ND	14	94
STREPTOMYCIN	92	94	ND	59	94	ND	91	95	ND
KANAMYCIN	86	93	ND	4	96	ND	3	87	ND
AMOXICILLIN	88	91	ND	93	96	ND	93	96	ND
PENICILLIN G	60	88	ND	81	92	ND	87	92	ND
CHLORAMPHENICOL	27	86	ND	0	74	ND	0	31	ND
NOVOBIOCIN	ND	0	77	ND	6	77	ND	4	79
NEOMYCIN	87	84	ND	94	87	ND	85	90	ND
POLYMIXIN B	84	85	ND	76	84	ND	0	93	ND

ND= Not Determined

The percentage inhibition for the antibiotics tested are shown in Table 5.5. There was a close similarity between Wai33 A1, *T.aquaticus* and "*T.thermophilus*". Of ten antibiotics used all three organisms showed nearly identical results for six of them (vancomycin, streptomycin, amoxicillin, penicillin G, novobiocin and neomycin). Wai33 A1 was more sensitive to phosphomycin at 20 μg/ml, to kanamycin at 0.2 μg/ml and chloramphenicol at 0.2μg/ml than the other organisms, while "T.thermophilus" was more resistant to polymixin B at 0.2 μg/ml than the other two. Wai33 A1 therefore exhibited the same unusual sensitivity toward β-lactam antibiotics as that shown by Thermus spp.

5.4 Discussion.

Table 5.6 shows a comparison of Wai33 A1 with other organisms with which it might be identified. Wai33 A1 can be clearly distinguished on a number of criteria from these other organisms.

Features which distinguish Wai33 A1 from "Tx.thiopara" are the differences in ability to grow autotrophically or anerobically and in ability to reduce nitrate to nitrite aerobically, pigmentation, motility and morphology as reported in the results section.

The G+C mol% values distinguish Wai33 A1 from C.aurantiacus and H.geysericola. C.aurantiacus also has a lower temperature optimum for growth, is motile, photoheterotrophic with the production of bacteriochlorophylls and is facultatively anaerobic while H.geysericola has a much lower temperature optimum, is motile, and has larger diameter cells. The two Bacillus species can be readily distinguished on the basis of their motility, Gram reaction, ability to sporulate, lower temperature optima and their ability to grow anaerobically.

TABLE 5.6

CHARACTER	WAI33 A1	Thermus (1)	"Thermothrix thioparus" (3)	Chloroflexus aurantiacus (4)	Heliothrix oregonensis (5)	Herpetosiphon Spp.	Bacillus thermocatenulatus (11)	Bacillus thermoflavus (12)
G+C MOL %	65	60-70	NA	55	NA	48-53 (6)	69	61
pH OPTIMUM	7.4	7.5-7.8	NA	7.6-8.4	NA	6.8-7.2 (7)	NA	6.2
pH MAXIMUM	8.6	9.5	NA	NA	NA	NA	NA	NA
pH MINIMUM	6	6	NA	7	NA	NA	NA	5.5
TEMPERATURE OPTIMUM	73	70-72	70-73	52-60	40-45	25 (7)	65-70	60
TEMPERATURE MINIMUM	37	40	62	30-35	NA	15 (7)	35	30
TEMPERATURE MAXIMUM	80	79	77	65-70	56-60	37 (7)	78	70
CELL WIDTH	0.8-1.7	0.5-0.8	0.5-1.0	0.6-0.7	1.5	1-1.5 (8)	0.9	0.85
CELL LENGTH	4-11	5-10	3-20	2-6	10	5-10 (8)	6-8	2.3-7.1
BULLATE	+	SWELLINGS(2)	-	-	-	+ (9)	-	-
ANAEROBE	-	-	F	F	-	- (7)	F	F
MOTILE	-	-	+	+	+	+ (8)	+	+
CAROTENOIDS	+	+	-	+	+	+ (6)	NA	+
GRAM REACTION	-	-	-	-	-	- (8)	+	+
AUTOTROPHY	-	-	+	+	+	- (7)	+	+
SPORULATION	-	-	-	-	-	-	+	+
MAJOR QUINONE	MK 8	MK 8	NA	NA	NA	MK 6 (10)	NA	NA

ABBREVIATIONS: NA, NOT AVAILABLE; F, FACULTATIVE

REFERENCES: (1) Brock, 1984 (2) Brock & Freeze, 1969 (3) Caldwell et al., 1976 (4) Pierson & Castenholz, 1974 (5) Pierson et al., 1985 (6) Lewin, 1970 (7) Trick & Lingens, 1984 (8) Holt & Lewin, 1968 (9) Reichenbach & Golecki, 1975 (10) Reichenbach et al., 1978 (11) Golovacheva et al., 1974 (12) Heinen & Lauwers, 1982

Comparison of Wai33 A1 with the Other Organisms as Which it Might be Identified.

From Table 5.6 however the general similarity with *Thermus* spp. can be seen, in fact from this table there are no major differences. It is the morphology of the isolate which permits its distinction from other *Thermus* isolates. Therefore a more detailed comparison of wai33 A1 and *Thermus* spp. is required.

It is hypothesised here that the filamentous form of Wai33 A1 is the result of the two extra layers in the cell wall. The cell wall of Wai33 A1 and that of *T.aquaticus* have features which appear to be in common, i.e. all the components of a Gram negative cell wall and the unusual corrugated outer cell wall layer reported by Brock & Edwards (1970). Wai33 A1 possessed two extra cell wall layers which appeared to run the whole length of the filament and played no part in septum formation. Thus they may have formed a sheath-like structure which served to form the trichomes observed. The possession of an extra layer exterior to the outer membrane is not unprecedented for *Thermus* strains. In a survey of cell envelope composition of *Thermus* isolates Pask-Hughes & Williams (1978) described an isolate, I1, which possessed such a layer. Figure 5.15 shows an interpretation of the cell wall of Wai33 A1 in comparison to the *Thermus* cell wall composition as given by Brock & Edwards (1970).

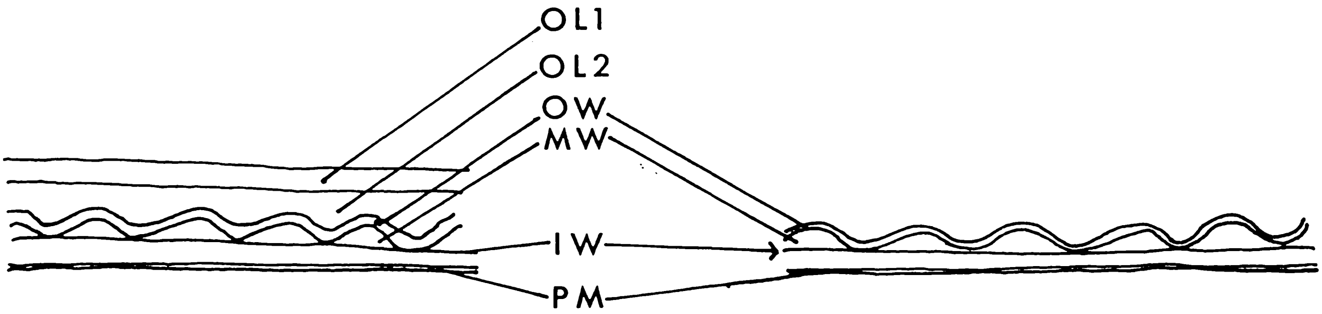


Figure 5.15.

