

<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:

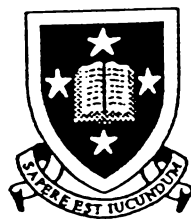
- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of the thesis, and due acknowledgement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

A Study of Diversity in Anaerobic Cellulolytic Thermophilic Bacteria

A thesis submitted in partial fulfilment
of the requirements for
the degree of
Doctor of Philosophy
in Biological Sciences

by

Frederick Andrew Rainey



University of Waikato
Hamilton, New Zealand
1992

ABSTRACT

Enrichments were set up using anaerobic bicarbonate buffered medium with cellulose and various other polysaccharides as the enrichment substrate. The inocula were water and sediment samples from freshwater and brackish thermal springs. These enrichments yielded anaerobic, cellulolytic, thermophilic bacteria with three distinct morphologies. Two of these isolates, a spirochete and a *Thermotoga*-like organism, were easily distinguished on the basis of their characteristic morphologies. The remaining isolates were rod-shaped and not easily distinguishable. The spirochete and the *Thermotoga*-like organism were fully characterised and their taxonomic position determined.

The majority of the rod-shaped isolates had temperature optima of 70°C, in contrast to the previously described cellulolytic, thermophilic species of the genus *Clostridium*, which have temperature optima of 60-65°C. The degree of diversity within this group of rod-shaped bacteria and previously described isolates from various geographic locations was determined using three methods, numerical taxonomy, protein electrophoresis and phylogenetic analysis of the 16S ribosomal RNA. The results from these three studies showed the rod-shaped organisms to be a diverse group, not closely related to the cellulolytic, thermophilic members of the genus *Clostridium*. The phylogenetic study of representative isolates demonstrated this group of organisms to be a new sub-line of descent within the *Clostridium/Bacillus* subdivision of the gram positive bacteria. The results of this polyphasic approach allowed suggestions to be made as to the taxonomic position of these organisms.

The isolation of the thermophilic spirochete and its designation to the genus *Spirochaeta* has extended the phenotypic and phylogenetic diversity of the organisms classified within the *Spirochaetales*. The phylogenetic study demonstrated the incoherence of the present taxonomy of the genus *Spirochaeta*.

The *Thermotoga*-like isolate was shown to be a member of the genus *Thermosipho* on the basis of both phenotypic and phylogenetic characteristics. The isolation of this organism extended the geographical locations from which the members of the *Thermotogales* have been isolated.

ACKNOWLEDGEMENTS

I would like to thank Professor Hugh W. Morgan and Dr Peter H. Janssen for supervising this project - their help and, above all, their encouragement, was much appreciated. Special thanks goes to Greg Cook, whose hard work ethic motivated me when the lab was otherwise deserted. The staff of Thermophile Research Unit, Lynne, Colin and Hazel, are also thanked.

The work described in this thesis was carried out in various geographical locations, including:

1. Institute of Microbiology, Academy of Sciences of the U.S.S.R., Moscow. I would like to thank Dr Lisa Bonch-Osmolovskya for arranging my visit to the laboratory of Professor G. Zavarzin. The staff of his laboratory, Dr L Bonch-Osmolovskya, Dr M Miroshnechenko, Dr H Yu Aksenova and Dr V. Svetlichny, are thanked for their help and hospitality.
2. Agricultural and Food Microbiology Research Division, Department of Agriculture Northern Ireland, Belfast. I would like to thank Mr Brian Finn for his help with the AMBIS system; also Dr Martin Collins for arranging access to the AMBIS system.
3. Department of Microbiology, University of Queensland, Brisbane, Australia. I would like to extend my gratitude to Professor Erko Stackebrandt for inviting me to his laboratory, for providing access to the facilities required for phylogenetic studies, and for instructing me in sequence alignment and phylogenetic tree construction. I would also like to thank Dr Matthias Dorsch for introducing me to 16S rDNA sequencing, and Dr Werner Liesack for valuable ideas and discussion.
4. Kermadec archipelago Expedition, 1990. I would like to thank the Captain, expedition leader, crew and scientific personnel of R/V Akademik "Alexander Nesmeyanov" (Far East Branch, Academy of Sciences of the U.S.S.R.) for their assistance during the expedition. I also acknowledge the help and friendship of Misha Zobokof, Dimar Starenin and Dimar Sorokin.

Lastly, I must acknowledge the many people whose work and writings have stimulated my interest in microbiology.

This work was possible due to funding from Pacific Enzymes.

LIST OF CONTENTS

	Page
ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
LIST OF CONTENTS	iv
LIST OF TABLES	xii
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS	xvi
 CHAPTER 1 INTRODUCTION	 1
 CHAPTER 2 A REVIEW OF THE LITERATURE	 3
 2.1 Cellulose	 4
2.1.1 Structure of cellulose	4
2.1.2 Biodegradation of cellulose	4
2.1.3 Cellulose hydrolyzing enzymes	5
2.1.4 Mechanism of enzymatic hydrolysis of cellulose	6
2.2 Bacterial Cellulases	7
2.2.1 An overview	7
2.2.2 Characteristics of bacterial cellulases	8
2.3 Thermophilic Bacteria	9
2.3.1 The terminology of thermophily	9
2.3.2 Thermophilic environments	10
2.3.3 Diversity of the thermophilic microorganisms	11
2.4 Aerobic Cellulolytic Thermophilic Bacteria	11
2.5 Anaerobic Cellulolytic Thermophilic Bacteria	12
2.5.1 Clostridial types	12
2.5.1.1 Early isolation studies	12
2.5.1.2 <i>Clostridium thermocellum</i> and <i>Clostridium thermocellulaseum</i>	12
2.5.1.3 <i>Clostridium stercorarium</i>	13
2.5.1.4 Strain T _x	15
2.5.1.5 <i>Clostridium thermolacticum</i>	15
2.5.1.6 <i>Clostridium josui</i>	15
2.5.1.7 <i>Clostridium thermocopriae</i>	15

2.5.1.8	<i>Clostridium thermopapyrolyticum</i> and <i>Clostridium cellulosi</i>	16
2.5.1.9	Isolation of clostridial types from thermal springs	16
2.5.2	Non clostridial types	17
2.5.2.1	Strain Tp8T. 6.3.3.1 ' <i>Caldocellum saccharolyticum</i> '	17
2.5.2.2	Strain NA 10	18
2.5.2.3	<i>Dictyoglomus turgidus</i>	18
2.5.2.4	Strain Z-1320 ' <i>Anaerocellum thermophilum</i> '	20
2.5.2.5	Strain H173	20
2.5.2.6	Isolation surveys	20
2.5.2.7	Cellulase activity in the <i>Thermotogales</i>	22
2.5.2.8	A study at the genomic level	23
2.6	Non-Cellulolytic Thermophilic Anaerobes	23
2.6.1	<i>Acetogenium kivui</i>	23
2.6.2	<i>Acetomicrobium flavidum</i>	23
2.6.3	<i>Acetothermus paucivorans</i>	23
2.6.4	<i>Clostridium</i> spp.	24
2.6.5	<i>Thermoanaerobium</i> spp.	25
2.6.6	<i>Thermoanaerobacter</i> spp.	26
2.6.7	<i>Thermobacteriodes</i> spp.	27
2.7	Systematics, Diversity, Classification and Taxonomy	27
2.7.1	Some definitions	27
2.7.2	Determination of diversity	28
2.7.2.1	Classical methods	29
2.7.2.2	Chemical methods	29
2.7.2.2.1	Lipid analysis	29
2.7.2.2.2	Cell wall components	31
2.7.2.2.3	End products of metabolism	31
2.7.2.2.4	Whole organism	32
2.7.2.3	Molecular methods	32
2.7.2.3.1	DNA base composition	33
2.7.2.3.2	DNA-DNA molecular hybridization	33
2.7.2.3.3	DNA-rRNA molecular hybridization	33
2.7.2.3.4	Other molecular methods	34
2.7.3	An overall strategy for determination of diversity and subsequent classification	35
2.8	Conclusion	37

CHAPTER 3	THE ISOLATION OF ANAEROBIC CELLULOLYTIC THERMOPHILIC BACTERIA	38
3.1	The Principles and Methods of Isolation	38
3.2	Methods Used in the Isolation of Anaerobic Cellulolytic Thermophilic Bacteria	40
3.3	An Isolation Study	41
3.3.1	Why more isolation ?	41
3.3.2	Methods and materials	41
3.3.2.1	Enrichment medium and culture conditions	41
3.3.2.2	Preparation of amorphous cellulose	42
3.3.2.3	Roll tube method	42
3.3.3	Results and discussion	42
3.3.3.1	Raoul Island samples	42
3.3.3.2	New Zealand samples	44
3.3.4	Conclusion	46
CHAPTER 4	A NUMERICAL CLASSIFICATION OF SOME ANAEROBIC, CELLULOLYTIC, THERMOPHILIC, ROD SHAPED BACTERIA	47
4.1	A Review of the Principles and Methods of Numerical Classification and Taxonomy Studies	48
4.1.1	Introduction to the definitions and terms used in taxonomy	48
4.1.2	The fundamental position of numerical taxonomy	49
4.1.3	The protocol of a numerical taxonomy study	49
4.1.3.1	Choice of strains	49
4.1.3.2	Test selection	50
4.1.3.2.1	Number of tests	50
4.1.3.2.2	Type of tests and test characters	51
4.1.3.3	Choice of tests	51
4.1.4	Numerical methods	52
4.1.4.1	Coding of tests	52
4.1.4.2	Test error and reproducibility	53
4.1.4.3	Resemblance	53
4.1.4.4	Clustering	55
4.1.4.5	Display of numerical taxonomy results	57
4.2	The Numerical Taxonomy Study	57

4.2.1	Introduction	57
4.2.2	Methods and materials	58
4.2.2.1	Source of strains	58
4.2.2.2	Culture conditions	61
4.2.2.3	Morphology and growth characteristics	61
4.2.2.4	Utilization of carbon sources	61
4.2.2.5	Antibiotic resistance	62
4.2.2.6	Degradation of polysaccharides	62
4.2.2.7	Growth in the absence of growth supplements	63
4.2.2.8	Tolerance tests	63
4.2.2.9	Fermentation end-products	63
4.2.2.10	Viability after 4d at optimum growth temperature	64
4.2.2.11	Hydrogen sulfide production	64
4.2.2.12	Presence DNAase	64
4.2.2.13	Calculation of test error.	64
4.2.2.14	Analysis of results	64
4.2.3	Results and discussion	65
4.2.3.1	Choice of tests	65
4.2.3.2	Experimental error	65
4.2.3.3	Clustering of the isolates	65
4.2.3.4	Composition and characteristics of the clusters	72
4.2.3.5	Tests and information which distinguish between the clusters	74
4.2.4	General conclusion	75
4.2.5	Taxonomic conclusion	76
CHAPTER 5	DIFFERENTIATION OF ANAEROBIC CELLULOLYTIC THERMOPHILIC BACTERIA BY ELECTROPHORESIS OF THEIR PROTEINS	78
5.1	A Review of the Principles and Methods of Classification and Identification of Bacteria Based on Electrophoresis of Their Proteins	79
5.1.1	Introduction	79
5.1.2	Methods	80
5.1.3	Use of PAGE in taxonomic studies	81
5.1.4	Comparison with other methods	81
5.1.4.1	Comparison with numerical taxonomy based on classical tests	81
5.1.4.2	Comparison with DNA-DNA hybridization	81

5.2	The Use of the Automated Microbiology Identification System (AMBIS) to Study the Degree of Diversity among Anaerobic, Cellulolytic, Thermophilic Bacteria	82
5.2.1	Introduction	82
5.2.2	The automated system	83
5.2.3	Methods and materials	83
5.2.3.1	Bacterial strains	83
5.2.3.2	Incorporation of ³⁵ S-methionine into bacterial proteins and sample preparation	83
5.2.3.3	Electrophoresis and analysis of the profiles	84
5.2.4	Results and discussion	86
5.3	The Use of the <i>PhastSystem</i> to Differentiate Between Anaerobic Cellulolytic Thermophilic Bacteria	87
5.3.1	Introduction	87
5.3.2	Methods and materials	87
5.3.2.1	Bacterial strains and culture conditions	87
5.3.2.2	Sample preparation	88
5.3.2.3	Electrophoresis and analysis of the profiles	88
5.3.3	Results and discussion	88
5.4	Conclusion	91
CHAPTER 6	PHYLOGENETIC ANALYSIS BASED ON 16S rDNA SEQUENCE DATA OF ANAEROBIC CELLULOLYTIC THERMOPHILIC ROD-SHAPED BACTERIA	92
6.1	A Review of the Principles and Methods of Phylogenetic Classification	93
6.1.1	Introduction	93
6.1.2	Restructuring systematics; the domains	93
6.1.3	Chronometers used in the determination of phylogenetic relationships	94
6.1.3.1	Molecular chronometers	94
6.1.3.2	Ribosomal RNAs as useful molecular chronometers	95
6.1.4	The methods applied to 16S ribosomal RNA in the determination of phylogenetic relationships	96
6.1.4.1	The 16S rRNA molecule	96
6.1.4.2	Oligonucleotide cataloging	97

6.1.4.3	Reverse transcriptase sequencing of 16S rRNA	97
6.1.4.4	Polymerase chain reaction (PCR) and direct sequencing of the PCR products	98
6.1.4.5	Sequence data analysis	100
6.1.5	Information provided by phylogenetic analysis of rRNA sequence data and its relevance to thermophilic bacteria	102
6.2	Phylogenetic Analysis of a Group of Anaerobic Cellulolytic Thermophilic Rod-Shaped Bacteria	104
6.2.1	Introduction	104
6.2.2	Methods and materials	104
6.2.2.1	Culture conditions	104
6.2.2.2	Isolation of genomic DNA	104
6.2.2.3	Amplification of 16S rDNA by polymerase chain reaction (PCR)	105
6.2.2.4	Purification of PCR products	105
6.2.2.5	Direct sequencing of PCR products	105
6.2.2.6	Electrophoresis and autoradiography	106
6.2.2.7	Data analysis	107
6.2.3	Results and discussion	107
6.2.4	Conclusion	110
CHAPTER 7	A DESCRIPTION OF THE CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF AN EXTREMELY THERMOPHILIC, POLYSACCHAROLYTIC SPIROCHETE	112
7.1	Characterization of an obligately anaerobic, polysaccharolytic, extremely thermophilic member of the genus <i>Spirochaeta</i>.	113
7.1.1	Introduction	113
7.1.2	Methods and materials	113
7.1.2.1	Medium and culture conditions	113
7.1.2.2	Electron microscopy	114
7.1.2.3	Light microscopy	115
7.1.2.4	Growth and metabolic characterization	115
7.1.2.5	G + C content	115
7.1.2.6	Chemicals	115
7.1.3	Results	116
7.1.3.1	Source of isolates	116

7.1.3.2	Enrichment and isolation	116
7.1.3.3	Morphology	116
7.1.3.4	Physiological and nutritional properties	119
7.1.3.5	Antibiotic sensitivity	121
7.1.3.6	DNA base composition	121
7.1.4	Discussion	121
7.2	<i>Spirochaeta thermophila</i>, sp. nov., an Obligately Anaerobic, Polysaccharolytic, Extremely Thermophilic Bacterium	123
7.2.1	Introduction	123
7.2.2	Methods and materials	123
7.2.2.1	Strains	123
7.2.2.2	Culture methods	123
7.2.2.3	Analytical techniques	123
7.2.3	Results and discussion	124
7.2.3.1	Thermophilic spirochete strains	124
7.2.3.2	Morphological characteristics	124
7.2.3.3	Nutritional characteristics	124
7.2.3.4	Cultural characteristics	125
7.2.3.5	Taxonomic position	125
7.2.3.6	Species description	127
7.3	The 16S rDNA analysis of <i>Spirochaeta thermophila</i>: its phylogenetic position and implications for the systematics of the order <i>Spirochaetales</i>	128
7.3.1	Introduction	128
7.3.2	Material and methods	128
7.3.2.1	Growth conditions	128
7.3.2.2	Obtaining sequence data	129
7.3.2.3	Data analysis	129
7.3.4	Results and discussion	129
7.3.5	Conclusion	136
7.4	General Conclusion	137
7.5	Addendum	138
CHAPTER 8	THE CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF AN EXTREMELY THERMOPHILIC BACTERIUM BELONGING TO THE "THERMOTOGALES"	139

8.1	Introduction to the <i>Thermotogales</i>	140
8.1.1	The genus <i>Thermotoga</i>	140
8.1.2	The genus <i>Thermosipho</i>	142
8.1.3	The genus <i>Fervidobacterium</i>	142
8.2	Characterization and phylogenetic analysis of strain RI 19. B2	143
8.2.1	Introduction	143
8.2.2	Methods and materials	143
8.2.2.1	Medium and culture conditions	143
8.2.2.2	Characterization methods	143
8.2.2.3	Phylogenetic analysis	143
8.2.3	Results and discussion	144
8.2.3.1	Source of isolate	144
8.2.3.2	Morphology	144
8.2.3.3	Physiological and nutritional properties	146
8.2.3.4	Polysaccharide degradation	146
8.2.3.5	Antibiotic sensitivity	148
8.2.3.6	DNA base composition	149
8.2.3.7	Phylogenetic analysis	149
8.2.4	Conclusion	151
CHAPTER 9	GENERAL CONCLUSION	153
LITERATURE CITED		157
APPENDIX 1	NUMERICAL TAXONOMY DATA SET	181
APPENDIX 2	CHAPTER 6 SEQUENCE ALIGNMENT	194
APPENDIX 3	CHAPTER 7 SEQUENCE ALIGNMENT	201
APPENDIX 4	CHAPTER 8 SEQUENCE ALIGNMENT	209

LIST OF TABLES

	Page
Table 2.1 Definition of organisms according to their cardinal growth temperatures (°C)	10
Table 2.2 Comparison of the characteristics of the spore-forming, anaerobic, cellulolytic, thermophilic bacteria described in the literature	14
Table 2.3 Comparison of the characteristics of the non spore-forming, anaerobic, cellulolytic, thermophilic bacteria described in the literature	19
Table 2.4 Minimum list of data for a description of a new species	36
Table 3.1 Source and enrichment conditions used in the isolation of some anaerobic, cellulolytic, thermophilic bacteria	40
Table 3.2 Site conditions and the isolates obtained from enrichments on CBM	46
Table 4.1 Anaerobic, cellulolytic, thermophilic isolates used	59
Table 4.2 Percentage distribution of positive characters to cluster groups defined at the 70% level	66
Table 5.1 Distribution of protein bands within the protein banding patterns of some isolates	90
Table 6.1 Sequence of primers used and the binding positions in the 16S rRNA primary structure	106
Table 6.2 Percentage similarity between partial 16S rDNA sequences of some anaerobic, cellulolytic, thermophilic bacteria and thermophilic <i>Clostridium</i> species (above the diagonal) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal)	108
Table 7.1 Substrates utilized as energy sources by strain RI 19. B1	119
Table 7.2 Fermentation stoichiometry and growth yields for strain RI 19. B1 grown on glucose	121

Table 7.3	The characteristics of the thermophilic spirochetes strains RI 19. B1, Z-1203 ^T and Rt3-BS1	126
Table 7.4	Percentage similarity between partial 16S rDNA sequences of <i>Spirochaeta thermophila</i> strain RI 19. B1 and other members of the order <i>Spirochaetales</i> (above the diagonal) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal)	131
Table 7.5	Distribution pattern of specific nucleotides showing the isolated, intermediate position of <i>S. thermophila</i> strain RI 19. B1 among the spirochetes from various genera	134
Table 7.6	Percentage similarity between partial 16S rDNA sequences (358 nucleotides) of <i>Spirochaeta thermophila</i> strain RI 19. B1, <i>Spirochaeta thermophila</i> strain Z-1203 ^T , type H sequences from Octopus Spring, other members of the genus <i>Spirochaeta</i> and an unrelated organism <i>Sporolactobacillus inulinus</i> (above the diagonal) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal)	135
Table 8.1	Comparison of the characteristics of the members of the <i>Thermotogales</i> and strain RI 19. B2	141
Table 8.2	Polysaccharolytic activity in <i>Thermotogales</i>	148
Table 8.3	Substrates enhancing the growth of strain RI 19. B2	149
Table 8.4	Percentage similarity between partial 16S rDNA sequences of strain RI 19. B2 and other members of the <i>Thermotogales</i> (above the diagonal) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal)	150

LIST OF FIGURES

	Page
Figure 3.1 a-c. Raoul Island, Kermadec archipelago	45
Figure 4.1 Simplified phenogram based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient	68
Figure 4.2 Detailed phenogram based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient. Clusters are defined at the 70% similarity level.	69
Figure 4.3 Detailed phenogram based on the UPGMA analysis of a similarity matrix derived from the S_J coefficient	70
Figure 4.4 A-E. Negatively stained preparations showing typical morphologies of representative isolates from clusters A-E.	71
Figure 5.1 The autoradiogram produced from the AMBIS gel	85
Figure 5.2 Dendrogram showing similarity of protein profiles of the isolates tested, the cluster designation of these isolates in the numerical taxonomy study is also shown	86
Figure 5.3 Typical protein banding patterns of representatives of three of the clusters as defined in the numerical taxonomy study (section 4.2)	89
Figure 6.2 Dendrogram indicating the position of the anaerobic, cellulolytic, thermophilic isolates among the thermophilic <i>Clostridium</i> species. The bar indicates 10 substitutions per 100 nucleotides	109
Figure 7.1 a-c. Strain RI 19. B1	117
Figure 7.2 a-c. Strain Ri 19. B1	118
Figure 7.3 Fermentation time course of strain RI 19. B1 in CBM4 containing glucose	120
Figure 7.4 Dendrogram indicating the position of <i>S. thermophila</i> strain RI 19. B1 among other representatives of the order <i>Spirochaetales</i>	130

Figure 7.5	Phylogenetic tree of the spirochetes, with <i>E. coli</i> as the outgroup. The scale bar represents a 10% difference in the nucleotide sequence as determined by taking the sum of all of the horizontal lines connecting two species. (Reproduced from Paster et al. 1991)	138
Figure 8.1	a,b. RI 19. B2	145
Figure 8.2	a-c. The typical radial diffusion assay plate results, showing polysaccharolytic activity	147
Figure 8.3	Dendrogram indicating the position of strain RI 19. B2 among the other representatives of the <i>Thermotogales</i>	150

LIST OF ABBREVIATIONS

CBM	bicarbonate buffered medium
dNTP	deoxynucleoside triphosphate
MIC	minimum inhibitory concentration
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
TYE	trypticase/yeast extract
UPGMA	unweighted pair group method using arithmetic averages
YE	yeast extract
μ	specific growth rate

CHAPTER 1

INTRODUCTION

The enormous abundance of cellulose and its potential as a renewable energy source has stimulated investigations into its degradation by both mesophilic and thermophilic microorganisms. For nearly 100 years, various attempts, both successful and unsuccessful, have been made to isolate anaerobic, cellulolytic, thermophilic bacteria. Until recently, the majority of anaerobic, cellulolytic, thermophilic bacteria isolated were shown to be sporogenous and assigned to the genus *Clostridium*. The use of thermal spring samples and higher temperature enrichments by various workers around the world has yielded a collection of extremely thermophilic, cellulolytic, asporogenous bacteria. Both the sporogenous and asporogenous isolates were obtained from enrichments on phosphate buffered medium containing yeast extract and trypticase. The majority of these organisms have rod-shaped morphology and no detailed investigations into their taxonomic position within the bacteria have been made.

The study described in this thesis had two aims: firstly to investigate the use of low phosphate bicarbonate buffered medium containing cellulose as the only carbon source for the isolation of anaerobic, cellulolytic thermophiles; secondly, and most importantly, to determine the degree of diversity within the rod-shaped, anaerobic, cellulolytic, thermophilic bacteria isolated from geographically distant thermal sites by various workers. A polyphasic approach using a numerical taxonomy study, a protein electrophoresis study and a study involving phylogenetic analysis based on 16S ribosomal RNA was followed.

Chapter 2 is a review of the large amount of literature that has been published on cellulose and its degradation by both mesophilic and thermophilic bacteria. The diversity and taxonomic position of the thermophilic, cellulose-degrading bacteria is considered. The methods available for determining microbial diversity and their application to microbial classification is also reviewed.

Chapter 3 describes an isolation study and the results obtained.

Chapter 4 is a numerical taxonomy study of the rod-shaped, anaerobic, cellulolytic, thermophilic bacteria. The methods and principles of numerical classification are also reviewed.

Chapter 5 reviews the methods, materials and applications of whole-cell protein electrophoresis in bacterial systematics, and describes two protein electrophoresis techniques as applied to the rod-shaped, anaerobic, cellulolytic, thermophilic bacteria.

Chapter 6 describes the use of phylogenetic analysis of 16S ribosomal RNA sequence data in bacterial systematics, and describes the application of this technique to the rod-shaped, anaerobic, cellulolytic, thermophilic bacteria.

Chapter 7 is a description of the characteristics and phylogenetic position of an extremely thermophilic, polysaccharolytic spirochete. A new species is assigned to the genus *Spirochaeta*.

Chapter 8 describes the characteristics of a *Thermotoga*-like isolate. On the basis of both phenotypic and phylogenetic characteristics, the isolate is identified as a member of the genus *Thermosipho*.

Chapter 9 details the general conclusions which were drawn from the work described in this thesis. Some suggestions are made as to the possible taxonomic position of the rod-shaped, anaerobic, cellulolytic, thermophilic bacteria.

CHAPTER 2

A REVIEW OF THE LITERATURE

INTRODUCTION

A large amount of literature has been published on cellulose, its degradation by microorganisms and the enzyme systems involved. The microbial degradation of cellulose at high temperatures has attracted considerable interest during the past one hundred years. Thermophilic bacteria capable of cellulose degradation have been isolated and in some cases characterized, and their taxonomic position determined. However, a large number remain uncharacterized.

In this chapter the structure of cellulose and its hydrolysis at high temperatures is reviewed, as well as the thermophilic bacteria capable of cellulose degradation. The diversity and current taxonomic position of these organisms is considered. Some of the methods which have been used in determining bacterial diversity and the application of the results to bacterial classification are reviewed. The criteria for designation of a novel isolate to a new taxon at the genus or species level is also considered.

The aim of this chapter is to review the published work on cellulolytic thermophilic anaerobes, to review the methods available for classification of new isolates and to provide a background for the study of the diversity of cellulolytic thermophilic anaerobes presented in the following chapters.

2.1 CELLULOSE

2.1.1 STRUCTURE OF CELLULOSE

Cellulose is found widely in the plant kingdom and in some groups of fungi, bacteria, invertebrates and protists (Richmond 1991). Due to its synthesis by all higher plants cellulose is the most abundant organic macromolecule on earth (Coughlan 1990).

Cellulose is a high molecular weight linear homopolymer composed of anhydro-D-glucose residues joined by β -1,4-glucosidic bonds. The number of residues i.e. the degree of polymerization depends on the source of the cellulose. In native cellulose the number of residues ranges between 10,000 and 14,000 (Fan et al. 1987; Coughlan 1990), in cellulose produced by bacteria eg. *Acetobacter xylinum* between 2,500 and 4,000 residues, while the number of residues in commercial celluloses ranges from 50-5,000 (Ljungdahl and Eriksson 1985). Data from x-ray diffraction and infrared spectral analysis indicate that adjacent glucose molecules alternate by 180° in orientation and so the basic repeating unit is cellobiose. Due to the rotation of adjacent residues and the β -1,4 linkages, cellulose has a ribbon-like structure which is stabilized by intrachain hydrogen bonds. By association with one another through interchain hydrogen bonds and van der Waals interactions, sixty to seventy adjacent chains form highly-ordered crystalline microfibrils (Rees et al. 1982). Analysis of x-ray and electron diffraction patterns has shown cellulose to exist in four recognised crystal structures designated as celluloses I, II, III, IV (Fan et al. 1987). Celluloses I and II are the most common, form I being found in nature while form II is obtained by the treatment of type I with concentrated solutions of sodium hydroxide. Celluloses III and IV are also artificial, being produced by treatments involving anhydrous ethanolamine (form III) or high temperatures (form IV).

UNIVERSITY OF WAIKATO
LIBRARY

2.1.2 BIODEGRADATION OF CELLULOSE

Weimer (1991) points out that cellulose degradation studies have been dominated by the potential applications of the process to the demands of the food, fuel, chemical and textile industries. Such studies have been carried out by researchers from a wide variety of disciplines including biochemistry, chemistry, engineering, genetics and microbiology, with the importance of cellulose as the most abundant biopolymer stressed in the majority of their publications.

Although reports of cellulose degradation studies can be found in the literature of the last century, it was the 'energy crisis' of the 1970s that increased interest in the potential of cellulose as a renewable source of fuel and chemicals and a replacement for fossil fuels. The large amounts of effort and funding put into the study of cellulose degradation led to the discovery of new cellulolytic microorganisms, both mesophiles and thermophiles, and gave insight into the cellulose enzyme complex at the molecular level. Most of the recent efforts in the field have been concentrated at the molecular level but still some workers have been involved in the isolation and characterization of new cellulolytic microorganisms.

It is the complex structure of cellulose and cellulosic materials that causes problems in its biodegradation. Physical and chemical parameters such as temperature, pH and composition of the reaction mixture determine the rate and extent of cellulose degradation by microorganisms and their cellulase enzymes. A number of substrate-related factors which contribute to the degradability of cellulose have been listed by Weimer (1991). These include the degree of polymerization, the degree of substitution, crystallinity, available surface area, pore volume and association with hemicelluloses and lignin. The structural properties are considered to be among the most important factors governing enzymatic hydrolysis of cellulose (Focher et al. 1991). Physical and chemical pretreatments of cellulosic materials can be used to aid enzymatic hydrolysis (Fan et al. 1987; Focher et al. 1991). Physical methods include dry and wet milling, high pressure treatment, high energy radiation, while chemical methods involve the intracrystalline swelling of cellulosic materials by chemical reagents such as NaOH solutions, liquid NH₃ or aqueous ZnCl₂ solutions.

2.1.3 CELLULOSE HYDROLYZING ENZYMES

Various studies involving the fractionation of culture filtrates (see Wood 1985) have revealed three classes of enzymes in both fungal and bacterial cellulase systems:

1. **Endoglucanases** (1,4- β -D-glucan-4-glucanohydrolases or endo-1,4- β -glucanases (EC 3.2.1.4) randomly cleave the internal cellulosic bonds producing a rapid decrease in polymer length along with a slow increase in reducing groups.
2. **Exoglucanases** (1,4- β -D-glucan cellobiohydrolases (EC 3.2.1.91) and 1,4- β -D-glucan glucohydrolases (EC 3.2.1.74)) act from the non-reducing end of cellulose chains of cellooligosaccharides, removing cellobiose or glucose resulting in a rapid increase in reducing sugar with little change in the overall chain length.

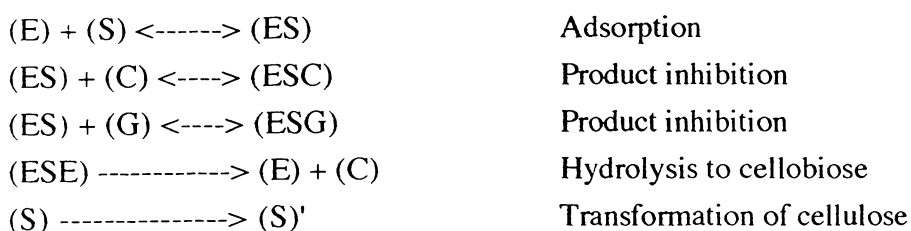
3. **β -glucosidases** (β -D-glucoside glucohydrolase or cellobiases EC 3.2.1.21) hydrolyse cellobiose and specifically cleave glucosyl units from the nonreducing ends of cello-oligosaccharides thus relieving feedback inhibition of endo- and exo- β -1,4-glucanases. Some bacteria do not have cellobiases and cleave cellobiose and cellodextrins by cellobiose phosphorylases (EC 2.4.1.20) eg. *Cellvibrio gilvus* (Sasaki et al. 1983), *Clostridium thermocellum* strain 651 (Alexander 1972a) and cellodextrin phosphorylases (EC 2.4.1.49) eg. *Clostridium thermocellum* strain 651 (Alexander 1972b) respectively.

2.1.4 MECHANISM OF ENZYMATIC HYDROLYSIS OF CELLULOSE

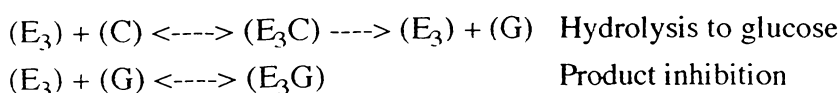
The majority of studies on the mechanism of enzymatic degradation of cellulose have been concerned with mesophilic fungi (see Wood 1985; Fan et al. 1987; Coughlan and Ljungdahl 1988). Three factors have been shown to affect the enzymatic hydrolysis of insoluble cellulose: the mode of action and properties of the cellulase, the cellulose structure, and the mode of interaction between the enzymes and the cellulose.

Fan and Lee (1983) devised a kinetic model for enzymatic hydrolysis of cellulose based on the assumption that the synergistic action of E_1 (endocellulase) and E_2 (exocellulase) considered as E hydrolyze the insoluble cellulose to cellobiose; the reaction yielding cellobiose is controlled by the extent of enzyme adsorption occurring at the surface of the cellulose, which is inhibited by cellobiose (C) and glucose (G); the cleavage of cellobiose to glucose (by E_3) in the liquid phase is competitively inhibited by glucose. The existence of cellulose as crystalline or amorphous was not considered but the conversion of cellulose (S) into a less hydrolyzable form (S') was considered. The mechanism of the proposed model results in the following steps:

Heterogenous and solid phase reactions:



Homogenous reactions:



Studies at the enzyme level (Wood and McCrae 1979; Chanzy and Henrissat 1985) show endoglucanases initiate the hydrolysis by randomly cleaving the cellulose polymer leaving exposed chain ends. From these ends exocellobiohydrolases remove cellobiose units. The secondary function of exoglucanase is the solubilization of the outermost chains on the surface of the microfibril allowing access of the endoglucanases to internal cellulose chains. Chang et al. (1981) suggested a model in which an individual cellulose molecule, once attacked by a cellulase complex, is hydrolyzed completely to cellobiose and glucose before the enzyme complex detaches and attacks another molecule.

2.2 BACTERIAL CELLULASES

2.2.1 AN OVERVIEW

The literature on bacterial cellulases is small in comparison to that on fungal cellulase systems. Coughlan and Ljungdahl (1988) pointed out that concepts and conclusions based on fungal studies have strongly influenced the bacteriologists' perception of what (and where) a cellulase is 'supposed' to be. Both aerobic and anaerobic bacteria capable of cellulose hydrolysis have been isolated from many different environments including the rumen (Hungate 1950), soils (Hagget et al. 1979; Blackall et al. 1985), compost (Madden 1983; Jin et al. 1988; Jin and Toda 1988; Sukhumavasi et al. 1988), manure (Lee and Blackburn 1975), municipal waste (Stutzenberger et al. 1970), sewage sludge (Patel et al. 1980; Murray et al. 1984), fermenting poplar wood (Sleat et al. 1984), reed beds (Mullings and Parish 1984), hot springs (Taya et al. 1985a,b; Mohagheghi et al. 1986; Sissons et al. 1987; Jones 1988; Svetlichny and Svetlichnya 1988; Jones and Lindsay 1989; Hudson et al. 1990a), marine sediments (Veiga et al. 1983), and the gland of *Deshayes* in shipworms (Waterbury et al. 1983; Green and Freer 1986). Only a few of the cellulases from these isolates have been studied. Bacteria producing enzymes with β -1,4-glucanase activity but unable to utilize cellulose as a carbon source have been described. These include *Clostridium acetobutylicum* (Lee et al. 1985), *Bacillus* spp. (Cantwell et al. 1988), *Erwinia* spp. (Chippaux 1988), and *Thermotoga* sp. (Ruttersmith and Daniel 1991).

The cellulase systems produced by cellulolytic bacteria are normally comprised of multiple activities, classified as exo- or endoglucanases. This classification is based on the activities of the enzymes on various model substrates:

Exoglucanases hydrolyze substrates such as *p*-nitrophenyl--D-cellobiose (*p*NPC) and methylumbelliferyl- β -D-cellobiose (MUC), they release cellobiose from microcrystalline cellulose (eg. Avicel) and they show weak activity on carboxymethylcellulose (CMC).

Endoglucanases do not hydrolyze the model substrates or exhibit weak activity on them, also exhibit weak activity on microcrystalline cellulose, but hydrolyze CMC.

2.2.2 CHARACTERISTICS OF BACTERIAL CELLULASES

The types of cellulases produced by a bacterium have been found to vary with the age of the culture and the growth substrate used. Various factors which may contribute to this include:

Glycosylation. *Clostridium thermocellum* has been demonstrated to produce glycosylated cellulases (Ng and Zeikus 1981). Lamed et al. (1987) presented preliminary evidence that various cellulolytic bacteria secrete glycoproteins. The change in cellulase pattern with time in the extracellular medium was suggested by Langsford et al. (1984) to be due to deglycosylation. The function of glycosyl groups is not clear but they may protect the enzymes against proteolytic enzymes (Langsford et al. 1987)

Proteolysis. Although some bacteria secrete both proteases and cellulases it has been shown that many cellulases retain their activity after truncation by proteases (Langsford et al. 1987).

Binding of cellulases to the substrate. Cellulases have been shown to adsorb strongly to the cellulose substrate (see Langsford et al. 1984; Coughlan and Ljungdahl 1988; Lamed and Bayer 1988). Lee and Blackburn (1975) and Langsford et al. (1984) found that growth of an organism in the presence of excess cellulose substrate can reduce the cellulase activity in the culture supernatant. A low molecular weight, water insoluble, yellow affinity substance that promotes the binding of an endoglucanase to the cellulose substrate was found to be produced by *Clostridium thermocellum* (Ljungdahl et al. 1983).

Aggregation of cellulases. The cellulase system of some bacteria has been shown to be produced as, or form, an aggregate. The best example and the most thoroughly investigated aggregate is the cellulosome of *Clostridium thermocellum* (Coughlan and Ljungdahl 1988; Lamed and Bayer 1988). The cellulosome of approximately 2×10^6 kDa, is comprised of at least 15 polypeptides, many of which have endoglucanase

activity. The cellulosome binds to and hydrolyzes crystalline cellulose. The cellulosomes form polycellulosomes which appear as blebs on the surface of *Clostridium thermocellum* during the early stages of growth on cellulose. The cells are bound to the cellulose by these blebs. Cellulase aggregates have been shown to be produced by other bacteria including *Bacteroides succinogenes* (Groleau and Forsberg 1981), *Ruminococcus albus* (Stack and Hungate 1984) and *Cellulomonas* sp. (Lamed et al. 1987).

All of these factors must be taken into consideration in studying or attempting to characterize the cellulase system of a given organism. Although some bacterial cellulases have been extensively investigated, for example that of *Clostridium thermocellum*, to date no cellulase system has been fully characterized.

2.3 THERMOPHILIC BACTERIA

2.3.1 THE TERMINOLOGY OF THERMOPHILY

Thermophiles have been defined as 'organisms living at high temperatures' (Brock 1978). This high temperature will depend on the group of organisms under consideration. The definition of a thermophile has been further refined by Brock (1986) to 'an organism capable of living at temperatures at or near the maximum for the taxonomic group of which it is a part'. Therefore a thermophilic insect would be one living at 45-50°C, a thermophilic alga one capable of living at 60°C, a thermophilic bacterium one capable of living at 80°C etc.. Wiegel (1990) points out that in recent years the upper and lower temperature limits for the growth of pure cultures of microorganisms has been extended to 110°C and -14°C, respectively. As no organism is capable of growth at both ends of this temperature range, organisms are grouped according to their growth temperature ranges. Table 2.1 shows how organisms have been defined according to their cardinal growth temperatures.

Table 2.1 Definition of organisms according to their cardinal growth temperatures (°C)

	T_{min}	T_{opt}	T_{max}
Psychrophiles	<0	<15	<20
Psychrotrophs	<5	>15	>20
Mesophiles	>5	<45	<50
Thermotolerants	-	<45	>50
Temperature tolerant thermophiles	<45	>65	>70
Thermophiles	>25	>45	>50
Temperature tolerant extreme thermophiles	<45	>65	>70
Extreme thermophiles	>45	>65	>70
Hyperthermophiles	?	<100	>100

From information of Wiegel (1990) and Stetter (1990).

2.3.2 THERMOPHILIC ENVIRONMENTS

The environments from which thermophilic organisms have been isolated are of two types, the 'natural' environments and the 'man-made' environments. Natural thermal environments can result from volcanic activity as in the case of thermal springs, geysers and deep-sea thermal vents, or non-volcanic factors as in the case of the Great Artesian Basin or solar heated lakes. The thermal environments associated with volcanic activity are widely dispersed throughout the world in areas which are tectonically active and where major crustal movements occur. The temperature and pH ranges of these thermal habitats cover the full range from 40->100°C and 1.0->10 respectively. The non-volcanic thermal habitats include solar heated lakes, river sediments, soils and decaying organic matter. Man-made thermal environments include deep oil reservoirs and pipelines, industrial fermentation vessels, food processing plants and hot water systems. It must also be noted that thermophilic organisms have been isolated from non-thermal environments, an example of this is the archaea *Methanobacterium thermoautotrophicum* which is found in high numbers in lake sediments that do not reach temperatures above 20°C but the T_{opt} of this organism is 68-69°C (Wiegel 1990).

2.3.3 DIVERSITY OF THE THERMOPHILIC MICROORGANISMS

Thermophilic microorganisms can be found within the domains Archaea and Bacteria as defined by Woese et al. (1990). Those of the domain Bacteria fall into the thermophilic and extremely thermophilic categories while those of the domain Archaea additionally fall within the growth temperature range of the hyperthermophile category. Both aerobic and anaerobic thermophilic bacteria and archaea have been isolated. In addition to growing at extreme temperatures, organisms with pH optima at either end of the scale from pH 1-10 have been isolated. These thermophilic organisms have been found to utilize a wide range of organic and inorganic compounds as energy sources, and produce a variety of end products in the aqueous and gaseous phases.

One group of thermophilic organisms which have been researched for nearly one hundred years are those capable of cellulose degradation. Both aerobic and anaerobic forms have been isolated and characterized. All of the cellulolytic thermophiles described fall within the domain Bacteria and as yet no cellulolytic archaea have been isolated.

2.4 AEROBIC CELLULOLYTIC THERMOPHILIC BACTERIA

The only aerobic thermophilic cellulolytic bacterium described in the literature is the moderate thermophile *Acidothermus cellulolyticus* (Mohagheghi et al. 1986). *Acidothermus cellulolyticus* was isolated from acidic thermal springs of Norris Geyser basin area of Yellowstone National Park (USA). The isolate was Gram reaction variable, asporogenous, non-pigmented with temperature and pH optima for growth of 55°C and 5, respectively. A study of the cellulase activity of *Acidothermus cellulolyticus* showed it to be the most thermostable of those studied, with the endoglucanase having a temperature optimum of 83°C (Tucker et al. 1989). Hudson et al. (1991) confirmed this finding when they compared the cellulase activity of strain H173 with those previously described in the literature. Recently a strain of *Bacillus* has been isolated from compost which degrades cellulose but has complex growth requirements (pers. comm. JA Hudson).

2.5 ANAEROBIC CELLULOLYTIC THERMOPHILIC BACTERIA

2.5.1 CLOSTRIDIAL TYPES

The most fully described anaerobic, cellulolytic, thermophilic bacteria are the spore forming clostridia which have been isolated from a wide variety of habitats including non-thermophilic ones. Table 2.2 shows a comparison of the properties of the spore forming, cellulolytic thermophiles isolated and described in the literature.

2.5.1.1 Early isolation studies

Since the turn of the century there has been a lot of interest in the potential use of thermophilic, cellulolytic bacteria to ferment cellulose directly to ethanol and organic acids. The first report of bacteria capable of fermenting cellulose at temperatures above 50°C was that of MacFayden and Blaxall (1896). They demonstrated an active fermentation of cotton and straw when mixed with mud from the River Thames (United Kingdom) and incubated at 55°C, but were unable to obtain pure cultures or colonies of the cellulolytic organisms involved. Various failed attempts to obtain pure cultures of thermophilic, cellulolytic bacteria in the first three decades of this century have been outlined by McBee (1950). These included (a) the 'pure cultures' of Langwell and Lymn (1923) which grew equally well at 37°C and 70°C either aerobically or anaerobically, (b) the isolation of an anaerobic cellulose fermenting thermophile by Viljoen, Fred and Peterson (1926) which they designated as *Clostridium thermocellum*; this culture lost its ability to ferment cellulose after having been cultured on media containing glucose, (c) studies by Rotmistrov (1939) who isolated a culture of a cellulolytic thermophile which he designated *Clostridium ellipso sporogenes*.; the extremely variable nature of this organism suggested that it was not a pure culture, a point made and demonstrated later by Imsenecki (1940). Rotmistrov (1940) did note that a low reduction potential was required for the satisfactory growth of this culture indicating its anaerobic physiology.

2.5.1.2 *Clostridium thermocellum* and *Clostridium thermocellulaseum*

Using the isolation technique developed by Hungate (1944). McBee (1948) obtained two cultures of thermophilic cellulose fermenting bacteria which were demonstrated to be pure by growing them on cellobiose agar plates followed by transfer to cellulose agar plates. The colonies were cellulolytic and had colony morphologies similar to the original colony. McBee (1950) compared seven different cultures obtained from various places, including one from Sweden, two from France and two from the United States,

with his own isolate. After purification of each culture using Hungate's technique and comparing the morphology and physiology he concluded that they all belonged to the same species, namely *C. thermocellum*. The characteristics of this species were then redescribed by McBee (1954). During this time Enebo (1951) had isolated another pure thermophilic, cellulolytic anaerobe using cellulose dextrin-agar plates and named it *Clostridium thermocellulaseum*. He also described two non-cellulolytic organisms which were closely associated with the cellulolytic strain and suggested that the mixture of these three organisms represented a symbiotic culture which had higher cellulolytic activity than that of the pure culture (Enebo 1949). *C. thermocellulaseum* had a very similar morphology to *C. thermocellum*, but differences were observed in the carbon recovery and the range of sugars fermented by the two species.

No other cellulolytic, thermophilic bacteria were described in the literature until 1975 when Lee and Blackburn (1975) isolated a cellulolytic thermophile from manure. This isolate, designated *Clostridium* strain M-7, had cell morphology and fermentative properties similar to *C. thermocellulaseum*. It was not possible for a direct comparison to be made of these strains as Enebo's strain had been lost by this time. Two strains of anaerobic cellulolytic thermophilic bacteria strains N1 and H1 were isolated by Ng et al. (1977) from sewage digester sludge and the effluent of a thermophilic dairy manure digester respectively. The comparison of strains N1 and H1 with strain LQ8 (provided by L.Y. Quinn of Iowa State University) showed them to be nearly identical in cellular and growth characteristics and it was concluded that they were the same species, namely *C. thermocellum*. A more detailed study of strain LQ8 was carried out by the working group of Zeikus who later indicated that it was not a pure culture and identified the contaminant as a non-cellulolytic organism. They re-isolated the cellulolytic organism re-designated as LQR1 from the mixed culture LQ8 (Lamed and Zeikus 1980a). Ljungdahl et al. (1981) isolated a strain of *C. thermocellum* (JW20) from a cotton bale; the strain was deposited as ATCC 31449.

2.5.1.3 *Clostridium stercorarium*

It was not until 1983 that a new species of anaerobic, cellulolytic thermophilic bacterium, isolated from compost, was added to the genus *Clostridium*, namely *C. stercorarium* (Madden 1983). *C. stercorarium* differed from *C. thermocellum* in not producing yellow pigmentation around cellulose hydrolyzing colonies, by fermenting hemicelluloses and by having a slower growth rate on cellulose.

Table 2.2 Comparison of the characteristics of the spore-forming, anaerobic, cellulolytic, thermophilic bacteria described in the literature.

Characteristic	<i>C. thermocellum</i>	<i>C. stercorarium</i>	<i>C. thermolacticum</i>	<i>C. josui</i>	<i>C. thermocopriae</i>	<i>C. thermopapyrolyticum</i>	<i>C. cellulosi</i>	Strain Tx	Strain 25A	Strain 3B
Cell size	0.5-0.6 x 4.0-5.0 μm	0.7-0.8 x 2.7-7.7 μm	0.3-0.4 x 2.0-4.0 μm	0.2-0.3 x 3.0-5.0 μm	0.5-0.7 x 2.2-6.0 μm	0.5-0.6 x 4.0-7.0 μm	0.3-0.6 x 2.0-15.0 μm	0.5-0.8 x 2.0-4.0 μm	0.5-1.0 x 2.0-4.0 μm	0.3-0.5 x 2.0-5.0 μm
Gram stain	-	-	-	+	-	+	-	+	-	-
Spore position	T ^a	T	T	T	T	ST	T	T,ST	T,ST	T
Flagella	+(ND ^b)	+(P ^c)	+(L ^d)	-	ND	+(ND)	+(LP ^e)	+(P)	+(P)	-
Temperature range (optima) °C	37-70 (60)	ND (65)	50-70 (60-65)	25-60 (45)	47-74 (60)	45-66 (59)	40-65 (55-60)	37-80 (60)	45-65 (60)	45-65 (60)
pH range (optima)	ND (7.0)	ND (7.3)	6.8-7.4 (7.0)	ND (7.0)	6.0-8.0 (6.5-7.3)	ND	6.2-8.5 (7.3-7.5)	4.0-8.0 (6.0-6.5)	5.0-9.5 (6.5-7.0)	5.0-9.5 (7.5-8.0)
Carbon source utilization :										
Cellulose	+	+	w	+	+	+	+	+	+	+
Xylan		+	+	+	+	ND	ND	+	ND	ND
Glucose	-	+	+	+	+	+	ND	+	ND	+ ^f
Fructose	-	-	+	-	+	+	+	+	ND	+
Raffinose	-	-	+	-	+	-	+	+	ND	ND
Rhamnose	-	+	+	-	-	ND	-	+	ND	ND
Starch	-	+	+	-	+	+	+	+	ND	ND
Sucrose	-	-	+	-	+	-	+	+	ND	ND
Mannitol		-	-	-	-	+	+	+	ND	ND
Melobiose	-	+	+	+	-	-	+	+	ND	ND
H ₂ S production	-	-	-	ND	+	+	-	-	-	-
mol% G+C	38-39	39.0	40.9	40.0	37.2	34.0	35.0	ND	ND	38.5
End products ^g	A, E, B, L	A, E, L	A, E, L	A, E, B, P	A, E, B, L	A, E, L, Bu	A, E	A, E, F, L	A, E	A, E

^a Position of endospore, T, terminal; ST, sub terminal. ^b ND, Not determined. ^c P, Peritrichous flagella. ^d L, Lateral flagella. ^e Lophotrichous flagella. ^f 6g/l yeast extract added.

^g End products A, acetate; B, butyrate; Bu, butanol; E, ethanol; F, formate; L, lactate; P, propionate.

2.5.1.4 Strain T_x

The strain T_x also isolated from compost by Taya et al. (1984) was identified as a *Clostridium* sp. on the basis of morphological and physiological characteristics. A few differences between strain T_x and *C. thermocellum* were noted but the isolate was not designated as a new species or further characterized.

2.5.1.5 *Clostridium thermolacticum*

Two strains of sporulating thermophiles strains TC21 and TX41 were isolated from methanogenic enrichment cultures on cellulose and xylan respectively (Le Ruyet et al. 1985). Both strains were designated as a new species of the genus *Clostridium*, namely *C. thermolacticum*, but only strain TC21 has the ability to weakly ferment cellulose. The two strains were differentiated from previously described *Clostridium* species on the basis of carbon utilization properties, the production of lactate as a main end product of fermentation and by comparison of its electrophoretic protein pattern with those of other thermophilic *Clostridia*.

2.5.1.6 *Clostridium josui*

An anaerobic, cellulolytic, moderately thermophilic (optimal temperature, 45°C), spore-forming bacterium was isolated from Thai compost and designated as a new species of the genus *Clostridium* namely *C. josui* (Sukhumavasi et al. 1988). The lower growth temperature range of *C. josui* was used to differentiate it from the previously described cellulolytic strains.

2.1.5.7 *Clostridium thermocopriae*

Jin and Toda (1988) described the isolation of six strains (JT) of spore-forming anaerobic thermophilic cellulolytic bacteria from camel feces, compost soil and hot spring water in Japan. These strains were classified as members of the genus *Clostridium* and designated as a new species, *C. thermocopriae* with strain JT-3^T as the type strain (Jin et al. 1988). Differences in optimum growth temperature, end products of fermentation and carbon source utilization patterns were used to differentiate the JT strains from *C. thermocellum* and *C. stercorarium* and justify their designation to a new species of the genus *Clostridium*. The utilization of fructose was used as a differentiating characteristic despite the fact that some of the JT strains gave a weak acid reaction on fructose.

2.5.1.8 *Clostridium thermopapyrolyticum* and *Clostridium cellulosi*

Recently two new thermophilic cellulolytic isolates were designated to the genus *Clostridium*, namely *C. thermopapyrolyticum* and *C. cellulosi*. *C. thermopapyrolyticum* was isolated from a riverside mud sample in Brazil (Mendez et al. 1991), while *C. cellulosi* was isolated in China from cow manure compost (Yanling et al. 1991). The former species was differentiated from the other cellulolytic clostridia on the basis of it being motile and the subterminal position of its spores in contrast to the terminal position of the spores in the previously described strains. The reasons for the designation of *C. cellulosi* as a new species concentrated on different substrate utilization properties and the isolate having lophotrichous flagella.

2.5.1.9 Isolation of clostridial types from thermal springs

The work of Khraptsova et al. (1984) was the first report of the isolation of anaerobic, cellulolytic, thermophilic bacteria from a thermal spring. Three strains were isolated from thermal spring samples collected in Buryatia (USSR). It was noted that these strains acquired a bright yellow pigment after two to four days growth and completely decomposed the filter paper substrate within seven days. Two of the isolates formed spores but no further characterization of these isolates was described in the literature.

The isolation of anaerobic, crystalline cellulose decomposing, thermophilic, spore-forming bacteria from an alkaline hot spring in Japan was reported by Taya et al. (1985a). Strain 25A was reported as being nearly identical to *C. thermocellum* and strain 3B as having some different characteristics. Strain 3B decomposed microcrystalline cellulose (Avicel) that had not been chemically or physically pretreated.

Recently a survey of cellulolytic anaerobic thermophiles isolated from hot springs was carried out using a variety of media and enrichment temperatures (Hudson et al. 1990a). Of the seven strains listed in this work only one was described as a strain of *C. thermocellum*, the others being described as non-clostridial types with higher growth temperature ranges.

All of the above named clostridial type cellulolytic thermophiles were assigned to the genus *Clostridium* on the basis of their sporogenous nature. The designation of species was on the basis of substrates utilized, end products of fermentation or growth temperature ranges. None of these strains of thermophilic clostridia were designated as new species on the basis of DNA-DNA hybridization or phylogenetic studies.

2.5.2 NON CLOSTRIDIAL TYPES

The clostridial type cellulolytic thermophilic anaerobes described above were, with the exception of strain T_x (Taya et al. 1984) found to have temperature maxima less than 75°C and temperature optima around 60-65°C. In contrast the asporogenous cellulolytic anaerobic thermophiles described in the literature have temperature maxima of >70°C and higher growth temperature ranges. These organisms have been described as asporogenous in that endospores have not been observed. Table 2.3 shows a comparison of the properties of these asporogenous isolates described in the literature.

Ljungdahl et al. (1981) first reported the existence of extremely thermophilic cellulolytic bacteria when he obtained from an Icelandic hot pool a culture that degraded cellulose at 84°C. Since the mid 1980s the isolation of a number of anaerobic thermophiles that degrade cellulosic materials optimally at temperatures of 70°C and above has been reported.

2.5.2.1 Strain Tp8T. 6.3.3.1 '*Caldocellum saccharolyticum*'

Three extremely thermophilic cellulolytic anaerobes isolated from New Zealand hot springs that grew at 70°C were compared with *C.thermocellum* and their cellulolytic potential evaluated (Reynolds et al. 1986). The isolation of these three strains, Tp8T. 6.3.3.1, Tp11 and KT8 along with the isolation of five other similar strains was described by Sissons et al. (1987). These isolates which grew at 75°C had a Gram-positive cell wall structure and did not form endospores. Reynolds et al. (1986) had suggested that the similarity of strain Tp8T. 6.3.3.1 to *C.thermocellum* in cellulase production and substrate binding properties and its ability to grow on a defined medium known to support clostridial group organisms may have made it more "*Clostridium-like*" than the other isolates from the study. The later study of Sissons et al. (1987) stated that it was unlikely that the isolates were clostridia as DNA-DNA hybridization studies had confirmed the lack of relationship between the isolates and *C.thermocellum* and that that result suggested the isolates belonged to a new genus. Although one of the isolates, strain Tp8T. 6.3.3.1, was deposited with the American Type Culture Collection, Rockville Md. as ATCC 43494 and it was reported that the name *Caldocellum saccharolyticum* gen. nov., sp. nov. had been proposed, there have been no subsequent reports on the taxonomic status of these isolates. Five of the isolates of Reynolds et al. (1986) and Sissons et al. (1987) including strain Tp8T. 6.3.3.1 were compared with respect to their ability to degrade lignocellulosic substrates (Donnison et al. 1989). The ability of these isolates to degrade the lignocellulosics tested appeared limited when they

were used as growth substrates and it was suggested that the use of either crude or partially purified enzymes may be more efficient in the hydrolysis of such compounds.

2.5.2.2 Strain NA 10

Taya et al. (1985b) in a short communication described the isolation of an anaerobic, cellulolytic, thermophile from a hot spring in the Nozawa district of Japan. This isolate which was designated as strain NA10 had a requirement for tungsten. The isolation and characterization of strain NA10 was reported in more detail in a later publication (Taya et al. 1988). A comparative study of the properties of NA10 was made with *Thermoanaerobacter ethanolicus*, *C.thermocellum*, *C. thermohydrosulfuricum* and strain Tp8T. 6.3.3.1. Strain NA10 was found to be similar to *C.thermocellum* in its ability to digest cellulose but differed in being asporogenous and having higher temperature and pH optima. The same working group had previously cloned a 4.8kb *Hind III* fragment from NA10 DNA into *Escherichia coli* and shown that it directed the synthesis of a thermostable β -glucanase (Honda et al. 1987). In this report they had assigned NA10 to the genus *Thermanaerobacter* as a species, namely *T. cellulolyticus*, but to date this species name has not been validated. They found that the gene coding the β -endo-glucanase activity did not hybridize with the chromosomal DNA of *C.thermocellum* suggesting that the cellulase produced by NA10 was different to that from *C.thermocellum*. They also found that ^{32}P -labelled chromosomal DNA of strain NA10 did not hybridise to chromosomal DNAs of *C.thermocellum*, *T. ethanolicus* or *Ruminococcus albus*. They suggested that strain NA10 was different to the other cellulolytic anaerobes and thermophilic anaerobes tested. Although detailed physiological data on the cellulolytic thermophile Tp8T. 6.3.3.1 (Sissons et al. 1987) was not available they suggested that NA10 was similar to this strain. The need for further studies, including a taxonomic comparison between strain NA10 and closely related anaerobes was recognised, but to date no further information on the taxonomic position of strain NA10 has been published.

2.5.2.3 *Dictyoglomus turgidus*

Svetlichny and Svetlichnya (1988) described the isolation and characterization of a new extremely thermophilic polysaccharolytic bacterium and assigned it to the genus *Dictyoglomus* as a new species *Dictyoglomus turgidus*. This morphologically distinct organism was reported to utilize starch, glycogen, pectin, cellulose, lignin and humic acid, had resistance to rifampin (100 $\mu\text{g/ml}$) and grew at temperatures up to 86°C. This is the only report of an anaerobic thermophile utilizing lignin and humic acid.

Table 2.3. Comparison of the characteristics of the non spore-forming, anaerobic, cellulolytic, thermophilic bacteria described in the literature.

Characteristic	Strain Tp8.T6.3.3.1	Strain NA10	Strain F2/1	<i>Anaerocellum thermophilum</i>	Strain H173
Cell size	0.6 x 3.0-4.0 μm	0.6-0.9 x 1.8-4.0 μm	ND ^a x 1.0-3.0 μm	0.7 x 2.5-4.0 μm	ND
Gram stain	-	-	-	+	-
Flagella	-	+(P ^b)	+(L ^c)	+(P)	ND
Temperature range (optima) °C	ND (72)	50-85 (75)	55-81 (~70)	40-83 (72-75)	51-74 (70)
pH range (optima)	ND	6.0-9.5 (8.0-8.3)	6.0-7.5 (ND)	5.0-8.3 (7.1-7.3)	5.7-7.7 (7.0)
Carbon source utilization :					
Xylan	+	+	+	+	+
Glucose	+	+	+	+	-
Fructose	+	-	+	+	-
Raffinose	-	+	+	W ^d	-
Rhamnose	+	-	-	ND	-
Starch	+	+	+	+	+
Sucrose	+	+	+	+	-
Mannitol	+	-	-	-	-
Melobiose		-	ND	ND	-
H ₂ S production	-	+	-	-	-
mol% G+C	ND	37.7	ND	36.7	ND
Resistance to Rifampin(100 $\mu\text{g/ml}$)	+	+	+	+	ND
End products ^e	A, L, E	A, L	A, E, L	A, E, L	A, E

^a ND, Not determined. ^b P, Peritrichous flagella. ^c L, Lateral flagella. ^d W, weak growth.

^e End products A, acetate; E, ethanol; L, lactate.

2.5.2.4 Strain Z-1320 '*Anaerocellum thermophilum*'

The first asporogenous extremely thermophilic, cellulolytic, anaerobe to be designated to a new genus was *Anaerocellum thermophilum* gen. nov., sp. nov. (Svetlichny et al. 1990a, 1990b). *A.thermophilum* strain Z-1320 was isolated from a hot spring in the Valley of Geysers, Kamchatka, Russia. A full description of the isolate was given and it was compared and contrasted with other cellulolytic and non-cellulolytic, anaerobic thermophiles. These comparisons are on the basis of phenotypic characteristics, and no DNA-DNA hybridization data was presented. It was suggested that *A.thermophilum* may be similar to *Caldocellum saccharolyticum*. Strain Z-1320 was also shown to constitutively produce extremely thermostable extracellular enzymes of the cellulase complex, which are not substrate-bound and have a specific activity greater than the analogous activity of *C. thermocellum*. The genus and species name *A.thermophilum* has not, as yet, been validated or added to the approved list of bacterial names.

2.5.2.5 Strain H173

Hudson et al. (1990b) described the cellulolytic properties of an extremely thermophilic anaerobe which they designated H173. This organism was isolated from a hot spring algal mat using the nutrient rich TYE medium described by Zeikus et al. (1979) with larchwood xylan substituted for glucose. Strain H173 grew optimally at 70°C and pH 7.0, utilizing a narrow range of carbon sources including cellobiose, Avicel, xylan, and starch but not glucose. The taxonomic position of strain H173 was not established, but it was noted that it appeared different from NA10 due to its pH optima difference and different from the other New Zealand strains isolated by Reynolds et al. (1986) due to differences in fermentation end-products. The fermentation end products of strain H173 were determined on a variety of basal media containing various amounts of cellulosic substrates. The fermentation end-products were not determined on minimal medium plus cellobiose.

2.5.2.6 Isolation surveys

A number of workers have carried out extensive isolation surveys in an attempt to determine the prevalence of anaerobic cellulolytic thermophiles and optimize the enrichment and isolation parameters.

In poster presentations Jones (1988) and Jones and Lindsay (1989) described some anaerobic, cellulolytic, thermophilic bacteria isolated from hot springs and unheated

soils. The results of an extensive sampling, enrichment program in various sites around the world were reported. They found that the moderately thermophilic (T_{opt} 50-55°C), cellulolytic anaerobes isolated from neutral hot springs were very similar to those isolated from the average unheated soil, in so much that they were spore-forming, rod-shaped bacteria. Such moderate thermophiles from hot springs were all ethanologenic while some non-ethanologenic types were found in unheated soils. The extremely thermophilic (T_{opt} 70-75°C), cellulolytic, anaerobic, bacteria were only found in samples from hot springs with pH values around neutral. No cellulolytic anaerobes were enriched at 85°C, or pH 4, or from acidic (solfataric) environments. Only one of the extremely thermophilic asporogenous strains isolated was described in detail and designated strain F2/1. Strain F2/1 was isolated from a sample of damp sand collected from a steaming beach at Fumarole, Italy. The isolate had a Gram positive wall structure and did not form endospores. Strain F2/1 has not been compared or contrasted with other isolates from that study or previous studies.

In a poster presentation Svetlichny et al. (1990a) described the diversity and distribution of extremely thermophilic, cellulolytic, anaerobes in both freshwater continental and shallow marine coastal hot springs of the Soviet Far East. From enrichment cultures with micro-crystalline cellulose as the sole carbon and energy source eleven pure cultures of freshwater anaerobic, rod-shaped, asporogenous bacteria were isolated. These were morphologically and physiologically similar to *A.thermophilum* and *Dictyoglomus* sp.. From the shallow coastal marine hydrotherms twelve strains of marine anaerobic, extremely thermophilic, asporogenous, cellulolytic eubacteria with morphologies similar to *Thermotoga* sp. were isolated from enrichment cultures with cellulose as the only carbon source. These isolates, with temperature optima of 80-85°C were identified as strains of *Thermotoga maritima* and *Thermotoga neapolitana*. This report not only described organisms from another family namely *Thermotogales* that are capable of anaerobic degradation of cellulose at high temperature but was the first report of cellulose degradation in the higher temperature range ie. at 85°C. The same poster described the isolation of a marine, obligately anaerobic spirochete which degraded cellulose at 68°C and pH 7.5. This was the first report of members of the genus *Spirochaeta* capable of growth at >60°C and of degrading polysaccharide, in this case cellulose. In general the study demonstrated that extremely thermophilic, cellulolytic, bacteria are widely distributed in freshwater and marine hot springs and great diversity exists within the organisms isolated. Extremely thermophilic, cellulolytic, anaerobes were not found in acidic hot springs or in those devoid of allochthonous organic matter. No archaea capable of cellulose utilization were isolated.

