Research Commons

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

Research Commons at the University of Waikato

Copyright Statement:

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

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

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

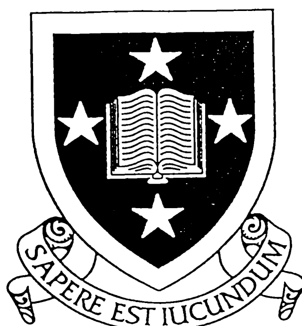
Control of Glucose and Xylose Utilization

by *Clostridium thermohydrosulfuricum* Rt8.B1

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

by

Gregory Murray Cook



University of Waikato
Hamilton, New Zealand
1992

ABSTRACT

Five extremely thermophilic organisms representing the Eubacterial and Archaebacterial Kingdoms were studied for controlled expression of β-galactosidase, β-glucosidase and α-glucosidase synthesis. Under the culture conditions used, these organisms exhibited a high constitutive level of enzyme synthesis which was modulated in the presence of added carbohydrate. The lack of observed classical induction in these organisms could either reflect a lack of control or a less elaborate control of enzyme synthesis, which may be advantageous to these bacteria in environments low in nutrients. This absence of highly regulated control is common in thermophilic organisms and may be a reflection of the limited evolutionary divergence of thermophiles as is suggested by phylogenetic studies.

Cl. thermohydrosulfuricum Rt8.B1 inhabits thermophilic environments where the natural concentrations of sugars would be expected to be quite low. The results of this study demonstrated that glucose and xylose were used simultaneously i.e. the bacterium exhibited hyperbolic growth when both glucose and xylose were supplied together at nonlimiting concentrations. Under conditions of hyperbolic growth, *Cl. thermohydrosulfuricum* Rt8.B1 exhibited neither catabolite repression nor inducer exclusion. Although classical control mechanisms were not seen, the utilization of glucose and xylose were tightly controlled at both low and high concentrations of single and multiple substrates. Such regulation to allow controlled hyperbolic growth is consistent with the idea of *Cl. thermohydrosulfuricum* Rt8.B1 being well adapted as an opportunist.

The transport of glucose and xylose across the bacterial cell membrane of *Cl. thermohydrosulfuricum* Rt8.B1 was governed by permeases which did not catalyze concomitant substrate transport and phosphorylation, and thus was not a phosphoenolpyruvate-dependent carbohydrate: phosphotransferase system. Glucose and xylose transport was not driven by a proton motive force ($\Delta\mu H^+$) nor coupled to sodium and potassium ion gradients. An involvement of ATP in the uptake process

was indicated by the reduction of glucose and xylose uptake by iodoacetate and sodium fluoride, both inhibitors of ATP synthesis.

The phosphorylation of glucose was carried out by a constitutive ATP-dependent glucokinase and that of xylose by an inducible ATP-dependent xylulokinase following isomerization by xylose isomerase. These enzymes not only initiated the metabolism of glucose and xylose in *Cl. thermohydrosulfuricum* Rt8.B1, but also served to "capture" sugars from the cytoplasm through their phosphorylation. Since glucokinase was regulated primarily by the concentration of ATP, ADP, and AMP, both glucose phosphorylation and uptake appeared to be dependent upon the energy status of the cell.

Xylulose inhibited glucokinase activity, indicating that xylose metabolism may regulate the activity of glucokinase in glucose and xylose-grown cells. Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 was mediated by two systems. Both systems were inducible and dependent on the xylose concentration used for cell growth. Cells grown on 5 mM xylose had a high-affinity, low-capacity system for xylose uptake which was saturable (low V_{max}). Cells grown on 50 mM xylose had a low-affinity, high-capacity (high V_{max}) system for xylose uptake and the kinetics were indicative of facilitated diffusion.

The xylose isomerase of *Cl. thermohydrosulfuricum* Rt8.B1 had a high K_m for xylose (low-affinity) and xylulokinase had a low K_m for xylose (high-affinity). The K_m for xylulokinase was lower than the K_t for the high-affinity xylose permease; this indicated that the xylulokinase phosphorylating system was fully saturated at external xylose concentrations near or above the K_t concentration for uptake and that the affinity of xylulokinase regulated both the rate of xylose uptake and its subsequent utilization.

When *Cl. thermohydrosulfuricum* Rt8.B1 was grown on high concentrations of xylose (low-affinity), xylulose leaked from the cell. This indicated that a futile cycle for xylose may be operative under conditions of high xylose and may explain the facilitated diffusion kinetics observed

A semi-defined minimal medium for the growth of *Clostridium thermohydrosulfuricum* Rt8.B1 was developed in this study. This medium was able to support the growth of several thermophilic anaerobes. Most strains studied showed morphological changes. In one instance, spores were seen in an apparently non-sporulating strain of *Thermoanaerobium brockii* DSM 1457 which could not be attributed to a contaminant. This observation has resulted in the species being renamed *Thermoanaerobacter brockii* comb. nov. and the genus *Thermoanaerobium* being removed due to the removal of the type strain.

ACKNOWLEDGEMENTS

I wish to thank my supervisors Professor Hugh Morgan and Dr Peter Janssen for their encouragement, guidance and enthusiasm during the practical work and writing of this thesis.

I would like to thank Dr Chris Harfoot for the proof reading and for his many helpful discussions and suggestions in the final stages of this thesis. Thanks are also due to Colin Monk and Lynne Parker for their technical assistance throughout the course of this project, Dr Keith Peek and Dr Leon Ruttersmith for their helpful advice and suggestions with the enzyme studies, Tracy Barea for her general helpfulness which was much appreciated, Frank Bailey for his draughting services and Dave Wild and Alf Harris for access and operation of the electron microscope.

Special thanks are due to Pete and Fred for their friendship and companionship on all the weekends and late nights spent in the lab. I would also like to thank my fellow students, especially Sally during the writing of this thesis for providing a relaxing lab in which to work.

I would like to say a special thank you to Sharron for her tolerance, encouragement and not letting me "stress" out too much.

Finally, I thank most of all my family for their support, encouragement and their willingness to help out with all the things that I never had time for in the last few months.

The work in this thesis was possible due to a University Grants Committee scholarship and Pacific Enzymes Limited.

TABLE OF CONTENTS

		Page
	ABSTRACT	ii
	ACKNOWLEDGEMENTS	v
	TABLE OF CONTENTS	vi
	LIST OF FIGURES	xiii
	LIST OF TABLES	xvii
	LIST OF ABBREVIATIONS	xx
CHAPTER ONE	**INTRODUCTION**	1
CHAPTER TWO	**LITERATURE REVIEW**	3
2.1	THERMOPHILIC ANAEROBIC BACTERIA	3
2.1.1	Thermophilic glycolytic anaerobic eubacteria	4
2.1.2	Thermophilic saccharide fermentations	7
2.2	CARBOHYDRATE UTILIZATION BY EXTREME THERMOPHILES	8
2.2.1	Pathways of hexose metabolism	8
2.2.2	Pathways of pentose metabolism	9
2.2.3	Formation of end products	10
2.3	DIAUXIC GROWTH	12
2.3.1	Control of diauxic growth	12
2.3.2	Diauxic growth in mesophilic organisms	13
2.3.3	Diauxic growth of thermophiles	14
2.4	HYPERBOLIC GROWTH KINETICS	14
2.5	SUGAR TRANSPORT SYSTEMS	16
2.5.2	Models of transport	16
2.6	PASSIVE DIFFUSION	17
2.7	FACILITATED DIFFUSION	18
2.7.1	Distribution of facilitated diffusion systems	19
2.8	ACTIVE TRANSPORT	21
2.8.1	Chemiosmotic hypothesis	22
2.8.2	Uniports, symports and antiports	23
2.8.3	Sodium-coupled active transport	25

		Page
2.9	OSMOTIC SHOCK-SENSITIVE ACTIVE TRANSPORT SYSTEMS	26
2.9.1	Structure	26
2.9.2	Energy coupling of periplasmic permeases	27
2.10	ENZYME-MEDIATED GROUP TRANSFER REACTIONS	28
2.10.1	Historical	28
2.10.2	Structure	29
2.10.3	Distribution of phosphotransferase systems in bacteria	31
2.10.4	Importance of the PTS system	33
2.10.5	Regulatory functions of the PTS	34
2.11	TRANSPORT SYSTEMS IN THERMOPHILES	34
2.12	THESIS OBJECTIVES AND OVERALL AIMS	36
CHAPTER THREE	**ENZYME EXPRESSION**	
3.1	INTRODUCTION	38
3.2	MATERIAL AND METHODS	39
3.2.1	Chemicals	39
3.2.2	Strains used in study	39
3.2.3	Preparation of media components	40
3.2.3.1	Sterilization	40
3.2.3.2	Reducing solution	40
3.2.3.3	Vitamin and trace element solutions	41
3.2.3.4	Carbon sources	42
3.2.3.5	Media preparation	42
3.2.4	Media and conditions for cultivation of bacteria	42
3.2.5	Assay methods	44
3.2.5.1	Chemicals	44
3.2.5.2	Preparation of cells for determination of enzyme activities	44
3.2.5.3	β-glucosidase, β-galactosidase and α-glucosidase assays	44
3.3	RESULTS	46
3.3.1	Thermophilic archaebacteria	47
3.3.1.1	*Thermococcus* AN1	47
3.3.1.2	*Sulfolobus solfataricus*	51
3.3.2	Thermophilic eubacteria	52
3.3.2.1	*Thermus* Rt41A	52
3.3.2.2	*Clostridium thermohydrosulfuricum* Rt8.B1	54
3.3.2.3	*Thermotoga* FjSS3.B1	55

		Page
3.4	DISCUSSION	57
3.4.1	Introduction	57
3.4.2	The expression of β-galactosidase in thermophilic organisms	58
3.4.3	The expression of β-glucosidase in thermophilic organisms	61
3.4.4	The expression of α-glucosidase in thermophilic organisms	62
3.4.5	Summary	63
CHAPTER FOUR	**DEVELOPMENT OF A DEFINED MINIMAL MEDIUM FOR ANAEROBIC GROWTH OF *Clostridium thermohydrosulfuricum* Rt8.B1**	
4.1	INTRODUCTION	67
4.2	MATERIALS AND METHODS	68
4.2.1	Chemicals	68
4.2.2	Bacterial strains	68
4.2.3	Media and cultivation conditions	68
4.2.4	Enzyme assays	69
4.3	RESULTS	69
4.3.1	Development of a defined minimal medium	69
4.4	DISCUSSION	81
CHAPTER FIVE	**ENDOSPORE FORMATION BY *Thermoanaerobium brockii* HTD4.**	
5.1	INTRODUCTION	84
5.2	MATERIALS AND METHODS	84
5.2.1	Bacterial strains	84
5.2.2	Media and cultivation conditions	84
5.2.3	Cellular characterization	85
5.2.4	Heat-treatment of cultures	85
5.3	RESULTS	86
5.3.1	Growth on a minimal medium containing xylose	86
5.3.2	Cellular features	86
5.3.3	Demonstration of heat resistance	87
5.3.4	Check of purity at end of heat-treatment	90
5.3.5	Dipicolinic acid	90

		Page
5.4	DISCUSSION	91
5.4.1	Sporulation in *T. brockii* and other related organisms	91
5.4.2	Recent developments	93
CHAPTER SIX	**GLUCOSE AND XYLOSE UTILIZATION BY *Clostridium thermohydrosulfuricum* Rt8.B1**	
6.1	INTRODUCTION	96
6.2	MATERIALS AND METHODS	97
6.2.1	Chemicals	97
6.2.2	Growth characteristics in minimal medium	97
6.2.3	Substrate utilization studies by cell-suspensions	99
6.2.3.1	Preparation of cell suspensions	99
6.2.3.2	Standard assay procedure	99
6.2.3.3	Protein assay	100
6.2.3.4	Kinetics of substrate utilization	101
6.2.4	ANALYTICAL METHODS	101
6.2.4.1	High performance liquid chromatography	101
6.2.5	MEASUREMENT OF ENZYME ACTIVITIES	102
6.2.5.1	Chemicals	102
6.2.5.2	Preparation of crude extracts	103
6.2.6	ASSAY METHODS	103
6.2.6.1	Protein determination of crude extracts	103
6.2.6.2	Assay of enzyme activities in crude extracts	104
6.3	RESULTS	106
6.3.1	Growth of *Clostridium thermohydrosulfuricum* Rt8.B1 in a minimal medium containing glucose + xylose	106
6.3.2	The effect of the glucose analogues 2-deoxy-glucose and methyl-α-D-glucoside on the growth of *Clostridium thermohydrosulfuricum* Rt8.B1	113
6.3.2.1	The effect of α-MG on the growth of *Clostridium thermohydrosulfuricum* Rt8.B1	113
6.3.2.2	The effect of 2-DG on the growth of *Clostridium thermohydrosulfuricum* Rt8.B1	118
6.3.2.3	Summary of results	127
6.3.3	Utilization studies using cell suspensions	127
6.3.3.1	Introduction	127
6.3.3.2	Glucose and xylose utilization by 50 mM glucose-grown cell suspensions of *Clostridium thermohydrosulfuricum* Rt8.B1	128

Page

6.3.3.3	Glucose and xylose utilization by 5 mM glucose-grown cell suspensions of *Clostridium thermohydrosulfuricum* Rt8.B1	129
6.3.3.4	Glucose and xylose utilization by 50 mM xylose-grown cell suspensions of *Clostridium thermohydrosulfuricum* Rt8.B1	131
6.3.3.5	Glucose and xylose utilization by 5 mM xylose-grown cell suspensions of *Clostridium thermohydrosulfuricum* Rt8.B1	136
6.3.3.6	Glucose and xylose utilization by 50 mM glucose + 50 mM xylose-grown cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1	137
6.4	DISCUSSION	142
6.4.1	Hyperbolic growth of *Cl. thermohydrosulfuricum* Rt8.B1	142
6.4.2	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose analogues	145
6.4.2.1	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose and xylose in the presence of α-MG	145
6.4.2.2	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose and xylose in the presence of 2-DG	146
6.4.3	Kinetics of glucose and xylose utilization of *Cl. thermohydrosulfuricum* Rt8.B1	148
6.4.3.1	Kinetics of substrate utilization by glucose-grown cells	148
6.4.3.2	Kinetics of substrate utilization by xylose-grown cells	150
6.4.3.3	Kinetics of substrate utilization by glucose + xylose-grown cells	153

CHAPTER SEVEN GLUCOSE AND XYLOSE TRANSPORT BY *Clostridium thermohydrosulfuricum* Rt8.B1

7.1	INTRODUCTION	155
7.2	MATERIALS AND METHODS	156
7.2.1	Chemicals	156
7.2.2	Preparation of cell suspensions	156
7.2.3	Preparation of toluenized cells and assay of PTS activity	157
7.2.4	Transport of radioactive labelled sugars	158
7.2.4.1	Standard method	158
7.2.4.2	Rapid sampling method	159
7.2.4.3	Kinetics of glucose and xylose transport	160
7.2.5	Competition experiments	161

Page

7.2.6	Metabolic inhibitors	161
7.2.7	Counterflow experiments and 2-deoxy-glucose transport	162
7.3	RESULTS	162
7.3.1	Phosphoenolpyruvate-dependent phosphotransferase activity	162
7.3.2	Kinetics of glucose and xylose transport	164
7.3.2.1	50 mM glucose-grown cell suspensions	164
7.3.2.2	5 mM glucose-grown cell suspensions	164
7.3.2.3	5 mM xylose-grown cell suspensions	164
7.3.2.4	50 mM xylose-grown cell suspensions	165
7.3.3	Competition studies	173
7.3.4	The effects of metabolic inhibitors on glucose and xylose transport	175
7.4	DISCUSSION	180
7.4.1	Glucose transport by a PEP-dependent phosphotransferase system	180
7.4.2	Kinetics of glucose transport by *Clostridium thermohydrosulfuricum* Rt8.B1	183
7.4.3	The effect of glucose analogues and competing sugars on glucose transport by *Clostridium thermohydrosulfuricum* Rt8.B1.	185
7.4.4	Kinetics of xylose transport by *Clostridium thermohydrosulfuricum* Rt8.B1	186
7.4.5	The effect of glucose analogues and competing sugars on xylose transport by *Clostridium thermohydrosulfuricum* Rt8.B1.	189
7.4.6	The effects of metabolic inhibitors on glucose and xylose transport by *Clostridium thermohydrosulfuricum* Rt8.B1	190
CHAPTER EIGHT	**CONTROL OF GLUCOSE AND XYLOSE UTILIZATION IN *Clostridium thermohydrosulfuricum* Rt8.B1**	
8.1	INTRODUCTION	195
8.2	MATERIALS AND METHODS	196
8.2.1	Chemicals	196
8.2.2	Enzyme assays	196
8.3	RESULTS	198
8.3.1	The kinetics of glucokinase activity in lysed cell crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1	198

Page

8.3.2 The effect of ATP concentration on glucokinase activity 201
8.3.3 The effect of competitive inhibitors on glucokinase activity 204
8.3.4 The effect of metabolic inhibitors on glucokinase activity 205
8.3.5 Kinetics of xylose isomerase and xylulokinase activity 206

8.4 DISCUSSION 211
8.4.1 Introduction 211
8.4.2 Kinetics of glucokinase activity 212
8.4.3 Control of glucokinase activity in *Clostridium thermohydrosulfuricum* Rt8.B1 214
8.4.4 The kinetics of xylulokinase and xylose isomerase 216

CHAPTER NINE GENERAL DISCUSSION AND SUMMARY

9.1 INTRODUCTION 219

9.2 REGULATION OF ENZYME ACTIVITY IN EXTREMELY THERMOPHILIC BACTERIA 219

9.3 HYPERBOLIC GROWTH IN *Clostridium thermohydrosulfuricum* Rt8.B1 221
9.3.1 Control of hyperbolic growth in *Clostridium thermohydrosulfuricum* Rt8.B1 222

9.4 GLUCOSE TRANSPORT AND UTILIZATION IN *Cl. thermohydrosulfuricum* Rt8.B1 223

9.5 XYLOSE TRANSPORT AND UTILIZATION IN *Cl. thermohydrosulfuricum* Rt8.B1 226

9.6 SUMMARY 229

APPENDIX 1

Endospore formation by *Thermoanaerobium brockii* HTD4 232

REFERENCES 237

LIST OF FIGURES

Figure		Page
2.1	Thermophilic hetero-ethanol fermentations	11
2.2	Binding protein-mediated transport	27
2.3	Components of the phosphoenolpyruvate:glucose phosphotransferase system in bacteria	30
5.1	Phase contrast photomicrograph of *T. brockii* HTD4 grown on TYEG with characteristic mini-cell present	88
5.2	Phase contrast photomicrograph of *T. brockii* HTD4 grown on minimal medium containing xylose	88
5.3	Electron micrograph ultrathin section of a spore in *T. brockii* HTD4 grown on minimal medium containing xylose showing the mono-layered cell wall and lamellar structure around the spore	89
5.4	Electron micrograph of ultrathin section of *T. brockii* HTD4 showing the typical constrictive or pinching off division with the monolayered cell wall and characteristic mesosomal structures	89
6.1	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose in pH-controlled batch culture	109
6.2	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose with 50 mM xylose added at the mid-log phase of growth in pH-controlled batch culture	109
6.3	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose with 50 mM glucose added at the mid-log phase of growth in pH-controlled batch culture	110
6.4	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose with 50 mM xylose added at the mid-log phase of growth in pH-controlled batch culture	110
6.5	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose with 50 mM glucose added at the mid-log phase of growth in pH-controlled batch culture	111
6.6	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose with 20 mM glucose added at the stationary phase of growth in pH-controlled batch culture	111
6.7	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 6 mM xylose with 30 mM glucose added at the mid-log phase of growth in pH-controlled batch culture	112
6.8	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 6 mM glucose with 18 mM xylose added at the mid-log phase of growth in pH-controlled batch culture	112
6.9	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM α-MG in batch culture	115
6.10	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose + 50 mM α-MG in batch culture	115

Figure		Page
6.11	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM α-MG in batch culture	116
6.12	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose with 30 mM α-MG added at the mid-log phase of growth in batch culture	116
6.13	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose with 45 mM α-MG added at the mid-log phase of growth in batch culture	117
6.14	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM 2-DG in batch culture	121
6.15	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM 2-DG in batch culture	121
6.16	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose + 50 mM 2-DG in batch culture	122
6.17	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose with 45 mM 2-DG added at the mid-log phase of growth in batch culture	122
6.18	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose with 20 mM 2-DG added at the mid-log phase of growth in batch culture	123
6.19	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose with 20 mM 2-DG added at the mid-log phase of growth in batch culture	123
6.20	The effect of 2-DG concentration on the lag phase in cultures of *Cl. thermohydrosulfuricum* Rt8.B1 grown on glucose or xylose	124
6.21	Growth of adapted glucose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM 2-DG in batch culture	124
6.22	Growth of adapted xylose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM 2-DG in batch culture	125
6.23	Growth of adapted glucose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose + 50 mM 2-DG in batch culture	125
6.24	Growth of adapted xylose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose + 50 mM 2-DG in batch culture	126
6.25	Growth of adapted glucose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM 2-DG in batch culture with glucose added at the lag phase of growth	126
6.26	Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose	133
6.27	Glucose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose with increasing glucose concentration	133

Figure		Page
6.28	Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM glucose	134
6.29	Glucose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM glucose with increasing glucose concentration	134
6.30	Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose	135
6.31	Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose with increasing xylose concentration	135
6.32	Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose	139
6.33	Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing xylose concentration	139
6.34	Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose + 50 mM xylose	140
6.35	Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose + 50 mM xylose with increasing glucose and xylose concentrations	140
6.36	Eadie-Hofstee plot of glucose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose + 50 mM xylose	141
6.37	Eadie-Hofstee plot of xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose + 50 mM xylose	141
6.38	Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM and 50 mM xylose with increasing xylose concentration	152
6.39	Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM and 50 mM xylose at low xylose concentrations	152
7.1	Glucose uptake with time by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose	168
7.2	Glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose with increasing glucose concentration	168
7.3	Glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM glucose with increasing glucose concentration	169
7.4	Glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing glucose concentration	169
7.5	Xylose uptake with time by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose	170
7.6	Xylose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing xylose concentration	170
7.7	2-DG uptake with time by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose	171
7.8	Counterflow of 2-DG by 2-DG and iodoacetate treated cells of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose	171

Figure		Page
7.9	Counterflow of 2-DG by 2-DG and iodoacetate treated cells of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose	172
7.10	Comparison of 2-DG uptake and glucose uptake with time by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose	172
8.1	Glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose with increasing concentrations of glucose	200
8.2	Glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing concentrations of glucose	200
8.3	The effect of ATP concentration on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose at a constant glucose concentration (0.50 mM)	202
8.4	The effect of ATP concentration on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose at a constant glucose concentration (0.50 mM)	202
8.5	The effect of ADP concentration on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose at a constant glucose concentration (0.50 mM) and various ATP concentrations	203
8.6	The effect of ADP and AMP concentration on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose at a constant glucose concentration (0.50 mM) and increasing ATP concentrations	203
8.7	The effect of D-xylulose on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose at a constant glucose concentration (0.50 mM) and various ATP concentrations	207
8.8	The effect of D-xylulose on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose at a constant glucose concentration (0.50 mM) and various ATP concentrations	207
8.9	Xylose isomerase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing concentrations of xylose	208
8.10	Xylose isomerase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose with increasing concentrations of xylose	208
8.11	Xylulokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing concentrations of xylose	210
8.12	Xylulokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose with increasing concentrations of xylose	210
9.1	Control of glucose utilization in *Cl. thermohydrosulfuricum* Rt8.B1	224
9.2	Control of xylose utilization in *Cl. thermohydrosulfuricum* Rt8.B1	227

LIST OF TABLES

Table		Page
2.1	Thermophilic glycolytic anaerobic bacteria	5
2.2	Growth of thermophilic anaerobes on mixed substrates	15
2.3	Distribution of the PTS in procaryotes	32
3.1	Thermophilic organisms used in study	39
3.2	Enzyme activities present in cell-free supernatants of *Thermococcus* AN1 grown on different carbon sources	47
3.3	Enzyme activities present in toluene-treated cells of *Thermococcus* AN1 grown on different carbon sources	49
3.4	Enzyme activities present in cultures of *Thermococcus* AN1 grown on different carbon sources	50
3.5	Enzyme activities present in cultures of *S. solfataricus* grown on different carbon sources	52
3.6	Enzyme activities present in cultures of *Thermus* Rt41A grown on different carbon sources	53
3.7	Enzyme activities present in cultures of *Cl. thermohydrosulfuricum* Rt8.B1 grown on different carbon sources	55
3.8	Enzyme activities present in cultures of *Thermotoga* FjSS3.B1 grown on different carbon sources	56
3.9	The expression of β-galactosidase in thermophilic organisms	59
3.10	The expression of β-glucosidase in thermophilic organisms	61
3.11	The expression of α-glucosidase in thermophilic organisms	62
4.1	Minimal medium used to determine the growth requirements of *Cl. thermohydrosulfuricum* Rt8.B1	69
4.2	Components of vitamin solutions used in study	70
4.3	The effect of vitamin addition on the growth of *Cl. thermohydrosulfuricum* Rt8.B1 in minimal medium containing glucose	72
4.4	The effect of growth supplements and amino acids on the growth of *Cl. thermohydrosulfuricum* Rt8.B1 on minimal medium containing glucose	74
4.5	The effect of different vitamin solutions on the growth of *Cl. thermohydrosulfuricum* Rt8.B1 in the presence of casamino acids and trypticase peptone	75
4.6	The effect of MM-vitamins and vitamin-free casamino acid concentrations on the growth of *Cl. thermohydrosulfuricum* Rt8.B1	76
4.7	Final medium composition used to grow *Clostridium thermohydrosulfuricum* Rt8.B1	77
4.8	Growth of thermophilic anaerobic bacteria on minimal medium containing glucose	78
4.9	Enzyme activities in cultures of *Cl. thermohydrosulfuricum* Rt8.B1 grown on different carbon sources and a defined medium	80
5.1	Proposed reclassification of some thermophilic isolates	94

Table		Page
6.1	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose and xylose in the presence of methyl-α-D-glucoside	113
6.2	Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose and xylose in the presence of 50 mM 2-deoxy-glucose	118
6.3	Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose	129
6.4	Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM glucose	130
6.5	Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose	132
6.6	Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose	136
6.7	Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on equal concentrations of glucose + xylose	137
6.8	Utilization rates and kinetic parameters for cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM or 50 mM glucose	149
6.9	Utilization rates and kinetic parameters for cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM or 50 mM xylose	150
6.10	Utilization rates and kinetic parameters for cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose + 50 mM xylose	153
7.1	Phosphoenolpyruvate-phosphotransferase and glucokinase activities in *Cl. thermohydrosulfuricum* Rt8.B1	163
7.2	The effect of unlabelled sugars on ^{14}C-glucose uptake by 50 mM glucose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1	173
7.3	The effect of unlabelled sugars on ^{14}C-glucose uptake by 5 mM xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1	174
7.4	The effect of unlabelled sugars on ^{14}C-xylose uptake by 5 mM xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1	175
7.5	The effect of various metabolic inhibitors on glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose	177
7.6	The effect of various metabolic inhibitors on glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose	178
7.7	The effect of various metabolic inhibitors on xylose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose	179
7.8	The kinetic parameters of glucose transport by glucose and xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1	183
8.1	Glucokinase activities in lysed cell crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1	199
8.2	The effect of various substrates on the activity of glucokinase in lysed cell crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1	204
8.3	The effect of metabolic inhibitors on the activity of glucokinase of lysed cell crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1	206

Table		Page
8.4	Glucose isomerase activity in crude extracts of *Clostridium thermohydrosulfuricum* Rt8.B1	211
8.5	The kinetic parameters of glucokinase activity in crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1	212
8.6	The kinetic parameters of xylulokinase and xylose isomerase from *Cl. thermohydrosulfuricum* Rt8.B1	216

LIST OF ABBREVIATIONS

2,4-DNP	2,4-dinitrophenol
2-DG	2-deoxy-glucose
α-MG	methyl-α-D-glucoside
h^{-1}	maximum growth rate
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
Ca.	*Caldocellum*
CCCP	carbonyl cyanide m-chlorophenylhydrazone
Cl.	*Clostridium*
DCCD	*N,N*-dicyclohexylcarbodiimide
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
MOPS	3-[morpholino]propanesulfonic acid
ρNPG	ρ-nitrophenyl substrate
ρNP	ρ-nitrophenol
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
PTS	phosphotransferase system
T.	*Thermoanaerobium*
Ta.	*Thermoanaerobacter*
Tb.	*Thermobacteroides*
TYEG	trypticase peptone-yeast extract-glucose medium

CHAPTER ONE

INTRODUCTION

The regulation of enzyme synthesis is vitally important to the survival of bacteria in natural environments, where nutrients are almost always limiting for bacterial growth. The ability of an organism to regulate its enzyme synthesis to those that are necessary for their metabolism under current physiological conditions is of obvious benefit as it not only prevents unnecessary protein synthesis, it also allows an organism to select ready utilizable substrates and therefore maximize growth. The regulation of enzyme synthesis has been well characterized in *Escherichia coli.* Lactose metabolism by *E. coli* is under negative genetic control, that is the *lac* operon containing the genes that code for lactose metabolism (lactose permease and β-galactosidase) is available for transcription, but transcription is prevented by a specific repressor. The repressor is removed by the inducer allolactose, a compound derived from lactose, which induces the formation of lactose permease and β-galactosidase. This process of substrate mediated enzyme synthesis is called induction. The control of this system prevents wasteful protein synthesis when lactose is absent. In natural environments an organism often has the alternative of using one or more substrates. For example, *E. coli* provided with glucose and lactose preferentially utilizes glucose before lactose. This is termed diauxie and is a widespread phenomenon in the control of carbohydrate utilization in bacteria. Diauxie is controlled by two related phenomena: catabolite repression and inducer exclusion. These general regulatory control mechanisms serve to control the activity of early reactions in carbohydrate metabolism and to ensure that *E. coli* uses the most favoured substrate (i.e., the substrate allowing the fastest growth rate) first, thus providing a competitive advantage in an environment where substrate availability may not be the only factor determining species success. Although these mechanisms have been well studied in *E. coli*, surprisingly little is known about the regulation of enzyme synthesis in other bacteria, especially thermophilic bacteria.

There is some support from phylogenetic evidence (largely based on 16S rRNA sequence homology data) that species of thermophilic bacteria have the greatest phylogenetic depth of several eubacterial lineages and that they constitute the least rapidly evolving form of that lineage. If that is the case, it is possible that substrate utilization in these organisms may be differently or less tightly controlled than in organisms which exhibit a greater degree of evolutionary divergence and are presumably better adapted to their environment. To investigate this possibility, the expression of enzyme synthesis in a number of thermophilic bacteria representing different genera and kingdoms was investigated for controlled enzyme expression of β-glucosidase, β-galactosidase and α-glucosidase synthesis in the hope of gaining some insight into the regulatory mechanisms present in these bacteria.

To date, the mechanism and regulation of carbohydrate uptake in thermophilic bacteria has been largely ignored, despite being an important step in the fermentation process of thermophilic fermentative bacteria. The reported simultaneous utilization of glucose and xylose by thermophilic glycolytic anaerobes might reflect either a lack of control in substrate uptake and metabolism or an adaptive response to a nutrient-poor environment such as a hot spring, where the ability to take up any substrate available outweighs any advantage in controlled use of substrate. This would represent survival at any cost, rather than survival of the fittest (best adapted). The simultaneous use of pentoses and hexoses suggests that pathways in the metabolism of these substrates may be constitutive at low substrate concentrations.

In attempts to gain more insight into how thermophilic bacteria regulate their substrate utilization the mechanism of glucose and xylose transport by the extremely thermophilic anaerobic bacterium *Clostridium thermohydrosulfuricum* Rt8.B1 was investigated. It is important to know whether transport is the rate-limiting step and how transport is controlled, energized and regulated. The key enzymes involved in the initial steps of glucose and xylose were investigated for their controlled expression and regulation. It was hoped that this study would lead to some understanding of the control processes involved in mixed substrate utilization of glucose and xylose by *Cl. thermohydrosulfuricum* Rt8.B1.

CHAPTER TWO

LITERATURE REVIEW

2.1 THERMOPHILIC ANAEROBIC BACTERIA

Interest in the study of thermophilic microorganisms has increased at an explosive rate in the last ten years. This increased interest stems from the ability of these organisms to live at extreme temperatures and the adaptations, both physical and metabolic, which they exhibit in order to survive in such environments. From a biotechnological view point, considerable interest in these bacteria has been generated for the conversion of renewable resources i.e. biomass and wastes, into liquid fuel and chemical feedstocks, and the search for thermostable enzymes which offer potential in industrial processes due to their stability. For the molecular taxonomist these bacteria are of interest to determine their phylogeny (relationship) with other life forms, especially when one considers that thermophilic environments may represent conditions similar to those under which life evolved (Achenbach-Richter *et al.,* 1987).

Until 1980 only three anaerobic organisms were reported to grow above 70°C, the archaeal (archaebacterium) *Methanobacterium thermoautotrophicum* (Zeikus and Wolfe, 1972), a sulfate reducer *Desulfovibrio thermophilus* (Rozanova and Khudyakova, 1974), and the saccharolytic spore forming *Clostridium thermohydrosulfuricum* (Wiegel *et al.*, 1979). In the last ten years many new thermophiles have been discovered especially among the Archaea, but also among the Bacteria (Eubacteria). The numbers of newly described organisms has increased dramatically with 27 species of anaerobic thermophilic eubacteria (Wiegel, 1992) and over 30 species of anaerobic Archaea having been described. The groups of published Archaea, thermophilic and extremely thermophilic sulfur reducers and the methanogens contain organisms capable of growth in extreme environments from pH 2 to around 9 and temperatures to above 100°C (Wiegel, 1992).

2.1.1 Thermophilic glycolytic anaerobic eubacteria

Thermophilic glycolytic anaerobic bacteria have been isolated from a variety of sources which include geothermal environments, e.g. hot springs, volcanic environments, decomposing thermal algal mats, heated soils, and non-geothermal environments, e.g. soils, lakes and in the food industry. These bacteria range in diversity from saccharolytic to cellulolytic species and both Gram-positive and Gram-negative species. The species diversity of these bacteria is somewhat limited with respect to the major genera present. The most widely studied members of the group include *Clostridium thermocellum, Clostridium thermohydrosulfuricum, Thermoanaerobium brockii, Thermoanaerobacter ethanolicus* and *Clostridium thermosaccharolyticum.*

To date, the taxonomic and systematic position of many of these organisms are still in doubt and require further studies involving the use of phylogenetically relevant properties such as DNA:DNA homology and 16S RNA sequence data in order to determine relationships and groupings. For the purposes of the following discussion, the organisms are described by their presently published names and can be grouped by their physiological characteristics i.e. growth temperature, pH optima and carbohydrate fermentation capabilities. Thermophilic glycolytic anaerobes can be divided into four groups according to their main fermentation end product (major organic compound) (Table 2.1).

1) Homoacetogenic (growing on H_2 and CO_2) and acetogenic (acetate, CO_2 and H_2-forming) organisms which produce > 1.8 mol acetate /mol glucose utilized.

2) Butyrate-forming organisms producing > 0.5 mol butyrate /mol glucose.

3) Lactate-forming organisms producing > 1.2 mol lactate /mol glucose utilized.

4) Organisms producing mixed fermentation products including acetate, lactate, propionate and ethanol. The last group includes some organisms capable of producing

> 1.5 mol ethanol /mol glucose utilized e.g. *Ta. ethanolicus* JW 200, *Thermoanaerobacter finii* and *Cl. thermohydrosulfuricum* E100-69 (Wiegel, 1992)

Table 2.1 Thermophilic glycolytic anaerobic eubacteria

Physiological Groupings	Gram type	Topt (°C)	pH optima	Fermentable carbohydrates
1. Homoacetogens and acetate producers				
a. Homoacetogenic species				
Clotridium thermoaceticum	positive	58	6.7	Ferments only glucose, fructose,galactose, xylose, and mannose (Fontaine et al., 1942)
Clostridium thermoautotrophicum	positive	58	6.6	Glucose (Wiegel et. al., 1981)
Acetogenium kivui	negative	65	6.6	Glucose, mannose and fructose (Leigh and Wolfe, 1983)
b. Acetate, carbon dioxide and hydrogen				
Clostridium fervidus	positive	68	7.0	Variety of sugars including glucose, maltose, xylan, mannose and starch (Patel et al., 1987)
Acetomicrobium flavidum	negative	58	7.0	Starch, glucose, fructose, maltose, cellobiose and glycerol (Soutschek et al., 1984)
Acetothermus paucivorans	negative	58	7.2	Glucose and fructose (Dietrich et al., 1988)
2. Butyrate forming organisms				
Clostridium thermosaccharolyticum	positive	55	7.0	Wide variety of carbohydrates starch, xylan, glucose, xylose and cellobiose (McClung, 1935)
Clostridium thermobutyricum	positive	57	7.0	Glucose, fructose, maltose, xylose, ribose and cellobiose (Wiegel et al., 1989)
3. Lactate producing organisms				
Clostridium thermolacticum	positive	65	7.0	Glucose, ribose, fructose, sucose and xylose (Le Ruyet et al., 1985)

Table 2.1 Thermophilic glycolytic anaerobic eubacteria (cont)

Physiological Groupings	Gram type	Topt (°C)	pH optima	Fermentable carbohydrates
4. Mixed fermentation end products				
Thermoanerobacter ethanolicus	positive	69	7.0	Various hexoses including cellobiose, sucrose, starch, xylose and ribose (Wiegel and Ljungdahl, 1981)
Clostridium thermohydrosulfuricum	positive	68	7.0	Wide variety of sugars including glucose, pentoses, maltose and mannose (Wiegel et al., 1979)
Thermoanaerobacter finnii	positive	65	7.0	Various hexoses, pentoses, cellobiose and mannitol (Schmid et al., 1986)
Thermoanaerobium brockii	positive	69	7.0	Various hexoses, cellobiose, ribose, sucrose and starch (Zeikus et al., 1979)
Thermoanaerobium lactoethylicum	positive	65	7.0	Glucose, fructose, galactose, pentoses, starch, maltose, lactose and cellobiose (Kondratieva et al., 1989)
Thermobacteroides acetoethylicus	negative	65	7.0	Variety of saccharides including glucose, mannose, starch and sucrose (Ben-Bassat and Zeikus, 1981)
Fervidobacterium nodosum	negative	65	7.0	Range of carbohydrates including sucrose, starch and lactose (Patel et al., 1985a)
Clostridium thermosulfurogenes	positive	60	7.0	Variety of sugars including pectin, starch, pentoses, mannose and melibiose (Schink and Zeikus, 1983)
Clostridium thermocellum	positve	60	7.0	Cellulose, cellodextrins, cellobiose, and glucose after cultural adaption (Freier et al., 1988)
Thermotoga maritima	negative	80	6.5	Variety of sugars including glucose, ribose, xylose, galactose, sucrose and maltose (Huber et al., 1986)
Thermotoga neapolitana	negative	80	7.0	Able to use ribose, xylose, glucose, sucrose, lactose, galactose and starch (Jannasch et al., 1988)
Thermosipho africanus	negative	75	7.2	Variety of carbohydrates (Huber et al., 1989)
Dictyoglomus thermophilum	negative	70	7.0	Numerous carbohydrates including xylan (Sakai et al., 1985)
Dictyoglomus turgidus	negative	72	7.1	Wide variety of carbohydrates including glucose, lactose, maltose, xylose (Svetlichnii and Svetlichnaya, 1988)

2.1.2 Thermophilic saccharide fermentations

Saccharides comprise the major form of biomass on earth with cellulose being the most abundant natural polymer. Biotransformations of renewable saccharides to chemicals or other vendable products (i.e., fuels, enzymes, etc) are of great importance to the fermentation industry. This is because of the eventual depletion of petroleum and other fossil sources of carbon substrates which are currently used by the chemical industry, to generate several products which can also be produced by biomass fermentations (Zeikus, 1979).

The thermophilic anaerobic saccharolytic ethanologens are proposed as a means of producing industrial alcohol from cellulosic and hemi-cellulosic wastes (Zeikus, 1979; Wiegel, 1980; Payton, 1984; Slapack *et al.*, 1987; Lacis and Lawford, 1991). *Ta. ethanolicus* JW200 and *Cl. thermohydrosulfuricum* E100-69, for example, are capable of producing greater than 1.5 mol ethanol per mol glucose utilized and offer particular promise (Wiegel, 1992). They may possess several advantages over mesophilic bacteria and yeasts in the production of ethanol.

These advantages include:

(1) As a group carrying out fermentations at high temperatures confers advantages with respect to volatility of some end-products (easier recovery) increased solubility of some substrates (smaller feed volumes or increased reaction rates), and possibly less contamination risk and lower costs for pumping and stirring.

(2) Some species are cellulolytic, able to hydrolyze pretreated lignocellulose and furthermore hydrolyze other hemicellulose polymers including xylan, thus making single stage fermentation possible.

(3) The glycolytic anaerobes can ferment glucose and xylose and small oligomers of these monosaccharides, and furthermore some, e.g. *Cl. thermohydrosulfuricum* appear to utilize both substrates simultaneously.

(4) By choosing a particular fermentation combination of bacteria it might be possible to optimize the reaction for a particular end-product e.g. ethanol, acetate, biomass etc.

To date, however, this potential has not yet been realized.

2.2 CARBOHYDRATE UTILIZATION BY EXTREME THERMOPHILES

2.2.1 Pathways of hexose metabolism

There are several fermentative routes available to bacteria for the conversion of sugar substrates to the key metabolic intermediate of bacterial metabolism, pyruvate. These include:

(1) The Embden Meyerhof Parnas pathway
(2) The pentose phosphate pathway.
(3) The Entner Doudoroff pathway.
(4) Heterofermentative pathway.

The Embden Meyerhof Parnas pathway is the most widespread pathway for the fermentative conversion of glucose to pyruvate, and is operative in many groups of bacteria. All previously reported species of thermophilic saccharolytic anaerobes convert hexoses during growth to pyruvate via the Embden Meyerhof Parnas pathway (Winter and Zellner, 1990). The elucidation of this pathway has been based on $^{14}CO_2$ production during growth on differentially labelled glucose from position C_3 or C_4 alone and the detection of catabolic levels of key metabolic enzymes involved in glucose utilization. This glycolytic pathway has been reported in *Cl. thermosaccharolyticum* (Lee and Ordal, 1967), *T. brockii* (Lamed and Zeikus, 1980a), *Cl. thermohydrosulfuricum* strains 39E and Rt8.B1 (Zeikus *et al.,* 1981; Cook, 1988),

Tb. acetoethylicus (Ben-Bassat and Zeikus, 1981), *Ta. ethanolicus* (Ljungdahl *et al.*, 1981), *Cl. thermocellum* (Patni and Alexander, 1971a), *Acetomicrobium* species (Soutschek *et al.*, 1984; Winter *et al.*, 1987), *T. lactoethylicum* (Krasilnikova *et al.*, 1987; Kondratieva *et al.*, 1989) and *Spirochaeta thermophila* strains RI 19.B1 and Z-1203 (Janssen and Morgan, 1992). The enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of the hexose monophosphate shunt have also been reported in resting cells of *Cl. thermosaccharolyticum* (Hsu and Ordal, 1970). These two enzymes were preferentially formed in sporulating cells of this bacterium.

Other pathways reported in thermophiles include the Entner Doudoroff pathway which has been found mainly in thermophilic aerobes. For example, the moderately thermophilic archaea *Thermoplasma acidophilum* (Budgen and Danson, 1986), and the extremely thermophilic archaea *Sulfolobus brieleyi* (Wood *et al.*, 1987) and *Sulfolobus solfataricus* (De Rosa *et al.*, 1984) have been found to possess this pathway.

2.2.2 Pathways of pentose metabolism

In addition to their central biosynthetic roles, pentoses may also serve as electron donors in fermentation and respiration in the absence of hexoses (Cook, 1988). Before catabolism, pentoses are isomerized and phosphorylated to xylulose-5-phosphate which is the central intermediate of pentose metabolism.

In the fermentation of pentoses two pathways are possible:

(1) Direct breakdown of the xylulose-5-phosphate involving a substrate level phosphorylation step with the enzyme phosphoketolase. This pathway is common among various lactobacilli and streptococci which carry out a heterofermentative metabolism forming multiple end products

(2) In the absence of phosphoketolase the xylulose-5-phosphate is not broken down directly but is converted first to hexose phosphate via reactions of the pentose

phosphate pathway. The resultant hexoses are metabolized by a hexose pathway of metabolism.

Pentose utilization by extreme thermophiles has been investigated in *Cl. thermohydrosulfuricum* Rt8.B1 (Cook, 1988) and *Ta. ethanolicus* (Lacis and Lawford, 1988a,b). Xylose utilization in *Cl. thermohydrosulfuricum* Rt8.B1 is mediated by the pentose phosphate pathway via xylose isomerase and xylulokinase with subsequent production of xylulose-5-phosphate and ribose-5-phosphate. These two pentose phosphates are then converted into hexose phosphates by a series of rearrangements brought about by the enzymes transketolase and transaldolase (Cook, 1988). The resultant hexose phosphates are then metabolized via the Embden Meyerhof Parnas pathway.

2.2.3 Formation of end products

The major catabolic intermediates of hexose and pentose metabolism via the Embden Meyerhof Parnas pathway are intracellular pyruvate and reduced pyridine nucleotides (Zeikus and Ng, 1982). The pyruvate formed can be metabolized in two ways. It can either be reduced to form lactate via lactate dehydrogenase which allows the re-oxidation of reduced pyridine nucleotides, or it can be decarboxylated by the phosphoroclastic pathway forming acetyl-CoA, carbon dioxide and reduced ferredoxin. The acetyl-CoA formed can be either reduced to ethanol via acetaldehyde dehydrogenase and alcohol dehydrogenase or hydrolyzed to acetic acid via phosphotransacetylase and acetate kinase with concomitant adenosine-5'-triphosphate (ATP) formation. The production of acetate linked to ATP formation does not re-oxidise the NADH or reduced ferredoxin produced. This regeneration is channelled through the oxidoreductase and hydrogenase system producing hydrogen.

All described thermophilic anaerobes that form ethanol as a fermentation product employ a hetero-ethanol fermentation pathway (figure 2.1) and produce a combination of common fermentation end products (e.g., lactate, acetate, CO_2, H_2) in addition to ethanol. Despite the fact that all ethanol producers possess the same glycolytic

pathway, the ethanol yields of hetero-fermentations differ depending on the specific growth conditions used. This is related to the specific activities, regulatory properties and the direction of specific oxidoreductases that control carbon and electron flow during fermentation (Lamed and Zeikus 1980b), which in turn may be influenced by changes in culture conditions such as temperature, chemical composition and pH of the growth medium.

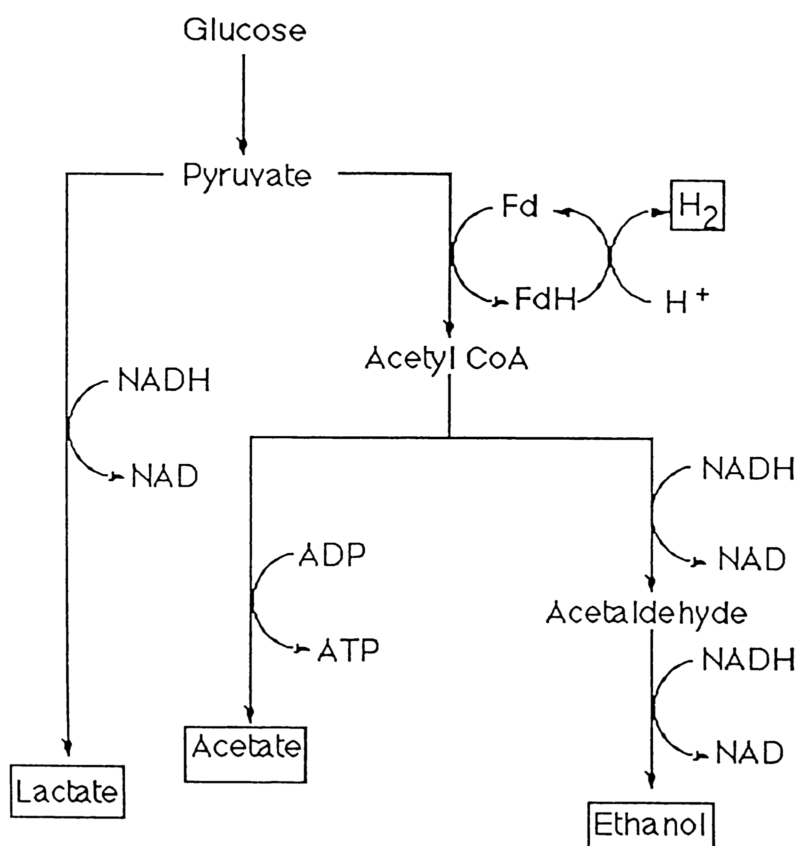


Figure 2.1 Thermophilic Hetero-Ethanol Fermentation

In some instances the effect of allosteric effectors has been reported to play a role in regulating end product formation e.g. fructose-1,6-diphosphate has been shown to be a positive allosteric effector of lactate dehydrogenase in *T. brockii* (Lamed and Zeikus, 1980a), *Ta. ethanolicus* (Carreira *et al.*, 1982), and *Cl. thermohydrosulfuricum* Rt8.B1 (Cook, 1988). But not all thermophilic bacteria behave similarly, thus Carreira *et al.* (1982) have shown that pyruvate was a positive allosteric effector of the alcohol dehydrogenase of *Ta. ethanolicus*, but Lamed and Zeikus (1980b) demonstrated that in

Cl. thermocellum it was inhibited by low concentrations of ethanol and NAD, whereas that from *T. brockii* was not.

2.3 DIAUXIC GROWTH

When bacteria are supplied with a growth medium containing a mixture of carbohydrates they often exhibit a sequential uptake. This phenomenon was first reported by Monod (1942) in *Bacillus subtilis* grown on glucose and arabinose. Glucose was utilized fully before metabolism of arabinose commenced. He termed this phenomenon "diauxic"growth or "polyauxic" growth depending on the number of sugars present in the growth medium (Monod, 1949).

In most cases, the substrate which permits the highest growth rate is used up first. Thus the more preferred and less preferred substrates are used sequentially and diauxic growth is generally characterized by a biphasic growth curve in which two exponential phases of growth are separated by a phase of no net increase in biomass.

2.3.1 Control of diauxic growth

Control of diauxic growth can be generally regarded as a consequence of those substrates which are utilized rapidly by constitutive enzymes causing catabolite repression of those pathways which are inducible. Thus the readily-utilizable substrates prevent the induction of the enzymes of other catabolic pathways. Diauxic growth can be explained on the basis of two related phenomena (1) catabolite inhibition and (2) inducer exclusion (Magasanik, 1961). Catabolite inhibition has been demonstrated in *Escherichia coli* during growth on lactose and glucose. The regulation of lactose utilization has been shown to involve catabolite repression of β-galactosidase synthesis by glucose. β-galactosidase is the enzyme responsible for the utilization of lactose. Glucose interferes with the synthesis of β-galactosidase in three ways (Magasanik, 1961). First, glucose acts to exclude the inducer, lactose, from the cells (termed inducer exclusion). Second, the enzyme is inducible and its synthesis is strongly repressed by glucose during balanced growth (termed catabolite repression),

i.e. when glucose remains in the medium β-galactosidase is not synthesized. Third, once the level of glucose decreases sufficiently then the catabolite inhibition is removed and after a short lag β-galactosidase is synthesized and growth on lactose can occur. Both of the repressive effects can be overcome by the addition of cyclic adenosine monophosphate (cAMP). The repressive effects have as their target the promoter site of the *lac* system and interfere with the initiation of transcription of the *lac* operon.

These general regulatory mechanisms serve to control the activity of early reactions in carbohydrate metabolism, allowing substrates to be preferentially or selectively utilized. They have been shown to be general regulatory mechanisms for controlling carbohydrate utilization in *E. coli* grown on combinations of glucose, lactose, galactose, maltose, mannose, xylose and arabinose (McGinnis and Paigen, 1969).

This control has evolved ecologically to solve the problem of choosing the most advantageous substrate when a mixture of carbon sources is available. This is of obvious advantage to an organism as it restricts the synthesis of enzymes to those that are necessary for their metabolism under current physiological conditions i.e. no need to synthesize an enzyme until it is needed. These general regulatory mechanisms have been well studied in the regulation of glucose and lactose in *E. coli.*

2.3.2 Diauxic growth in mesophilic organisms

Diauxic growth has been reported to occur in a wide variety of bacteria including photosynthetic bacteria (Solaiman and Uffen, 1982), halophilic lactic acid bacteria (Abe and Uchida, 1989), *Bacteroides* species (Janssen, 1991), rumen bacteria (Russell and Baldwin, 1978; Martin and Russell, 1986), free-living and symbiotic nitrogen-fixing bacteria (Ucker and Signer, 1978; George *et al.*, 1985; Tauchert *et al.*, 1990), spore-forming bacteria (Ounine *et al.*, 1985), *Pseudomonas* species (Lynch and Franklin, 1978; Dijkhuizen *et al.*, 1980), propionic acid bacteria (Lee *et al.*, 1974), yeasts (Bajpai and Ghose, 1978; Hsiao *et al.*, 1982; Emerich and Radler, 1983) and on

an equally diverse range of substrates which include carbohydrates, intermediates of glycolysis, sugar alcohols, organic acids and amino acids.

2.3.3 Diauxic growth of thermophiles

Although diauxic growth occurs almost universally in mesophilic bacteria and yeasts, it has been a little studied phenomenon in thermophilic bacteria and very few examples have been reported. Its occurrence has mainly been restricted to the moderate thermophiles i.e. those growing optimally around 50-60°C. Diauxic growth has been reported in the cellulolytic bacterium *Thermomonospora curvata* (Bernier and Stuzenberger, 1987) and with *Cl. thermoaceticum*, (Andreesen *et al.,* 1973) and *Cl. thermosaccharolyticum* (Aduse-Opoku and Mitchell, 1988).

The distribution of diauxic growth among extreme thermophiles i.e. those bacteria capable of growth at temperatures in excess of 70°C with an optimum above 65°C, has been restricted to cellulolytic bacteria and saccharolytic bacteria grown on substrate combinations involving glucose and cellobiose. Diauxie has been demonstrated in *Cl. thermocellum* LQRI (Ng and Zeikus, 1982), *Cl. thermohydrosulfuricum* Rt8.B1 and *T. brockii* Rt8G4.1 (Cook, 1988).

2.4 HYPERBOLIC GROWTH KINETICS

There is now a growing body of literature suggesting that some micro-organisms can utilize mixed substrates simultaneously (i.e. exhibit hyperbolic growth kinetics). Hyperbolic growth has been reported in some yeasts (Orlowski and Barford, 1987; Heredia and Ratledge, 1988; Johnston and Barford, 1991), thermophilic fungi (Maheshwari and Balasubramanyam, 1988) and mesophilic bacteria (Anthony and Guest, 1968; Mitchell *et al.,* 1987; Russell and Baldwin, 1978).

In thermophiles, hyperbolic growth has been best exemplified by the extreme thermophiles. Hyperbolic growth was first reported in *Ta. ethanolicus* mutant strain JW200 grown on xylose in combination with glucose, cellobiose or soluble corn starch

(Carreira *et al.*, 1983). Since this first report, hyperbolic growth kinetics have been reported in the cellulolytic strain "Caldocellum saccharolyticum" Tp8T grown on glucose and cellobiose (Cook, 1988), *Cl. thermohydrosulfuricum* Rt8.B1 (Cook, 1988; Patel *et al.*, 1988), *T. brockii* Rt8G4.1 (Cook, 1988) on xylose in combination with glucose or cellobiose. Hyperbolic kinetics have also been demonstrated in *Cl. thermosulfurogenes* (Hudson *et al.*, 1988) on glucose and xylose. A thermophilic aerobe, *Thermus* Rt41A has been shown to exhibit hyperbolic growth on L-glutamate and acetate (Janssen pers. comm.). A summary of these results is presented in Table 2.2.

Table 2.2 Growth of Thermophilic Anaerobes on Mixed Substrates

Organism	Glucose and xylose	Xylose and cellobiose	Glucose and cellobiose
Cl. thermocellum	ND	ND	Diauxic
Cl. thermohydrosulfuricum	Hyperbolic	Hyperbolic	Diauxic
Cl. thermosaccharolyticum	Diauxic	ND	ND
Cl. thermosulfurogenes	Hyperbolic	ND	ND
T. brockii	Hyperbolic	Hyperbolic	Diauxic
Ta. ethanolicus	Hyperbolic	Hyperbolic	ND
"Ca. saccharolyticum"	Hyperbolic	Hyperbolic	Hyperbolic

ND = not determined

An organism which can co-utilize two substrates has potential advantages over those organisms which prefer a sequential uptake of substrates (i.e grow diauxically). In a nutritionally poor environment there would be an obvious advantage for a bacterium to utilize the available carbon sources rapidly and simultaneously. Therefore, the ability of an organism to co-utilize two substrates might be an adaptive strategy for opportunistic growth in nature under conditions of low nutrient availability.

2.5 SUGAR TRANSPORT SYSTEMS

2.5.1 Introduction

In natural environments, energy sources are often limiting for bacterial growth and the acquisition of a nutrient uptake system is a key factor in an organism's ability to survive. As environments fluctuate in available nutrients, an organism must be able to adapt quickly and regulate its metabolic capabilities in accordance with such external changes. Nutrient acquisition and subsequent metabolism to generate biomass and energy are tightly integrated processes in bacteria, and transport systems are central for modulating these activities. The importance of transport systems as sites of metabolic regulation is evident from the extensive work done on these systems in bacteria (Saier, 1985).

2.5.2 Models of transport

To date much research has been directed toward determining molecular mechanisms by which hydrophilic compounds are transported through the cytoplasmic membrane (hydrophobic cell barrier) into the cell (Dills *et al.*, 1980). The transport systems studied in greatest detail are those of *E. coli, Salmonella typhimurium* and *B. subtilis*.

Models for transport systems normally involve a transport protein which undergoes a conformational change, so that the substrate binding site is alternately presented at the exo- and endo-facial sides of the membrane (Walmsley, 1988)

From biochemical and genetic approaches it appears that while some carrier proteins function as monomers, others operate as dimers. Despite this variation, all examples can be modelled as having a pair of membrane-embedded domains, each of which contains an array of (about) six transmembrane α-helical elements. The substrate diffusion pathway is likely defined by the space between these helical bundles

(Maloney, 1990a). The structure and evolution of permease diversity has been reviewed recently (Maloney, 1990a; Saier, 1990).

Bacteria have evolved a variety of mechanisms by which solutes are transported into and out of living cells. Certain solutes may be either in equilibrium across a biological membrane or concentrated on one side. There are five substrate transport mechanisms now known to occur in microorganisms (Dills *et al.,* 1980).

1. Passive diffusion
2. Facilitated diffusion
3. Active transport
4. Osmotic shock-sensitive active transport
5. Group translocation

The systems can be classified conveniently according to the source of energy coupled to the transport process. It should be mentioned that the same solute may be transported by different mechanisms in different genera and in a single species the same solute is often transported by several distinct transport processes, often depending on the external concentration of the solute.

The different mechanisms for sugar transport are discussed in detail in the following sections:- passive diffusion (2.6), facilitated diffusion (2.7), active transport (2.8), osmotic-shock sensitive systems (2.9) and group translocation (2.10).

2.6 PASSIVE DIFFUSION

Substrates transported by passive diffusion do not specifically interact with protein components of the cell membrane, but cross the lipid phase of the membrane until an equilibrium is reached between the concentration inside and that outside. In general passive diffusion involves hydrophobic compounds which are soluble in the lipid bilayer of cell membranes.

For passive diffusion the first enzyme of the metabolic pathway generally controls the rate of transport and determines the affinity of uptake (Russell *et al.,* 1990). This is the case for ammonia assimilation in the bacterium *Aerobacter aerogenes.* The glutamate dehydrogenase (GDH) responsible for the synthesis of amino acids from ammonia and intermediary metabolites has a high K_m for ammonia (5 mM) (Tempest *et al.,* 1970). Under conditions of ammonia limitation, the level of this enzyme decreases markedly and glutamate formation proceeds by another route involving the amidation of glutamate to glutamine and the reductive transfer of the glutamine amide nitrogen to 2-oxoglutarate to form a second glutamine molecule. This pathway has been shown to involve the participation of a second enzyme, glutamine synthetase, which has a K_m of < 1 mM for ammonia (high-affinity) and requires ATP for the formation of glutamine. The synthesis of this enzyme has been shown to be under the control of ammonia concentration (Meers and Tempest, 1970). Glutamate synthase which had a lower K_m (high-affinity) works in combination with glutamate synthetase at low ammonia concentrations. It now seems that some bacteria, notably marine bacteria, possess active transport systems for the ammonium ion NH_4 (Kleiner, 1985). Russell and Strobel (1987) have demonstrated a concentration gradient of ammonia across the cell membranes of mixed ruminal bacteria and this indicated that some ruminal bacteria may have active transport mechanisms for ammonia.

2.7. FACILITATED DIFFUSION

Facilitated diffusion is similar to passive diffusion in that neither requires metabolic energy i.e. they are energy independent processes and both aid in the rapid equilibration of a substrate across the bacterial membrane along preexisting gradients. Both are freely reversible such that equal concentrations of the substrate transported are found inside and outside the cell. However, unlike passive diffusion, facilitated diffusion is mediated by an integral membrane protein or permease, which exhibits stereospecificity towards its substrates and thus displays competition between the solute and structural analogues. Carrier-mediated transport is generally responsible for transport of solutes which are too lipophobic or large to pass freely across cell membranes. Because the translocation step is not coupled to metabolic energy, the

solute cannot be accumulated within the cell against a concentration gradient. However, rates of uptake are much more rapid than occurs for passive diffusion. Due to the involvement of a carrier protein, facilitated diffusion can exhibit saturation kinetics so resembling an enzyme reaction and thus the affinity of the transporter for its substrate can be measured (Russell *et al.*, 1990). The driving force for continued uptake is the transformation of the solute inside the cell, thus maintaining a concentration gradient across the membrane and allowing further solute transport.

2.7.1 Distribution of facilitated diffusion systems

Facilitated diffusion is an important transport mechanism in yeasts whereas in bacteria there have been few reports of its occurrence. It is generally agreed that yeasts employ simple facilitated diffusion for glucose, galactose and maltose in energy depleted cells or in cells lacking a functional kinase activity (Bisson and Fraenkel, 1983, 1984; McClellan and Bisson, 1988; Ramos *et al.*, 1989; Beullens and Thevelein, 1990; Cheng and Michels, 1991).

The properties of transport systems in fungi have been reported in just a few cases. Facilitated diffusion has been reported for glucose uptake in *Neurospora crassa* (Scarborough, 1970) and *Fusarium oxysporum* (Brandáo and Loureido-Dias, 1990).

There have been few reports of facilitated diffusions systems operating for the transport of carbohydrates in bacteria. The best characterized facilitated system is that for glycerol. Bacterial cell membranes are intrinsically permeable to glycerol as a consequence of its small size and neutral charge, and energy-dependent uptake would be wasteful due to rapid passive efflux. This intrinsic permeability of the membrane may have prevented the evolution of an active transport system for glycerol.

The uptake and dissimilation of glycerol in *E. coli* has been well characterized by Lin (1976). In wild-type *E. coli*, glycerol enters the cytoplasm via an inducible non-specific proteinaceous pore known as a glycerol facilitator (Sanno *et al.*, 1968; Richey and Lin, 1972; Heller *et al.*, 1980) and is then phosphorylated at the expense of ATP in

the rate-limiting glycerokinase catalyzed reaction. The facilitator thus serves to supply saturating amounts of glycerol to glycerokinase. Glycerokinase activity normally limits the growth rate when glycerol is supplied in abundance (Zwaig *et al.,* 1970). The specificity of the glycerol facilitator allowed the entry of other polyols, glycerol and erythritol, pentitols and hexitols. Urea and glycine could also use this pathway to enter the cell (Heller *et al.,* 1980).

Evidence for this mechanism was obtained using mutants of *E. coli*; those lacking glycerokinase were not capable of accumulating glycerol. The K_m of glycerokinase for its substrate and the K_m of intact cells for the uptake of glycerol were similar, suggesting that any accumulation of glycerol was due to the trapping of glycerol by the action of glycerokinase (phosphorylation) (Hayashi and Lin, 1965).

Facilitator diffusion systems for glycerol appear to be widespread. In addition to the systems in *E.coli* and *S. typhimurium*, glycerol facilitators occur in *Pseudomonas aeruginosa* (Tsay *et al.,* 1971), *Klebsiella aerogenes* (Ruch *et al.,* 1974), *Shigella flexneri* (Richey and Lin, 1972), *S.faecalis* (Lin, 1976), *B. subtilis* (Saheb, 1972) and *Nocardia asteroides* (Calmes and Deal, 1972).

Other reports of facilitated diffusion systems in bacteria have been less frequent. Button *et al.* (1973) reported that uptake of D-maltose by *Staphylococcus aureus* was not coupled to phosphoenolpyruvate-dependent phosphorylation (group translocation) and concluded that uptake occurred by a diffusion process with hydrolysis of the non-phosphorylated molecule to form glucose. The rumen bacterium *Streptococcus bovis* JB1 has been reported to have a constitutive glucose PTS for glucose (Martin and Russell, 1987), but this mechanism of transport could not account for glucose consumption rates of rapidly growing cultures. A PTS-deficient mutant displayed no high-affinity, low-capacity glucose uptake system, but retained its ability to take up glucose at high substrate concentrations (low-affinity, high-capacity system). Low-affinity transport was directly proportional to the external substrate concentration and exhibited counterflow kinetics. From this it was concluded that a facilitated

mechanism was responsible for glucose transport at high substrate concentrations (Russell, 1990).

Similar findings have been reported for the transport of D-glucose in *Zymomonas mobilis*. Glucose transport does not involve a PEP-PTS and active transport of the non-metabolizable analogue 2-deoxy-glucose could not be demonstrated (Romano *et al.*, 1979). Glucose uptake in this organism has been shown to involve a constitutive, stereospecific, carrier-mediated facilitated diffusion system (DiMarco and Romano, 1985).

Transport of glutamate and glutamine by strain F, an amino acid fermenting ruminal bacterium was found to involve facilitated diffusion at high concentrations of these two amino acids. Ammonia production by washed cells was shown to be directly proportional to the glutamate or glutamine concentration (Chen and Russell, 1990). Transport of glutamine at high concentrations has been shown to be via facilitated diffusion in *S. bovis* JB1 (Chen and Russell, 1989a).

In most cases, facilitated transport systems for solute transport can be explained on the basis of the natural occurrence of the organism. For example, *Z. mobilis* is found in warm, tropical climates in fermentations of plant saps with high sugar, and the acquisition of this low-affinity, high-capacity facilitated transport system allows for the rapid conversion of glucose to metabolic products with low energetic expenditure.

2.8 ACTIVE TRANSPORT

Active transport is an energy-dependent process coupled to chemical or electrical energy. Active transport allows substrate saturation of the cell's metabolic enzymes at much lower external substrate concentrations than do processes dependent on diffusion. A several-fold to 100-fold concentration of compounds (lactose and β-galactosides) has been observed in *E. coli* (Winkler and Wilson, 1966). The acquisition of an active transport system thus enables cells to inhabit environments containing substrates in low concentrations, a situation common in nature.

Active transport can be classified either as primary active transport or as secondary active transport. Primary active transport involves the direct utilization of chemical energy to drive solute transport. In contrast, secondary active transport is coupled to an electrochemical gradient of ions (H^+, Na^+ etc) which are in turn generated by primary pumps via proton extrusion (the conservation of light, electrical or chemical energy into electroosmotic energy). The coupling of this energy source to the influx of a compound occurs so that an endergonic process (the accumulation of a nutrient in the cell against a concentration gradient) can be coupled to an exergonic process (protons moving down a concentration gradient).

2.8.1 Chemiosmotic hypothesis

Involvement of the membrane potential in energizing active transport is universally accepted. The prevailing theory of ATP production and the establishment of a proton motive force is that proposed by Mitchell (1961). The basic postulate of Mitchell's concept of energy conservation is that hydrogen and electron carriers of the respiratory chain are arranged in loops across the cytoplasmic membrane in such a way that oxidation of substrate brings about the transport of protons outward across the membrane resulting in the generation of an electrochemical gradient. The hydrolysis of ATP in the membrane-bound F_1F_0-ATPase is also able to translocate protons outward, similarly generating an H^+ gradient. Thus oxidation of substrate via the respiratory chain and ATP hydrolysis can both result in the same form of conserved energy, i.e. an electrochemical potential, designated the proton motive force ($\Delta\mu H^+$) (Mitchell, 1963). In addition, gradient formation can also be mediated by ion pumps linked to metabolic reactions or to product excretion (Michels *et al.*, 1979; Gätje *et al.*, 1991). The gradient thus established comprises a proton gradient termed ΔpH (chemical potential) and a electrical potential ($\Delta\psi$). The gradient arises due to the asymmetric arrangement of the electron transport system carrier molecules across the membrane and the impermeability of the membrane to H^+ and OH^-.

The proton gradient is a potential source of energy which can be used to do work. Energy can be recovered by allowing the protons to flow back across the membrane in a controlled manner to complete the coupling. Movement of molecules or ions can proceed spontaneously only in a direction which tends to reduce a concentration gradient or electrical gradient i.e. from a high concentration to a low concentration. In the case of respiring bacteria, the work may be the generation of ATP, the transport of solutes and cell motility. In the case of obligate fermenters, ATP is generated via substrate level phosphorylation only, but an H^+ gradient is still required for transport and motility. This gradient is established by the energy released following hydrolysis of ATP in the same type of F_1F_0-ATPase complex found in respiring bacteria; in one case an inflow of H^+ generates ATP from ADP + P_i; in the other the hydrolysis of ATP to ADP + P_i results in extrusion of protons (Hinkle and McCarty, 1978).

2.8.2 Uniports, symports and antiports

In most systems the $\Delta\mu H^+$ is taken advantage of by carriers having binding sites for protons and a particular substrate, and both (protons and substrate molecules) are transported into the cell. The $\Delta\mu H^+$ drives transport by several different mechanisms depending on the nature of the substrates (Kaback, 1986). The following terms are used to define the mechanisms by which metabolites are linked to ion transport: symport, antiport and uniport and are discussed in relevance to the systems present in bacteria.

Uniport are processes whereby a solute moves across a membrane but is not tightly coupled to the movement of another species (Ambudkar and Rosen, 1990). For example, transport of cationic substrates such as lysine or K^+ occurs by a uniport, a mechanism dependent specifically on $\Delta\psi$ (interior negative and alkaline) (Kaback, 1990).

Symport are processes involving two or more substrates translocated obligately, by the same carrier, and in the same direction (Ambudkar and Rosen, 1990). For Substrates

such as sugars and neutral amino the carrier is presumed to bind substrate and H^+ independently and couples the energy released from the downhill translocation of H^+ in response to $\Delta\mu H^+$ (interior negative and alkaline) to uphill transport of substrate against the concentration gradient (Kaback, 1990).

The most extensively studied active transport system is the β-galactoside or *lac* permease of *E. coli* originally described by Cohen and Rickenberg in 1955 (Cohen and Monod, 1957). Mitchell (1963) postulated that lactose accumulation was coupled to chemical energy via a proton symport on the basis of experiments with the non-metabolizable sugar analogue thiomethyl-β-D-galactopyranoside (TMG) transported by the lactose permease (West, 1970; West and Mitchell, 1973). The studies of West (1970), later confirmed by West and Mitchell (1973), demonstrated that the initial rate of effective inflow of H^+ is equal to the initial rate of lactose inflow and that the two flows are stoichiometrically coupled to the symporter with a ratio of 1:1 (West and Mitchell, 1973). The substrates D-galactose, L-arabinose, and D-xylose, D-glucose or sucrose are similarly transported in *E. coli* (Henderson, 1974; Heller and Wilson, 1979; Lam *et al.*, 1980).

Antiports can de defined as countertransports or exchanges where the two substrates (the driving and the driven species) are translocated in opposite directions (Ambudkar and Rosen, 1990). Solutes such as Na^+ or Ca^{2+} which are pumped out of the cell are transported by antiport systems (Kaback, 1990). A transmembrane antiporter couples the downhill translocation of H^+ to the efflux of substrate against a concentration gradient. Novel antiporters have also been reported to play a role in energy conservation in lactic acid bacteria (Maloney, 1990b).

Mutant analyses and sequence comparisons as well as functional considerations, suggest that uniporters, antiporters, and symporters function by essentially the same type of carrier mechanism with respect to the translocation step, and that they may differ from each other only with respect to the number and nature of species translocated (Botfield *et al.*, 1990; Maloney, 1990a ;Mitchell, 1990; Saier, 1990).

2.8.3 Sodium-coupled active transport

An increasing body of evidence has accumulated indicating bacteria use Na^+-ions in addition to or instead of protons to couple exergonic reactions with endergonic reactions in the membrane. The first substrate shown to be transported by a Na^+ symport (cotransport) was melibiose. This was first reported in the enteric bacterium *Salmonella typhimurium* (Stock and Roseman, 1971).

Analogous to H^+ cotransport systems, Na^+ cotransport systems use the energy of the Na^+ electrochemical gradient to drive substrate accumulation. Bacteria have been found to generate a $\Delta\mu Na^+$ in a number of ways (Maloy, 1990). These include the generation of Na^+ electrochemical potential by using the $\Delta\mu H^+$ generated by bacterial respiration or ATPase to generate the Na^+ gradient by Na^+/H^+-antiport. A Na^+ gradient can also be established in bacteria by a decarboxylase or a NADH-oxidase Na^+ pump (Dimroth, 1980, 1982; Dimroth and Thomer, 1989). These Na^+-ion gradients are taken advantage of to drive solute transport, flagellar motion or ATP synthesis (Dimroth, 1990).

Sodium symporters have been reported in alkalophilic bacteria (Krulwich *et al.*, 1982), marine bacteria (Droniuk *et al.*, 1987), *E. coli* (Tsuchiya *et al.*, 1977), *S. typhimurium* (Cairney *et al.*, 1984), rumen bacteria (Russell *et al.*, 1988; Chen and Russell, 1989b; Chen and Russell, 1990), thermophilic bacteria (De Vrij *et al.*, 1989, 1990; Speelmans *et al.*, 1989; Heyne *et al.*, 1991) and halophilic bacteria (Severina *et al.*, 1991a). These systems are common to bacteria which inhabit environments rich in Na^+ and require Na^+ ions for growth. Symport of both Na^+ and H^+ ions with the same solute has been observed for the L-glutamate transport system of *E. coli* (Fujimura *et al.*, 1983) and *Bacillus stearothermophilus* (De Vrij *et al.*, 1989; Heyne *et al.*, 1991).

2.9 OSMOTIC SHOCK-SENSITIVE ACTIVE TRANSPORT SYSTEMS

There is a second active transport system present in *E. coli* and other microorganisms (particularly in Gram-negative bacteria). It is involved in the transport of amino acids, peptides, some sugars, organic acids, and inorganic cations and anions (Gottschalk, 1986). This second class includes more complex systems which are sensitive to the mild cold osmotic shock procedure of Neu and Heppel (1965) and hence are termed shock-sensitive permeases. Loss of uptake activity is the result of the loss of substrate binding proteins from the periplasmic space (periplasmic component) (Ames. 1986; Higgins, 1990).

2.9.1 Structure

Many binding-protein-dependent transport systems, and their equivalents from eucaryotic cells have now been characterized and all share a similar basic organisation (Ames, 1986).