Comparison of the cell walls of Wai 33A1 and Thermus showing the proposed comparable layers between the two organisms and the extra layers (OL1 and OL2) found in Wai 33A1.

OL1	:	Outer Layer 1
OL2	:	Outer Layer 2
OW	:	Outer Dense Layer
MW	:	Middle Light Zone
IW	:	Inner dense Layer
PM	:	Plasma membrane

In support of a relationship between Wai33 A1 and Thermus comes good evidence from the anomolous position of Thermus as regards Gram reaction and Gram type (see chapter 6). Like T.aquaticus and "T.thermophilus" the major respiratory quinone component is menaquinone 8; like T.aquaticus, T.ruber and "T.thermophilus, Wai33 A1 is aminopeptidase negative and like T.aquaticus and T.thermophilus Wai33 A1 has an unusual susceptibility to β-lactam antibiotics. More general evidence comes from the fact that Wai33 A1, like Thermus isolates shows neither an oxidative or fermentative reaction in the Hugh & Leifson test (Degryse et al., 1978).

If Wai33 A1 is closely related to Thermus then some features should be described which distinguish Wai33 A1 from being just another Thermus isolate. There are a number of such characteristics. The morphology of the organism is a constant and clear character with the formation of trichomes of indeterminate length, bullae, necridia and coiling of trichomes. The clustering at a low level with other Thermus isolates (Figures 3.6A and 3.6B) is also a strong indicator of phenetic differences. Other characters could have been included which would further reduced the similarity between Wai33 A1 and the other Thermus strains. These characters would be primarily morphological e.g. growth in broth culture as a clump and production of necridia. Unlike most Thermus isolates tested Wai33 A1 was non-proteolytic by the tests used. Other diagnostic tests include non-reduction of nitrate to nitrite, non-reduction of methylene blue, and non-production of DNAase. Less discriminatory but a more comprehensive range of tests are given in Table 5.3.

In summary it is suggested that Wai33 A1 should be considered as a new species of Thermus, namely Thermus filiformis. There is good evidence that the isolate is a member of the genus and it can be clearly distinguished from other Thermus strains on the basis of morphology. The isolate did not form a separate cluster at the 65% S_{SM} level which was the suggested level drawn in section 3.3.11 to define a species. However other characters, in which all other Thermus isolates tested would have been different from Wai33 A1, could be added to reduce the level at which Wai33 A1 clusters with the other isolates, and so this would not necessarily prevent its inclusion as a new species.

CHAPTER 6

The Gram Type of Thermus.

6.1 Introduction.

As indicated in chapter 1 a number of features of *Thermus* are more consistent with it being a Gram type positive organism rather than Gram type negative which Gram staining and electron microscopy indicate. This evidence together with the phylogenetic tree produced by Hori & Osawa (1979), which shows *T.aquaticus* as an ancient group diverging from the other eubacteria at about the same time as the major eubacterial divisions were occurring, was very interesting and it was decided to examine further the question of the *Thermus* Gram type.

Wiegel (1981) proposed the replacement of the terms "Gram positive, negative and variable" with the terms "Gram reaction positive, negative and variable" and "Gram type positive, negative and zero", so that there would be no ambiguity when such a term was used. He suggested that the classification of bacteria by the Gram type should be on biochemical grounds using the presence of characteristic cell wall compounds and enzyme properties such as the regulatory properties of citrate synthase and the presence or absence of aminopeptidase.

Two tests which had not been carried out on *Thermus* which could add to the data on the Gram type were the aminopeptidase test and the KOH lysis test.

The test for the presence of aminopeptidase was reported by Cerny (1976) and at that time the reactions of all the bacteria tested were consistent with their Gram reaction i.e. Gram reaction negative bacteria were positive for aminopeptidase while Gram reaction positive bacteria gave a negative result or were weakly negative and could be distinguished by limiting the reaction time to 5 min. Cerny (1978) again provided evidence for the consistency of the test. It was also noted that Gram reaction positive bacteria which gave a positive result in times greater than 5 min. had L-alanine in position 1 of the interpeptide bridge.

The KOH lysis test was evaluated by Gregersen (1978) using 71 Gram reaction positive and 55 Gram reaction negative bacteria. The method was shown to be reliable in all but 1 case. Buck (1982) tested 400 isolates. Of these 81% were Gram reaction negative, 11% were Gram reaction positive and 9% were Gram reaction variable. All of the Gram reaction negative and Gram reaction positive bacteria conformed to the expected KOH lysis results. The Gram reaction variable bacteria showed a mixture of negatives and positives by the KOH lysis method.

An overview of both of these techniques was undertaken by Carlone et al. (1983). Of 88 strains of non-fermentative Gram reaction negative bacteria all were positive for the aminopeptidase test, while 4 gave negative KOH tests. A few other discrepencies were observed, the main one being that nonsporulating anaerobic bacteria did not show good correlation between Gram reaction, the KOH lysis test and the aminopeptidase test.

These two tests are therefore useful tests to use on the aerobic Gram reaction negative genus Thermus to obtain more data on the Gram type of the genus.

6.2 Materials and Methods.

6.2.1 Strains Used.

The thermophiles used were T.aquaticus (ATCC 25104^T), "T.thermophilus" (ATCC 27634), T.ruber (DSM 1279^T) and Wai33 A1 (see chapter 5). As a control for each test Escherichia coli C (ATCC 13706) was used.

6.2.2 The Aminopeptidase Test.

The aminopeptidase test was performed according to the method of Cerny (1976). Test reagent comprised 4 g of L-alanine-4-nitroanilide hydrochloride (Sigma) dissolved in 100 ml of 50 mM Tris-maleate buffer (pH 7.0). Whatman GF/C filter papers were soaked in the reagent and allowed to dry. They were used immediately by placing them directly onto bacterial colonies grown on CMD agar for 24 h at their optimum growth temperature, and then transferring back to the appropriate incubator to allow the reaction to take place except for E.coli C, which was kept at room temperature. Plates were examined after 5 min. and periodically after that for the production of a yellow colouration around the colonies.

6.2.3 The KOH Lysis Test.

The KOH lysis test was by the method in Buck (1982). An inoculating loop was used to transfer a visible amount of growth from a CMD plate (bacteria had been incubated for 24 h at their optimum growth temperature) to 10μl of 3% (w/v) KOH solution followed by mixing of the cells continuously for up to 1 min. The mixture was examined for gelling or increased viscosity by pulling the loop away from the slide and checking for "stringiness".

6.3 Results.

E.coli showed a positive result very quickly with the aminopeptidase test while none of the thermophiles was positive after 5 min. or 15 min. However on extended incubation (6 h) there was some colour change but it was very weak compared to that produced by E.coli. Thus the Thermus strains and Wai33 A1 (also considered to be a Thermus isolate) were all negative for the test, a Gram type positive characteristic.

With the KOH test E.coli showed a quick positive result whereas the thermophiles took longer (Wai33 A1 10s, T.ruber 15-20s, T.aquaticus and "T.thermophilus" 40-45s) but were all positive for the test. This was a Gram type negative characteristic.