A similar survey of thermophilic, cellulolytic, anaerobes from hot springs was carried out on samples from New Zealand and the Azores (Hudson et al. 1990a). This study demonstrated the influence of substrate on the success of the sampling and enrichment program. Avicel, steam exploded poplar (SEP) and alkali washed steam exploded poplar (AWS) were used as cellulosic substrates in a number of basal media. The highest number of positive enrichments were obtained with Avicel and AWS as the cellulosic substrates. As in the surveys of Jones (1988) and Svetlichny (1990a) no acidophilic cellulolytic thermophiles were isolated. The isolates from this study were mainly rod-shaped asporogenous eubacteria similar to strain Tp8T. 6.3.3.1 (Sissons et al. 1987) or the strain NA10 (Taya et al. 1988). Only one clostridial type isolate was obtained from the large number of pools sampled and it was suggested that the temperatures of enrichment incubation 65 and 75°C were too high to allow enrichment of *C. thermocellum*. If *C. thermocellum* can be enriched at 65°C then it was suggested that the organism was not common in hot springs of New Zealand and the Azores.

In the abstract of an oral presentation at the 5th European Congress on Biotechnology, Copenhagen July 1990, Mathrani and Ahring (1990) described the isolation of anaerobic, thermophilic, cellulose and hemicellulose degrading bacteria from several natural and man-made anaerobic high-temperature environments, including sludge from a paper bound mill and geothermal springs. They reported the isolation of typical rod-shaped organisms and a long thin hemicellulose degrading rod which formed unusual spherical structures. Although not indicated in the abstract the later organism may be a *Dictyoglomus* species.

2.5.2.7 Cellulase activity in the *Thermotogales*

In addition to the report of cellulolytic activity in the *Thermotogales* (Svetlichny et al. 1990a), Huber et al. (1990) described a new species of *Fervidobacterium*, *Fervidobacterium islandicum* and noted that growth was stimulated by the addition of cellulose. The type of cellulose used was not reported. Ruttersmith and Daniel (1991) reported the purification and properties of a cellobiohydrolase from a *Thermotoga* sp., strain FjSS3 B1 which does not grow on cellulose as a sole carbon source.

2.5.2.8 A study at the genomic level

In a study of the genotypic properties of several anaerobic, thermophilic chemoorganotrophic bacteria a thermophilic asporogenous bacterium capable of hydrolyzing cellulose and designated strain ATP1 was used in DNA-DNA hybridization studies (Krivenko et al. 1987). The source, morphological or physiological properties of this isolate were not reported but it was found to have a high homology to the non-cellulolytic *Thermoanaerobium brockii*.

2.6 NON-CELLULOLYTIC THERMOPHILIC ANAEROBES

It is worth considering the properties of the non-cellulolytic glycolytic thermophilic anaerobic bacteria as the organisms described above (section 2.5) may be closely related to them or be cellulolytic species of described genera. The non-cellulolytic glycolytic thermophilic anaerobes will be listed and a brief description given for the described species of each genus.

2.6.1 *Acetogenium kivui*

This asporogenous, homoacetate fermenter was isolated from Lake Kivu (S.E. Africa) and characterized by Leigh and Wolfe (1983). *Ac. kivui* ferments a range of carbon sources and grows autotrophically with H_2/CO_2 . The T_{opt} and pH range for growth are 65°C and 5.0-7.7, respectively. Phylogenetically it was found not to be closely related to *C. thermoaceticum* and *C. thermoautotrophicum* but still within the clostridial group (Tanner et al. 1982).

2.6.2 *Acetomicrobium flavidum*

This Gram-type negative asporogenous acetate forming thermophilic anaerobe was isolated from a sewage sludge digester at 60°C, in which the organism made up 40% of the microbial population (Soutschek et al. 1984). From 1 mol hexose this organism produces 2 mol acetic acid, 2 mol CO_2 and 4 mol H_2 and was considered an ideal organism for use in thermophilic biogas digesters, since it hydrolyses several polymers to methanogenic substrates.

2.6.3 *Acetothermus paucivorans*

Another Gram-type negative acetate forming thermophilic anaerobe isolated from a sewage sludge digester at 60°C (Dietrich et al. 1988). Although similar to

Acetomicrobium flavidum in physiology and morphology, DNA-DNA hybridization showed only 24% homology. *Acetothermus paucivorans* has the very limited substrate utilization range of glucose and fructose and a requirement for vitamin B₁₂.

2.6.4 *Clostridium* spp.

The genus *Clostridium* contains eight non-cellulolytic, thermophilic species: *C. thermosaccharolyticum*, *C. thermoaceticum*, *C. thermohydrosulfuricum*, *C. thermoautotrophicum*, *C. thermosulfurogenes*, *C. fervidus*, *C. thermolacticum* (strain TX41), and *C. thermobutyricum*. These species have been isolated from a wide range of thermal environments, both natural and man-made.

C. thermosaccharolyticum was first isolated from 'blown' food cans (McClung 1935) and subsequently from soils, sewage sludge, and beet sugar juices (see DeCampos et al. 1986). The end products of fermentation have been shown to vary depending on the growth medium composition and pH (Freier et al. 1989). *C. thermosaccharolyticum* sporulates well and sporulation is enhanced when grown at pH values slightly above the pH_{min} (Hsu and Ordal 1969). *C. thermosaccharolyticum* differs from *C. thermohydrosulfuricum* in forming only small amounts of H₂S and in having a lower T_{max} ie. 67°C.

C. thermoaceticum exists as only one strain which was isolated from horse manure (Fontaine et al. 1942). The physiology and metabolism of this homo-acetogenic organism has been extensively researched. Like *C. thermosaccharolyticum*, *C. thermoaceticum* has a T_{max} in the lower temperature range ie. 65°C, but differs in not degrading polysaccharides.

C. thermohydrosulfuricum was first isolated from an Austrian sugar factory (Hollaus and Klaushofer 1973) and later from hot springs, river sediments, sewage sludge and animal manure (Patel et al. 1988; Wiegel et al. 1979). The majority of the research on this organism has been on two strains, a sugar factory isolate (the neotype) strain E100-69 (Matteuzzi et al. 1978) and a thermal spring isolate strain 39E (Zeikus et al. 1980). *C. thermohydrosulfuricum* utilizes a wide range of substrates including polysaccharides such as glycogen, pectin and xylan. The end products of glucose fermentation include ethanol, acetate, lactate, CO₂ and H₂ (Wiegel et al. 1979). This organism has the highest T_{max} of the thermophilic clostridia at 78°C and grows optimally at 69°C.

C. thermoautotrophicum has been isolated from hot springs, sediment samples, thermal soil and sewage sludge fermenters (Wiegel et al. 1981; Ilarionow 1985). This

homoacetogen differs from *C. thermoaceticum* in its higher sporulation, and wider pH and temperature growth ranges; DNA-DNA hybridization showed the two organisms to have 50% homology. The study of Bateson et al. (1989) showed the partial 16S rRNA sequences of *C. thermoautotrophicum* and *C. thermoaceticum* to be 99.8% similar.

C. thermosulfurogenes. Two pectinolytic strains have been isolated from thermal springs (Schink and Zeikus 1983). The characteristic that distinguishes this organism from the other thermophilic clostridia is its ability to produce large amounts of sulfur from thiosulfate. This organism grows optimally at 60°C in the pH range 4.0-7.6 on a wide range of carbon sources.

C. fervidus was isolated from a New Zealand hot spring and to date only one strain has been described (Patel et al. 1987). The organism produces mainly acetate, CO₂ and H₂ from hexoses and pentoses but differs from the other acetogenic clostridia in not forming sulfur from thiosulfate and its inability to grow autotrophically. The phylogenetic relationship of this organism to the other thermophilic clostridia is as yet unknown.

C. thermolacticum (strain TX41) was isolated from a methanogenic enrichment culture on xylan and designated the type species (Le Ruyet et al. 1985). The organism, which produces lactate as the main fermentation end product, was found to have a characteristically different protein pattern compared to the other thermophilic clostridial species.

C. thermobutyricum was isolated from a cellulose degrading enrichment culture inoculated with horse manure (Wiegel et al. 1989). This organism, which produces butyrate as the main end product of glucose fermentation, has a growth temperature range similar to *C. thermosaccharolyticum* but differs from it in not forming ethanol and having a higher GC content. *C. thermobutyricum* differs from the other moderately thermophilic clostridial species in its inability to hydrolyze polymers such as xylan, pectin and starch.

2.6.5 *Thermoanaerobium* spp.

Thermoanaerobium brockii was isolated from a hot spring in the Yellowstone National Park, USA (Zeikus et al. 1979). The organism had a T_{opt} of 70°C, a T_{max} of <85°C and fermented a range of carbohydrates including pectin and starch to ethanol, lactate, acetate, CO₂ and H₂. It was assigned to the new genus on the basis of it being asporogenous. Recently Cook et al. (1991) induced sporulation in the type strain of *T. brockii* when it was grown on minimal salts medium. This finding, along with that of

Bateson et al. (1989) that *T. Brockii* showed high similarity to *C. thermohydrosulfuricum* strain 39E at the 16S rRNA level, brings into question the taxonomic position of this organism.

***Thermoanaerobium lactoethylicum*.** The isolation from a hot spring in Kamchatka of *Thermoanaerobium lactoethylicum* was first described by Zacharova et al. (1987) and later by Kondratieva et al. (1989). *T. lactoethylicum* was found to be most similar to *Thermoanaerobium Brockii* but differed from *T. Brockii* in that it could not reduce acetone to isopropanol and the level of DNA-DNA homology between the two organisms was only 14%. The DNA-DNA homology studies of Krivenko et al. (1987) and Kondratieva et al. (1989) lead them to suggest that the genera *Thermoanaerobium* and *Thermoanaerobacter* be combined and the genus name *Thermoanaerobium* retained. As *Thermoanaerobacter ethanolicus* was not included in their study further taxonomic data, possibly at the phylogenetic level would be required before such a change could be made.

2.6.6 *Thermoanaerobacter* spp.

Thermoanaerobacter ethanolicus was isolated and characterized by Wiegel and Ljungdahl (1981). Two strains JW200 and JW201 were isolated from slightly acidic and slightly alkaline hot springs, respectively. Strain JW200 was designated the type strain of the species. *T. ethanolicus* was placed in a genus separate from *Thermoanaerobium Brockii* on the basis of differences in cell wall structure and Gram-stain reaction. A wide range of substrates are utilized by both strains of *T. ethanolicus* and the fermentation end products from glucose and starch are mainly ethanol and CO₂ with minor amounts of lactate and acetate. Growth occurs over a wide temperature range (37-78°C) and a wide pH range (4.4-9.9).

Thermoanaerobacter finnii was isolated from a continuous acetate-to-methane enrichment culture of a sediment sample from Lake Kivu (Schmid et al. 1986). *T. finnii* utilizes a range of substrates and the main products of glucose fermentation are ethanol and CO₂. DNA-DNA hybridization showed 75% homology between *T. finnii* and *T. ethanolicus* but no clostridial type strains were included in the study. The fact that *T. finnii* forms heat resistant spores differentiates it from *T. ethanolicus*. No further taxonomic studies have been carried out on this organism, so its taxonomic position remains unknown at present.

2.6.7 *Thermobacteriodes* spp.

Thermobacteroides acetoethylicus was isolated from a thermal spring and was determined by dilution enumerations to be one of the most prevalent thermophilic bacteria in the hot springs of Yellowstone National Park (Ben-Bassat and Zeikus 1981; Zeikus et al. 1980). The organism grows on a range of substrates in the temperature range 40-80°C and ferments glucose to ethanol, acetate, CO₂, H₂, butyrate and isobutyrate but not lactate. *Thermobacteroides acetoethylicus* was designated a new genus on the basis of its distinctive cell wall characteristics, not forming lactate and being inhibited by 2% NaCl but not H₂. No DNA-DNA hybridization data has been presented for this organism.

Thermobacteroides proteolyticus was isolated from a thermophilic digester that was fermenting tannery waste and cattle manure at 55°C (Oliver et al. 1985). This organism differed from *Thermobacteriodes acetoethylicus* in being proteolytic but was similar in not producing lactate from glucose fermentation.

Thermobacteroides leptospartum was isolated from cattle compost and showed characteristics similar to *Bacteroides* strains, for which reason it was placed in the genus *Thermobacteroides* (Toda et al. 1988). The production of ethanol as the main product of glucose fermentation along with the absence of lactate are characteristics similar to *T. acetoethylicus* and *T. proteolyticus*. The relatedness of this organism to these species is not clear at present.

2.7 SYSTEMATICS, DIVERSITY, CLASSIFICATION AND TAXONOMY

2.7.1 SOME DEFINITIONS

Systematics is defined by Stackebrandt and Goodfellow (1991) as 'the scientific study of the diversity of organisms and their relationships'. Systematics involves classification, taxonomy, nomenclature and identification.

Diversity can be defined simply as 'variety'. It is soon clear to anyone embarking on a study of microorganisms that diversity is a key factor. It is the estimation and recognition of the degree of this diversity that leads to a useful system of classifying the organisms under investigation.

Classification is defined by Sneath and Sokal (1973) as "the ordering of organisms into groups (or sets) on the basis of their relationships." The term relationship may indicate a resemblance or overall similarity on the basis of the characteristics of the organisms but not implying any relationship by ancestry or it may imply a phylogenetic relationship. The term "phenetic relationship" can be used to distinguish between relationships judged from phenotypic studies and those from phylogenetic studies. Classification is both the name of a process and the end result of that process, as the result of classification is a classification.

Taxonomy. Simpson (1961) used the term taxonomy to mean "the theoretical study of classification including its bases, principles, procedures and rules." The term *taxon* (plural *taxa*) is used as an abbreviation for taxonomic group of any nature or rank as suggested by Rickett (1958).

Taxonomies can be of two types, phenotypic or phylogenetic. Phenetic taxonomies are based on numerous observed properties of organisms and are the result of an overall resemblance measured in units of similarity or dissimilarity. Phenotypic taxonomies have no evolutionary implications other than displaying the end product of evolution. Phylogenetic classifications are expressions of evolutionary relationships between organisms and reflect the degree of change in lines of descent.

2.7.2 DETERMINATION OF DIVERSITY

Stackebrandt and Goodfellow (1991) have stated 'there is no choice but to classify, identify, and name'. They consider it necessary for microbiologists to know what organisms they are working with before they can disclose information in the literature about them. Equally, it is important for microbiologists who are involved in work that will not be disclosed in the literature to establish the type of organism they are working with. Establishing the diversity in a group of organisms under investigation not only enables classification and taxonomy but allows organisms with desirable properties to be selected for further study.

The methods available for use in determination of diversity will be briefly reviewed in this chapter. Those methods used in this thesis, namely numerical taxonomy, whole cell protein electrophoresis and 16S rRNA sequencing will be reviewed and described in more detail in the relevant sections.

2.7.2.1 Classical methods

In the past, taxonomic studies were performed in order to generate classifications which could be used in the identification of new isolates. Such studies were initially applied to organisms relevant to disease and food spoilage, where identification methods were important. Phenotypic characteristics such as morphology, pigmentation, nutritional requirements, susceptibility to antimicrobial compounds were used to assign organisms to particular groups. The data obtained was subjected to numerical analysis and used to establish relationships between closely related organisms, normally below the genus level. Although providing no phylogenetic information such studies are still useful in providing a data base of the general characteristics of a group of test organisms and demonstrating the degree of phenotypic diversity within them.. A full review of the principles and methods of numerical taxonomy is given in section 4.1.

2.7.2.2 Chemical methods

The study of chemical variation in living organisms and the use of chemical characters in classification and identification has been termed chemosystematics, chemo-taxonomy and chemical taxonomy (Goodfellow and Minnikin 1985). Komagata and Suzuki (1987) suggested that any chemical component that : (i) is distributed universally among the organisms to be studied; (ii) the data are homologous among strains within a taxon yet showing significant differences between the taxa to be differentiated; (iii) can be easily analyzed on a large number of samples, is useful in bacterial systematics. The chemical characters or components which have been used in classification include nucleic acids, proteins, lipids, cell wall components, end products of metabolism and the whole organism. Chemotaxonomy has benefited from the rapid developments in automated analytical instruments. Automated taxonomic methods have the advantage of reducing the error found by Sneath and Johnson (1972) to be introduced between individual workers and between laboratories in classical taxonomy studies. Some of the chemical methods used in taxonomic studies are described below. The study of nucleic acids will be considered under molecular methods, while the principles and methods of classification based on electrophoretic whole-cell protein patterns will be described in chapter 5.

2.7.2.2.1 Lipid analysis

Fatty acids, mycolic acids, polar lipids, and isoprenoid quinones are lipids that have been studied and found useful in bacterial systematics.

Fatty acids are an essential component of cellular lipids and the types and relevant proportions found in bacterial cells have been shown to be characteristic of particular groups of organisms. Langworthy (1982) demonstrated the presence of isopranyl glycerol ether lipids in archaea in place of the glycerol esters of fatty acids found in most bacteria. The presence of isopranyl glycerol ether lipids in an organism can be used as one of the criteria for designation to the domain archaea. Bacteria can be divided into two groups on the basis of cellular fatty acid composition. One group contains iso- and/or anteiso-branched fatty acids as the main component while the fatty acids of the other group are mainly composed of straight chain acids. Gram positive aerobes such as *Bacillus* (Kaneda 1977) and *Micrococcus* (Girard 1971) which contain branched acids, especially anteiso-acids of $C_{15:0}$ and $C_{17:0}$, as major components can be differentiated from Gram negative organisms in which this pattern is not found. A similar pattern of straight-chain saturated and mono-unsaturated fatty acids is found in both Gram negative and Gram positive bacteria; groups for which this has been shown include the lactic acid bacteria (Veerkamp 1971), *Clostridium* (Johnson and Goldfine 1983), *Enterobacteriaceae* (Bergen 1983) and *Pseudomonas* (Ikemoto et al. 1978). Fatty acid analysis has been applied to thermophilic organisms, for example the organisms of order *Thermotogales* have been shown to have nearly identical monobasic and dibasic fatty acid compositions (Huber et al. 1990), branched fatty acids have been shown to be major components in the fatty acids of *Thermus* (Oshima and Miyagawa 1974).

Mycolic acids, which are 2-alkyl-3-hydroxy fatty acids with long alkyl chains, have been found to be characteristic lipid components of organisms belonging to the genera *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Corynebacterium* (Lechevalier et al. 1973; Minnikin et al. 1975; Goodfellow et al. 1976). Mycolic acid analysis has been useful in classification of these organisms at the genus level since the total carbon number for each of these genera is different.

The phospholipids which are components of the cell membrane are involved in permeability and regulation at the membrane. Fingerprint comparisons of two dimensional thin layer chromatograms of phospholipids have been used in bacterial classification and found to show good correlation with other chemotaxonomic methods (Goodfellow et al. 1980; Collins et al. 1980).

Isoprenoid quinones have an important role in the electron transport system in respiration. Various types of quinones are found in microbial cells. The majority of aerobic bacteria possess isoprenoid menaquinone and/or isoprenoid ubiquinone. Some bacteria have been found to possess unusual quinones. Quinone analysis has been used

to differentiate *Acetobacter* and *Gluconobacter* (Yamada et al. 1969) and as evidence for the transfer of *Pseudomonas maltophilia* to the genus *Xanthomonas* (Swings et al. 1983)

2.7.2.2.2 Cell wall components

The majority of bacteria are surrounded by a wall envelope, composed of murein (peptidoglycan). Murein is the main cell wall polymer of bacteria and is found in both Gram positive and Gram negative types. The *Mycoplasmatales* and the archaea are known to lack murein (Kandler 1982; Schleifer and Stackebrandt 1983). Peptidoglycan consists of a glycan moiety, which is an alternating polymer of *N*-acetylglucosamine and *N*-acetylmuramic acid, and a peptide moiety that links the glycan chain. The existence of almost one hundred murein types has been demonstrated by the investigation of the chemical structure of thousands of bacterial strains (Schleifer and Kandler 1972; Schleifer and Stackebrandt 1983). The chemical structure of the murein of Gram negative bacteria is rather uniform and it is in the differentiation of Gram positive types that it is a more useful marker. Both the chemical type and the amino acid composition of the murein can be used as chemotaxonomic criteria. The presence of specific amino acids in the murein has been used in classifications of bacterial groups including *Microbacterium*, *Arthrobacter* and *Pimelobacter* (see Schleifer and Kandler 1972).

The sugar composition of cell walls has been used in the classification and identification of some bacteria, for example some actinomycetes (Lechevalier and Lechevalier 1970). Many bacterial taxa do not have a uniform sugar composition and so this method has not been widely applied in bacterial systematics.

2.7.2.2.3 End products of metabolism

When fermentative bacteria metabolize carbohydrates or nitrogenous substrates a complex range of metabolic end products is usually formed. These end products can include organic acids, alcohols, ketones, and amines. End products are known to vary, both qualitatively and quantitatively, in response to variations in cultural conditions including availability of growth substrate, growth rate, pH and gaseous atmosphere (Shah et al. 1985). Gas liquid chromatography has been used for end product analysis in the identification of many different types of bacteria (Holdeman et al. 1977; Deacon et al. 1978; Hammann and Werner 1980; Cresci et al. 1988). High performance liquid

chromatography has also been used in the identification of end products of bacterial metabolism.

2.7.2.2.4 Whole organism

One method in which the composition of the whole organism is analyzed is pyrolysis mass spectrometry. In pyrolysis mass spectrometry pyrolysis fragments are detected and quantified by mass spectrometry. The mass spectra produced are treated as chemical profiles or 'fingerprints' of the organisms analyzed. Unknown isolates can be identified by comparison of their profile with reference spectra held in a data base. Extensive pyrolysis mass spectrometry studies have been carried out on mycobacteria (Wieten et al. 1981, 1982), selected yeast species (Windig and Haverkamp 1982), lactic acid bacteria (Shaw et al. 1985), *Bacillus* (Shute et al. 1984), and *Legionella* (Kajioka and Tang 1984). One study of thermophilic bacteria of the genus *Thermus* was compared with the numerical taxonomy data of the same group of isolates (Donnison et al. 1986).

2.7.2.3 Molecular methods

The methods described under classical and chemical methods have been used to classify bacteria on the basis of phenotypic characteristics. Harvey and Pickett (1980) estimate that conventional phenotypic tests measure at best only 10% of the chromosomally coded capability of the microbial cell, therefore studies at the DNA level are especially important for organisms for which very few tests are available for species differentiation. In many cases these methods are not precise enough for distinguishing superficially similar organisms. Johnson (1989) outlined several of the advantages to be gained by basing classifications on genomic relatedness: '(i) a more unifying concept of a bacterial species is possible; (ii) classifications based on genomic relatedness tend to be stable, that is, they accommodate new information; (iii) reliable identification schemes can be prepared once organisms have been classified on the basis of genomic relatedness; (iv) information can be obtained that is useful for understanding how various bacterial groups have evolved and how they can be classified according to their ancestral relationships'.

The greatest source of information at the genomic level lies in the nucleotide sequence of the complete genome but at present sequencing the latter is not a practical proposition. Since the early 1960s, analysis of the chromosomal DNA of microorganisms has been used in classification. Two approaches that have been widely used in bacterial classification are the estimation of the base composition of DNA and the estimation of

the extent of base pairing in homology studies. The genome size expressed in daltons or in number of nucleotide pairs has also been used to distinguish between organisms (Owen and Hill 1979).

2.7.2.3.1 DNA base composition

The first feature of DNA that was recognized as being useful in differentiating between organisms was the ratio of adenine (A) plus thiamine (T) to guanine (G) plus cytosine (C), or the mol% guanine plus cytosine [$\%GC = 100(G+C)/(A+T+G+C)$]. The value of %G+C in bacteria varies between different taxa within the range 23-78 (Hollander and Pohl 1980). In the interpretation of mol% G+C values other types of taxonomic data must be considered along side the G+C data as similarity in % G+C of DNA samples does not necessarily imply biological relatedness since unrelated organisms can have identical GC ratios. Goodfellow and Minnikin (1985) suggest that bacteria with DNA differing by more than 5% G+C should not be classified in the same species and those showing differences of over 10% should not be assigned to the same genus.

2.7.2.3.2 DNA-DNA molecular hybridization

DNA homology or nucleic acid similarity has been determined by measurement of the extent to which single-stranded DNA from one bacterial strain reassociates or hybridizes with single-stranded DNA from another strain. Stackebrandt and Goodfellow (1991) point out that the experience of the last thirty years has demonstrated that DNA-DNA hybridization data are most relevant when investigating relationships within and between bacterial species. Bacteria which have DNA homology above 70% are considered to be the same genomic species (Wayne et al. 1987). Various methods have been used in determination of DNA-DNA homology, these include, membrane-filter method, hydroxyapatite batch procedure, S1 endonuclease assay, fluorometric assay and spectrophotometric method. The methods of DNA-DNA hybridization are in general laborious and time-consuming and so not applicable to large-scale studies of diversity in large numbers of new isolates but have been widely used in the description of new genera and species.

2.7.2.3.3 DNA-rRNA molecular hybridization

Ribosomal RNA genes have been found to be more highly conserved than the bulk of the DNA (Doi and Igarashi 1965; Dubnau et al. 1965), making them useful in taxonomic and phylogenetic investigations at the generic and suprageneric levels. The two most

widely used DNA-rRNA hybridization techniques are the competition method and the saturation hybridization method. DNA-rRNA hybridization has been used in the taxonomic studies of a range of bacteria including enterobacteria and myxobacteria (Moore and McCarthy 1967), *Desulfovibrio* species (Pace and Campbell 1971.), *Agrobacterium* species (De Smedt and De Ley 1977), streptococci, enterococci and lactococci (Schleifer and Kilpper-Balz 1987).

2.7.2.3.4 Other molecular methods

Plasmids are found in a wide range of bacteria but their presence or profiles have not been widely used in taxonomic studies. Plasmid profiles have been used in the identification of lactic streptococci (Davies et al. 1981) and in epidemiological studies of *Staphylococcus epidermis* (Parisi and Hecht 1980) and Gram-negative bacilli (Schaberg et al. 1981).

A number of DNA fingerprinting methods involving the analysis of restriction fragments have been developed and used for taxonomic purposes (Grimont and Grimont 1991). These methods are based on the fact that short sequences of double-stranded DNA are recognised by restriction endonucleases as cleavage sites. Depending on the number and position of these sites on the DNA molecule a varying number of fragments of differing sizes are generated. The separation of these fragments on an agarose gel provides a restriction profile which can be compared between organisms. Restriction profile analysis can be applied to both plasmid (Meyers et al. 1976) and chromosomal DNA (Bove and Saillard 1979). Bacterial restriction endonuclease DNA analysis (BRENDA) has been applied to a wide range of bacteria including *Legionella pneumophila* (van Ketel et al. 1984), *Campylobacter* (Penner et al. 1983; Kakoyiannis et al. 1988), *Vibrio cholerae* (Wachsmuth 1986) and *Leptospira* (Marshall et al. 1981; Ellis et al. 1988).

The analysis of restriction patterns of rRNA genes, known as ribotyping, has also been applied to bacterial taxonomy. The patterns are obtained by hybridizing Southern-blotted DNA fragments with end-labelled 16S and 23S rRNA from *E.coli* (Grimont and Grimont 1986), with end-labelled oligonucleotides complementary to conserved sequence portions of rRNA (Moureau et al. 1989), nick-translated cloned rRNA genes (Brosius et al. 1981), or nick-translated total DNA from a phylogenetically remote bacterium (Grimont and Grimont 1986). The specificity of rRNA gene restriction patterns has been found to vary with the species studied and the restriction endonuclease chosen (Grimont and Grimont 1991). This method has been used in taxonomic and

epidemiological studies of a range of organisms including *Brucella* species (Verger et al. 1987), *Legionella* species (Saunders et al. 1988), *Pasteurella multocida* (Snipes et al. 1989), and *Mycobacterium leprae* (Sela et al. 1989).

2.7.3 AN OVERALL STRATEGY FOR DETERMINATION OF DIVERSITY AND SUBSEQUENT CLASSIFICATION

The constraints of time and expenditure prevent the majority of microbiologists from applying more than two or three of the above methods to a group of isolates in which they want to determine the degree of diversity. The methods applied depend in many cases not only on the purpose of the study but also on the available facilities and expertise. It is therefore not uncommon to find working groups which specialize in one particular technique and apply it to a wide range of organisms or in some cases to a wide range of isolates of a particular taxonomic group.

In most cases a newly isolated strain or group of strains is not isolated randomly and some strategy has already been applied with respect to the isolation technique before pure cultures are obtained. This could involve isolation from a particular environment (eg. thermal springs, food products), isolation using a particular enrichment substrate (cellulose, acetate), isolation under a particular physiological condition (pH, temperature, anaerobiosis). When the isolates from this isolation strategy have been obtained in pure culture then the process of classification can begin. Before applying those methods described above, the majority of which apply to organisms with some degree of relatedness, some general characteristics of the isolates should be determined. Those characteristics which are useful at this level of classification include morphological and structural features, physiology and metabolism, and pathogenicity. Organisms which show similarities at this level can be further characterized using the methods described above. The information obtained may then be used to define and describe new taxa or demonstrate diversity within existing taxa.

Assigning an isolate to a previously defined or new genus is the first step in identification of microorganisms. Murray et al. (1990) put forward ways of approaching the taxonomy of the *Proteobacteria*, but such ideas can be applied to any microbial group. They recognised that a polyphasic approach, including phenotypic and phylogenetic data is essential and recommended that 'descriptions of new genera should, whenever possible, include either sequencing or hybridization data'. Below the genus in the taxonomic hierarchy is the species. Truper and Kramer (1981) outlined the minimal requirements for a description of a new species, as shown in Table 2.4.

Table 2.4 Minimum list of data for a description of a new species

	Required	Required if applicable
Cell morphology	Cell shape ^a	Color
	Cell size (diameter, length)	Flagellation ^a
	Motility	
	Visible internal and external structures ^a	Spores ^a , appendages ^a , capsules ^a , sheaths ^a
	Formation of typical cellular aggregates ^a	
	Occurrence of cell differentiation ^a	Life cycle ^a , heterocysts ^a , hormogonia ^a
	Ultrastructure (genera) ^b	Ultrastructure(flagella, envelope, cell wall) ^b
Colonial morphology	Appearance of cell suspensions	Color of suspensions
	Appearance of colonies	Color of colonies
		Motility of colonies
		Formation of fruiting bodies ^a
		Formation of mycelia ^a
Staining behavior	Gram stain	Acid-fast, spore stain, flagella stain
Cell constituents	DNA base ratio	Nucleic acid homologies
	Reserve materials	Cellular pigments
		Cell wall constituents
		Typical enzymes
Physiology	Temperature range	Salinity or osmolarity requirements
	Optimal temperature	Vitamin requirements
	pH range	Typical metabolic products formed
	pH optimum	Tolerances
	Modes of energy metabolism	
	Relation to oxygen	
	List of electron acceptors	
	List of carbon sources	
	List of nitrogen sources	
Ecology	List of sulfur sources	
	Natural habitat(s)	Pathogenicity, host range
		Antigen formation
		Serology, phage susceptibility

^a To be demonstrated by photograph. ^b To be demonstrated by electron micrograph
 Reproduced from Truper and Kramer (1981).

This data can then be used to explain why a new species should be defined by comparing the *new* and the known species. Wayne et al. (1987) stated that 'the phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m . Both values must be considered'. They recommended that 'a distinct genospecies that cannot be differentiated from another genospecies on the basis of any known phenotypic property not be named until they can be differentiated by some phenotypic property.' A species can be divided into two or more subspecies based on minor but consistent phenotypic differences or on genetically determined clusters of strains within a species. The subspecies is the lowest taxonomic rank that has official standing in bacterial nomenclature but infrasubspecific ranks can be used to further differentiation, these include biovar, serovar, pathovar, phagovar and morphovar (Staley and Krieg 1984)..

2.8 CONCLUSION

The structure of cellulose and the factors influencing its degradation either by microbial cultures or microbial enzymes was found to have been extensively investigated and reported in the literature. Reviewing the literature of both the cellulolytic and the non-cellulolytic thermophilic anaerobes reveals the great degree of diversity within this group. The clostridial types have been assigned to the genus *Clostridium* on the basis of their sporogenous nature but the criteria used in the designation of species seems less convincing and therefore a full taxonomic study at the genomic level may be useful to complement the phenotypic data already available. The taxonomy of the asporogenous thermophilic anaerobes, both cellulolytic and non-cellulolytic, would seem to be in chaos and an extensive study at the genomic level would be required before a taxonomy can be established and genera and species designations revised. The methods available to investigate the taxonomy of any group of microorganisms are numerous. The choice of method will be determined by the availability of the equipment, expertise and not least on the basis of the aims of the study to be undertaken. A pathway involving both phenotypic and phylogenetic methods would be required if new taxa are to be described.

CHAPTER 3

THE ISOLATION OF ANAEROBIC CELLULOLYTIC THERMOPHILIC BACTERIA

INTRODUCTION

The literature review of chapter 2 described the anaerobic cellulolytic thermophiles that have been isolated by various working groups around the world using a variety of enrichment media and techniques. In order to increase the number of isolates from geographically distant sites in New Zealand and investigate the use of an alternative enrichment medium a limited isolation study was undertaken. Samples from a thermal site that had never before been sampled for microbiological purposes were included in this study.

3.1 THE PRINCIPLES AND METHODS OF ISOLATION

Numerous different kinds of microorganism live side by side in the natural environment, this is also true for the thermophilic environment. The degree of diversity in a thermophilic environment may be less than that of many nutrient rich mesophilic environments. In any isolation study the physical conditions of the sample site must be measured and taken into consideration in the design of an isolation strategy. No single culture medium will allow the growth of all bacteria occurring in the environment to be sampled. In the majority of cases microbiologists have some idea as to the type of organism they want to isolate, this may be a particular nutritional type or physiological type. Enrichment medium composition is probably the most important factor after the physical parameters such as pH and temperature have been decided.

In any batch enrichment culture with high substrate concentrations the organisms that are enriched are the fastest growing under those conditions. Organisms that may be present in relatively high numbers but that are slow to grow under the conditions of the enrichment culture may be out-grown by other organisms and subsequently not isolated. In order to isolate organisms capable of utilizing a particular carbon source it is normal to include that substance in the enrichment medium as the sole carbon source. Earlier workers in anaerobic microbiology additionally added large amounts of yeast extract and peptones which may have resulted in the enrichment of large numbers of clostridial type isolates. Pfennig (1984) points out that the use of a nutritionally rich medium or a medium resembling the conditions of the natural environment influences the type of organism that is enriched. In recent years the working groups of N. Pfennig, F. Widdel and B. Schink have used bicarbonate buffered low phosphate medium to isolate numerous new genera and species of sulphate-reducing and fermentative anaerobes. Other factors that may influence the type of anaerobic bacteria isolated include the reducing agent used and the size of the inoculum (see Janssen 1989).

The literature regarding the isolation of anaerobic thermophiles indicates that the majority of isolates have come from batch enrichment cultures. A recent example of the use of *in situ* enrichment techniques to isolate acetate-utilizing bacteria from a thermal spring involved the semi-continuous feeding of ammonium acetate into the spring over a period of several weeks (Konradsdottir et al. 1991). Normal enrichment techniques were then used to isolate the acetate utilizing organisms the numbers of which had presumably been increased in the natural environment due to the abundance of acetate.

3.2 METHODS USED IN THE ISOLATION OF ANAEROBIC CELLULOLYTIC THERMOPHILIC BACTERIA

As pointed out in chapter 2 the anaerobic cellulolytic thermophiles isolated to date have all been members of the domain Bacteria and have fallen into two categories, the clostridial sporogenous types and the non-clostridial asporogenous types. All of these strains were isolated by conventional enrichment techniques using various medium compositions and enrichment substrates. Hudson et al. (1990a) described an *in situ* enrichment method using filter units containing steam exploded poplar which were submerged in thermal springs for periods of 2-7 months. Although some of these enrichments produced positive cultures, on subculturing none yielded pure cultures when plated out on solid medium. Table 3.1 summarizes the information from the literature regarding the enrichment and isolation of some of the described strains of anaerobic cellulolytic thermophilic bacteria.

Table 3.1 Source and enrichment conditions used in the isolation of some anaerobic cellulolytic thermophilic bacteria

Strain	Source	Substrate	Temperature (°C)	YE (g/l)	Reductant
<i>C. stercorarium</i>	compost	filter paper	60°C	1.0	cysteine HCl
<i>C. thermolacticum</i>	compost	xylan	65°C	5.0	cysteine HCl
<i>C. thermocopriae</i>	various	cellulose	55-60°C	6.0	cysteine HCl
Tp8T 6331	hot spring	Sigmacell 50	70°C	3.0	cysteine HCl
NA10	hot spring	cellulose (floc)	80°C	1.0	sulfide
F2/1	hot spring	amorphous cellulose	70°C	1.0	NA
<i>A. thermophilum</i>	hot spring	filter paper	75°C	1.0	sulfide
H173	hot springs	xylan	70°C	3.0	cysteine HCl

The three clostridial isolates in Table 3.1 were enriched at lower temperatures than the non clostridial types. This difference in enrichment incubation temperature may have had some influence on the type of organism enriched. In the isolation study of Hudson et al. (1990a) the temperature of incubation of the enrichment culture was found to influence the number of positive enrichments. Using the same media and samples twenty four positive enrichments were obtained at 75°C compared to forty three at 65°C. The non

clostridial strains in Table 3.1 were all isolated from thermal springs while the source of the clostridial types was compost. The five strains of *C. thermocopriae* described by Jin et al. (1988) were isolated by the same enrichment procedure but from varying sources, including camel feces, soil, compost and hot spring. The strain designated the type species was a compost isolate. Yeast extract was used in all of the enrichments at concentrations from 1-6g/l.

3.3 AN ISOLATION STUDY

3.3.1 WHY MORE ISOLATION ?

The reader may wonder why additional isolation work was undertaken when a large number of isolates requiring characterization were available from previous isolation studies. There were three reasons which prompted further isolation work. Firstly in order to obtain additional isolates from New Zealand hot springs previously not sampled or not giving a positive enrichment. Such isolates were required to add geographical diversity within the New Zealand isolates since seven isolates had been isolated from the same hot spring namely Rt8 and this may have put a bias on the numerical taxonomy study to be carried out. The second reason was partly curiosity by the author who after hearing of the potential of isolating anaerobes using low phosphate sulfide reduced medium (pers comm Bernhard Schink) wanted to test the method. Thirdly the possibility to sample a new thermal site, that of Raoul Island, Kermadec archipelago, New Zealand.

3.3.2 METHODS AND MATERIALS

3.3.2.1 Enrichment medium and culture conditions

The medium used for enrichment was bicarbonate-buffered medium (CBM), containing (per litre): NaCl, 0.9 g; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 g; KCl, 0.5 g; NH_4Cl , 0.3 g; KH_2PO_4 , 0.2 g; 0.15 % (w/v) FeCl_3 , 1 ml; 0.1 % (w/v) resazurin, 1 ml; trace element solution SL10 (without FeSO_4) and selenite-tungstate solution (Widdel et al. 1983) 1ml each. Sodium bicarbonate (2.5 g/litre) was added to boiled, cooled medium which was dispensed in 10 ml aliquots in 23 ml anaerobic culture tubes (Bellco) gassed with N_2/CO_2 mixture (80%/20% v/v) and sealed with butyl rubber stoppers. Sulfide-calcium solution (containing $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ 36 g/litre; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 15 g/litre; pH 7.2) at 2 ml/litre and a 7-

vitamin solution (Widdel and Pfennig 1981) at 1 ml/litre were added to the autoclaved, cooled medium from sterile stock solutions. When changing the salinity of the medium, NaCl and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ were varied at a fixed ratio of 20 g to 2.7 g/litre respectively in the medium CBM. The final medium pH was 6.9 to 7.1 unless otherwise specified. The enrichment substrates were added from anaerobic sterile neutralized stock solutions.

3.3.2.2 Preparation of amorphous cellulose

Amorphous cellulose was prepared by adding 25 g of Avicel to 1,000 ml 5 M ZnCl_2 . The suspension formed was added to 4,000 ml vigorously stirred distilled water. The resulting white flocculent was recovered by centrifugation at 8,000 rpm. for 20 min. The combined pellets were washed three times with distilled water and suspended overnight in 1,500ml 25 mM Na_2EDTA . The flocculent was centrifuged again and washed four times with distilled water. The washed amorphous cellulose was stored as a slurry at 4°C. Amorphous cellulose was added to bicarbonate-buffered medium at 5g/l of the slurry.

3.3.2.3 Roll tube method

The roll tube method was as described by Hungate (1969). The appropriate bicarbonate-buffered medium was solidified by the addition of purified agar (Oxoid) at 18g/l. The medium was dispensed in 5ml aliquots in 23ml anaerobic culture tubes. Serial dilutions of positive enrichments were made in the molten medium maintained at 55°C. After mixing the tubes were continuously mechanically rolled at 4°C until the agar solidified. The tubes were then incubated at 70°C at a 45° angle in order to stop the agar sliding to the bottom at the high temperature and to allow the condensate to collect in the bottom of the tube. Single colonies were removed from the agar as small cores using a pasteur pipette which had 5mm of the end bent at a right angle. These agar plugs were placed in liquid medium and crushed against the side of the tube to release the cells.

3.3.3 RESULTS AND DISCUSSION

3.3.3.1 Raoul Island samples

During the 1990 visit of the R/V "Akademik Alexander Nesmeyanov" (Far East Branch, Academy of Sciences of the USSR) to the Kermadec archipelago, sediment and water samples were collected anaerobically from springs on Raoul Island. This is the largest

island of the group and lies 1,000 km to the north-east of New Zealand (Figure 3.1a,b). Spring RI 19, one of the many hot water springs on the south-west shore of Green Lake on Raoul Island (Figure 3.1c), had a pH of 7.0 and temperature of 65°C. Before the most recent volcanic eruption at the site of Green Lake in 1964 the water had a sodium content of 20 ppm and a chloride content of 35 ppm. The boiling water which discharged into the lake after the 1964 eruption was essentially sea-water modified by dilution by meteoric groundwater and enriched in Li, Ca, F, B, As, and HCO₃ with respect to diluted sea-water (Weissberg and Sarbutt 1966). The sample of water from spring RI 19 used in this study had a sodium content of 2,920 ppm and a chloride content of 4,800 ppm (Na and Cl levels in sea-water are 11,000 ppm and 19,000 ppm respectively). The conductivity of the sample RI 19 was 15.3 mS (20°C), equivalent to salt concentration of 0.76% (w/v) NaCl. Other hot springs (RI 1 and RI 2) in the surrounding rock of Green Lake are fresh water and contain algal mat. The algal mat from springs RI 1 and RI 2 had a pH of 6.5 for both samples and were at 66 and 67°C respectively. To the best of my knowledge the samples collected and used in this study were the first from Raoul Island to be investigated microbiologically and definitely the first from this site to be used for the enrichment of thermophilic bacteria. The area of Green Lake is an interesting thermal site with both brackish and freshwater thermal springs in a small area. It is also rich in organic matter as can be seen from Figure 3.1c and therefore an ideal sample source for the enrichment of cellulolytic or polysaccharolytic thermophilic organisms.

Ten milliliters of CBM10 (CBM with the NaCl and MgCl₂·6H₂O increased to 10 g/litre and 1.5 g/litre respectively) plus amorphous cellulose was inoculated with 1 ml of sample RI 19 at the sample site and incubated at 70°C without shaking on return to the laboratory. These cellulose enrichments did not show any signs of growth after 5 days incubation. CBM10 containing 0.5 g/litre glycogen was also inoculated with 1 ml of sample RI 19, and after 3-5 d incubation gas production and visible turbidity were observed indicating substrate utilization. Microscopic examination of the glycogen enrichment culture revealed two distinct morphologies: *Thermotoga*-like cells with characteristic sheath-like structure (Windberger et al. 1989), and thin helical organisms. The two cell types were separated into pure cultures by serial dilution in agar roll tubes as described above. The cells which were shown by electron microscopy to be spirochetes had fluffy white colony morphology in the agar roll tubes while those of the *Thermotoga*-like cells were cream in color with entire edges. The isolated spirochete was designated RI 19. B1, and the *Thermotoga*-like isolate RI 19.B2. It is worth noting that enriching sample RI 19 in phosphate buffered medium containing yeast extract and trypticase with glycogen as the substrate and reduced with cysteine HCl gave only the

Thermotoga-like organism, and the spirochete was not observed in the enrichment culture. A number of reasons could be put forward to explain this finding including inhibition of the spirochete by phosphate or cysteine HCl, the yeast extract and trypticase allowing the *Thermotoga*-like organism to completely out grow the spirochete. An extensive enrichment study would be required in order to establish the true reason and this was thought to be outside the scope of this thesis.

Samples of algal mat from two freshwater hot springs on Raoul Island were used to inoculate 10ml CBM containing amorphous cellulose as the enrichment substrate. These enrichments were incubated at 70°C for 3-5d when gas production and visible turbidity were observed indicating substrate utilization. Microscopic examination of these enrichment cultures revealed rod shaped organism of one morphotype. These positive enrichments were processed by the roll tube technique using agar containing amorphous cellulose and the colonies showing zones of cellulose hydrolysis were further purified. This resulted in two rod shaped cellulolytic organisms being designated strains RI 1.B1 and RI 2.B1.

3.3.3.2 New Zealand samples

Samples from New Zealand freshwater thermal springs (Table 3.2) were processed as for the Raoul Island samples with CBM plus amorphous cellulose or Avicel being used as enrichment substrates. The cellulolytic organisms from positive enrichments were isolated using the roll tube method. Pure cultures of cellulolytic isolates were obtained from eight thermal springs which had either not been sampled before or that did not yield positive enrichments from previous samples on other enrichment media. It should also be noted that the medium used to isolate these organisms did not contain yeast extract in contrast to that used in the isolation of the strains listed in Table 3.1.

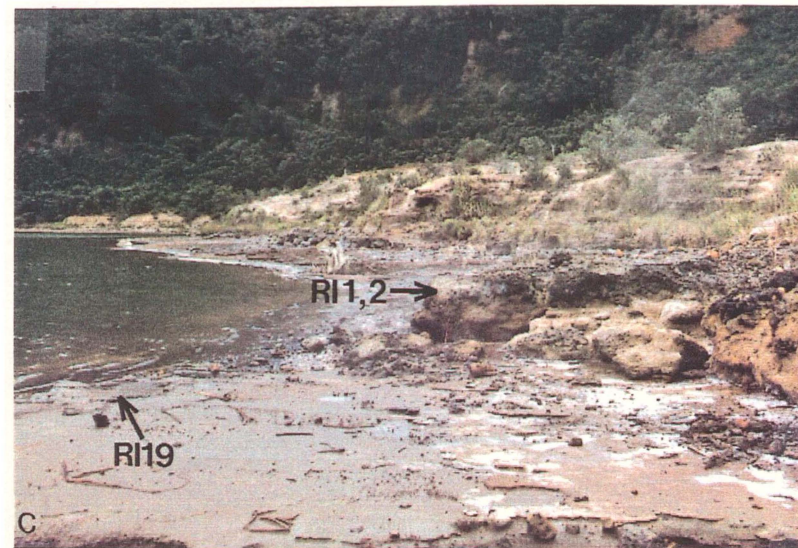


Figure 3.1 a-c Raoul Island, Kermadec archipelago, New Zealand. **a.** The geographical location of Raoul Island, Kermadec archipelago. **b.** Map of Raoul Island, with the location of Green Lake indicated (*GL*). **c.** The sampling sites on the south-west shore of Green Lake (*RI 1*, *RI 2*, *RI 19*).

Table 3.2 Site conditions and the isolates obtained from enrichments on CBM.

Isolate	Enrichment substrate	Source temp (°C)	Source pH
Ok1. B1	amorphous cellulose	50	9.6
Ok2. B1	amorphous cellulose	73	8.1
Ok9. B1	Avicel	79	7.6
Rt4. B3	amorphous cellulose	67	7.1
Rt UK4	Avicel	60	8.7
Tok9. B1	amorphous cellulose	56	6.4
Wai28. B1	amorphous cellulose	64	ND
Wai35. B1	amorphous cellulose	69	ND

ND, not determined

3.3.4 CONCLUSION

This limited isolation demonstrated the use of one particular enrichment medium in the isolation of cellulolytic and polysaccharolytic thermophilic anaerobes. The organisms isolated did not require yeast extract for growth. In the case of the RI 19 sample bicarbonate buffered medium (CBM) was used to enrich a thermophilic spirochete not enriched in phosphate buffered medium. The usefulness of the roll tube method (not previously applied by our laboratory to thermophilic anaerobes) for obtaining pure cultures of cellulolytic organisms on the basis of cellulose hydrolysis and in obtaining pure cultures of two differing colony types was demonstrated.

The study provided the first information, although limited, on the microbiology of the thermal springs around Green Lake, Raoul Island. The one sampling trip and the limited enrichment work carried out on the Raoul Island samples yielded three morphologically distinct thermophilic anaerobes. Further work on samples from this site may produce isolates with various morphologies and with different physiological characteristics. The isolates from the freshwater New Zealand thermal springs added to the geographical diversity of the rod shaped cellulolytic anaerobes.

The ten anaerobic cellulolytic thermophilic rod shaped organisms could not be easily differentiated and so were added to the collection of previously isolated and in some cases described anaerobic cellulolytic thermophilic rod shaped bacteria. The degree of diversity within these isolates was determined by a numerical taxonomy study as described in chapter 4. The full characterization of the two morphologically distinct isolates RI 19. B1 and RI 19. B2 is described in chapters 7 and 8.

CHAPTER 4

A NUMERICAL CLASSIFICATION OF SOME ANAEROBIC, CELLULOLYTIC, THERMOPHILIC, ROD SHAPED BACTERIA

INTRODUCTION

Most major groups of microorganisms have been studied by numerical taxonomic methods. It was over 30 years ago that P. H. A. Sneath first applied numerical taxonomic methods to bacteria (Sneath 1957a,b). Jones and Sackin (1980) noted that from 1957-1978 ca. 500 publications of numerical taxonomy studies could be found in the literature. A large number also appeared in the 1980s, but recently such studies are becoming less popular with the increasing use of molecular techniques in taxonomic studies. Sneath (1989) has pointed out that the aim of taxonomy is 'to give a classification that is useful for varied scientific purposes, including identification, and also (a point often overlooked) to produce the data bases that summarize as much relevant information about organisms as possible'. The information obtained from molecular methods, for example 16S rRNA sequence data, can be used to show the relatedness of a group of organisms but does not provide information on the physiology of the organisms. Numerical taxonomy studies although not providing phylogenetic information do demonstrate the degree of relatedness of the organisms in the study and also provides a lot of information on their general characteristics and physiology.

Numerical taxonomy studies of thermophilic organisms have been restricted to the genus *Thermus*. In three studies Hudson et al. (1986, 1987, 1989) examined the phenotypic and geographic diversity of New Zealand, Icelandic and global hot spring isolates of the genus *Thermus*. A similar study was carried out on *Thermus* isolates from hot springs in Portugal (Santos et al. 1989). To date no numerical taxonomy studies of thermophilic anaerobes have been described in the literature.

This chapter is divided into two sections. In section 4.1 the principles and methods of numerical taxonomy are reviewed. These principles and methods are applied in a study of the diversity of a group of anaerobic, cellulolytic, thermophilic bacteria which is described in section 4.2.

4.1 A REVIEW OF THE PRINCIPLES AND METHODS OF NUMERICAL CLASSIFICATION AND TAXONOMY STUDIES.

4.1.1 INTRODUCTION TO THE DEFINITIONS AND TERMS USED IN TAXONOMY

Taxonomy. Simpson (1961) used the term taxonomy to mean "the theoretical study of classification including its bases, principles, procedures and rules." The term *taxon* (plural *taxa*) is used as an abbreviation for a taxonomic group of any nature or rank as suggested by Rickett (1958).

Taxonomic relationships may be phenetic or cladistic. Phenetic relationship (Cain and Harrison 1960) is based on numerous observed properties of organisms, is equivalent to an overall resemblance and is measured in units of similarity or dissimilarity. Cladistic relationship (Cain and Harrison 1960) is the inferred relationship by ancestry shown by phylogenetic branching patterns or cladograms.

Numerical Taxonomy was defined by Sneath and Sokal (1973) as "the grouping by numerical methods of taxonomic units into taxa on the basis of their character states. The term includes the drawing of phylogenetic inferences from the data by statistical or other mathematical methods to the extent to which this is possible." A similar definition was put forward by Jones and Sackin (1980), defining numerical taxonomy as "the computer assisted numerical evaluation of similarities between groups of organisms and the ordering or arrangement of these groups into clusters or taxa based on these similarities. On the basis of this clustering a classification can be constructed. The groups or taxa can be quantitatively defined and their relationships to each other measured by their intra- and inter-group similarity or dissimilarity values." They also stated that as "numerical taxonomy is based on phenetic evidence ie. similarities between organisms based on observable and recordable characters without regard to ancestry, the relationship between organisms and any hierarchies constructed from these relationships are phenetic not phylogenetic." Sokal (1985) noted that due to a lack of faith in phylogenetic interpretation of numerical taxonomy data microbiologists had practised phenetic taxonomy in the great majority of cases.

4.1.2 THE FUNDAMENTAL POSITION OF NUMERICAL TAXONOMY

The fundamental position of numerical taxonomy has been summarized by Sneath and Sokal (1973) in a set of principles which can be traced to concepts of Michel Adanson a French botanist of the 18th century:

- " 1. The greater the content of information in the taxa of a classification and the more characters on which it is based, the better a given classification will be.
2. *A priori*, every character is of equal weight in creating a natural taxa.
3. Overall similarity between any two entities is a function of their individual similarities in each of the many characters in which they are being compared.
4. Distinct taxa can be recognized because correlations of characters differ in the group of organisms under study.
5. Phylogenetic inferences can be made from the taxonomic structures of a group and from character correlations, given certain assumptions about evolutionary pathways and mechanisms.
6. Taxonomy is viewed and practiced as an empirical science.
7. Classifications are based on phenetic similarity."

4.1.3 THE PROTOCOL OF A NUMERICAL TAXONOMY STUDY

Below are listed the series of steps carried out in the following sequence for a numerical taxonomy study:

4.1.3.1 Choice of strains

The term applied to the strains to be classified by a numerical taxonomy study is *Operational Taxonomic Units* (OTUs). The type of study to be carried out and its aims will influence the number and type of strains (OTUs) included. Jones and Sackin (1980) outlined some considerations in the choice of strains for numerical taxonomy studies. To enable comparisons to be made, strains should be obtained from other collections, these should include reference strains and type strains. When type strains are not available authentic representatives of species or subspecies should be included; this may be important if the designated type strain is found not to be typical of the taxon. The inclusion of new isolates in studies concerned with the taxonomic revision of known structures is useful as greater evidence is added to the conclusions if several strains of a taxon form tight clusters. Cultures used in numerical taxonomy studies should be pure

and this purity should be checked during the course of the study either microscopically or by plating on non-selective media.

4.1.3.2 Test selection

The questions of how many and which type of tests to employ in a numerical taxonomy study must be considered in the early planning stages.

4.1.3.2.1 Number of tests

The decision of how many tests to use in a numerical taxonomy study will be influenced by both the amount of useful information they will supply and the physical constraints of carrying out a very large number of tests. In all the reviews and studies published no definitive answer as to the number of tests to use in a numerical taxonomy study has been given, however, some recommendations have been made. It would seem logical that the larger the number of tests the better, as a better measure of the phenome (reflecting the genome) would be made with a larger number of tests used. Lockhart (1967) makes the logical assumption that any adequately large sample of the total properties of two strains will yield an estimate of their phenetic similarity that gets close to the true value which would be obtained if all their properties were compared. The use of a large number of tests is suggested by Harwood (1980) who claims that when a large number of independent characters are employed the effect of a plasmid-borne trait would normally be expected to be small. The use of large (>100) numbers of tests also has negative aspects as outlined by O'Brien and Colwell (1987) who suggest it can lead to overrepresentation of some classes of the tests or to large gaps in the data due to the fact that not all tests are applicable to all strains. Sneath (1978) suggests that with higher numbers the gain in information falls off disproportionately to the effort involved in obtaining the data. An upper limit of 200 characters (<200 tests) has been recommended.

The lower limit of the number of tests in a numerical taxonomy study has also been the focus of much debate. This lower limit may be dictated in some part by the sampling error which is significant when the number of tests is low (Sneath and Johnson 1972). Numerical taxonomists such as Lockhart (1967) claim that 40 characters are sufficient while others recommend a minimum of 50-60 test characters (Sokal and Sneath 1963, Skerman 1967). Austin et al. (1981) state that no fewer than 40 tests should be included. Similarly, less than 50 tests is considered by Jones and Sackin (1980) not to supply enough information to be discriminatory.

With the use of disposable items such as test tubes, petri dishes and microtitre plates and the use of techniques for replica plating and multipoint inoculation most laboratories should be capable of handling 50-200 tests. This number may also vary depending on the number of strains to be tested.

4.1.3.2.2 Type of tests and test characters

The tests chosen should reflect as much phenetic information as possible. A *test character* has been defined as 'any property or attribute that can vary between strains and the values assigned to them as *character states* each of unit weight and ideally each mutually exclusive; each character state should contribute one piece of new information' (Sneath 1962). So *growth at 80°C* is a character with the states *positive* and *negative* usually coded numerically as 1 (or +) or 0 (or -) respectively. Such *two-state* or *binary* characters are the most common type used in numerical taxonomy studies. Less commonly used *multistate* characters include qualitative (eg. pigmentation) and quantitative (eg. enzyme activity levels) features. Qualitative multistate characters are normally scored as plus for the character state observed and minus for the alternatives. The additive method of Sneath and Sokal (1973) can be used to score quantitative multistate characters. Tests used in numerical taxonomy studies can be categorized into various groups. Examples of these groups and of the tests include: cytological (eg. cell dimensions, spore formation), cultural (eg. colonial morphology, particulate growth), physiology (eg. pH or temperature growth range), biochemical (eg. urease production), nutritional (eg. utilization of carbon sources), chemical constituents (eg. lysine in the cell wall), antimicrobial susceptibility (eg. sensitivity to rifampin), tolerance tests (eg. ethanol, sodium chloride), serological (eg. agglutination by an antiserum to a reference culture), molecular genetic (eg. mol % G+C). Less frequently used test categories may include ecological (eg. habitats, hosts) and behavioural (eg. chemotactic response).

4.1.3.3 Choice of tests

O'Brien and Colwell (1987) point out that tests selected for use in a numerical taxonomy study should represent a broad range of the biological attributes of the organism i.e. reflect as much phenetic information as possible and that provide information about a single property. Some tests and test characters are by their nature inadmissible in numerical taxonomy studies as they do not reflect the nature of the organism (eg. strain names or culture collection numbers) and are therefore meaningless. Lapage (1974) points out the properties of an organism which are a logical consequence of another

property should be omitted from analysis (eg. detection of the first enzyme in the metabolic pathway of a particular carbon source should not be included with the utilization of the same carbon source). This point is also made by Russek-Cohen and Colwell (1986) who suggest that many catabolic properties may be involved in only a limited number of biochemical pathways. A test may be inapplicable if under certain conditions a result cannot be determined; an example used by Lapage (1974) is that of the oxidase reaction of *Chromobacterium* being confused with the organism's violet pigmentation. A test may be meaningless if a result is required for a test that cannot be determined (eg. testing of cellulose hydrolysis at 75°C when some of the strains being tested have $T^{\circ}C_{\max}$ of 65°C). The aim of the numerical taxonomy study should also be considered when choosing tests. Tests involving cell wall constituents may be useful in separation of genera while carbon source utilization tests or fermentation end product tests may be useful in differentiation at the species level. The use of criteria for choosing of tests does to some extent introduce an element of character weighting. Tests which prove to give the same result with all strains tested (either a positive or negative result in all cases) should not be included in the final analysis as this introduces a weighting in the taxonomy.

4.1.4 NUMERICAL METHODS

4.1.4.1 Coding of tests

The method of coding of data will depend on the type of computer program used to carry out the numerical analysis. There are many different programs as outlined by Sackin (1987), including, Genstat (Statistics Department, Rothamstead Experimental Station, Harpenden, England), Clustran (D. P. C. Wishart, Kingsburgh Rd, Edinburgh, UK), Taxan (R. R. Colwell, University of Maryland, USA), Taxpak (M. J. Sackin Department of Microbiology, University of Leicester, Leicester, England) which all carry out the same type of analysis. They tend to vary in the types of characters which are permitted in the entry of data. Some programs require all characters to be 2-state i. e. 'all or none' characters eg. 0, 1 or -, +. Other programs allow multi-state characters eg. 0, 1, 2. or the use of missing-data entries termed NC ('no comparison') and entered as * or 9 for example.

4.1.4.2 Test error and reproducibility

Sneath and Johnson (1972) pointed out that a large degree of error could occur in characterization tests. Errors may be of two types (Lapage 1974), firstly mistakes (eg. misnumbering or cross-contamination) and secondly, those due to failure of test reproducibility whether between laboratories or within a laboratory. Lapage (1974) has shown that under most circumstances the contribution of mistakes to test error is much less than that due to the lack of test reproducibility. The studies of both Sneath and Johnson (1972) and Lapage (1974) generally agree that test error can be significantly high even when the test methods have been standardized. O'Brien and Colwell (1987) outlined some of the factors which may act singly or in combination to affect test reproducibility, these included time of reading of the tests, incubation temperature, culture media, mutations, source and size of inoculum and substrate concentration.

For statistical purposes, test error p may be defined as the probability that a test is erroneous averaged over n tests, taking it as equally probable that a negative will be misread as a positive and *vice versa*, and also assuming that the probabilities are the same for both strains (Sneath and Johnson 1972). Sneath and Johnson (1972) recommended that tests with >10-15% error should be rejected. According to Jones and Sackin (1980) it is usual practice to repeat the tests on at least 25% of the strains used in order to assess the reliability of each test. The figure of the number of strains duplicated in a study is found to vary eg. 9.3% (Williams et al. 1983), 27.4% (Hudson et al. 1986), 7.6% (Priest et al. 1988), 11.2% (Goodfellow et al. 1990), 9.6% (Dobson et al. 1991) and no firm criteria for this seem to have been established.

4.1.4.3 Resemblance

There are two types of resemblance measures, similarity and dissimilarity coefficients and as pointed out by Sokal (1985) all commonly used coefficients were established during the early studies of numerical taxonomy. Sneath and Sokal (1973) define a *similarity coefficient* as 'a quantification of the resemblance between the elements in the two columns of the data matrix representing the character states of the two OTUs in question.'

Similarity coefficients are computed with two-state characters coded 0 or 1. When character states are compared over pairs of columns in a conventional data matrix the outcome can be summarized in a conventional 2x2 frequency table:

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

Here a = the number of characters coded 1 in both OTUs, while d = the number of characters coded 0 in both OTUs; b and c register the number of characters in which the two OTUs disagree being coded 1 for the OTU **j** and 0 for the OTU **k** or *vice versa*; n = the sample size.

Many different similarity coefficients have been devised but only a few have been used routinely in numerical taxonomy studies of microorganisms. These include:

Simple matching coefficient, S_{SM} :

$$S_{SM} = \frac{a + d}{a + b + c + d}$$

This coefficient was introduced by Sokal and Michener (1958) and has been used in the majority of microbiological numerical taxonomy studies carried out to date. It is the proportion of characters that have the same state (both negative or both positive) in the pair of OTUs being compared. Characters for which either or both OTUs have a missing entry (NC) are ignored.

Jaccard's coefficient, S_J :

$$S_J = \frac{a}{a + b + c}$$

It was in Sneath's first numerical taxonomy study (1957a) that Jaccard's coefficient was used. Jaccard's coefficient is the same as the simple matching coefficient except that it omits the consideration of negative matches. Although Sneath (1957b) excluded negative matches by using *Jaccard's coefficient* most studies using similarity coefficients since 1957 have included negative matches in their coefficients. Jones and

Sackin (1980) suggest that the choice between S_{SM} and S_j is somewhat arbitrary, but suggest that valuable information may be lost when using S_j rather than S_{SM} . Sneath and Sokal (1973) note that it can be argued that basing similarity between two species on the mutual absence of a certain character is improper and use the example of the absence of wings among a group of distantly related organisms (such as camel, louse and nematode) would be an absurd indication of similarity. They suggest that the exclusion of negative matches may be a safe procedure in bacteriological studies since a large group of negatives may on occasion be due to an unrecognised metabolic block which in turn prevents the expression of many other characters.

Gower's coefficient, S_G : the similarity for any one character was given by:

$$S_{ijk} = 1 - \frac{|X_{ij} - X_{ik}|}{R}$$

where S_{ijk} is the similarity between OTUs j and k for the i th character, X_i being the state of the i th character and R is its range. This coefficient proposed by Gower (1971) is a combination of both S_{SM} and S_j which allows the use of multistate characters. In contrast to S_{SM} and S_j where each character contributes either a similarity or no similarity, in S_G a character may contribute a partial similarity. If a certain character can have the states negative, weakly positive and strongly positive, coded as 0, 1, 2 respectively, then a pair of identical states will yield a complete similarity, a 0 compared with a 2 will give no similarity, and a 0 compared with a 1, or a 1 compared with a 2, will give half similarity. S_G also allows the use of non-ordered multistate characters eg. pigmentation, with states red, yellow, orange, brown in which a match (eg. same pigmentation) counts as a whole similarity and a mismatch counts as no similarity, so there are no partial similarities. As such characters are rarely of bacteriological application it is usually more useful to break them down into several two state characters as outlined by Sneath and Sokal (1973).

