



THE UNIVERSITY OF  
**WAIKATO**  
*Te Whare Wānanga o Waikato*

Research Commons

<http://researchcommons.waikato.ac.nz/>

## Research Commons at the University of Waikato

### Copyright Statement:

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

The thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of the thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from the thesis.

**Characterisation of Four Strains  
of Thermophilic Spirochetes  
and  
Characterisation of Phosphofructokinase  
from  
Isolate Rt 118.B2**

A Thesis  
submitted in partial fulfilment  
of the requirements for the Degree  
of  
Master of Science in Biological Sciences  
at the University of Waikato  
by

**LISA APIMERIKA**

**University of Waikato**

**1996**

## Abstract

Four strains of spirochetes were purified by the selective use of antibiotics. Microscopy revealed the strains have typical spirochete morphology. The strains were obligate anaerobes and all four strains utilised a variety of carbohydrates including polysaccharides -but not amino acids- as energy sources. The optimum pH of the isolates was between 6.5 and 7.0. The cardinal growth temperatures varied between the different strains from 52°C to 59°C. The lowest temperature any of the four strains could grow at was 37°C and the highest was 62°C. Doubling times of the isolates varied from 221 minutes to 333 minutes. The major products of glucose fermentations were acetate and lactic acid, no ethanol was formed. All strains were resistant to rifampicin, naldixic acid, novobiocin and polymyxin B. Determination of the G+C mol% of DNA revealed that the strains have the lowest G+C % of the species.

Cell free extracts from the isolates were assayed for pyrophosphate dependent phosphofructokinase (PFK) activity and the results were compared with those from other members of the species. Attempts were made to purify PFK from one of the isolates. The partially purified enzyme was characterised and was found to have an optimum pH of 7 and optimum temperature of 60°C. The thermostability of the enzyme at various temperatures was established. The kinetics of the enzyme were examined and the  $K_m$  of fructose-6-phosphate, pyrophosphate and tripolyphosphate established. Various polyphosphates were used as phosphoryl donors; this is the first report of polyphosphates being used as a phosphoryl donor for PFK.

## ACKNOWLEDGEMENTS

My thanks to Professor Hugh Morgan for his supervision, guidance and for providing such an interesting project.

Special thanks to Ron Ronimus and Lynne Parker for all their assistance.

Extra-special thanks and gratitude to Colin Monk without whom none of the equipment would work.

Thanks to all the members of the Thermophile Research Unit for your help and companionship. Especially Dorothy for dancing in the rain.

Lastly, thanks to my best friend and husband, David Apimerika, who kept me sane in the midst of crisis and who spent many hours with me in the publishing of this thesis.

# TABLE OF CONTENTS

<b>ABSTRACT</b>	<b>I</b>
<b>ACKNOWLEDGEMENTS</b>	<b>II</b>
<b>TABLE OF CONTENTS</b>	<b>III</b>
<b>LIST OF FIGURES</b>	<b>VIII</b>
<b>LIST OF TABLES</b>	<b>X</b>
<b>CHAPTER ONE</b>	<b>1</b>
<b>LITERATURE REVIEW</b>	<b>1</b>
<b>1.1 Taxonomy of order <i>Spirochaetales</i></b>	<b>1</b>
1.1.1 Rifampicin resistance	1
1.1.2 Morphology of spirochetes	1
1.1.3 Movement	2
1.1.4 Phylogeny	2
<b>1.2 Free Living Saccharolytic Spirochetes, genus <i>Spirochaeta</i>:</b>	<b>4</b>
<b>1.3 Thermophily</b>	<b>7</b>
1.3.1 Thermophilic <i>Spirochaeta</i>	7
<b>1.4 Carbohydrate metabolism</b>	<b>9</b>
1.4.1 The glycolytic enzyme phosphofructokinase	11
1.4.2 Pyrophosphate	11
1.4.3 Pyrophosphate dependent Phosphofructokinase	12
1.4.4 Polyphosphates	14
1.4.5 Classification of P <sub>Pi</sub> -PFK	14

1.4.6	The evolutionary role of Poly Phosphates	15
<b>CHAPTER TWO</b>		<b>17</b>
<b>MATERIALS AND METHODS</b>		<b>17</b>
<b>2.1</b>	<b>Chemicals</b>	<b>17</b>
<b>2.2</b>	<b>Media and cultivation conditions</b>	<b>17</b>
2.2.1	Carbonate-Buffered Medium	17
2.2.2	SE (spirochete enrichment) media	18
2.2.3	<i>S.stenostrepta</i> media	19
<b>2.3</b>	<b>Culture conditions and measurement of growth</b>	<b>19</b>
<b>2.4</b>	<b>Strains used in this study</b>	<b>20</b>
<b>2.5</b>	<b>Isolation</b>	<b>21</b>
2.5.1	Roll tubes	21
2.5.2	Phosphate supply in the SE medium	21
2.5.3	Enrichment by filtration	22
2.5.4	Enrichment with antibiotics	22
<b>2.6</b>	<b>Preservation</b>	<b>22</b>
2.6.1	Cryopreservation	22
2.6.2	Freeze drying	23
<b>2.7</b>	<b>Microscopy</b>	<b>24</b>
2.7.1	Phase contrast microscopy	24
2.7.2	Electron microscopy	24
<b>2.8</b>	<b>pH Optima</b>	<b>25</b>
<b>2.9</b>	<b>Cardinal growth temperatures</b>	<b>25</b>
<b>2.10</b>	<b>Growth curves</b>	<b>26</b>
<b>2.11</b>	<b>Fermentation End Products</b>	<b>26</b>

<b>2.12</b>	<b>Growth on different carbon sources</b>	<b>27</b>
<b>2.13</b>	<b>Antibiotic Sensitivity</b>	<b>27</b>
<b>2.14</b>	<b>G+C mol% Determination.</b>	<b>28</b>
2.14.1	DNA extraction	28
2.14.2	DNA melting curves	29
<b>2.15</b>	<b>Random amplification of polymorphic DNA (RAPDs)</b>	<b>30</b>
<b>2.16</b>	<b>Phosphofructokinase assays</b>	<b>31</b>
2.16.1	Preparation of cell free extract	32
2.16.2	Materials and Methods for the Assay System	32
2.16.3	Controls	34
<b>2.17</b>	<b>Protein Estimations</b>	<b>36</b>
<b>2.18</b>	<b>Enzyme Purification</b>	<b>37</b>
2.18.1	Bulk cultivation of Rt 118.B2	37
2.18.2	Harvesting bulk cultures	38
2.18.3	Purification of PFK from Rt 118.B2	38
2.18.3.1	First Attempt at purification of PFK from isolate Rt 118.B2	39
2.18.3.2	Preparation of cell free extracts	39
2.18.3.3	Fractionation with Ammonium Sulphate	39
2.18.3.4	Cellulose Phosphate Chromatography	39
2.18.3.5	Ultrafiltration	40
2.18.3.6	Loading onto the cellulose phosphate column.	40
2.18.3.7	Elution with fructose-1,6-P <sub>2</sub>	40
2.18.3.8	Mono-Q column	40
2.18.3.9	SDS Page Electrophoresis	41
2.18.3.10	Silver staining method for SDS gels (Phastgel)	41
2.18.4	Second attempt at purification of PFK from isolate Rtl 18.B2	41
2.18.4.1	Preparation of cell free extract	41
2.18.4.2	Phenol sepharose column	42
2.18.4.3	Mono Q column	42
2.18.4.4	Phenol sepharose column	42
2.18.4.5	Rotofor System	43
2.18.5	Third attempt at purification of PFK from isolate Rt118.B2	43

2.18.5.1	Fractogel	43
2.18.6	Fourth attempt at purification of PFK from isolate Rt118.B2	44
<b>2.19</b>	<b>Characterisation of the partially purified PFK</b>	<b>45</b>
2.19.1	Optimum pH of the partially purified PFK	45
2.19.2	Optimum temperature of the partially purified PFK	45
2.19.3	Thermostability of the partially purified PFK	46
2.19.4	Kinetic properties of the partially purified PFK	46
2.19.4.1	$K_m$ and $V_{max}$ of Fructose-6-phosphate	46
2.19.4.2	Phosphate donors used by the partially purified PFK and their kinetics	46
<b>CHAPTER THREE</b>		<b>48</b>
<b>RESULTS</b>		<b>48</b>
<b>PART A: CHARACTERISATION OF FOUR STRAINS OF FREE-LIVING THERMOPHILIC SPIROCHETES</b>		<b>48</b>
<b>3.1</b>	<b>Purification of contaminated stock cultures</b>	<b>48</b>
3.1.1	Roll Tubes	48
3.1.2	Phosphate supply in the SE medium	48
3.1.3	Enrichment by filtration	49
3.1.4	Purification by serial dilution	49
3.1.5	Purification with antibiotics	49
<b>3.2</b>	<b>Microscopy</b>	<b>50</b>
<b>3.3</b>	<b>pH Optima</b>	<b>52</b>
<b>3.5</b>	<b>Cardinal growth temperatures</b>	<b>53</b>
<b>3.6</b>	<b>Growth curves</b>	<b>54</b>
<b>3.7</b>	<b>Fermentation End Products</b>	<b>56</b>
<b>3.8</b>	<b>Growth on different carbon sources</b>	<b>58</b>
<b>3.9</b>	<b>Antibiotic sensitivity.</b>	<b>60</b>

<b>3.10</b>	<b>G+C mol% determination</b>	<b>62</b>
3.11.1	Random Amplification of Polymorphic DNA	65
<b>3.12</b>	<b>Part A : Discussion</b>	<b>66</b>
	Summary	69
 <b>PART B: CHARACTERISATION OF PHOSPHOFRUCTOKINASE FROM RT 118.B2</b>		 <b>70</b>
<b>3.13</b>	<b>PFK activity from cell-free extracts</b>	<b>70</b>
3.13.1	Protein estimations	70
3.13.2	PFK assay results	71
3.13.1	PFK assay discussion	76
<b>3.14</b>	<b>Purification of PFK from Rt 118.B2</b>	<b>78</b>
<b>3.15</b>	<b>Characterisation of the partially purified PFK</b>	<b>80</b>
3.15.1	Optimum pH	80
3.15.2	Optimum temperature of PFK from isolate Rt 118.B2	81
3.15.3	Thermostability of PFK of isolate Rt 118.B2	81
3.15.4	$K_m$ and $V_{max}$ of PFK from isolate Rt 118.B2 in various fructose-6-phosphate concentrations	82
3.15.5	The $K_m$ and $V_{max}$ of PFK from Rt 118.B2 with either Pyrophosphate or Polyphosphate at various concentrations	84
3.15.5.6	Specific Activity of PFK from isolate Rt 118.B2 using alternative polyphosphates.	86
<b>3.16</b>	<b>Discussion</b>	<b>87</b>
3.16.1	Classification of PPi-PFK	87
3.16.2	Kinetics	87
3.16.3	Phosphoryl donors	88
 <b>REFERENCES</b>		 <b>91</b>

# LIST OF FIGURES

Figure 1.1: Phylogenetic dendrogram indicating the positions of the <i>Spirochaeta</i> species within the radiation of the spirochetes.	3
Figure 1.2: The Glycolytic Pathway	10
Figure 2.1: The assay system to measure the disappearance of NADH by phosphofructokinase activity	33
Figure 3.1: Electron micrographs of isolates Rt 118.B2 and GAB 76.	51
Figure 3.2: Growth curve of GAB 76. Doubling time was 309 minutes	54
Figure 3.3: Growth curve of Rt 118.B1. Doubling time was 300 minutes	55
Figure 3.4: Growth curve of Rt 118.B2. Doubling time was 221 minutes	55
Figure 3.5: Growth curve of Wai 21.B2. Doubling time was 333 minutes	56
Figure 3.6: Chromatogram of uninoculated SE media.	57
Figure 3.7: Chromatogram of SE media after incubation with spirochete strain Rt 118.B1.	57
Figure 3.8: DNA melting curve of GAB 76.	62
Figure 3.9: The DNA melting curve of Rt 118.B1.	63
Figure 3.10: The DNA melting curve of Rt 118.B2.	63
Figure 3.11: The DNA melting curve of Wai 21.B2.	64
Figure 3.12: DNA melting curve from the <i>E.coli</i> standard.	64
Figure 3.13: Banding patterns of DNA from the four spirochete isolates after PCR amplification and digestion with EcoR1.	65
Figure 3.14: Protein standard curve	70
Figure 3.15: PFK assay of GAB 76 extract with no-phosphoryl donor	72
Figure 3.16: PFK assay of GAB 76 extract with ATP as the phosphoryl donor	72
Figure 3.17: PFK assay of GAB 76 extract with PPi as the phosphoryl donor	73
Figure 3.18: Optimum pH of partially purified PFK	80
Figure 3.19: Optimum temperature for the partially purified PFK	81
Figure 3.20: Thermostability of partially purified PFK	82
Figure 3.21: Rate of activity of PFK from isolate Rt 118.B2 at increasing fructose-6-phosphate concentrations.	83
Figure 3.22: Lineweaver Burk plot from the rate of activity of PFK from isolate Rt 118.B2 at increasing fructose-6-phosphate concentrations.	83
Figure 3.23: Rate of activity of PFK from isolate Rt 118.B2 at increasing pyrophosphate concentrations.	84
Figure 3.24: Lineweaver-Burk plot from the rate of activity of PFK from isolate Rt 118.B2 at increasing pyrophosphate concentrations.	85
Figure 3.25: Rate of activity of PFK from isolate Rt 118.B2 at increasing tripolyphosphate concentrations.	85

Figure 3.26: Lineweaver-Burk plot from the rate of activity of PFK from isolate Rt 118.B2 at increasing tripolyphosphate concentrations



## LIST OF TABLES

Table 1.1: Optimum growth temperature, habitat, oxygen requirement and G+C% of members from the genus <i>Spirochaeta</i> .	5
Table 1.2: Cell size, optimum pH, pigmentation, and NaCl requirements of members of the genus <i>Spirocheata</i> .	6
Table 1.3: Distribution of PPi dependent PFK.	13
Table 2.1: Primer sequences used for RAPD analysis of spirochete isolates GAB 76, Rt 118.B1, Rt 118.B2, and Wai 21.B2	31
Table 3.1: Growth response of four spirochete isolates in buffered media at different pH values	52
Table 3.2: Cardinal growth temperatures and temperature range of four spirochete isolates	53
Table 3.3: Growth of four strains of spirochetes on different carbon sources.	59
Table 3.4: Antibiotic sensitivity of four strains of spirochetes to various antibiotics and concentrations, expressed as the percent growth recorded against media which contains no antibiotics.	61
Table 3.5: Characteristics of GAB 76, Rt 118.B1, Rt 118.B2 and Wai 21.B2 compared to those of <i>S.caldaria</i> and <i>S.thermophila</i> .	67
Table 3.6: Protein concentrations of cell free extracts of spirochetes used in PFK assays.	71
Table 3.7: Specific activity of PFK from different spirochete strains with different phosphoryl donors.	74
Table 3.8: Comparison of specific activities of PFK from the four isolates and from other members of the genus <i>Spirocheata</i>	77
Table 3.9: Purification table from the fourth attempt at purifying PFK.	79
Table 3.10: Specific activity of PFK from isolate Rt 118.B2 with various phosphate donors.	86
Table 3.11: Comparison of $K_m$ of F-6-P, PPi and Tripolyphosphate from organisms which have PPi-PFK activity. From O'Brien et al., 1975; Mertens et al., 1993; Denton et al., 1994; and Li et al., 1995	88

# CHAPTER ONE

## Literature review

### 1.1 Taxonomy of order *Spirochaetales*

The spirochetes are defined by their characteristic morphology and cellular structure. They are ubiquitous in the environment, found free-living in aqueous environments and associated with a wide range of hosts, from protozoa to termites to molluscs to mammals. The most well studied of these bacteria are those which are the pathogenic spirochetes, causing syphilis, relapsing fever, swine dysentery, Lyme disease and leptospirosis. (Harwood et al., 1984)

Within the order there are five genera: *Leptospira*, *Spirochaeta*, *Cristispira*, *Treponema*, and *Borrelia*. Each Genus is distinguished by morphological, ecological and physiological differences, eg the guanine-plus-cytosine (G + C) content of DNA ranges from 22 to 66 mol%. (Harwood et al., 1984)

#### 1.1.1 Rifampicin resistance

Rifampicin resistance is a general characteristic of spirochetes, and has become a very useful selective agent for isolating spirochete cultures. A comparison of spirochetes and other bacteria showed spirochetes were 10-fold more resistant to rifampicin than *Escherichia coli* and 10 000-fold more resistant than *Staphylococcus aureus*. This resistance may be due to a lower affinity of spirochete RNA polymerase to this antibiotic. (Leschine et al., 1986)

#### 1.1.2 Morphology of spirochetes

The outer membrane of the cell encloses the protoplasmic cylinder (coiled cell body). This 'cylinder' contains the cytoplasmic and nuclear regions of the cell, and the peptidoglycan-cytoplasmic complex which maintains the helical shape of the spirochete. Between the outer membrane and the protoplasmic cylinder are the organelles which give the spirochetes their unique motility. These periplasmic flagella (also known as axial fibrils, periplasmic fibrils, flagella, and endoflagella) have one end inserted near a pole in the protoplasmic cylinder,

whilst the other end is free. All *Spirochaeta* have two periplasmic flagella per cell, one inserted by each pole, with the exception of *S.plicatilis* which has any-where between 18 - 20 inserted near each end of the protoplasmic cylinder (Canale-Parola 1991). The flagella run for most of the cells length, wound around the protoplasmic cylinder. The protoplasmic cylinder and attached periplasmic flagella are completely surrounded by the outer membrane. This makes spirochetes motility unique among all bacteria. (Canale-Parola., 1991, Harwood et al., 1984)

### 1.1.3 Movement

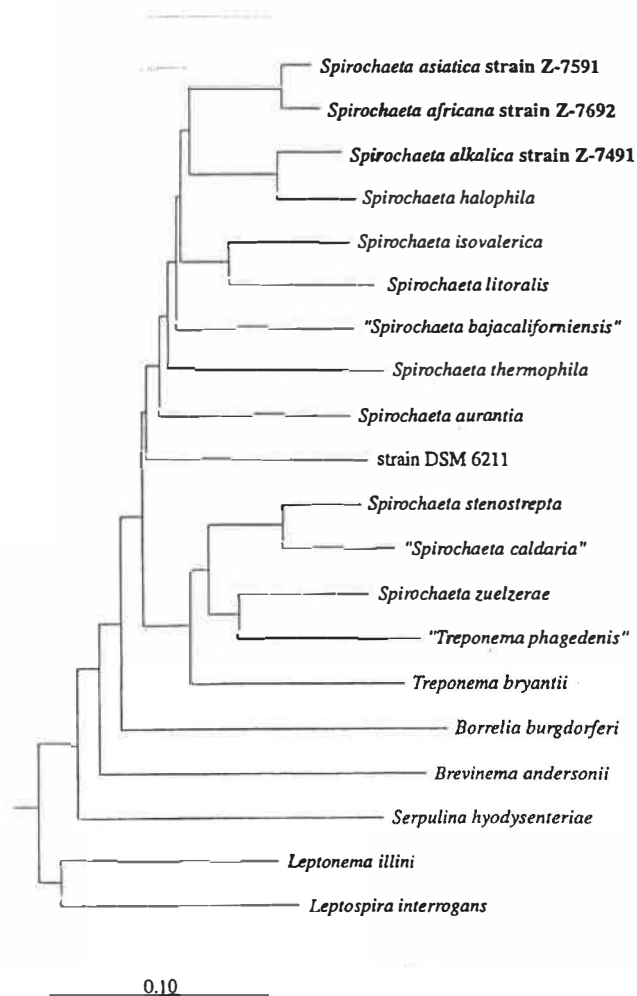
Because the periplasmic fibrils are completely surrounded by the outer membrane, they are not in direct contact with the external environment. Spirochetes move by translational motion, by rotation of the cell around its longitudinal axis and by flexing movements when they swim in liquid environments. (Harwood et al., 1984 Canale-Parola., 1991). They can move through higher viscosity and lipids than most flagellated bacteria. In 1977 Blakemore and Canale-Parola demonstrated that spirochetes could move though a fluid with viscosity of 500 centipoise whilst flagellated bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* were immobilised at viscosities of or above 60 centipoise.. (Canale-Parola 1991) Spirochetes can also move on solid surfaces by “creeping or crawling”, in a similar manner to that of gliding bacteria. (Harwood et al, 1984). This ability to move through a more viscous environment than most micro-organisms can be exploited when attempting to isolate spirochetes.

### 1.1.4 Phylogeny

The phylogenetic dendrogram indicating the positions of the *Spirochaeta* species within the radiation of the spirochetes of the *Spirocheata* is shown in figure 1.1. 16S RNA sequencing allows a reliable genotypic classification tree to be constructed instead of the traditional phenotypic tree. Genotypic classification enables the examination of a species evolution. 16s RNA studies have shown that on cellular and physiological levels the prokaryotes don't always have characteristics which allow reliable phylogenetic ordering, however their rRNAs are excellent for this purpose.

16S rRNA sequencing shows spirochetes and their relatives form a deep evolutionary grouping. Spirochetes are one of the few groupings correctly identified by classical (morphological) criteria; with the exception of a psychrophilic Antarctic isolate. Their common spiral shape and axially coiled fibrils, lying between inner and outer cell envelopes are

distinctly characteristic. The sequence signature of the group is also quite distinctive, for example the U residue at position 47 of the 16S RNA, the A residue at position 52 and the C residue at position 1415 are found in all species of this group but occur nowhere else among eubacteria. (Woese., 1987)



**Figure 1.1:** Phylogenetic dendrogram indicating the positions of the *Spirochaeta* species within the radiation of the spirochetes. Scale bar, 10 substitutions per 100 nucleotides. The root was determined by the inclusion of *E.coli* as an out-group organism. From Zhilna et al 1996.

16s RNA also allows organisms to be identified directly in their niches without isolation, through a combination of rRNA gene cloning and sequencing, and through the design and use of rRNA directed "phylogenetic stains". Using these rRNA probes will allow the identification of strains which have hitherto been unclassified because they have been (so far) unculturable.

At the roots of many phylogenetic trees are thermophiles, which suggests life arose in very warm environments. Among the *Archaea* the deepest euryarchaeal branchings are represented exclusively by thermophiles. Among the *Bacteria*, the deepest known branchings are again represented exclusively by thermophiles. (Olsen et al., 1994). *Leptospira* are one of the deepest branches of the spirochetes and *Leptospira biflexa* var *thermophila* may represent one of the earliest members of the order, but in contrast more thermophilic members of the order have been isolated which are not as deeply rooted.

## **1.2 Free Living Saccharolytic Spirochetes, genus *Spirochaeta*:**

Free living spirochetes live in aquatic environments such as deep sea hydrothermal vents, salt marsh sediments, freshwater and thermal pools and other aquatic environments where decaying plant matter is present. They are all either anaerobic or facultatively anaerobic, they are all motile, and Gram negative. Because they are free living they are not pathogenic, and perhaps because of this they are less well studied than other members of the spirochete family. There are thirteen species currently known, however *S. plicatilis* has not been grown in pure culture, but was observed in mud from a brackish marsh. This demonstrates the difficulty researchers have had in culturing these bacteria. Their phylogenetic relationship to each other is shown in figure 1.1

The characteristics of members of *Spirochaeta* are set out in Tables 1.1 and 1.2. Spirochetes utilise carbohydrates including hexoses, disaccharides and pentoses which are found in the local environment from the metabolism of plant material by other bacteria. They do not utilise amino acids as fermentable substrates for growth, but do produce de novo all their cellular lipids (unlike host-associated spirochetes). No strains of the genus *Spirochaeta* have been reported to be polysaccharolytic, except that of *S.thermophila*. and the recently discovered *S.caldaria*. (Aksenova et al., 1992, Harwood et al, 1984, Rainey et al., 1992, Zhilina et al 1996)

**Table 1.1:** Optimum growth temperature, habitat, oxygen requirement and G+C% of members from the genus *Spirochaeta*.

Species	Optimum growth temperature	Habitat	O <sub>2</sub>	G+C percent
<i>S. litoralis</i>	30	Marine	AnO <sub>2</sub>	50.5
<i>S.stenostrepta</i>	30-37	Freshwater	AnO <sub>2</sub>	60.2
<i>S. plicatilis</i>	unknown	unknown	unknown	unknown
<i>S. zuelzerae</i>	37-39	Freshwater	AnO <sub>2</sub>	56.1
<i>S. thermophila</i>	64-66	Brackish water	AnO <sub>2</sub>	52
<i>S.bajacaliforniensis</i>	36	Fresh water	AnO <sub>2</sub>	50.1
<i>S. isovalerica</i>	15-35	Marine	AnO <sub>2</sub>	63.6-65.6
<i>S. aurantia</i>	25-30	Freshwater	FAnO <sub>2</sub> <sup>1</sup>	62.2-65.3 <sup>2</sup>
<i>S.caldaria</i>	48-52	Freshwater	AnO <sub>2</sub>	45
<i>S.asiatica</i>	33-37	Alkaline lakes	AnO <sub>2</sub>	49.2
<i>S.africana</i>	30-37	Alkaline lakes	AnO <sub>2</sub> <sup>3</sup>	56.1
<i>S.alkalica</i>	33-37	Alkaline lakes	AnO <sub>2</sub>	57.1
<i>S. halophila</i>	35-40	High Salinity	FAnO <sub>2</sub>	62

(Fracek et al 1985, Canale-Parola 1991, Rainey *et al* 1991, Pohlschoeder et al 1993, Zhilina et al 1996).

<sup>1</sup> AnO<sub>2</sub> denotes obligate anaerobe

FAnO<sub>2</sub> denotes facultative anaerobe

<sup>2</sup> *S.aurantia* var *aurantia* has a G+C % of 62.2-65.3, whilst *S.aurantia* var *stricta* has a G+C % of 61.2

<sup>3</sup> Aerotolerant

**Table 1.2:** Cell size, optimum pH, pigmentation, and NaCl requirements of members of the genus *Spirocheata*.

Species	Size ( $\mu\text{m}$ )	Optimum pH	Pigmentation of colonies	NaCl requirement
<i>S. litoralis</i>	0.4-0.5 x 5-7	7.0-7.5	none	opt 2%
<i>S. stenostrepta</i>	0.2-0.3 x 15-45	7.0-7.5	none	none
<i>S. plicatilis</i>	0.75 x 80-250	unknown	unknown	unknown
<i>S. zuelzeræ</i>	0.2-0.4 x 8-16	7.0-8.0	none	none
<i>S. thermophila</i> <sup>1</sup>	0.2-0.25 x 16-20	7.5	none	opt 1.5%
<i>S. Bajacali-forniensis</i>	0.2-0.3 x 15-45	7.5	none	opt 2.8%
<i>S. aurantia</i>	0.3 x 10-20	7.0-7.3	yellow-orange	none
<i>S. caldaria</i>	0.2-0.3 x 15-45	7.2-7.5	none	opt 2.5%
<i>S. asiatica</i>	0.2-0.25 x 15-22	8.4-9.4	none	min 2%
<i>S. africana</i>	0.25-0.3 x 15-30	8.8-9.75	orange	min 3%
<i>S. alkalica</i>	0.4-0.5 x 9-18	8.7-9.6	orange	min 3%
<i>S. halophila</i>	0.4 x 15-30		red or white <sup>2</sup>	opt 4.4%

(Fracek et al 1985, Canale-Parola 1991, Rainey et al 1991, Pohlschroeder et al 1993, Zhilina et al 1996).