6.4 Discussion.

The results presented here, like the data available in the literature, are equivocal. One test gave results characteristic of Gram type positive organisms and the other gave results characterisitc of Gram type negative organisms. The anomolous behaviour of Thermus in so many respects with regard to the gram type indicates that there is something unusual about this aspect of the genus.

It may be that these ambiguities represent an adaptation to the high temperature environment. If so then it would be expected that other caldoactive eubacterial Gram reaction negative bacteria would show similar anomolies. However there is little information available in the literature on this aspect of caldoactive eubacteria. It may be significant that Thermomicrobium roseum , another caldoactive aerobic heterotrophic rod and exhibiting a negative Gram reaction has a cell wall which lacks peptidoglycan entirely (Merkel et al., 1980).

Another possible explanantion may lie in the phylogeny of the genus. The early divergence by Thermus aquaticus from other eubacteria is evident in the phylogenetic tree produced by Hori & Osawa (1979). Stahl et al. (1985) found that the Thermus line of descent is at least as ancient as any of the purple photosynthetic bacteria primary divisions. By 16s rRNA sequence analysis the phylogeny of the eubacteria is complex (Fox *et al.*, 1980) with the Gram-positive organisms forming a reasonabely cohesive group, but with the Gram-negative organisms following many separate lines of development. However 16s rRNA analysis of *Thermus* isolates might reveal whether the genus diverged from the Gram-positive line or whether it diverged at the same time as the other divisions seem to have occurred. The anomolous features of *Thermus* therefore appear to be phenetic indicators of a separate line of descent. A study by DNA doublet analysis (Subak-Sharpe *et al.*, 1974) has also shown *Thermus* to represent a discrete group when compared to other eubacteria.

CHAPTER 7: GENERAL DISCUSSION

Towards the end of this project when results were beginning to be analysed it became clear that the questions raised by the work were likely to exceed the questions which may have been answered. In this final chapter it is intended to summarise the conclusions of the preceeding chapters and to discuss them in terms of what further work can be carried out to answer the questions raised (and probably raise more).

Numerical classification of New Zealand and Icelandic isolate sets together with a classification carried out on these isolates plus some from Yellowstone Park, New Mexico and the South Island of New Zealand showed that both geography and the temperatures and pHs of the isolate sources were related to the clusters produced. Clusters produced showed strong regional bias so that major clusters could clearly be identified with a particular geographical source. Within any one limited thermal region (e.g. New Zealand North Island) the clusters produced had isolate source pH and temperature distributions which differed significantly from the null hypothesis. It may be that the correlation between pH and temperature to the clusters is merely a reflection of regional variations of pool parameters which contain similar isolates. Whether the isolate phenotype differences are primarily due to the different physical and chemical parameters of the regions' pools or purely to the geography cannot be resolved with certainty from the data presented. Is it therefore the pool parameters or geography which is more important in influencing phenotype distribution? It would seem unlikely that just pH and temperature would influence the distribution, as overlapping of pool parameters measured in this work would seem to exceed the overlapping of phenotypes observed. More information needs to be gathered on hot pool parameters and correlated with phenotypes present. It may also be the case that the distribution of phenotypes is influenced by pool types within any one thermal region and that this pattern is superimposed on a more general influence on phenotype by geography. An interesting piece of work would be to determine the phenotype composition of pools, say in percentage terms, and to try and correlate these data with a quantitative analysis of an extensive range of biologically important ions such as sulphide and heavy metals. Comparisons between chemical compositions of springs from different thermal regions may also yield significant differences which may explain why phenotypes vary between them.

From another perspective the differences between chemistry of pools from different areas could be assumed to be unimportant. In this case differences of phenotypes could be attributed to different lines of evolutionary progress (also assuming minimal cross contamination between thermal areas). If this is the case then there are several approaches which could be used to demonstrate it. Analysis by DNA/DNA hybridization should show that isolates from within a region should have higher homology than those from other thermal areas. Sequence comparisons of 16s rRNA should also show a similar pattern i.e. isolates from one area would diverge from one another more recently than all of the isolates obtained from different thermal regions. If geographic isolation is not important then neither of these patterns should emerge.

The geographical variation of phenotypes has confused the taxonomic structure of the genus, as broad phenetic groups would contain isolates from different areas which would have different character correlations. There is also little precedent in the literature for the way classifications of previously taxonomically unstructured groups should be divided up. The question is at what level of similarity is a species defined? (with the assumption that all of the isolates are in the same genus). To comply with the current classification of the genus a division could be drawn at 55% S_{SM} in Figure 3 to give the two species T.aquaticus and T.ruber. If this were done however the range of isolates in each species would be so great that there would be no way of establishing a set of diagnostic characters to distinguish between them. At 60% S_{SM} 3 clusters are formed but they still differ in only a few characters (because of the many intermediate values present) and would not be satisfactorally distinguishable. At 65% S_{SM} 8 clusters are produced which can be discriminated fairly easily. This was the value decided upon in Chapter 3 as being a convenient division. As there is currently little structure to the genus this system could be adopted and tested experimentally to see if the classification is stable. In this thesis it is already proposed to add one more species to the list even though it does not form a cluster at 65% S_{SM} (see below). It is assumed that "T.flavus" is a typical Japanese isolate and other isolates from Japan would cluster with it, and that T.ruber is similarly typical of isolates from the USSR. The classification should be tested to confirm this. To be accepted as new species the type strain from each group should have its G+C mol% value determined as a formality, although these data are likely to be somewhat similar.

Data from other techniques could aslo be treated phenetically. Such methods as restriction endonuclease pattern analysis of organismic DNA can be interpreted mathematically (e.g. Sorensen et al., 1985) to reveal taxonomic structure. Using this and similar techniques the classification presented here could be supported or refuted. Here the potential phyletic differences may cause the phenetic pattern to be masked and so non-genetic techniques such as fatty acid methyl ester analysis, serotyping, phagetyping, or a mixture of techniques might be preferred. Pyrolysis mass spectroscopy has already been used on Thermus (Donnison et al., 1986) and there is only limited agreement with this classification. In pyrolysis mass spectroscopy there may be a strong influence on the peak heights obtained by cell components such as carotenoids which could adversely affect the groupings produced.

Two approaches to assess the stability of phenotypes were used, and it was concluded that there was no evidence for the proposal of Cometta et al. (1982b) that Thermus is genetically unstable. A continuous culture was run where the conditions of temperature and pH were altered and the phenotypes examined by using the methods developed in this project. Isolates taken from the continuous culture vessel, after periods of environmental parameter changes, did not differ greatly from one another above a level which could be attributed to test error. A time course experiment, where selected hot pools were sampled periodically, showed that phenotypes isolated at the beginning of the sample period could still be isolated from the same pool after 1 year and these strains were designated "persistent" by analogy with Escherichia coli ecology of the human gut.