The coefficients of resemblance described above all give equal weighting to the characters in accordance with the Adansonian principles.

4.1.4.4 Clustering

The calculation of similarity values for each pair of organisms enables the formation of a symmetrical matrix of similarity values. The two fundamental methods of clustering are hierarchical and non-hierarchical systems. Non-hierarchical techniques are not used frequently in biology. When using a hierarchical method clusters may be formed either

by the gradual addition of OTUs to the cluster (agglomerative) or by the gradual splitting of the OTUs into clusters (divisive). Due to the ease of computer programming agglomerative techniques have become the most commonly used (Sneath and Sokal 1973). The choice of the use of non-overlapping (no OTU may belong to more than one cluster at any taxonomic rank) or overlapping (an OTU may belong to more than one cluster) clustering has to be made. Rohlf (1970) suggests that overlapping methods may permit a better representation of the similarity matrix, but the non-overlapping technique is considered the more conventional method and results in the formation of nested clusters.

The starting point in the process of clustering is finding the pair of closest OTUs which then form a cluster. The similarities between this cluster and each of the remaining OTUs are computed and the process starts again treating the newly formed cluster as an OTU, to find the new highest similarity. This new highest similarity may be either between two single OTUs or between an OTU and the cluster just formed during the first clustering cycle. The clustering process is complete when all the OTUs have been joined to form one cluster.

Various methods of clustering can be used, the most common belonging to the SAHN (Sequential Agglomerative Hierarchical Non-overlapping) type. These include single linkage, complete linkage and average linkage clustering:

Single linkage clustering was used in early numerical taxonomy studies by Sneath (1957b) and is also called the nearest neighbour technique (Lance and Williams 1967). Single linkage defines similarity between two clusters as the similarity of the two most similar OTUs, one from each cluster. Connections between OTUs and clusters and between two clusters are established by single links between pairs of OTUs.

Complete linkage clustering is the direct opposite of the single linkage method and is also known as farthest neighbour clustering (Lance and Williams 1967). An OTU that is a candidate for admission to an existing cluster has similarity to that cluster equal to its similarity to the farthest member of the cluster. When two groups or clusters join, their similarity is that existing between the two most distant members, one in each cluster.

Average linkage clustering. Sneath and Sokal (1973) describe four methods of average linkage clustering, arithmetic average clustering, centroid clustering, weighted average clustering and unweighted average clustering. An unweighted average clustering method, UPGMA (unweighted pair-group method using arithmetic averages)

is the most frequently used clustering strategy in microbiological numerical taxonomy studies. Sokal (1985) points out that taxa constructed by numerical taxonomy must have members that resemble each other more than they do those of other taxa at the same or at other rank levels in the hierarchy.

On reviewing these various methods one is faced with the question as to which technique to use? The literature does not give a definitive answer. Dunn and Everitt (1982) state that the method used depends on the situation, while single linkage is the most mathematically respectable it is the least satisfactory in practice and they suggest that the average linkage methods provide the best solution. Sokal (1985) is in agreement based on the intuitive notion that within a taxon, similarity encompasses an average measure rather than the maximum or minimum thresholds. In reviewing the various numerical taxonomy studies presented in the literature it is clear that average linkage clustering (usually UPGMA) has become the method of choice.

4.1.4.5 Display of numerical taxonomy results

The results of numerical taxonomy studies are most commonly displayed as dendrograms or phenograms. Sneath and Sokal (1973) prefer the term phenogram over the term dendrogram which can include phenograms (which display phenetic similarity) and cladograms (which represent evolutionary positions). Simplified phenograms are often used in publications (eg. Hudson et al. 1986) and have the advantage of making the interpretation of large numerical taxonomy studies much easier. Another method used is the shaded triangle in which the organisms are arranged so that clustered organisms are adjacent. Graded shades of black/grey from black (showing high similarity) to white (showing low similarity) are used to represent the similarity values between pairs of organisms (eg. McCarthy and Cross (1984)). A triangle can be constructed with the clusters represented along the diagonal edge as dark triangles.

4.2 THE NUMERICAL TAXONOMY STUDY

4.2.1 INTRODUCTION

The potential use of anaerobic, thermophilic bacteria in the hydrolysis of cellulosic wastes has recently lead to extensive isolation studies and surveys of both thermophilic and non-thermophilic ecosystems (Jin and Toda 1988; Jones and Lindsay 1989; Hudson et al 1990a; Svetlichny et al 1990a). As described in chapters 1 and 2 the

anaerobic, cellulolytic, thermophilic organisms isolated from these studies have fallen into two categories, the thermophilic organisms isolated from compost or non-thermophilic environments and the extremely thermophilic organisms isolated from thermal springs. The majority of the organisms in the thermophilic category have been assigned to the genus *Clostridium* on the basis of their sporogenous nature, these have included *C. thermocellum* (Ng et al 1977), *C. stercorarium* (Madden 1983), *C. thermolacticum* (LeRuyet et al 1985), *C. thermocopriae* (Jin et al 1988) and *C. thermopapyrolyticum* (Mendez et al. 1991). The isolates in the extremely thermophilic category, with the exception of strain Z-1320 (*Anaerocellum thermophilum*) have not been fully characterized or assigned to a taxon. All of these isolates were rod-shaped and therefore not morphologically distinct as in the case of the thermophilic, cellulolytic spirochete described in chapter 7.

In order to investigate the diversity within this group of rod-shaped organisms and determine their relatedness if any to the clostridial type strains, a numerical taxonomy study was planned and carried out as described below. An additional reason for undertaking this study was to build up a data base of phenotypic characteristics of this group of isolates for use in the selection of organisms with desirable properties for future research and biotechnological purposes.

4.2.2 METHODS AND MATERIALS

4.2.2.1 Source of strains

Fifty-one strains were obtained from public and private culture collections as shown in Table 4.1. The temperature and pH of the sample sites are included where available. Strains Z-1320, 2713-32, 2706-27, 2711-28, 2805-34, 2906-25, 2705-55 were provided by V A Svetlichny and H Yu Aksenova (Institute of Microbiology, Academy of Sciences of the USSR, Moscow). B E Jones (Royal Gist-Brocades, Delft, The Netherlands) provided strain F2/1.

Each strain was examined for 92 unit characters (Table 4.2). All tests were done in triplicate on each strain.

Table 4.1 Anaerobic, cellulolytic, thermophilic isolates used

The isolates are arranged in the order of the clusters shown in Figure 4.1, with their source temperature and pH when available.

Cluster	Isolate*	Source	Country of origin	Source temp(°C)	Source pH
A	AZ2. B1	Hot spring	Azores	75	7.1
A	Z-1320	Hot spring	Russia	90	6.5
A	COMP. B1	Compost	New Zealand	NA	NA
A	COMP. B4	Compost	New Zealand	NA	NA
A	Tok9. B1	Hot spring	New Zealand	59.5	6.4
A	Tp8T. 6.3.3.1	Wood in hot spring	New Zealand	78	NA
A	2713-32	Hot spring sludge	Russia	80	6.5
A	Rt8.B2	Hot spring	New Zealand	63	8.8
A	2706-27	Hot spring	Russia	97	5.0
A	2711-28	Soil	Russia	56	6.5-7.0
A	2805-34	Hot spring algae	Russia	50	5.0
A	F2/1	Hot spring	Italy	58	7.0
A	NA10 (IFO 14436)	Hot spring	Japan	75-85	9.0
A	Rt8. B11	Hot spring	New Zealand	63	8.8
A	Ok1. B1	Hot spring	New Zealand	53	8.7
B	Tp8B3. 2	Wood in hot spring	New Zealand	78	NA
B	Tp8T2. 3	Wood in hot spring	New Zealand	78	NA
B	Tp11. W1	Hot spring	New Zealand	88	7.0
B	Wai 35. B1	Hot spring	New Zealand	NA	NA
B	RI1. B1	Hot spring algae	Raoul Island	66	6.5
B	RI2. B1	Hot spring algae	Raoul Island	67	6.5
B	2906-25	Hot spring	Russia	82	6.5
B	2705-55	Hot sand	Russia	70	7.0
C	AZ3. B1	Hot spring	Azores	72	8.6
C	Rt8. B7	Hot spring	New Zealand	63	8.8
C	Rt8. B4	Hot spring	New Zealand	63	8.8
C	Rt8. B6	Hot spring	New Zealand	63	8.8
C	Rt8. B8	Hot spring	New Zealand	63	8.8

Cluster	Isolate*	Source	Country of origin	Source temp(°C)	Source pH
D	Rt8. B15	Hot spring	New Zealand	63	8.8
D	Rt613. B1	Hot spring	New Zealand	NA	NA
D	Rt613. B2	Hot spring	New Zealand	NA	NA
D	Tok8. B1	Hot spring	New Zealand	52	6.2
D	RtUK4. B1	Hot spring	New Zealand	55	7.6
D	Tok9. B3	Hot spring	New Zealand	56	6.4
D	Ok9. B1	Hot spring	New Zealand	79	7.6
D	Ok2. B1	Hot spring	New Zealand	73	8.2
D	Wai28. B1	Hot spring	New Zealand	80	7.9
D	Rt69. B2	Hot spring	New Zealand	79	7.2
D	Rt69 B3	Hot spring	New Zealand	79	7.2
D	Wai21. W1	Hot spring	New Zealand	52	5.2
D	Rt426. B1	Hot spring	New Zealand	NA	NA
D	Rt4. B3	Hot spring	New Zealand	64	6.5
D	Ok8. B1	Hot spring	New Zealand	65	6.4
E	Rt51. B1	Hot spring	New Zealand	44	8.3
E	NCIMB 11754 <i>C. stercoarium</i>	Compost	NA	NA	NA
E	DSM 2911 <i>C. thermolacticum</i>	Compost	France	NA	NA
E	Tp10. B1	Hot spring	New Zealand	55	7.0
E	IAM 13577 <i>C. thermocopriae</i>	Compost	Japan	NA	NA
-	Rt69. B1	Hot spring	New Zealand	79	7.2
-	Rt69. B4	Hot spring	New Zealand	79	7.2
-	DSM 1237 <i>C. thermocellum</i>	Compost	NA	NA	NA

NA, Not available.

*Abbreviations used: *C.*, *Clostridium*. New Zealand thermal sites: Tok, Tokaanu; Rt, Rotorua; Wai, Waimangu Valley; Ok, Orakei Korako; Tp, Taupo. Other thermal sites: AZ, Azores. Culture collections: IFO, Institute of Fermentation, Osaka, Japan; NCIMB, National Collection of Industrial and Marine Bacteria, United Kingdom; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG; IAM, Institute of Applied Microbiology, Tokyo, Japan.

4.2.2.2 Culture conditions

Strains were routinely cultured on 2/1 cellobiose medium. The 2/1 basal medium contained (per litre): NH_4Cl , 0.9g; NaCl , 0.9g; MgCl_2 , 0.2g; KH_2PO_4 , 0.75g; K_2HPO_4 , 1.5g; cysteine-HCl, 0.75g; trypticase peptone, 2g; yeast extract, 1g; 0.2%(w/v) resazurin, 1ml; 0.15%(w/v) FeCl_3 , 1ml; trace element solution SL10 (without FeSO_4) and selenite-tungstate solution (Widdel et al 1983), 1ml each. The 2/1 cellobiose medium was prepared by the addition of cellobiose (1g/litre) to 2/1 basal medium. For solid medium, purified agar (Oxoid) was added at 15g/l. The medium with a final pH of 7.0 was dispensed as 10ml amounts in Hungate tubes under an atmosphere of N_2/H_2 (95%/5%). All manipulations of agar plates and microtitre plates were carried out in an anaerobic chamber (Coy, USA) with an atmosphere of N_2/H_2 (95%/5%). Inoculated media were incubated at the optimal growth temperature of the test organism which was either 65°C or 70°C. Strains were maintained at room temperature on 2/1 cellobiose medium. Subculturing and microscopic checking of purity strains was performed twice monthly on all strains.

4.2.2.3 Morphology and growth characteristics

Colonial morphology was examined on isolated colonies grown on 2/1 cellobiose agar for 3d. Cellular morphology was examined on negatively-stained preparations by electron microscopy. Negatively-stained specimens were prepared as described in section 4.1.2.2. The presence of particulate growth and slimy cell pellet formation were determined after 3d growth on 2/1 cellobiose medium. The presence of a slimy cell pellet was determined after the test culture had been standing at room temperature for 2d following incubation for 3d at the optimal growth temperature. Lysis of cells in 3% KOH was tested using the method of Gregersen (1978).

4.2.2.4 Utilization of carbon sources

Carbon source utilization was tested using a microtitre plate method for soluble carbon sources and a tube method for insoluble carbon sources. All carbon sources were obtained from Sigma Chemical Co. (USA) and were the best grade available. Avicel (export grade) was obtained from Asahi Chemical Industry Co. Ltd (Osaka, Japan). Amorphous cellulose was prepared as described in section 3.3.2.2. Soluble carbon sources were autoclaved as 10% (w/v) solutions. The microtitre plates were of the U-bottomed type which enabled the visualization of cell pellet formation. Each well contained 100 μl of 2/1 basal medium containing 0.2% (w/v) test carbon source and 0.01% (w/v) phenol red. The inoculum was 100 μl of a 1/50 dilution of an 18h liquid

culture. After inoculation 100 μ l of sterile liquid paraffin was added. The edges of the microtitre plates were sealed with masking tape and placed in plastic bags before heat sealing and incubation. Microtitre plates were incubated for 24h and growth was scored by examining for the presence of cell pellets and acid production indicated by the phenol red indicator. For insoluble carbon sources 2/1 basal medium containing 1g/litre insoluble carbon source and 0.01% (w/v) phenol red was dispensed in 10ml amounts in Hungate tubes and inoculated with 100 μ l of an 18h liquid culture. After incubation the tubes were examined for visible growth and acid production after 24h and 72h..

4.2.2.5 Antibiotic resistance

Antibiotics were obtained from Sigma Chemical Co. (USA). Stock solutions were prepared in preboiled distilled water and filter sterilized into sterile N₂ flushed serum vials. Minimum inhibitory concentrations (MICs) of antibiotics were determined using a microtitre plate technique. To each well was added 100 μ l 2/1 cellobiose medium containing 0.02% (w/v) phenol red, which had been inoculated with 2%(v/v) of a 18h liquid culture. This was followed by 100 μ l of the appropriate filter sterilized antibiotic solution. diluted in 2/1 cellobiose medium. The inoculation, microtitre plate sealing, incubation and examination were as for the carbon source utilization tests. MIC values differentiating between the strains were chosen as the test values.

4.2.2.6 Degradation of polysaccharides

The radial diffusion assay described by Williams (1983) was modified for use in detecting polysaccharide degradation by the test strains. The dyes and polysaccharides were obtained from Sigma Chemical Co. and BDH Chemical Co. Ltd. The polysaccharide substrate (1g/litre) was dissolved/suspended in 2/1 basal medium and dispensed in 10ml amounts in Hungate tubes. Inoculated tubes were incubated at the optimum growth temperature of the test organism for 42h. The visual presence or absence of growth was recorded and 50 μ l of the whole culture used in the radial diffusion assay.

For the radial diffusion assay the polysaccharide substrate (2g/litre) was dissolved/suspended in 2/1 basal medium minus cysteine-HCl and resazurin. Purified agar (Oxoid) (15g/litre) was added and the medium dispensed as 20ml amounts in 9cm petri dishes. Wells (4mm dia.) were cut in the agar plates. Whole culture (50 μ l) was applied to the wells and the plates were incubated at the optimum temperature of the test organism for 12h. Uninoculated medium was used as a control for each substrate tested.

The zones of hydrolysis were visualised by flooding the plates with an aqueous solution of Congo red (1g/litre) for carboxymethyl cellulose, locust bean gum, guar gum, *Cetraria islandica* lichenan, *Laminaria digitata* laminarin; Gram's iodine (I₂, 1g/litre; KI, 6.6g/litre) for crab shell chitin, larchwood arabinogalactan, karaya gum, dahlia tuber inulin, rabbit glycogen, oat spelts xylan, soluble starch, *Saccharomyces cerevisiae* mannan; 95% (v/v) ethanol for *Aureobasidium pullulans* pullulan, each for 15min. The staining/precipitating solution was poured off and the plates flooded with 1M NaCl for a further 15min. The width of the zone of hydrolysis was measured using a micrometer. Zones of hydrolysis >2mm were scored as positive degradation of the test polysaccharide.

4.2.2.7 Growth in the absence of growth supplements

2/1 basal medium was prepared without the addition of trypticase or yeast extract. Growth on cellobiose, amorphous cellulose and Avicel was tested in the absence of trypticase and yeast extract; growth was recorded after 3d. Cultures showing growth were subcultured to the same medium and incubated for 3d to eliminate the possibility of growth due to 'carry over' of yeast extract and trypticase from the initial inoculum.

4.2.2.8 Tolerance tests

2/1 cellobiose medium was used as the basal medium. Growth at 45°C was recorded after 10d, and growth at 75°C and 80°C after 3d. Growth at pH 5.5, 7.5 and 8.0 was determined in media adjusted to the appropriate pH with conc. HCl or 10M NaOH before autoclaving; growth was recorded after 3d. Growth in the presence of NaCl (0.5 and 1.0%w/v) and ethanol (2.0 and 3.0% v/v) was recorded after 3d.

4.2.2.9 Fermentation end-products

2/1 cellobiose (10mM) medium was used as the growth medium. Cultures were incubated for 3d. Acetate, lactate and ethanol were determined by HPLC using the method of Patel et al. (1987); GLC was used to determine the presence of butyrate and iso-butyrate by the method of Patel et al. (1985a). Acetate and lactate were recorded as main products when the other end-products were present at concentrations less than 3mM. Ethanol was recorded as a major end-product at concentrations greater than 3mM and as a minor end-product at concentrations less than 3mM. Butyrate and iso-butyrate were recorded as end-products at concentrations greater than 0.5mM.

4.2.2.10 Viability after 5d at optimum growth temperature

Liquid cultures of 2/1 cellobiose were incubated at optimum growth temperature for 5d and 0.1ml transferred to a fresh 2/1 cellobiose tube which was incubated for 3d before growth was recorded.

4.2.2.11 Hydrogen sulfide production

The production of H₂S was determined using SIM medium (Baltimore Biological Laboratory). SIM medium was dispensed anaerobically and the agar concentration increased to 1.5g/l.

4.2.2.12 Presence of DNAase

The method of Jeffries et al. (1957) was followed. Calf thymus DNA (BDH) was added to 2/1+cellobiose agar to a concentration of 2g/l before autoclaving. Plates were incubated anaerobically for 3d before being flooded with 1M HCl. DNA which had not been degraded precipitated as an opaque white precipitate. Cultures were scored as positive for DNAase production where a zone of clearing was observed around the area of growth.

4.2.2.13 Calculation of test error.

At the beginning of the study six cultures were each transferred to two tubes and each replicate was treated as a separate isolate during testing. These replicates were used to estimate the test error by the method of Sneath and Johnson (1972) which calculates the probability of obtaining an erroneous result from the proportion of mismatches between duplicate strains. Sneath and Johnson (1972) considered an error of <5% to be achievable within one laboratory.

4.2.2.14 Analysis of results

The 92 unit characters used were scored as one of two mutually exclusive states, positive or negative, coded as 1 and 0 respectively. The cluster analysis facilities of the GENSTAT program (The Numerical Algorithms Group, Rothamsted Experimental Station, Harpenden, United Kingdom) were used to process the data.

The simple matching (S_{SM} , Sokal and Michener 1958) and Jaccard (S_J , Sneath 1957a) coefficients were used to calculate similarity matrices. Clustering was achieved using the unweighted pair group method with arithmetic averages (UPGMA) algorithm (Sneath and Sokal 1973). The results were displayed as both simplified and detailed phenograms.

4.2.3 RESULTS AND DISCUSSION

4.2.3.1 Choice of tests

The characters tested are listed in Table 4.2. These tests were chosen to represent a wide range of characteristics of this group of organisms. The full data set obtained in the study is presented in appendix 1. In order to obtain information on the cellulolytic and polysaccharolytic nature of the isolates a large number of the tests used represented these characteristics. Tests which were found to give a uniform result (either negative or positive) for all isolates tested were not included in the numerical analysis, these included Gram reaction (negative), utilization of cellobiose (positive), production of indole (negative), growth at 65°C (positive) and growth at pH7.0 (positive).

4.2.3.2 Experimental error

The experimental error was calculated from the data collected on the 6 duplicate strains. The average probability (p) of an erroneous test result was 3.5% which is within the acceptable range suggested by Sneath and Johnson (1972). The 6 pairs of duplicate strains showed a mean observed similarity of 94.8 % S_{SM} .

4.2.3.3 Clustering of the isolates

From the S_{SM} /UPGMA analysis, five clusters containing five or more isolates were formed at or above the 70% similarity level as shown in the simplified phenogram (Figure 4.1), these have been labelled A to E. In addition there was one two-membered cluster and one single-membered cluster.

Table 4.2. Percentage distribution of positive characters to cluster-groups defined at the 70% level (S_{SM})

Cluster group Number of strains	A 15	B 8	C 5	D 15	E 5
Character					
Colonial morphologies:					
Flat/raised	27	50	0	0	0
Convex	7	12	20	87	60
Umbonate	67	37	80	13	40
Entire	27	25	60	93	0
Undulate/lobate	73	75	40	7	100
Cellular characteristics:					
Length >2.5 μ m	53	75	0	13	100
Width >0.4 μ m	60	50	60	60	60
Flagella	73	62	100	60	80
Ends blunt	47	37	80	67	20
KOH Lysis	100	100	100	100	60
Growth in liquid culture:					
Particulate	0	0	0	13	0
Slimy sediment	7	25	0	0	0
Utilization of soluble carbon sources:					
Amygdalin	27	0	0	0	20
Arabinose	87	100	0	40	80
Raffinose	33	75	0	13	20
Galactose	93	100	0	27	100
Glucose	100	100	0	47	100
Sorbitol	0	0	0	7	60
Lactose	100	87	60	80	60
Xylose	93	100	0	40	80
Ribose	20	0	20	27	80
Melezitose	20	25	0	0	0
Fructose	100	100	0	40	80
Sucrose	80	100	0	13	60
Mannose	93	100	0	53	80
Rhamnose	40	50	0	0	40
Mannitol	27	0	0	7	60
Maltose	73	100	0	20	60
Melibiose	20	50	0	13	60
Utilization of polysaccharide carbon sources:					
Laminarin (72h)	100	100	100	73	100
Lichenin (72h)	100	100	100	100	100
Gum Locust Bean (72h)	93	100	100	93	100
Pullulan (72h)	100	100	40	93	100
Glycogen (72h)	93	87	40	93	100
GumGuar (72h)	100	100	100	100	80
Inulin (72h)	80	100	0	40	80
Pectin (72h)	87	87	0	33	100
Amorphous cellulose (24h)	93	100	100	100	0
Amorphous cellulose (72h)	100	100	100	100	46
Avicel (24h)	80	75	60	100	0
Sigmacell 20 (24h)	73	100	80	100	20
Sigmacell 20 (72h)	100	100	100	100	40
Sigmacell 50 (24h)	80	100	80	100	20
Sigmacell 50 (72h)	93	100	100	100	20
Sigmacell 100 (24h)	20	12	40	40	0
Sigmacell 100 (72h)	67	62	80	93	0

Cluster group Number of strains	A 15	B 8	C 5	D 15	E 5
Character					
Degradation of polysaccharides:					
Carboxymethylcellulose	87	100	100	93	80
Starch	73	100	20	80	100
Xylan	93	100	60	87	80
Laminarin	73	100	100	53	80
Lichenin	100	100	100	87	60
Gum Locust Bean	93	100	100	87	80
Gum Karaya	7	100	0	7	20
Chitin	7	100	0	0	0
Pullulan	100	100	40	67	100
Glycogen	67	100	0	47	100
Mannan	13	100	0	20	20
Arabinogalactan	13	100	0	7	0
Gum Guar	93	100	100	93	40
Inulin	0	100	0	0	0
Growth on medium without growth supplements:					
Cellobiose (24h)	73	25	80	27	0
Cellobiose (72h)	100	87	100	87	20
Amorphous cellulose (24h)	27	25	100	27	0
Amorphous cellulose (72h)	100	75	100	93	0
Avicel (24h)	7	12	60	0	0
Antibiotic MICs ($\mu\text{g/ml}$):					
Penicillin-G (MIC>7.8)	87	100	100	93	100
Bacitracin (MIC>1.95)	80	50	80	20	100
Polymyxin (MIC>7.8)	53	62	60	53	100
Streptomycin (MIC>15.6)	93	87	60	73	100
Novobiocin (MIC>15.6)	60	50	20	13	100
Cycloserine (MIC>62.5)	93	87	80	87	100
Rifampin (MIC>50)	93	100	100	100	0
Growth inhibited by:					
Ethanol (2% (v/v))	7	0	0	7	40
Ethanol (3% (v/v))	80	37	80	53	60
NaCl (0.5% (w/v))	13	37	80	100	0
NaCl (1.0% (w/v))	73	87	100	100	40
Growth at:					
pH 5.5	100	100	60	73	40
pH 7.5	93	75	60	80	100
pH 8.0	33	0	40	0	40
45°C (10d)	80	62	80	53	80
75°C (3d)	100	100	100	100	60
80°C (3d)	73	75	80	53	0
Fermentation end products:					
Acetate/lactate (1:1)	40	25	100	13	0
Acetate (main end product)	53	75	0	87	60
Lactate (main end product)	7	0	0	0	40
Ethanol (major end product)	20	0	0	0	100
Ethanol (minor end product)	60	37	40	7	0
<i>iso</i> -Butyrate	20	25	0	0	20
Butyrate	7	12	0	0	60
Other tests:					
Viability after 5d at optimum temperature	100	100	100	93	80
H ₂ S production	7	12	0	0	20
DNAase	87	75	40	80	60

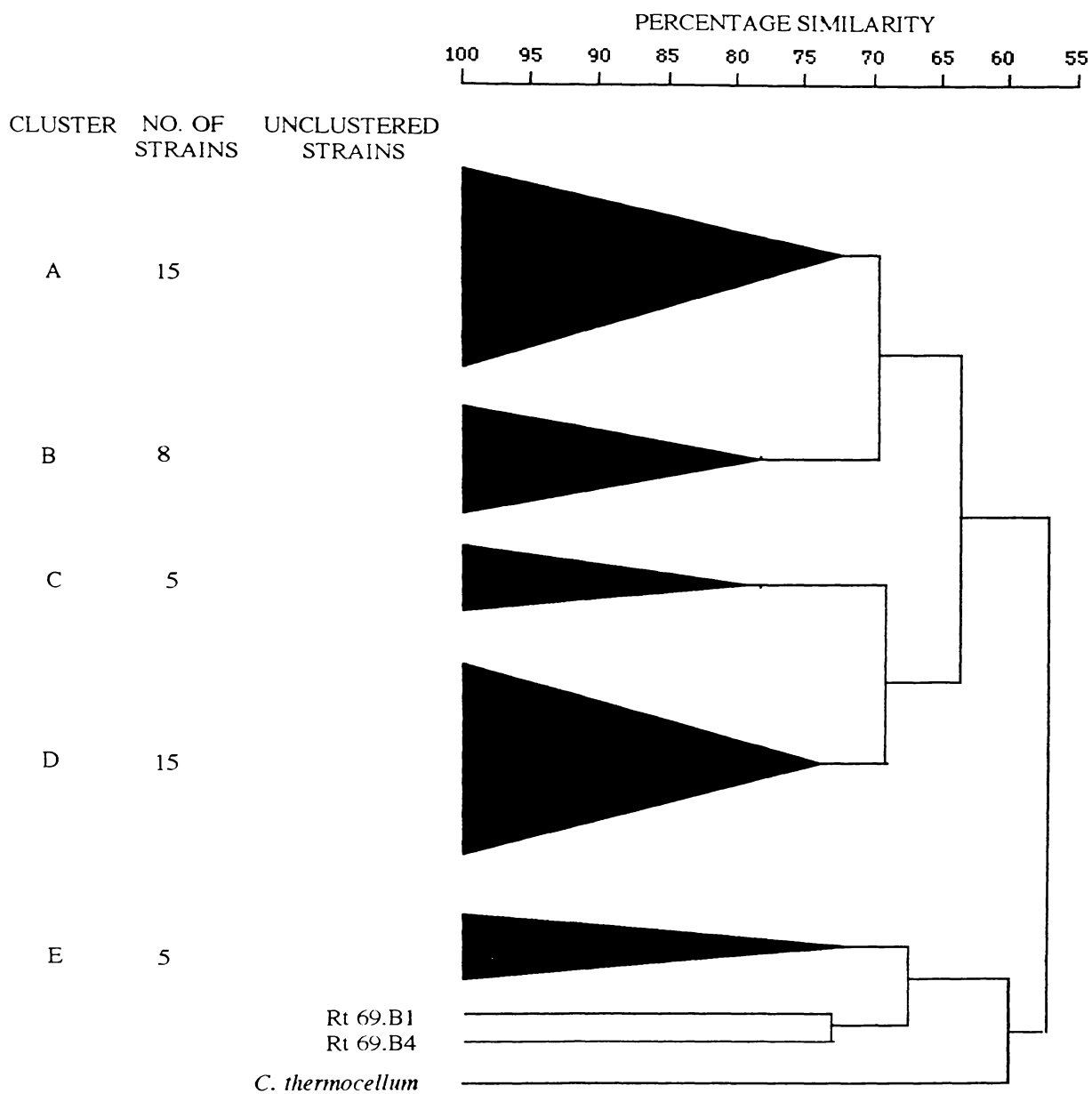


Figure 4.1 Simplified phenogram based on the UPGMA analysis of a similarity matrix derived from the S_{SM} coefficient.

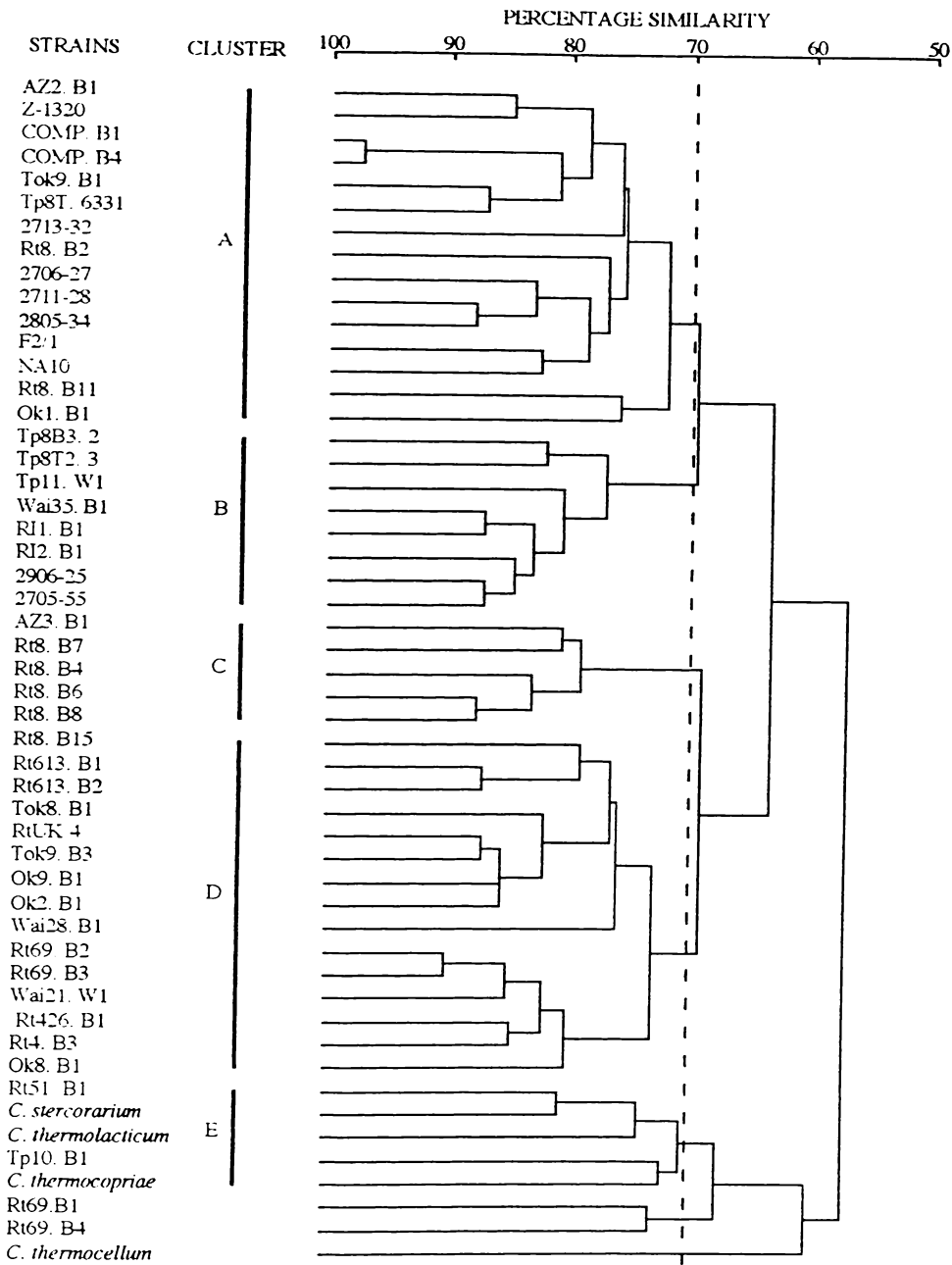


Figure 4.2 Detailed phenogram based on the UPGMA analysis of a similarity matrix derived from the *SSM* coefficient. Clusters are defined at 70% similarity level.

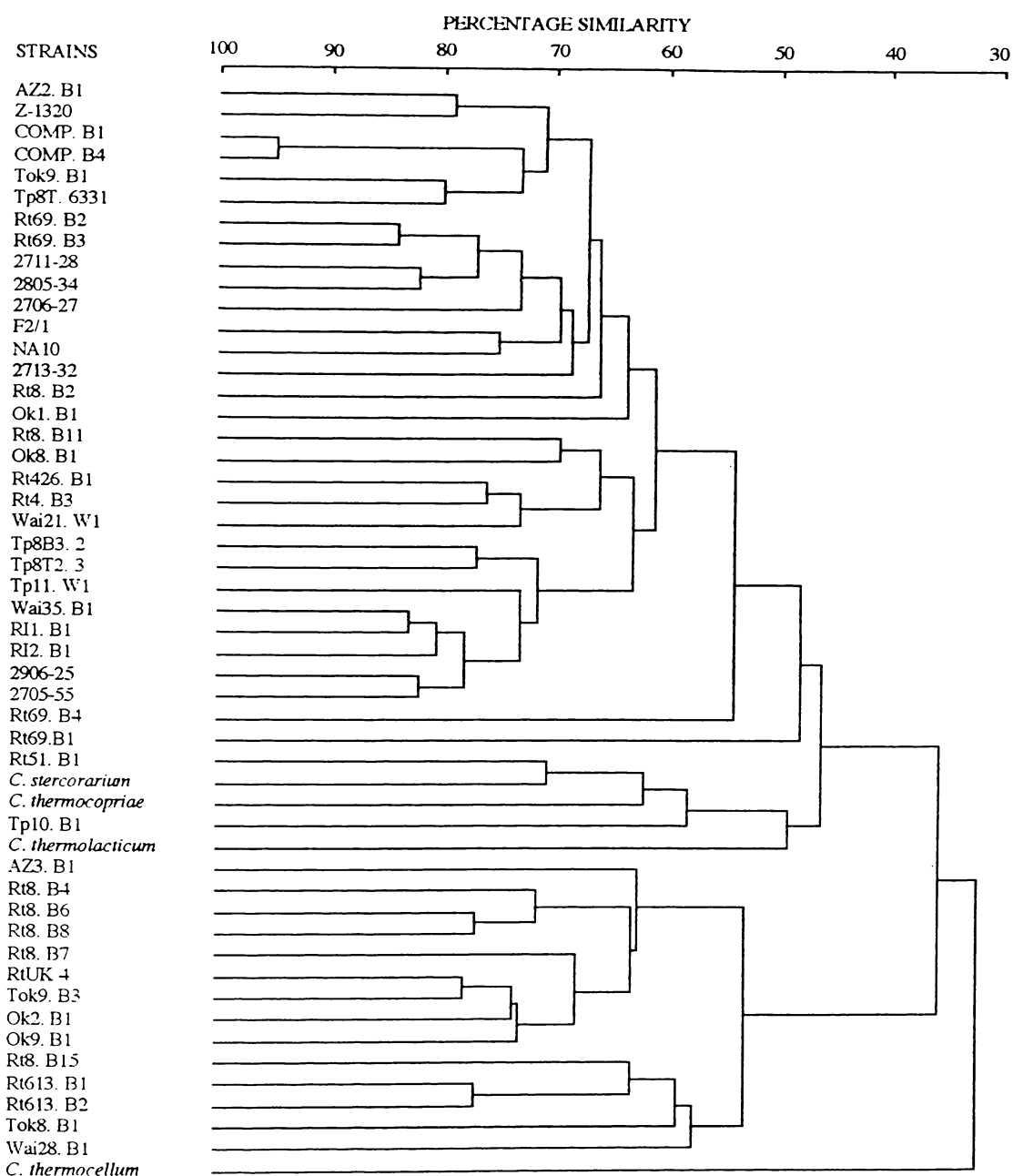


Figure 4.3 Detailed phenogram based on the UPGMA analysis of a similarity matrix derived from the S_j coefficient.

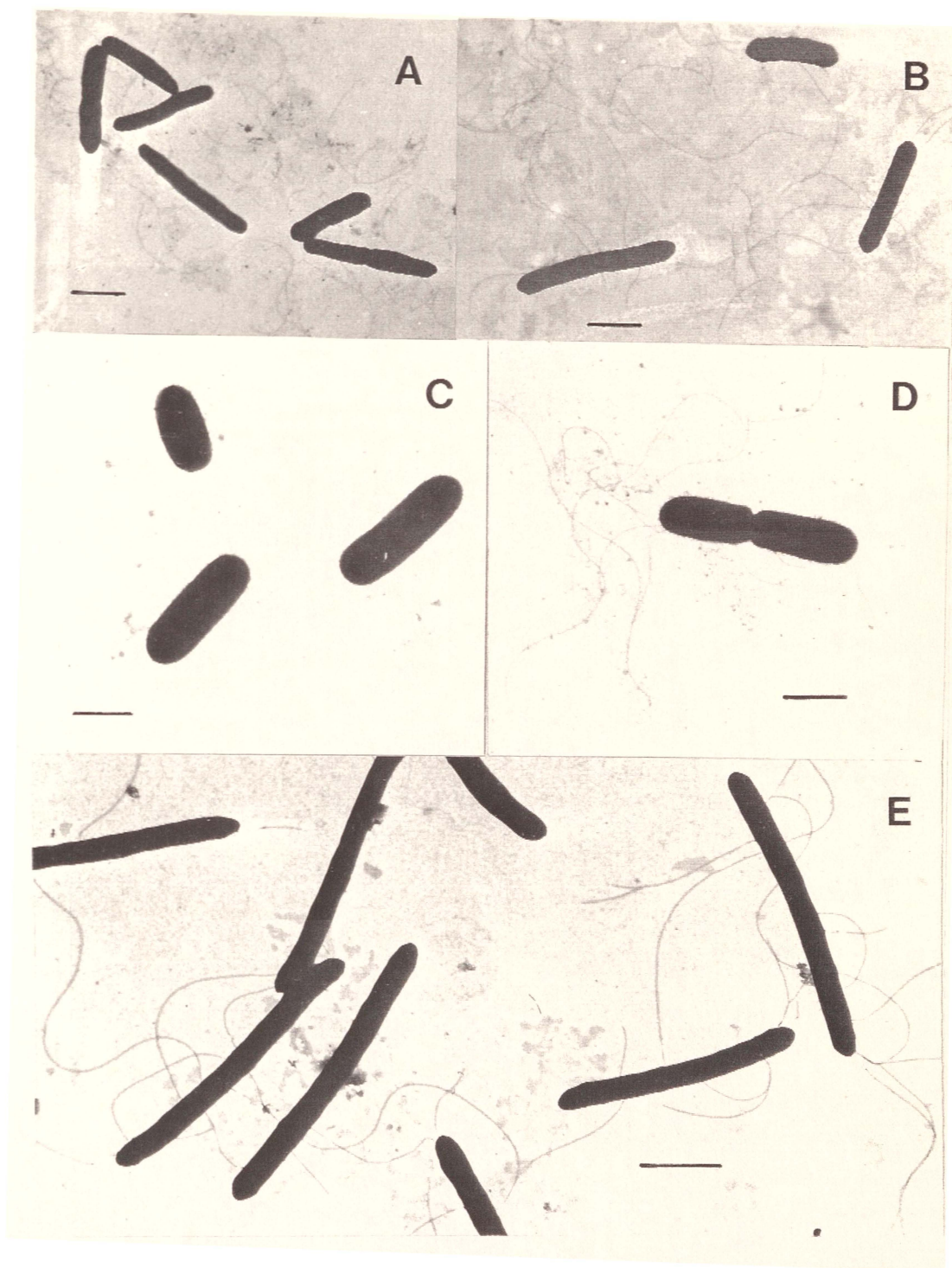


Figure 4.4 A-E Negatively stained preparations showing typical morphologies of representative isolates from clusters A-E. **A.** COMP. B4. **B.** Wai 35. B1. **C.** Rt8. B4. **D.** Ok8. B1. **E.** Rt51. B1. Bar = 1.0 μ m.

The S_{SM} /UPGMA detailed phenogram (Figure 4.2) showed some differences from the S_J UPGMA detailed phenogram (Figure 4.3). These differences indicate that some of the clusters formed in Figure 4.3 may reflect a significant component of negative matches. Clusters A and B remain identical irrespective of the similarity coefficient used. In the S_J analysis cluster D is split to form two additional clusters of five isolates each, the remaining isolates become ungrouped with the exception of strains Rt69. B2 and Rt69. B3 which moved to cluster A. One member of cluster A, strain Rt8. B11 moved to group with organisms from the split cluster D. In both cluster analyses the isolates of cluster C group together, in the case of S_J analysis clustering with one group of cluster D isolates (those with limited carbon source utilization range). The five members of cluster E in the S_{SM} /UPGMA still cluster together and strains Rt69. B1, Rt69. B4 and *C. thermocellum* remain unclustered.

Since negative matches provide as much information and are as important as positive ones in this type of study further comments are restricted to the S_{SM} /UPGMA phenogram.

4.2.3.4 Composition and characteristics of the clusters

The composition of the clusters formed with the S_{SM} /UPGMA analysis is shown in Figure 4.2. The distribution of positive characters among the clusters with the S_{SM} /UPGMA analysis is displayed in Table 4.2. Wide ranges of source temperature are found within the clusters eg. cluster A 50-90°C, cluster D 52-80°C. Typical morphologies of representative isolates from clusters A-E are shown in Figure 4.4.

Cluster A contained fifteen strains including all the isolates described in the literature as asporogenous organisms (strains Z-1320, Tp8T. 6.3.3.1, F2/1 and NA10) with the exception of strain H173 (Rt8. B6). This cluster contained isolates from a mix of geographically isolated (Russia, New Zealand, Italy and Japan) thermal sites with temperatures and pH values ranging from 50-90°C and 5.0-9.0 respectively. Two highly similar isolates (strains COMP. B1 and COMP. B4) from commercially prepared compost are also contained in cluster A. Thirteen isolates formed the main part of the cluster at 76% S_{SM} , while a further two isolates joined to form a homologous phenon at 72% S_{SM} . The isolates within cluster A utilized a wide range of carbon sources and degraded the majority of the polysaccharides tested. Eighty percent of the isolates formed ethanol as a fermentation end-product, 20% as a major and 60% as a minor end-product.

Cluster B contained eight isolates from New Zealand, Raoul Island and Russian thermal sites which formed a homologous phenon at 77% S_{SM} . The isolates in cluster B utilized a narrower range of soluble carbon sources, sugars not utilized included amygdalin, sorbitol, ribose and mannitol. All of the polysaccharides tested were degraded by all of the isolates of cluster B. The isolates of cluster B were found to be unique to the organisms tested in their ability to degrade the fructose polymer inulin. None of the isolates of cluster B grew at pH 8.0. The majority of the isolates in cluster B formed acetate as the main fermentation end product, no members of the cluster formed lactate or ethanol as major end products. Cluster B merged with cluster A at below 70% S_{SM} .

Cluster C contained five isolates of which four were from the same sample site, namely thermal spring Rt8 (New Zealand). The fifth isolate was from an Azores thermal spring (AZ3). The isolates of cluster C form a homologous phenon at 79% S_{SM} . The main characteristic of the cluster C isolates is their extremely limited soluble carbon source utilization range. Three isolates were found to utilize only lactose and one isolate only ribose in addition to cellobiose (which was utilized by all isolates in all clusters). The only soluble carbon source utilized by strain Rt8. B7 was cellobiose. This narrow utilization range with respect to soluble carbon sources was not the case with the polysaccharides tested since a large number of the polysaccharides tested were utilized by all of the isolates of cluster C. A cell length of $<2.5\mu\text{m}$ and the presence of flagella were found to be characteristics of the isolates of cluster C. Acetate/lactate in a ratio of 1:1 were produced as the fermentation end-products of the cluster C isolates. Two of the isolates in cluster C produced ethanol as a minor fermentation end-product; butyrate and iso-butyrate were not produced by members of this cluster.

Cluster D was made up of fifteen isolates all of New Zealand thermal spring origin. These isolates were from thermal sites with a wide range of source temperature (52-80°C) and pH (5.2-8.8) values. The cluster was composed of two subclusters (one containing nine isolates and one six isolates) which formed a homologous phenon at 73% S_{SM} . The colonial morphology of the majority of the isolates of cluster D was convex with entire edges. Morphologically the majority of the isolates were short rods ($<2.5\mu\text{m}$ in length). The isolates of the nine member subcluster are similar to the isolates of cluster C in their utilization of a very narrow range of carbon sources; the isolates of the six member subcluster utilized a wider range of carbon sources. A wide range of polysaccharides were utilized and degraded by the cluster D isolates. All of the isolates of cluster D were inhibited by 0.5% (w/v) NaCl; growth at pH 8.0 was not observed in any of these strains. Acetate was the main end-product of fermentation of

most of the isolates of cluster D with only one isolate producing ethanol as a minor end-product; butyrate and iso-butyrate were not produced by any members of cluster D. Clusters C and D merged at below 70% S_{SM} .

Cluster E was made up of three clostridial type strains (*C. stercorarium*, *C. thermolacticum*, *C. thermocopriae*) and two New Zealand isolates forming a homologous phenon at 71% S_{SM} . Cluster E joined clusters A, B, C, D to form a homologous phenon at 58% S_{SM} . The two New Zealand strains were thermal spring isolates in contrast to the clostridial type strains which were isolated from compost. All of the isolates of cluster E had undulate/lobate colony morphology and had a cell length of $>2.5\mu\text{m}$. A wide range of carbon sources were utilized and polysaccharides degraded by the isolates of cluster E. The results from the utilization of the insoluble carbon sources (eg. amorphous cellulose, Avicel and Sigmacell 20, 50, 100) show that the isolates of cluster E utilize these substrates more slowly than the isolates of the other clusters. With the exception of one isolate, the members of cluster E require growth supplements for growth on cellobiose, amorphous cellulose and Avicel. Rifampin was found to inhibit all of the isolates of cluster E at concentrations less than $50\mu\text{g/ml}$ this is in contrast to the isolates of the other clusters all of which have MICs $>50\mu\text{g/ml}$ for rifampin. The isolates of cluster E displayed a greater resistance to NaCl than the isolates of the other clusters. None of the isolates of cluster E grow at 80°C . Ethanol was produced as a major end-product of fermentation by all of the isolates of cluster E; butyrate was produced by 60% of the cluster E isolates.

4.2.3.5 Tests and information which distinguish between the clusters

From the test results the identification of isolates and assigning them to a cluster group is based more on general characteristics than on specific tests. The fact that the majority of isolates in clusters C and D were $<2.5\mu\text{m}$ in length makes the cell length a useful characteristic for identification. Isolates with a cell length of $<2.5\mu\text{m}$ can be excluded from belonging to cluster E in which all the isolates were found to have a cell length of $>2.5\mu\text{m}$. Isolates can be identified as members of cluster C or the nine member sub cluster D on the basis of their utilization of a narrow range of carbon sources, but it should be noted that the unclustered type strain *C. thermocellum* also utilized a limited range of carbon sources. The fact that all isolates of cluster E are inhibited by rifampin at $50\mu\text{g/ml}$ clearly distinguishes them from the other isolates and makes sensitivity to rifampin a useful identification test. Inulin was only degraded by the isolates of cluster B and can be used to identify strains of cluster B. The production of ethanol as a major end-product of fermentation could be used in the identification of cluster E isolates. The

cluster E isolates did not grow at 80°C in contrast to the majority of isolates in clusters A-D and so growth at 80°C could be used for identification purposes.

Many of the tests suggested as being useful in identification of isolates do not stand alone as specific for a particular cluster but could be used in combination to exclude an isolate from, or assign an isolate to a particular cluster.

4.2.4 GENERAL CONCLUSION

The results of this study have shown that the anaerobic, cellulolytic thermophiles represent a phenotypically diverse group of bacteria. The isolates which have been described in the literature as asporogenous cellulolytic thermophiles (strains Z-1320, Tp8T. 6.3.3.1, F2/1 and NA10) were all found to be members of cluster A, forming a homologous phenon at the 76% S_{SM} level. The exception was strain Rt6. B6 (strain H173) described by Hudson et al. (1990b) which was a member of cluster C. The clustering pattern demonstrated that with the exception of strains Rt51. B1, Tp10. B1, Rt69. B1 and Rt69. B4 the isolates tested are not closely related to the clostridial type strains. None of the isolates were found to cluster with *C. thermocellum* suggesting either that this organism is not widespread in thermal springs or that the isolation methods used do not enrich for this organism. The one thermal spring isolate (strain Rt51. B1 (H197)) described as "*C. thermocellum* -like" by Hudson et al. (1990a) was found to cluster with *C. stercorarium*, a compost isolate. The two non-type strain, compost isolates COMP. B1 and COMP. B4 clustered with the thermal spring isolates of cluster A indicating that the non-clostridial type organisms are not exclusive to thermal spring environments.

The isolates of cluster C and the majority of those in cluster D were found to utilize or degrade a wide range of polysaccharides but not simple carbon sources, such as glucose. It could be considered that in the natural environment they have a hydrolytic role, providing simple carbon sources for commensal organisms. None of the previously described anaerobic, thermophilic bacteria have utilized a restricted range of carbon sources, making the organisms of clusters C and D unique in this respect. The isolates of cluster B were found to degrade all the polysaccharides tested in this study thus demonstrating that enrichment on cellulose can yield organisms not only cellulolytic but generally polysaccharolytic.

Clusters A and B were found to contain isolates from geographically distant thermal sites. A subcluster of cluster B contained one isolate from New Zealand, two isolates from Raoul Island and two isolates from Russia, forming a homologous phenon at 83% S_{SM} . With the exception of strain AZ3. B1 (Azores isolate) all of the isolates in clusters C and D were from New Zealand thermal springs. The sample of isolates from locations other than New Zealand was not large enough to determine if the clustering was influenced by the geographical source of the isolates. The fact that the seven Russian isolates fall in clusters A and B only, may suggest that the isolates of cluster D are unique to New Zealand thermal sites. The results obtained do show that isolates with similar phenotypes exist in geographically distant thermal sites.

The results of this study demonstrated that there is diversity within this group of organisms, thus enabling unrelated organisms to be chosen for future enzymatic and biotechnological studies. The study also provided a useful data base on these isolates. This information has been used in choosing organisms with required characteristics eg. a strain which grows at pH 8 and 80°C.

4.2.5 TAXONOMIC CONCLUSION

It is difficult to make taxonomic conclusions from a study such as that described above. Few criteria have been laid down in the literature for the designation with respect to the level of similarity at which isolates are assigned to genera or species. The majority of numerical taxonomy studies have been of groups of organisms of known or assumed genera eg. the genus *Thermus* (Hudson et al. 1989), the genus *Bacillus* (Priest et al. 1988) and the genus *Streptomyces* (Williams et al. 1983). An example of a numerical study of a group of isolates of unknown generic position is that of some leuconostocs and related bacteria from distilleries (Priest and Pleasants 1988) in which the genera *Leuconostoc* and *Lactobacillus* are separated at the 57% S_{SM} level. In a similar study of moderately halophilic Gram-negative rods from an inland saltern the genus *Vibrio* was separated from the genera *Alteromonas*, *Deleya*, *Acinetobacter* and *Pseudomonas* at the 59% S_{SM} level, the later three genera separated at the 66% S_{SM} level (Del Moral et al. 1988).

The problem with this study of anaerobic, cellulolytic, thermophiles is that the majority of isolates were found not to cluster with the clostridial type strains which were included on the basis of being thermophilic and cellulolytic. The isolates of clusters A-D could constitute one or more new genera depending on the levels chosen to designate genera

and species levels. Another possibility is that they could be cellulolytic species of non-cellulolytic thermophilic genera, for example *Thermoanaerobium* or *Thermoanaerobacter*. Organisms from these non-cellulolytic genera were not included in this study as many of the characteristics tested were restricted to cellulolytic organisms and would therefore have weighted the study. In order to determine the taxonomic position of the isolates of clusters A-D, studies should be carried out at the genomic level and representatives of these anaerobic, thermophilic bacteria should be included.

CHAPTER 5

DIFFERENTIATION OF ANAEROBIC CELLULOLYTIC THERMOPHILIC BACTERIA BY ELECTROPHORESIS OF THEIR PROTEINS

INTRODUCTION

The electrophoresis of microbial proteins has been used in taxonomic, systematic and identification studies. The limiting factors in previous studies have been the degree of reproducibility and the time-consuming nature of the techniques. In this chapter, two electrophoresis methods, both of which were semi-automated to some extent and one of which gave a result within twenty-four hours, were used to determine the degree of diversity within a group of anaerobic, cellulolytic thermophiles. The potential to use one of the methods as an identification system was investigated.

5.1 A REVIEW OF THE PRINCIPLES AND METHODS OF CLASSIFICATION AND IDENTIFICATION OF BACTERIA BASED ON ELECTROPHORESIS OF THEIR PROTEINS

5.1.1 INTRODUCTION

Workers such as P. Jackman, K. Kersters and J. De Ley have over the years demonstrated the use of polyacrylamide gel electrophoresis (PAGE) as a convenient and valuable tool in microbial systematics and identification. The application of whole-cell protein PAGE in microbial classification, identification and typing has been reviewed by Kersters and De Ley (1980) and Jackman (1985, 1987).

Jackman (1985) estimated that the expression of the microbial genome results in the synthesis of about 2000 protein molecules that make up the microbial cell. Kersters and De Ley (1980) point out that a bacterial strain growing under standardized conditions always produces the same set of proteins. Applying electrophoresis to mixtures of cell proteins under standardized conditions produces protein banding patterns often referred to as electrophoretograms. These protein banding patterns can be considered 'finger prints' of the bacterial strains under investigation. The early work of Kersters and De Ley (1975) applied numerical taxonomic analysis and demonstrated that bacterial strains that were closely related displayed similar or almost identical protein banding patterns. On the basis of this finding the technique has been widely used in taxonomic and identification studies.

Before a large taxonomic study of a group of organisms is embarked upon it is worth considering the drawbacks of this particular taxonomic or identification technique. One-dimensional protein patterns have a similar level of taxonomic differentiation to DNA-DNA hybridization data, and therefore are most useful for discriminating between strains at the species level. This has been demonstrated in a large number of taxonomic groups. Jackman (1987) indicates that the technique is not normally useful for the study of taxonomic relationships at the genus level and so some preselection of extremely diverse groups of organisms is required. At the other end of the taxonomic ladder very closely related organisms eg. serovars, are not well discriminated by the technique, and in such cases 2-D techniques have been applied more successfully. It is essential to investigate the degree of diversity within a group of isolates using some classical techniques before starting an electrophoresis study.

5.1.2 METHODS

The electrophoresis of bacterial proteins has been carried out in one of the following ways or by using a modification of these methods:

1. Solubilized proteins from cell-free extract or from total cell mass have been submitted to electrophoresis in polyacrylamide gels. These gels have been stained before the complete banding patterns are examined and compared with the patterns of other strains. A miniaturized, rapid, standardized method useful in the comparison and identification of bacterial strains is described in section 5.3.

2. Native proteins, usually from cell-free extracts have been separated by electrophoresis in a non-denaturing system and stained for specific enzyme activities. The result is a zymogram from which comparisons of specific enzymes on the basis of their electrophoretic mobilities can be made.

3. Radiolabelled proteins from total cell mass have been separated by electrophoresis and after autoradiography the pattern and relative quantities of labelled proteins analyzed. A fully automated system based on this method has been developed and marketed for taxonomic and identification studies; it will be described in section 5.2.

Jackman (1985) proposed that an electrophoresis method based on that of Laemmli (1970) was the most suitable for use as a standard 1D technique in taxonomic studies. The culture medium used to grow the organisms under investigation should support good growth in all cases and the culture conditions should be kept constant within a study and between studies if they are to be compared. The majority of organisms can be lysed by boiling in SDS buffer but others have been found to require lysozyme treatment or mechanical disruption. The method should however be kept constant for all organisms in a particular study. Soluble proteins are separated by SDS-PAGE using a 10mm long 5% stacking gel and 100mm long 10% separation gel for 3-4 hours at constant current of 30mA. Gels are stained overnight in a stirred Coomassie brilliant blue solution before destaining and drying. Analysis of the protein banding patterns can be carried out visually, noting differences between strains, or scanned using a scanning densitometer linked to a computer. Numerical taxonomic analysis can be applied to densitometer traces, similarities calculated and cluster patterns produced.

5.1.3 USE OF PAGE IN TAXONOMIC STUDIES

The numerical analysis of whole-cell protein patterns has been applied to a wide range of microorganisms, examples include *Yersinia pestis* (Hudson et al. 1976), *Zymomonas* species (De Ley and Swings 1976), *Clostridium* species (Cato et al. 1982), *Corynebacterium* (Carlson and Vidaver 1982), *Streptobacillus moniliformis* (Costas and Owen 1987), *Streptomyces* (Manchester et al. 1990), *Campylobacter* species (Vandamme et al. 1990) and *Acidovorax* species (Willems et al. 1992). Simple visual comparisons of protein banding patterns are now widely used in description of new isolates or groups of isolates. This is demonstrated by the fact that in the first issue of the International Journal of Systematic Bacteriology of 1992 there are three papers in which protein banding patterns are used as evidence in the description of new species or in reclassifying particular organisms (Busse et al. 1992; Pot et al. 1992; Willems et al. 1992). One example of protein banding pattern analysis in the description of a thermophilic bacterium is *Clostridium thermolacticum* where the profile of the new isolate was compared with other thermophilic clostridial species (Le Ruyet et al. 1985).

5.1.4 COMPARISON WITH OTHER METHODS

5.1.4.1 Comparison with numerical taxonomy based on classical tests

One of the main advantages of a numerical taxonomy based on whole-cell protein patterns over one based on classical tests is that in the former case all the data is obtained at one time from a single 'test'. The fact that the results of this single 'test' can be read automatically is an additional advantage as it reduces the error involved in reading large numbers of tests. The ability to adjust the level of discrimination by selecting tests to match the level of variation found in the group of organisms being studied is an advantage of taxonomies based on conventional tests. The level of discrimination in studies based on whole-cell protein analysis is fixed and this may be too high or too low for some studies.

5.1.4.2 Comparison with DNA-DNA hybridization

Many examples can be found in the literature where the taxonomy resulting from whole-cell protein analysis has been compared with that resulting from DNA-DNA hybridization studies on the same set of isolates (eg. Kersters and De Ley 1975; Cato et al. 1982; Dicks et al. 1990; Dijkshoorn et al. 1990). In a study of the protein profiles of

Acinetobacter by Dijkshoorn et al. (1990) many of the DNA groups were found to correspond to the electrophoretic clusters. It was suggested that the whole-cell protein method may be useful in routine identification as it had the advantage of providing rapid results and was more easily accomplished than DNA-DNA hybridization. The study of the *Clostridium* species by Cato et al. (1982) found that generally, strains with greater than 80% DNA-DNA homology produced identical protein patterns while major differences were observed in the protein patterns of strains unrelated by DNA-DNA homology. It was also noted that strains with similar phenotypic properties were often more similar than those with dissimilar phenotypic properties. One disadvantage of both whole-cell protein analysis and DNA-DNA hybridization is that both methods require cell breakage which in some cases can be difficult.

5.2 THE USE OF THE AUTOMATED MICROBIOLOGY IDENTIFICATION SYSTEM (AMBIS) TO STUDY THE DEGREE OF DIVERSITY AMONG ANAEROBIC, CELLULOLYTIC, THERMOPHILIC BACTERIA

5.2.1 INTRODUCTION

An alternative approach to the staining of whole-cell protein patterns is the radiolabelling of the proteins and detection of the protein banding patterns by autoradiography. This method was applied by Howard et al. (1981) in the differentiation of serovars of *Ureaplasma ureolyticum* of human and bovine origin. Extensive studies comparing the protein banding patterns of ³⁵S-methionine labelled proteins of a wide range of medically important microorganisms were carried out by Tabaqchali (1985), Tabaqchali et al. (1984; 1986), Stephenson and Tabaqchali (1986), and Stephenson et al. (1986). The radiolabelled protein patterns were found to be significantly different between species and these differences could be easily identified. The usefulness of the method in differentiating below the species level was also demonstrated (Tabaqchali et al. 1986; Stephenson and Tabaqchali 1986; Stephenson et al. 1986). Although sensitive, the autoradiography approach is slow, requiring one to five days depending on the signal strength. This problem was overcome by the design of a scanner to detect beta emission (Tabaqchali et al. 1987), an instrument which was later patented by Automated Microbiology Systems (AMBIS), (San Diego, USA). AMBIS financed the development of a complete system for the automated pattern matching of proteins labelled with ³⁵S-methionine. Due to the expense of the AMBIS it is as yet not widely used in routine

laboratories. A recent study by Rowe and Finn (1991) demonstrated the use of the AMBIS in differentiating biovars of *Pseudomonas fluorescens*. The opportunity to use an AMBIS was provided at the Agriculture and Food Microbiology Division, The Department of Agriculture, Northern Ireland, but due to time constraints only a limited number of isolates could be analysed. An outline of the AMBIS and the results of the study carried out are presented below.

5.2.2 THE AUTOMATED SYSTEM

The system consists of three main components: a computer/VDU system, an automated electrophoresis unit and a scanner. The electrophoresis unit and the scanner are controlled by an IBM pc/AT computer with a high quality graphics screen. The computer also collects, stores, and manipulates the scan data. A flat bed computer controlled electrophoresis unit with special sensors that automatically terminate the end of a run is used for the separation step. Precast gels are used to avoid interlaboratory variations in preparation. A wide range of data analysis features are available in the software package including molecular weight calibration, pattern recognition against a data base of known isolates, selective analysis of protein banding pattern, and cluster analysis.

5.2.3 METHODS AND MATERIALS

5.2.3.1 Bacterial strains

Strains were selected on the basis of the results of the numerical taxonomy study described in chapter 4. Isolates from the phenons A-D of the numerical taxonomy study were included along with two clostridial type strains. The isolates included were Z-1320, Tp8T. 6.3.3.1, Wai 35. B1, Rt8. B4, Rt8. B6, Rt8. B8. AZ3. B1, Ok8. B1, Rt613. B1, *Clostridium stercorarium*, and *Clostridium thermocopriae*. The source, source temperature and pH of these strains are given in Table 4.1 (chapter 4).

5.2.3.2 Incorporation of ³⁵S-methionine into bacterial proteins and sample preparation

The isolates were grown overnight in 2/1 cellobiose medium as described in section 4.2.2.2 at 65°C. The methionine free-medium for the labelling step consisted of 2/1

cellobiose medium without the trypticase and yeast extract and with an eighteen amino acid solution added. The final concentration of each amino acid was 0.01g/l. To 50 μ l of methionine-free medium in a 0.5ml eppendorf tube was added 1 μ l overnight culture and 1 μ l L-³⁵S-methionine (10 μ Ci) (Amersham International plc, UK) and overlaid with 80 μ l sterile liquid paraffin. The manipulation of cultures and labelling assay was carried out in an anaerobic chamber (Coy Ltd, USA). The cultures were incubated with the labelled methionine for 12h at 65°C.

After incubation the liquid paraffin was removed and an equal volume (50 μ l) of lysis buffer containing 0.125mol/l Tris-HCl (pH 6.8), 10% v/v 2-mercaptoethanol, 4% w/v sodium dodecyl sulphate, 20% v/v glycerol and 0.002% w/v bromophenol blue was added to the culture and the mixture boiled for 2min.

5.2.3.3 Electrophoresis and analysis of the profiles

SDS-polyacrylamide gel electrophoresis (SDS PAGE) was carried out as described by Tabaqchali et al. (1987). The cell lysate (8 μ l) was loaded into pre-cast 12% w/v polyacrylamide gels (AMBIS Systems, San Diego) using an eighteen well applicator strip. Electrophoresis was carried out at 8°C and the gel was run at 20W constant power for 20min followed by a resolving step of 60W constant power for 120min. Prior to scanning gels were dried for 1h on an integral scanning unit.

The scan data were visualized on the VDU screen in the form of an image resembling an autoradiogram. The lanes were normalised against a reference strain (strain Tp8T. 6.3.3.1) so eliminating any variations across the gel introduced by the electrophoresis step. The normalized profiles were compared using the Pearson Product Moment (PPM) correlation coefficient. The correlation coefficient data was subjected to cluster analysis using the UPGMA to produce a dendrogram showing the similarity between the different strains. A autoradiogram can be produced from the dried gel and is useful for making visual comparisons of the protein bandings patterns of the strains tested. The autoradiogram from this study is shown in Figure 5.1.