<sup>1</sup> Type strain Z-1203

<sup>2</sup> Colonies were red if grown aerobically and white if grown anaerobically

## 1.3 Thermophily

Thermophilic environments are often remarkably constant, allowing organisms to colonise which have specific temperature requirements.

High temperature has many effects on the environment in which micro-organisms live. The pH of water decreases with increasing temperature as the pH of anions and cations decrease. Increasing water temperature results in lower oxygen solubility. Water with a temperature of 90°C will dissolve only 2% of the oxygen that would be dissolved in water at a temperature of 20°C (Brock 1978). This may explain why many anaerobic bacteria are isolated at high temperatures.

A thermophile is 'an organism capable of living at temperatures at or near the maximum for the taxonomic group of which it is a part.' (Brock 1986). This means a thermophile has a  $T_{\min}$  of >25°C,  $T_{\text{opt}}$  of > 45°C and a  $T_{\max}$  of >50°C (Rainey 1992).

In 1978 T. D. Brock described the only known thermophilic spirochete as being *Leptospira biflexa* var. *thermophila*. . So it was thought that the upper limit of growth for spirochaetes was 54°C (Brock., 1978). However Rainey et al (1992) have since isolated a member of the genus *Spirochaeta* (*S. thermophila*) which has an optimum growth temperature of 65°C and a maximum of 75°C. The work the present author has undertaken with strains isolated from New Zealand and the Great Artesian Basin in Australia has revealed further thermophilic spirochetes, and although they exhibit a lower temperature optimum and maximum than *S. thermophila*, they still exceed those described by Brock. (see section 3.5).

### 1.3.1 Thermophilic *Spirochaeta*

Until recently only mesophilic (15°-40°C) species of *Spirocheata* had been isolated. Helical, motile bacteria living at 52° - 56°C were observed as far back as 1910 in thermal springs at Dax, France, however they were not isolated and it is not certain that they were spirochetes. (Pohlschroeder et al 1994).

In 1985 Patel et al, wrote the first publication on thermophilic spirochetes of the genus *Spirochaete*. These strict anaerobes were isolated from four thermal springs in Rotorua and Waimangu, New Zealand, and grew optimally between 45-50°C. They did not grow above 60°C, and showed an optimum pH of 7.0-7.5 with no growth occurring at pH 5.5 or 8.5.

Acetate was the major end product of glucose fermentation, with ethanol and traces of lactate being produced. An interesting finding from this study was the first evidence of phage in spirochete cultures. Phage particles could be observed under electron microscopy attached to spirochetes in culture, though not attached to rods from the same enrichment. Although morphologically similar to *S.stensostrepta* and with similar end products, the spirochetes discovered in these pools appeared to be quite different. They produce far more acetate in relation to ethanol than *S.stensostrepta*, and have a higher temperature optimum and maximum for growth. (Patel et al., 1985). These strains did not survive long term storage, and so have not been characterised further (Aksenova et al., 1992).

Before the work of Aksenova et al (1992) no members of the *Spirochaeta* genus were known to grow at temperatures above 60°C or use polysaccharides. Then two were found, one from a brackish (26% seawater) hot spring in Raoul Island (Kermadec Archipelago, New Zealand) named strain RI 19. B1, and the other from a marine hot spring in Shiashkotan Island (Kuril Islands, Kamchatka, the former USSR) named strain Z-1203.

The two strains were found to be strictly anaerobic, chemoorganotrophic, had a fermentative metabolism and grew on a variety of mono-, di-, and polysaccharides, including cellulose. Strain RI 19 B1 required CO<sub>2</sub>. It had a temperature optima of 64-66°C, pH optimum of 6.95 and salinity optimum of 0.4%. Glucose was fermented to lactate, acetate, carbon dioxide and hydrogen. It did not produce ethanol as an end product, unlike previously described species (all *Spirocheata* were thought to produce ethanol bar *S.zuelzeriae* which doesn't produce ethanol but does produce succinate) RI 19.B1 had resistance to rifampicin which is typical of all spirochetes, and the mol % G + C was 52%. The New Zealand and the Russian strains demonstrated very similar morphological characteristics, and a high level of homogeneity (DNA-DNA hybridisation showed a homogeneity of 87%) On the basis of the extremely thermophilic and polysaccharolytic nature of both the Russian and New Zealand strains, which distinguished them from previously described species, they were introduced in the genus of *Spirochaeta* as a new species, *S.thermophila*. (Aksenova et al., 1992, Rainey et al., 1991). *S.thermophila* was the only spirochete know to be polysaccharolytic. We now know that *S.caldaria* is also capable of metabolising some polysaccharides, and in 1996 Zhilina et al described three alkaliphilic spirochetes (*S.alkalica*, *S.africana*, and *S.asiatica*) which were all able to utilise starch which demonstrates that the polysaccharolytic nature of *S.thermophila* is not limited to that species. (Pohlschroeder et al 1994).

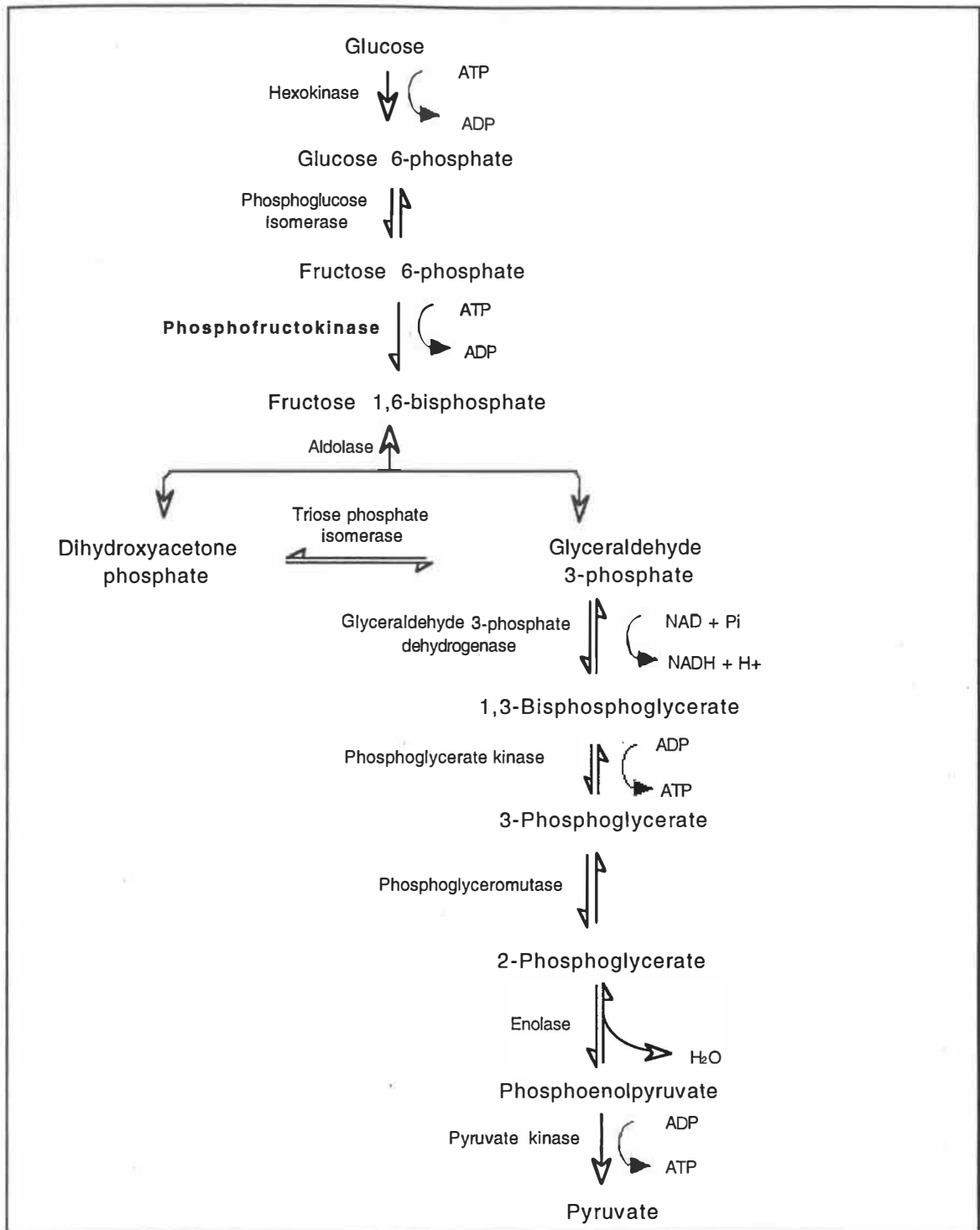
More recently, Pohlschroeder et al 1994 published results describing *S.caldaria* which grows optimally between 48° and 52°C. Two strains of this organism were isolated from cyanobacterial samples collected from freshwater hot springs in Oregon and Utah, USA. The end-products of D-glucose fermentation were H<sub>2</sub>, CO<sub>2</sub>, acetate and lactate. One of the strains

was grown in co-culture with *Clostridium thermocellum* a thermophilic cellulolytic bacterium with cellulose as the only fermentable substrate. Cellulose was broken down at a faster rate in this co-culture than in the clostridial mono-culture. This provided evidence for the suggestion that interactions between cellulolytic and non-cellulolytic spirochetes enhance cellulose breakdown. The mol % G+C of the DNA was 45 (thermal denaturation) which is lower than all other free living spirochetes which range from 49.2 - 65 % (Table 1.1).

Weller et al (1992) investigated the microbial population of the thermal (50-55°C) Octopus Spring using 16s rRNA analysis of cDNA sequences from the cyanobacterial mat. This revealed two uncultured spirochete-like organism. One of these can be placed in the leptospira subdivision of the spirochete group, however the other has so little relationship to the spirochete group that it may be an unrecognised subdivision or even a new eubacterial line of descent. Spirochetes have been observed microscopically in the cyanobacterial mat, but have not been cultured. It would seem possible that there are free-living spirochetes within this mat.

## 1.4 Carbohydrate metabolism

Studies on carbohydrate catabolism of thermophilic anaerobes have mainly been carried out on *Clostridium* spp and *Thermoanaerobium* spp, *Acetomicrobium* spp and recently (Janssen and Morgan 1992) on *S.thermophila*. All of these eubacteria use the glycolytic (Embden-Meyerhof-Parnas) pathway of glucose fermentation (Figure 1.2).



**Figure 1.2:** The Glycolytic Pathway (Stryer 1988)

Glycolysis is the conversion of glucose to pyruvate with the net production of ATP. It is a vital metabolic pathway in almost all biological species, and is the principal means of energy generation for anaerobic spirochetes.

### 1.4.1 The glycolytic enzyme phosphofructokinase

The rate limiting step of glycolysis is the synthesis of fructose 1,6-diphosphate (figure 2). This step is catalysed in most organisms by ATP dependent phosphofructokinase (PFK). The reaction is essentially irreversible and strongly regulated by a variety of activators and inhibitors. (Li et al., 1995, Stryer., 1988).

PFKs are categorised into five groups as described by Li et al., 1995:

1. allosteric ATP dependent PFK found in prokaryotes
2. allosteric ATP-PFK of higher animals
3. non-allosteric ATP-PFK that is structurally unrelated to the ATP-PFK above
4. allosteric P<sub>Pi</sub>-PFK found in several of the higher plants
5. non-allosteric P<sub>Pi</sub>-PFK found in some anaerobic eukaryotes and bacteria

### 1.4.2 Pyrophosphate

Until recently it was generally assumed that the P<sub>Pi</sub> produced in many biosynthetic reactions was not utilised in energy-driven reactions. Any P<sub>Pi</sub> in the cell was thought to be rapidly hydrolysed by pyrophosphatase, which maintains P<sub>Pi</sub> concentrations at low levels. This hydrolysis was thought to favour a thermodynamic pull for anabolic processes and so this was the only role attributed to the generated P<sub>Pi</sub>. There are now many examples (see table 3) where it is known that P<sub>Pi</sub> and other polyphosphates are the energy source of an enzymatic reaction. Furthermore all protists with P<sub>Pi</sub>-PFK activity examined so far do not have an inorganic pyrophosphatase. (Denton et al., 1994, Mertens et al., 1993). H. G. Wood., (1985) found that enzymatic reactions requiring phosphate are thermodynamically favourable regardless of how low the P<sub>Pi</sub> concentration is maintained.

P<sub>Pi</sub> has three main roles in metabolism:

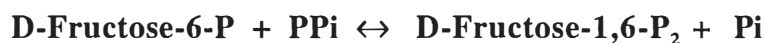
1. P<sub>Pi</sub> is used in some reactions in place of ATP
2. as a nutritional source of energy
3. it is synthesised during photosynthesis and oxidative phosphorylation. (H. G. Wood., 1985)

The more organisms that researchers examine for pyrophosphate dependent enzymes, the more ubiquitous it seems to be, suggesting that use of PPi as an energy source is not only far more wide-spread than originally thought, but also that it may have been the first energy source available to evolving life forms. (Kornberg., 1995)

### 1.4.3 Pyrophosphate dependent Phosphofructokinase

In most organisms ATP-PFK catalyses the first committed step of glycolysis, and this is a major control point. When PPi is used instead of ATP, glycolysis becomes readily reversible and so less useful as a metabolic control point. This appears to be reflected in the lack of allosteric properties associated with many PPi-PFKs.

The reaction catalysed by PPi-PFK is:



Pyrophosphate dependent phosphofructokinase (PPi-PFK) has been found in a broad range of species, in both micro-organisms and higher plants. (table 3.) Species which can use PPi as a phosphate donor generally show a much higher PFK activity when PPi is supplied than if ATP is the donor. The activity in pineapple leaves is 10 to 20 times higher with PPi than with ATP. PPi-PFK from plant sources also contain significant amounts of an allosteric ATP-PFK in contrast to many PPi-PFKinases (such as the Giardial enzyme, and the *Eimeria tenella* enzyme ) which lack an ATP specific PFK activity altogether. (Denton et al., 1994, Li et al., 1995, Wood., 1985). The enzyme has yet to be found in mammals. However animals can utilise oxygen as an electron acceptor which allows them to produce more ATP than anaerobes, so it might make sense for them to favour electron transport.

**Table 1.3:** Distribution of PPI dependent PFK.

<b>Organism</b>	<b>Species</b>
Protozoa	<i>Eimeria tenella</i>
	<i>Toxoplasma gondii</i>
	<i>Entamoeba histolytica</i>
	<i>Naegleria fowleri</i>
	<i>Giardia lamblia</i>
	<i>Trichomonas vaginalis</i>
	<i>Tritrichomonas foetus</i>
	<i>Isotricha prostoma</i>
Obligate anaerobic bacteria	<i>Spirochaeta thermophila</i>
Bacteroides species	<i>Bacteroides fragilis</i>
Facultative anaerobic bacteria	<i>Propionibacterium shermanii</i>
Aerobic marine bacteria	<i>Alcaligenes</i>
	<i>Alteromonas communis</i>
	<i>Pseudomonas marina</i>
Photosynthetic bacteria	<i>Rhodospirillum rubrum</i>
	<i>Rhodopseudomonas galatinosa</i>
Mycoplasma	<i>Acholeplasma laidlawii</i>
	<i>Anaeroplasma</i>
Plant tissues	pineapple leaves
	spinach leaf cytoplasm
	mung bean hypocotyl
	potato tubers
	castor bean seedlings

(Aksenova et al., 1992, Denton et al., 1994, Li et al., Mertens et al., 1993, 1995, Siu et al., 1962)

PPI-PFK was first described in 1962, when Siu and Wood demonstrated that PPI was used in place of ATP as a phosphorylating agent in *Propionibacterium shermanii*. (Siu et al., 1962). In 1992 Janssen et al found that *S.thermophila* strain RI 19.B1 and the type strain Z-

1203 also utilised pyrophosphate instead of ATP as the phosphoryl donor for phosphofructokinase. The PPi was probably being formed by pyrophosphoclastic cleavage of ATP, and the AMP produced was recovered by adenylate kinase activity. Phosphofructokinase was the only *S. thermophila* kinase activity measured which used PPi instead of ATP as the phosphoryl donor.

#### 1.4.4 Polyphosphates

Polyphosphates are linear phosphoryl anhydrides : Poly P(n) where n is the number of phosphoryl groups which vary from 3 to over a thousand residues. Poly P occurs in practically all life forms. (H .G. Wood., 1985; Wood et al., 1988). Polyphosphates are used as

1. a store for phosphate bond energy
2. as a phosphorylating agent in metabolic pathways including PFK.
3. as a phosphate reserve
4. as regulators through their chelation of metals, binding to histones and destabilisation of chromatin.

The phosphoanhydride bond is high-energy, with a free energy approximately equivalent to that of ATP.

H. G Wood., 1985, reported that Poly P accumulated in *P.shermanii* grown in lactate but not in glucose. It is thought that when these cells were grown with glucose as the substrate, Poly P did not accumulate because it was utilised as rapidly as it was formed by phosphorylation of the glucose being metabolised.

#### 1.4.5 Classification of PPI-PFK

PPI-PFK belongs to two broadly classified categories: the type I found in anaerobic protozoa or in bacteria and the type II enzyme found in plants and in *Euglena gracilis*.

The type I enzyme is a 'non-regulated enzyme' which displays a rather low  $K_m$  for fructose-6-phosphate (< 50 $\mu$ M). It is found in some anaerobic protozoa and in bacteria which have no ATP-PFK or fructose-1,6-bisphosphatase. The activity of this type of PPI-PFK is usually quite high in crude extracts (approximately 0.5 units/mg of protein). Subunits are usually 45-

50 kDa, and optimum pH is acidic. It is not regulated by fructose 2,6-bisphosphate. (O'Brien et al., 1975; Mertens et al., 1993)

The type II PPI-PFK is regulated by fructose 2,6-bisphosphate which is present with fructose-1,6-bisphosphate in the cytosol of plants and in *E. gracilis*. The fructose 2,6-bisphosphate greatly increases  $V_{\max}$  and the affinity to fructose-6-phosphate. The subunits are larger than those of type I and the pH optimum is neutral. (O'Brien et al., 1975; Mertens et al., Li et al., 1995)

*N.fowleri* and *G.lambli*a fit into neither type I nor type II categories. *N. fowleri* PPI-PFK has properties of type I in that it does not have an ATP-PFK, has a low  $K_m$  for Fructose-6-phosphate and an acidic optimum pH. It has properties of type II as it is allosterically regulated, this time by AMP. The enzyme found in *Giardia* is unregulated by fructose 2,6-bisphosphate and unlike other PPI-PFK, the enzyme is a monomer of mol mass 64-67 kDa without any subunits. (O'Brien et al., 1975; Mertens et al., Li et al., 1995). These differences would indicate that there may be many types of PPI-PFK awaiting classification.

#### 1.4.6 The evolutionary role of Poly Phosphates

It is likely that PPI and Poly P were present in the earth's crust and the first form of life were anaerobes and used these sources of high energy phosphate. The life which evolved able to use oxygen (ie animals) may not require PPI and through evolution lost some ability to use these forms of energy. (H. G. Wood., 1985)

The more phylogenetically ancient species of eubacteria appear to show a preference for pyrophosphate over ATP (Kornberg., 1995). In nature polyphosphate can occur from the dehydration and condensation of Pi at high temperatures and is found in volcanic condensates and oceanic steam vents. This is especially interesting when considering that life is thought to have evolved at high temperatures.

It is interesting to speculate that the reason that *Naegleria* has a PPI dependent PFK is due to its more ancient origins (ancient compared to *Acanthamoeba castellanii*.) *A. castellanii* has an ATP dependent PFK. rRNA sequence comparisons show *Naegleria* is a relatively early branch in eukaryotic evolution, while *Acanthamoeba* is more closely related to animals and plants. (Mertens et al., 1993).

Studies on *E.tenella* (Denton et al., 1994) have led to the suggestion that P<sub>Pi</sub>-PFK is an adaptation towards life in environments containing low concentrations of oxygen (such as that found in early evolution before plants or that of an animal host). All the protists that have P<sub>Pi</sub>-PFK have fermentative metabolism's. In *E.tenella*, the P<sub>Pi</sub>-PFK reaction is reversible and not subject to metabolic control. However pyruvate kinase is strongly controlled by allosteric activation by glucose-6-phosphate, fructose-6-phosphate and AMP. It is suggested the allosteric control evolved to compensate for glycolysis not being controlled at the P<sub>Pi</sub> PFK step. So perhaps some organisms started to utilise P<sub>Pi</sub> as a phosphate donor late in their evolutionary history, rather than early. The phylogenetic distribution of P<sub>Pi</sub>-PFK among protists indicates that the enzyme may have evolved on more than one occasion as an adaptation for anaerobiosis. (Denton et al., 1994)

# CHAPTER TWO

## Materials and Methods

### 2.1 Chemicals

Enzymes and chemicals were routinely purchased from Sigma, Chemical CO, St Louis, MO, USA. All other reagents were analytical grade. Nitrogen gas and carbon dioxide gas were purchased from New Zealand Industrial Gases (Wellington, New Zealand).

### 2.2 Media and cultivation conditions

#### 2.2.1 Carbonate-Buffered Medium

For cultivation of *S.thermophila* strain RI 19 B1 (Rainey et al., 1991)

<u>Ingredients</u>	<u>g/l</u>
NaCl	4.0
MgCl <sub>2</sub> .6H <sub>2</sub> O	0.8
KCl	0.5
NH <sub>4</sub> Cl	0.3
KH <sub>2</sub> PO <sub>4</sub>	0.2
FeCl <sub>3</sub> (0.15% w/v)	1ml
SL10	1ml
0.1%w/v Resazurin	1ml
Selenite Tungstate	1ml

Make up to 1 litre volume with MQ water. Boil to remove oxygen. Cool, then add :

NaHCO <sub>3</sub>	2.5 g
--------------------	-------

pH to 7.0. Dispense under N<sub>2</sub>/CO<sub>2</sub> mixture (80/20)

Before inoculation add filter sterilised:

$\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ (36g/l)/ $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ (15g/l)	2 ml/l
7 Vitamin mix	1 ml/l
glucose or cellobiose	5 g/l

7 vitamin mix (from the 1993 DSM Catalogue pg 400)

Cyanocobalamine	100mg
p-Aminobenzoic acid	80mg
D(+) Biotin	20mg
Nicotinic acid	200mg
Calcium panthothenate	100mg
Pyridoxine hydrochloride	200mg
Thiamine hydrochloride	200mg
Distilled water to	1000ml

Filter sterilise

## 2.2.2 SE (spirochete enrichment) media

For cultivation of isolates GAB 73, GAB 76, Rt 118.B1, Rt 118. B2 and Wai 21 B1.

<u>Ingredients</u>	<u>g/l</u>
Yeast extract	0.5
Tryptone	0.5
Maltose	3.0
Hepes	2.4
Resazurine (20mg/l)	1ml
$(\text{NH}_4)_2\text{SO}_4$	1.3
$\text{KH}_2\text{PO}_4$	0.28
$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$	0.247

CaCl <sub>2</sub> .2H <sub>2</sub> O	0.074
Distilled water to	1000ml
Na <sub>2</sub> S.9H <sub>2</sub> O	7-9ml

Boil to remove oxygen. pH to 7.2 with NaOH. Dispense while hot under N<sub>2</sub>, Seal and autoclave.

Hepes is an expensive buffer, so an alternative was sought for bulk (80 litres) cultivation. KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> buffer was made at 50mM concentration and substituted for Hepes buffer in the SE media. The KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> buffer was inhibitory to the isolates, and as the other potential alternative buffers were the same price, if not more expensive than hepes, hepes was always the buffer in SE media.

### 2.2.3 *S.stenostrepta* media

For cultivation of *S.stenostrepta*

<u>Ingredients</u>	<u>g/l</u>
Glucose	5.0
Yeast extract	2.0
Peptone	2.0
L-Cysteine	0.5
Resazurin (20mg/l)	1ml
Distilled water to	1000ml

pH was adjusted to 7.3 - 7.6 and Hungate tubes containing the dispensed media flushed with nitrogen before autoclaving.

## 2.3 Culture conditions and measurement of growth

Cultures (other than *S.thermophila* strain RI 19.B1) were always incubated at 55°C unless indicated otherwise. *S.thermophila* strain RI 19. B1 was incubated at 65°C. All experiments

were carried out in duplicate (unless indicated otherwise) and results represent the mean values.

Additions and inoculations were made and samples taken using sterile plastic syringes and 25-gauge hypodermic needles.

Growth was measured by visual inspection of the media for turbidity. Confirmation of growth was by examination under phase contrast microscopy (samples were examined using an Olympus BH-2 microscope with phase contrast optics under oil immersion). Optical densities were also used as a measure of growth using a PYE Unicam SP 6-450 UV/VIS Spectrophotometer, at 400nm.

## 2.4 Strains used in this study

GAB 76 (TG 643), and GAB 73 (TG 642) were obtained from the Great Artesian Basin in Australia. These strains in pure culture were a gift from Dr B. Patel from Griffith University Queensland.

Wai 21.B2 (TG 646) came from a New Zealand Geothermal pool in Waimangu Valley

Rt 118.B1 (TG 653) and Rt 118.B2 were isolated from an outlet (pool Rt 118) which drains into Lake Rotorua in Ohinemutu.

*S.thermophila* strain RI 19.B1 (DSM 6192) came from a brackish thermal spring on Raoul Island, Kermadec Archipelago, New Zealand. (Rainey et al., 1991)

All of the above were obtained in pure culture from the culture collection at the Thermophile Research Unit, at the University of Waikato, Hamilton, with the exception of Rt 118.B2 which was enriched and purified from the native pool in this study.

The mesophile *Spirochaeta stenostrepta* (DSM 2028) was obtained from the DSM, and frozen pellets of *Leptospira interrogans* serotype *pomona* and of *L. interrogans* strain RGA were obtained from Australia.

Strains GAB 76, Rt 118.B1, Rt 118.B2 and Wai 21. B2 were used in phenotypic and genotypic characterisation studies. All these strains plus GAB 73, *L. interrogans*, *S.thermophila* strain RI 19.B1 and *S.stenostrepta* were used in PPI dependent PFK studies.

## 2.5 Isolation

Although supplied as a 'pure culture', rod contaminants were found in strains GAB 76, *S.thermophila* strain RI 19.B1, Rt 118.B1, and Wai 21.B2. Many attempts were made to obtain a pure culture from pool Rt 118 (isolate Rt 118.B2), and to purify *S.thermophila* strain RI 19 B1, GAB 76 and Wai 21 B1.