What other methods could be used to demonstrate genetic instability in Thermus? Presumably the ability to show variation depends on the possession of a set of genes coding for the same kind of protein which can be expressed or, as Cometta et al. (1982b) suggested, lost to provide genetic variability. In this case there could be a large genome present in these orgainsms so that the extra copies of genes can be accommodated. It would therefore be interesting to repeat a continuous culture experiment but this time analyse the DNA of the organism directly. Overall differences in size of the chromosome, restriction endonuclease digest patterns and plasmid stability might reveal genetic changes brought about by changing environmental conditions. It might be assumed that conditions extreme for the organism would accelerate any changes which might occur as these conditions would apply the maximum selective pressure to them. In the natural population in hot pools these techniques could also be used to assess the stability of the genetic structure of the organisms over a period of time.

Continuous culture of Thermus isolates on single carbon sources is still problematic unless yeast extract is used as the energy source. Presumably therefore there is some fraction of the yeast extract which enables Thermus to grow well when compared to growth on defined medium. The characterisation of this/these compound(s) would be of interest as not only would it allow for better carbon source testing, and the approach attempted by Cometta et al. (1982a) would be simplified there may also be some further insight on the nature of thermophily in this organism if perhaps the compound(s) in the yeast extract enhanced thermostability (as Thermus can be recovered from hot pools with temperatures above the maximum laboratory growth temperature).

A new isolate (Wai33 A1) has been characterised and appears to be a member of the genus Thermus. Although it shows little morphological similarity with other members of the genus its other phenetic characteristics show that it is very similar to other isolates in the genus. Some of the anomolous features of Thermus are shared by Wai33 A1, further indicating a relationship. When Wai33 A1 was included in the all isolate clasification (section 3.4) it remained as an unclustered strain at 73% S_{SM}, but was clustered in the 65% S_{SM} phena. Why then should it be considered as a new species? The classification was derived from tests normally used for the standard Thermus strains, so with Wai33 A1 further tests could be included which would serve to separate it from the rest. Such characters would include growth as a single clump in liquid culture, production of necridia and production of swellings at trichome termini, i.e. they are all morphological, and it is this feature which distinguishes this isolate from other Thermus strains.

Further work which would help to show that Wai33 A1 is a member of the genus includes DNA/DNA hybridization studies, 16s rRNA analysis (as Thermus has apparently followed a unique line of descent), citrate synthase regulation and size studies and fatty acid analysis (to show the same unusual composition as Thermus). It would also be of interest to know the chemical nature of the wall layer(s) which hold Wai33 A1 cells in long trichomes, and to determine what causes the coiling evident in this organism.

Results from literature review and a small amount of experimental work has shown that the Gram type of Thermus is not clear, and also indicates that exceptions to the formerly rigid dichotomy of the eubacteria into Gram negative and Gram positive organisms exists. The literature evidence presented in chapter 1 and the experimental results reported in chapter 6 show that Thermus possesses features common to both Gram negative and Gram positive organisms. Further to this the phylogeny of Thermus as determined by 5s rRNA comparisons has shown that Thermus has followed a line of descent apparently differnt from other eubacteria, branching from them at the same time as the other major divisions in the phylogeny of eubacteria occurred.

Recently other reports have appeared in the literature of similar anomolies in mesophilic organisms. For example a gliding bacterium *Filibacter limicola* (Clausen *et al.*, 1985) was shown by 16s rRNA sequence analysis to be more closely related to Gram positive organisms, and has therefore cast doubts on the general assumption which has been made (Reichenbach, 1981) that all gliding bacteria are Gram type negative.

It may be that the anomolies shown by *Thermus* are in some way adaptations to growth at elevated temperatures. To provide more information on this suggestion the Gram type of other caldoactive Gram reaction negative organisms warrants examination. This would establish if there is a general trend for Gram reaction negative organisms with Gram type negative cell wall appearance to have the same biochemical features more consistent with Gram type positive organisms as is the case with *Thermus*.

The evidence that has come to light with *Thermus* and with other recent publications (see above) on Gram type discrepencies reinforces the suggestion of Wiegel (1981) that descriptions of organisms should contain corroborative biochemical evidence to support the description of a Gram stain so that the organism can be clearly defined by Gram type.

The results reported here have shown that *Thermus* is a much more interesting genus than it may appear on preliminary inspection. Phenotypic distribution has shown strong signs of a geographical influence and perhaps influence by the chemical and physical parameters of the hot spring. The geographic isolation of thermal areas may have allowed *Thermus* to evolve separately in each of these areas producing parochial phenotypes. Environmental influence on the phenotypes of *Vibrio* isolates has also been shown (Simidu & Tsukamoto, 1985). The phylogeny of *Thermus* shows a distinct line of evolution which has probably led to the anomolous Gram type characteristics which it exhibits. Further work suggested above would help to show whether geography and/or pool type influences the phenotypes present, if separate phyletic development has occurred between thermal regions, if genetic instability can be demonstrated by genetic analysis and whether mixed Gram type characteristics are common in Gram reaction negative thermophiles.

APPENDIX 1

Carbon Source Utilisation Data for New Zealand Isolates By Method 1 (See Chapter 2).

Results for 14 carbon sources tested are given for the isolates tested in the following order:-

CARBON SOURCES

----

1) MELIBIOSE
2) ACETATE
3) SUCCINATE
4) GLUCOSE
5) ACETAMIDE
6) GALACTOSE
7) PYRUVATE
8) MALTOSE
9) MANNITOL
10) SORBITOL
11) LACTOSE
12) PROLINE
13) MANNOSE
14) GLUTAMATE

Kt 4	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Rt 34	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OK G	1	1	0	0	0	0	0	1	0	0	0	0	1	1
Tok23	0	1	0	0	1	0	0	0	0	0	0	0	1	1
Oh A1	0	1	0	1	0	1	0	1	0	1	1	1	1	1
Rt4	0	0	1	0	0	0	1	0	0	0	1	1	0	1
Wai33 A3	1	1	1	0	1	1	1	1	0	0	1	1	1	1
WN4	1	1	1	0	0	0	1	1	0	0	1	1	0	1
Tok 20	1	1	0	0	0	1	0	1	0	0	1	1	1	1
OKJ	1	1	0	0	1	1	0	1	0	0	1	1	1	1
Wai 6	1	1	1	0	0	1	0	1	0	0	1	1	1	1
WKV3	0	1	0	0	0	1	0	0	0	0	0	0	0	1
OK4	0	1	1	0	1	0	0	1	0	1	0	1	1	1
Tok1	0	1	0	0	0	0	0	1	0	0	0	0	0	0
WKV2	0	0	0	0	0	0	0	0	0	0	0	1	0	0
TP10	0	0	1	1	0	0	0	1	0	0	0	0	1	1
11247	0	0	0	1	1	1	1	1	0	0	1	1	0	1
WN5	1	1	1	1	1	1	1	1	0	1	1	1	1	1
Tok21	1	1	1	0	0	1	1	1	0	0	1	1	1	1
11245	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rt 35	0	0	0	0	0	0	1	0	0	0	0	1	1	1
Wai33 A2	0	0	0	0	0	0	0	0	0	0	0	1	0	0
T351	1	1	0	0	0	1	0	1	1	1	1	1	1	1
Rt6 A2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
W28 AT	0	1	0	0	0	0	0	0	0	0	0	0	0	1
WN8	1	1	1	0	0	1	0	1	0	0	1	1	0	1
Rt6 A1	1	1	0	0	0	1	1	1	0	0	1	1	1	1
Tok3	1	1	0	0	0	1	0	0	0	0	0	1	1	1
27737	0	0	0	0	0	0	0	0	0	0	1	1	0	0