These systems have a complex organization and require three or more protein components to operate (Figure 2.2). Periplasmic permeases are composed of a membrane-bound complex, usually comprising between two and four membrane-bound proteins of which two are hydrophobic and embedded in the cytoplasmic membrane (Q and M), whereas the third has a hydrophilic sequence and is thought to be bound to the inner surface of the cytoplasmic membrane (P). The fourth component is a soluble periplasmic protein (periplasmic component) (J). In all cases studied, this periplasmic component is a protein consisting of a substrate-specific binding site that binds the transported solute with high-affinity and an ATP-requiring transport unit. Substrate molecules are bound by binding proteins in the periplasmic space and then interact with membrane-embedded components of the transport system, and at the expense of ATP or another high-energy phosphate compound the substrate is actively taken up (Ames and Higgins, 1983). In contrast, to the *lac* permease active transport

system, the binding protein dependent systems exhibit asymmetry i.e. entry and exit are mediated by different systems (e.g., maltose) (Ferenci *et al.*, 1977).

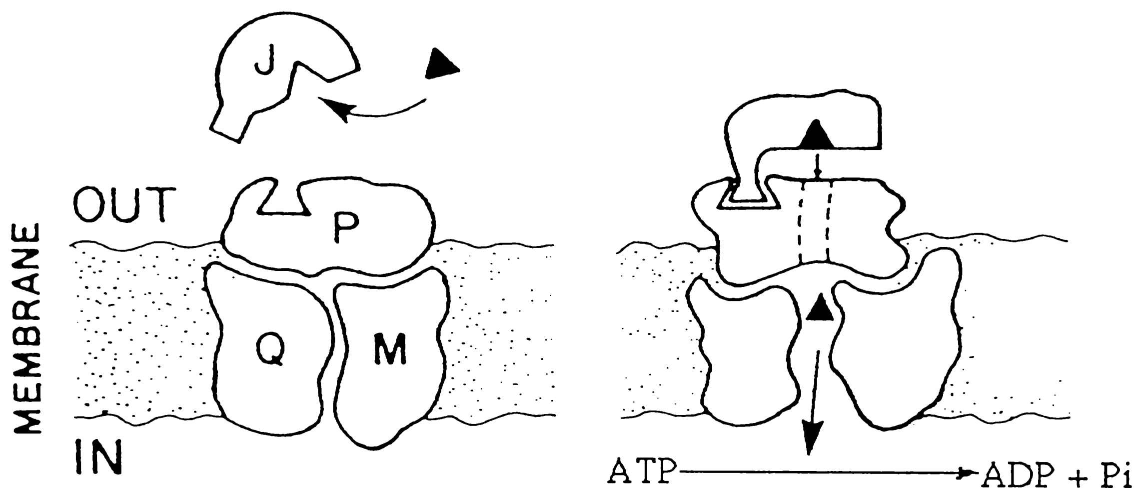


Fig. 2.2 Binding protein-mediated transport. P, Q, and M are the membrane-bound components of the transport system, J is the substrate-binding protein (periplasmic) and ▲ the substrate molecule (modified from Ames and Higgins, 1983).

This system typically transports with high affinity, accumulating solute against large concentration gradients. Examples include the maltose uptake (Kellerman and Szmelcman, 1974; Ferenci *et al.*, 1977; Saurin *et al.*, 1989), histidine (Ames and Lever, 1972), or oligopeptide permeases (Sussman and Gilverg, 1971; Ames *et al.*, 1973) of enteric bacteria.

2.9.2 Energy coupling of periplasmic permeases

There has been much controversy surrounding the energetics of the periplasmic binding-protein-dependent transport systems (Dean *et al.*, 1990). Shock-sensitive systems are considered to be primary active transport systems because chemical energy is directly coupled to the uptake process. It was initially postulated these systems were linked to substrate level phosphorylation via ATP (Berger and Heppel, 1974) and similar energy coupling characteristics were reported by Curtis (1974). Recent advances in membrane technology, for example, the measurement of maltose uptake in both membrane vesicles (Dean *et al.*, 1989) and proteoliposomes (Davidson and Nikaido, 1990) has provided convincing evidence that it is the hydrolysis of ATP alone

that drives transport. This view has been expressed by Joshi *et al.* (1989), Higgins (1990) who state that direct hydrolysis of ATP by components of the transport systems themselves provide the mechanism by which energy is coupled to the accumulation of substrate in those multicomponent binding-protein-dependent permeases such as those specific for histidine, maltose and oligopeptides (driven by ATP hydrolysis) (Joshi *et al.*, 1989; Ames, 1990; Dean *et al.*, 1990; Higgins, 1990). Neither $\Delta\psi$ or ΔpH are involved in energizing periplasmic transport (Joshi *et al.*, 1989).

2.10 ENZYME-MEDIATED GROUP TRANSFER REACTIONS

2.10.1 Historical

All carrier-mediated active transport systems or facilitated diffusion systems have one property in common: the solute is transported through the membrane in an unchanged form. Group translocation processes are unique in that the solute is altered chemically during transport and appears inside the cell in a chemically modified form, usually as a phosphate ester. The best-characterized group translocation systems are the phosphoenolpyruvate-dependent sugar phosphotransferases (PEP-PTS), which were first discovered by Kundig *et al.* (1964) in cell-extracts of *E. coli* (Kundig *et al.*, 1964). PTSs have been the subject of recent reviews (Postma and Lengeler, 1985; Saier, 1989a; Postma *et al.*, 1989; Meadows *et al.*, 1990; Saier and Chin, 1990).

It was originally thought that the PTS was simply a "trapping system"; sugars were transported across the membrane by a translocator and were trapped inside as their phospho-derivatives by the PTS (Roseman, 1989). The issue was resolved by Simoni *et al.* (1967) who demonstrated that a single mutation in *S. typhimurium* resulting in the loss of enzyme I caused a defect in the ability to transport sugars into the cell. From these results and other studies (Ghosh and Ghosh, 1968) they concluded that the PTS was intimately involved in sugar transport.

Since its discovery the PTS has become the focus of intense study. The system is of interest as it is essential for the growth of many pathogens, and is of importance in the

bacteria that carry out economically important processes such as milk, cheese, soy sauce and rumen fermentations. It is also of importance in oral microbiology since oral streptococci transport sugars via the PTS. *Streptococcus mutans* has been implicated as the principle agent of dental caries and has been shown to operate a PTS for the uptake of various carbohydrates (Calmes, 1978; Schachtele and Mayo, 1973; Slee and Tanzer, 1979; St. Martin and Wittenberger, 1979). The PTS is also widely distributed in the most common marine bacteria, the vibrios. This is of great importance for the marine ecosystem as these bacteria play a key role in marine carbon and nitrogen cycles (Gee *et al.*, 1975; Meadow *et al.*, 1987).

2.10.2 Structure

The PTS is a complex enzyme system which is responsible for the detection, transmembrane transport and phosphorylation of its numerous sugar substrates in Gram-positive and Gram-negative bacteria. The uptake and phosphorylation of all PTS sugars is dependent on the constitutive activity and participation of a number of soluble and membrane-bound enzymes. It is composed of two general PTS soluble cytoplasmic proteins, enzyme I (EI) and a heat-stable protein (HPr), and two carbohydrate-specific proteins, the cytoplasmic enzyme III (EIII) and the membrane-bound enzyme II (EII). Their relationship to the cytoplasmic membrane is shown schematically in figure 2.3 (Postma and Lengeler, 1985).

These proteins catalyse the transfer of the phosphoryl moiety of phosphoenolpyruvate (PEP) to the sugar substrate. Transfer of the phosphoryl group from PEP to the sugar can be summarized as follows (see figure 2.3):

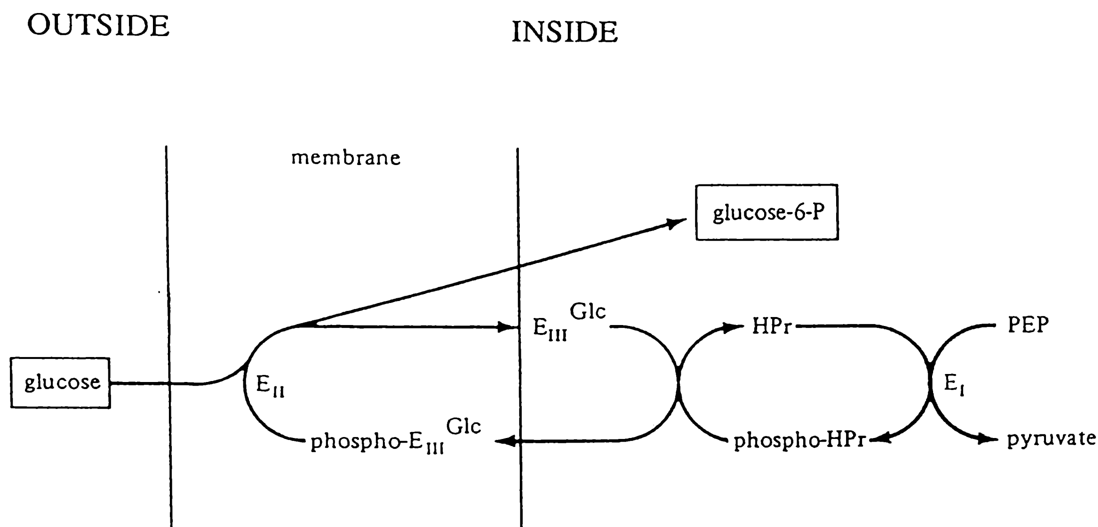


Fig. 2.3 Components of the phosphoenolpyruvate:glucose phosphotransferase system in bacteria (from Gottschalk, 1986).

The phosphate donor is not directly transferred from PEP to sugar, but is sequentially transferred down the phosphate transfer chain of the PTS first from PEP to EI and then to HPr with the concomitant formation of pyruvate. The next step is a sequence of phosphoryl transfer reactions involving transfer of the phosphoryl moiety of phospho-HPr, to a sugar-specific component, an EIII. EIII serves as a phosphocarrier to the membrane-bound EII, but in many cases HPr-P transfers its phosphoryl group directly to EII without the involvement of EIII. The last step in the sequence of reactions results in sugar phosphorylation. The phosphoryl moiety of phospho-enzyme III is transferred to sugar in a process that requires the participations of the membrane-associated EII complex (figure 2.3). The reaction is accompanied by transmembrane sugar translocation. The transfer from the permease to the sugar substrate is catalyzed by the permeases themselves. The overall chemical reaction catalyzed by the PTS requires Mg^{2+} and the products of this reaction are sugar phosphate and pyruvate.

The consequence of transport by this mechanism is that the sugar is trapped within the membrane by reaction with a phosphorylated enzyme (EIII) and that the phosphate

ester formed is released into the cytoplasm. The phosphorylated enzyme is generated using PEP as the principle source of phosphate-bound energy. Thus an ATP equivalent (as PEP) is used as the energy source driving the otherwise endergonic accumulation of the sugar against a concentration gradient.

2.10.3 Distribution of phosphotransferase systems in bacteria

The PTS has been found widely distributed in procaryotes (Romano *et al.*, 1970; 1979; Saier, 1977; Dills *et al.*, 1980; Meadows *et al.*, 1990), but has not yet been found in eucaryotes (Postma and Lengeler, 1985). Table 2.3 lists the bacterial genera in which PTSs have been found to date.

In general, the glucose PTS has been shown in most bacteria tested which ferment glucose via the Embden Meyerhof Parnas pathway (Romano *et al.*, 1970, 1979; Dills *et al.*, 1980), whereas those that normally metabolize sugars aerobically via the Entner Doudoroff pathway generally accumulate sugars by an active transport mechanism. This observation can be explained in terms of energetic requirements of aerobic and anaerobic bacteria. A principle end-product of anaerobic glycolysis is PEP; 2 mol of PEP are generated per mol of sugar metabolized via this pathway. Consequently, if the sugar is metabolized via glycolysis, energy for uptake of additional sugar molecules by the PTS will always be available i.e. providing a self-fueling system for sugar transport and supplies energy as PEP (1 mol of PEP /mol glucose is consumed by the PTS the other is available for ATP formation via pyruvate kinase). In contrast, the principle energy source generated by alternative sugar metabolic pathways are ATP and a proton electrochemical gradient. Since these two forms of energy are available to the aerobically grown cell, they serve to energize active transport systems and therefore accumulate carbohydrates by active transport mechanisms.

Table 2.3. Distribution of the PTS in procaryotes

Genera	Gram stain Reaction	Subtrates
Beneckea	-	General
Escherichia	-	General
Klebsiella	-	General
Photobacterium	-	General
Salmonella	-	General
Serratia	-	General
Staphylococcus	+	General
Streptococcus	+	General
Bacillus	+	General
Clostridium	+	Fructose, mannitol
Lactobacillus	+	General
Arthrobacter	+	Fructose, rhamnose
Mycoplasma		General
Ancalomicrobium	-	General
Rhodopseudomonas	-	Fructose
Rhodospirillum	-	Fructose
Thiocapsa	-	Fructose
Thiocystis	-	Fructose
Alcaligenes	-	Fructose
Spirochaeta	-	Mannitol
Pseudomonas	-	Fructose
Bacteroides	-	Tartrate

(modified from Dills *et al.*, 1980).

There are several exceptions to this facultatively anaerobic pattern. PEP-glucose PTS activity has been found in all those homofermentative lactic acid bacteria tested which ferment glucose via the Embden Meyerhof Parnas pathway (Hickey *et al.*, 1986), but in none of a group of heterofermentative species of *Lactobacillus* or *Leuconostoc*, which ferment glucose via the phosphoketolase pathway (Romano *et al.*, 1979).

Similarily, the anaerobic bacterium *Zymomonas mobilis*, which employs the Entner-Doudoroff pathway for hexose catabolism, does not possess the PTS (Romano *et al.*, 1979).

Among bacteria lacking PTS one finds an equally diverse group of Gram-positive and Gram-negative organisms. Generally strict aerobes, such as *Micrococcus*, *Mycobacterium*, and *Azotobacter vinelandii* (Romano *et al.*, 1970), *Nocardia* (Saier, 1977), *Thiobacillus* (Wood and Kelly, 1982), *Rhizobium* species (Bigwaneza *et al.* 1990; De Vries *et al.*, 1982) and *Bradyrhizobium* species (SanFrancisco and Jacobson, 1986) do not possess PTSs. There are some exceptions; PEP-dependent glucose PTS has been found in a number of species of *Bacillus* (Romano *et al.*, 1979) e.g. *Bacillus subtilis* (Delobbe *et al.*, 1976) and several *Pseudomonas* species (Sawyer *et al.*, 1977).

In general, pentoses are not transported by the PTS, but novel pentitol-specific PTSs have been reported in the homofermentative organism *Lactobacillus casei* (London and Chace, 1977; London and Chace, 1979; London and Hausman, 1982). These bacteria transport xylitol, arabitol, and ribitol via a PEP-dependent PTS route. Pentoses are not transported by PTS in other bacteria (Shamanna and Sanderson, 1979; Lam *et al.*, 1980; Martin and Russell, 1988; Williams and Martin, 1990).

2.10.4 Importance of the PTS system

The PTS is an energetically favourable pathway for the bacteria that carry it out as the external sugar is simultaneously transported and converted to the first catabolic product, internal sugar phosphate, in one step without expending additional ATP in a kinase reaction (Postma and Roseman 1976). The product of PTS translocation is the corresponding sugar phosphate; these phosphates do not normally cross the cell membrane so that, in this way, the product of transport is trapped inside the cell. By contrast, solutes that are actively concentrated have the ability to flow out of the cell (down the chemical gradient) so that it is likely that energy must be continuously expended to maintain high intracellular levels of such sugars. It is perhaps this reason that anaerobic bacteria, which do not gain as much metabolic energy from sugar catabolism as do aerobic bacteria, generally utilize the PTS for sugar uptake.

2.10.5. Regulatory functions of the PTS

The PTS not only catalyses uptake of its own sugar substrates, but it also functions in the regulation of various bacterial physiological processes and is itself subject to regulatory control (Postma and Lengeler, 1985; Reizer *et al.*, 1988; Saier, 1989b). Sugar substrates of the PTS generally inhibit the uptake of non-PTS carbohydrates in several Gram-negative bacterial species (Saier, 1977). This unidirectional regulation allows the bacterium to select preferred carbon sources when more than one is in the extracellular environment.

Regulation via PTS consists of proteins that can be phosphorylated and dephosphorylated (Postma *et al.*, 1989). In vitro studies with the purified glycerokinase from *Streptococcus faecalis* demonstrated that the enzyme is activated by phosphorylation of a histidyl residue in the protein, catalyzed by the PEP-PTS (Romano *et al.*, 1990).

Other functions of the PTS include: chemoreception (Adler and Epstein, 1974), regulation of gluconeogenesis in *E. coli* and *S. typhimurium* (Chin *et al.*, 1987, 1989), aerobic metabolism (Robertson *et al.*, 1988)), gene transcription (Aymerich *et al.*, 1986) and virulence (Saier, 1989b). For a review of regulation in Gram-positive bacteria see recent reviews by Reizer and Peterkofsky (1987), Reizer *et al.* (1988) and Reizer (1989).

2.11 TRANSPORT SYSTEMS IN THERMOPHILES

Most studies on thermophilic organisms to date have focussed on routes of carbohydrate metabolism which are well known. The mechanism and regulation of accumulation of carbohydrates has been largely ignored, despite being an important step in the fermentation process of saccharolytic thermophilic bacteria. Transport studies with extremely thermophilic organisms have been restricted to a few genera and species.

The most intensively studied thermophile in terms of bacterial energetics and transport is the aerobic bacterium *Bacillus stearothermophilus* (DeVrij *et al.*, 1990). The presence of membrane associated dehydrogenases and σ and aa_3-type terminal oxidases have been found (De Vrij *et al.*, 1988).

Membrane vesicles were used to study the secondary transport of amino acids in *B. stearothermophilus* (De Vrij *et al.*, 1989). Different amino acids were accumulated in response to a $\Delta\mu H^+$. Membrane vesicles of *B. stearothermophilus* possessed a high-affinity transport system for L-glutamate which was competitively inhibited by L-aspartate. Both amino acids were transported by a Na^+-H^+-solute-symport mechanism in a 1:1:1 stoichiometry (DeVrij *et al.*, 1989). Uptake of neutral (branched chain) amino acids is also facilitated by Na^+-solute symport mechanism in this organism (Heyne *et al.*, 1991).

The extremely thermophilic obligate anaerobe *Cl. fervidus* was shown to have a sodium-ion **dependent** transport of neutral, acidic and aromatic amino acids. These were transported in symport with one Na^+ or Li^+ ion (Speelmans *et al.*, 1989).

For the transport of carbohydrates, studies have concentrated on glucose and cellobiose uptake, but no studies have been made of xylose transport in extreme thermophiles. Patni and Alexander (1971b) demonstrated low levels of PEP-PTS activity for fructose and mannitol in extracts of *Cl. thermocellum* strain 651. Glucose and cellobiose transport has been investigated in *Cl. thermocellum* LQR1 and *Cl. thermohydrosulfuricum* 39E (Ng and Zeikus, 1982). The uptake of glucose and cellobiose in these organisms was shown to involve glucose permease and cellobiose phosphorylase with ATP rather than a PEP-PTS. Hernandez (1982) also confirmed these findings for glucose transport with *Cl. thermocellum* ATCC-27405. Glucose uptake in *Spirochaeta thermophila* strain RI 19.B1 was by a glucose permease followed by ATP-dependent phosphorylation by glucokinase (Janssen and Morgan, 1992). No PEP-PTS activity for glucose uptake could be demonstrated.

The moderately thermophilic bacterium(optimum growth temperature 60°C) *Cl. thermosaccahrolyticum* has been reported to have an inducible permease for the transport of xylose. The glucose permease was constitutive and growth on a glucose + xylose substrate mix was diauxic, with glucose used preferentially (Aduse-Opoku and Mitchell, 1988).

2.12 THESIS OBJECTIVES AND OVERALL AIMS

The main objectives of this thesis are threefold.

First, to carry out a general study of the means whereby extremely thermophilic bacteria control their enzyme expression in response to carbohydrate utilization. These results are presented in chapter three. The development of a minimal medium used to grow the thermophilic bacterium *Cl. thermohydrosulfuricum* Rt8.B1 is presented in chapter four.

Second, to extend the preliminary findings of Cook (1988), in which it was found that extreme thermophiles have a tendency to utilize substrates simultaneously i.e. exhibit hyperbolic growth kinetics. Growth on mixed substrates can be controlled at a number of levels. The level of substrate transport into the cell and the level of expression and activation /inactivation of enzymes involved in the dissimilation of substrates. The level at which this control takes place in *Cl. thermohydrosulfuricum* Rt8.B1 was investigated. The results are presented in chapter six.

Third, to investigate the mechanisms which exist for the uptake of glucose and xylose into the cells of the extremely thermophilic anaerobe *Cl. thermohydrosulfuricum* Rt8.B1. Very little is known of such processes in extreme thermophiles. *Cl. thermohydrosulfuricum* has been proposed as a means of producing industrial alcohol from hemi-cellulosic wastes. These features are important to understand as they are fundamental to the development of sugar fermentation processes. This knowledge is of further importance in planning adaptive strategies to introduce new sugar utilisation

capacities by genetic manipulation. It is important to know whether transport is the rate-limiting step and how transport is controlled, energized and regulated. It was hoped that this study would lead to some understanding of the control processes involved in mixed substrate utilization of glucose and xylose by *Cl. thermohydrosulfuricum* Rt8.B1. Chapter seven deals with the transport systems present for glucose and xylose and how this transport is energized and the effects of metabolic inhibitors and analogues on the rate of transport. Chapter eight covers the role of intracellular enzymes in controlling the uptake of glucose and xylose in *Cl. thermohydrosulfuricum* Rt8.B1.

An overall discussion of the results, and the conclusions drawn from the study are given in chapter 9.

CHAPTER THREE
ENZYME EXPRESSION IN THERMOPHILIC BACTERIA

3.1 INTRODUCTION

To date, most studies on enzymes from thermophilic bacteria have concentrated on the purification, characterization, and thermostability of these enzymes (Cowan *et al.*, 1984, Patchett *et al.*, 1987; Plant *et al.*, 1988; Ruttersmith and Daniel, 1991) but in very few instances have detailed studies been reported on the expression and regulation of the enzyme in the whole organism.

An understanding of the control of expression would allow for greater enzyme yield and increase the potential for application in biotechnology processes. Enzymes from thermophilic bacteria have significant biotechnological potential, since they may be able to advantageously replace enzymes in existing applications and extend the range of enzyme-catalyzed industrial processes. The control of enzyme synthesis and therefore production in thermophilic organisms is important to understand. This is of benefit to industrial processes where these enzymes are required in high yield and purity.

Several species of thermophilic anaerobic bacteria have been shown to ferment pentoses and hexoses simultaneously under the culture conditions used (Carreira *et al.*, 1983; Cook, 1988; Hudson *et al.*, 1988; Patel *et al.*, 1988). This simultaneous utilization could be a growth strategy which has evolved in oligotrophic environments or it may simply reflect a lack of highly regulated control of substrate utilization and enzyme synthesis. This simultaneous utilization may reflect enzymes and pathways which are synthesized constitutively, although perhaps at only low levels, until substrate utilization results in the synthesis of more enzyme (i.e., the phenomenon is one of partial repression). In the light of these data it was of interest to determine whether other thermophilic bacteria also displayed similar strategies of enzyme expression.

To investigate this further a number of organisms representing different thermophilic genera and kingdoms were studied for their regulation of enzyme expression. A list of these organisms is presented in table 3.1.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals

Unless otherwise stated, chemicals were of analytical grade and obtained from well-known chemical distributors. The distributors of special chemicals are indicated in the appropriate section describing their use. All gases were purchased from New Zealand Industrial Gases Limited, Wellington, New Zealand. The buffer 3-[morpholino]propanesulfonic acid (MOPS) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3.2.2 Strains used in study

All bacteria used in this study are currently held in the Thermophile Research Unit Culture Collection (TRUCC) at Waikato University. The strains used in this study together with their source and physiological characteristics are shown in Table 3.1.

Table 3.1 Thermophilic organisms used in study

Genus /species	**strain designation**	Growth pH	Temp (°C)	Medium
Archaea				
Sulfolobus solfataricus	DSM 1616	4	80	DSM 182
Thermococcus	AN1	7.3	75	AN1 medium
Eubacteria				
Thermus	Rt41A	7.5	70	162
Cl. thermohydrosulfuricum	Rt8.B1	7	70	TYEG
Thermotoga	FjSS3.B1	7	80	MSM medium

Thermococcus strain AN1 was a S°-reducing anaerobe and *Sulfolobus solfataricus* is a S°-oxidising aerobe. *Thermus* strain Rt41A is an O_2-respiring heterotroph and *Thermotoga* strain FjSS3.B1 an obligate anaerobe. The other organism chosen was the glycolytic anaerobe *Clostridium thermohydrosulfuricum* strain Rt8.B1.

To study the level of expression of enzyme synthesis in these organisms, enzymes common to all strains were chosen. The following enzymes, which are, in general, inducible and highly regulated in mesophilic bacteria were assayed: β-galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23), β-glucosidase (β-D-glucoside glucohydrolase EC 3.2.1.21) and α-glucosidase (α-D-glucoside glucohydrolase EC 3.2.1.20). The organisms described were assayed for both extracellular and intracellular enzyme production. The enzymes were assayed at the growth temperature and pH used to support growth of the thermophilic strain being tested. The following growth substrates were used to test for induction or repression of these enzymes: lactose, cellobiose, glucose, galactose, maltose, and starch. These substrates were added individually to the growth medium at a final concentration of 0.1 and 1.0 % (w/v)

3.2.3 Preparation of media components

3.2.3.1 Sterilization

All autoclaving was at 121°C and one atmosphere overpressure for 20 min. Filter-sterilization was through 0.20 μM pore size disposable filter units (Sartorius, Göttingen, Germany).

3.2.3.2 Reducing solution

Sulfide reductant consisted of: degassed distilled H_2O, 100 ml; $Na_2S.9H_2O$, 10.0 g; pH adjusted to 7.0 with 5 N H_2SO_4. This was added at 5 ml per litre.

3.2.3.3 Vitamin and trace element solutions

Vitamin solutions were prepared in distilled water degassed under vacuum, and were stored in the dark at 4°C after filter-sterilization into sealed sterile serum vials. The vitamin solutions were prepared so that the addition of 0.1 ml by syringe to 10 ml of medium gave the final required concentration.

The vitamin solution of Wolin (1963) contained ($mg.\ell^{-1}$ distilled water) biotin, 2.0; folic acid, 2.0; pyridoxine-HCl, 10.0; riboflavin, 5.0; thiamine-HCl, 5.0; nicotinic acid, 5.0; pantothenic acid, 5.0; vitamin B_{12}, 0.1, ρ-aminobenzoic acid, 5.0 and thioctic acid, 5.0. This solution was filter sterilized through a 0.2 µm disposable filter and stored in a cold room until required. Vitamin solution was added aseptically after sterilization of media.

Trace mineral solution: The trace mineral solution contained ($g\ell^{-1}$ distilled water) nitrilotriacetic acid, 12.5 adjusted to pH 6.5 with KOH, $FeCl_3.4H_2O$, 0.2; $MnCl_2.4H_2O$, 0.1; $CoCl_2.6H_2O$, 0.017; $CaCl_2.2H_2O$, 0.1; $ZnCl_2$, 0.1; $CuCl_2$, 0.02; H_3BO_3, 0.01; $NaMoO_4.2H_2O$, 0.01; NaCl, 1.0 and Na_2SeO_3, 0.02. The pH was adjusted to 7.0 at room temperature.

Nitsch's trace elements (Brock *et al.*, 1972) contained ($g\ell^{-1}$) distilled water: $MnCl_2.4H_2O$, 1.80; $Na_2B_4O_7.10H_2O$, 4.50; $ZnSO_4.7H_2O$, 0.22; $CuCl_2.2H_2O$, 0.05; $Na_2MoO_4.2H_2O$, 0.03; $VSO_4.2H_2O$, 0.03; $CoSO_4.7H_2O$, 0.01. The pH was adjusted to 7.0 at room temperature.

Zeikus trace element solution (Zeikus *et al.,* 1979) contained ($g\ell^{-1}$): nitrilotriacetic acid, 12.8; $FeCl_3.6H_2O$, 0.20; $MnCl_2.4H_2O$, 0.10; $CoCl_2.6H_20$, 0.017; $CaCl_2.2H_2O$, 0.10; $ZnCl_2$, 0.10; $CuCl_2$ 0.02, H_3BO_3, 0.01; $Na_2MoO_4.2H_2O$, 0.01; NaCl, 1.0; Na_2SeO_4, 0.02. The pH was adjusted to pH 6.75 at room temperature.

All trace element solutions were added to media prior to autoclaving.

3.2.3.4 Carbon sources

All carbon sources were added to medium separately after autoclaving from 10 % sterile stock solutions prepared anaerobically and filter sterilized. The carbon sources were prepared so that the addition of 0.5 ml by syringe to 10 ml of medium gave the final required concentration.

3.2.3.5 Media preparation

The Hungate technique (Hungate, 1950) with minor modifications was used to prepare anaerobic media (Patel *et al.,* 1985b). Medium was placed in an Oxoid dispensing jar (Model S-A) and put in a pressure cooker (Wisconsin Aluminium Foundry Co., Inc. Wisconsin, USA) for 15 min at 121°C to boil off oxygen thereby increasing the effectiveness of the reducing agent. The medium was allowed to cool under a steady stream of oxygen-free nitrogen gas through an inlet in the dispenser via a bent needle syringe. Upon cooling 5 ml of a 10 % neutralized $Na_2S.9H_2O$ reducing solution was added and mixed. The medium was dispensed in 10 ml aliquots under oxygen-free nitrogen conditions into medium-walled Bellco tubes fitted with rubber stoppers before autoclaving in a press.

Medium prepared aerobically was dispensed in 100 ml aliquots into 500 ml total volume Schott bottles with plastic screw caps and then autoclaved.

3.2.4 Media and conditions for cultivation of bacteria

Thermus Rt41A was maintained on modified 162 medium (Janssen *et al*., 1991). The medium had the following composition ($g\ell^{-1}$): nitrilotriacetic acid, 0.10; $MgSO_4.7H_2O$, 0.05; $MgCl_2.6H_2O$, 0.16; NH_4Cl, 0.53; KH_2PO_4, 0.68; Fe(III)citrate.$3H_2O$, 0.6 mg; d-biotin, 10 mg; Nitsch's trace element solution (Brock *et al*., 1972), 1 ml. The pH was adjusted to pH 7.5 with 1 N NaOH.

Cl. thermohydrosulfuricum Rt8.B1 was grown on the trypticase peptone-yeast extract-glucose (TYEG) medium described by Zeikus *et al.*, (1979). This medium contained ($g\ell^{-1}$ distilled water) NH_4Cl, 0.9; $MgCl_2$, 0.2; NaCl, 0.9; KH_2PO_4, 0.75; K_2HPO_4, 1.50; trace mineral solution, 9 ml; 10 % $FeSO_4$, 0.03 ml; 0.2% resazurin (redox indicator), 1 ml; Wolin's vitamin solution, 5 ml; yeast extract, 3.0 and trypticase peptone. The pH was adjusted to pH 7 by the addition of 1 N NaOH.

Thermotoga FjSS3.B1 was maintained on mineral salt medium (MSM) (Huser *et al.*, 1986). The medium had the following composition ($g\ell^{-1}$): $(NH_4)_2SO_4$, 0.65; $CaCl_2.2H_2O$, 0.074; $MgSO_4.7H_2O$, 0.28; KH_2PO_4, 0.28; NaCl, 7.0, resazurin, 0.0005; $Na_2S.9H_2O$, 0.24; Nitsch's trace elements, 1.0; yeast extract, 2.0; casamino acids, 2.0. The pH was adjusted to 6.8 at 25°C prior to autoclaving.

Thermococcus AN1 was grown in AN1 medium (Klages, 1991). The medium had the following composition ($g\ell^{-1}$): $MgCl_2.6H_2O$, 0.30; K_2HPO_4, 1.50; NaCl, 2.50; Wolin's vitamin solution, 1 ml, Zeikus trace elements, 5 ml; trypticase peptone, 8.0; sodium-thioglycollate, 1.0; elemental sulphur, 2.0. The medium was adjusted to pH 7.3 prior to autoclaving.

Sulfolobus solfataricus DSM 1616 was grown in DSM 182 medium (Zillig *et al.*, 1980). The medium had the following composition ($g\ell^{-1}$):KH_2PO_4, 3.1; $(NH_4)_2SO_4$, 2.50; $MgSO_4.7H_2O$, 0.20; $CaCl_2.2H_2O$, 0.25; yeast extract, 1.0, casamino acids, 1.0, Nitsch's trace elements, 1.0 ml. The medium was adjusted to pH 4.0 with 10 N H_2SO_4 at room temperature.

Anaerobic growth was carried out in Bellco tubes (total volume 23 ml) containing 10 ml of medium and added growth substrate. Aerobic growth was carried out in Schott bottles (total volume 500 ml) containing 100 ml of medium and added growth substrate. Incubations were at the optimum growth temperature of the organism (table 3.1) and with shaking. The period of incubation was for 24 hours (or until the organism had reached the mid-log phase of growth) in order to compensate for the

different rates of growth on the different carbon sources tested. All incubations were carried out in duplicate.

3.2.5 ASSAY METHODS

3.2.5.1 Chemicals

The chromogenic substrates ρ-nitrophenyl-β-D-galactopyranoside, ρ-nitrophenyl-β-D-glucopyranoside and ρ-nitrophenyl-α-D-glucopyranoside were obtained from Sigma Chemical Co. (St Louis, Mo, USA). Bovine serum albumin of 99.9 % purity was obtained from Sigma.

3.2.5.2 Preparation of cells for determination of enzyme activities

Cells (10 ml) were harvested by centrifugation at 8000 X g for 20 min. Cells were washed and resuspended in 50 mM MOPS (1.0 ml) For growth at pH 4.0, citric acid buffer was used (pH 4.0). The pH of 50 mM MOPS was 7.0.

3.2.5.3 β-Glucosidase, β-galactosidase and α-glucosidase assays

β-galactosidase, β-glucosidase, α-glucosidase activity were assayed by the methods of Cowan *et al.* (1984), Patchett *et al.* (1987) and Plant *et al.* (1988), respectively. The assays involved the spectrophotometric measurement at 400 nm of ρ-nitrophenol (ρNP) released from ρ-nitrophenyl substrate (ρNPG).

Both intracellular and extracellular activity were assayed as follows:

1. Intracellular activity (toluenized cells) were assayed by taking cells centrifuged from 10 ml of culture and the cell pellet resuspended in 1.0 ml of 50 mM MOPS buffer and adding 100 μl of toluene:acetone (1:1 v/v), mixing, and standing at room temperature for 10 min. Aliquots of 200 μl were removed and assayed, cellular material was removed by centrifugation prior to reading the absorbance at 400 nm.

2. Cell-free supernatants were assayed by taking cells centrifuged from 10 ml of culture and assaying the supernatant. A 500 μl sample was added to the assay mix.

The following standard assay procedure was used for all three enzymes:

The assay was begun by equilibrating 500 μl 40 mM ρNPG substrate in 100 mM MOPS (citrate buffer at pH 4.0) at the appropriate pH (pH for growth of organism tested) at the appropriate temperature (growth temperature of organism) for 5 min in sealed Eppendorf tubes. After equilibration, tubes were taken from the water bath and injected with 200 μl of cells or 500 μl of supernatant using a 500 μl automatic pipetter (Socorex) with rapid mixing and returned to the water bath. After 10 min incubation at the appropriate temperature, the reaction was stopped by the addition of 0.5 ml of 1 M Na_2CO_3 which raised the pH of the assay solution. This resulted in the conversion of virtually all the ρNP product to its yellow coloured conjugate base. The solutions were then allowed to cool to room temperature and the absorbance at 400 nm read on a DMS-80 UV-Visible Spectrophotometer (Varian Techtron Pty, Mulgrave, Australia).

Each assay was run with a sample blank to allow for any interference in the ρNPG substrate assay of buffer /solution used, and also to correct for thermal hydrolysis of ρ-NPG substrates during the incubation period. ρNPG thermal breakdown at temperatures below 70°C was negligible during the standard assay incubation time, but increased rapidly at temperatures above 80°C. Absorbance values were converted to molar concentrations of ρNP using a molar extinction coefficient for ρNP of 18300 l mol^{-1} cm^{-1} (Ruttersmith and Daniel, 1992). Enzyme activity was expressed as the amount of enzyme required to release 1 nanomole of ρNP per min per milligram of cellular protein. All assays were performed in triplicate and repeated if the two absorbance values differed by more than 5 %.

The protein content of the cell suspension was determined by incubating 1 ml of the cell suspension with 1 ml of 1 M NaOH at 100°C for 15 min. After neutralization with

1 ml of 1 M HCl, the protein content was determined by the Biuret reaction as described by Scopes (1982), without modification. Bovine serum albumin of 96.99 % purity was used to construct a standard curve which was linear from 0 to 5 $mg.ml^{-1}$. All assays were carried out in triplicate.

3.3 RESULTS

The results of these studies are presented in the following tables. In all tables, substrates added at 1 % (w/v) final concentration are in uppercase type and those added at 0.1 % (w/v) are in lower case type.

The aim of the experiments in this chapter was originally to investigate control of enzyme expression in a range of thermophilic bacteria. The premise under which the results were to be interpreted was that formulated for classical induction /repression response as defined for β-galactosidase control in *Escherichia coli*. It soon became apparent that results with thermophilic organisms were not as clear cut and easily interpreted. Generally the degree of response was never as great, the cultures always possessed a high constitutive level of the enzyme and substrates did not conform to patterns of inducers or repressors. In order to rationalize the results from the data obtained some arbitrary decisions have had to be made. The most important of these was to decide when an organisms response to an added substrate was simply a small variation in modulated enzyme concentration or an "inductive" response leading to a significant increase. I have chosen a value of 3 times the constitutive level as being indicative of a significant response to a substrate addition. I should stress the value is arbitrary and one of convenience in interpreting results. Possible reasons for the absence of classical induction /repression response in thermophilic organisms will be discussed later in the chapter.

3.3.1 Thermophilic archaebacteria

3.3.1.1 *Thermococcus* AN1

The extracellular and intracellular β-galactosidase, β-glucosidase and α-glucosidase enzyme activities of the extremely thermophilic organism *Thermococcus* AN1 are presented in table 3.2 and 3.3.

Table 3.2 Enzyme activities present in cell-free supernatants of *Thermococcus* AN1 grown on different carbon sources

	ß-galactosidase			ß-glucosidase			α-glucosidase		
Carbon source	S.Act	% Act	I	S.Act	% Act	I	S.Act	% Act	I
Basal	0.8	100	-	0.62	100	-	0.69	100	-
LACTOSE	5.86	733	Y	1.38	223	N	0.8	116	N
lactose	7.19	899	Y	1.23	198	N	0.42	<100	N
STARCH	9.77	1221	Y	1.61	260	N	0.89	129	N
starch	9.61	1201	Y	2.26	365	Y	0.8	116	N
CELLOBIOSE	7.75	969	Y	0.92	148	N	0.42	<100	N
cellobiose	7.69	961	Y	0.88	142	N	0.29	<100	N
MALTOSE	6.32	790	Y	1.55	250	N	1.01	146	N
maltose	2.32	290	N	1.85	298	N	1.18	171	N
GALACTOSE	NG	-	-	NG	-	-	NG	-	-
galactose	0.74	<100	N	0.28	<100	N	5.1	739	Y
GLUCOSE	NG	-	-	NG	-	-	NG	-	-
glucose	0.34	<100	N	0.22	<100	N	4.3	623	Y

S.Act = specific activity expressed as nmoles per minute per milligram of protein at 75°C; % Act = percentage activity of basal level; I = significant response (Y), no significant response (N); NG = no growth. The basal medium contained no added carbohydrate.

With *Thermococcus* AN1, extracellular β-galactosidase, β-glucosidase and α-glucosidase activities were detected in the absence of added carbohydrate suggesting that the enzymes are constitutive. No growth was present on high concentrations of glucose and galactose.

For β-galactosidase a significant response was observed on starch, lactose, cellobiose and maltose. No significant change in the level of enzyme was detected with the other substrates. A similar level of β-glucosidase activity was present on all substrates tested with growth on 0.1 % starch resulting in a significant response in enzyme activity. Both glucose and galactose produced a significant response of α-glucosidase expression.

Intracellular enzyme activities for *Thermococcus* AN1 are summarized in table 3.3.

Growth of *Thermococcus* AN1 on all substrates resulted in a significant response of β-galactosidase activity. The greatest increase in β-galactosidase activity was on 0.1 % lactose.

The level of β-glucosidase was increased on 0.1 % glucose, galactose, maltose and lactose. No β-glucosidase activity was detected on high concentrations lactose, starch, cellobiose and maltose.

A constitutive level of α-glucosidase was present on lactose, galactose and glucose which was absent on the other growth substrates tested.

Table 3.3 **Enzyme activities present in toluene-treated cells of *Thermococcus* AN1 grown on different carbon sources.**

	ß-galactosidase			ß-glucosidase			α-glucosidase		
Carbon source	S.Act	% Act	I	S.Act	% Act	I	S.Act	% Act	I
Basal	3.94	100	-	5.53	100	-	7.35	100	-
LACTOSE	8.1	206	N	NA	-	N	NA	-	N
lactose	38.49	977	Y	25	452	Y	7.31	<100	N
STARCH	13.6	345	Y	NA	-	N	NA	-	N
starch	18.9	480	Y	13	235	N	NA	-	N
CELLOBIOSE	6.51	165	N	NA	-	N	NA	-	N
cellobiose	23	584	Y	3.33	<100	N	NA	-	N
MALTOSE	15	381	Y	NA	-	N	NA	-	N
maltose	14	355	Y	19	344	Y	NA	-	N
GALACTOSE	NG	-	-	NG	-	-	NG	-	-
galactose	12	305	Y	25	452	Y	8.01	109	N
GLUCOSE	NG	-	-	NG	-	-	NG	-	-
glucose	24	609	Y	39	705	Y	3.46	<100	N

S.Act = specific activity expressed as nmoles per minute per milligram of protein at 75°C; % Act = percentage activity of basal level; I = significant response (Y), no significant response (N); NG = no growth; NA = no activity detected.

The intracellular levels of all three enzymes were higher than levels detected in cell-free supernatants. For example, 84 % of the total β-galactosidase activity on 0.1 % lactose was intracellular, suggesting that in this organism the enzyme has a cytoplasmic or periplasmic origin. For the remainder of the results in this study the total enzyme activity (extracellular + intracellular activity) is summarized in the following tables.

A summary of total (extracellular plus intracellular) activities of β-galactosidase, β-glucosidase and α-glucosidase for the extremely thermophilic organism *Thermococcus* AN1 are presented in table 3.4.

Table 3.4 Enzyme activities present in cultures of *Thermococcus* AN1 grown on different carbon sources.

	ß-galactosidase			ß-glucosidase			α-glucosidase		
Carbon source	S.Act	% Act	I	S.Act	% Act	I	S.Act	% Act	I
Basal	4.74	100	-	6.15	100	-	8.04	100	-
LACTOSE	13.96	295	N	1.38	<100	N	0.8	<100	N
lactose	45.68	964	Y	26.23	426	Y	7.73	<100	N
STARCH	23.37	493	Y	1.61	<100	N	0.89	<100	N
starch	28.51	601	Y	15.26	248	N	13.8	172	N
CELLOBIOSE	14.26	301	Y	0.92	<100	N	0.42	<100	N
cellobiose	30.69	647	Y	4.21	<100	N	3.62	<100	N
MALTOSE	21.32	450	Y	1.55	<100	N	1.01	<100	N
maltose	16.32	344	Y	20.85	339	Y	20.18	251	N
GALACTOSE	NG	-	-	NG	-	-	NG	-	-
galactose	12.74	268	N	25.28	411	Y	13.1	163	N
GLUCOSE	NG	-	-	NG	-	-	NG	-	-
glucose	24.34	514	Y	39.22	638	Y	7.76	<100	N

S.Act = specific activity expressed as nmoles per minute per milligram of protein at 75°C; % Act = percentage activity of basal level; I = significant response (Y), no significant response (N); NA = no activity detected.

In summary, all three enzymes were expressed constitutively and most sugar substrates caused an increase in the level of β-galactosidase activity. Growth on low concentrations (0.1 %) of glucose, maltose, galactose and lactose produced a

significant response of increasing β-glucosidase activity. No significant α-glucosidase above the constitutive level was detected on any of the other substrates tested.

The medium used to grow *Thermococcus* AN1 contained high levels of trypticase peptone (8 g /litre) which contains carbohydrates of unknown composition which may act as inducers of enzyme synthesis. *Thermococcus* AN1 has been shown to have an obligate growth requirement for trypticase peptone (Klages, 1991); the level of this medium constituent can be reduced to 0.50 $g\ell^{-1}$ in the presence of amino acids. In order to test if a constituent in the medium was inducing enzyme synthesis, *Thermococcus* AN1 was grown on 0.50 $g\ell^{-1}$ trypticase peptone medium supplemented with carbohydrates as before and the levels of β-galactosidase, β-glucosidase and α-glucosidase measured. The results were similar to those obtained previously (table 3.4); all three enzymes were synthesized constitutively.

3.3.1.2 *Sulfolobus solfataricus*

A summary of total extracellular and intracellular levels of β-galactosidase, β-glucosidase and α-glucosidase for the extremely thermophilic aerobic organism *S. solfataricus* are presented in table 3.5.

In *S. solfataricus* a high constitutive level of β-galactosidase, α-glucosidase and β-glucosidase were detected in the absence of any added inducer. The activity of all three enzymes was reduced or absent in the presence of added carbohydrate.

This organism could not be grown in the absence of yeast extract with the medium used in this study and therefore the effects of gratuitous inducers in the medium cannot be ruled out.

Table 3.5 **Enzyme activities present in cultures of *S. solfataricus* grown on different carbon sources.**

	ß-galactosidase			ß-glucosidase			α-glucosidase		
Carbon source	S.Act	% Act	I	S.Act	% Act	I	S.Act	% Act	I
Basal	28.8	100	-	21.68	100	-	6.79	100	-
LACTOSE	5.31	<100	N	7.16	<100	N	NA	-	N
lactose	7.64	<100	N	11	<100	N	NA	-	N
STARCH	8	<100	N	13	<100	N	NA	-	N
starch	NA	-	N	8.22	<100	N	NA	-	N
CELLOBIOSE	1.34	<100	N	8.46	<100	N	NA	-	N
cellobiose	NA	-	N	10	<100	N	NA	-	N
MALTOSE	9.08	<100	N	12	<100	N	NA	-	N
maltose	5.26	<100	N	NA	-	N	NA	-	N
GALACTOSE	NA	-	N	NA	-	N	NA	-	N
galactose	NA	-	N	NA	-	N	NA	-	N
GLUCOSE	NA	-	N	NA	-	N	NA	-	N
glucose	NA	-	N	MA	-	N	NA	-	N

S.Act = specific activity expressed as nmoles per minute per milligram of protein at 80°C; % Act = percentage activity of basal level; I = significant response (Y), no significant response (N); NA = no activity detected.

3.3.2 Thermophilic Eubacteria

The following section is a summary of results for β-galactosidase, β-glucosidase and α-glucosidase synthesis for the thermophilic eubacteria used in this study.

3.3.2.1 *Thermus* Rt41A

A summary of β-galactosidase, β-glucosidase and α-glucosidase activities for the obligate aerobe *Thermus* Rt41A are presented in tables 3.6.

Table 3.6 **Enzyme activities in cultures of *Thermus* Rt41A grown on different carbon sources.**

	ß-galactosidase			ß-glucosidase			α -glucosidase		
Carbon source	S.Act	% Act	I	S.Act	% Act	I	S.Act	% Act	I
Basal	16.4	100	-	18.81	100	-	38	100	-
LACTOSE	NG	-	-	NG	-	-	NG	-	-
lactose	87	530	Y	27.36	145	N	41	108	N
STARCH	16	<100	N	36.41	194	N	34	<100	N
starch	14	<100	N	30.41	162	N	33.6	<100	N
CELLOBIOSE	NG	-	-	NG	-	-	NG	-	-
cellobiose	134	817	Y	176	936	Y	109	287	N
MALTOSE	NG	-	-	NG	-	-	NG	-	-
maltose	14	<100	N	7.64	<100	N	60	158	N
GALACTOSE	NA	-	N	33	175	N	NA	-	N
galactose	60	366	Y	57	303	Y	25	<100	N
GLUCOSE	NA	-	N	NA	-	N	NA	-	N
glucose	5.82	<100	N	NA	-	N	94	247	N

S.Act = specific activity expressed as nmoles per minute per milligram of protein at 70°C; % Act = percentage activity of basal level; I = significant response (Y) no significant response (N); NG = no growth; NA = no activity detected. The basal medium contained sodium L-glutamate at 2 g/l.

In *Thermus* Rt41A β-galactosidase, β-glucosidase and α-glucosidase were synthesized constitutively in the absence of added carbohydrate. No growth of this organism was observed on high concentrations of lactose, cellobiose and maltose.

Growth of *Thermus* Rt41A on 0.1 % cellobiose, lactose and galactose produced a significant response of increasing β-galactosidase activity. High concentrations (1.0 %) of glucose and galactose totally repressed the constitutive synthesis of this enzyme. A significant increase in β-glucosidase activity was seen on 0.1 % cellobiose and galactose. A constitutive level of activity was detected on the other substrates tested.

The constitutive level of β-glucosidase was repressed on glucose. A level of α-glucosidase similar to the constitutive level was detected on all substrates tested. High concentrations (1.0 %) of glucose and galactose repressed the synthesis of constitutive enzyme.

Thermus Rt41A was grown on the defined 162 medium described by Janssen *et al.* (1991), which contains no yeast extract or trypticase peptone. The effect of a medium component inducing enzyme synthesis can therefore be ruled out.

3.3.2.2 *Clostridium thermohydrosulfuricum* Rt8.B1

The total β-galactosidase, β-glucosidase and α-glucosidase enzyme activities for the thermophilic glycolytic anaerobe *Cl. thermohydrosulfuricum* Rt8.B1 are presented in table 3.7.

Cl. thermohydrosulfuricum Rt8.B1 contained a constitutive level of β-galactosidase, β-glucosidase and α-glucosidase in the absence of added carbohydrate.

Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on galactose glucose, lactose and starch produced a significant increase in β-galactosidase activity. The β-glucosidase level increased significantly during growth of *Cl. thermohydrosulfuricum* Rt8.B1 on galactose, cellobiose and starch. The α-glucosidase was increased by growth on galactose, glucose, starch and maltose. No activity was detected on the other substrates tested.

Cl. thermohydrosulfuricum Rt8.B1 was grown on TYEG medium described by Zeikus *et al.* (1979), which contains high levels of yeast extract and trypticase peptone and therefore the effect of a medium component inducing enzyme synthesis can't be ruled out.

Table 3.7 Enzyme activities in cultures of *Cl. thermohydrosulfuricum* Rt8.B1 grown on different carbon sources

	ß-galactosidase			ß-glucosidase			α-glucosidase		
Carbon source	S.Act	% Act	I	S.Act	% Act	I	S.Act	% Act	I
Basal	8.79	100	-	5.37	100	-	3.39	100	-
LACTOSE	40.31	459	Y	3.21	<100	N	NA	-	N
lactose	9.77	111	N	2.27	<100	N	NA	-	N
STARCH	34	387	Y	25.57	476	Y	26.5	782	Y
starch	31.3	356	Y	27	503	Y	33	973	Y
CELLOBIOSE	14.06	160	N	25.19	469	Y	NA	-	N
cellobiose	4.3	<100	N	17.85	332	Y	NA	-	N
MALTOSE	12.7	144	N	3.46	<100	N	28.5	841	Y
maltose	2.74	<100	N	2.54	<100	N	29.6	873	Y
GALACTOSE	95	1080	Y	55	1024	Y	52	1534	Y
galactose	71	808	Y	39	726	Y	14	413	Y
GLUCOSE	4.57	<100	N	2.5	<100	N	45	1327	Y
glucose	45	512	Y	5.2	<100	N	27	796	Y

S.Act = specific activity expressed as nmoles per minute per milligram of protein at 70°C; % Act = percentage activity of basal level; I = significant response (Y), no significant response (N); NA = no activity detected. The basal medium contained xylose at 0.1 % (w /v).

3.3.2.3 *Thermotoga* FjSS3.B1

A summary of β-galactosidase, β-glucosidase and α-glucosidase activities for the extremely thermophilic anaerobic eubacterium *Thermotoga* FjSS3.B1 is presented in table 3.8.

Table 3.8 **Enzyme activities in cultures of *Thermotoga* FjSS3.B1 grown on different carbon sources**

	ß-galactosidase			ß-glucosidase			α-glucosidase		
Carbon source	S.Act	% Act	I	S.Act	% Act	I	S.Act	% Act	I
Basal	35.76	100	-	37	100	-	9.13	100	-
LACTOSE	15.99	<100	N	7.96	<100	N	4.01	<100	N
lactose	25.42	<100	N	8.85	<100	N	3.2	<100	N
STARCH	19.17	<100	N	12.88	<100	N	5.2	<100	N
starch	16.06	<100	N	9.5	<100	N	5.5	<100	N
CELLOBIOSE	43.32	121	N	29.78	<100	N	6.78	<100	N
cellobiose	51.22	143	N	38.67	105	N	6.44	<100	N
MALTOSE	23.53	<100	N	14.21	<100	N	7.14	<100	N
maltose	19.95	<100	N	12.79	<100	N	5.44	<100	N
GALACTOSE	137.72	385	Y	39	105	N	4.85	<100	N
galactose	163.23	456	Y	52	141	N	18.2	199	N
GLUCOSE	70.61	197	N	64	173	N	7.1	<100	N
glucose	87.02	243	N	68	184	N	8.68	<100	N

S.Act = specific activity expressed as nmoles per minute per milligram of protein at 80°C; % Act = percentage activity of basal level; I = significant response (Y), no significant response (N).

Thermotoga FjSS3.B1 produced a constitutive level of β-galactosidase, β-glucosidase and α-glucosidase. This activity was modified depending on the growth substrate supplied.

The enzyme β-galactosidase was detected on all growth substrates tested. The maximum response was observed with galactose. The enzymes β-glucosidase and α-glucosidase were present at a constitutive level, with no significant response observed with any of the carbohydrates tested.

3.4 DISCUSSION

3.4.1 Introduction

A large number of inducible metabolic systems have been described and studied in bacteria. Most of the fundamental characteristics of the induction effect have been established in the study of the lactose system of *Escherichia coli* (Pardee *et al.,* 1959; Jacob and Monod, 1961). Wild type *E. coli* grown in the absence of lactose contains about 1 to 10 units of β-galactosidase. In the presence of lactose, β-galactosidase is increased to 10,000 units representing a 1000-fold increase and this is known as the induction effect (Jacob and Monod, 1961). This phenomenon is not a result of activation or conversion of pre-existing protein precursors, but results from the complete *de novo* synthesis of enzyme molecules. Induction is defined as activation by inducer of enzyme protein synthesis (Jacob and Monod, 1961).

Not all enzymes are inducible or repressible. Some are synthesized continuously regardless of the presence of an inducer or repressor and are called constitutive enzymes. "Constitutive" is an operational term and the level of some cellular proteins formerly thought to be constitutive are now known to fluctuate according to the carbon source used for growth and to the stage of growth at which the cells are harvested (Pardee *et al.*, 1959).

The majority of mesophilic organisms have been reported to regulate their enzyme synthesis and substrate utilization in accordance with their nutritional and physiological needs. It is reasonable and economical that organisms do not synthesize all the enzymes they are able to continuously, but only those that are necessary for their metabolism under current physiological conditions.

It might be assumed that growth in an oligotrophic environment such as a thermophilic hot pool would have necessitated selection for bacteria that were able to regulate tightly their substrate utilization, thus resulting in the controlled synthesis of enzymes as opposed to their continual expression. Continual synthesis is wasteful in terms of

energy demands, as the organism has to continually expend energy to produce an enzyme which is not needed. In mesophilic organisms this regulation is tightly controlled and accomplished by induction and repression which are complementary to each other.

Little is known about the regulation of enzyme synthesis in thermophilic bacteria. Induction and repression mechanisms have been reported for extracellular enzyme synthesis. For example, extracellular amylase and pullulanase enzyme synthesis in some thermophilic bacteria is controlled by induction and catabolite repression; enzyme expression is accomplished when maltose or maltose-containing carbohydrates are used as substrates (Antranikian, 1990). Glucose has been shown to repress the synthesis of extracellular amylase and pullulanases in *Thermoanaerobacter finii* and *Clostridium* sp. EM1 (Madi *et al.*, 1987; Antranikian *et al.*, 1987). In contrast, the synthesis of α-amylase and pullulanase in *Cl. thermohydrosulfuricum* 39E is only partially repressed by glucose, whereas α-glucosidase synthesis is not (Melasniemi, 1987) and indicates that intracellular and extracellular enzymes are regulated differently in some thermophilic bacteria.

The enzymes β-galactosidase, β-glucosidase and α-glucosidase which have a periplasmic or cytosol origin in thermophilic bacteria (Ait *et al.*, 1979, 1982; Buonocore *et al.*, 1980; Saha and Zeikus, 1991; Saito *et al.*, 1989) were investigated for controlled synthesis of enzyme expression.

3.4.2 The expression of β-galactosidase in thermophilic organisms

A generalized summary of the results obtained for β-galactosidase expression in this study and other studies is given in table 3.9.

Table 3.9 The expression of β-galactosidase in thermophilic organisms

Organism	strain	expression	Reference
B. stearothermophilus	AT-1 to AT-8	Constitutive	Goodman and Pederson (1976)
T. aquaticus	T2	Inducible	Ulrich et al. (1972)
Thermoanaerobacter	TP6-B1	Constitutive	Lind et al. (1989)
Caldariella acidophila	MT-4	Constitutive	Buonocore et al. (1980)
Desulfurococcus	Tok12S.1	Constitutive	Bryant (1986)
Thermococcus	AN1	Constitutive	Bryant (1986), this study
S. solfataricus	DSM 182	Constitutive	This study
Thermus	Rt41A	Constitutive	This study
Cl. thermohydrosulfuricum	Rt8.B1	Constitutive	This study
Thermotoga	FjSS3.B1	Constitutive	This study

As with most other thermophilic bacteria studied (table 3.9), the expression of β-galactosidase synthesis by the five thermophilic organisms studied was constitutive. The results of this study demonstrate that some substrates enhanced the synthesis of β-galactosidase present in the cells over and above the constitutive level, which was already quite high. In wild type *E. coli* grown in the absence of lactose the basal level of β-galactosidase is extremely low, about 1 to 10 units of β-galactosidase. Following exposure to the inducer lactose, β-galactosidase is increased to 10,000 units and this is known as the induction effect (Jacob and Monod, 1961). In the present study, the maximum increase in the expression of β-galactosidase was about 10-fold in *Thermococcus* AN1 grown on lactose (table 3.4) and *Cl. thermohydrosulfuricum* Rt8.B1 grown on galactose (table 3.7). Although an enhanced synthesis of β-galactosidase was observed this did not represent an increase to suggest classical induction of the basal level in which the level of induction is far greater (100-fold). In some cases the increase in the basal enzyme was at maximum 10-fold and in the majority of experiments only 3-fold or less. Classical induction has been shown in some *Thermus* isolates. *Thermus aquaticus* T2 was found to have an inducible β-galactosidase which was induced 40-100 fold by lactose and galactose (Ulrich *et al.*, 1972).

Other studies with thermophilic bacteria have also reported a constitutive synthesis of β-galactosidase which is modified in the presence of added carbohydrate. Bryant (1986) studied the β-galactosidase from *Thermococcus* AN1 and *Desulfurococcus* Tok12S.1 and found that when these organisms were grown without sugars in the growth medium a high constitutive level of β-galactosidase was detected. Lactose was shown to stimulate a small increase in β-galactosidase activity in these two organisms as opposed to classical induction. The thermophilic bacteria *Caldariella acidophila* and *Thermoanaerobacter* sp. TP6.B1 have been reported to have a constitutive β-galactosidases whose levels were unaffected by the inclusion of lactose or galactose in the growth medium (Buonocore *et al.*, 1980; Lind *et al.*, 1989).

In this study, lactose, the classical inducer of β-galactosidase activity in *E. coli*, was shown to produce a significant increase in β-galactosidase activity with some of the strains; however a number of different substrates also produced a similar response, including glucose which is a repressor of the synthesis of this enzyme in *E. coli*. Magasanik (1961) has shown that glucose interferes with the synthesis of β-galactosidase in *E. coli* in three ways:

1. It excludes the inducer from the cells (inducer exclusion).

2. It represses the β-galactosidase strongly but transiently, when added to cells growing on another carbon source (transient repression).

3. It represses the enzyme weakly but permanently during balanced growth (catabolite repression).

Repression like induction is highly specific and the repressing molecules generally are the product of the sequence (Jacob and Monod, 1961). In the present study, high concentrations of glucose and galactose were shown to repress the constitutive expression of β-galactosidase in *Thermus* Rt41A (table 3.6); therefore in this organism catabolite repression may control synthesis of β-galactosidase.

3.4.3 The expression of β-glucosidase in thermophilic organisms

Glucosidases have a wide distribution having been reported in animal tissues, plants, microbes including microorganisms such as fungi, yeasts, and bacteria. In this study, a constitutive level of β-glucosidase was found in all five thermophilic strains and these results along with those from previous studies are summarized in table 3.10.

Table 3.10 The expression of β-glucosidase in thermophilic organisms

Organism	strain	expression	Reference
Cl. thermocellum	NCIB 10682	Constitutive	Ait et al. (1982)
Thermococcus	AN1	Constitutive	This study
S. solfataricus	DSM 182	Constitutive	This study
Thermus	Rt41A	Constitutive	This study
Cl. thermohydrosulfuricum	Rt8.B1	Constitutive	This study
Thermotoga	FjSS3.B1	Constitutive	This study

Increases above this constitutive level, i.e. a significant response were seen in the presence of added carbohydrate for most of the strains studied. The maximum increase in the expression of β-glucosidase was about 10-fold in *Thermus* Rt41A grown on cellobiose (table 3.6) and *Cl. thermohydrosulfuricum* Rt8.B1 grown on galactose (table 3.7). Both results reflected an increase in the constitutive activity as opposed to classical induction.

Previous studies have shown the thermophilic organism *Clostridium thermocellum* LQR1 to produce a constitutive level of cellulases i.e. cellulase and **exocellobiohydrolase** on cellobiose and cellulose. The level of β-glucosidase was also constitutive (Ait *et al.*, 1982). The β-glucosidase was not inhibited by glucose and exhibited a wide substrate specificity. Similar results were seen in this study with the β-glucosidase of *Cl. thermohydrosulfuricum* Rt8.B1 which was increased on cellobiose, but the constitutive level was unaffected by glucose (table 3.7). In one

instance, glucose was shown to be a strong repressor of the constitutive β-glucosidase in *Thermus* Rt41A (table 3.6) indicating some catabolite repression was occurring.

3.4.4 The expression of α-glucosidase in thermophilic organisms

The enzyme α-glucosidase is widely distributed in nature and is found in microorganisms, plants and in animals, where it is generally found in association with amylases (Kelly and Fogarty, 1983). This enzyme is produced by many microorganisms in response to growth on starch-containing media and is involved in the degradation of oligosaccharides produced by the action of amylolytic enzymes on starch. The enzyme has also been shown to be induced by maltose, maltotriose and maltosaccharides (Kelly and Fogarty, 1983).

In this study a constitutive level of α-glucosidase was found in the five thermophilic organisms studied and these results along with previous studies are summarized in table 3.11.

Table 3.11 The expression of α-glucosidase in thermophilic organisms

Organism	strain	expression	Reference
Pyrococcus furiosus	DSM 3638	Constitutive	Costantino et al. (1990)
Thermococcus	AN1	Constitutive	This study
S. solfataricus	DSM 182	Constitutive	This study
Thermus	Rt41A	Constitutive	This study
Cl. thermohydrosulfuricum	Rt8.B1	Constitutive	This study
Thermotoga	FjSS3.B1	Constitutive	This study

An increase above the constitutive level, i.e. an inductive response was seen in the presence of added carbohydrate for some of the strains tested. The maximum increase in the expression of α-glucosidase was 15-fold in *Cl. thermohydrosulfuricum* Rt8.B1 grown on galactose (table 3.7). The results again reflected an increase of the constitutive level as opposed to classical induction.

Other studies have reported a constitutive synthesis of α-glucosidase in thermophilic organisms. Costantino *et al.* (1990) have reported the production of a constitutive intracellular α-glucosidase in the hyperthermophilic archaea *Pyrococcus furiosus*. When grown in the absence of carbohydrate or in the presence of glucose, the specific activity of α-glucosidase was lower than when grown in the presence of carbohydrate containing α-1-4 linkages. Glucose added to medium containing starch did not repress the production of α-glucosidase to constitutive levels. Glucose was shown not to repress the synthesis of α-glucosidase in *Cl. thermohydrosulfuricum* Rt8.B1 in this study. With *Thermus* Rt41A high glucose concentrations (1.0 %) repressed the constitutive level of α-glucosidase again indicative of some catabolite repression by glucose.

3.4.5 Summary

In summary, induction and catabolite repression are classical control mechanisms of enzyme expression in *E. coli* and ensure that *E. coli* only synthesizes additional protein if an appropriate substrate is present and only if a better substrate is not available. With the thermophilic bacteria investigated in this study, a high constitutive level of enzyme synthesis was present under the culture conditions used, which was modified in the presence of added carbohydrate. This could either reflect a lack of control or a less elaborate control of enzyme synthesis in these organisms, which may be advantageous to these bacteria in environments low in nutrients. Such a mechanism may save valuable energy for growth which would be otherwise required in the synthesis of genetic machinery for such intricate control mechanisms such as occurs in *E. coli*. The widespread absence of highly regulated control may also be a reflection of the limited evolutionary divergence of thermophiles suggested by phylogenetic relationships or both.

Strobel and Russell (1987) have reported a different form of regulation than classical induction and repression for β-glucosidase in *Bacteroides ruminicola*. In this organism β-glucosidase synthesis was not under inductive control, β-glucosidase was high even

when cellobiose was absent. β-glucosidase synthesis showed catabolite repression by energy sources which yielded a high growth rate but at slow growth rates synthesis was controlled by a novel process termed growth rate-dependent derepression. Such a mechanism may play a role in regulating enzyme synthesis in thermophilic organisms, but more detailed studies are required to establish this in these bacteria.

Classically with induction studies, not only is the enzyme induced by its substrate but also repressed by a down-stream product or catabolite repressor which is frequently glucose. In this study, a significant increase was frequently seen with substrates which are not classical inducers of the enzyme system in mesophilic bacteria nor was there evidence of repression by an expected product. For example, the β-glucosidase was rarely inhibited by glucose in this study. Some classical regulation was seen with *Thermus* Rt41A where the constitutive level of β-glucosidase, β-galactosidase and α-glucosidase was repressed in the presence of high glucose concentrations indicative of catabolite repression.

The constitutive nature of enzyme synthesis observed in this study could be a direct consequence of the media used to grow these organisms. With many of the thermophilic organisms it is a major problem in studying enzyme induction and repression, as these organisms have complex nutritional requirements which in most instances can be satisfied only by the addition of yeast extract or trypticase peptone. Both these growth factors contain carbohydrates of unknown composition which could act as gratuitous inducers of enzyme synthesis. Only for *Thermus* Rt41A could this possibility be ruled out. Bryant (1986) reported that the constitutive level of β-galactosidase of *Thermococcus* sp. AN1 increased with increasing amounts of trypticase peptone (5 $g\ell^{-1}$ to 10 $g\ell^{-1}$) in the growth medium, suggesting that some induction was occurring with this growth supplement.

The enzymes β-galactosidase, β-glucosidase and α-glucosidase in the thermophilic organisms studied were shown to exhibit a broad range of substrates which increased their synthesis. For example, the β-galactosidase of *Thermococcus* AN1 was found to be present in high activity on lactose, starch, maltose, glucose and cellobiose (table

3.4). This could reflect a broad substrate specificity of the enzymes involved, that is the β-galactosidase may have the ability to hydrolyze cellobiose and lactose or maltose. Another example was the β-galactosidase of *Thermus* Rt41A which was present in high levels during growth on cellobiose, galactose and lactose. This organism exhibited a high level of β-glucosidase activity on cellobiose (table 3.6) and the β-galactosidase was sensitive to glucose, which in general represses β-glucosidase synthesis. These observations suggest that the β-glucosidase enzyme from this organism may have cross-reactivity for galactosides.

Other studies have shown that the enzymes β-glucosidase and β-galactosidase from thermophilic organisms have low affinities for their substrates and Goodman and Pederson (1976) suggest that the high k_m values observed with thermophilic enzymes may be due to a broad substrate specificity. They further suggest that although these enzymes may be primarily β-galactosidases, they may also be glycosidases with a specificity broader than is common with equivalent mesophilic enzymes. Patchett (1985) has reported that the β-galactosidase and β-glucosidase from the extreme thermophile Wai21W.2 has a broad substrate specificity, including the ability to hydrolyze both lactose and cellobiose. A recent survey of *Sulfolobus* isolates showed all to contain thermostable enzyme activities hydrolyzing various glycosidic compounds (Grogan, 1991). The study provided conclusive evidence that the β-galactosidase and β-glucosidase activities from *S. solfataricus* P2 were derived from the same enzyme. The β-glucosidase was found to have the same kinetics of inactivation and other biochemical properties of the β-galactosidase enzyme present in this strain. The chromogenic substrates β-D-galactosides and β-D-glucosides were hydrolyzed at a common site. From these observations the authors concluded that the activity of the β-galactosidase in this organism is only one of several activities of thermostable β-D-glycosidase.

The enzyme activities measured in this study were determined by measuring the activity on ρNPG substrates and assuming they are specific. The use of such synthetic substrates can be misleading. In this respect, β-glucosidase activity of the extremely thermophilic eubacterium *Thermotoga* FjSS3.B1 was shown to have a broad substrate

specificity (Ruttersmith and Daniel, 1992). Despite having activity against ρ-nitrophenyl β-D-glucopyranoside and ρ-nitrophenyl β-D-xylopyranoside, the β-glucosidase was not active on xylobiose. The reported specificities of many β-glucosidases described in the literature are based entirely on enzyme assays using synthetic substrates. The results reported by Ruttersmith and Daniel (1992) suggest that this can be misleading. Similar broad substrate specificities were observed in this study.

The results of this study are preliminary and it is difficult to draw firm conclusions in terms of the classical definition of induction. This is due to a combination of factors; the undefined nature of the growth media and the possibility of gratuitous induction even in the control cultures and the low specificity of some thermophilic glycosidases which hampers the interpretation of results. Until a medium can be developed which allows the growth of these organisms in the absence of complex growth factors, conclusions about enzyme induction and repression are difficult to make. Only for *Cl. thermohydrosulfuricum* Rt8.B1 was it possible to develop a defined minimal medium. The other thermophilic organisms could not grow in the absence of complex growth factors. Because of this *Cl. thermohydrosulfuricum* Rt8.B1 was chosen for further study.

CHAPTER FOUR

DEVELOPMENT OF A DEFINED MINIMAL MEDIUM FOR ANAEROBIC GROWTH OF *Clostridium thermohydrosulfuricum* Rt8.B1

4.1 INTRODUCTION

To carry out a study of the key metabolic pathways and uptake systems involved in carbohydrate utilization it is necessary to develop a simple defined medium with no or little organic supplement. Such a medium is essential if induction or repression of transport proteins or metabolic enzymes are to be studied. Most metabolic and physiological studies on thermophilic bacteria to date have employed complex media (e.g., Lamed and Zeikus, 1980b; Ben-Bassat *et al.*, 1981; Hernandez, 1982; Ng and Zeikus, 1982; Cook, 1988; DeVrij *et al.*, 1989; Heyne *et al.*, 1991) which contain high levels of yeast extract and trypticase peptone. This is necessary because of the fastidious vitamin and amino acid requirements of most thermophilic organisms, which can be met by the inclusion of complex nutritional sources in the growth medium. For example, the recommended medium for the thermophilic glycolytic anaerobe *Clostridium thermohydrosulfuricum* Rt8.B1 is the trypticase peptone-yeast extract-glucose (TYEG) as described by Zeikus *et al.* (1979). This medium contains 3.0 g of yeast extract ℓ^{-1} and 10.0 of trypticase peptone ℓ^{-1}. Both components contain carbohydrates of unknown composition, with yeast extract containing 15 % carbohydrate and trypticase peptone trace levels. These carbohydrates could act as gratuitous inducers of metabolic pathways and transport proteins.

This chapter outlines the development of a defined minimal medium capable of supporting growth of *Cl. thermohydrosulfuricum* Rt8.B1 and suitable for physiological studies on its carbohydrate utilization.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

Vitamin-free casamino acids were purchased from Difco Laboratories (Michigan, USA). Yeast extract and trypticase peptone were obtained from Becton Dickinson Microbiology Systems (Maryland, USA).

4.2.2 Bacterial strains

Thermoanerobium brockii DSM 1457, *Clostridium thermosaccharolyticum* DSM 571, *Thermobacteroides acetoethylicus* DSM 2359, *Thermoanaerobacter ethanolicus* DSM 2246, *Clostridium thermolacticum* DSM 2910 and *Thermoanaerobacter finnii* DSM 3389 were obtained from the Deutsche Sammlung von Mikroorganismen (DSM, Braunschweig, Germany). *Cl. thermohydrosulfuricum* Rt8.B1, *Thermoanaerobium brockii* Rt8G4.1, *Thermoanerobium brockii* Tok6.B1, *Thermotoga* FjSS3.B1, "Caldocellum saccharolyticum" Tp8T 6.3.3.1 and *Clostridium fervidus* Rt4.B1 were from the collection of our laboratory.

4.2.3 Media and cultivation conditions

Cl. thermohydrosulfuricum Rt8.B1 was maintained by fortnightly subculture on trypticase peptone-yeast extract-glucose (TYEG) medium (Zeikus *et al.*, 1979). The other organisms used in this study were all grown on the minimal medium described in this chapter. All media were prepared anaerobically as described in section 3.2.3.

All vitamin and trace element solutions were prepared as previously described (see section 3.2.3).

4.2.4 Enzyme assays

β-galactosidase, β-glucosidase and α-glucosidase were assayed as described in section 3.2.5.

4.3 RESULTS

4.3.1 Development of a defined minimal medium

A minimal medium was developed using the basic components of TYEG medium (Zeikus *et al.*, 1979), with the omission of yeast extract and trypticase peptone. The composition of the minimal medium is shown in table 4.1.

Table 4.1 Minimal medium used to determine the growth requirements of *Cl. thermohydrosulfuricum* Rt8.B1

Component	g/l
NH_4Cl	0.90
$MgCl_2.6H_2O$	0.20
NaCl	0.90
KH_2PO_4	0.75
K_2HPO_4	1.50
Trace element solution	9.0 ml
10 % $FeSO_4$	0.03 ml
0.2 % resazurin	1.0 ml
MOPS	10.0
Neutralized Na_2S	7.0 ml

Carbon sources were added to this medium from filter sterilized stock solutions to give the appropriate concentration required. For all experiments involving the optimization of growth factors, D-glucose was added at a final concentration of 5 $g\ell^{-1}$.

In general, the medium contains a mix of simple inorganic salts essential for various metabolic functions and a trace element solution consisting of various metal ions. Due

to the absence of yeast extract and trypticase peptone, which have some buffering capacity, a buffer, MOPS, was included to give a final concentration of 50 mM.