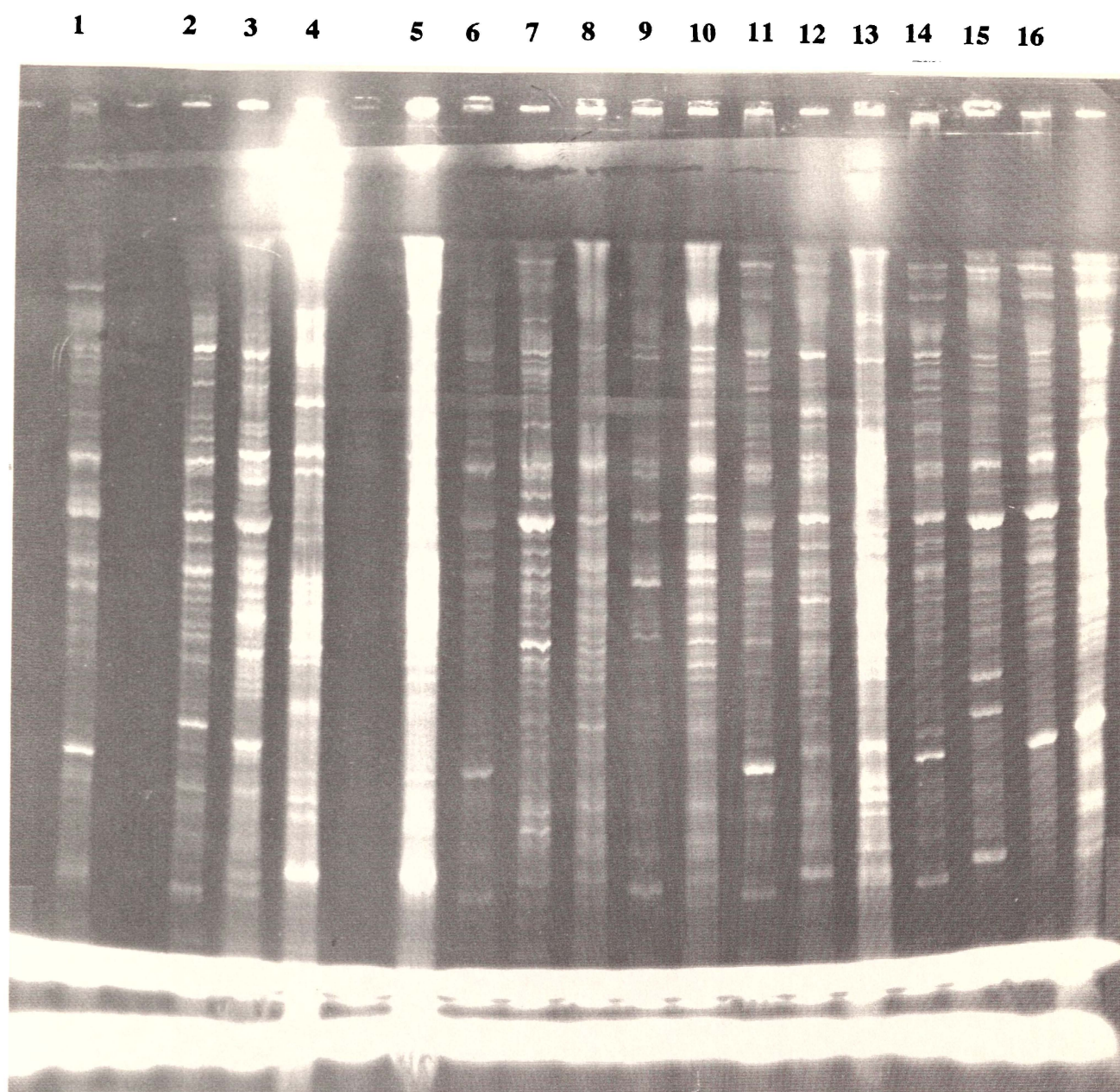


Figure 5.1 The autoradiogram produced from the AMBIS gel. **Lane 1.** Rt8. B11. **Lane 2.** Ok8. B1. **Lane 3.** Rt613. B2. **Lane 4.** *C. thermocopriae*. **Lane 5.** *C. stercorarium*. **Lane 6.** AZ3. B1. **Lane 7.** AZ2. B1. **Lane 8.** Rt8. B2. **Lane 9.** Rt8. B8. **Lane 10.** Tp8T. 6.3.3.1. **Lane 11.** Rt8. B4. **Lane 12.** Z-1230. **Lane 13.** Wai35. B1. **Lane 14.** Rt8. B6. **Lane 15.** NA10. **Lane 16.** Rt8. B10.

5.2.4 RESULTS AND DISCUSSION

The dendrogram produced from the study of a limited number of anaerobic, cellulolytic thermophiles is shown in Figure 5.2. The cluster designations from the numerical taxonomy data are listed alongside the strains to allow a comparison to be made. Before interpreting the results obtained, the level of discrimination involved in the AMBIS technique should be considered. The only data showing the level of discrimination is that of Rowe and Finn (1991). Their study of the protein banding patterns of *Pseudomonas fluorescens* biovars demonstrated that duplicate runs could differ by as much as 12% in respect to similarity values obtained, this is below the value of 20% recommended by Jackman (1985). The level of similarity between different biovars of *P. fluorescens* was as low as 63%. Since no data was presented showing the level of similarity between other *Pseudomonas* species, the potential use of AMBIS in differentiation at the species level cannot be determined.

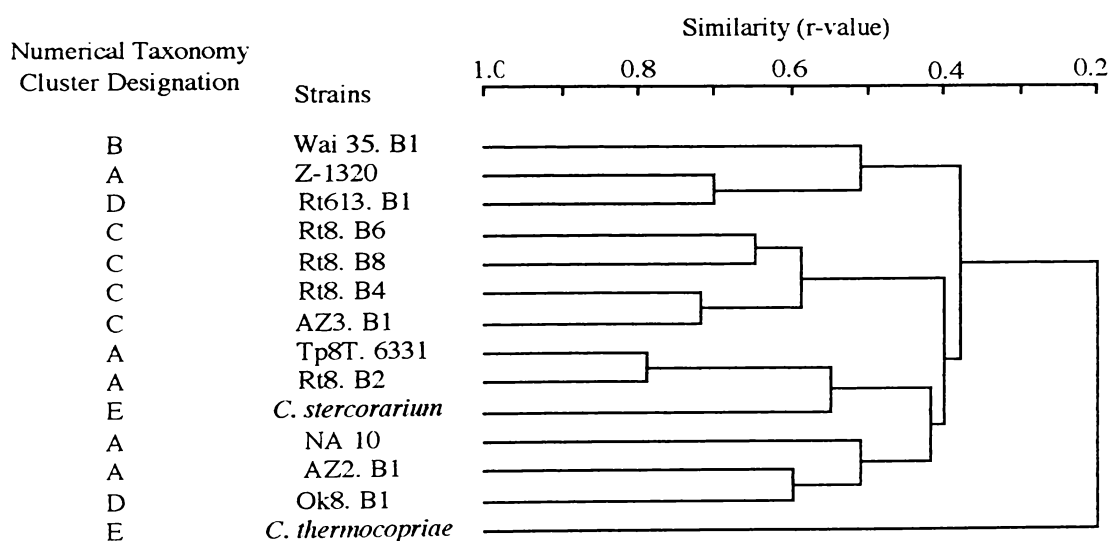


Figure 5.2 Dendrogram showing similarity of protein profiles of the isolates tested, the cluster designation of these isolates in the numerical taxonomy study is also shown.

Due to the limited time scale of this study it was not possible to run large numbers of duplicates of the strains under investigation. Duplicates of strain Tp8T. 6.3.3.1 gave a similarity of 90%. From the dendrogram (Figure 5.2) it can be seen that the levels of similarity between any of the isolates are less than 80%. Those organisms of cluster group C in the numerical taxonomy are also found to cluster together in this protein banding pattern study. It is interesting to note that strains Tp8T. 6.3.3.1 and Rt8. B2

cluster at similar levels in both studies. The differences found between this study and the numerical taxonomy study cannot be explained fully but the fact that two different similarity coefficients were used in the calculation of similarity may contribute to the differences observed. Both methods have however demonstrated that the isolates of cluster C are closely related, and using the information of Rowe and Finn (1991) it could be considered that these organisms represent one species.

5.3 THE USE OF THE *PHASTSYSTEM* TO DIFFERENTIATE BETWEEN ANAEROBIC, CELLULOLYTIC, THERMOPHILIC BACTERIA

5.3.1 INTRODUCTION

From the methods described in section 5.2.1, it is clear that some of the steps involved in SDS-PAGE are time consuming and open to operator-introduced errors which may affect the reproducibility. The *PhastSystem* marketed by Pharmacia LKB Biotechnology AB (Uppsala, Sweden) claims to give 'reproducible, high resolution, publication-quality results quickly, and with a minimum of effort'. The *PhastSystem* consists of a separation unit and a development unit and uses pre-cast gels (3.5x4.0 cm). The high degree of reproducibility claimed by the manufacturer is due to the strict control of separation temperatures, sample application, power settings, and staining parameters. The total process for SDS-PAGE on a *PhastGel* gradient 10-15, including staining, is less than 90 min. The *PhastSystem* was used to compare the protein banding patterns of a selection of anaerobic, cellulolytic, thermophilic isolates which had previously been studied using classical numerical techniques as described in chapter 4. The protein banding patterns were visually compared and a group of bands shown to be useful in the identification of one group within the isolates investigated.

5.3.2 METHODS AND MATERIALS

5.3.2.1 Bacterial strains and culture conditions

The strains used in this study are listed in Table 5.1. The isolates were grown on 2/1 cellobiose medium as described in section 4.2.2.2. Cultures were incubated for 18h at the optimum growth temperature, 65°C or 70°C, depending on the particular strain.

To determine the degree of reproducibility of the system a duplicate culture, the identity of which was unknown to the operator, was included.

5.3.2.2 Sample preparation

The cells were recovered by centrifugation at 5, 000 rpm for 15min, before washing with fresh 2/1 cellobiose medium. The cell suspension was diluted to a OD_{650} 1.0. To the cells recovered from 50 μ l of this cell suspension was added 150 μ l of lysis buffer (150mM Tris-HCl (pH6.8), 10% v/v 2-mercaptoethanol, 4% w/v SDS, 20% v/v glycerol and 0.002% bromophenol blue) and the mixture boiled for 10 min before cooling on ice. The size range of the proteins in the protein banding patterns were determined using molecular weight markers in the range 10-180 kD (Pharmacia LKB, Sweden and Sigma, USA).

5.3.2.3 Electrophoresis and analysis of the profiles

The samples were applied to the *PhastGel* gradient 10-15% using the *PhastGel* sample applicator 8/1 which applies eight 1 μ l samples automatically and simultaneously when the separation program is started. The electrophoresis was carried out in the separation unit as recommended by the manufacturers for 65Vh. After separation the *PhastGel* was placed in the development unit and stained using the *PhastGel Blue R* Coomassie blue staining procedure. The gel was then automatically destained using methanol:acetic acid:water (50:20:30). After drying the gel was viewed and photographed using FP4 film (Ilford, UK).

All samples were run in duplicate and the protein extract of strain Rt8. B6 was run on all gels as a standard. The protein banding pattern of Rt8. B6 was used to set the start of the profiles for the calculation of R_f values, the solvent front was detected as the marker dye. The typical protein banding patterns obtained are shown in Figure 5.3 and the distribution of specific protein bands for each strain is shown in Table 5.1.

5.3.3 RESULTS AND DISCUSSION

The initial aim of this study was to produce protein banding patterns using the automated *PhastSystem*, which was available in our laboratory, scan the gels and carry out numerical analysis of the data. Obtaining the required software for a single study was

outside the budget of this project and so the protein banding patterns were visually analysed.

The protein banding patterns obtained contained numerous protein bands ranging in size from greater than 180kD to 10kD. It was clear to see that in some regions of the protein banding pattern it was not possible to differentiate between the individual protein bands, while in other regions individual bands could be seen clearly. After looking at the overall protein banding patterns of the various strains and calculating the R_f values for certain bands, a set of bands were selected and their presence or absence recorded for each strain tested. The results are shown in Table 5.1.

By comparing the bands present in the various strains it was found that some protein bands are specific to particular groups of organisms. These groupings were found to correspond in most cases to the groupings obtained in the numerical taxonomy study described in chapter 4. The organisms of clusters A, B, E had protein bands unique to the strains within those groups. The organisms of clusters C and D in the numerical taxonomy were found to have three high molecular weight proteins which are not found in the strains of other groups. It is interesting to note that the cluster A strains do not have a homologous banding pattern within the overall cluster, but that strains 2805-34 and NA10 which were part of a subcluster within cluster A have the same protein bands in this study. Using this method of differentiation the organisms of clusters C and D are found to have the same proteins within their banding pattern. It should be considered that there may be other proteins within the protein banding patterns of these organisms which were not considered in this set of results that may differentiate between the strains of clusters C and D. The duplicate culture was identified as a member of the cluster group C by this method and was revealed to the operator after the results had been analysed to be a duplicate of strain Rt8. B8.



Rt8. B6 Cluster C isolate

Tp8T 6.3.3.1 Cluster A isolate

Wai 35. B1 Cluster B isolate

Figure 5.3 Typical protein banding patterns of representatives of three of the clusters as defined in the numerical taxonomy study (section 4.2)

Table 5.1 Distribution of protein bands within the protein banding patterns of some isolates.

Strain ^a	Cluster ^b	Protein bands (R _f value)											
		0.02	0.04	0.05	0.11	0.12	0.13	0.15	0.46	0.47	0.50	0.52	0.53
AZ2. B1	A	-	-	-	+	-	-	-	+	+	+	-	+
COMP. B4	A	-	-	-	-	-	-	+	+	+	+	-	+
Tp8T. 6.3.3.1	A	-	-	-	+	-	-	-	+	+	+	-	+
Rt8. B2	A	-	-	-	+	-	-	-	+	+	+	-	+
2805-34	A	-	-	-	-	-	+	-	+	+	+	-	+
NA10	A	-	-	-	-	-	+	-	+	+	+	-	+
Tp8T2.3	B	-	-	-	-	-	-	+	+	+	+	-	+
Tp11. W1	B	-	-	-	-	-	-	+	+	+	+	-	+
Wai35. B1	B	-	-	-	-	-	-	+	+	+	+	-	+
RI1. B1	B	-	-	-	-	-	-	+	+	+	+	-	+
Rt8. B4	C	+	+	+	-	-	+	-	+	+	+	-	+
Rt8. B6	C	+	+	+	-	-	+	-	+	+	+	-	+
Rt8. B8	C	+	+	+	-	-	+	-	+	+	+	-	+
Rt8. B15	D	+	+	+	-	-	+	-	+	+	+	-	+
RtUK4	D	+	+	+	-	-	+	-	+	+	+	-	+
Ok9. B1	D	+	+	+	-	-	+	-	+	+	+	-	+
Wai28. B1	D	+	+	+	-	-	+	-	+	+	+	-	+
Rt426. B1	D	+	+	+	-	-	+	-	+	+	+	-	+
Ok8. B1	D	+	+	+	-	-	+	-	+	+	+	-	+
Rt51. B1	E	-	-	-	-	+	-	-	+	+	-	+	+
<i>C.stercorarium</i>	E	-	-	-	-	+	-	-	+	+	-	+	+
<i>C.thermolacticum</i>	E	-	-	-	-	+	-	-	+	+	-	+	+
<i>C.thermocopriae</i>	E	-	-	-	-	+	-	-	+	+	-	+	+
<i>C.thermocellum</i>		-	-	-	-	+	-	-	+	+	-	+	+

^aStrain abbreviations as in chapter 4. ^bClusters as determined by the numerical taxonomy study described in chapter 4.

5.4 CONCLUSION

This study demonstrated the use of semi-automated electrophoresis of cell proteins for determination of diversity within a group of environmental isolates. The limited study using the AMBIS procedure confirmed the finding that the isolates of cluster C (formed in the numerical taxonomy study of chapter 4) are closely related. The AMBIS system would be useful for building up a data base of protein banding patterns for a wide range of organisms. It could be used for the determination of differences at the subspecies level but its value for differentiation at the species or genus level has as yet not been determined. Although automated, the AMBIS procedure still requires two cultivation steps before the radiolabelled proteins can be separated by electrophoresis, followed by drying and scanning of the gel. The *PhastSystem*, also automated at the electrophoresis step, gives the protein banding pattern in a much shorter time than the AMBIS system but its suitability for scanning and numerical analysis has not been determined. Visual analysis of the protein banding patterns from the *PhastSystem* gels was shown to be useful in differentiating between isolates. By selecting bands that are both similar to all isolates and bands unique to certain isolates, it was possible to design an identification scheme for grouping the isolates. The groupings were generally found to correspond with clusters formed in the numerical taxonomy study described in chapter 4. The method was also shown to be applicable to the identification of subclusters. The controlled conditions and pre-cast gels make the system very reproducible as was found in this study. This simple and rapid method has been shown to be useful in determining the degree of diversity within a group of isolates. It may also be a valuable tool in assigning isolates to groups defined on the basis of numerical taxonomy data.

CHAPTER 6

PHYLOGENETIC ANALYSIS BASED ON 16S rDNA SEQUENCE DATA OF ANAEROBIC CELLULOLYTIC THERMOPHILIC ROD-SHAPED BACTERIA

INTRODUCTION

The results of the numerical taxonomy study described in chapter 4 gave some indication that the rod-shaped, anaerobic, cellulolytic thermophiles studied constituted a heterogeneous group. The majority of the isolates did not cluster with the clostridial-type strains. Due to the nature of the numerical taxonomy study and the limited conclusions that could be made from it, a phylogenetic study of selected isolates was undertaken.

This chapter is divided into two sections. Section 6.1 describes the principles and methods used in phylogenetic analysis, while section 6.2 describes the phylogenetic study and the results obtained.

6.1 A REVIEW OF THE PRINCIPLES AND METHODS OF PHYLOGENETIC CLASSIFICATION

6.1.1 INTRODUCTION

As discussed in section 2.7.1, microbial classifications can be of two types, phenetic and phylogenetic. The phenetic classifications are derived from similarities and differences in phenotypic characteristics; such classifications do not provide information on the evolutionary pathway but only show the end product of the process. The evolutionary relationships between organisms are expressed in phylogenetic classifications. In addition, such classifications reflect the degree of change in evolutionary lines. The first classifications of microorganisms were based on phenotypic characteristics and the taxonomic structures proposed the result of numerical analysis of this data. It later became clear that reliable phylogenetic classifications were not possible using numerical analysis of phenotypic characteristics. The introduction of molecular methods of analysis such as rRNA sequencing techniques was a major breakthrough in determining the evolution and phylogeny of microorganisms.

6.1.2 RESTRUCTURING SYSTEMATICS; THE DOMAINS

Since it was realised that molecular structures and sequences give a better insight into the evolutionary relationships between organisms than the classical phenetic methods, the basis for the definition of taxa has moved from the level of the whole organism via the cellular level to its current position at the molecular level. Molecular comparisons had shown that living things can be divided into three primary groupings known as the eubacteria, the archaebacteria, and the eukaryotes (Woese et al. 1985: 1987). Such comparisons revealed that the evolutionary differences among eubacteria, archaebacteria and eukaryotes are greater than those used to distinguish traditional kingdoms such as animals and plants. Woese et al. (1990) added a new taxonomic rank at the top of the hierarchy, the 'domain'. For the eubacteria, the formal name *Bacteria* was suggested. The term *Eucarya* was proposed for the eukaryotes, reflecting the group's cytological characteristic of having well-defined encapsulated nuclei. The term *Archaea*, proposed for the archaebacteria, indicates their apparent primitive nature. At the same time, the domain *Archaea* was divided into two kingdoms, namely *Euryarchaeota* and *Crenarchaeota*. The three groupings previously used ie. archaebacteria, eubacteria and eukaryotes had been defined on the basis of relatively few small subunit rRNA

sequences. Recently the domains *Bacteria*, *Archaea* and *Eucarya* have been defined in terms of the characteristics of a greater number and range of small subunit rRNA made available through the ribosomal RNA database project, University of Illinois (Winker and Woese 1991).

6.1.3 CHRONOMETERS USED IN THE DETERMINATION OF PHYLOGENETIC RELATIONSHIPS

6.1.3.1 Molecular chronometers

The idea of using molecular chronometers to determine phylogenetic relationships was proposed in detail by Zuckerkandl and Pauling (1965), yet nearly a decade passed before microbial taxonomists tested out their ideas. A chronometer is any molecule whose sequence changes randomly in time. The amount of change that a chronometer accumulates is a product of the rate at which mutations occur and the time period over which the changes have occurred. The comparison of some original state to some final state would be a measure of this change, but this is not possible since the original state is not available. To overcome this problem, the fact that two (or more) versions of a given sequence occurring in existing representatives of two or more lineages have initially come from the same common ancestral pattern has been applied. Thus, by measuring the sequence difference between the two or more existing versions, which is approximately twice the amount of change occurring in each of the lineages (assuming similar rates of change), the evolutionary distance can be determined.

Woese (1987) outlined the criteria of a useful chronometer based on certain specifications with respect to '(i) clockwise behavior (changes in its sequence have to occur as randomly as possible), (ii) range (rates of change have to be commensurate with the spectrum of evolutionary distances being measured), (iii) size (the molecule has to be large enough to provide an adequate amount of information.' Molecules that have been used as chronometers include cytochrome *c*, ferredoxin, ribosomal RNAs, β -subunit of the ATPase (*atpD* gene) and the *tuf* gene (Ludwig 1991). Those most extensively studied are cytochrome *c* and the rRNAs. Molecular chronometers should measure, and be representative of, the overall rate of evolutionary change in a line of descent. The more useful molecules for use in phylogenetic analysis are those representing highly constrained functions. In some cases these sequences change slowly enough to span the full evolutionary spectrum.

Cytochrome *c* was the first molecule used in phylogenetic measurements and was found to be useful in the phylogenetic analysis of eukaryotes (Zuckerkindl and Pauling 1965; Fitch and Margoliash 1967). It was found to be less useful in the study of bacterial phylogeny because of the enormous scope of bacterial evolution. In bacterial phylogeny its effective range is restricted to the level of subdivision, since in the case of the α -purple bacteria the molecule changes in size and thus distorts the true phylogenetic situation (Amber et al. 1979). This is an example of a molecular chronometer that is too fast to be used accurately in bacterial phylogeny. Additionally, cytochrome *c* does not remain functionally constrained across the evolutionary distances found among the bacteria, and is not found in all bacterial groups.

The 5S rRNA sequence has been used as a molecular chronometer in bacterial phylogeny studies (eg. Woese et al 1976; Hori and Osawa 1979; Kuntzel 1982; MacDonell et al. 1986; Dams et al. 1987). This molecule is small in size and comprises only 4-5 separate double helical elements. Woese et al. (1976) showed several examples for the 5S rRNA in which one of these regions was found to have changed in size, introducing non-clock-like changes into the sequence which results in a distorted phylogeny with overestimated phylogenetic distances. For large molecules like the 16S with about fifty helical regions (Woese et al. 1980), a change in size or structure of one such region would not have such a drastic effect on the phylogenetic analysis as in the case of the 5S rRNA.

6.1.3.2 Ribosomal RNAs as useful molecular chronometers

Woese (1987) wrote 'rRNAs are at present the most useful and most used of the molecular chronometers', five years later this statement is still valid. As essential components of the protein synthesis machinery, rRNAs are found universally in cellular life forms and are therefore useful in studies of microorganisms (with the exception of viruses). The two large rRNAs, the 16S rRNA and the 23S rRNA, are the most useful since they are extremely constant in function, large in size, universal in distribution, and moderately to highly conserved in sequence across extensive distances (Woese 1982). The large size of these molecules means that they constitute a larger number of functionally defined subunits or domains (50 in the case of 16S rRNA and about 100 in 23S rRNA) than the smaller molecules. The phylogenetic categories defined using the larger molecules appear to be more consistent than those defined using the 5S rRNA (Woese et al. 1985).

One important characteristic of rRNA as a chronometer is that even though some changes in rRNA would seem to have a neutral effect, it is the combination of changes that results in the viable lines of descent. Woese (1985) used the following example to demonstrate this: 'it is unlikely that a change in one member of a base pair in a helix is a selectively neutral event; however altering both members of a pair thus retaining pairing can be'. Another characteristic of this chronometer is the frequency of changes which can vary by at least two orders of magnitude at different positions in the molecule. Positions are found to change from species to species within a genus, and while other positions show little difference across an entire primary kingdom, some appear not to vary at all (Woese et al. 1983). This characteristic makes the rRNA a useful chronometer for measuring the full range of phylogenetic relationships. The great disparity in the rates at which mutations become fixed in different parts of the molecule is another characteristic of the rRNA chronometer. From this it can be considered that the rRNA chronometer is a clock with many hands, but unlike a conventional clock, when this clock speeds up (due to increased mutation rate in a particular line of descent) the other hands of the clock do not speed up at the same rate but rather speed proportionally. This characteristic improves the determination of branching orders since relative clock speeds within groups of organisms can be determined intrinsically.

6.1.4 THE METHODS APPLIED TO 16S RIBOSOMAL RNA IN THE DETERMINATION OF PHYLOGENETIC RELATIONSHIPS

6.1.4.1 The 16S rRNA molecule

The rRNA molecule most characterized for phylogenetic analysis to date is the 16S rRNA subunit. Comparative analysis has shown the rRNA molecules to have a highly conserved secondary structure. The primary structure is composed of a number of domains, the primary nucleotide sequence of which is found to vary. Near universal conservation is found in some domains, and this can be used to advantage in the design of sequencing primers, and in the designation of hybridization probes for 16S rRNAs of all organisms for use in techniques such as ribotyping. In the majority of the molecule, primary sequence differences occur but the secondary structure remains conserved. These regions may contain domains that are conserved in specific phylogenetic groups and can be used in sequencing or for probing groups of related organisms. Highly variable regions are also found, these can be used in the design of oligonucleotide probes for identification of specific microorganisms.

6.1.4.2 Oligonucleotide cataloging

Oligonucleotide cataloging was the first method applied to the measurement of phylogenetic relationships by 16S rRNA in the late 1970s and early 1980s (Fox et al. 1977; Fox et al. 1980; Stackebrandt et al. 1981). The original method as described by Uchida et al. (1974) involved the addition of $^{32}\text{PO}_4$ to cultures in the exponential phase of growth followed by the isolation of the labelled 16S rRNA. This labelled RNA was then digested with T1 ribonuclease to produce a set of oligonucleotides usually up to 15-20 bases in length, each ending in a G residue and preceded by a number of non-G residues. These oligonucleotides were then separated by two-dimensional paper electrophoresis. The individual oligonucleotides were then sequenced by a combination of endonuclease digestion steps to produce a catalog of sequences that is characteristic of the organism under investigation. The alternative method described by Stackebrandt et al. (1980) involved digestion of unlabelled 16S rRNA with T1 ribonuclease, removal of the terminal phosphates and enzymatic addition of a ^{32}P labelled phosphate on the 5' end of each oligonucleotide, followed by separation and sequencing. The resulting catalogs were analysed by a binary method in which each catalog was compared individually to every other catalog and the oligonucleotides of six residues or larger common to any two catalogs are scored to produce a S_{AB} value characteristic of that pair of organisms (Fox et al. 1977). The S_{AB} values ranged from 1.0 for identical RNAs to about 0.03 for randomly related sequences. To these S_{AB} values standard clustering methods were applied and dendrograms produced.

The data provided by oligonucleotide cataloging laid the basis for bacterial phylogeny and was used to define most of the major lines of descent. In general the technique failed to resolve the branching orders within the lines of descent and their subdivisions. Oligonucleotide cataloging was replaced by full sequencing of 16S rRNA, a more powerful tool in phylogenetic analysis.

6.1.4.3 Reverse transcriptase sequencing of 16S rRNA

The reverse transcriptase sequencing protocol evolved from the dideoxynucleotide chain-terminating, copying method of Sanger et al. (1977). Lane et al. (1985) described a protocol and a series of universally conserved primers that simplified the Sanger method. The use of low salt buffers and higher temperatures to overcome the ambiguities sometimes associated with 'N-banding' or 'pausing' in Klenow sequencing was suggested by Ornstein and Kashdan (1985). These concepts were applied by Lane

et al. (1988) and Shimomaye and Salvato (1989) to reverse transcriptase sequencing of rRNA.

Reverse transcriptase sequencing involves the annealing of an oligonucleotide to the template nucleic acid under conditions of stringency that facilitate its binding at a particular location. This oligonucleotide serves as a primer for the synthesis of a DNA copy of the template extending from the 3' end of the primer which becomes incorporated into the reverse transcript. By including low levels of one dideoxynucleotide triphosphate in the reaction, random terminations of reverse transcription occur at positions corresponding to the nucleotide complement of that dideoxynucleotide in the template strand. Four separate reactions are run, each containing one of the four dideoxynucleotide triphosphates. Lane (1991) notes that, in general, high stringency conditions are applied and this results in 'clean sequence' with respect to its readability. Lower stringency conditions can be useful in situations where a particular primer does not completely complement the target site. After electrophoresis and autoradiography of the reverse transcriptase reactions, the sequence data can be recorded in a suitable data base before analysis. The reverse transcriptase technique has been widely used in the last six years to determine the 16S rRNA sequence of numerous microorganisms. One of the limitations of the technique is the requirement to isolate RNA, which requires a 500ml cell culture and numerous large volume purification steps. The introduction of the polymerase chain reaction (PCR) and the direct sequencing of the PCR products (Gyllenstein 1989; Hultman et al. 1989) has already relegated reverse transcriptase sequencing to a secondary position.

6.1.4.4 Polymerase chain reaction (PCR) and direct sequencing of the PCR products

The polymerase chain reaction (PCR) first described by Saiki et al. (1985) provides a rapid means to obtain multiple copies of specific DNA regions. Target regions up to 10 kilobases have been amplified (Jeffreys et al. 1988), which is within the size range of the 16S rDNA fragment. The fact that sequence information is required about the target region to be amplified makes PCR useful in the amplification of rRNA genes about which much is known.

The PCR reaction is composed of three steps which are repeated in multiple cycles: (i) the double stranded DNA sample is melted, (ii) two primers are annealed to opposite DNA strands and (iii) the primers are enzymatically extended by the addition of nucleotides to produce a copy of the targeted gene. It is the oligonucleotide primers that

introduce specificity to PCR and the repetition of the cycles that produces the multiple copies of the targeted gene. As the number of copies increases exponentially, with 30–40 cycles it is theoretically possible to amplify the target a million-fold in a few hours. The enzyme involved in the addition of nucleotides to the primers is a DNA polymerase. In the first PCR experiments, the Klenow fragment of DNA polymerase I from *E. coli* was used (Saiki et al. 1985). As this enzyme was irreversibly denatured at 94°C, fresh enzyme had to be added for each PCR cycle, making the overall process extremely tedious. These problems were overcome by the use of the thermostable DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) (Saiki et al. 1988). The use of the thermostable polymerase not only simplified the PCR procedure but allowed the introduction of high temperature annealing steps and, more importantly, allowed automation of the process. The methodology of a typical PCR process for the amplification of 16S rDNA is described below.

The *Taq* polymerase possesses no 3' exonuclease activity and this is thought to contribute to the high processing rate of the enzyme (Giovannoni 1991) but a drawback of this is the misincorporation of nucleotides due to reading errors. Such errors are a problem when PCR products are to be sequenced and the information used in phylogenetic analyses. Various rates of misincorporation have been described in the literature, for example 1 in 4000–5000 base pairs after 35 cycles (Innes et al. 1988). A much higher rate of 0.3% was reported by Dunning et al. (1988). The effect that such errors could have on microbial systematic studies is open to speculation. Another problem with PCR is contamination, this has been studied by various workers (see Kwok and Higuchi 1989). The high sensitivity of PCR allows the detection of single gene copies, therefore the slightest contamination of reagents or materials could result in the amplification of the 16S rDNA of the contaminant or the generation of a PCR product containing a mixture of products. To reduce the possibility of contamination, sterile working practices should be used and control reactions set up routinely.

In sequencing of double stranded DNA the oligonucleotide primer is annealed to the denatured template DNA. The complementary DNA strand is synthesized *in vitro* by elongation of the primer using a DNA polymerase. The incorporation of a nucleotide analogue (2',3'-dideoxynucleoside 5'-triphosphate) that does not support continued DNA synthesis (due to it lacking a 3'-OH group) terminates the elongation reaction. The polymerase synthesises random-length copies of the DNA template. By performing four reactions, one for each of the ddNTPs, full sequence information can be obtained. DNA polymerases which can be used in chain termination DNA sequencing include: (i) the large fragment (Klenow fragment) of *E. coli* DNA polymerase I, (ii) the T7 DNA

polymerase (Tabor and Richardson 1987) and its genetic variant *Sequenase* marketed by USB, Ohio, (iii) reverse transcriptase, and (iv) *Taq* polymerase. Ludwig (1991) suggests that the modified T7 DNA polymerase gives the most uniform band patterns. The sequencing reaction products are separated by acrylamide urea gel electrophoresis and detected by autoradiography.

6.1.4.5 Sequence data analysis

Before a set of sequences can be phylogenetically analysed they must be aligned. The high degree of primary sequence conservation among rRNAs and the conservation of secondary structure aids the process of sequence alignment. It is more difficult to align sequences in variable regions and in most cases these highly variable regions are eliminated from the comparative analysis, an approach known as 'restricted' or 'masked' analysis. When sequences that are being compared are very closely related the variable regions can be included in the analysis which is unmasked or unrestricted.

It is possible to use the similarity between any pair of aligned sequences for identification purposes, or to suggest possible phylogenetic position. The percentage similarity value will vary depending on the number and position of the nucleotides included in the analysis. Absolute percentage similarity values cannot be defined for use in determination of different taxonomic levels for all groups of microorganisms.

It is the phylogenetic tree that is used to compare sets of sequence data and infer phylogenetic relationships. A large number of methods of reconstructing phylogenetic trees have been used and compared in the literature (see Olsen 1987; Lake 1987; Gouy and Li 1989; Penny 1991). The results obtained using these methods have been shown to be influenced by various factors including the amount of sequence data (Gouy and Li 1989), the alignment and assumptions made about mutational rates at each base position (Olsen 1987). It is generally accepted that the more sequence data included in an analysis the greater the validity of the phylogenetic result.

Analysis of the information contained in large numbers of the sequences has revealed features of primary and secondary structure that can be used to identify certain groups of organisms. These signature sequences can be used to verify the phylogenetic position to which an organism has been assigned on the basis of either sequence similarity or treeing (Woese 1987; Woese et al. 1985).

The majority of literature on the various methods of reconstructing phylogenetic trees is weighed down with complicated mathematics and statistics. Woese (1987) to some extent simplified the situation and described three methods used in phylogenetic analysis:

Distance matrix methods use only the sequence distances between pairs of sequences, which is the fraction of positions in which the sequences differ. Such distances are considered to be an underestimation of the true evolutionary distance between sequences. Since all lineages and positions in a sequence are not changing at the same rate, correction for multiple changes is a problem. In distance matrix treeing, the evolutionary distances are assumed to conform to a tree topology. To the matrix of evolutionary distances various algorithms can be applied to generate the 'best' tree.

In maximum parsimony analysis the tree that requires the smallest number of changes along the edges of the tree is accepted. Such a tree is considered the most parsimonious one. The process of finding the correct tree can be extremely computationally intense and a large number of algorithms have been developed to attempt to reduce this problem.

Cluster analysis methods can also be used in the analysis of sequence data. The methods involve the grouping of sequences on the basis of their similarity to one another or to other groups of sequences. This method is considered the least accurate and is very sensitive to the problem of different parts of a sequence evolving at different rates.

Sneath (1989) pointed out some of the problems associated with phylogenetic analysis of sequence data. The various methods for sequence alignment, the different similarity coefficients and the numerous treeing techniques can yield slightly different results but in general the differences are minor (Sneath 1989). One of the main sources of errors in phylogenetic analysis is the sampling error, since a tree from one semantide can differ from one produced from another semantide. This sampling error will be greater for smaller sequences therefore the use of more complete sequence information is desirable.

6.1.5 INFORMATION PROVIDED BY THE PHYLOGENETIC ANALYSIS OF rRNA SEQUENCE DATA AND ITS RELEVANCE TO THERMOPHILIC BACTERIA

The last five years have seen a large amount of rRNA sequence data obtained in various laboratories around the world, the majority of this has now been deposited in accessible data bases including that of the ribosomal RNA database project at the University of Illinois. The collection of bacterial, archaeal, and eucaryal small subunit rRNA sequences available is roughly 400, 40 and 80 respectively (Winker and Woese 1991). This information was used to define the domains as described in section 6.1.2. Phylogenetic information is becoming common place in descriptions of new bacterial and archeal genera and species.

The bacterial (then eubacteria) phylogenetic tree (Figure 6.1), based on 16S rRNA sequence comparison, was shown to have various lines of descent, of which *Thermotoga maritima* was the deepest (Woese 1987). Nine of the ten branches were found to branch from the same region of the tree. The exception to this was the green non-sulfur bacteria. The rooting of the tree with an archeal consensus sequence placed *Thermotoga maritima* as the most ancient member of the tree, well separated from the other lines of descent. On the basis of this tree, the slowly evolving nature of *Thermotoga maritima*, and its position as the deepest branch, Achenbach-Richter et al. (1987) suggested that the original bacteria (then eubacteria) were thermophiles. The fact that the other deep branch of the tree, that of the green non-sulfur bacteria and their relatives, contained thermophilic members (ie. *Chloroflexus* and *Thermomicrobium*) added weight to this suggestion. The organisms since isolated and assigned to the same branch as *Thermotoga maritima* have all been thermophiles and the branch has been designated as the order *Thermotogales* (Huber et al. 1989; Huber et al. 1990). There is always a special interest in the phylogenetic position of new thermophilic isolates and what the analysis can tell us about the phylogeny of thermophilic bacteria.

Three phylogenetic studies were carried out on thermophilic bacteria in this thesis, one on a group of rod-shaped cellulolytic thermophiles is described in section 6.2, the phylogenetic position of a thermophilic spirochete is described in section 7.3 and in chapter 8 the analysis of a member of the *Thermotogales* is described.

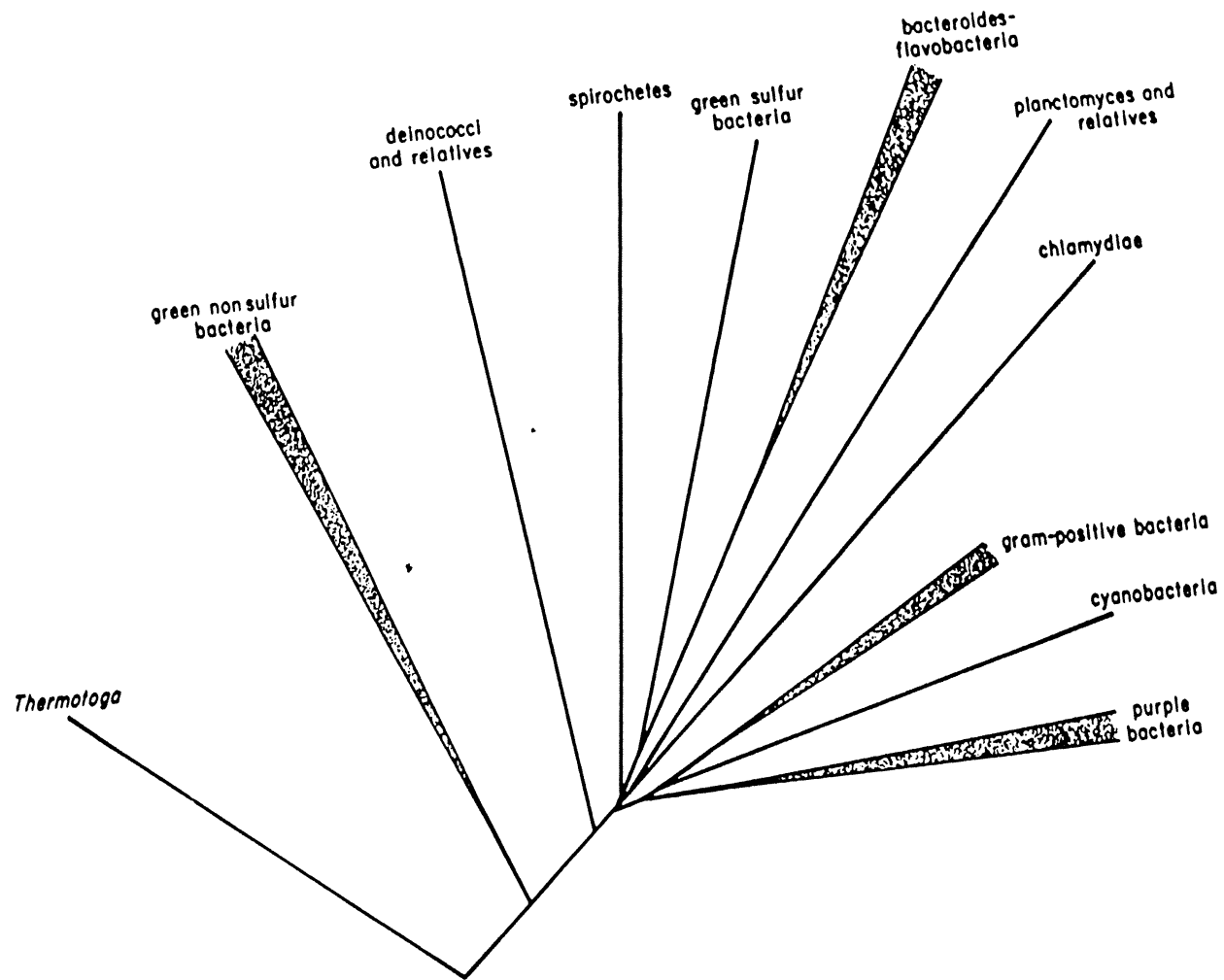


Figure 6.1 Bacterial phylogenetic tree based on 16S rRNA comparisons
(Reproduced from Woese, 1987).

6.2 PHYLOGENETIC ANALYSIS OF A GROUP OF ANAEROBIC CELLULOLYTIC THERMOPHILIC ROD SHAPED BACTERIA

6.2.1 INTRODUCTION

A phylogenetic study was carried out on a group of anaerobic, cellulolytic, thermophilic bacteria. The isolates were selected on the basis of their position in the numerical taxonomy study. Strains were selected from the various clusters, two from cluster A (Tp8T. 6.3.3.1 and NA10), two from cluster B (RI2. B1 and Wai35. B1), one from clusters C and D (Rt8. B7 and Rt8. B15 respectively) and one isolate from cluster E (Rt51. B1). In the case of the two isolates of clusters A and B they were chosen from distinct sub clusters, so as to provide the most diverse isolates from the same group. Partial 16S rDNA sequences were obtained for these isolates and also for some of the clostridial type strains including, *C. thermocellum*, *C. stercorarium*, *C. thermocopriae*. Partial sequence was also obtained for *Thermoanaerobium brockii* in order to include a asporogenous representative of the Gram type positive thermophiles.

6.2.2 METHODS AND MATERIALS

6.2.2.1 Culture conditions

All of the isolates and type cultures were grown in 2/1 cellobiose medium as described in section 4.2.2.2.

6.2.2.2 Isolation of genomic DNA

1 ml of an overnight culture was transferred to an Eppendorf tube, cells were harvested and resuspended in 400 μ l saline-EDTA. After the addition of 10 μ l of lysozyme (0.1mg/ml), cells were incubated for 15min at 60°C. Lysis was completed by adding 10 μ l proteinase K (1.0 % w/v) and 10 μ l SDS (25 % w/v). The crude extract was purified with equal volumes of phenol, phenol-chloroform (1:1) and chloroform-isoamylalcohol (24:1). Nucleic acids were precipitated with ammonium acetate (Brow 1990), washed with 70 % ethanol, dried and dissolved in sterile distilled water. The concentration was determined spectrophotometrically.

6.2.2.3 Amplification of 16S rDNA by polymerase chain reaction (PCR)

400ng of bacterial DNA was used per reaction in a total volume of 100 μ l 50mM KCl, 10mM Tris/HCl pH 8.4, 2.5mM MgCl₂, 0.1% gelatine, 400 μ M of each dNTP (dATP, dCTP, dGTP, dTTP) and 0.5 μ M of each amplification primer. The first denaturation step was carried out at 98°C for 2min. Samples were then chilled on ice, 2.5 units of *Taq* polymerase (Perkin-Elmer Cetus) was added and the reaction mixtures overlayed with 80 μ l of light mineral oil (Sigma). The thermal profile included repeated cycles of primer annealing at 48°C for 1min, extension for 2min at 72°C, and denaturation for 1min at 93°C. After 28 cycles, primer annealing was repeated followed by a final extension step at 72°C for 10min. Amplification of 16S rDNA between positions 29 and 1521 (*E. coli* numbering system [Woese et al. 1983]) was performed with the primer combination 5'GAG TTT GAT CCT GGC TCA G' (forward primer) and 5'AGA AAG GAG GTG ATC CAG CC3' (reverse primer).

6.2.2.4 Purification of PCR products

The aqueous phase of the PCR was extracted with chloroform to remove any residual mineral oil, sodium acetate precipitated, dried and the pellet dissolved in 20 μ l of distilled water. Fragments were separated from bacterial genomic DNA, excess primer and nucleotides by 0.6 % (w/v) agarose gel electrophoresis. Agarose blocks containing the DNA of the desired size were cut from the agarose gel. Products were recovered by the freeze/squeeze method of Dretzen et al. (1981), using Millipore Filter Units Ultrafree MC (0.22 μ m, Millipore, Göttingen, Germany). DNA was precipitated with ammonium acetate (Brow 1990), washed with 70 % ethanol, dried and dissolved in 14 μ l of distilled water. Towards the end of this study a *Prep-a-Gene* DNA purification kit (Biorad, Australia) was used for the purification of PCR products, following the manufacturers instructions.

6.2.2.5 Direct sequencing of PCR products

The nucleotide compositions of the sequencing primers have been recently published (Stackebrandt and Charfreitag 1990) and are listed in Table 6.1. The sequencing protocol was modified as follows: 7 μ l of the dissolved template DNA was mixed with 2 μ l 5x reaction buffer and 1 μ l sequencing primer (50ng/ μ l in distilled water), denatured by boiling for 2 min and snap-frozen by chilling the tube in a mixture of dry ice and ethanol for 15 sec (Casanova et al. 1990). The labelling solution containing 2 μ l 5x diluted labelling mix, 1 μ l 0.1 M dithiothreitol, 3 units *Sequenase* (*Sequenase* Kit

Version 2.0, United States Biochemicals) and 10 μ Ci of [α - 32 P]dATP was added immediately to the denatured DNA/primer mix, and the labelling reaction carried out at 20°C for 45-60sec. Aliquots of 3.2 μ l were added to the prewarmed termination solutions and incubated for 4min at 37°C. The reactions were stopped by the addition of 4 μ l of the stop solution provided with the *Sequenase* kit. The reaction products were denatured by boiling for 2min and chilled on ice.

Table 6.1 Sequences of primers used and the binding positions in the 16S rRNA primary structure.

Binding position*	Sequence of primer
105-124	5'CACGTGTTACTACCCGTCC3'
343-357	5'CTGCTGCCTCCCGTA3'
519-536	5'GWATTACCGCGGCKGCTG3'
691-704	5'TCTGCGCATTCCAC3'
786-803	5'CTACTSGGGTATCTAATC3'
907-926	5'CCGTCAATTCMTTTRAGTTT3'
1056-1073	5'ACGAGCTGACGACRGCCA3'
1100-1115	5'AGGGTTGCGCTCGTTG3'
1224-1242	5'CATTGTAGCATGCGTGAAG3'
1282-1401	5'CGGTGTGTRCAAGGCC3'
1492-1510	5'GGTTACCTTGTTACGACTT3'

*Nucleotide positions according to the *E. coli* numbering system (Brosius et al. 1978)
Non-standard symbols refer to wobble bases abbreviated as follows: K = G/T, M = A/C.
R = A/G, W = A/T, S = G/C (Lane et al 1985)

6.2.2.6 Electrophoresis and autoradiography

Electrophoresis and autoradiography of the sequencing reactions was done as described previously (Rogall et al. 1990). The DNA fragments from the sequencing procedure were separated by acrylamide urea gel electrophoresis. Gels were poured between a thermostatic and a notched glass plate, which were separated by 0.2 mm Teflon spacers and fixed by clamps. The gel solution was prepared using PAGE pre-mix (Pharmacia, FRG), which was added to 8M urea solution in 0.1M Tris-borate pH 8.0; 0.001M EDTA; and 0.08% (w/v) ammonium persulphate to give a 8.0% acrylamide solution.

Gels were run at 55°C at 2500V until the tracking dyes had migrated the desired distance. The electrode buffer was 0.1M Tris-borate buffer (pH 8.0) and 0.001M EDTA. The bromophenol blue comigrates with fragments of about 24 bases and the xylene cyanol FF marker with fragments of about 110 bases. Both long (6h) and short (2-3h) electrophoresis runs were performed in order to maximize the amount of information obtained per sequencing reaction.

After electrophoresis the gels were fixed for 20min in 10% (v/v) glacial acetic acid solution, rinsed with water and dried with a hot air heater. Exposure of x-ray film was normally overnight or 2-3d for weak sequence reactions.

6.2.2.7 Data analysis

The sequences were aligned against a representative alignment of bacterial 16S rRNA sequences. Equally weighted Hamming distances and percent similarities were calculated between each pair of sequences. The phylogenetic analysis was carried out using the least squares distance method of De Soete (1983).

6.2.3 RESULTS AND DISCUSSION

Partial 16S rDNA sequence data was obtained within the region between positions 12 and 1377 (*E. coli* numbering system). The regions used in the analysis included positions 189-451, 571-906, 1045-1377, a total stretch of 929 nucleotides. Initially the sequences were included in an alignment with representatives of the main lines of descent - this showed that the isolates and thermophilic clostridial type strains grouped with the low GC Gram positive organisms, including the mesophilic members of the genus *Clostridium*. An alignment of the new sequences and those of a number of mesophilic and thermophilic clostridia clearly showed that these organisms were members of the general clostridial group. Finally an alignment was constructed using just the thermophilic clostridia, namely *C. thermoaceticum* and *C. thermosaccharolyticum* and the sequences obtained in the study. A more restricted alignment of 800 nucleotides which included *Thermoanaerobium brockii* was also analysed. A general alignment containing the sequence data obtained in this study is shown in appendix 2.

A matrix of binary similarity values and Hamming distances for the rod shaped isolates and the thermophilic *Clostridium* species is shown in Table 6.2.

Table 6.2 Percentage similarity between partial 16S rDNA sequences of some anaerobic, cellulolytic, thermophilic bacteria and thermophilic *Clostridium* species (above the diagonal) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal)

		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
1.	Tp8T.6.3.3.1		95.2	94.9	95.3	95.9	94.3	82.3	80.7	82.0	82.8	83.3	83.1
2.	R12. B1	43		99.7	95.3	95.7	94.4	82.3	80.4	81.4	81.0	83.4	82.3
3.	Wai35. B1	46	3		95.1	95.6	94.3	82.2	80.3	81.5	81.1	83.7	82.2
4.	NA10	42	42	44		97.4	96.2	82.1	80.7	81.7	81.0	83.8	81.7
5.	Rt8. B15	37	39	40	23		98.2	83.3	81.4	83.5	82.0	84.8	83.0
6.	Rt8. B7	51	50	51	34	16		84.1	81.8	82.0	80.4	83.4	84.1
7.	Rt51. B1	160	160	160	161	150	143		95.6	87.5	79.8	81.9	84.5
8.	<i>C. stercorarium</i>	174	177	178	178	168	164	40		88.5	78.3	81.2	83.0
9.	<i>C. thermocellum</i>	162	168	167	165	149	162	113	104		81.2	83.5	80.5
10.	<i>C. thermosaccharolyticum</i>	155	171	170	171	162	177	182	196	169		85.6	83.6
11.	<i>C. thermoaceticum</i>	151	150	147	146	137	150	163	169	149	130		86.5
12.	<i>C. thermocopriae</i>	152	160	160	165	153	143	140	153	176	148	122	

The Hamming distances (lower left triangle) were used for the generation of an unrooted phylogenetic dendrogram (Figure 6.2). The branching pattern remained stable whether *Arthrobacter globiformis* or *Thermotoga maritima* were used as a reference organism.

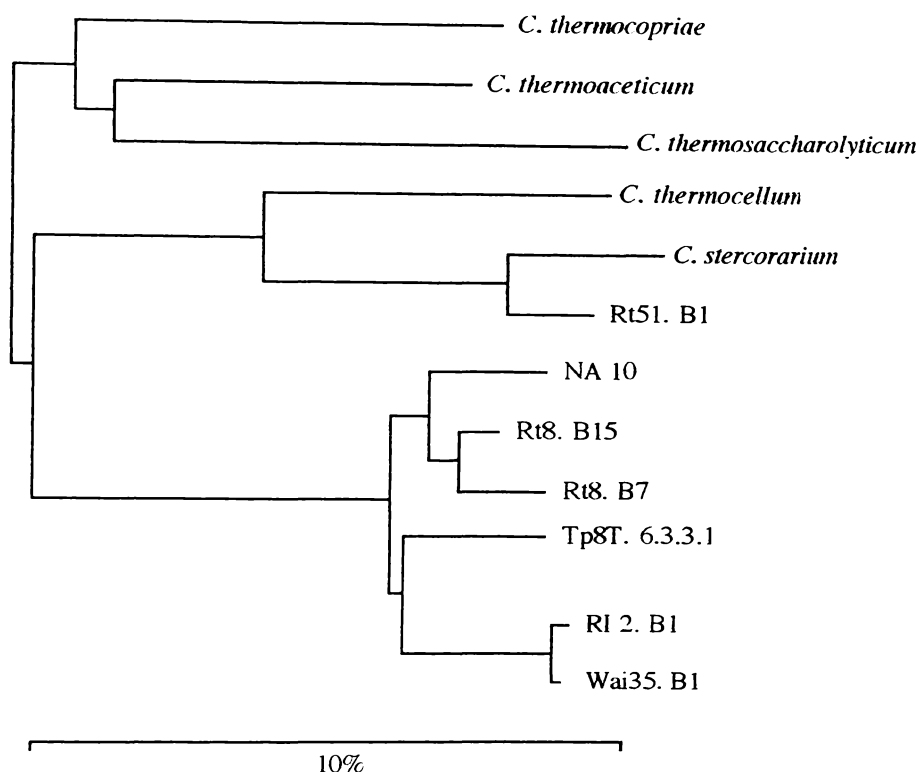


Figure 6.2 Dendrogram indicating the position of the anaerobic cellulolytic thermophilic isolates among the thermophilic *Clostridium* species. The scale bar indicates 10 substitutions per hundred nucleotides.

It is clear from the percentage similarity values and the dendrogram that the rod-shaped, anaerobic, cellulolytic, thermophilic isolates form a cluster within the *Clostridium* group. Strain Rt51. B1 is most similar to *C. stercorarium* at 95.6% similarity. With the exception of *C. thermocopriae*, all of the cellulolytic clostridia are in one group. Although not shown it should be noted that *Thermoanaerobium Brockii* clustered with *C. thermocopriae*, *C. thermoaceticum*, and *C. thermosaccharolyticum*, and was shown not to be closely related to the new anaerobic, cellulolytic, thermophilic isolates (data not shown). On the basis of data derived from 16S rRNA catalogs, Cato and Stackebrandt (1989) showed that the organisms assigned to the genus *Clostridium* did not constitute a phylogenetically coherent taxon and were found in six sublines within the

Clostridium/Bacillus subdivision of the Gram-positive bacteria. The organisms that constituted group VI included *C. thermoaceticum*, *C. thermosaccharolyticum*, *Thermoanaerobium brockii* and *Acetogenium kivui*. This finding is backed up by the sequence data of this study, as three of these organisms were found to cluster together, along with *C. thermocopriae*, which can now be added to this group. This data adds credence to the discovery of spore formation by *T. brockii* (Cook et al. 1991). This study provides the first phylogenetic information on *C. thermocellum* and *C. stercorarium* and shows them to form a separate group from the non cellulolytic thermophilic *Clostridium* species. It is interesting to note that the sporogenous organisms from similar habitats and with very similar phenotypic characteristics fall into different phylogenetic groups, which show them to be at the least species of the same genera. The 16S rRNA similarity level at which a species is defined has recently been discussed by Fox et al. (1992), using examples from the genera *Bacillus* and *Serpulina* - they have shown that a high percentage similarity at the 16S rRNA level is not always reflected in the DNA-DNA hybridization data. In the case of *B. psychrophilus* and *B. globisporus*, a percentage similarity of 99.8% is shown for their 16S rRNA, but yet at the DNA-DNA level values of 23 and 50% are given.

It could be suggested that the anaerobic, cellulolytic, thermophilic organisms form a new subline within the *Clostridium/Bacillus* subdivision. Evidence for this comes from the similarity values of less than 85.0% between the anaerobic, cellulolytic, thermophilic organisms and the other sporeforming organisms. There is very high similarity between the isolates in this group, and in some cases (for example strains RI2. B1 and Wai 35. B1) a number of differences may fall within the experimental error. Those isolates with similarity values around 95% may constitute new species.

The branching positions within the anaerobic, cellulolytic, thermophilic organisms and strain Rt51. B1 correlate with the data obtained from the numerical taxonomy study (chapter 4) and the PAGE studies (chapter 5) and this will be discussed in chapter 9. The possible taxonomic designation of these organisms will also be discussed.

6.2.4 CONCLUSION

The phylogenetic position of a group of anaerobic, cellulolytic, thermophilic bacteria has been determined. The majority of these organisms form a new subline within the *Clostridium/Bacillus* subdivision. Strain Rt51. B1 has been found to be highly related to the thermophilic sporogenous *C. stercorarium*. The clustering patterns were found to correlate with the groupings obtained by the numerical taxonomy study and the PAGE

electrophoresis studies, described in Chapters 4 and 5 respectively. By combining the phylogenetic information obtained on these isolates with the phenotypic data it may be possible to designate these isolates to taxa either new or previously described.

CHAPTER 7

A DESCRIPTION OF THE CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF AN EXTREMELY THERMOPHILIC POLYSACCHAROLYTIC SPIROCHETE.

INTRODUCTION

The order *Spirochaetales* at present contains the families *Spirochaetaceae* and *Leptospiraceae*, classified as the genera *Spirochaeta*, *Borrelia*, *Cristispira*, *Serpulina*, *Treponema*, *Leptonema*, and *Leptospira* (Canale-Parola 1981, 1984; Stanton et al. 1991; Stanton 1992). Ribosomal RNA oligonucleotide cataloging and 16S rRNA sequencing have shown the spirochetes to form a deep and coherent evolutionary group (Paster et al. 1984; Woese 1987; Stanton et al. 1991). The genus *Spirochaeta* contains both obligately and facultatively anaerobic free-living forms (Canale-Parola 1981). Eight species have been assigned to the genus *Spirochaeta* (the type species *S. plicatilis*, *S. stenostrepta*, *S. zuelzeriae*, *S. litoralis*, *S. aurantia*, *S. isovalerica*, *S. halophila*, *S. bajacaliforniensis*) on the basis of their phenotypic characteristics (see Canale-Parola 1984; Fracek and Stolz 1985).

This chapter is divided into three sections. In section 7.1 the characterization of an extremely thermophilic spirochete is described. A comparison of this isolate with a similar isolate from the Soviet Far East and their designation to the genus *Spirochaeta* is described in section 7.2. Finally in section 7.3 a phylogenetic study is described in which the phylogenetic position of the thermophilic spirochete is determined and the implication of this on the general systematics of the *Spirochaetales* is considered.

7.1 CHARACTERIZATION OF AN OBLIGATELY ANAEROBIC POLYSACCHAROLYTIC EXTREMELY THERMOPHILIC MEMBER OF THE GENUS *SPIROCHAETA*.

7.1.1 INTRODUCTION

The genus *Spirochaeta* contains both obligately and facultatively anaerobic free-living forms (Canale-Parola 1981). They have a unique and complex helical morphology and possess internal organelles of motility, the periplasmic flagella. Resistance to the antibiotic rifampin is another characteristic of this group. Members of the genus *Spirochaeta* have been isolated from various environments, including thermal springs (Patel et al. 1985b; Svetlichny et al. 1990a; Aksenova et al. 1990). A recent study of 16S rDNA sequences isolated from a thermal spring cyanobacterial mat demonstrated the presence of a sequence exhibiting 94.3 % similarity with the sequence for *S. halophila*. It has been suggested that this sequence may be contributed by an as yet uncultivated spirochete inhabitant of the mat (Ward et al. 1990). The species of *Spirochaeta* that have been isolated utilize carbohydrates including hexoses, disaccharides and pentoses commonly released from plant material through the metabolic activities of bacteria other than spirochetes (Harwood and Canale-Parola 1984). Until the recent description of strain Z-1203 (Svetlichny et al. 1990a; Aksenova et al. 1990) no polysaccharolytic strains of the genus *Spirochaeta* have been reported. The isolation of a thermophilic spirochete was described in chapter 3. Below is a description of the characterization of this obligately anaerobic, polysaccharolytic, extremely thermophilic spirochete, *Spirochaeta* sp., strain RI 19. B1.

7.1.2 METHODS AND MATERIALS

7.1.2.1 Medium and culture conditions

The new isolate was grown in bicarbonate-buffered medium 4 (CBM4), containing (per litre): NaCl, 4.0 g; MgCl₂.6H₂O, 0.8 g; KCl, 0.5 g; NH₄Cl, 0.3 g; KH₂PO₄, 0.2 g; 0.15 % (w/v) FeCl₃, 1 ml; 0.1 % (w/v) resazurin, 1 ml; trace element solution SL10 (without FeSO₄) and selenite-tungstate solution (Widdel et al. 1983) 1ml each. Sodium bicarbonate (2.5 g/litre) was added to boiled, cooled medium which was dispensed in 10 ml aliquots in 23 ml anaerobic culture tubes (Bellco) gassed with N₂/CO₂ mixture (80%/20% v/v) and sealed with butyl rubber stoppers. Volumes of 100 ml and 750 ml were similarly prepared and dispensed in 160 ml and 1,000 ml vessels respectively.

Sulfide-calcium solution (containing $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ 36 g/litre; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 15 g/litre; pH 7.2) at 2 ml/litre and a 7-vitamin solution (Widdel and Pfennig 1981) at 1 ml/litre were added to the autoclaved, cooled medium from sterile stock solutions. When changing the salinity of the medium, NaCl and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ were varied at a fixed ratio of 20 g to 2.7 g/litre respectively in the medium CBM4. The final medium pH was 6.9 to 7.1 unless otherwise specified. Substrates were added from anaerobic sterile neutralized stock solutions. Growth on amorphous cellulose as a sole carbon source was determined by adding amorphous cellulose at 5 g/l to CBM4, the method of preparation of amorphous cellulose is described in section 3.3.2.2.

Antibiotics were obtained from Sigma Chemical Co. (USA). Stock solutions were prepared in preboiled distilled water and filter sterilized into sterile N_2 flushed serum vials. Antibiotics were added at concentrations of 100 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$. Additions and inoculations were made and samples taken using plastic syringes and 25-gauge hyperdermic needles. All incubations were at 65°C unless otherwise stated. Temperatures permitting growth were determined using a gradient incubator.

Solid media were prepared by the addition of 20 g/litre purified agar (Oxoid). To determine the pH parameters of growth, appropriately poised buffers adjusted with NaOH (10 M) were added to medium at 50 mM. All pH values were determined at 70°C. The buffers were 2-(N-morpholino) ethanesulfonic acid (MES), 3-(N-morpholino) propanesulfonic acid (MOPS) and N-(2-hydroxyethyl)-piperazine-N'-(3-propanesulfonic acid) (EPPS), purchased from Sigma (St Louis, Mo. USA).

7.1.2.2 Electron microscopy

Negatively-stained specimens were prepared as follows. A drop of culture was placed on a 200 mesh formvar- coated grid for 1 min. Excess culture was drawn off the grid with filter paper and the specimen fixed by applying a drop of 2.5% (v/v) glutaraldehyde to the grid for 1 min. After drawing of the excess fixative the grid was stained by applying a drop of a saturated solution of uranyl acetate for 1 min. Excess stain was then removed. Thin sections were prepared and stained as described by Patel et al. (1985a). Both negatively-stained specimens and thin sections were examined using a Philips EM 400 electron microscope at an accelerating voltage of 80kV.

7.1.2.3 Light microscopy

Examination of enrichment cultures and routine checking of purity was carried out using an Olympus BH-2 microscope with phase contrast optics. Phase contrast photomicrographs were taken using a Reichert-Jung Polyvar microscope (C. Reichert, Vienna, Austria) with FP4-135 film (Ilford, Cheshire, UK). Immobilization of cultures was aided by the use of agar coated slides prepared as described by Pfennig and Wagener (1986).

7.1.2.4 Growth and metabolic characterization

Substrates supporting growth were tested by visible increase in turbidity, substrate disappearance (for insoluble substrates) and HPLC analysis of culture supernatants. Growth curves on glucose were followed by measuring the increase in turbidity at 650 nm by direct insertion of the anaerobic culture tubes into a Pye Unicam SP-450 UV/VIS spectrophotometer. All individual experiments were in triplicate and represent mean values.

Fermentation end products were analyzed by gas chromatography (Patel et al. 1985a) and HPLC (Patel et al. 1987). Cell dry weights were determined by drying centrifuged cell pellets to constant weight at 85°C. Sulfide production from 20 mM Na₂SO₄, approximately 1 M S⁰, or 2 mM L-cysteine was measured by a micromethod adaptation of the method of Truper and Schlegel (1964). Nitrate reduction and nitrite production were tested using Uristix (Miles, Musgrave, Victoria, Australia), converting nitrate to nitrite using zinc powder. Fumarate reduction was determined by HPLC. Indole from 5 mM L-tryptophan, urea and esculin hydrolysis were tested as described by Janssen and Harfoot (1990). All tests were carried out on cultures grown on 10 mM glucose.