Oh A2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
27978	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Rt366 A1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fiji3	0	1	0	1	1	1	1	1	1	1	0	1	1	1
Tok8	0	1	0	0	0	0	0	0	0	0	0	0	0	0
OK6	1	1	1	1	0	1	0	1	1	1	1	1	1	1
Rt41A	1	1	1	1	0	1	0	1	0	0	1	1	1	1
WN2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tok22	0	0	1	0	0	0	1	0	0	0	0	0	1	1
HWBS A2	0	0	1	0	1	1	1	1	0	0	0	1	0	1
HWBS A1	0	0	0	1	0	0	0	0	0	0	0	1	1	0
Rt8 A1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rt39	0	1	0	0	0	0	0	0	0	0	0	1	0	1
T351	1	1	0	0	0	1	0	1	1	1	1	1	1	1
OK2	1	1	1	1	0	0	1	1	0	0	0	0	1	1
B2	0	1	0	0	0	0	0	1	0	0	0	0	1	1
B5	0	0	0	0	0	0	0	0	0	0	0	1	0	1
B7	0	0	1	0	1	0	0	1	0	0	0	1	0	1
Rt1	1	1	1	0	1	1	0	1	1	1	1	1	1	1
Rt355	0	1	0	0	0	1	1	1	0	0	0	1	0	1
Rt358 A1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

APPENDIX 2A

Test Results for all Organisms Classified in Section 3.4

Results are shown in two rows of 34 tests, * representing missing data or no comparison. The tests used are given below in the order for the data shown.

1) Tellurite reduction
2) DNAase round growth
3) DNAase under growth
4) Viable after 28 d at 25°C
5) Nitrate reduction, 24 h
6) Nitrate reduction, 7 d
7) Triple Sugar Iron Agar Reddened
8) Growth at pH 6
9) Growth at pH 9.5
10) Methylene blue reduced
11) Colony > 3mm
12) Growth in 2.5% peptone
13) Colony forms "pitting"
14) Broth pH > 7.6
15) α-Glucosidase produced by cells 6 h
16) α-Glucosidase produced by cells 18 h
17) ß-Glucosidase produced by cells 6 h
18) ß-Glucosidase produced by supernatant 18 h
19) Laurate degraded, supernatant 3 h
20) Laurate degraded, supernatant 1 h
21) Palmitate degraded, supernatant 3 h
22) Palmitate degraded, cells 1 h

23) palmitate degraded, supernatant 1 h
24) Growth at 80°C
25) Growth at 45°C
26) Growth at 75°C
27) Orange/yellow colonies
28) ß-Galactosidase production
29) Hide powder azure hydrolysed 24 h
30) 48 h
31) Fibrin hydrolysed 24 h
32) 48 h
33) 72 h
34) Elastin hydrolysed 24 h
35) 48 h
36) 72 h
37) Casein hydrolysed
38) Viability after 14 d at 70°C
39) Aesculin hydrolysis
40) Tween 80 hydrolysis
41) Arbutin hydrolysis
42) Growth in 2% NaCl
43) Growth on acetate
44) melibiose
45) glutamate
46) maltose
47) succinate
48) pyruvate
49) glucose
50) dextrin
51) mannitol

52) sucrose

53) sorbitol

54) mannose

55) acetamide

56) proline

57) galactose

58) lactose

59) Resistance to chloramphenicol

60) phosphomycin

61) spectinomycin

62) kanamycin

63) cycloserine

64)α-Galactosidase production

65) Growth on cellobiose

66) trehalose

67) raffinose

68) salicin

All methods are given in detail in chapter 2.

```
NMX2 A4
0 0 1 1 1 1 0 1 1 0 0 1 0 0 0 1 1 0 1 0 0 0 0 0 1 1 1 0 1 1 1 1 1 0
0 0 0 1 1 1 1 0 1 0 1 1 1 0 1 0 0 1 0 0 0 1 0 0 0 0 0 0 0 1 0 1 0 0
NMX2 A3
0 0 1 0 0 0 0 1 1 1 0 1 0 0 0 1 1 0 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 0
0 0 0 0 1 0 1 * 0 0 0 0 0 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0
NMX2 A1
0 0 1 1 1 1 0 1 1 0 0 1 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 0 1 1 1 1 1 0
0 1 0 1 0 1 0 0 1 0 1 1 1 0 1 1 0 1 0 0 0 1 0 0 1 0 1 0 0 1 0 1 0 0
HS1 A1
0 0 1 1 0 0 0 0 0 0 0 1 0 0 * 1 * * 0 0 0 0 0 0 1 1 0 0 1 1 1 1 1 0
0 0 0 1 1 1 1 0 1 0 1 0 0 1 1 0 1 0 1 0 0 1 0 1 0 0 1 0 1 1 0 1 0 0
HS2 A1
0 0 1 1 0 1 0 0 0 0 0 1 0 1 * 1 * * 0 0 0 0 0 0 1 1 0 0 1 1 0 1 1 0
1 1 0 1 1 1 1 0 1 0 1 0 1 0 1 1 0 1 0 0 0 0 0 0 0 0 1 0 1 1 0 1 0 0
HS2 A2
0 0 1 1 1 1 0 0 0 0 0 1 0 1 * 1 * * 1 0 0 0 0 0 1 1 0 0 1 1 0 1 1 0
1 1 0 1 1 1 1 0 1 0 1 0 1 1 0 0 1 0 1 0 0 1 0 0 1 0 1 0 1 1 0 0 0 0
HS3 A1
0 0 1 1 0 0 0 0 1 0 0 1 0 0 * 1 * * 1 0 0 0 0 0 1 1 0 0 1 1 0 1 1 0
0 0 0 1 1 1 1 0 1 0 1 0 1 1 0 0 1 0 1 0 0 1 0 0 1 0 1 0 1 1 0 0 0 0
HS5 A1
```