The initial aims of this work were to replace the complex growth factors yeast extract and trypticase with a simple vitamin solution. It was not the intentions of this study to specify the exact individual vitamin requirements of *Cl. thermohydrosulfuricum* Rt8.B1. Three vitamin solutions were chosen for their ability to support growth in the absence of yeast extract and trypticase peptone. These were: Wolin's vitamins (Wolin, 1963), SL-7 (Janssen, 1989) and a modified Wolin's vitamin solution termed MM vitamins. Wolin's vitamins have been used as a vitamin supplement for some thermophilic bacteria in media containing yeast extract and /or trypticase peptone. The vitamin mix SL-7 has been used as a vitamin supplement to grow various organic acid-fermenting bacteria from anaerobic sediments (Janssen, 1989). The vitamin solution SL-7 which contains lower concentrations of growth factors was included in this study as a comparative source of vitamins. The components of each vitamin solution are listed in Table 4.2.

Table 4.2 Components of vitamin solutions used in study

Component[a]	MM vitamins	Wolin's vitamins	SL-7 vitamins
d-biotin	1.0	2.0	0.01
ρ-aminobenzoic acid	1.0	5.0	0.10
Nicotinic acid	10.0	5.0	1.0
Pantothenate (hemi-Ca^{2+} salt)	5.0	5.0	0.50
Pyridoxamine.$(HCl)_2$	5.0	-	0.50
Thiamine.HCl	10.0	-	1.0
Pyridoxine.HCl	5.0	10.0	-
Pyridoxal.HCl	5.0	-	-
Flavin adenine dinucleotide	1.0	-	-
DL-6,8 thioctic acid	1.0	5.0	-
Folic acid	3.0	2.0	-
Vit B_{12}	-	0.10	-
Cyanocobalamin	-	-	0.20
Riboflavin	-	-	-

[a]All concentrations are mg.ℓ^{-1} distilled water

Yeast extract and trypticase peptone contain the vitamins thiamine.HCl, pyridoxal.HCl and pyridoxamine.$(HCl)_2$ hence the inclusion of these in the MM vitamin mix. These vitamins and their phosphorylated derivatives comprise the vitamin B_6 group and are important for various anabolic and catabolic functions in bacteria. For example, pyridoxal.HCl is the most widely used growth factor and pyridoxal-5-phosphate and pyridoxamine-5'-phosphate participate in a large number of reactions involving the synthesis and degradation of the naturally occurring α-amino acids. Thiamine pyrophosphate functions in the decarboxylation of α-keto acids and in transketolase reactions. A large number of bacteria are auxotrophic for flavin adenine dinucleotide (FAD) which is a major co-enzyme form of riboflavin that functions in oxidoreduction reactions. FAD was included in the MM vitamin mix in place of riboflavin.

In all experiments the following protocol was followed: To determine vitamin and growth requirements, an initial inoculum was grown in TYEG medium for 24 hours at 70° and added as a 2 % (v/v) inoculum to minimal medium containing vitamins but lacking yeast extract and trypticase peptone. Cells were cultured for 24 hours or until growth was apparent, the carry-over of growth factors allowing growth to occur in the first transfer. These cells were used to inoculate (2 % v/v) 10 ml amounts of minimal medium containing various concentrations of growth factors (second transfer). The growth in these tubes was subcultured a third time (2 % v/v) and growth (absorbance at 650 nm) was measured after 24-48 hours.

Experiments were set up to determine if *Cl. thermohydrosulfuricum* Rt8.B1 could grow in the absence of yeast extract and trypticase peptone. Tubes were set up containing individual vitamin solutions at concentrations between 0.25 and 1.0 ml per 10 ml of minimal medium containing glucose. The addition of vitamins at 1.0 ml /10 ml represents a 20-fold concentration excess above the level at which they are normally incorporated in other media supplemented with yeast extract and trypticase peptone. The results of these experiments are shown in table 4.3.

Table. 4.3 **The effect of vitamin addition on the growth of *Cl. thermohydrosulfuricum* Rt8.B1 in minimal medium containing glucose.**

		Optical density (650 nm)		
Vitamin solution	Vol (ml)	1st transfer (24 hours)	2nd transfer (36 hours)	3rd transfer (36 hours)
MM vitamins	0.10	0.08	NG	NG
	0.25	0.15	NG	NG
	0.50	0.24	0.09	NG
	1.00	0.36	0.17	NG
Wolin's vitamins	0.10	0.06	NG	NG
	0.25	0.06	NG	NG
	0.50	0.08	NG	NG
	1.00	0.24	0.14	NG
SL-7	0.10	0.06	NG	NG
	0.25	0.07	NG	NG
	0.50	0.07	NG	NG
	1.00	0.13	NG	NG

NG = no growth.

For all three vitamin solutions, growth of *Cl. thermohydrosulfuricum* Rt8.B1 was seen in the absence of yeast extract and trypticase peptone. Growth was evident after the first transfer from TYEG medium into minimal medium containing the individual vitamin solutions. The greatest amount of growth was obtained with the MM vitamin mix and Wolin's vitamins. A small amount of growth was detected with SL-7. The second transfer from minimal medium to minimal medium resulted in a decreased level of growth for all vitamin solutions and no growth was seen with SL-7. The final transfer resulted in no growth in any of the vitamin solutions. These results are consistent with a carryover effect of growth factors from the initial inoculum developed in TYEG medium. Growth was not detected after these growth factors were diluted out.

From these results it was concluded that the addition of a concentrated vitamin solution alone to minimal medium containing glucose could not replace yeast extract or trypticase peptone for the growth of *Cl. thermohydrosulfuricum* Rt8.B1. This suggested that additional growth factors were required for the growth of this organism.

Yeast extract and trypticase peptone contain amino acids at various concentrations. In the absence of yeast extract and trypticase peptone, growth may be limited by some essential amino acids or peptides. Experiments were set up to determine the effects of different sources of amino acids on the growth of *Cl. thermohydrosulfuricum* Rt8.B1. The following sources of amino acids were tested: Vitamin-free casamino acids (produced from the acid hydrolysis of casein to the amino acid stage and containing no added carbohydrate) and the amino acids present in yeast extract and trypticase peptone prepared as separate stock solutions and added to minimal medium at a final concentration equal to that of added yeast extract or trypticase peptone. The amino acid compositions of yeast extract and trypticase peptone were obtained from the manual of BBL products and laboratory procedures (Sixth Edition, 1988). Inocula and transfers were done exactly as for the vitamin experiments. All amino acid sources were added in combination with the MM vitamin solution. Growth on trypticase peptone was included for a comparison. The results of these experiments for the 3rd and final transfer are shown in table 4.4.

The results of these experiments showed that amino acids are important as growth factors for *Cl. thermohydrosulfuricum* Rt8.B1 when grown in minimal medium containing glucose. The greatest amount of growth was seen with the growth supplements trypticase peptone and vitamin-free casamino acids. In comparison, growth on the amino acid stock solutions resulted in lower final optical densities.

These results demonstrate that vitamin-free casamino acids are a good source of amino acids for the growth of *Cl. thermohydrosulfuricum* Rt8.B1. A mixture of amino acids in the same proportions as found in yeast extract and trypticase peptone can serve as a source of amino acids. The fact that better growth was detected with trypticase peptone and vitamin-free casamino acids alone, suggested that other growth factors or

different amino acids are required for growth of this organism and these cannot be replaced solely by the amino acids present in trypticase peptone and yeast extract.

Table 4.4 The effect of growth supplements and amino acids on the growth of *Cl. thermohydrosulfuricum* Rt8.B1 on minimal medium containing glucose

Growth supplement ¶	Optical density (650 nm)
Trypticase peptone	0.68
Trypticase peptone amino acids	0.32
Yeast extract amino acids	0.25
Vitamin-free casamino acids	0.63

¶ = All growth factors and the MM vitamin solution were added as 1.0 ml solutions to 10.0 ml of minimal medium. Trypticase peptone and casamino acids were added at 1.0 $g\ell^{-1}$. Optical densities are the means of three values.

On the basis of these results, it was decided to use vitamin-free casamino acids as the added source of amino acids for the growth of *Cl. thermohydrosulfuricum* Rt8.B1 on the minimal medium. A series of experiments similar to those described for the vitamins and amino acid studies were set up to test the three individual vitamin solutions in the presence of vitamin-free casamino acids. Growth on trypticase peptone was included for a comparison. The results of these experiments are presented in table 4.5.

The greatest amount of growth for *Cl. thermohydrosulfuricum* Rt8.B1 occurred on minimal medium containing glucose and either vitamin-free casamino acids or trypticase peptone together with the MM vitamin solution. The final optical density obtained with this vitamin mix was 0.62 with casamino acids and 0.78 with added trypticase peptone. The overall final optical density for these vitamin solutions was higher on trypticase peptone than casamino acids.

Table 4.5 **The effect of different vitamin solutions on the growth of *Cl. thermohydrosulfuricum* Rt8.B1 in the presence of casamino acids and trypticase peptone**

Vitamin solution ¶	Casamino acids (g/l)	Trypticase peptone (g/l)	Optical density (650 nm) (24 hours)
Wolin's	1.00	-	0.28
	-	1.00	0.43
MM vitamins	1.00	-	0.62
	-	1.00	0.78
SL-7	1.00	-	0.07
	-	1.00	0.11

¶ = All vitamin solutions were added at 1.0 ml per 10 ml of minimal medium. All optical densities are shown for the 3rd and final transfer.

MM vitamin mix was therefore chosen as the source of vitamins used to grow *Cl. thermohydrosulfuricum* Rt8.B1 with minimal medium. In order to determine the optimum levels of added vitamins and vitamin-free casamino acids, *Cl. thermohydrosulfuricum* Rt8.B1 was grown on a range of vitamin and vitamin-free casamino acid concentrations and the final optical density measured. The results of these experiments are presented in table 4.6.

Table 4.6 **The effect of MM-vitamins and vitamin-free casamino acid concentrations on the growth of *Cl. thermohydrosulfuricum* Rt8.B1**

MM vitamins (ml /10 ml)	Casamino acids (g/l)	Optical Density (650 nm) (24 hours)
0.10	0.50	0.17
0.25	0.50	0.36
0.50	0.50	0.44
1.00	0.50	0.47
0.10	1.00	0.32
0.25	1.00	0.54
0.50	1.00	0.52
1.00	1.00	0.53
0.10	2.00	0.41
0.25	2.00	0.72
0.50	2.00	0.78
1.00	2.00	0.89
0.10	3.00	0.41
0.25	3.00	0.78
0.50	3.00	0.90
1.00	3.00	1.15

The results demonstrate that increasing the concentration of vitamin-free casamino acids from 0.5 to 3.0 $g\ell^{-1}$ produced an overall increase in the final optical density. At concentrations of vitamin-free casamino acids from 0.50 to 1.0 $g\ell^{-1}$, increasing the level of MM vitamins to 0.50 ml /10 ml of minimal medium increased the final optical density. Vitamin concentrations above this level did not significantly result in further growth. At vitamin-free casamino acids concentrations of 2.0 $g\ell^{-1}$, an increase in the volume of added vitamins up to 1.0 ml increased the final optical density up to 0.89. Similar results were seen with vitamin-free casamino acid concentrations of 3.0 $g\ell^{-1}$ with a final optical density of 1.15. The effect of casamino acids on growth was not due to a buffering effect on the final pH limiting growth. All final pH values were

between 5.5 and 6.0 and not dependent on the level of vitamin-free casamino acids present. Therefore the effect of vitamin-free casamino acids was a direct stimulation of growth

In summary, added vitamin solution and casamino acids increased the final optical density with increasing concentrations. There were however, optimum concentrations where the addition of further MM vitamins did not increase the final optical density significantly. This concentration was 50 ml per litre for the vitamin stock solution. With vitamin-free casamino acids good growth was shown with 3.0 $g\ell^{-1}$ and this was the final concentration used.

On the basis of the above results, the medium tabulated in Table 4.7 was used to grow *Cl. thermohydrosulfuricum* Rt8.B1 in further physiological studies on this organism.

Table 4.7 **Final medium composition used to grow *Cl. thermohydrosulfuricum* Rt8.B1**

Component	g/l
NH_4Cl	0.90
$MgCl_2.6H_2O$	0.20
NaCl	0.90
KH_2PO_4	0.75
K_2HPO_4	1.50
Trace element solution	9.0 ml
10 % $FeSO_4$	0.03 ml
0.2 % resazurin	1.0 ml
MOPS	10.0
Neutralized Na_2S	7.0 ml
MM vitamin solution	50.0 ml
Vitamin-free casamino acids	3.0

To demonstrate the defined nature of this medium in contrast to TYEG, *Cl. thermohydrosulfuricum* Rt8.B1 was grown on both media in the absence of added carbon source. The final optical density on the minimal medium was 0.08 and with TYE medium (TYEG minus glucose) the optical density was 0.21. The growth rates

were significantly higher on TYEG (0.55 h^{-1}) compared with the minimal medium containing glucose (0.33 h^{-1}).

The defined minimal medium developed was tested with a number of thermophilic isolates for their ability to grow on this medium. A summary of these results is presented in table 4.8.

Table 4.8 Growth of thermophilic anaerobic bacteria on minimal medium containing glucose

Thermophilic isolate	Strain	Growth	Sporulation
Cl. thermohydrosulfuricum	Rt8.B1	+++	spores
Cl. thermosaccharolyticum	DSM 571	++	spores
Cl. thermolacticum	DSM 2910	+	spores
Cl. fervidus	Rt4.B1	-	negative
T. brockii	DSM 1457	+++	spores
T. brockii	Rt8G4.B1	+++	negative
T. brockii	Tok6.B1	+++	negative
Ta. finii	DSM 3389	+	spores
Ta. ethanolicus	DSM 2246	++	negative
Tb. acetoethylicus	DSM 2359	+	negative
Caldocellum saccharolyticum	Tp8T 6.3.3.1	+	negative
Thermotoga	FjSS3.B1	+	negative

All strains were grown on 5 $g\ell^{-1}$ of D-glucose.
(+) = poor growth, (++) = good growth, (+++) excellent growth.

The only thermophilic isolate tested unable to grow on this minimal medium was *Clostridium fervidus* Rt4.B1. *Cl. fervidus* Rt4.B1 has been shown to have complex nutritional needs and has an absolute requirement for the addition of yeast extract or trypticase peptone. These growth factors could not be substituted by vitamins or casamino acids (Patel *et al.*, 1987).

A major observation when the thermophilic anaerobic isolates listed in table 4.8 were grown with this defined minimal medium was that most strains exhibited a change in morphology. For example, *T. brockii* HTD4 grown on TYEG medium produced cells which were short rods with characteristic mini-cells (see chapter 5, figure 5.1). When *T. brockii* HTD4 was transferred from TYEG into the defined minimal medium cells became longer and thinner (see chapter 5, figure 5.2).

Another important observation was the incidence of sporulation for sporulating strains which was markedly increased on this minimal medium. No spores were seen when sporulating strains were grown on TYEG. Selected strains were tested for their ability to utilize D-xylose as the sole carbon source when grown on this medium. When grown on this medium an apparently non-sporeforming species *T. brockii* HTD4 produced unusual terminal swellings which appeared to be spores. This was significant because this organism has frequently been referred to as a *Clostridium*-like isolate, but previous and extensive attempts to induce sporulation have been unsuccessful. Although not directly related to the main thrust of this thesis, this observation was thought to be of sufficient interest to merit further investigation. The results of this investigation are reported in chapter five.

The results for enzyme expression of *Cl. thermohydrosulfuricum* Rt8.B1 presented in chapter three (see section 3.3.2.2) showed that β-galactosidase, β-glucosidase and α-glucosidase were synthesized constitutively. In these preliminary studies, *Cl. thermohydrosulfuricum* Rt8.B1 was grown on TYEG medium containing high levels of trypticase peptone and yeast extract both of which contain carbohydrate of unknown composition which could induce enzyme synthesis.

The defined minimal medium developed as described above was therefore used to grow *Cl. thermohydrosulfuricum* Rt8.B1 and the assays described in chapter three repeated. The results of these experiments are shown in table 4.9

Table 4.9 **Enzyme activities in cultures of *Cl. thermohydrosulfuricum* Rt8.B1 grown on different carbon sources and a defined medium**

	ß-galactosidase			ß-glucosidase		
Carbon source	S.Act	% Act	I	S.Act	% Act	I
Basal	8.60	100	-	5.25	100	-
LACTOSE	44.80	521	Y	42.30	805	Y
lactose	NA	-	N	-	NA	N
STARCH	12.20	147	N	69.50	1322	Y
starch	NA	-	N	-	NA	N
CELLOBIOSE	12.20	251	N	83.60	1590	Y
cellbiose	NA	-	N	-	NA	N
MALTOSE	10.70	124	N	28.00	533	Y
maltose	38.40	447	Y	26.50	504	Y
GALACTOSE	34.90	406	Y	33.81	644	Y
galactose	172.00	2000	Y	-	-	N
GLUCOSE	6.76	<100	N	5.16	<100	N
glucose	34.80	404	Y	27.20	518	Y

S.Act = specific activity expressed as nanomoles per minute per mg of protein at 70°C; % Act = percentage activity of basal level; I = significant response (Y), no significant response (N); NA = no activity detected. D-xylose was used as the carbon source in the basal medium. Carbon sources at 1 % (w/v) are in uppercase type and 0.1 % (w/v) lowercase type.

Cl. thermohydrosulfuricum Rt8.B1 grown on minimal medium containing different carbon sources exhibited constitutive β-glucosidase and β-galactosidase. No α-glucosidase activity was detected. A significant increase in β-galactosidase activity was seen with galactose, lactose, maltose and glucose.

A significant response of β-glucosidase activity in *Cl. thermohydrosulfuricum* Rt8.B1 was shown with all carbon sources used for growth.

4.4 DISCUSSION

In many of the anaerobic thermophiles some growth factors are required for optimum growth and these are frequently supplied by the addition of yeast-extract and /or trypticase peptone. To study the true anabolic and catabolic capabilities of an organism it is desirable to know the nutritional requirements of an organism and to use chemically defined medium. In the literature there are few reports of minimal medium used in this respect for thermophilic bacteria. A defined minimal medium for the growth of *Cl. thermohydrosulfuricum* Rt8.B1 was developed in this study. The minimal medium contained no yeast extract or trypticase peptone. These complex growth factors were replaced with a simple vitamin solution and vitamin-free casamino acids.

The defined minimal medium was able to support the growth of several thermophilic anaerobes. Most thermophilic strains showed morphological changes, normally from short fat rods to thinner longer rods. In one instance spores were seen with an apparently non-sporulating strain *T. brockii* DSM 1457.

Other studies with thermophilic bacteria have demonstrated that yeast extract and trypticase peptone can be replaced with simple growth factors. For example, *Spirochaeta thermophila* strain Z-1203 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 (Aksenova *et al.*, 1992). The results of this study demonstrated that amino acids are important for the growth of *Cl. thermohydrosulfuricum* Rt8.B1 and that these could be supplied in the form of vitamin-free casamino acids.

Detailed studies of other workers have shown that individual vitamins can replace yeast extract. This has been reported for the thermophilic acetogens *Clostridium thermoautotrophicum* JW 701-3 (Savage and Drake, 1986) and *Cl. thermoaceticum*

(Lundie and Drake, 1984). These authors were able to demonstrate that nicotinic acid was the sole essential vitamin required for growth on glucose. Biotin was shown to be stimulatory to cell lines grown on glucose. Growth on such a minimal medium was shown to involve a relatively long adaptation time and involved a sequential passage from undefined medium containing yeast extract and trypticase peptone to minimal defined medium supplemented with amino acids and vitamins. Similarly, Johnson *et al.* (1981) were able to replace the yeast extract of complex media to yield a minimally defined medium for growth of *Clostridium thermocellum* ATCC 27405. *Cl. thermocellum* was shown to require four vitamins biotin, pyridoxamine, B_{12} and ρ-aminobenzoic acid. Amartey *et al.* (1991) described a defined medium for the aerobic growth of *Bacillus stearothermophilus* LLD-15 in genetic and physiological studies. These authors demonstrated that methionine, biotin, nicotinic acid and thiamine were absolute requirements for growth, completely replacing yeast extract. The aim of this study was not to specify the exact individual vitamin requirements of this organism, but simply substitute for the yeast extract and trypticase peptone.

The defined minimal medium developed in this study was used to study the expression of β-galactosidase, β-glucosidase and α-glucosidase of *Cl. thermohydrosulfuricum* Rt8.B1.

The results of this study demonstrate that β-galactosidase and β-glucosidase activity were constitutive in *Cl. thermohydrosulfuricum* Rt8.B1 grown on a defined minimal medium. These results are in agreement with those shown on the undefined medium TYEG (see section 3.3.2.2) and suggest that the effects of gratuitous inducers can be ruled out. A 20-fold increase in the level of β-galactosidase was shown during growth on galactose and represents a significant increase above pre-existing activity. The classical inducer of β-galactosidase, lactose, increased the level of enzyme 5-fold. Glucose is a classical repressor of β-galactosidase activity in *Escherichia coli*. In *Cl. thermohydrosulfuricum* Rt8.B1 glucose (0.1 %) increased the level of β-galactosidase and therefore classical repression by this carbon source was absent for the control of this enzyme.

The enzyme β-glucosidase was increased maximally on cellobiose (0.1 %) which is a classical inducer of this enzyme in other bacteria. A significant response was shown on glucose (0.1 %) again demonstrating a lack of classical control in enzyme synthesis of β-glucosidase.

The results obtained using the defined medium support the view that classical control mechanisms of enzyme synthesis are absent or not expressed under the culture conditions used for *Cl. thermohydrosulfuricum* Rt8.B1 and that presumably other factors operate to control the expression of enzyme synthesis.

Previous studies (Cook, 1988) demonstrated that hyperbolic growth kinetics were common with thermophilic bacteria grown on a glucose + xylose substrate mix. This feature could be a consequence of the undefined medium used in that study. It was therefore decided to investigate this feature in more detail using the defined minimal medium developed here and determine at what level of control dual substrate utilization is regulated. The studies on uptake and utilization of glucose and xylose, and aspects of their control are described in chapters 6-8.

The following chapter is a digression; it describes a brief study of the sporulation which was observed to occur in *Thermoanaerobium brockii* HTD4 on the defined medium.

CHAPTER FIVE

ENDOSPORE FORMATION BY *Thermoanaerobium brockii* HTD4

5.1 INTRODUCTION

Thermoanaerobium brockii HTD4 was first isolated from thermal areas of Yellowstone National Park by Zeikus *et al.* (1979). The organism is a Gram-positive, obligately anaerobic thermophile with a mol %G+C of 30-32 and was originally described as a non-spore forming species (Zeikus *et al.*, 1979). Subsequent isolates of the genus have also failed to produce observable spores (Morgan *et al.*, 1985; Kondratieva *et al.*, 1989).

In the course of growing the type strain of *T. brockii* (strain HTD4, DSM 1457) on the minimal medium developed to study enzyme expression (see chapter four) the formation of endospores was observed which could not be attributed to a contaminant. This observation lead to an investigation of this feature.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains

T. brockii HTD4 (DSM 1457), and *Clostridium thermosaccharolyticum* DSM 571 were obtained from the Deutsche Sammlung von Mikroorganismen (DSM, Braunschweig, Germany). *Clostridium thermohydrosulfuricum* strain Rt8.B1 was from the Thermophile Research Unit culture collection (Patel *et al.*, 1986).

5.2.2 Media and cultivation conditions

T. brockii HTD4 was maintained by fortnightly subculture on the trypticase peptone-yeast extract-glucose (TYEG) medium of Zeikus *et al.* (1979). Media were prepared

anaerobically as described in section 3.2.3. The defined minimal medium (MM) used to grow *T. brockii* for sporulation studies was as described in section 4.3.1.

Carbon sources were prepared anaerobically in distilled water as concentrated stock solutions and sterilized by autoclaving. They were added to sterile MM to give a final concentration of 5 $g\ell^{-1}$. All sugars were D-isomers unless noted otherwise. Soil extract was prepared as described by Norris *et al.* (1981), boiled and autoclaved under a nitrogen atmosphere.

Incubations were at 65°C unless otherwise noted. Plates were incubated in anaerobic jars under an N_2 gas atmosphere.

5.2.3 Cellular characterization

Phase contrast photomicrographs were taken using a Reichert-Jung Polyvar microscope (C. Reichert, Vienna, Austria). Cells were immobilized on agar-coated microscope slides as described by Pfennig and Wagener (1986). The methods used for preparing cells for thin sectioning and electron microscopic examination were as described by Zeikus and Bowen (1975). Thin sections were stained with 1 % uranyl acetate followed by 1 % lead citrate, then examined under a Phillips EM 400 electron microscope at an accelerating voltage of 80kV.

5.2.4 Heat-treatment of cultures

Cl. thermohydrosulfuricum Rt8.B1 and *T. brockii* HTD4 grown on TYEG medium were inoculated into sets of tubes containing 5 ml of either TYEG or minimal medium containing xylose (MMX). These cultures were incubated into stationary phase at 65°C. The tubes were then immersed completely in an oil bath at 115°C. The time taken for control tubes containing a thermistor probe immersed in 5 ml of medium to reach this temperature was 5 min. At intervals, tubes for both cultures were removed and quickly cooled to room temperature. Viability of the cultures was determined by inoculating 0.1 ml aliquots into tubes of both TYEG and MMX and incubating at

65°C. From all tubes showing growth, samples were streaked onto TYEG agar and incubated anaerobically in anaerobic jars. Well-isolated colonies were checked for cell morphology, and inoculated into TYEG broth for end product analysis by HPLC (Patel *et al.*, 1987).

5.3 RESULTS

5.3.1 Growth on minimal medium containing xylose

Spore formation was observed when cultures of *T. brockii* HTD4 grown on TYEG were transferred into MMX. During the late logarithmic phase of growth on MMX, some cells formed terminal swellings which became phase-refractile and resembled mature spores. These structures were not observed when transferring from TYEG medium to TYEG medium, or from MMX to MMX. There seemed to be an optimum period of 24-48 hours in MMX to produce maximum sporulation, after which time the number of cells sporulating decreased. The maximum level of sporulation in MM was obtained using D-xylose as the sole carbon source (MMX). In some instances an increase was seen in the number of observable spores when 0.5 mM theophylline, 0.2% (v/v) soil-extract or L-xylose (5 $g\ell^{-1}$) were added in conjunction with D-xylose. Sporulation was not detected on MM containing the following growth-supporting carbon sources: glucose, cellobiose, lactose, maltose, mannose, pullulan, starch, galactose, sodium pyruvate and sucrose. Pectin, dextran, cellulose, sodium L-tartrate, sodium DL-lactate, ethanol, tryptone, casamino acids, L-arabinose and L-arginine did not support growth. Attempts to induce sporulation as described by Zeikus *et al.* (1979) i.e. growth on the medium of Duncan and Strong, (1968), GC medium and D-xylose /soil-extract medium (Hollaus and Sleytr, 1972) gave negative results.

5.3.2 Cellular features

The morphology of *T. brockii* HTD4 varied depending on the culture conditions. Growth on TYEG broth produced single and paired cells, and cells in chains with characteristic mini-cells present (figure 5.1). When grown on MMX, cells were longer

and thinner and sometimes contained spores (figure 5.2). The spores were round, terminal, distended the cell, and were brightly refractile under phase contrast microscopy. Non-refractile structures could be observed in the same position within the cell; these were assumed to be pre-spores. Dark inclusion bodies were also observed within the cells (figure 5.2) which were readily distinguishable from both spores and pre-spores by their shape and position. Under the appropriate conditions, spores were observed in approximately one cell in a thousand, while pre-spores were more numerous, approximately 7 cells in a thousand.

Electron microscopic observation of thin sections of sporulating cells showed a typical mono-layered cell wall (figure 5.3) as described by Zeikus *et al.* (1979), quite distinct from the double layered cell wall type found in *Cl. thermohydrosulfuricum* (Sleytr and Glauert, 1976). Around the spore could be seen a number of concentric membrane-like structures (termed "lamellar" stuctures, figure 5.3) which were probably part of the spore coat. Cell division appeared to be by a constrictive or "pinching off" type of division (figure 5.4) typical of *T. brockii* (Zeikus *et al.*, 1979) together with characteristic mesosomal structures also observed by Zeikus *et al.* (1979) at the division site of *T. brockii.*

5.3.3 Demonstration of heat resistance

To ascertain if the structures seen had the heat resistant property of true endospores, cultures were incubated at 115°C. Cultures of *T. brockii* HTD4 and *Cl. thermohydrosulfuricum* Rt8.B1 grown on TYEG broth at 65°C survived an exposure of 5 min at 115°C but no viable cells remained after 10 min exposure. In contrast, cultures of both organisms grown on MMX at 65°C (conditions found to induce sporulation) survived over 80 min exposure to 115°C.

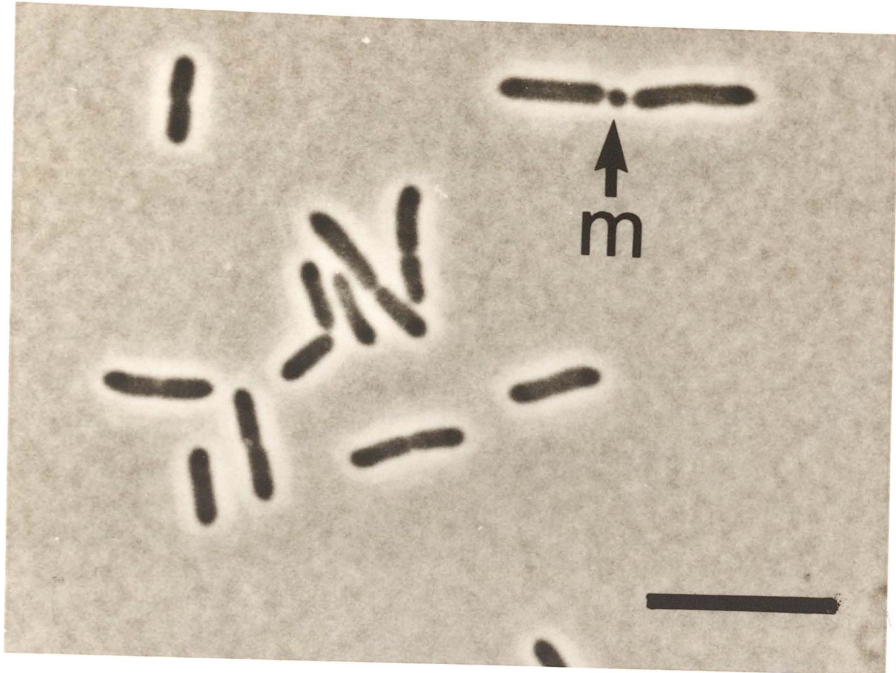


Figure 5.1 Phase contrast photomicrograph of *T. brockii* HTD4 grown on TYEG with characteristic mini-cell (m) present. Bar = 10μm.

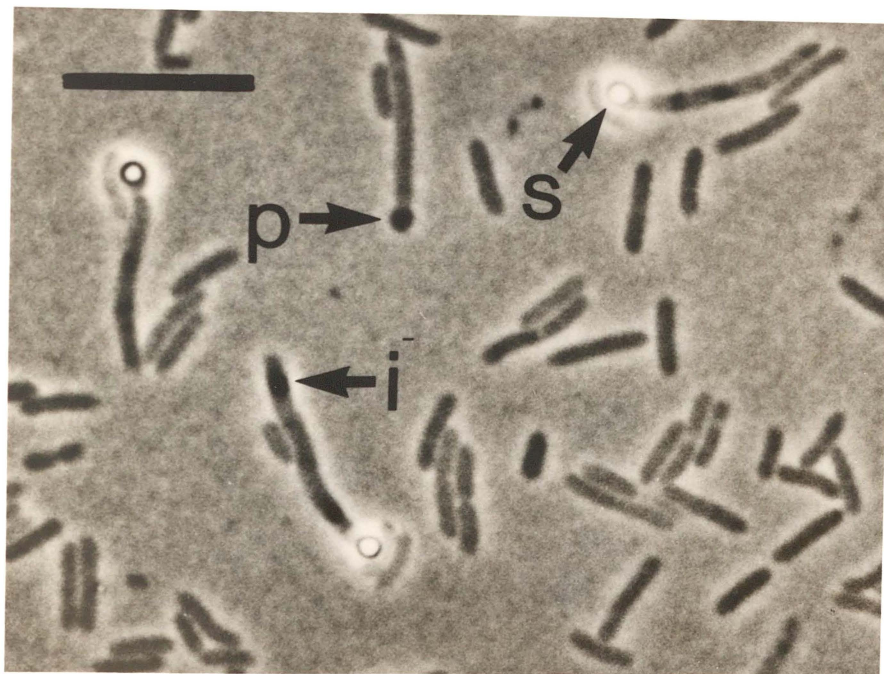


Figure 5.2 Phase contrast photomicrograph of *T. brockii* HTD4 grown on minimal medium containing xylose. Features observed in cells grown under these conditions include refractile spores (s), non-refractile pre-spores (p), and inclusion bodies (i). Bar = 10μm.

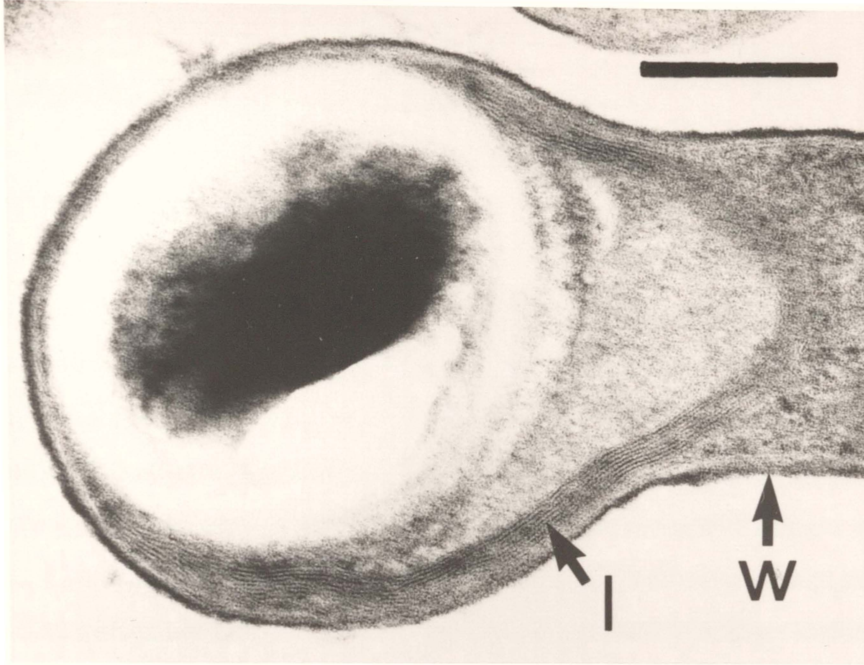


Figure 5.3 Electron micrograph ultrathin section of a spore in *T. brockii* HTD4 grown on minimal medium containing xylose, showing the mono-layered cell wall (w) and lamellar structure (l) around the spore. Bar = 0.2μm.

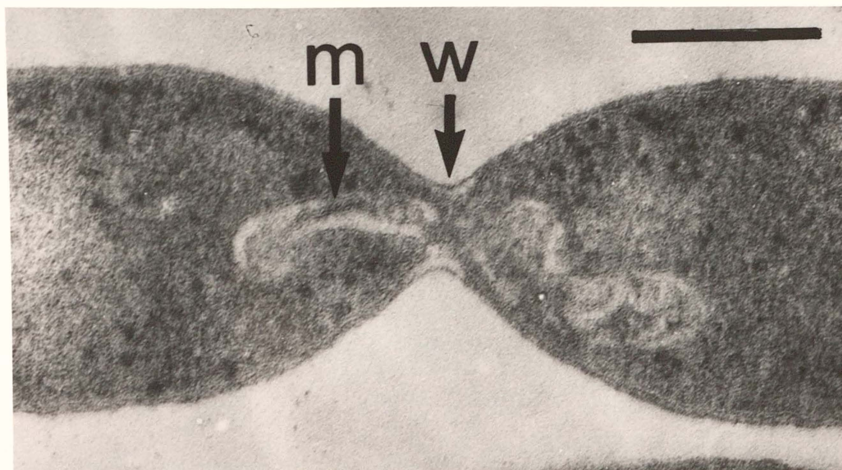


Figure 5.4 Electron micrograph of ultrathin section of *T. brockii* HTD4 showing the typical constrictive or pinching off division with the mono-layered cell wall (w) and characteristic mesosomal structures (m). Bar = 0.2 μm.

5.3.4 Check of purity at end of heat-treatment

To check for purity of heat-resistant cultures obtained from the heat-treatment experiments, end-product analysis by HPLC was done to establish the major end-product types and their ratios. For *T. brockii* HTD4 the major end-products from TYEG fermentation were always ethanol and lactate (typically 36 mM and 30 mM respectively), while for *Cl. thermohydrosulfuricum* Rt8.B1 the end-products were acetate (18 mM) and ethanol (36 mM). Survivors of the heat treatment from all time intervals were inoculated onto solid TYEG and MMX to determine colony and cell morphology. Colonies grown on TYEG or MMX agar plates were non-pigmented, flat, mucoid and uniformly circular. When sub-cultures from these heat treated tubes were inoculated into TYEG broth they always produced lactate and ethanol, and acetate and ethanol at the concentrations listed above. When heat-treated cultures were streaked on TYEG agar and MMX agar, colony and cell morphology was the same as cultures which had not been heat treated.

If the observed spores were due to a contaminant, then one would expect that the heat treatment would enrich for this strain. When subcultures from such heat-treated cultures were transfered from TYEG to MMX, a higher proportion of sporulating cells would therefore be expected. However, heat-treated subcultures displayed the same degree of sporulation (about 1 cell in 10^3) as the original culture did.

To confirm these findings, a fresh culture of *T. brockii* HTD4 was purchased from the DSM and treated by the same protocol. Identical results were obtained. Again, spores that survived exposure to 115°C for 80 min produced typically 36 mM ethanol, 30 mM lactate and 2 mM acetate when grown on TYEG, identical to the original cultures.

5.3.5 Dipicolinic acid

Dipicolinic acid (DPA) has been reported only to occur in bacterial spores and is released from spores during germination (Janssen *et al.*, 1958). DPA was assayed in sporulating cultures of *T. brockii* HTD4 to substantiate the finding that the terminal

structures were indeed endospores. Assays were attempted using the method of Janssen *et al.* (1958) for both *Cl. thermohydrosulfuricum* Rt8.B1 and *Cl. thermosaccharolyticum* DSM 571 (positive controls) and for *T. brockii* HTD4 grown under conditions producing spores. For all organisms the resolution of this technique proved too low for reliable results. A more sensitive technique using capillary gas chromatography and gas chromatography mass spectroscopy was attempted which produced reliable results with sporulating cultures of *C. thermosaccharolyticum* DSM 571 but the technique was again unsuccessful for *T. brockii* HTD4, possibly due to the low percentage of sporulating cells (0.1 %) compared with cultures of *C. thermosaccharolyticum* DSM 571 (90 % sporulating cells).

5.4 DISCUSSION

5.4.1 Sporulation in *T. brockii* and other related organisms

This study represents the first demonstration of heat-resistant endospores in the previously non-sporulating thermophilic anaerobe *T. brockii* HTD4. The results of this study indicate that growth of *T. brockii* HTD4 on MMX after pre-growth on the richer TYEG medium induces cells to sporulate. The ability of transfer to MMX to induce sporulation in this strain is of interest but the mechanism remains unresolved. It was confirmed that the methods used by Zeikus *et al.* (1979) on the original isolate failed to induce sporulation. Thus long term laboratory culture has not altered these characteristics. However, the type strain used in this study utilised xylose and mannose, observations in disagreement with Zeikus *et al.* (1979) and Morgan *et al.* (1985). Xylose utilisation has however been reported by other authors (Schmid *et al.* 1986).

Zeikus *et al.* (1979) initially commented that *T. brockii* HTD4 might be an asporogenous strain of *Cl. thermohydrosulfuricum* or *C. thermosaccharolyticum.* This view has been expressed again recently by Krivenko *et al.* (1987) and Kondratieva *et al.* (1989). The lack of spore formation by *T. brockii* on a medium which supports sporulation of *Cl. thermohydrosulfuricum* cannot be used alone to designate a new

species. Some clostridia form spores only rarely and some strains hardly ever produce observable spores unless a sporulation medium is developed (Duncan and Strong, 1968; Sacks and Thompson, 1978; Gottschalk *et al.*, 1981). In the light of the findings reported in this communication, it might seem reasonable to reclassify *T. brockii* as a new species of *Clostridium*.

Since the original characterization of *T. brockii* HTD4 a number of studies have reported the relationship of *T. brockii* to *Clostridium* and other gram-positive glycolytic anaerobes. *T. brockii* HTD4 and *Cl. thermohydrosulfuricum* 39E have been shown by partial 16S rRNA sequence comparisons to be very similar (Bateson *et al.*, 1989), and the two species show weak serological crossreactivity (Morgan *et al.*, 1985). DNA-DNA hybridisation of the two species gave a 50 % hybridisation value (Kondratieva *et al.*, 1989). DNA-DNA homology studies by Krivenko *et al.* (1987) also suggest a cluster of *Thermoanaerobium* species with *Cl. thermohydrosulfuricum* separate from *Cl. thermosaccharolyticum*. *T. brockii* and *Cl. thermohydrosulfuricum* also have similar metabolic patterns (Ben-Bassat *et al.*, 1981, Lamed and Zeikus, 1980b). *T. brockii* forms a closely related group with *Thermoanaerobacter ethanolicus, Thermobacteroides acetoethylicus* and *Cl. thermohydrosulfuricum* (Wiegel, 1986; Bateson *et al.*, 1989) and the closely related genus *Thermoanaerobacter* contains a spore-forming member in *Thermoanaerobacter finnii* (Schmid *et al.*, 1986).

The occurrence of spore formation in *T. brockii* HTD4 reinforces its relationship with *Cl. thermohydrosulfuricum*, and strengthens the case for reclassification. While assigning *T. brockii* to the genus *Clostridium* might appear appropriate, there is a need for reclassification of this genus (Cato *et al.*, 1986) and the likelihood of further change. In the meantime, it is appropriate to suggest that *T. brockii* remains in the genus *Thermoanaerobium*. Such an arrangement does not conflict with the taxonomic groupings suggested by Bateson *et al.* (1989) or Kondratieva *et al.* (1989) which include spore-forming species (*T. brockii, Cl. thermohydrosulfuricum, Ta. finnii*) and species for which spore formation has not yet been demonstrated (*Tb. acetoethylicus, Ta. ethanolicus*). Thus sporulation *per se* does not become the sole characteristic for

assignment to a genus, and this seems reasonable in the light of current evidence of mixed sporogenous /asporogenous phylogeny (Tanner *et al.*, 1982; Bateson *et al.*, 1989; Hermann *et al.*, 1987). The exact taxonomic and nomenclatural status of these species still remains a problem which needs to be addressed, but preferably in conjunction with a review (at least of the thermophilic members) of the genus *Clostridium*.

5.4.2 Recent developments

Since the documentation of spore formation in *T. brockii* (Cook *et al.*, 1991 see appendix 1) a number of publications and ideas on the taxonomic relationships of *T. brockii* and related organisms have emerged. The species has been renamed (by Zeikus *et al.*, in prep/subm) *Thermoanaerobacter brockii* comb. nov. and the genus *Thermoanaerobium* has been removed due to the removal of the type strain (Wiegel pers. comm.).

For a better taxonomic classification of the thermophilic anaerobic bacteria it appears that additional properties need to be more closely examined. Spore formation is generally regarded as a strong marker for systematic (phylogenetic) classification (Wiegel, 1992). Due to both the frequently observed difficulties in demonstrating spore formation, the usual grouping into spore-formers and non-sporeformers now needs to be revised (Wiegel, 1992). This property as a taxonomic and systematic marker is presently in doubt for use within the groups of thermophilic anaerobic bacteria. This is due to the frequently unobservable spore formation in apparently spore forming species and a repression of sporulation at the time of isolation and description of the isolate. With newly isolated strains from hot springs, it is often very difficult to demonstrate unequivocally whether the strains can form spores (Wiegel, 1992).

In strains of thermophiles that sporulate, Wiegel (1992) discusses the possibility that sporulation has developed cryptically. He proposes that this could have occurred in a number of ways. For instance, spore formation could have been impaired through

(point) mutations in genes responsible for one or more of the various sporulation stages. Then, under repeated batch growth conditions *in vitro*, the block could be eventually restored. Another possibility is that the expression of the sporulation genes in these organisms became tightly repressed or that the sporulation-related genes became a loop of supercoiled DNA. In such cases, some stress condition such as found in the late log or stationary growth phase or growth on a minimal medium in a batch culture, may initiate gene expression for spore formation, thus conferring a survival advantage for the organism. This could indeed explain the sporadic occurrence of spore formation in cultures of *T. brockii* (Cook *et al.*, 1991; Wiegel pers. comm.) and *Thermoanaerobacter* (Wiegel, 1992). Obviously the development of a genetic probe to test for the presence or absence of sporulation genes would help to resolve the problem (Wiegel, 1992).

A number of organisms need to be tested in this way including *Thermoanaerobacter* spp., and *Thermoanaerobium* spp. The other problem that needs to be addressed is the grouping of non-sporulating and sporulating organisms into one genus. Zeikus and associates have begun to rename some of the thermophilic anaerobic bacteria currently belonging to *Ta. ethanolicus* (type species), *T. brockii* (type species), *Cl. thermohydrosulfuricum* (type strain and similar strains and the unusual strain 39E). The following names have been suggested:

Table 5.1 Proposed reclassification of some thermophilic isolates

Thermophilic isolate	Strain	Suggested new designation
Cl. thermosulfurogenes	4B	*Thermoanaerobacterium thermosulfurogenes*
T. brockii	HDT4	*Thermoanaerobacter brockii*
Cl. thermohydrosulfuricum	E100	*Thermoanaerobacter thermosulfuricus*
Cl. thermohydrosulfuricum	39E	*Thermoanaerobacter ethanolicus*

(Modified from Wiegel, 1992)

All strains listed in table 5.1 under new designation will remain the type strain for the new species [n. comb] except for strain *Ta. ethanolicus* 39E. The new assignments were based primarily on DNA:DNA hybridization, enzyme and protein patterns and presence of unusual lipids components (molecular criteria).

Additional phylogenetic studies are required to establish a useful classification system at the genus and species level. This is important with the isolation of new species in the future. Using phenotypic properties, such isolates may appear physiologically relatively similar, but may be phylogenetically unrelated. To take a more phylogenetic approach, one must ask the question: does the isolate have a corresponding gene, regardless of whether it leads to functioning gene product(s)? Thus in respect to spore formation, the question should be: can one detect a a set of genes for sporulation, regardless of the phenotype? (i.e. whether heat stable spores can be detected under the tested culture conditions). To establish relationships between related organisms, these must be based on relevant taxonomic properties such as DNA-DNA homology data.

CHAPTER SIX

GLUCOSE AND XYLOSE UTILIZATION BY *Clostridium thermohydrosulfuricum* Rt8.B1

6.1 INTRODUCTION

A number of studies (Carreira *et al.*, 1983; Cook, 1988; Hudson *et al.*, 1988; Patel *et al.*, 1988) have demonstrated that hyperbolic growth kinetics for glucose and xylose utilization are a common property of some extremely thermophilic bacteria. All these studies have used undefined media containing high levels of trypticase peptone and yeast extract which contain carbohydrates of unknown composition. It is possible that these high levels of yeast extract and trypticase peptone caused the induction of metabolic pathways and enzymes resulting in the observed hyperbolic growth kinetics. Although the observation of hyperbolic growth of thermophilic bacteria has been made, how these organisms regulate their substrate uptake and what mechanisms exist for the control of dual substrate utilization has not been investigated. In the present study it was therefore decided to investigate mixed substrate utilization in a defined minimal medium and to study the control mechanisms which exist for glucose and xylose utilization by the extremely thermophilic anaerobic bacterium *Clostridium thermohydrosulfuricum* Rt8.B1.

Cl. thermohydrosulfuricum was first isolated from sugar beet extraction juices (Hollaus and Klaushofer, 1973) and then from soil (Wiegel *et al.*, 1979) and thermal volcanic features (Wiegel *et al.*, 1979; Patel *et al.*, 1986). The best physiologically-characterized strains ferment hexoses, pentoses, cellobiose, and starch to ethanol, H_2/CO_2, acetic and lactic acid. In a previous study (Cook, 1988), it was shown that growth of *Cl. thermohydrosulfuricum* Rt8.B1 on a mixed glucose + xylose substrate resulted in higher ethanol:acetate ratios than occurred during growth on single

substrates. Whether this was a consequence of hyperbolic growth or the medium used was investigated using the defined minimal medium described in section 4.3.1.

6.2 MATERIALS AND METHODS

The methods specific to this chapter are outlined in this section. Where methods and cultures have been previously used, reference is made to the appropriate section.

6.2.1 Chemicals

All chemicals were of analytical grade. The glucose analogues 2-deoxy-glucose (2-DG) and methyl-α-D-glucoside (α-MG) were purchased from Sigma Chemical Co. (St Louis, Mo, USA). Chloramphenicol was also purchased from Sigma.

6.2.2 Growth characteristics in minimal medium

For fermentation studies, the defined minimal medium described in section 4.3.1 was used. Carbon sources and glucose analogues were all prepared anoxically and added from separate stock solutions to media to the final concentration desired.

Fermentation studies with *Cl. thermohydrosulfuricum* Rt8.B1 grown on a minimal medium containing glucose + xylose were carried out in pH-controlled batch culture using a 1000 ml (working volume) fermenter (Gallenkamp, West Sussex, England). This was equipped with an automatic pH control unit (model FBL-720) with an autoclavable pH electrode (Ingold, Urdorf-Zurich, Switzerland), thermometer pocket, inoculation and sampling port, silicon rubber seal, direct drive shaft stirrer and temperature control module (model FBL-360) with autoclavable heating and thermostat probes. The culture vessel was sterilized by autoclaving for 30 min at 121°C. After autoclaving, the pre-sterilized minimal medium containing carbon sources was added to the culture vessel (aseptically in a laminar flow cabinet) and sealed. The culture vessel was then heated up to 70° under a continuous flow of oxygen-free nitrogen which was used to flush the head space until growth was

established. The gas was delivered to the culture vessel by oxygen-impermeable butyl rubber hosing through sterile filters (0.22 μm). After the culture vessel had reached the required temperature, 7 ml of a neutralized 10 % (w/v) aqueous $Na_2S.9H_2O$ solution was injected via a rubber septum into the culture vessel. After reducing conditions were obtained (resazurin decolourized), a 40 ml inoculum of a 24 hour glucose-grown culture was injected to give a 4 % inoculum. In all cases the initial pH was 7 and this was maintained throughout growth by the automatic addition of sterile 2N NaOH or H_2SO_4 via a peristaltic pump and delivered to the fermentation vessel via silicone tubing. Constant stirring (100 rpm) was maintained during the entire experiment.

The effect of glucose analogues on the growth of *Cl. thermohydrosulfuricum* was studied under batch conditions without pH control using 100 ml working volumes contained in 125 ml serum vials dispensed anaerobically, as previously described (section 3.2.3.5). Incubations where the pH was not controlled are referred to below as pH-uncontrolled. Incubations where the pH was maintained at pH 7.0 are referred to as pH-controlled. A 1 % 24 hour glucose-grown inoculum was used for pH-uncontrolled batch cultures. In all cases the initial pH was 7. Cultures were incubated at 70°C in an orbital shaking incubator (L.H Fermentation Ltd, Stoke Poges, Buckinghamshire, England) at 150 rpm. Glucose analogues were added in the medium to the desired concentration prior to growth unless otherwise stated.

To check the purity of culture when studying growth characteristics in batch culture, samples were routinely taken throughout the growth cycle and examined using an Olympus BH phase contrast microscope.

The following sampling procedure was used for both pH-uncontrolled and pH-controlled batch culture: 5 ml samples were withdrawn aseptically as required. To each of two 1 ml subsamples, 10 μl of saturated oxalic acid was added to stop any further metabolism occurring. Samples were then centrifuged in a bench top centrifuge (Runne Heidelberg, RS 85-1) at 6500 x g for 10 min and stored in a -20°C freezer for subsequent analysis. The remaining 3 ml was used to determine cell density by measuring the optical density at 650 nm using a Pye Unicam SP6-450 UV/VIS

spectrophotometer (Pye Unicam, Cambridge, England) with distilled water as a blank. These samples were diluted with distilled water if the optical density was greater than 0.8. The growth rate constant (k) for the log phase of growth was determined by plotting the optical density (log cycle) against time (linear cycle). The relationship between the growth rate constant (k), the doubling time (t_d), and the specific growth rate (μ) is given by Pirt (1975). The maximum growth rate was expressed as h^{-1}.

6.2.3 Substrate utilization studies by cell suspensions

6.2.3.1 Preparation of cell suspensions

For substrate utilization studies, cells of *Cl. thermohydrosulfuricum* Rt8.B1 from 1 litre of a mid-logarithmic phase culture grown on minimal medium containing either glucose alone, xylose alone or glucose + xylose together at the required concentrations were collected by centrifugation (8000 x g for 20 min). The supernatant fluid was removed, and the cell pellet washed twice with anaerobic minimal salts having the following composition: ($g\ell^{-1}$ distilled water) NH_4Cl, 0.9; $MgCl_2.6H_20$, 0.2; NaCl, 0.9; KH_2PO_4, 0.75; K_2HPO_4, 1.5; 0.2% resazurin, 1 ml; and MOPS, 10.0; Na_2S, 7 ml, and pH 7.0 at room temperature. The washed cells were then resuspended in 20 ml of the same anaerobic minimal salts under an oxygen-free nitrogen atmosphere. All cell manipulations were carried out in an anaerobic chamber (Coy, Inc., USA) containing an atmosphere of hydrogen and oxygen-free nitrogen (NZ Industrial Gases Ltd). Freshly harvested cells were used for each determination of substrate utilization.

6.2.3.2 Standard assay procedure

In the standard utilization procedure, 2.0 ml resting cells (cell suspensions) were transferred anaerobically to serum vials (total volume 60 ml) containing 20 ml of sterile anaerobic minimal salts (pH 7 at 70°C) containing 100 µg chloramphenicol /ml under an oxygen-free N_2 atmosphere. Minimal salts were used as opposed to buffer alone as ion requirements for *Cl. thermohydrosulfuricum* Rt8.B1 have not been determined and may be important for the utilization of glucose and xylose.

Chloramphenicol was added to prevent the induction of systems not present at the time of harvest and to prevent growth (and a consequent change in protein concentration) occurring. Minimal salts alone in the presence of added carbon source could not support the growth of *Cl. thermohydrosulfuricum* Rt8.B1 at the concentrations of cell suspensions used. Cell suspensions (between 10 and 20 mg of protein per ml) were incubated at 70°C in an orbital shaking incubator (L.H Fermentation Ltd, Stoke Poges, Buckinghamshire, England) at 100 rpm. Cells were incubated for 20 min with gentle agitation to equilibrate and in order to exhaust any endogenous energy source present. Utilization was then initiated by adding 1.0 ml of the required carbon source to give the required concentration. Aliquots of the cell suspension (1.0 ml) were withdrawn periodically (15-30 min intervals) with a 1 ml syringe and placed in a 1.5 ml total volume Eppendorf tube. Further metabolism was prevented by cooling on ice and the addition of 20 μl of saturated oxalic acid. The samples were then frozen for subsequent analysis by HPLC.

6.2.3.3 Protein assay

The protein content of the cell suspension was determined by incubating 1 ml of the cell suspension with 1 ml of 1 M NaOH at 100°C for 15 min. After neutralization with 1 ml of 1 M HCl, the protein content was determined by the Biuret reaction as described by Scopes (1982). Bovine serum albumin (Sigma) of 96.99 % purity was used to construct a standard curve which was linear from 0 to 5 $mg.ml^{-1}$. All assays were carried out in triplicate. Protein assays were performed on cultures added to assay vials (originals) and on cultures from assay vials at the start and end of the experiment. This was done to ensure that the protein content of the assay was constant throughout the time course of the assay.

6.2.3.4 Kinetics of substrate utilization

Substrate utilization was determined by the difference between the substrate concentration in samples taken immediately after inoculation, and samples taken throughout the sampling period. For all experiments the rate of substrate utilization was calculated from the initial linear plot of substrate disappearance versus time (lasting up to 180 min) and is expressed as nanomoles of substrate utilized per minute per milligram of protein at 70°C. For all cell suspension experiments the rates calculated were the means of a minimum of 2 independent experiments. For kinetic analysis of data, the results were analyzed by Eadie-Hofstee plots of the rate of enzyme activity (v) against the rate of enzyme activity over the substrate concentration (v/[S]). Such plots are useful in determining departures from linearity. Plots of v /[S] versus v are linear and the y intercept gives the V_{max} and the x axis (v/[S]) intercept gives V_{max} /K_m (Dixon and Webb, 1979). The term K_s was used to express the apparent K_m for substrate utilization.

6.2.4 ANALYTICAL METHODS

6.2.4.1 High Performance Liquid Chromatography

Fermentation products and substrate concentrations in cell-free extracts of fermentation broth were determined by high performance liquid chromatography (HPLC) using an Aminex HPX-87H organic analysis column (300 X 7.8 mm) (Bio-Rad, Richmond, California, USA), operated at 50°C using a temperature control module (Waters associates, Milford, Massachusetts, USA) and fitted with a micro-guard ion exclusion and anion/OH cartridge guard column (Bio-Rad, Richmond, California, USA). The mobile liquid phase consisted of 0.01 N H_2SO_4 at a flow rate of 0.5 ml/min. The solvent was prepared daily from a stock solution of 1 N H_2SO_4 and Milli-Q water {deionised, reagent grade water from the Milli-Q water purification system (Millipore)}, filtered through a 0.45 µm filter (Durapore) and vacuum degassed. Elution was monitored with a refractive index detector (Erma Optical Works Ltd, Tokyo, Japan). Data were collected via a series interface (Nelson Analytical Inc,

Cupertino, California, USA) linked to an Exzel XT computer (Computer Imports Ltd, Auckland, New Zealand) using 3000 series chromatography data system software (Nelson Analytical Inc, Cupertino, California, USA) and printed via a Panasonic Impact dot matrix printer (Matsushita Electric Trading Co, Osaka, Japan)

For analysis, cell-free supernatants were loaded directly onto the column using a 20 μl model 7125 syringe loaded sample injector (Rheodyne Inc, Cotati, California, USA) and injected with a 50 μl glass syringe (Hamilton ,Reno, Nevada). Automatic injections were carried out using a modified Phillips PU 4700 Pye-Unicam Autoinjector (Pye Unicam Ltd, Cambridge, England). Standard stock solutions of substrates were run at the beginning and end of a series of samples to be analyzed. Concentrations of substrates and end-products were run at concentrations where linearity of response had been determined (0.1 mM to 100 mM) and reproducibility was within 2 %.

6.2.5 MEASUREMENT OF ENZYME ACTIVITIES

6.2.5.1 Chemicals

All chemicals were reagent grade. Reduced nicotinamide adenine dinucleotide (β-NADH), nicotinamide adenine dinucleotide phosphate (β-NADP), adenosine 5-triphosphate (ATP), D-xylulose, dithiothreitol and D-glucose-6-phosphate were all obtained from Sigma Chemical Company (St Louis, Mo, USA).

The following enzymes were also obtained from Sigma Chemical Company (St Louis, Mo, USA):- glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucokinase (EC 2.7.1.2) and sorbitol dehydrogenase (EC 1.1.1.14).

6.2.5.2 Preparation of crude extracts

Cells used for analysis of enzyme activities were grown in pH-controlled batch cultures (working volumes 1 litre) using Gallenkamp fermentation equipment under conditions identical to those previously discussed (section 6.2.2). Cells were collected throughout the growth cycle and were harvested by centrifuging at 7500 x g for 20 min at 4°C in a Sorvall SS-34 centrifuge (Du Pont Company, Wilmington, Delaware, USA). The cells were washed and resuspended in 20 mM MOPS buffer, pH 7.0, containing: $MgSO_4$, 20 mM; EDTA, 0.01 mM; and dithiothreitol, 0.2 mM, and re-centrifuged for 10 min at 4500 x g. The cell pellets were then resuspended in the above buffer (10 ml) and frozen in liquid nitrogen and stored at -70°C until required.

When required, centrifuged cells was allowed to thaw at room temperature and placed in a 50 ml beaker for sonication. The suspension was kept on ice and the cells disrupted by sonication using an Artek 300 series ultrasonic dismembranator (Artek Systems Corporation, Farmingdale, New York, USA). After 5 min sonication (45 second bursts with 1 min cooling on ice in between bursts) using a standard 1/2 inch titanium tip at a setting of 50 %, maximum cell lysis as confirmed by microscopic examination occurred. The sonicate was then poured into Beckman polycarbonate centrifuge tubes (13 x 51 mm) and centrifuged for 45 min at 4°C and 40 000 x g using a Beckman TL-100 table top ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California, USA) to remove cell debris. The supernatant was frozen in 1 ml aliquots in liquid nitrogen and stored at-70°C. Where possible the preparation of crude-extracts was done anaerobically. All enzyme assays were done anaerobically and this was the important step in which anaerobic conditions were necessary for enzyme activity.

6.2.6. ASSAY METHODS

6.2.6.1 Protein determination of crude extracts

The protein content of crude extracts was assayed by the Biuret reaction as described by Scopes (1982). All assays were carried out in duplicate.

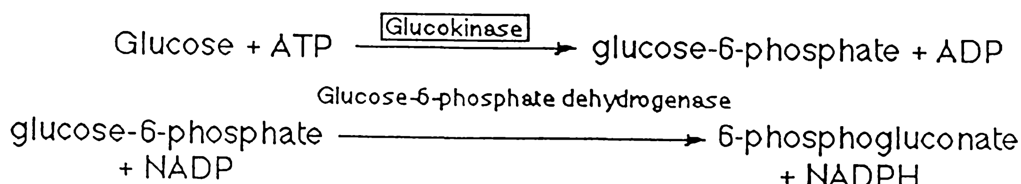
6.2.6.2 Assay of enzyme activities in crude extracts

All enzyme assays were carried out at the temperature specified in text under anaerobic conditions essentially as described by Zeikus *et al.* (1977). All activities were measured by modifications of standard assay methods, Lamed and Zeikus (1980a,b) and Bergmeyer (1974). Specific activities were determined in a range where linearity of activity with protein concentration was shown and expressed as the amount of enzyme catalyzing the conversion of 1 μmol of substrate per min per mg of protein into specific products. All assays were carried out in triplicate with appropriate controls. Where available, commercial enzymes were used as a positive control.

All enzyme activities were assayed by measuring the change in optical density due to pyridine nucleotide oxidation or reduction at 340 nm ($\varepsilon 340 = 6.22\ mM^{-1}\ cm^{-1}$.) Enzyme assays were carried out in quartz cuvettes with a 1.0 cm light path, 3.0 ml total volume, using a Shimadzu double monochromator recording spectrophotometer model UV-250 (Shimadzu Corporation, Kyoto, Japan) equipped with a Shimadzu temperature controlled cell holder (Model TCC-240A) and computer controlled graphic printer (Model PR-1).

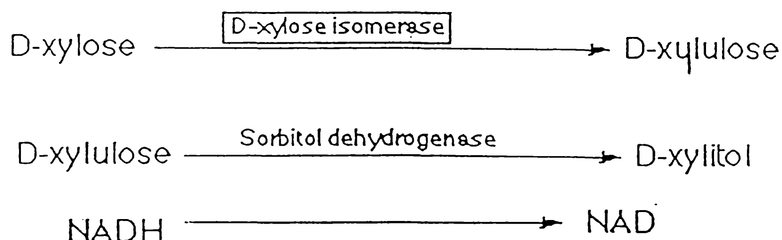
Glucokinase (EC 2.7.1.2) was assayed by the coupling of NADP reduction to glucose phosphorylation and measuring the increase in optical density at 340 nm. The reaction mix contained: MOPS, pH 7.0, 50 mM; $MgCl_2$, 60 mM; dithiothreitol, 5 mM; NADP, 0.5 mM; ATP, 5 mM; glucose, 15 mM; glucose-6-phosphate dehydrogenase, 5.0 U, and 50 μl of crude extract. Control assays were performed in the absence of 1. Glucose to correct for nonspecific ATPase; 2. ATP to show that glucose phosphorylation is ATP-dependent; 3. Boiled crude extract. All controls were subtracted from actual activity.

Glucokinase assay principle:



Xylose isomerase (EC 5.3.1.5) activity was assayed by a modification of the method of Schellenberg *et al.* (1984), a two step procedure where the xylulose formed during the first step was reduced in the second step to xylitol by sorbitol dehydrogenase with concomitant oxidation of NADH.

Xylose isomerase assay principle:



In the first step, 60 µl of crude extract was added to 240 µl of a reaction mixture containing MOPS, pH 7.0, 50 mM; D-xylose, 333 mM; and $MnCl_2$, 5 mM in a 1.5 ml Eppendorf tube. This reaction mix was incubated at 70°C for time intervals of 1-2 min over 10 min. The reaction was stopped by boiling the sample for 1 min and then centrifuged for 2 min in a bench top centrifuge (Runne Heidelberg, RS 85-1) at 6500 x g to precipitate coagulated protein. The xylulose present in the supernatant was quantified in a second reaction which contained (in a final volume of 2.0 ml):- sorbitol dehydrogenase, 0.5 U; NADH, 0.35 mM; MOPS, pH 7.0, 50 mM and 100 µl of supernatant. Controls assays were performed in the absence of 1. xylose to correct for nonspecific activity; 2. Boiled crude extract. All controls were subtracted from actual activity. Xylose isomerase activity was expressed as µmoles of xylulose formed /min /milligram of protein at 70°C.

6.3 RESULTS

6.3.1 Growth of *Cl. thermohydrosulfuricum* in a minimal medium containing glucose + xylose

Cl. thermohydrosulfuricum Rt8.B1 grown in minimal medium containing equal concentrations of glucose + xylose under pH-controlled batch culture exhibited hyperbolic growth kinetics i.e. both substrates were utilized simultaneously (figure 6.1). The major end-products formed were ethanol and acetate in a ratio of 2:1. A small amount of xylulose was detected in the growth medium presumably due to leakage from the cell. The maximum growth rate was 0.26 h^{-1}.

The results of this experiment indicate that there was no repression of either uptake or catabolism for one substrate by the other. From the data it cannot be ascertained how glucose and xylose are taken up, but two mechanisms can be postulated:

1. A common constitutive uptake system exists for both substrates which has equal affinities for glucose and xylose.

2. Separate uptake systems are responsible for glucose and xylose uptake.

To investigate this further, cultures of *Cl. thermohydrosulfuricum* Rt8.B1 were grown on 50 mM glucose alone and an equivalent concentration of xylose was injected at the mid-logarithmic phase of growth (figure 6.2). Xylose utilization began immediately with no lag as observed in classical diauxic growth. The rate of xylose utilization increased over the first 3-5 hours which coincided with xylose isomerase induction (figure 6.2). Glucose had no effect on the induction of xylose isomerase and xylose had no effect on the rate of glucose uptake and utilization. The growth rate on glucose alone was 0.27 h^{-1} and the after the depletion of glucose, when only xylose remained the growth rate decreased to 0.105 h^{-1}. Similar results were obtained when glucose was added to cultures growing on xylose (figure 6.3). The growth rate on xylose alone

was 0.074 h^{-1} and after the addition of glucose the growth rate increased to 0.125 h^{-1}. In both experiments the major end products were ethanol and acetate in a ratio of 2:1 (figures 6.4 and 6.5). When xylose was added to cultures growing on glucose (figure 6.2) xylose utilization continued after growth had ceased. This was also accompanied by a shift in metabolism from acetate (ATP generation) and ethanol production to predominantly ethanol fermentation (figure 6.4).

In both experiments the level of key metabolic enzymes involved in the initial steps of glucose and xylose utilization were followed. The enzymes glucokinase (EC 2.7.1.2) (responsible for the phosphorylation of glucose) and xylose isomerase (EC 5.1.3.5) (responsible for the conversion of D-xylose to D-xylulose) were assayed by the methods outlined in section 6.2.6.

The changes in the activities of these enzyme are shown in figures 6.2 and 6.3. For the metabolism of glucose, a high constitutive level of glucokinase activity was detected in cells grown on glucose or xylose. The constitutive level of glucokinase increased in the presence of glucose. When glucose was added to cultures growing on xylose (figure 6.3) the level of glucokinase activity increased 3-fold and was unaffected by the level of xylose in the culture medium. On the depletion of glucose, the level dropped to the constitutive level. Similar results for glucokinase activity were seen when xylose was added to cultures growing on glucose (figure 6.2). Experiments were carried out in which, after the depletion of glucose from the growth medium, a low concentration of glucose (20 mM) was injected into the culture vessel in the absence of xylose and the rate of glucose disappearance and glucokinase activity were followed (figure 6.6). The level of glucokinase increased rapidly and glucose was consumed, thus demonstrating the constitutive nature of glucokinase.

Cl. thermohydrosulfuricum Rt8.B1 contained a low level of xylose isomerase activity (< 0.01 µmoles /min /mg of protein) when grown on medium containing glucose (figure 6.2). A 24-fold increase in the level of xylose isomerase activity was detected upon the addition of xylose to the culture medium (figure 6.2). The observation that xylose isomerase was induced in the presence of glucose but was unaffected by the

addition of glucose to cultures actively growing on xylose (figure 6.3) suggested a lack of modulation of xylose isomerase activity by glucose.

The observation that glucose- or xylose-grown cultures were capable of taking up the added substrate with no apparent lag suggested that any preference for the initial growth substrate, be it glucose or xylose, does not dominate to the exclusion of the added substrate.

To investigate this further, the ratio of added substrate to the initial growth substrate was adjusted to create an excess of one substrate over the other. When cells were grown on a low concentration of xylose (6 mM) and a high concentration of glucose (30 mM) was added at the mid-logarithmic phase of growth, a significant inhibition of xylose utilization was seen (figure 6.7). The growth rate on xylose alone was 0.04 h^{-1} and this was increased significantly by the addition of glucose (0.12 h^{-1}). In direct contrast, the addition of 18 mM xylose to cultures growing on low concentrations of glucose (6 mM) had no effect on the rate of glucose uptake and utilization (figure 6.8). The results of these experiments suggest that some competition exists between glucose and xylose for a transport protein or some key enzyme or metabolite such as ATP which is not apparent when both substrates are at equally high concentrations. This result suggests that different uptake systems operate at low concentration of substrate which are then repressed /switched off when a substrate is added at high concentration. If this is correct, it suggests that for glucose uptake the same uptake system is used at both high and low concentrations of glucose as neither are affected by the addition of xylose.

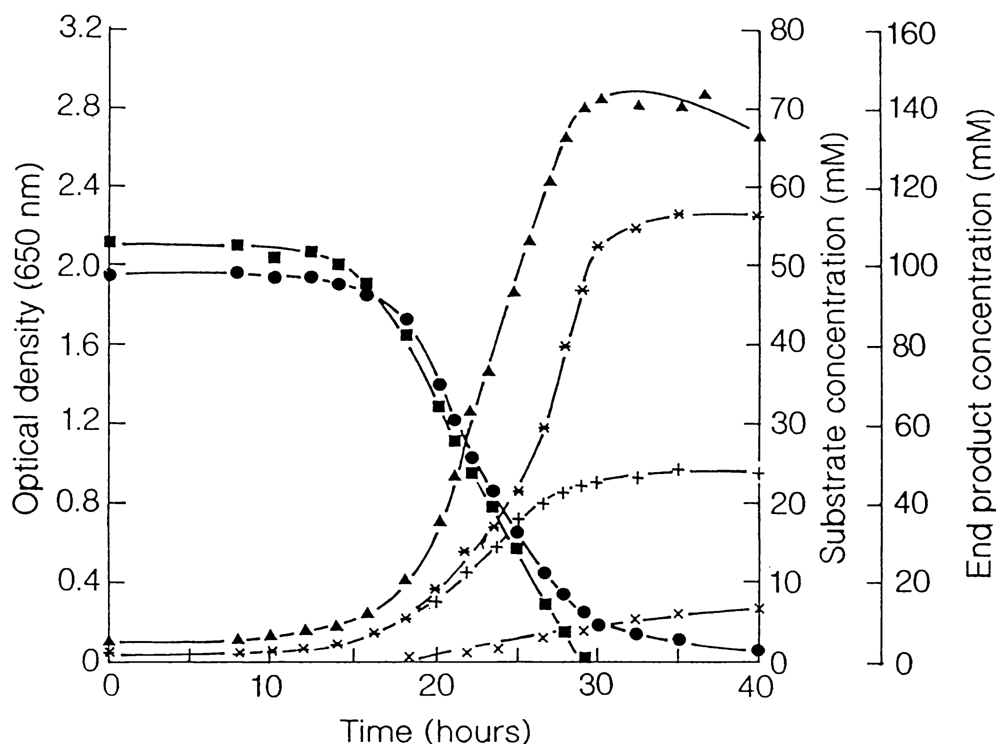


Figure 6.1 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose in pH-controlled batch culture. Optical density (▲), glucose (■), xylose (●), xylulose (x), acetate (+) and ethanol (⋇).

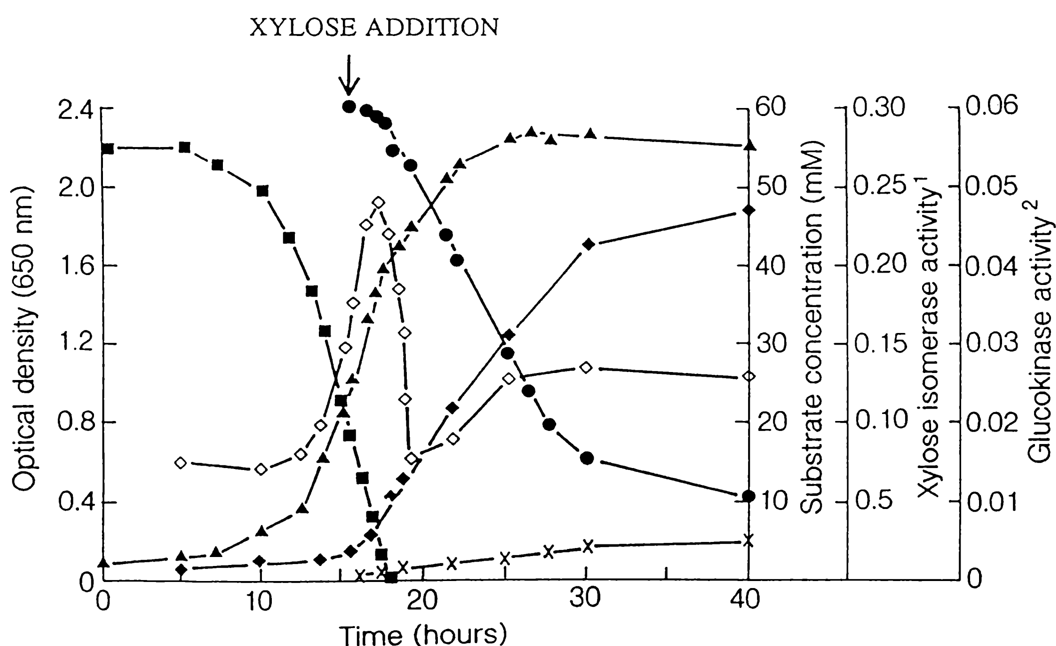


Figure 6.2 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose with 50 mM xylose added at the mid-log phase of growth in pH-controlled batch culture. Optical density (▲), glucose (■), xylose (●), xylulose (x), glucokinase (◇), xylose isomerase (♦).