7.1.2.5 G + C content

DNA was isolated and purified using the method of Egli et al. (1988). The T_m of the DNA was determined as described by Patel et al. (1985a). *Escherichia coli* DNA (Sigma, St. Louis, MO, USA) was used as a standard.

7.1.2.6 Chemicals

All chemicals were analytical grade. Oxygen-free nitrogen and carbon dioxide (food-grade) were obtained from New Zealand Industrial Gases.

7.1.3 RESULTS

7.1.3.1 Source of isolates

A brackish, thermal spring sample from Green Lake, Raoul Island, Kermadec archipelago, New Zealand was the source of the described spirochete. The site from which this sample was taken is described in section 3.3.3.1.

7.1.3.2 Enrichment and isolation

The methods of enrichment and subsequent purification of the thermophilic spirochete are described in section 3.3.3.1. The isolated thermophilic spirochete was designated RI 19. B1 and was the subject of this study.

7.1.3.3 Morphology

Cells of RI 19. B1 measured $0.2\ \mu\text{m}$ by $10\text{--}50\ \mu\text{m}$ during growth in CBM4 with glucose (10 mM) as a substrate. The coiling was regular with coils having a wavelength of about $0.75\ \mu\text{m}$ and amplitude of $0.35\ \mu\text{m}$ (Figure 7.1 a,c). Spherical bodies similar to those observed in other spirochetes (Breznak and Canale-Parola 1975) and structural features, described here as tails were present in stationary phase cultures (Figure 7.1 b). The tails consisted of the outer sheath enclosing the periplasmic flagella with the protoplasmic cylinder absent from this portion of the cell. Electron microscopy showed morphological features similar to those observed in other spirochetes (Greenberg and Canale-Parola 1976; Fracek and Stolz 1985). These features included two periplasmic flagella and a central coiled protoplasmic cylinder both surrounded by an outer sheath (Figure 7.2 a,b). Irregular crenulations were observed in the outer sheath similar to those reported by Fracek and Stolz (1985). Thin sections showed the periplasmic flagella were anchored by insertion discs (Figure 7.2 c) at one end and ended subterminally at the other end, overlapping in the central area of the cell (Figure 7.2 a) in the typical 1-2-1 arrangement found in *Spirochaeta*. The Gram-staining reaction was negative. Cells did not lyse in 3 % KOH.

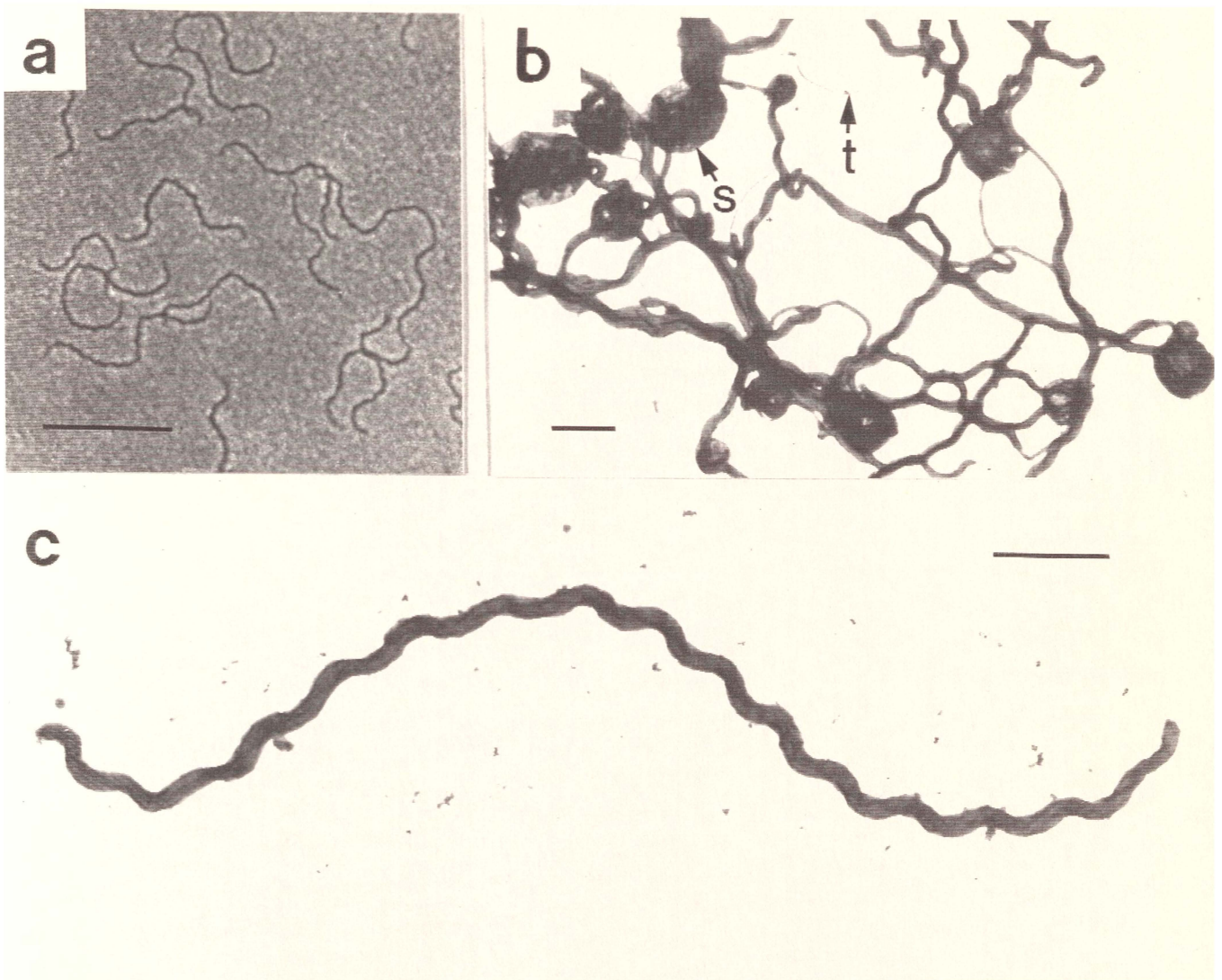


Figure 7.1 a-c Strain RI 19. B1 **a.** Phase-contrast photomicrograph. Bar = $10\mu\text{m}$. **b.** Negatively stained preparation of cells in stationary phase showing spherical bodies (*s*) and tail structures(*t*). Bar = $1\mu\text{m}$. **c.** Negatively stained preparation showing regular coiling of the cell. Bar = $1\mu\text{m}$

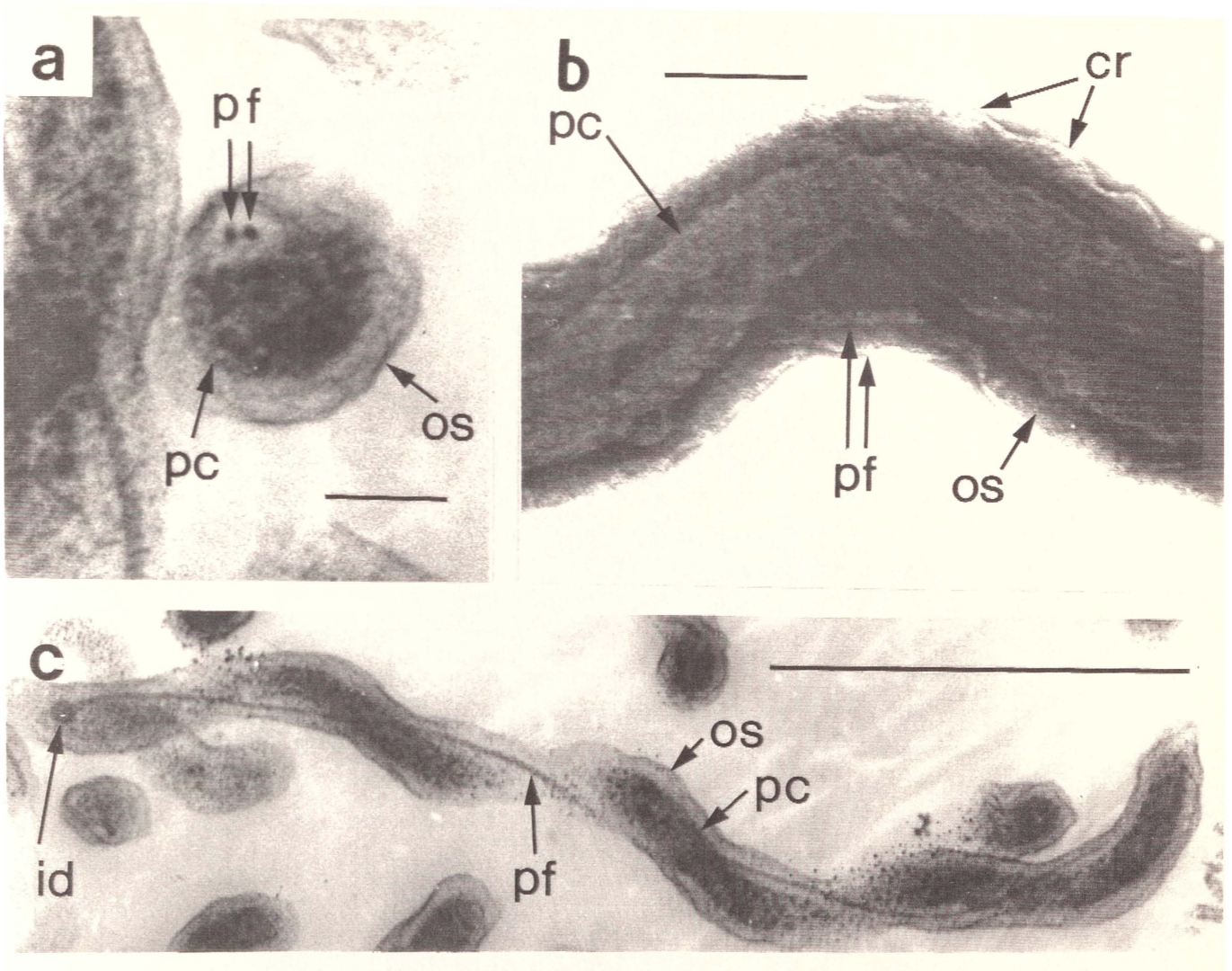


Figure 7.2 a-c Strain RI 19. B1 **a.** Thin section showing outer sheath (*os*), protoplasmic cylinder (*pc*) and two periplasmic flagella (*pf*). Bar = $0.1\mu\text{m}$. **b.** Negatively stained preparation showing protoplasmic cylinder(*pc*), two periplasmic flagella (*pf*) and outer sheath (*os*), with crenulations (*cr*). Bar = $0.1\mu\text{m}$. **c.** Longitudinal thin section showing outer sheath (*os*), protoplasmic cylinder (*pc*) and a periplasmic flagellum (*pf*) anchored by an insertion disc (*id*). Bar = $1.0\mu\text{m}$.

7.1.3.4 Physiological and nutritional properties

RI 19. B1 did not grow in CBM4 minus the sulfide reductant. Growth occurred in CBM4 reduced with sulfide (3mM), cysteine (2mM) or Ti-NTA (Moench and Zeikus 1983) but no growth was observed when thioglycollate (6.6mM) was used as a reductant. These results indicated the strict anaerobic nature of RI 19. B1.

Growth occurred over the pH range 6.25 to 7.15 but not at 6.05 or 7.25. The optimum pH for growth was 6.95. The temperature range over which growth occurred was 43 to 73°C with an optimum between 64 and 66°C. No growth was detected at 42 or 76°C. Growth occurred at NaCl concentrations between 0.1 % (the lowest tested) and 2.5 %, with an optimum around 0.4 %. No growth occurred at 2.8 % NaCl.

Growth did not require the presence of the vitamin supplement or yeast extract. No growth was detected on CBM4 containing no fermentable carbon source. Monosaccharides, disaccharides and polysaccharides served as fermentable substrates (Table 7.1) producing good growth in all cases. On cellulose, cell numbers in the medium above the substrate remained low until the cellulose was hydrolysed, indicating association with the substrate. The sugar alcohols, organic acids and amino acids tested did not serve as energy sources. Growth did not occur via the reduction of fumarate, nitrate, oxygen, sulfate or sulfur with glucose as the carbon source. Indole was not formed from L-tryptophan. Urea was not hydrolysed, nor was sulfide produced from L-cysteine. Esculin was hydrolysed.

Table 7.1 Substrates^a utilized as energy sources by strain RI 19. B1.

AMYGDALIN	GALACTOSE	SUCROSE	GLYCOGEN
ARABINOSE	GLUCOSE	XYLOSE	PULLULAN
CELLOBIOSE	MALTOSE	AVICEL	STARCH
FRUCTOSE	MANNOSE	XYLAN	CELLULOSE

Substrates tested but not utilized included : erythritol, glycerol, inositol, lactose, mannitol, melezitose, raffinose, rhamnose, ribose, sorbitol, sorbose, acetoin, trisodium citrate, sodium formate, disodium fumarate, disodium malate, sodium pyruvate, sodium succinate, L-arginine, sodium L-glutamate, L-lysine ^a Substrate was added (to 0.1 %) to CBM4

A typical growth curve for RI 19. B1 is shown in Figure 7.3. The doubling time on 10mM glucose under optimal conditions of NaCl concentration, pH and temperature was 199 min ($\mu = 0.21 \text{ h}^{-1}$). The pH of the media at the end of growth was 6.4 with complete utilization of 10 mM glucose. Strain RI 19. B1 fermented glucose to lactate, acetate, carbon dioxide and hydrogen. End-products not detected in culture supernatants included formate, succinate and ethanol. The increase in acetate and lactate with growth and glucose consumption is shown in Figure 7.3. The fermentation products of glucose metabolism by RI 19. B1 determined after 40 h growth at 65°C are shown in Table 7.2. The amount of CO_2 produced could not be measured due to the high background as a consequence of using CO_2 /bicarbonate as the pH buffering system. Growth in the absence of CO_2 was poor and inconsistent. Replacement of the CO_2 /bicarbonate by a phosphate (5 mM) or MOPS (50 mM) buffering system produced only poor or no growth. MOPS was not inhibitory at the same concentration when used in conjunction with CO_2 /bicarbonate.

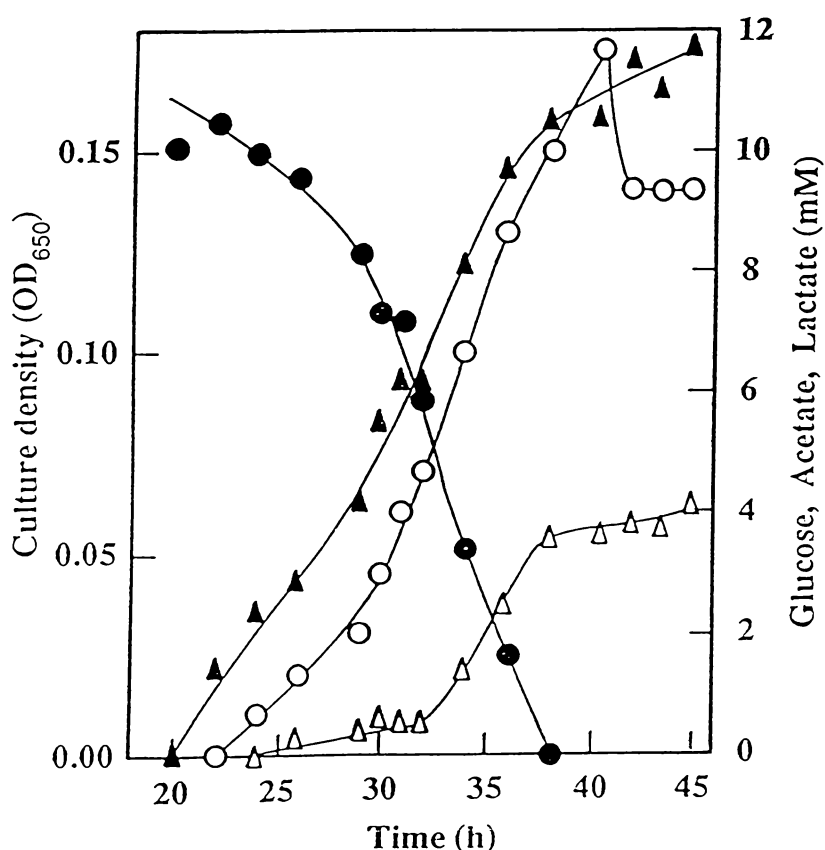


Figure 7.3 Fermentation time course of strain RI 19. B1 in CBM4 containing glucose as substrate. Experiments were performed in 160 ml serum bottles. Symbols: ○ culture density, ▲ acetate, △ lactate, ● glucose. OD_{650} : optical density at 650 nm.

Table 7.2 Fermentation stoichiometry and growth yields for strain RI 19. B1 grown on glucose

Expt	Glucose used (mmol/l)	Cell dry weight (mg/l)	End-products (mmol/l)				Dry weight yield (g/mol)
			lactate	acetate	H ₂	cell matter ^a	
1	5.9	111.0	1.4	6.0	12.1	0.7	19
2	9.0	184.0	4.3	9.1	17.1	1.2	20
3	7.8	160.0	3.4	7.4	14.7	1.0	21

^a Dry weight was converted to a mmol/l value using C₆H_{10.84}N_{1.4}O_{3.07} as the empirical formula for cell material (Stouthamer 1979)

7.1.3.5 Antibiotic sensitivity

Growth of RI 19. B1 was inhibited by penicillin, neomycin and polymyxin B at 10 µg/ml and by novobiocin at 100 µg/ml. Rifampin was not inhibitory at either of these concentrations.

7.1.3.6 DNA base composition

The guanine-plus-cytosine ratios of the DNA was 52.0±1.0 mol%.

7.1.4 DISCUSSION

The isolate described here has been placed in the genus *Spirochaeta* on the basis of it being free-living, anaerobic, resistant to rifampin and having a morphology typical of *Spirochaeta* species (Canale-Parola 1984). Electron microscopy revealed the typical outer sheath enclosing the helical protoplasmic cylinder and two overlapping periplasmic flagella. Strain RI 19. B1 is similar to the other *Spirochaeta* species in its ability to grow on a wide variety of sugars but not on amino acids or organic acids. Growth in the extremely thermophilic temperature range and utilization of polysaccharides by this isolate extends the growth and metabolic activities of the genus *Spirochaeta*.

The glucose fermentation products differ from previously described species which produce ethanol as a major end product (the single exception is *S. zuelzeriae*, which does not produce ethanol but produces succinate (Canale-Parola 1984)). Strain RI 19. B1

does not produce either ethanol or succinate from the fermentation of glucose. Strain RI 19. B1 apparently has a requirement for CO₂ to allow optimum growth. Such requirements for many organisms have been documented and growth is often poor at low CO₂ concentrations (Pirt 1975). Once growth has been initiated CO₂ production from glucose should meet any requirement for biosynthetic reactions.

Future enrichments using the bicarbonate buffered medium described and used in this study may yield strains of *Spirochaeta* similar to RI 19. B1 from other thermophilic environments such as the cyanobacterial mat from which a 16S rcDNA sequence having 94.3 % similarity with the sequence of *S. halophila* was isolated (Ward et al. 1990). Members of the genus *Spirochaeta* may also exist in marine thermal springs from which *Thermotoga* strains have been isolated.

On the basis of the extremely thermophilic and polysaccharolytic nature of strain RI 19. B1 which distinguishes it from previously described species we propose it be included in the genus *Spirochaeta* as a new species. Before assigning strain RI 19. B1 a species name it was compared with strain Z-1203 (Svetlichny et al. 1990a; Aksenova et al. 1990) (see section 7.2).

7.2 SPIROCHAETA THERMOPHILA, SP. NOV., AN OBLIGATELY ANAEROBIC POLYSACCHAROLYTIC EXTREMELY THERMOPHILIC BACTERIUM

7.2.1 INTRODUCTION

The isolation and characterization of an obligately anaerobic, polysaccharolytic, extremely thermophilic spirochete, *Spirochaeta* sp. strain RI 19. B1, from a brackish (0.76 % NaCl) hot spring of neutral pH, on Raoul Island, Kermadec archipelago, New Zealand is described above (see chapter 3). A similar organism, strain Z-1203, was isolated from a marine hot spring on the beach of Shiashkotan Island, Kuril Islands, Kamchatka USSR (Svetlichny et al. 1990a; Aksenova et al. 1990). The two strains were further characterized and strain Z-1203^T (T=type strain) designated the type strain of a new species, *Spirochaeta thermophila*.

7.2.2 MATERIALS AND METHODS

7.2.2.1 Strains

Strain RI 19. B1 (= DSM 6192) and strain Z-1203^T (= DSM 6578) were obtained from culture collections in New Zealand and the USSR, respectively. The isolates described by Patel et al (1985b) did not survive long term storage and could not be used in this study.

7.2.2.2 Culture methods

Strain RI 19. B1 was grown on bicarbonate buffered medium CBM4 as described above (see section 7.1.2.1). Strain Z-1203^T was grown on bicarbonate buffered medium CBM15, which had the same composition as CBM4 with the NaCl and MgCl₂·6H₂O concentrations raised to 15 g/litre and 2 g/litre respectively.

7.2.2.3 Analytical techniques

The physiological and metabolic properties, antibiotic sensitivity and the DNA base composition were determined as described above (see section 7.1.2.1). DNA-DNA homology between the two strains was calculated from the data of optical reassociation kinetics using the methods of DeLey et al. (1971).

7.2.3 RESULTS AND DISCUSSION

7.2.3.1 Thermophilic spirochete strains

Strains RI 19. B1 was isolated as described in chapter 3. Strain Z-1203^T was isolated from a marine hot spring near the beach of Shiashkotan Island, Soviet Far East (Svetlichny et al. 1990a; Aksenova et al. 1990). The salient characteristics of the two strains and those of strain Rt3-BS1 described by Patel et al (1985b) are shown in Table 7.3. The morphology of strains RI 19. B1 and Z-1203^T, together with their free-living nature and obligately anaerobic metabolism, places them in the genus *Spirochaeta*. The two isolates have guanine-plus-cytosine ratios of 52 mol %, their level of homology as determined by DNA-DNA hybridization is 87 %.

7.2.3.2 Morphological characteristics

The helical cells of both strains exhibited regular coiling. Spherical bodies and tails similar to those observed in other spirochetes (Breznak and Canale-Parola 1975) were present in stationary phase. Electron microscopy revealed morphological features similar to those observed in other spirochetes (Fracek and Stolz 1985; Greenberg and Canale-Parola 1976), including two periplasmic flagella and a central coiled protoplasmic cylinder, both of which were surrounded by a crenulated outer sheath. The periplasmic flagella were present in a 1-2-1 arrangement and were subterminally anchored by insertion discs. Thus, the two strains had morphological properties consistent with the properties of members of the genus *Spirochaeta* (Canale-Parola 1981; Canale-Parola 1984).

7.2.3.3 Nutritional characteristics

Both strains utilized a wide range of monosaccharides, disaccharides and polysaccharides as energy sources; these included D-amygdalin, L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, sucrose, D-xylose, amorphous cellulose, microcrystalline cellulose (Avicel), glycogen, pullulan, starch and xylan. Substrates that were not utilized included erythritol, glycerol, inositol, lactose, D-mannitol, melezitose, melibiose, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, L-sorbose, acetoin, trisodium citrate, sodium formate, disodium fumarate, disodium DL-malate, sodium pyruvate, disodium succinate, L-arginine, sodium L-glutamate and L-lysine. Growth did not occur via the reduction of fumarate, nitrate, oxygen, sulfate or

sulfur. Indole was not formed from L-tryptophan. Urea was not hydrolysed, nor was sulfide produced from L-cysteine. Esculin was hydrolysed.

Glucose was fermented to acetate, carbon dioxide, hydrogen and lactate. Strain Z-1203^T also produced isobutyrate as a minor end-product when grown in the presence of yeast extract. Studies of the enzymology of the metabolic pathways of these strains (Janssen and Morgan unpublished results) showed glucose was fermented via Embden-Meyerhof-Parnas pathway and involved a pyrophosphate-dependent phosphofructokinase (EC 2.7.1.90) rather than an ATP-dependent phosphofructokinase (EC 2.7.1.11). Strain Z-1203^T has a growth requirement for yeast extract which can be replaced by single amino acids including L-alanine, L-glutamate, L-valine and L-isovaline.

7.2.3.4 Cultural characteristics

Growth of both strains was inhibited by penicillin, neomycin, erythromycin, tetracycline, polymyxin B and novobiocin at 100 µg/ml. Rifampin and streptomycin at 100 µg/ml were not inhibitory to either strain. The differences in the temperature and NaCl ranges shown in Table 7.3 may reflect the conditions prevailing at the site of isolation. The isolation of a similar strain from both a marine and a brackish thermal spring would encourage the search for further strains from freshwater thermal springs. The strains of Patel et al (1985b) did not grow above 60°C while strains RI 19. B1 and Z-1203^T grew to 73°C.

7.2.3.5 Taxonomic position

The morphology of the strains RI 19. B1 and Z-1203^T, together with their free-living nature and obligately anaerobic metabolism places them in the genus *Spirochaeta*. These organisms differ from previously described strains in their extremely thermophilic and polysaccharolytic nature. None of the previously described members of the genus *Spirochaeta* grow at temperatures above 60°C and none have been shown to be polysaccharolytic.

Table 7.3 The characteristics of thermophilic spirochetes strains RI19. B1, Z-1203^T and Rt3-BS1

Strain	Cell size μm	Temperature range (opt) $^{\circ}\text{C}$	NaCl range (opt) %	pH range (opt)	Ethanol from glucose	Streptomycin MIC ($\mu\text{g/ml}$)	G+C mol %	Source of isolate
Z-1203 ^T	0.2 - 0.25 x 16 - 20	40 - 73 (66 - 68)	0.5 - 4.5 (1.5)	5.9 - 7.7 (7.5)	-	>100	52	Marine hot spring
RI 19.B1	0.2 x 10 - 50	44 - 73 (64 - 66)	0.1 - 2.5 (0.4)	6.2 - 7.1 (6.9)	-	>100	52	Brackish hot spring
Rt 3-BS1 ^a	0.15 - 0.2 x 15 - 25	20 - <60 (45 - 50)	NA	6.0 - 8.0 (7.0 - 7.5)	+	<10	NA	Freshwater hot spring

^a Data from Patel et al. 1985b. ^T Type strain. NA = Data not available.

The glucose fermentation end products differ from the previously described species, most of which produce ethanol as a major end product, the single exception being *S. zuelzeriae*, which does not produce ethanol but produces succinate (Canale-Parola 1984). Strains RI 19. B1 and Z-1203^T do not produce either ethanol or succinate from the fermentation of glucose. The trace amount of isobutyrate produced by strain Z-1203^T has been shown to be a product of growth in the presence of yeast extract. Isobutyrate production has been demonstrated in the marine mesophilic spirochete *S. isovalerica* (Harwood and Canale-Parola 1983). The differences between strains RI 19. B1 and Z-1203^T and the other previously described members of the genus *Spirochaeta* (Canale-Parola 1984) are great enough to warrant a new species. A new species, *Spirochaeta thermophila*, is proposed for these isolates, with strain Z-1203^T (= DSM 6578) as the type strain.

7.2.3.6 Species description

***Spirochaeta thermophila* sp. nov.** ther.mo'phi.la. Gr. n. *therme* heat; Gr. adj. *phila* loving; M. L. adj. *thermophila* heat-loving. Single helical cells 0.2-0.25 μm by 16-50 μm . Negative Gram stain reaction. No lysis of the cells occurs in 3 % KOH. An outer sheath encloses a protoplasmic cylinder; two periplasmic flagella are present in a 1-2-1 arrangement and are subterminally anchored by an insertion disc. Strictly anaerobic chemoorganotroph. The temperature range for the type strain is 40 to 73°C (optimum, 66 to 68°C). The pH range for the type strain is 5.9 to 7.7 (optimum, 7.5). The NaCl range for the type strain is 0.5 to 4.5 % (optimum, 1.5 %) (doubling time, 70 min). The temperature, pH, and salinity parameters vary for different strains, reflecting the environmental conditions prevailing at the site of isolation. No reduction of fumarate, nitrate, oxygen, sulfate or sulfur occurs. Utilizes various mono-, di- and polysaccharides but not sugar alcohols, organic acids, or amino acids. Inhibited by penicillin, neomycin, erythromycin, tetracycline, polymyxin B, and novobiocin but resistant to rifampin and streptomycin. Fermentation end-products from glucose are acetate, carbon dioxide, hydrogen and lactate. Glucose fermented via Embden-Meyerhof-Parnas pathway, involving a pyrophosphate-dependent phosphofructokinase. Ethanol and succinate are not produced. Indole is not formed; urea is not hydrolysed; sulfide is not produced from cysteine, and esculin is hydrolysed. The guanine-plus-cytosine content of the DNA is 52 mol % (as determined by the thermal denaturation method). Type strain Z-1203^T (= DSM 6578) was isolated from a marine hot spring near the beach of Shiashkotan Island, Kuril Islands, Soviet Far East, USSR.

7.3 THE 16S rDNA ANALYSIS OF *SPIROCHAETA THERMOPHILA*: ITS PHYLOGENETIC POSITION AND IMPLICATIONS FOR THE SYSTEMATICS OF THE ORDER *SPIROCHAETALES*

7.3.1 INTRODUCTION

Ribosomal RNA oligonucleotide cataloging and 16S rRNA sequencing have shown the spirochetes to form a deep and coherent evolutionary group (Paster et al. 1984; Woese 1987; Stanton et al. 1991). Two subgroups have been demonstrated within the phylum, one containing the leptospiras and the other the spirochetes, treponemas etc. From a phylogenetic point of view, however, the genus *Spirochaeta* is not coherent (Paster et al. 1984) as two *Spirochaeta* species (*S. stenostrepta* and *S. zuelzeriae*) were shown to cluster in the *Treponema* group. The phenotypic diversity of this group was increased with the description of two strains of a thermophilic spirochete, i.e. *S. thermophila* (see section 7.2) one strain isolated from a brackish thermal spring on Raoul Island, New Zealand (see chapter 3), the other from a marine thermal spring, Kuril Islands, Soviet Far East (Svetlichny et al. 1990a; Aksenova et al. 1990) that have ability to grow optimally at 65°C. The phylogenetic position of extreme- and hyperthermophilic organisms has attracted considerable interest in that many of these organisms, i.e. thermotogas, thermi, thermomicrobia and the many archaeas actually represent descendants of ancient organisms. Here the phylogenetic position of *S. thermophila* strain RI 19. B1 is determined its 16S rDNA sequence is compared with the spirochete like sequences of Ward et al. (1990) and some aspects of the systematics of the order *Spirochaetales* are considered.

7.3.2 MATERIAL AND METHODS

7.3.2.1 Growth conditions

Spirochaeta thermophila strain RI 19. B1 and *Spirochaeta thermophila* strain Z-1203^T were cultivated in bicarbonate buffered medium 4 (CBM4) and 15 (CBM15) respectively as described above (see section 7.1.2.1).

7.3.2.2 Obtaining sequence data

The isolation of genomic DNA, amplification of 16S rDNA, purification of PCR products and direct sequencing of PCR products was carried out as described in section 6.2.2.

7.3.2.3 Data analysis

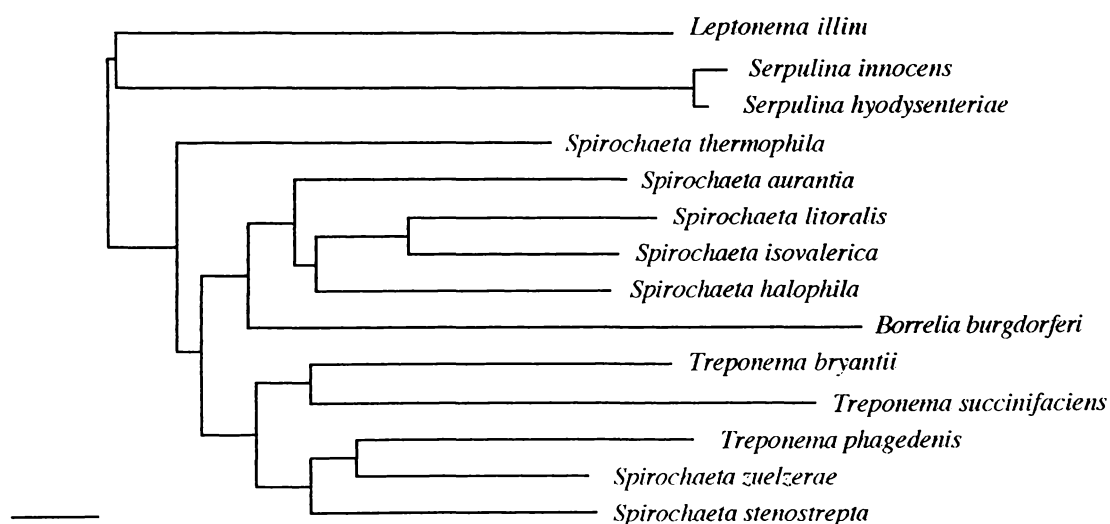
The partial 16S rDNA sequence of *S. thermophila* strain RI 19. B1 was aligned to the 16S rDNA/RNA sequences of 13 species from six spirochete genera which have been either published (Stanton et al. 1991) or are being made available through the Ribosomal Data Project, Urbana, Illinois. The GenBank accession numbers are as follows: *Spirochaeta thermophila* (submitted), *S. halophila* M34262, *S. aurantia* M57740, *S. litoralis* M34263, *S. isovalerica* M34260, *S. zuelzeriae* M34265, *S. stenostrepta* M34264, *Treponema phagedenis* M57739, *T. succinifaciens* M57738, *T. bryantii* M57737, *Serpulina hyodysenteriae* M57743, *Se. innocens* M57744, and *Leptonema illini* M24118. Unsequenced regions of *S. thermophila* (1 through 37 and 1371 through 1542, *E. coli* nomenclature [Woese et al. 1983] and regions of alignment uncertainties due to length variations (68 through 102, 198 through 219, 453 through 479, 904 through 968, 1024 through 1045, 1133 through 1145) were omitted from the analysis. Equally weighted (Hamming) distances and percent similarities were calculated between each pair of sequences. The phylogenetic analysis was carried out by using the neighborliness method (Sattath and Tversky 1977; Fitch 1981). The algorithm is implemented as part of the program package SAGE (Technoma, GmbH, Heidelberg, FRG).

7.3.3 RESULTS AND DISCUSSION

The total length of the analysed 16S rDNA stretch of *S. thermophila* strain RI 19. B1 contained 1333 nucleotides, covering the region between positions 38 and 1370 (*E. coli* numbering system). Attempts to determine the 3' terminus of the gene using the same primer as successfully used in the gene amplification step yielded unsatisfactory sequence information which was therefore omitted from the sequence analysis. A short stretch (position 980 through 1370) of the 16S rDNA sequence of a second strain of *S. thermophila*, namely strain Z-1203^T was analyzed and compared to the homologous region of strain RI 19. B1. The high degree of relatedness between the two strains, originally observed by the finding that their DNAs have 87 % similarity (see section

7.2.3.1), is supported by high sequence similarity of 99.5 % over this region. Consequently, no further attempts were made to obtain the full sequence of strain Z-1203^T.

A matrix of binary similarity values and Hamming distances for *S. thermophila* strain RI 19. B1 and 13 spirochete reference strains from 6 genera is shown in Table 7.4. The Hamming distances (lower left triangle) were used for the generation of an unrooted phylogenetic dendrogram (Figure 7.4). The branching pattern remained stable whether *Sporolactobacillus inulinus*, *Leptotrichia buccalis* or *Thermotoga maritima* were used as a non-spirochete reference organism.



The scale represents 20 nucleotide exchanges.

Figure 7.4 Dendrogram indicating the position of *S. thermophila* strain RI 19. B1 among other representatives of the order *Spirochaetales*.

Table 7.4 Percentage similarity between partial 16S rDNA sequences of *Spirochaeta thermophila* strain RI19. B1 and other members of the order *Spirochaetales* (above the diagonal) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal)

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.
1. <i>Spirochaeta aurantia</i>		86.0	86.0	85.7	78.9	81.2	77.4	82.5	81.0	85.5	82.9	74.7	75.2	77.4
2. <i>Spirochaeta litoralis</i>	165		90.5	86.7	78.1	81.0	76.2	83.5	80.1	82.6	84.4	75.3	75.3	76.9
3. <i>Spirochaeta isovalerica</i>	165	112		87.8	78.3	81.6	77.5	84.8	81.2	83.9	84.5	76.5	76.7	77.5
4. <i>Spirochaeta halophila</i>	169	157	144		78.9	81.6	78.1	83.6	81.5	84.8	86.1	79.2	78.8	77.0
5. <i>Borrelia burgdorferi</i>	249	249	256	249		76.3	71.0	83.5	77.4	77.1	75.2	72.5	72.7	72.2
6. <i>Treponema bryantii</i>	222	224	217	217	280		81.8	86.4	82.5	84.7	80.9	76.3	76.8	75.9
7. <i>Treponema succinifaciens</i>	267	281	265	259	343	215		79.4	79.1	80.6	79.7	71.5	72.1	73.4
8. <i>Spirochaeta zuelzeriae</i>	207	195	179	194	194	161	243		88.1	89.6	83.5	77.1	77.6	76.1
9. <i>Treponema phagedenis</i>	224	235	222	219	264	207	247	140		84.9	79.9	76.4	76.5	74.7
10. <i>Spirochaeta stenostrepta</i>	171	205	190	179	270	181	229	123	178		84.4	72.3	76.7	76.9
11. <i>Spirochaeta thermophila</i>	202	184	183	165	293	225	240	194	237	184		72.2	76.8	80.0
12. <i>Serpulina hyodysenteriae</i>	298	291	277	245	325	280	336	270	279	280	281		99.0	75.9
13. <i>Serpulina innocens</i>	293	291	275	250	322	274	329	264	277	275	274	12		75.9
14. <i>Leptonema illini</i>	267	273	265	272	328	284	314	282	299	273	236	284	284	

All spirochetes constitute one of the several main lines of descent within the domain Bacteria as defined by Woese et al. (1990) in which members show a higher degree of relationships among each other than to members of any other major lineage (Paster et al. 1984; Woese 1987). In addition, spirochete strains are characterized by a unique set of signature nucleotides of their 16S rRNA/DNA (Woese et al. 1985; Woese 1987). The dendrogram displays the presence of several sublines of descent; three of these, *Leptonema illini*, *Serpulina* species and *Borrelia burgdorferi*, contain one or two species only. The majority of spirochetes, i.e. species of *Spirochaeta* and *Treponema*, form two neighbouring clusters which, as determined by their position within the dendrogram, embrace the most recently evolved spirochete species. As observed previously by 16S rRNA cataloging (Paster et al. 1984) certain organisms which have been assigned to the genus *Spirochaeta*, i.e. *S. stenostrepta* and *S. zuelzeriae*, appear to be more closely related to members of *Treponema* than they are to the main *Spirochaeta* group (Figure 7.4). Members of the latter group will in the following be referred to as the authentic *Spirochaeta* species, i.e. *S. aurantia*, *S. litoralis*, *S. isovalerica* and *S. halophila*. This is done despite the fact that no information is available on the phylogenetic position of the uncultured type species *S. plicatilis* (Canale-Parola 1981; Wayne 1982) since it has not as yet been subjected to rRNA analysis.

16S rDNA analysis clearly confirms the species status of *S. thermophila* as described above (see section 7.2). It branches off close to the common root of the two clusters of treponemas and authentic *Spirochaeta/Borrelia*. *S. thermophila* shows the highest similarities with *Spirochaeta* species (82.9 to 86.15 %) while those with *Treponema* species are slightly lower (79.7 to 80.9 %) (Table 7.4). *S. thermophila* possesses almost all signature nucleotides of spirochete 16S rRNA/DNA (Woese et al. 1985), with two exceptions. The first exception refers to position 52, where strain RI 19. B1 contains a G-residue instead of the A-residue which is unique for spirochetes. A second significant exception from the conserved sequence is the presence of a G-residue at position 1212, usually occupied by a U-residue. There is no explanation for these differences, but the possibility of errors introduced by either *Taq* polymerase or *Sequenase* cannot be excluded. It should also be mentioned that in contrast to the published data (Woese 1987) position 698 is occupied by a C-residue and not by a G-residue in all spirochetes investigated.

The isolated position of *S. thermophila* between the *Leptonema/Serpulina* and the *Spirochaeta/Borrelia/Treponema* groups is supported by the presence of nucleotides unique to this species (position 182-G; 359-C; 578-G; 763-C; 1216-C, 1257-G, and an inserted C between positions 1361 and 1362). Its almost equidistant relationship to

members of neighbouring sublines is indicated by the distribution pattern of specific nucleotides shared by this species and members of adjacent groups (Table 7.5). It is worth noting that the majority of those nucleotides common to *S. thermophila* and members of the more ancient genera *Serpulina* and *Leptonema* are either G- or C-residues. Apparently they constitute the ancestral character in these positions because many of them are also found in the 16S rDNA sequence of *Thermotoga maritima*, an organism representing an ancient line of descent (Achenbach-Richter et al. 1987). The phylogenetic position of *S. thermophila* in relation to the *Spirochaeta*/*Treponema*/*Borrelia* cluster is supported by the presence of a long version of region 452 to 480 (*E. coli* nomenclature), absent in members of *Leptonema* and *Serpulina*. Genetic and metabolic properties support the intermediate position of the thermophilic strain, eg. the mol% G+C value of the DNA of *S. thermophila* is 52%, falls between the values described for the members of *Spirochaeta* (50 to 66 %) and *Treponema* (37 to 46 %) (see **Smibert** 1984), and *S. thermophila* and *T. succinifaciens* are the only two spirochetes reported to have polysaccharolytic activities.

A stretch of 21 nucleotides located between positions 290 and 310 (GGG GUC UGA GAG GAU GGC CCC) is so unique to *S. thermophila* that it could be used for species identification via sequencing, PCR assay or oligonucleotide probing of pure isolates or environmental samples. Taxon-specific sequence information is becoming increasingly useful in molecular ecology studies where initial information is in the form of DNA or RNA sequences (Giovannoni et al. 1990; Stackebrandt 1992).

Recently, Ward et al. (1990) described the recovery of 16S rcDNA sequences from a cyanobacterial mat of Octopus Spring (Yellowstone, Mo, USA). We have used 358 nucleotides (position 990 through 1370, excluding ambiguous sequence stretches) of three type H sequences as defined by Ward et al. (1990), for comparison with *S. thermophila*. The matrix of binary similarity values and Hamming distances for *S. thermophila* strains RI 19. B1, Z-1203^T, the type H sequences and five *Spirochaeta* species is shown in Table 7.6. On the basis of this rather small data set, the three type H sequences were almost identical to each other (95.3 %) showing an almost equidistant relationship to the sequences of *S. halophila* (90.8 %) and *S. thermophila* (88.9 %). The latter two species shared 87.4 % similarity in this region. Sequence differences together with the ecological niche from which these sequences were recovered were interpreted as belonging to an as yet uncultivated thermophilic spirochete (Ward et al. 1990). This work has confirmed their observation but results of this study exclude the possibility that these Octopus spring sequences of type H belong to *S. thermophila*.

Table 7.5 Distribution pattern of specific nucleotides showing the isolated, intermediate position of *S. thermophila* strain RI19. B1 among the spirochetes from various genera.

Position	Nucleotide	Organisms													
		<i>Sp. t.</i>	<i>Sp. h.</i>	<i>Sp. a.</i>	<i>Sp. i.</i>	<i>Sp. l.</i>	<i>Sp. z.</i>	<i>Sp. s.</i>	<i>T. b.</i>	<i>T. p.</i>	<i>T. s.</i>	<i>B. b.</i>	<i>Se. i.</i>	<i>Se. h.</i>	<i>Ln. i.</i>
¹ Position															
48	C						+	+	+	+	+	+	+	+	+
108	G						+	+	+		+				
111	G		+	+	+	+									+
241-285	C-G						+	+	+	+		+	+	+	+
269	U		+	+	+	+			+		+	+	+	+	
366	C		+	+	+	+					+	+	+	+	+
409-433	G-C										+	+			
442-492	C-G								+	+					
443-491	C-G							+	+						+
449	A						+	+		+		+	+	+	+
489	C														+
490	U														+
534	U											+	+	+	+
564	C												+	+	
681-709	C-G												+	+	+
713	G												+	+	+
840-846	G-C		+		+	+									
986-1219	A-U		+	+	+	+					+				
1145	C									+		+	+	+	
1154	G		+				+	+	+	+		+	+	+	+
1155	G						+	+	+	+		+	+	+	+
1295	U		+	+	+	+			+		+				+

Abbreviations: *Sp. t.*, *Spirochaeta thermophila*, *Sp. h.*, *S. halophila*, *Sp. a.*, *S. aurantia*, *Sp. i.*, *S. isovalerica*, *Sp. l.*, *S. litoralis*, *Sp. z.*, *S. zuelzeriae*, *Sp. s.*, *S. stenostrepta*, *T. b.*, *Treponema bryantii*, *T. p.*, *Treponema phagedenis*, *T. s.*, *Treponema succinifaciens*, *B. b.*, *Borrelia burgdorferi*, *Se. i.*, *Serpulina innocens*, *Se. h.*, *Se. hyodysenteriae*, *Ln. i.*, *Leptonema illini*. ¹ Positions refer to the *E. coli* numbering system (Woese et al. 1983).

Table 7.6 Percentage similarity between partial 16S rDNA sequences (358 nucleotides) of *Spirochaeta thermophila* strain RI19. B1, *Spirochaeta thermophila* strain Z-1203^T, type H sequences from Octopus Spring, other members of the genus *Spirochaeta* and an unrelated organism *Sporolactobacillus inulinus* (above the diagonal) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal).

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1. <i>Spirochaeta halophila</i>		87.4	87.1	90.5	91.1	90.2	87.1	87.4	87.7	79.3
2. <i>Spirochaeta thermophila</i> (6192)	45		99.7	88.8	89.1	88.5	86.9	90.5	88.5	82.1
3. <i>Spirochaeta thermophila</i> (6578)	46	1		88.5	88.8	88.3	86.6	90.2	88.3	81.8
4. Type H sequence OS	34	40	41		99.7	99.2	88.3	88.0	87.7	81.3
5. Type H sequence OSI	32	39	40	1		99.4	88.8	88.3	88.0	81.8
6. Type H sequence OSII	35	41	42	3	2		88.3	87.7	87.4	81.0
7. <i>Spirochaeta aurantia</i>	46	47	48	42	40	42		89.9	90.2	82.4
8. <i>Spirochaeta litoralis</i>	45	34	35	43	42	44	36		94.1	84.1
9. <i>Spirochaeta isovalerica</i>	44	41	42	44	43	45	35	21		83.1
10. <i>Sporolactobacillus inulinus</i>	74	64	65	67	65	68	63	57	61	

7.3.4 CONCLUSION

The finding that validly-described species of the genus *Spirochaeta* occupy three different positions in the phylogenetic tree of spirochetes brings into question the present taxonomy of this group. In order to obtain phylogenetic coherence, two alternative solutions to this problem exist. Firstly, all non-*Spirochaeta* species, i.e. borrelias, treponemas and eventually *Cristispira* (once its phylogenetic position becomes available) included in the confines of the phylogenetically defined *Spirochaeta* cluster, must be transferred into the genus. In this case, the family-specific characters could be used to circumscribe the amended genus *Spirochaeta*. On the other hand, the definition of this genus would then be extremely broad, containing both free -living and host-associated organisms, obligate and facultative anaerobes as well as aerobes, and a DNA mol% G+C range of 36 to 65 %. Such a solution would not improve the taxonomy of this group. Secondly, the genus *Spirochaeta* could be split into two genera, one containing the authentic species, and a second for *S. thermophila*. Significant phenotypic characteristics such as extreme thermophily, polysaccharolytic activity, and the lack of ethanol and/or succinate formation from the fermentation of glucose, along with its phylogenetic position may enable *S. thermophila* to be assigned to a separate genus. *S. zuelzeri* should be reclassified as a member of *Treponema*, the genus to which it was originally assigned on the basis of serology (Veldkamp 1960). In order to maintain the phylogenetic coherency of *Treponema* (Figure 7.4), *S. stenostrepta* should also be transferred into this genus.

The problem we face at present is the lack of information regarding which group the type species of both *Spirochaeta* and *Treponema*, (*S. plicatilis* and *T. pallidum*) belong phylogenetically (although the the 16S rRNA of the latter species has apparently been sequenced [Stanton et al. 1991], the sequence has not as yet been made available). Previous attempts to replace the uncultured type species *S. plicatilis* by the cultured organism *S. stenostrepta* failed, because of rule 18a of the Bacteriological Code (Lapage et al. 1975), which "does permit a description to serve in place of a type culture for a taxon that has not yet been cultivated in the laboratory". On the basis of the phylogenetic information now available the choice of *S. stenostrepta* as a neotype would have been an unwise decision. Until the phylogenetic position of these uncultured type strains is determined (as recently done for a *Cristispira* strain [Pelletier et al. 1991]) no further progress in the systematics of the *Spirochaetales* is possible.

7.4 GENERAL CONCLUSION

The isolation of *S. thermophila* (Strain RI 19. B1) has extended the phenotypic diversity of the organisms classified within the *Spirochaetales*. The designation of strains Z-1203^T and RI 19. B1 to the genus *Spirochaeta* extends the diversity of this genus to include organisms that are extremely thermophilic in their growth temperature range and organisms that have the ability to utilize polysaccharides including cellulose. The ability of *S. thermophila* to degrade polysaccharides raises some questions about the role of spirochetes in the natural environment where they were previously considered to utilize only the carbohydrates released from the metabolic activity of other microorganisms. Future studies may show that spirochetes do play a role in the degradation of polysaccharides in non-thermophilic environments. If this is not the case then it may be considered that the more ancient member of the genus *Spirochaeta* is the only member of this group with the ability to metabolize more complex carbohydrates.

It has been found that two isolates from geographically distant sites have very high similarity both in phenotypic and genotypic characteristics, the only differences being their NaCl and pH optima which reflect the environment from which they have been isolated. In the case of strain RI 19. B1 it may have adapted to the brackish conditions when introduced from the marine to the lower NaCl environment as a result of the physical changes in its habitat.

The phylogenetic study in which the 16S rRNA/DNA sequence information on a large range of organisms from the *Spirochaetales* was brought together for analysis supported the previous phylogenetic tree obtained from catalogue information. *S. thermophila* was found to have most of the signature nucleotides of spirochete 16S rRNA/DNA. The 16S rDNA analysis clearly confirmed the species status of *S. thermophila* which branches off close to the common root of the two clusters of *treponema* and authentic *Spirochaeta/Borrelia*. The results of Ward et al (1990) that showed sequences recovered from a thermophilic cyanobacterial mat were from an as yet uncultured spirochete have been confirmed and the possibility that *S. thermophila* was that organism has been excluded. A stretch of 21 nucleotides unique to *S. thermophila* has been identified which could be used in identification of pure isolates or the detection of this organism in environmental samples. Such studies would be useful in determining the distribution of *S. thermophila* in the thermal spring environment. The present taxonomy of the *Spirochaetales* is brought into question by the finding that validly described species of the genus *Spirochaeta* occupy three different positions in the phylogenetic tree.

7.5 ADDENDUM

Paster et al. (1991) carried out a full phylogenetic analysis of the spirochetes. 16S rRNA sequences were determined for species of the genera *Spirochaeta*, *Treponema*, *Borrelia*, *Leptospira*, *Leptonema*, *Serpulina*, and various unidentified spirochetes including a moderately thermophilic isolate. The analysis showed the spirochetes to form a coherent taxon composed of six distinct and deeply branching groups: *Treponema*, *Spirochaeta*, *Borrelia*, *Serpulina*, the leptospirae and a single member group containing the shrew isolate. The phylogenetic tree produced in the Paster et al. (1991) study (Figure 7.7) shows a high correlation with the tree produced in the study described in section 4.3 (Figure 7.4). The inclusion of a moderately thermophilic spirochete strain H1 (not previously described in the literature), and its grouping within the treponema group further extends the diversity of thermophilic microorganisms. The phylogenetic position of strain H1 clearly differentiates it from *Spirochaeta thermophila* strain RI 19. B1.

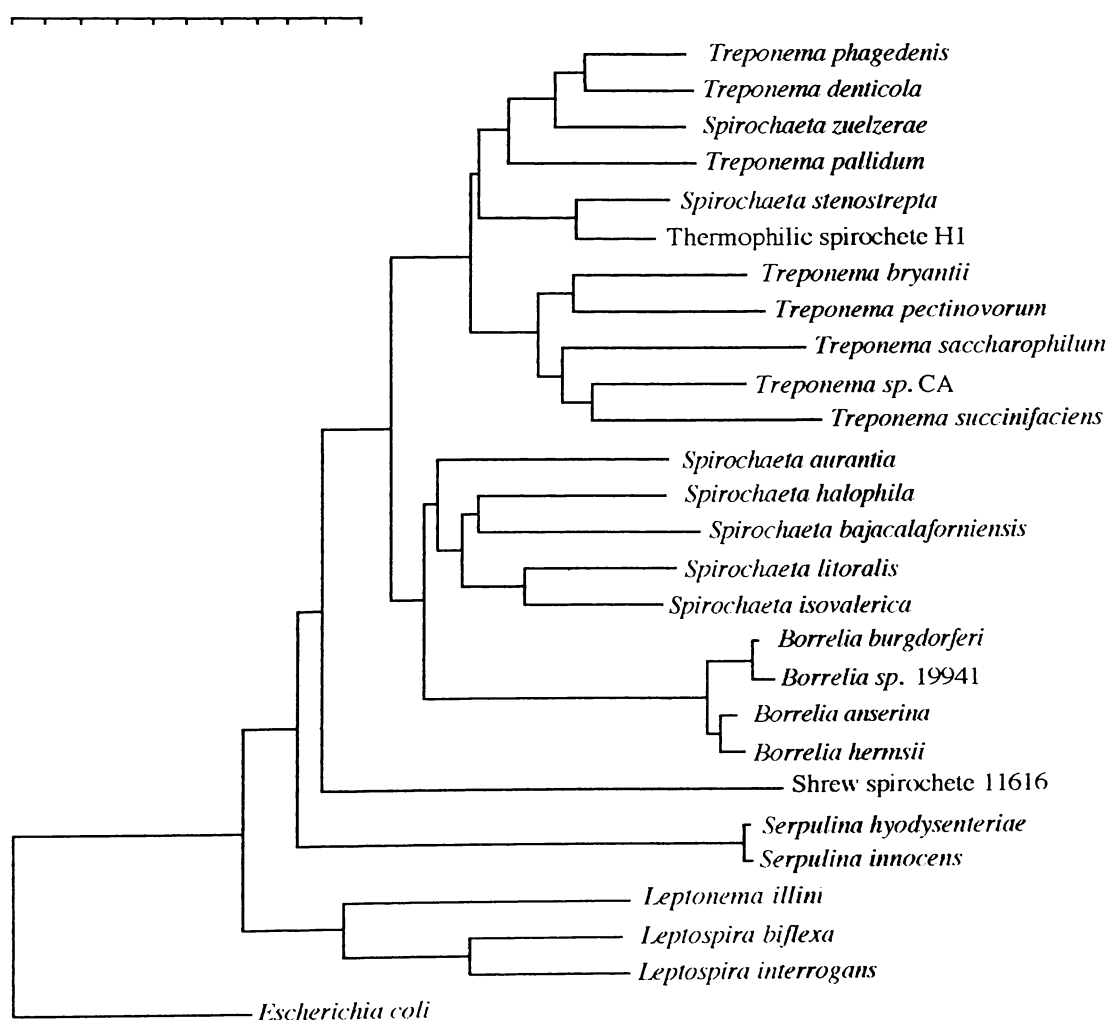


Figure 7.5 Phylogenetic tree of the spirochetes, with *E. coli* as the outgroup. The scale bar represents a 10% difference in the nucleotide sequence as determined by taking the sum of all of the horizontal lines connecting two species. (Reproduced from Paster et al. 1991)

CHAPTER 8

THE CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF AN EXTREMELY THERMOPHILIC BACTERIUM BELONGING TO THE "*THERMOTOGALES*"

INTRODUCTION

The order *Thermotogales* at present contains three genera, *Thermotoga*, *Thermosipho*, and *Fervidobacterium* (Patel et al. 1985a; Huber et al. 1989; Huber et al. 1990). These genera have been defined using both phenotypic and phylogenetic approaches. Phylogenetic studies have shown these genera to represent the deepest branch and the slowest evolving lineage within the bacteria (Huber et al. 1986; Achenbach-Richter 1987; Woese 1987; Huber et al. 1989; Huber et al. 1990).

This chapter describes the characterization of an extremely thermophilic *Thermotoga*-like organism strain RI 19. B2, the determination of its phylogenetic position and its designation to the genus *Thermosipho*. The full description of this organism is included in this thesis on cellulolytic thermophiles because it was found to have cellulase activity, but was not able to grow on cellulose as a sole carbon source.

8.1 INTRODUCTION TO THE *THERMOTOGALES*

The organisms assigned to the order *Thermotogales* are Gram reaction negative, rodshaped, anaerobic thermophilic bacteria. A sheath-like envelope referred to as a "toga" surrounds the cells and usually balloons over the ends. Lipid analysis has shown this group of organisms to have C₂₈ to C₃₄-dicarboxylic fatty acids containing either a single internal 15-methyl or 15, 16-dimethyl branches. The characteristics of these organisms, along with those of the isolate described in this chapter, are shown in Table 8.1.

8.1.1 THE GENUS *THERMOTOGA*

The genus *Thermotoga* contains the species *Thermotoga maritima*, *Thermotoga neapolitana*, and *Thermotoga thermarum* (Huber et al. 1986; Jannasch et al. 1988; Windberger et al. 1989). The ability to grow at temperatures up to 90°C makes *T. maritima* and *T. neapolitana* the most extremely thermophilic microorganisms within the bacteria so far isolated. All of the *Thermotoga* species have been isolated from saline environments, *T. maritima* from marine hydrothermal systems, *T. neapolitana* from both marine and low salt continental hot springs and *T. thermarum* which grows at low salinity from low salt continental hot springs.

T. maritima was the first species assigned to the genus *Thermotoga* on the basis of its unique morphology, growth at 90°C and its phylogenetic position as shown by 16S rRNA sequence analysis (Huber et al. 1986). Jannasch et al. (1988) described a similar organism, strain NS-E, isolated from a submarine thermal vent near Naples, as a second species of the genus *Thermotoga*, namely *T. neapolitana*. The designation of a new species was on the basis of its immotility, growth rate, DNA base composition and DNA homology when compared with *T. maritima*. The third species of the genus *Thermotoga*, *T. thermarum*, was isolated from a geothermally heated continental, low salinity environment in Djibouti and was designated a new species on the basis of the lack of significant DNA homology with *T. maritima* and *T. neapolitana*. It also differed in its ability to grow at low salinity and its high sensitivity to rifampin (1µg/ml). A strain of *T. neapolitana* was also isolated from the same low saline environment (Windberger et al. 1989).

Table 8.1 Comparison of the characteristics of the members of the *Thermotogales* and strain RI 19. B2

	<i>Thermotoga maritima</i>	<i>Thermotoga neopolitana</i>	<i>Thermotoga thermarum</i>	<i>Thermosipho africanus</i>	Strain RI 19. B2	<i>Fervidobacterium nodosum</i>	<i>Fervidobacterium islandicum</i>
Cell size	0.6 x 1.5-11.0µm	0.6 x 1.5-11.0µm	0.6 x 1.5-11.0µm	av. 0.5 x 3.0-4.0µm	0.6-0.7x 2.5-6.0µm	0.5-0.55 x 1.0-2.5µm	0.6 x 1.0-4.0µm
Flagella	+	-	+	-	+	ND ^a	+
Temperature range (optima) °C	55-90 (80)	55-90 (80)	55-84 (70)	35-77 (75)	37-76 (63)	41-79 (70)	50-80 (65)
pH range (optima)	5.5-9.0 (6.5)	5.5-9.0 (7.0)	5.5-9.0 (7.0)	6.0-8.0 (7.2)	6.0-8.0 (7.0)	6.0-8.0 (7.0)	6.8-8.0 (7.2)
NaCl range (optima) %	0.25-3.75 (2.7)	ND	0.2-0.55 (0.35)	0.11-3.6 (ND)	0.1-2.5 (1.0)	ND	ND
H ₂ S produced	+	+	-	+	+	+	+
H ₂ inhibition	+	ND	+	+	+	+	+
YE requirement	+	ND	+	+	+	ND	+
Cysteine requirement	-	-	-	+	-	-	-
Resistance to rifampin (100µg/ml)	+	+	-	-	-	ND	-
mol% G+C	46	41	40	30	32	34	41
End products ^b	L, A	ND	ND	ND	L, A	A, L, E, b ^c , v	A, L, E, b, v

^a ND, Not determined. ^b End products A, acetate; L, lactate; E, Ethanol; b, n-butyrate; v, n-valerate. ^c Trace amounts

8.1.2 THE GENUS *THERMOSIPHO*

Huber et al. (1989) described a new genus within the *Thermotogales* and designated strain Ob7 a new species, namely *Thermosipho africanus*. Strain Ob7 was isolated from a shallow marine thermal system in Djibouti. The cells were morphologically similar to *Thermotoga* species in possessing a sheath-like envelope with ballooning ends. In contrast to *Thermotoga* one tube-like envelope surrounded up to twelve *Thermosipho* cells. *Ts. africanus* showed other differences to the previously described *Thermotoga* species, these included a much lower growth temperature range (35–77°C), a lower G+C content of the DNA (30 mol%) and no serological cross-reaction of the RNA polymerase with the same enzyme from *T. maritima*. Phylogenetic studies of the 16S rRNA sequence were reported to show *Ts. africanus* to exist at the level of a new genus within the *Thermotogales* branch. The fact that *Ts. africanus* required cysteine for growth and was unable to grow on carbohydrates also differentiated it from the *Thermotoga* species.

8.1.3 THE GENUS *FERVIDOBACTERIUM*

The genus *Fervidobacterium* was described by Patel et al. (1985a), the type species *F. nodosum* having been isolated from a freshwater hot spring in New Zealand. This rod-shaped, Gram-reaction negative, anaerobic thermophilic bacterium was shown to form spheroids in the late phase of growth. It was not until Huber et al. (1990) isolated a second species of the genus *Fervidobacterium*, namely *F. islandicum*, that the phylogenetic position of the genus was determined. 16S rRNA sequence analysis showed *F. nodosum* and *F. islandicum* to belong to the order *Thermotogales*. Other similarities between the *Fervidobacterium* species and the other members of the *Thermotogales* include the spheroid-forming outer layer which may be a structural homologue of the toga, the possession of lipids up to now only found in members of the *Thermotogales*, and inhibition by hydrogen. The differences reported between *F. nodosum* and *F. islandicum* include the growth of *F. islandicum* being stimulated by the addition of cellulose, the 6% higher G+C content of *F. islandicum* (40 mol%) and the lack of significant DNA homology between the two species (Huber et al. 1990).

8.2 CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF STRAIN RI 19. B2

8.2.1 INTRODUCTION

The isolation of a *Thermotoga*-like organism designated strain RI 19. B2 was described in chapter 3 section 3.3. The cells had the characteristic sheath-like structures of members of the *Thermotogales*. Below is a description of the full characterization and phylogenetic analysis of strain RI 19. B2, on the basis of which it was assigned to the genus *Thermosipho*.

8.2.2 METHODS AND MATERIALS

8.2.2.1 Medium and culture conditions

The new isolate was grown in bicarbonate-buffered medium 10 (CBM10). CBM10 had the same composition as CBM4 (section 7.1.2.1) with the NaCl and the $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ increased to 10.0g/l and 1.5g/l respectively. To enhance the growth of strain RI 19. B2 yeast extract and trypticase were added to CBM10 at 1g/l and 2g/l respectively (CBM10YT). All incubations were at 65°C unless otherwise stated.

8.2.2.2 Characterization methods

The methods used for electron and light microscopy, growth and metabolic characterization, and determination of G+C content were as described in chapter 7, section 7.1. The utilization of carbon sources was tested in CBM10YT, the test substrate being added at 1g/l. After 18h incubation the OD_{650} was recorded. Polysaccharolytic degradation was tested by the radial diffusion method as described in section 4.2.2.6.

8.2.2.3 Phylogenetic analysis

The genomic DNA isolation, the amplification and sequencing of the 16S rDNA was as described in chapter 6. The partial 16S rDNA sequence of strain RI 19. B2 was aligned with the 16S rDNA/RNA sequences of four members of the *Thermotogales* which were made available through the Ribosomal Data Project, Urbana, Illinois. Unsequenced regions of RI 19. B2 (1 through 12 and 1371 through 1542, *E. coli* nomenclature

(Woese et al. 1983) and regions of alignment uncertainties due to length variation (70 through 97, 190 through 225, 406 through 481, 839 through 845, 999 through 1037, 1128 through 1145, 1198 through 2012) were omitted from the analysis. Equally weighted (Hamming) distances and percent similarities were calculated between each pair of sequences. The phylogenetic analysis was carried out by using the neighborliness method (Sattath and Tversky 1977; Fitch 1981). The algorithm is implemented as part of the program package SAGE (Technoma, GmbH, Heidelberg, FRG).

8.2.3 RESULTS AND DISCUSSION

8.2.3.1 Source of isolate

Strain RI 19. B2 was isolated from sample spring RI 19 taken at the edge of Green Lake, Raoul Island, Kermadec archipelago, New Zealand as described in chapter 3. The characteristics of strain RI 19. B2 are compared in Table 8.1 with those of the members of the *Thermotogales* previously described.