### 2.5.1 Roll tubes

Roll tubes were made by the method of Hungate. SE medium had purified agar (Oxoid) added at 18g/l. This was heated in the microwave until the mixture had dissolved and all oxygen had been removed by boiling. The medium was dispensed in 5ml aliquots into Bellco tubes, flushed with N<sub>2</sub>, sealed then autoclaved. The tubes were placed into a waterbath at 60°C, while the agar was still molten. The filter sterilised components of the media were added. Serial dilutions (10<sup>1</sup>-10<sup>3</sup>) of cultures were made in the molten agar. The tubes were then rolled at room temperature on an automatic roller, at high speed and at a slight angle to try to prevent agar from solidifying at the entrance of the tube. Tubes were placed at 4°C for 20 minutes to ensure the agar had set. RI 119. B1 was incubated at 65°C while GAB 76, Rt 118.B1, Rt 118.B2 and Wai 21.B2 were incubated at 55°C. Cultures were incubated at a 45° angle to allow condensation to accumulate at the bottom of the tubes. After incubation, colonies were removed aseptically, and inoculated into fresh media. The media was then examined microscopically for spirochetes.

### 2.5.2 Phosphate supply in the SE medium

The strains that were being isolated were suspected to have a P<sub>Pi</sub>-PFK activity. To further enrich the SE media the phosphate supply (KH<sub>2</sub>PO<sub>4</sub>) was replaced with sodium pyrophosphate in the same proportions as the KH<sub>2</sub>PO<sub>4</sub>. It was hoped that this would give a competitive advantage for the spirochetes over the other microbes in the media, and thus help select for spirochaetes. Both standard SE media and sodium pyrophosphate media were inoculated with cultures in their log phase and incubated overnight. Growth was examined microscopically, and cell numbers counted.

### 2.5.3 Enrichment by filtration

Spirochetes are able to pass through filtration disks (which retain most other bacteria) because of their relative small cell diameter. (Canale-Parola 1991). Contaminated cultures of GAB 76, RI 19.B1, Rt 118.B2 and Wai 21.B2 were filtered through a 0.45- $\mu\text{m}$ -pore size sterile Millipore filter, inoculated into SE or CBM media, then incubated overnight. Cultures were examined microscopically for spirochete growth and contaminants.

### 2.5.4 Enrichment with antibiotics

Various combinations and strengths of antibiotics were added to the spirochete medium. These included:

- Rifampicin at a final concentration of 80 $\mu\text{g/ml}$
- Rifampicin at 100 $\mu\text{g/ml}$  and sodium azide at 500 $\mu\text{g/ml}$
- Tetracycline at 20 $\mu\text{g/ml}$ , rifampicin 100 $\mu\text{g/ml}$  and sodium azide at 500 $\mu\text{g/ml}$ .

Serial dilutions ( $10^1$ - $10^3$ ) of cultures in log phase growth were inoculated into media containing antibiotics and incubated for 12 hours. Inoculations into media containing the third combination of antibiotics (tetracycline, rifampicin and sodium azide) were allowed to sit at room temperature for 24 hours, before incubation for 12 hours. After incubation cultures were subbed to an antibiotic free media and examined microscopically.

## 2.6 Preservation

Pure cultures were preserved by both cryopreservation and freeze drying techniques.

### 2.6.1 Cryopreservation

Centrifuge bottles were sterilised by autoclaving. Cultures in late log phase of growth were added to the centrifuge bottles and centrifuged at 7000 rpm for 15 minutes at 5°C.

Supernatant was discarded immediately to prevent the soft pellet from re-suspending. 1 ml of anaerobic cyroprotectant medium was added to resuspend the pellet.

Anaerobic cyroprotectant medium:

Trypticase peptone	10 g
NaCl	5 g
Beef extract	3 g
Yeast Extract	5 g
Cysteine hydrochloride	1 g
Glucose	1 g
Na <sub>2</sub> HPO <sub>4</sub>	4 g
Glycerol	150 ml
R/O water	to 1 litre

pH to 7.0 and dispense under O<sub>2</sub> free nitrogen in Bellco tubes. Sterilise by autoclaving at 121°C for 15 minutes.

The re-suspended pellet was dispensed into sterile cyropreserving tubes and plunged into liquid nitrogen to snap freeze before being stored at -70°C. After one week, an aliquot was removed and revived to check the viability of the culture.

## 2.6.2 Freeze drying

Method as used by Lynne Parker (Thermophile Research Unit, Waikato University)

### 1) Preparation of cell suspension

Late log phase cultures were centrifuged at 5000 rpm for 20 minutes to obtain a good cell pellet. The cell material was then resuspended in anaerobic suspending fluid, which contained a reducing agent.

### 2) Suspending fluids

10 ml of 5% mesoinositol was added to a serum vial of desiccated Difco horse serum. This was allowed to reconstitute and 2ml removed to a sterile bottle per culture. 10 ml of amorphous ferrous sulphide (1:20 dilution) was added immediately before use.

### 3) Preparation of ampoules

Six ampoules were used per culture. Labels were prepared by typing the identification on to blotting paper. Labels were folded, added to the ampoules, and ampoules plugged

with non-absorbent cotton wool. They were then sterilised by autoclaving for 30 minutes.

#### 4) Freeze drying

0.2 ml of suspension was delivered into the bottom of each ampoule using a sterile Pasteur pipette. The sterile cotton wool was pushed a short way into the ampoule. The ampoules were then frozen in liquid nitrogen before being placed onto the freeze drier and left to dry overnight. After removal from the drier, ampoules were heated with a hand torch to obtain a short capillary section. Ampoules were returned to the drier at full suction for another 30 minutes. Ampoules were sealed with a hand torch while still on the drier. The freeze dried cultures were stored at room temperature. One ampoule from the batch was removed and revived to check the viability of the cultures.

## 2.7 Microscopy

### 2.7.1 Phase contrast microscopy

The growth of cultures was observed using an Olympus BH-2 microscope with phase contrast optics. Spirochetes were observed under 1000x magnification using oil immersion.

### 2.7.2 Electron microscopy

Pure cultures of Rt 118.B2, and GAB 76 were washed in SE media salts to remove debris which might interfere with the negatively stained samples. A drop of the culture was placed on a 200 mesh formvar - coated grid for 1 minute. Filter paper was used to remove excess sample. The culture was fixed for 1 minute using a drop of 2.5% (v/v) glutaraldehyde. Excess fixative was removed, and a drop of saturated solution of uranyl acetate was applied for a minute. After removal of excess stain, grids were examined under a Philips EM 400 electron microscope with an accelerating voltage of 80 kv.

## 2.8 pH Optima

SE media was prepared using various buffers to give varying pH levels. Buffer concentrations of 10 mM (standard SE medium strength) and 100mM strength were used. The pH of the medium was adjusted at 55°C before autoclaving. Buffers and pH used were:

	<u>pH</u>
Bis Tris	6.0
	6.5
	7.0
Hepes	7.0
	7.5
	8.0
Tris	7.5
	8.0
	8.5

0.2 ml of well grown cultures of GAB 76, Rt 118.B1, Rt 118.B2 or Wai 21.B2, were inoculated separately into Bellco tubes containing 10ml each of medium and incubated at 55°C for 48 hours.

Optical densities were read at 400nm at 12 hour intervals for 48 hours.

## 2.9 Cardinal growth temperatures

0.2 ml of well grown cultures of GAB 76, Rt 118. B1, Rt 118.B2, or Wai 21 B2 were inoculated separately into Bellco tubes containing 10 ml of SE medium. The Bellco tubes were incubated in triplicate in a temperature gradient incubator, which had been allowed to stabilise for 2-3 days. The temperature gradient of the incubator spanned the range 32 - 68°C. Tubes were placed at approximately one degree intervals along the gradient where optimum growth was expected to be found and every two degrees above or below this temperature to determine maximum and minimum temperatures which would support growth.

Tubes were slowly oscillated to maintain even temperature and mixing. Optical density readings were taken hourly after the initial 6 hour lag phase was over, until the OD had stopped increasing in tubes at the optimum temperature (typically 28 hours). Cultures were left in the incubator for four days after this to establish maximum and minimum temperatures at which the bacteria would grow.

The spectrophotometer was a PYE Unicam SP 6-450 UV/VIS Spectrophotometer, which allowed the Bellco tubes to be inserted and read at 400 nm. Blanks were used at various temperatures to determine the effect of temperature on medium optical density.

## 2.10 Growth curves

When the optimum temperature of each species was found, growth curves and doubling times were ascertained. GAB 76, Rt 118.B1, Rt 118.B2, and Wai 21 B2 were incubated in triplicate at their optimum growth temperature and recordings were taken hourly (after the initial six hour lag phase) until OD (measured by a PYE Unicam SP 6-450 UV/VIS Spectrophotometer) had stopped increasing. The results were plotted and the linear portion of the graph was used to estimate doubling time by using the equation :

$$K = \frac{(\log_{10}t_2 - \log_{10}t_1)}{0.3010 \times \Delta \text{ time}}$$

where K = the number of divisions per hour

t<sub>1</sub> = the optical density at time 1

t<sub>2</sub> = the optical density at time 2

## 2.11 Fermentation End Products

Well grown cultures of GAB 76, Rt 118.B1, Rt 118.B2, and Wai 21 B2 were centrifuged for 10 min at 10000 rpm. The supernatant was decanted off, and stored at -20°C until needed.

Fermentation end products were analysed by High Pressure Liquid Chromatography (HPLC). A Bio-Rad HPX-87.H column at 50°C was used. The carrier liquid was 0.1N

Sulphuric Acid at an elution rate of 0.5ml/min. 20µl of supernatant was loaded onto the column and the peaks were detected using refractive index detection.

## 2.12 Growth on different carbon sources

Four strains of spirochetes were grown on a variety of carbon sources. 0.5 ml of the 10% w/v filter sterilised carbon source was injected into 9.5 ml sugar free SE media. 0.2 ml of well grown cultures of GAB 76, Rt 118.B1 Rt 118.B2, or Wai 21 B2 were inoculated in duplicate into Hungate tubes containing 10 ml of SE medium.

Oat spelt xylan contains 10% arabinose and 15% glucose, both substrates that all four species grew well on. In order to remove free arabinose and glucose, the xylan was dialysed against 3 changes of MQ water for 48 hours.

After inoculation and incubation at 55°C for 24 hours, growth was assessed by observing turbidity and confirmed by microscopic examination. Optical density readings (at 400nm) were taken to record growth. When the carbon source interfered with optical density measurement, numbers of spirochetes were counted.

## 2.13 Antibiotic Sensitivity

Stock solutions were prepared in pre-boiled distilled water (to remove dissolved oxygen) and filter sterilised into sterile vials. The vials were N<sub>2</sub> flushed before being sealed. Stock solutions of Chloramphenicol, D-Cycloserine, Nalidixic Acid, Neomycin, Novobiocin, Penicillin, Polymyxin B, Rifampicin, Streptomycin and Tetracycline were prepared at concentration of 500µg/ml and 2000µg/ml.

Inoculations of antibiotics were made into SE media at final concentrations of 0, 5, 10, 20, 50, 100, and 200 µg/ml. 0.2 ml of well grown GAB 76, Rt 118.B1, Rt 118.B2 or Wai 21 B2, were inoculated separately into Hungate tubes containing 10 ml of SE media and the antibiotics. The cultures were incubated in duplicate for 36 hours and optical densities were read at 400nm at regular intervals to determine growth. Both Tetracycline and Rifampicin interfere with optical densities measurement at 400nm, so cell counts were made microscopically when these antibiotics were used. Where solvents were used to dissolve the antibiotic (ie methanol is used to dissolve rifampicin), the same amount of solvent was added to the control cultures which contained 0 µg/ml antibiotic. Growth of a culture in the

antibiotic containing media is expressed in terms of growth measured in medium containing 0 $\mu$ g antibiotic.

## **2.14 G+C mol% Determination.**

The G+C% of GAB 76, Rt 118.B1, Rt 118.B2, and Wai 21.B2 was determined by the thermal denaturation method. (Marmur., 1961; Mandel et al., 1968; Owen et al., 1979; Gerhardt et al., 1994)

### **2.14.1 DNA extraction**

DNA from strains was extracted by a modified method of Marmur (1961)

Cells were grown in a litre of medium for 48 hours and harvested by centrifugation. Cells were washed twice with 0.1 M EDTA/0.15M NaCl pH 8, and resuspended in 25 ml 0.1M EDTA/0.15M NaCl containing 10 mg lysozyme.

They were then incubated for 45 min at 37°C. 2 ml of 25%(w/v) sodium dodecyl sulphate was added and the cells were incubated again for 3 hrs until lysed (lysis was checked by examining under phase contrast microscopy).

The suspension was heated for 10 mins at 60°C. An equal volume of 24:24:1 (v/v) chloroform : phenol : isoamyl alcohol was added, and inverted until well mixed. The solution was centrifuged for 5 min at 10,000g. Three layers formed, the top aqueous layer contained the nucleic acid, and the middle white band the protein.

The extraction was repeated on the aqueous layer until no more protein was visible. Then twice the volume of cold ethanol was added. The DNA that precipitated at the liquid interface was collected with a glass rod. The DNA was washed with 70% ethanol, spun down to remove the ethanol layer, dried under vacuum, then redissolved in 1 ml TE buffer overnight.

RNase (DNase free) was added at 50 $\mu$ g/ml and incubated at 37°C for 20 - 30 minutes. SDS was added to 0.5% final concentration, and EDTA was added to 20mM final concentration. Proteinase K was added to 50 $\mu$ g/ml and incubated at 50°C for 30 minutes. The phenol chloroform extract and ethanol precipitation was repeated. NaCl was added to 0.2M. The DNA was washed with 70% ethanol, dried and resuspended in 500 $\mu$ l TE buffer.

The DNA was stored in a fridge if used immediately, and at -20°C if used at a later date. An OD measurement of 260/280 was taken to determine the concentration and purity using very clean (chromic acid washed) matched quartz cuvettes. Pure DNA had a ratio of 1:8. If the ratio was 2 or above the DNA contained RNA. If 1.7 or below it contained protein. The concentration was determined using the formula :

$$1 \text{ OD}_{260} = 50\mu\text{g/ml}$$

### 2.14.2 DNA melting curves

DNA had been stored in TE buffer. Because salt has an affect on the  $T_m$  of a melting curve, the DNA was dialysed in 2 changes of SSC (standard saline citrate) buffer at 4°C overnight.

Before the DNA was used it was checked again for purity, by running 1µl of DNA on an agarose gel and examining the bands on the gel for signs of shearing or RNA.

Melting curves were carried out in a Perkin Elmer Lambda 3B UV/Vis Spectrophotometer at  $\lambda$  260nm, using a blank of SSC buffer. Temperature was set on the spectrophotometer to increase from 30°C to 110°C at a continuous rate of increase. A matched pair of quartz cuvettes were used which were sealed to prevent concentration of the DNA by evaporation. To ensure that no air bubbles would interfere with the absorbance readings, the temperature was raised from 30°C to 50°C, and the cuvettes tapped to removed any air bubbles. 1 ml of sample was added to the sample cuvette, at a concentration which gave approximately 0.400 absorbance units. A temperature probe was inserted into a cuvette next to the sample and OD was recorded for every 0.1°C increase. All the melting curves were carried out in duplicate, as were the extractions.

*Escherichia coli*, strain B, DNA (ultrapure) was obtained from Sigma Co. USA, and used as reference DNA.

%G+C was calculated using the equation (from Owen et al., 1979):

$$\%G+C = 51 + 2.24 (T_m \text{ unknown} - T_m \text{ reference})$$

where:  $T_m$  is the midpoint temperature of the thermal melting profile

51 is the G+C mol % value of the reference *E.coli* DNA

## 2.15 Random amplification of polymorphic DNA (RAPDs)

Rt 118.B1 and Rt 118.B2 were isolated from the same pool, and have similar characteristics. In order to determine if they are the same organism or different strains, RAPD analysis was performed. In a second repeat experiment, DNA from GAB 76 and WAI 21 B2 was also used to compare genetic differences between the strains.

DNA was extracted by the method laid out in section 2.13.1 of this thesis. DNA was diluted in MQ water until it was at a concentration of 6ng/ml using the formula:

$$OD_{260} \times \text{Dilution} \times 50\mu\text{g/ml} = \mu\text{g/ml DNA}$$

(knowing that 1  $OD_{260}$  = 50  $\mu\text{g/ml DNA}$ ).

The reaction mixture used was :

50 $\mu\text{l}$  10x PCR buffer

50 $\mu\text{l}$  25mM  $\text{MgCl}_2$

50 $\mu\text{l}$  dNTP

5 $\mu\text{l}$  Taq polymerase

275  $\mu\text{l}$  MQ

2  $\mu\text{l}$  primer

5  $\mu\text{l}$  DNA

The first 5 reagents were mixed and dispensed into sterile Eppendorph tubes as 2 x 21.5  $\mu\text{l}$  aliquots, and then the last two items were added to each Eppendorph. All additions were carried out in a laminar flow unit, under the most sterile conditions possible to reduce the chance of DNA contamination from the environment.

Primers were obtained from Operon Technologies, Alameda California. Many different primers were used in order to find a primer which would produce enough bands to be able to differentiate between species, but not so many bands that a smear would occur. The primers used to anneal to the spirochete DNA were OPR 11, 12, 14-20 (see table 2.1).

**Table 2.1:** Primer sequences used for RAPD analysis of spirochete isolates GAB 76, Rt 118.B1, Rt 118.B2, and Wai 21.B2

Primer	Sequence (5'-3')
OPR 11	GTAGCOGTCT
OPR 12	ACAGGTGOGT
OPR 14	CAGGATTOOC
OPR 15	GGACAAOGAG
OPR 16	CTCTGOGOGT
OPR 17	COGTAOGTAG
OPR 18	GGCTTTGCCA
OPR 19	OCTOCTCATC
OPR 20	ACGGCAAGGA

After cyclic amplification for 30 cycles, the DNA underwent a restriction digest before being separated by gel electrophoresis. The samples had 5 $\mu$ l EcoR1 and 2 $\mu$ l of EcoR1 buffer added to 1 $\mu$ l of the amplified DNA. This was loaded onto a 1% agarose gel and run for approximately one hour. The gel was then stained with ethidium bromide (50 $\mu$ l of 10 mg/ml solution in 500 ml MQ water) for 30 - 40 minutes. The gel was destained with water for 30 minutes, and photographed under a UV light.

## 2.16 Phosphofructokinase assays

The aim of this set of experiments was to find out if the isolates have a PPi dependent PFK activity, and if so, the specificity of the enzyme for PPi as a phosphoryl donor compared to ATP as a phosphoryl donor.

### 2.16.1 Preparation of cell free extract

Cell free extract was prepared from GAB 73, GAB 76, *L. interrogans* serotype *pomona*, *L. interrogans* strain RGA, *S.thermophila* strain RI 19.B1, Rt 118.B1, Rt 118.B2, *S.stensostrepta*, and Wai 21.B2

I was unable to revive *S.stensostrepta*, so used cell pellets frozen by Branwyn Morgan, a former student at the Thermophile Research Unit. *L. interrogans* cultures were supplied as cell pellets. The remaining strains of spirochetes were grown in 1 litre batches. The log phase growth was harvested by centrifugation for 20 minutes at 8000 rpm in a Sorvall SS-3 Automatic centrifuge using a GSA rotor. The pellets were used immediately or frozen at -70°C.

0.5 ml of SE salts was added to the thawed or fresh cell pellets. The resulting suspension was sonicated using the micoprobe of a Dynatech Sonic Dismembrator sonicator at 30% maximum intensity, with 30 second bursts of sonication followed by 30 seconds in ice water. This was repeated until lysis was observed by examination of the cells under the microscope (lysis usually occurred after one minute of sonication).

Sonicated cells had 1 ml of SE salts added to them and were centrifuged at full speed for 20 minutes on a bench centrifuge. The supernatant was removed and diluted with 60mM imadazole buffer pH 7.4 to the appropriate dilution. Duplicate 200 µl aliquots were taken to determine the protein concentration of each sample. These were frozen until required.

### 2.16.2 Materials and Methods for the Assay System

The assay was modified by Janssen and Morgan (1992) from the method of O'Brien et al (1975).

#### Reagents

60 mM Imidazole buffer (pH 7.4 with HCL)

3.5 mM MgCl<sub>2</sub>

1.5 mM Fructose-6-phosphate (F-6-P)

0.2 mM NADH

Linker enzymes (obtained from Sigma Co.)

0.35 U aldolase

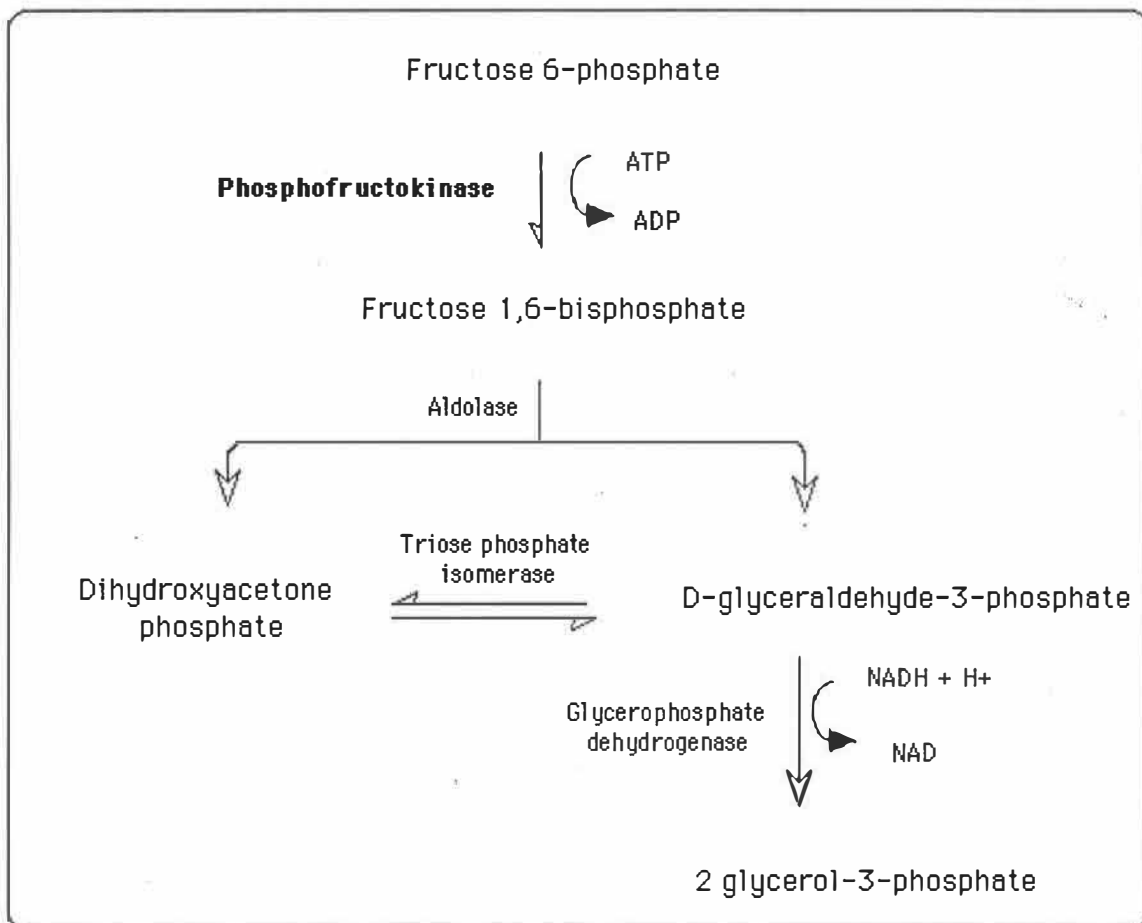
5.0 U  $\alpha$ -glycerol-3-phosphate dehydrogenase

50.0 U triose phosphate isomerase

1.5 mM Fructose-1,6-diphosphate (F-1,6-P)

1mM ATP

1mM  $\text{Na}_4\text{P}_2\text{O}_7$



**Figure 2.1:** The assay system to measure the disappearance of NADH by phosphofructokinase activity

All the reagents were made up at a 10x concentration and had 100µl added to the reaction mixture, which had a final volume of 1000µl. The reaction used either ATP or Na<sub>4</sub>PPi as the phosphoryl donor. F-1,6-P was only used in the linker test (see section 2.16.4). Each assay had 100µl of cell free extract added. The cell free extract was diluted to a concentration that allowed the reduction of the NADH to be measured over a one minute period. If the disappearance of NADH was too fast, the linker enzymes would become the rate limiting step, so the extract would need to be diluted.

Figure 2.1 shows diagrammatically how PFK activity is measured by the catalysis of NADH to NAD.

The MgCl<sub>2</sub>, NADH, imidazole buffer, and linker enzymes were stored at room temperature, whilst ATP, Na<sub>4</sub>PPi, F-6-P and F-1,6-P were frozen in aliquots at -20°C.

The reagents were added to a quartz cuvette containing pre-warmed imidazole buffer. The cuvette was inverted to mix the reagents and allowed to reach 50°C (except for extracts from RI 19.B1 which were carried out at 60°C, *L. interrogans* strains which were carried out at 37°C and *S.stensostrepta* which were carried out at 35°C). 100µl of the pre-warmed cell free extract was added and mixed by inversion of the cuvette. The reduction of NADH to NAD was continuously recorded over a two minute period.

Assays were carried out in triplicate in a Perkin Elmer Lambda 3B UV/Vis Spectrophotometer at the required temperature for each extract,  $\lambda = 340\text{nm}$  using a blank of imadazole buffer. The resulting graph was plotted and the change in absorbance per second on the sloping section of the graph was calculated.

### 2.16.3 Controls

Several controls were carried out to test that the assay system was working before experiments started using the cell free extracts.

In order to test that the linkers were working, a reaction was set up using 100µl of MgCl<sub>2</sub>, F-1,6-P, NADH, and 600µl of imidazole buffer (all at the concentrations described in 2.15.3). Reagents were mixed by inversion and allowed to equilibrate to 50°C, before the

addition of 100µl of linker enzymes. This test should give a rapid drop in absorbance as the NADH is reduced to NAD. When necessary F-1,6-P was added to a cell free extract assay when no change in absorbance was detected to ensure that the lack of NADH reduction was because the enzyme could not utilise the substrate, rather than a problem with the assay system.

The linker test was performed before each day's assays. This ensured that all the reagents were working, and also demonstrated the speed at which the linker enzymes were able to perform. It was important to ensure that the rate limiting step of the reaction was the PFK enzyme from the cell free extracts rather than the linker enzymes.

Pyrophosphate dependent F-6-P kinase extracted from *Propionibacterium freudenreichii* (*shermanii*) was bought from Sigma Co. USA to be used as a positive control. 100µl containing 2 units of this enzyme was used in the reaction mixture instead of the cell free extract. A rapid drop in absorbance was expected when PPi was the phosphoryl donor, but no change in absorbance when ATP was the phosphoryl donor.

ATP dependent F-6-P kinase type III from rabbit muscle was also bought from Sigma, and again 2 units was used in the reaction instead of the cell free extract. This test reduced NADH to NAD when ATP was the phosphoryl donor, but no reduction when PPi was the donor.