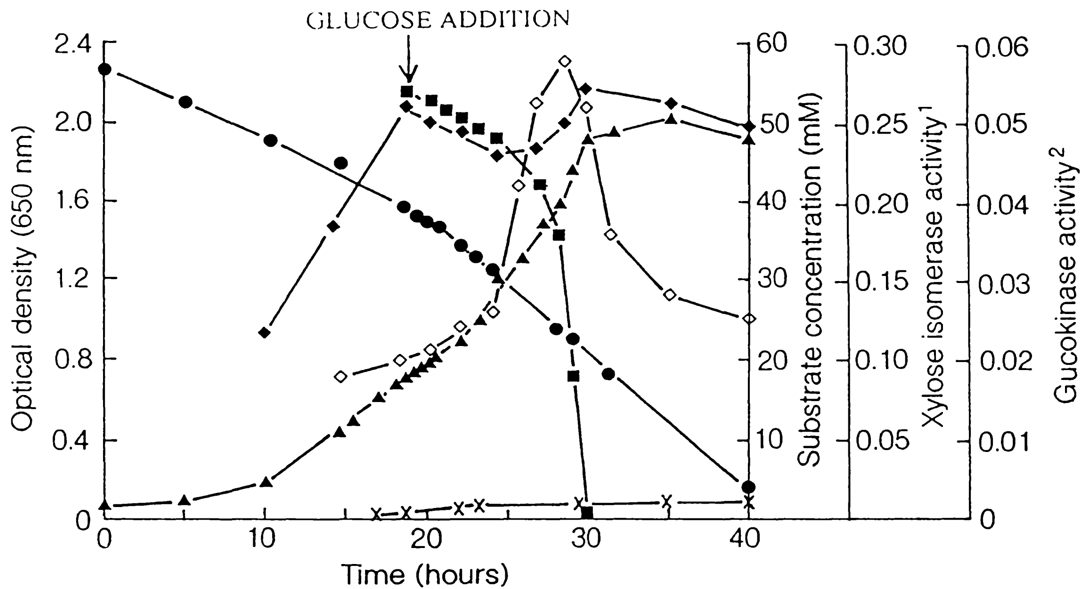


Figure 6.3 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose with 50 mM glucose added at the mid-log phase of growth in pH-controlled batch culture. Optical density (▲), glucose (■), xylose (●), xylulose (x), glucokinase (◇), xylose isomerase (♦).

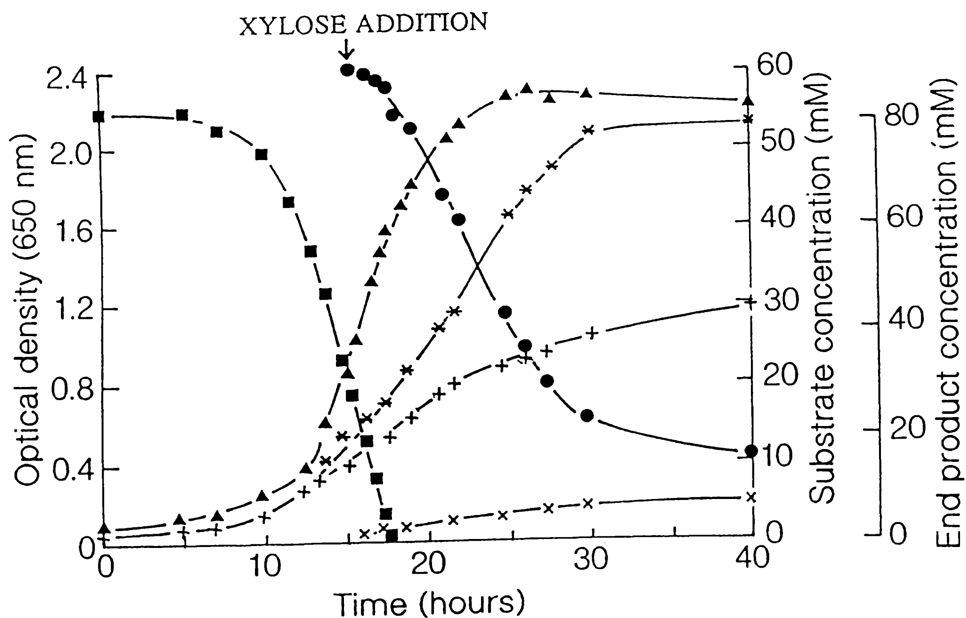


Figure 6.4 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose with 50 mM xylose added at the mid-log phase of growth in pH-controlled batch culture. Optical density (▲), glucose (■), xylose (●), xylulose (x), acetate (+) and ethanol (✶)

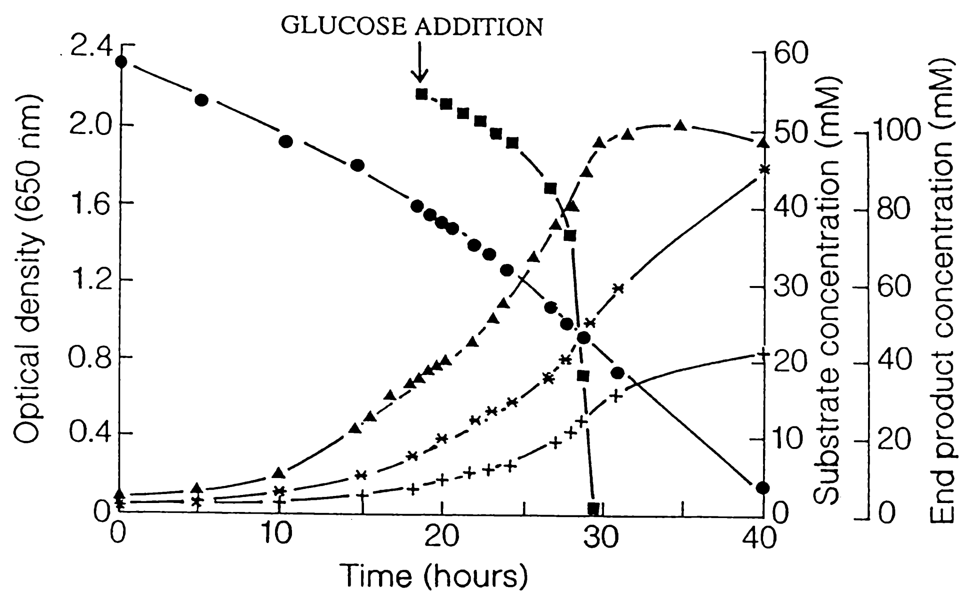


Figure 6.5 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose with 50 mM glucose added at the mid-log phase of growth in pH-controlled batch culture. Optical density (▲), glucose (■), xylose (●), acetate (+) and ethanol (✱).

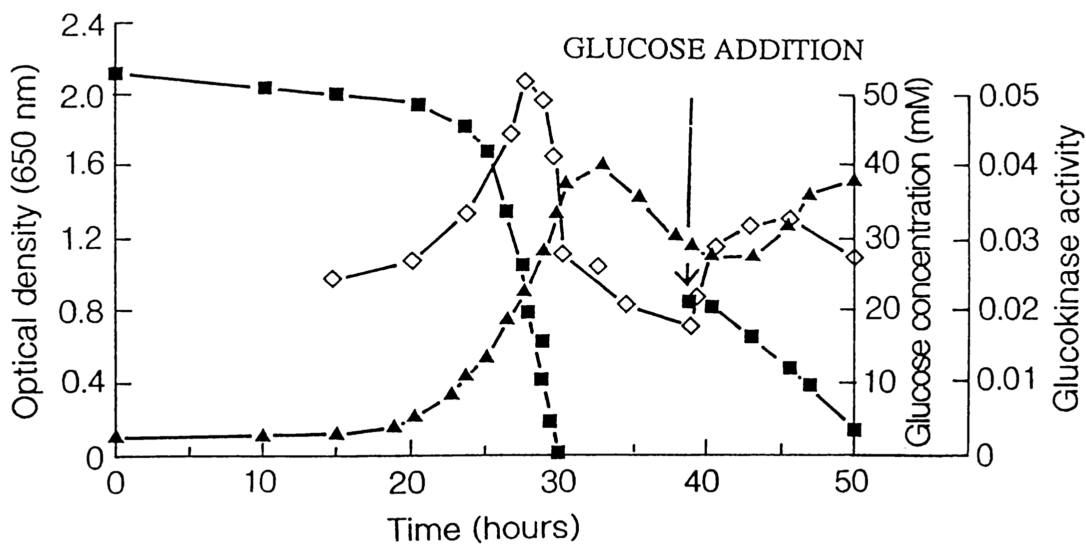


Figure 6.6 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose with 20 mM glucose added at the stationary phase of growth in pH-controlled batch culture. Optical density (▲), glucose (■), glucokinase (◇).

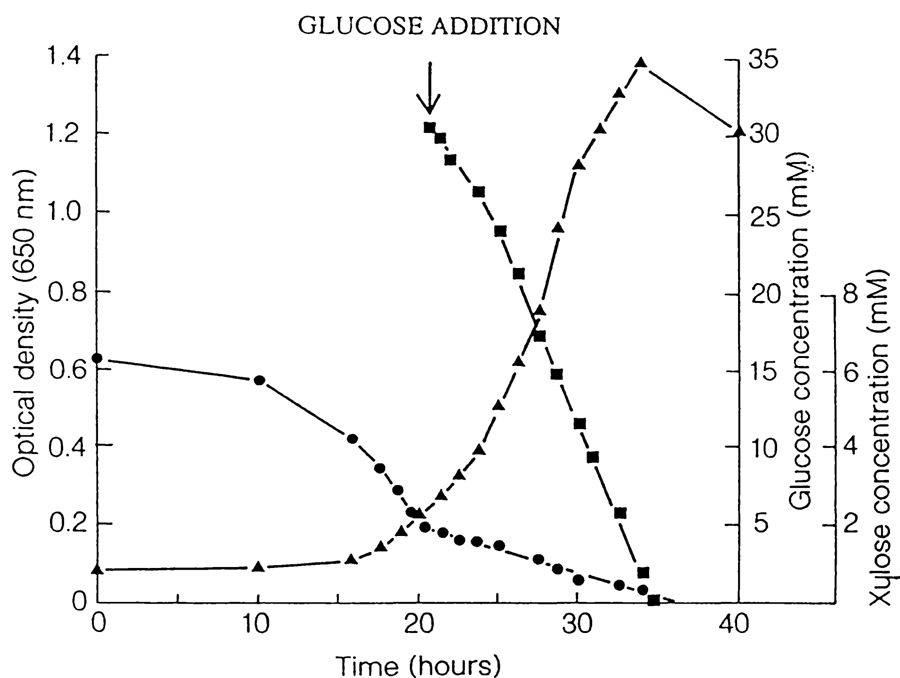


Figure 6.7 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 6 mM xylose with 30 mM glucose added at the mid-log phase of growth in pH-controlled batch culture. Optical density (▲), glucose (■) and xylose (●).

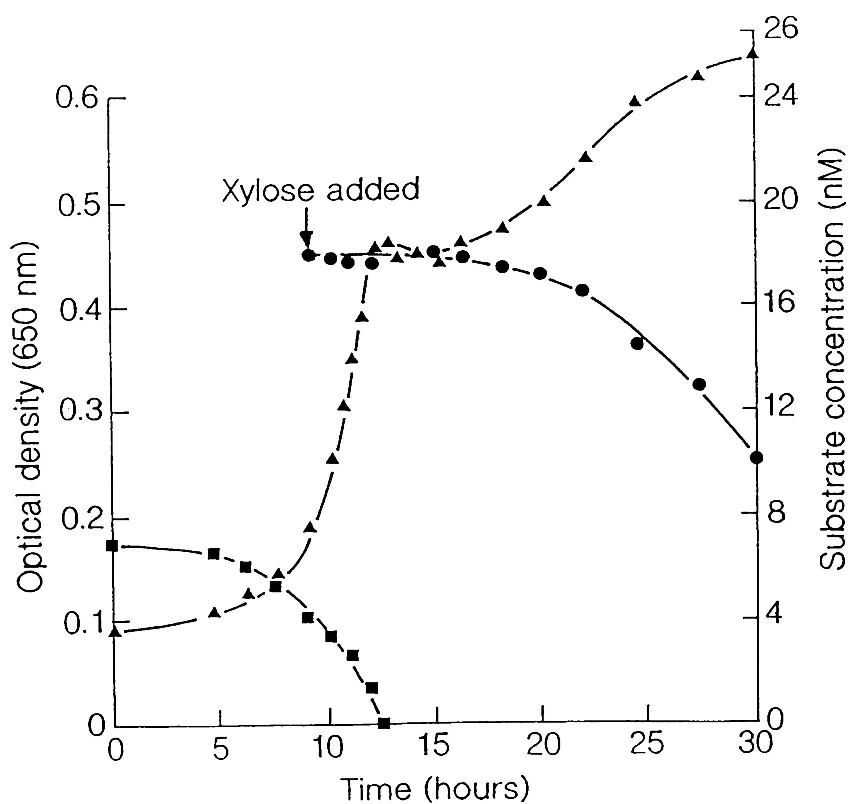


Figure 6.8 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 6 mM glucose with 18 mM xylose added at the mid-log phase of growth in pH-controlled batch culture. Optical density (▲), glucose (■) and xylose (●).

6.3.2. The effect of the glucose analogues 2-deoxy-glucose and methyl-α-D-glucoside on the growth of *Cl. thermohydrosulfuricum* Rt8.B1

To investigate the possibility that glucose and xylose share a common transport protein or metabolic enzyme, the glucose analogues 2-DG and α-MG were added to the growth medium at the beginning of the growth cycle. Unless otherwise stated, the final concentrations of the analogues were 50 mM. These analogues are reputed to have a high affinity for the glucose carrier and have been commonly used to study transport across bacterial membranes.

6.3.2.1 The effect of α-MG on the growth of *Cl. thermohydrosulfuricum* Rt8.B1

The results for the effects of α-MG on the growth of *Cl. thermohydrosulfuricum* Rt8.B1 are summarized in table 6.1.

Table 6.1 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose and xylose in the presence of methyl-α-D-glucoside

Growth substrate (s)	Final optical density (650 nm)	Lag period (hours)	Growth rate (h^{-1})	Figure number
50mM α-MG	NG	-	-	-
50 mM Glucose	1.92	15.00	0.32	6.90
50 mM Glucose + α-MG	1.95	36.00	0.18	6.90
50 mM Glucose + α-MG added at mid-log	1.93	15.00	0.33	-
50 mM Xylose	1.59	12.00	0.09	6.11
50 mM Xylose + 50 mM α-MG	1.20	22.00	0.05	6.11
50 mM Xylose + 45 mM α-MG added at mid-log	1.16	15.00	0.08	6.13
50 mM Glucose + 50 mM xylose	1.90	12.00	0.29	6.10
50 mM G + 50 mM X + 50 mM α-MG	1.74	28.00	0.23	6.10
50 mM G + 50 mM X + 30 mM α-MG added at mid-log	1.95	12.00	0.29	6.12

NG = no growth; G = glucose, X = xylose. When analogues were added to cultures at mid-log, then the growth rates are calculated after the addition of the analogue.

When *Cl. thermohydrosulfuricum* Rt8.B1 was grown on medium containing 50 mM α-MG and a fermentable carbohydrate (glucose or xylose) a significant decrease in the growth rate and substrate consumption was observed. The lag phase of growth was also extended. No growth or utilization was observed on α-MG in the absence of a fermentable carbon source. The greatest inhibition by α-MG was seen with glucose-grown cells where the growth rate was effectively halved and glucose utilization was decreased as a result. These results are summarized in table 6.1 for all substrates tested.

For cultures of *Cl. thermohydrosulfuricum* Rt8.B1 grown on glucose + α-MG (figure 6.9) and glucose + xylose + α-MG (figure 6.10) no α-MG uptake could be detected. Cells grown on 50 mM xylose + α-MG showed a small amount of α-MG was taken up (figure 6.11). The addition of α-MG at the mid-log phase of growth to cells growing on glucose (table 6.1) and glucose + xylose (6.12) did not have any significant effect on substrate utilization or growth. The addition of α-MG to mid-logarithmic cultures growing on xylose caused a transient decrease in the rates of growth and xylose utilization (figure 6.13). Only with xylose-grown cells was α-MG taken up from the culture medium when added at the mid-logarithmic phase of growth. Whether this was metabolized or only accumulated is not known.

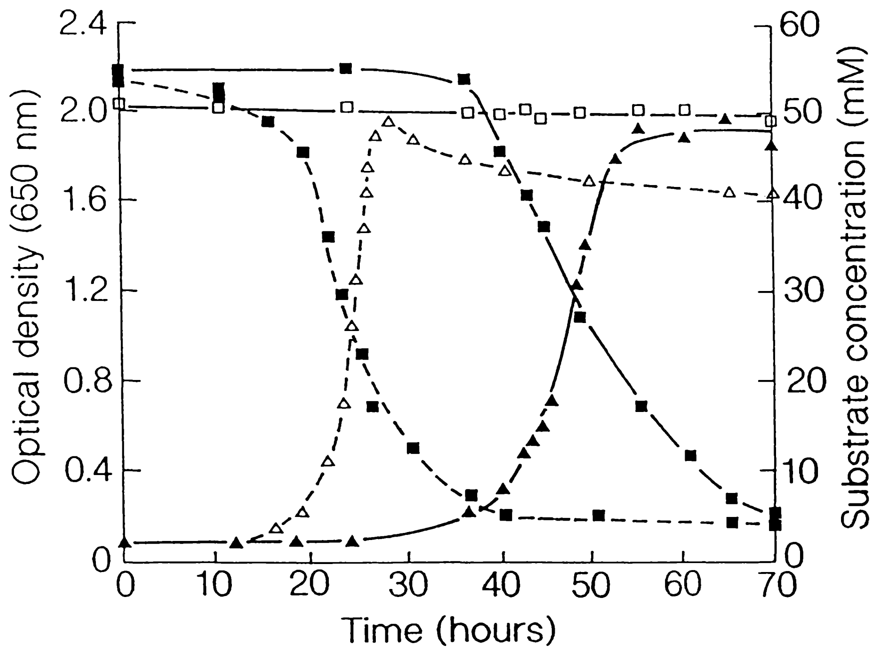


Figure 6.9 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM α–MG in batch culture. Optical density (▲), glucose (■), α–MG (□). Broken lines (---) are controls representing optical density (Δ) and glucose consumption (■) by cultures growing on 50 mM glucose alone.

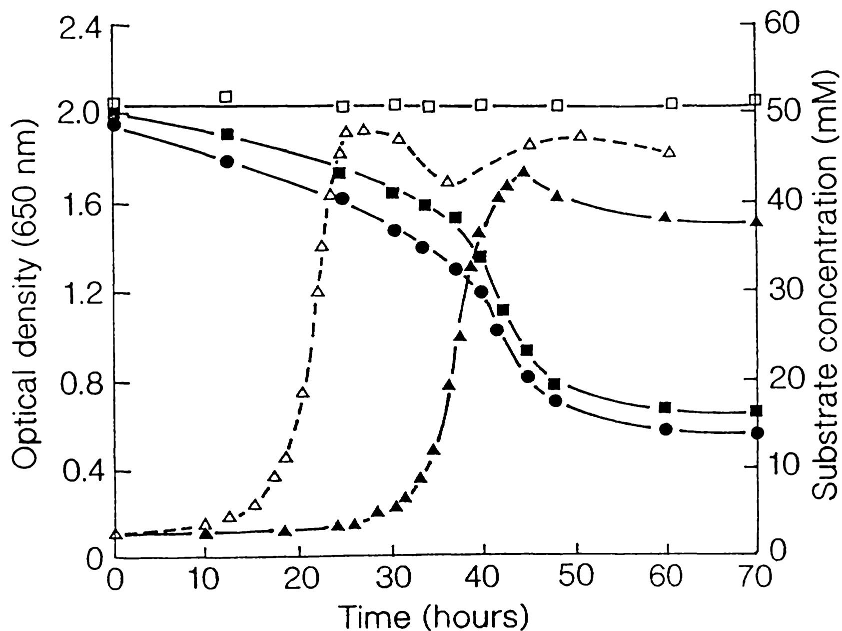


Figure 6.10 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM α–MG + 50 mM xylose in batch culture. Optical density (▲), glucose (■), α–MG (□), xylose (●). Broken line (---) is control representing optical density (Δ) for cultures grown on 50 mM glucose + 50 mM xylose.

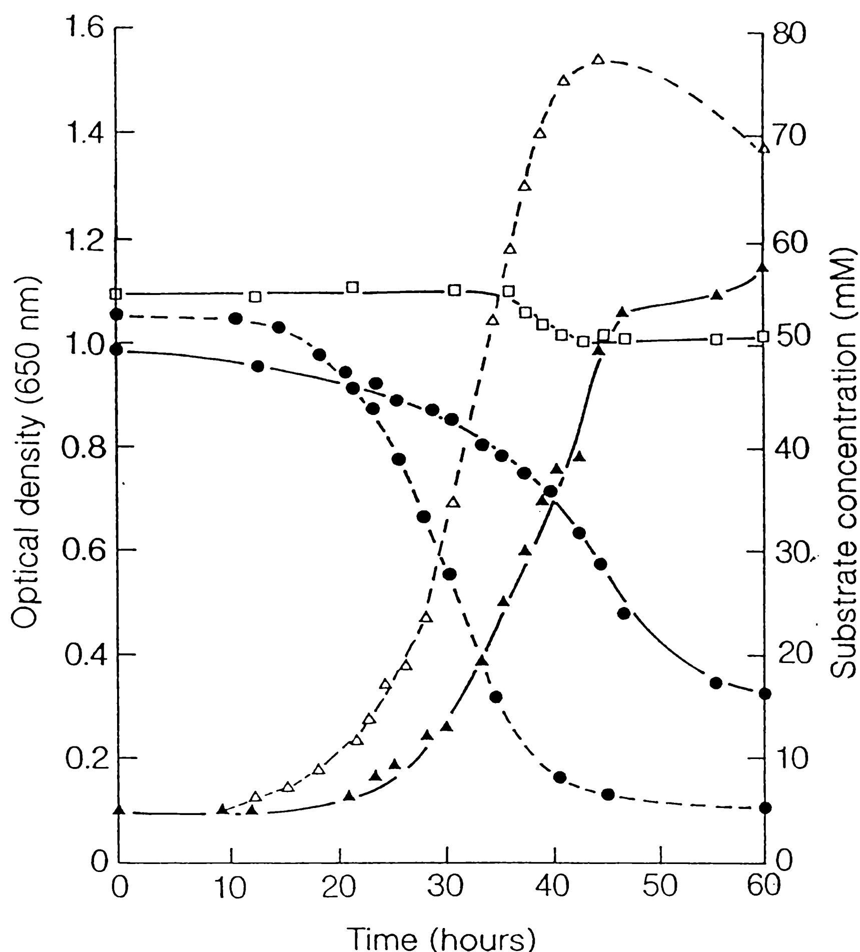


Figure 6.11 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM α–MG in batch culture. Optical density (▲), xylose (●), α–MG (□). Broken lines (---) are controls representing optical density (Δ) and xylose (●) consumption by cultures grown on 50 mM xylose.

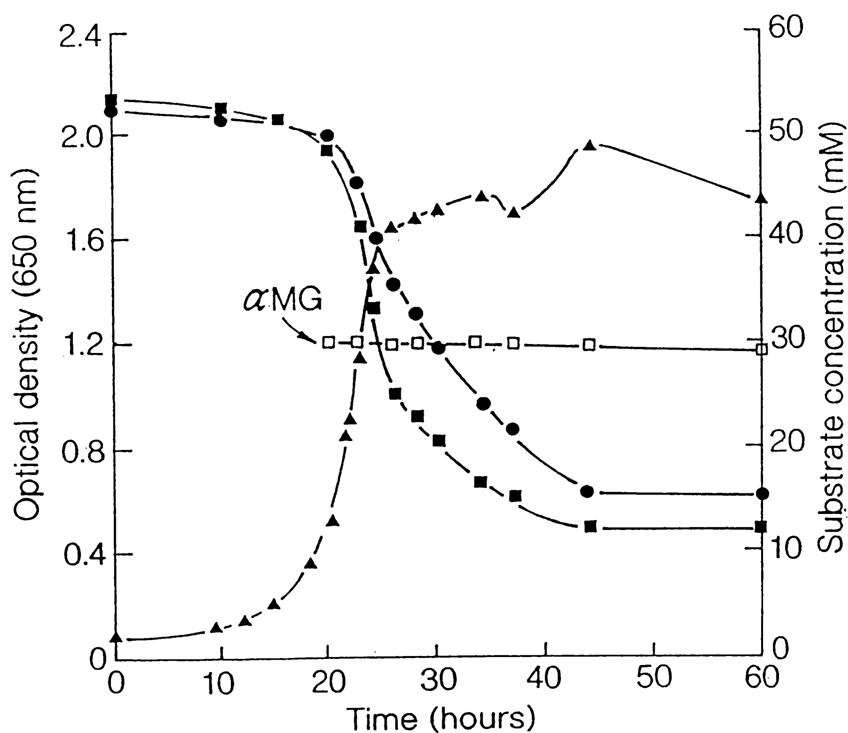


Figure 6.12 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose with 30 mM α–MG added at the mid-log phase of growth in batch culture. Optical density (▲), glucose (■), α–MG (□) and xylose (●).

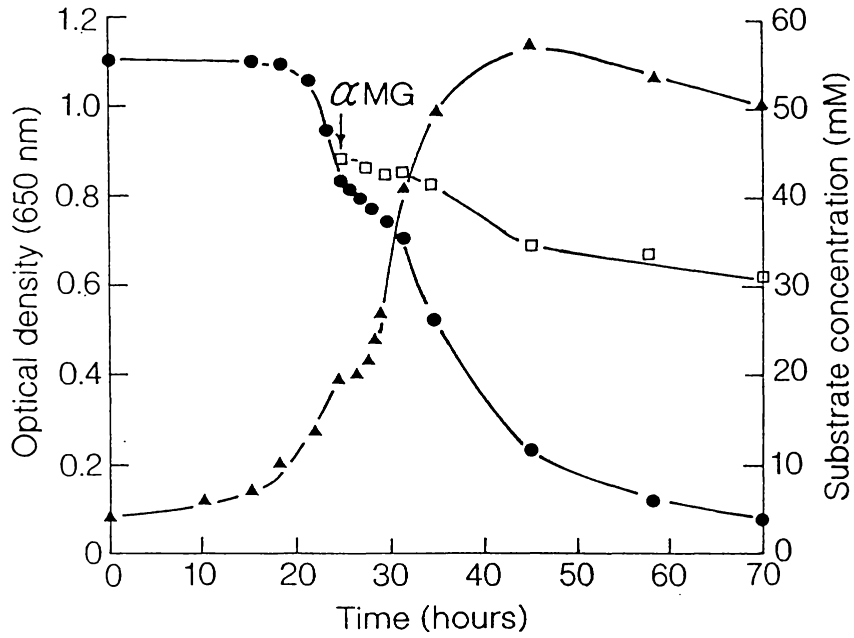


Figure 6.13 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose and 45 mM α–MG added at the mid-log phase of growth in batch culture. Optical density (▲), α–MG (□) and xylose (●).

6.3.2.2 The effect of 2-DG on the growth of *Cl. thermohydrosulfuricum* Rt8.B1

Experiments with 2-DG were carried out under identical conditions to the above. 2-DG inhibited both the growth rate and the substrate utilization and extended the growth lag phase. A summary of these results for all substrates is presented in table 6.2. Xylose-grown cells exhibited longer lag phases than did glucose-grown cells. No growth or utilization was observed with 2-DG in the absence of added substrate.

Table 6.2 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose and xylose in the presence of 50 mM 2-deoxy-glucose

Growth substrate (s)	Final optical density (650 nm)	Lag period (hours)	Growth rate (h^{-1})	Figure number
50 mM 2-DG	NG	-	-	-
50 mM Glucose	1.92	15.00	0.32	6.14
50 mM Glucose + 50 mM 2-DG	1.10	40.00	0.04	6.14
50 mM Glucose + 45 mM 2-DG added at mid-log	1.80	12.00	0.23	6.17
50 mM G + 50 mM 2-DG (G-DG)	1.85	12.00	0.19	6.21
50 mM G + 50 mM 2-DG (X-DG)	1.60	40.00	0.07	-
50 mM Xylose	1.59	12.00	0.08	6.15
50 mM Xylose + 50 mM 2-DG	0.95	90.00	0.04	6.15
50 mM Xylose + 20 mM 2-DG added at mid-log	0.78	15.00	0.01	6.18
50 mM X + 50 mM 2-DG (G-DG)	1.65	50.00	0.11	-
50 mM X + 50 mM 2-DG (X-DG)	1.60	15.00	0.09	6.22
50 mM Glucose + 50 mM xylose	1.90	12.00	0.29	6.16
50 mM G + 50 mM X + 50 mM 2-DG	1.10	25.00	0.03	6.16
50 mM G + 50 mM X + 20 mM 2-DG added at mid-log	1.60	12.00	0.09	6.19
50 mM G + 50 mM X + 50 mM 2-DG (G-DG)	1.76	12.00	0.18	6.23
50 mM G + 50 mM X + 50 mM 2-DG (X-DG)	1.85	18.00	0.09	6.24

NG = no growth; G-DG = glucose + 2-DG adapted strain; X-DG = xylose + 2-DG adapted strain. When analogues were added to cultures at mid-log, then the growth rates are calculated after the addition of the analogue.

Cl. thermohydrosulfuricum Rt8.B1 grown on 2-DG + glucose consumed both substrates simultaneously, with glucose being taken up at a greater rate than 2-DG

(figure 6.14). There was a significant decrease in the growth rate (table 6.2). Cultures grown on xylose + 2-DG also consumed 2-DG and xylose simultaneously with no apparent preference for either substrate (figure 6.15). *Cl. thermohydrosulfuricum* Rt8.B1 grown on glucose + 2-DG + xylose utilized all three simultaneously (6.16). For cultures grown in the presence of 2-DG + glucose (figure 6.14), a 19.0 mM decrease in the concentration of 2-DG was seen; this was presumably due to 2-DG uptake. A 36.50 mM change in the concentration of 2-DG was seen with cultures grown on xylose + 2-DG (figure 6.15), this concentration difference was 17.0 mM for glucose + xylose + 2-DG growing cultures (figure 6.16). Whether these changes in the concentration of 2-DG were due to accumulation or metabolism of the analogue is not known.

The addition of 2-DG to a mid-log phase culture growing on glucose did not affect either the growth rate or the rate of substrate utilization significantly (figure 6.17). In contrast, the addition of 2-DG to cells growing on xylose had a pronounced effect both on the growth rate and on xylose utilization (figure 6.18). When 2-DG was added to cultures growing on glucose + xylose ,the rates of growth and of substrate utilization were slowed but both substrates were still utilized simultaneously and some 2-DG was taken up (figure 6.19).

The effect of 2-DG on cell growth was shown to be concentration- dependent with increasing concentrations of 2-DG increasing the lag phase proportionately (figure 6.20). For cells grown on glucose, concentrations of 2-DG below 5 mM had no effect on the growth of *Cl. thermohydrosulfuricum*. In contrast, xylose-grown cells were inhibited by concentrations of 1 mM 2-DG.

An extended lag phase of growth was exhibited by *Cl. thermohydrosulfuricum* Rt8.B1 when grown in the presence of 2-DG. This could be overcome by sequential transfer of *Cl. thermohydrosulfuricum* Rt8.B1 on medium containing either 2-DG + glucose or 2-DG + xylose. After 10 transfers on medium containing 2-DG + glucose, there was no extended lag phase and the amount of 2-DG utilized was also reduced (figure 6.21). Similar results were seen with xylose + 2-DG (figure 6.22); again the lag phase was

reduced and the amount of 2-DG utilized was slightly reduced. For both adapted cultures (G-DG and X-DG), the growth rate and utilization of glucose or xylose was lower than in cultures grown on glucose or xylose alone in the absence of 2-DG. Cells adapted to grow on 2-DG + glucose (G-DG adapted strain) were not adapted to grow on xylose + 2-DG, where a long lag was observed (table 6.2). Corresponding results were obtained with cells adapted to grow on xylose + 2-DG (X-DG adapted strain); these cultures exhibited the same lag phase as did unadapted cells when first grown on glucose + 2-DG (table 6.2).

These adapted strains were tested for their ability to grow on glucose + xylose together. The G-DG adapted strain when transferred to medium containing glucose + xylose + 2-DG exhibited hyperbolic growth kinetics with glucose and xylose utilized simultaneously (figure 6.23). X-2-DG adapted cultures grown on glucose + xylose + 2-DG consumed all substrates simultaneously with xylose being used at a faster rate than either glucose or 2-DG, both of which were taken up at a similar rate (figure 6.24).

Cells of *Cl. thermohydrosulfuricum* Rt8.B1 adapted to grow on medium containing 2-DG + glucose (G-DG strain) exhibited a characteristically long lag when transferred to medium containing 2-DG + xylose. This inhibition was relieved if glucose was added during the lag phase (figure 6.25). When glucose was not added, the lag phase was greatly extended, which suggested that glucose addition may block the effect of 2-DG on xylose utilization, thereby allowing growth on xylose to occur.

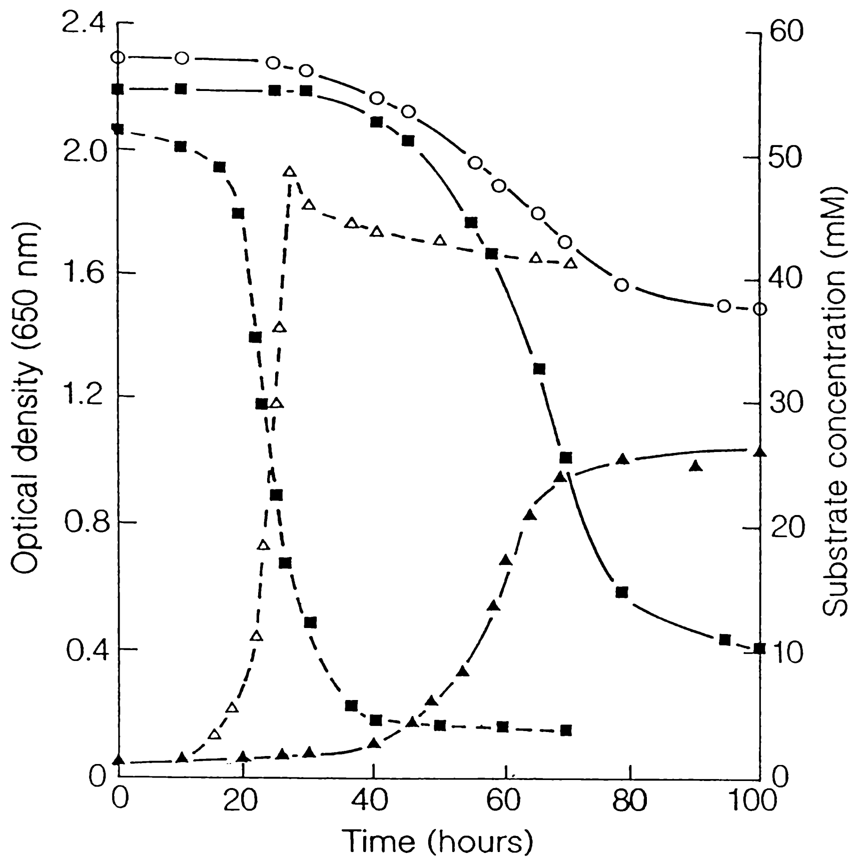


Figure 6.14 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM 2-DG in batch culture. Optical density (▲), glucose (■), 2-DG (O). Broken lines (---) are controls representing optical density (Δ) and glucose (■) consumption by cultures grown on 50 mM glucose alone.

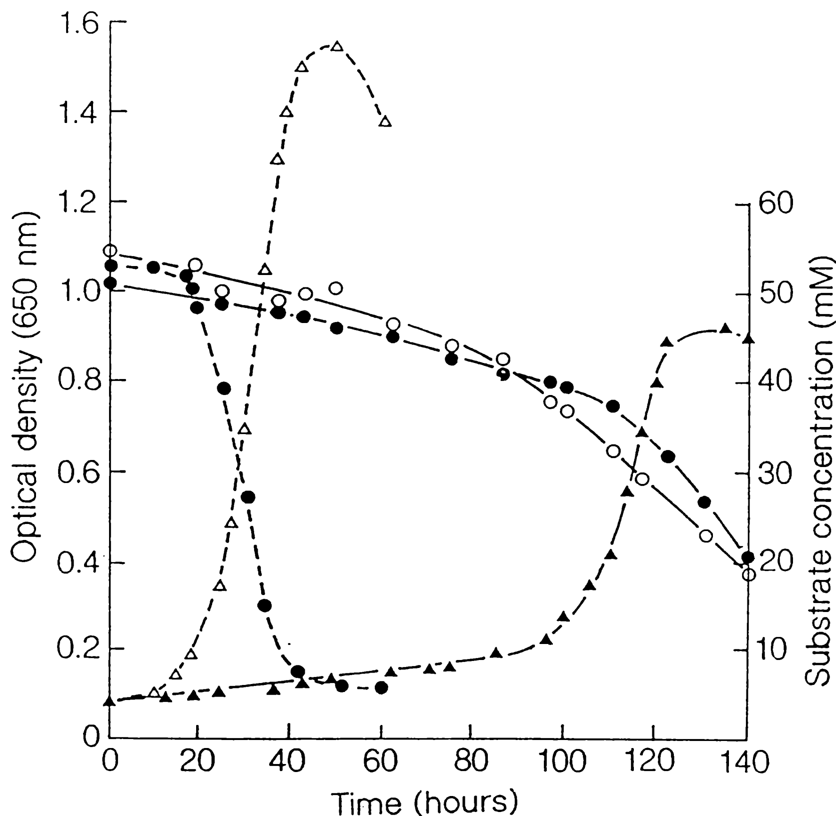


Figure 6.15 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM 2-DG in batch culture. Optical density (▲), xylose (●), 2-DG (O). Broken lines (---) are controls representing optical density (Δ) and xylose (●) consumption by cultures grown on 50 mM xylose alone.

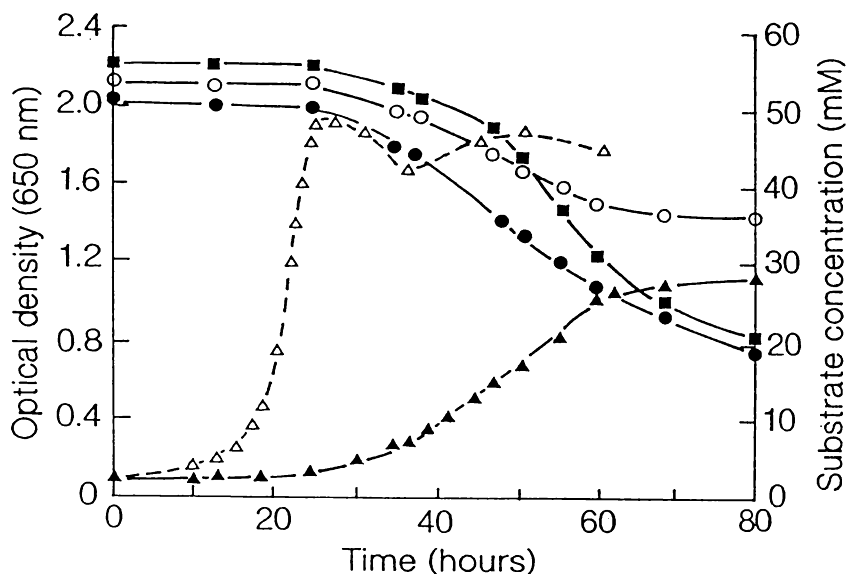


Figure 6.16 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM glucose + 50 mM 2-DG in batch culture. Optical density (▲), xylose (●), glucose (■) and 2-DG (O). Broken line (---) is control representing optical density (Δ) for cultures grown on 50 mM glucose + 50 mM xylose.

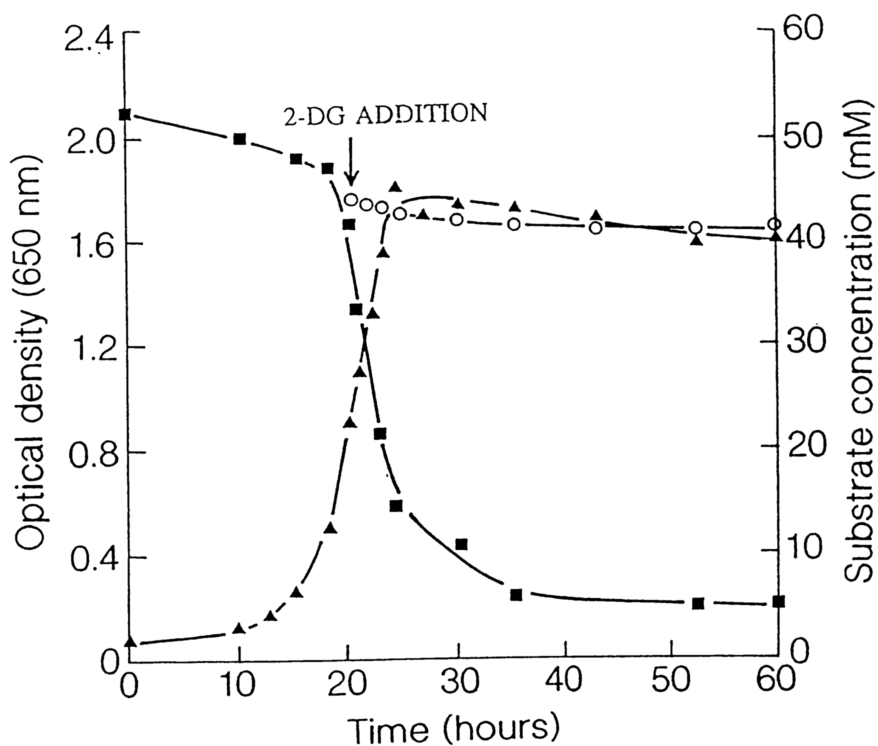


Figure 6.17 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose with 45 mM 2-DG added at the mid-log phase of growth in batch culture. Optical density (▲), glucose (■) and 2-DG (O).

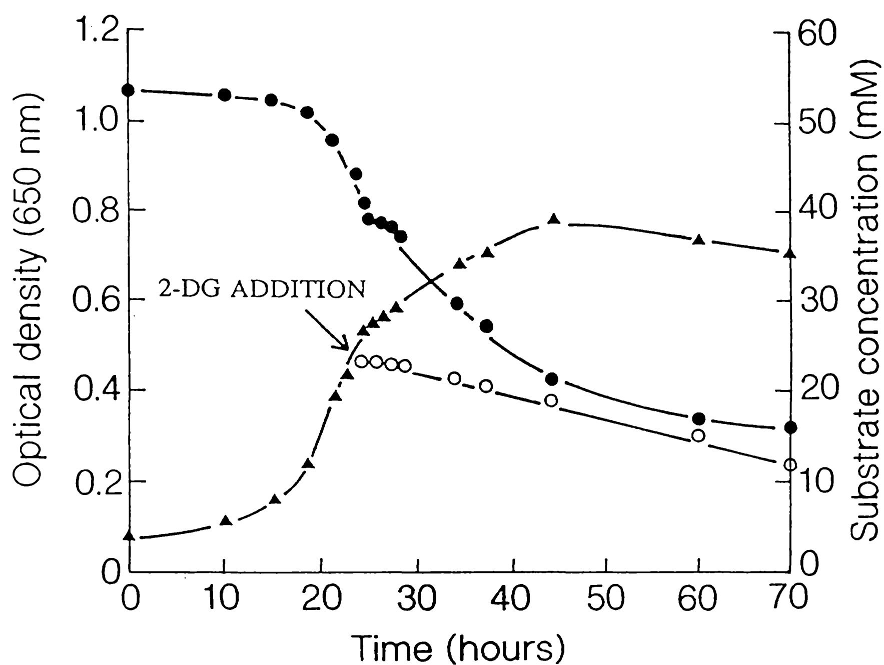


Figure 6.18 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose with 20 mM 2-DG added at the mid-log phase of growth in batch culture. Optical density (▲), xylose (●) and 2-DG (O).

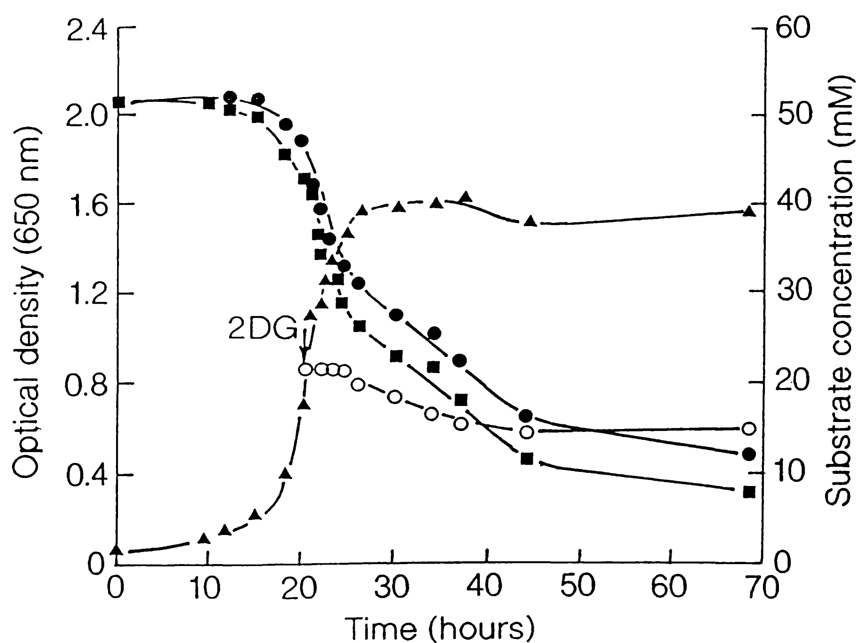


Figure 6.19 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM glucose with 20 mM 2-DG added at the mid-log phase of growth in batch culture. Optical density (▲), xylose (●), glucose (■) and 2-DG (O).

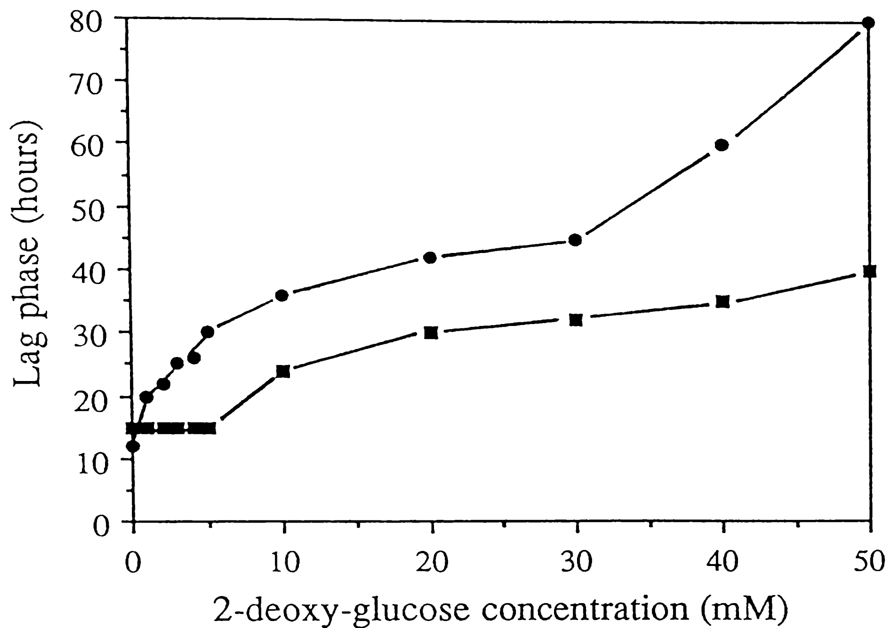


Figure 6.20 The effect of 2-DG concentration on the lag phase of growth in cultures of *Cl. thermohydrosulfuricum* Rt8.B1 grown on glucose or xylose. Glucose-grown (■) and xylose-grown (●).

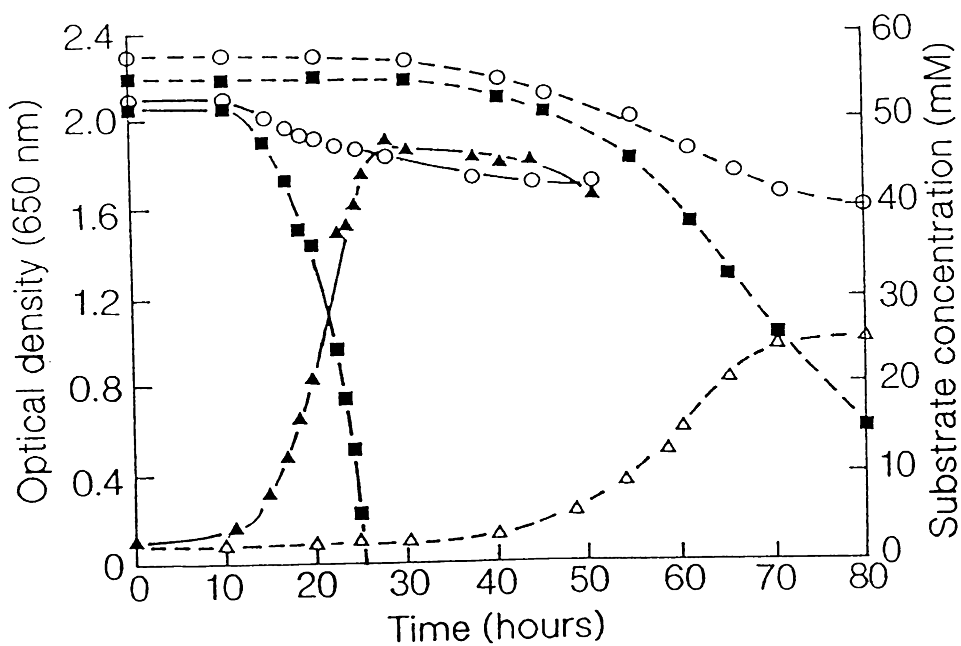


Figure 6.21 Growth of adapted glucose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM 2-DG in batch culture. Optical density (▲),glucose (■) and 2-DG (O). Broken lines (---) are controls representing optical density (Δ) and 2-DG (O) + glucose (■) consumption for cultures without adaption.

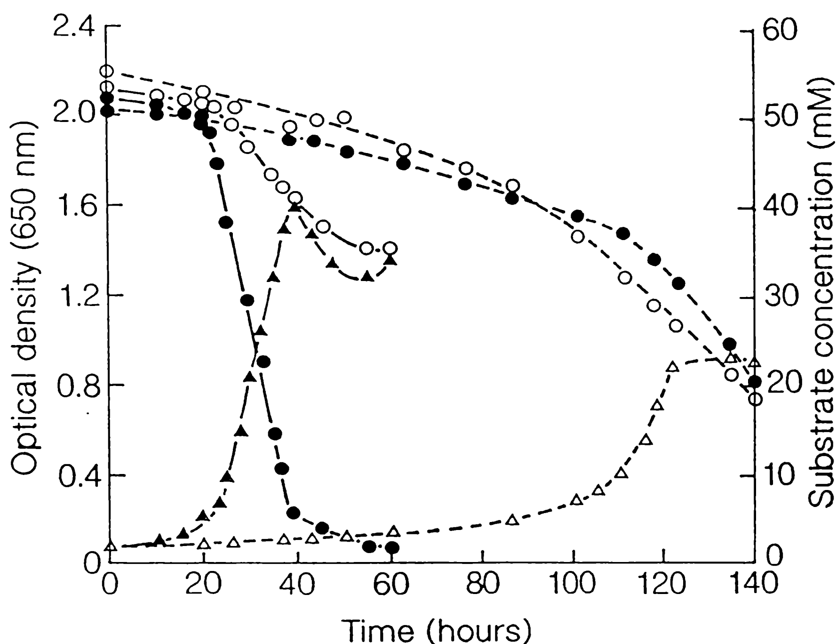


Figure 6.22 Growth of adapted xylose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM 2-DG in batch culture. Optical density (▲),xylose (●) and 2-DG (O). Broken lines (---) are controls representing optical density (Δ) and 2-DG (O) + xylose (●) consumption for cultures without adaption.

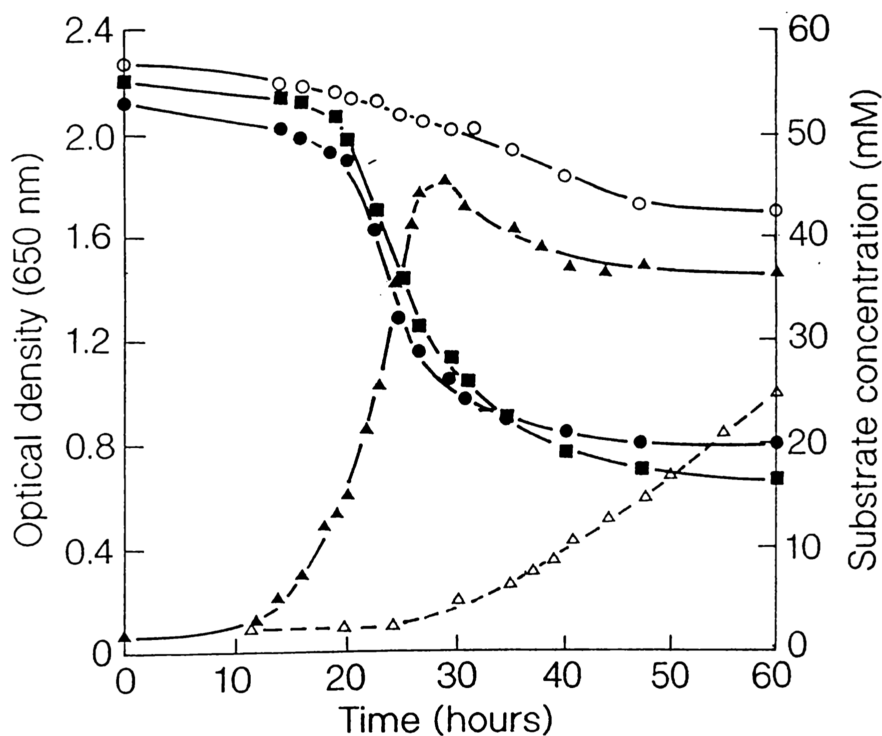


Figure 6.23 Growth of adapted glucose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose + 50 mM 2-DG in batch culture. Optical density (▲),glucose (■), xylose (●) and 2-DG (O). Broken line (---) is control representing optical density (Δ) for cultures grown on 50 mM glucose + 50 mM xylose + 50 mM 2-DG without adaption.

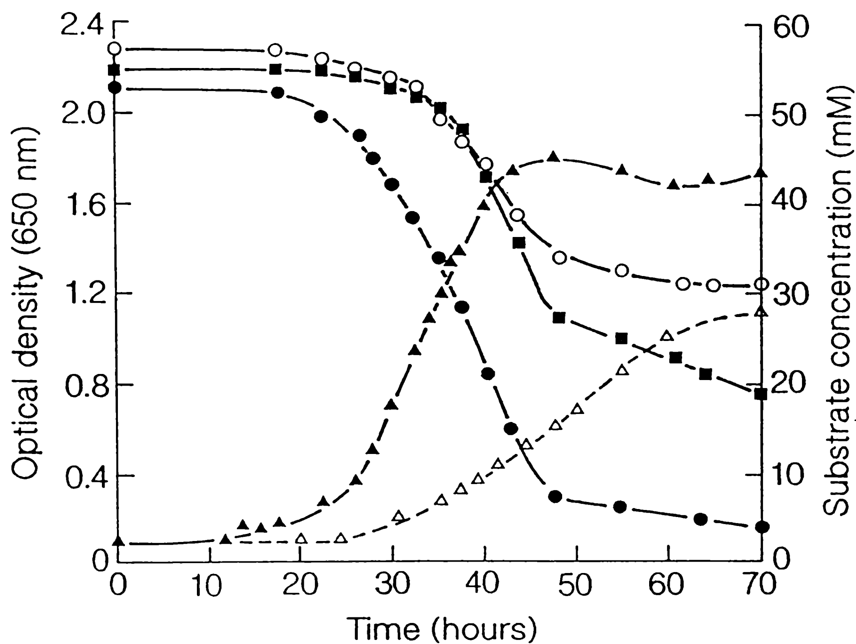


Figure 6.24 Growth of adapted xylose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM glucose + 50 mM xylose + 50 mM 2-DG in batch culture. Optical density (▲),glucose (■), xylose (●) and 2-DG (O). Broken line (---) is control representing optical density (Δ) for cultures grown on 50 mM glucose + 50 mM xylose + 50 mM 2-DG without adaption.

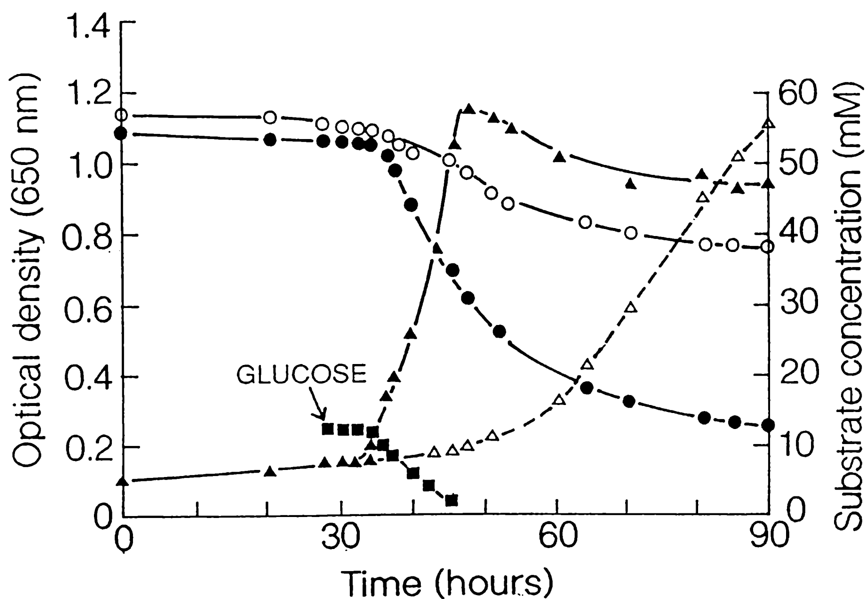


Figure 6.25 Growth of adapted glucose + 2-DG cultures of *Cl. thermohydrosulfuricum* Rt8.B1 on 50 mM xylose + 50 mM 2-DG in batch culture with glucose added at the lag phase of growth. Optical density (▲),glucose (■), xylose (●) and 2-DG (O). Broken line (---) is control representing optical density (Δ) for cultures grown on 50 mM glucose + 50 mM xylose + 50 mM 2-DG without glucose addition.

6.3.2.3 Summary of results

The results of experiments using 2-DG and α-MG demonstrated that these analogues inhibit growth and subsequent substrate utilization. This could be a result either of competition between glucose and xylose for the same transport protein or the inhibition of key enzymes involved in the utilization of these substrates. The greatest effect was seen on the lag phase of growth, which was greatly increased. There was some specificity with the analogues in terms of the effects on growth and substrate utilization. Glucose-grown cells were more susceptible to inhibition by α-MG whereas xylose-grown cells were inhibited more by 2-DG. This suggests that differences may exist in the systems used for glucose and xylose utilization by *Cl. thermohydrosulfuricum*. The fact that both analogues inhibited both glucose and xylose utilization did not provide any conclusive evidence towards the existence of dual uptake systems for glucose and xylose. From these results it was decided to investigate mechanisms operating for glucose and xylose utilization in more detail, using cell suspensions prepared from cultures grown on glucose, xylose and glucose + xylose.

6.3.3 Utilization studies using cell suspensions

6.3.3.1 Introduction

In order to study the utilization (the sum of transport and metabolism) of carbohydrates by *Cl. thermohydrosulfuricum* Rt8.B1 in the absence of induction and increasing growth rates, experiments were carried out using cell suspensions prepared from the mid-logarithmic phase of growth. Cell suspensions were resuspended in buffered minimal salts with chloramphenicol added to prevent protein synthesis. These were tested for their ability to utilize added substrates in the absence of growth. Under these conditions the utilization rate is linear, and expresses the uptake capabilities divorced from growth rate and the consequent continual changes in cellular protein and total uptake capacity encountered in growing cultures.

Cell suspensions were tested for substrate utilization using the following carbohydrates:

1. 50 mM glucose alone (single)
2. 50 mM xylose alone (single)
3. 50 mM 2-DG alone (single)
4. 50 mM 2-DG + 50 mM glucose or 50 mM xylose (mix)
5. 50 mM glucose + 50 mM xylose together (mix)

6.3.3.2 Glucose and xylose utilization by 50 mM glucose-grown cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1

Figure 6.26 shows a typical response when 50 mM glucose or 50 mM xylose were added as single substrates (not in combination) to suspensions of cells grown on 50 mM glucose. The results show that the rate of glucose utilization was linear with time (180 min) and no lag in the utilization of substrate was observed. In contrast to glucose utilization, xylose was not utilized at a significant measurable level thus confirming the inducible nature of xylose metabolism. Thus glucose-grown cells do not have the ability to utilize xylose (figure 6.26), but are able to induce a system for xylose catabolism rapidly when growing. For 50 mM glucose-grown cells, the rates of utilization for all substrates tested are summarized in table 6.3.

The results in table 6.3 show that when either 50 mM 2-DG or 50 mM xylose is supplied in combination (mix) with 50 mM glucose, the rate of glucose utilization is reduced when compared with glucose supplied as a single substrate. Interestingly, in the presence of glucose, a significant level of xylose utilization was detected. The fact that 2-DG and xylose reduced the rate of glucose utilization suggests that competition exists between these substrates for a common uptake system or catabolic enzymes.

Table 6.3 **Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose**

Substrates supplied	Rate of utilization (nmol /min /mg protein)
50 mM glucose (single)	44.15
50 mM xylose (single)	0.89
50 mM 2-DG (single)	0.38
50 mM glucose + 50 mM 2-DG (mix)	33.28 1.24
50 mM glucose + 50 mM xylose (mix)	28.52 6.02

With increasing concentrations of glucose from 0 to 50 mM, glucose utilization exhibited saturation kinetics (figure 6.27). The Eadie-Hofstee plot with increasing glucose concentration was biphasic (inset figure 6.27). Biphasic kinetics (non-Michaelis Menten kinetics) are usually explained as due to multiple uptake systems acting in concert (Button, 1985). The two systems are usually described as a high-affinity (low K_m)-low V_{max} system plus a low-affinity (high K_m)-high-V_{max} system (Button, 1985). For glucose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 at low glucose concentrations there was a low rate of glucose utilization and the K_s (apparent K_m) was 1.15 mM and the y intercept (V_{max}) was 26.50 nmoles of glucose /min /mg protein. With increasing concentrations of glucose, the rate of utilization increased and the K_s rose to 7.67 mM with a V_{max} of 54.50 nmoles of glucose /min /mg protein.

6.3.3.3 Glucose and xylose utilization by 5 mM glucose-grown cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1

To determine whether a second system was operative for glucose utilization when cells were grown on low concentrations of glucose, cell suspensions were prepared from

mid-logarithmic 5 mM glucose-grown cultures and tested for their ability to utilize glucose and xylose.

Figure 6.28 shows that the rates of glucose and xylose utilization were linear over the time period of the assay. The results show that cells grown on low concentrations of glucose do not utilize xylose, which is in agreement with the observation for cells grown on high concentrations of glucose. The rate of glucose utilization when cells were grown on 5 mM glucose was about 70 % of that obtained for cells grown on 50 mM glucose, reflecting a reduced capacity to utilize glucose. A summary of utilization rates for 5 mM glucose-grown cells are presented in table 6.4.

Table 6.4 Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM glucose

Substrate supplied	Rate of utilization (nmol /min /mg protein)
50 mM glucose (single)	36.23
50 mM xylose (single)	0.65
50 mM glucose + 50 mM xylose (mix)	24.93 3.46

The results in table 6.4 show that in the presence of 50 mM xylose the rate of glucose utilization is reduced. There was some xylose utilization in the presence of glucose; as this was also seen in the cell suspensions from *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose (table 6.3).

When supplied with increasing concentrations of glucose (0-50 mM), saturation kinetics were observed (figure 6.29). The Eadie-Hofstee plot was biphasic with increasing concentrations of glucose (inset 6.29). At low glucose concentrations, where there was a low rate of glucose utilization and the K_s was 0.84 mM glucose and

the V_{max} was 21.0 nmoles of glucose /min /mg protein. With increasing concentrations of glucose, the rate of utilization increased, but the rate of glucose utilization to substrate concentration decreased (low-affinity) and the K_s was 6.29 mM glucose with a V_{max} of 36.50 nmole of glucose /min /mg protein.

The results of these experiments suggest that glucose utilization by glucose-grown cells is mediated by two systems. At low concentrations of glucose the system has a high-affinity for glucose (low-capacity), but with increasing glucose concentrations, glucose utilization is mediated by a system which has a low-affinity, but with an increased capacity (high rate). The fact that both 5 mM glucose and 50 mM glucose-grown cells exhibited biphasic kinetics and that the relative affinities (K_s) for glucose were similar suggested that the same systems were operative in both instances.

Similar studies were carried out with cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1 grown on xylose.

6.3.3.4 Glucose and xylose utilization by 50 mM xylose-grown cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1

The results of glucose or xylose utilization by cell suspensions of 50 mM xylose-grown cells are presented in figure 6.30. The results showed that cells grown on 50 mM xylose utilized both glucose or xylose when supplied as single substrates at a linear rate over the time period of the assay (150 min) and that no lag period occurred. Xylose-grown cells were capable of utilizing glucose, thus confirming the constitutive nature of glucose metabolism. The results of substrate utilization for cell suspensions of 50 mM xylose-grown cultures are summarized in table 6.5.

Table 6.5 **Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose**

Substrates supplied	Rate of utilization (nmol /min /mg protein)
50 mM glucose (single)	30.95
50 mM xylose (single)	72.93
50 mM 2-DG (single)	0.46
50 mM xylose + 50 mM 2-DG (mix)	51.78 2.43
50 mM glucose + 50 mM xylose (mix)	8.29 20.95

The results presented in table 6.5 demonstrate that the rate of xylose utilization was reduced in the presence of 50 mM glucose or 50 mM 2-DG when compared with xylose as the single substrate. The presence of 50 mM xylose also reduced the rate of glucose utilization.

The effect of xylose concentration (0-50 mM) on the rate of xylose utilization was also investigated. With increasing concentrations of xylose, the rate of xylose utilization was proportional to the external substrate concentration (figure 6.31); this suggested that a diffusion or facilitated diffusion mechanism was operating with increasing concentrations of xylose, a linear Eadie-Hofstee plot was seen which did not intercept the y axis. This was indicative of some diffusion process (inset figure 6.31).

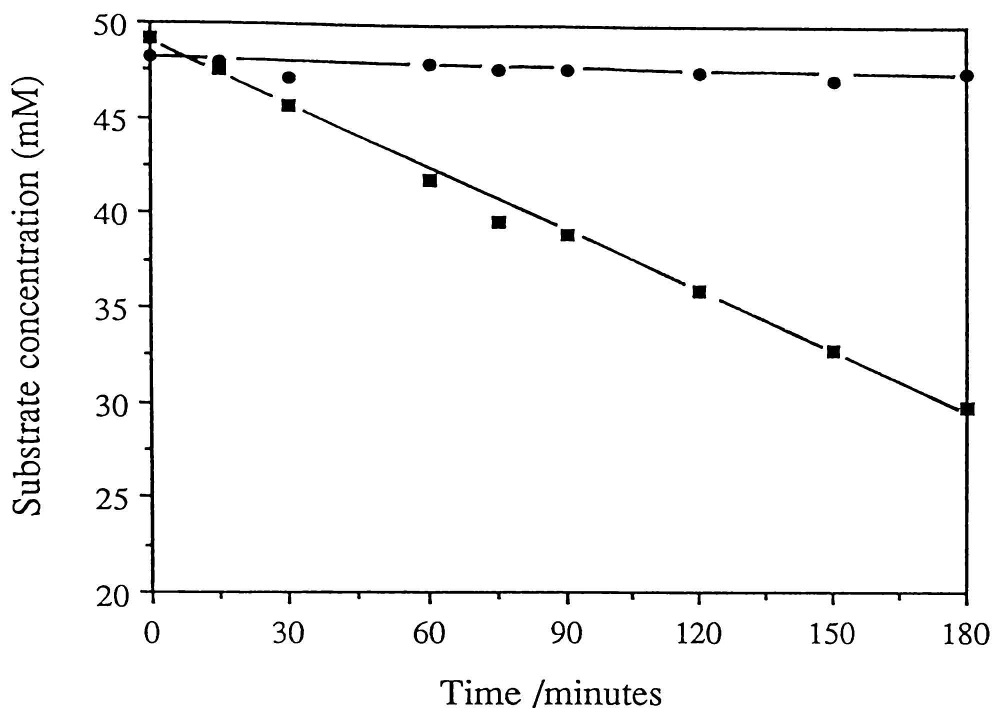


Figure 6.26 Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose. Glucose = (■), xylose = (●).

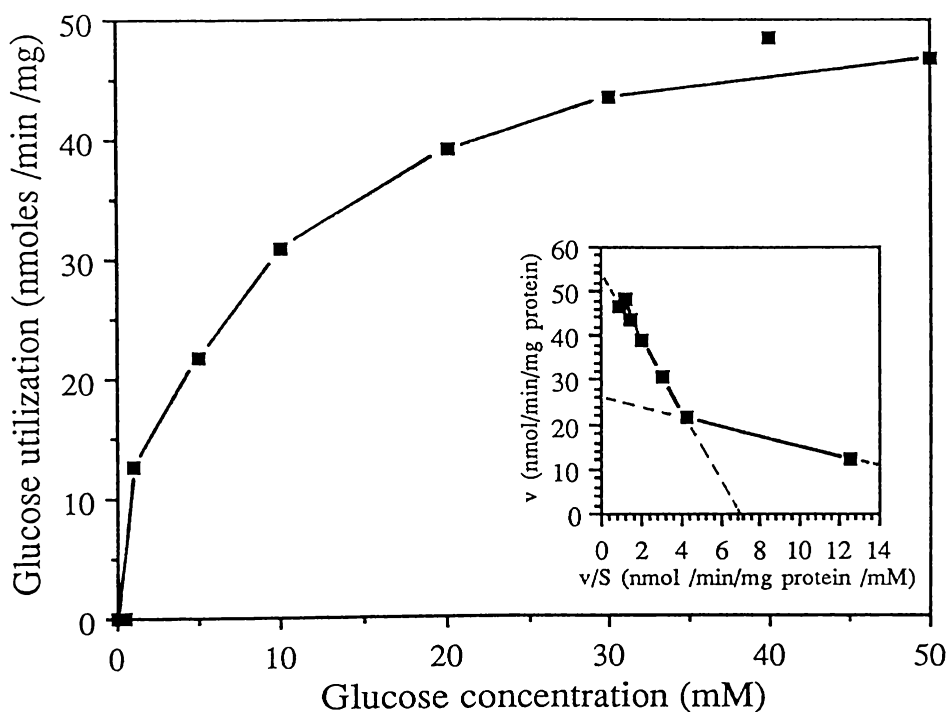


Figure 6.27 Glucose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose with increasing glucose concentration. Eadie-Hofstee plot of glucose utilization is shown in inset.

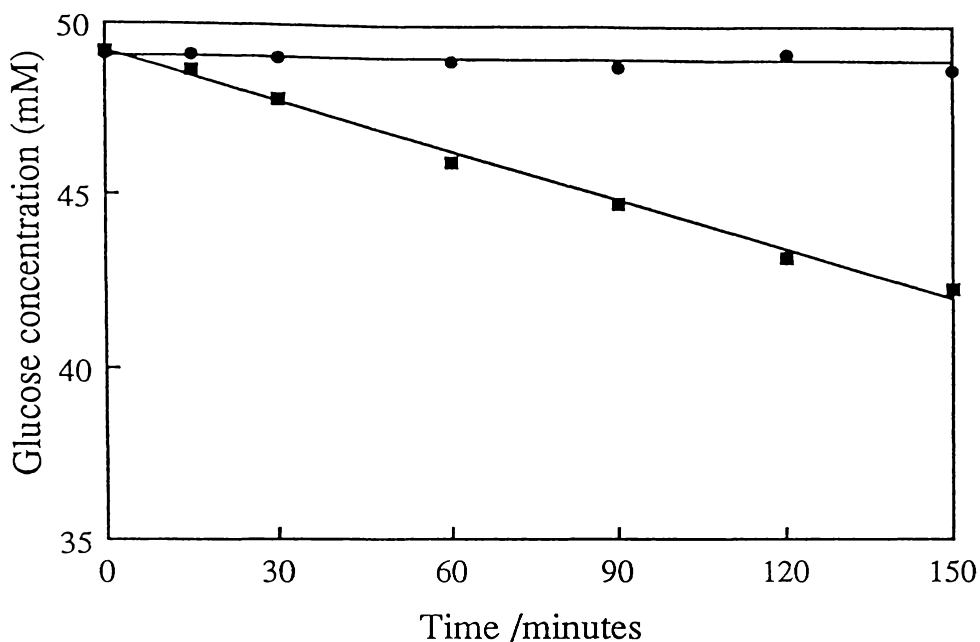


Figure 6.28 Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM glucose. Glucose = (■), xylose = (●).

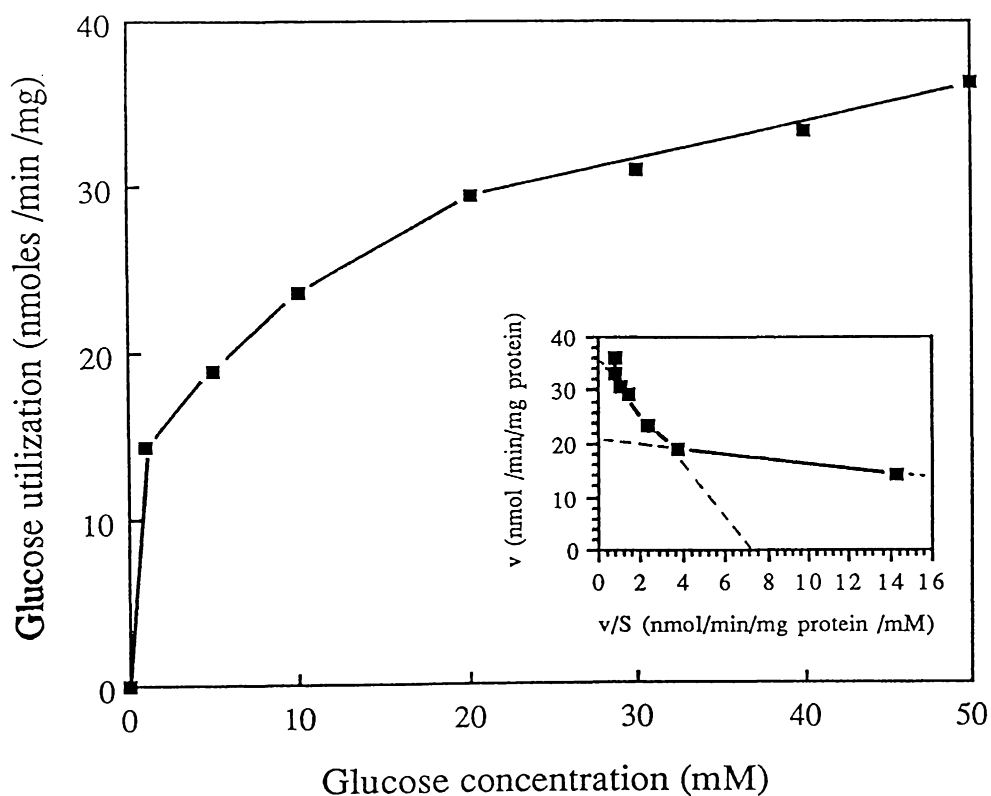


Figure 6.29 Glucose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM glucose with increasing glucose concentration. Eadie-Hofstee plot of glucose utilization is shown in inset.