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0 0 1 1 0 0 0 1 0 0 0 1 0 1 * 1 * * 0 0 0 0 0 0 1 1 0 1 1 1 1 1 1 0
0 0 0 1 1 1 1 0 1 1 1 0 0 1 0 0 0 0 1 0 0 1 0 0 1 0 1 0 1 1 0 1 0 0
HS6 A1
0 0 1 1 0 0 0 1 0 0 0 1 0 0 * 1 * * 0 0 0 0 0 0 1 0 0 1 1 1 0 1 1 0
0 0 0 1 1 1 1 0 1 0 1 0 0 1 0 0 1 0 1 0 0 1 0 0 1 0 1 0 1 1 0 1 0 1
HS7 A1
0 0 1 1 0 0 0 1 0 0 0 1 0 0 * 1 * * 0 0 0 0 0 0 1 1 0 1 1 1 1 1 1 0
0 1 0 1 1 1 1 0 1 0 1 0 1 1 0 0 1 0 1 0 0 1 0 0 1 0 1 0 1 1 0 1 0 1
HS8 A1
0 0 1 1 0 0 0 1 0 0 0 0 0 0 * 1 * * 0 0 0 0 0 0 1 1 0 0 1 1 0 1 1 1
0 1 0 1 1 1 1 0 1 1 1 0 0 1 0 0 0 0 1 0 0 1 0 0 0 0 1 0 1 1 0 1 0 0
YSP1A A1
1 0 1 1 0 0 1 1 0 1 0 1 0 0 * 0 * * 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1
1 1 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 0 0 1 0 0 1 0 0 0 0
YSP1B A1
1 0 1 0 0 0 1 1 1 1 0 1 0 0 * 0 * * 1 1 0 1 0 0 1 1 1 0 1 1 1 1 1 1
1 1 1 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 1 1 0 1 1 0 0 1 0 0 0
YSP1C A1
1 0 1 0 0 0 1 1 1 1 0 1 0 0 * 0 * * 0 0 0 0 0 0 1 1 1 0 1 1 1 1 1 1
1 1 1 1 0 1 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 1 0 1 1 0 1 1 1 0 0 0 0 0
YSP1D A1
0 0 1 1 0 0 0 1 0 0 0 1 0 0 * 1 * * 0 0 0 0 0 0 1 1 1 1 0 1 0 1 1 0
0 0 0 1 * 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 0 1 0 1 * 0 0 * 0 1 0 0 0 0
YSP1D A2
1 0 1 0 0 0 1 1 1 1 0 1 0 0 * 0 * * 0 0 0 0 0 0 1 1 1 0 1 1 1 1 1 1
1 1 1 0 0 1 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 1 0 1 1 0 1 1 1 0 0 1 0 0
YSP1D A3
1 0 1 0 0 0 1 1 1 1 0 1 0 1 * 0 * * 0 0 0 0 0 0 1 1 1 0 1 1 1 1 1 1
1 1 1 1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 1 1 0 0 1 1 0 0 0 0 0
YSP1E A1
0 0 1 1 0 0 0 1 1 0 0 1 0 1 * 1 * * 0 0 0 0 0 0 1 1 1 1 0 0 1 1 1 0
0 0 0 1 * 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 * 0 * * 0 1 0 0 0 0
YSP1E A2
1 0 1 0 1 0 1 1 0 0 0 1 0 1 * 0 * * 0 0 0 0 0 0 1 1 1 0 1 1 1 1 1 1
1 1 1 1 0 1 0 0 0 0 1 0 1 0 0 0 1 0 0 0 0 1 0 0 1 0 1 0 1 0 0 0 0 0
YSP2A A1
1 0 1 0 0 0 1 1 1 1 0 1 0 0 * 0 * * 1 0 0 0 0 1 1 1 1 0 1 1 1 1 1 1
1 1 1 1 1 1 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 1 0 1 1 1 0 0 0 0 0
YSP2A A2
1 1 1 1 0 0 1 1 0 1 0 1 0 0 * 0 * * 1 0 0 0 0 1 1 1 1 0 1 1 1 1 1 1
1 1 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 1 0 0 0 0 0 0
YSP2B A1
1 0 1 0 0 0 0 1 1 1 0 1 0 0 * 0 * * 0 0 0 0 0 1 1 1 1 0 1 1 1 1 1 1
1 1 1 1 0 1 0 0 1 0 1 0 1 0 0 0 0 0 1 0 0 1 0 1 1 0 0 1 1 0 0 1 1 0
YSP2B A2
1 0 1 1 0 0 1 1 1 1 0 1 0 1 * 0 * * 0 0 0 0 0 1 1 1 1 0 1 1 1 1 1 1
1 1 1 1 0 1 0 0 1 0 1 0 0 0 0 0 1 0 1 0 0 1 1 0 1 0 0 1 1 0 0 1 0 0
YSP3A A1
1 0 1 1 0 0 0 1 1 1 0 1 0 0 * 0 * * 0 0 0 0 0 0 1 1 1 0 1 1 1 1 1 1
1 1 1 1 0 1 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 1 0 0 1 0 0 1 0 0 0 0 0 0
ZF2 A1
1 0 1 0 0 1 0 1 0 0 0 1 0 0 0 0 1 0 1 0 0 1 0 1 1 1 1 1 0 1 0 0 0 0
0 0 0 1 1 1 1 0 0 1 1 1 1 1 0 0 1 0 0 1 0 1 1 1 1 0 1 0 0 1 0 1 0 0
ZF1 A1
1 0 1 1 0 0 0 1 1 0 0 1 0 0 0 1 1 0 0 0 0 0 0 1 1 1 1 1 0 1 0 0 0 0
0 0 0 0 1 1 1 1 0 1 1 1 1 0 1 0 0 1 0 1 0 1 1 1 1 1 1 0 0 1 0 0 0 0
ZF1 A2
```

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0 0 1 1 1 1 0 1 1 0 0 1 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1
1 1 1 0 0 1 1 0 0 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 1 1 0 1 0 0 0 0 0 0
ZF1 A3
0 0 1 0 1 1 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 0
1 1 1 0 * 1 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0
ZG6 A1
1 0 1 1 1 1 0 1 1 0 0 1 0 0 0 1 1 0 1 0 0 0 0 1 1 1 1 1 1 1 0 0 0 0
1 1 0 1 1 1 1 0 0 1 1 1 1 1 1 1 0 1 0 1 0 1 0 1 1 0 * 0 0 1 1 1 0 1
ZG6 A2
1 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1
1 1 1 1 0 1 1 0 1 0 0 0 1 1 0 0 1 0 1 0 0 0 1 0 * * * * * 0 0 1 0 0
ZG5 A2
1 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1
1 1 1 0 1 1 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 1 0 0 0 0 0 0
ZV4B A1
0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 0
1 1 0 1 1 1 1 0 1 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0
ZV4B A2
0 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1
1 1 1 1 1 0 1 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 1 0 1 1 0 0 0 0 0 0
ZF2B A1
1 0 1 0 0 0 0 1 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 0 0 0
0 0 0 1 1 0 1 0 1 1 1 0 1 1 0 1 0 1 0 1 0 1 1 1 1 0 0 0 0 1 1 1 0 1
ZF2B A2
0 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1
1 1 1 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 1 0 0 0 0 0 0
ZV4C A1
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ZV1F A1