To demonstrate that the reduction in NADH was from an enzyme in the cell free extract rather than some other component, 100µl of the extract was boiled for 5 minutes to denature the enzyme and then added to the reaction mixture.

Assays were carried out in triplicate:

1. with ATP as the phosphoryl donor
2. with PPi as the phosphoryl donor
3. with 100µl of imidazole buffer instead of a phosphoryl donor.

## 2.17 Protein Estimations

Adapted from the Lowry Assay (Bollag et al, 1986, Morgan 1994)

### Reagent A

Water	100ml
CuSO <sub>4</sub> (5H <sub>2</sub> O)	0.5g
Sodium citrate	1.0g

### Reagent B

Water	100ml
Na <sub>2</sub> CO <sub>3</sub>	2.0g
NaOH	0.4g

### Reagent C

Reagent A	1ml
Reagent B	50ml

Make reagent C immediately before use and discard after one hour.

### Reagent D

1:3 Folin Ciocalteu reagent (Sigma): Water

### Method

1) 200µl of protein sample or standard was added to 1000µl of reagent C, mixed and left to stand at room temperature for 5 - 10 minutes

2) 200µl of solution D was added and vortexed

3) After 20 - 30 minutes the samples were read at A<sub>750</sub> on a Varian DMS 80 UV/Vis spectrophotometer.

Blanks were made containing 200µl of distilled water instead of protein.

A standard curve was made using BSA at concentrations of 0, 50, 100, 150, 200, 250 and 300 µg/ml.

Protein concentrations of unknowns were calculated from the standard curve.

## **2.18 Enzyme Purification**

In order to study the enzyme phosphofructokinase further it was decided to attempt to purify it from Rt 118.B2. Rt 118.B2 was the fastest growing of the spirochetes with the exception of R1 19.B1 which was not always reliable (ie two out of four subcultures would regularly not grow).

### **2.18.1 Bulk cultivation of Rt 118.B2**

To obtain the cell mass required to harvest the enzyme it was necessary to cultivate 80 l of Rt118.B2.

4 x 20 litre vessels were autoclaved containing 1 l of water with 20 ml each of resazurine (20mg/ml).

Media (which did not contain calcium) was made up in a 40 l container with tap water. 280 ml of 20%  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  was added to the SE media. This was pumped through a sterile 0.2 µm filter into two of the 20 l vessels.  $\text{CaCl}_2$  (5.92g in solution) was added last to the vessels, as it would precipitate the media if added before this step.

Sterile rubber stoppers with a glass air lock were fitted to the vessels, and the air lock was partially filled with 5 ml 20% sodium sulphite to act as an anti-microbial agent. The spirochetes produced gas while growing, and the air lock allowed this to escape without the introduction of microbial contaminants.

The vessels were placed in a water bath at 55°C to warm for several hours. Paraffin was flooded over the water to prevent evaporation, and polystyrene balls were floated on this to retain the heat in the bath.

N<sub>2</sub> was sparged through a sterile 0.2µm filter fitted to each 20 litre vessel for at least 30 minutes before inoculation, and for 10 minutes after inoculation. N<sub>2</sub> sparging provided an anaerobic atmosphere and aided in the circulation of the media, which helped to bring the media to the correct temperature.

Each vessel was inoculated with 800ml of log phase Rt 118.B2 culture and allowed to grow until high cell numbers (50 cells or more per field of view) of log phase growth was observed under the phase contrast microscope. Vessels were then removed from the water bath and cooled to 4°C overnight.

### **2.18.2 Harvesting bulk cultures**

The first attempt at harvesting 80 l of Rt 118.B2 used a Sharple centrifuge. This centrifuge works by a continuous intake of culture which is spun so the pellet forms a smear against the bowl of the centrifuge. The supernatant continuously exits from the opposite end of the centrifuge. After the 80 l of culture had been processed this way, the centrifuge bowl must then be scraped out. This method gave a poor cell yield (only 32 g) and also contaminated the pellet with flecks of rust from the aging centrifuge bowl. Microscopic examination of the supernatant showed that it still contained many spirochetes. The pellet was washed with SE media salts, but was very cohesive and it was not possible to resuspend it in the media.

The subsequent harvestings of bulk cultures used an Amicon Hollow Fibre Filtration system. This system has two advantages over the Sharple centrifuge, it can be operated at 4°C and it is not stressful on the cells. When the total volume was reduced to 5 l, 10 l of SE salts were added, once this had reduced to 5 l, a further 10 l of SE salts was added. This washed the cells, as once they have formed a pellet after centrifugation the cells were extremely difficult to re-suspend. Once volumes were down again to 5 l the cells were drained out and spun down in a GSA rotor for 30 min at 9000 rpm. This method resulted in 52 g of cells.

### **2.18.3 Purification of PFK from Rt 118.B2**

All columns were at room temperature and were run at the maximum flow rate possible without causing pressure build up or compaction of the columns.

### **2.18.3.1 First Attempt at purification of PFK from isolate Rt 118.B2**

The initial protocol used to try to purify PFK from Rt 118.B2 was based on the paper by O'Brien et al 1975.

### **2.18.3.2 Preparation of cell free extracts**

Cells were re-suspended (as best they could) in 60 ml Imadazole 0.2M buffer pH 6.8. The suspension was sonicated by a Dynatech Sonic Dismembrator sonicator at 50% maximum intensity for 1 minute, and then allowed to cool in an ice bath for 1 minute. This process was repeated six times, and the suspension was examined microscopically to ensure all the cells had been disrupted. Sonicated cells were centrifuged for 20 minutes at 10, 000 rpm. Supernatant was decanted and stored at 4°C. The pellet was resuspended in 40 ml imadazole buffer and centrifuged again at 12 000 rpm for 30 minutes. Supernatant was decanted again and pooled with the first supernatant. The total volume of cell free extract measured 146ml.

### **2.18.3.3 Fractionation with Ammonium Sulphate**

The crude extract was placed in an ice bath in a 4°C cold room and ammonium sulphate was slowly added with stirring until 55% saturation was reached. The sample was allowed to precipitate over night. The extract was centrifuged at 12 000 rpm for 25 minutes. The pellet was resuspended in imadazole buffer pH 6.8. At this point according to the protocol of O'Brien et al 1975, the supernatant could be discarded. However both the pellet and the supernatant contained PFK activity in almost equal amounts, so they were re-pooled.

### **2.18.3.4 Cellulose Phosphate Chromatography**

Cellulose phosphate had been allowed to soak overnight to allow swelling of the particles. It was washed several times with 0.02 M imadazole buffer (pH 6.8) to remove very fine particles that could block the column. The cellulose phosphate column had a final volume of 74.3 ml (140mm). The column was equilibrated with 0.02 M imadazole buffer before loading on the crude extract. O'Brien reports that 90% of enzyme activity binds to the column under these conditions, however the results obtained were closer to 5% of activity. The enzyme that had bound to the column was eluted off with 0.02 M imadazole/HCL buffer, pH 6.8, containing  $5 \times 10^{-4}$  M fructose-1,6-P<sub>2</sub>.

### **2.18.3.5 Ultrafiltration**

The ionic strength of the extract was very high because of the ammonium sulphate content which was possibly why the enzyme did not bind to the cellulose phosphate column. The sample was ultrafiltered using an Amicon PM 10 membrane to concentrate the sample and to dilute out the ammonium sulphate. Ultrafiltration took three days, used three changes of 0.02 M imadazole buffer (pH 6.8) and was carried out at 4°C.

### **2.18.3.6 Loading onto the cellulose phosphate column.**

Once the extract had the same conductivity as the 0.02M imadazole buffer, the extract was again loaded onto the cellulose phosphate column. The loading was at 10% maximum pump rate as any higher than this caused compaction of the column. Approximately 50% of the enzyme did not bind to the column.

### **2.18.3.7 Elution with fructose-1,6-P<sub>2</sub>**

0.05 mM F-1,6-P<sub>2</sub> was pumped onto the column to elute the PFK. Fractions were run through a UV detector at 280 nm to observe protein peaks, and results were plotted. Fractions were collected and assayed for activity. Fractions with high activity (ie activity which could be observed at 1/50 dilution) were pooled. The enzyme did not elute off the column in a sharp peak, but in a very wide band. 5mM F-1,6-P<sub>2</sub> was used to attempt to elute the remainder of the enzyme off the column in a sharp band. The column was only able to run very slowly (0.5 ml/min) as pressure caused compaction which necessitated the plunger be removed and the column mixed to re-suspend the cellulose-phosphate particles.

### **2.18.3.8 Mono-Q column**

The extract was loaded onto a Pharmacia HR 10/10 Mono Q column (an anion exchange column) at 4 ml/min using 20mM imadazole buffer pH 6.8 to wash/equilibrate the column. 1M NaCl was used to create a salt elution gradient. The gradient was created over 60 minutes from 0% NaCl at time 0, to 100% NaCl at time 60 minutes. The column was washed for a further 10 minutes with 1M NaCl. Fractions were collected and measured for activity. Fractions with activity were pooled.

### 2.18.3.9 SDS Page Electrophoresis

Pooled fractions which contained activity were loaded onto an SDS Page mini-gel from Pharmacia Ltd. A PhastSystem Separation and Control Unit was used to run the minigels in accordance with the manufacturer's specifications.

The sample was prepared by adding 10 $\mu$ l of sample to 5 $\mu$ l SDS buffer, centrifuged to mix, and autoclaved for 5 minutes to denature the protein. 1 $\mu$ l was loaded onto the minigel.

### 2.18.3.10 Silver staining method for SDS gels (Phastgel)

Once the SDS buffer had run to the end of the gel, the gel was stained using the following protocol:

1. 40% methanol, 10% acetic acid 30 minutes
2. 20% ethanol, 5% acetic acid 2 x 15 minutes
3. 8.3% glutaraldehyde 5 minutes
4. rinse with MQ water
5. 0.25% AgNO<sub>3</sub> 20 minutes
6. rinse with MQ water
7. developer 0.04% formaldehyde, 2.5% Na<sub>2</sub>CO<sub>3</sub>
8. stop: 5% acetic acid
9. store: 10% acetic acid, 5% glycerol

## 2.18.4 Second attempt at purification of PFK from isolate Rt118.B2

### 2.18.4.1 Preparation of cell free extract

47.2 g (wet-weight) of Rt 118.B2 was suspended in 125 ml 20mM imadazole buffer pH 6.8. The suspension was sonicated for 1 minute at 50% maximum intensity, then cooled on ice for 1 minute. This cycle was repeated eight times until cells were lysed completely (as observed under phase contrast microscopy). The lysed cells were ultracentrifuged for 1 hour at 35 000 rpm. 90% of the activity was found in the supernatant.

#### 2.18.4.2 Phenol sepharose column

Instead of using an ammonium sulphate fractionation step, a phenol sepharose column was used (Hi load 26/10 Phenyl Sepharose column from Pharmacia). The 73 ml of cell free extract had 7.9g of  $(\text{NH}_4)_2\text{SO}_4$  added to raise the ionic strength to 1M  $(\text{NH}_4)_2\text{SO}_4$ . This was loaded onto a phenol sepharose column with the loading buffer : 20mM imadazole buffer + 1M  $(\text{NH}_4)_2\text{SO}_4$  adjusted to pH 6.8 with HCL at 4 ml/min. The extract bound to the column and was eluted off with imadazole buffer. The eluting buffer contained 20 mM imadazole buffer + 1M  $(\text{NH}_4)_2\text{SO}_4$  which was changed to 20mM imadazole buffer + 0%  $(\text{NH}_4)_2\text{SO}_4$  with a linear gradient over 60 minutes. Activity was found in fractions 1-47, which were pooled. The enzyme eluted off in the first fraction, which indicates that it was only weakly bound to the column.

#### 2.18.4.3 Mono Q column

The  $(\text{NH}_4)_2\text{SO}_4$  in the sample from the phenol sepharose column was removed by ultrafiltration with 20mM imadazole buffer pH 6.8. The sample was then loaded onto a Mono Q column. The PFK activity eluted with the solvent front. Although the pH was satisfactory (pH 6.75), the ionic strength was a little high at 1.9 mS which is equivalent to 20mM  $(\text{NH}_4)_2\text{SO}_4$ . So the sample was diluted from 100ml to 400 ml with 20mM imadazole buffer pH 6.8 to dilute any residual  $(\text{NH}_4)_2\text{SO}_4$  which may have interfered with the PFK binding to the column.

The diluted extract was loaded onto the column again, using the same elution conditions. The PFK did not bind to the column.

#### 2.18.4.4 Phenol sepharose column

460 ml of sample was loaded back onto the phenol sepharose column to further remove non-PFK proteins and to concentrate it. Because the PFK did not bind well at 1M  $(\text{NH}_4)_2\text{SO}_4$  the first time the phenol sepharose column was used (section 2.18.4.2),  $(\text{NH}_4)_2\text{SO}_4$  was added to the sample until a 1.5M concentration was reached. A linear gradient was established, from 20mM imadazole buffer containing 1.5M  $(\text{NH}_4)_2\text{SO}_4$ , to 20mM imadazole buffer containing 0 mM  $(\text{NH}_4)_2\text{SO}_4$  after 60 minutes. The enzyme was eluted off the column in fractions 33-39 which were pooled.

#### **2.18.4.5 Rotofor System**

The sample was ultrafiltered using an Amicon YM 10 membrane in 3 changes of imadazole buffer to remove the  $(\text{NH}_4)_2\text{SO}_4$ . The rotofor is designed to separate proteins on the basis of their isoelectric point. The rotofor was a Bio-Rad model 3000/300. Before loading on the sample a gradient was established using 2ml of 4N urea and 18ml of 0.5% electrolytes. Once the voltage and milli Ampage were constant (approximately 1 hour) the sample was loaded. After 40 minutes the sample was collected in 20 fractions, and each fraction was assayed for PFK activity. Fraction 10-14 had PFK activity which was a wider spread than foreseen.

#### **2.18.5 Third attempt at purification of PFK from isolate Rt118.B2**

Cells were sonicated using the protocol in section 2.18.4.1 and ultracentrifuged at 34 000 rpm for 75 minutes. The supernatant contained some particulate matter so was filtered through a 0.2 $\mu\text{m}$  Millipore filter before loading onto a Mono Q (anion exchange) column. Elution and sample conditions used the same protocol as section 2.18.4.3. The PFK did not bind. An enzyme which won't bind onto an anion exchange column will usually bind onto a cation exchange column and vice versa. So the sample was loaded onto a cation exchange column (Mono S HR 5/5 from Pharmacia) at 1 ml/min using 20mM imadazole buffer pH 6.8 as the equilibrating/loading buffer. The PFK eluted off with the solvent front.

The sample was then loaded onto a phenol sepharose column under the conditions outlined in section 2.18.4.4. The fractions with activity were pooled and then ultrafiltered using a YM 10 membrane in three changes of imadazole buffer to remove  $(\text{NH}_4)_2\text{SO}_4$ . An SDS page gel was made of the sample and the bands on the gel seemed well separated.

##### **2.18.5.1 Fractogel**

The fractogel was used to try to separate the remaining proteins in the sample by size. The gel was prepared by using fractogel TSK HW-55 (Merck) which has a pre-swollen particle size of 0.032 - 0.063 mm. The gel had been pre-washed in 20 mM imadazole buffer prior to packing. The column was 1 meter long and had a diameter of 1cm. The flow rate was 1.2 ml/min. 10 ml of the sample was loaded onto the fractogel. The sample eluted with the void volume, along with the six other proteins associated with it. A 50mM solution of cytochrome

C and blue dextran was loaded onto the column to see if the column gave adequate separation of bands. Separation was adequate which leads to the speculation that the remaining proteins in the sample were interacting somehow with each other. 0.10 ml of sample was loaded on to an HPLC column (Bio-gel HPHT 100 x 7.8 mm) at a flow rate of 1.0 ml/min to try to separate the proteins by size. All the proteins came out in one peak. Finally the enzyme was loaded onto the rotofor system as in section 2.18.4.5. This gave very poor separation as activity was found in fractions 5 - 14 (ideally activity should have been in one or a maximum of two fractions).

### **2.18.6 Fourth attempt at purification of PFK from isolate Rt118.B2**

The final attempt of purification was based on protocols by Li et al 1995.

80l of Rt 118.B2 was grown up as in section 2.18.1. Cells were harvested by an Amicon Hollow Fibre Filtration System using the same protocol as in section 2.18.2. The cell free extract was extracted as in 2.18.4.1.

120 ml of cell free extract was loaded onto a Mono Q column using the protocol in section 2.18.3.7. All the PFK activity eluted off with the void volume.

$\text{NH}_4\text{SO}_4$  was added to the 140 ml of sample until the concentration reached 1.5 M. The sample was loaded onto a fast flow phenol sepharose column and eluted off with the same conditions used in section 2.18.4.4. Fractions containing PFK activity (fractions 28-37) were pooled (total volume was 40 ml) and the ammonium sulphate removed by ultrafiltration (section 2.18.3.4).

The 15 ml of enzyme from ultrafiltration was loaded onto a cellulose phosphate column which was prepared and run following the protocol in section 2.18.3.3. Again the enzyme did not bind to this column, despite Li et al reporting good recovery from using this column.

The recovered enzyme was loaded onto a mono S column. The elution buffer was potassium phosphate buffer 20mM pH 7.2 (20 mM  $\text{KH}_2\text{PO}_4$  and 20 mM  $\text{K}_2\text{HPO}_4$  are mixed to give a pH of 7.2). This buffer was used to ascertain if the imadazole buffer or the pH which was used in section 2.18.5 was interfering with the enzyme binding to the column. The enzyme eluted off with the void volume.

The sample was ultrafiltered in three changes of 0.025M Bis-Tris Buffer pH 7.1 to equilibrate it with the buffer which is used in the Mono P column, and to concentrate the sample down to 6ml. Mono P is used for chromatofocusing, a technique for separating molecules on the basis of their pI. The Mono P HR 5/20 4 ml column was bought from Pharmacia. The loading buffer was 0.025M Bis-Tris pH 7.1 and the elutant was a 3M saturated solution of iminodiacetic acid pH 4.0. A linear gradient was run over one hour at a flow rate of 1.5 ml/minute to prepare the column gradient. The sample was loaded onto the Mono-P column and PFK was eluted off with the void volume. Other proteins bound to the column and were eluted off.

The 15.7ml of PFK from the Mono-P column was loaded onto a Phenol-Sepharose column (see section 2.18.4.2) to further purify the sample and an SDS-Page gel was run (see section 2.18.3.8) to see how pure the fractions with activity were.

## **2.19 Characterisation of the partially purified PFK**

### **2.19.1 Optimum pH of the partially purified PFK**

Imadazole buffer (60mM) was prepared to cover the pH range of 6.0-8.0

The reagents used in the PFK assay (section 2.16.2) were also prepared over the range of 6.0-8.0 with the exception of the partially purified PFK which was at pH 7.4. The assay was carried out as in section 2.16.3. Protein estimation of the PFK was assayed as in section 2.17.

### **2.19.2 Optimum temperature of the partially purified PFK**

The linker enzyme in the PFK assay becomes inactivated at high temperatures as the protein starts to precipitate. To avoid this problem reactions were carried out at the required temperatures without the linker enzyme and NADH present. Reaction mixtures containing 102  $\mu$ l partially purified PFK, 102  $\mu$ l  $MgCl_2$  3.5 mM, 102 $\mu$ l  $Na_4PPi$  1mM, 102  $\mu$ l F-6-P<sup>1</sup> 1.5 mM and 408 $\mu$ l imadazole buffer 60 mM were made up in ependorf tubes. The reagents were incubated in water baths at temperatures from 50°C- 70°C, in sealed ependorf tubes to minimise losses due to evaporation. The assays were then plunged into a water bath at 95°C

for two minutes to inactivate the enzymes and spun for 30 seconds at maximum on a bench microfuge. 800 $\mu$ l of the assay mixture was pipetted into a quartz cuvette with 100 $\mu$ l of NADH. Once the assay temperature had equilibrated to 50°C, 100  $\mu$ l of linker enzyme was added and the change in absorbance read on the spectrophotometer at 400nm. 1.5 mM F-1,6-P was incubated at 85°C to ensure that it was not affected by high temperature, as F-1,6-P is the product of PFK activity. The protein content of the PFK was estimated using the method in section 2.17.

### **2.19.3 Thermostability of the partially purified PFK**

400  $\mu$ l of partially purified PFK was mixed with an equal volume of imadazole buffer 20mM pH 7.4 and incubated in a water baths at 65°C, 75°C and 85°C. The temperature inside the ependorf was measured with a thermocouple probe to ensure the contents were at the required temperature. The ependorfs were sealed with parafilm to minimise evaporation. At various time points an aliquot of the enzyme was removed and assayed for activity.

### **2.19.4 Kinetic properties of the partially purified PFK**

#### **2.19.4.1 $K_m$ and $V_{max}$ of Fructose-6-phosphate**

Fructose-6-Phosphate was prepared at concentrations from 1.5 to 0.0125mM in 60mM Imadazole buffer pH 7.4. The activity of the partially purified enzyme preparation was assayed by standard procedure and the initial rate of reaction at each substrate concentration was used to obtain a  $K_m$  rate from a Lineweaver-Burk plot of results.

#### **2.19.4.2 Phosphate donors used by the partially purified PFK and their kinetics**

Na<sub>4</sub>PPi was prepared at concentrations from 10mM to 0.1mM in 60mM Imadazole buffer pH 7.4. The activity of the partially purified enzyme preparation was assayed by standard procedure and the initial rate of reaction at each substrate concentration was used to obtain a  $K_m$  rate from a Lineweaver-Burk plot of results.

Alternative phosphate donors were used in the PFK assay to determine if the PFK extracted from Rt 118.B2 was able to use a phosphate donor other than  $\text{Na}_4\text{P}_2\text{O}_7$ . 1mM solutions of Type 15 Phosphate Glass (average chain length of  $\text{Na}_{20}\text{P}_{18}\text{O}_{55}$ ) and type 75+ phosphate glass (average chain length of  $\text{Na}_{79}\text{P}_{75}\text{O}_{226}$ ) were prepared in 60mM Imadazole buffer pH 7.4 and substituted for the phosphoryl donor in the PFK assay.

Tripolyphosphate (pentasodium tripolyphosphate  $\text{Na}_5\text{P}_3\text{O}_{10}$ ) was prepared at concentrations of 20mM - 0.1mM in 60mM Imadazole buffer pH 7.4. The activity of the partially purified enzyme preparation was assayed by standard procedure and the initial rate of reaction at each substrate concentration was used to obtain a  $K_m$  rate from a Lineweaver-Burk plot of results.

## CHAPTER THREE

### Results

#### Part A: Characterisation of four strains of free-living thermophilic spirochetes

##### 3.1 Purification of contaminated stock cultures

Purification of contaminated stock cultures of GAB 76, *S.thermophila* strain RI19.B1, Rt 118.B1 and Wai 21.B2 and purification of a spirochete from pool Rt 118 (designated as strain Rt 118.B2) was required.

###### 3.1.1 Roll Tubes

All attempts at retrieving pure cultures of spirochetes from contaminated stock cultures using the roll tube method were unsuccessful. The only visible colonies that developed were always the contaminating rod, even though spirochetes were sometimes more numerous in the inoculum. Because agar can be inhibitory to some bacteria this approach was repeated with gelrite as the gelling agent, but successful isolation was still not obtained.

###### 3.1.2 Phosphate supply in the SE medium

Sodium pyrophosphate was substituted as the phosphate source in SE media in place of  $\text{KH}_2\text{PO}_4$  to attempt give the spirochetes a competitive advantage over the contaminants, assuming that the spirochetes had a PPI dependent PFK while the contaminants did not.

There was no significant increase in spirochetes numbers when inoculated into the sodium pyrophosphate media, compared to inoculations in normal SE media.

It is likely that many organisms (including the contaminant) contain a PFK independent pyrophosphatase for phosphate uptake so that no advantage was conferred.

### **3.1.3 Enrichment by filtration**

None of the spirochetes passed through the 0.45 $\mu$ m filter. Although this is often a very efficient method for isolation of spirochetes it is not always successful as Harwood et al 1982 found when they tried to isolate spirochetes from deep sea sediments and water from Sargasso Sea.

### **3.1.4 Purification by serial dilution**

Serial dilution did not remove the rod contaminants, even if the spirochetes appeared to be in greater numbers than the rods initially. The spirochetes seemed to require a minimum number to grow well at low concentrations, whilst the rods had no such inhibitions. Diluting the inoculum seemed to select for the rods.

### **3.1.5 Purification with antibiotics**

Attempts to obtain pure cultures using single antibiotic in the media (rifampicin at 80 $\mu$ g/ml or sodium azide at 500 $\mu$ g/ml) failed. Although the spirochetes are resistant to rifampicin, at these concentrations they do not grow well. The rods contaminating the culture were much more resistant to this antibiotic, and in fact were being selected for. A similar situation was created using a combination of rifampicin and sodium azide at the above concentrations.

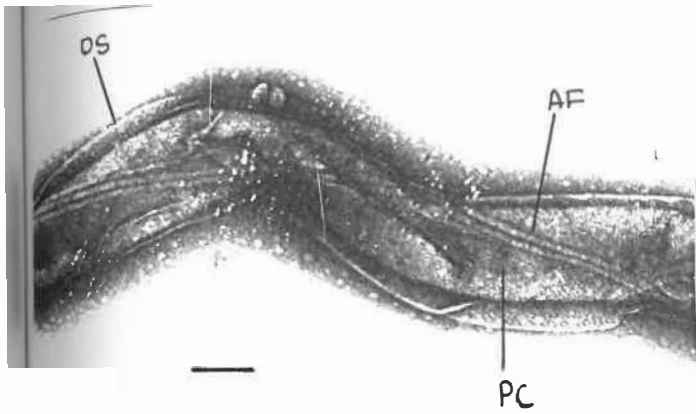
The combination of tetracycline at 20 $\mu$ g/ml, rifampicin 100 $\mu$ g/ml and sodium azide at 500 $\mu$ g/ml was successful in obtaining a pure culture, when allowed to sit at room temperature for 24 hours after inoculation with the cultures but before incubation. All four contaminated cultures and Rt 118.B2 from the raw pool sample were successfully isolated using this method. The contaminants were probably able to grow slowly at room temperature, which allowed them to have a longer exposure to the high levels of antibiotics than the spirochetes.

## 3.2 Microscopy

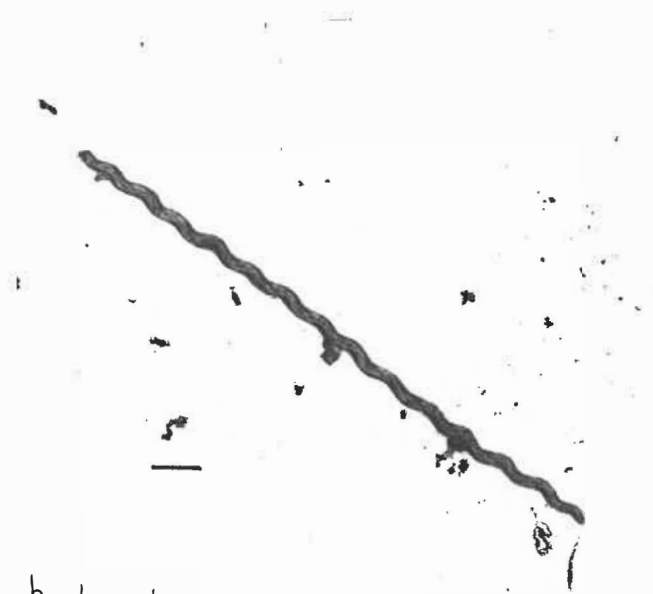
Figures 3.1 a-d show negative stained preparations of isolates Rt 118.B2 and GAB 76. They are representative of all four isolates, as the morphology of all four strain are very similar. Both electron and phase contrast microscopy revealed the strains examined have typical spirochete morphology. Spirochetes were thin, long and coiled and the Gram staining reaction was negative.