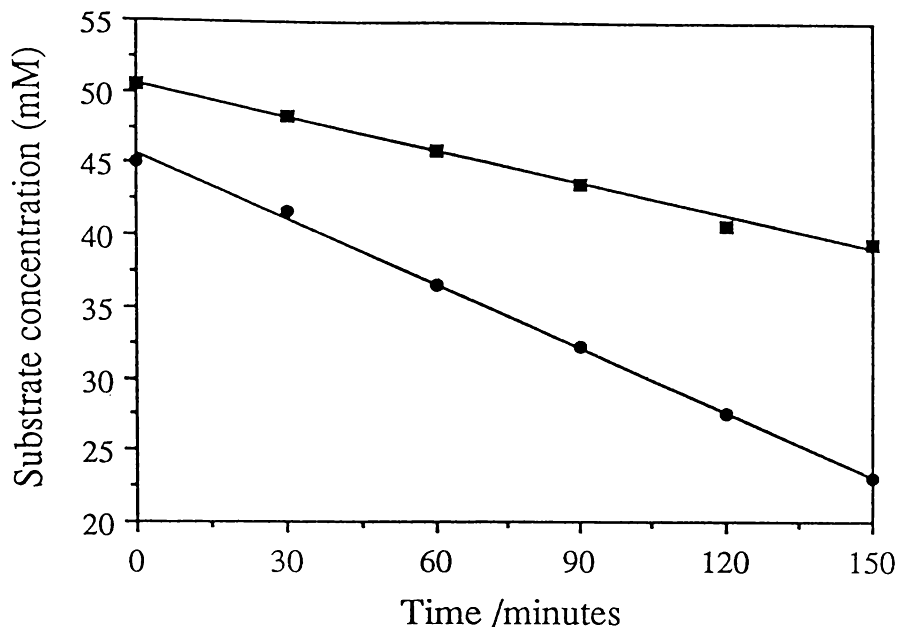


Figure 6.30 Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose. Glucose = (■), xylose = (●).

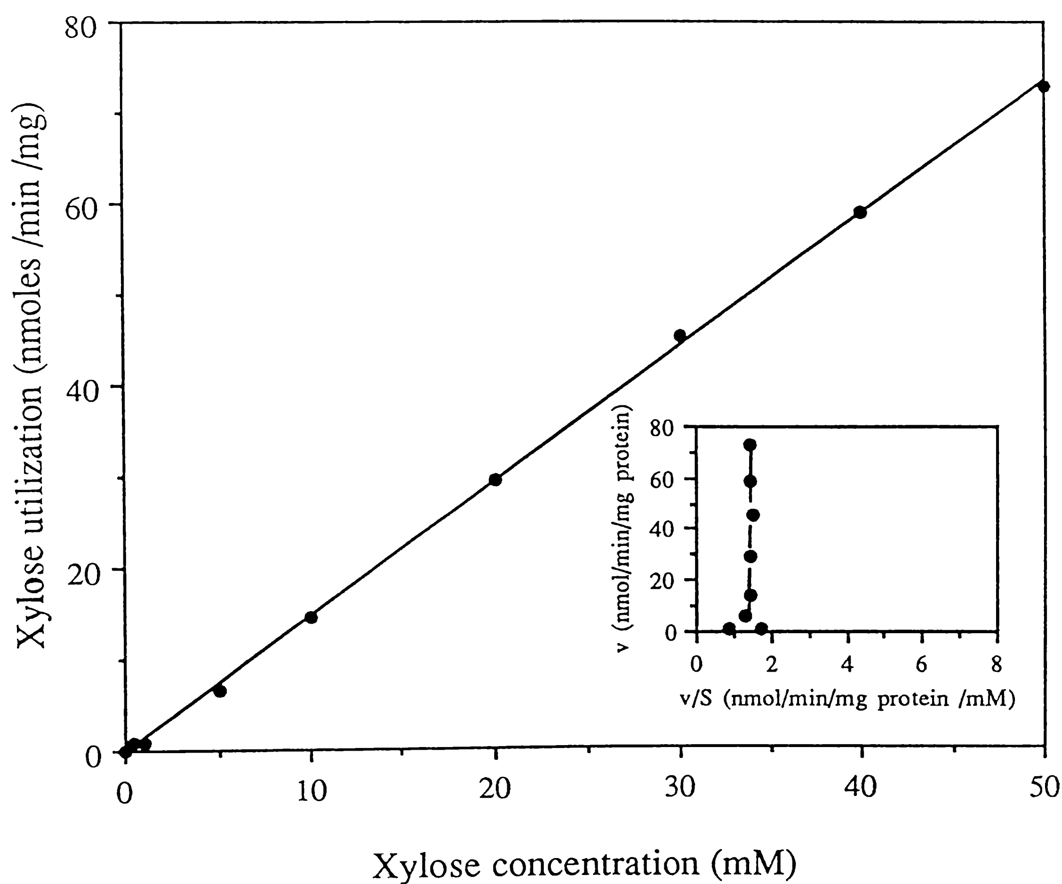


Figure 6.31 Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose with increasing xylose concentration. Eadie-Hofstee plot of xylose utilization is shown in inset.

6.3.3.5 Glucose and xylose utilization by 5 mM xylose-grown cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1

To determine if a second system for the utilization of xylose was present when *Cl. thermohydrosulfuricum* Rt8.B1 was grown on low concentrations of xylose, cell suspensions were prepared from mid-logarithmic 5 mM xylose-grown cultures.

The results for the utilization of glucose or xylose when added as single substrates to 5 mM xylose-grown cells are shown in figure 6.32. Both rates were linear over the period of the assay and no lag in substrate utilization was observed. Both glucose and xylose were utilized at a lower rate compared with that for cells grown on 50 mM xylose. A summary of utilization rates for 5 mM xylose-grown cells are presented in table 6.6.

Table 6.6 **Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose**

Substrate supplied	Rate of utilization (nmol /min /mg protein)
50 mM glucose (single)	7.55
50 mM xylose (single)	18.68
50 mM glucose + 50 mM xylose (mix)	0.42 10.78

When compared with single substrates, the rates of glucose and xylose utilization were significantly reduced when both substrates were supplied together, with a low level of glucose utilization detected.

When supplied with increasing concentrations of xylose (0-50 mM), saturation kinetics were exhibited (figure 6.33). The Eadie-Hofstee plot was linear and the K_s was 2.76

mM xylose with the intercept to the y axis (V_{max}) 20.0 nmole of xylose /min/mg protein (inset figure 6.33).

6.3.3.6 Glucose and xylose utilization by 50 mM glucose + 50 mM xylose-grown cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1

To determine which systems were present when cells were grown on a glucose + xylose substrate mix, the rates of utilization for glucose and xylose were measured in cell suspensions from cultures grown on equal concentrations of glucose + xylose (50 mM each).

Figure 6.34 shows that both glucose or xylose (added as single substrates) were utilized at similar rates which were linear over the time period of the assay. The results of substrate utilization for glucose + xylose grown cells are summarized in table 6.7.

Table 6.7 **Rates of substrate utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on equal concentrations of glucose + xylose**

Substrate supplied	Rate of utilization (nmol /min /mg protein)
50 mM glucose (single)	28.36
50 mM xylose (single)	25.32
50 mM 2-DG (single)	0.50
50 mM 2-DG + 50 mM glucose + 50 mM xylose (mix)	1.36 20.13 22.60
50 mM glucose + 50 mM xylose (mix)	23.39 23.51

The results summarized in table 6.7 show that when glucose and xylose were supplied together (mix) the rates for their utilization were not significantly different to the rates of glucose and xylose added individually (single). In the presence of 2-DG the rates for glucose and xylose utilization were reduced, with greater inhibition of glucose utilization.

The kinetics of substrate utilization with increasing concentrations of glucose or xylose (0-50 mM of each substrate) were investigated (figure 6.35). From these results 2 major trends are apparent. For glucose utilization, saturation kinetics are observed and the Eadie-Hofstee plot is biphasic reflecting the presence of two systems (figure 6.36). At low concentrations of glucose where the rate of glucose utilization was low the K_s was 0.98 mM glucose and the V_{max} 6.67 nmoles of glucose /min /mg protein. With increasing concentrations of glucose, the rate increased greatly and the V_{max} was 80.0 nmoles of glucose /min /mg protein. For xylose, the rate of utilization was directly proportional to the substrate concentration suggesting facilitated diffusion is involved. The Eadie-Hofstee plot for xylose utilization was linear and did not intercept the y axis (maximum velocity) (figure 6.37). The results demonstrate that the systems for glucose and xylose utilization are different in their relative affinities for their substrates.

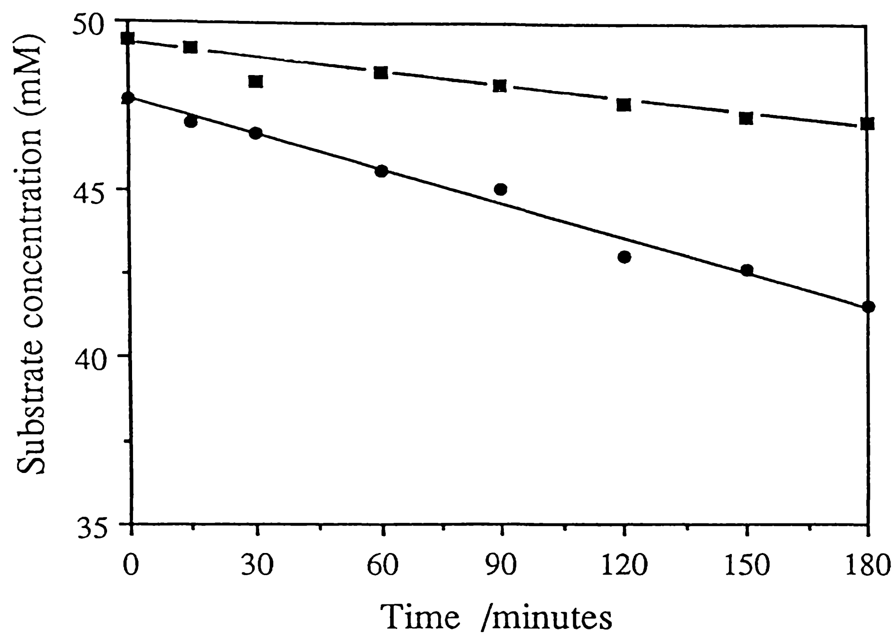


Figure 6.32 Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose. Glucose = (■), xylose = (●).

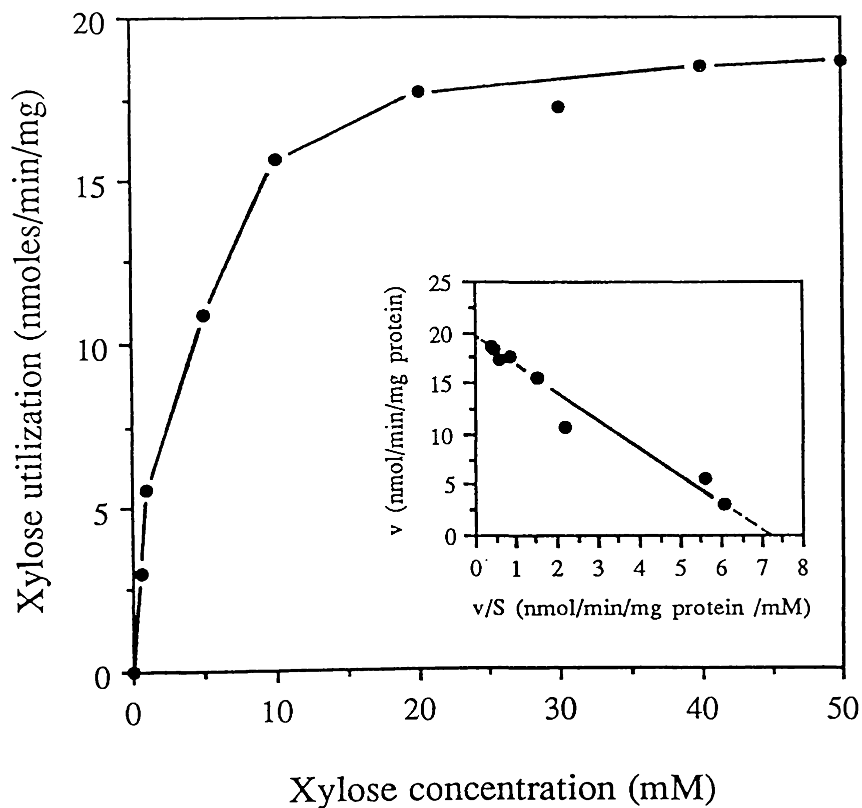


Figure 6.33 Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing xylose concentration. Eadie-Hofstee plot of xylose utilization is shown in inset.

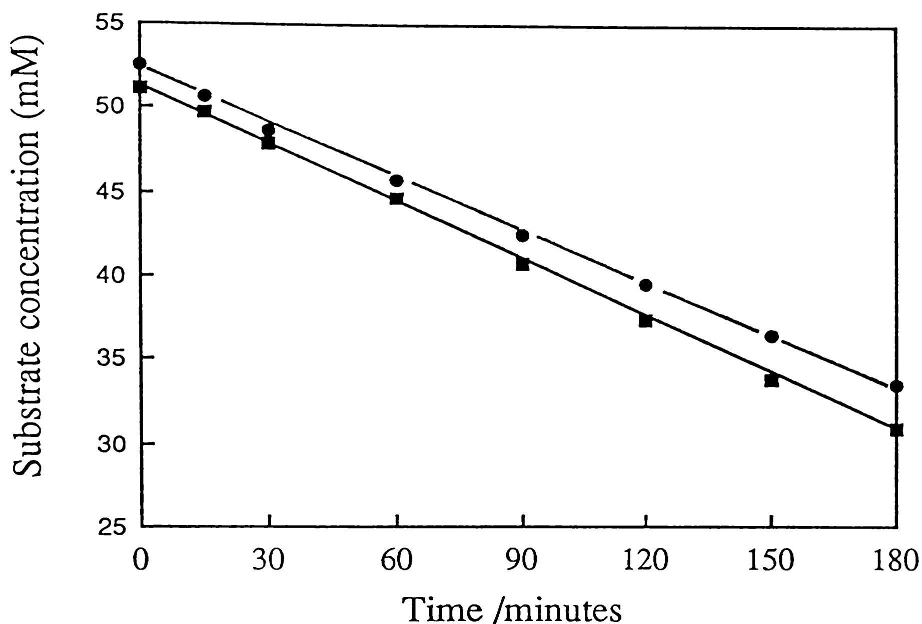


Figure 6.34 Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose + 50 mM xylose. Glucose = (■), xylose = (●).

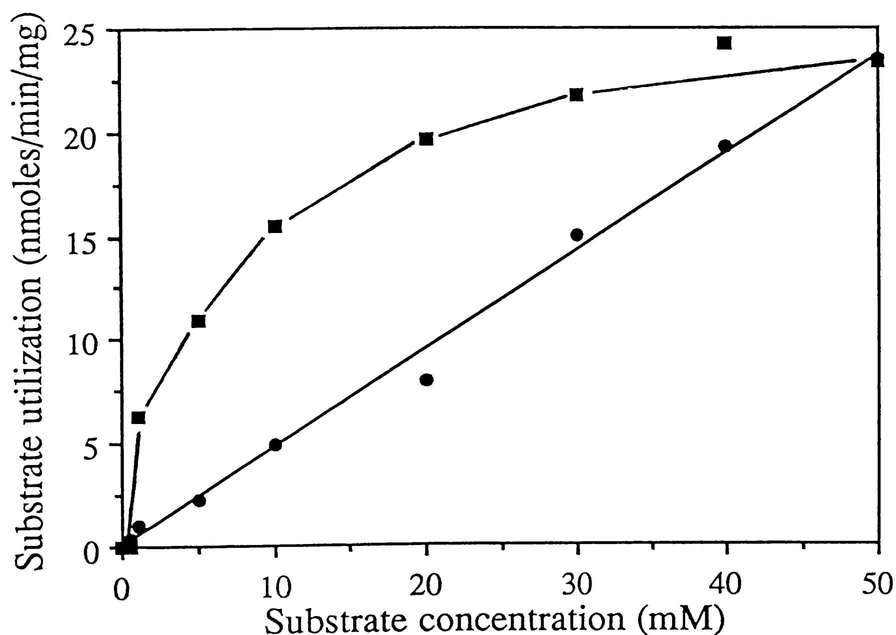


Figure 6.35 Glucose or xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose + 50 mM xylose with increasing glucose and xylose concentrations. Glucose = (■), Xylose = (●).

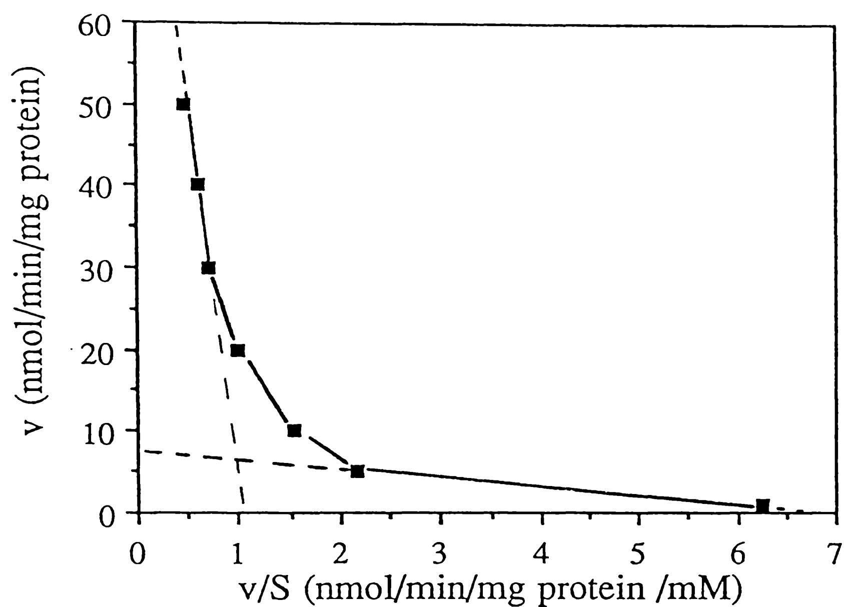


Figure 6.36 Eadie-Hofstee plot of glucose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose + 50 mM xylose.

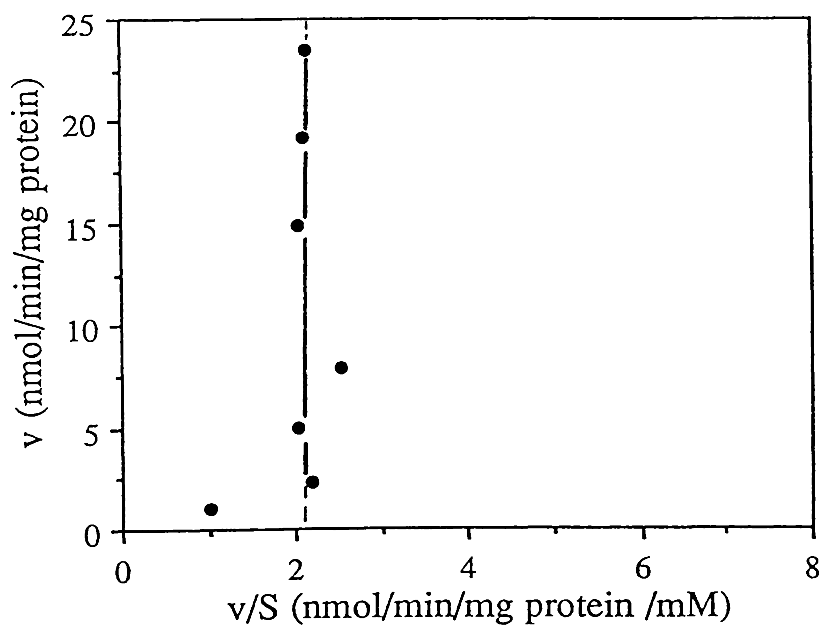


Figure 6.37 Eadie-Hofstee plot of xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1grown on 50 mM glucose + 50 mM xylose.

6.4 DISCUSSION

6.4.1 Hyperbolic growth of *Cl. thermohydrosulfuricum* Rt8.B1

Cook (1988) reported hyperbolic growth kinetics for some extremely thermophilic bacteria grown in an undefined medium containing glucose and xylose in both pH-controlled and pH-uncontrolled batch culture. These findings have been confirmed in this study with *Cl. thermohydrosulfuricum* Rt8.B1 grown in defined minimal medium containing glucose + xylose (figure 6.1). The present results suggest that hyperbolic growth kinetics are not a consequence of high levels of yeast extract and trypticase peptone present in the undefined medium used in previous studies, but a physiological property of this extreme thermophile.

From the data obtained on the studies of *Cl. thermohydrosulfuricum* Rt8.B1 grown on glucose + xylose substrate combinations it was not possible to distinguish between substrate uptake by a common carrier for both sugars (but with a very high total uptake capacity with similar affinities for each sugar) or whether independent uptake systems operate. Even where the addition of an equal amount of substrate was added to a culture actively growing on an alternate substrate,the added substrate was used immediately and co-utilized with no obvious classical lag (figures 6.2 and 6.3). These results lend further support to the conclusions in chapter three on the lack of induction /repression in thermophilic organisms.

When glucose was added to cultures growing on low concentrations of xylose (5 mM), xylose utilization was inhibited. In the reverse situation xylose had no inhibitory effect on glucose utilization (figures 6.7 and 6.8), which indicates that glucose and xylose might share a common transport protein or uptake system when grown on low concentrations of xylose. The common transport protein would appear to have a greater affinity for glucose than for xylose. However, the results could also be interpreted in terms of competition for available ATP for glucose and xylose phosphorylation by intracellular kinases. If this second explanation is true, then the

rate of substrate turnover (and consequently ATP formation) was limiting under the growth conditions used.

Inhibition of xylose uptake by glucose might still be observed if a separate transport system existed for each sugar. If the maximum rate of sugar uptake by the cell is less than the sum of the maximum rates for each individual sugar, then the control of the transport of either sugar to a lower rate might occur. This might take place by virtue of the fact that although separate transport systems are available for the individual sugars, there is a limit to the total amount of utilization of sugar species: the result would be an inhibition in the rate of transport of one or both of the sugars.

If a common transporter does exist for glucose and xylose then *Cl. thermohydrosulfuricum* Rt8.B1 should be able to switch from growth on one sugar to growth on another without a lag provided that the enzymes for the metabolism of glucose and xylose are constitutive. Cook (1988) has reported that glucose metabolism in *Cl. thermohydrosulfuricum* Rt8.B1 is mediated by the Embden Meyerhof Parnas pathway. In this study, the enzyme glucokinase responsible for the phosphorylation of glucose was constitutive in *Cl. thermohydrosulfuricum* Rt8.B1. A constitutive glucokinase has been reported in *Clostridium thermosaccharolyticum* (Aduse-Opoku and Mitchell, 1988) and *Clostridium acetobutylicum* (Ounine *et al.*, 1985) also grown on glucose + xylose substrate mixes. In the present study, the constitutive level of glucokinase in *Cl. thermohydrosulfuricum* Rt8.B1 increased only 3-fold in the presence of added glucose and was not repressed or inhibited by high concentrations of xylose.

The major route of xylose dissimilation in *Cl. thermohydrosulfuricum* Rt8.B1 is the pentose phosphate pathway (Cook, 1988). The results of the present study showed that xylose isomerase activity was inducible in *Cl. thermohydrosulfuricum* Rt8.B1, with a low level detected in glucose-grown cells. This was increased 24-fold in the presence of xylose and the level of induction and the specific activity of the enzyme were unaffected by the level of glucose in the growth medium.

Diauxic growth on glucose + xylose substrate mixes has been reported in the mesophilic bacteria *Pediococcus halophilus* (Abe and Uchida, 1989), *Cl. acetobutylicum* (Ounine *et al.*, 1985) and the moderately thermophilic (60°C) bacterium *Cl. thermosaccharolyticum* (Aduse-Opoku and Mitchell, 1988). Diauxic growth in the two clostridial strains was explained on the basis of the inducible nature of xylose metabolism i.e., xylose isomerase and xylose permease activity, both of which were repressed by glucose (catabolite repression), and when glucose was added to cells actively growing on xylose. In direct contrast to these other studies, the results of the present study of growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose + xylose indicated that growth was hyperbolic and that enzymes for the catabolism of both substrates were present (figures 6.1, 6.2 and 6.3). This could provide an explanation for hyperbolic growth in that the 2 main factors resulting in diauxic growth were both absent when *Cl. thermohydrosulfuricum* Rt8.B1 was grown in glucose + xylose containing medium. That one of these factors (catabolite repression) was absent was shown by the observation that glucose did not repress xylose metabolism when added to cells growing on xylose (figure 6.3). That the other factor (inducer exclusion) was absent was shown by the observation that glucose did not prevent xylose uptake since both substrates were taken up at equal rates when supplied together (figures 6.1).

In general, substrates allowing fast growth are degraded by constitutive enzymes, while induced enzymes show catabolite repression by these better substrates (Gottschalk, 1986). In this study, the highest growth rates for *Cl. thermohydrosulfuricum* Rt8.B1 were obtained on glucose and the metabolism of glucose was constitutive. The lowest growth rates were seen on xylose and the metabolism of xylose was inducible. Therefore even though glucose does not repress the synthesis of xylose isomerase or inhibit the uptake of xylose (at high xylose concentrations), an effect is seen on the overall growth rate. This was illustrated by the observation that when cultures were initially grown on glucose the growth rate was high; after the addition of xylose the growth rate decreased (figure 6.2). When cultures were initially grown on xylose the growth rate was low and this was increased by the addition of glucose. Similar growth rates which are intermediate between the substrate which results in the highest growth rate (glucose) and that which results in a lowered growth rate (xylose) has been

reported for growth of rumen bacteria on substrate combinations (Russell *et al.*, 1979). These authors suggested that the lowered growth rate is caused by a specific interference of the poorer substrate (i.e., xylose) with the utilization of the better substrate (i.e., glucose). However, substrates such as glucose and xylose which yield the same glycolytic intermediates in *Cl. thermohydrosulfuricum* Rt8.B1 (glyceraldehyde-3-phosphate; Cook, 1988) would not be likely to cause interference and it might therefore be expected that the level of inhibition would be at the level of transport into the cell if the uptake systems for glucose and xylose shared a common transport protein. The other explanation is that the utilization of xylose results in a lower level of ATP for cell protein synthesis.

6.4.2 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose analogues

6.4.2.1 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose and xylose in the presence of α-MG

The glucose analogue α-MG is a typical substrate of the glucose:phosphotransferase system in enteric bacteria (Postma and Roseman, 1976) or *Streptococcus mutans* (Liberman and Bleiweis, 1984). Growth of *Cl. thermohydrosulfuricum* Rt8.B1 in medium containing α-MG reduced growth and substrate utilization. The lag phase of growth was also greatly extended (table 6.1). However, a similar response was observed with xylose, a typically non-PTS sugar, and so at least in *Cl. thermohydrosulfuricum* Rt8.B1 can be considered to be to non-specific inhibition of growth and metabolism. During growth in the presence of xylose, α-MG was accumulated or metabolized by cells (figure 6.11). This did not occur in cultures grown on glucose (figure 6.90) or glucose + xylose (figure 6.10). The observation that only xylose-grown cells were capable of accumulating α-MG might indicate that the uptake systems of *Cl. thermohydrosulfuricum* Rt8.B1 for glucose and xylose are structurally different and have different affinities for α-MG. The result could also be due to the high affinity of the glucose uptake for glucose which means that, in the presence of glucose, α-MG cannot compete for the uptake site. In either case, in the absence of glucose (xylose-grown cells) α-MG is either effectively taken up by the

constitutive glucose uptake system or the xylose permease. There is also evidence for a toxic effect of α-MG, which may be due to the competitive effect on substrate uptake. This is shown by the increased lag phase and by the transient decrease in growth rate and xylose utilization when added at the mid-log phase of growth to cultures growing on xylose (figure 6.13).

6.4.2.1 Growth of *Cl. thermohydrosulfuricum* Rt8.B1 on glucose and xylose in the presence of 2-DG

The non-metabolizable glucose analogue 2-DG is a potent inhibitor of sugar transport and growth (Thompson and Chassy, 1982). The analogue 2-DG is a typical substrate of the mannose:PTS in lactic streptococci (Thompson and Chassy, 1985) and has been used to select mutants of *Streptococcus* or *Lactobacillus* species defective in the PTS mediated transport of specific sugars (Thompson and Chassy, 1983; Slee and Tanzer, 1982; Vadeboncoeur, 1984). Growth of *Cl. thermohydrosulfuricum* Rt8.B1 in media containing either 2-DG + glucose or 2-DG + xylose was slowed and occurred only after a long lag. The longest lag periods were seen with xylose-grown cultures and this difference could be a reflection of their uptake system. It was hoped that the addition of 2-DG to glucose + xylose grown cultures would inhibit the uptake of glucose, thereby providing evidence for the existence of separate uptake systems for these sugars. However, in the presence of 2-DG both glucose and xylose were utilized simultaneously, along with 2-DG. This accumulation of 2-DG was shown to vary depending on the growth substrate supplied but was always greatest for xylose-grown cultures.

That 2-DG is toxic to some bacteria is widely recognized but little is known of the mechanisms whereby the analogue inhibits growth in bacteria in general. *Cl. thermohydrosulfuricum* Rt8.B1 could be adapted to grow on 2-DG with glucose or 2-DG + xylose by sequential transfer on medium containing 2-DG. The usual extended lag phase was absent and the accumulation of 2-DG by both glucose and xylose grown cultures was greatly reduced. These observations may reflect a change in the affinity of the uptake system for 2-DG. When glucose + 2-DG adapted cultures were grown on

2-DG + xylose, an extended lag phase was observed (table 6.2). This inhibition could be overcome by the addition of glucose during the lag phase of the growth cycle which suggested that the glucose uptake system was unaffected by 2-DG and once growth began inhibition of xylose uptake and subsequent metabolism was relieved. Thus the adaption period resulted in changes in only one of the uptake systems which again suggested that the uptake systems for glucose and xylose were different. If a common uptake system was present, then inhibition by 2-DG should be overcome by adaption on one substrate for both glucose and xylose. Thompson and Chassy (1982) have demonstrated that 2-DG inhibits the growth of *S. lactis* 133 and this inhibition was a consequence of the PEP-dependent futile recycling of 2-DG at the expense of PEP (an ATP equivalent). The cycle causes the dissipation of PEP and the generation of ATP via pyruvate kinase. PEP and ATP are both required for biosynthetic, chemiosmotic and transport functions of the growing cell.

In summary, both 2-DG and α-MG inhibited both the growth rate and the utilization of substrate by *Cl. thermohydrosulfuricum* Rt8.B1. The results of these experiments were difficult to interpret as it was not known whether the reduction of growth was a consequence of the analogues being toxic to the cell or a consequence of substrate transport being reduced and therefore resulting in a lowered rate of substrate uptake and hence growth. The analogues once transported into the cell may have produced other effects on key metabolic enzymes. In general, 2-DG is not phosphorylated by ATP-dependent bacterial kinases (Ghosh and Ghosh, 1968; Romano *et al.*, 1970; Romano *et al.*, 1979) but is phosphorylated by the PEP-PTS (Postma and Roseman, 1976). The fact that reports of glucokinases capable of phosphorylating 2-DG have been demonstrated (Martin and Russell, 1986; Abe and Uchida, 1991) could explain the inhibition of growth and substrate utilization seen with *Cl. thermohydrosulfuricum* Rt8.B1 grown on medium containing 2-DG. If 2-DG is inhibiting glucokinase activity, then an overall inhibition of growth would be seen due to a decrease in the rate of effective phosphorylation of glucose and therefore metabolism.

6.4.3 Kinetics of glucose and xylose utilization of *Cl. thermohydrosulfuricum* Rt8.B1

To investigate glucose and xylose utilization in more detail the utilization of these substrates was studied using cell suspensions from mid-logarithmic harvested cultures of *Cl. thermohydrosulfuricum* Rt8.B1.

6.4.3.1 Kinetics of substrate utilization by glucose-grown cells

Glucose utilization by glucose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 was biphasic. This was indicated by the relative affinities for glucose at high and low concentrations of glucose. At low concentrations of glucose (< 5 mM) a high-affinity system (low K_s) was present and with increasing concentrations of glucose the rate increased greatly, but the rate of utilization to substrate concentration decreased (low-affinity). The change in kinetics with increasing glucose concentration could be due to a single system which changes its relative affinity (K_s) and maximum velocity for glucose depending on the concentration of glucose present. This was not dependent on the concentration of glucose used for cell growth as kinetics of utilization in 5 and 50 mM glucose-grown cells were similar. The fact that saturation kinetics were observed with increasing concentrations of glucose suggested that the uptake system or the first enzyme of the metabolic sequence limits the rate of glucose utilization.

A summary of kinetic parameters and utilization rates for glucose-grown cells are summarized in table 6.8.

Table 6.8 **Utilization rates and kinetic parameters for cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM or 50 mM glucose**

Substrates supplied and kinetic parameter	Rate of utilization (nmoles /min /mg of protein)	
	5 mM Glucose grown	50 mM Glucose grown
1 mM glucose (single)	14.34	13.80
50 mM glucose (single)	36.23	44.15
50 mM xylose (single)	0.65	0.89
50 mM Glucose + 50 mM xylose (mix)	24.93 3.46	28.52 6.02
Relative affinity (Ks)		
Low glucose concentrations	0.84 mM	1.15 mM
High glucose concentrations	6.29 mM	7.67 mM
Maximum velocity (Vmax)		
Low glucose concentrations	21.00	26.50
High glucose concentrations	36.50	54.50

Low glucose concentrations = 0 to 5 mM

High glucose concentrations = 5 to 50 mM.

Glucose utilization was reduced in the presence of xylose and 2-DG indicating a competitive effect at the level of uptake or metabolism. If the xylose transporter is inducible, this result may indicate that, in its absence, some xylose is transported by the glucose uptake system in the presence of glucose, thereby decreasing the rate of glucose uptake and metabolism. The low level of xylose isomerase present in glucose-grown cells could explain the low level of xylose utilization observed. The sum of glucose and xylose utilization was 78 % of the total glucose utilization when glucose was added as the sole substrate, demonstrating that *Cl. thermohydrosulfuricum* Rt8.B1

has a greater capacity to utilize glucose. With 2-DG a toxicity effect cannot be ruled out and inhibition of glucokinase may also be occurring which would also reduce the rate of utilization.

6.4.3.2 Kinetics of substrate utilization by xylose-grown cells

The kinetic parameters and utilization rates for *Cl. thermohydrosulfuricum* grown on 5 mM and 50 mM xylose are summarized in table 6.9.

Table 6.9 Utilization rates and kinetic parameters for cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM or 50 mM xylose

Substrates supplied and kinetic parameter	Rate of utilization (nmoles /min /mg of protein)	
	5 mM Xylose grown	50 mM Xylose grown
1 mM xylose (single)	5.57	0.89
50 mM xylose (single)	18.68	72.93
50 mM glucose (single)	7.55	30.95
50 mM glucose + 50 mM xylose (mix)	0.42 10.78	8.29 20.95
Relative affinity (Ks)	2.76 mM	ND
Maximum velocity (Vmax)	20.00	ND

ND = not able to be determined due to the rate of utilization being proportional to the substrate concentration. The rate of utilization did not intercept the y axis (maximum velocity) over the substrate concentrations investigated.

In *Cl. thermohydrosulfuricum* Rt8.B1, xylose utilization was mediated by two systems which were dependent on the xylose concentration used for cell growth. Cells grown on low concentrations of xylose (< 5 mM) possessed a system which has a high affinity for xylose (low K_s) and with increasing concentrations of xylose exhibited saturation kinetics reflecting a low capacity. When grown on high concentrations of xylose (50 mM), a system was present which had a high capacity i.e. exhibited high rates of xylose utilization and with increasing concentrations of xylose the rate of utilization was directly proportional to the external substrate concentration. This suggested that some diffusion process or facilitated diffusion mechanism is involved (low-affinity).

Although the acquisition of a low-affinity, high-capacity system may seem of no advantage to an organism in a natural environment where substrate is limiting, it would be of benefit at high substrate concentrations where the selection is for rapid growth and therefore the ability to accumulate substrate is important. At low concentrations of substrate the selection is for affinity rather than capacity. The fact that 2 systems are present for the utilization of xylose suggest that some form of control is present, as both systems appeared inducible i.e. biphasic kinetics were not observed (figure 6.38). Biphasic kinetics would have been observed if the systems were constitutive.

Plots of xylose utilization versus time at low concentrations of xylose (< 1.0 mM) also provided evidence for the relative affinity of the two systems (figure 6.39). Cells grown on 50 mM xylose were unable to utilize xylose at low concentrations (< 0.40 mM) reflecting the presence of a low-affinity uptake system. In contrast cells grown on 5 mM xylose were able to utilize xylose at concentrations < 0.40 mM reflecting the higher affinity of the system. Whether the different affinity of these two systems is at the level of transport or due to key metabolic enzymes changing their relative affinity for xylose at different concentrations remains to be investigated.

Competitive effects were observed when the rate of xylose utilization was measured in the presence of 2-DG and glucose. The presence of glucose reduced xylose utilization by 71 % (50 mM xylose-grown), suggesting that some competition at the level of transport or metabolism was occurring.

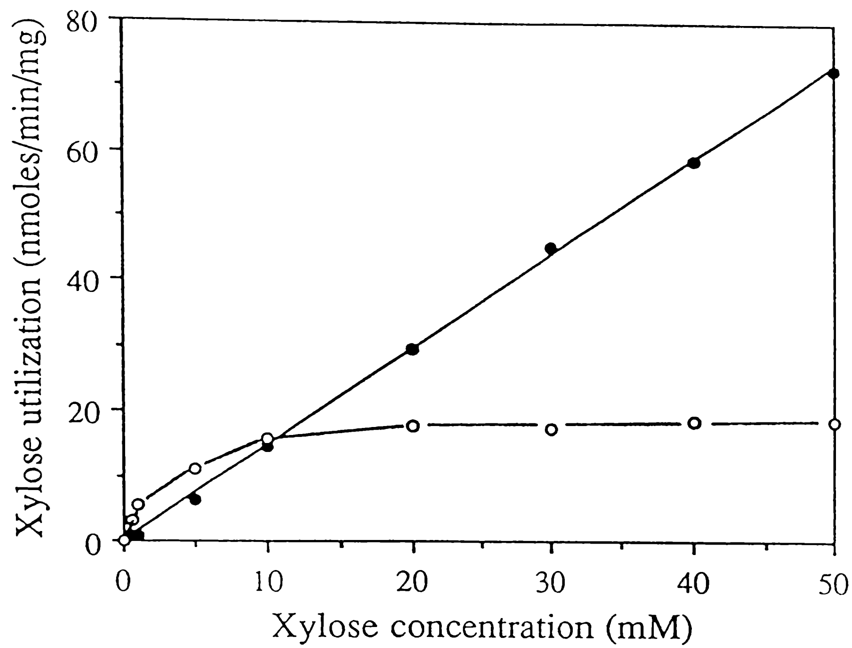


Figure 6.38 Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM and 50 mM xylose with increasing xylose concentration. 5 mM xylose = (O), 50 mM xylose = (●)

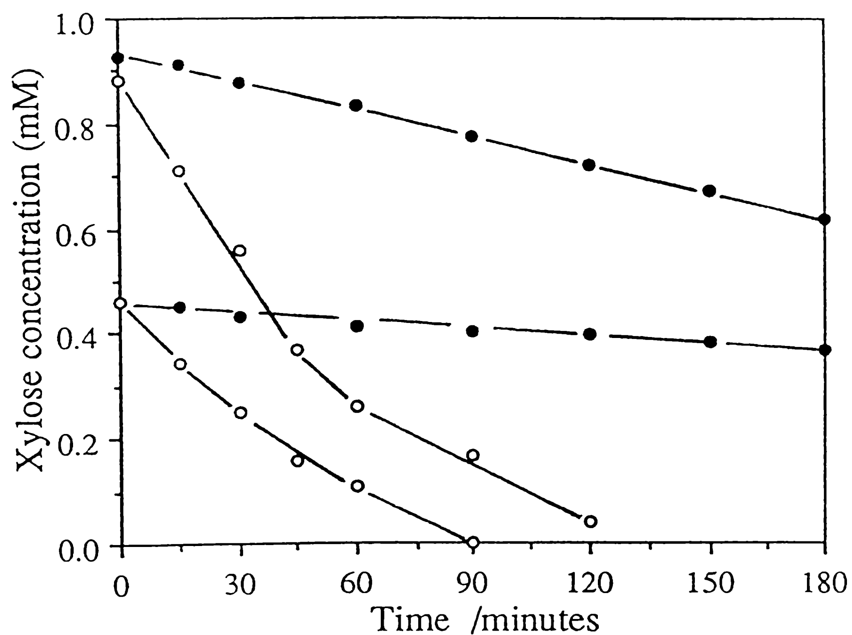


Figure 6.39 Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM and 50 mM xylose at low xylose concentrations. 5 mM xylose = (O) and 50 mM xylose = (●).

6.4.3.3 Kinetics of substrate utilization by glucose + xylose-grown cells

To determine what mechanisms were operative for glucose + xylose grown cells, utilization of substrate was studied with cell suspensions grown on a mix of two substrates. A summary of the kinetic parameters and utilization rates for glucose + xylose grown cells are presented in table 6.10.

Table 6.10 Utilization rates and kinetic parameters for cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose + 50 mM xylose

Substrates supplied and kinetic parameter	Rate utilization (nmol /min /mg protein)
1 mM glucose (single)	6.26
50 mM glucose (single)	23.39
1 mM xylose (single)	0.98
50 mM xylose (single)	23.51
50 mM glucose + 50 mM xylose (mix)	28.36 25.32
Relative affinity (Ks) Maximum velocity	0.98 mM glucose 6.67

Kinetic parameters were not able to be determined at high substrate concentrations (> 10 mM). Kinetics of glucose and xylose utilization were linear and did not intercept the y axis (maximum velocity).

The results of utilization studies with glucose + xylose grown cells demonstrated the differences between the systems responsible for glucose and xylose utilization. Glucose utilization was mediated by a system which was similar, if not identical, to that present in cells grown on glucose as a single substrate. Again, the system was

saturable and exhibited biphasic kinetics. The rate of xylose utilization was directly proportional to the external xylose concentration, again suggesting the presence of facilitated diffusion as for 50 mM xylose-grown cells. When the rates of glucose and xylose utilization at low concentrations of substrate are compared (table 6.10 and figure 6.35), the different affinities of the two systems became apparent. At low concentrations of substrate (1 mM), the rate of glucose utilization was 6-fold greater than for xylose utilization, reflecting a system with a higher affinity. With increasing concentrations of substrate, the rate of xylose utilization increases to the same level of glucose utilization at saturation concentrations of glucose (50 mM). Due to the inducible nature of the high-affinity xylose system present in 5 mM xylose-grown cells, the kinetics would be presumably similar in cultures actively growing on a glucose + xylose substrate mix.

The results of utilization studies with cell suspensions demonstrated clearly the ability of *Cl. thermohydrosulfuricum* Rt8.B1 to regulate its substrate utilization in response to the concentration of substrate and that the rate of glucose and xylose utilization in a substrate mix is precisely controlled. The rate of substrate utilization following growth on single substrates was always greater than following growth on mixed substrates. This could reflect an increase in activity of whichever enzyme constitutes the major kinetic control point in the catabolic pathway, thereby increasing the overall activity of the pathway which in turn could be determined by the rate at which the catabolic pathway synthesizes ATP or the rate at which ATP so formed is catalyzed by the cell.

The results of utilization studies suggest that the level of control for glucose and xylose utilization occurs either at the level of transport or involves the initial enzymes involved in the metabolism of glucose and xylose. In either case, the transport system or the enzymes may change their relative affinities for glucose and xylose dependent on the external concentration of substrate. These possibilities are further investigated at the level of transport in chapter seven and at the level of metabolism in chapter eight.

CHAPTER SEVEN

GLUCOSE AND XYLOSE TRANSPORT BY *Clostridium thermohydrosulfuricum* Rt8.B1

7.1 INTRODUCTION

Physiological studies on thermophilic organisms to date have focused on the pathways of carbohydrate catabolism. The mechanism and regulation of accumulation of carbohydrates has been largely ignored, despite being an important step in the fermentative metabolism of saccharolytic thermophilic bacteria. The importance of uptake systems as sites of metabolic regulation is clear from the extensive literature dealing with other bacterial groups (Saier, 1985).

Transport studies with extremely thermophilic bacteria have been restricted to a few genera and species. Amino acid transport in the thermophilic aerobe *Bacillus stearothermophilus* and the fermentative bacterium *Cl. fervidus* has been the focus of intense study (Speelmans *et al.*, 1989; DeVrij *et al.*, 1989, 1990; Heyne *et al.*, 1991).

For the transport of carbohydrates, studies have concentrated on glucose and cellobiose uptake, and no studies have been made of xylose transport in extreme thermophiles. Patni and Alexander (1971b) demonstrated low levels of phosphoenolpyruvate-dependent sugar:phosphotransferase (PEP-PTS) activity for fructose and mannitol in extracts of *Cl. thermocellum* 651. Glucose and cellobiose transport has been investigated in *Cl. thermohydrosulfuricum* 39E and *Cl. thermocellum* LQR1 (Ng and Zeikus, 1982). The uptake of glucose and cellobiose in these organisms was shown to involve glucose permease and cellobiose phosphorylase using ATP, rather than involving a PEP-PTS. Hernandez (1982) also confirmed these findings for glucose transport with *Cl. thermocellum* ATCC-27405. *Cl. thermosaccharolyticum*, a moderately thermophilic bacterium (optimum growth temperature 60°C), has been reported to have an inducible permease for the transport of xylose. The glucose

permease was constitutive and growth on a glucose + xylose substrate mix was diauxic with glucose used preferentially (Aduse-Opoku and Mitchell, 1988).

Except for these few findings, the transport of solutes and export of metabolic end products have received little attention in thermophilic fermentative organisms. This chapter deals with an investigation into the mechanisms of solute transport by the extremely thermophilic anaerobic organism *Cl. thermohydrosulfuricum* strain Rt8.B1.

7.2 MATERIALS AND METHODS

7.2.1 Chemicals.

D-[U-^{14}C]glucose (^{14}C-glucose), D-[U-^{14}C]xylose (^{14}C-xylose) and 2-deoxy-D-[1-^{3}H]glucose (^{3}H-2-DG) were from Amersham International (Amersham Laboratories, Buckinghamshire, England). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), monensin, iodoacetate, phosphoenolpyruvate (PEP) and lactate dehydrogenase (EC 1.1.1.28) were obtained from Sigma Chemical Co. (St Louis, Mo, USA). *N,N*-dicyclohexylcarbodiimide (DCCD) 2,4-dinitrophenol (2,4-DNP) and sodium azide were from BDH Chemicals Ltd (Poole, England). Sodium fluoride was obtained from May and Baker Australia Pty Ltd (Victoria, Australia). Sodium arsenate and potassium cyanide were obtained from Ajax chemicals (Sydney, Australia).

7.2.2 Preparation of cell suspensions

Resting cells (cell suspensions) of *Cl. thermohydrosulfuricum* Rt8.B1 were prepared by growing the cells in minimal medium containing the appropriate carbon source until the mid-exponential growth phase and then harvested by centrifugation at 8000 X g for 20 min at room temperature using a Sorvall SS-34 centrifuge (Du Pont Company, Wilmington, Delaware, USA). The harvested cells were washed twice with pre-reduced (Na_2S) buffered (50 mM MOPS) minimal salts (pH 7.0) containing 10 mM $MgCl_2$, recentrifuged and resuspended in the same buffer to give a protein concentration of 20 mg.ml^{-1}. The whole process was performed under anaerobic

conditions by maintenance of a N_2 headspace inside the culture vessels and addition and removal of fluids via a syringe. Cell manipulations were carried out in an anaerobic chamber (Coy, Inc., USA).

7.2.3 Preparation of toluenized cells and assay of PTS activity

Toluenized (decryptified) cells for the measurement of PEP-dependent glucose PTS activity were prepared immediately before the assay by the method of Kornberg and Reeves (1972). Cells were transferred by syringe injection (1 ml) of cell-suspension into a sealed anoxic test tube, followed by addition of 10 µl of a toluene-ethanol mixture (1:9, vol/vol), and vigorous blending in a Vortex mixer for 1 min at room temperature.

PEP-glucose PTS activity was assayed by two methods:

(1) PEP-glucose PTS activity was assayed in toluene-treated cells by the direct assay of Kornberg and Reeves (1972). The reaction mix contained (1 ml): 100 mM MOPS (pH 7.0), 10 mM $MgCl_2$, 10 mM PEP, 0.5 mM NADH, 4 U of lactate dehydrogenase, 10 mM glucose and 5 mM dithiothreitol. The production of pyruvate from PEP-dependent glucose phosphorylation was followed spectrophotometrically by lactate dehydrogenase-dependent NADH reduction (Bergmeyer, 1974).

(2) PEP-dependent phosphorylation of glucose to glucose-6-phosphate was assayed with the following reaction mix (1 ml): 100 mM MOPS (pH 7.0), 10 mM $MgCl_2$, 10 mM glucose, 10 mM PEP, 1 mM NADP, 5 U of glucose-6-phosphate dehydrogenase, and 5 mM dithiothreitol. For ATP-dependent glucose-6-phosphotransferase (glucokinase activity) PEP was replaced with 5 mM ATP.

Both reactions were initiated by the addition of 100 µl of toluenized cells (approximately 1 mg of cells wet weight) to 0.9 ml of a reaction mixture, and the production of NADPH (NADP $\Rightarrow$ NADPH) or NAD (NADH $\Rightarrow$ NAD) was monitored continuously using a double monochromator recording spectrophotometer model UV-

250 (Shimadzu Corporation, Kyoto, Japan) equipped with a Shimadzu temperature controlled cell holder (Model TCC-240A) and computer controlled graphic printer (Model PR-1) at 50°C. Assays were performed at 50°C, rather than at the growth temperature of 70°C, due to the instability of the linking enzymes lactate dehydrogenase and glucose-6-phosphate dehydrogenase at the higher temperature. All pyridine nucleotide oxidation or reduction reactions were measured at 340 nm (ε_{340} = 6.22 $mM^{-1}cm^{-1}$).

Decryptified cells were pre-incubated at 70°C for 10 min to consume any endogenous energy source that may have been present. Specific activities were determined where linearity with protein concentration was established and these activities expressed as nanomoles of glucose-6-phosphate formed per minute per milligram of protein as calculated from the initial velocity of NADPH or NADH production. All assays were performed in triplicate and the mean of these determinations (within 5 % of each other) presented. Controls were used for all assays and all values shown have been corrected using appropriate controls and blanks. In all assays a positive control was included as a test of PEP-dependent phosphorylation activity. The anaerobic organism 16Lt1 was used as it had been shown to have PEP-PTS activity for glucose (Janssen, 1991).

7.2.4 Transport of radioactive labelled sugars

7.2.4.1 Standard method

Resting cells were prepared as described above (section 7.2.2). Cell suspensions (10 ml each) were transferred anaerobically to vials with a 100 % N_2 gas phase. In the standard uptake procedure, 0.5 ml of cell-suspension was transferred anaerobically to serum vials containing 10 ml of pre-sterilized, buffered (50 mM MOPS, pH 7 at 70°C) minimal salts and flushed with N_2 gas. Cells were incubated for 15 min at 70°C with gentle agitation in a shaking water bath (Julabo, Labortechnik, GMBH, Germany) to equilibrate. To measure xylose transport, uptake was initiated by adding 0.5 ml of 0.5 mM xylose containing 2.5 µCi D-[U-^{14}C]xylose. For glucose transport, uptake was initiated by adding 0.50 ml of 0.1 mM glucose containing 1.0 µCi D-[U-

^{14}C]glucose. Transport of the glucose analogue 2-deoxy-glucose (2-DG) was initiated by adding 0.2 mM 2-DG containing 2 μCi of 2-deoxy-D-[1-^{3}H]glucose. Where the concentration of substrate differed, the amount of added isotope was corrected for dilution to achieve the same final specific activity for all concentrations. All uptake experiments were conducted at 70°C in a shaking water bath. Aliquots of the cell suspension (0.8 ml) were withdrawn periodically (10 second intervals) with a 1 ml syringe that was pre-rinsed in 10 % sodium sulphide. The suspension was then filtered through a 0.45 μm pore-size cellulose nitrate membrane filter (Millipore Corp., Bedford, Mass. USA) and washed twice with 5 ml of ice-cold 50 mM MOPS (pH 7.0 at 20°C). Cell suspensions were used at concentrations which allowed rapid filtration (< 30 seconds). The filters were dried under heat lamps and then placed in scintillation vials (Wheaton, Millville, NJ, USA) containing 5 ml of Ready Safe liquid scintillation cocktail (Beckman Instruments, Inc., Fullerton, Ca, USA) and counted in an LS-3801 scintillation counter (Beckman Instruments, Inc., Fullerton, Ca, USA)

7.2.4.2 Rapid sampling method

The transport of ^{3}H-2-DG uptake by cell suspensions was usually measured using the previously outlined method; the following method was used to allow more rapid sampling. The method involved mixing of individual small volumes of cell-suspension and isotopic sugar solutions for periods of 2 seconds to 1 min and stopping uptake by addition of ice-cold buffer, followed by rapid filtration. Uptake was initiated by injecting 0.25 ml of cell-suspension with a 1 ml syringe into 0.25 ml of 2-DG and ^{3}H-2-DG isotopic sugar solution. Contents were mixed on a vortex mixer and the reaction stopped at appropriate times by rapid addition of 5 ml of ice-cold buffer, filtration on a membrane filter (0.45 μM), and washing with an additional 5 ml of ice-cold buffer.

For all experiments, the initial rates of ^{14}C- or ^{3}H-labelled substrate uptake (0-60 seconds; minimum of 6 sampling points) were used to calculate the initial rate of uptake. The rate of ^{14}C-labelled substrate uptake with time was linear over the time period of the assay. All experiments were carried out in triplicate with appropriate blanks and controls. In all experiments, the observed number of disintegrations per

minute (DPM) was corrected for background and quenching. Data points were the mean of three experiments in which the standard error of the mean did not differ by more than 5 %.

Filters were counted for 10 min using channel window settings 0-400 for ^{3}H-2-DG and 0-670 for ^{14}C-glucose or ^{14}C-xylose single labelled experiments. The scintillation counter was calibrated at the beginning of each experiment to ensure that a given window setting for an isotope covered the energy spectrum of that isotope by using the unquenched calibration standard of ^{3}H obtained from Beckman Instruments, Inc. (Fullerton, Ca, USA). Results were corrected for quench by a stored calibration curve under the assay conditions used, using the external standard H number ($H^\#$) method based on ^{137}Cs Compton spectrum. Results were expressed as DPM (measure of absolute activity within the sample) to ensure the same counting efficiency of all samples. For all experiments the $H^\#$ did not change throughout the course of an experiment or when comparing different experiments, thereby confirming that all samples were counted with a similar efficiency.

The protein content of the cell suspension was determined by incubating 0.50 ml of the cell suspension with 0.50 ml of 1 M NaOH at 100°C for 15 min. After neutralization with 0.50 ml of 1 M HCl, the protein content was determined by the Biuret method (Scopes, 1982). All protein assays were carried out in triplicate and repeated where results differed by 5 %.

7.2.4.3 Kinetics of glucose and xylose transport

The data for ^{14}C-xylose and ^{14}C-glucose uptake were analyzed by the Eadie-Hofstee plot as outlined in section 6.2.3.4. The Michaelis constant (K_t or saturation constant) for transport was used to express the relative affinity of the uptake process (Button, 1991). The term V_{max} was used to define the maximal rate of substrate uptake (initial rate of uptake at saturating concentrations) expressed as nanomoles of substrate taken up per minute per milligram of protein (Button, 1991).

7.2.5 Competition experiments

For these experiments the volume of buffered minimal salts in the assay vial was reduced by 0.50 ml to accommodate the volume of competing sugar added. To investigate the effects of non-labelled sugars as potential inhibitors of glucose and xylose uptake, isotope uptake experiments were carried out in which cells were pre-incubated with buffered minimal salts and unlabelled sugars at a final concentration of 5 mM for 10 min prior to the addition of radioactive labelled substrate. The results of these experiments were expressed as the mean of three determinations ± the standard error of the mean.

7.2.6 Metabolic inhibitors

For these experiments the volume of buffered minimal salts in the assay vial was reduced by 50 µl to accommodate the volume of inhibitor added. Metabolic inhibitors, tested as potential inhibitors of glucose and xylose uptake, were added to the transport assay medium 10 min prior to initiating uptake. The following inhibitors were added at 100 µM final concentration: monensin, 2,4-DNP, CCCP and DCCD. The following inhibitors were all added to 5 mM final concentration: sodium azide, sodium fluoride, iodoacetate, potassium cyanide and sodium arsenate. All of the water-insoluble inhibitors were dissolved in 95 % ethanol and compared with ethanol-treated controls. The results of these experiments were expressed as the mean of three determinations ± the standard error of the mean and the level of inhibition expressed as the per cent inhibition on the initial rate of uptake as compared to controls (nominally 100 %) in the absence of inhibitor. For inhibitors added at final concentrations of 100 µM, the effective inhibitor concentration per milligram of protein was 10 µM under all conditions. For inhibitors added at a final concentration of 5 mM, the concentration of inhibitor per milligram of protein was 500 µM.

7.2.7 Counterflow experiments and 2-deoxy-glucose transport

Counterflow experiments were determined with cell suspensions in buffered minimal salts. Cells were preloaded in buffered minimal salts containing 5 mM 2-DG and 5 mM iodoacetate for 30 min at 70°C. This resulted in 2-DG uptake and the cells being loaded with 2-DG. The 2-DG loaded cells were harvested by centrifugation (6500 X g for 5 min at room temperature) in a bench top centrifuge to remove external 2-DG and were diluted 20-fold into buffered minimal salts containing 2 μCi of ^{3}H-2-DG (external concentration 0.24 mM 2-DG) at 70°C. Control cells were not loaded with 2-DG but were diluted in buffer containing 2 μCi of ^{3}H-2-DG and 0.24 mM unlabelled 2-DG (external concentration). The rate of 2-DG uptake was measured as described in section 7.2.4.1 (standard assay procedure).

7.3 RESULTS

7.3.1 Phosphoenolpyruvate-dependent phosphotransferase activity

ATP-dependent glucose-6-phosphotransferase (glucokinase) and PEP-dependent glucose:phosphotransferase activities were examined in toluene-treated cells of *Cl. thermohydrosulfuricum* Rt8.B1 by measuring the rates of ATP- and PEP-dependent phosphorylation of glucose by the procedure of Kornberg and Reeves (1972). The results of experiments with PEP or ATP as the phosphoryl donor are summarized in table 7.1.

The lack of significant PEP-dependent phosphorylation of glucose by toluene-treated cells of *Cl. thermohydrosulfuricum* Rt8.B1 grown on high and low concentrations of glucose, xylose and glucose + xylose indicated that a PEP-dependent phosphoenolpyruvate phosphotransferase system (PTS) was not used for the transport of glucose under the assay conditions used.

The phosphorylation of glucose was ATP-dependent, indicative of glucokinase or hexokinase activity. A similar level of glucokinase activity occurred in cells grown on

glucose, xylose and glucose + xylose (table 7.1). The bacterium 16Lt1 used as a positive control, exhibited a high level of PEP-dependent phosphorylation, indicative of PTS activity and showing that the experimental protocol was reliable. No activity with ATP was detected with strain 16Lt1. Xylose uptake by a PEP-dependent PTS mechanism was not investigated as the first enzyme in the metabolic pathway, xylose isomerase does not phosphorylate xylose but converts xylose to xylulose. It is the second step in the pathway which involves the conversion of xylulose to xylulose-5-phosphate by the enzyme xylulokinase and which involves phosphorylation at the expenditure of ATP

Table 7.1 Phosphoenolpyruvate-phosphotransferase and glucokinase activities in *Cl. thermohydrosulfuricum* Rt8.B1

	Carbon source used to grow cells				
Phosphoryl donor	5 mM G	50 mM G	5 mM X	50 mM X	50 mM G-X
ATP (glucokinase)	12.63	19.20	8.43	10.50	18.60
PEP (PTS)	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0

All results expressed as nanomoles of glucose-6-phosphate produced /minute /milligram of protein at 50°C.
G = glucose-grown, X = xylose-grown, G-X = glucose + xylose-grown.

Since there is no evidence for the existence of a PEP-PTS driven transport system for glucose in *Cl. thermohydrosulfuricum* Rt8.B1 the kinetics of glucose and xylose uptake were investigated at low concentrations of substrate using isotopic sugars.

7.3.2 Kinetics of glucose and xylose transport

7.3.2.1 50 mM glucose-grown cell suspensions

Cell suspensions prepared from exponential-phase cells of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose and incubated with ^{14}C-labelled glucose exhibited a high initial rate of uptake with no lag in uptake (figure 7.1). The rate of ^{14}C-glucose accumulation was linear over the first 90-120 seconds of the assay. When the rate of glucose uptake was measured at increasing concentrations of glucose (0.05 mM to 1.0 mM), saturation kinetics were observed (figure 7.2). The Eadie-Hofstee plot showed linear kinetics, thus suggesting the existence of only one uptake system at low concentrations of glucose (inset figure 7.2). The K_t (affinity constant for transport) for glucose was 0.15 mM glucose, and the V_{max} approximately 140.0 nmoles of glucose /min /mg protein. No accumulation of ^{14}C-labelled xylose could be demonstrated, indicating that the capacity for xylose uptake was absent in 50 mM glucose-grown cells, and thus confirming its inducibility.

7.3.2.2 5 mM glucose-grown cell suspensions

Similar cell suspensions grown on 5 mM glucose were also capable of transporting glucose at a high rate. The response was similar to that shown in figure 7.1. The rate of glucose uptake with increasing concentrations of glucose showed saturation kinetics (figure 7.3). The Eadie-Hofstee plot was again linear, indicating that one uptake system was present at low glucose concentrations (inset figure 7.3). Glucose uptake had a K_t of 0.09 mM and a V_{max} of 70.0 nmoles of glucose /min/ mg protein. No uptake of ^{14}C-xylose could be demonstrated, again indicating that xylose uptake is not constitutive in 5 mM glucose-grown cells.

7.3.2.3 5 mM xylose-grown cell suspensions

The rate of glucose uptake was measured in 5 mM xylose-grown cells. The uptake kinetics were similar to those shown in figure 7.1, demonstrating that a constitutive

uptake system for glucose exists when *Cl. thermohydrosulfuricum* is grown on low concentrations of xylose (5 mM). With increasing concentrations of glucose, saturation kinetics were observed (figure 7.4) and the Eadie-Hofstee plot was linear (inset figure 7.4). The K_t for glucose uptake was 0.08 mM and the V_{max} was approximately 30.0 nmoles of glucose /min /mg protein.

The rate of ^{14}C-xylose uptake was measured in 5 mM xylose-grown cells. The rate of xylose uptake with time is shown in figure 7.5. The rate was linear over the time course of the assay and no lag in uptake was observed. When the rate of uptake was measured at increasing concentrations of xylose, saturation kinetics were observed (figure 7.6). The Eadie-Hofstee plot was biphasic and at low concentrations of xylose the kinetics indicated that a diffusion process was operating (inset figure 7.6). The K_t for xylose uptake at concentrations above 0.5 mM xylose was 0.78 mM and the V_{max} was approximately 85.0 nmoles of xylose /min /mg protein.

7.3.2.4 50 mM xylose-grown cell suspensions

Cell suspensions prepared from exponential-phase cells of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose and incubated with ^{14}C-labelled glucose showed a response similar to that shown in figure 7.1. The rate of glucose uptake was 22.60 nmoles of glucose /minute /milligram of protein at 0.1 mM glucose. No kinetics with increasing concentrations of glucose were determined with these cells.

Cell suspensions of 50 mM xylose-grown cells were incubated with ^{14}C-labelled xylose. No uptake of xylose was detected. With increasing xylose concentrations up to 5 mM no significant level of accumulation above background was detected. The uptake of ^{14}C-xylose was in this case almost equal to the level of non-specific adsorption of labelled compound by filter membranes and inactive cells. This was surprising, as cell suspensions were capable of utilizing low concentrations of xylose (1 mM) at a slow rate (see section 6.4 figure 6.39). However, these utilization assays were done over a 180 min period and in order to determine the initial rate of substrate uptake (transport), short-term assays are necessary. In order to determine xylose

transport at higher external xylose concentrations e.g. above 5 mM a greater concentration of labelled substrate would have to be used and the cost becomes prohibitive. If a higher concentrations of ^{14}C-xylose is used then isotopic dilution increases experimental error making accurate measurement difficult. The results do indicate that the K_t was presumably much higher (low-affinity) and this result is consistent with the idea that a diffusion mechanism was responsible for low-affinity xylose uptake at high concentrations.

Isotopic sugars are metabolizable and therefore the incorporation of sugar measured is the result of transport, metabolism and incorporation into cellular materials. In order to investigate transport specifically in the absence of metabolism, the glucose analogue 2-DG was used to investigate glucose transport by 50 mM glucose-grown cells and 5 mM xylose-grown cells. When cell suspensions were incubated with ^{3}H-2-DG, the rate of uptake was low and presumably the uptake of this analogue was rapid with equilibration occurring in the first sampling interval (15 seconds) (figure 7.7). Similar results were seen with 5 mM xylose-grown cells. These results are consistent with the model that 2-DG is not phosphorylated by the glucose transport protein nor presumably by glucokinase as no accumulation of 2-DG could be measured.

The sampling procedure used in the investigation of ^{3}H-2-DG uptake proved inadequate to measure uptake kinetics where equilibrium conditions had been reached within 15 seconds, since the sampling, filtration and washing steps took approximately 30 seconds. A rapid uptake assay was therefore used as described in section 7.2.4.2 in which sampling was possible after 2 seconds. No significant differences in results were seen using this technique, again demonstrating the rapid uptake or equilibration of 2-DG across the membrane.

A major characteristic of facilitated diffusion systems and which provides strong evidence for the operation of a mobile carrier in such systems is the phenomenon of influx counterflow (or countertransport) and provides good evidence to reject a pore model of transport (Stein, 1981). When cells are pre-loaded with a non-radioactive transport substrate and then transferred to a solution containing a lower concentration

of the radioactive substrate, there is an enhanced rate of inward flux of the radioactive species and an apparent transient accumulation against a concentration gradient. This is because the outward flux of the radioactive species is decreased by its displacement from the carrier by the non-radioactive species present in higher concentration on the internal side of the cytoplasmic membrane (Stein, 1981).

The results of counterflow experiments for ^{3}H-2-DG uptake by 50 mM glucose-grown cells are shown in figure 7.8. The level of 2-DG accumulation was two-fold greater than the accumulation seen with unloaded cells. For 5 mM xylose-grown cells a similar level of accumulation was observed compared to unloaded cells (figure 7.9). The results of these experiments suggested that glucose uptake in glucose and xylose-grown cells was carrier-mediated. As no analogue is available to measure xylose transport in the absence of metabolism similar experiments were done using ^{14}C-xylose as the test substrate in 5 mM xylose + iodoacetate (10 mM) treated cells. Cells were diluted into buffered minimal salts containing 2 μCi of ^{14}C-xylose and 5 mM iodoacetate to prevent metabolism of xylose. The level of ^{14}C-xylose uptake was similar to cells that were not preloaded, thus no conclusive evidence for carrier involvement in xylose uptake could be demonstrated.

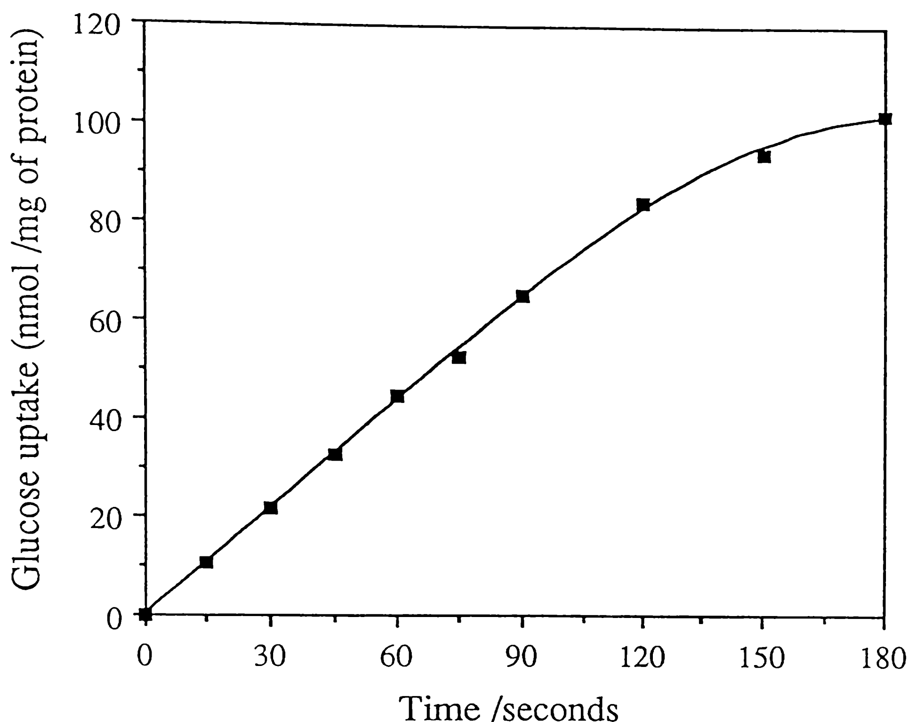


Figure 7.1 Glucose uptake with time by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose. The assay mix was 10 ml of buffered minimal salts containing 0.1 mM glucose (1.0 μCi of ^{14}C-glucose) and 100 μl of cell suspension.

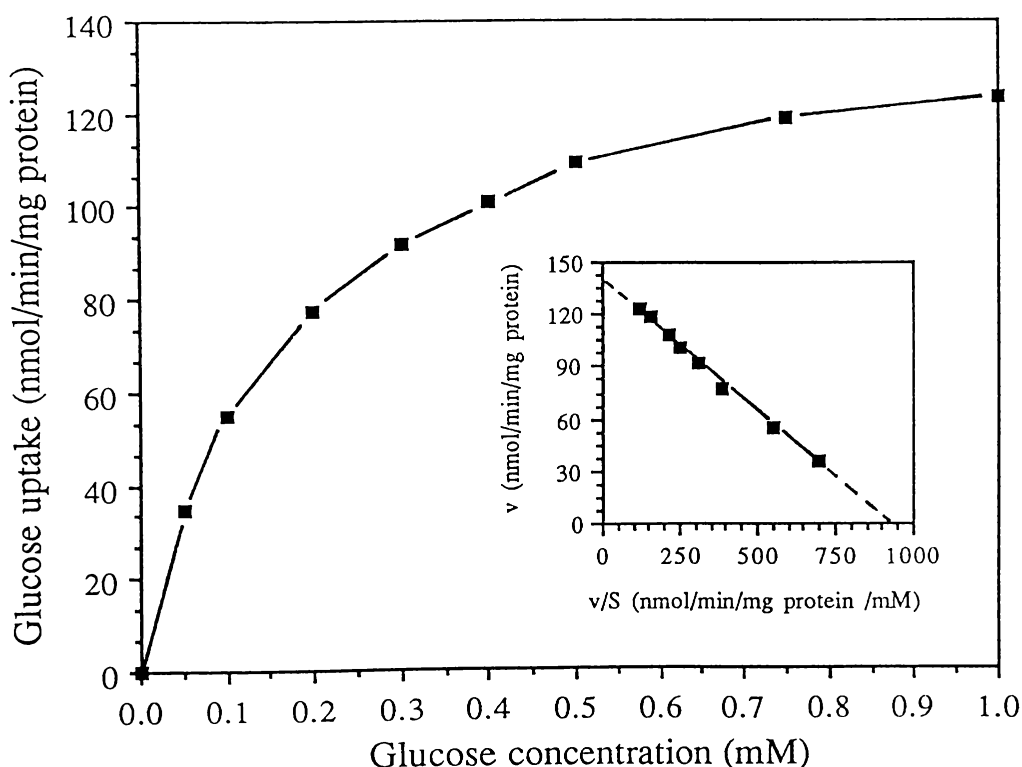


Figure 7.2 Glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose with increasing glucose concentration. Eadie-Hofstee plot of glucose uptake is shown in inset.

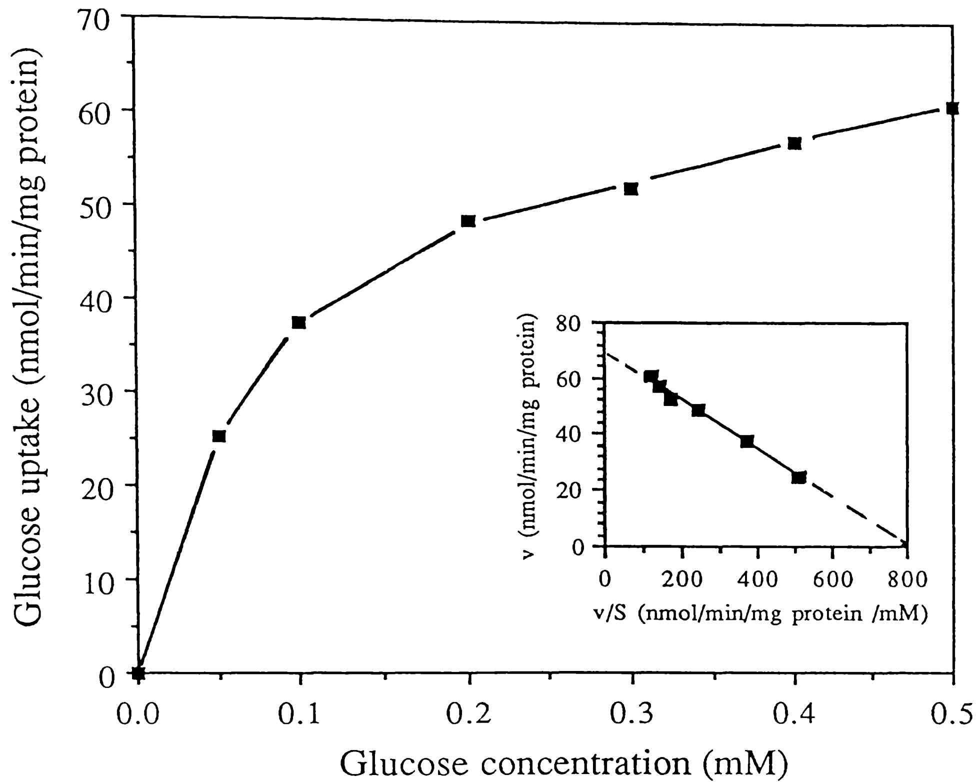


Figure 7.3 Glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM glucose with increasing glucose concentration. Eadie-Hofstee plot of glucose uptake is shown in inset.