8.2.3.2 Morphology

The new isolate was a rod-shaped bacterium measuring 0.6-0.7 μ m by 2.5-6.0 μ m during growth on CBM10YT with cellobiose (10mM) as a substrate (Figure 8.1 a). The cells gave a negative Gram-staining reaction. The cells were surrounded by a sheath-like structure, ballooning over the ends (Figure 8.1 b). This structure was visible in all phases of growth. Single or pairs of rods were dominant in the early log-phase while chains of four to six cells were present in the mid-log-phase. In the early stationary-phase chains of bacterial cells up to sixteen in length could be observed, all of which were surrounded by the sheath-like structure (Figure 8.1 a). In the late stationary-phase of growth the majority of cells became large spheres. The formation of long chains in early stationary-phase and spheres in late stationary-phase was also reported in *Ts. africanus* (Huber et al. 1989), and used as a characteristic to differentiate it from the isolates of the genus *Thermotoga*.



Figure 8.1 a, b. Strain RI 19. B2. **a.** Negatively stained preparation of early stationary-phase cells in chain formation, surrounded by a sheath-like structure. Bar = $10\mu\text{m}$. **b.** Thin section showing sheath-like structure ballooning over the ends. Bar = $1.0\mu\text{m}$.

8.2.3.3 Physiological and nutritional properties

Strain RI 19. B2 grew over the pH range 6.0 to 8.0 but not at 5.5 or 8.5. The optimum pH for growth was 7.0. Growth occurred over the temperature range 37 to 77°C with an optimum of 63°C. No growth was detected at 34 or 79°C. The NaCl concentration range over which growth occurred was 0.1 to 2.5%, with an optimum around 1.0%. No growth occurred at 3.0%. It can be seen from Table 8.1 that the temperature and salinity ranges permitting growth vary between the different genera and species of the *Thermotogales*. The optimum temperature and salinity values in most cases reflect the environmental conditions prevailing at the sample site. *Ts. africanus* was isolated from a site with a temperature range of 58-70°C (Huber et al. 1989), while *Thermotoga* was isolated from a sample site with a higher temperature range (70-100°C) (Huber et al. 1986). Strain RI 19. B2 was isolated from a sample at 65°C which is within the temperature range of the sample from which *Ts. africanus* was isolated. The NaCl was greater than that of strain *Spirochaeta thermophila* strain RI 19. B1 which was isolated from the same sample indicating that the environmental conditions at the sample site may not be optimal for the microorganisms surviving in them.

Only slight growth occurred in the absence of yeast extract, which indicates that there was a growth factor in the original enrichment sample since the enrichment culture did not contain yeast extract. Strain RI 19. B2 did not grow in CBM10YT minus the sulfide reductant, but both cysteine-HCl (2mM) and Ti-NTA served as reducing agents. Cysteine-HCl was not required for the growth of strain RI 19. B2. This is in contrast to *Ts. africanus* which requires cysteine-HCl for growth (Huber et al. 1989). A range of carbon sources were found to enhance the growth of strain RI 19. B2 as shown in Table 8.2. Substrates tested that did not enhance growth included amino acids, sugar alcohols, cellulose and xylan. Huber et al. (1989) reported that *Ts. africanus* did not grow on a range of carbon sources but did not specify if this was in the presence or absence of yeast extract, therefore a comparison cannot be made. Glucose was fermented to lactate and acetate, ethanol was not detected.

8.2.3.4 Polysaccharide degradation

The ability of strain RI 19. B2 to degrade various polysaccharides was compared with that of the other members of the *Thermotogales*. The results are shown in Table 8.1. Figure 8.2 shows the typical radial diffusion assay plate results, indicating the strong polysaccharolytic activity detected.

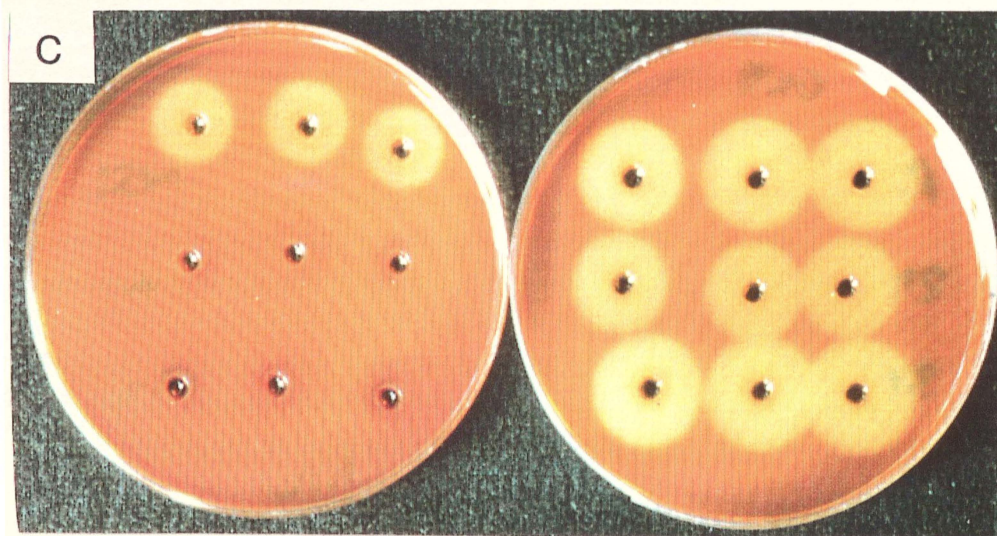
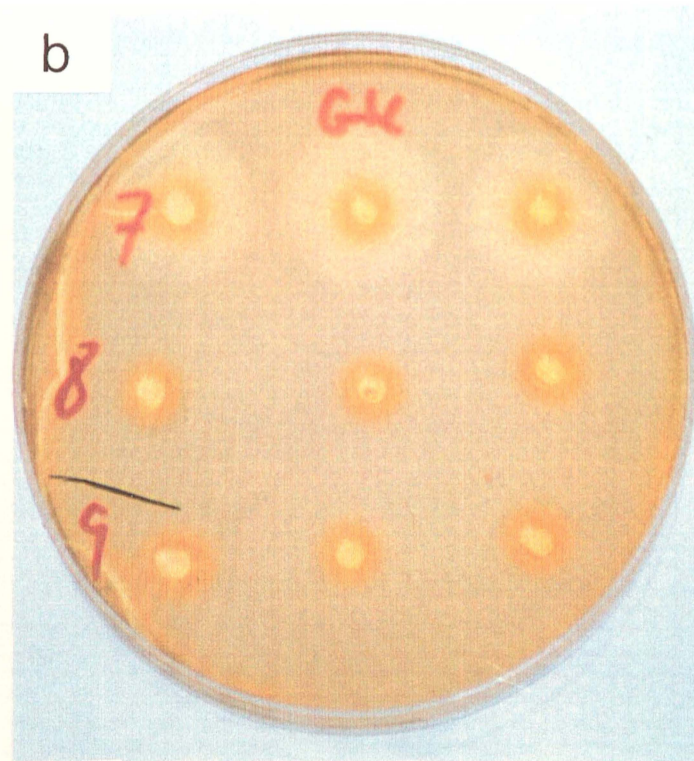
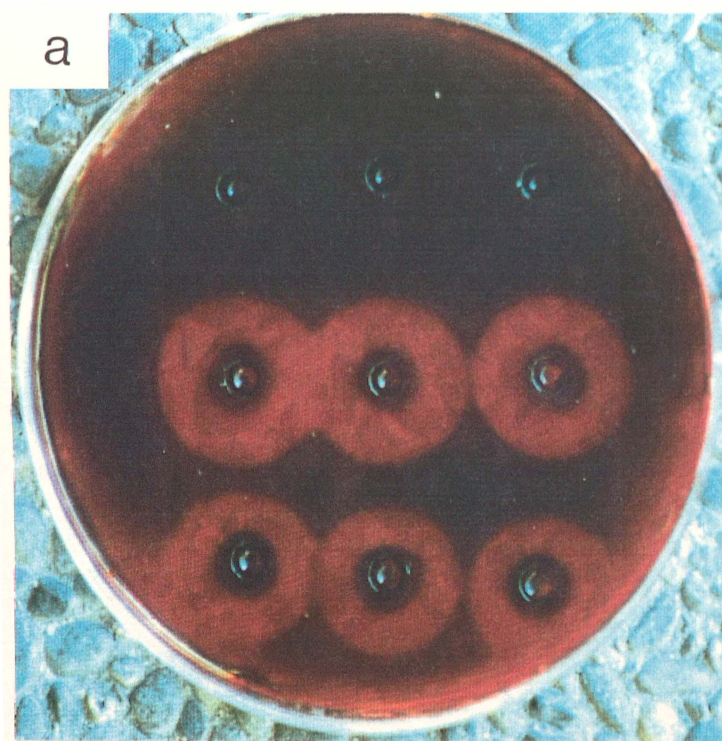


Figure 8.2 a-c. Typical radial diffusion assay plate results, showing polysaccharolytic activity. **a.** CMC plate. **b.** Gum karaya plate. **c.** Glycogen plate. Plates stained as described in section 4.2.2.6.

These results show RI 19. B2 to have a range of polysaccharolytic activities which are also present in other organisms of the *Thermotogales*. These results correlate with the reports of cellobiohydrolase activity in a *Thermotoga* species (Ruttersmith and Daniel 1991) and the study of amylase activity in *T. maritima* (Schumann et al 1991).

Table 8.2 Polysaccharolytic activity in *Thermotogales*

Polysaccharide	<i>T. mar</i>	<i>T. neap</i>	<i>Ts. afric</i>	RI 19. B2
Starch	-	+	+	+
Xylan	-	-	-	-
Gum Karaya	-	-	+	+
Mannan (yeast)	-	-	-	-
Arabanogalactan	-	-	+	+
Gum Locust Bean	+	+	+	-
Lichenan	+	+	+	+
Laminarin	+	+	+	+
CMC	+	+	+	+

8.2.3.5 Antibiotic sensitivity

Growth of strain RI 19. B2 was inhibited by penicillin, streptomycin, neomycin, and polymyxin at 10 μ g/ml. No inhibition was observed when rifampin was added at 10 μ g/ml but at 100 μ g/ml it was found to be inhibitory. This inhibitory effect of rifampin at 100 μ g/ml was also found in *Ts. africanus* (Huber et al. 1989). *T. maritima* and *T. neapolitana* were both found to be resistant to rifampin at 100 μ g/ml (Huber et al. 1986; Jannasch et al. 1988).

Table 8.3 Substrates enhancing the growth of strain RI 19. B2

Substrate	OD ₆₅₀ at 18h
No addition	0.13
Cellobiose	0.50
D-Glucose	0.62
D-Xylose	0.19
D-Fructose	0.65
D-Galactose	0.25
Maltose	0.20
Starch	0.40

8.2.3.6 DNA base composition

The DNA base composition of strain RI 19. B2 was 32 mol% GC as determined by thermal denaturation. This value is similar to that of *Ts. africanus* at 30 mol% GC (Huber et al. 1989) and much lower than the *Thermotoga* species with values in the range 40-46 mol% GC (Huber et al. 1986; Jannasch et al. 1988; Windberger et al. 1989).

8.2.3.7 Phylogenetic analysis

The total length of the 16S rDNA stretch of RI 19. B2 sequenced was 1358 nucleotides, covering the region between positions 13 and 1370 (*E. coli* numbering system). A matrix of binary similarity values and Hamming distances for strain RI 19. B2 and four other members of the *Thermotogales* is shown in Table 8.3. The Hamming distances (lower left triangle) were used for the generation of the phylogenetic dendrogram (Figure 8.2). The branching pattern remained stable when either *Thermococcus celer* or *Thermus thermophilus* were used as non-*Thermotogales* reference organisms.

Table 8.4 Percentage similarity between partial 16S rDNA sequences of strain RI 19. B2 and other members of the *Thermotogales* (above the diagonal) and equally weighted (Hamming) distances between the 16S rDNA sequences (below the diagonal).

	1.	2.	3.	4.	5.
1. <i>F. nodosum</i>		95.8	90.8	91.7	89.5
2. <i>F. islandicum</i>	48		90.3	91.4	88.6
3. RI 19. B2	105	111		99.8	91.5
4. <i>Ts. africanus</i>	95	99	5		92.2
5. <i>T. maritima</i>	120	131	97	89	

On the basis of 16S rDNA sequence RI 19. B2 is most similar to *Ts. africanus*, with 99.8% homology between the two strains. A difference of 8.5% was found over the stretch of sequence analysed between strain RI 19. B2 and *T. maritima*. Huber et al. (1989) in reference to the difference between *T. maritima* and *Ts. africanus* suggested that due to the slow evolving nature of the *Thermotogales* lineage, differences in this range are more significant and would correspond to larger differences in any other eubacterial group. The 16S rDNA sequences of strain RI 19. B2 and *Ts. africanus* were found to differ in only five base positions.

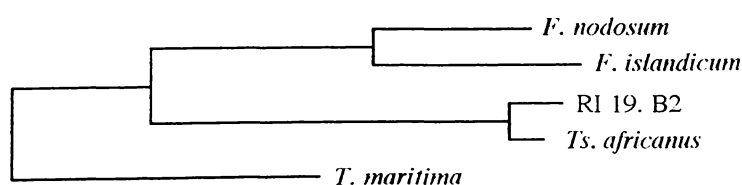


Figure 8.3 Dendrogram indicating the position of strain RI 19. B2 among the other representatives of the *Thermotogales*.

From this data it is clear that strain RI 19. B2 is a member of the genus *Thermosipho* but if it is a new species is not clear. As discussed in chapter 6 organisms with 16S rDNA similarities in the 99.5-99.8 range were shown to have DNA-DNA homology values of less than 50% which is considered within the species definition. DNA-DNA hybridization data may help to determine the true position of strain RI 19. B2 within the genus *Thermosipho*.

8.2.4 CONCLUSION

The new isolate described in this study is a rod-shaped thermophilic bacterium with a surrounding sheath-like envelope characteristic of the members of the *Thermotogales*. Both phenotypic and phylogenetic characteristics have been used in the characterization of strain RI 19. B2. Chain formation of strain RI 19. B2 cells in the early stationary-phase was similar to that observed in *Ts. africanus*. The temperature range of strain RI 19. B2 (37-76°C) was in the range of that described for *Ts. africanus* (35-77°C) and was much lower than that of *T. maritima* (55-90°C). Strain RI 19. B2 had a GC content of 32 mol% which is 14 mol% lower than that of *T. maritima* and similar to that of *Ts. africanus* (30 mol%). In contrast to the resistance of *Thermotoga* species to rifampin at 100 µg/ml, strain RI 19. B2 and *Ts. africanus* are resistant at concentrations of 10 µg/ml but totally inhibited at 100 µg/ml. Strain RI 19. B2 was found to be similar to other members of the *Thermotogales* in expressing polysaccharolytic activities but not utilizing the polysaccharide as the sole carbon source.

Phylogenetic studies of the 16S rDNA sequence revealed that the new isolate is a member of the *Thermotogales* branch and a member of the genus *Thermosipho*. The high degree of homology between strain RI 19. B2 and *Ts. africanus* (99.8%) was in contrast to the lower level of homology between strain RI 19. B2 and *T. maritima* (91.5%). In order to determine the status of strain RI 19. B2 at the species or subspecies level, DNA-DNA hybridization data will be required.

Some phenotypic differences were found between strain RI 19. B2 and *Ts. africanus*. These included the growth enhancement of strain RI 19. B2 by a range of carbon sources, while *Ts. africanus* was reported not to utilize defined carbon sources. The cysteine-HCl requirement of *Ts. africanus* was not found in strain RI 19. B2.

It is interesting that very similar thermophilic strains can be isolated from geographically distant thermal sites; this was also found to be the case with the two strains of *Spirochaeta thermophila* described in chapter 7. It can be suggested that in both cases the differences in the temperature, salinity and pH optima are due to the conditions prevailing at the site of enrichment. It is also interesting to note that the salinity optimum of strain RI 19. B2 (1.0%) is greater than the salinity of the environmental sample (0.7%) and may suggest that the environment from which this strain was isolated was not optimal for its growth. Huber et al. (1989) suggested that isolates similar to *Ts. africanus* may be present in other low saline environments and proposed further isolation studies in order to show the global distribution of this organism.

In this study we described the characterization of a member of the *Thermotogales*, and using a polyphasic approach assigned it to the genus *Thermosipho*. We have shown the genus *Thermosipho* not to be restricted to the original site of isolation (Africa) but to exist in a low salinity thermal spring in the Kermadec archipelago, New Zealand.

CHAPTER 9

GENERAL CONCLUSION

Although conclusions were included at the end of each of the previous chapters, a general conclusion, bringing together the results and their significance, is given below.

The limited isolation study described in chapter 3 demonstrated the use of bicarbonate buffered medium in the isolation of cellulolytic and polysaccharolytic thermophilic anaerobes. This medium enriched a diverse group of organisms. With the exception of the two morphologically distinct isolates, the spirochete and the *Thermosipho* species, of which only one strain was isolated, the rod-shaped isolates from bicarbonate buffered medium enrichments clustered with isolates enriched using phosphate buffered medium containing yeast extract and trypticase. This finding indicates that the presence of yeast extract, trypticase and phosphate buffer did not select for a particular type of organism (i.e. clostridial types). However, this result may have been influenced by the higher incubation temperature of the enrichment cultures, i.e. 70°C.

This study has demonstrated diversity among anaerobic cellulolytic thermophiles, with such organisms being found in three of the major lines of descent: the spirochetes, the *Thermotogales* and the gram-positive bacteria. Highly similar, if not identical, organisms have been found in geographically distant thermal sites, as seen in the case of the two strains of *S. thermophila*, one from the Soviet Far East and one from Raoul Island, New Zealand, with 99.5% rRNA sequence homology and 87% DNA-DNA homology. A similar situation was found with the *Thermosipho* species, strain RI 19. B2, also isolated from Raoul Island and having 99.8% rRNA sequence homology with *Ts. africanus*, which was isolated from Africa. High similarity between isolates from geographically distant sites was also seen in the rod-shaped isolates, with organisms from the Azores, Russia, Italy and New Zealand grouping in the same clusters of the numerical taxonomy study.

The distinct morphological type of the spirochete and the *Thermosipho* species isolates facilitated their identification and their designation to taxonomic groups. The phylogenetic analysis of these organisms confirmed the classification based on phenotypic characteristics.

Spirochaeta thermophila is the first extremely thermophilic polysaccharolytic member of the genus *Spirochaeta* to be described and validly named. The isolation of strain RI 19. B1, its characterization and comparison with strain Z-1203 has not only added to the diversity of the spirochetes as a group, but has also added to the known diversity of thermophilic bacteria. The isolation of strain RI 19. B2 *Thermosipho* species confirmed the suggestion of Huber et al. (1989) that isolates similar to *Ts. africanus* may exist in other low saline environments. The ability of the members of the *Thermotogales* to degrade polysaccharides has been confirmed. The ability of these organisms to degrade but not actively utilize polysaccharides as sole carbon sources will no doubt be the subject of further investigations, since these polysaccharolytic enzymes from the most extremely thermophilic bacteria may be of use in the biotechnology industry

In contrast to the thermophilic spirochete and *Thermosipho* species, the rod-shaped anaerobic cellulolytic thermophilic bacteria could not be distinguished by their morphotype, so the degree of diversity within this group was demonstrated using a polyphasic approach. The numerical taxonomy study demonstrated diversity within this group of organisms and showed the majority of them to be unrelated to the clostridia. But the degree of this unrelatedness could not be determined from the phenotypic data. The numerical taxonomy study provided a large data base of the characteristics of these organisms, which will hopefully be useful to future workers, selecting organisms with desirable characteristics.

The results obtained in the numerical taxonomy study were found to correlate well with the two protein electrophoresis studies described in chapter 5. This was especially true in the case of the isolates of cluster C, which formed a tight cluster in both the numerical taxonomy and protein electrophoresis studies. Visual analysis of the protein banding patterns on the *PhastSystem* gels allowed differentiation between the isolates and designation to clusters similar to those of the numerical taxonomy study. It may be possible to use the *PhastSystem* as a rapid identification system for anaerobic cellulolytic thermophilic bacteria.

A phylogenetic study showed that the representative isolates of clusters A, B, C, D in the numerical study constituted a new sub-line within the *Clostridium/Bacillus* subdivision of the gram-positive bacteria. The similarity values obtained showed the new isolates to be dissimilar to the previously described cellulolytic and non-cellulolytic members of the genus *Clostridium*. They were also shown to be dissimilar to the glycolytic thermophile *Thermoanaerobium brocki*. Although their relationship to the

other glycolytic thermophiles was not determined, if one considers the results of the work of Bateson et al. (1989), these organisms would not be expected to group together.

Strain Rt51. B1 was shown to be similar and closely related to the cellulolytic thermophilic clostridia, *C. stercorarium* by both phenotypic and phylogenetic studies. It is interesting that an organism isolated from a thermal spring is highly similar to *C. stercorarium* which was isolated from a compost system.

In order to assign the isolates from the numerical taxonomy clusters, A, B, C, D to defined taxa, the results of the numerical taxonomy study and the protein electrophoresis should be considered together. It should first be noted that strain NA 10 is in a different position on comparison of the numerical taxonomy results with those of the phylogenetic and protein electrophoresis studies. Strain NA 10 utilizes a wide range of substrates but is morphologically similar to the isolates of clusters C and D. The phylogenetic study showed strain NA 10 to cluster between the representatives of clusters C and D and those of cluster A and B. Although the isolates of Clusters A-D were shown to cluster separately from the other thermophilic clostridia, the degree of dissimilarity is no more than that found between separate clostridial species. This is not surprising, since Cato and Stackebrandt (1989) have demonstrated that the genus *Clostridium* does not constitute a phylogenetically coherent taxon.

On the basis of the phylogenetic data, it could be considered that the non-clostridial type isolates of clusters A, B, C, D constitute a new genus within the *Clostridium/Bacillus* subdivision. This could be substantiated by the phenotypic data, using characteristics such as extreme thermophily, resistance to rifampin and an asporogenous nature. Within this group, differences of approximately 5% separate A, B, C and D isolates at the 16S rRNA level. It could be argued that these groups constitute new species, again phenotypic characteristics could be used to support this. These characteristics include limited substrate utilization range and short cell length, in the case of clusters C and D, or the utilization of specific substrates, for example inulin, in the case of cluster B isolates. Other phenotypic data from the protein electrophoresis study could also be used to support this argument. DNA-DNA hybridization data, when available, may help in the designation of these new isolates to taxa.

It is interesting that the three methods used to determine the diversity and subsequent taxonomic position of a group of anaerobic cellulolytic thermophilic bacteria complement each other and result in a convincing polyphasic taxonomy. Unfortunately, the time

constraints imposed on this study did not permit a DNA-DNA hybridization study to be carried out. This will be the subject of further investigation in time and space!

LITERATURE CITED

- Achenbach-Richter L, Gupta R, Stetter KO, Woese CR** (1987) Were the original eubacteria thermophiles? *Systematic and Applied Microbiology* **9**:34-39
- Aksenova HYu, Svetlichny VA, Zavarzin GA** (1990) *Spirochaeta thermophila* sp. nov., a thermophilic marine spirochete isolated from a littoral hydrotherm of Shiashkotan Island. *Mikrobiologiya* **59**:735-741 (English translation)
- Aksenova HYu, Rainey FA, Jansen PH, Morgan HW, Zavarsin GA** (1992) *Spirochaeta thermophila* sp. nov., an obligately anaerobic polysaccharolytic member of the genus *Spirochaeta*. *International Journal of Systematic Bacteriology* **42**:175-177
- Alexander JK** (1972a) Cellobiose phosphorylase from *Clostridium thermocellum*, p. 944-948. *In* V Ginsburg (ed.), *Methods in Enzymology*, vol. 28. Academic Press, New York
- Alexander JK** (1972b) Cellodextrin phosphorylase from *Clostridium thermocellum*, p. 948-953. *In* V Ginsburg (ed.), *Methods in Enzymology*, vol. 28. Academic Press, New York
- Austin B, Hussong D, Weiner RM, Colwell RR** (1981) Numerical taxonomy analysis of bacteria isolated from the completed "most probable numbers" test for coliform bacilli. *Journal of Applied Bacteriology* **51**:101-112
- Bateson MM, Weigel J, Ward DM** (1989) Comparative analysis of 16S ribosomal RNA sequences of thermophilic fermentative bacteria isolated from hot spring cyanobacterial mats. *Systematic and Applied Microbiology* **12**:1-7
- Ben-Bassat A, Zeikus JG** (1981) *Thermobacteriodes acetoethylicus* gen. nov. sp. nov., a new chemoorganotrophic, anaerobic, thermophilic bacterium. *Archives of Microbiology* **128**:365-370
- Bergen Tb, Grimont PAD, Grimont F** (1983) Fatty acids of *Serratia* determined by gas chromatography. *Current Microbiology* **8**:7-11
- Blackall LL, Hayward AC, Sly LI** (1985) Cellulolytic and dextrinolytic gram-negative bacteria; revival of the genus *Cellvibrio*. *Journal of Applied Bacteriology*. **59**:81-98
- Bove JM, Saillard C** (1979) Cell biology of spiroplasma, p. 83-153. *In* RF Whitcomb, JG Tully (eds.), *The Mycoplasma*, vol. 3. Academic Press, New York
- Breznak JA, Canale-Parola E** (1975) Morphology and physiology of *Spirochaeta aurantia* strains isolated from aquatic habitats. *Archives of Microbiology* **105**:1-12
- Brock TD** (1978) *Thermophilic Microorganisms and Life at High Temperatures*, Springer-Verlag, New York

- Brock TD** (1986) Introduction: an overview of the thermophiles, p. 1-16. *In* TD Brock (ed.), *Thermophiles: General, Molecular, and Applied Microbiology*, Wiley and Sons, New York
- Brosius J, Ullrich A, Raker MA, Gray A, Dull TJ, Gutell RR, Noller HF** (1981) Construction and fine mapping of recombinant plasmids containing the *rrnB* ribosomal RNA operon of *E. coli*. *Plasmid* **6**:112-118
- Brow, MAD** (1990) Sequencing with Taq DNA polymerase, p. 189-196. *In* MA Innis, DH Gelfand, JJ Sninsky, TJ White (eds.), *PCR Protocols. A guide to methods and applications*, Academic Press, San Diego
- Busse HJ, El-Banna T, Oyaizu H, Awling G** (1992) Identification of xenobiotic-degrading isolates from the beta subclass of the *Proteobacteria* by a polyphasic approach including 16S rRNA partial sequencing. *International Journal of Systematic Bacteriology* **42**:19-26
- Cain AJ, Harrison GA** (1960) Phyletic weighting. *Proceedings of the Zoological Society London* **135**:1-31
- Canale-Parola E** (1981) Free-living anaerobic and facultatively anaerobic spirochetes: The genus *Spirochaeta*, p. 538-547. *In* MP Starr, H Stolp, HG Truper, A Balows, HG Schlegel (eds.), *The prokaryotes, A handbook on habitats, isolation and identification of bacteria*, vol. 1. Springer-Verlag, New York
- Canale-Parola E** (1984) The Spirochetes, p. 38-46. *In* NR Krieg, JG Holt (eds.), *Bergey's Manual of Systematic Bacteriology*, vol. 1. Williams and Wilkins, Baltimore
- Cantwell BA, McConnell DJ** (1988) Molecular cloning and expression of a *Bacillus subtilis* β -glucanase gene in *Escherichia coli*. *Gene* **23**:211-219
- Carlson RR, Vidaver AK** (1982) Taxonomy of *Corynebacterium* plant pathogens, including a new pathogen of wheat based on polyacrylamide gel electrophoresis of cellular proteins. *International Journal of Systematic Bacteriology* **32**:315-326
- Casanova JL, Pannetier C, Jaulin C, Kourilsky P** (1990) Optimal conditions for directly sequencing double-stranded PCR products with sequenase. *Nucleic Acids Research* **18**:4028
- Cato EP, Hash DE, Holdeman LV, Moore WEC** (1982) Electrophoretic study of *Clostridium* species. *Journal of Clinical Microbiology* **15**:688-702
- Cato EP, Stackebrandt E** (1989) Taxonomy and Phylogeny, p. 1-22. *In* NP Minton, DJ Clarke (eds.), *Clostridia, Biotechnology handbooks* 3, Plenum, London
- Chang MM, Chou TYC, Tsao GT** (1981) Structure, pretreatment and hydrolysis of cellulose. *Advances in Biochemical Engineering* **20**:15-42
- Chanzy H, Henrissat B** (1985) Undirectional degradation of *Valonia* cellulose microcrystals subjected to cellulose action. *Federation of European Biochemistry Societies Letters* **184**:285-288

- Chippaux M** (1988) Genetics of cellulase in *Erwinia chrysanthemi*, p. 219-234. In JP Aubert, P Beguin, J Millet (eds.), *Biochemistry and Genetics of Cellulose Degradation*, FEMS Symposium 43, Academic Press, London
- Collins MD, Shah HN, Minnikin DE** (1980) A note on the separation of natural mixtures of bacterial menaquinones using reverse-phase partition thin-layer chromatography. *Journal of Applied Bacteriology* **48**:277-282
- Cook GM, Janssen PH, Morgan HW** (1991) Endospore formation by *Thermoanaerobium brockii* HTD4. *Systematic and Applied Microbiology* **14**:240-244
- Costas M, Owen RJ** (1987) Numerical analysis of electrophoretic protein patterns of *Streptobacillus moniliformis* strains from human, murine and avian infections. *Journal of Medical Microbiology* **23**:303-311
- Coughlan MP** (1990) Cellulose degradation by fungi, p. 1-36. In WM Fogarty, CT Kelly (eds.), *Microbial Enzymes and Biotechnology* (2nd ed.). Elsevier Applied Science, London
- Coughlan MP, Ljungdahl LG** (1988) Comparative biochemistry of fungal and bacterial cellulolytic enzyme systems, p. 11-30. In JP Aubert, P Beguin, J Millet (eds.), *Biochemistry and Genetics of Cellulose Degradation*, Academic Press, London
- Cresci A, Orpianesi C, La Rosa F, Pannelli F, Saltalamacchia G, Scaloni G, Trotta F, Mastrandrea V** (1988) Characterization of some *Clostridium* species by gas-liquid chromatography using numerical analysis. *Microbiologica* **11**:179-199
- Dams E, Huysmans E, Vandenberghe A, De Wachter R** (1987) Structure of clostridial 5S ribosomal RNAs and bacterial evolution. *Systematic and Applied Microbiology* **9**:54-61
- Davies FL, Underwood HM, Gassom MJ** (1981) The value of plasmid profiles for strain identification in lactic streptococci and the relationship between *Streptococcus lactis* 712, ML3 and C2. *Journal of Applied Bacteriology* **51**:325-337
- De Campos SL, De Oliveria EPT, Higashi HG, Lorenzetti MR, Niniz AV, Zappa D** (1986) The genus *Clostridium*, p. 1141-1200 In EP Cato, WL George, SM Finegold (eds.), *Bergey's Manual of Systematic Bacteriology*, vol. 2, Williams and Wilkins, Baltimore
- De Ley JH, Cattoir H, Reynaerts A** (1971) The quantitative measurement of DNA hybridization from renaturation rates. *European Journal of Biochemistry* **12**:133-142.
- De Ley J and Smedt J** (1975) Improvements of the membrane filter method for DNA:rRNA hybridization. *Antonie van Leeuwenhoek Journal of Microbiology and Serology* **41**:287-307
- De Ley J, Swings J** (1976) Phenotypic distribution, numerical analysis, and proposal for an improved taxonomy and nomenclature of the genus *Zymomonas* Kluyver and van Niel 1936. *International Journal of Systematic Bacteriology* **26**:146-157

- De Smedt J, De Ley J** (1977) Intra- and intergeneric similarities of *Agrobacterium* ribosomal ribonucleic acid cistrons. *International Journal of Systematic Bacteriology* **27**:222-240
- Deacon AG, Duerden BI, Holbrook WP** (1978) Gas-liquid chromatographic analysis of metabolic products in the identification of *Bacteroidaceae* of clinical interest. *Journal of Medical Microbiology* **11**:81-99
- Del Moral A, Prado B, Quesada E, Garcia T, Ferrer R, Ramos-Cormenzana A** (1988) Numerical taxonomy of moderately halophilic Gram-negative rods from an inland saltern. *Journal of General Microbiology* **134**:733-741
- Dicks LMT, van Vuuren HJJ, Dellaglio F** (1990) Taxonomy of *Leuconostoc* species, particularly *Leuconostoc oenos*, as revealed by numerical analysis of total soluble cell protein patterns, DNA base compositions, and DNA-DNA hybridizations. *International Journal of Systematic Bacteriology* **40**:83-91
- Dietrich G, Weiss N, Winter J** (1988) *Acetothermus paucivorans*, gen. nov., sp. nov., a strictly anaerobic, thermophilic bacterium from sewage sludge, fermenting hexoses to acetate, CO₂ and H₂. *Systematic and Applied Microbiology* **10**:174-179
- Dijkshoorn L, Tjernberg I, Pot B, Michel MF, Ursing J, Kersters K** (1990) Numerical analysis of cell envelope protein profiles of *Acinetobacter* strains classified by DNA-DNA hybridizations. *Systematic and Applied Microbiology* **13**:338-344
- Dobson SJ, James SR, Franzmann PD, McMeekin TA** (1991) A numerical taxonomic study of some pigmented bacteria isolated from Organic Lake, an antarctic hypersaline lake. *Archives of Microbiology* **156**:56-61
- Doi RH, Igarashi RT** (1965) Conservation of ribosomal and messenger ribonucleic acid cistrons in *Bacillus* species. *Journal of Bacteriology* **90**:384-390
- Donnison AM, Gutteridge CS, Norris JR, Morgan HW, Daniel RM** (1986) A preliminary grouping of New Zealand *Thermus* strains by pyrolysis mass spectrometry. *Journal of Analytical and Applied Pyrolysis* **9**:281-285
- Donnison AM, Brockelsby CM, Morgan HW, Daniel RM** (1989) The degradation of lignocellulosics by extremely thermophilic microorganisms. *Biotechnology and Bioengineering* **33**:1495-1499
- Dretzen G, Bellard M, Sassone-Corsi P, Chambon P** (1981) A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. *Analytical Biochemistry* **112**:295-298
- Dubnau D, Smith I, Morell P, Marmur J** (1965) Gene conservation in *Bacillus* species. I. Conserved genetic and nucleic acid base sequence homologies. *Proceedings of the National Academy of Sciences, USA* **54**:491-498
- Dunn G, Everitt BS** (1982) *An Introduction to Mathematical Taxonomy*, Cambridge University Press, Cambridge, United Kingdom

- Dunning A, Talmud P, Humphries S** (1988) Errors in the polymerase chain reaction. *Nucleic Acids Research* **16**:10393
- Egli T, Weilenmann HU, El-Banna T, Auling G** (1988) Gram-negative, aerobic, nitrilotriacetate-utilizing bacteria from wastewater and soil. *Systematic and Applied Microbiology* **10**:297-305
- Ellis WA, Thiermann AB, Montgomery J, Handsaker A, Winter PJ, Marshall RB** (1988) Restriction endonuclease analysis of *Leptospira interrogans* serovar *hardjo* isolates from cattle. *Research in Veterinary Science* **44**:375-379
- Enebo L** (1949) Symbiosis in thermophilic cellulose fermentation. *Nature* **163**:805
- Enebo L** (1951) On three bacteria connected with thermophilic cellulose fermentation. *Physiologia Plantarum* **4**: 652-666
- Fan LT, Lee Y-H** (1983) Kinetic studies of enzymatic hydrolysis of insoluble cellulase: derivation of a mechanistic kinetic model. *Biotechnology and Bioengineering* **25**:2707-2733
- Fan LT, Gharpuray MM, Lee Y-H** (1987) *Cellulose Hydrolysis*, Springer-Verlag, Berlin
- Fitch WM** (1981) A non-sequential method for constructing trees and hierarchical classifications. *Journal of Molecular Evolution* **18**:30-37
- Focher B, Marzetti A, Beltrame PL, Carniti P** (1991) Structural features of cellulose and cellulose derivatives and their effects on enzymatic hydrolysis, p. 293-310. *In* CH Haigler, PJ Weimer (eds.), *Biosynthesis and Biodegradation of Cellulose*, Marcel Dekker, New York
- Fontaine FE, Peterson WH, McCoy E, Johnston MJ, Ritter GJ** (1942) A new type of glucose fermentation by *Clostridium thermoaceticum*. *Journal of Bacteriology* **43**:701-703
- Fox GE, Peckman KJ, Woese CR** (1977) Comparative cataloging of 16S ribosomal ribonucleic acid: molecular approach to prokaryotic systematics. *International Journal of Systematic Bacteriology* **27**:44-57
- Fox GE, Stackebrandt E, Hespell RB, Gibson J, Maniloff J, Dyer TA, Wolfe RS, Balch WE, Tanner R, Magrum L, Zablen LB, Blakemore R, Gupta R, Bonen L, Lewis BJ, Stahl DA, Luehrsen KR, Chen KN, Woese CR** (1980) The phylogeny of prokaryotes. *Science* **209**:457-463
- Fracek SP, Stolz JF** (1985) *Spirochaeta bajacaliforniensis* sp. n. from a microbial mat community at Laguna Figueroa, Baja, California Norte, Mexico. *Archives of Microbiology* **142**: 317-325.
- Freier D, Mothershed CP, Wiegel J** (1988) Characterization of *Clostridium thermocellum* JW 20. *Applied and Environmental Microbiology* **54**:204-211
- Giovannoni SG** (1991) The polymerase chain reaction, p. 177-203. *In* E Stackebrandt, M Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*, Wiley and Sons, London

- Giovannoni SG, Britschgi TB, Moyer CL, Field KG** (1990) Genetic diversity of Sargasso Sea bacterioplankton. *Nature* **345**:60-63
- Girard AE** (1971) A comparative study of the fatty acids of some micrococci. *Canadian Journal of Microbiology* **17**:1503-1508
- Goodfellow M, Collins MD, Minnikin DE** (1976) Thin-layer chromatographic analysis of mycolic acids and other long-chain components in whole-organism methanolysates of coryneform and related taxa. *Journal of General Microbiology* **96**:351-358
- Goodfellow M, Collins MD, Minnikin DE** (1980) Fatty acid and polar lipid composition in the classification of *Kurthia*. *Journal of Applied Bacteriology* **48**:269-276
- Goodfellow M, Minnikin DE** (1985) Introduction to chemosystematics, p. 1-8. *In* M Goodfellow, DE Minnikin (eds.), *Chemical Methods in Bacterial Systematics*, Academic Press, London
- Goodfellow M, Stanton LJ, Simpson KE, Minnikin DE** (1990) Numerical and chemical classification of *Actinoplanes* and some related actinomycetes. *Journal of General Microbiology* **136**:19-36
- Gouy M, Li W-H** (1989) Phylogenetic analysis based on rRNA sequences supports the archaeobacterial rather than the eocyte tree. *Nature* **339**:145147
- Gower JC** (1971) A general coefficient of similarity and some of its properties. *Biometrics* **27**:857-871
- Green RV, Freer SN** (1986) Growth characteristics of a novel nitrogen-fixing cellulolytic bacterium. *Applied and Environmental Microbiology* **53**:982-986
- Greenberg EP, Canale-Parole E** (1976) *Spirochaeta halophila* sp. n., a facultative anaerobe from a high salinity pond. *Archives of Microbiology* **110**:185-194.
- Gregersen T** (1978) Rapid method for distinction of Gram-negative from Gram-positive bacteria. *European Journal of Applied Microbiology and Biotechnology* **5**:123-127
- Grimont PAD, Popoff MY, Grimont F, Coynault C, Lemelin M** (1980) Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Current Microbiology* **4**:325-330
- Grimont F, Grimont PAD** (1986) Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Annales d'Institut Pasteur Microbiology* **137B**:165-175
- Grimont F, Grimont PAD** (1991) DNA fingerprinting, p. 249-279. *In* E Stackebrandt, M Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*, Wiley and Sons, London
- Groleau D, Forsberg CW** (1981) Cellulolytic activity of the rumen bacterium *Bacteroides succinogenes*. *Canadian Journal of Microbiology* **27**:517-530.

- Gyllensten U** (1989) Direct sequencing of *in vitro* amplified DNA, p. 37-64 *In* HA Ehrlich (ed.), PCR Technology - Principles and Applications of DNA Amplification, Stockton Preess, New York
- Hagget KD, Gray PP, Dunn NW** (1979) Crystalline cellulose degradation by a strain of *Cellulomonas* and its mutant derivatives. European Journal of Applied Microbiology and Biotechnology **8**:183-190
- Hammann R, Werner H** (1980) Fermentation products (using g.l.c.) in the differentiation of non-sporing anaerobic bacteria, p. 257-271. *In* M Goodfellow, RG Board (eds.), Microbiological Classification and Identification, Academic Press, London
- Harvey S, Pickett MJ** (1980) Comparison of Adansonian analysis and deoxyribonucleic acid hybridization results in the taxonomy of *Yersinia enterocolitica*. International Journal of Systematic Bacteriology **30**:86-102
- Harwood CR** (1980) Plasmids, p. 27-53. *In* M Goodfellow, RG Board (eds.), Micro-biological Classification and Identification, Academic Press, London
- Harwood CS, Canale-Parola** (1983) *Spirochaeta isovalerica* sp. nov., a marine anaerobe that forms branch-chained fatty acids as fermentation products. International Journal of Systematic Bacteriology **33**:573-579
- Harwood CS, Canale-Parole E** (1984) Ecology of spirochetes. Annual Review of Microbiology **38**:161-192
- Holdeman LV, Cato EP, Moore WEC** (1977) Anaerobic laboratory manual, 4th edn. Blacksburg, Virginia. Virginia Polytechnic Institute and State University Anaerobic Laboratory
- Hollander R, Pohl S** (1980) Deoxyribonucleic acid base composition of bacteria. Zentralblatt fur Bakteriologie, Mikrobiologie, Hygiene. Abteilung original A **246**:236-275
- Hollaus F, Klaushofer H** (1973) Identification of hyperthermophilic obligate anaerobic bacteria from extraction juices of beet sugar factories. International Sugar Journal **75**:237-242
- Honda H, Naito H, Taya M, Iijima S, Kobayashi T** (1987) Cloning and expression in *Escherichia coli* of a *Thermoanaerobacter cellulolyticus* gene coding for heat-stable B-glucanase. Applied Microbiology and Biotechnology **25**:480-483
- Hori H, Osawa S** (1979) Evolutionary change in 5S RNA secondary structure and a phylogenetic tree of 54 5S rRNA species. Proceedings of the National Academy of Sciences, U.S.A. **76**:381-385
- Howard CJ, Pocock DH, Gourley RN** (1981) Polyacrylamide gel electrophoretic comparison of polypeptides from ureaplasma isolated from cattle and humans. International Journal of Systematic Bacteriology **32**:125-131
- Hsu EJ, Ordal ZJ** (1969) Sporulation of *Clostridium thermosaccharolyticum*. Applied Microbiology **18**:958-960
- Huber R, Langworthy TA, Konig H, Thomm M, Woese CR, Sleytr UB, Stetter KO** (1986) *Thermotoga maritima* sp. nov., represents a new

genus of unique extremely thermophilic eubacteria growing up to 90°C. Archives of Microbiology **144**:324-333

Huber R, Woese CR, Langworthy TA, Fricks H, Stetter KO (1989) *Thermosipho africanus* gen. nov., represents a new genus of thermophilic eubacteria within the *Thermotogales*. Systematic and Applied Microbiology **12**:32-37

Huber R, Woese CR, Langworthy TA, Kristjansson JK, Stetter KO (1990) *Fervidobacterium islandicum* sp. nov., a new extremely thermophilic eubacterium belonging to the "*Thermotogales*". Archives of Microbiology **154**:105-111

Hudson BW, Quan TJ, Bailey RE (1976) Electrophoretic studies of the geographic distribution of *Yersinia pestis* protein variants. International Journal of Systematic Bacteriology **26**:1-16

Hudson JA, Morgan HW, Daniel RM (1986) A numerical classification of some *Thermus* isolates. Journal of General Microbiology **132**:531-540

Hudson JA, Morgan HW, Daniel RM (1987) Numerical classification of some *Thermus* isolates from Icelandic hot springs. Systematic and Applied Microbiology **9**:218-223

Hudson JA, Morgan HW, Daniel RM (1989) Numerical classification of *Thermus* isolates from globally distributed hot springs. Systematic and Applied Microbiology **11**:250-256

Hudson JA, Morgan HW, Daniel RM (1990a) A survey of cellulolytic anaerobic thermophiles from hot springs. Systematic and Applied Microbiology **13**:72-76

Hudson JA, Morgan HW, Daniel RM (1990b) Cellulolytic properties of an extremely thermophilic anaerobe. Applied Microbiology and Biotechnology **33**:687-691

Hudson JA, Morgan HW, Daniel RM (1991) The cellulase activity of an extreme thermophile. Applied Microbiology and Biotechnology **35**:270-273

Hultman T, Stahl S, Hornes E, Uhlen M (1989) Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as a solid support. Nucleic Acids Research **17**:4937-4946

Hungate RE (1944) Studies on cellulose fermentation. I. The culture and physiology of an anaerobic cellulose-digesting bacterium. Journal of Bacteriology **48**:499-513

Hungate RE (1950) The anaerobic mesophilic cellulolytic bacteria. Bacteriological Reviews **14**:1-50

Hungate RE (1969) A roll tube method for cultivation of strict anaerobes, p. 117-132. In JR Norris, DW Ribbons (eds.), Methods in Microbiology, vol. 3B. Academic Press, London

Ikemoto S, Kuraishi H, Komagata K, Azuma R, Shuto T, Murooka H (1978) Cellular fatty acid composition in *Pseudomonas* species. Journal of General and Applied Microbiology **24**:199-213

- Ilarionow SA** (1985) Isolation of *Clostridium thermoautotrophicum*, an anaerobic methylotrophic spore-forming organism. *Mikrobiologiya* **54**:533-538 (English translation)
- Imsenecki AA** (1940) Microbiology of anaerobic degradation of cellulose V Isolation of thermophilic cellulolytic bacteria. *Mikrobiologiya* **9**:433-443
- Innis MA, Myambo KB, Gelfand DH, Brow MAD** (1988) DNA sequencing with *Thermus aquaticus* DNA polymerase, and direct sequencing of PCR amplified DNA. Proceedings of the National Academy of Sciences, U.S.A. **85**:9436-9440
- Jackman PJH** (1985) Bacterial taxonomy based on whole cell protein patterns, p. 125-129. In M Goodfellow, DE Minnikin (eds.), *Chemical Methods in Bacterial Systematics*, Academic Press, London
- Jackman PJH** (1987) Microbial systematics based on electrophoretic whole cell protein patterns, p. 209-225. In RR Colwell, R Grigorova (eds.), *Methods in Microbiology*, vol. 19. Academic Press, London
- Jannasch HW, Huber R, Belkin S, Stetter KO** (1988) *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. *Archives of Microbiology* **150**:103-104
- Janssen PH** (1989) Isolation and characterization of organic acid-fermenting bacteria from anaerobic sediments, D. Phil. thesis, University of Waikato, New Zealand
- Janssen PH, Harfoot CG** (1990) Isolation of a *Citrobacter* species able to grow on malonate under strictly anaerobic conditions. *Journal of General Microbiology* **136**:1037-1042
- Janssen PH, Morgan HW**. Unpublished results.
- Jeffries CD, Holtman DF, Guse DG** (1957) Rapid method for determining the activity of microorganisms on nucleic acids. *Journal of Bacteriology* **73**: 590-591
- Jeffries AJ, Wilson V, Neumann R, Keyle J** (1988) Amplification of human minisatellites by the polymerase chain reaction. Towards DNA fingerprinting of single cells. *Nucleic Acids Research* **16**:10953-10971
- Jin F, Toda K** (1988) Isolation of a new anaerobic, thermophilic and cellulolytic bacteria-JT and their cellulase production. *Journal of Fermentation Technology* **66**:389-395
- Jin F, Yamasato K, Toda K** (1988) *Clostridium thermocopriae* sp. nov., a cellulolytic thermophile from animal feces, compost, soil and a hot spring in Japan. *International Journal of Systematic Bacteriology* **38**:277-281
- Johnson JL** (1989) Nucleic acids in bacterial classification, p. 2306-2309. In ST Williams, ME Sharpe, JG Holt (eds.), *Bergey's Manual of Systematic Bacteriology*, vol. 4. Williams & Wilkins, Baltimore

- Johnson NC, Goldfine H** (1983) Lipid composition in the classification of the butyric acid-producing clostridia. *Journal of General Microbiology* **129**:1075-1081
- Jones BE** (1988) Thermophilic anaerobic bacteria from hot springs. *Proceedings: FEMS Meeting, Portugal 1988*
- Jones BE, Lindsay JM** (1989) An extreme thermophilic, anaerobic eubacterium which hydrolyses cellulose. *Proceedings: Academy Workshop on Adaptation of Microorganisms to Extreme Environments, Haren, Netherlands, June 5-7 1989*
- Jones D, Sackin MJ** (1980) Numerical methods for the classification and identification of bacteria with especial reference to the enterobacteriaceae, , p. 73-106. *In* M Goodfellow, RG Board (eds.), *Microbiological Classification and Identification*, Academic Press, London
- Kajioka R, Tang PW** (1984) Curie-point pyrolysis-mass spectrometry of *Legionella* species. *Journal of Analytical and Applied Pyrolysis* **6**:59-68
- Kakoyiannis CK, Winter PJ, Marshall RB** (1988) The relationship between intestinal *Campylobacter* species isolated from animals and humans as determined by BRENDAs. *Epidemiology and Infection* **100**:379-387
- Kandler O** (1982) Cell wall structures and their phylogenetic applications. *Zentralblatt für Bakteriologie, Mikrobiologie, Hygiene. Abteilung original* **c3**:149-160
- Kaneda T** (1977) Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriological Reviews* **41**:391-418
- Kerstens K, De Ley J** (1975) Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. *Journal of General Microbiology* **87**:333-342
- Kerstens K, De Ley J** (1980) Classification and identification of bacteria by electrophoresis of their proteins, p. 273-297. *In* M Goodfellow, R Board (eds.), *Microbiological Classification and Identification*, Academic Press, London
- Khraptsova GI, Tsaplina IA, Seregina LM, Loginova LG** (1984) Thermophilic bacteria of the hot springs of Buryatia. *Microbiology* **53**:137-141
- Komagata K, Suzuki K** (1987) Lipid and cell-wall analysis in bacterial systematics, p. 161-207. *In* RR Colwell, R Grigorova (eds.), *Methods in Microbiology*, vol. 19. Academic Press, London
- Kondratieva EN, Zacharova EV, Duda VI, Krivenko VV** (1989) *Thermoanaerobium lactoethylicum* spec. nov. a new anaerobic bacterium from a hot spring of Kamchatka. *Archives of Microbiology* **151**:117-122
- Konradsdottir M, Perttula M, Pere J, Viikari L, Kristjansson JK** (1991) *In situ* enrichment of thermophilic acetate-utilizing bacteria. *Systematic and Applied Microbiology* **14**:190-195
- Krivenko VV, Chernykh NA, Zakhrova EV, Duda VI** (1987) Characterization of genotypical properties of several anaerobic thermophilic chemoorganotrophic bacteria. *Mikrobiologiya* **56**: 855-859 (English translation)

- Kuntzel H** (1982) Phylogenetic trees derived from mitochondrial, nuclear, eubacterial and archaebacterial rRNA sequences: Implications on the origin of eukaryotes. *Zentralblatt fur Bakteriologie, Mikrobiologie, Hygiene. Abteilung original* **c3**:31-39
- Kwok S, Higuchi R** (1989) Avoiding false positives with PCR. *Nature* **339**:237
- Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**:680-685
- Lake JA** (1987) A rate-independent technique for analysis of nucleic acid sequences: evolutionary parsimony. *Molecular Biology Evolution* **4**:167-191
- Lamed R, Zeikus JG** (1980) Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. *Journal of Bacteriology* **144**: 569-578.
- Lamed R, Zeikus JG** (1980) Glucose fermentation pathway of *Thermoanaerobium brockii*. *Journal of Bacteriology* **141**:1251-1257
- Lamed R, Setter E, Bayer EA** (1983) Characterization of a cellulose-binding, cellulose containing complex in *Clostridium thermocellum*. *Journal of Bacteriology* **156**:828-836
- Lamed R, Naimark J, Uozumi T, Beppu T** (1987) Specialized cell surface structures in cellulolytic bacteria. *Journal of Bacteriology* **169**:3792-3800
- Lamed R, Bayer EA** (1988) The cellulosome of *Clostridium thermocellum*. *Advances in Applied Microbiology* **33**:1-46
- Lance GN, Williams WT** (1967) A general theory of classificatory sorting strategies. I. Hierarchical systems. *Computer Journal* **9**:373-380
- Lane DJ** (1991) 16S/23S rRNA sequencing, p. 115-147 *In* E Stackebrandt, M Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*, Wiley and Sons, London
- Lane DJ, Pace B, Olsen G, Stahl DA, Sogin ML, Pace NR** (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proceedings of the National Academy of Sciences, U.S.A.* **82**:6955-6959
- Lane DJ, Field KG, Olsen GJ, Pace NR** (1988) Reverse transcriptase sequencing of ribosomal RNA for phylogenetic analysis. *Methods in Enzymology* **167**:138-144
- Langford ML, Gilkes NR, Wakarchuk WW, Kilburn DG, Miller RC, Warren RAJ** (1984) The cellulase system of *Cellulomonas fimi*. *Journal of General Microbiology* **130**:1367-1376
- Langford ML, Gilkes NR, Singh NR, Moser B, Miller RC, Warren RAJ, Kilburn DG** (1987) Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains. *Federation of European Biochemistry Societies Letters* **225**:163-167

- Langwell H, Lymn A** (1923) Cellulose fermentation. *Journal of the Society of the Chemical Industry* **42**:280-283
- Langworthy TA** (1982) Lipids of *Thermoplasma*, p. 396-406. *In* SP Colowick, NO Kaplan (eds.), *Methods in Enzymology*, vol. 88. Academic Press, New York
- Lapage SP** (1974) Practical aspects of probabilistic identification of bacteria. *International Journal of Systematic and Applied Microbiology* **24**: 500-507
- Lapage SP, Sneath PHA, Lessel EF, Skerman VBD, Seeliger HRP, Clark WA** (1975) *International Code of Nomenclature of Bacteria*. American Society for Micro-biology, Washinton, DC.
- Lechevalier MP, Lechevalier H** (1970) Chemical composition as a criterion in the classification of aerobic actinomycetes. *International Journal of Sytematic Bacteriology* **20**:435-443
- Lechevalier MP, Lechevalier H, Horen AC** (1973) Chemical characteristics and classification of nocardiae. *Canadian Journal of Microbiology* **19**:965-972
- Lee BH, Blackburn TH** (1975) Cellulase production by a thermophilic *Clostridium* species. *Applied Microbiology* **30**:346-353
- Lee SF, Forsberg CW, Gibbins LN** (1985) Cellulolytic activity of *Clostridium acetobutylicum*. *Applied and Environmental Microbiology* **50**:220-228
- Leigh JA, Wolfe RS** (1983) *Acetogenium kivui*, a thermophilic acetogenic bacterium. *International Journal of Systematic Bacteriology* **33**:886
- LeRuyet P, Dubourguier HC, Albagnac G, Prensier G** (1985) Characterization of *Clostridium thermolacticum* sp. nov. a hydrolytic, thermophilic anaerobe producing high amounts of lactate. *Systematic and Applied Microbiology* **6**:196-202.
- Ljungdahl LG, Carreira L, Wiegel J** (1981) The Ekman Days. *International Symposium on Wood and Pulping Chemistry Vol 4*, June 9-12, pp23-28.
- Ljungdahl LG, Pettersson B, Eriksson KE, Wiegel J** (1983) A yellow affinity substance involved in the cellulolytic system of *Clostridium thermocellum*. *Current Microbiology* **9**:195-200
- Ljungdahl LG, Eriksson KE** (1985) Ecology of microbial cellulose degradation, p. 237-299. *In* KC Marshall (ed.), *Advances in Microbial Ecology*, vol. 8. Plenum Press, New York
- Lockhart WR** (1967) Factors affecting reproducibility of numerical classifications. *Journal of Bacteriology* **94**:826-831
- Ludvig W** (1991) DNA sequencing in bacterial systematics, p. 69-94. *In* E Stackebrandt, M Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*, Wiley and Sons, London

- MacDonell MT, Swartz DG, Ortiz-Conde A, Last GA, Colwell RR** (1986) Ribosomal RNA phylogenies for the vibrio-enteric group of eubacteria. *Microbiological Science* **3**:172-179
- MacFadden A, Blaxall FR** (1896) Thermophilic bacteria. *Journal of Pathology and Bacteriology* **3**:87-99
- Madden RH** (1983) Isolation and characterization of *Clostridium stercorarium* sp. nov., a cellulolytic thermophile. *International Journal of Systematic Bacteriology* **33**:837-840
- Manchester L, Pot B, Kersters K, Goodfellow M** (1990) Classification of *Streptomyces* and *Streptovorticillium* species by numerical analysis of electrophoretic protein patterns. *Systematic and Applied Microbiology* **13**:333-337
- Marshall RB, Wilton BE, Robinson AJ** (1981) Identification of *Leptospira* serovars by restriction endonuclease analysis. *Journal of Medical Microbiology* **14**:163-166
- Mathrani IM, Ahring BK** (1990) Anaerobic, thermophilic, cellulose and hemicellulose degrading bacteria. 5th European Congress on Biotechnology, Copenhagen, July 8-13, Abstract p 41
- Matteuzzi D, Hollaus J, Biavati B** (1978) Proposal of neotype for *Clostridium thermohydrosulfuricum* and the merging of *Clostridium tartarivorum* with *Clostridium thermosaccharolyticum*. *International Journal of Systematic Bacteriology* **28**:528-531
- McBee RH** (1948) The culture and physiology of a thermophilic cellulose-fermenting bacterium. *Journal of Bacteriology* **56**:653-663
- McBee RH** (1950) The anaerobic thermophilic cellulolytic bacteria. *Bacteriological Reviews* **14**:51-63
- McBee RH** (1954) The characteristics of *Clostridium thermocellum*. *Journal of Bacteriology* **67**:505-506
- McCarthy AJ, Cross T** (1984) A taxonomic study of *Thermomonospora* and other monosporic Actinomycetes. *Journal of General Microbiology* **130**:5-25
- McClung LS** (1935) Studies on anaerobic bacteria. IV. Taxonomy of cultures of thermophilic species causing "swells" of canned food. *Journal of Bacteriology* **29**:189-193
- Mendez BS, Pettinari MJ, Ivanier SE, Ramos CA, Sineriz F** (1991) *Clostridium thermopapyrolyticum* sp. nov., a cellulolytic thermophile. *International Journal of Systematic and Applied Microbiology* **41**: 281-283
- Meyers JA, Sanchez D, Elwell LP, Falkow S** (1976) Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *Journal of Bacteriology* **127**:1529-1537
- Minnikin DE, Alshamaony L, Goodfellow M** (1975) Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. *Journal of General Microbiology* **88**:200-204

- Moench TT, Zeikus JG** (1983) An improved preparation method for a titanium(III) media reductant. *Journal of Microbiological Methods* **1**:199-202
- Mohagheghi A, Grohmann K, Himmel M, Leighton L, Updegraff DM** (1986) Isolation and characterization of *Acidotherrmus cellulolyticus* gen. nov., sp. nov., a new genus of thermophilic, acidophilic, cellulolytic bacteria. *International Journal of Systematic and Applied Microbiology* **36**: 435-443
- Moore RL, McCarthy B** (1967) Comparative study of ribosomal RNA. *Methods in Enzymology* **164**:793-812
- Moureau P, Derclaye I, Gregoire D, Janssen M, Cornelis GR** (1989) *Campylobacter* species identification based on polymorphism of DNA encoding rRNA. *Journal of Clinical Microbiology* **27**:1514-1517
- Mullings R, Parish JH** (1984) Mesophilic aerobic Gram-negative cellulose degrading bacteria from aquatic habitats and soil. *Journal of Applied Bacteriology* **57**:455-468
- Murray RGE, Brenner DJ, Colwell RR, De Voss P, Goodfellow M, Grimont PAD, Pfennig N, Stackebrandt E, Zavarzin GA** (1990) Report of the Ad Hoc Committee on approaches to taxonomy within the Proteobacteria. *International Journal of Systematic Bacteriology* **40**:213-215
- Murray WD, Sowden LC, Colvin JR** (1984) *Bacteroides cellulosolvens* sp. nov., a cellulolytic species from sewage sludge. *International Journal of Systematic and Applied Microbiology* **34**:185-187
- Ng TK, Weimer PJ, Zeikus JG** (1977) Cellulolytic and physiological properties of *Clostridium thermocellum*. *Archives of Microbiology* **114**:1-7
- Ng TK, Zeikus JG** (1981) Purification and characterization of an endoglucanase (1,4- β -D-glucan glucanohydrolase) from *Clostridium thermocellum*. *Biochemical Journal* **199**:341-350
- O'Brien M, Colwell R** (1987) Characterization tests for numerical taxonomy, p. 69-104. In RR Colwell, R Grigorova (eds.), *Methods in Microbiology*, vol. 19. Academic Press, London
- Oliver BM, Mah RA, Ferguson TJ, Boone DR, Garcia JL, Robinson R** (1985) Emendation of the genus *Thermobacteroides*: *Thermobacteroides proteolyticus* sp. nov., a proteolytic acetogen from a methanogenic enrichment. *International Journal of Systematic Bacteriology* **35**:425-428
- Olsen GJ** (1987) Earliest phylogenetic branchings: comparing rRNA-based evolutionary trees inferred with various techniques. *Cold Spring Harbor Symposium on Quantitative Biology* **52**:825-837
- Ornstein DL, Kashdan MA** (1985) Sequencing DNA using ³⁵S-labeling: a trouble-shooting guide. *Biotechniques* **3**:476-483
- Oshima M, Miyagawa A** (1974) Comparative studies of the fatty acid composition of moderately and extremely thermophilic bacteria. *Lipids* **9**:476-480

- Owen RJ, Hill LR** (1979) The estimation of base compositions, base pairing and genome sizes of bacterial deoxyribonucleic acids, p. 277-296. *In* FA Skinner, DW Lovelock (eds.), *Identification Methods for Microbiologists*, 2nd ed., Academic Press, London
- Pace B and Campbell LL** (1971) Homology of ribosomal ribonucleic acid of *Desulfovibrio* species with *Desulfovibrio vulgaris*. *Journal of Bacteriology* **106**:717-719
- Parisi JT, Hecht DW** (1980) Plasmid profiles in epidemiological studies of infections by *Staphylococcus epidermis*. *Journal of Infectious Diseases* **141**:637-643
- Paster BJ, Stackebrandt E, Hespell RB, Hahn CM, Woese CR** (1984) The phylogeny of the spirochetes. *Systematic and Applied Microbiology* **5**:337-351
- Paster BJ, Dewhirst FE, Weisburg WG, Tordoff IA, Fraser GJ, Hespell RB, Stanton TB, Zablen L, Mandelco L, Woese CR** (1991) Phylogenetic analysis of the spirochetes. *Journal of Bacteriology* **173**:6101-6109
- Patel BKC, Morgan HW, Daniel RM** (1985a) *Fervidobacterium nodosum* gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. *Archives of Microbiology* **141**:63-69
- Patel BKC, Morgan HW, Daniel RM** (1985b) Thermophilic anaerobic spirochetes in New Zealand hot springs. *FEMS. Microbiology Letters* **26**:101-106.
- Patel BKC, Monk C, Littleworth H, Morgan HW, Daniel RM** (1987) *Clostridium fervidus* sp. nov., a chemoorganotrophic acetogenic thermophile. *International Journal of Systematic and Applied Microbiology* **37**:123-126
- Patel BKC, Hudson JA, Morgan HW, Daniel RM** (1988) Non-dioxic fermentation of acid hydrolysed pine by a strain of *Clostridium thermohydrosulfuricum* isolated from a New Zealand hot spring. *FEMS Microbiology Letters* **56**:285-288
- Patel GB, Khan AW, Agnew BJ, Colvin JR** (1980) Isolation and characterization of an anaerobic cellulolytic microorganism *Acetovibrio cellulolyticus* n. gen., n. sp. *International Journal of Systematic Bacteriology* **30**:179-185
- Pelletier DA, Paster BJ, Weisburg WG, Dewhirst FE, Dannenberg S, Schroeder I** (1991) *Cristispira* phylogeny by 16S rRNA sequence comparison of amplified bacterial DNA from crystalline styles. 91st. General Meeting of the American Society of Microbiology Abstract R 14
- Penner JL, Hennessy JN, Mills SD, Bradbury WC** (1983) Application of serotyping and chromosomal restriction endonuclease digest analysis in investigating a laboratory-acquired case of *Campylobacter jejuni* enteritis. *Journal of Clinical Microbiology* **18**:1427-1428
- Penny D** (1991) From macromolecules to trees, p. 281-324. *In* E Stackebrandt, M Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*, Wiley and Sons, London