Cultures in stationary phase or those exposed to oxygen had many spherical bodies associated with the culture. These bodies were similar in appearance to those formed by other cultures in stationary phase (Canale-Parola., 1991; Pohlschroeder et al., 1993) Stressed cultures would also curl into themselves forming hooks or circles. Very old cultures (ie several months old) would begin to straighten out and become more rod shaped as would cultures which had been spun hard in a centrifuge. Cultures always had high numbers of spherical bodies and hooked cells if the media had been contaminated with oxygen. Cultures in this state were non-viable.

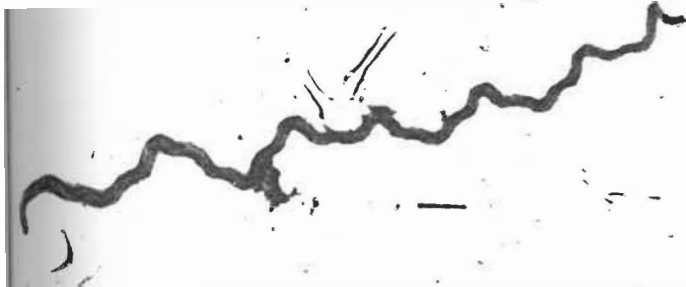
GAB 73 and GAB 76 were highly motile. The other strains were less motile, which is probably due to the rapid cooling of the spirochetes on the microscope slides. Regular coiling was observed.



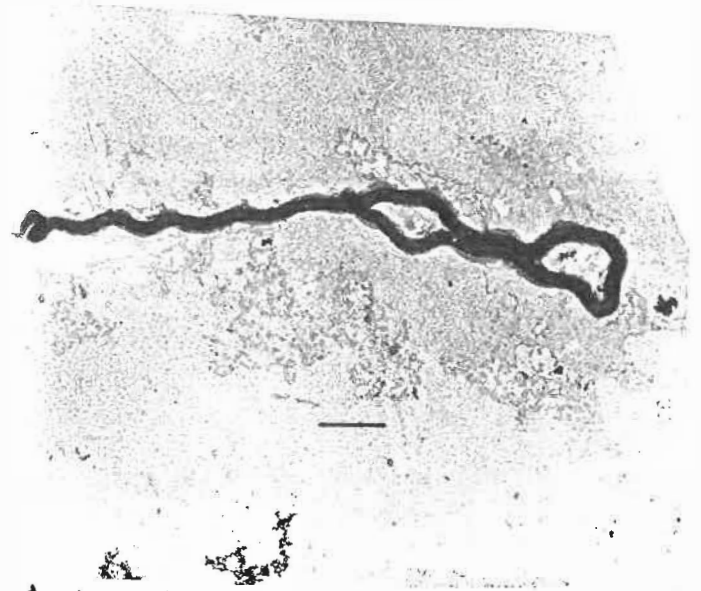
a. bar = 10  $\mu$ m



b. bar = 1  $\mu$ m



bar = 1  $\mu$ m



d. bar = 1  $\mu$ m

**Figure 3.1:** a-d Electron micrographs of isolates Rt 118.B2 and GAB 76. (a) Rt 118.B2 showing the outer sheath (OS), axil fibril (AF), and protoplasmic cylinder (PC). (b) isolate Rt 118.B2 in log phase growth. (c) isolate GAB 76 in log phase growth. (d) a dying cell of isolate Rt 118.B2. Note the formation of a spherical body at the end of the cell and that the cell is starting to curl up upon itself.

### 3.3 pH Optima

Table 3.1 shows the growth response of the four spirochete isolates at various pH values. Growth was measured as O.D. at 400nm. The optimal growth for all four species was exhibited when the initial pH was 6.5 in 100 mM Bis Tris buffer and pH 7.0 in 100 mM Hepes buffer (Table 3.1). Growth for all species was possible when the initial pH was 7.5 to pH 6.5. Growth in Tris buffer (pH 7.5, 8.0, 8.5) was not evident until late in the 48 hour incubation period. Tris buffer is probably inhibitory to the spirochetes, as growth was observed at pH 7.5 in Hepes buffer. Because of this, Tris results are not included in table 3.1.

**Table 3.1:** Growth response of four spirochete isolates in buffered media at different pH values

Buffer	pH	GAB 76	Rt 118. B1	Rt 118. B2	Wai 21. B2	Final pH of the medium
Bis Tris	6.0	0.1	0.1	0.0	0.1	5.3
	6.5	0.9	1.0	0.8	0.8	6.0
	7.0	0.5	0.5	0.7	0.5	6.4
Hepes	7.0	0.7	0.7	0.7	0.3	6.0
	7.5	0.2	0.2	0.2	0.2	7.1
	8.0	0.0	0.0	0.0	0.0	7.9

Buffer strength was increased from 10mM (used in standard SE media) to 100mM to ensure pH remained stable through out growth. In 10mM, buffer the pH dropped (after growth) to around 4.8 regardless of the initial pH. This drop is most likely due to the production of acetic acid as an end product of fermentative growth. When 10mM buffer was used the optimum pH was higher than with 100mM buffer. Optima pH were:

Bis Tris:           pH 7.0 for Wai 21.B2, Rt 118.B1 and Rt 118.B2.  
                           pH 6.5 for GAB 76

Hepes                      pH 7.2 for Rt 118.B1  
                                  pH 7.5 for Rt 118.B2, Wai 21. B2, GAB 76

These higher pH optimums reflect the poorer buffering capacity at 10 mM concentration. A higher pH at the start (but not so high as to be inhibitory) gives longer growing time before acid builds up to an inhibitory level. When 100mM buffer was used the OD readings were also considerably higher, which reflects the longer time the spirochetes have at their optimum pH before acid production becomes inhibitory.

### 3.5 Cardinal growth temperatures

The cardinal temperatures for each isolate are shown in Table 3.2. The optimal temperature was the temperature that the species grew the fastest and to the highest OD. All the species exhibited growth almost as high and fast as the optimum a degree higher and lower than the temperature recorded in Table 3.2.

**Table 3.2:** Cardinal growth temperatures and temperature range of four spirochete isolates

	GAB 76	Rt 118.B1	Rt 118.B2	Wai 21 B2
Optimal Temperature	57°C	52°C	55°C	59°C
Temperature range	40-61°C	38-63°C	30-61°C	37-62°C

Minimum temperatures are difficult to measure precisely. Over the five day period in which readings were taken it became apparent that the longer the cultures were left to incubate, the lower the minimum growth temperature was recorded. So if the experiment was allowed to run for a greater length of time (ie ten days) it is possible that even lower temperatures capable of supporting the isolates growth would be discovered, as the organisms slowly grew to numbers high enough to be measured. After the five day incubation period the lowest temperature in which growth was measured was 40°C for GAB 76, 38°C for Rt 118.B1, 30°C for Rt 118.B2 and 37°C for Wai 21.B2.

## 3.6 Growth curves

Figures 3.2 - 3.5 display the growth curves of GAB 76, Rt 118.B1, Rt 118.B2, and Wai 21. B2. Doubling times were 309 minutes for GAB 76, 300 minutes for Rt 118.B1, 221 minutes for Rt 118.B2, and 333 minutes for Wai 21 B2. Each isolate was incubated at its optimal temperature (Table 3.2). These times reflect well the experimenter's experiences in culturing these strains in the laboratory. Rt 118.B2 was always the fastest growing, with the highest OD of the group, while Wai 21 B2 and GAB 76 were always the slowest and most difficult to grow.

The doubling time for *S.thermophila* strain RI 19.B1 was 199 min under optimum conditions of NaCl concentration, pH and temperature (Rainey et al., 1991). The doubling time of Rt 118. B2 (221 minutes) is comparable to that of *S.thermophila* strain RI 19.B1.

Normal SE media was used in the growth curve experiments, which means that the pH was at 7.2, not at the individual optima pH of the isolates. The buffer concentration was at 10mM not at 100mM. pH experiments (section 3.3) showed a 100mM buffer concentration dramatically increased cell yield. Altering the SE media to optimal pH and buffering conditions would probably decrease the doubling times of these strains.

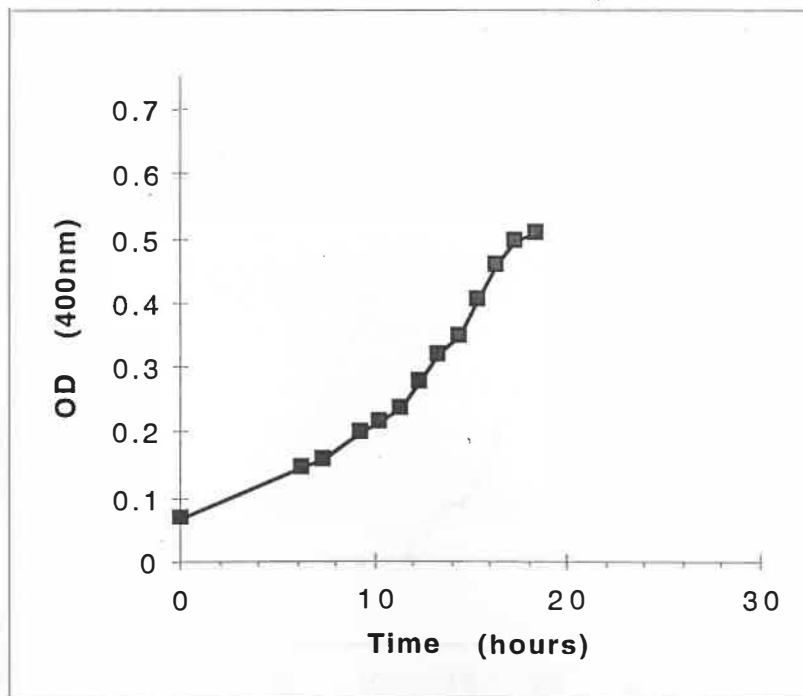
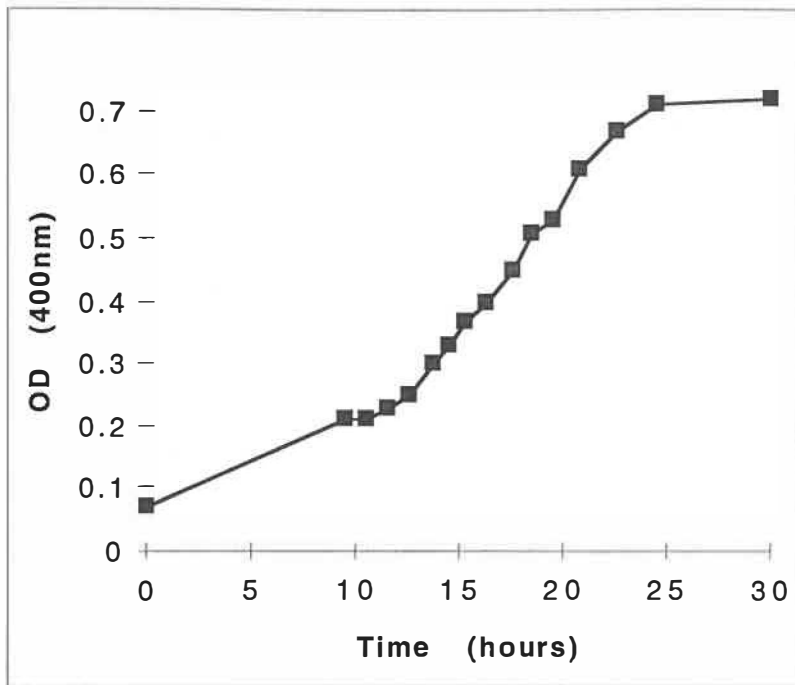
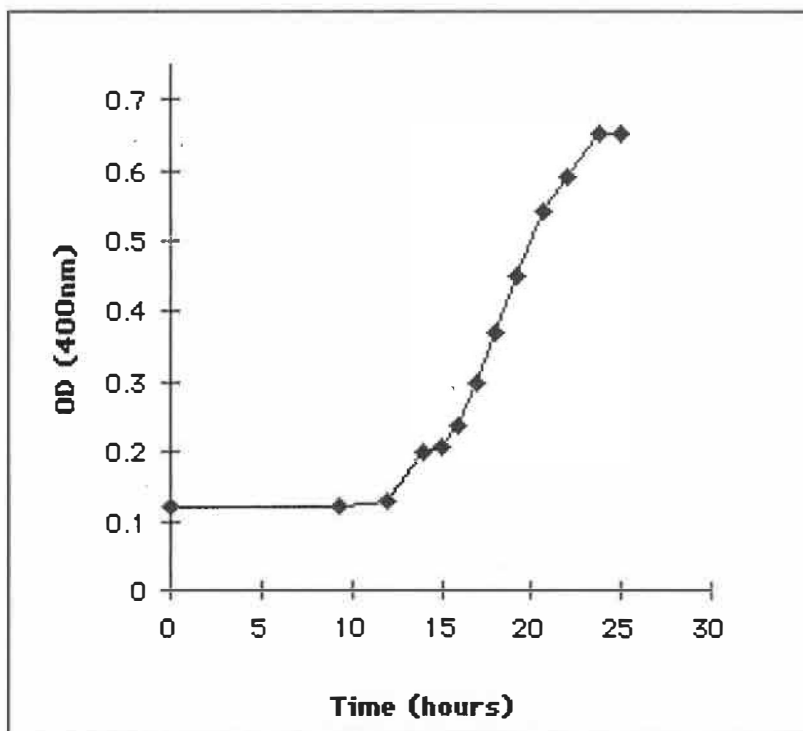


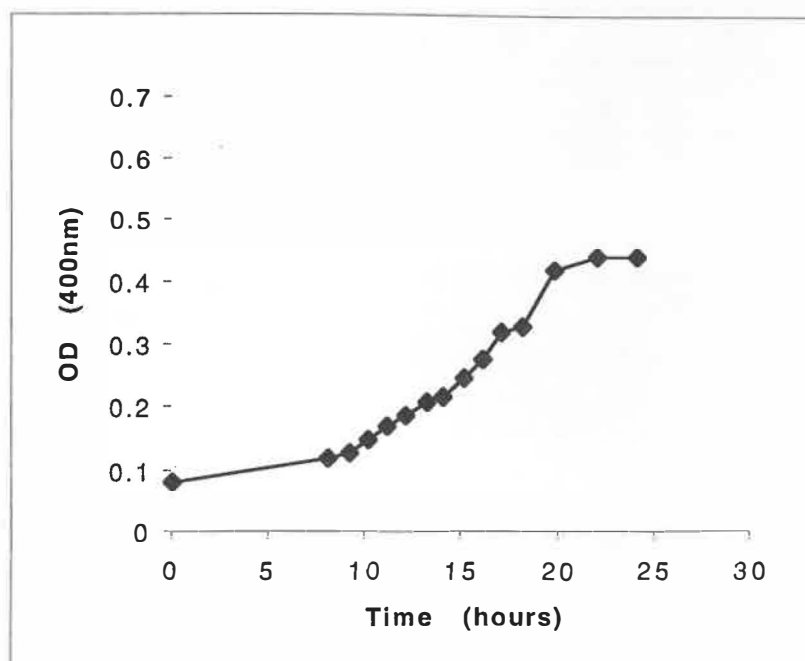
Figure 3.2: Growth curve of GAB 76. Doubling time was 309 minutes



**Figure 3.3:** Growth curve of Rt 118.B1. Doubling time was 300 minutes



**Figure 3.4:** Growth curve of Rt 118.B2. Doubling time was 221 minutes



**Figure 3.5:** Growth curve of Wai 21.B2. Doubling time was 333 minutes

Doubling times measured in anaerobic cultures of other members of the *Spirochaeta* genus vary from 2 hours (*S.thermophila* strain RI 19.B1) to 12 hours (*S.bajacaliforniensis*). (Fracek et al 1985, Canale-Parola 1991, Rainey et al 1991).

### 3.7 Fermentation End Products

Figures 3.6 and 3.7 are typical examples of chromatographs obtained for control and inoculated media after incubation for 48 hours at 55°C with 0.3% maltose. All strains fermented maltose to lactate, acetate (and presumably carbon dioxide and hydrogen).

All the maltose present (3mg/ml) in the media was used up by the isolates. No propionic acid or ethanol peaks were found. Yeast extract peaks came through almost unaltered, so this is probably used for trace elements rather than as a carbon source.

PEAK NUM	RET TIME	PEAK NAME	CONCENTRATION in	NORMALIZED CONC	AREA	HEIGHT	AREA/ HEIGHT BL	REF PEAK	% DELTA RET TIME	CONC/AREA
1	7.50	YEAST EXTRACT	2.5707	24.0735%	860825	36786	23.4	2	0	2.9863E-06
2	9.02	MALTOSE	7.0278	65.8129%	2353356	76657	30.7	2	1.851	2.9863E-06
3	10.54	glucose	1.0800	10.1136%	181197	4867	37.2	2	1.580	5.9602E-06

TOTAL AMOUNT = 10.6784

PEAKS NOT FOUND IN THIS RUN

NAME	ADJUSTED RET.TIME.	REFERENCE PEAK
LACTIC ACID	14.60	MALTOSE
ACETIC ACID	16.90	MALTOSE
ETHANOL	25.00	MALTOSE

Start time: 0.00 Stop time: 30.00 Offset: 10  
 Low Value: 1 High Value: 76607 Scale factor: 1

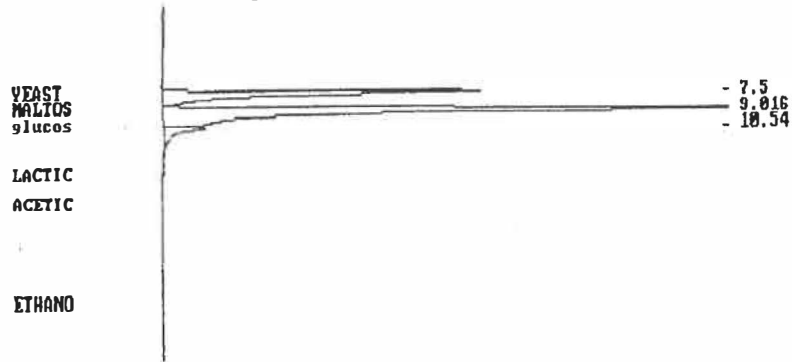


Figure 3.6: Chromatogram of uninoculated SE media.

PEAK NUM	RET TIME	PEAK NAME	CONCENTRATION in	NORMALIZED CONC	AREA	HEIGHT	AREA/ HEIGHT BL	REF PEAK	% DELTA RET TIME	CONC/AREA
1	7.44	YEAST EXTRACT	3.4192	7.8012%	1144963	48465	23.6	2	0	2.9863E-06
2	8.93	MALTOSE	0.2317	0.5286%	77588	2366	32.8	2	0	2.9863E-06
3	9.95		14.5324	33.2026%	145524	1330	109.4	2		1.0000E-04
4	14.55	LACTIC ACID	17.6151	40.1905%	887676	26110	34.0	2	0	1.9844E-05
5	16.83	ACETIC ACID	6.6280	15.1223%	193270	5021	38.5	2	0	3.4294E-05
6	27.38		1.3827	3.1547%	13827	2208	6.3	2		1.0000E-04

TOTAL AMOUNT = 43.8290

PEAKS NOT FOUND IN THIS RUN

NAME	ADJUSTED RET.TIME.	REFERENCE PEAK
glucose	10.54	MALTOSE
ETHANOL	25.00	MALTOSE

Start time: 0.00 Stop time: 30.00 Offset: 10  
 Low Value: 3365 High Value: 52103 Scale factor: 1

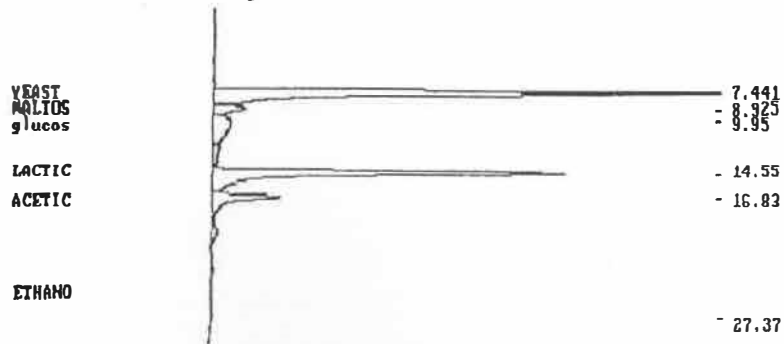


Figure 3.7: Chromatogram of SE media after incubation with spirochete strain Rt 118.B1.

### 3.8 Growth on different carbon sources

The growth response of the four spirochete isolates on different carbon sources is shown in table 3.3 as the percent growth recorded against a maltose-SE medium control.

Enumeration in some substrates (marked with an asterisk on table 3.3) was difficult due to their particulate nature. Dense clumps of spirochetes would mass where there was substrate, with very few free swimming spirochetes to be found in the media. When this occurred ten samples were taken and cells numbers counted, the average number of these samples was used.

When growth was minimal, the culture underwent at least one transfer to ensure that growth was due to utilisation of the carbon source tested and not carry over of maltose from the inoculum.

**Table 3.3:** Growth of four strains of spirochetes on different carbon sources.

Carbon Source	Growth (% compared to maltose)			
	GAB 76	Rt 118.B1	Rt 118.B2	Wai 21.B2
Amorphous cellulose	0	0	0	0
Amylopectin	156	160	122	149
Arabinose	79	99	90	54
Cas-amino acids	0	0	0	0
Cellobiose	106	104	64	117
Chitin	0	0	0	0
Dextran	0	0	0	0
Galactose	85	96	60	83
Galactomannan (Locust Bean Gum)*	83	69	44	119
Glucomannan (Guar Gum)*	165	51	64	164
Glucomannan (Konjac)*	89	69	89	112
Glucose	82	90	70	105
Glutamate	0	0	0	0
Inulin	0	0	0	0
Lactose	31	71	45	42
Maltose	100	100	100	100
Mannose	115	100	66	148
Mannitol	0	8	6	8
Soluble Starch*	69	67	59	75
Sucrose	108	105	82	108
Trypticase	43	56	52	41
D (+) Xylose	53	35	0	0
Xylan (Birchwood)*	110	82	99	90
Xylan (Oat Spelt)*	55	110	59	60

\* Spirochetes become entangled in these carbon sources making enumeration very difficult.

### **3.9 Antibiotic sensitivity.**

Table 3.4 shows the sensitivity of GAB 76, Rt 118.B1, Wai 21 B2, and Rt 118.B2 to several antibiotics at different concentrations. Results are expressed in terms of percentage of growth in antibiotic free SE media compared to growth with antibiotics.

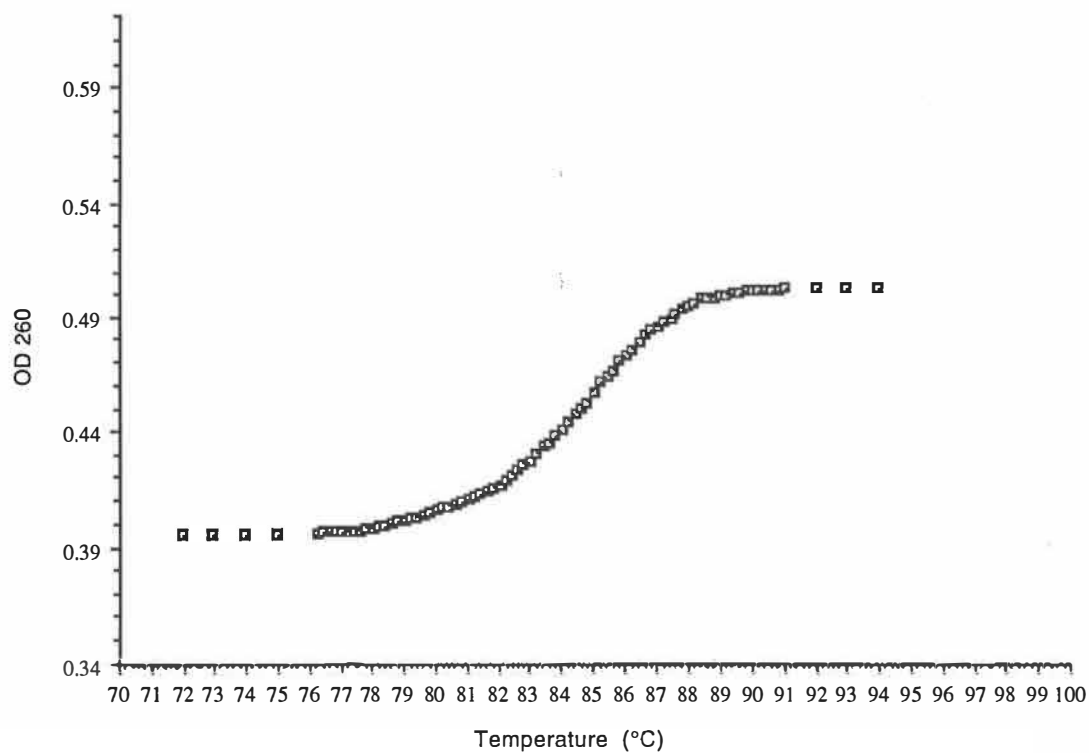
All four strains of spirochetes were sensitive to Chloramphenicol, D-Cycloserine, Neomycin, and Penicillin. All were resistant to Nalidixic Acid, Novobiocin, Polymyxin B, Rifampicin (which is characteristic of the species), and had some resistance to Streptomycin and Tetracycline.

**Table 3.4:** Antibiotic sensitivity of four strains of spirochetes to various antibiotics and concentrations, expressed as the percent growth recorded against media which contains no antibiotics.