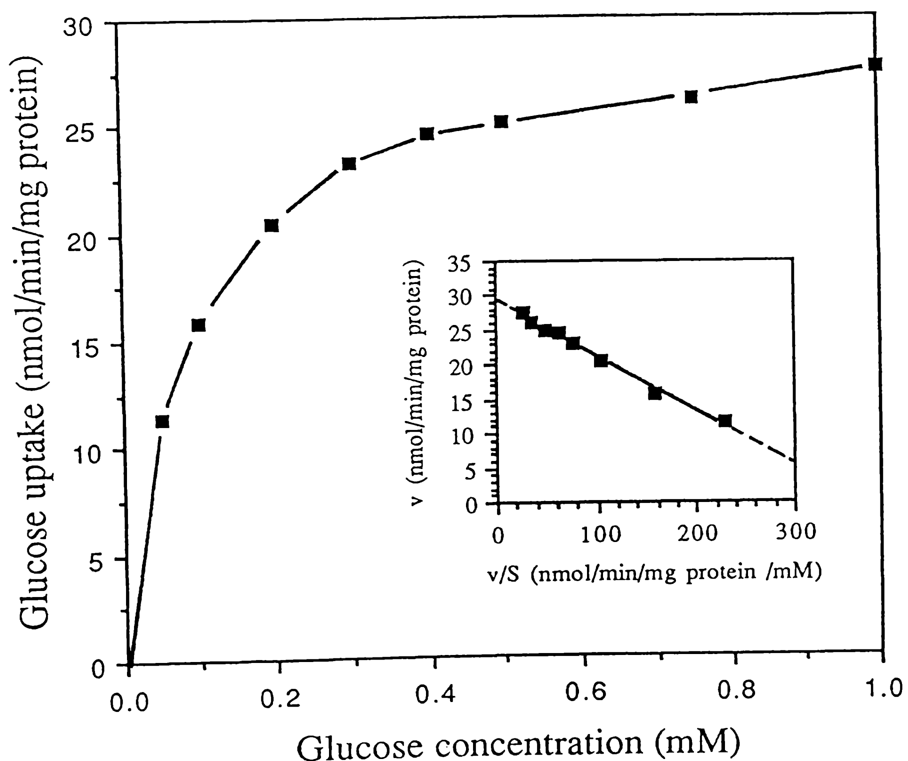


Figure 7.4 Glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing glucose concentration. Eadie-Hofstee plot of glucose uptake is shown in inset.

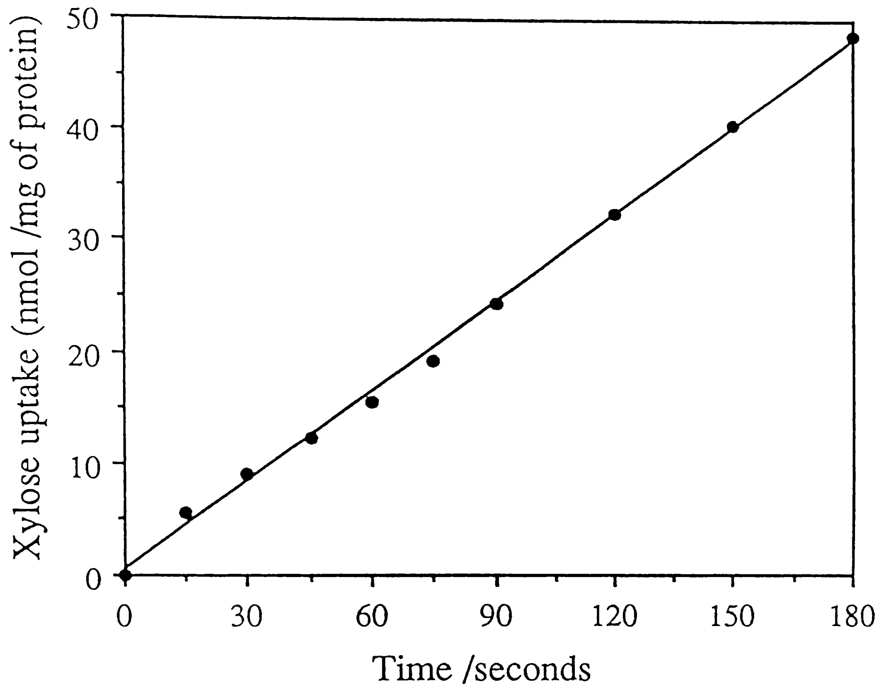


Figure 7.5 Xylose uptake with time by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose. The assay mix was 10 ml of buffered minimal salts containing 0.2 mM xylose (2.5 μCi of ^{14}C-xylose) and 100 μl of cell suspension.

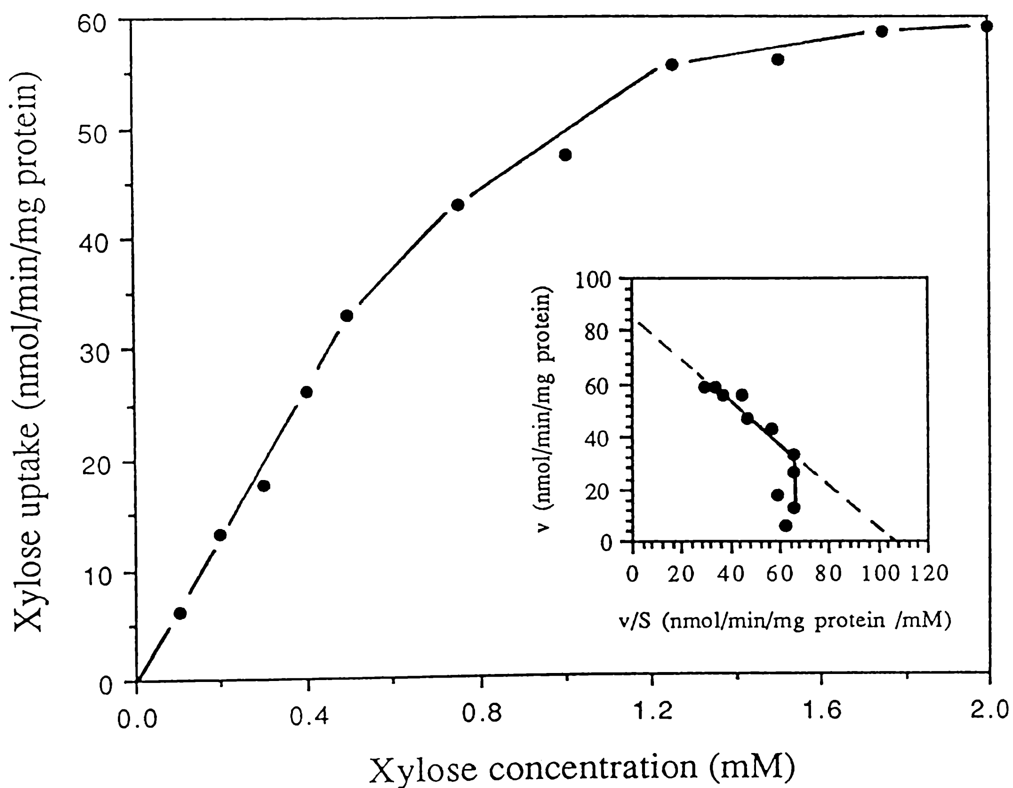


Figure 7.6 Xylose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing xylose concentration. Eadie-Hofstee plot of xylose uptake is shown in inset.

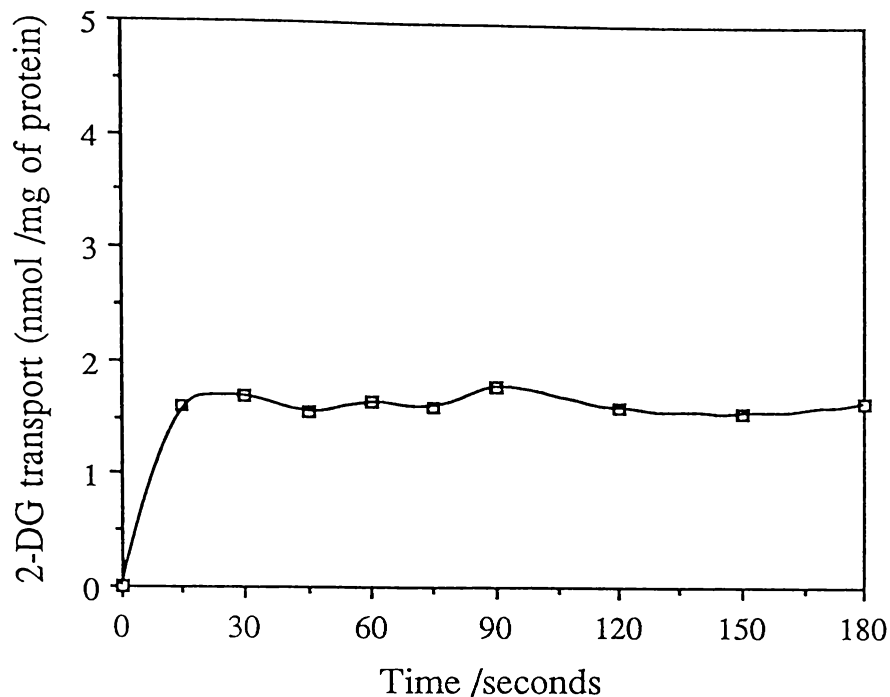


Figure 7.7 2-DG uptake with time by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose. The assay mix was 10 ml of buffered minimal salts containing 0.2 mM 2-DG (2.0 μCi of ^{3}H-2-DG) and 100 μl of cell suspension.

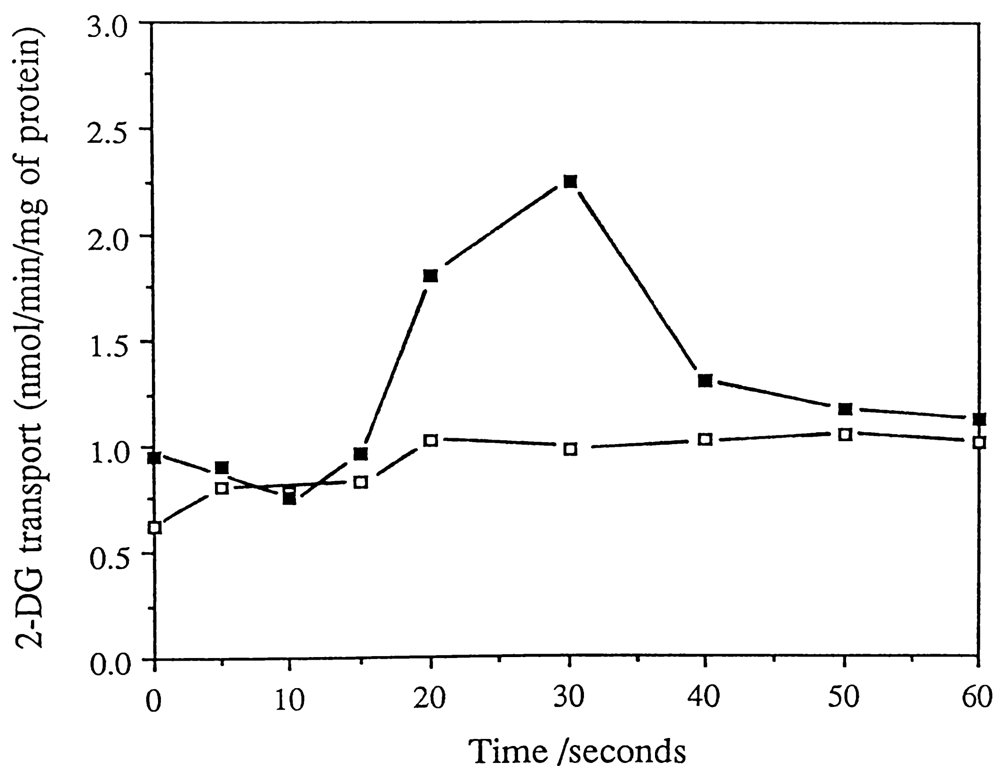


Figure 7.8 Counterflow of 2-DG by 2-DG and iodoacetate treated cells of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose. Cells were loaded (■) with 5 mM 2-DG and diluted 20-fold into buffered minimal salts containing 2 μCi of ^{3}H-2-DG (external 2-DG concentration of 0.24 mM). Unloaded cells (□) were diluted 20-fold into buffer containing 2 μCi of ^{3}H-2-DG and 0.24 mM 2-DG (external concentration).

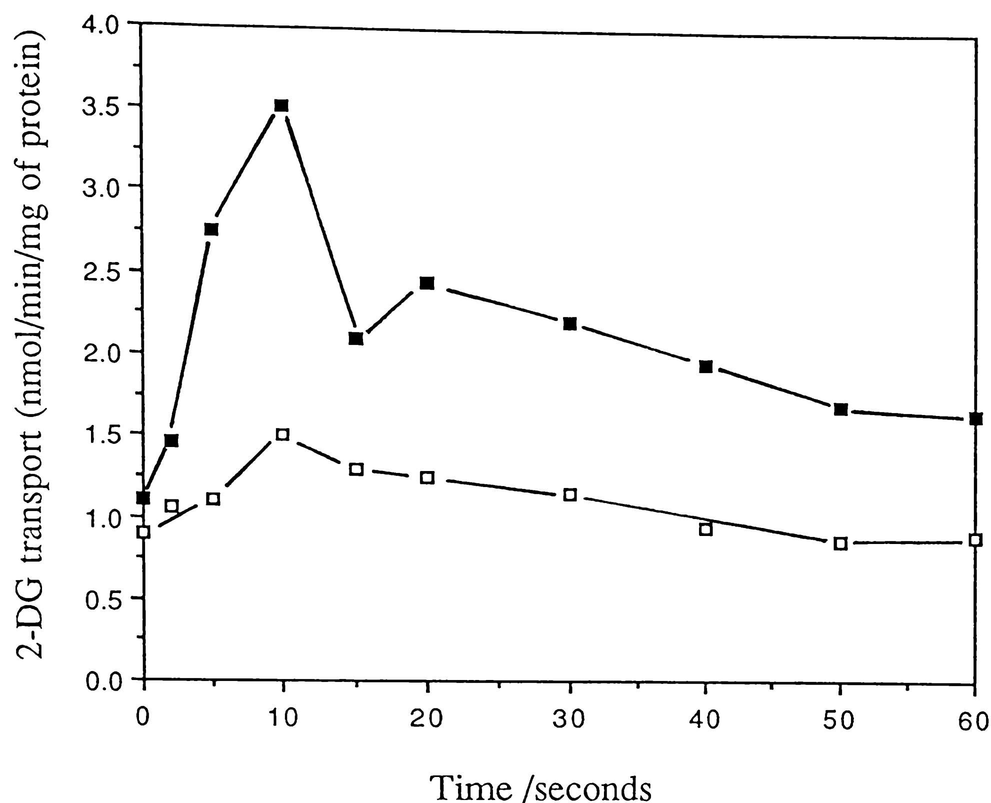


Figure 7.9 Counterflow of 2-DG by 2-DG and iodoacetate treated cells of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose. Cells were loaded (■) with 5 mM 2-DG and diluted 20-fold into buffered minimal salts containing 2 µCi of ^{3}H-2-DG (external 2-DG concentration of 0.24 mM). Unloaded cells (□) were diluted 20-fold into buffer containing 2 µCi of ^{3}H-2-DG and 0.24 mM 2-DG (external concentration).

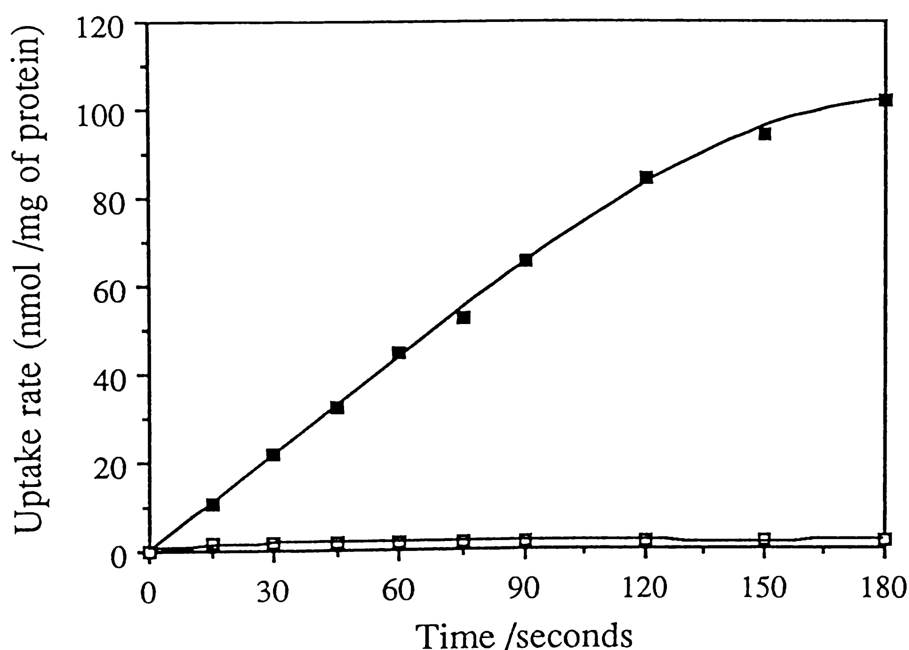


Figure 7.10 Comparison of 2-DG uptake and glucose uptake with time by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose. 2-DG uptake = (□) and glucose uptake = (■).

7.3.3 Competition studies

A variety of sugars and glucose analogues were tested as potential inhibitors of glucose and xylose uptake in *Cl. thermohydrosulfuricum* Rt8.B1.

The effects of unlabelled sugars on glucose uptake by 50 mM glucose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 are summarized in table 7.2.

Table 7.2 The effect of unlabelled sugars on ^{14}C-glucose uptake by 50 mM glucose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1

Sugar added (5 mM) ¶	Specific Activity §	% Inhibition of control
Control	59.36 ± 4.60	
D-glucose	1.05 ± 0.42	98.23
L-glucose	58.67 ± 3.25	1.16
D-xylose	52.47 ± 6.52	11.60
L-xylose	60.58 ± 5.38	0.00
D-xylulose	16.98 ± 3.58	71.40
α–MG	56.89 ± 5.60	4.16
2-deoxy-glucose	9.74 ± 2.90	83.60

¶ = Each reaction mix contained 10 ml of buffered minimal salts (pH 7 at 70°C), 5 mM of unlabelled competing sugar, 0.1 mM glucose containing 1 μCi of ^{14}C-glucose, and 100 μl of glucose-grown cell suspension.

§ = Specific activity expressed as nanomoles of glucose uptake /minute /milligram of protein ± the standard error of the mean.

With 50 mM glucose-grown cells, a 50-fold excess of unlabelled D-glucose, D-xylulose and 2-DG inhibited uptake of ^{14}C-glucose by 98.23, 71.40 and 83.6 %, respectively (table 7.2). Unlabelled D-xylose, L-xylose, L-glucose and α-MG did not inhibit glucose uptake significantly (< 15 %). The inhibition by D-glucose (98.23) was an amount close to the theoretical dilution (98.0).

The effects of unlabelled sugars on glucose uptake by 5 mM xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 are summarized in table 7.3.

Table 7.3 The effect of unlabelled sugars on ^{14}C-glucose uptake by 5 mM xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1

Sugar added (5 mM) ¶	Specific activity §	% Inhibition of control
Control	15.65 ± 2.30	
D-glucose	0.37 ± 0.20	97.60
L-glucose	16.63 ± 3.25	0.00
D-xylose	1.22 ± 0.56	92.20
L-xylose	15.98 ± 3.79	0.00
D-xylulose	0.68 ± 0.32	95.65
α–MG	6.53 ± 1.20	58.27
2-deoxy-glucose	1.03 ± 0.45	93.40

¶ = Each reaction mix contained 10 ml of buffered minimal salts (pH 7 at 70°C), 5 mM of unlabelled competing sugar, 0.1 mM glucose containing 1 μCi of ^{14}C-glucose, and 100 μl of xylose-grown cell suspension.

§ = Specific activity expressed as nanomoles of glucose uptake /minute /milligram of protein ± the standard error of the mean.

The uptake of ^{14}C-glucose by cells grown on 5 mM xylose was inhibited by unlabelled D-glucose, D-xylose, D-xylulose, α-MG and 2-DG by 97.60, 92.20, 95.65, 58.27 and 93.40 %, respectively (table 7.3). Unlabelled L-xylose and L-glucose had no effect on glucose uptake. Again the inhibition by D-glucose (97.60) was an amount close to the theoretical dilution (98.0).

The effects of unlabelled sugars on xylose uptake by 5 mM xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 are summarized in table 7.4.

Table 7.4 **The effect of unlabelled sugars on ^{14}C-xylose uptake by 5 mM xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1**

Sugar added (5 mM) ¶	Specific Activity §	% Inhibition of control
Control	29.60 ± 3.30	
D-glucose	3.45 ± 1.65	88.34
L-glucose	30.35 ± 4.20	0.00
D-xylose	12.64 ± 4.80	57.30
L-xylose	26.32 ± 2.45	11.08
D-xylulose	6.36 ± 3.10	78.51
α–MG	23.43 ± 2.90	20.85
2-deoxy-glucose	4.44 ± 1.80	85.00

¶ = Each reaction mix contained 10 ml of buffered minimal salts (pH 7 at 70°C), 5 mM of unlabelled competing sugar, 0.50 mM xylose containing 2 µCi of ^{14}C-xylose, and 100 µl of 5 mM xylose-grown cell suspension.

§ = Specific activity expressed as nanomoles of xylose uptake /minute /milligram of protein ± the standard error of the mean.

Uptake of ^{14}C-xylose by cells grown on 5 mM xylose was inhibited by unlabelled D-glucose, D-xylose, D-xylulose, α-MG and 2-DG by 88.34, 57.30, 78.51, 20.85 and 85.0, respectively (table 7.4). Unlabelled L-xylose and L-glucose had no significant effect on xylose uptake.

7.3.4 The effect of metabolic inhibitors on glucose and xylose transport

Metabolic inhibitors can provide useful information for identifying the uptake system present in a bacterium and the driving force for this uptake. The relationship between the source of energy and the active transport process can be determined by experiments involving interference at different sites of energy metabolism by selected substrates and inhibitors.

In order to determine whether a proton-motive force ($\Delta\mu H^+$) was responsible for the non-PTS glucose and xylose transport by *Cl. thermohydrosulfuricum* Rt8.B1, ^{14}C-glucose and ^{14}C-xylose uptake were measured in the presence of the ionophores (protonophores) CCCP and 2,4-DNP and the F_1F_0-ATPase inhibitor, DCCD, all of which dissipate the energized state of the membrane. To determine if sodium or potassium ions were important in glucose and xylose transport the effect of the ionophore monensin, which is capable of disrupting sodium or potassium gradients or both across bacterial cell membranes, was used.

Other inhibitors of bacterial metabolism including oxygen were also used to determine what effects metabolic activity had on glucose and xylose uptake by *Cl. thermohydrosulfuricum* Rt8.B1.

The effects of metabolic inhibitors on glucose uptake by 50 mM glucose-grown cells are summarized in table 7.5.

Incubation of 50 mM glucose-grown cells in buffered minimal salts under an oxygen atmosphere almost completely inhibited (92.33 %) glucose uptake. The protonophores CCCP and DNP, and the F_1F_O-ATPase inhibitor DCCD had no significant effect on the rate of ^{14}C-glucose uptake. The bacterial ionophore monensin had no effect on glucose uptake.

The most potent inhibitors of glucose uptake were the metabolic inhibitors sodium fluoride (60.0 %) and iodoacetate (65.97 %). Inhibition of glucose uptake was also seen with sodium arsenate (33.49) and potassium cyanide (44.60).

Table 7.5 **The effect of various metabolic inhibitors on glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose**

Inhibitor added ¶	Specific Activity §	% Inhibition of control
Control	62.60 ± 5.30	
Control (ethanol)	55.20 ± 3.80	
Oxygen	4.80 ± 2.30	92.33
2,4-Dinitrophenol (100 µM)	52.74 ± 4.20	4.45
CCCP (100 µM)	53.60 ± 6.80	2.90
DCCD (100 µM)	51.36 ± 2.60	6.96
Monensin (100 µM)	56.30 ± 8.50	0.00
Sodium fluoride (5 mM)	25.04 ± 7.80	60.00
Sodium arsenate (5 mM)	41.63 ± 6.70	33.49
Sodium azide (5 mM)	53.68 ± 2.10	14.24
Iodoacetate (5 mM)	21.30 ± 3.20	65.97
Potassium cyanide (5 mM)	34.68 ± 6.90	44.60

¶ = Each reaction mix contained 10 ml of buffered minimal salts (pH 7 at 70°C), inhibitor at specified concentration, 0.1 mM glucose containing 1 µCi of ^{14}C-glucose, and 100 µl of glucose-grown cell suspension. CCCP, 2,4-DNP, DCCD and monensin were all dissolved in ethanol.

§ = Specific activity expressed as nanomoles of glucose uptake /minute /milligram of protein ± the standard error of the mean.

The effect of metabolic inhibitors on the uptake of ^{14}C-glucose by 5 mM xylose grown cells are summarized in table 7.6.

The effects of metabolic inhibitors on glucose uptake by 5 mM xylose-grown cells produced similar results to those obtained for 50 mM glucose-grown cells. Oxygen was a potent inhibitor of glucose uptake (93.60 %) in agreement with previous results for glucose uptake. The inhibitors DNP, CCCP, DCCD and monensin had no effect on glucose uptake.

Again the metabolic inhibitors potassium cyanide and iodoacetate were good inhibitors of glucose uptake, inhibiting uptake by 52.49 and 62.33 % respectively. A small amount of inhibition was seen with sodium fluoride (21.43 %). Sodium arsenate and sodium azide had no significant effect on the rate of glucose uptake by 5 mM xylose-grow cells.

Table 7.6 The effect of various metabolic inhibitors on glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose

Inhibitor added ¶	Specific Activity §	% Inhibition of control
Control	18.90 ± 2.60	
Control (ethanol)	16.85 ± 3.76	
Oxygen	1.20 ± 0.76	93.60
2,4-Dinitrophenol (100 μM)	15.85 ± 1.20	5.93
CCCP (100 μM)	17.60 ± 5.35	0.00
DCCD (100 μM)	15.96 ± 2.21	5.28
Monensin (100 μM)	21.36 ± 6.80	0.00
Sodium fluoride (5 mM)	14.85 ± 3.20	21.43
Sodium arsenate (5 mM)	17.60 ± 2.20	6.88
Sodium azide (5 mM)	18.30 ± 4.60	3.17
Iodoacetate (5 mM)	7.12 ± 2.00	62.33
Potassium cyanide (5 mM)	8.98 ± 3.30	52.49

¶ = Each reaction mix contained 10 ml of buffered minimal salts (pH 7 at 70°C), inhibitor at specified concentration, 0.1 mM glucose containing 1 μCi of ^{14}C-glucose, and 100 μl of glucose-grown cell suspension. CCCP, 2,4-DNP, DCCD and monensin were all dissolved in ethanol.

§ = Specific activity expressed as nanomoles of glucose uptake /minute /milligram of protein ± the standard error of the mean.

For xylose uptake the effect of metabolic inhibitors are summarized in table 7.7.

Xylose uptake was inhibited by oxygen which was a potent inhibitor (96.70 %). No level of significant inhibition was seen with DNP, CCCP, DCCD, and monensin.

Iodoacetate inhibited xylose uptake by 54.68 % and a small level of inhibition (< 15 %) was seen with the inhibitors potassium cyanide, sodium azide, sodium arsenate and sodium fluoride.

Table 7.7 The effect of various metabolic inhibitors on xylose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose

Inhibitor added ¶	Specific Activity §	% Inhibition of control
Control	28.35 ± 2.80	
Control (ethanol)	25.45 ± 3.15	
Oxygen	0.94 ± 0.48	96.70
2,4-Dinitrophenol (100 μM)	23.32 ± 2.65	8.36
CCCP (100 μM)	32.68 ± 8.50	0.00
DCCD (100 μM)	26.80 ± 5.54	0.00
Monensin (100 μM)	27.35 ± 6.70	0.00
Sodium fluoride (5 mM)	25.58 ± 3.10	9.76
Sodium arsenate (5 mM)	24.86 ± 2.35	12.31
Sodium azide (5 mM)	28.68 ± 1.95	0.00
Iodoacetate (5 mM)	12.84 ± 3.36	54.68
Potassium cyanide (5 mM)	27.00 ± 6.85	4.76

¶ = Each reaction mix contained 10 ml of buffered minimal salts (pH 7 at 70°C), inhibitor at specified concentration, 0.5 mM xylose containing 2 μCi of ^{14}C-xylose, and 100 μl of 5 mM xylose-grown cell suspension. CCCP, 2,4-DNP, DCCD and monensin were all dissolved in ethanol.

§ = Specific activity expressed as nanomoles of xylose uptake /minute /milligram of protein ± the standard error of the mean.

7.4 DISCUSSION

7.4.1 Glucose transport by a PEP-dependent phosphotransferase system

The phosphoenolpyruvate-dependent carbohydrate:phosphotransferase system (PEP-PTS) is an energetically favourable transport mechanism for bacteria. This is because, the external sugar is simultaneously transported and converted to the first catabolic product, internal sugar phosphate, in one step without expending additional ATP in a kinase reaction (Postma and Roseman 1976). The product of PTS translocation is the corresponding sugar phosphate; these phosphates do not normally transverse the cell membrane so that, in this way, the product of transport is trapped inside the cell. By contrast, solutes that are actively concentrated have the ability to leave the cell (down the chemical gradient) so that it is likely that energy must be continuously expended to maintain high intracellular levels of such sugars. It is perhaps for this reason, the conservation of metabolic energy, that anaerobic bacteria, which are less efficient in oxidizing sugars to yield useful chemical energy, generally utilise the PTS for sugar uptake.

To date, most mesophilic bacteria that metabolize glucose by the Embden Meyerhof Parnas pathway have been shown to possess a PEP-PTS for the transport of glucose (Romano *et al.,* 1970, 1979; Dills *et al.,* 1980; Saier, 1977; Meadow *et al.*, 1990). The only report of a PEP-PTS system in thermophilic bacteria is that of *Cl. thermocellum* 651 (Patni and Alexander, 1971b), which contained low levels of PEP-dependent PTS activities for fructose and mannitol. However, no transport studies were performed and the level of activity was so low that the authors cast doubt on the role of PTS in the uptake of carbohydrate during growth. Studies on the differential metabolism of glucose and cellobiose by *Cl. thermocellum* LQR1 and *Cl. thermohydrosulfuricum* 39E demonstrated that a PEP-PTS mechanism was not responsible for the uptake of these substrates (Ng and Zeikus, 1982) and this was confirmed for glucose transport by a different strain of *Cl. thermocellum*, ATCC 27405 (Hernandez, 1982). Recently, Janssen and Morgan (1992) have shown a thermophilic spirochaete *Spirochaeta*

thermophila to also lack PTS activity for the uptake of glucose and glucose was taken up by a permease followed by ATP-dependent phosphorylation by glucokinase.

In this study, the lack of significant PEP-dependent phosphorylation of glucose by toluene-treated cells of *Cl. thermohydrosulfuricum* Rt8.B1 grown on high and low concentrations of glucose, xylose and glucose + xylose (table 7.1) indicated that a PEP-dependent PTS was not used for the transport of glucose. Glucose phosphorylation was shown to be ATP-dependent and was mediated by an active constitutive glucokinase. In contrast to thermophilic clostridia studied, the majority of mesophilic clostridia transport many sugars, including glucose, by a PEP-PTS (Groves and Gronlund, 1969; Hugo and Gottschalk, 1974; Booth and Morris, 1982; Mitchell and Booth, 1984; Hutkins and Kashket, 1986; Mitchell *et al.*, 1991).

The uptake of xylose by a PEP-dependent PTS mechanism was not investigated in *Cl. thermohydrosulfuricum* Rt8.B1. Xylose metabolism in this organism does not involve an initial phosphorylative step and xylose is transported in an unmodified form. The first step in xylose metabolism involves an isomerization reaction by the enzyme xylose isomerase converting xylose into xylulose which is subsequently phosphorylated to xylulose-5-phosphate at the expense of ATP by the enzyme xylulokinase. Pentoses are not transported by PTS in other bacteria (Shamanna and Sanderson, 1979; Lam *et al.,* 1980; Martin and Russell, 1988; Williams and Martin, 1990). Novel pentitol-specific PTSs have been reported in the homofermentative organism *Lactobacillus casei* (London and Chace, 1977, 1979; London and Hausman, 1982). These bacteria transport xylitol, arabitol, and ribitol via a PEP-dependent PTS route.

The use of sugar analogues have been widely used for the determination of the substrate specificity of sugar uptake systems and indicative of a PEP-PTS. The most widely used analogue for studies of glucose transport are 2-DG and α-MG which compete with glucose for a specific carrier. The analogue 2-DG is specific for mannose:PTS and α-MG for glucose:PTS activity (Postma and Roseman, 1976). In general terms, bacteria which exhibit a high rate of PEP-dependent glucose

phosphorylation also use PEP to phosphorylate 2-DG. The fact that 2-DG is used as a substrate for the PEP:glucose PTS assay is based upon the observation that most bacterial hexokinases and glucokinases do not catalyse the ATP-dependent phosphorylation of this analogue (Ghosh and Ghosh, 1968; Romano *et al.*, 1979). However, some bacteria have been shown to contain a glucokinase able to phosphorylate 2-DG. For example, the rumen bacterium *Selenomonas ruminantium* HD4 has been shown to have an ATP-dependent 2-DG kinase (Martin and Russell, 1986). A mannose:PTS defective strain of *Pediococcus halophilus* was also shown to exhibit ATP-dependent 2-DG-6-P formation which was thought to occur with cytoplasmic glucokinase (Abe and Uchida, 1991).

The lack of PEP-PTS activity in *Cl. thermohydrosulfuricum* Rt8.B1 for glucose transport was further supported by the fact that uptake of 2-DG could not be demonstrated in cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1 grown on either glucose or xylose. The lack of 2-DG accumulation also indicated that the glucokinase in *Cl. thermohydrosulfuricum* Rt8.B1 is unable to phosphorylate 2-DG in common with the majority of other bacteria investigated (Romano *et al.*, 1979). Similar findings have been reported for *Zymomonas mobilis* which has a carrier-mediated facilitated diffusion system for glucose transport; active uptake of 2-DG could not be demonstrated in this bacterium (Romano *et al.*, 1979; DiMarco and Romano, 1985).

The observation that 2-DG is not accumulated by *Cl. thermohydrosulfuricum* Rt8.B1 indicates that metabolism is very important in the uptake process and highlights the importance of glucose phosphorylation by glucokinase in the process of glucose uptake. This is further illustrated in figure 7.10. Although 2-DG was accumulated in *Cl. thermohydrosulfuricum* Rt8.B1 when actively grown on glucose + 2-DG or xylose + 2-DG (see section 6.3.2.2) this may represent a different form of metabolism in the growing cell as opposed to cell suspensions (non-growing). In this respect a low level of 2-DG utilization was seen in utilization studies with non-growing cell suspensions (see section 6.3.3.1) at high (50 mM) 2-DG concentrations and no 2-DG utilization was seen in the absence of glucose or xylose in these cells (see section 6.3.3.1 table 6.3).

Other studies have also demonstrated glucose uptake in the absence of PEP-PTS activity. These studies have been shown in bacteria which were defective in a functional PTS. In these studies glucose uptake has been shown to involve facilitated diffusion (Russell, 1990), $\Delta\mu H^+$-driven glucose uptake acting in concert with either glucokinase or hexokinase (Abe and Uchida, 1990; Keevil *et al.*, 1984, 1986) and simple diffusion (Dashper and Reynolds, 1990).

7.4.2 Kinetics of glucose transport by *Cl. thermohydrosulfuricum* Rt8.B1

A summary of the kinetic parameters for glucose uptake by glucose and xylose grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 are shown in table 7.8.

Table 7.8 The kinetic parameters of glucose transport by glucose and xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1

	Carbon source used for growth			
Kinetic parameter	5 mM G	50 mM G	5 mM X	50 mM X
Kt (affinity constant)	0.09 mM	0.15 mM	0.08 mM	ND
Vmax (maximal velocity)	70.00	140.00	30.00	ND

V_{max} = expressed as nmoles of glucose transport /min /milligram of protein at 70°; ND = not determined. G = glucose-grown, X = xylose-grown.

Glucose uptake was present in glucose and xylose-grown cells demonstrating the constitutive nature of the transport process in *Cl. thermohydrosulfuricum* Rt8.B1. The fact that glucose metabolism is constitutive in *Cl. thermohydrosulfuricum* Rt8.B1 indicates that uptake is tightly coupled to metabolism.

The K_t for glucose transport varied depending on the culture conditions used, but was not significantly different between glucose and xylose-grown cells, indicating that the

glucose uptake system was essentially the same (if not identical) in both glucose- and xylose-grown cells. The V_{max} also varied depending on the culture conditions used. It would be expected that cells grown on high concentrations of glucose would contain a greater level of transport proteins and glucose metabolizing enzymes as opposed to those grown on either 5 mM glucose or 5 mM xylose and this is reflected in the lower V_{max} values in these cells. The lower capacity (V_{max}) of xylose-grown cells could also suggest a xylose-induced inactivation and repression of glucose uptake activity. Since the cell suspensions were prepared from washed cells with presumably a very low intracellular xylose pool, the previous hypothesis seems more likely. These results suggest that glucose uptake is limited by the level of metabolic enzyme present for the phosphorylation of glucose and the removal of substrate from the cytoplasm to products.

The kinetics of glucose uptake were linear (insets in figures 7.2, 7.3 and 7.4) at the glucose concentrations from 0 to 1.0 mM. This was in contrast to utilization studies where biphasic kinetics were seen, indicative of 2 systems with high and low affinity for glucose (see section 6.3.3 figures 6.27 and 6.29). However, the kinetics of glucose utilization below concentrations of 5.0 mM glucose were linear, indicating the presence of one system as for glucose uptake at low glucose concentrations. The observation that glucose uptake was saturable under all conditions indicates that at higher concentrations of glucose a low-affinity system may be operative. Biphasic kinetics indicative of two uptake systems with high- and low-affinity for glucose have been shown in other bacteria. Examples include *H. saccharovorum* (Severina *et al.*, 1991a), *Sulfurococcus mirabilis* (Severina *et al.*, 1991b), *Rhizobium* spp. (Bigwaneza *et al.*, 1990), *Pediococcus halophilus* (Abe and Uchida, 1991), *Streptococcus mutans* (Keevil *et al.*, 1984, 1986) and *Streptococcus bovis* JB1 (Russell, 1990). In most instances the high affinity is usually a PTS system while the low affinity system is a $\Delta\mu H^{+}$-driven permease (Abe and Uchida, 1991 and Keevil *et al.*, 1984, 1986), although diffusion systems have also been implicated in high-capacity, low-affinity systems (Dashper and Reynolds, 1990; Russell, 1990).

7.4.3 The effect of glucose analogues and competing sugars on glucose transport by *Cl. thermohydrosulfuricum* Rt8.B1

The rate of glucose uptake by glucose- and xylose-grown cell suspensions was reduced greater than 80 % in the presence of 2-DG. The involvement of a membrane carrier in glucose uptake was demonstrated in both glucose- and xylose-grown cells (figure 7.8 and 7.9) and competition with 2-DG for this carrier could explain the observed reduction in glucose uptake. However, the possibility of a toxicity effect on blocking metabolism cannot be ruled out. Other studies on thermophilic bacteria have also confirmed the inhibitory effect of 2-DG on glucose uptake by *Cl. thermocellum* LQR1 and *Cl. thermohydrosulfuricum* 39E (Ng and Zeikus, 1982), and *S. mirabilis* (Severina *et al.,* 1991b).

The glucose analogue α-MG is frequently an analogue of the glucose:PTS as observed in *E. coli* (Postma and Roseman, 1976) or *S. mutans* (Liberman and Bleiweis, 1984; Vadeboncoeur, 1984). In this study, α-MG reduced glucose uptake in 5 mM xylose-grown cells by 58.27 %, yet no inhibition was seen with α-MG on glucose uptake by 50 mM glucose-grown cells (tables 7.2 and 7.3). These results indicate that the glucose permease has a higher affinity for glucose and α-MG cannot effectively compete with glucose for the carrier, hence the low inhibition in these cells. In xylose-grown cells, the analogue is able to be transported on the inducible xylose permease in the absence of xylose and once inside the cell it inhibits glucose uptake in some non-specific way. Glucose-grown cells do not contain xylose permease, therefore no α-MG accumulates within the cell and so no inhibition of glucose uptake is seen. This analogue has also been shown not to affect glucose uptake in other thermophilic bacteria investigated (Ng and Zeikus, 1982).

In 50 mM glucose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 L-glucose, D-xylose and L-xylose had no significant effect on glucose uptake, demonstrating that the glucose carrier is stereospecific for D-glucose. However, glucose uptake in 5 mM xylose-grown cells was inhibited by 92.20 %, again suggesting that xylose uptake through the xylose permease is able to reduce the uptake of glucose by competing with

glucose catabolic enzymes for available phosphorylation (ATP) potential. In addition, if xylose went in on any uptake system then its inhibition could be due to the conversion of xylose to metabolic intermediates which may inhibit glucose metabolic activity.

An interesting finding in this study was the inhibition of glucose uptake in the presence of D-xylulose. D-xylulose (an intermediate of xylose metabolism) inhibited glucose uptake, with the greatest inhibition shown with 5 mM xylose-grown cells. Under normal conditions the equilibrium favours the production of D-xylulose from D-xylose and the intermediate is able to move across the membrane as shown by the accumulation of D-xylulose in the medium when cells are grown on 50 mM xylose (see section 6.3, figures 6.1 and 6.2). This suggests a number of possibilities for the reduced glucose uptake seen in the presence of xylulose:

1. Xylulose goes in on the glucose permease and thereby lowers glucose uptake.

2. Xylulose goes in on the glucose permease and inhibits glucose metabolism (by affecting, say, glucokinase).

3. Xylulose is taken up on any uptake system and inhibits glucose utilization (by affecting, say, glucokinase).

7.4.4 Kinetics of xylose transport by *Cl. thermohydrosulfuricum* Rt8.B1

Xylose uptake was not detected in glucose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 indicating that the xylose permease was inducible. Cells grown on 5 mM xylose exhibited high rates of xylose uptake and the kinetics of uptake were biphasic on an Eadie-Hofstee plot, suggesting the existence of two systems. At low xylose concentrations (< 0.5 mM), the rate of xylose transport was directly proportional to the external concentration suggesting a diffusion process (inset figure 7.6). This was surprising, as diffusion of substrate at such low concentrations would not be expected due to the small concentration gradient present. However, with increasing

concentrations, saturation kinetics were observed (figure 7.6) and the K_t was 0.78 mM xylose with a V_{max} of 85.0 nmoles of xylose /min /mg of protein, indicating the saturation of an active system be it metabolic enzyme or transport protein. The fact that saturation kinetics were observed with increasing concentrations of xylose suggested that a low-affinity uptake system was operative at high concentrations of xylose.

Importantly, 50 mM xylose-grown cells were not capable of accumulating xylose at a significant level above the level of background at low concentrations of xylose (< 5 mM). These results reflect the presumably low affinity (high K_t) of the uptake system in 50 mM xylose-grown cells. The presence of a low-affinity system for xylose was demonstrated for xylose utilization in 50 mM xylose-grown cells (see section 6.3.3, figure 6.31). A high-affinity system was shown for xylose utilization in 5 mM xylose-grown cells (see section 6.3.3, figure 6.33). The fact that both systems were inducible suggests that either the affinity for xylose is reflected in the uptake system for xylose or that key enzymes of xylose metabolism are capable of changing their relative affinity for xylose depending on the concentration. This remains to be investigated. The presence of a facilitated system for xylose suggests that in the range of concentrations encountered in the log phase of growth, cellular catabolic activity is not limited by xylose permeation and is controlled by the maximal activity of xylose catabolic enzymes and the rate of catabolic activity determines the rate of xylose utilization.

More than one system for the transport of pentoses has been reported in other bacteria. For example, L-arabinose and D-ribose are transported by two transport systems a low-affinity and a high-affinity transport system (Aksamit and Koshland, 1972; Brown and Hogg, 1972; Willis and Furlough, 1974). In both cases high affinity transport was associated with binding proteins. Williams and Martin (1990) reported nonlinear kinetics for xylose transport by the rumen bacterium *S. ruminantium* HD4 and suggested that either more than one mechanism for the uptake of xylose may be involved or that one mechanism of xylose uptake exists in which two proteins, such as a periplasmic binding protein and a permease protein, with different affinities for

xylose may be present. These explanations can also be applied here to *Cl. thermohydrosulfuricum* Rt8.B1; alternatively this could also be attributed to separate transport systems, but having different conformations (high- and low-affinity) of the same transport protein which result in similar kinetics.

A major characteristic of facilitated diffusion systems which provides strong evidence for the operation of a mobile carrier in such systems is the phenomenon of influx counterflow (or countertransport) and provides good evidence to reject a pore model of transport (Stein, 1981). In bacteria, counterflow has been demonstrated for galactose transport in *Streptococcus lactis* (Kashket and Wilson, 1972), and glucose transport in *S. bovis* JB1 (Russell, 1990) and *Z. mobilis* (DiMarco and Romano, 1985). In this study the involvement of a carrier protein in glucose uptake was demonstrated for glucose- and xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 (figure 7.8 and 7.9). The unavailability of a non-metabolizable xylose analogue to measure xylose transport and thus demonstrate the existence of a mobile carrier made such determinations difficult.

To date there have been no reports of facilitated diffusion operating for carbohydrate transport in thermophilic bacteria. Indeed, few reports of bacterial facilitative diffusion systems operating for the transport of carbohydrates have been reported. The best characterized facilitated system is that of glycerol utilization by *Escherichia coli* (Lin, 1976; Richey and Lin, 1972; Heller *et al.*, 1980). Facilitated diffusion has been reported for maltose uptake in *Staphylococcus aureus* (Button *et al.,* 1973), glucose uptake in a PTS-deficient mutant of *S. bovis* JB1 (Russell, 1990) and glucose and fructose uptake in *Z. mobilis* (DiMarco and Romano, 1985; Vikarii *et al.*, 1988).

In most cases, facilitated transport systems for solute transport can be explained on the basis of the natural occurrence of the organism. For example, *Z. mobilis* is found in warm, tropical climates in fermentations of plant saps with high sugar content and the acquisition of this low-affinity, high-capacity facilitated transport system allows for the rapid conversion of glucose to metabolic products with low energetic yield. The rumen bacterium *S. bovis* JB1 inhabits the rumen where energy sources fluctuate. The dual

mechanism of glucose transport explains why *S. bovis* JB1 can grow very rapidly when soluble carbohydrates accumulate in the rumen and yet persists even if sugars are present at low concentrations. *Cl. thermohydrosulfuricum* Rt8.B1 inhabits thermal environments where the availability of carbohydrate would be expected to be quite low, and therefore the acquisition of a transport system able to accumulate nutrients against a concentration gradient would be of obvious advantage. The fact that a facilitated diffusion system was present for xylose uptake in *Cl. thermohydrosulfuricum* Rt8.B1 is difficult to explain in relation to the organism's natural environment. However, the presence of a high-affinity transport system for xylose explains its ability to compete effectively at low xylose concentrations.

7.4.5 The effect of glucose analogues and competing sugars on xylose transport by *Cl. thermohydrosulfuricum* Rt8.B1

The uptake of xylose by 5 mM xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 was reduced in the presence of glucose. Similar results were seen when glucose was added to cultures of *Cl. thermohydrosulfuricum* Rt8.B1 growing on low concentrations of xylose (5 mM) (see section 6.3.1, figure 6.7). No inhibition of xylose utilization was seen when glucose was added to cultures growing on high concentrations of xylose (50 mM) (see section 6.3.1, figure 6.3). These results suggest that the high-affinity uptake system for xylose present in 5 mM xylose-grown may have some affinity for glucose and competes with the xylose carrier. Presumably the low-affinity xylose uptake system in 50 mM xylose-grown cells is not affected by glucose. An effect of glucose at the level of the xylose carrier protein was also indicated by the observation that 2-DG reduced xylose transport by a similar level of inhibition (table 7.4), suggesting some competition at the level of the xylose carrier protein. An effect at the level of metabolism may also act to inhibit xylose uptake by glucose reducing the availability of key metabolites which may be essential for xylose uptake.

The glucose analogue α-MG inhibited xylose uptake by 20.85 %, suggesting that this analogue is transported by the xylose permease which has greater affinity for xylose as suggested by the lack of α-MG inhibition of glucose uptake in glucose-grown cells.

L-xylose had no effect on D-xylose transport suggesting the carrier or phosphorylating enzyme was specific for D-xylose. D-xylulose inhibited D-xylose uptake by 78.51 %, indicating a competition effect at the level of the membrane and an accumulation of substrate preventing any further uptake of D-xylose.

7.4.6 The effects of metabolic inhibitors on glucose and xylose transport by *Cl. thermohydrosulfuricum* Rt8.B1

The ultimate source of energy for all transport processes (apart from diffusion) is metabolism i.e. the energy derived from respiration or fermentation. As a rule, the driving forces for transport processes are not directly coupled to the ultimate oxygen consuming or glycolytic reactions, but the transfer of energy is, rather, mediated through a chain of intermediate reactions (Geck and Heinz, 1981). The reaction to which the transport process is coupled directly can be either a chemical reaction, such as the hydrolysis of energy rich phosphates (ATP-driven), or an osmotic process e.g. the downhill flow of another solute, predominantly H^+ (proton motive force driven) or Na^+ in bacteria.

Oxygen was shown to be a potent inhibitor of glucose and xylose uptake by *Cl. thermohydrosulfuricum* Rt8.B1. This is not surprising given the strictly anaerobic nature of this bacterium (Patel *et al.*, 1986). Oxygen has also been shown to be a potent inhibitor of glucose and cellobiose transport in other strictly anaerobic thermophilic bacteria (Ng and Zeikus, 1982).

The $\Delta\mu H^+$ (and therefore the energized state of the membrane) can be abolished by proton conductors or uncouplers such as 2,4-DNP and CCCP (Kaback *et al.*, 1974). CCCP and DNP are protonophores, catalyzing the transfer of protons across the membrane (Pressman, 1976). Both inhibitors will, therefore, interfere with both H+-

solute symport systems and generation of ATP via a membrane bound H+-translocating ATPases (Heytler, 1979) i.e. those transport systems driven by a $\Delta\mu H^+$.

Both glucose and xylose uptake by *Cl. thermohydrosulfuricum* Rt8.B1 were unaffected by the presence of DNP and CCCP. These results indicate that the energized state of the membrane was not required for the uptake of glucose and xylose. Similar findings have been shown for glucose and cellobiose uptake in *Cl. thermocellum* LQR1 and *Cl. thermohydrosulfuricum* 39E (Ng and Zeikus, 1982) and another strain of *Cl. thermocellum* ATCC-27405 (Hernandez, 1982).

DCCD covalently interacts with a proteolipid portion of F_0F_1-ATPase. The interaction of one molecule of DCCD with one DCCD-binding subunit per ATPase complex inhibits the enzyme (Linnet and Beechey, 1979). DCCD had no effect on the uptake of glucose and xylose by *Cl. thermohydrosulfuricum*, Rt8.B1 demonstrating the absence of $\Delta\mu H^+$-driven transport established by ATP hydrolysis through the F_1F_0-ATPase. DCCD has been shown to inhibit glucose uptake in *Cl. thermocellum* LQR1 and *Cl. thermohydrosulfuricum* 39E by 46 % (Ng and Zeikus, 1982). The fact that glucose and xylose transport by *Cl. thermohydrosulfuricum* Rt8.B1 were unaffected by the protonophores DNP and CCCP, and by the F_1F_0-ATPase inhibitor DCCD demonstrates that neither glucose or xylose transport is energized by a transmembrane electrochemical gradient of protons and not driven by a $\Delta\mu H^+$.

Monensin is a carboxylic ionophore. It catalyzes the exchange of Na+ or K^+ for H^+ with a selectivity of Na^+:K^+ of 10:1 (Schönheit and Beimborn, 1986). Monensin is thus capable of disrupting sodium or potassium gradients or both across bacterial membranes. Monensin would, therefore affect Na+-solute symport systems. Monensin was shown to have no effect on the uptake of glucose and xylose by *Cl. thermohydrosulfuricum* Rt8.B1 suggesting that transport was not dependent on Na^+ or K^+ ions. The ability of *Cl. thermohydrosulfuricum* Rt8.B1 to grow in the near absence of sodium (< 0.10 g/l) also suggested that this ion was not involved in transport. Sodium has been shown to be important in some thermophilic bacteria for the transport of amino acids. Membrane vesicles of *B. stearothermophilus* transported the acidic

amino acids L-glutamate and L-aspartate by a Na^+-H^+-solute symport mechanism (DeVrij *et al.*, 1989) and neutral (branched chain) amino acids by a Na^+-solute symport mechanism (Heyne *et al.*, 1991). *Cl. fervidus* has been shown to have a sodium-ion dependent transport of neutral, acidic and aromatic amino acids (Speelmans *et al.*, 1989). No sodium-dependent transport has to date been demonstrated for carbohydrate transport systems in thermophilic bacteria.

Potassium cyanide has been shown to inhibit electron transport steps by reacting with iron-containing proteins (Franklund and Glass, 1987). Glucose uptake by glucose- and xylose-grown cells was inhibited by 44.60 and 52.49 % respectively in the presence of potassium cyanide. Xylose uptake was not inhibited. The effect of potassium cyanide is difficult to explain given the glycolytic nature of this organism. The fact that xylose uptake was not inhibited suggests that a non-specific toxicity effect on the cells can be ruled out. In *Cl. thermohydrosulfuricum* Rt8.B1 the inhibition observed may involve an effect on the level of electron transfer from substrate to hydrogenase or at pyruvate synthase. This could be a due to the inhibition of ferredoxins linked to hydrogenase perhaps because there is more reducing power lost as H_2 when cells are grown on glucose compared with xylose. The ratio of acetate to ethanol did not change significantly between glucose or xylose-grown cells therefore this possibility may be ruled out.

Sodium arsenate had little inhibitory effect on glucose and xylose uptake by 5 mM xylose-grown cells. Glucose uptake by 50 mM glucose-grown cells was inhibited 33.49 %. Sodium arsenate is a potent inhibitor of shock-sensitive or periplasmic binding active transport systems i.e. transport systems that use high-energy phosphates directly as a source of energy (Berger and Heppel, 1974; Wilson, 1974). The inhibition of glucose uptake in 50 mM glucose-grown cells suggests that phosphate bond energy may play a role in glucose uptake by *Cl. thermohydrosulfuricum* Rt8.B1. Sodium arsenate also inhibits substrate level phosphorylation: acetyl-CoA + arsenate $\Rightarrow$ acetyl-arsenate + CoA catalyzed by phosphotransacetylase instead of acetyl-CoA + $P_i \Rightarrow$ acetyl-P which can then be used to form ATP from ADP (Stadtman, 1955). This suggests that in *Cl. thermohydrosulfuricum* Rt8.B1, glucose uptake in 50 mM glucose-

grown cells is dependent more on substrate level phosphorylation for energy. Sodium arsenate has also been shown to inhibit glucose uptake in *Cl. thermocellum* strains LQR1, ATCC 27405 and *Cl. thermohydrosulfuricum* 39E (Hernandez, 1982; Ng and Zeikus, 1982) by a similar level.

The metabolic inhibitor sodium fluoride inhibited glucose uptake in both glucose- and xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 by 60.0 and 21.43 % respectively. Studies with oral streptococci have indicated that the enzyme enolase which converts 2-phosphoglycerate to phosphoenolpyruvate is fluoride sensitive and thus in effect, reduces the amount of PEP available to drive the PTS and the production of endogenous ATP (Hamilton and Ellwood, 1978; Kanapka and Hamilton, 1971). The fact that *Cl. thermohydrosulfuricum* Rt8.B1 did not operate a PEP-PTS for glucose uptake indicates that the inhibition by sodium fluoride acts on other enzymes in the glycolytic sequence. Sodium fluoride has been shown to inhibit certain glycolytic enzymes of the Embden Meyerhof Parnas pathway and inhibits a wide variety of ATPases (Ng and Zeikus, 1982; Sturr and Marquis, 1990). Therefore the effect of sodium fluoride is likely due to inhibition directed at an enzyme involved in either uptake or utilization that is ATP-requiring (glucokinase). Sodium fluoride has been shown to inhibit glucose uptake 50 % in *Cl. thermocellum* LQR1 and *Cl. thermohydrosulfuricum* 39E (Ng and Zeikus, 1982).

Iodoacetate is an inhibitor of glycolysis and glyceraldehyde-3-phosphate dehydrogenase and therefore is capable of abolishing the generation of ATP by substrate level phosphorylation (Strobel and Russell, 1989). Iodoacetate was a potent inhibitor of glucose and xylose uptake in *Cl. thermohydrosulfuricum* Rt8.B1. The fact that glucose and xylose were inhibited by iodoacetate suggests that the energy for glucose and xylose uptake is derived from substrate level phosphorylation and that inhibition of this process reduced the energy supply or transduction of energy for the non-PTS glucose uptake which was driven by ATP-dependent glucokinase in *Cl. thermohydrosulfuricum* Rt8.B1. Similar inhibition by iodoacetate on low-affinity glucose uptake in *Pediococcus halophilus* has been shown (Abe and Uchida, 1991) and glucose uptake in this organism was driven by $\Delta\mu H^+$ acting in concert with

glucokinase. If the ATP requirements for maximal substrate uptake in *Cl. thermohydrosulfuricum* Rt8.B1 were small, then it is possible that a low rate of substrate level phosphorylation could provide enough ATP to support a substantial level of glucose uptake.

In summary, although the data obtained from experiments with metabolic inhibitors are insufficient to establish the direct energy source for transport, ATP generated by substrate level phosphorylation appears to be involved in energizing transport. The experimental results using transport inhibitors and uncouplers suggest that glucose transport involves a glucose permease and ATP rather than PEP-PTS or a respiration linked transport system. A similar transport system for glucose has been shown in other thermophilic bacteria which lack PEP-PTS (Hernandez, 1982; Ng and Zeikus, 1982; Janssen and Morgan, 1992).

Glucose and xylose (5 mM xylose-grown cells) transport under all conditions was shown to be a saturable function of the substrate concentration. All known mechanisms of the accumulation of substrates involve a saturable protein, permease or other enzyme system (Button, 1983). The saturation kinetics observed for glucose and xylose transport could reflect a regulatory effect of glucose or xylose and /or their metabolites on the glucose and xylose transport systems or the saturation of the first enzyme in the metabolic pathway for the degradation of glucose and xylose.

The rates of glucose and xylose transport were higher than the rates of utilization (transport + metabolism) at similar concentrations. Therefore, the rate of glucose and xylose transport appears to depend on the context and kinetic characteristics of enzymes involved in the removal of glucose and xylose in the cytoplasm. The concentration of these rate-limiting enzymes plays a key role. To investigate this possibility the kinetic parameters and relative affinities of key enzymes involved in the breakdown of glucose and xylose were determined.

CHAPTER EIGHT

CONTROL OF GLUCOSE AND XYLOSE UTILIZATION IN *Clostridium thermohydrosulfuricum* Rt8.B1

8.1 INTRODUCTION

The rates of glucose and xylose utilization by *Clostridium thermohydrosulfuricum* Rt8.B1 (chapter six) were lower than their rates of uptake as measured using ^{14}C-glucose and ^{14}C-xylose (chapter seven), and no concentrative uptake of the glucose analogue 2-deoxy-glucose (2-DG) could be demonstrated. This suggests that *Cl. thermohydrosulfuricum* Rt8.B1 lacks a concentrative uptake system for glucose. Thus, the rate of substrate removal from the cytoplasm appears to be the overall rate-limiting step in the utilization process. The first step of glucose metabolism in *Cl. thermohydrosulfuricum* Rt8.B1 is the phosphorylation of glucose by the enzyme glucokinase (Cook, 1988) and it is possible that the relative affinities of this enzyme for glucose may control the overall rate of glucose uptake in *Cl. thermohydrosulfuricum* Rt8.B1.

The first step of xylose metabolism in *Cl. thermohydrosulfuricum* Rt8.B1 involves the isomerization of D-xylose to D-xylulose by the enzyme xylose isomerase. D-xylulose is phosphorylated by the enzyme D-xylulokinase to D-xylulose-5-phosphate at the expense of ATP (Cook, 1988). The fact that no xylose uptake could be measured in cells grown on 50 mM xylose suggests that the affinity of xylose isomerase and xylulokinase may act as a dragging force for xylose removal from the external medium.

Both glucokinase and xylulokinase are ATP-requiring and therefore are energy driven, and thus represent the first opportunity for the cell to remove substrates against a mass flow equilibrium. The aim of this section of work was to determine what regulates the activity of key enzymes involved in the utilization of glucose and xylose and to determine the K_m (relative affinity) and V_{max} of these enzymes.

8.2 MATERIALS AND METHODS

8.2.1 Chemicals

All chemicals were reagent grade. Adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), D-xylulose-5-phosphate, pyruvate kinase (EC 2.7.1.40) and D-glucose-6-phosphate were all obtained from Sigma Chemical Company (St Louis, Mo, USA).

8.2.2 Enzyme assays

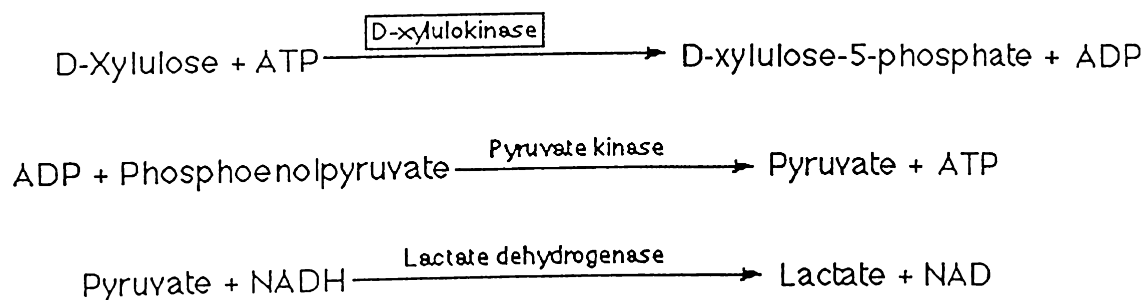
Glucokinase (EC 2.7.1.2) was assayed by the procedure outlined in section 6.2. The assay mix contained: MOPS, pH 7.0, 50 mM; $MgCl_2$, 60 mM; dithiothreitol, 5 mM; NADP, 0.5 mM; ATP, 5 mM; glucose at concentrations ranging from 0 to 1.0 mM; glucose-6-phosphate dehydrogenase, 5.0 U and 50 µl of crude extract.

Xylose isomerase (D-xylose ketol isomerase, EC 5.3.1.5) was assayed by measuring the formation of D-xylulose from D-xylose using the colorimetric assay of Dische and Borenfreund (1951) in which 100 µl of a test mixture containing D-xylose and 100 mM MOPS (pH 7 at 70°C) containing 0.4 mM $MnSO_4$ and 1 mM dithiothreitol were incubated together with 50 µl of crude extract for 3 min at 70°C. After this period, the samples were put on ice to stop the enzyme reaction and once cooled were centrifuged at 6500 X g for 2 min. The supernatant was assayed for D-xylulose by adding 50 µl 1.5 % (w/v) cysteine hydrochloride in water, 50 µl 0.12 % (w/v) carbazole in ethanol, and 1.2 ml 70 % (v/v) sulphuric acid. After standing for 20 min, the absorbance at 540 nm was determined. The concentration of D-xylulose was determined from a standard curve of D-xylulose. Xylose isomerase activity was expressed as nmoles of xylulose produced /minute /mg of protein at 70°C. All assays were corrected for non-specific activity.

Glucose isomerase activity was assayed by the same method as used for xylose isomerase, with the exception that the test mixture contained in 100 µl: D-glucose and 100 mM MOPS (pH 7 at 70°C) containing 0.4 mM $MgSO_4$ and 1 mM dithiothreitol. The incubation period for the development of the colour with carbazole was extended to 30 min and the absorbance was measured at 560 nm. The enzyme activity was calculated on the basis of a standard curve obtained with D-fructose. Glucose isomerase activity was expressed as nmoles of D-fructose produced /minute /mg of protein at 70°C.

Xylulokinase (EC 2.7.1.17) activity was measured by the method of Shamanna and Sanderson (1979) by measuring the production of ADP as a result of the D-xylulokinase reaction. This was coupled with the pyruvate kinase reaction and lactate dehydrogenase with concomitant NADH oxidation. The reaction was carried out in the following assay mixture (total volume 2.0 ml):- MOPS, pH 7.0, 50 mM; ATP, 5 mM; $MgCl_2$, 60 mM; dithiothreitol, 5 mM; xylulose at concentrations 0 to 2.0 mM; NADH, 0.25 mM; phosphoenolpyruvate, 1.0 mM; lactate dehydrogenase, 300 µg; and pyruvate kinase, 300 µg and 50 µl of crude extract. The change in absorbance at 340 nm due to NADH conversion to NAD was followed spectrophotometrically. Xylulokinase activity was expressed as nmoles of D-xylulose-5-phosphate produced /minute /mg of protein. The following controls were performed: xylulose phosphorylation in the absence of ATP, non-specific ATP activity in the absence of xylulose and boiled extract added to the assay mix.

Reaction scheme:



Assays of glucokinase and xylulokinase activity were carried out at 50°C under anaerobic conditions as described by Zeikus *et al.* (1977). Specific activities were determined in a range where linearity with protein concentration was established. All assays were carried out in triplicate with appropriate controls. Where available, commercial enzymes were purchased as a check of the assay system for a particular enzyme.

Enzyme assays were carried out in quartz cuvettes with a 1.0 cm light path, total volume 2.0 ml, using a Shimadzu double monochromator recording spectrophotometer model UV-250 (Shimadzu Corporation, Kyoto, Japan) equipped with a Shimadzu temperature-controlled cell holder (MODEL TCC-240A) and computer controlled graphic printer (MODEL PR-1). All pyridine nucleotide oxidation or reduction reactions were measured at 340 nm ($\varepsilon 340 = 6.22\ mM^{-1}\ cm^{-1}$.)

The protein concentration of crude-extracts was determined by the Biuret method as described by Scopes (1982). Bovine serum albumin was used to construct a standard curve of protein concentration which was linear to 5 $mg.ml^{-1}$.

The apparent Michaelis constant (K_m) and maximal activity (V_{max}) for enzyme activity were estimated from Eadie-Hofstee plots by measuring the initial rates of enzyme reaction.

8.3 RESULTS

8.3.1 The kinetics of glucokinase activity in lysed cell crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1

The levels of glucokinase (EC 2.7.1.2) found in crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1 grown on high and low concentrations of glucose and xylose are summarized in table 8.1.

Table 8.1 **Glucokinase activities in lysed cell crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1**

Carbon source used to grow cells	5 mM G	50 mM G	5 mM X	50 mM X
Glucokinase activity ¶	26.20	39.03	23.90	19.00

¶ = All activities are expressed as nmoles of glucose-6-phosphate produced /minute /mg of protein at 50°C. X = xylose-grown cells, G = glucose-grown cells.

ATP-dependent phosphorylation of glucose (glucokinase activity) was detected in crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1 grown on glucose and on xylose. To study the kinetics of glucokinase activity, two different growth conditions were investigated, 5 mM xylose-grown and 50 mM glucose-grown cells.

The glucokinase of 50 mM glucose-grown cells exhibited saturation kinetics with increasing concentrations of glucose (figure 8.1) and the Eadie Hofstee plot was biphasic (inset figure 8.1). The K_m of glucokinase at low glucose concentrations (< 0.20 mM) was 0.21 mM with a V_{max} of 29.0 nmoles of glucose /minute /mg of protein. At high glucose concentrations (> 0.20 mM) the K_m was 0.86 mM and the V_{max} 74.0 nmoles of glucose /minute /mg of protein.

The glucokinase from 5 mM xylose-grown cells showed saturation kinetics with increasing glucose concentrations (figure 8.2) and the Eadie-Hofstee plot was biphasic (inset figure 8.2). At low concentrations of glucose (< 0.20 mM) the K_m was 0.17 mM with a V_{max} of 18.50 nmoles /minute /mg of protein. At high concentrations of glucose (> 0.20 mM) the K_m was 0.87 mM and the V_{max} 52.0 nmoles /minute /mg of protein.

Saturation kinetics were not a consequence of ATP or glucose-6-phosphate dehydrogenase limiting the rate of glucokinase activity at increasing concentrations of substrate as all reagents were added in excess. Adding more ATP (> 5 mM) or glucose-6-phosphate dehydrogenase did not result in increased activities.

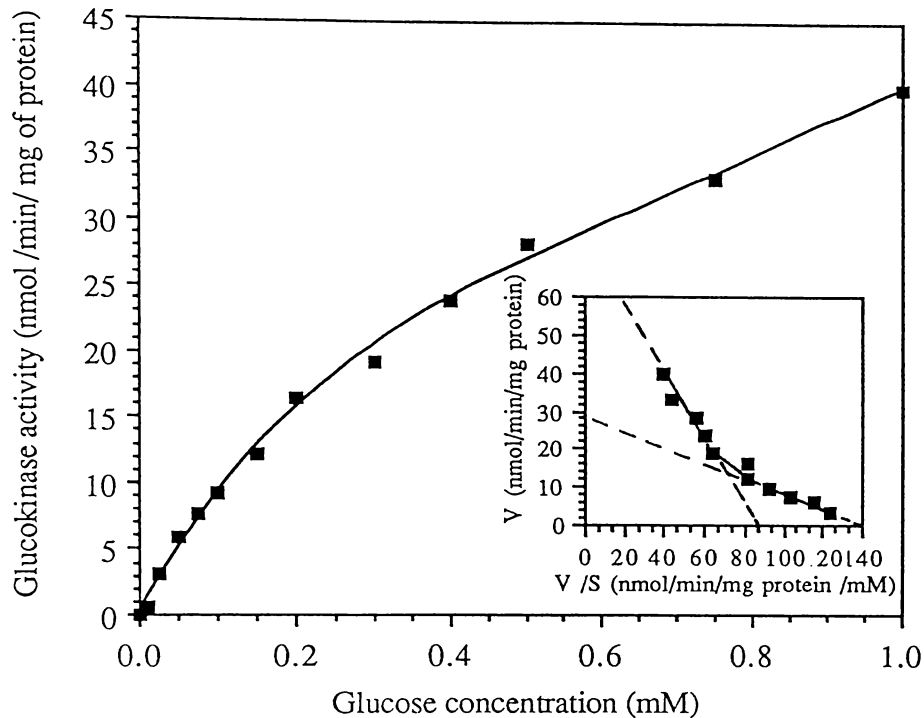


Figure 8.1 Glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose with increasing concentrations of glucose. Eadie-Hofstee plot of glucokinase activity is shown in inset.

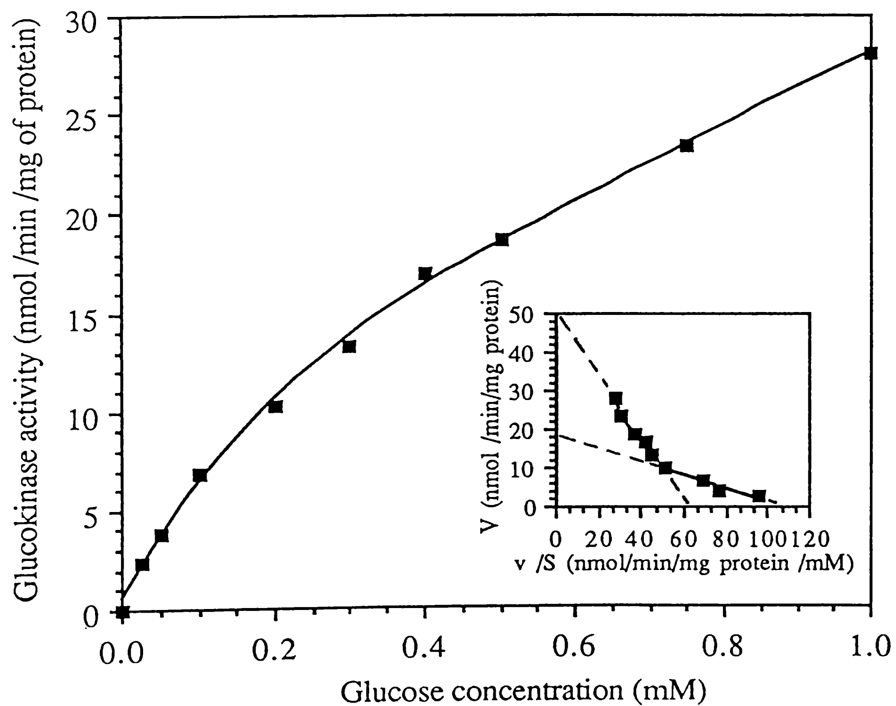


Figure 8.2 Glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing concentrations of glucose. Eadie-Hofstee plot of glucokinase activity is shown in inset.

The kinetics of glucokinase activity were biphasic, indicating high and low-affinity of glucokinase for glucose. The change in kinetics with increasing glucose concentration suggests either the presence of isoenzymes with different affinities for glucose or glucokinase activity is controlled by allosteric effectors which modifies the affinity of glucokinase for glucose. Glucokinase is an ATP-dependent enzyme and therefore it might be expected that the concentration of ATP or other adenylates may regulate glucokinase activity.

8.3.2 The effect of ATP concentration on glucokinase activity

The effect of ATP concentration on the rate of glucokinase activity at a constant glucose concentration of 0.5 mM was investigated.

With 50 mM glucose-grown cells using different concentrations of ATP (0.5-5.0 mM), typical substrate saturation curves were observed (figure 8.3). An increase in ATP concentration above 5 mM did not result in a significant increase in enzyme activity. A K_m of 2.38 mM ATP was calculated from the Eadie-Hofstee plot (inset figure 8.3).

For 5 mM xylose-grown cells saturation kinetics were observed with increasing concentrations of ATP (figure 8.4). The K_m for ATP was 2.05 mM calculated from the Eadie-Hofstee plot (inset figure 8.4).

Glucokinase is an enzyme which requires ATP for phosphorylation of glucose. The nucleotide ADP was investigated for its effect on glucokinase activity in 50 mM glucose-grown cells at constant glucose concentrations of 0.5 mM. At a constant ATP concentration of 1 mM, glucokinase activity was not affected by increasing concentrations of ADP up to 5 mM (figure 8.5). At a high concentration of ATP (5 mM), increasing concentrations of ADP inhibited the glucokinase activity suggesting that the level of ATP and ADP within the cell may regulate the enzyme (figure 8.5).

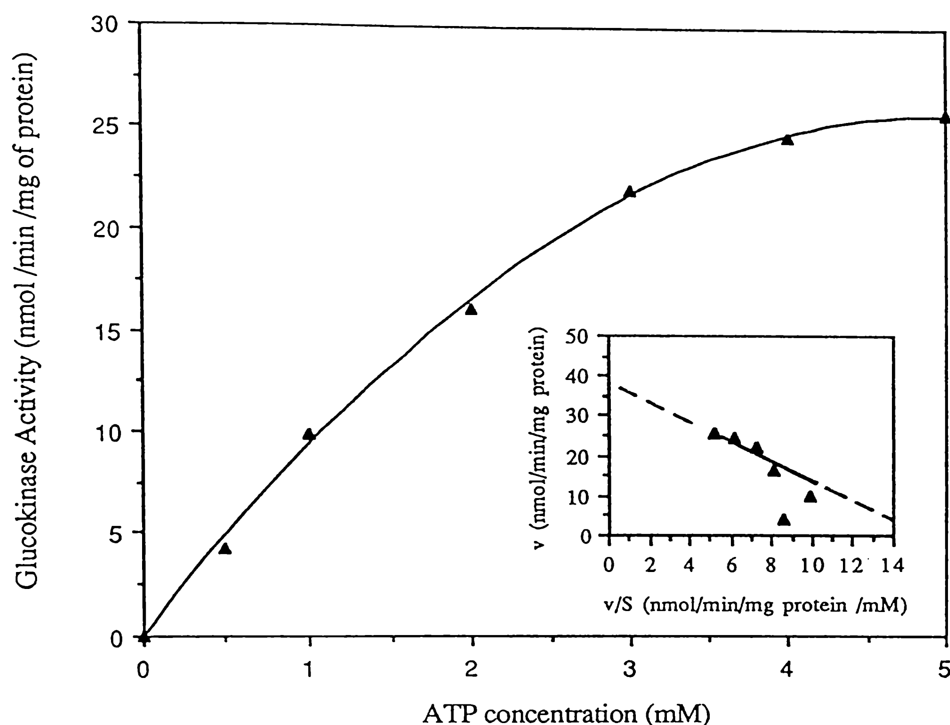


Figure 8.3 The effect of ATP concentration on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose at a constant glucose concentration (0.50 mM).