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1 1 1 1 0 1 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0
ZG5 A1
0 0 1 1 1 1 0 1 1 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 1 0 1 1 1 1 1 1 1 1
1 1 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 1 0 0 0 0 0 0
ZV1C A2
0 0 1 1 0 1 0 1 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 1
1 1 1 0 0 1 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0
ZV1E A1
0 0 1 1 1 1 0 1 1 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 0
0 0 0 0 1 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 1 0 * 1 0 0 0 0 0 0
ZV2 A2
1 0 1 0 1 1 0 1 1 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 0 0 0
0 0 0 1 0 1 1 0 1 1 1 1 0 1 0 1 0 1 0 0 0 0 0 1 1 0 0 0 0 1 0 1 1 1
ZT1 A2
1 0 1 0 1 1 0 1 1 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 1 1 1 1 0 0 0 0 0 0
0 0 0 1 1 1 1 0 0 1 1 0 1 1 0 1 0 0 0 0 0 0 1 1 1 0 0 0 0 1 1 1 1 1
ZD2 A1
1 0 1 0 1 1 0 1 1 0 0 1 0 0 0 0 1 0 1 0 0 0 0 1 1 1 1 1 0 0 0 0 0 0
0 0 0 1 1 1 1 0 1 0 0 1 1 0 0 1 0 1 0 0 0 1 1 1 1 0 * 0 0 1 1 1 0 1
ZD3 A1
1 0 1 1 1 1 0 1 0 0 0 1 0 0 0 0 1 1 1 0 0 0 0 0 1 1 1 1 1 1 0 1 1 0
0 0 0 1 1 1 1 0 1 1 1 1 0 1 0 0 0 1 0 1 0 1 0 1 1 0 0 0 0 1 1 1 1 1
ZT1 A1
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ZS1 A1
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ZA2 A2
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"T.lacteus"
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"T.thermophilus"
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T.aquaticus
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Tok21
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WN4
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T351
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Tok8
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W28 AT
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Rt6 A1
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OK6
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Ramaley X-1
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NCIB 11245
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NCIB 11247
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Wai33 A2
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Wai33 A3
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HWBS A2
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Rt6 A2
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Oh A1
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Oh A2
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ATCC 27737
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OK2
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OK4
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Tok20
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Tok22
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Tok23
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WN5
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WN6
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WN8
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Kt4
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B2
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B5
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Rt1
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Rt4
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Rt34
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Rt35
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Rt366
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Rt39
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Rt355
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Rt358
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HWBS A1
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WKV2
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WKV3
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Rt8 A1
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Wai33 A1
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0 0 0 * 1 * 0 1 1 * 0 1 0 1 0 1 1 0 0 0 1 1 0 1 1 1 0 0 1 * 1 0 0
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APPENDIX 2B

Test Results for Time Course Organisms.

Results for the 62 tests used are given in the order presented below.

1) Tellurite reduction
2) DNAase production around growth
3) DNAasa production under growth
4) Viability after 28 d at 25°C
5) Nitrate reduced after 1 d
6) Nitrate reduced after 7d
7) Triple sugar iron agar reddened
8) Growth at pH 6
9) Growth at pH 9.5