- Pfennig N** (1984) Microbial behaviour in natural environments, p. 25-30. *In* DP Kelly, NG Carr (eds.), *The Microbes 1984, Part II, Prokaryotes and Eukaryotes*, Cambridge University Press, Cambridge, United Kingdom
- Pfennig N, Wagener S** (1986) An improved method of preparing wet mounts for photomicrographs of microorganisms. *Journal of Microbiological Methods* **4**:303-306
- Pirt SJ** (1975) *Principles of Microbe and Cell Cultivation*. Blackwell, Oxford
- Plant AR, Patel BKC, Morgan HW, Daniel RM** (1986) Starch degradation by thermophilic anaerobic bacteria. *Systematic and Applied Microbiology* **9**:158-162
- Pot B, Willems A, Gillies M, De Ley J** (1992) Intra- and intergeneric relationships of the genus *Aquaspirillum*: *prolinoborus*, a new genus for *Aquaspirillum fasciculus*, with the species *Prolinoborus fasciculus* comb. nov. *International Journal of Systematic Bacteriology* **42**:44-57
- Priest FG, Goodfellow M, Todd C** (1988) A numerical classification of the genus *Bacillus*. *Journal of General Microbiology* **134**:1847-1882
- Priest FG, Pleasants JG** (1988) Numerical taxonomy of some leuconostocs and related bacteria from Scotch whisky distilleries. *Journal of Applied Bacteriology* **64**:379-387
- Rees DA, Morris ER, Thom D, Madden JK** (1982) *Polysaccharides*, Vol. I, Academic Press, New York
- Reynolds PHS, Sissons CH, Daniel RM, Morgan HW** (1986) Comparison of cellulolytic activities in *Clostridium thermocellum* and three thermophilic, cellulolytic anaerobes. *Applied and Environmental Microbiology* **51**:12-17
- Rohlf FJ** (1970) Adaptive hierarchical clustering schemes. *Systematic Zoology* **19**:58-82
- Richmond PA** (1991) Occurrence and function of native cellulose, p. 5-23. *In* CH Haigler, PJ Weimer (eds.), *Biosynthesis and Biodegradation of Cellulose*, Marcel Dekker, New York
- Rickett HW** (1958) So what is a taxon ? *Taxon* **7**:37-38
- Rogall T, Flohr T, Bottger EC** (1990) Differentiation of *Mycobacterium* species by direct sequencing of amplified DNA. *Journal for General Microbiology* **136**:1915-1920
- Rotmistrov MN** (1940) Isolation of anaerobic cellulolytic bacteria. *Microbiologiya* **9**:341-343
- Rowe MT, Finn B** (1991) A study of *Pseudomonas fluorescens* biovars using the Automated Microbiology Identification System (AMBIS). *Letters in Applied Microbiology* **13**:238-242

- Russek-Cohen E, Colwell RR** (1986) Application of numerical taxonomy procedures in microbial ecology, p. 178-194. *In* RL Tate (ed.), *Microbial Autecology*, Willey, New York
- Ruttersmith LD, Daniel RM** (1991) Thermostable cellobiohydrolase from the thermophilic eubacterium *Thermotoga* sp. strain FjSS3-B.1- Purification and properties. *Biochemical Journal* **277**:887-890
- Sackin MJ** (1987) Computer programs for classification and identification, p. 459-494. *In* RR Colwell, R Grigorova (eds.), *Methods in Microbiology*, vol. 19. Academic Press, London
- Saiki RK, Scharf S, Faloona F, Mullis Kb, Horn GT, Ehrlich HA, Arnheim NA** (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1350-1354
- Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Ehrlich HA** (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487-491
- Sanger F, Nicklen S, Coulson AR** (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, U.S.A.* **74**:5463-5467
- Santos MA, Williams RAD, Da Costa MS** (1989) Numerical taxonomy of *Thermus* isolates from hot springs in Portugal. *Systematic and Applied Microbiology* **12**:310-315
- Sasaki T et al** (1983) Purification and properties of *Cellvibrio gilvis* cellobiose phosphorylase. *Biochemical Journal* **209**:803-807
- Sattath S, Tversky A** (1977) Additive similarity trees. *Psychometrika* **42**:319-345
- Saunders NA, Harrison TJ, Kachwalla N, Taylor AG** (1988) Identification of species of the genus *Legionella* using a cloned rRNA gene from *Legionella pneumophila*. *Journal of General Microbiology* **134**:2363-2374
- Schaberg DR, Tompkins LS, Falkow S** (1981) Use of agarose gel electrophoresis of plasmid deoxyribonucleic acid to fingerprint Gram-negative bacilli. *Journal of Clinical Microbiology* **13**:1105-1108
- Schink B, Zeikus JG** (1983) *Clostridium thermosulfurogenes* sp. nov., a new thermophile that produces elemental sulfur from thiosulfate. *Journal of General Microbiology* **129**: 1149-1158
- Schleifer KH, Kandler O** (1972) The peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological Reviews* **36**:407-477
- Schleifer KH, Stackebrandt E** (1983) Molecular systematics of prokaryotes. *Annual Review of Microbiology* **37**:143-187
- Schleifer KH, Kilpper-Balz** (1987) Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: a review. *Systematic and Applied Microbiology* **10**:1-19

- Schmid U, Geisel H, Schoderth SM, Sahm H** (1986) *Thermoanaerobacter finnii* spec. nov., a new ethanologenic sporogenous bacterium. *Systematic and Applied Microbiology* **8**:80-85
- Sela S, Clark-Curtiss JE, Bercovier H** (1989) Characterization and taxonomic implications of the rRNA genes of *Mycobacterium leprae*. *Journal of Bacteriology* **171**:70-73
- Shah HN, Nash RA, Hardy JM, Weetman DA, Geddes DA, Macfarlane TW** (1985) Detection of acidic end products of metabolism of anaerobic Gram-negative bacteria. p 317-340. *In* M Goodfellow, DE Minnikin (eds.), *Chemical Methods in Bacterial Systematics*, Academic Press, London
- Shaw BG, Puckey DJ, MacFie HJH, Bolt SJ** (1985) Classification of some lactic acid bacteria from vacuum-packed meats by direct probe mass spectrometry. *Journal of Applied Bacteriology* **59**:157-165
- Shimomaye E, Salvato M** (1989) Use of avian myeloblastosis virus reverse transcriptase at high temperature for sequence analysis of highly structured RNA. *Gene Analytical Techniques* **6**:25-28
- Shute LA, Gutteridge CS, Norris JR, Berkeley RCW** (1984) Curie-point pyrolysis mass spectrometry applied to the classification of *Bacillus* species. *Journal of General Microbiology* **130**:343-355
- Simpson GG** (1961) *Principles of Animal Taxonomy*. Columbia University Press, New York.
- Sissons CH, Sharrock KR, Daniel RM, Morgan HW** (1987) Isolation of cellulolytic anaerobic extreme thermophiles from New Zealand thermal sites. *Applied and Environmental Microbiology* **53**:832-838
- Skerman VBD** (1967) *A Guide to the Identification of the Genera of Bacteria*. 2nd ed. Williams and Wilkins, Baltimore, MD.
- Sleat R, Mah RA, Robinson R** (1984) Isolation and characterization of an anaerobic, cellulolytic bacterium *Clostridium cellulovorans* sp. nov. *Applied and Environmental Microbiology* **48**:88-93
- Sneath PHA** (1957a) Some thoughts on bacterial classification. *Journal of General Microbiology* **17**:184-200
- Sneath PHA** (1957b) The application of computers to taxonomy. *Journal of General Microbiology* **17**:201-226
- Sneath PHA** (1962) The construction of taxonomic groups, p. 289-332. *In* GC Ainsworth, PHA Sneath (eds.), *Microbial Classification*, 12th Symposium of the Society for General Microbiology, Cambridge University Press, Cambridge.
- Sneath PHA** (1978) Classification of microorganisms p. 14-22. *In* JR Norris and MH Richmond (eds.), *Essays in Microbiology*, John Wiley, London
- Sneath PHA** (1989) Analysis and interpretation of sequence data for bacterial systematics: the view of a numerical taxonomist. *Systematic and Applied Microbiology* **12**:15-31

- Sneath PHA, Johnson R** (1972) The influence on numerical taxonomic similarities of errors in microbiological tests. *Journal of General Microbiology* **72**:377-392
- Sneath PHA, Sokal RR** (1973) *Numerical Taxonomy: The Principles and Practice of Numerical Classification*. W.H. Freeman, San Francisco
- Snipes KP, Hirsh DC, Kasten RW, Hansen LN, Hird DW, Carpenter TE, McCapes RH** (1989) Use of a rRNA probe and restriction endonuclease analysis to fingerprint *Pasteurella multocida* isolated from turkeys and wildlife. *Journal of Clinical Microbiology* **27**:1847-1853
- Sokal RR** (1985) The principles of numerical taxonomy: twenty-five years later, p. 1-14. *In* M Goodfellow, D Jones, FG Priest (eds.), *Computer-assisted Bacterial Systematics*, Special Publication for the Society for General Microbiology 15, Academic Press, London
- Sokal RR, Michener CD** (1958) A statistical method for evaluating systematic relationships. *University of Kansas Science Bulletin* **38**:1409-1438
- Sokal RR, Sneath PHA** (1963) *Principles of Numerical Taxonomy*. W.H. Freeman, San Francisco.
- Soutschek E, Winter J, Schindler F, Kandler O** (1984) *Acetomicrobium flavidum* gen. nov., sp. nov., a thermophilic anaerobic bacterium from sewage sludge, forming acetate, CO₂ and H₂ from glucose. *Systematic and Applied Microbiology* **5**:377-390
- Stack RJ, Hungate RE** (1984) Effect of 3-phenyl-propanoic acid on capsule and cellulases of *Ruminococcus albus* 8. *Applied and Environmental Microbiology* **48**:218-223
- Stackebrandt E** (1992) Molecular microbial ecology. *In* J Lederberg (ed.), *Encyclopedia of Microbiology*, Academic Press, San Diego (in press)
- Stackebrandt E, Lewis B, Woese CR** (1980) The phylogenetic structure of the coryneform group of bacteria. *Zentralblatt für Bakteriologie, I Abteilung Originale* **c1**:137-149
- Stackebrandt E, Wummer-Fuessl B, Fowler V, Schleifer KH** (1981) DNA homologies and ribosomal RNA similarities among spore-forming members of the order *Actinomycetales*. *International Journal of Systematic Bacteriology* **31**:420-431
- Stackebrandt E, Charfreitag O** (1990) Partial 16S RNA primary structure of five *Actinomyces* species: phylogenetic implications and development of an *Actinomyces israelii*-specific oligonucleotide probe. *Journal for General Microbiology* **136**:37-43
- Stackebrandt E, Goodfellow M** (1991) Introduction, p. xix-xxix. *In* E Stackebrandt, M Goodfellow (eds.), *Nucleic Acid Techniques in Bacterial Systematics*, Wiley and Sons, London
- Stanton TB, Jensen NS, Casey TA, Tordoff LA, Dewhirst FE, Paster BJ** (1991) Reclassification of *Treponema hyodysenteriae* and *Treponoma innocens* in a new genus *Serpula* gen. nov., as *Serpula hyodysenteriae* comb.

nov. and *Serpula innocens* comb. nov. International Journal of Systematic Bacteriology **41**:50-58

- Stephenson JR, Tabaqchali S** (1986) New methods for typing coagulase-negative staphylococci. Journal of Clinical Pathology **39**:1271-1275
- Stephenson JR, Crook SJ, Tabaqchali S** (1986) New method for typing *Staphylococcus aureus* resistant to methicillin based on sulphur-35 methionine labelled proteins; its application in an outbreak. British Medical Journal **293**:581-583
- Stetter KO, Fiala G, Huber G, Huber R, Segerer A** (1990) Hyperthermophilic microorganisms. FEMS Microbiology Reviews **75**:117-124
- Stouthamer AH** (1979) The search for correlation between theoretical and experimental growth yields, p. 1-47. In JR Quayle (ed.). International Review of Biochemistry, vol 21, Microbial Biochemistry, University Park Press, Baltimore
- Stutzenberger FJ, Kaufman AJ, Lossin RD** (1970) Cellulolytic activity in municipal solid waste compost. Canadian Journal of Microbiology **16**:553-560
- Sukhumavasi J, Kunio O, Shoichi S, Kazue U** (1988) *Clostridium josui* sp. nov., a cellulolytic moderate thermophilic species from Thai compost. International Journal of Systematic Bacteriology **38**:179-182
- Svetlichny VA, Svetlichnya TP** (1988) *Dictyoglomus turgidus* sp. nov., a new extreme thermophilic bacterium isolated from hot springs in the Uzon volcano crater. Mikrobiologiya **57**:435-441 (English translation)
- Svetlichny V, Aksenova HYu, Zavarzin G** (1990a) Extremely thermophilic cellulolytic anaerobes from hot springs of the Soviet Far East. Poster Abstract. Thermophily Today. International meeting on cell constituents, genetics and biotechnological applications of thermophilic microorganisms. Viterbo, Italy. May 7-9, 1990.
- Svetlichny VA, Svetlichnya TP, Chernykh NA, Zavarzin GA** (1990b) *Anaerocellum thermophilum* gen., nov., sp. nov., an extreme thermophilic cellulolytic eubacterium isolated from hot springs in the Valley of Geysers. Mikrobiologiya **59**:871-879 (English translation)
- Swings J, De Vos P, Van den Mooter M, De Ley J** (1983) Transfer of *Pseudomonas maltophilia* Hugh 1981 to the genus *Xanthomonas maltophilia* (Hugh 1981) comb. nov. International Journal of Systematic Bacteriology **33**:409-413
- Tabaqchali S** (1985) Advances in diagnosis I., p. 65-79. In DAJ Tyrrell (ed.), Clinical Investigation of Infectious Diseases, Royal Society Medical Services Ltd, London
- Tabaqchali S, O'Farrell S, Holland D, Silman R** (1984) Typing scheme for *Clostridium difficile*: its application in clinical and epidemiological studies. Lancet **1**:935-938
- Tabaqchali S, O'Farrell S, Holland D, Silman R** (1986) Method for the typing of *Clostridium difficile* based on PAGE ³⁵S-methionine-labelled proteins. Journal of Clinical Microbiology **23**:197-198

- Tabaqchali S, Silman R, Holland D** (1987) Automation in clinical microbiology: A new approach to identify microorganisms by automated pattern matching of proteins labelled with ^{35}S -methionine. *Journal of Clinical Pathology* **40**:1070-1087
- Tabor S, Richardson CC** (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proceedings of the National Academy of Sciences, U.S.A.* **84**:4767-4771
- Tanner RS, Stackebrandt E, Fox GE, Gupta R, Magnum LG, Woese CR** (1982) A phylogenetic analysis of anaerobic eubacteria capable of synthesizing acetate from carbon dioxide. *Current Microbiology* **7**:127-132
- Taya M, Suzuki Y, Kobayashi T** (1984) A thermophilic anaerobe (*Clostridium* species) utilizing various biomass-derived carbohydrates. *Journal of Fermentation Technology* **62**:229-236
- Taya M, Hinok H, Suzuki Y, Yagi T, Yap M, Kobayashi T** (1985a) New thermophilic anaerobes that decompose crystalline cellulose. *Journal of Fermentation Technology* **63**:383-387
- Taya M, Hinok H, Kobayashi T** (1985b) Tungsten requirement of an extremely thermophilic cellulolytic anaerobe (NA10). *Agricultural and Biological Chemistry* **49**:2513-2515
- Taya M, Hinok H, Suzuki Y, Yagi T, Kobayashi T** (1988) Isolation and characterization of an extremely thermophilic, cellulolytic, anaerobic bacterium. *Applied Microbiology and Biotechnology* **29**:474-479
- Toda Y, Saiki T, Uozumi T, Beppu T** (1988) Isolation and characterization of a protease-producing, thermophilic, anaerobic bacterium, *Thermobacteroides leptospartum* sp. nov.. *Agricultural and Biological Chemistry* **52**:1339-1344
- Truper HG, Schlegel HG** (1964) Sulphur metabolism in *Thiorhodaceae*. I. Quantitative measurements on growing cells of *Chromatium okenii*. *Antonie van Leeuwenhoek Journal of Microbiology and Serology* **30**:63-65
- Truper HG, Kramer J** (1981) Principles of characterization and identification of prokaryotes, p. 176-193. *In* MP Starr, H Stolp, HG Truper, A Balows, HG Schlegel (eds.), *The prokaryotes, A handbook on habitats, isolation and identification of bacteria*, vol. 1. Springer-Verlag, New York
- Tucker MP, Mohagheghi A, Grohmann K, Himmel ME** (1989) Ultrathermostable cellulases from *Acidothermus cellulolyticus*: comparison of temperature optima with previously reported cellulases. *Biotechnology* **7**:817-820
- Uchida T, Bonen L, Schaup HW, Lewis BJ, Zablen L, Woese CR** (1974) The use of ribonuclease U₂ in RNA sequence determination. *Journal of Molecular Evolution* **3**:63-77
- van Ketel RJ, ter Schegger JL, Zanen HC** (1984) Molecular epidemiology of *Legionella pneumophila* serogroup I. *Journal of Clinical Microbiology* **20**: 362-364

- Vandamme P, Pot B, Falsen E, Kersters K, De Ley J** (1990) Intro- and inter-specific relationships of veterinary campylobacters revealed by numerical analysis of electrophoretic protein profiles and DNA:DNA hybridizations. *Systematic and Applied Microbiology* **13**:295-303
- Veerkamp JH** (1971) Fatty acid composition of *Bifidobacterium* and *Lactobacillus* strains. *Journal of Bacteriology* **108**:861-867
- Veiga M, Esparis A, Fabregas J** (1983) Isolation of cellulolytic actinomycetes from marine sediments. *Applied and Environmental Microbiology* **46**:286-287
- Veldkamp H** (1960) Isolation and characteristics of *Treponema zuelzeri* nov. spec., an anaerobic, free living spirochete. *Antonie van Leeuwenhoek Journal of Microbiology and Serology* **26**:103-125
- Verger JM, Grimont F, Grimont PAD, Grayon M** (1987) Taxonomy of the genus *Brucella*. *Annales d'Institut Pasteur Microbiology* **138**:235-238
- Viljoen JA, Fred EB, Peterson WH** (1926) The fermentation of cellulose by thermophilic bacteria. *Journal of Agricultural Science* **16**:1-17
- Wachsmuth K** (1986) Molecular epidemiology of bacterial infections: Examples of methodology and investigations of outbreaks. *Review of Infectious Diseases* **18**:682-692
- Ward DM, Weller R, Bateson MM** (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**:63-65
- Waterbury JB, Calloway CG, Turner RD** (1983) A cellulolytic nitrogen fixing bacterium cultured from the gland of *Deshayes* in shipworms (*Bivalvia*: *Teredinidae*). *Science* **221**:1401-1403
- Wayne LG** (1982) Action of the Judicial Commission of the International Committee on Systematic Bacteriology on requests for opinions published between July 1979 and April 1982. *International Journal of Systematic Bacteriology* **32**:464-465
- Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Truper HG** (1987) Report of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology* **37**:463-464
- Weimer PJ** (1991) Quantative and semiquantative measurements of cellulose biodegradation, p. 263-291. *In* CH Haigler, PJ Weimer (eds.), *Biosynthesis and Biodegradation of Cellulose*, Marcel Dekker, New York
- Weimer PJ, Wagner W, Knowlton S, NgTK** (1984) Thermophilic anaerobic bacteria which ferment hemicellulose: characterization of organisms and identification of plasmids. *Archives of Microbiology* **138**:31-36
- Weissberg BG, Sarbutt J** (1966) Chemistry of the hydrothermal waters of the volcanic eruption on Raoul, November 1964. *New Zealand Journal of Science* **9**:426-432

- Widdel F, Pfennig N** (1981) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of a new sulfate-reducer enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov. sp. nov. Archives of Microbiology **129**:395-400
- Widdel F, Kohring GW, Mayer F** (1983) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. Arch Microbiol **134**:286-294
- Wiegel J** (1990) Temperature spans for growth: hypothesis and discussion. FEMS Microbiology Reviews **75**:155-170
- Wiegel J, Ljungdahl LG, Rawson JR** (1979) Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum*. Journal of Bacteriology **139**:800-810
- Wiegel J, Ljungdahl** (1981) *Thermoanaerobacter ethanolicus* gen. nov., sp. nov., a new extreme thermophilic, anaerobic bacterium. Archives of Microbiology **128**:343-348
- Wiegel J, Kuk S, Kohring GW** (1989) *Clostridium thermobutyricum* sp. nov., a moderate thermophile isolated from a cellulolytic culture that produces butyrate as the major end product. International Journal of Systematic Bacteriology **39**:199-204
- Wieten G, Haverkamp J, Meuzelaar HLC, Engel HBW, Berwald LG** (1981) Pyrolysis mass spectrometry: a method to differentiate between the mycobacteria of 'Tuberculosis' complex and other mycobacteria. Journal of General Microbiology **122**:109-118
- Wieten G, Haverkamp J, Berwald LG, Groothuis DG, Draper P** (1982) PMS: its application to mycobacteriology, including *Mycobacterium leprae*. Annales de Microbiologie **133B**:15-27
- Willems A, Goor M, Thielemans S, Gillies M, Kersters K, De Ley J** (1992) Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. International Journal of Systematic Bacteriology **42**:107-119
- Williams AG** (1983) Staining reactions for the detection of hemicellulose-degrading bacteria. FEMS Microbiology Letters **20**:253-258
- Williams ST, Goodfellow M, Alderson G, Wellington EMH, Sneath PHA, Sackin MJ** (1983) Numerical Classification of *Streptomyces* and related genera. Journal of General Microbiology **129**:1743-1813
- Windberger E, Huber R, Trincone A, Fricke H, Stetter KO** (1989) *Thermotoga thermarum* sp. nov. and *Thermotoga neapolitana* occurring in African continental solfataric springs. Archives of Microbiology **151**:506-512
- Windig W, Haverkamp J** (1982) Pyrolysis mass spectrometry of selected yeast species. I. *Rhodospiridium*. Studies in Mycology **22**:56-59

- Winker S, Woese CR** (1991) A definition of the domains Archaea, Bacteria and Eucarya in terms of small subunit ribosomal RNA characteristics. *Systematic and Applied Microbiology* **14**:305-310
- Woese CR** (1987) Bacterial evolution. *Microbiological Reviews* **51**:221-271
- Woese CR, Sogin ML, Stahl DA, Lewis BJ, Bonen L** (1976) A comparison of the 16S ribosomal RNAs from mesophilic and thermophilic bacilli. *Journal of Molecular Evolution* **7**:197-213
- Woese CR, Gibson J, Fox GE** (1980) Do genealogical patterns in purple photosynthetic bacteria reflect interspecific gene transfer? *Nature* **283**:212-214
- Woese CR, Gutell R, Gupta R, Noller HF** (1983) A detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. *Microbiology Reviews* **47**:621-669
- Woese CR, Stackebrandt E, Macke TJ, Fox GE** (1985) A phylogenetic definition of the major eubacterial taxa. *Systematic and Applied Microbiology* **6**:143-151
- Woese CR, Kandler O, Wheelis ML** (1990) Towards a natural system of organisms: Proposal for the domains *Archaea*, *Bacteria* and *Eukarya*. *Proceedings of the National Academy of Sciences, USA*. **87**: 4576-4569
- Wood TM** (1985) Properties of cellulose enzyme systems. *Biochemical Society Transactions* **13**:407-410
- Wood TM, McCrae SI** (1979) Synergism between enzymes involved in the solubilization of native cellulose, p. 181-209. *In* RD Brown, L Jurasek (eds.), *Advances in Chemistry Series 181*, American Chemical Society, Washington, DC
- Yamada Y, Aida K, Uenura T** (1969) Enzymatic studies on the oxidation of sugar and sugar alcohol. V. Ubiquinone of acetic acid bacteria and its relation to classification of the genera *Gluconobacter* and *Acetobacter*, especially of the so-called intermediate strains. *Journal of General and Applied Microbiology* **15**:181-196
- Yanling H, Youfang D, Yanquan L** (1991) Two cellulolytic *Clostridium* species: *Clostridium cellulosi* sp. nov. and *Clostridium cellulofermentans* sp. nov. *International Journal of Systematic Bacteriology* **41**:306-309
- Zacharova EV, Krivenko VV, Mitjuschina LL, Duda VI** (1987) A new obligately thermophilic anaerobic bacterium. *Mikrobiologiya* **56**:288-293
- Zeikus JG, Hegge PW, Anderson MA** (1979) *Thermoanaerobium brockii* gen. nov. and sp. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. *Archives of Microbiology* **122**:41-48
- Zeikus JG, Ben-Basset A, Hegge PW** (1980) Microbiology of methanogenesis in thermal, volcanic environments. *Journal of Bacteriology* **143**:423-440
- Zuckerkindl E, Pauling L** (1965) Molecules as documents of evolutionary history. *Journal of Theoretical Biology* **8**:357-366

ADDITIONAL LITERATURE CITED

- Ambler RP, Daniel M, Hermoso J, Meyer TE, Bartsch RG, Karmen MD** (1979) Cytochrome c_2 sequencing variation among the recognised species of purple nonsulphur photosynthetic bacteria. *Nature* **278**:659-660
- Bergen TB, Grimont PAD, Grimont F** (1983) Fatty acids of *Serratia* determined by gas chromatography. *Current Microbiology* **8**:7-11
- Brosius J, Palmer JL, Kennedy JP, Noller HF** (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proceedings of the National Academy of the United States of America* **75**:4801-4805
- De Soete G** (1983) A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**:621-626
- Fitch WM, Margoliash E** (1976) Construction of Phylogenetic trees. *Science* **155**:279-284
- Fox GE, Wisotzkey JD, Jurtshuk P** (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *International Journal of Systematic Bacteriology* **42**:166-170
- Schumann J, Alexander W, Rainer J, Stetter KO** (1991) Topographical and enzymatic characterization of amylases from the extremely thermophilic eubacterium *Thermotoga maritima*. *Federation of European Biochemical Societies* **282**:122-126
- Smibert RM** (1984) Genus III *Treponema*, p. 49-57. *In*: NR Krieg, JG Holt (eds.), *Bergey's Manual of Systematic Bacteriology*, vol. 1. Williams and Wilkins, Baltimore
- Staley JT, Krieg NR** (1984) Classification of procaryotic organisms: an overview, p. 1-7. *In*: NR Krieg, JG Holt (eds.), *Bergey's Manual of Systematic Bacteriology*, vol. 1. Williams and Wilkins, Baltimore
- Stanton TB** (1992) Proposal to change the genus designation *Serpula* to *Serpulina* gen. nov. containing the species *Serpulina hyodysenteria* comb. nov. and *Serpulina innocens* comb. nov.. *International Journal of Systematic Bacteriology* **42**:189-190
- Woese CR** (1982) Archaeobacteria and cellular origins: an overview. *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene I. Abt Originale* **C3**:1-17

APPENDIX 1

NUMERICAL TAXONOMY DATA SET

		TEST CHARACTERS															
STRAIN	CLUSTER	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16
01	AZ2. B1	A	0	1	0	1	1	0	1	1	0	0	1	1	1	0	1
02	Z-1320	A	0	1	0	1	1	0	1	1	1	1	1	1	1	0	1
03	COMP. B1	A	1	1	1	1	1	0	1	1	0	1	1	1	1	0	1
04	COMP. B4	A	1	1	1	1	1	0	1	1	0	1	1	1	0	0	1
05	Tok9. B1	A	1	1	1	1	1	0	1	1	0	0	1	1	0	0	1
06	Tp8T.6331	A	0	1	0	1	1	0	1	1	0	0	1	1	1	0	1
07	2713-32	A	0	1	0	1	1	0	1	1	1	0	1	1	1	0	0
08	Rt8. B2	A	0	1	0	1	1	0	1	1	0	0	1	1	1	0	1
09	2706-27	A	0	1	0	1	1	0	1	1	0	0	1	0	1	0	1
10	2711-28	A	0	1	0	1	1	0	1	1	0	0	1	0	0	0	0
11	2805-34	A	0	1	0	1	1	0	1	1	0	0	1	0	1	0	0
12	F2/1	A	0	1	1	1	1	0	1	1	0	0	1	1	1	0	0
13	NA10	A	0	0	1	1	1	0	1	1	0	0	1	1	1	0	0
14	Rt8. B11	A	1	1	0	0	1	0	1	1	1	0	1	1	1	0	1
15	Ok1. B1	A	0	0	0	1	1	0	1	0	0	0	1	1	1	0	1
16	Tp8B3.2	B	0	1	1	1	1	0	1	1	0	1	1	1	1	1	0
17	Tp8T2.3	B	0	1	1	1	1	0	1	1	0	1	1	1	1	1	0
17a	Duplicate		1	1	1	1	1	0	1	1	0	1	1	1	1	1	0
18	Tp11. W1	B	0	1	1	1	1	0	0	1	0	0	1	1	1	1	0
19	Wai35. B1	B	0	1	1	1	1	0	1	1	0	0	1	1	1	0	0
20	RI1. B1	B	0	1	1	1	1	0	1	1	0	0	1	1	1	0	0
21	RI2. B1	B	0	1	1	1	1	0	1	1	0	0	1	1	1	1	0
22	2906-25	B	0	1	0	1	1	0	1	1	0	0	1	1	1	0	0
23	2705-55	B	0	1	0	1	1	0	1	1	0	0	1	1	1	0	0
24	AZ3. B1	C	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
24a	Duplicate		0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
25	Rt8. B7	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	Rt8. B4	C	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
27	Rt8. B6	C	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
28	Rt8. B8	C	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
28a	Duplicate		0	0	0	0	0	0	1	0	0	0	0	0	0	0	0

		TEST CHARACTERS																
	STRAIN	CLUSTER	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16
29	Rt8. B15	D	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
29a	Duplicate		0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
30	Rt613. B1	D	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
31	Rt613. B2	D	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
32	Tok8. B1	D	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0
32a	Duplicate		0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0
33	RtUK4	D	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0
34	Tok9. B3	D	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
35	Ok9. B1	D	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
36	Ok2. B1	D	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
37	Wai28. B1	D	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1
38	Rt69. B2	D	0	1	0	1	1	0	1	1	0	0	1	0	1	0	0	0
39	Rt69. B3	D	0	1	0	1	1	0	1	1	0	0	1	0	1	0	0	0
40	Wai21. W1	D	0	1	0	0	1	0	1	1	0	0	0	0	1	0	0	0
41	Rt426. B1	D	0	1	0	1	1	0	1	1	0	0	0	0	1	0	0	0
42	Rt4. B3	D	0	1	1	0	1	0	1	1	0	0	0	1	1	0	0	1
43	Ok8. B1	D	0	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1
44	Rt51. B1	E	0	1	0	1	1	0	1	1	1	0	1	0	1	1	0	1
44a	Duplicate		0	1	0	1	1	0	1	1	1	0	1	0	1	1	0	1
45	<i>C. stercorarium</i>	E	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1
46	<i>C. thermolacticum</i>	E	0	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0
47	Tp10. B1	E	0	0	0	1	1	1	0	1	1	0	1	0	1	0	0	0
48	<i>C. thermocopriae</i>	E	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1
49	Rt69. B1	E	0	1	0	1	1	0	1	1	1	0	1	0	1	0	0	0
50	Rt69. B4	E	0	1	0	1	1	0	1	1	0	0	1	0	1	0	0	0
51	<i>C. thermocellum</i>	E	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0

01= AMYGDALIN, 02= ARABINOSE, 03= RAFFINOSE, 04= GALACTOSE, 05= GLUCOSE, 06= SORBITOL, 07= LACTOSE, 08= XYLOSE, 09= RIBOSE, 10= MELEZITOSE, 11= FRUCTOSE, 12= SUCROSE, 13= MANNOSE, 14= RHAMNOSE, 15= MANNITOL, 16= MALTOSE

		TEST CHARACTERS																
	STRAIN	CLUSTER	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
01	AZ2. B1	A	0	1	1	0	1	1	1	1	0	1	1	1	1	1	0	1
02	Z-1320	A	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
03	COMP. B1	A	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1
04	COMP. B4	A	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1
05	Tok9. B1	A	1	1	1	1	1	1	1	0	1	1	1	0	0	1	0	1
06	Tp8T.6331	A	0	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1
07	2713-32	A	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
08	Rt8. B2	A	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
09	2706-27	A	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
10	2711-28	A	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
11	2805-34	A	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	F2/1	A	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1
13	NA10	A	0	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1
14	Rt8. B11	A	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
15	Ok1. B1	A	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
16	Tp8B3.2	B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
17	Tp8T2.3	B	0	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1
17a	Duplicate		0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
18	Tp11. W1	B	0	1	1	1	1	1	1	1	0	1	1	0	1	1	0	1
19	Wai35. B1	B	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1
20	RI1. B1	B	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1
21	RI2. B1	B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
22	2906-25	B	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	2705-55	B	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
24	AZ3. B1	C	0	1	1	1	0	0	1	0	0	1	1	0	0	0	0	0
24a	Duplicate		0	0	1	1	1	1	1	0	1	1	1	0	0	0	0	1
25	Rt8. B7	C	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1
26	Rt8. B4	C	0	1	1	1	0	0	1	0	0	1	1	1	1	0	0	0
27	Rt8. B6	C	0	1	1	1	0	0	1	0	0	1	1	1	0	1	0	1
28	Rt8. B8	C	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1
28a	Duplicate		0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1

	STRAIN	CLUSTER	TEST CHARACTERS															
			17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
29	Rt8. B15	D	0	0	1	1	0	0	1	0	0	1	1	0	0	0	0	0
29a	Duplicate		0	0	1	1	0	1	1	1	0	1	1	0	0	0	0	1
30	Rt613. B1	D	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1
31	Rt613. B2	D	0	1	1	1	1	1	1	0	0	1	0	0	0	1	0	1
32	Tok8. B1	D	0	1	1	1	1	1	1	1	0	0	1	0	1	0	0	1
32a	Duplicate		0	1	1	1	1	1	1	1	0	0	1	0	1	0	0	1
33	RtUK4	D	0	1	1	1	1	1	1	0	0	1	1	0	1	1	0	1
34	Tok9. B3	D	0	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1
35	Ok9. B1	D	0	1	1	1	1	1	1	0	0	1	1	0	1	1	0	1
36	Ok2. B1	D	0	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1
37	Wai28. B1	D	0	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0
38	Rt69. B2	D	0	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1
39	Rt69. B3	D	0	0	1	1	1	1	1	0	1	1	1	0	1	1	0	1
40	Wai21. W1	D	0	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1
41	Rt426. B1	D	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1
42	Rt4. B3	D	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	1
43	Ok8. B1	D	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0	1
44	Rt51. B1	E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
44a	Duplicate		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
45	<i>C. stercorarium</i>	E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
46	<i>C. thermolacticum</i>	E	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1
47	Tp10. B1	E	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
48	<i>C. thermocopriae</i>	E	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
49	Rt69. B1	E	0	1	0	0	1	1	0	1	0	1	1	0	0	1	1	1
50	Rt69. B4	E	0	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1
51	<i>C. thermocellum</i>	E	0	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1

17= MELIBIOSE, 18= GROWTH ON LAMINARIN, 19= GROWTH ON LICHENIN, 20= GROWTH ON GUM LOCUST BEAN, 21= GROWTH ON PULLULAN, 22= GROWTH ON GLYCOGEN, 23= GROWTH ON GUM GUAR, 24= GROWTH ON INULIN, 25= GROWTH ON PECTIN
26= VIABILITY AFTER 5 d AT OPTIMUM GROWTH TEMPERATURE, 27= PENICILLIN-G (MIC>125µg/ml), 28= BACITRACIN (MIC>1.95µg/ml), 29= POLYMYXIN (MIC>7.8µg/ml), 30= STREPTOMYCIN (MIC>15.6µg/ml), 31= NOVOBIOCIN (MIC>15.6µg/ml)
32=CYCLOSERINE(MIC>62.5µg/ml)

		TEST CHARACTERS																
	STRAIN	CLUSTER	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
01	AZ2. B1	A	1	0	1	0	1	1	1	1	1	1	1	1	1	0	0	1
02	Z-1320	A	1	1	1	0	1	1	1	1	1	0	1	1	1	0	0	1
03	COMP. B1	A	1	0	1	0	1	1	0	1	1	0	1	1	0	0	0	1
04	COMP. B4	A	1	0	1	0	1	1	0	1	1	0	1	1	0	0	0	1
05	Tok9. B1	A	1	0	1	0	1	1	1	1	1	0	1	1	1	0	0	1
06	Tp8T.6331	A	1	0	1	0	1	1	1	1	1	0	1	1	1	0	0	1
07	2713-32	A	0	0	1	0	0	1	1	1	1	1	1	1	1	0	0	1
08	Rt8. B2	A	1	0	1	0	0	1	1	1	1	0	1	1	1	1	0	0
09	2706-27	A	1	0	1	0	0	1	1	1	1	0	1	1	1	1	0	0
10	2711-28	A	1	0	1	0	1	1	1	1	0	0	0	1	1	1	0	0
11	2805-34	A	1	0	1	0	0	1	1	1	1	0	0	1	1	0	0	1
12	F2/1	A	1	0	0	0	1	1	1	1	1	0	1	1	1	0	0	1
13	NA10	A	1	0	0	0	1	1	1	1	1	1	0	1	1	0	1	0
14	Rt8. B11	A	1	0	0	1	1	1	1	1	1	1	1	1	0	1	0	0
15	Ok1. B1	A	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1
16	Tp8B3.2	B	1	0	0	0	1	1	1	1	0	0	1	1	1	1	0	0
17	Tp8T2.3	B	1	0	0	0	1	1	1	1	1	0	0	1	1	0	1	0
17a	Duplicate		1	0	0	0	1	1	1	1	1	0	0	1	1	0	1	0
18	Tp11. W1	B	1	0	0	1	1	1	0	1	1	0	1	1	1	0	0	1
19	Wai35. B1	B	1	0	1	0	1	1	1	1	1	0	1	1	1	0	0	1
20	RI1. B1	B	1	0	0	0	1	1	1	1	1	0	1	1	1	0	0	1
21	RI2. B1	B	1	0	0	0	1	1	0	1	1	0	0	1	0	1	0	0
22	2906-25	B	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	0
23	2705-55	B	1	0	1	1	1	1	1	1	0	0	0	1	0	1	0	0
24	AZ3. B1	C	1	0	1	1	1	1	0	1	1	1	0	1	1	0	0	1
24a	Duplicate		1	0	1	1	1	1	0	1	1	0	0	1	1	0	0	1
25	Rt8. B7	C	1	0	0	0	1	1	1	1	1	0	1	1	1	0	0	1
26	Rt8. B4	C	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1
27	Rt8. B6	C	1	0	1	1	1	1	0	0	0	0	1	1	1	0	0	1
28	Rt8. B8	C	1	0	1	1	1	1	0	0	0	0	1	1	0	0	1	0
28a	Duplicate		1	0	1	1	1	1	1	0	0	0	1	1	0	0	1	0

			TEST CHARACTERS															
	STRAIN	CLUSTER	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
29	Rt8. B15	D	1	0	0	1	1	1	0	0	0	0	1	1	0	0	1	0
29a	Duplicate		1	0	0	1	1	1	0	0	0	0	1	1	0	0	1	0
30	Rt613. B1	D	1	0	1	1	1	1	1	1	1	0	1	1	0	0	1	0
31	Rt613. B2	D	1	0	1	1	1	1	1	1	0	0	1	1	0	0	1	0
32	Tok8. B1	D	1	0	1	1	1	1	1	0	1	0	0	1	0	0	1	0
32a	Duplicate		1	0	1	1	1	1	1	0	1	0	0	1	0	0	1	0
33	RtUK4	D	1	0	1	1	1	1	1	1	1	0	1	1	0	0	1	0
34	Tok9. B3	D	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	0
35	Ok9. B1	D	1	0	0	1	1	1	1	1	1	0	0	1	0	0	0	1
36	Ok2. B1	D	1	0	0	1	1	1	0	1	1	0	0	1	1	0	1	0
37	Wai28. B1	D	1	0	1	1	1	1	1	1	1	0	1	1	0	0	1	0
38	Rt69. B2	D	1	0	1	1	1	1	1	1	1	0	0	1	0	0	1	0
39	Rt69. B3	D	1	0	1	1	1	1	1	0	1	0	1	1	1	0	1	0
40	Wai21. W1	D	1	0	0	1	1	1	1	0	1	0	1	1	1	0	1	0
41	Rt426. B1	D	1	0	0	1	1	1	1	1	1	0	0	1	1	0	0	1
42	Rt4. B3	D	1	0	0	1	1	1	1	1	0	0	1	1	0	0	1	0
43	Ok8. B1	D	1	0	0	1	1	1	0	1	1	0	0	1	1	0	1	0
44	Rt51. B1	E	0	1	1	0	1	1	0	0	1	0	0	0	0	0	0	1
44a	Duplicate		0	1	1	0	1	1	0	0	1	0	0	0	0	0	0	1
45	<i>C. stercorarium</i>	E	0	1	1	0	0	1	1	1	1	1	1	1	0	0	1	0
46	<i>C. thermolacticum</i>	E	0	0	1	0	1	1	0	0	1	0	1	0	0	0	1	0
47	Tp10. B1	E	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	1
48	<i>C. thermocopriae</i>	E	0	0	0	0	0	0	1	0	1	1	1	1	0	0	1	0
49	Rt69. B1	E	1	0	0	1	1	1	0	1	0	0	1	1	0	0	1	0
50	Rt69. B4	E	1	0	1	1	1	1	0	0	1	0	0	1	0	0	1	0
51	<i>C. thermocellum</i>	E	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0

33= RIFAMPIN (MIC>50µg/ml), 34= GROWTH INHIBITED BY 2% ETHANOL, 35= GROWTH INHIBITED BY 3% ETHANOL, 36= GROWTH INHIBITED BY 0.5% NaCl, 37= GROWTH INHIBITED BY 1.0% NaCl, 38= KOH LYSIS, 39= DNAase PRODUCTION, 40= GROWTH @ pH5.5
41= GROWTH @ pH7.5, 42= GROWTH @ pH 8.0, 43= GROWTH AFTER 10 d @ 45°C, 44= GROWTH AFTER 3 d @ 75°C, 45= GROWTH AFTER 3 d @ 80°C, 46= FLAT/RAISED COLONY ELEVATION, 47= CONVEX COLONY ELEVATION, 48= UMBONATE COLONY ELEVATION

		TEST CHARACTERS																
	STRAIN	CLUSTER	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
01	AZ2. B1	A	0	1	0	0	0	1	0	0	0	1	1	0	1	0	1	1
02	Z-1320	A	0	1	0	0	1	1	1	1	0	1	1	0	1	0	1	1
03	COMP. B1	A	0	1	0	0	0	0	0	1	1	1	1	1	1	0	1	1
04	COMP. B4	A	0	1	0	0	0	0	0	1	0	1	1	1	1	0	1	1
05	Tok9. B1	A	0	1	0	0	0	1	0	0	0	1	1	0	1	0	1	1
06	Tp8T.6331	A	0	1	0	0	0	1	1	0	0	1	1	0	1	0	1	1
07	2713-32	A	0	1	0	0	0	1	0	1	1	0	1	0	1	0	1	1
08	Rt8. B2	A	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	1
09	2706-27	A	0	1	0	0	0	0	1	0	0	1	1	1	1	0	1	1
10	2711-28	A	1	0	0	0	0	0	1	1	1	0	1	0	1	0	1	1
11	2805-34	A	1	0	1	0	0	0	1	1	1	1	1	1	1	1	1	1
12	F2/1	A	1	0	0	0	0	1	1	1	1	1	1	0	1	0	1	1
13	NA10	A	1	0	0	0	0	1	1	1	0	0	1	0	1	0	1	1
14	Rt8. B11	A	0	1	0	0	0	0	1	1	0	0	1	0	1	0	1	1
15	Ok1. B1	A	0	1	0	0	0	1	1	1	1	1	1	0	1	0	1	1
16	Tp8B3.2	B	0	1	1	0	0	1	1	0	0	0	0	0	0	0	1	1
17	Tp8T2.3	B	1	0	0	0	0	1	0	1	1	0	1	0	0	0	1	1
17a	Duplicate		1	0	0	0	0	1	0	1	1	0	1	0	0	0	1	1
18	Tp11. W1	B	1	0	0	0	1	1	0	0	0	0	1	0	1	0	1	1
19	Wai35. B1	B	0	1	0	0	1	1	0	1	0	0	1	1	1	1	1	1
20	RI1. B1	B	0	1	0	0	0	0	1	1	1	1	1	1	1	0	1	1
21	RI2. B1	B	0	1	0	0	0	1	0	1	0	1	1	0	1	0	1	1
22	2906-25	B	0	1	0	0	0	0	1	1	1	0	1	0	1	0	1	1
23	2705-55	B	0	1	0	0	0	1	1	0	0	0	1	0	1	0	1	1
24	AZ3. B1	C	1	0	0	0	0	0	1	1	1	1	1	1	1	0	1	1
24a	Duplicate		1	0	0	0	0	0	1	1	1	1	1	0	1	0	1	1
25	Rt8. B7	C	0	1	0	0	0	0	1	1	1	1	1	1	1	0	1	1
26	Rt8. B4	C	0	1	0	0	0	0	0	1	0	1	1	1	1	1	1	1
27	Rt8. B6	C	1	0	0	0	0	0	0	1	1	0	1	1	1	1	1	1
28	Rt8. B8	C	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
28a	Duplicate		1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1

			TEST CHARACTERS															
	STRAIN	CLUSTER	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64
29	Rt8. B15	D	1	0	0	1	0	0	0	1	1	0	1	1	1	0	1	1
29a	Duplicate		1	0	0	1	0	0	0	1	1	1	1	1	1	1	1	1
30	Rt613. B1	D	1	0	0	0	0	0	1	0	0	1	1	1	1	0	1	1
31	Rt613. B2	D	1	0	0	0	0	0	0	1	0	1	1	1	1	0	1	1
32	Tok8. B1	D	1	0	0	0	0	1	0	0	1	0	1	0	1	0	1	1
32a	Duplicate		1	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1
33	RtUK4	D	1	0	0	1	0	0	1	1	1	0	1	0	1	0	1	1
34	Tok9. B3	D	1	0	0	0	0	1	1	1	0	0	0	0	1	0	1	1
35	Ok9. B1	D	0	1	0	0	0	0	1	1	1	1	1	0	0	0	1	1
36	Ok2. B1	D	1	0	0	0	0	0	1	1	1	0	1	0	1	0	1	1
37	Wai28. B1	D	1	0	0	0	0	0	0	1	0	0	1	0	1	0	1	1
38	Rt69. B2	D	1	0	0	0	0	0	1	1	1	0	1	0	1	0	1	1
39	Rt69. B3	D	1	0	0	0	0	0	1	0	1	1	1	0	1	0	1	1
40	Wai21. W1	D	1	0	0	0	0	0	1	0	0	0	1	1	1	0	1	1
41	Rt426. B1	D	1	0	0	0	0	1	0	0	1	0	0	0	1	0	1	1
42	Rt4. B3	D	1	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1
43	Ok8. B1	D	1	0	0	0	0	0	1	1	1	0	1	0	1	0	1	1
44	Rt51. B1	E	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	1
44a	Duplicate		0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	1
45	<i>C. stercorarium</i>	E	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0
46	<i>C. thermolacticum</i>	E	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
47	Tp10. B1	E	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0
48	<i>C. thermocopriae</i>	E	0	1	1	0	0	1	1	1	0	0	1	0	0	0	0	1
49	Rt69. B1	E	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0
50	Rt69. B4	E	0	1	0	1	0	0	0	1	1	0	1	0	1	0	0	1
51	<i>C. thermocellum</i>	E	1	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0

49= ENTIRE COLONY MARGIN, 50= UNDULATE/LOBATE COLONY MARGIN, 51= H₂S PRODUCED, 52= PARTICULATE GROWTH, 53= SLIMEY PELLET, 54= CELL LENGTH >2.5 µm 55= CELL WIDTH >0.4 µm, 56= FLAGELLA, 57= BLUNT CELL ENDS, 58= GROWTH ON BASAL MEDIUM PLUS CELLIBIOSE @ 24 h, 59= GROWTH ON BASAL MEDIUM PLUS CELLIBIOSE @ 72 h, 60= GROWTH ON BASAL MEDIUM PLUS AMORPHOUS CELLULOSE @ 24 h, 61= GROWTH ON BASAL MEDIUM PLUS AMORPHOUS CELLULOSE @ 72 h, 62= GROWTH ON BASAL MEDIUM PLUS AVICELL @ 24 h, 63= GROWTH ON AMORPHOUS CELLULOSE @ 24 h, 64= GROWTH ON AMORPHOUS CELLULOSE @ 72 h

		TEST CHARACTERS																
	STRAIN	CLUSTER	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
01	AZ2. B1	A	1	1	1	1	1	0	1	1	1	1	1	1	0	0	0	1
02	Z-1320	A	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1
03	COMP. B1	A	1	1	1	1	1	0	0	1	1	1	0	1	1	0	0	1
04	COMP. B4	A	1	1	1	1	1	0	0	1	1	1	0	1	1	0	0	1
05	Tok9. B1	A	0	0	1	0	0	0	0	1	1	1	1	1	1	0	0	1
06	Tp8T.6331	A	0	0	1	1	1	0	1	1	1	1	1	1	1	0	0	1
07	2713-32	A	1	1	1	1	1	0	1	1	1	1	0	1	1	0	0	1
08	Rt8. B2	A	0	0	1	0	1	0	0	1	0	0	1	1	1	0	0	1
09	2706-27	A	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1
10	2711-28	A	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1
11	2805-34	A	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1
12	F2/1	A	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1
13	NA10	A	1	1	1	1	1	0	0	1	0	1	1	1	1	0	0	1
14	Rt8. B11	A	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	1
15	Ok1. B1	A	1	0	1	0	1	0	1	0	1	1	1	1	1	1	1	1
16	Tp8B3.2	B	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1
17	Tp8T2.3	B	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1
17a	Duplicate		0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1
18	Tp11. W1	B	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
19	Wai35. B1	B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
20	RI1. B1	B	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
21	RI2. B1	B	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
22	2906-25	B	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1
23	2705-55	B	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
24	AZ3. B1	C	0	1	1	0	1	0	1	1	0	0	1	1	1	0	0	0
24a	Duplicate		0	0	1	0	1	0	0	1	0	0	0	1	1	0	0	1
25	Rt8. B7	C	0	0	1	1	1	0	0	1	1	0	1	1	1	0	0	1
26	Rt8. B4	C	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0
27	Rt8. B6	C	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0
28	Rt8. B8	C	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1
28a	Duplicate		1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	0

			TEST CHARACTERS																
	STRAIN	CLUSTER	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	
29	Rt8. B15	D	1	1	1	1	1	1	1	1	0	1	0	1	1	0	0	0	
29a	Duplicate	50	1	1	1	1	1	0	1	1	0	1	0	1	1	0	0	0	
30	Rt613. B1	D	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	
31	Rt613. B2	D	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	
32	Tok8. B1	D	1	1	1	1	1	0	0	1	1	1	0	1	1	0	0	1	
32a	Duplicate		1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	1	
33	RtUK4	D	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	
34	Tok9. B3	D	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1	
35	Ok9. B1	D	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	
36	Ok2. B1	D	1	1	1	1	1	0	1	1	1	0	0	1	1	0	0	1	
37	Wai28. B1	D	1	1	1	1	1	0	1	0	0	0	1	0	0	0	0	1	
38	Rt69. B2	D	1	1	1	1	1	0	1	1	1	1	0	1	1	0	0	1	
39	Rt69. B3	D	1	1	1	1	1	0	1	1	1	1	0	1	1	0	0	1	
40	Wai21. W1	D	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1	
41	Rt426. B1	D	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	
42	Rt4. B3	D	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	
43	Ok8. B1	D	1	1	1	1	1	0	1	1	1	1	0	1	1	0	0	1	
44	Rt51. B1	E	0	1	1	0	0	0	0	1	1	1	1	1	1	0	0	1	
44a	Duplicate		0	1	1	0	0	0	0	1	1	1	1	1	1	0	0	1	
45	<i>C. stercorarium</i>	E	0	0	1	0	1	0	0	1	1	1	1	1	1	0	0	1	
46	<i>C. thermolacticum</i>	E	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	1	
47	Tp10. B1	E	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1	
48	<i>C. thermocopriae</i>	E	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0	1	
49	Rt69. B1	E	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	
50	Rt69. B4	E	0	0	1	0	0	0	0	1	1	1	1	1	0	0	0	1	
51	<i>C. thermocellum</i>	E	0	0	1	0	1	0	1	1	0	0	1	1	0	0	0	0	

65= GROWTH ON AVICEL @ 24 h, 66= GROWTH ON SIGMA CELL 20 @ 24 h, 67= GROWTH ON SIGMA CELL 20 @ 72 h, 68= GROWTH ON SIGMA CELL 50 @ 24 h, 69= GROWTH ON SIGMA CELL 50 @ 72 h, 70= GROWTH ON SIGMA CELL 100 @ 24 h, 71= GROWTH ON SIGMA CELL 100 @ 72 h, 72= DEGRADATION OF CMC, 73= DEGRADATION OF STARCH, 74= DEGRADATION OF XYLAN, 75= DEGRADATION OF LAMINARIN, 76= DEGRADATION OF LICHENIN, 77= DEGRADATION OF GUM LOCUST BEAN, 78= DEGRADATION OF GUM KARAYA 79= DEGRADATION OF CHITIN, 80= DEGRADATION OF PULLULAN

	STRAIN	CLUSTER	TEST CHARACTERS											
			8 1	8 2	8 3	8 4	8 5	8 6	8 7	8 8	8 9	9 0	9 1	9 2
01	AZ2. B1	A	1	0	0	0	0	1	0	0	1	0	0	0
02	Z-1320	A	1	0	1	1	0	1	0	0	1	0	0	0
03	COMP. B1	A	1	0	0	1	0	1	0	0	0	1	1	0
04	COMP. B4	A	1	0	0	1	0	1	0	0	0	1	1	0
05	Tok9. B1	A	0	0	0	1	0	1	0	0	0	1	1	0
06	Tp8T.6331	A	0	0	0	1	0	0	1	0	0	1	0	0
07	2713-32	A	1	0	0	1	0	0	0	1	0	1	0	0
08	Rt8. B2	A	0	0	0	1	0	0	1	0	0	1	0	0
09	2706-27	A	1	0	0	1	0	0	1	0	0	1	0	0
10	2711-28	A	1	0	0	1	0	0	1	0	0	0	0	0
11	2805-34	A	1	0	0	1	0	0	1	0	0	0	0	0
12	F2/1	A	0	0	0	1	0	0	1	0	0	1	0	0
13	NA10	A	0	0	0	1	0	0	1	0	0	1	0	0
14	Rt8. B11	A	1	1	1	1	0	0	1	0	0	0	0	0
15	Ok1. B1	A	1	1	0	1	0	1	0	0	1	0	0	1
16	Tp8B3.2	B	1	1	1	1	1	1	0	0	0	1	0	0
17	Tp8T2.3	B	1	1	1	1	1	1	0	0	0	1	0	1
17a	Duplicate		1	1	1	1	1	1	0	0	0	1	0	0
18	Tp11. W1	B	1	1	1	1	1	0	1	0	0	0	1	0
19	Wai35. B1	B	1	1	1	1	1	0	1	0	0	0	0	0
20	RI1. B1	B	1	1	1	1	1	0	1	0	0	0	1	0
21	RI2. B1	B	1	1	1	1	1	0	1	0	0	0	0	0
22	2906-25	B	1	1	1	1	1	0	1	0	0	1	0	0
23	2705-55	B	1	1	1	1	1	0	1	0	0	0	0	0
24	AZ3. B1	C	0	0	0	1	0	1	0	0	0	1	0	0
24a	Duplicate		0	0	0	1	0	1	0	0	0	1	0	0
25	Rt8. B7	C	0	0	0	1	0	1	0	0	0	0	0	0
26	Rt8. B4	C	0	0	0	1	0	1	0	0	0	1	0	0
27	Rt8. B6	C	0	0	0	1	0	1	0	0	0	0	0	0
28	Rt8. B8	C	0	0	0	1	0	1	0	0	0	0	0	0
28a	Duplicate		0	0	0	1	0	1	0	0	0	0	0	0

	STRAIN	CLUSTER	TEST CHARACTERS											
			81	82	83	84	85	86	87	88	89	90	91	92
29	Rt8. B15	D	0	0	0	1	0	0	1	0	0	0	0	0
29a	Duplicate		0	1	0	1	0	0	1	0	0	0	0	0
30	Rt613. B1	D	0	1	0	1	0	0	1	0	0	0	0	0
31	Rt613. B2	D	0	1	1	1	0	0	1	0	0	0	0	0
32	Tok8. B1	D	0	0	0	1	0	0	1	0	0	1	0	0
32a	Duplicate		0	0	0	1	0	0	1	0	0	1	0	0
33	RtUK4	D	0	0	0	1	0	0	1	0	0	0	0	0
34	Tok9. B3	D	0	0	0	1	0	1	0	0	0	0	0	0
35	Ok9. B1	D	0	0	0	1	0	0	1	0	0	0	0	0
36	Ok2. B1	D	1	0	0	1	0	0	1	0	0	0	0	0
37	Wai28. B1	D	1	0	0	1	0	0	1	0	0	0	0	0
38	Rt69. B2	D	1	0	0	1	0	0	1	0	0	0	0	0
39	Rt69. B3	D	1	0	0	1	0	0	1	0	0	0	0	0
40	Wai21. W1	D	1	0	0	1	0	1	0	0	0	0	0	0
41	Rt426. B1	D	1	0	0	1	0	0	1	0	0	0	0	0
42	Rt4. B3	D	1	0	0	1	0	0	1	0	0	0	0	0
43	Ok8. B1	D	1	1	0	1	0	0	1	0	0	0	0	0
44	Rt51. B1	E	1	0	0	1	0	0	1	0	1	0	1	1
44a	Duplicate		1	0	0	1	0	0	1	0	1	0	1	1
45	<i>C. stercorarium</i>	E	1	0	0	0	0	0	1	0	1	0	0	0
46	<i>C. thermolacticum</i>	E	1	0	0	1	0	0	0	1	1	0	0	0
47	Tp10. B1	E	1	0	0	0	0	0	1	0	1	0	0	1
48	<i>C. thermocopriae</i>	E	1	1	0	0	0	0	0	1	1	0	0	1
49	Rt69. B1	E	0	0	0	0	0	0	1	0	0	0	0	0
50	Rt69. B4	E	1	1	0	0	0	1	0	0	0	0	0	0
51	<i>C. thermocellum</i>	E	1	1	0	0	0	0	1	0	1	0	0	0

81= DEGRADATION OF GLYCOGEN, 82= DEGRADATION OF MANNAN, 83= DEGRADATION OF ARABINOGALACTAN
84= DEGRADATION OF GUM GUAR, 85= DEGRADATION OF INULIN, 86= LACTATE/ACETATE PRODUCED (1:1),
87= ACETATE MAIN END PRODUCT, 88= LACTATE MAIN END PRODUCT, 89= ETHANOL MAJOR END PRODUCT
90= ETHANOL MINOR END PRODUCT, 91= *iso*-BUTYRATE PRODUCED, 92= BUTYRATE PRODUCED

APPENDIX 2

CHAPTER 6 SEQUENCE ALIGNMENT

Abbreviations of strains:

C.tellum:		Rem: Def: small subunit ribosomal RNA.
Date: 29-JAN-1992	DEFINITION C; small subunit ribosomal RNA.	Ok9.B1:
Rem: KEYWORDS:small subunit ribosomal RNA.		Name: Ok9.B1; small
C.thaceti:		Date: Thu Feb 13 16:02:46 1992
Name: Clostridium thermoaceticum		Who: Erko Stackebrandt
Date: 13-MAY-1991		Rt8.D1:
Rem: KEYWORDS:small subunit ribosomal RNA.		Name: Rt8.D115; small
C.thsacch:		Date: 1-JAN-1992
Name: Clostridium thermosaccharolyticum		Rem: KEYWORDS:small subunit ribosomal RNA.
Date: 13-MAY-1991		NA10:
Rem: KEYWORDS:small subunit ribosomal RNA.		Name: Na; small
C.sticklan:		Date: 1-JAN-1992
Name: Clostridium sticklandii		Rem: KEYWORDS:small subunit ribosomal RNA.
Date: 13-MAY-1991		Rt8.C1:
Rem: KEYWORDS:small subunit ribosomal RNA.		Name: Rt8.C1B7; small
C.coccoids:		Date: 1-JAN-1992
Name: Clostridium coccoides		Rem: KEYWORDS:small subunit ribosomal RNA.
Date: 13-MAY-1991		RI2.B1:
Rem: KEYWORDS:small subunit ribosomal RNA.		Name: RI2.B1; small
C.clostrid:		Date: 1-JAN-1992
Name: Clostridium clostridioforme		Rem: KEYWORDS:small subunit ribosomal RNA.
Date: 13-MAY-1991		Rt51.B1:
Rem: KEYWORDS:small subunit ribosomal RNA.		Name: Rt51.B1; small
C.aminoval:		Date: 1-JAN-1992
Name: Clostridium aminovalericum		Rem: KEYWORDS:small subunit ribosomal RNA.
Date: 13-MAY-1991		C.ster:
Rem: KEYWORDS:small subunit ribosomal RNA.		Name: C.stercorarium; small
C.linsebu:		Date: 1-JAN-1992
Name: Clostridium linsebaurens		Ctcop:
Date: 13-MAY-1991		Name: C.thermocopriac; small
Rem: KEYWORDS:small subunit ribosomal RNA.		Date: 1-JAN-1992
C.pasturi:		Rem: KEYWORDS:small subunit ribosomal RNA.
Name: Clostridium pasteurianum		Tbroc:
Date: 13-MAY-1991		Name: Theroanaerobium brockii; small
Rem: KEYWORDS:small subunit ribosomal RNA.		Date: 1-JAN-1992
C.purinoly:		Rem: KEYWORDS:small subunit ribosomal RNA.
Name: Clostridium purinolyticum		Wai35.B1:
Date: 13-MAY-1991		Name: Wai35.B1; small
Rem: KEYWORDS:small subunit ribosomal RNA.		Date: 1-JAN-1992
C.butyric:		Rem: KEYWORDS:small subunit ribosomal RNA.
Name: Clostridium butyricum		
Date: 13-MAY-1991		
Rem: KEYWORDS:small subunit ribosomal RNA.		
T.maritim:		
Name: Thermotoga maritima str. MSB8		
Date: 13-MAY-1991		
Rem: Keywords:small subunit ribosomal RNA.		
Def: small subunit ribosomal RNA.		
Arb.globif:		
Name: Arthrobacter globiformis		
Date: 13-MAY-1991		
Rem: KEYWORDS:small subunit ribosomal RNA.		
Tp8T:		
Name: Tp8		
Date: 13-MAY-1991		
Rem: Keywords:small subunit ribosomal RNA.		