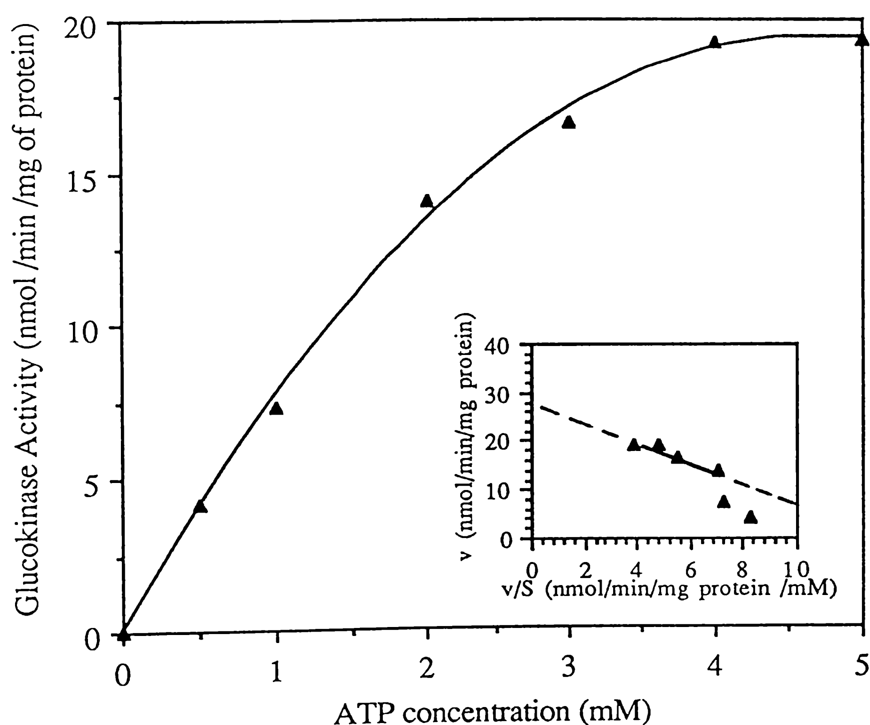


Figure 8.4 The effect of ATP concentration on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose at a constant glucose concentration (0.50 mM).

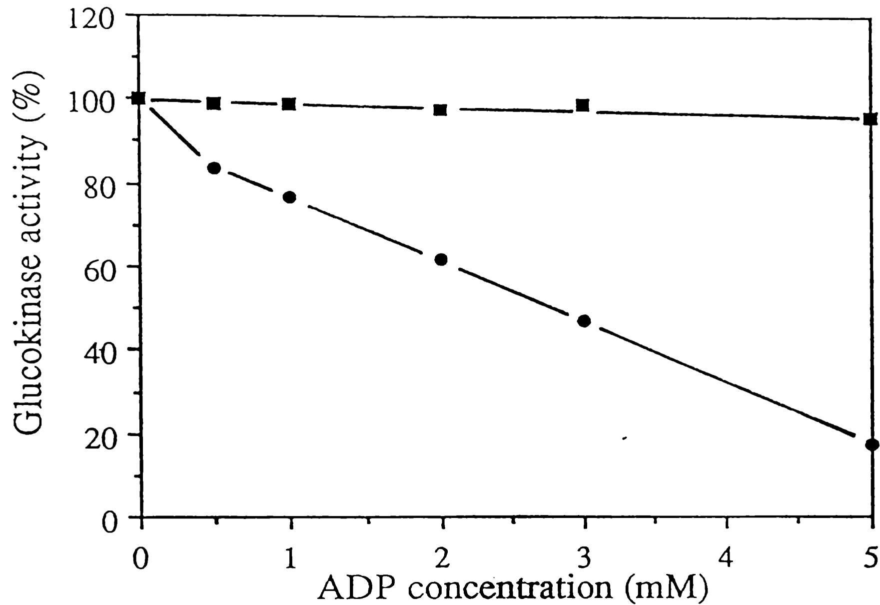


Figure 8.5 The effect of ADP concentration on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose at a constant glucose concentration (0.50 mM) and various ATP concentrations. (■) = 1 mM ATP and (●) = 5 mM ATP.

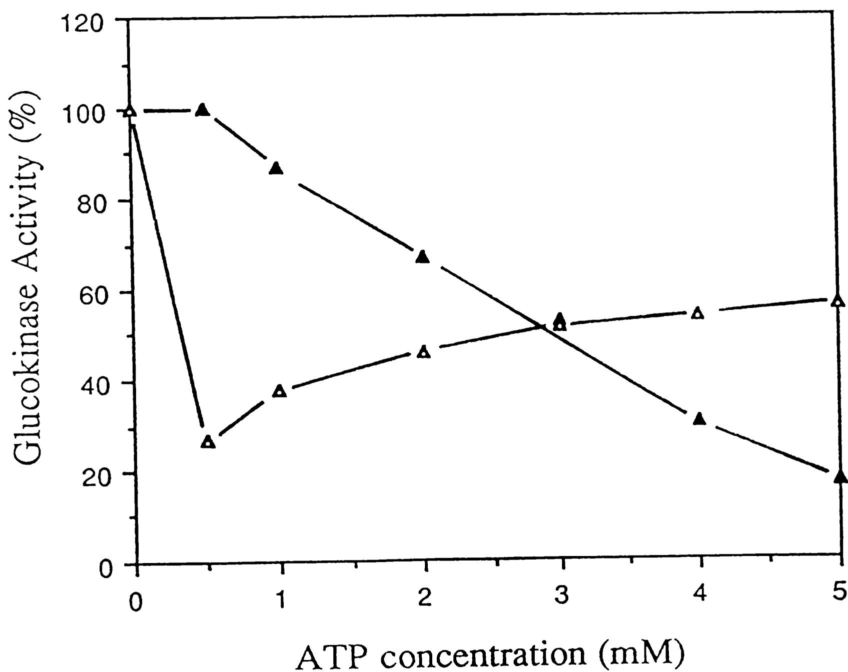


Figure 8.6 The effect of ADP and AMP concentration on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose at a constant glucose concentration (0.50 mM) and increasing ATP concentrations. (▲) = 5 mM ADP and (Δ) = 5 mM AMP.

When the concentration of ADP was kept constant (5 mM), glucokinase activity was inhibited with increasing ATP concentrations above 0.5 mM (figure 8.6). The nucleotide AMP at constant concentrations of 5 mM inhibited the activity of glucokinase at low ATP concentrations and with increasing concentrations of ATP some alleviation of the inhibition was observed (figure 8.6).

The nucleotides ADP and AMP showed a strong inhibitory effect on *Cl. thermohydrosulfuricum* Rt8.B1 glucokinase activity. These results indicate that in *Cl. thermohydrosulfuricum* Rt8.B1 glucokinase is under the control of ATP, AMP and ADP concentrations within the cell. The effect of ADP and AMP were not due to inhibition of the linking enzyme glucose-6-phosphate dehydrogenase.

8.3.3 The effect of competitive inhibitors on glucokinase activity

A series of different substrates were tested to explore possible effects on ATP-dependent glucose phosphorylation by glucokinase. A summary of these results for 5 mM xylose- and 50 mM glucose-grown cells is presented in table 8.2. All competitive inhibitors were also tested for their ability to inhibit the linking enzyme glucose-6-phosphate dehydrogenase.

Table 8.2 The effect of various substrates on the activity of glucokinase in lysed cell crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1

	% inhibition of glucokinase activity	
Inhibitor tested ¶	50 mM glucose-grown	5 mM xylose-grown
D-xylose	17.00	11.00
L-xylose	0.00	0.00
methyl- -D-glucoside	0.00	0.00
2-deoxy-glucose	0.00	0.00
L-glucose	0.00	0.00
D-xylulose	49.06	82.10

¶ = All inhibitors were added at a final concentration of 5 mM in the assay mixture.

The glucokinase of 50 mM glucose-grown and 5 mM xylose-grown cells was not affected by 2-DG, α-MG, L-xylose and L-glucose at final concentrations of 5 mM in the assay mixture. A small amount of inhibition was seen with D-xylose. D-xylulose was a potent inhibitor of glucokinase activity. The concentration of xylulose added here was 5 mM. This suggested that when grown in the presence of high concentrations of xylose, the xylulose formed by the action of xylose isomerase may serve to regulate the activity of glucokinase. The effect of D-xylulose on the activity of glucokinase was therefore investigated under conditions of high (5 mM) and low (1.0 mM) concentrations of ATP. With both low and high concentrations of ATP the presence of xylulose was not inhibitory until its concentration exceeded 0.2 mM in 50 mM glucose-grown cells (figure 8.7).

At high concentrations of ATP (5 mM), the glucokinase of 5 mM xylose-grown cells showed inhibition by low concentrations of xylulose (< 0.10 mM) and at 2 mM xylulose the glucokinase was inhibited 60 % (figure 8.8). At low concentrations of ATP (1.0 mM), glucokinase was inhibited by low concentrations of xylulose (< 1.0 mM). At concentrations of 2 mM xylulose the level of inhibition was 80 % (figure 8.8).

8.3.4 The effect of metabolic inhibitors on glucokinase activity

Having investigated the effect of ADP and ATP and the effect of competitive inhibitors, various metabolic inhibitors were tested for their ability to inhibit glucokinase activity in crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1. A summary of these results for 5 mM xylose-grown and 50 mM glucose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 is presented in table 8.3.

The inhibitors potassium cyanide, sodium azide, sodium fluoride, and sodium arsenate had no effect on glucokinase activity from either 50 mM glucose-grown or 5 mM xylose-grown cells. Iodoacetate was a potent inhibitor of glucokinase activity in 5 mM

xylose (57.30) and 50 mM glucose-grown (62.20 %) cells. This inhibition was not attributed to inhibition of glucose-6-phosphate dehydrogenase by iodoacetate.

Table 8.3 The effect of metabolic inhibitors on the activity of glucokinase in lysed cell crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1

	% inhibition of glucokinase activity	
Inhibitor tested ¶	50 mM glucose-grown	5 mM xylose-grown
Potassium cyanide	0.00	0.00
Sodium azide	0.00	0.00
Sodium arsenate	0.00	0.00
Iodoacetate	62.20	57.30
Sodium fluoride	0.00	0.00

¶ = All inhibitors were added at a final concentration of 5 mM in the assay mixture.

8.3.5 Kinetics of xylose isomerase and xylulokinase activity

The results in chapter seven showed that xylose uptake was a saturable function of the xylose concentration and this indicated the saturation of an active system.

D-xylose isomerase and D-xylulokinase activity were not detected in 5 mM and 50 mM glucose-grown cells confirming the inducible nature of these enzymes.

The xylose isomerase from 5 mM xylose-grown cells showed saturation kinetics with increasing concentrations of xylose (figure 8.9) and the Eadie Hofstee plot was biphasic (inset figure 8.9). At xylose concentrations below 10 mM, the K_m for xylose isomerase was 3.37 mM and the V_{max} 56.0 nmoles /minute /mg of protein. At xylose concentrations above 10 mM, the K_m was 9.71 and the V_{max} 85.0 nmoles /minute /mg of protein.

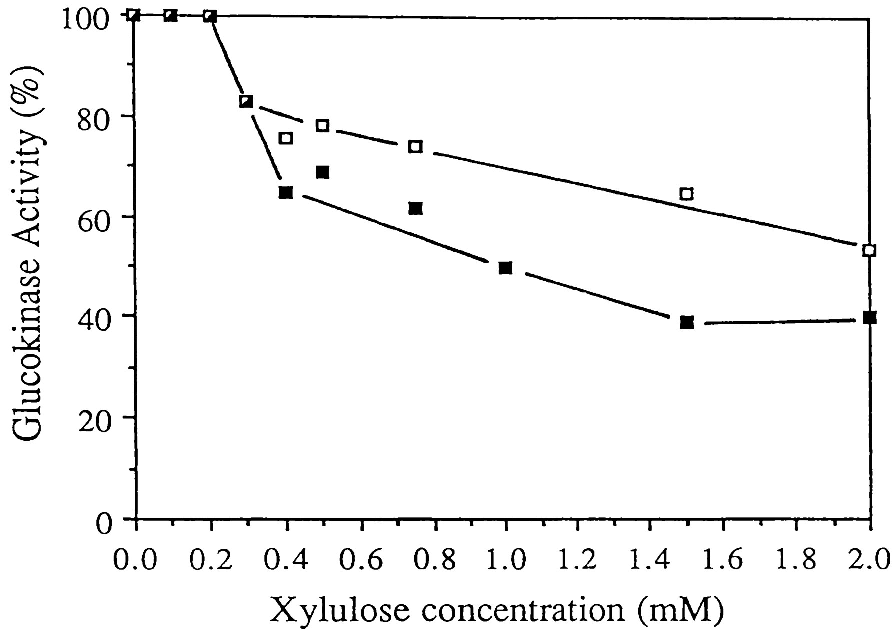


Figure 8.7 The effect of D-xylulose on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM glucose at a constant glucose concentration (0.50 mM) and various ATP concentrations. (□) = 5 mM ATP and (■) = 1 mM ATP.

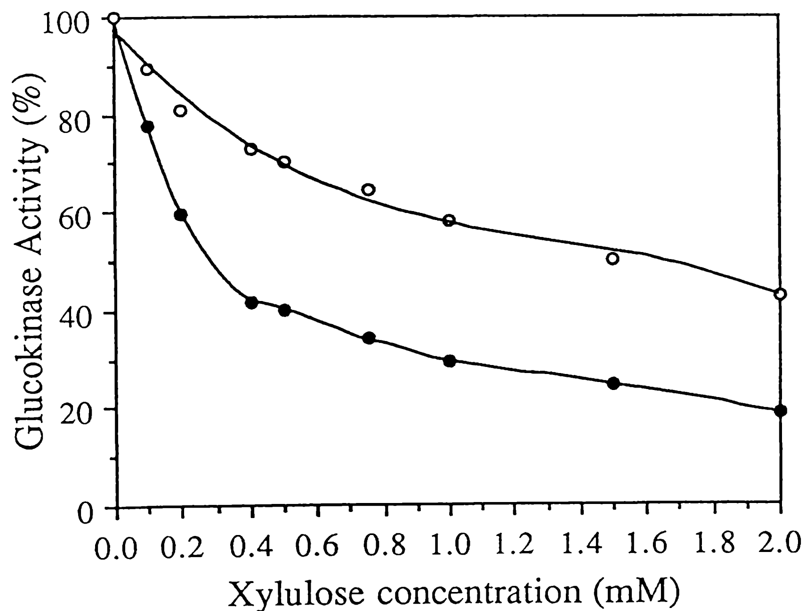


Figure 8.8 The effect of D-xylulose on glucokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose at a constant glucose concentration (0.50 mM) and various ATP concentrations. (O) = 5 mM ATP and (●) = 1 mM ATP.

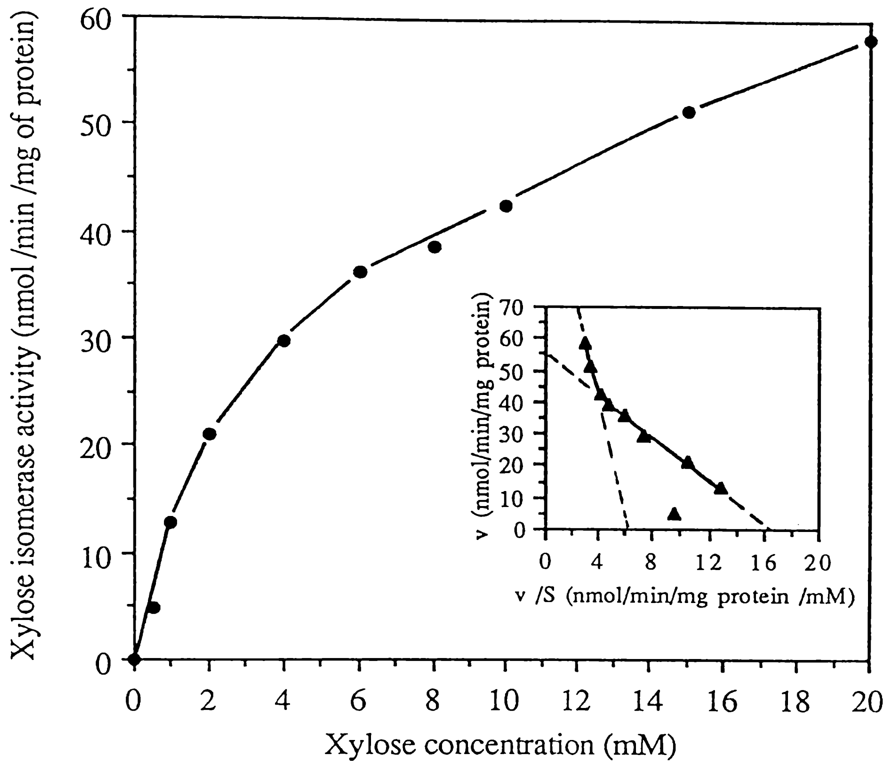


Figure 8.9 Xylose isomerase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing concentrations of xylose. Eadie-Hofstee plot of xylose isomerase activity is shown in inset.

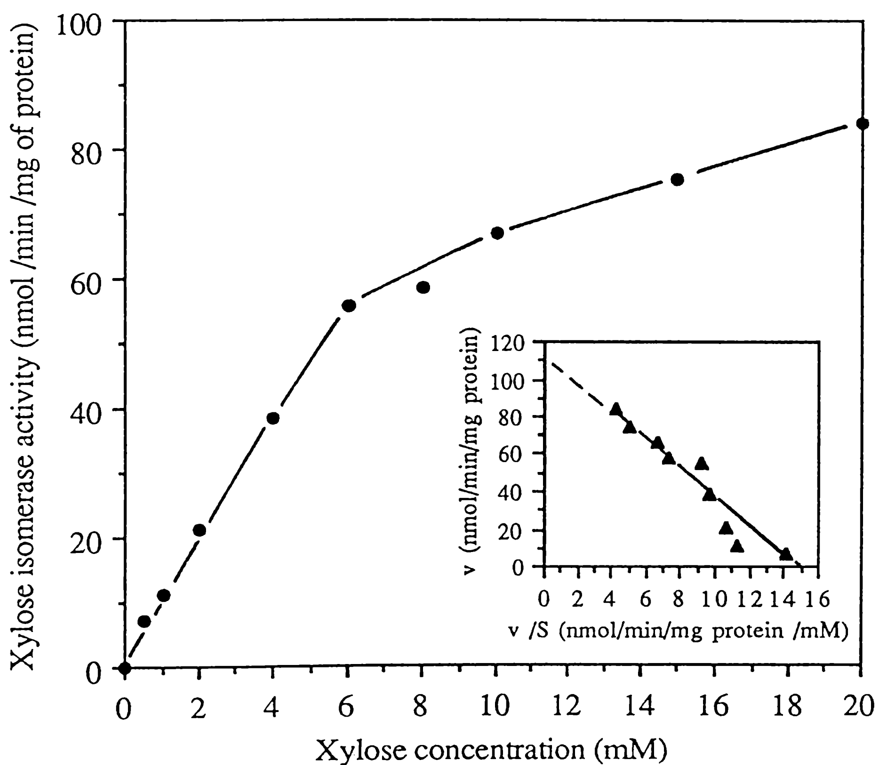


Figure 8.10 Xylose isomerase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose with increasing concentrations of xylose. Eadie-Hofstee plot of xylose isomerase activity is shown in inset.

The xylose isomerase of 50 mM xylose-grown cells exhibited saturation kinetics with increasing concentrations of xylose (figure 8.10) and the Eadie-Hofstee plot was linear (inset figure 8.10). The K_m for xylose was 7.60 mM and the V_{max} 114.0 nmoles /minute /mg of protein.

The enzyme kinetics of xylulokinase (catalyzing the ATP-dependent phosphorylation of D-xylulose to D-xylulose-5-phosphate) were investigated in xylose-grown cells.

Xylulokinase activity in 5 mM xylose-grown cells exhibited saturation with increasing concentrations of xylulose (figure 8.11). The Eadie-Hofstee plot was linear with a K_m for xylulose of 0.27 mM and a V_{max} of 62.0 nmoles of xylulose /minute /mg of protein (inset figure 8.11).

Xylulokinase activity in 50 mM xylose-grown cells showed saturation kinetics with increasing concentrations of xylulose (figure 8.12) and the Eadie hofstee plot was linear above concentrations of 0.20 mM with a K_m for xylulose of 0.25 mM and a V_{max} of 52.0 nmoles of xylulose /minute /mg of protein (inset figure 8.12).

The effect of high concentrations of glucose and glucose and glucose-6-phosphate were investigated for inhibition of xylose isomerase and xylulokinase activity. High concentrations (100 mM) of glucose and glucose-6-phosphate had no effect on the rate of xylose isomerase or xylulokinase activity supporting the view that these enzymes are not subject to catabolite inhibition (production not activity) by glucose.

In addition to the reversible isomerization between D-xylose and D-xylulose, xylose isomerase can convert D-glucose into D-fructose, hence the enzyme is often referred to as glucose isomerase (Lee *et al.*, 1990). A small amount of glucose isomerase activity was detected in crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1 grown on xylose (table 8.4).

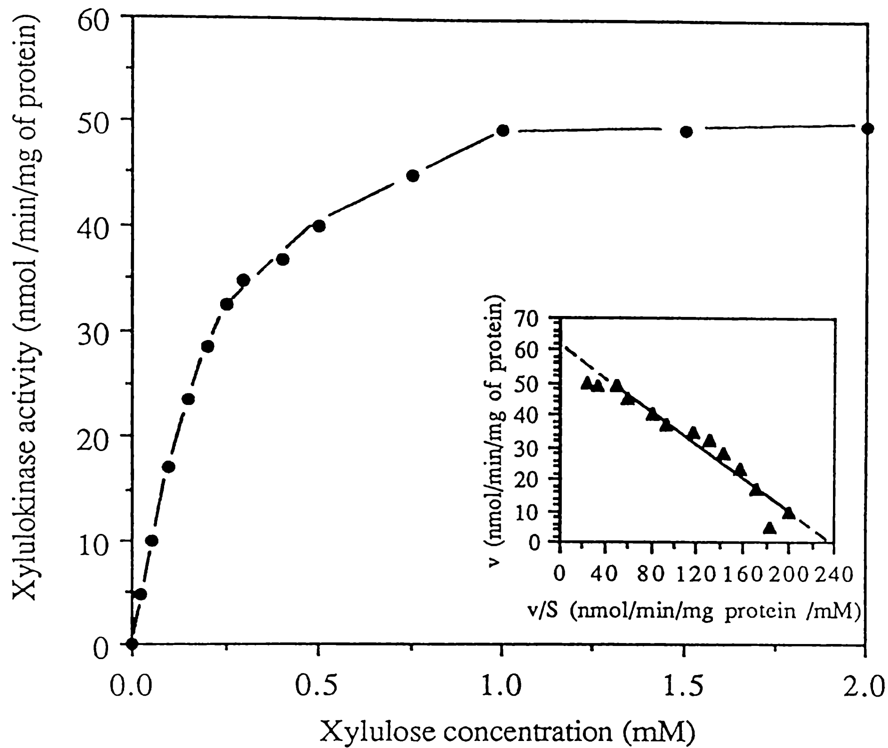


Figure 8.11 Xylulokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose with increasing concentrations of xylose. Eadie-Hofstee plot of xylulokinase activity is shown in inset.

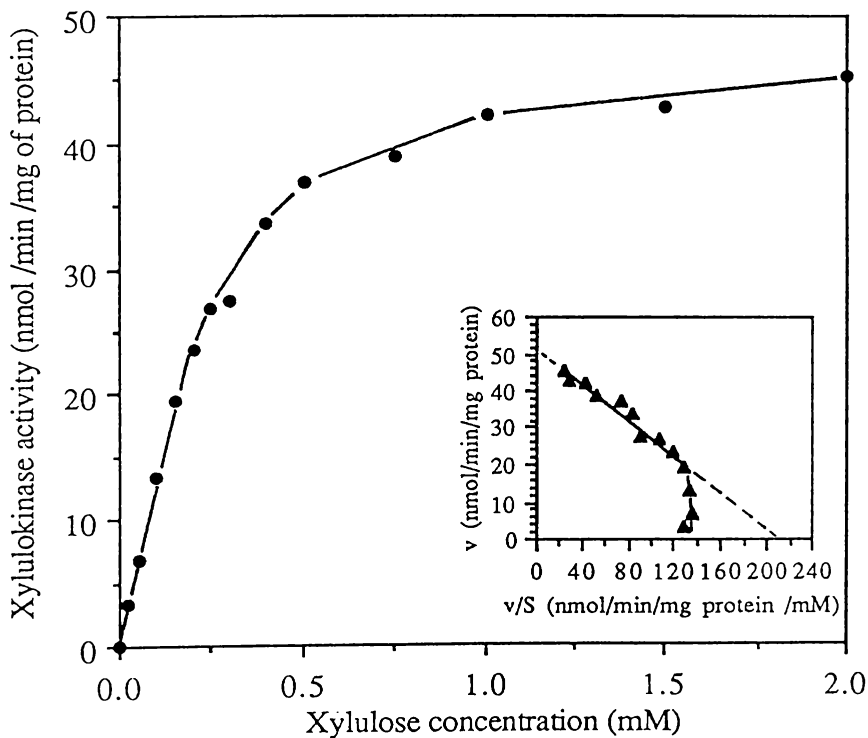


Figure 8.12 Xylulokinase activity of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 50 mM xylose with increasing concentrations of xylose. Eadie-Hofstee plot of xylulokinase activity is shown in inset.

Table 8.4 Glucose isomerase activity in crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1

Carbon source used to grow cells	5 mM G	50 mM G	5 mM X	50 mM X
Glucose isomerase activity §	0.00	0.00	3.65	5.12
Xylose isomerase activity ¶	0.00	0.00	42.00	66.00

§ = Glucose isomerase specific activity expressed as nmoles of D-fructose produced /minute /mg of protein.

¶ = Xylose isomerase specific activity expressed as nmoles of D-xylulose produced /min /mg of protein.

No glucose isomerase activity was detected in glucose grown cells suggesting the enzyme was induced by growth on xylose and was indeed the xylose isomerase.

8.4 DISCUSSION

8.4.1 Introduction

The first step in the metabolism of D-glucose in bacteria is the phosphorylation of D-glucose at the 6-position. This reaction can be catalyzed either by cytoplasmic hexokinase or by the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PEP-PTS), which concurrently transports and phosphorylates the sugar.

With few exceptions, strictly aerobic bacteria use an active transport system making use of the proton motive force ($\Delta\mu H^+$) derived from aerobic respiration or by ATP hydrolysis via the F_1F_0 ATPase and subsequently use intracellular kinases for phosphorylation. Among anaerobes, which generate their ATP and PEP via substrate level phosphorylation, specific PEP-PTS exist for each sugar transported and transport occurs with concomitant phosphorylation (Romano *et al.*, 1970, 1979). An organism may possess different transport systems for the same sugar. For example, *Escherichia*

coli has multiple enzymes capable of phosphorylating glucose. One known pathway is via the constitutive soluble ATP-dependent glucokinase (Curtis and Epstein, 1975).

8.4.2 Kinetics of glucokinase activity

Cl. thermohydrosulfuricum Rt8.B1 was shown not to possess a PEP-PTS for the phosphorylation of glucose (section 7.3.1). The phosphorylation of glucose in *Cl. thermohydrosulfuricum* Rt8.B1 is mediated by a constitutive cytoplasmic ATP-dependent glucokinase reaction.

Studies of the enzymes affinities of the glucokinase present in *Cl. thermohydrosulfuricum* Rt8.B1 for glucose revealed in all cases, kinetics of the Michaelis-Menten type. A summary of the kinetic parameters for glucokinase are presented in table 8.5.

Table 8.5 The kinetic parameters of glucokinase activity in crude extracts of *Cl. thermohydrosulfuricum* Rt8.B1

	Carbon source used for growth	
Kinetic parameter	50 mM glucose	5mM xylose
Km (low glucose)	0.21 mM	0.17 mM
Km (high glucose)	0.86 mM	0.87 mM
Vmax (low glucose)	29.00	18.50
Vmax (high glucose)	74.00	52.00
Km (ATP)	2.38	2.05 mM

V_{max} = nmoles of glucose-6-phosphate produced /minute /mg of protein. Low glucose = glucose concentrations < 0.2 mM, High glucose = glucose concentrations > 0.2 mM.

There have been few reports on the kinetics of glucokinase activity in thermophilic bacteria. The glucokinase from the moderately thermophilic bacterium *Bacillus stearothermophilus* was purified and the kinetics determined (Tomita *et al.*, 1990). The enzyme had a K_m for glucose of 0.10 mM and and had a small amount of activity towards 2-DG. The glucokinase of *Cl. thermohydrosulfuricum* Rt8.B1 had a similar K_m value at low concentrations of glucose, but did not exhibit any activity towards 2-DG. The fact that 2-deoxy-glucose had no effect on glucokinase activity is consistent with the view that the glucokinase from *Cl. thermohydrosulfuricum* Rt8.B1 does not phosphorylate 2-DG, as in other bacteria (Romano *et al.*, 1979). The glucokinase from *Cl. thermohydrosulfuricum* Rt8.B1 was stereospecific for D-glucose.

Bacterial glucokinases have been reported as an alternative route for the production of glucose-6-phosphate in oral streptococci that lack significant PEP-PTS activity. Glucokinase in these bacteria has been shown to be responsible for the low-affinity pathway of glucose uptake and PTS responsible for the high-affinity pathway (Keevil *et al.*, 1984, 1986). Vadeboncoeur *et al.* (1982) reported *Streptococcus mutans, Streptococcus* ***sanguis*** and *Streptococcus salivarius* had glucokinase activity and Michaelis-Menten kinetics were observed with increasing concentrations of glucose. The K_m for glucose varied with the species, ranging from 0.90 to 2.47 mM (high K_m-low-affinity). In the present study, the K_m values for glucokinase activity at glucose concentrations > 0.20 mM were in the range 0.86-0.87 mM glucose and these values indicate a low-affinity for glucose.

Streptococcus bovis JB1 and *Zymomonas mobilis* have been shown to operate a facilitated diffusion system for glucose transport at high glucose concentrations (Russell, 1990; DiMarco and Romano, 1985). From studies with crude extracts the K_m values of glucokinase from these organisms were determined (Martin and Russell, 1986; DiMarco and Romano, 1985). Both glucokinases had K_m values of 0.25 mM glucose which is similar to the values obtained in this study with *Cl. thermohydrosulfuricum* Rt8.B1 which also lacked a concentrative uptake system for glucose.

The ability of *Cl. thermohydrosulfuricum* Rt8.B1 to regulate glucokinase activity in response to the glucose concentration is also reflected in the kinetics of glucose utilization, which were biphasic with increasing glucose concentrations (see section 6.3.3). The K_s for glucose utilization at low glucose concentrations (< 5.0 mM) was 0.84-1.15 mM glucose (section 6.3.3). These values are similar to the K_m for glucokinase at high glucose concentrations and indicate that the glucokinase is the key enzyme controlling the rate of glucose utilization in *Cl. thermohydrosulfuricum* Rt8.B1. The K_t for glucose uptake at low glucose concentrations was in the range 0.08-0.15 mM glucose (section 7.3.1) which is similar to the K_m of glucokinase at low glucose concentrations. These results suggest that glucose uptake is controlled by glucokinase.

8.4.3 Control of glucokinase activity in *Cl. thermohydrosulfuricum* Rt8.B1

The ability of *Cl. thermohydrosulfuricum* Rt8.B1 to regulate the affinity of glucokinase in response to glucose concentration suggests some form of modulation of enzyme activity. Control of enzyme activity is modulated in a variety of ways, but the most important are inhibition or activation of allosteric proteins by their specific effectors, and variation in the rate of reaction in response to concentration of substrate (Ingraham *et al.*, 1983). In allosteric enzymes, their activation is modified by effectors thereby inducing or favouring a conformational change in the enzyme that alters the enzyme activity either by changing K_m or V_{max}. Allosteric inhibition and activation by metabolic intermediates plays an important role in regulating the flow through fueling pathways. In general, the target enzymes for the control of reaction sequences usually catalyzes reactions that are irreversible under physiological conditions. For example, PEP an intermediate of glycolysis, inhibits phosphofructokinase an allosteric enzyme in *E. coli*. In this study, glucokinase activity was inhibited by AMP and ADP and stimulated by ATP. Because ATP synthesis and utilization involve a cyclic flow through ADP and /or AMP, it is not surprising that all three adenylates play regulatory roles in glucose catabolism (glucokinase) in *Cl. thermohydrosulfuricum* Rt8.B1.

These observations suggest that under conditions where the supply of glucose is low (i.e. below the K_m of glucokinase), the efficacy of glucose capture is dependent on the phosphorylation capability of glucokinase which has a high-affinity for glucose. As the glucose concentration increases, the phosphorylation capability of the cell is improved by the production of additional glucokinase and the modification of glucokinase from a high-affinity into a low-affinity state. Thus the rate of catabolic activity (quantity of substrate utilized per unit time) depends on the external concentration of glucose. Glucose uptake is therefore solely controlled by the maximal activity of catabolic enzymes and in turn the energy balance of the cell i.e. if the energy balance is the allosteric effector. Fluctuations in the steady state of ATP, ADP and AMP will modulate the rate of catabolism. Thus any disturbance in environmental conditions which alters the balance of ATP and ADP concentration will immediately affect the rate of glucose utilization. Presumably an increase in the rate of ATP turnover, thus lowering the ratio of ATP to ADP ratio, will also influence the catabolic pathway by alleviating any allosteric inhibition by ADP and/ or enhancing the availability of ATP for glucose phosphorylation. The glucokinase of *Z. mobilis* has also been reported to be regulated by the level of ADP and ATP within the cell (Doelle, 1982).

The xylose intermediate D-xylulose had a strong inhibitory effect on glucokinase activity. The glucokinase from 5 mM xylose-grown cells was strongly inhibited by D-xylulose with inhibition being greatest at low concentrations of ATP. The fact that xylulose is able to inhibit glucokinase activity suggests that in the cell there may be some regulation of glucose utilization by xylose metabolic activity.

Iodoacetate is a known inhibitor of glyceraldehyde-3-phosphate dehydrogenase. Surprisingly, the glucokinase of *Cl. thermohydrosulfuricum* Rt8.B1 was strongly inhibited by iodoacetate and this result could explain the inhibition of glucose uptake by iodoacetate as measured by ^{14}C-glucose uptake in cell-suspensions of *Cl. thermohydrosulfuricum* Rt8.B1 (section 7.3.4).

8.4.4 The kinetics of xylulokinase and xylose isomerase

The kinetic parameters of xylose isomerase and xylulokinase are summarized in table 8.6.

Table 8.6 The kinetic parameters of xylulokinase and xylose isomerase from *Cl. thermohydrosulfuricum* Rt8.B1

	Carbon source used for growth	
Kinetic parameter and Enzyme	50 mM xylose	5mM xylose
Xylose Isomerase §		
Km (low xylose)	7.6 mM	3.37 mM
Km (high xylose)	7.6 mM	9.71 mM
Vmax (low xylose)	114.00	56.00
V max (high xylose)	114.00	85.00
Xylulokinase ¶		
Km	0.25 mM	0.27 mM
Vmax	52.00	62.00

Low xylose = < 10 mM xylose, High xylose = > 10 mM xylose.

§ = Xylose isomerase V_{max} expressed as nmoles of xylulose produced /minute /mg of protein.

¶ = Xylulokinase V_{max} expressed as nmoles of xylulose-5-phosphate produced /minute /mg of protein.

The xylose isomerase of *Cl. thermohydrosulfuricum* Rt8.B1 grown on 5 mM xylose exhibited biphasic kinetics. The high K_m values of xylose isomerase reflects the low-affinity of this enzyme for xylose which would therefore not be expected to play a key role in the uptake of xylose at low concentrations either in the absence of an active transport system for xylose or in the presence of an active removal system for xylulose.

The high K_m values of xylose isomerase suggest that this enzyme does not limit the rate of xylose utilization and that the equilibrium lies in the favour of xylulose formation. The xylulokinase in 5 and 50 mM xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 has a low K_m (high-affinity) for xylulose in the range 0.24 to 0.27 mM xylulose. The fact that the K_m of xylose isomerase was significantly greater than the K_m for xylulokinase suggests that the rate-limiting step of xylose utilization is at the level of substrate phosphorylation by the enzyme xylulokinase.

The K_m for xylulokinase was lower than the K_t for xylose uptake which was 0.78 mM in 5 mM xylose-grown cells (see section 7.3.2). This suggests that the phosphorylating system for xylulose (xylulokinase) would be fully saturated at external xylose concentrations near or above the K_t concentration for uptake and the affinity of xylulokinase regulates the rate of xylose uptake and subsequent utilization. Presumably at concentrations greater than those used to measure xylulokinase activity and uptake (> 2 mM xylose), either xylulokinase is able to regulate its activity in accordance with the external xylose concentration and available ATP within the cell or the cell is able to regulate xylulokinase level. The fact that xylose uptake in 50 mM xylose-grown cells was by facilitated diffusion (section 6.3.3 and 7.3.1) and that the xylulokinase had a similar K_m to that of 5 mM xylose-grown cells indicates that if xylulokinase is rate-limiting or inhibited, then xylose can still go to xylulose by xylose isomerase (low-affinity /high K_m) since the equilibrium would lie in this direction; thus xylulose may leave the cell at rate equal to xylose influx. In this situation there would be no net carbohydrate accumulation, but an apparent xylose utilization, the rate of which would be related to the external concentration of xylose consequently a facilitated diffusion effect would be observed and this may explain the kinetics observed.

Glucose and glucose-6-phosphate at high concentrations (100 mM) had no effect on xylulokinase or xylose isomerase activity suggesting that neither enzyme is subject to allosteric or competitive inhibition and that once xylose is added to the growth medium these enzymes are induced whether glucose or glucose-6-phosphate is present or not.

Xylose isomerase can also convert D-glucose into D-fructose, hence the enzyme is often referred to as glucose isomerase (Lee *et al.*, 1990). Glucose isomerase has been reported to occur in the thermophilic microorganisms *Thermoanaerobacter* strain B6A (Lee *et al.*, 1990), *Clostridium thermosulfurogenes* strain 4B, *Thermoanaerobacter* strain B6A, *Clostridium thermohydrosulfuricum* strain 39E (Meng *et al.*, 1991) and *Thermus thermophilus* (Dekker *et al.*, 1991). Xylose isomerases in general display lower rates of activity and higher K_m values for glucose than for xylose (Meng *et al.*, 1991). The results of this study demonstrated that glucose isomerase activity in *Cl. thermohydrosulfuricum* Rt8.B1 was induced during growth on xylose and had a lower activity for glucose compared with activity for xylose. The fact that xylose isomerase has activity towards glucose explains the observation that glucose does not repress the synthesis of this enzyme or inhibits its activity when present in *Cl. thermohydrosulfuricum* Rt8.B1.

CHAPTER NINE

GENERAL DISCUSSION AND SUMMARY

9.1 INTRODUCTION

Five decades of study on control processes, beginning with Monod (1942), have revealed that mesophilic eubacteria have evolved a range of control mechanisms, some complex, which control the expression of the enzymes and transport systems involved in the assimilation of substrates. These studies have concentrated largely on *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtilis*. To date, study of these features in thermophilic eubacteria have largely been neglected.

The main objectives of this thesis were threefold. First, to carry out a general study of the means whereby extremely thermophilic bacteria control their enzyme expression in response to carbohydrate utilization. Second, to extend the preliminary findings of Cook (1988), in which it was found that extreme thermophiles have a tendency to utilize substrates simultaneously when supplied together. Third, to investigate the mechanisms which exist for the transport of glucose and xylose into the cells of the extremely thermophilic anaerobe *Clostridium thermohydrosulfuricum* Rt8.B1. Very little is known of such processes in extreme thermophiles. It was hoped that this study would lead to some understanding of the control processes involved in the mixed-substrate utilization of glucose and xylose.

9.2 REGULATION OF ENZYME ACTIVITY IN EXTREMELY THERMOPHILIC BACTERIA

Thermophilic bacteria inhabit environments low in available nutrient sources. Growth in such oligotrophic environments presumably would select for bacteria that are able to tightly regulate their substrate utilization and to control the synthesis of the enzymes involved rather than produce them continuously. Continuous synthesis is wasteful in

terms of energy, as the organism has to continually expend energy to produce an enzyme, the substrate for which may not be present.

A number of thermophilic bacteria representing different genera, kingdoms and physiological types were investigated for the controlled expression of β-galactosidase, β-glucosidase and α-glucosidase synthesis. From the results of this study it was difficult to draw firm conclusions in terms of the classical definition of induction. This was due to a combination of factors:

1. The undefined nature of the growth medium used to grow these organisms. Only for two thermophilic organisms was a defined medium used. Defined media which do not contain yeast extract and trypticase peptone both of which may contain gratuitous inducers, are necessary as otherwise conclusions about enzyme induction and repression are difficult to make.

2. In this study, enzyme activities were determined by measuring the activity using p-NP substrates and assuming they are specific substrates for the enzyme. The low specificity of thermophilic glycosidases reported here suggest that this can be misleading.

Given the difficulties mentioned, the results showed that the thermophilic organisms studied here exhibited a high level of constitutive β-galactosidase, β-glucosidase and α-glucosidase activity, which was increased by growth on various substrates. In general, these enzymes were not under the control of classical inducers and repressors of enzyme synthesis as in mesophilic organisms and in general did not exhibit classical induction under the culture conditions used. The high constitutive activity of these enzymes indicated that induction played a minor role in the regulation of these enzymes and presumably other mechanisms must control enzyme expression by a less elaborate array of mechanisms than the *E. coli lac* operon.

Although the constitutive synthesis of these enzymes might seem wasteful, the overall strategy may not be so. If the synthesis of these enzymes was dependent on induction,

the initial rates of enzyme production might be insufficient for efficient substrate utilization at the low substrate concentrations which occurs in an oligotrophic environments. The strategy adopted might allow thermophilic bacteria to match the rate of substrate availability with enzyme synthesis. As substrate depletes due to utilization, more enzyme might be synthesized, thus increasing the ability of the bacterium to scavenge substrate. This would afford a means of control different to that of the classical induction commonly found in mesophilic bacteria.

9.3 HYPERBOLIC GROWTH IN *Cl. thermohydrosulfuricum* Rt8.B1

Matin (1979) has reasoned that the preferential utilization of one compound would be of little value in a nutritionally poor environment and that selective pressure would favour microorganisms that are capable of making use of different substrates simultaneously when these substrates are present at low concentrations. There is now an increasing body of literature that microorganisms are capable of utilizing mixed substrates simultaneously at low nutrient concentrations (Matin, 1979; Harder and Dijkhuizen, 1982).

Cl. thermohydrosulfuricum Rt8.B1 inhabits thermophilic environments where the natural concentrations of sugars would be expected to be quite low. The results of this study demonstrated that glucose and xylose were used simultaneously, i.e. exhibited hyperbolic growth, when supplied together at nonlimiting concentrations. The lack of diauxic growth in *Cl. thermohydrosulfuricum* Rt8.B1 would suggest that this organism is a "generalist", able to use substrates simultaneously in an oligotrophic environment with low cell numbers present and therefore less competition. In contrast, diauxic growth would appear to be a feature of "specialists", where in a population with a high cell density it is advantageous to specialize in the catabolism of a limited range of substrates.

9.3.1. Control of hyperbolic growth in *Cl. thermohydrosulfuricum* Rt8.B1

It would be expected that bacteria capable of hyperbolic growth may show a different level of control from those which grow diauxically on glucose and xylose. Control of diauxic growth can be generally regarded as a consequence of one substrate being utilized rapidly by constitutive enzymes and thus bringing about catabolite repression of inducible pathways. When all the substrate has disappeared, the catabolite repression ceases, permitting induction of the enzyme for the utilization of the second substrate. The evolution of such control mechanisms solves the problem of choosing the most advantageous substrate for growth when a mixture of carbon sources is available. This is of obvious advantage to an organism as it restricts the synthesis of enzymes to those that are necessary for their cellular metabolism under current physiological conditions.

In order for xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 to proceed rapidly in the presence of glucose, xylose metabolic activity (i.e. xylose permease, xylose isomerase and xylulokinase) would be expected to be insensitive to catabolite repression or repressed only minimally by glucose. In *Cl. thermohydrosulfuricum* Rt8.B1, under conditions of hyperbolic growth when grown on glucose + xylose, both catabolite repression and inducer exclusion were absent. This was shown by the following observations:

1. Glucose did not repress xylose metabolic activity i.e. xylose permease and xylose isomerase were induced in the presence of glucose and were not subject to catabolite inhibition when glucose was added to cultures growing on high concentrations of xylose.

2. Glucose did not prevent xylose uptake, as both substrates were taken up at equal rates when supplied together. Further evidence was shown by the addition of glucose to xylose-grown cultures and by addition of xylose to glucose-grown cultures. Neither addition had any effect on the rate of utilization of the initial substrate at high concentrations.

Further evidence for a lack of catabolite inhibition by glucose or glucose intermediates on xylose metabolic activity was indicated by addition of 100 mM glucose and glucose-6-phosphate having no effect on xylose isomerase and xylulokinase activity in lysed cell crude extracts. These results also indicated that no allosteric modulation by glucose or glucose-6-phosphate was present. The fact that xylose isomerase has low activity towards glucose (glucose isomerase) explains the observation that glucose does not repress the synthesis of this enzyme or inhibit its activity when induced (present) in *Cl. thermohydrosulfuricum* Rt8.B1.

In *Cl. thermohydrosulfuricum* Rt8.B1 the genetic or biochemical features which determines the insensitivity of xylose permease and xylose isomerase to catabolite repression by glucose is not known. Recently, studies on the thermophilic xylose isomerase from *Cl. thermosulfurogenes* indicated that the switching preference of the thermophilic xylose isomerase from D-xylose to D-glucose was by a redesigning of the substrate-binding pocket of the active site (Meng *et al.*, 1991).

9.4 GLUCOSE TRANSPORT AND UTILIZATION IN *Cl. thermohydrosulfuricum* Rt8.B1

The transport of glucose by *Cl. thermohydrosulfuricum* Rt8.B1 did not involve a phosphoenolpruvate-dependent glucose:phosphotransferase system (PEP-PTS). The lack of PEP-PTS activity in *Cl. thermohydrosulfuricum* Rt8.B1 for glucose transport was further supported by the fact that a concentrative uptake of 2-DG and α-MG, both substrates of the PTS system in mesophilic bacteria, could not be demonstrated in glucose-grown cell suspensions of *Cl. thermohydrosulfuricum* Rt8.B1.

The lack of a PTS for glucose transport in *Cl. thermohydrosulfuricum* Rt8.B1 may provide a possible reason for the presence of glucose having no effect on xylose utilization. In general, catabolite inhibition usually involves sugars that are transported by a PTS (Postma and Lengeler, 1985). In general terms, PTS repression prevents the synthesis of induced enzymes by inhibiting the transport of inducer (or its precursor)

into the cell (McGinnis and Paigen, 1969). With *Cl. thermohydrosulfuricum* Rt8.B1 the highest growth rates occurred on glucose as opposed to growth on xylose where low growth rates were observed. From these observations it would be expected that glucose (the better substrate) would regulate xylose utilization by catabolite repression and this lack of regulation may be due to the absence of a PTS system for glucose. In this respect, the lack of catabolite inhibition in mutant strains (lacking mannose:PTS) of *Pediococcus halophilus* growing on glucose + xylose was attributed to a component of the mannose:PTS participating directly to effect catabolite inhibition (Abe and Uchida, 1989, 1991).

The lack of PEP-PTS activity led to an investigation of glucose utilization (transport and subsequent metabolism) in *Cl. thermohydrosulfuricum* Rt8.B1. A summary of the regulatory processes and kinetic parameters in glucose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 is shown in figure 9.1.

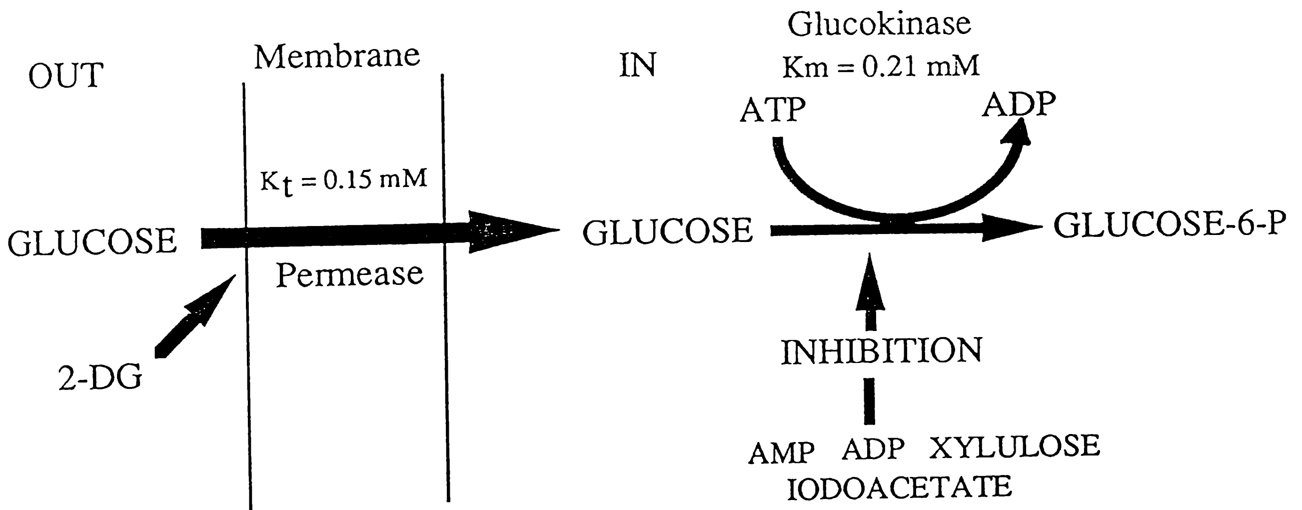


Figure 9.1 Control of glucose utilization in *Cl. thermohydrosulfuricum* Rt8.B1

Glucose transport by *Cl. thermohydrosulfuricum* Rt8.B1 was mediated by a stereospecific constitutive carrier-mediated permease which was competively inhibited by the glucose analogue 2-DG. In addition to 2-DG, glucose uptake in xylose-grown cells was inhibited by α-MG and xylose. These results suggest that for glucose entry, 2 permeases may exist:

1. In glucose-grown cells: a glucose-specific carrier (no xylose or α-MG inhibition) which is competitively inhibited by 2-DG.

2. In xylose-grown cells: a xylose carrier which may have affinity for glucose and is inhibited by xylose and glucose analogues.

These results may also indicate a non-specific inhibition of glucose uptake i.e. 2-DG and α-MG may be toxic to the cell. In xylose-grown cells xylose may compete for available ATP or key metabolites necessary for glucose transport.

On the basis of the insensitivity of glucose uptake to CCCP, 2,4-DNP, monensin and the F_1F_0-ATPase inhibitor DCCD, glucose transport was not driven by a proton motive force ($\Delta\mu H^+$) and not coupled to sodium and potassium ion gradients. An involvement of ATP in glucose transport was suggested by the reduction of glucose uptake in the presence of iodoacetate and sodium fluoride, both inhibitors of ATP synthesis by substrate level phosphorylation.

Glucose phosphorylation was ATP dependent and was mediated by an active constitutive glucokinase. *Cl. thermohydrosulfuricum* Rt8.B1 lacked a concentrative transport system for glucose and this indicated that glucokinase was responsible for this function. This conclusion was also supported by the following observations:

1. Glucokinase had a high affinity for glucose and the K_m for glucokinase was similar to the K_t for glucose uptake at similar concentrations (figure 9.1).

2. Glucose uptake was inhibited by iodoacetate and sodium fluoride. These compounds inhibit ATP synthesis by substrate level phosphorylation. Glucokinase is an ATP-dependent enzyme and therefore the inhibition of ATP synthesis would decrease the supply of ATP for glucose phosphorylation by glucokinase and therefore reduce glucose uptake. Both glucose uptake and glucokinase were inhibited by D-xylulose, which indicated that the inhibition of glucose uptake is due to the inhibition

of glucokinase. Glucokinase was not inhibited by 2-DG, indicating that the inhibition of glucose uptake by 2-DG was at the level of transport.

Glucokinase exhibited both high and low affinity for glucose with increasing concentrations of glucose. The change in affinity is most likely due to an allosteric activation of this enzyme in response to either glucose concentration or the concentration of key metabolites such as ATP and ADP. Glucokinase activity was inhibited by AMP and ADP in the presence of high concentrations of ATP. Thus the concentrations of ATP, ADP and AMP may serve to regulate glucokinase activity in response to the energy charge within the cell. Thus both glucose phosphorylation and glucose uptake are dependent upon the energy status of the cell. Any disturbance in environmental conditions which leads to the accumulation of ATP or ADP would presumably immediately affect the rate of glucose utilization.

Xylulose inhibited glucokinase activity at both high and low concentrations of ATP and may have a regulatory role. When both substrates are available the flow of substrate through the hexose and pentose metabolic routes may be regulated by a subtle interaction of permease affinities, xylose isomerase concentration i.e. conversion rate of xylose to xylulose, and the energy status of the cell. In xylose-grown cells the glucokinase was particularly sensitive to low concentrations of xylulose at low ATP concentrations.

9.5 XYLOSE TRANSPORT AND UTILIZATION IN *Cl. thermohydrosulfuricum* Rt8.B1

Xylose utilization by *Cl. thermohydrosulfuricum* Rt8.B1 was mediated by two systems. Both systems were inducible and dependent on the xylose concentration used for cell growth.

1. Cells grown on 5 mM xylose had a high-affinity, low-capacity system for xylose utilization which was saturable (low V_{max}).

2. Cells grown on 50 mM xylose had a low-affinity, high-capacity (high V_{max}) system for xylose utilization indicative of facilitated diffusion.

A summary of the regulatory processes and kinetic parameters in a xylose-grown cells of *Cl. thermohydrosulfuricum* Rt8.B1 is shown in figure 9.2.

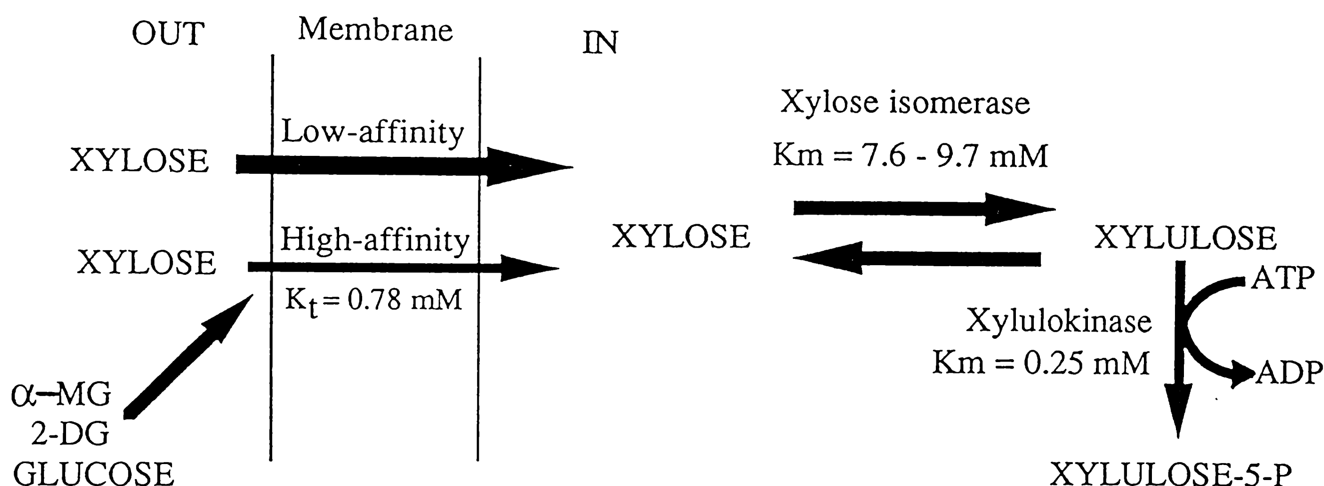


Figure 9.2 Control of xylose utilization in *Cl. thermohydrosulfuricum* Rt8.B1

High-affinity xylose uptake was inhibited by the glucose analogues 2-DG and α-MG suggesting some involvement of a carrier protein in xylose transport. Xylose uptake by the high-affinity transport system was also reduced in the presence of glucose. This was also demonstrated in cultures of *Cl. thermohydrosulfuricum* Rt8.B1 actively growing on 5 mM xylose. Although this could be interpreted as competition by intracellular glucose for ATP, since a similar level of inhibition was shown by 2-DG, which is not phosphorylated, suggests that glucose does compete for the xylose carrier.

Due to the insensitivity of xylose uptake to CCCP, 2,4-DNP, monensin and DCCD it can be concluded that xylose transport was not dependent on $\Delta\mu H^+$ and not coupled to sodium or potassium ion gradients. As for glucose uptake, xylose uptake was reduced in the presence of iodoacetate and sodium fluoride, which suggested that ATP was involved in this high-affinity xylose uptake.

The xylose isomerase of *Cl. thermohydrosulfuricum* Rt8.B1 had a high K_m for xylose (low-affinity) and therefore does not limit the rate of xylose uptake at high xylose concentrations. The xylulokinase of *C. thermohydrosulfuricum* Rt8.B1 had a low K_m for xylose (high-affinity) (figure 9.2). The K_m for xylulokinase was lower than the K_t for the high-affinity xylose permease (figure 9.2) which indicates that the phosphorylating system for xylose i.e. xylulokinase would be fully saturated at external xylose concentrations near or above the K_t concentration for uptake and thus the affinity of xylulokinase regulates that rate of xylose uptake and subsequent metabolism.

The fact that xylose uptake in 50 mM xylose-grown cells took place by facilitated diffusion and that the xylulokinase had a similar K_m to that of 5 mM xylose-grown cells implies that if xylulokinase was saturated, the xylose could still be converted to xylulose by xylose isomerase (high K_m) (the equilibrium would lie in this direction) and the xylulose could flow from the cell at a rate equal to xylose influx. In this situation there would be no net carbohydrate accumulation, but an apparent xylose utilization, the rate of which would be related to the external concentration of xylose. The high-affinity system would be able to regulate the uptake of xylose to prevent an excess of xylose saturating the cell; under these conditions no xylulose accumulation was observed as was seen in cultures grown on low concentrations of xylose.

This resembles the futile cycle thought to be active when organisms contain two uptake systems for a particular nutrient (one with high-affinity, the other with low-affinity for its substrate). The high-affinity system is responsible for uptake of the nutrient, some of which is subsequently lost to the medium again via leakage through the low-affinity system (Neijssel *et al.*, 1990). In this study, growth on high concentrations of xylose resulted in a low level of xylulose accumulation in the growth medium and this may be due to the futile cycling of xylose through xylulose.

The acquisition of a facilitated diffusion system for xylose in *Cl. thermohydrosulfuricum* Rt8.B1 is difficult to explain given the natural oligotrophic environment of this organism in which the low-affinity of this system would seem

disadvantageous. On the other hand, the high-affinity system for xylose would be expected to occur in such an environment.

Discussion of ecological advantages also raises the question of laboratory selection. The classic enrichment technique in batch culture with comparably high substrate concentrations always selects for those bacteria that can grow the fastest under the respective conditions (Schink, 1990). *Cl. thermohydrosulfuricum* Rt8.B1 was enriched and isolated and subsequently maintained under unnatural conditions where the selection pressure is for organisms with rapid growth rates and able to accumulate substrates at a high capacity and at concentrations where a high-affinity systems is of little use. Therefore the conditions use to grow *Cl. thermohydrosulfuricum* Rt8.B1 in this study at nonlimiting substrate concentrations, are possibly only of relevance in the laboratory and do not necessarily reflect the natural environment.

9.6 SUMMARY

In summary, the transport of both glucose and xylose across the bacterial cell membrane of *Cl. thermohydrosulfuricum* Rt8.B1 was governed by permeases which do not catalyze concomitant substrate transport and phosphorylation, and thus not a PEP-PTS. The phosphorylation of glucose was carried out by an ATP-dependent glucokinase and that of xylose by an ATP-dependent xylulokinase after isomerization by xylose isomerase. Thus both glucokinase and xylulokinase are potential control points for regulating the rates of glucose and xylose utilization. These enzymes not only initiate the metabolism of glucose and xylose, but also serve to extract the compound from the medium by phosphorylation. In *Cl. thermohydrosulfuricum* Rt8.B1 glucose is metabolized by the Embden Meyerhof Parnas pathway which yields 2 moles of PEP. One mole of PEP can be converted to ATP by pyruvate kinase and subsequently used by glucokinase thus effectively leaving a mole of PEP for further ATP production. The energetics of this system are therefore similar to those found in bacteria which possess a PTS, which is regarded as an energetically favourable pathway for glucose transport in fermentative bacteria which generate their ATP via substrate level phosphorylation.

The rates of glucose and xylose utilization when supplied together were also under cellular control. This was clearly shown in cells grown on 50 mM glucose + 50 mM xylose where identical rates of glucose and xylose utilization were seen at high substrate concentrations. The rate of substrate utilization from growth on single substrates was always greater than on a substrate mix. This could reflect an increase in activity of whichever enzyme constitutes the major kinetic control point in the catabolic pathway thereby increasing the overall activity of the pathway. This in turn could be determined by the rate at which the catabolic pathway synthesizes ATP or the rate at which ATP so formed is catalyzed by the cell.

Regulation to decrease the uptake and flux of both sugars to allow controlled hyperbolic growth is consistent with the idea of *Cl. thermohydrosulfuricum* Rt8.B1 being well adapted as a generalist. It is able to control substrate utilization at low and high concentrations of single and multiple substrates.

APPENDIX 1

System. Appl. Microbiol. 14, 240–244 (1991)
© Gustav Fischer Verlag, Stuttgart/New York

Endospore Formation by *Thermoanaerobium brockii* HTD4

GREGORY M. COOK, PETER H. JANSSEN, and HUGH W. MORGAN

Thermophile and Microbial Biochemistry and Biotechnology Unit, University of Waikato, Hamilton, New Zealand

Received November 11, 1990

Summary

The formation of heat-resistant endospores could be induced in *Thermoanaerobium brockii* HTD4 by cultivation on a minimal medium with D-xylose as the sole carbon source (MMX) using cultures pre-grown on trypticase-yeast extract-glucose (TYEG) as an inoculum. Sporulation was not observed when transferring from TYEG to TYEG, or from MMX to MMX. The spores were shown to be heat-resistant, and after killing vegetative cells at 115 °C for 80 mins, germinated to produce cultures with morphological and physiological characteristics identical with the original culture. This finding is interesting in the light of mixed sporogenous and asporogenous taxonomic and phylogenetic groupings reported by other workers among the thermophilic glycolytic anaerobic bacteria. It is suggested that *Thermoanaerobium brockii* remains a valid taxon, rather than reassigning the species to the genus *Clostridium*.

Key words: *Thermoanaerobium brockii* – *Clostridium thermohydrosulfuricum* – Endospore formation – Thermophilic glycolytic anaerobes

Introduction

Thermoanaerobium brockii HTD4 was first isolated from thermal environments of Yellowstone National Park by *Zeikus* et al. (1979). The organism is a Gram-positive, obligately anaerobic thermophile with a mol% G+C of 30–32 and was originally described as a non-spore forming species (*Zeikus* et al., 1979). Subsequent isolates of the genus have also failed to produce observable spores (*Morgan* et al., 1985; *Kondratieva* et al., 1989).

In the course of growing the type strain of *T. brockii* (strain HTD4, DSM 1457) on a minimal medium we observed the formation of endospores which we could not attribute to a contaminant. This observation led to an investigation of this feature.

Materials and Methods

Bacterial strains. Thermoanaerobium brockii HTD4 (DSM 1457) and *Clostridium thermosaccharolyticum* DSM 571 were obtained from the Deutsche Sammlung von Mikroorganismen (DSM, Braunschweig, Germany). *Clostridium thermohydrosulfuricum* Rt8.B1 was from the collection of our laboratory (*Patel* et al., 1986).

Chemicals. All chemicals were reagent grade. Oxygen-free nitrogen (N_2) was obtained from New Zealand Industrial Gases Limited (Wellington, New Zealand). The buffer 3-[morpholino]propanesulfonic acid (MOPS) was obtained from Sigma (St Louis, MO, USA).

Media and cultivation conditions. T. brockii HTD4 was maintained by fortnightly subculture on the trypticase peptone-yeast extract-glucose (TYEG) medium of *Zeikus* et al. (1979). Media were prepared anaerobically as described by *Patel* et al. (1985). The pH of the medium was adjusted to pH 7 by the addition of 1N NaOH and autoclaved at 121 °C for 20 min. Minimal medium (MM) contained: ($g\ell^{-1}$ distilled water) NH_4Cl, 0.9; $MgCl_2 \cdot 6H_2O$, 0.2; NaCl, 0.9; KH_2PO_4, 0.75; K_2HPO_4, 1.5; trace mineral solution, 9 ml; 10% $FeSO_4$, 0.03 ml; 0.2% resazurin, 1 ml; vitamin solution, 0.5 ml; vitamin-free casamino acids, 3.0; and MOPS, 10.0. The trace mineral solution contained ($g\ell^{-1}$ distilled water) nitrilotriacetic acid, 12.5 adjusted to pH 6.5 with KOH, $FeCl_3 \cdot 4H_2O$, 0.2; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2$, 0.017; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnCl_2$, 0.1; $CuCl_2$, 0.02; H_3BO_3, 0.01; $NaMoO_4 \cdot 2H_2O$, 0.01; NaCl, 1.0 and Na_2SeO_3, 0.02. The vitamin solution contained; ($mg\ell^{-1}$ distilled water) d-biotin, 1; p-aminobenzoic acid, 1; nicotinic acid, 10; pantothenate (hemicalcium salt), 5; pyridoxamine. $(HCl)_2$, 5; thiamine · HCl, 10; pyridoxine · HCl, 5; pyridoxal · HCl, 5; FAD, 1; DL-6,8 thioctic acid, 1; folic acid, 3. This solution was filter sterilised through a 0.2 µm disposable Minisart filter (Sartorius, Göttingen, Germany) and stored at 4 °C. Vitamin solution was added aseptically after sterilisation of medium. For solidified media 20.0 $g\ell^{-1}$ of purified agar (Oxoid Ltd, Hampshire, England) was added. Carbon sources were prepared anaerobically in distilled water as concentrated stock solutions and sterilised by autoclaving. They

were added to sterile MM to give a final concentration of 5 $g\ell^{-1}$. All sugars were D-isomers unless noted otherwise. Soil extract was prepared as described by *Norris* et al. (1981), boiled and autoclaved under a nitrogen atmosphere.

Incubations were at 65 °C unless otherwise noted. Plates were incubated in anaerobic jars under an N_2 gas atmosphere.

Cellular characterization. Phase contrast photomicrographs were taken using a Reichert-Jung Polyvar microscope (C. Reichert, Vienna, Austria). Cells were immobilized on agar coated microscope slides as described by *Pfennig* and *Wagener* (1986). The methods used for preparing cells for thin sectioning and electron microscopic examination were as described by *Zeikus* and *Bowen* (1975). Thin sections were stained with 1% uranyl acetate followed by 1% lead citrate, then examined under a Phillips EM 400 electron microscope at an accelerating voltage of 80kV.

Heat-treatment of cultures. C. thermohydrosulfuricum Rt8.B1 and *T. brockii* HTD4 grown on TYEG medium were inoculated into sets of tubes containing 5 ml of either TYEG or minimal medium containing xylose (MMX). These cultures were incubated into stationary phase at 65 °C. The tubes were then immersed completely in an oil bath at 115 °C. A heat-up time for control tubes containing a thermistor probe immersed in 5 ml of medium was 5 min. At intervals tubes for both cultures were removed and quickly cooled to room temperature. Viability of the cultures was determined by inoculating 0.1 ml aliquots into tubes of both TYEG and MMX and incubating at 65 °C. From all tubes showing growth, samples were streaked onto TYEG agar. Well isolated colonies were checked for cell morphology, and inoculated into TYEG broth for end product analysis by HPLC (*Patel* et al., 1987).

Results

Growth on minimal medium containing xylose

Spore formation was observed when cultures of *T. brockii* HTD4 grown on TYEG were transferred into MMX. During the late logarithmic phase of growth on MMX, some cells formed terminal swellings which became phase refractile and resembled mature spores. These structures were not observed when transferring from TYEG medium to TYEG medium, or from MMX to MMX. There seemed to be an optimum period of incubation in MMX for maximum sporulation i.e. 24–48 h, after which time the number of cells sporulating decreased. The maximum level of sporulation in MM was obtained using D-xylose as the sole carbon source (MMX). In some instances an increase was seen in the number of observable spores when 0.5 mM theophylline, 0.2% (v/v) soil-extract or L-xylose (5 $g\ell^{-1}$) were added in conjunction with D-xylose. Sporulation was not detected on MM containing the following growth-supporting carbon sources: glucose, cellobiose, lactose, maltose, mannose, pullulan, starch, galactose, sodium pyruvate and sucrose, while pectin, dextran, cellulose, sodium L-tartrate, sodium DL-lactate, ethanol, tryptone, casamino acids, L-arabinose and L-arginine did not support growth. Attempts to induce sporulation as described by *Zeikus* et al. (1979) i.e. growth on the medium of *Duncan* and *Strong* (1968), GC medium and D-xylose/soil-extract medium (*Hollaus* and *Sleytr*, 1972) gave negative results.

Cellular features

The morphology of *T. brockii* HTD4 varied depending on the culture conditions. Growth on TYEG broth produced single and paired cells, and cells in chains with characteristic mini-cells present (Fig. 1). When grown on MMX, cells were longer and thinner and sometimes contained spores (Fig. 2). The spores were round, terminal, distended the cell, and were brightly refractile under phase contrast microscopy. Non-refractile structures could be observed in the same position within the cell; these were assumed to be pre-spores. Dark inclusion bodies were also observed within the cells (Fig. 2) which were readily distinguishable from both spores and pre-spores by their shape and position. Under the appropriate conditions, spores were observed in approximately one cell in a thousand,

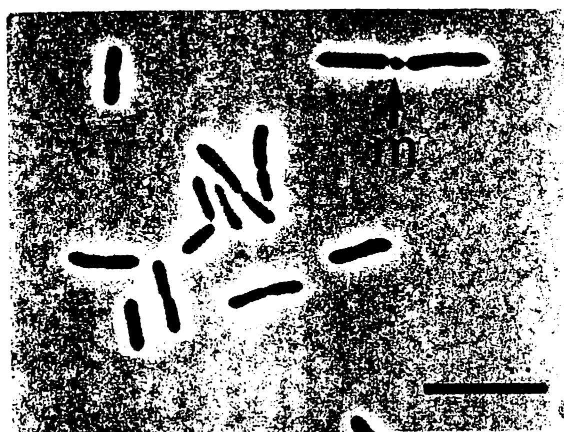


Fig. 1. Phase contrast photomicrograph of *Thermoanaerobium brockii* HTD4 grown on TYEG with characteristic mini-cell (m) present. Bar = 10 μm.

Fig. 2. Phase contrast photomicrograph of *Thermoanaerobium brockii* HTD4 grown on minimal medium containing xylose. Features observed in cells grown under these conditions include refractile spores (s), non-refractile pre-spores (p), and inclusion bodies (i). Bar = 10 μm.

while pre-spores were more numerous, approximately 7 cells in a thousand.

Electron microscopic observation of thin sections of sporulating cells showed a typical mono-layered cell wall (Fig. 3) as described by *Zeikus* et al. (1979), quite distinct from the double layered cell wall type found in *C. thermohydrosulfuricum* (*Sleytr* and *Glauert*, 1976). Around the spore could be seen a number of concentric membrane-like structures (termed by us "lamellar" structures, Fig. 3) which were probably part of the spore coat. Cell division appeared to be by a constrictive or "pinching off" type of division (Fig. 4) typical of *T. brockii* (*Zeikus* et al., 1979) together with characteristic mesosomal structures also observed by *Zeikus* et al. (1979) at the division site of *T. brockii*.

Demonstration of heat resistance

To ascertain if the structures seen had the heat resistant property of true endospores, cultures were incubated at 115 °C. Cultures of *T. brockii* HTD4 and *C. thermohydrosulfuricum* Rt8.B1 grown on TYEG broth at 65 °C survived an exposure of 5 min at 115 °C but no viable cells remained after 10 min exposure. In contrast, cultures of both organisms grown on MMX at 65 °C (condititons found to induce sporulation) survived over 80 min exposure to 115 °C.

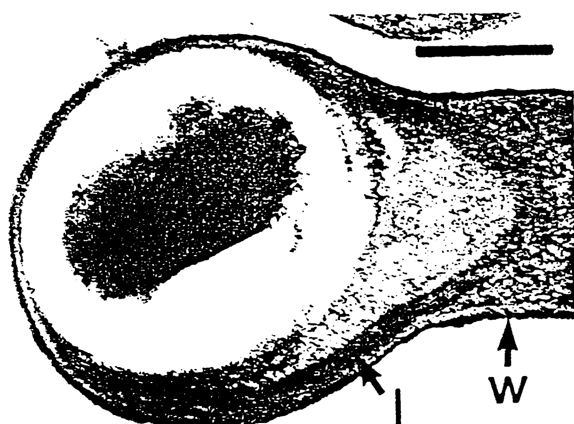


Fig. 3. Electron micrograph ultrathin section of a spore in *Thermoanaerobium brockii* HTD4 grown on minimal medium containing xylose, showing the mono-layered cell wall (w) and lamellar structure (l) around the spore. Bar = 0.2 μm.

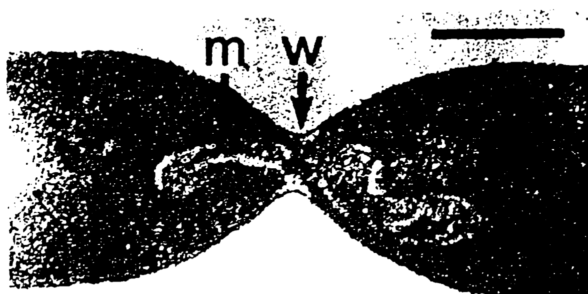


Fig. 4. Electron micrograph of ultrathin section of *Thermoanaerobium brockii* HTD4 showing the typical constrictive or pinching off division with the mono-layered cell wall (w) and characteristic mesosomal structures (m). Bar = 0.2 μm.

Check of purity at end of heat-treatment

To check for purity of heat-resistant cultures obtained from the heat-treatment experiments, end-product analysis by HPLC was done to establish the major end-product types and their ratios. For *T. brockii* HTD4 the major end-products from TYEG fermentation were always ethanol and lactate (typically 36 mM and 30 mM respectively), while for *C. thermohydrosulfuricum* Rt8.B1 the end-products were acetate (18 mM) and ethanol (36 mM). Survivors of the heat treatment from all time intervals were inoculated onto solid TYEG and MMX to determine colony and cell morphology. Colonies grown on TYEG or MMX agar plates were non-pigmented, flat, mucoid and uniformly circular. When sub-cultures from these heat treated tubes were inoculated into TYEG broth they always produced lactate and ethanol, and acetate and ethanol at the concentrations listed previously. When heat-treated cultures were streaked on TYEG agar and MMX agar, colony and cell morphology was the same as cultures which had not been heat treated.