10) Methylene blue reduction
11) Colony size greater than 3 mm
12) Growth in 2.5% peptone
13) Culture pH > 7.6
14) β-Glucosidase production by cells after 18 h
15) α-Glucosidase production by cells after 6 h
16) α-Glucosidase production by supernatant after 18 h
17) Laurate degradation by supernatant after 3 h
18) Laurate degradation by supernatant after 1 h
19) Palmitate degradation by supernatant after 3 h
20) Palmitate degradation by cells after 1 h
21) Palmitate degradation by superanatant after 1 h
22) Growth at 80°C
23) Growth at 45°C
24) β-Galactosidase production

25) Hide powder azure hydrolysis 24 h

26) 48 h

27) Fibrin hydrolysis 24 h

28) 48 h

29) Elastin hydrolysis 24 h

30) 48 h

31) Casein hydrolysis

32) Viability after 14 d at 70°C

33) Aesculin hydrolysis

34) Tween 80 hydrolysis

35) Arbutin hydrolysis

36) Growth in 2% NaCl

37) Growth on acetate

38) melibiose

39) glutamate

40) maltose

41) succinate

42) pyruvate

43) glucose

44) dextrin

45) mannitol

46) sucrose

47) sorbitol

48) mannose

49) proline

50) galactose

51) lactose

52) Resistance to chloramphenicol

53) phosphomycin

54) spectinomycin

55) kanamycin

56) cycloserine

57) α-Galactosidase production

58) Growth on cellobiose

59) trehalose

60) raffinose

61) salicin

62) D-asparagine

Rt6 A6
1 0 1 1 0 1 0 1 1 1 0 1 0 1 1 0 1 0 0 0 0 0 1 0 1 1 0 0 0 0 0
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Rt4 A6
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Rt366 A4
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Rt358 A3
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Rt8 A1
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Rt6 A9
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APPENDIX 3

Percentage Positive Results for 65% S_{SM} Phena for the all Isolate Classification.

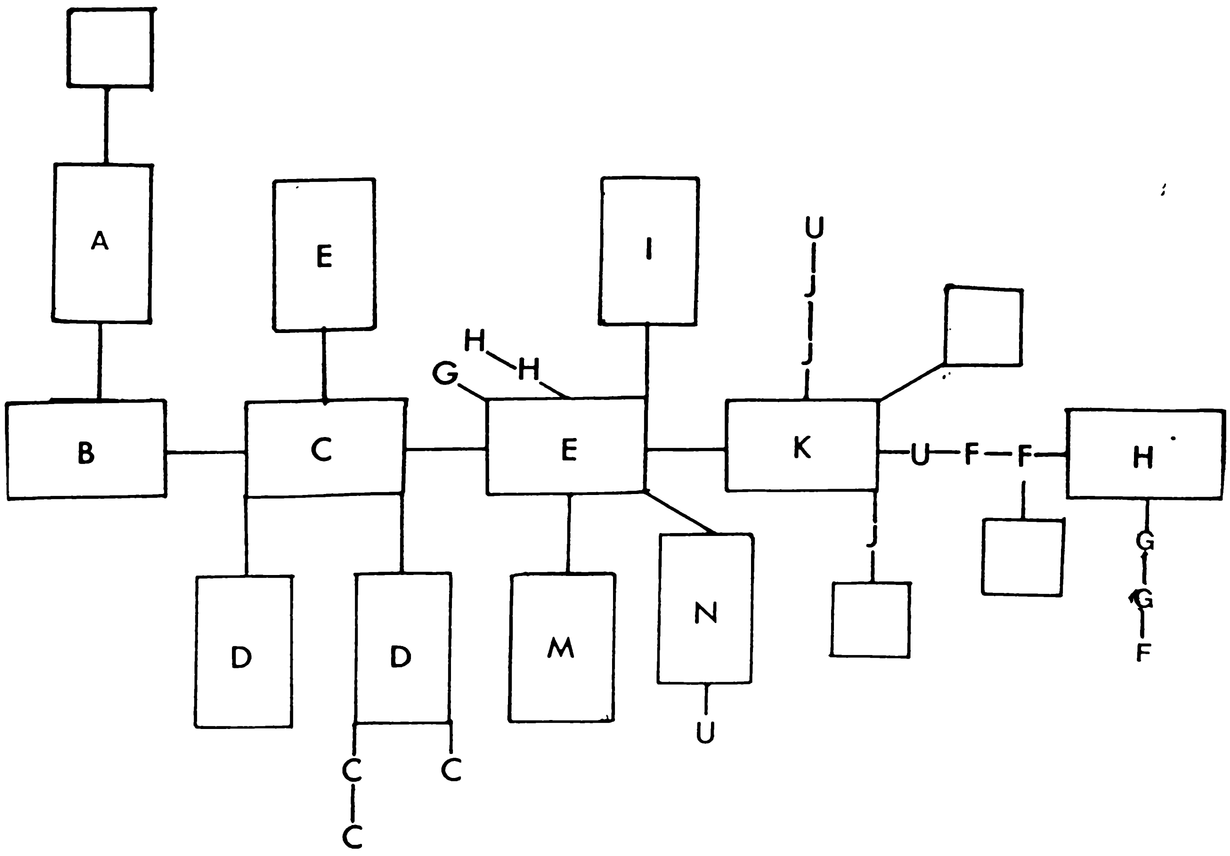
The tests used are those of Appendix 2A, and results are presented in that order.

TEST	CLUSTER A	B	C	D	E	F	G	H
1)	18	67	100	91	100	95	92	100
2)	0	11	0	0	8	0	7	0
3)	90	100	100	64	100	90	93	50
4)	93	89	100	91	71	50	64	100
5)	57	100	100	73	8	40	15	0
6)	59	84	50	54	0	65	38	50
7)	0	11	0	0	83	5	17	0
8)	77	78	100	100	100	100	57	100
9)	50	11	100	27	75	55	29	50
10)	24	44	0	73	83	20	64	0
11)	5	11	0	9	0	0	7	0
12)	43	44	50	45	100	80	28	50
13)	5	0	0	9	0	10	21	0
14)	10	11	0	9	25	0	21	0
15)	18	33	100	18	*	0	36	50
16)	75	88	0	50	0	30	61	50
17)	98	88	0	100	*	100	85	50
18)	6	38	0	10	*	10	0	0
19)	26	89	50	82	25	45	93	0
20)	3	22	0	73	8	5	29	0
21)	11	11	0	82	0	10	64	0
22)	15	33	0	73	8	15	43	0
23)	3	22	0	73	33	40	14	0
24)	5	0	50	18	33	40	17	50
25)	98	78	100	91	92	90	71	100
26)	93	100	100	91	100	100	100	50
27)	80	89	100	100	100	100	93	100
28)	77	89	50	100	8	95	43	100
29)	97	100	0	100	100	25	29	0
30)	98	100	100	100	100	60	78	0
31)	67	89	0	73	100	0	7	0
32)	90	100	50	91	100	5	14	0
33)	93	100	100	100	100	5	36	0
34)	44	44	0	91	100	0	9	0
35)	61	78	0	91	100	5	14	0
36)	70	78	50	100	100	5	21	0
37)	46	89	0	91	100	5	17	0
38)	75	67	100	100	83	65	79	0
39)	68	100	50	64	17	84	79	0
40)	84	89	100	82	92	65	71	0
41)	87	100	100	64	0	84	79	0
42)	2	0	100	18	0	5	0	0
43)	43	100	100	82	58	75	29	0
44)	7	33	0	45	0	95	0	100
45)	31	67	100	82	67	84	43	50

46)	13	67	50	82	8	70	21	50
47)	26	100	0	36	17	65	29	100
48)	43	78	100	73	0	60	21	0
49)	20	89	100	64	0	40	8	50
50)	8	67	100	100	0	80	9	100
51)	17	0	0	22	17	10	0	0
52)	13	*	*	*	0	88	*	0
53)	16	0	0	22	42	0	0	0
54)	10	75	100	73	8	55	14	0
55)	0	11	0	0	0	52	0	0
56)	25	67	100	100	75	65	29	0
57)	7	44	0	60	17	80	17	100
58)	7	44	0	100	42	85	0	0
59)	84	78	50	91	83	80	36	0
60)	36	33	50	45	0	15	17	100
61)	70	78	100	82	67	44	21	50
62)	25	11	100	45	83	5	21	100
63)	18	11	0	0	58	5	7	100
64)	31	22	0	82	8	95	21	50
65)	8	63	100	80	8	79	8	100
66)	28	75	50	90	25	95	25	0
67)	2	25	0	100	8	58	0	0
68)	12	50	100	40	8	80	0	0

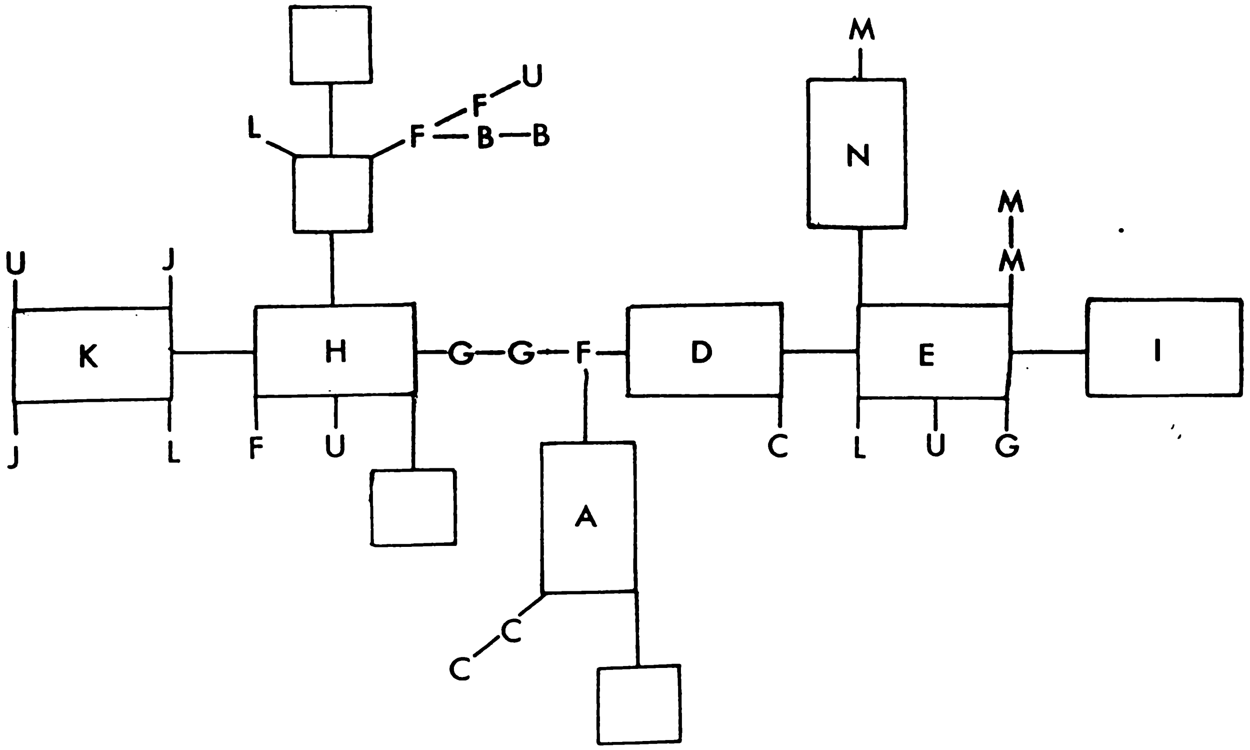
APPENDIX 4A

Simplified minimum spanning tree for all isolates based on the single linkage analysis of the S_{SM} similarity matrix. Cluster labelling is as for Figure3.6. Unclustered organisms have been labelled u, and those in pairs shown as square boxes. Rectangles represent groups of organisms with their cluster letter. Individual letters show cluster members not falling into the same groups as Figure 3.6 in this analysis.



APPENDIX 4B

Simplified minimum spanning tree for all isolates based on the single linkage analysis of the S_J similarity matrix. Labelling is as for Appendix 4A.



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