Page 195

Page 196

0
600
661
618
627
630
631
648
604
609
628
613
644
619
634
368
616
573
506
606
369
557
623
486
605

0
700
761
718
729
730
731
748
704
709
728
713
744
719
734
468
716
673
606
707
468
657
723
586
706

0
800
860
817
828
830
830
848
804
808
827
813
844
819
833
567
815
772
705
806
568
757
823
586
805

Page 198

0
1100
1162
1091
1130
1128
1126
1147
1103
1106
1125
1085
1152
1120
1149
758
998
983
924
1033
827
1016
1075
935
1002

0
1200
1262
1190
1229
1228
1226
1246
1202
1205
1224
1184
1251
1220
1249
859
1098
1083
1024
1133
927
1116
1175
982
1102

0
1300
1363
1291
1330
1329
1327
1347
1303
1305
1325
1284
1351
1321
1351
960
1199
1184
1125
1234
1027
1216
1276
1083
1203

APPENDIX 3

CHAPTER 7 SEQUENCE ALIGNMENT

10 20 30 40 50 60 70 80 90 100

1 GGC G G C G - U G - C - C U A A G A C A U G C A A G U C G U G C G C G G C G G - - A C G G G U G A G U - - - A A C - G C G U G G G U G A - - C C U A C - C C G G A A G A G G G G G A C A A C C C G G G T.thermoph
1 G G C G G C A - C G - U - U U U A A G n A U G C A A G U C G A A U A U G G C G A - - A C G G G U G A G U - - - A A C - A C G U A G G U G A - - U C U G C - C U G A C G G A U G G G G A U A G C U U U G S p i . a u r a n t
1 G G C G G C G - C G - U - U U U A A G n A U G C A A G U C G A A C G U G G C G A - - A C G G G U g A G U - - - A A C - G C G U A G G U G A - - U C U A C - C U U U U A G U U G G G G A U A G C C C A U G S p i . l i t o r a
1 G G C g G C G - C G - U - U U U A A G n A U G C a a G U C G A a C G U G G C G A - - a C G G n n A G U - - - A A C - A C G U A G A u G A - - U C U G C - C U u A A G U U G G G G A U A G C C C A U A S p i . i s o v a l
1 G G C A G U G - C G - U - C U U A A G C A U G C A A G U C A A A C G - - - - - - - A C G G G U G A G U - - - A A C - G C G U G G A U G A - - U C U A C - C U A U G A G A U G G G G A U A A C U A U U A B o r . b u r g d o r f e r i
1 G G C G G C G - C G - U - C U U A G G C A U G C A A G U C G A G C G C G G C G G - - A C G G G U G A G U - - - A A C - A C G U A G G U G A - - C C U G U - C C U C A G G U U G G G G A U A G C C C G U G R I 19 B 1
1 G G C G G C G - C G - U - C U U A A G C A U G C A A G U C G A G C G C G G C G G - - A C U G G U G A G U - - - A A C - G C G U G G G U G A - - C G U A C - C U U U G U G A C G G G G A C A G C U C C U A T r p . b r y a n t
1 G G C G G C G - C G - U - C U U A A G C A U G C A A G U C G G G - - C G G C G G - - A C U G G C G A G U - - - A A C - A C G U G G G C G A - - C G C G C - C C U C C G G A C G G G A A U A G C C U G C A T r e p o n e m a s u c c i n i f a c i e n s
1 G G C G G C G - C G - U - C U U A A G C A U G C A A G U C G G A C G u g g c g g - - a c u g n u n a g u - - - a a c - n c g u g g g u G A - - C C U A C - C C U U G g n u n g g g a a u a G C C A U U A S p i . z u e l z e
1 G G C G G C G - C G - U - C U U A A G C A U G C A A G U C G A A C - - - - - - - A C U G G U G A G U - - - A A C - G C G U G G G U G A - - U C U A C - C C U U A A G A U G G G G A U A G C U C C U A T r e p o n e m a p h a g e d e n i s
1 G G C G G C G - C G - U - C U U A A G n A U G C A A G U C G A g C G C G G C G G - - A C U G G U G A G U - - - A A C - A C G U G G G U G A - - U C U A C - C U U U A G G U U G G G G A U A G C C A U U A S p i . s t e n o s
1 G G C G A U G - C G - U - C U U A A G C A U G C A A G U C G A G C - C G G C G A - - A C U G G U G A G U - - - A A C - A C G U A G G U A A - - U C U G C - C G U A G A G U G G G G G A U A A C C C A U G S . i n n o c e n s
1 G G C G A U G - C G - U - C U U A A G C A U G C A A G U C G A G C - C G G C G A - - A C U G G U G A G U - - - A A C - A C G U A G G U A A - - U C U G C - C G U A G A G U G G G G G A U A A C C C A U G S . h y o d y s e n t e r i a e
1 G G C G G C G - C G - U - C U U A A A C A U G C A A G U C G A G C G C G G C G A - - A C G G G U G A G U - - - A A C - A C G U A G G U A A - - U U U G C - C C A C G G A U G G G G G A U A A C C U U U C L p n . i l l i n i
1 G A C A G A A - U G - C - U U A A C A C A U G C A A G U C U - U U n A G G C G G - - A C G G G U G A G U - - - A A C - G C G U A A A G A A - - C U U G C - C C U G C A G A G A G G G A U A A C A G A C G L p t . b u c c a l
1 G G C G G C G - U G - C - C U A A U A C A U G C A A G U C G A G C g c g G C g g - - A U g G G U G A G U - - - A A C - A C G U g G G C A A - - C C U G C - C U G U a A G A C G G G G A U A A C U U C G G S p l . i n u l i n
1 G G C G G C G - U G - C - C U A A C A C A U G C A A G U C G A G C G C G G C G G - - A C G G g U G A G U - - - A A C - A C g U G G g U A A - - C C U G c - C C U C C G g A G G G G G A U A A C c A G G T t . m a r i t i m a

* *

89 G A A A C U C G G G C U A A U C C C C A U - G U G G A C C C - - G - - - - C C - C U U G - G G G - - - - U - G U G U C - C A A A - C G - - C U U C C G G - A U G G G C C C G C G U C C C A U C A - G - T.thermoph
89 G A A A C A C A G G G U A A U A C C G A A U - G A G C U G A C - - G A - - - G C - U U U G - G U U - - - U U - G U U A G - U A A A - C G - - C C G A C A G - A G G A G C C U G C G G C U G A U U A - G - S p i . a u r a n t
89 G A A A C A U G G G G U A A U A C C G A A U - A A U C U C A U - - U - - - - G U - U U U G - U U U - - - - A - A U G A G - G A A A - C G - - C U G A A A G - A U G A G C C U G C G U A U G A U U A - G - S p i . l i t o r a
89 G A A A U A U G G G G U A A U A C C G G A U - A A U C U C U U - - U U - - - U C - U U U G - G U G - - - A A - U A G A G - G A A A - c G - - C U U U r A G - A U G A G U C U G C G G C U G A U U A - G - S p i . i s o v a l
83 G A A A U A G U A G C U A A U A C C G A A U - A A G G U C A - - G U U - - A - - U U U G - U U - - - - a A - U U G A U - G A A A - c g - - C U U G U A G A U G A G - U C U G C G U C U A U U A - G - B o r . b u r g d o r f e r i
89 G A A A C A C G G G G U A A U A C C G A A U - G G C C U C C - - U U U - - G C - U G U G - G C C - - U G A - - G G A G - G A A A - C G - - C C U G A G G - A G N N N N N U G C G G C C C A U U A - G - R I 19 B 1
89 G A A A U A G G A G A U A A U A C C G G A U - A C G C U G C A - - U G U - - U U - C A G - - A G G - - A C A - U G C A G - G A A A - C G - - C A C G A A G - A G C G G C C C G C G U A C U A U U A - G - T r p . b r y a n t
87 G A A A U G C A G G G U A A U G C C G G A U - G C G A A C A - - C A C - - G G - C U G G - A G C - C G U G - - C G U G - G A A A G G G - - G C G C C G G - A G G A G C G G C C C G C G G C C C A - U - T r e p o n e m a s u c c i n i f a c i e n s
89 G A A A U A G u g G A U A A U A C C G A A U - A C G C u C U U - - U U - - - U c - u u u g - g C n - - - g a - g a a a g - G A A A - n n - - C U C G A G G - A U G G G C C C g C G U C C C A U U A - G - S p i . z u e l z e
82 G A A A U A G G A G G U A A U A C C G A A U - A C G C U U A - - - U A C - - G A - U A A A - G C C - G U A U - - ? A A G - G A A A - N G - - C U U G A G G - A U G A G C C C G C G U C C C A U U A - G - T r e p o n e m a p h a g e d e n i s
89 G A A A U A G U g G G U A A U A C C G A A U - G U g g U U G C - - G G - - - G C - U U U G - G C C - - - U U - G U A A A - G A A A - C G - - C C U G A A G - A U G A G C C C n C G U C C C A U U A - G - S p i . s t e n o s
88 G A A A C A U G G A C U A A U A C C G C A U - A U A C U C U - - - U G C - - U C - A C A A - G U A - G A G U - - - A G A G - G A A A - C G - - C U U U A C G - A U G A G C C U G C G G U C U A U U A - G - S . i n n o c e n s
88 G A A A C A U G G A C U A A U A C C G C A U - A U A C U C U - - - U G C - - U C - A U A A - G U A - G A G U - - - A G A G - G A A A - C G - - C U U U A C G - A U G A - C C U G C G G C C U A U U A - G - S . h y o d y s e n t e r i a e
89 G A A A G G A A G G C U A A U A C C G C A U - A A C A C C A U - - - G G U - - U C - C A C G - G U A - - A U U - A U G G U - C A A A - C G - - - u C U G U G G - A G A A G C C U g C G U C C G A U U A - G - L p n . i l l i n i
88 G A A A C G A C U G A U A A U A C C U g A U - A n A A U U G C - - C A G - - C C - G C A U - G U G - C C C G - G C A A U - G A A A - U G - - C U G C A G G - A G A G C U U U G C G U C C U A U U A - G - L p t . b u c c a l
89 G A A A C C G g A G C U A A U A C C G G A U - A A U C C C U C - - G C - - - A C - G C n U - G G U - - - G C - G a G g u G A A A - C A - - C U U A C A G - A U G G G C C C G C G G U G C A U U A - G - S p l . i n u l i n
89 G A A A C C C U G G C U A A U A C C C C A U - A C G C U C C A - - U C - - - A C - G C A A - G U U - - - G G - U G G A G - G A A A - C G - - C C G G A G G - A g g g g C C C G C G C C C A U C A - G - T t . m a r i t i m a

* *

168	CUAG---UUGGUG-GGGUAAUGGCCACCAAGGCACGA ⁺ C ⁺ GCGGUAGCCGGUCUGAGAGGAU-GGCCGGCCACAGGGGCACUGAGACACGGGCCCCACUC-	T.thermoph
170	CUAG---UUGGUG-GGGUAAAGGCCUACCAAGGCACGAUCAGUAGCCGGCCUGAGAGGGU-GUACGGCCACACUGGAACUGAGACACGGUCCAGACUC-	Spi.aurant
168	CUaG---UUGGUG-GGGUAAUUGCCUACCAAGGCACGAUCAUAGCCGGCCUGAGAGGGU-GACCGGCCACA <u>U</u> UGGGACUGAGACACGGCCCAGACUC-	Spi.litora
170	CUaG---UUGGUA-GGGUAAAGGCCUACCAAGGCACGAUCAGUAGCCGGCCUGAGAGGGU-GACCGGCCACA <u>U</u> UGGGACUGAGACACGGCCCCAACUC-	Spi.isoval
162	CUaG---UUGGUA-GGGUAAAUGCCUACcaAGGCAAUGAUAGUAACC ⁺ GGCCUGAGAGGGU- ⁺ GAACGGUCACACUGGAACUGAGA <u>U</u> ACGGUCCaGnCUC-	Bor.burgdorferi
170	CUAG---UUGGUG-GGGUACGGCCUACCAAGGCACGAUGGGUAGGGGGUCUGAGAGGAU-GGCCCCCACACUGGGACUGAGACACGGCCCAGACUC-	R119 B1
171	CUAG---UUGGUG-AGGUACGGCCCACCAAGGCAACGAUAGUUACCCGGCCUAAGAGGGU-GAUCGGGCACA <u>U</u> UGGGACUGAGAUACGGCCCAGACUC-	Trp.bryant
170	CAGC-UGGUAGGCGGUGCAAGGGACCACCUAGGCUACGACGGGUA ⁺ CCCGGCUAAGAGGGC-GGACGGGCACA <u>U</u> UGGGACUGAGAUACGGCCCAGACUC-	Treponema succinifaciens
170	CUAG---UUGGUG-AGGUACGGCCCACCAAGGCACGAU ⁺ GGGUAV ⁺ CCGGCCUGAGAGGGU-GUACGGGACA <u>U</u> UGGGACUGAGAUACGGCCCCAACUC-	Spi.zuelze
164	CUUG---UUGGUG-AGGUACGGCUVACCAAGGCUACGAUUGNAUCCGGCCUGAGAGGGU-GGACGGACACA <u>U</u> UGGGACUGAGAUACGGCCCCAACUC-	Treponema phagedenis
170	CUAG---UUGGGG-GUGUACGGACCACCAAGGCGAUGAUGGGnAGCCGGCCUGAGAGGGU-gAACGGCCACACUGGGACUGAGAUACGGCCCAGACUC-	Spi.stenos
170	CCUG---UUGGUG-GGGUAACGGCCUACCAAGCVACGAUAGGUAGCCGACCUGAGAGGGU-GACCGGCCACA <u>U</u> UGGGACUGAGAUACGGCCCAGACUC-	S.innocens
169	CCUG---UUGGUG-GGGUAACGGCCUACCAAGCVACGAUAGGUAGCCGACCUGAGAGGGU-GACCGGCCACA <u>U</u> UGGGACUGAGAUACGGCCCNAGACUC-	S.hyodysenteriae
172	GUAG---UUGGUG-AGGUACGGCVCACCAAGCCAGCGAUCGGUAGCCGGCCUGAGAGGGU-GAACGGCCACACUGGAACUGGGACACGGUCCAGACUC-	Lpn.illini
172	CUUG---UUGGUG-AGGUAA ⁿ GGCUCACcnAGGCGAUGAUAGGuAGCCGGCCUGAGAGGGU-GAACGGCCACAAGGGgACUGAGAUACGGCCnCnUACUC-	Lpt.buccal
171	UUAG---UUGGCG-GGGUACGGCCCACCAAGACCGGAUGCAUAGCCGACCUGAGAGGGU-GAUCGGCCACA <u>U</u> UGGGACUGAGACACGGCCCCAACUC-	Spl.inulin
170	GUAG---UUGGUG-GGGUACGGCCCACCAAGCCGACGACGGGUAGCCGGCCUGAGAGGGU-GGUCGGCCACAGGGGCACUGAGACACGGGCCCCACUC-	Tt.maritima

* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

262	CUACG--GGAGGCAGCA-G-UUAGGAAUCUCCGCAAU-GGGC-GCAA-GCCUGACGGAGCGACGCCGCU-UGG-AGGAAGAAGCCC--UUCG-GGGUGU	T.thermoph
264	CUACG--GGAGGCAGCA-G-CUAAGAAUCUCCGCAAU-GGGC-GAAA-GCCUGACGGAGCGAUGCCGCG-UGA-UCGAUGAAGGUC--GAGA-GAUUGU	Sp.aurant
262	CUACG--GGAGGCAGCA-G-CUAAGAAUCUCCGCAAU-GGAC-GCAA-GUCUgACGGAGCGACGCcGCG-UGU-GCGAAGAAUGUC--GAGA-GAUUGU	Sp.litora
264	CUACG--GGAGGCAGCA-G-CUAAGAAUCUCCGCAAU-GGGC-GCAA-GccugACGGAGCGACGCCGCG-UGG-ACGAAGAAUGCC--GAGA-GGUUGU	Sp.isoal
256	cUACG--GGAGGCAGCA-G-CUAAGAAUCUCCGCAAU-GGGC-GAAA-GccugACGGAGCGAcaCUGCG-UGA-AUGAAGAAGGUC--GAAA-GAUUGU	Bor. burgdorferi
264	CUACG--GGAGGCAGCA-G-CCAAGAAUCUCCGCAAU-GGGC-GAAA-GCCUGACGGAGCGACGCCGCG-UGG-GGGAAGAAGGCC--GGAA-GGUUGU	RI19 B1
265	CUACG--GGAGGCAGCA-G-CUAAGAAUAUCCGCAAU-GGAC-GAAA-GUCUGACGGAGCGACGCCGCG-UGG-AUGAUGAAGGCC--GGAA-GGUUGU	Trp.bryant
267	NUACG--GGAGGCAGCA-G-CUAAGAAUAUCCGCAAU-GGGG-GGAA-CNNUGACGGAGCGACGCCGCG-UGG-GCGAGGAAGGCC--GGAA-GGUUGU	Treponema succinifaciens
264	CUACG--GGAGGCAGCA-G-CUAAGAAUAUCCGCAAU-GGAC-GAAA-GUCUgACGGAGCGACGCcGCG-UGG-AUGAAGAAGGCC--GAAA-GGUUGU	Sp.zuelze
258	CUACG--GGAGGCAGCA-G-CUAAGAAUAUCCGCAAU-GGAC-GGAA-GUCUGACGGAGCGACGCCGCG-UGG-ACGAAGAAGGCC--GAAA-GGUUGU	Treponema phagedenis
264	CUACG--GGAGGCAGCA-G-CUAAGAAUAUCCGCAAU-GGAC-GAAA-GUCUGACGGAGCGACGCCGCG-UGG-AUGAUGAAGGCC--GAAA-GGUUGU	Sp.stenos
264	CNACG--GGAGGCAGCA-G-CUGAGAAUCUCCACAAU-GGAC-GAAA-GUCUGAUGGAGCGACAUCGCG-UGA-GGGAUGAAGGCC--UUCG-GGUUGU	S.innocens
263	CNACG--GGAGGCAGCA-G-CUGAGAAUCUCCACAAU-GGAC-GAAA-GUCUGAUGGAGCGACAUCGCG-UGA-GGGAUGAAGGCC--UUCG-GGUUGU	S.hyodysenteriae
266	CUACG--GGAGGCAGCA-G-UUAAGAAUCUUGCGCAAU-GGAG-GCAA-CUCUGACGCAGCGACGCCGCG-UGU-GCGAUGAAGGCC--CUCG-GGUUGU	Lpn.illini
266	CUACG--GGAGGCAGCA-G-UGGGGAAUAUUGGACAAU-GGGG-GCAA-CCCUGAUCCAGCAAUUCUGUG-UGC-ACGAAGAAGGUU--UUCG-GAUUGU	Lpt.buccal
265	CUACG--GGAGGCAGCA-G-UAGGGAAUCUCCACAAU-GGAC-GAAA-GUCUGAUGGAGCAACGCCGCG-UGA-GCGAAGAAGGUU--UUCG-GAUUGU	Sp.inulin
264	CUACG--GGAGGCAGCA-G-UGGGgAAUCUUGGACAAU-GGGG-gAAA-CCCUGAUCCAGCGACGCCGCG-UGC-GGGACGAAgccc--UUCG-GGGUGU	Tt.maritima

Page 203

350 AAA-----CUCCUGAA-CCCGGGACG--AAUGACGGUACCG-G-GGU-AAU-AGCGCC-GGCCAACUCCGUGCCAGCAGCCGCGGUAAUACG T.thermoph
 352 AAA-----GAUCUUUUCUACAUGAGG--AAUGACGUUAGUG-U-AGG-AAUAAAGCCCC-GGCUAAUACGUGCCAGCAGCCGCGGUAAACACG Spi.aurant
 350 AAA-----GCACUUUUUAUUGUGAGG--AAUGACGUUAGCA-U-AUG-AAUAAAGCUCC-GGCCAAUACGUGCCAGcAGCCGCGGUAAACACG Spi.litora
 352 AAA-----GUCCnUUUAUUGCGAAG--AAUGACGUUAGCU-U-AUG-AAUAAAGCUCC-GGCCAAUACGUGCCAGCAGCCGCGGUAAACACG Spi.isoval
 344 AAA-----AUUCUUUUUAUAAUGAGG--AAUGACGUUAAUU--UAUG-AAUAAAGCCCC-GGCUAAUACGUGCCAGnAgCcGcGGuaAUACg Bor.burgdorferi
 352 AAA-----CCCCUUUCCAGGGAAG--AAUGACGUUACCU-G-GGG-AAUAAAGCCCC-GGCUAACUACGUGCCAGCAGCCGCGGUAAUACG RI19 B1
 353 AAA-----AUCCUUUAUGACUGAGG--AAUGACUGUAGGU-C-AUG-AAUAAAGCAAC-GGCUAAUACGUGCCAGCAGCCGCGGUAAACACG Trp.bryant
 355 AAA-----GCCCUUUUGCGCGCGAGG--AGUGACUGUAGCG-C-GCG-AAUAAAGCGCC-GGCUAAUACGUGCCAGCAGCCGCGGUNACACG Treponema succinifaciens
 352 AAA-----AUCCUUUUGCUGAUGAAG--AAUGACAUUAGUC-G-GUG-AAUAAAGCCCC-GGCUAACUACGUGCCAGCAGCCGCGGUAAACACG Spi.zuelze
 346 AAA-----GUUCUUUUGCCGAUGAAG--AAUGACGGUAGUC-GAGCG-AAUAAAGCCCC-GGCUAAUACGUGCCAGCAGCCGCGGUAAACACG Treponema phagedenis
 352 AAA-----GUCCUUUUGUCGACGAAG--AAUGACGUUAGUC-G-GCG-AAUAAAGCCCC-GGCUAAUACGUGCCAGcAGCCGCGGUAAACACG Spi.stenos
 352 AAA-----CCUCGGAUUUAUCGAAG--AAUGACAGUAGAU--??AA-UGUAAGCCUC-GGCUAACUACGUGCCAGCAGCCGNNNUNAUACG S.innocens
 351 AAA-----CCUCGGAUUUAUCGAAG--AAUGACAGUAGAU-C-UAA-UGUAAGCCUC-GGCUNACUNCUGGCCAGCNGCCGNNNUNNUNCG S.hyodysenteriae
 354 AAA-----GCACAACAAACAGGGAAG--AAUGACGGUACCU-G-UCU-GA--AGCACC-GGCUAACUACGUGCCAGCAGCCGCGGUAAUACG Lpn.illini
 354 AAA-----GUGCUUUCAGCAGGGAAG--AAUGACGGUACCU-G-CAG-AAGAAGCGAC-GGCUAAUACGUGCCAGCAGCCGCGGUAAUACG Lpt.buccal
 353 AAA-----GCUCUGUUGCCGGAGAAG--AAUGACGGUAUCC-G-GCC-AGAAAGCCAC-GGCUAACUACGUGCCAGCAGCCGCGGUAAUACG Spl.inulin
 352 AAA-----ccgCUGUGGCGGGGGAAG--AAUGACGGUACCC-C-GCU-AGAAAGCCCC-GGCUAACUACGUGCCAgCagCCgCGGUAAUACG Tt.maritima

***** * ** **** * * * * * * * * * * *

429 GAG-GGCGCGAGCGUUAACCGGAUUCACUGGGCGUAAAGGGCGUGUAGGCGGCCU-GGGGCGUCCC-AUGUGAAAGACCACGGCUCAACCGUGGGGG-AG T.thermoph
 433 UAA-GGGGcNAGCGUUGUUCGGAUUUAUUGGGCGUAAAGGGCACGCAGGCGGUUA-UGCAAGUCUG-AUGUGAAAUUCCGGGCUUAAUCUGGAAUC-CG Spi.aurant
 431 UAA-GGAgCnAGCGUUGUUCGGAUUUAUUGGGCGUAAAGGGCAUGUAGGCGGUUU-UGUAAGUCCG-AUGUGAAAGCGUGCAGCUUAAUCUGCAUUGU-UG Spi.litora
 433 UAA-GGAGCgAGCGUUGUUCGGAUUUAUUGGGCGUAAAGGGCAUGUAGGCGGUUU-UGUAAGUCUG-GUGUGAAAUACUACAGCUUAAUCUGUGGGAA-CG Spi.isoval
 425 UAA-GGGGnnAGCGUUGUUCGGAUUUAUUGGGCGUAAAGGGGUGAGUAGGCGGAUA-UUAAGUCUA-UGCAUAAAAUACCACAGCUcAACUGUGgaCCUA Bor.burgdorferi
 433 UAN-GGGGCGAGCGUUGUUCGGAUUACUGGGCGUAAAGGGGGCGCAGGCGGCCU-GGCAAGUCUG-GUGUAAAAGGCUACGGCUCAACCGUAGUGU--G RI19 B1
 434 UAA-GUUGCGAGCGUUGUUCGGAUUUAUUGAGCGUAAAGGGCAUGUAGGCGGUUC-UGCAAGUCUG-GUGUGAAAUAGCCGGGCUCAACCCCGGAAC-UG Trp.bryant
 436 UAA-GGCGcNAGCGUUGUNCGGAUUUAUUGGGCGUAAAGGGCGUGUAGGCGGCC-UGCAAGCCUN-GCGUGAAAUCCCGGGGcNCAACCCCGNAAC-CG Treponema succinifaciens
 433 UAG-GGGGcNAGCGUUGUUCGGAUUUAUUGGGCGUAAAGGGCAUGUAGGCGGUUU-GGUAAGCCUg-GUGUGAAAUCCUGCAGCUUAAUCUGUAGAAU-UG Spi.zuelze
 428 UAA-GGGGNNAGCGUUGUUCGGAUUUAUUGGGCGUAAAGGGCACGCAGGCGGGUU-GGUAAGCCUG-NUGUGAAAUACUCAAGCUUAAUCUGAGAAU-UG Treponema phagedenis
 433 UAA-GGGGcnAGCGUUGUUCGGAUUUAUUGGGCGUAAAGGGUACGUAGGCGGUCC-UGUAAGCCcN-ACGUGAAAUACUGGAGCUUAAUCUCCAGAAC-UG Spi.stenos
 433 UAG-GAGGCAAAAGUUGCUCGGAUUUAUUGGGCGUAAAGGGUGAGUAGGCGGACU-UUAAGUCUA-AGGUGAAAGACCGAAGCUCAACUUCGGGAA-CG S.innocens
 432 UAG-GAGGcNAACGUUGCUCGGAUUUNCGGGCGUNAAGGGUGAGUAGGCGGACU-UUAAGUCUA-AGGUGAAAGACCGAAGCUCAACUUCGGAAA-CG S.hyodysenteriae
 433 UAU-GGUGCAAGCGUUGUUCGGAUUCACUGGGCGUAAAGGGUGCGCAGGCGGACU-GUUAAGUCGG-GAGUGAAAUACUGAGGGCUCAACCCUCAGCC-UG Lpn.illini
 435 UAU-GUCGcNAGCGUUAUCCGGAUUUAUUGGGCAUAAAGGGCAUCUAGGCGGCCa-GACAAGUCUG-GGGUGAAAUACUUGCGGCUCAACCGcNAGCC-UG Lpt.buccal
 434 UAG-GUGGcNAGCGUUGUCCGGAUUUAUUGGGCGUAAAGCGCGCGCAGGCGGCUU-CUUAAGUCUG-AUGUGAAAUUUGCGGCUCAACCGCAAAAG-GU Spl.inulin
 433 UAG-GGGGCAAGCGUUAACCGgAUUUAUUGGgCGUAAAGGGGGCGUAGGCGGCCU-GGUGUGUCGG-AUGUGAAAUCCCACGGCUCAACCGUGGGG-UG Tt.maritima

* * * * * * * * * * *

525	CGUGGGAUACGCUCA-GGCUAGACGGUGGGAGAGGGU-GGUGGAAUUC	----	GGAGUAGCGGUGAAAUG-CGCAGAUACCGGGAGGAACGCCGA--U	T.thermoph
529	CGUUGGAAACUGCAU-GACUAGAGUAUUAGAGGGGGA-GUCGGAAUUCU	----	AGUGUAGGGGUGAAAUC-UGUUGAUUUAGGAAGAACACCGG--U	Spi.aurant
527	CGUUGGAAACUGCGA-GACUAGAAUCCGAGAGGGGUA-AGUGGAAUUCU	----	AGUGUAGGGGUGAAAUC-UGUUGAUUUAGGAAGAACACCGA--A	Spi.litora
529	CACUGGAAACUGUGA-GACUAGAAUCAGUGAGGGGUA-ACCGGAAUUCU	----	UGUGUAGGGGUGAAAUC-UGUUGAUUAAGGAAGAACACCGA--U	Spi.isoal
522	UGUUGGAAACUAUUAU-GUCUAGAGUCUGAUAGAGGAA-GUUAGAAUUCU	----	GGUGUAAGGGUGgAAUC-UGUUGAUUACAGaAAGAAUACCGG--A	Bor. burgdorferi
528	CACCGGAAACUGCUG-GGCUGGAGUCCCGAGGGGGA-GCUGGAAUUC	----	GGUGUAGGGGUGAAAUC-UGUAGAUUACGGGAGGAACGCCGG--A	RI19 B1
530	CGUUGGAAACUGCAG-AACUUGAGUCGCUGAGGGGcG-GCCAGAAUCCA	----	GGUGUAGGGGUGAAAUC-UGUAGAUUACUGGAAGAAUACCAA--U	Trp.bryant
532	CGCUGGGAACUGCUG-GGCUUGAGCCGCUGUGGCGCA-GCCGGAUUNCA	----	GGUGUAGGGGUGAAAUC-UGUAGAUUACUGGAAGAACACCGA--U	Treponema succinifaciens
529	CAUUGGGUACUGCCA-GACUUGAAUCACAGAGGGGAA-ACUAGAAUCCA	----	GGUGUAGGGGUGGAAUC-UGUAGAUUACUGGAAGAAUACCGG--U	Spi.zuelze
524	CAUUGGGUACUGCCA-GUCUUGAAUCACGAGGGGAA-ACCGGAAUCCA	----	AGUGUAGGGGUGGAAUC-UGUAGAUUUUGGAAGAACACCGG--U	Treponema phagedenis
529	CGCCGGGAACUGUAG-GACUUGAGUCAUGGAAGGGGA-GUUGGAAUCCA	----	GGUGUAGGGGUGAAAUC-UGUAGAUUACUGGAAGAACACCGG--U	Spi.stenos
529	CCUCGGAUACUGUGA-GUCUUGGAUAUUGUAGGGGAU-GAUGGAAUUCNC	----	GGUGUAGCGGUGGAAUG-CGCAGAUUACGAGAGGAACACCUA--U	S.innocens
528	CCUCGGAUACUGUAA-GUCUUGGAUAUUGUAGGGGAU-GAUGGAAUUCUC	----	GGUGUAGCGGUGGAAUG-CGCAGAUUACGAGAGGAACANCUA--U	S.hyodysenteriae
529	CUUUCGAAACUGGCA-GUCUGGAGUCCCGAGAGGCA-GGCGGAAUUC	----	GGUGUAGCGGUGAAAUG-CACAGAUUACGGGAGGAACACCAA--U	Lpn.illini
531	CCCUGGAAACUGUUU-GGcuAGAGUGCUGGAGAGGUG-GACGGAACUGCA	----	CGAGUAGAGGUGAAAUC-CGUAGAUUAGUGCAGGAUUGCCGA--U	Lpt.buccal
530	CAUUGGAAACUGGGG-aGcuUGAGUGCAGAAGAGGAG-AGUAGAAUUCa	----	CGUGUnCGGUGAAAUG-CGUnGAGAUGUGGAGGAUACCAG--U	Spl.inulin
529	CAUCCGAAACUACCA-GGCUUGGGGGCGGUAGAGGGA-GACGGAACUGCC	----	GGUGUAGGGGUGgAAAUC-CGUAGAUUACGGCAGGAACGCCGG--U	Tt.maritima

* ** *

615	GGCGAAGGCAGCCACCUGGUCCACCCUGACGCUGAGGCGCAAAGCGUGGG	-GAGCAAACCGGAUUAAGAUACCCGGGUAUCCACGCCCUAAACGAUGC	T.thermoph
619	GGCGAAGGCGGACUCCUGGCUGAAUACUGACGCUGAGGUGCGAAAGCAUAGG	-GAGCAAACAGGAUUAAGAUACCCUGGUAGUCUAUGCUGUAAACGAUGU	Spi.aurant
617	GgCGAAGGCGACUUGCUGGCUCUGGAUUGACGCUGAGGUGCGAAAGCGUAGG	-UAGCGAACAGGAUUAAGAUACCCUGGUaGUCUACGCCGUAACGAUGU	Spi.litora
619	GGCGAAGGCAGGUUACUGGCGCAUGAUUGACGCUGAUGUGCnnAGCGUAGG	-GAGCGAACAGGAUUAAGAUACCCUGGUGnGUCUACGCUGUAAACGAUGU	Spi.isoal
612	GGCGAAGGCGAACUUCUGGGUCAAGACUgACGCUgAGUCACgaAAGCGUAGG	-GAGCAAACAGGAUUAAGAUACCCUGGUAUAGUCUACGCUGUAAACGAUGC	Bor. burgdorferi
618	GGCGAAGGCGAGCUCCUGGCCGAGACUGACGCUGAGGCCCGAAAGCGUGGG	-UAGCGAACAGGAUUAAGAUACCCUGGUAGUCCACGCCGUAACGAUGU	RI19 B1
620	GGCGAAGGCAGGCUGCCAGCAGAUACUGACGCUGAGGUGCGAAGGUGCGGG	-GAGCAAACAGGAUUAAGAUACCCUGGUAGUCCGCGCAGUAAACGAUGC	Trp.bryant
622	GGCGAAGGCAGGCUGCGAGCGGACGGCUGACGCUGAGGCGCGAAGGCGCGGG	-GAGCGAACAGGAUUAAGAUACCCUGGUAGUCCGCGCAGUAAACGAUGC	Treponema succinifaciens
619	GGCGAAGGCGGGUUUCUAGCUGAUGAUUGACGCUGAGGUGCGaAAGUGCGGG	-GAGCAAACAGGAUUAAGAUACCCUGGUAGUCCGACCGUAAACGAUGU	Spi.zuelze
614	GGCGAAGGCGGGUUUCUGGCCGAUGAUUGACGCUGAGGUGCGAAGGUGUGGG	-GAGCAAACAGGAUUAAGAUACCCUGGUAGUCCACACAGUAAACGAUGU	Treponema phagedenis
619	GGCGAAGGCGAACUCCUGGCCAAUGACUgACGCUgAGGUACGAAAGUGCGGG	-GAGCAAACAGGAUUAAGAUACCCUGGUaGUCCGCGCAGUAAACGAUGU	Spi.stenos
619	AGCGAAGGCAGUCAUCUGGGCAUUUAUCGACGCUGAAUACGAAAGCUAGGG	-GAGCAAACAGGCUUAGAUACCCUGGUAGUCCUAGCCGUAACGUUGU	S.innocens
618	AGCGAAGGCAGUCAUCUGGGCAUUUAUCGACGCUGAAUACGAAAGCUAGGG	-GAGCAAACAGGCUUAGAUACCCUGGUAGUCCUAGCCGUAACGUUGU	S.hyodysenteriae
619	GGCGAAGGCAGCCUGCUGGACGGAGACUGACGCUCAUGCACGAAAGCGUGGG	-GAUCAAACAGGAUUAAGAUACCCUGGUAGUCCACGCCCUAAACGUUGU	Lpn.illini
621	GAUGAAGAUAGUUCACUGGACGGUAACUGACGCUGAAGUGCGAAAGCUGGGG	-GAGCGAACAGGAUUAAGAUACCCUGGUAGUCCAGCCGUAACGAUGA	Lpt.buccal
620	GGCGAAGGCGGCUCUCUGGUCUGUACUGaCGCUGAGGUGCGaAAGcgUGGG	-GAGCAAACAGGAUUAAGAUACCCUGGUAGUCCAGUCCcGUAACGAUGA	Spl.inulin
619	GGGGAAGCCGGUCUCCUGGGCCGACCCCGACGCUGAGGCCCGAAAGCCAGGG	-GAGCAAACCGGAUUAAGAUACCCGGGUAUCCUGGCUGUAAACGAUGC	Tt.maritima

**** *

714	GCG-CUAGGUCUCUGGG-----UCU-----CCUGGGGGCCGAAGCUAACGCGUUAAGCGCGCCGCCUGGGGAGUACGGCC-GCAAGACGCGAAGAA	T.thermoph
718	GCA-CUUGGUGUUGGGCC-----GAUUA-----GGUUCAGUGCCGUAGCUAACGCGAUAAAGUGCACCGCCUGGGGAGUAUGCCC-GCAAGACGCGAGAAA	Spi.aurant
716	ACA-CUUGGUGUCUGGAUC----AUGA-----GAUCUAGGUGCCGUAGCUAACGCGUUAAGUGUACCGCCUGGGGAGUAUGCCG-GCAaCACGCGAGAAA	Spi.litora
718	GCA-CUUGGUGUUGGGGUU----ACUA-----AGCCUCAGUGCCgAAGCUAACGCGUUAAGUGCACCGCCUGGGGAGUAUGCCG-GCAACaCGCGAGAAA	Spi.isoal
711	ACA-CUUGGUGUU-----AACUAAAAGUU-----AGUACCGAAGCUAA-CGUGUUAAGUgUGCaGCCugGGGAGuAUGCUC-GCAAGACGCGAGGAA	Bor. burgdorferi
717	GCA-CUAGGCGUUGGGCC-----UUGA-----GGCUCGGUGCCGAAGCUAACGCGUUAAGUGCACCGCCUGGGGAGUAUGCCG-GCAACACGCGAGGAA	RI19 B1
719	ACA-CUGGGUGUCCGUUC-----AUGA-----GAAUGGGUGCCGAAGCAAACGCGAUAAAGUGUACCGCCUGGGGAGUAUGCCC-GCAAGACGCGAGGAA	Trp.bryant
721	ACA-CUGGGUGNUCCGG---CAUGAG-----CCGGNUGCCGAAGCGAACGCGUUAAGUGUGCCGCCUGGGGAGUAUGCCC-GCAANUAGCGAGGAA	Treponema succinifaciens
718	ACA-CUAGGUGUUGGGGC-----AUGA-----GUCUCAGUGCCAAAGCUAACGUGAUAAAGUGUACCGCCUGGGAaAGUAUGCCc-GCaAGACGCGAGGAA	Spi.zuelze
713	ACA-CUAGGUGUUGGGG---CAAGAGCUU-----CAGUGCCGGCGCAAACGCAAUAAGUGUACCGCCUGGGGAGUAUGCCC-GCAAGACGCGAGGAA	Treponema phagedenis
718	GCA-CUAGGUGUUGGGCC-----GAGC-----GGUUCAGUGCCGAAGCUAACGUGAUAAAGUGCACCGCCUGGGGAGUAUGCCC-GCAAGACGCGAGGAA	Spi.stenos
718	ACA-CUAGGUGCUUCUA----UUUAAAU-----AGGAGUGCCGUAGCUAACGUCUUAAGUGUACCGCCUGAGGAGUAUGCCC-GCAAGACGCGAAAAA	S.innocens
717	ACA-CUAGGUGCUUCUN----UUUNA AU-----AGGAGUGCNGUAGCUNACGUCUUNAGUGUNCCGCCUGAGGAGUAUGCCC-GCAAGACGCGAAAAA	S.hyodysenteriae
718	AGA-CCAGACGUUGGAGG---CAUCAACC---CCUUCAGUGUCAAAACCAAACGGAUUAAGUCUACCGCCUGGGGACUAUGCUC-GCAAGACGCGAAAAA	Lpn.illini
720	UUA-CUGGGUGUGGGCAUG----AAG-----AGUGUCCGUGCCGAAGCUAAUGCGAUAAAGUAAUCCGCCUGGGGAGUACGGCC-GCAAGACGCGAGGAA	Lpt.buccal
719	AUg-CuAGGuGUnAgggGGG---UCCA----ACCCCUUAGUGCUGAAGUUAACACAUUAAGCAUUCGCCUGGGGAGUACGACC-GCAAGACGCGAAGAA	Spl.inulin
718	CCA-CUAGGUGUGGGGGGG---UAA-----UCCCUCCGUGCUGAAGCUAACGCGUUAAGUGGGCCGCCUGGGGAGUACGCCC-GCAAGAAGCCAAGAA	Tt.maritima

$\frac{1}{2}$ $\frac{1}{3}$ $\frac{1}{4}$ $\frac{1}{5}$ $\frac{1}{6}$ $\frac{1}{7}$ $\frac{1}{8}$ $\frac{1}{9}$ $\frac{1}{10}$ $\frac{1}{11}$ $\frac{1}{12}$ $\frac{1}{13}$ $\frac{1}{14}$ $\frac{1}{15}$ $\frac{1}{16}$ $\frac{1}{17}$ $\frac{1}{18}$ $\frac{1}{19}$ $\frac{1}{20}$ $\frac{1}{21}$ $\frac{1}{22}$ $\frac{1}{23}$ $\frac{1}{24}$ $\frac{1}{25}$ $\frac{1}{26}$ $\frac{1}{27}$ $\frac{1}{28}$ $\frac{1}{29}$ $\frac{1}{30}$ $\frac{1}{31}$ $\frac{1}{32}$ $\frac{1}{33}$ $\frac{1}{34}$ $\frac{1}{35}$ $\frac{1}{36}$ $\frac{1}{37}$ $\frac{1}{38}$ $\frac{1}{39}$ $\frac{1}{40}$ $\frac{1}{41}$ $\frac{1}{42}$ $\frac{1}{43}$ $\frac{1}{44}$ $\frac{1}{45}$ $\frac{1}{46}$ $\frac{1}{47}$ $\frac{1}{48}$ $\frac{1}{49}$ $\frac{1}{50}$ $\frac{1}{51}$ $\frac{1}{52}$ $\frac{1}{53}$ $\frac{1}{54}$ $\frac{1}{55}$ $\frac{1}{56}$ $\frac{1}{57}$ $\frac{1}{58}$ $\frac{1}{59}$ $\frac{1}{60}$ $\frac{1}{61}$ $\frac{1}{62}$ $\frac{1}{63}$ $\frac{1}{64}$ $\frac{1}{65}$ $\frac{1}{66}$ $\frac{1}{67}$ $\frac{1}{68}$ $\frac{1}{69}$ $\frac{1}{70}$ $\frac{1}{71}$ $\frac{1}{72}$ $\frac{1}{73}$ $\frac{1}{74}$ $\frac{1}{75}$ $\frac{1}{76}$ $\frac{1}{77}$ $\frac{1}{78}$ $\frac{1}{79}$ $\frac{1}{80}$ $\frac{1}{81}$ $\frac{1}{82}$ $\frac{1}{83}$ $\frac{1}{84}$ $\frac{1}{85}$ $\frac{1}{86}$ $\frac{1}{87}$ $\frac{1}{88}$ $\frac{1}{89}$ $\frac{1}{90}$ $\frac{1}{91}$ $\frac{1}{92}$ $\frac{1}{93}$ $\frac{1}{94}$ $\frac{1}{95}$ $\frac{1}{96}$ $\frac{1}{97}$ $\frac{1}{98}$ $\frac{1}{99}$ $\frac{1}{100}$

889	UAAGUCCC	GCAACGAGCGCAACCCCG	-CCGUUAG	---	UUGCCAGCACUCUAACGGGACUGCCCGCG	-AAA-GCGG	-GAGG-A-AGGAGGGG-ACGAC	T.thermoph
897	UAAGUCCC	GCAACGAGCGCAACCCCUA	-UUGCCAG	---	UUACCAUACACUCUGGCGAGACUGCCGGUGA	-CAA-ACCG	-GAGG-A-AGGUGGGG-AUGAC	Spi.aurant
896	UAAGUCCC	GAAACGAGCGCAACCCUG	-UUGUAUG	---	UUACCAUACACUAUGcAAGACUGCcGGuGA	-CAA-ACCG	-GAGG-A-AGGUGGGG-AUGAC	Spi.litora
898	UAAGUCCC	GCAACGAGCGCAACCCCUA	-UUGUCUG	---	UUACCAUACACUCAGGCAAGACUGCCGGUGA	-CAA-ACCG	-GAGG-A-AGGUGGGG-ACGAC	Spi.isoal
887	UAAGUCC	CgcAACGAGCGCAACccUUG	-UUAUCUG	---	UUACCAGCaCUCAGAUAAAGACUGCCGGUGA	-UAA-GUCG	-GAGG-A-AGGUGAGG-AUGAC	Bor. burgdorferi
895	UAAGUCCC	GCAACGAGCGCAACCCUG	-CCGUCUG	---	UUGCCAUCACUCAGGCGGGACUGCCGGUGA	-CAA-ACCG	-GAGG-A-AGGUGGGG-AUGAC	RI19 B1
898	UAAGUCCC	GCAACGAGCGCAACCCCUA	-CUGCCAG	---	UUACUAGCACUCUGGCGGAACUGCCGGUGA	-CAA-ACCG	-GAGG-A-AGGUGGGG-AUGAC	Trp.bryant
899	UAAGUCCC	GCAACGAGCGCAACCCUG	-CCGCCAG	---	UUGCCAGCACUCUGGCGGAACUGCCGGCGA	-CAA-GCAG	-GAGG-A-AGGCGGGG-ACGAC	Treponema succinifaciens
896	UAAGUCCC	GCAACGAGCGCAACCCCUA	-CUGCCAG	---	UUACUAACACUCUGgCGGAACUGCcGGUGA	-CAA-ACCG	-GAGG-A-aGGunGGG-aUGAC	Spi.zuelze
892	UAAGUCCC	GCAACGAGCGCAACCCCUA	-CUGUCAG	---	UUGCUNACACUCUGGCGGAACUGCCGAUGA	-CAA-AUCG	-GAGG-A-AGGUGGGG-AUGAC	Treponema phagedenis
896	UAAGUCCC	GCAACGAGCGCAACCCCUA	-CUGCCAG	---	UUACUAACACUCUGGcGnGAACUGCCGGUGA	-CAA-ACCG	-GAGG-A-AGGUGGGG-AUGAC	Spi.stenos
896	UAAGUCCC	GCAACGAGCGCAACCCUCA	-CCCUUUG	---	UUGCUACCACUCUUAGGGGACUGCCUACGU	-UCA-AGUA	-GGAGG-A-AGGUGGGG-AUGAC	S.innocens
895	UNAGUCCC	GCAACGAGCGCAACCCUCA	-CCNNUUG	---	UUGCUACCACUCUUAGGGGACUGCCUACGU	-UCN-AGUA	-GGAGG-A-AGGUNGNGN-AUGAU	S.hyodysenteriae
900	UAAGUCCC	GCAACGAGCGCAACCCCUA	-CCGUGUG	---	UUACCAUACACUUGCACGGAACUGCCGGUGA	-UAA-ACCG	-GAGG-A-AGGUGGGG-AUGAC	Lpn.illini
898	UAAGUCCC	GCAACGAGCGCAACCCCUA	-UCGCAAG	---	UUGCCAUCACUCUAGCGAGACUGCCUGCGA	-AGA-GCAG	-GAGG-A-AGGUGGGG-AUGAC	Lpt.buccal
900	UAAGUCCC	GCAACGAGCGCAACCCUUG	-AUCUUAG	---	UUGCCAGCACUCUAAGGUGACUGCCGGUGA	-CAA-ACCG	-GAGG-A-AGGUGGGG-AUGAC	Spl.inulin
899	UAAGUCCC	GCAACGAGCGCAACCCUG	-CCCCUAG	---	UUGCCAGCACUCUAGGGGGACUGCCGGCGA	-CGA-gCCG	-GAGG-A-AGGAGGGG-AUGAC	Tt.maritima

***** ***** * ***** * * ***** * * * * ***** ** * *

1058	--GAGCUAAUCGCA-AAAAGGUGGGCCAGUUCGGAUUGGGGUCUGCAACCCGACCCCAUGAAGCCGGAUUCGCUAGUAAUCGCGGAUCAGCCAUGCCGC	T.thermoph
1067	--AAGCUAAUCUCA-AAAAACCGAUCUCAGUUCGGAUUGGAGUCUGAAACUCGACUCCAUGAAGGUGGAAUCGCUAGUAAUCGCACAUCAG-CAUGGUGC	Spi.aurant
1066	--AAGCAAAACGCA-UAAAGCCgGUCUCAGUUCGGAUUGGAGUCUGAAACUCGACUCCAUGAAGGUGGAAUCGCUAGUAAUCGCGCAUCAG-CAUGGCGC	Spi.litora
1068	--GAGCCAAACGCA-AAAAGCCGGUCUCAGUUCGGAUUGGAGUCUGAAACUCGACUCCAUGAAGGUGGAAUCGCUAGUAAUCGCGCAUCAG-CAUGGCGC	Spi.isoal
1057	--AAGCAAAACGCA-UAAAGCAGGgCUCAGUCCGGAUUGAAGUCUGAAACUCGACUCCAUGAAGUUGGAAUCGCUAGUAAUCGUAAUUCAG-AAUGAUAC	Bor. burgdorferi
1065	--GAGCAAAUCCCA-GAAAGCCGGUCUCAGUACGGAUUGGAGUCUGAAACCCGACUCCAUGAAGGUGGAAUCGCUAGUAAUCGCGCAUCAGCCAUGGCGC	RI19 B1
1068	--GAGCAAAACGCA-GAAAGCCUGCCUCAGUCCGGAUAGGAGUCUGAAACCCGACUCCAUGAAGUUGGAAUCGCUAGUAAUCGCACAUCAG-CACGGUGC	Trp.bryant
1069	--GAGCGAAACGCA-GAAAACCGUCCGUAGUCCGGAUCGGAGUCUGAAACCCGACUCCAUGAAGCUGGAAUCGCUAGUAAUCGCGCAUCAG-CACGGCGC	Treponema succinifaciens
1066	--ACGCAAAACGCA-AAAAAGCUACCGUAGUUCGGAUUGAAGUCUGAAACCCGACUCCAUGAAGUUGGAAUCGCUAGUAAUCGCGCAUCAG-CACGGCGC	Spi.zuelze
1062	--AAGCAAAUCGCA-AAAAAGCAAUCGUAGUUCGGAUUGAAGUCUGAAACUCGACUCCAUGAAGUUGGAAUCGCUAGUAAUCGCACAUCAG-CACGGUGC	Treponema phagedenis
1067	--aAGCGAACCGCA-AAAAGCCGGCgGUAGUUCGGAUUGAAGUCUGAAACCCGACUCCAUGAAGGUGGAAUCGCUAGUAAUCGCGCAUCAG-CACGGCGC	Spi.stenos
1067	--GAGCAAAACUCA-AAAAAGUUGCCUCAGUUCGGAUUGGAGUCUGAAACUCGACUCCAUGAAGUUGGAAUCACUAGUAAUCGUAGAUCAG-AACGCUAC	S.innocens
1066	--GAGCAAAACUCA-AAAAAGUUGCCUCAGUUCGGAUUGGAGUCUGAAACUCGACUCCAUGAAGUUGGAAUCACUAGUAAUCGUAGAUCAG-AACGCUAC	S.hyodysenteriae
1070	--GAGCAGAUCCGA-GAAAGCCGAUCUCAGUUCGGAUUGGGGUCUGCAACUCGACUCCAUGAAGCUGGAAUCGCUAGUAAUCGCGGAUCAG-CAUGCCGC	Lpn.illini
1068	--UAGCCAAUCUUU--AAAGCCGGUCCAAGUUCGGAUUGCAGGCUGCAACUCGCCUGCAUGAAGCCGGAUUGCUAGUAAUCGCGGAUCAG-CAUGCCGC	Lpt.buccal
1070	--AAGCCAAUCCCA-UAAAGCCGCCCCAGUUCGGAUUGCAGGCUGCAACUCGCCUGCAUGAAGCCGGAUUGCUAGUAAUCGCGGAUCAG-CAUGCCGC	Spl.inulin
1069	--GAGCCAAUCCCC--AAAGCCGCCUCAGUUCGGAUCGCAGGCUGCAACCCGCCUGCGUGAAGCCGGAUUGCUAGUAAUCGCGGAUCAGCCAUGCCGC	Tt.maritima
	** *	

1155	G	T.thermoph
1163	G	Spi.aurant
1162	G	Spi.litora
1164	G	Spi.isoal
1153	G	Bor. burgdorferi
1162	G	RI19 B1
1164	G	Trp.bryant
	-	Treponema succinifaciens
1162	G	Spi.zuelze
1158	G	Treponema phagedenis
1163	G	Spi.stenos
1163	G	S.innocens
1162	G	S.hyodysenteriae
1166	G	Lpn.illini
1164	G	Lpt.buccal
1166	G	Spl.inulin
1165	G	Tt.maritima

APPENDIX 4

CHAPTER 8 SEQUENCE ALIGNMENT

	10	20	30	40	50	60	70	80	90	100	
1	UUU-GAUCCUGGCUCAGGG-UGAACGCUGGCGGCGUGC----	CUAACACAUGCAAGUCGAGCGGcAUAGCGGCGGACGGGUGCGUAACACGUAGG-AACG	FERV.NOD								
1	uUu-gAUCCUGGCUCAGGG-UgAACGCUGGCGGCGUGC----	cuAACACAUGCAAGUCGAGCGGcGcAUAGCGGcGAACGGGUGCGUAACCGUAGG-AACG	FERV.ICE								
1	UUU-GAUCCUGGCUCAGGG-UGAACGCUGGCGGCGUGC----	CUUACACAUGCAAGUCGAGCGACCCAGCGGCGGACGGGUGAGUAACCGUAGGUGACC	RI 19.B2								
1	UUU-GAUCCUGGCUCAGGG-UgAACGCUGGCGGCGUGC----	CUUACACAUGCAAGUCGAGCGACcAGCGGCGGcAGGGUGAGUAACCGUAGGUGACC	TSIPH. AFRICANA								
1	UUU-GAUCCUGGCUCAGGG-UGAACGCUGGCGGCGUGC----	CUAACACAUGCAAGUCGAGCGGCCAGCGGCGGACGGGUGAGUAACACGUGGGUAACC	Thermotoga maritima								
94	UGCCCCUgGA-----	GGCGGAUAGCUACGGGAAA--CUGUAGGUaAUCCnnnAUA---GACUCGGGC	FERV.NOD								
94	UGCCcnCUGGA-----	GGCGGAUAGCCUGGGaAA--CUGCGGUAACCGcnaAUA---GACUCGGGc	FERV.ICE								
95	UACCCUCAGA-----	GGGGGAUAACUGGGGAAA--CCUCAGCUAAUACCCCAUA---CGUUCUAG	RI 19.B2								
95	UACCCUCAGA-----	GGGGgAUAACUGGGGgAA--CCUCAGCUAAUACCCnAUA---CGUUCUAn	TSIPH. AFRICANA								
95	UGCCUCCGA-----	GGGGGAUAACCAGGGGAAA--CCCUGGCUAAUACCCCAUA---CGCUCCAUG	Thermotoga maritima								
152	AGGgnGCCngCG--GCCCAUc-----	AGGU--AGUUGGUAGG-GUAACGGCCUACnn---AGCCGACGACGGGU--AGCCGAUCUGAGA-GG	FERV.NOD								
152	AGGgggCCUgCG--UCCCAUc-----	AGGU--aguUGGUAGG-GUAACGGccuaCna---AGCCuaCgACGGGu--AGCCGUCUGaGA-GG	FERV.ICE								
153	GAGGGGCCUGCG--ACCCAUC-----	AGGU--AGUUGGUGAG-GUAACGGCUCACCA---AGCCUACGACGGGU--AGCCGUCUGAGA-GG	RI 19.B2								
153	nnGGGGCCUGCG--ACCCAUC-----	AGGU--AGUUGGUGAG-GUAACGGCUCACna---AGCCuaCGAcGGGU--AGCCgGUCUGAGA-GG	TSIPH. AFRICANA								
153	GAGGGGCCCGCG--GCCCAUC-----	AGGU--AGUUGGUGG-GUAACGGCCCACCA---AGCCGACGACGGGU--AGCCGGCCUGAGA-GG	Thermotoga maritima								
228	AUg-GUCGgcCACAAGGGCA-CUGAGACACGGGCCnnnCUCcUAC----	GG-GAGGCAGCAGUGGGGGAUAUUGGACAAUGGGCGAA-----AGCCunA	FERV.NOD								
228	AUG-GCcGgccACAAGGGCA-CUGAGACACggGCCcuaCUccUAC----	GG-GAGgcAGCaUGGGGGAUAUUGGAcAAUGggCGAA-----AGCCUgA	FERV.ICE								
229	AUG-GCCGGCGACAAGGGCA-CUGAGACACGGGCCCCACUCCUAC----	GG-GAGGCAGCAGUGGGGGAUUUUGGACAAUGGGCGAA-----AGCCUGA	RI 19.B2								
229	AUG-GcCgnCnACAAGGGCA-CUGAGACaCGGGCCnnaCUccUAC----	GG-GAGgCAGCAGUGGGGGAUUUUGGACAAUGGGCGAA-----AGCCUnA	TSIPH. AFRICANA								
229	GUG-GUCGGCCACAGGGGCA-CUGAGACACGGGCCCCACUCCUAC----	GG-GAGGCAGCAGUGGGGAUCUUGGACAAUGGGGGAA-----ACCCUGA	Thermotoga maritima								
315	UCCAGCGaCGCCGcGUAUGGU-ACCUAGCGAAU----	AAGCCCCGGCUAACU--ACGUGCCAGCAGCCGCGGUAUUAACGU-AGGGgGcnAGCGUUAACCCG	FERV.NOD								
315	UCCaGCGacGCCGCGUAUGGU-ACCCgACGAAU----	AAGCCCCGGCUAaCU--ACGUGCCAGCAGcCGCGGuaAUACGU-AgGGGncnAGCGUUAACCCg	FERV.ICE								
316	UCCAGCGACGCCGCGUACGGU-AUCCACUAGA----	AAGCCCCGGCUAACU--ACGUGCCAGCAGCCGCGGUAUUAACGU-AGGGGGCGAGCGUUAACCCG	RI 19.B2								
316	UCCAGCGACGccGngUACGgU-AUCCACUAGA----	AAGCCCCGGCUAACU--ACGUGCCAGCAGCCGCGGUAUUAACGU-AGGGGgcnAGCGUUAACCCG	TSIPH. AFRICANA								
316	UCCAGCGACGCCGCGUACGGU-ACCCCGCUAGA----	AAGCCCCGGCUAACU--ACGUGCCAGCAGCCGCGGUAUUAACXU-AGGGGGCAAGCGUUAACCCG	Thermotoga maritima								
407	GAAUCACUGGGCGUA----	AAGGGUGCGUAGG-UG----GCCUGUGnGUCUGGCGUAAAUUACCACGG-CUCAACUGUgGGAUUG-CGCU-GGAA-ACU	FERV.NOD								
407	GAAUcACUGGgCGUa----	aaGGgUGCGuAGG-CG----GcGaGUGuGUCUGGCGUaAAAUUcCACGG-CUcAACuGUGGgUAUG-CgCU-GGAA-ACU	FERV.ICE								
408	GAUUUACUGGGCGUA----	AAGGGGGCGUAGG-CG----GCCGUGAAAGUCCGGUGUGAAAACUCACGG-CUCAACCGUGGGACUG-CGCU-GGAA-ACU	RI 19.B2								
408	GAUUUACUGGGCGUA----	AAGGGGgCGUAGG-CG----GCCGUGAAAGUCCGGUGuGAAAACUCACGG-CUCAACCGUGGGACUG-cGCU-GGAA-ACU	TSIPH. AFRICANA								
408	GAUUUACUGGGCGUA----	AAGGGGGCGUAGG-CG----GCCUGGUGUGCGGAUGUGAAAUCCACGG-CUCAACCGUGGGGUG-CAUC-CGAA-ACU	Thermotoga maritima								

494	GCAG-GGcuUGGGUGC---GGC-AGA-----GGCAGAU---GGAACUGCUGGU--GUAGGGGUnAAUCC-CGUAUAUACAGCAgnAACGCCG	FERV.NOD
494	aCUC-GGcuUGGGUGC---GGC-aGA-----GGCAGAC---GGAACUGCUGGU--GUAGGGGUGAAAUCC-GUAGUAUACAGCaGGaACgCCG	FERV.ICE
495	ACAC-GGCUUGAGGAC---GGU-AGA-----GGGAGAC---GGAACUGCUGGU--GUAGGGGUGAAAUCC-GUAGUAUACAGCAGGAACGCCG	RI 19.B2
495	ACaC-GGCuUGAGGAC---GgU-AGA-----GGGAGAC---GGAACUGCUGGU--GUAGGGGUGAAAUCC-GUAGUAUACAGCAGgAACGCCG	TSIPH. AFRICANA
495	ACCA-GGCUUGGGGGC---GGU-AGA-----GGGAGAC---GGAACUGCCGGU--GUAGGGGUGAAAUCC-GUAGUAUACGGCAGGAACGCCG	Thermotoga maritima
571	GU--GGA-----GAAG-----UCG-----GUCUGCUnGGCcgunACCgA-CGC-UGAGGC-aCnA--AAGCUAG	FERV.NOD
571	GU--GGA-----GAAG-----UCG-----GUCUGCUGGGCCGUAACCGA-CGC-UGAGGC-aCga--AAGcuAG	FERV.ICE
572	GU--GGA-----GAAG-----UCG-----GUCUCCUGGGCCGUUCCUGA-CGC-UGAGGC-CCGA--AAGCUAG	RI 19.B2
572	GU--GGA-----GAAG-----UCG-----GUCUCCUGGGCCGunCCUGA-CGC-UGAGGC-CcnA--AAGCUAG	TSIPH. AFRICANA
572	GU--GGG-----GAAG-----CCG-----GUCUCCUGGGCCGACCCCGA-CGC-UGAGGC-CCGA--AAGCCAG	Thermotoga maritima
623	GGGAGC-GAACCGgaUUAGAUACCCGGGUnGUCCU--aGCCGUaAACGAUGCUCACUAgGUGUGGGGGCUCcGUGCUgAA--gc-UaACGCGAUAAAGUGA	FERV.NOD
623	GGGAGC-GaACCGGAUUAGAUUnCCCGGGUaGUCCU--AGCCGUAAACGAUGCUCACUaGGUGngGGGCCUCCGugcugAA--GC-naAcgCGGaUAAgUg	FERV.ICE
624	GGGAGC-AAACCGGAUUAGAUACCCGGGUAGUCCU--AGCCGUAAACGAUGCCCACUAGGUGUGGGGCCUCCGUGCUNAA--GC-UAACGCGAUAAAGUGG	RI 19.B2
624	GGGAGC-AAACCGGAUUAGAUACCCGGGUAGUCCU--AGCCGUaAACGAUGCCCnCUAGguGUGGGGCCUCCGUGCUGAA--gc-uAACGCGAUAAAGUGG	TSIPH. AFRICANA
624	GGGAGC-AAACCGGAUUAGAUACCCGGGUAGUCCU--GGCUGUAAACGAUGCCCACUAGGUGUGGGGCCUCCGUGCUGAA--GC-UAACGCGUUAAGUGG	Thermotoga maritima
717	GC-----nGCCUGGG--GAGUAUGC-CCG-CA-AGGGUGAAACUCAAAAGGAUUGACgGGGGCCng--CACAA-GCgUGGAGCGUGUGUUUAAUUGG	FERV.NOD
717	AG-----cGCcUggg--gaGUACGc-ccG-CA-AggGUGAAACUCAAAgqAAUUGACggGGnCCG--CACAA-GCGgUGGAGCGUGUGGUUUAAUUGg	FERV.ICE
718	GC-----CGCCUGGG--GAGUAUGC-CCG-CA-AGGGUGAAACUCAAAAGGAUUGACGGGACCCCG--CACAA-GCGGUGGAGCGUGUGGUUUAAUUGG	RI 19.B2
718	Gc-----cGCCUGGG--GAGUAUGC-CCG-CA-AGGGUGAAACUCAAAAGGAUUGACGGGACCCng--cACAA-GcGGUGGAGCGUGUGGUUUAAUUGG	TSIPH. AFRICANA
718	GC-----CGCCUGGG--GAGUACGC-CCG-CA-AGGGUGAAACUCAAAAGGAUUGACGGGGGCCG--CACAA-GCGGUGGAGCGUGUGGUUUAAUUGG	Thermotoga maritima
803	AAgcUAAGCCAAGAACCUUA-CCAGGGCUUGACAUGGCCAGcn-----CAGGUGGuGCACGGUCGUC-GUCAGCUCG-UG-CCG-UGAGGUGUUGGGUUA	FERV.NOD
803	aAncUAAGCCAAGAACCUUA-CCAGGGCUUGACAUGGCCagca-----CAGGUGgugcaCgGUCGUC-GUCAGCUCG-UG-CCg-ugAGGUGuuGGGUUA	FERV.ICE
804	ACGCUAAGCCAAGAACCUUA-CCAGGGCUUGACAUGGCCAGCA-----CAGGUGGUGCACGGUCGUC-GUCAGCUCG-UG-CCG-UGAGGUGUUGGGUUA	RI 19.B2
804	AcgcUAAGCCAAGAACCUUA-CcAGGGCUUGACAUGGCCAgca-----CAGGUGGuGCACGGUCGUC-GUCAGCUCG-UG-CCG-UGAGGUGUUGGGUUA	TSIPH. AFRICANA
804	AUGCUAAGCCAAGAACCUUA-CCAGGGCUUGACAUGGCCGGCA-----CAGGUGGUGCACGGCCGUC-GUCAGCUCG-UG-CCG-UGAGGUGUUGGGUUA	Thermotoga maritima
893	AGUCCcgC--AACGAGCGCA-----ACCCUG--CCCUUAGUUG-aC-UCUAAGGnn-ACU-Gc-cGGC-----GACgA-----	FERV.NOD
893	aGUCCcgC--AACGAGCGCa-----ACCCUG--CCcUUAGUUG-aC-UCUAAGGgg-ACU-GC-CGGc-----gnnnA-----	FERV.ICE
894	AGUCCCGC--AACGAGCGCA-----ACCCUG--CCCUUAGUUG-AC-UCUAAGGGG-ACU-GC-CGGC-----GANGA-----	RI 19.B2
894	AGUCCcgC--AACGAGCGCa-----ACCCUG--CCCUUAGUUG-aC-UCUAAGGgn-ACU-GC-cGGC-----GACgA-----	TSIPH. AFRICANA
894	AGUCCCGC--AACGAGCGCA-----ACCCUG--CCCUUAGUUG-AC-UCUAGGGG-ACU-GC-CGGC-----GACGA-----	Thermotoga maritima

953	-GCCG-gAGGAAGGAGGGnnUGACGUCA-GAUGUCCUgGGCGACACACGCGCUACAaUG---GGGCGGACAGC-----	FERV.NOD
953	-gCCG-GAGGAAGGagGGgaUGACGUCA-GAUGUCCUGGgcUACnncGCGCUacnaUG---GGUGGAUagC-----	FERV.ICE
954	-GCCG-GAGGAAGGUGGGGAUGACGUGA-GAUGCCCUGGGCGACACACGCGCUACAAUG---GGUGGGACAGC-----	RI 19.B2
954	-GCcG-GAGGAAGGAGGGGaUGACGUCA-GAUGCCCunGGCGACACACGCGCUACaAUG---GGUGGGACAGC-----	TSIPH. AFRICANA
954	-GCCG-GAGGAAGGAGGGGAUGACGUCA-GGUGCCCUGGGCGACACACGCGCUACAAUG---GGCGGUACAAU-----	Thermotoga maritima
1020	-----GGGaAGCGA--GCCAGU--GA-UGGUG-AGCAAAACCC-----UCnAACC	FERV.NOD
1020	-----GGgAagCgA--GGCAgc--gA-UGCUG-AGCaAAaCCC-----UgaAACC	FERV.ICE
1021	-----GGGAAGCGA--GCCAGC--GA-UGGUG-AGCGAAGAAA-----UUAaACC	RI 19.B2
1021	-----GGGAaGCGA--GCCAGC--GA-UGGUG-AGCGAAGCCC-----UUAaACC	TSIPH. AFRICANA
1021	-----GGGUUGCGA--CCCCGC--GA-GGGGG-AGCCAAUCCC-----CAAAGC	Thermotoga maritima
1059	GUCC--CCCAGUUCAGAUUGUGG-GC-----UGAAACCCGCCAC-AUGAAGCCGGAAU	FERV.NOD
1059	AcCC--CCcAGUUCAGAUUGUGG-GC-----UGAAACCCGCCaC-aUGAAGCCGGAAU	FERV.ICE
1060	CACC--CUCAGUUCGGAUUGCAG-GC-----UGAAACCCGCCUGC-AUGAAGCCGGAAU	RI 19.B2
1060	CACC--CUCaGUUCGGAUUGCAG-GC-----UGAAACCCGCCUGC-AUGAAGCCGGAAU	TSIPH. AFRICANA
1059	CGCC--CUCAGUUCGGAUCGCAG-GC-----UGCAACCCGCCUGC-GUGAAGCCGGAAU	Thermotoga maritima
1109	CGCUAGUAAUCGCGGAUCAGCCACGCCGCG	FERV.NOD
1109	CGCUAGUAAUCGCGGAUCAGCCaCGCCGCG	FERV.ICE
1110	CGCUAGUAAUCGCGGAUCAGCCACGCCGCG	RI 19.B2
1110	CGCUAGUAAUCGCGGAUCAGCCACGCCGCG	TSIPH. AFRICANA
1109	CGCUAGUAAUCGCGGAUCAGCCAUGCCGCG	Thermotoga maritima