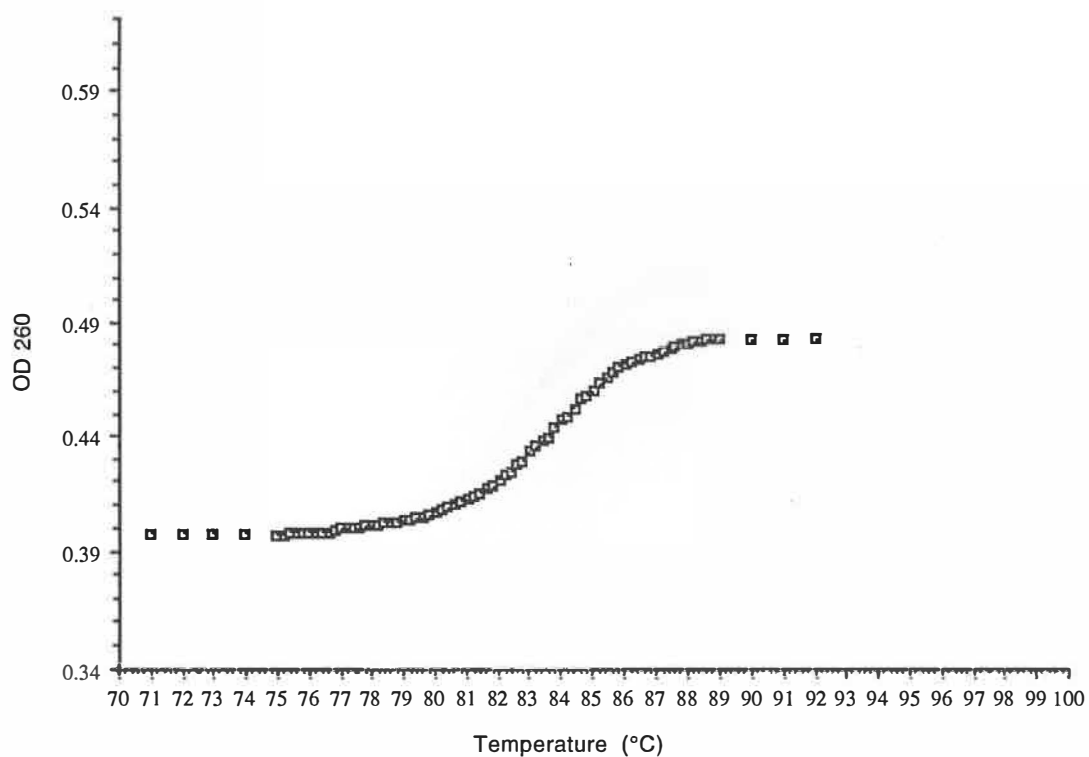
Antibiotic	Growth (%)				
	$\mu\text{g/ml}$	GAB 7 6	Rt 118. B 1	Wai 21. B 2	Rt 118. B 2
Chloramphenicol $\mu\text{g/ml}$	5	0	0	0	0
	10	0	0	0	0
D-Cycloserine $\mu\text{g/ml}$	5	0	0	0	0
	10	0	0	0	0
Nalidixic Acid $\mu\text{g/ml}$	20	90	85	70	85
	50	65	50	46	59
	100	15	0	0	10
	200	0	0	0	0
Neomycin $\mu\text{g/ml}$	5	0	0	0	0
	10	0	0	0	0
Novobiocin $\mu\text{g/ml}$	5	60	74	60	47
	10	52	70	48	28
	20	32	56	4	5
	50	5	9	0	0
	100	0	0	0	0
Penicillin $\mu\text{g/ml}$	5	0	0	0	0
	10	0	0	0	0
Polymyxin B $\mu\text{g/ml}$	5	88	139	96	68
	10	56	117	75	47
	20	0	26	46	3
	50	0	0	6	0
	100	0	0	0	0
Rifampicin $\mu\text{g/ml}$	10	87	72	68	70
	20	54	61	45	45
	50	19	48	13	5
	100	0	0	0	0
Streptomycin $\mu\text{g/ml}$	5	25	65	20	89
	10	0	0	0	0
Tetracycline $\mu\text{g/ml}$	5	63	48	59	51
	10	10	8	5	7
	20	0	0	0	0

### 3.10 G+C mol% determination

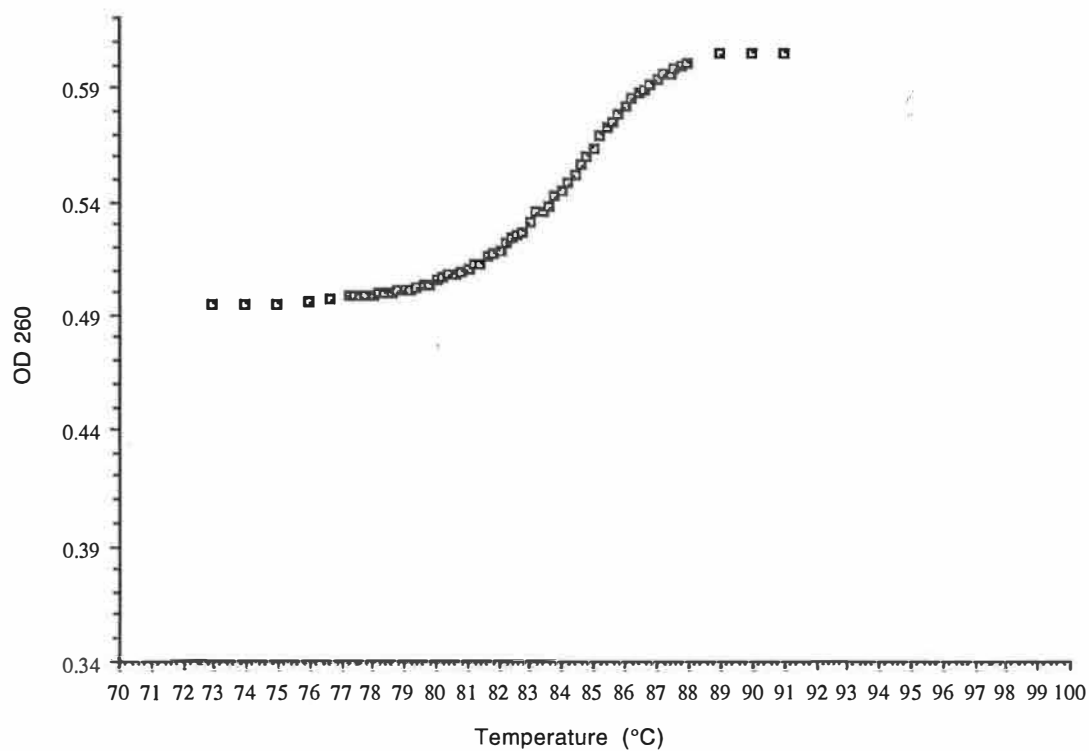
DNA melting curves of the four isolates and their G+C% are displayed in figures 3.8- 3.12.



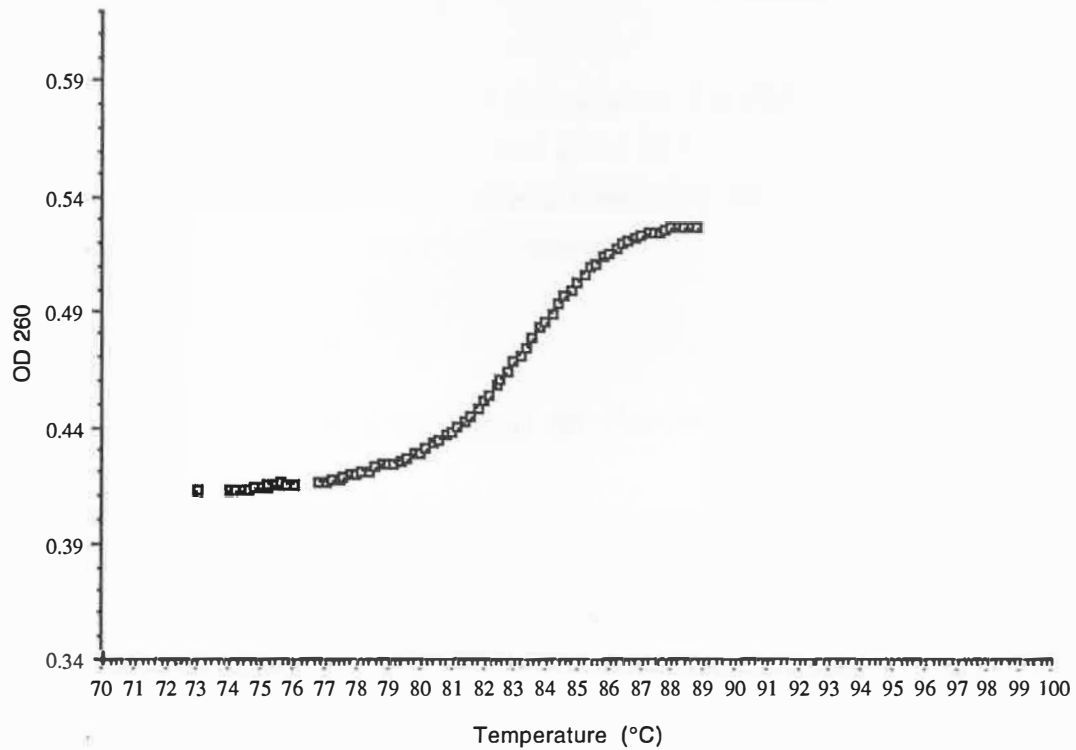
**Figure 3.8:** DNA melting curve of GAB 76. The  $T_m$  of GAB 76 was 83.8°C and the G+C mol % was 46.5%



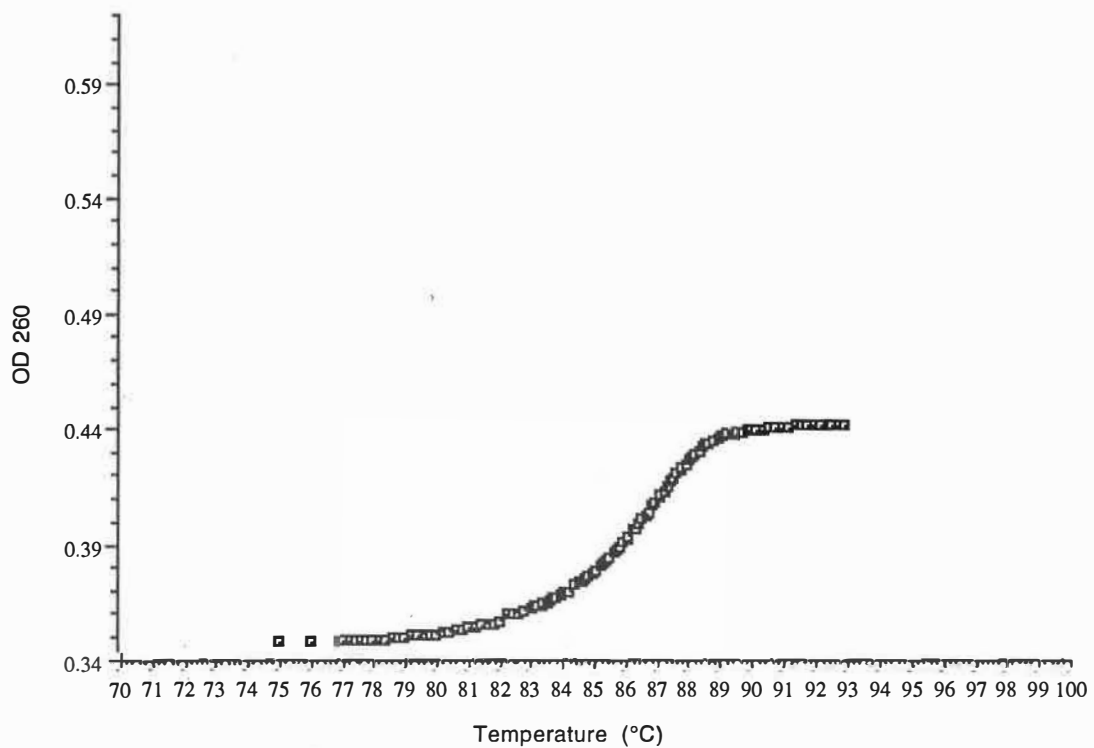
**Figure 3.9:** The DNA melting curve of Rt 118.B1. The  $T_m$  of Rt 118.B1 was 83.4°C and the G+C mol % was 45.6%



**Figure 3.10:** The DNA melting curve of Rt 118.B2. The  $T_m$  of Rt 118.B2 was 84.2°C and the G+C mol % was 47.3%



**Figure 3.11:** The DNA melting curve of Wai 21.B2. The  $T_m$  of Wai 21. B2 was 83°C and the G+C% was 44.7 mol%



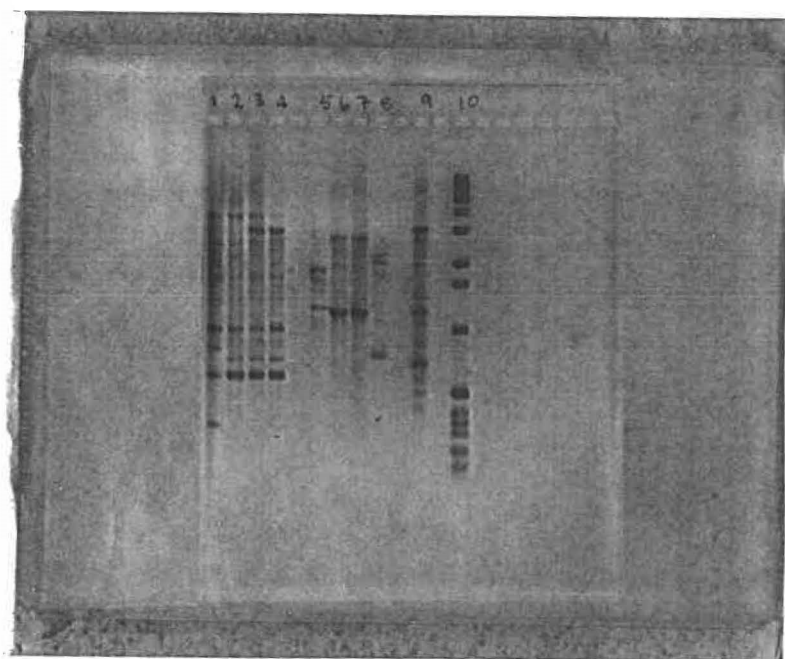
**Figure 3.12:** DNA melting curve from the *E.coli* standard. The  $T_m$  was 85.8°C and the G+C mol % was 51.

Isolate GAB 76 had a G+C of 46.5 mol%, Rt 118.B1 had 45.6 mol%, Rt 118.B2 had 47.3 mol%, and Wai 21.B2 had 44.7 mol%.

Until Pohlschroeder et al (1994) published their work on *S.caldaria* no member of the genus *Spirocheata* was known to have a G+C value under 50.5. *S.caldaria* has a G+C % of 45 (by thermal denaturation), and recently Zhilina et al (1996) described *S.asiatica* as having a G+C % of 49.2, which indicates that it is by no means unusual to discover a spirochete with a mol G+C % under 50.

### 3.11.1 Random Amplification of Polymorphic DNA

In a preliminary trial three primers were found to give good amplified product on all four strains (OPR 11,12 and 20). Primer OPR 20 was selected for subsequent use because it clearly produced different band patterns between isolates Rt 118.B1 and Rt 118.B2. Results with this primer are shown in Figure 3.13 and demonstrates that the two isolates are not identical.



<u>lane &amp; isolate</u>	<u>primer</u>
1. GAB 76	OPR 20
2. Rt 118.B1	OPR 20
3. Rt 118.B2	OPR 20
4. Wai 21.B2	OPR 20
5. Rt 118.B1	OPR 11
6. Rt 118.B2	OPR 11
7. Rt 118.B1	OPR 12
8. Rt 118.B2	OPR 12
9. Rt 118.B2	OPR 14
10. Rt 118.B2	OPR 15

**Figure 3.13:** Banding patterns of DNA from the four spirochete isolates after PCR amplification and digestion with EcoR1.

The banding patterns produced using OPR 20 primer were different in all four isolates (GAB 76, Rt 118 B1, Rt 118 B2, and Wai 21 B2).

OPR 11 5' -GTAGCCGTCT- 3'  
OPR 12 5' -ACAGGTGCGT- 3'  
OPR 20 5' -ACGGCAAGGA- 3'

OPR 11 annealed to Rt 118.B2, but not to Rt 118. B1. OPR 16 annealed very poorly to both Rt 118 B1 and Rt 118.B2, but seemed to show more bands in Rt 118.B1 than Rt 118.B2. OPR 12 Annealed to both Rt 118. B1 and Rt 118. B2, however only two discrete bands could be identified, which does not provide enough resolution to differentiate between strains. OPR 14 and 15 were only trialed against Rt 118. B2 DNA. OPR 15 would seem to be promising as it gave bright discrete bands.

### **3.12 Part A : Discussion**

The major characteristics of the four isolates are shown in table 3.5, where they are compared to those of the other known thermophiles in the genus.

The four strains characterised (GAB 76, Rt 118.B1, Rt 118.B2 and Wai 21. B2) can be placed in the genus *Spirochaeta* as they were found to be free living, rifampicin resistant and had a typical *Spirochaeta* morphology. They were obligate anaerobes and failed to grow if the resazurin indicator was not reduced.

**Table 3.5:** Characteristics of GAB 76, Rt 118.B1, Rt 118.B2 and Wai 21.B2 compared to those of *S.caldaria* and *S.thermophila*.

	<b>GAB 76</b>	<b>Rt 118. B1</b>	<b>Rt 118. B2</b>	<b>Wai 21. B2</b>	<b>S.Cal- daria</b>	<b>S.therm -ophila<sup>1</sup></b>
O <sub>2</sub> Requirement	AnO <sub>2</sub>	AnO <sub>2</sub>	AnO <sub>2</sub>	AnO <sub>2</sub>	AnO <sub>2</sub>	AnO <sub>2</sub>
NaCl requirement	None	None	None	None	None	0.5%
Habitat	Fresh water	Fresh water	Fresh water	Fresh water	Fresh water	Brackish water
Doubling time (min)	309	300	221	333	240	119
pH optima	6.5-7.0	6.5-7.0	6.5-7.0	6.5	7.2-7.5	7.5
Acid end products	lactate + acetate	lactate + acetate	lactate + acetate	lactate + acetate	lactate + acetate	lactate + acetate
Resistance to Rifampicin	√	√	√	√	√	√
Growth Temperature Optimum (°C)	57	52	55	59	48-52	64-66
Utilisation of polysaccharides as a carbon source	√	√	√	√	√	√
G+C mol %	46.5	45.6	47.3	44.7	45	52

The four isolates are essentially identical with respect to their phenotypic characteristics, but differ significantly from the other known spirochete species with the exception of *S.caldaria*. As mentioned in the literature review all previously known spirochetes with the exception of

<sup>1</sup> Strain RI 19.B1

*S.thermophila* and *S.caldaria* are mesophiles. The four isolates presented in table 3.5 have the highest optimal growth temperatures of any species except for *S.thermophila*.

They also have the lowest G+C content of DNA of any species except for *S.caldaria*, significantly lower than that of *S.thermophila*.

Unlike *S.thermophila* the isolates show no requirement for salt.

The strains do not form ethanol as an end product of glucose metabolism unlike all the known mesophilic strains of *Spirochaeta* (with the exception of *S. zuelzerae* which forms succinate). Like the thermophilic *S.thermophila* and *S. caldaria* the isolates produce acetate, lactate, CO<sub>2</sub> and H<sub>2</sub>. It is interesting to note that only the thermophilic members of the *Spirocheata* Genus do not form ethanol as an end product, this absence could be an indication of thermophily in spirochetes.

The strains show great similarity to *S.caldaria* especially Rt 118.B1 which has a similar temperature optimum and G+C mol% to *S.caldaria*. However the temperature optimum of *S.caldaria* was lower than that of GAB 76, Rt 118.B2 and Wai 21.B2. The maximum temperature that *S.caldaria* would grow to was 59°C, where as growth was still seen for GAB 76 at 61°C, Rt 118.B1 at 63°C Rt 118.B2 at 61°C and Wai 21.B2 at 62°C.

*S.caldaria* cannot ferment plant wall polysaccharides such as cellulose and xylan. All four isolates grow extremely well on xylan, but were not able to metabolise cellulose. *S.thermophila* can utilise both xylan and amorphous cellulose. Using xylan as a sole carbon source would be a good method for differentiating GAB 76, Rt 118.B1, Rt 118.B2 and Wai 21.B2 from strains of *S.caldaria* . Only thermophilic strains of *Spirocheata* are known to metabolise polysaccharides with the exception of the alkalophilic species. *S.alkalica*, *S.africana* and *S.asiatica* which have a poor ability to metabolise starch.

GAB 76, Rt 118.B1, Rt 118.B2 and Wai 21.B2 are similar to other spirochetes in that they grow on a wide variety of sugars but no amino acids or mannitol. They have no requirement for CO<sub>2</sub>. Carbon sources that all strains grew well on were amylopectin, arabinose, cellobiose, galactose, galactomannan, glucomannan, glucose, lactose, maltose, mannose, trypticase, soluble starch, sucrose, trypticase, and xylan. Strains were not able to utilise amorphous cellulose, cas-amino acids, chitin, dextran, glutamate, inulin, or mannitol.

All four strains were inhibited by Chloramphenicol, D-Cycloserine, Neomycin, and Penicillin. All were resistant to Nalidixic, Rifampicin, Streptomycin and Tetracycline as is *S.caldaria* and *S.thermophila*.

GAB 76, Rt 118.B1, Rt 118.B2 and Wai 21.B2 have a pH optima of 6.5-7.0 which is the lowest in the Genus (see table 2), and lower than the pH 7.2 - 7.5 optimum of *S.caldaria* and pH 7.5 of *S.thermophila*.

## Summary

The four isolates differ from mesophilic spirochete strains by their temperature optimum, ability to utilise polysaccharides as a carbon source, their low G+C mol%, their acid end products and their low pH optimum.

The isolates are distinguishable from the thermophile *S.thermophila* by their low DNA G+C mol%, they do not require salt for growth, their low pH optimum, their inability to ferment cellulose, their ability to ferment lactose, their slow growth, and their lower optimal temperatures.

The isolates differ from the thermophile *S.caldaria* by their higher maximum growth temperature, their higher optimal growth temperatures, their lower optimal pH, their ability to ferment xylan, and the different mol G+C % of the strains:

Because of the many differences that these strains have to the other members the strains might form a different species. The four strains are similar enough to be considered varieties of the new species. However similarities to *S.caldaria* are great enough that 16s RNA or DNA:DNA hybridisation will need to be carried out to clarify their relationship to this species.

## Part B: Characterisation of Phosphofructokinase from Rt 118.B2

### 3.13 PFK activity from cell-free extracts

#### 3.13.1 Protein estimations

Cell free extracts of isolate Rt 118.B1 (prepared as outlined in section 2.15.1) were diluted to obtain absorbance readings in the range of the Bovine Serum Albumin (BSA) standard curve (Figure 3.14). The estimations of the amount of protein in the cell free extracts are presented in Table 3.6.

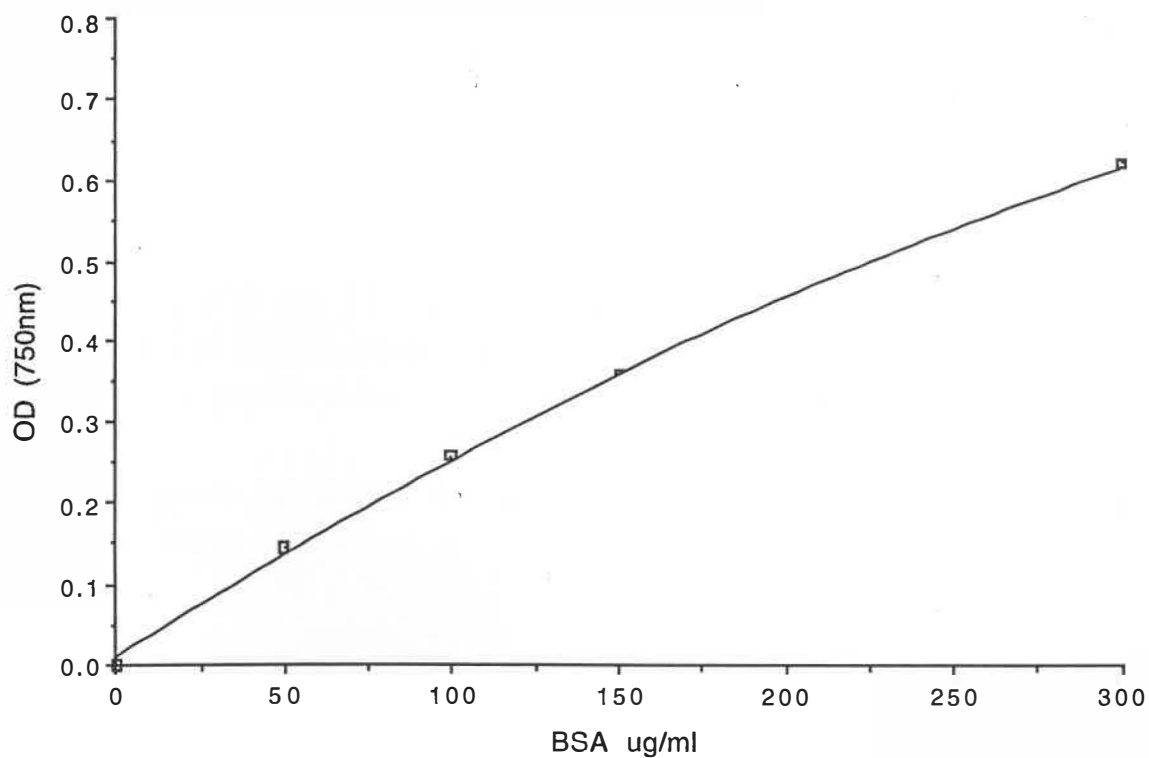


Figure 3.14: Protein standard curve

**Table 3.6:** Protein concentrations of cell free extracts of spirochetes used in PFK assays.

Strain	Abs 750nm	Protein $\mu\text{g/mlBSA}$ standard	Dilution (fold)	Protein mg/ml	Protein used in PFK assay ( $\mu\text{g}$ )
GAB 73	0.253	110	200	22.0	11
GAB 76	0.235	98	200	19.6	9.8
Rt 19.B1	0.358	169	100	16.9	16.9
Rt 118.B1	0.252	109	200	21.8	10.9
Rt 118.B2	0.400	176	100	17.6	17.6
<i>S.stenosstrepta</i>	0.359	152	10	1.52	152 *
Wai 21.B2	0.275	116	200	23.2	11.6

\* although it was necessary to dilute this sample to fit on the standard curve, it was used undiluted in the PFK assay because the sample had low PFK activity.

### 3.13.2 PFK assay results

Figures 3.15, 3.16 and 3.17 show typical PFK enzymatic activity with no-phosphoryl donor, with ATP as a phosphoryl donor, and with PPi as a phosphoryl donor. The PFK activity in these graphs was in cell free extracts from isolate GAB 76.

Table 3.7 shows the specific activity of PFK from different spirochete strains and phosphoryl donors.

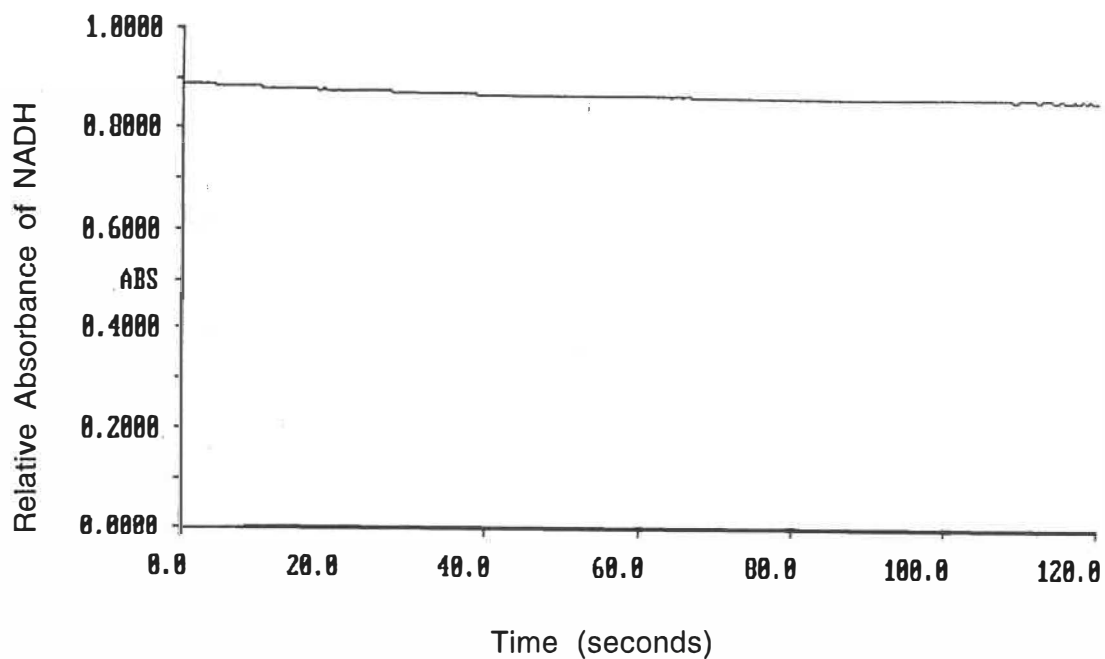


Figure 3.15: PFK assay of GAB 76 extract with no-phosphoryl donor

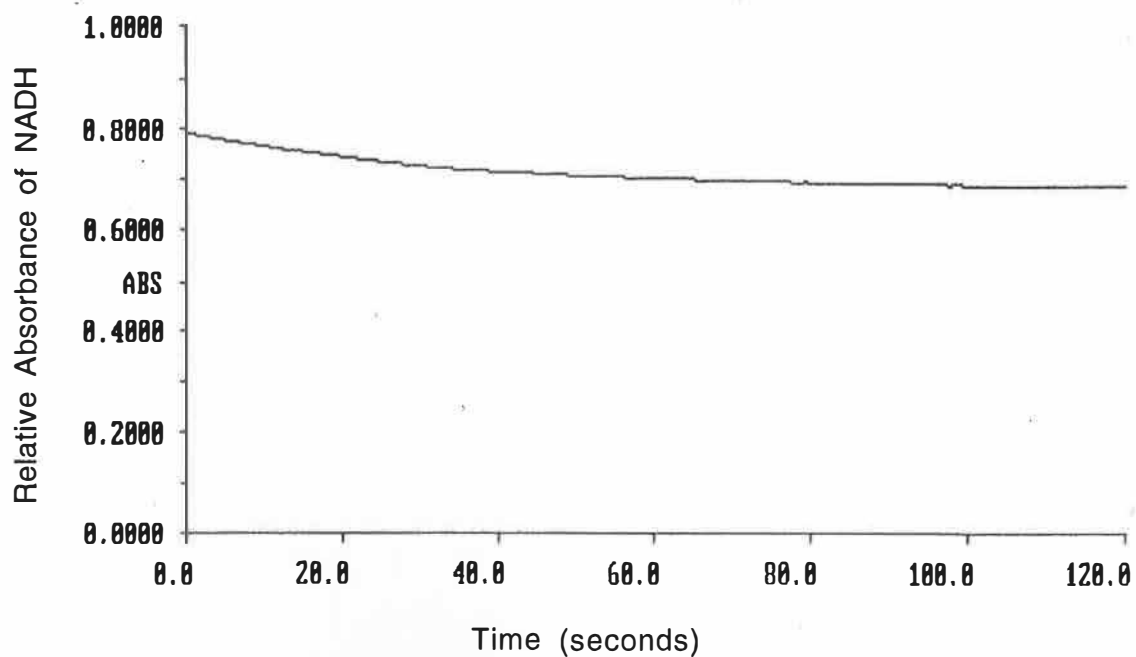
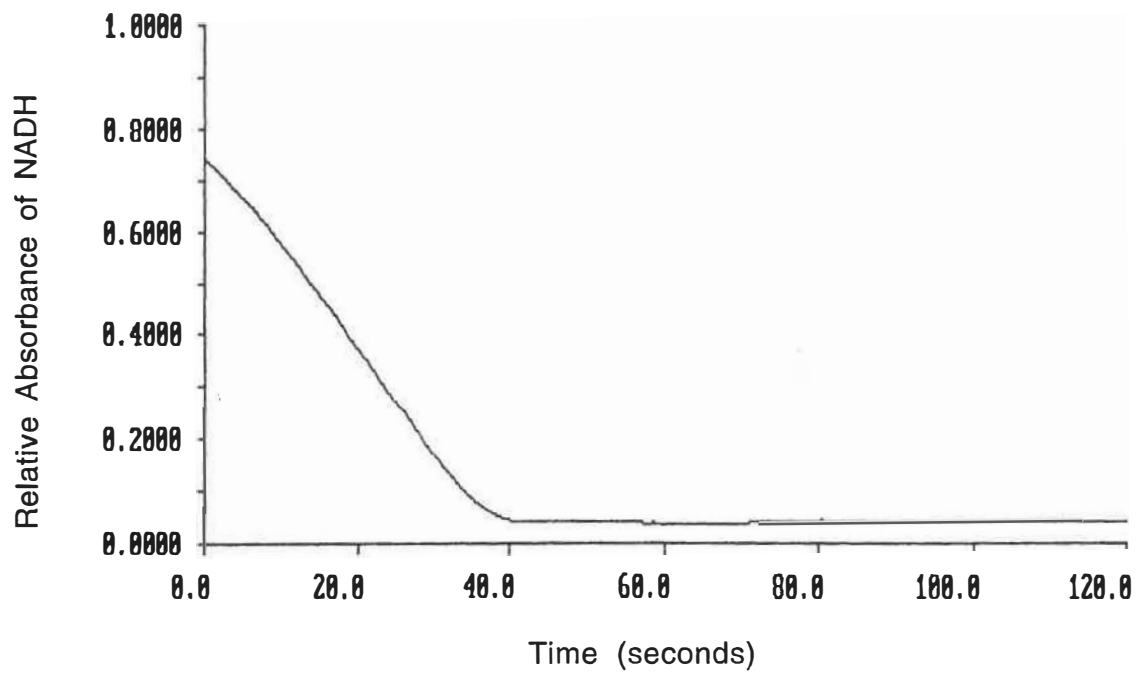


Figure 3.16: PFK assay of GAB 76 extract with ATP as the phosphoryl donor



**Figure 3.17:** PFK assay of GAB 76 extract with PPI as the phosphoryl donor

**Table 3.7:** Specific activity of PFK from different spirochete strains with different phosphoryl donors.

Strain	Phosphoryl donor	ABS 340/min	corrected ABS	A nmol/min NAD	amt protein in assay ( $\mu\text{g}$ )	sp. activity $\mu\text{mol/m in/mg}$
GAB 73	ATP	0.017	0.009	0.72	11.0	0.07
	PPi	1.688	1.638	131.65	22.0	5.98
	no donor	0.008	0.000	0.00	11.0	0.00
	no donor	0.050	0.000	0.00	22.0	0.00
GAB 76	ATP	0.058	0.016	1.29	9.8	0.13
	PPi	0.590	0.548	44.05	9.8	4.50
	no donor	0.042	0.000	0.00	9.8	0.00
RI 19.B1	ATP	0.063	0.046	3.70	16.9	0.22
	PPi	0.480	0.463	37.2	16.9	2.20
	no donor	0.017	0.000	0.00	16.9	0.00
Rt	ATP	0.042	0.017	1.37	10.9	0.13
118.B1	PPi	1.120	1.095	87.98	10.9	8.07
	no donor	0.025	0.000	0.00	10.9	0.00
Rt	ATP	0.017	0.008	0.65	17.6	0.04
118.B2	PPi	0.529	0.520	41.80	17.6	2.38
	no donor	0.009	0.000	0.00	17.6	0.00
<i>S.stenosost-repta</i>	ATP	0.240	0.040	3.22	152	0.02
	PPi	0.312	0.112	9.00	152	0.06
	no donor	0.200	0.000	0.00	152	0.00
Wai	ATP	0.050	0.013	1.01	11.6	0.09
21.B2	PPi	0.411	0.391	31.39	5.8	5.41
	no donor	0.020	0.000	0.00	5.8	0.00
		0.037	0.000	0.00	11.6	0.00

The molar absorbance coefficient for NADH is  $6.22 \times 10^3 \text{ cm}^{-1}\text{M}^{-1}$ . It must be remembered that for every mole of substrate converted, two moles of NAD are formed. The corrected

ABS (absorbance) is the absorbance for each sample minus the background (the background is the reaction mixture with no phosphoryl donor). I was unable to culture *S.stenostrepta*, so pellets frozen at  $-20^{\circ}\text{C}$  were used. As these pellets had been stored for approximately six months, it possibly accounts for the low activity of this strain.

Boiling the cell free extracts inactivated the PFK. Extracts from frozen cells showed approximately half the activity of freshly harvested cells. Rt 118.B1 when freshly harvested had an activity of  $8.07\ \mu\text{mol}/\text{min}/\text{mg}$  whereas when it had been frozen for one week at  $-20^{\circ}\text{C}$  and then thawed, activity was  $3.86\ \mu\text{mol}/\text{min}/\text{mg}$ .

Results from assays on *L. interrogans* cell free extract are not included in the table. *L. interrogans* strain RGA had a specific activity of  $0\ \mu\text{mol}/\text{min}/\text{mg}$  because the rate with no phosphoryl donor was the same rate that it had with either P<sub>i</sub> or ATP as a donor. This indicates there was probably no enzyme activity and the measured decrease in absorbance (change in absorbance was  $0.023\ \text{abs}/\text{min}$ ) was most likely settling of the high protein content of the extract.

Strain *L. interrogans L.pomona* had almost identical activities whether ATP, P<sub>i</sub> or no phosphoryl donor was used. Activity with P<sub>i</sub> as the phosphoryl donor on one occasion was  $0.005\ \mu\text{mol}/\text{min}/\text{mg}$ , and  $-0.001\ \mu\text{mol}/\text{min}/\text{mg}$  when ATP was the phosphoryl donor. However in another trial the specific activity for both phosphoryl donors was  $0\ \mu\text{mol}/\text{min}/\text{mg}$  as the assay containing no phosphoryl donor had a higher change in absorbance than that of the assays containing ATP and P<sub>i</sub>.

The *L. interrogans* samples came from Australia and were probably frozen before shipment, thawed on the transit to New Zealand and then once reaching the Thermophile Research Unit were frozen again at  $-20^{\circ}\text{C}$ . Because the temperature of the thawed samples is likely to be close to the temperatures at which the organism is metabolically active, it also means the enzyme will be degraded quickly by proteases etc in the cells. Constant freeze thawing has very adverse effects on enzymes. These experiments should be repeated with freshly grown and harvested cells.

### 3.13.1 PFK assay discussion

The assay demonstrated that all the thermophilic strains assayed have PPi dependent PFK activity. The increase in activity between ATP as the phosphoryl donor to PPi as the donor ranged from an 85.4 fold increase by GAB 73 to a ten fold increase by RI 19 B1. The mesophilic PFK activity of *S.stensostrepta* demonstrated only a three fold increase when PPi was the donor. The increase in activity of PFK with PPi as the phosphoryl donor may reflect on how gentle the extraction procedure was on the crude enzyme. Poor storage techniques and handling of the enzyme will cause some of the PFK to become inactive, resulting in a lower specific activity. Certainly this would seem to be the case with *L. interrogans*. As PFK is allosterically regulated, other substances in the cell supernatant may also affect its activity. Table 3.8 compares the specific activities for PFK from various spirochetes, and also shows different specific activities achieved by the PFK from the same organism by different researchers.

**Table 3.8:** Comparison of specific activities of PFK from the four isolates and from other members of the genus *Spirocheata*

Strain	Specific activity ( $\mu\text{mol}/\text{min}/\text{mg}$ ) with ATP as donor	Specific activity ( $\mu\text{mol}/\text{min}/\text{mg}$ ) with PPi
GAB73	0.07	5.98
GAB76	0.13	4.50
Rt 118.B1	0.13	8.07
Rt118.B2	0.04	2.38
Wai 21.B2	0.09	5.41
<i>S.thermophila</i> strain RI 19.B1 <sup>1</sup>	0.22	2.20
<i>S.thermophila</i> strain RI 19.B1 <sup>2</sup>	0.03	4.63
<i>S.thermophila</i> strain Z- 1203 <sup>2</sup>	0.00	0.30
<i>S.stenostrepta</i> <sup>3</sup>	0.02	0.06
<i>S.stenostrepta</i> <sup>4</sup>	0.01	0.08

In later experiments when attempts were made to purify PFK from Rt 118.B2, the more pure the enzyme became the less ATP activity was evident, until no PFK activity with ATP was detectable.

There is only slight evidence that *L. interrogans* may have PPi-PFK. In one experiment there was a small PPi activity and no activity with ATP, however in another experiment no activity at all was observed. The results are too ambiguous to be able to say with any certainty that this organism has a PPi-dependent PFK.

<sup>1</sup> Result from this work

<sup>2</sup> Result from Jansen et al (1992)

<sup>3</sup> Result from this work

<sup>4</sup> Result from Morgan (1994)

### 3.14 Purification of PFK from Rt 118.B2

Purification by chromatography was difficult because the enzyme did not bind to either anion or cation exchange columns. However many other proteins did bind to these column so it is still a useful separation technique. Specific activity of PFK increased from 0.38  $\mu\text{mol}/\text{mg}/\text{min}$  to 1.52  $\mu\text{mol}/\text{mg}/\text{min}$  after the sample was loaded onto a Mono Q column. The PFK did not bind to the column but other proteins did (total protein in the sample reduced from 4620 mg to 2520)

In the first attempt at purification PFK bound to a Mono Q column (section 2.18.3.7), and the enzyme was eluted off the column at a concentration of 15mM NaCl. In subsequent purification steps using a Mono Q column, the PFK came off with the void volume. The sample in the first attempt contained some F-1,6-P from the eluting solvent used in the cellulose phosphate column. The F-1,6-P in the sample may have bound to the Mono Q column by the  $\text{PO}_4^-$  group, and the PFK may have bound to another of the phosphate groups on its substrate rather than to the column.

The PFK did not bind to a Mono S column but eluted off with the void volume. On each occasion the amount of protein in the sample increased by about 6 fold (eg from 630 mg to 3842 mg) after the sample had passed through the column. The column must have been contaminated by protein which did not elute off when the column was washed with solvent, but did elute off when the PFK sample was passed through. It is possible that the proteins on the Mono S column interacted with the proteins in the sample, as the buffer in the sample and the buffer used to wash the column was the same. Because of the contamination of the sample with foreign proteins it is not possible to draw up a meaningful purification table. The purification is shown in Table 3.9.

Phenol sepharose columns gave good purification. Specific activity of PFK increased from 0.10  $\mu\text{mol}/\text{mg}/\text{min}$  to 55.43  $\mu\text{mol}/\text{mg}/\text{min}$  after purification with this column. Protein was reduced from 372 mg to 30 mg.

As the only column that the PFK bound to was the phenol sepharose column, a column was required which the PFK would bind to, and then be eluted off with specific substrate (eg F - 1,6-P). The cellulose-phosphate column has been used successfully by many researchers for this purpose, however the PFK from isolate Rt118.B2 bound so weakly to the column that it was washed off with the buffer. The cellulose-phosphate that was available at the laboratory was very compacted and approximately 10 years old, which leads me to believe that it had

lost its functional properties over time. Unfortunately it would have taken two months to obtain another supply from England, so it was not possible to repeat the experiment with fresh cellulose-phosphate within the time constraints of the project.

SDS-page gel revealed 7 bands after the third purification attempt and 6 bands after the fourth purification attempt. It was not possible to estimate a molecular weight for the enzyme as it could not be ascertained from looking at the gel which band contained the PFK activity. An activity stain could have revealed which band contained the PFK. Several techniques were tried (paranitrophenol and 4-methyl-umbelliferyl phosphate) but were unsuccessful. A native page gel was made from the sample from the fourth purification to see if all the six protein bands were subunits of the enzyme. The gel revealed two bands which indicates that there is more than one protein present, but as the gel was past its 'use by' date it cannot be said with certainty that there were only two proteins present.

It was decided to use the partially purified enzyme from the fourth purification which had 6 protein bands on the sds-page gel for characterisation studies. Although there were 6 proteins on the sds-page gel, probably only 4 are contaminants, as PFK is usually a dimer.

**Table 3.9:** Purification table from the fourth attempt at purifying PFK.

<b>step</b>	<b>total protein (mg)</b>	<b>specific activity (<math>\mu\text{mol}/\text{min}/\text{mg}</math>)</b>
sonication	4620	0.382
mono Q	2520	1.519
phenol sepharose	90	45.76
cellulose phosphate	2	7.41 <sup>1</sup>
mono S	396	0.076
mono P	372	- <sup>2</sup>
phenol sepharose	30	55.43

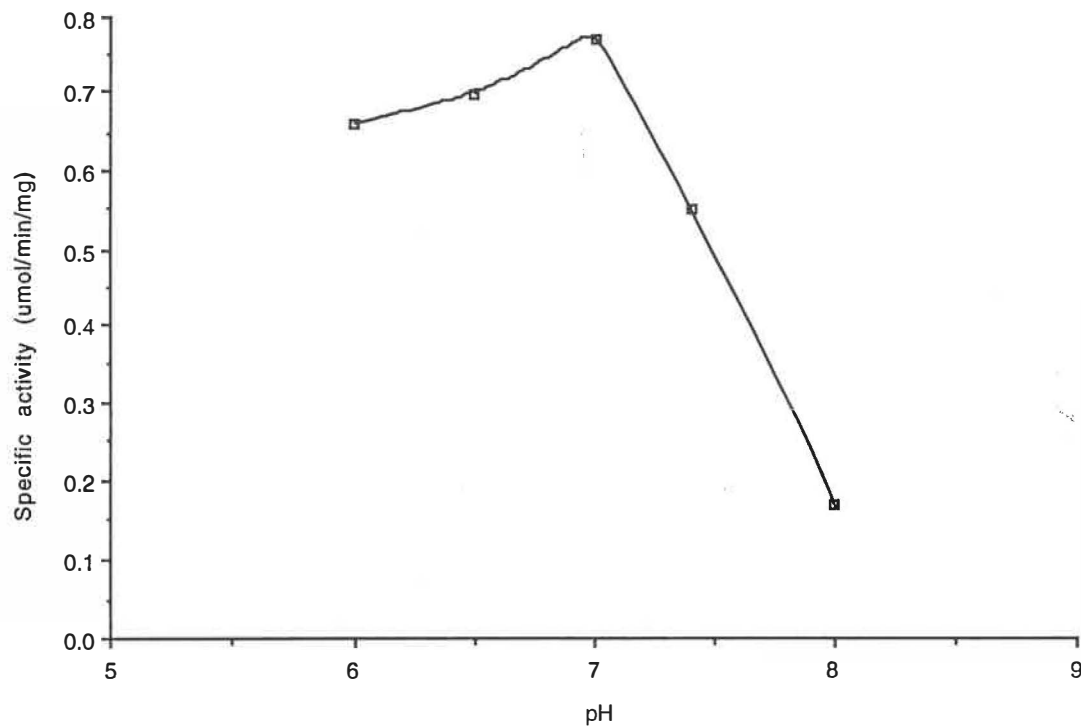
<sup>1</sup> Some of the PFK was lost in this step, possible binding onto the column, and not eluting off.

<sup>2</sup> Sample was not assayed for activity

## 3.15 Characterisation of the partially purified PFK

### 3.15.1 Optimum pH

The partially purified PFK from isolate Rt 118.B2 had an optimum pH of 7.0 (figure 3.18).



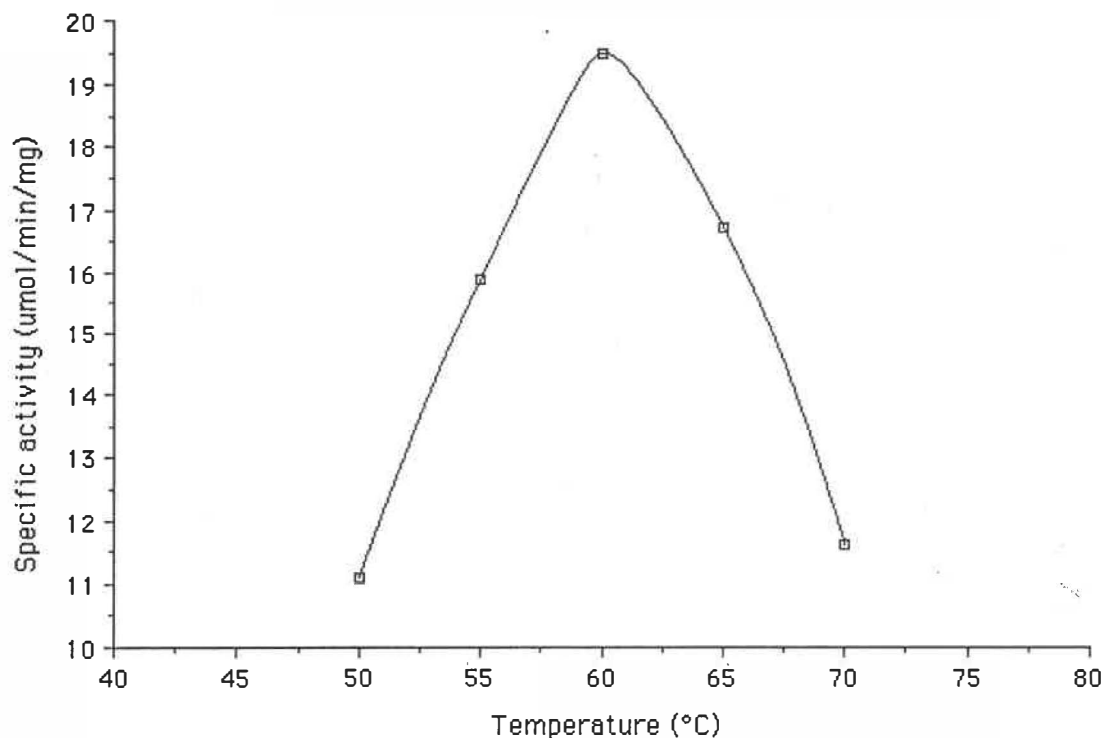
**Figure 3.18:** Optimum pH of partially purified PFK

The sharp decline of activity on the alkaline side of the enzymes optimum compared to the acidic side is similar to results obtained by Li et al 1995. They found that the Giardial enzyme had an optimum pH of 7.0 but at pH 8 activity was reduced by one third. At pH 6 the enzyme retained 90% of its activity.

The low specific activity in this experiment is because the PFK had been stored at 4°C for two months before use.

### 3.15.2 Optimum temperature of PFK from isolate Rt 118.B2

The optimum temperature of the partially purified PFK was 60°C (see figure 3.19)



**Figure 3.19:** Optimum temperature for the partially purified PFK

The PFK exhibited a maximum specific activity when incubated at 60°C, with activity dropping rapidly at either side of this temperature. A ten degree drop in temperature resulted in a ten fold drop in specific activity. As mentioned in the materials and method section the assay was incubated at the required temperature without the linker enzymes so that the assay would measure the affect of temperature on the PFK rather than on the linker enzymes. Isolate Rt 118.B2 grew at an optimum temperature of 55°C and a maximum of 61°C, so the enzyme was active above the maximum temperature that supports growth of this isolate.

### 3.15.3 Thermostability of PFK of isolate Rt 118.B2

Figure 3.20 displays the thermostability of partially purified PFK from Rt 118.B2. PFK was most thermostable at 65°C. At 85°C the enzyme was rapidly inactivated and at 95°C there was no activity. It is to be expected that at 55°C enzymatic activity would barely decrease. The half life at 75°C was 108 minutes. When enzyme was stored at 4°C for 4 months it retained

approximately 5% of its activity. Freezing at  $-70^{\circ}\text{C}$  resulted in minimal loss of activity, while freezing at  $-20^{\circ}\text{C}$  lost 90% of the enzymes activity within two days.

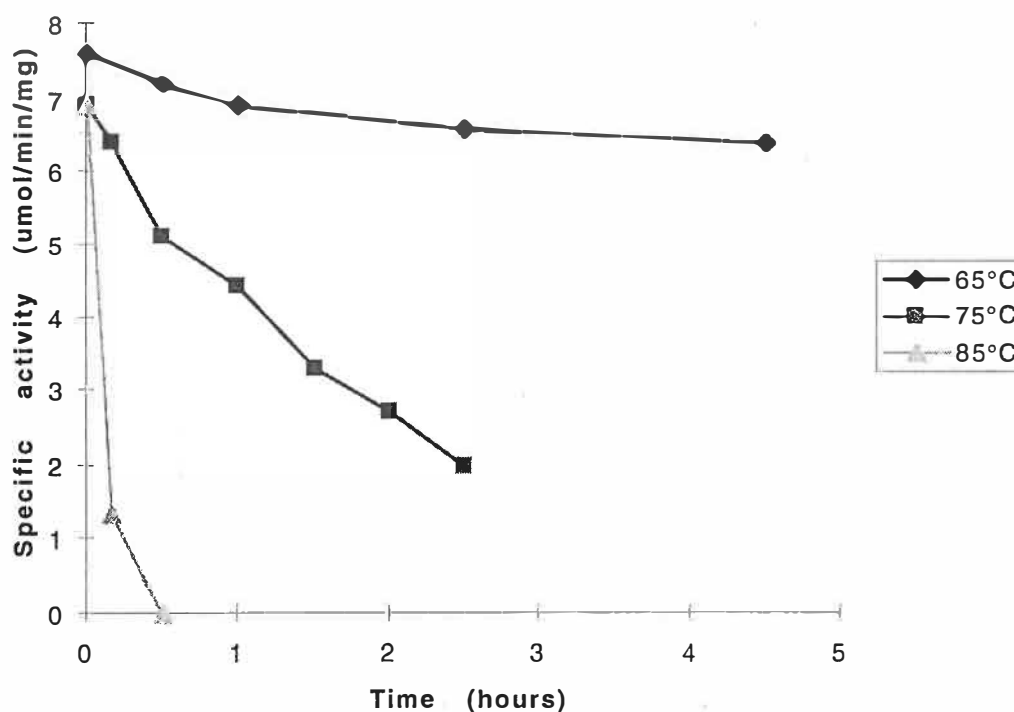
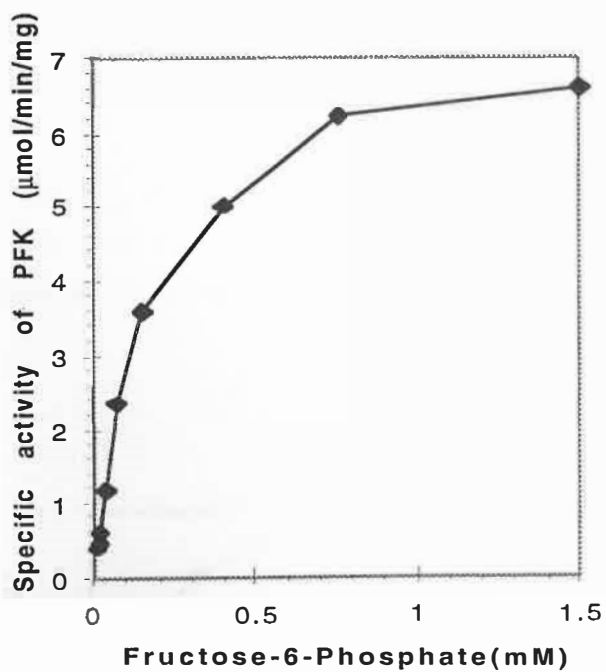


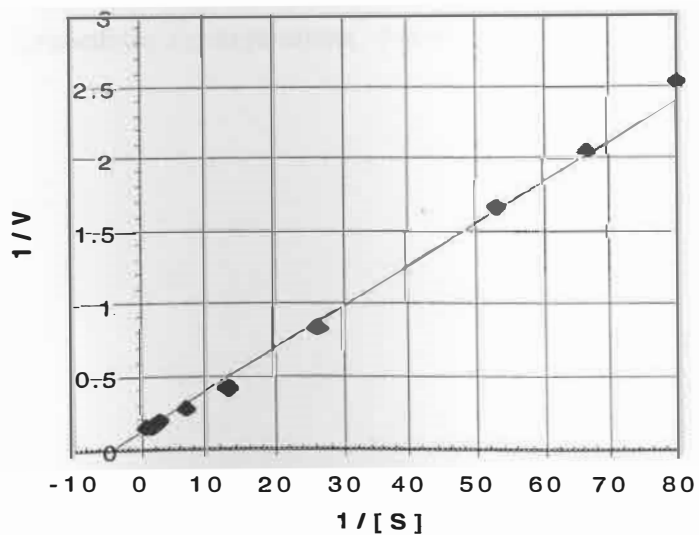
Figure 3.20: Thermostability of partially purified PFK

### 3.15.4 $K_m$ and $V_{max}$ of PFK from isolate Rt 118.B2 in various fructose-6-phosphate concentrations

The  $K_m$  and  $V_{max}$  results for PFK at various F-6-P concentrations are displayed in figures 3.21 and 3.22



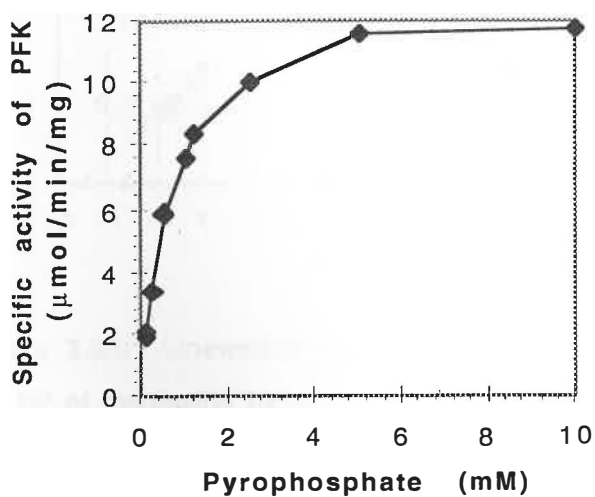
**Figure 3.21:** Rate of activity of PFK from isolate Rt 118.B2 at increasing fructose-6-phosphate concentrations. PPI concentration was 10mM



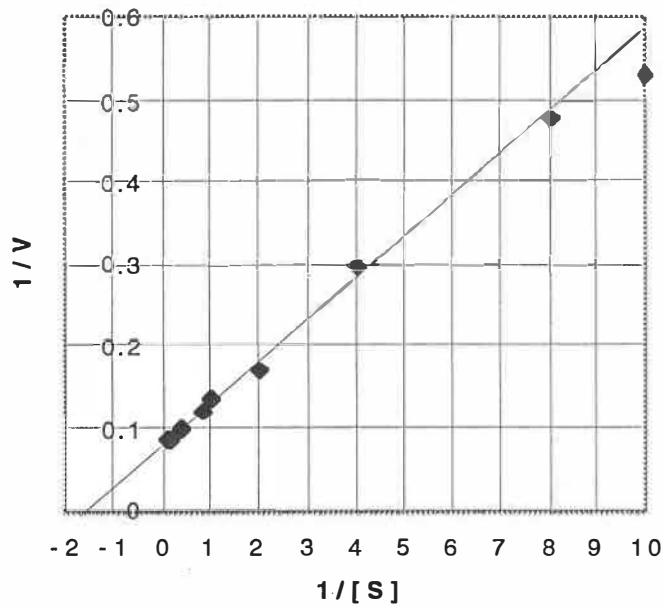
**Figure 3.22:** Lineweaver Burk plot from the rate of activity of PFK from isolate Rt 118.B2 at increasing fructose-6-phosphate concentrations.  $K_m$  for F-6-P was 0.25mM and the  $V_{max}$  was 8.49

### 3.15.5 The $K_m$ and $V_{max}$ of PFK from Rt 118.B2 with either Pyrophosphate or Polyphosphate at various concentrations

The  $K_m$  and  $V_{max}$  results for PFK at various pyrophosphate concentrations are displayed in figures 3.23 and 3.24. The  $K_m$  and  $V_{max}$  results for PFK at various tripolyphosphate concentrations are displayed in figures 3.25 and 3.26.

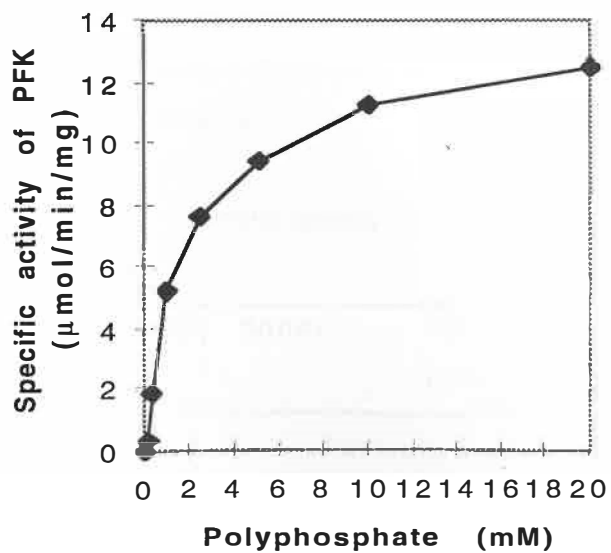


**Figure 3.23:** Rate of activity of PFK from isolate Rt 118.B2 at increasing pyrophosphate concentrations. F-6-P concentration was 1.5mM.

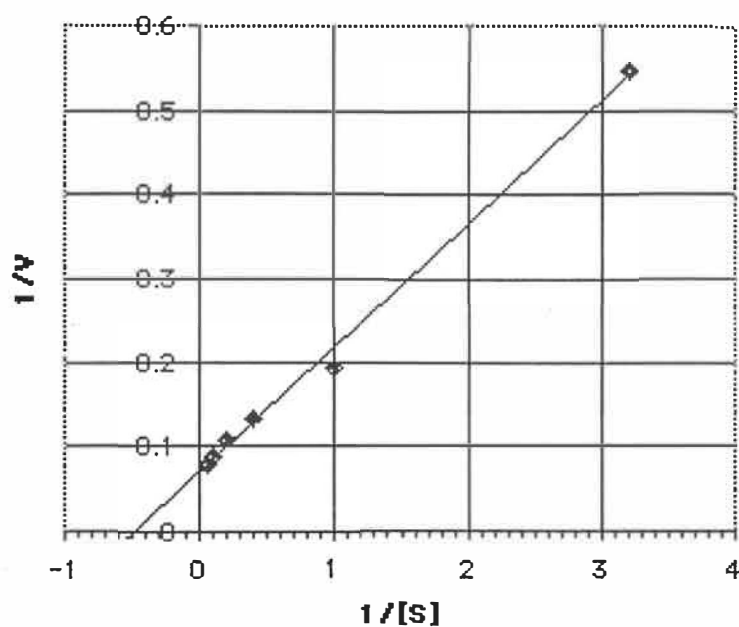


**Figure 3.24:** Lineweaver-Burk plot from the rate of activity of PFK from isolate Rt 118.B2 at increasing pyrophosphate concentrations.

$K_m$  for pyrophosphate was 0.63mM and the  $V_{max}$  was 12.99



**Figure 3.25:** Rate of activity of PFK from isolate Rt 118.B2 at increasing tripolyphosphate concentrations. F-6-P concentration was 1.5mM



**Figure 3.26:** Lineweaver-Burk plot from the rate of activity of PFK from isolate Rt 118.B2 at increasing tripolyphosphate concentrations

$K_m$  was 2.01mM and the  $V_{max}$  was 13.89

### 3.15.5.6 Specific Activity of PFK from isolate Rt 118.B2 using alternative polyphosphates.

Table 3.10 displays the specific activities of PFK with polyphosphates and pyrophosphate at 10mM concentrations.

**Table 3.10:** Specific activity of PFK from isolate Rt 118.B2 with various phosphate donors.

Phosphoryl donor	Specific activity ( $\mu\text{mol/ml/mg}$ )
Sodium glass type 75	0.06
Sodium glass type 15	0.63
Tripolyphosphate	11.25
Pyrophosphate	11.70

## 3.16 Discussion

### 3.16.1 Classification of PPI-PFK

The PPI-PFK enzyme isolated from Rt 118.B2 would appear to be a type II enzyme rather than a type I. (See section 1.4.5) Unlike type I enzymes, it has a  $K_m$  of above  $50\mu\text{M}$ . Like type II enzymes it has a pH optimum of 7. However it does not appear to require fructose 2,6-bisphosphate, which regulates type II enzymes. Until this chemical is added to the assay and is shown to greatly increase the  $V_{\text{max}}$  and affinity to fructose-6-phosphate no real conclusions on the enzymes classification can be drawn. Perhaps like *N.fowleri* and *G.lambli*a it will be found that the Rt 118.B2 enzyme will fit into neither type I nor type II categories.

The enzyme displayed an optimum pH of 7, which is also the optimum pH for growth of the organism. The optimum temperature for PFK activity was  $60^\circ\text{C}$  which was at the higher limit of the organisms growth range (Rt 118.B2 had a maximum growth temperature of  $61^\circ\text{C}$ ). Specific activity was still very high (around  $11.5\mu\text{mol}/\text{min}/\text{mg}$ ) at  $70^\circ\text{C}$ . This is to be expected, as the organism must be able to survive higher temperatures than its optimum as temperatures in a thermal area may not always remain constant. The enzyme was able to retain activity at  $75^\circ\text{C}$  for 4 hours (extrapolation of figure 3.18). Most enzymes from all organisms are resistant to higher temperatures than the organism can grow at. This is probably more related to the turnover rate of protein in a cell than a reflection on the enzyme being required to function at this temperature.

### 3.16.2 Kinetics

$K_m$  is the substrate concentration at which the rate of enzyme activity is half maximal. So the lower the  $K_m$  the more affinity an enzyme has for that substrate. The enzyme showed greatest affinity for F-6-P as a substrate, and less affinity for tripolyphosphate than for pyrophosphate as a substrate.

$V_{\text{max}}$  is the rate when the enzyme is fully saturated with substrate. The rate was faster when fully saturated with pyrophosphate than polyphosphate. The rate when fully saturated with F-6-P was slower than that when fully saturated with pyrophosphate, which indicates that the amount of pyrophosphate used in the standard assay system needs to be increased to reach

the enzymes maximal rate possible. Figure 3.23 show that at the normal concentration of PPI used in the assay (1mM), only 64% of the maximum activity is being achieved (specific activity was 11.7 $\mu$ mol/ml/mg at 10mM PPI and 7.6  $\mu$ mol/ml/mg at 1mM PPI).

Table 3.11 compares the  $K_m$  of F-6-P, PPI and tripolyphosphate from organisms which have PPI-PFK activity.

**Table 3.11:** Comparison of  $K_m$  of F-6-P, PPI and Tripolyphosphate from organisms which have PPI-PFK activity. From O'Brien et al., 1975; Mertens et al., 1993; Denton et al., 1994; and Li et al., 1995

Organism	$K_m$ of F-6-P (mM)	$K_m$ of PPI (mM)	$K_m$ of Tri poly- phosphate (mM)
Rt 118.B2	0.25	0.63	2.01
<i>N.fowleri</i>	0.01	0.015	n/a <sup>4</sup>
<i>G.lamblia</i>	0.25	0.039	n/a
<i>P.shermanii</i>	0.10	0.069	n/a
<i>E.tenella</i>	0.077	n/a	n/a

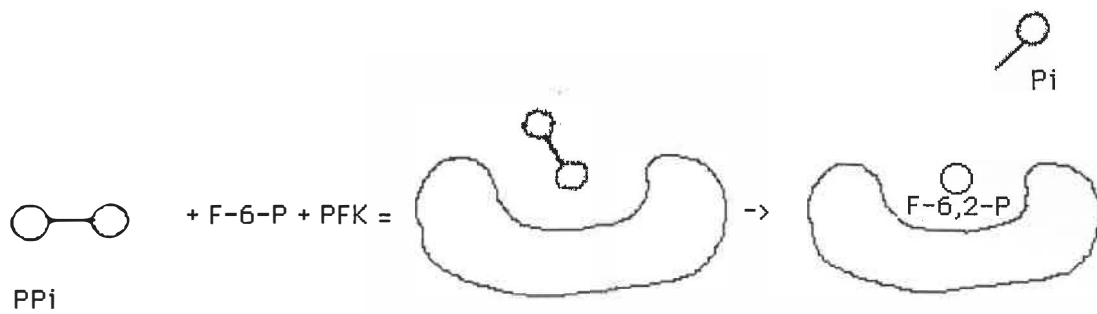
### 3.16.3 Phosphoryl donors

The partially purified PPI-PFK from Rt 118.B2 was able to utilise a number of phosphoryl donors, including polyphosphates. The fastest rates were achieved when PPI was the donor. As the number of phosphate residues increased, the specific activity decreased, until with a chain length of approximately 75 phosphates the rate was 0.06  $\mu$ mol/ml/mg.

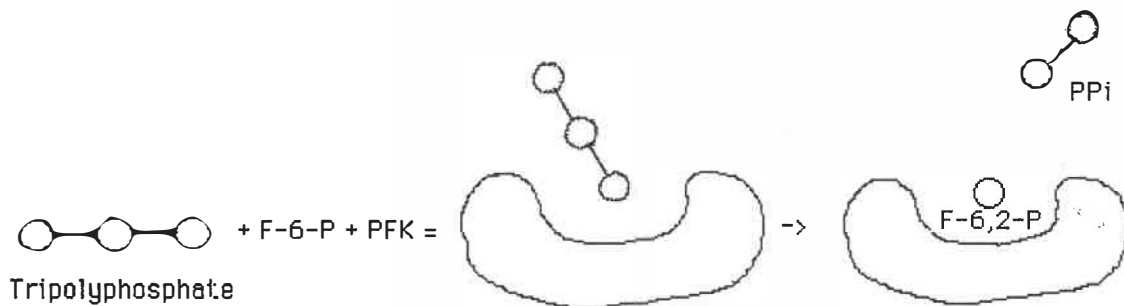
This is the first report of polyphosphate being used as a phosphoryl donor for PFK. The lower  $k_m$  of polyphosphates mean that if PPI and Poly phosphates are added together in a reaction mixture, then PPI is the preferred substrate - thus phosphate that is stored as Poly P would not be used if PPI was present.

<sup>4</sup> n/a = not assayed for or not recorded in the references that the results were taken from

The rate which tripolyphosphate could be an amalgam of 2 independent rates eg with PPi the following happens:

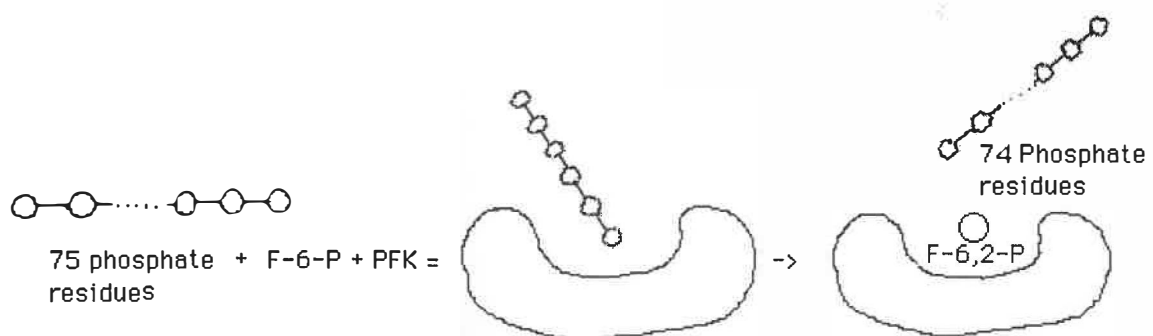


with tripolyphosphate the following happens:

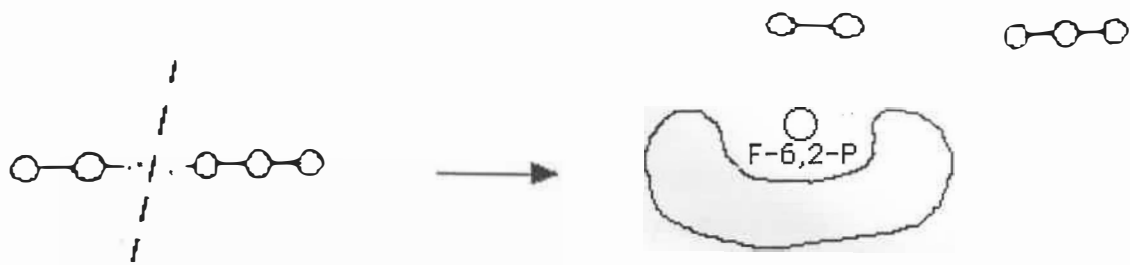


The PPi product that is released will then be the preferred substrate and compete with excess tripolyphosphate.

But with 75 Polyphosphate probably the following occurs:



Here the product (74 polyphosphate) would have basically the same affinity as the 75 polyphosphate so no difference would be noted. This assumes an exo acting polyphosphate which is probably the case - but an endo acting enzyme cannot be ruled out eg:



Site of enzyme attack

In this case the rate of reaction would speed up with time as more smaller fragments accumulate.

## References

Aksenova, H. Y., Rainey, F. A., Jansen, P. H., Morgan, H. W., Zavarzin, G. A. (1992). *Spirochaeta thermophila* sp. nov., an obligately anaerobic, polysaccharolytic, extremely thermophilic bacterium. *International Journal of Systematic Bacteriology*. **42**:175-177.

Aksenova, H. Y., Rainey, F. A., Janssen, P. H., Zavarzin, G. A., Morgan, H. W. (1992). *Spirochaeta thermophila* sp. nov., an Obligately anaerobic, polysaccharolytic, extremely thermophilic bacterium. *International journal of systematic bacteriology*. **42**:175-177.

Amann, R. I., Ludwig, W., Schleifer, K. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbial reviews*. **59**: 143-169.

Bollag, D. M., Edelstein, S. J. (1986). Protein Methods. Wiley-Liss. New York, Chichester, Brisbane, Toronto, Singapore. 56-65

Breznak, J. A., Canale-Parola, E. (1975). Morphology and physiology of *Spirochaeta aurantia* strains isolated from aquatic habitats. *Archives of microbiology*. **105**: 1-12.

Brock, T.D., (1978). Thermophilic micro-organisms and life at high temperatures. Springer - Verlag. New York.

Canale-Parola, E. (1977). Physiology and evolution of spirochetes. *Bacteriological reviews*. **41**:181-204.

Canale-Parola, E., (1991). Free-Living saccharolytic spirochetes: The genus *Spirochaeta*. In The Prokaryotes second edition, Balows, A., Truper, H. G., Dworkin, M., Harder, W., Schleifer, K. (eds). Springer - Verlag. New York. 3524 - 3536.

Denton, H., Thong, K., Coombs, G. (1994). *Eimeria tenella* contains a pyrophosphate-dependent phosphofructokinase and a pyruvate kinase with unusual allosteric regulators. *FEMS Microbiology Letters* **115**: 87-92.

Fracek, S. P., Stolz, J. F. (1985). *Spirochaeta bajacaliforniensis* sp. n. from a microbial mat community at Laguna Figueroa, Baja California Norte, Mexico. *Archives of microbiology*. **142**: 317-325.

Fontana, A., (1991). How nature engineers protein (thermo) stability. *In: Life under extreme conditions*. Guido di Prisco (ed). Springer - Verlag. 89 - 113

Gerhardt, P. (ed) *et al* (1994). *Methods for general and molecular bacteriology*. American Society for Microbiology. Washington, DC.

Greenburg, E. P., Canale-Parola, E. (1976). *Spirochaeta halophila* sp. n., a facultative anaerobe from a high salinity pond. *Archives of microbiology*. **110**:185-194

Harwood, C. S.; Canale-Parola, E. (1984). Ecology of spirochetes. *Annual review of microbiology*. **38**:161-189.

Harwood, C. S., Jannasch, H. W., Canale-Parola, E. (1982). Anaerobic spirochete from a deep-sea hydrothermal vent. *Applied and environmental microbiology*. **44**: 234-237.

Hespell, R. B., Canale-Parola, E. (1973). Glucose and pyruvate metabolism of *Spirochaeta litoralis*, an anaerobic marine spirochete. *Journal of bacteriology*. **116**:931-937.

Hudson, J.A. (1986). Taxonomy and Ecology of genus *Thermus*. Unpublished PHD thesis. School of Biological Science, Waikato University, New Zealand.

Kornberg, A. (1995). Inorganic polyphosphate: toward making a forgotten polymer unforgettable. *Journal of Bacteriology*. **177**:491-496.

Janssen, P. H., Morgan, H. W. (1992). Glucose catabolism by *Spirochaeta thermophila* RI 19.B1. *Journal of bacteriology* **174**:2449-2453.

Konings, W. N., Tolner, B., Speelmans, G., Elferink, M. G. L, de Wit, J. G., Driessen, J. M. (1992) Energy transduction and transport process in thermophilic bacteria. *Journal of Bioenergetics and Biomembranes*. **24**: 601- 609.

Leschine, S. B., Canale-Parola, E. (1986). Rifampin-resistant RNA polymerase in spirochetes. *FEMS Microbiology letters*. **35**:199-204

Li, Z., Phillips, F. B. (1995). Pyrophosphate-dependent phosphofructokinase from *Giardia lamblia*: Purification and characterization. *Protein expression and purification*. **6** 319-328.

Mandel, M., Marmur, J. (1968). Use of Ultraviolet Absorbance-Temperature Profile for Determining the Guanine plus Cytosine content of DNA. *Methods in enzymology*. **12**: 195-206

Marmur, J. (1961). A procedure for the isolation of Deoxyribonucleic Acid from Microorganisms. *Journal of molecular biology*. **3**: 208-218.

Mertens, E., De Jonckheere, J., Van Schaftingen, E. V. (1993). Pyrophosphate-dependent phosphofructokinase from the amoeba *Naegleria fowleri*, an AMP-sensitive enzyme. *Biochemical Journal*. **292**: 797-803.

Morgan, B. (1994) Unpublished results. Thermophile Research Unit. Waikato University, New Zealand.

O'Brien, W. E., Bowien, S., Wood, H. G. (1975). Isolation and characterization of a pyrophosphate-dependent phosphofructokinase from *Propionibacterium shermanii*. *The journal of biological chemistry*. **250**: 8690-8695.

Olsen, G. J., Woese, C. R., Overbeek, R. (1994). The winds of (evolutionary) change: breathing new life into microbiology. *Journal of bacteriology*. **176**: 1-6.

Owen., Hill. (1979). Estimation of base composition, base pair and genome sizes of bacterial deoxyribose acid. *In*: Skinner, F.A., Lovelock D.W. (1979). Identification methods for microbiologists. Academic press. London. 280-285

Patel, B. K. C., Morgan, H. W., Daniel, R. M., (1985). Thermophilic anaerobic spirochetes in New Zealand hot springs. *FEMS Microbiology letters*. **26**:101-106.

Pohlschroeder, M., Leschine, S. B., Canale-Parola, E. (1994). *Spirochaeta caldaria* sp. nov., a thermophilic bacterium that enhances cellulose degradation by *Clostridium thermocellum*. *Archives of microbiology*. **161**: 17-24.

Rainey, F. A., Dorsch, M., Morgan, H. W., Stackebrandt, E. (1992). 16S rDNA Analysis of *Spirochaeta thermophila*: Its phylogenetic position and implications for the systematics of the order *Spirochaetales*. *Systematics of applied microbiology*. **15**:197-202.

Rainey, F. A., Janssen, P. H., Wild, D. J. C., Morgan, H. W. (1991). Isolation and characterization of an obligately anaerobic, polysaccharolytic, extremely thermophilic member of the genus *Spirochaeta*. *Archives of Microbiology*. **155**:396-401.

Rainey, F. A. (1992) Unpublished data. Thermophile Research Unit. Waikato University, New Zealand.

Ram, A. (1993) Unpublished results. Thermophile Research Unit. Waikato University, New Zealand.

Sambrook *et al* (1989) *Advances in Molecular cloning*. 2nd edition.

Siu, P. M. , Wood, H. G. (1962). *Journal of Biological Chemistry*. **237**:3044-3051.

Stryer, L. (1988). *Biochemistry*. W. H. Freeman & Co. New York.

Uyeda, K. (1979). Phosphofructokinase. *Advances in enzymology*. **48**:193-241.

Weller, R., Bateson, M. M., Heimbuch, B. K., Kopczynski, E. D., Ward, D. M. (1992). Uncultivated Cyanobacteria, *Chloroflexus*-like inhabitants, and spirochete-like inhabitants of a hot spring microbial mat. *Applied and environmental microbiology*. **58**: 3964-3969.

Woese, C. R. (1987). Bacterial evolution. *Microbiological reviews*. **51**:221-271.

Wood, H. G. (1985). Inorganic pyrophosphates and polyphosphates as sources of energy. *Current topics in cellular regulation*. **26**:355-369.

Wood, H. G., Clark, J. E. (1988). Biological aspects of inorganic polyphosphates. *Annual review of biochemistry*. **57**: 235-260.

Zhilina, T. N., Zavarzin, G. A., Rainey, F., Kevbrin, V. V., Kostrikina, N. A., Lysenko, A. M. (1996). *Spirochaeta alkalica* sp. nov., *Spirochaeta africana* sp. nov., and *Spirochaeta asiatica* sp. nov., Alkaliphilic anaerobes from the continental soda lakes in central asia and the East African Rift. *International Journal of Sytematic Bacteriology*. **46**: 305-312.