If the observed spores were due to a contaminant then the heat treatment would enrich for this strain. When sub-cultures from heat-treated cultures are transfered from TYEG to MMX, a higher proportion of sporulating cells could be expected if this were the case. However, heat-treated subcultures displayed the same degree of sporulation as the original culture did.

To confirm our findings, a fresh culture of *T. brockii* HTD4 was purchased from the DSM and treated by the same protocol. Identical results were obtained. Again, spores that survived exposure to 115 °C for 80 min produced typically 36 mM ethanol, 30 mM lactate and 2 mM acetate when grown on TYEG, identical to the original cultures.

Dipicolinic acid

Dipicolinic acid (DPA) has been reported only to occur in bacterial spores and is released from spores during germination (*Janssen* et al., 1958). We therefore assayed for DPA in sporulating cultures of *T. brockii* HTD4 to substantiate the finding that the terminal structures were indeed endospores. Assays were attempted using the method of *Janssen* et al. (1958) for both *C. thermohydrosulfuricum* Rt8.B1 and *C. thermosaccharolyticum* DSM 571 (positive controls) and for *T. brockii* HTD4 grown under conditions producing spores. For all organisms the resolution of this technique proved too low for reliable results. A more sensitive technique using capillary gas chromatography and gas chromatography mass spectroscopy was attempted which produced reliable results with sporulating cultures of *C. thermosaccharolyticum* DSM 571 (*G. M. Cook* and *A. L. Wilkins*, unpublished results)

but the technique was again unsuccessful for *T. brockii* HTD4, possibily due to the low percentage of sporulating cells (0.1%) compared with cultures of *C. thermosaccharolyticum* DSM 571 (90% sporulating cells).

Discussion

This study represents the first demonstration of heat-resistant endospores in the previously non-sporulating thermophilic anaerobe *T. brockii* HTD4. The results of this study indicate that growth of *T. brockii* HTD4 on MMX after pre-growth on the richer TYEG medium induces cells to sporulate. The ability of transfer to MMX to induce sporulation in this strain is of interest but the mechanism remains unresolved. We have confirmed that the methods used by *Zeikus* et al. (1979) on the original isolates failed to induce sporulation. Thus long term laboratory culture has not altered these characteristics. However, the type strain used in this study utilised xylose and mannose, observations in disagreement with *Zeikus* et al. (1979) and *Morgan* et al. (1985). Xylose utilisation has however been reported by other authors (*Schmidt* et al., 1986).

Zeikus et al. (1979) initially commented that *T. brockii* HTD4 might be an asporogenous strain of *C. thermohydrosulfuricum* or *C. thermosaccharolyticum*. This view has been expressed again recently by *Krivenko* et al. (1987) and *Kondratieva* et al. (1989). The lack of spore formation by *T. brockii* on medium which supports sporulation of *C. thermohydrosulfuricum* cannot be used alone to designate a new species. Some clostridia form spores only rarely and some strains hardly ever produce observable spores unless a sporulation medium is developed (*Duncan* and *Strong*, 1968; *Sacks* and *Thompson*, 1978; *Gottschalk* et al., 1981). In the light of the findings reported in this communication, it might seem reasonable to reclassify *T. brockii* as a new species of *Clostridium*.

Since the original characterisation of *T. brockii* HTD4 a number of studies have reported the relationship of *T. brockii* to *Clostridium* and other Gram-positive glycolytic anaerobes. *T. brockii* HDT4 and *C. thermohydrosulfuricum* have been shown by partial 16S rRNA sequence comparisons to be very similar (*Bateson* et al., 1989), and the two species show weak serological crossreactivity (*Morgan* et al., 1985). DNA-DNA hybridisation of the two species gave a 50% hybridisation value (*Kondratieva* et al., 1989). DNA-DNA homology studies by *Krivenko* et al. (1987) also suggest a cluster of *Thermoanaerobium* species with *C. thermohydrosulfuricum* separate from *C. thermosaccharolyticum*. *T. brockii* and *C. thermohydrosulfuricum* also have similar metabolic patterns (*Ben-Bassat* et al., 1981, *Lamed* and *Zeikus*, 1980). *T. brockii* forms a closely related group with *Thermoanaerobacter ethanolicus*, *Thermobacteroides acetoethylicus* and *C. thermohydrosulfuricum* (*Wiegel*, 1986; *Bateson* et al., 1989) and the closely related genus *Thermoanaerobacter* contains a spore-forming member in *Thermoanaerobacter finnii* (*Schmidt* et al., 1986).

The occurrence of spore formation in *T. brockii* HTD4 reinforces its relationship with *C. thermohydrosulfuricum*, and strengthens the case for reclassification. While assigning *T. brockii* to the genus *Clostridium* might appear appropriate, we note the need for reclassification of this genus (*Cato* et al., 1986) and the likelihood of further change. In the meantime, we suggest that *T. brockii* remains in the genus *Thermoanaerobium*. Such an arrangement does not conflict with the taxonomic groupings suggested by *Bateson* et al. (1989) or *Kondratieva* et al. (1989) which include spore-forming species (*T. brockii*, *C. thermohydrosulfuricum*, *Thermoanaerobacter finnii*) and species for which spore formation has not yet been demonstrated (*Thermobacteroides acetoethylicus*, *Thermoanaerobacter ethanolicus*). Thus sporulation *per se* does not become the sole characteristic for assignment to a genus, and this seems reasonable in the light of current evidence of mixed sporogenous/asporogenous phylogeny (*Tanner* et al., 1982; *Bateson* et al., 1989; *Hermann* et al., 1987). The exact taxonomic and nomenclatural status of these species still remains a problem which needs to be addressed, but preferably in conjunction with a review (at least of the thermophilic members) of the genus *Clostridium*.

Acknowledgements. This work was funded by Pacific Enzymes Ltd. *G. M. Cook* was supported by a University Grants Committee Scholarship. The authors thank the Meat Industry Research Institute of New Zealand for access to electron microscopy facilities, and *D. Wild* and *A. Harris* for specimen preparation.

References

Bateson, M. M., Wiegel, J., Ward, D. M.: Comparative analysis of 16S ribosomal RNA sequences of thermophilic fermentative bacteria isolated from hot spring cyanobacterial mats. System. Appl. Microbiol. *12*, 1–7 (1989)

Ben-Bassat, A., Lamed, R., Zeikus, J. G.: Ethanol production by thermophilic bacteria: metabolic control of end product formation in *Thermoanaerobium brockii*. J. Bact. *146*, 192–199 (1981)

Cato, E. P., George, W. L., Finegold, S. M.: Genus *Clostridium*, pp. 1141–1200. In: *Sneath, P. H. A., Mair, N. S., Sharpe, M. E., Holt, J. G.* (eds.), Bergey's manual of systematic bacteriology, vol. 2. Baltimore/MD, Williams and Wilkins 1986

Duncan, C. L., Strong, D. H.: Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. *16*, 82–89 (1968)

Gottschalk, G., Andressen, J. R., Hippe, H.: The genus *Clostridium* (nonmedical aspects), pp. 1767–1803. In: *Starr, M. P., Stolp, H., Trüper, H. G., Balows, A., Schlegel, H. G.* (eds.), The prokaryotes. A handbook on habitats, isolation and identification of bacteria, vol. 2. Berlin–Heidelberg–New York, Springer-Verlag 1981

Hermann, M., Popoff, M. L., Sebald, M.: *Sporomusa paucivorans* sp. nov., a methylotrophic bacterium that forms acetic acid from hydrogen and carbon dioxide. Int. J. System. Bact. *37*, 93–101 (1987)

Hollaus, F., Sleytr, U.: On the taxonomy and fine structure of some hyperthermophilic saccharolytic clostridia. Arch. Mikrobiol. *86*, 129–146 (1972)

Janssen, F. W., Lund, A. J., Anderson, L. E.: Colorimetric assay for dipicolinic acid in bacterial spores. Science *127*, 26–27 (1958)

Kondratieva, E. N., Zacharova, E. V., Duda, V. I., Krivenko, V. V.: *Thermoanaerobium lactoethylicum* spec. nov. a new anaerobic bacterium from a hot spring of Kamchatka. Arch. Microbiol. *151,* 117–122 (1989)

Krivenko, V. V., Chernykh, N. A., Zakharova, E. V., Duda, V. I.: Characterization of genotypical properties of several anaerobic thermophilic chemoorganotrophic bacteria. Microbiology (English translation of Mikrobiologya) *56,* 678–681 (1987)

Lamed, R., Zeikus, J. G.: Ethanol production by thermophilic bacteria: relationship between fermentation product yields and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaeerobium brockii.* J. Bact. *144,* 569–578 (1980)

Morgan, H. W., Patel, B. K. C., Daniel, R. M.: Comparison of a *Thermoanaerobium* sp. from a New Zealand hot spring with *Thermoanaerobium brockii.* FEMS Microbiol. Lett. *30,* 121–124 (1985)

Norris, J. R., Berkeley, R. C. W., Logan, N. A., O'Donnell, A. G.: The genera *Bacillus* and *Sporolactobacillus,* pp. 1711–1742. In: *Starr, M. P., Stolp, H., Trüper, H. G., Balows, A., Schlegel, H. G.* (eds.), The prokaryotes. A handbook on habitats, isolation and identification of bacteria, vol. 2. Berlin–Heidelberg–New York, Springer-Verlag 1981

Patel, B. K. C., Morgan, H. W., Daniel, R. M.: A simple and efficient method for preparing and dispensing anaerobic media. Biotechnol. Lett. *7,* 277–278 (1985)

Patel, B. K. C., Morgan, H. W., Daniel, R. M.: Studies on some thermophilic glycolytic anaerobic bacteria from New Zealand hot springs. System. Appl. Microbiol. *8,* 128–136 (1986)

Patel, B. K. C., Monk, C., Littleworth, H., Morgan, H. W., Daniel, R. M.: *Clostridium fervidus* sp. nov., a new chemoorganotrophic acetogenic thermophile. Int. J. System. Bact. *37,* 123–126 (1987)

Pfennig, N., Wagener, S.: An improved method of preparing wet mounts for photomicrographs of microorganisms. J. Microbiol. Meth. *4,* 303–306 (1986)

Sacks, L. E., Thompson, P. A.: Clear, defined medium for the sporulation of *Clostridium perfringens.* Appl. Environ. Microbiol. *35,* 405–410 (1978)

Schmid, U., Giesel, H., Schoberth, S. M., Sahm, H.: *Thermoanaerobacter finnii* spec. nov., a new ethanologenic sporogenous bacterium. System. Appl. Microbiol. *8,* 80–85 (1986)

Sleytr, U. B., Glauert, A. M.: Ultrastructure of the cell walls of two closely related clostridia that possess different regular arrays of surface subunits. J. Bact. *126,* 869–882 (1976)

Tanner, R. S., Stackebrandt, E., Fox, G. E., Gupta, R., Magnum, L. J., Woese, C. R.: A phylogenetic analysis of anaerobic eubacteria capable of synthesizing acetate from carbon dioxide. Curr. Microbiol. *7,* 127–132 (1982)

Wiegel, J. K. W.: Genus *Thermoanaerobacter* pp. 1379–1383. In: *Holt, J. G.* (ed.), Bergey's manual of systematic bacteriology, vol. 2. Baltimore/MD, Williams and Wilkins 1986

Zeikus, J. G., Bowen, V. G.: Fine structure of *Methanospirillum hungatii.* J. Bact. *121,* 373–380 (1975)

Zeikus, J. G., Hegge, P. W., Anderson, M. A.: *Thermoanaerobium brockii* gen. and sp. nov., a new chemoorganotrophic, caldoactive, anaerobium bacterium. Arch. Microbiol. *122,* 41–48 (1979)

Gregory M. Cook, Thermophile and Microbial Biochemistry and Biotechnology Unit, University of Waikato, Private Bag 3105, Hamilton, New Zealand

REFERENCES

Abe, K., and K. Uchida. 1989. Correlation between depression of catabolite control of xylose metabolism and a defect in the phosphoenolpyruvate:mannose phosphotransferase system in *Pediococcus halophilus*. J. Bacteriol. **171**:1793-1800.

Abe, K., and K. Uchida. 1990. Non-PTS uptake and subsequent metabolism of glucose in *Pediococcus halophilus* as demonstrated with a double mutant defective in phosphoenolpyruvate:mannose phosphotransferase system and in phosphofructokinase. Arch. Microbiol. **153**:537-540.

Abe, K., and K. Uchida. 1991. Release of glucose-mediated catabolite repression due to a defect in the membrane fraction of phosphoenolpyruvate:mannose phosphotransferase system in *Pediococcus halophilus*. Arch. Microbiol. **155**:517-520.

Achenbach-Richter, R. Gupta, K.O. Stetter, and C.R. Woese. 1987. Were the original eubacteria thermophiles? System. Appl. Microbiol **9**:34-39.

Adler, J., and W. Epstein. 1974. Phosphotransferase-system enzymes as chemoreceptors for certain sugars in *Escherichia coli* chemotaxis. Proc. Nat. Acad. Sci. USA **71**:2895-2899.

Aduse-Opoku, J., and W.J. Mitchell. 1988. Diauxic growth of *Clostridium thermosaccharolyticum* on glucose and xylose. FEMS Microbiol. Lett. **50**:45-49.

Aït, N., N. Creuzet, and J. Cattaneo. 1979. Characterisation and purification of β-glucosidase from *Clostridium thermocellum*. Biochem. Biophys. Res. Commun. **90**:537-546.

Aït, N., N. Creuzet, and J. Cattaneo. 1982. Properties of β-glucosidase purified from *Clostridium thermocellum*. J. Gen. Microbiol. **128**:569-577.

Aksamit, R., and D.E. Koshland, Jr. 1972. A ribose binding protein of *Salmonella typhimurium* LT2. Biochem. Biophys. Res. Commun. **48**:1348-1353.

Aksenova, H. Yu., F.A. Rainey, P.H. Janssen, H.W. Morgan, and G.A. Zavarsin. 1992. *Spirochaeta thermophila* sp. nov., an obligately anaerobic polysaccharolytic member of the genus *Spirochaeta*. Int. J. Syst. Bacteriol. **42**:175-177.

Amartey, S.A., D.J. Leak, and B.S. Hartley. 1991. Development and optimization of a defined medium for aerobic growth of *Bacillus stearothermophilus* LLD-15. Biotech. Lett. **13**:621-626.

Ambudkar, S.V. and B.P. Rosen. 1990. Ion exchange systems in procaryotes, p. 247-266. *In* T.A. Krulwich (ed.), Bacterial energetics. Academic press, Inc., San Diego.

Ames, G.F.-L. 1986. Bacterial periplasmic transport systems: structure, mechanism, and evolution. Annu. Rev. Biochem. **55:**397-425.

Ames, G.F.-L. 1990. Energy coupling in periplasmic permeases: the histidine permease as a model system. Res. Microbiol. **141:**341-348.

Ames, B.N., G.F.-L. Ames, J.D. Young, D. Tsuchiya, and J. Lecocq. 1973. Illicit transport: the oligopeptide permease. Proc. Nat. Acad. Sci. USA **70:**456-458.

Ames, G.F.-L., and C.F. Higgins. 1983. The organization, mechanism of action, and evolution of periplasmic transport systems. Trends Biochem. Sci. **8:**97-100.

Ames, G.F.-L., and J.E. Lever. 1972. The histidine-binding protein J is a component of histidine transport. J. Biol. Chem. **247:**4309-4316.

Andreesen, J.R., A. Schaupp, C. Neurauter, A. Brown, and L.G. Ljungdahl. 1973. Fermentation of glucose, fructose and xylose by *Clostridium thermoaceticum*: effect of metals on growth yield, enzymes and the synthesis of acetate from carbon dioxide. J. Bacteriol. **114:**743-751.

Anthony, C., and J.R. Guest. 1968. Deferred metabolism of glucose by *Clostridium tetanomorphum*. J. Gen. Microbiol. **54:**277-286.

Antranikian, G. 1990. Physiology and enzymology of thermophilic anaerobic bacteria degrading starch. FEMS Microbiol. Rev. **75:**201-218.

Antranikian, G., C. Herzberg, and G. Gottschalk. 1987. Production of thermostable α-amylase, pullulanase, and α-glucosidase in continuous culture by a new *Clostridium* isolate. Appl. Environ. Microbiol. **53:**1668-1673.

Aymerich, S., G. Gonzy-Tréboul, and M. Steinmetz. 1986. 5'-Noncoding region *sacR* is the target of all identified regulation affecting the levansucrase gene in *Bacillus subtilis*. J. Bacteriol. **166:**993-998.

Bajpai, R.K., and T.K. Ghose. 1978. An induction-repression model for growth of yeasts on glucose/cellobiose mixtures. Biotechnol. Bioeng. **20:**927-935.

Bateson, M.M., J. Wiegel, and D.M. Ward. 1989. Comparative analysis of 16S ribosomal RNA sequences of thermophilic fermentative bacteria isolated from hot spring cyanobacterial mats. System. Appl. Microbiol. **12:**1-7.

Ben-Bassat, A., R. Lamed, and J.G. Zeikus. 1981. Ethanol production by thermophilic bacteria: metabolic control of end product formation in *Thermoanaerobium brockii*. J. Bacteriol. **146:**192-199.

Ben-Bassat, A. and J.G. Zeikus. 1981. *Thermobacteroides acetoethylicus* gen. nov. and spec. nov., a new chemoorganotrophic, anaerobic thermophilic bacterium. Arch. Microbiol. **128:**365-370.

Berger, E.A., and L.A. Heppel. 1974. Different mechanisms of energy coupling for the shock-sensitive and shock-resistant amino acid permeases of *Escherichia coli*. J. Biol. Chem. **249:**7747-7755.

Bergmeyer, H.U. 1974. Methods of enzymatic analysis. 2nd edn. Academic press, Inc., New York.

Bernier, R., and F. Stutzenberger. 1987. Preferential utilisation of cellobiose by *Thermomonospora curvata*. Appl. Environ. Microbiol. **53:**1743-1747.

Beullens, M., and J.M. Thevelein. 1990. Investigation of transport-associated phosphorylation of sugar in yeast mutants (*snf3*) lacking high-affinity glucose transport and in a mutant (*fdp1*) showing deficient regulation of initial sugar metabolism. Curr. Microbiol. **21:**39-46.

Bigwaneza, P.C., D. Prévost, L.M. Bordeleau, and H. Antoun. 1990. Glucose transport by an artic and a temperate strain of rhizobia. Can. J. Microbiol. **37:**105-109.

Bisson, L., and D.G. Fraenkel. 1983. Involvement of kinases in glucose and fructose uptake by *Saccharomyces cerevisiae*. Proc. Nat. Acad. Sci. USA **80:**1730-1734.

Bisson, L., and D.G. Fraenkel. 1984. Expression of kinase-dependent glucose uptake in *Saccharomyces cerevisiae*. J. Bacteriol. **159:**1013-1017.

Booth, I.R., and J.G. Morris. 1982. Carbohydrate transport in *Clostridium pasteurianum*. Biosci. Rep. **2:**47-53.

Botfield, M.C., D.M. Wilson, and T. H. Wilson. 1990. The melibiose carrier of *Escherichia coli*. Res. Microbiol. **141:**328-331.

Brandáo, R., and M.C. Loureiro-Dias. 1990. Regulation of sugar transport systems in *Fusarium oxysporum* var. lini. Appl. Environ. Microbiol. **56:**2417-2420.

Brock, T.D., K.M. Brock, R.T. Belly, and R.L. Weiss. 1972. *Sulfolobus*: a new genus of sulfur-oxidising bacteria living at low pH and high temperature. Arch. Microbiol. **84:**54-68.

Brown, C.E., and R.W. Hogg. 1972. A second transport system for L-arabinose in *Escherichia coli* B/r controlled by the *araC* gene. J. Bacteriol. **111:**606-613.

Bryant, K.J. 1986. The β-galactosidases of two archaebacteria. M.Sc thesis, University of Waikato, Hamilton, New Zealand.

Budgen, N., and M.J. Danson. 1986. Metabolism of glucose via a modified Entner-Doudoroff pathway in the thermoacidophilic archaebacterium *Thermoplasma acidophilum*. FEBS lett. **196:**207-210.

Buonocore, V, O. Sgambati, M. De Rosa, E. Esposito, and A. Gambacorta. 1980. A constitutive β-galactosidase from the extreme thermoacidophile archaebacterium *Caldariella acidophila*: properties of the enzyme in the free state and in the immobilized whole cells. J. Appl. Biochem. **2**:390-397.

Button, D.K. 1983. Differences between the kinetics of nutrient uptake by micro-organsims, growth and enzyme kinetics. Trends Biochem. Sci. **8**:121-124.

Button, D.K. 1985. Kinetics of nutrient-limited transport and microbial growth. Microbiol. Rev. **49**:270-297.

Button, D.K. 1991. Biochemical basis for whole-cell uptake kinetics: specific affinity, oligotrophic capacity, and the meaning of the Michaelis constant. Appl. Environ. Microbiol. **57**:2033-2038.

Button, D.K., J.B. Egan, W. Hengstenberg, and M.L. Morse. 1973. Carbohydrate transport in *Staphylococcus aureus*. IV. Maltose accumulation and metabolism. Biochem. Biophys. Res. Commun. **52**:850-855.

Cairney, J., C.F. Higgins, and I.R. Booth. 1984. Proline transport through the major transport system of *Salmonella typhimurium* is coupled to sodium ions. J. Bacteriol. **160**:22-27.

Calmes, R. 1978. Involvement of phosphoenolpyruvate in the catabolism of caries-conducive disaccharides by *Streptococcus* mutans: lactose transport. Infect. Immun. **19**:934-942.

Calmes, R., and S.J. Deal. 1972. Glycerol transport by *Nocardia asteroides*. Can. J. Microbiol. **18**:1703-1708.

Carreira, L.H., L.G. Ljungdahl, F. Bryant, M. Szulczynski, and J. Wiegel. 1982. Control of product formation with *Thermoanaerobacter ethanolicus*, enzymology and physiology, p. 351-355. *In* Y. Ikeda (ed.), Genetics of industrial microorganisms. American Society for Microbiology, Washington D.C.

Carreira, L.H., J. Wiegel, and L.G. Ljungdahl. 1983. Production of ethanol from biopolymers by anaerobic, thermophilic, and extreme thermophilic bacteria. I. Regulation of carbohydrate utilisation in mutants of *Thermoanaerobacter ethanolicus*. Biotechnol. Bioeng. symp. **13**:183-191.

Cato, E.P., W.L. George, and S.M. Finegold. 1986. Genus *Clostridium*, p. 1141-1200. *In* P.H.A Sneath, N.S. Mair, M.E. Sharpe, and J.G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams and Wilkins, Baltimore /Md.

Chen, G., and J.B. Russell. 1989a. Transport of glutamine by *Streptococcus bovis* and conversion of glutamine to pyroglutamic acid and ammonia. J. Bacteriol. **171**:2981-2985.

Chen, G., and J.B. Russell. 1989b. Sodium-dependent transport of branched-chain amino acids by a monensin-sensitive ruminal *Peptostreptococcus*. Appl. Environ. Microbiol. **55**:2658-2663.

Chen, G., and J.B. Russell. 1990. Transport and deamination of amino acids by a Gram-positive, monensin-sensitive ruminal bacterium. Appl. Environ. Microbiol. **56**:2186-2192.

Cheng, Q., and C.A. Michels. 1991. *MAL11* and *MAL61* encode the inducible high-affinity maltose transporter of *Saccharomyces cerevisiae*. J. Bacteriol. **173**:1817-1820.

Chin, A.M., D.A. Feldheim, and M.H. Saier, Jr. 1989. Altered transcriptional patterns affecting several metabolic pathways in strains of *Salmonella typhimurium* which overexpress the fructose regulon. J. Bacteriol. **171**:2424-2434.

Chin, A.M., B.U. Feucht, and M.H. Saier, Jr. 1987. Evidence for the regulation of gluconeogenesis by the fructose phosphotransferase system in *Salmonella typhimurium*. J. Bacteriol. **169**:897-899.

Cohen, G.N., and J. Monod. 1957. Bacterial permeases. Bacteriol. Rev. **21**:169-194.

Cohen, G.N., and H.V. Rickenberg. 1955. Etude directe de la fixation d'un inducteur de la B-galactosidase par les cellules d'*Escherichia coli*. C. R. Acad. Sci. Ser. D **240**:466-468.

Cook, G.M. 1988. Carbohydrate metabolism of thermophilic glycolytic anaerobic bacteria. M.Sc thesis, University of Waikato, New Zealand.

Cook, G.M., P.H. Janssen, and H.W. Morgan. 1991. Endospore formation by *Thermoanaerobium brockii* HTD4. Syst. Appl. Microbiol. **14**:240-244.

Costantino, H.R., S.H. Brown, and R.M. Kelly. 1990. Purification and characterization of an α-glucosidase from a hyperthermophilic archaebacterium, *Pyrococcus furiosus*, exhibiting a temperature optimum of 105 to 115°C. J. Bacteriol. **172**:3654-3660.

Cowan, D.A., R.M. Daniel, A.M. Martin, and H.W. Morgan. 1984. Some properties of a β-galactosidase from an extremely thermophilic bacterium. Biotech. Bioeng. **26**:1141-1145.

Curtis, S.J. 1974. Mechanism of energy coupling for transport of D-ribose in *Escherichia coli*. J. Bacteriol. **120**:295-303.

Curtis, S.J., and W. Epstein. 1975. Phosphorylation of D-glucose in *Escherichia coli* defective in glucosephosphotransferase, mannosephosphotransferase and glucokinase. J. Bacteriol. **122**:1189-1199.

Dashper, S.G., and E.C. Reynolds. 1990. Characterization of transmembrane movement of glucose and glucose analogs in *Streptococcus mutans* Ingbritt. J. Bacteriol. **172**:556-563.

Davidson, A.L., and H. Nikaido. 1990. Overproduction, solubilization, and reconstitution of the maltose transport system from *Escherichia coli*. J. Biol. Chem. **265**:4254-4260.

Dean, D.A., A.L. Davidson, and H. Nikaido. 1989. Maltose transport in membrane vesicles of *Escherichia coli* is linked to ATP hydrolysis. Proc. Nat. Acad. Sci. USA **86**:9134-9138.

Dean, D.A., A.L. Davidson, and H. Nikaido. 1990. The role of ATP as the energy source for maltose transport in *Escherichia coli*. Res. Microbiol. **141**:348-352.

Dekker, K., H. Yamagata, K. Sakaguchi, and S. Udaka. 1991. Xylose (glucose) isomerase from the thermophile *Thermus thermophilus*: cloning, sequencing, and comparison with other thermostable xylose isomerases. J. Bacteriol. **173**:3078-3083.

Delobbe, A.H., H. Chalumeau, J.-M. Claverie, and P. Gay. 1976. Phosphorylation of intracellular fructose in *Bacillus subtilis* mediated by phosphoenolpyruvate-1-fructose phosphotransferase. Eur. J. Biochem. **66**:485-491.

De Vries, G.E., A.A.N. Van Brussel, and A. Quispel. 1982. Mechanism and regulation of glucose transport in *Rhizobium leguminosarum*. J. Bacteriol. **149**:872-879.

De Vrij, W., R,A. Bulthuis, and W.N. Konings. 1988. Comparative study of energy-transducing properties of cytoplasmic membranes from mesophilic and thermophilic bacteria. J. Bacteriol. **170**:2359-2366.

De Vrij, W., R,A. Bulthuis, P.R. van Iwaarden, and W.N. Konings. 1989. Mechanism of L-glutamate transport in membrane vesicles from *Bacillus stearothermophilus*. J. Bacteriol. **171**:1118-1125.

De Vrij, W., G. Speelmans, R.I.R. Heyne, and W.N. Konings. 1990. Energy transduction and amino acid transport in thermophilic aerobic bacteria and fermentative bacteria. FEMS Microbiol. Rev. **75**:183-200.

De Rosa, M., A. Gambacorta, B. Nicolaus, P. Giardina, E. Poerio, and V. Buonocore. 1984. Glucose metabolism in the extreme thermoacidophilic archaebacterium *Sulfolobus solfataricus*. Biochem. J. **224**:407-414.

Dietrich, G., N. Weiss, and J. Winter. 1988. *Acetothermus paucivorans*, gen. nov. sp. nov., a strictly anaerobic, thermophilic bacterium from sewage sludge, fermenting hexoses to acetate, CO_2 and H_2. Syst. Appl. Microbiol. **10**:174-179.

Dijkhuizen, L., B. van der Warf, and W. Harder. 1980. Metabolic regulation in *Pseudomonas oxalaticus* OX1. Diauxic growth on mixtures of oxalate and formate or acetate. Arch. Microbiol. **124**:261-268.

Dills, S.S., A. Apperson, M.R. Schmidt, and M.H. Saier, Jr. 1980. Carbohydrate transport in bacteria. Microbiol. Rev. **44**:385-418.

DiMarco, A.A., and A.H. Romano. 1985. D-glucose transport system of *Zymomonas mobilis*. Appl. Environ. Microbiol. **49**:151-157.

Dimroth, P. 1980. A new-sodium transport system energized by the decarboxylation of oxaloacetate. FEBS Letters **122**:234-236.

Dimroth, P. 1982. The generation of an electrochemical gradient of sodium ions upon decarboxylation of oxaloacetate by the membrane-bound and Na+-activated oxaloacetate decarboxylase from *Klebsiella aerogenes*. Eur. J. Biochem. **121**:443-449.

Dimroth, P. 1990. Bacterial energy transductions coupled to sodium ions. Res. Microbiol. **141**:332-336.

Dimroth, P., and A. Thomer. 1989. A primary respiratory Na+ pump of an anaerobic bacterium: the Na^+-dependent NADH:quinone oxidoreductase of *Klebsiella pneumoniae*. Arch. Microbiol. **151**:439-444.

Dische, Z., and E. Borenfreund. 1951. A new spectrophotometric method for the detection and determination of keto sugars and trioses. J. Biol. Chem. **192**:583-587.

Dixon, M., and E.C. Webb. 1979. Enzymes. 3rd edn. Longman Group Limited, London.

Doelle, H.W. 1982. Kinetic characteristics and regulatory mechanisms of glucokinase and fructokinase from *Zymomonas mobilis*. Eur. J. Appl. Microbiol. Biotechnol. **14**:241-246.

Droniuk, R., P.T.S. Wong, G. Wisse, and R.A. MacLeod. 1987. Variation in quantitative requirements for Na^+ for transport of metabolizable compounds by the marine bacteria *Alteromonas haloplanktis* 214 and *Vibrio fischeri*. Appl. Environ. Microbiol. **53**:1487-1495.

Duncan, C.L., and D.H. Strong. 1968. Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. **16**:82-89.

Emmerich, W., and F. Radler. 1983. The anaerobic metabolism of glucose and fructose by *Saccharomyces bailii*. J. Gen. Bacteriol. **129**:3311-3318.

Ferenci, T., W. Boos, M. Schwartz, and S. Szmelcman. 1977. Energy coupling of the transport system of *Escherichia coli* dependent on maltose binding protein. Eur. J. Biochem. **75**:187-193.

Fontaine, F.E., W.H. Peterson, E. McCoy, and M.J. Johnson. 1942. A new type of glucose fermentation by *Clostridium thermoaceticum* n.sp. J. Bacteriol. **43:**701-715.

Franklund, C.V., and T.L. Glass. 1987. Glucose uptake by the cellulolytic ruminal anaerobe *Bacteroides succinogenes*. J. Bacteriol. **169:**500-506.

Freier, D., C.P Mothershed, and J. Wiegel. 1988. Characterisation of *Clostridium thermocellum* JW 20. Appl. Environ. Microbiol. **54:**204-211.

Fujimura, T., I. Yamato, and Y. Anraku. 1983. Mechanism of glutamate transport in *E. coli* B. 1. Proton-dependent and sodium-ion dependent binding of glutamate to a glutamate carrier in the cytoplasmic membrane. Biochemistry **22:**1954-1959.

Gätje, G., V. Müller, and G. Gottschalk, G. 1991. Lactic acid excretion via carrier-mediated facilitated diffusion in *Lactobacillus helveticus*. Appl. Microbiol. Biotechnol. **34:**778-782.

Geck, P., and E. Heinz. 1981. Coupled transport of metabolites, p. 285-310. *In* S.L. Bonting and J.J.H.H.M. de Pont (ed.), Membrane transport. Elseiver /North-Holland Biomedical Press, Amsterdam.

Gee, D.L., P. Baumann, and L. Baumann. 1975. Enzymes of D-fructose catabolism in species of *Beneckea* and *Photobacterium*. Arch. Microbiol. **103:**205-207.

George, S.E., C.J. Costenbader, and T. Melton. 1985. Diauxic growth in *Azotobacter vinelandii*. J. Bacteriol. **164:**866-871.

Ghosh, S., and D. Ghosh. 1968. Probable role of membrane-bound phosphoenolpyruvate-hexose phosphotransferase system of *Escherichia coli* in the permeation of sugars. Indian J. Biochem. **5:**49-52.

Goodman, R.E., and D.M. Pederson. 1976. β-galactosidase from *Bacillus stearothermophilus*. Can. J. Microbiol. **22:**817-825.

Gottschalk, G. 1986. Bacterial metabolism. Springer-Verlag, New York.

Gottschalk, G., J.R. Andreesen, and H. Hippe. 1981. The genus *Clostridium* (nonmedical aspects), p. 1767-1803. *In* M.P. Starr, H. Stolp, H.G. Trüper, A. Balows, and H.G. Schlegel (ed.), The prokaryotes. A handbook on habitats, isolation and identification of bacteria, vol. 2. Springer-Verlag, New York.

Grogan, D.W. 1991. Evidence that β-galactosidase of *Sulfolobus solfataricus* is only one of several activities of a thermostable β-D-glycosidase. Appl. Environ. Microbiol. **57:**1644-1649.

Groves, D.J., and A.F. Gronlund. 1969. Carbohydrate transport in *Clostridium perfringens* type A. J. Bacteriol. **100:**1256-1263.

Hamilton, I.R., and D.C. Ellwood. 1978. Effects of fluoride on carbohydrate metabolism by washed cells of *Streptococcus mutans* grown at various pH values in a chemostat. Infect. Immun. **19:**434-442.

Harder, W., and L. Dijkhuizen. 1982. Strategies of mixed substrate utilization in microorganisms. Philos. Trans. R. Soc. London Ser. B **297:**459-480.

Hayashi, S.-I, and E.C.C. Lin. 1965. Capture of glycerol by cells of *Escherichia coli.* Biochim. Biophys. Acta. **94:**479-487.

Heller, K.B., E.C.C. Lin, and T.H. Wilson. 1980. Substrate specificity and transport properties of the glycerol facilitator of *Escherichia coli.* J. Bacteriol. **144:**274-278.

Heller, K.B., and T.H. Wilson. 1979. Sucrose transport by the *Escherichia coli* lactose carrier. J. Bacteriol. **140:**395-399.

Henderson, P.J.F. 1974. Application of the chemiosmotic theory to the transport of lactose, D-galactose, and L-arabinose by *Escherichia coli*, p 409-424. *In* L. Bolis, K. Bloch, S.E. Luria, and F. Lynen (ed.), Comparative biochemistry and physiology of transport. North-Holland Publishing Co., Amsterdam.

Heredia, L., and C. Ratledge. 1988. Simultaneous utilisation of glucose and xylose by *Candida curvata* D in continuous culture. Biotechnol. Lett. **10:**25-30.

Hermann, M., M.L. Popoff, and M. Sebald. 1987. *Sporomusa paucivorans* sp. nov., a methylotrophic bacterium that forms acetic acid from hydrogen and carbon dioxide. Int. J. System. Bacteriol. **37:**93-101.

Hernandez, P.E. 1982. Transport of D-glucose in *Clostridium thermocellum* ATCC-27405. J. Gen. Appl. Microbiol. **28:**469-477.

Heyne, R.I.R., W. De Vrij, W. Crielaard, and W.N. Konings. 1991. Sodium ion-dependent amino acid transport in membrane vesicles of *Bacillus stearothermophilus.* J. Bacteriol. **173:**791-800.

Heytler, P.G. 1979. Uncouplers of oxidative phosphorylation, p. 462-472. *In* S. Fleischer and L. Packer (ed.), Methods in enzymology vol. 55, Biomembranes. Academic Press, Inc., New York.

Hickey, M.W., A.J. Hillier, and G.R. Jago. 1986. Transport and metabolism of lactose, glucose, and galactose in homofermentative lactobacilli. Appl. Environ. Microbiol. **51:**825-831.

Higgins, C.F. 1990. The role of ATP in binding-protein-dependent transport systems. Res. Microbiol. **141:**353-359.

Hinkle, P.C., and R.E. McCarty. 1978. How cells make ATP. Sci. Am. **238:**104-123.

Hollaus, F. and H. Klaushofer. 1973. Identification of hyperthermophilic obligate anaerobes from extraction juices of beet sugar factories. Int. Sugar J. **75**:237-241.

Hollaus, F., and U. Sleytr. 1972. On the taxonomy and fine structure of some hyperthermophilic saccharolytic clostridia. Arch. Microbiol. **86**:129-146.

Hsiao, H.Y., L.C. Chiang, P.P. Ueng, and G.T. Tsao. 1982. Sequential utilisation of mixed monosaccharides by yeast. Appl. Environ. Microbiol. **43**:840-845.

Hsu, E.J., and Z.J. Ordal. 1970. Comparative metabolism of vegetative and sporulating cultures of *Clostridium thermosaccharolyticum*. J. Bacteriol. **102**:369-376.

Huber, R., T.A. Langworthy, H. Konig, M. Thomm, C.R. Woese, U.B. Sleytr, and K.O. Stetter. 1986. *Thermotoga maritima* sp. nov., represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. **144**:324-333.

Huber, R., C.R. Woese, T.A. Langworthy, H. Fricke, and K.O. Stetter. 1989. *Thermosipho africanus* gen. nov., represents a new genus of the thermophilic eubacteria within the *Thermotogales*. System. Appl. Microbiol. **12**:32-37.

Hudson, J.A., G.M. Cook, H.W. Morgan, and R.M. Daniel. 1988. Carbohydrate utilisation preferences among extremely thermophilic glycolytic anaerobes. Poster presentation, A.S.M. Annual Scientific Meeting, Canberra, Australia.

Hugo, H. Von, and G. Gottschalk. 1974. Distribution of 1-phosphofructokinase and PEP:fructose phosphotransferase in clostridia. FEBS lett. **46**:106-109.

Hungate, R.E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1-9.

Huser, B.A., B.K.C. Patel, R.M. Daniel, and H.W. Morgan. 1986. Isolation and characterisation of a novel extremely thermophilic anaerobic, chemo-organotrophic eubacterium. FEMS Microbiol. lett. **37**:121-127.

Hutkins, R.E., and E.R. Kashket. 1986. Phosphotransferase activity in *Clostridium acetobutylicum* from acidogenic and solventogenic phases of growth. Appl. Environ. Microbiol. **51**:1121-1123.

Ingraham, J.L., O. Maaloe, and F.C. Neidhardt. 1983. Growth of the bacterial cell. Sinauer Associates, Inc. Sunderland, Massachusetts.

Jacob, F., and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. **3**:318-356.

Jannasch, H.W., R. Huber, S. Belkin, and K.O. Stetter. 1988. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. Arch. Microbiol. **150**:103-104.

Janssen, P.H.. 1989. Isolation and characterization of organic acid-fermenting bacteria from anaerobic sediments. D.Phil thesis, University of Waikato, New Zealand.

Janssen, P.H. 1991. Fermentation of L-tartrate by a newly isolated Gram-negative glycolytic bacterium. Antonie van Leeuwenhoek **59:**191-198.

Janssen, F.W., A.J. Lund, and L.E. Anderson. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127:**26-27.

Janssen, P.H., and H.W. Morgan. 1992. Glucose catabolism of *Spirochaeta thermophila* strain RI 19.B1. Submitted to J. Bacteriol.

Janssen, P.H., H.W. Morgan, and R.M. Daniel. 1991. Effect of medium composition on extracellular proteinase stability and yield in batch cultures of a *Thermus* species. Appl. Microbiol. Biotechnol. **34:**789-793.

Johnston, J.H., and J.P. Barford. 1991. Continuous growth of *Saccharomyces cerevisiae* on a mixture of glucose and fructose. J. Gen. Appl. Microbiol. **37:**133-140.

Johnson, E.A., A. Madi, and A.L. Demain. 1981. Chemically defined minimal medium for growth of the anaerobic cellulolytic thermophile *Clostridium thermocellum*. Appl. Environ. Microbiol. **41:**1060-1062.

Joshi, A., S. Ahmed, and G.F.-L. Ames. 1989. Energy coupling in bacterial periplasmic transport systems: studies in intact *Escherichia coli* cells. J. Biol. Chem. **264:**2126-2133.

Kaback, H.R. 1986. Active transport in *Escherichia coli*: passage to permease. Ann. Rev. Biophys. Biophys. Chem. **15:**279-319.

Kaback, H.R. 1990. Active transport: membrane vesicles, bioenergetics, molecules, and mechanisms, p. 151-203. *In* T.A Krulwich (ed.), Bacterial energetics, Academic press, Inc., San Diego.

Kaback, H.R., J.P. Reeves, S.A. Short, and F.J. Lombardi. 1974. Mechanisms of active transport in isolated bacterial membrane vesicles XVIII. The mechanism of action of carbonylcyanide m-chlorophenylhydrazone. Arch. Biochem. Biophys. **160:**215-222.

Kanapaka, J.A., and I.R. Hamilton. 1971. Fluoride inhibition of enolase activity *in vivo* and its relationship to the inhibition of glucose-6-P formation in *Streptococcus salivarius*. Arch. Biochem. Biophys. **146:**167-174.

Kashket, E.R., and T.H. Wilson. 1974. Proton motive force in fermenting *Streptococcus lactis* 7962 in relation to sugar accumulation. Biochem. Biophys. Res. Commun. **59:**879-886.

Keevil, C.W., A.S. McDermid, P.D. Marsh, and D.C. Ellwood. 1986. Proton-motive force driven 6-deoxyglucose uptake by the oral pathogen, *Streptococcus mutans* Ingbritt. Arch. Microbiol. **146:**118-124.

Keevil, C.W., M.I. Williamson, P.D. Marsh, and D.C. Ellwood. 1984. Evidence that glucose and sucrose uptake in oral streptococci bacteria involves independent phosphotransferase and proton-motive force-mediated mechanisms. Arch. Oral. Biol. **29:**871-878.

Kellermann, O., and S. Szmelcman. 1974. Active transport of maltose in *Escherichia coli* K12. Eur. J. Biochem. **47:**139-149.

Kelly, C.T., and W.M. Fogarty. 1983. Microbial α-glucosidases. Process Biochem. **18:**6-12.

Klages, K.U. 1991. Characterization of an extremely thermophilic, anaerobic sulphur-dependent archaebacterium. D.Phil thesis, University of Waikato, Hamilton, New Zealand.

Kleiner, D. 1985. Bacterial ammonium transport. FEMS Microbiol. Rev. **32:**87-100.

Kondratieva, E.N., E.V. Zakharova, V.I. Duda, and V.V. Krivenko. 1989. *Thermoanaerobium lactoethylicum* spec. nov. a new anaerobic bacterium from a hot spring of Kamchatka. Arch. Microbiol. **151:**117-122.

Kornberg, H.L., and R.E. Reeves. 1972. Inducible phosphoenolpyruvate-dependent hexose phosphotransferase activities in *Escherichia coli*. Biochem. J. **128:**1339-1344.

Krasilnikova, E.N., N.A. Zorin, and E.V. Zakharova. 1987. The glucose metabolism and hydrogenase activity of *Thermoanaerobium lactoethylicum*. Microbiol. (English translation of Mikrobiologiya) **56:**533-536.

Krivenko, V.V., N.A. Chernykh, E.V. Zakharova, and V.I. Duda. 1987. Characterization of genotypical properties of several anaerobic thermophilic chemoorganotrophic bacteria. Microbiol. (English translation of Mikrobiologiya) **56:**678-681.

Krulwich, T.A., A.A. Guffanti, R.F. Bornstein, and J. Hoffstein. 1982. A sodium requirement for growth, solute transport, and pH homeostasis in *Bacillus firmus* RAB. J. Biol. Chem. **257:**1885-1889.

Kundig, W., S. Ghosh, and S. Roseman. 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. Proc. Nat. Acad. Sci. USA **52:**1067-1074.

Lacis, L.S., and H.G. Lawford. 1988a. Effect of growth rate on ethanol production by *Thermoanaerobacter ethanolicus* in glucose- or xylose-limited continuous culture. Biotechnol. Lett.**10:**603-608.

Lacis, L.S., and H.G. Lawford. 1988b. Ethanol production from xylose by *Thermoanaerobacter ethanolicus* in batch and continuous culture. Arch. Microbiol.**150:**48-55.

Lacis, L.S., and H.G. Lawford. 1991. *Thermoanaerobacter ethanolicus* growth and product yield from elevated levels of xylose or glucose in continuous cultures. Appl. Environ. Microbiol. **57:**579-585.

Lam, V.M.S, K.R. Daruwalla, P.J.F. Henderson, and M.C. Jones-Mortimer. 1980. Proton-linked D-xylose transport in *Escherichia coli.* J. Bacteriol. **143:**396-402.

Lamed, R., and J.G. Zeikus. 1980a. Glucose fermentation pathway of *Thermoanaerobium brockii.* J. Bacteriol. **141:**1251-**1257.**

Lamed, R., and J.G. Zeikus. 1980b. Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii.* J. Bacteriol. **144:**569-578.

Lee, I.H., A.G. Fredrickson, and H.M. Tsuchiya. 1974. Diauxic growth of *Propionibacterium shermanii.* Appl. Microbiol. **28:**831-835.

Lee, C.K. and Z.J. Ordal. 1967. Regulatory effect of pyruvate on the glucose metabolism of *Clostridium thermosaccharolyticum.* J. Bacteriol. **94:**530-536.

Lee, C., B. Saha, and J.G. Zeikus. 1990. Characterization of *Thermoanaerobacter* glucose isomerase in relation to saccharidase synthesis and development of single-step processes for sweetener production. Appl. Environ. Microbiol. **56:**2895-2901.

Leigh, J.A., and R.S. Wolfe. 1983. *Acetogenium kivui*, a thermophilic acetogenic bacterium. Int. J. Syst. Bacteriol. **33:**886-901.

Le Ruyet, P., H.C. Dubourguier, G. Albagnac, and G. Prensier. 1985. Characterization of *Clostridium thermolacticum* sp. nov., a hydrolytic thermophilic anaerobe producing high amounts of lactate. Syst. Appl. Microbiol. **6:**196-202

Liberman, E.S., and A.S. Bleiweis. 1984. Transport of glucose and mannose by a common phosphoenolpyruvate-dependent phosphotransferase system in *Streptococcus mutans* GS5. Infect. Immun. **43:**1106-1109.

Lin, E.C.C. 1976. Glycerol dissimilation and its regulation in bacteria. Annu. Rev. Microbiol. **30:**535-578.

Lind, D.L., R.M. Daniel, D.A. Cowan, and H.W. Morgan. 1989. β-galactosidase from a strain of the anaerobic thermophile *Thermoanaerobacter*. Enzyme. Microb. Technol. **11:**180-186,

Linnet, P.E., and R.B. Beechey. 1979. Inhibitors of the ATP synthetase system, p. 472-518. *In* S. Fleischer and L. Packer (ed.), Methods in enzymology vol. 55, Biomembranes. Academic Press, Inc., New York.

Ljungdahl, L.G., F. Bryant, L.H. Carreira, T. Saiki, and J. Wiegel. 1981. Some aspects of thermophilic and extreme thermophilic anaerobic microorganisms, p. 397-419. *In* A. Hollaender (ed.), Trends in the biology of fermentations for fuels and chemicals, vol. 18. Plenum Publishing Corporation, New York.

London, J., and N.M. Chace. 1977. New pathway for metabolism of pentitols. Proc. Nat. Acad. Sci. USA **74**:4296-4300.

London, J., and N.M. Chace. 1979. Pentitol metabolism in *Lactobacillus casei*. J. Bacteriol. **140**:949-954.

London, J., and S. Hausman. 1982. Xylitol-mediated transient inhibition of ribitol utilization by *Lactobacillus casei*. J. Bacteriol. **150**:657-661.

Lundie, L.L., Jr., and H.L. Drake. 1984. Development of a minimally defined medium for the acetogen *Clostridium thermoaceticum*. J. Bacteriol. **159**:700-703.

Lynch, W.H., and Franklin, M. 1978. Effect of temperature on diauxic growth with glucose and organic acids in *Pseudomonas fluoresens*. Arch. Microbiol. **118**:133-140.

Madi, E., G. Antranikian, K. Ohmiya, and G. Gottschalk. 1987. Thermostable amylolytic enzymes from a new *Clostridium* isolate. Appl. Environ. Microbiol. **53**:1661-1667.

Magasanik, B. 1961. Glucose effects: inducer exclusion and repression, p. 189-219. *In* J.R. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Maheshwari, R., and P.V. Balasubramanyam. 1988. Simultaneous utilisation of glucose and sucrose by thermophilic fungi. J. Bacteriol. **170**:3274-3280.

Maloney, P.C. 1990a. A consensus structure for membrane transport. Res. Microbiol. **141**:374-383.

Maloney, P.C. 1990b. Microbes and membrane biology. FEMS Microbiol. Rev. **87**:91-102.

Maloy, S.R. 1990. Sodium-coupled cotransport, p. 203-224. *In* T.A. Krulwich (ed.), Bacterial energetics. Academic Press, Inc., San Diego.

Martin, S.A., and J.B. Russell. 1986. Phosphoenolpyruvate-dependent phosphorylation of hexoses by ruminal bacterium: evidence for the phosphotransferase transport system. Appl. Environ. Microbiol. **52**:1348-1352.

Martin, S.A., and J.B. Russell. 1987. Transport and phosphorylation of disaccharides by the ruminal bacterium *Streptococcus bovis*. Appl. Environ. Microbiol. **53:**2388-2393.

Martin, S.A., and J.B. Russell. 1988. Mechanisms of sugar transport in the rumen bacterium *Selenomonas ruminantium*. J. Gen. Microbiol. **134:**819-827.

Matin, A. 1979. Microbial regulatory mechanisms at low nutrient concentrations as studied in chemostat, p. 323-339. *In* M. Shilo (ed.), Strategies of microbial life in extreme environments. Verlag Chemie, Weinheim, Germany.

McClellan, C.J., and L.F. Bisson, 1988. Glucose uptake in *Saccharomyces cerevisiae* grown under anaerobic conditions: effect of null mutations in the hexokinase and glucokinase structural genes. J. Bacteriol. **170:**5396-5400.

McClung, L.S. 1935. Studies on anaerobic bacteria. IV. Taxonomy of cultures of thermophilic species causing "swells" of canned food. J. Bacteriol. **29:**189-193.

McGinnis, J.F., and K. Paigen. 1969. Catabolite inhibition: a general phenomenon in the control of carbohydrate utilisation. J. Bacteriol. **100:**902-913.

Meadow, N.D., D.K. Fox, and S. Roseman. 1990. The bacterial phosphoenolpyruvate:glycose phosphotransferase system. Annu. Rev. Biochem. **59:**497-542.

Meadow, N.D., R. Revuelta, V.N. Chen, R.R. Colwell and S. Roseman. 1987. Phosphoenolpyruvate:glycose phosphotransferase system in species of *Vibrio*, a widely distributed marine bacterial genus. J. Bacteriol. **169:**4893-4900.

Meers, J.L., and D.W. Tempest. 1970. Regulation of glutamine synthetase synthesis in some Gram-negative bacteria. Biochem. J. **119:**603-605.

Melasniemi, H. 1987. Effect of carbon source on production of thermostable α-amylase, pullulanase and α-glucosidase by *Clostridium thermohydrosulfuricum*. J. Gen. Microbiol **133:**883.

Meng, M., C. Lee, M. Bagdasarian, and J.G. Zeikus. 1991. Switching substrate preference of thermophilic xylose isomerase from D-xylose to D-glucose by redesigning the substrate binding pocket. Proc. Nat. Acad. Sci. USA **88:**4015-4019.

Michels, P.A.M., J.P.J. Michels, J. Boonstra, and W.N. Konings. 1979. Generation of an electrochemical gradient in bacteria by the excretion of metabolic end-products. FEMS Microbiol. Lett. **5:**357-364.

Mitchell, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature (London) **191:**144-148.

Mitchell, P. 1963. Molecule, group, and electron translocation through natural membranes. Biochem. Soc. Symp. **22:**142-168.

Mitchell, P. 1990. Osmochemistry of solute translocation. Res. Microbiol. **141**:286-289.

Mitchell, W.J., and I.R. Booth. 1984. Characterization of *Clostridium pasteurianum* phosphotransferase system. J. Gen. Microbiol. **130**:2193-2200.

Mitchell, W.J., M.S. Roohi, M.J. Mosely, and I.R. Booth. 1987. Regulation of carbohydrate utilisation in *Clostridium pasteurianum*. J. Gen. Microbiol. **133**:31-36.

Mitchell, W.J., J.E. Shaw, and L. Andrews. 1991. Properties of the glucose phosphotransferase system of *Clostridium acetobutylicum* NCIB 8052. Appl. Environ. Microbiol. **57**:2534-2539.

Monod, J. 1942. Recherches sur la croissance des cultures bacteriennes, Herman et Cie, Paris.

Monod, J. 1949. The growth of bacterial cultures. Ann. Rev. Microbiol. **3**:371-394.

Morgan, H.W., B.K.C. Patel, and R.M. Daniel. 1985. Comparison of a *Thermoanaerobium* sp. from a New Zealand hot spring with *Thermoanaerobium brockii*. FEMS Microbiol. Lett. **30**:121-124.

Neijssel, O.M., E.T. Buurman, and M.J.T. de Mattose. 1990. The role of futile cycles in the energetics of bacterial growth. Biochim. Biophys. Acta **1018**:252-255.

Neu, H.C., and L.A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. **240**:3685-3692.

Ng, T.K., and J.G. Zeikus. 1982. Differential metabolism of cellobiose and glucose by *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. J. Bacteriol. **150**:1391-1399.

Norris, J.R., R.C.W. Berkeley, N.A. Logan, and A.G. O'Donnell. 1981. The genera *Bacillus* and *Sporolactobacillus*, p. 1711-1742. *In* M.P. Starr, H. Stolp, H.G. Trüper, A. Balows, and H.G. Schlegel (ed.). The prokaryotes. A handbook on habitats, isolation and identification of bacteria, vol. 2. Springer-Verlag, New York.

Orlowski, J.H., and J.P. Barford. 1987. The mechanism of uptake of multiple sugars by *Saccharomyces cerevisiae* in batch culture under fully aerobic conditions. Appl. Microbiol. Biotechnol. **25**:459-463.

Ounine, K., H. Petitdemange, G. Raval, and R. Gay. 1985. Regulation and butanol inhibition of D-xylose and D-glucose uptake in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **49**:874-978.

Pardee, A.B., F. Jacob, and J. Monod. 1959. The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by *E. coli*. J. Mol. Biol. **1**:165-178.

Patchett, M.L. 1985. β-glucosidase from an extreme thermophile. M.Sc thesis, University of Waikato, Hamilton, New Zealand.

Patchett, M.L., R.M. Daniel, and H.W. Morgan. 1987. Purification and properties of a β-glucosidase from an extremely thermophilic anaerobic bacterium. **243**:779-787.

Patel, B.K.C., J.A. Hudson, H.W. Morgan, and R.M. Daniel. 1988. Non-diauxic fermentation of acid hydrolysed pine by a strain of *Clostridium thermohydrosulfuricum* isolated from a New Zealand hot spring. FEMS Microbiol. lett. **56**:285-288.

Patel, B.K.C., C. Monk, H. Littleworth, H.W. Morgan, and R.M. Daniel. 1987. *Clostridium fervidus* sp. nov., a new chemoorganotrophic acetogenic thermophile. Int. J. System. Bacteriol. **37**:123-126.

Patel, B.K.C., H.W. Morgan, and R.M. Daniel. 1985a. *Fervidobacterium nodosum* gen. nov. and sp. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch. Microbiol. **141**:63-69.

Patel, B.K.C., H.W. Morgan, and R.M. Daniel. 1985b. A simple and efficient method for preparing and dispensing anaerobic media. Biotechnol. Lett. **7**:277-278.

Patel, B.K.C., H.W. Morgan, and R.M. Daniel. 1986. Studies on some thermophilic glycolytic anaerobic bacteria from New Zealand hot springs. System. Appl. Microbiol. **8**:128-136.

Patni, N.J., and J.K. Alexander. 1971a. Utilisation of glucose by *Clostridium thermocellum*: presence of glucokinase and other glycolytic enzymes in cell extracts. J. Bacteriol. **105**:220-225.

Patni, N.J., and J.K. Alexander. 1971b. Catabolism of fructose and mannitol in *Clostridium thermocellum*: presence of phosphoenolpyruvate: fructose phosphotransferase, fructose-1-phosphate kinase, phosphoenolpyruvate: mannitol phosphotransferase, and mannitol 1-phosphate dehydrogenase in cell extracts. J. Bacteriol. **105**:226-231.

Payton, E. 1984. Production of ethanol by thermophilic bacteria. Trends Biotechnol. **2**:153-158.

Pirt, S.J. 1975. Principles of microbe and cell cultivation. Blackwell, Oxford, UK.

Pfennig, N., and S. Wagener. 1986. An improved method of preparing wet mounts for photomicrographs of microorganisms. J. Microbiol. Meth. **4**:303-306.

Plant, A.R., S. Parratt, R.M. Daniel, and H.W. Morgan. 1988. A cell-associated oligo-1,6-α-glucosidase from an extremely thermophilic anaerobic bacterium, *Thermoanaerobium* Tok6-B1. Biochem. J. **255**:865-868.

Postma, P.W., C.P. Broekhuizen, and R.H. Geerse. 1989. The role of PEP:carbohydrate phosphotransferase system in the regulation of bacterial metabolism. FEMS Microbiol. Rev. **63**:69-80.

Postma, P.W., and J. Lengeler. 1985. Phosphoenolpyruvate:carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. **49**:232-269.

Postma, P., and S. Roseman. 1976. The bacterial phosphoenolpyruvate:sugar phosphotransferase system. Biochim. Biophys. Acta. **457**:213-257.

Pressman, B.C. 1976. Biological applications of ionophores. Ann. Rev. Biochem. **45**:501-530.

Ramos, J., K. Szkutnicka, and V.P. Cirillo. 1989. Characteristics of galactose transport in *Saccharomyces cerevisiae* cells and reconstituted lipid vesicles. J. Bacteriol. **171**:3539-3544.

Reizer, J. 1989. Regulation of sugar uptake and efflux in Gram-positive bacteria. FEMS Microbiol. Rev. **63**:149-156.

Reizer, J., J. Deutscher, F. Grenier, J. Thompson, W. Hengstenberg, and M.H. Saier, Jr. 1988. The phosphoenolpyruvate:sugar phosphotransferase system in Gram-positive bacteria: properties, mechanism, and regulation. Crit. Rev. Microbiol. **15**:297-338.

Reizer, J., and A. Peterkofsky. 1987. Regulatory mechanisms for sugar transport in Gram-positive bacteria, p. 333-364. *In* J. Reizer and A. Peterkofsky (ed.), Sugar transport and metabolism in Gram-positive bacteria. Ellis Horwood, Chichester.

Richey, D.P., and E.C.C. Lin. 1972. Importance of facilitated diffusion for effective utilization of glycerol by *Escherichia coli*. J. Bacteriol. **112**:784-790.

Robertson, E.F., J.C. Hoyt, and H.C. Reeves. 1988. Evidence of histidine phosphorylation in isocitrate lyase from *Escherichia coli*. J. Biol. Chem. **263**:2477-2482.

Romano, A.H., S.J. Eberhard, S.L. Dingle, and T.D. McDowell. 1970. Distribution of the phosphoenolpyruvate:glucose phosphotransferase system in bacteria. J. Bacteriol. **104**:808-813.

Romano, A.H., M.H. Saier, Jr., O.T. Harriott, and J. Reizer. 1990. Physiological studies on regulation of glycerol utilization by the phosphoenolpyruvate:sugar phosphotransferase system in *Enterococcus faecalis*. J. Bacteriol. **172**:6741-6748.

Romano, A.H., J.D. Trifone, and M. Brustolon. 1979. Distribution of the phosphoenolpyruvate:glucose phosphotransferase system in fermentative bacteria. J. Bacteriol. **139**:93-97.

Roseman, S. 1989. Sialic acid, serendipity, and sugar transport: discovery of the bacterial phosphotransferase system. FEMS Microbiol. Rev. **63**:3-12.

Rozanova, E.P., and A.I. Khudyakova. 1974. A new spore-forming thermophilic sulfate-reducing organism, *Desulfovibrio thermophilus* sp. nov. Microbiol. (English translation of Mikrobiologiya) **43**:1069-1075.

Ruch, F.E., J. Lengeler, and E.C.C. Lin. 1974. Regulation of glycerol catabolism in *Klebsiella aerogenes*. J. Bacteriol. **119**:50-56.

Russell, J.B. 1990. Low-affinity, high-capacity system of glucose transport in the ruminal bacterium *Streptococcus bovis*: evidence for a mechanism of facilitated diffusion. Appl. Environ. Microbiol. **56**:3304-3307.

Russell, J.B., and R.L. Baldwin. 1978. Substrate preferences in rumen bacteria: evidence of catabolite regulatory mechanisms. Appl. Environ. Microbiol. **36**:319-329.

Russell, J.B., F.J. Delfino, and R.L. Baldwin. 1979. Effects of combinations of substrates on maximum growth rates of several rumen bacteria. Appl. Environ. Microbiol. **37**:544-549.

Russell, J.B., and H.J. Strobel. 1987. Concentration of ammonia across cell membranes of mixed rumen bacteria. J. Dairy Sci. **70**:970-976.

Russell, J.B., H.J. Strobel, A.J.M. Driessen, and W.N. Konings. 1988. Sodium-dependent transport of neutral amino acids by whole cells and membrane vesicles of *Streptococcus bovis*, a ruminal bacterium. J. Bacteriol. **170**:3531-3536.

Russell, J.B., H.J. Strobel, and S.A. Martin. 1990. Strategies of nutrient transport by ruminal bacteria. J. Dairy Sci. **73**:2996-3012.

Ruttersmith, L.D., and R.M. Daniel. 1991. Thermostable cellobiosehydrolase from the thermophilic eubacterium *Thermotoga* sp. strain FjSS3.B1. Biochem. J. **277**:887-890.

Ruttersmith, L.D., and R.M. Daniel. 1992. Thermostable β-glucosidase and β-xylosidase from *Thermotoga* sp. strain FjSS3.B1. (submitted to J. Biochem).

Sacks, L.E., and P.A. Thompson. 1978. Clear, defined medium for the sporulation of *Clostridium perfringens*. Appl. Environ. Microbiol. **35**:405-410.

Saha, B.C., and J.G. Zeikus. 1991. Characterization of thermostable α-glucosidase from *Clostridium thermohydrosulfuricum* 39E. Appl. Microbiol. Biotechnol. **35**:568-571.

Saheb, S.A. 1972. Permeation du glycerol et sporulation chez *Bacillus subtilis*. Can. J. Microbiol. **18:**1307-1313.

Saier, M.H., Jr. 1977. Bacterial phosphoenolpyruvate:sugar phosphotransferase systems: structural, functional and evolutionary interrelationships. Bacteriol. Rev. **41:**856-871.

Saier, M.H., Jr. 1985. Mechanisms and regulation of carbohydrate transport in bacteria. Academic Press, Inc., New York.

Saier, M.H., Jr. 1989a. Current studies on the bacterial phosphotransferase system in the Saier laboratory (La Jolla, California, Summer, 1988). FEMS Microbiol. Rev. **63:**183-192.

Saier, M.H., Jr. 1989b. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate:sugar phosphotransferase system. Microbiol. Rev. **53:**109-120.

Saier, M.H., Jr. 1990. Evolution of permease diversity and energy-coupling mechanisms: an introduction. Res. Microbiol. **141:**281-286.

Saier, M.H., Jr., and A.M. Chin. 1990. Energetics of the bacterial phosphotransferase system in sugar transport and the regulation of carbon metabolism, p. 273-300. *In* T.A. Krulwich (ed.), Bacterial energetics. Academic Press, Inc., San Diego.

Saito, T., H. Honda, S. Iijima, and T. Kobayashi. 1989. Isolation of thermostable β-galactosidase gene from a thermophilic anaerobe and its expression in *Escherichia coli*. Enzyme Microb. Technol. **11:**302-305.

Sakai, T., Y. Kobayashi, K. Kawagoe, and T. Beppu. 1985. *Dictyoglomus thermophilum* gen. nov., a chemoorganotrophic, anaerobic thermophilic bacterium. Int. J. System. Bacteriol. **35:**253-259.

San Francisco, M.J.D., and G.R. Jacobson. 1986. Glucose uptake and phosphorylating activities in two species of slow-growing *Rhizobium*. FEMS Microbiol. Lett. **35:**71-74.

Sanno, Y., T.H. Wilson, and E.C.C. Lin. 1968. Control of permeation to glycerol in cells of *Escherichia coli*. Biochem. Biophys. Res. Commun. **32:**344-349.

Saurin, W., E. Francoz, P. Martineau, A. Charbit, E. Dassa, P. Duplay, E. Gilson, A. Molla, G. Ronco, S. Szmelcman, and M. Hofnung. 1989. Periplasmic binding protein dependent transport system for maltose and maltodextrins: some recent studies. FEMS Microbiol. Rev. **63:**53-60.

Savage, M.D., and H.L. Drake. 1986. Adaption of the acetogen *Clostridium thermoautotrophicum* to minimal medium. J. Bacteriol. **165:**315-318.

Sawyer, M.H., P. Baumann, L. Baumann, S.M. Berman, J.L. Canovas, and R.H. Berman. 1977. Pathways of D-fructose catabolism in species of *Pseudomonas*. Arch. Microbiol. **112:**49-55.

Scarborough, G.A. 1970. Sugar transport in *Neurospora crassa*. J. Biol. Chem. **245:**1694-1698.

Schachtele, C.F., and J.A. Mayo. 1973. Phosphoenolpyruvate-dependent glucose transport in oral streptococci. J. Dent. Res. **52:**1209-1215.

Schellenberg, G.D., A. Sarthy, A.E. Larson, M.P. Backer, J.W. Crabb, M. Lidstrom, B.D. Hall, and C.E. Furlong. 1984. Xylose isomerase from *Escherichia coli*. J. Biol. Chem. **259:**6826-6832.

Schink, B. 1990. Selecting and developing new microbes: anaerobes, p. 21-31. *In* D. Kamely, A. Chakrabarty, and G.S. Omenn (ed.), Biotechnology and biodegradation advances in applied biotechnology series 4. Gulf Publishing Co., Houston.

Schink, B., and J.G. Zeikus. 1983. *Clostridium thermosulfurogenes* sp. nov., a new thermophile that produces elemental sulfur from thiosulphate. J. Gen. Microbiol. **129:**1149-1158.

Schmid, U., H. Giesel, S.M. Schoberth, and H. Sahm. 1986. *Thermoanaerobacter finnii* spec. nov., a new ethanologenic sporogenous bacterium. Syst. Appl. Microb. **8:**80-85.

Schönheit, P., and D.B. Beimborn. 1986. Monensin and gramicidin stimulate CH_4 formation from H_2 and CO_2 in *Methanobacterium thermoautotrophicum* at low external Na^+ concentration. Arch. Microbiol. **146:**185.

Scopes, R.K. 1982. Protein purification, principles and practice. Springer-Verlag. New York.

Severina, L.O., N.V. Pimenov, and V.K. Plakunov. 1991a. Glucose transport into the extremely halophilic archaebacteria. Arch. Microbiol. **155:**131-136.

Severina, L.O, N.V. Zhilina, and V.K. Plakunov. 1991b. Some characteristics of glucose transport systems in extreme thermoacidophilic archaebacteria belonging to the genus *Sulfurococcus*. Microbiol. (English translation of Mikrobiologiya) **60:**413-418.

Shamanna, D.K., and K.E. Sanderson. 1979. Uptake and catabolism of D-xylose in *Salmonella typhimurium* LT2. J. Bacteriol. **139:**64-70.

Simoni, R.D., M. Levinthal, F.D. Kundig, W. Kundig, B. Anderson, P.E. Hartman, and S. Roseman. 1967. Genetic evidence for the role of a bacterial phosphotransferase system in sugar transport. Proc. Nat. Acad. Sci. USA. **58:**1963-1970.

Slapack, G.E., I. Russell, and G.G. Stewart. 1987. Thermophilic microbes in ethanol production. CRC Press, Inc., Boca Raton, Florida.

Slee, A.M., and J.M. Tanzer. 1979. Phosphoenolpyruvate-dependent sucrose phosphotransferase activity in *Streptococcus mutans* NCTC 10449. Infect. Immun. **24**:821-828.

Slee, A.M., and J.M. Tanzer. 1982. Sucrose transport by *Streptococcus mutans* evidence for multiple transport systems. Biochim. Biophys. Acta. **692**:415-424.

Sleytr, U.B., and A.M. Glauert. 1976. Ultrastructure of the cell walls of two closely related clostridia that possess different regular arrays of surface subunits. J. Bacteriol. **126**:869-882.

Solaiman, D., and R.L. Uffen. 1982. Pyruvate-dependent diauxic growth of *Rhodospirillum rubrum* in light. J. Bacteriol. **152**:1175-1187.

Soutschek, E., J. Winter, F. Schindler, and O. Kandler. 1984. *Acetomicrobium flavidum*, gen. nov. sp. nov., a thermophilic anaerobic bacterium from sewage sludge, forming acetate, CO_2 and H_2 from glucose. Syst. Appl. Microbiol. **5**:377-390.

Speelmans, G., W. De Vrij, and W.N. Konings. 1989. Characterization of amino acid transport in membrane vesicles from the thermophilic fermentative bacterium *Clostridium fervidus*. J. Bacteriol. **171**:3788-3795.

Stadtman, E.R. 1955. Phosphotransacetylase from *Clostridium kluyveri*, p. 596-599. *In* S.P. Colowick and N.O. Kaplan (ed.) Methods in enzymology, vol. 1. Academic press, Inc., New York.

Stein, W.D. 1981. Concepts of mediated transport, p. 123-157. *In* S.L. Bonting and J.J.H.H.M. de Pont (ed.), Membrane transport. Elseiver/North-Holland Biomedical Press, Amsterdam.

St. Martin, E.J., and C.L. Wittenberger. 1979. Characterization of a phosphoenolpyruvate-dependent sucrose phosphotransferase system in *Streptococcus mutans*. Infect. Immun. **24**:865-868.

Stock, J., and S. Roseman. 1971. A sodium-dependent sugar transport system in bacteria. Biochem. Biophys. Res. Commun. **44**:132-138.

Strobel, H.J., and J.B Russell. 1987. Regulation of β-glucosidase in *Bacteroides ruminicola* by a different mechanism: growth rate-dependent derepression. Appl. Environ. Microbiol. **53**:2505-2510.

Strobel, H.J., and J.B Russell. 1989. Non-proton-motive-force-dependent sodium efflux from the ruminal bacterium *Streptococcus bovis*: bound versus free pools. Appl. Environ. Microbiol. **55**:2664-2668.

Sturr, M.G., and R.E. Marquis. 1990. Inhibition of proton-translocating ATPases of *Streptococcus mutans* and *Lactobacillus casei* fluoride and aluminium. Arch. Microbiol. **155:**22-27.

Sussman, A.J., and C. Gilvarg. 1971. Peptide transport and metabolism in bacteria. Annu. Rev. Biochem. **40:**397-408.

Svetlichnii, V.A., and T.P. Svetlichnaya. 1988. *Dictyoglomus turgidus* sp. nov., a new extreme thermophilic bacterium isolated from hot springs in the Uzon volcanic crater. Microbiol. (English translation of Mikrobiologiya) **57:**435-441.

Tanner, R.S., E. Stackebrandt, G.E. Fox, R. Gupta, L.J. Magnum, and C.R. Woese. 1982. A phylogenetic analysis of anaerobic eubacteria capable of synthesizing acetate from carbon dioxide. Curr. Microbiol. **7:**127-132.

Tauchert, K., A. Jahn, and J. Oelze. 1990. Control of diauxic growth of *Azotobacter vinelandii* on acetate and glucose. J. Bacteriol. **172:**6447-6451.

Tempest, D.W., J.L. Meers, and C.M. Brown. 1970. Synthesis of glutamate in *Aerobacter aerogenes* by a hitherto unknown route. Biochem. J. **117:**405-407.

Thompson, J., and B.M. Chassy. 1982. Novel phosphoenolpyruvate-dependent futile cycle in *Streptococcus lactis*: 2-deoxy-glucose uncouples energy production from growth. J. Bacteriol. **151:**1454-1465.

Thompson, J., and B.M. Chassy. 1983. Regulation of glycolysis and sugar phosphotransferase activities in *Streptococcus lactis*: growth in the presence of 2-deoxy-D-glucose. J. Bacteriol. **154:**819-830.

Thompson, J., and B.M. Chassy. 1985. Intracellular phosphorylation of glucose analogs via the phosphoenolpyruvate:mannose phosphotransferase-system in *Streptococcus lactis*. J. Bacteriol. **162:**224-234.

Tomita, K., K. Nagata, H. Kondo, T. Shiraishi, H. Tsubota, H. Suzuki, and H. Ochi. 1990. Thermostable glucokinase from *Bacillus stearothermophilus* and its analytical application. Enzyme Engineering **10:**421-425.

Tsay, S.S., K.K. Brown, and E.T. Gaudy. 1971. Transport of glycerol by *Pseudomonas aeruginosa*. J. Bacteriol. **108:**82-88.

Tsuchiya, T., S.M. Hasan, and J. Raven. 1977. Glutamate transport driven by an electromechanical gradient of sodium ions in *Escherichia coli*. J. Bacteriol. **131:**848-853.

Ucker, D.S., and E.R. Signer. 1978. Catabolite-repression-like phenomenon in *Rhizobium meliloti*. J. Bacteriol. **136:**1197-1200.

Ulrich, J.T., G.A. McFeters, and K.L. Temple. 1972. Induction and characterization of β-galactosidase in an extreme thermophile. J. Bacteriol. **110:**691-698.

Vadeboncoeur, C. 1984. Structure and properties of the phosphoenolpyruvate:glucose phosphotransferase system of oral streptococci. Can. J. Microbiol. **30**:495-502.

Vadeboncoeur, C., D. Mayrand, and L. Trahan. 1982. A comparative study of enzymes involved in glucose phosphorylation in oral streptococci. J. Dent. Res. **61**:60-65.

Viikari, L. 1988. Carbohydrate metabolism in *Zymomonas*. CRC Crit. Rev. Biotech. **7**:237-261.

Walmsley, A.R. 1988. The dynamics of the glucose transporter. Trends Biochem. Sci. **13**:226-231.

West, I.C. 1970. Lactose transport coupled to proton movements in *Escherichia coli*. Biochem. Biophys. Res. Commun. **41**:655-661.

West, I.C., and P. Mitchell. 1973. Stoichiometry of lactose-proton symport across the plasma membrane of *Escherichia coli*. Biochem. J. **132**:587-592.

Wiegel, J. 1980. Formation of ethanol by bacteria. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. Experimentia **36**:1434-1446.

Wiegel, J.K.W. 1986. Genus *Thermoanaerobacter*, p. 1379-1383. *In* J.G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. Williams and Wilkins, Baltimore /Md.

Wiegel, J. 1992. The obligately anaerobic thermophilic bacteria (in preparation).

Wiegel, J., M. Braun, and G. Gottschalk. 1981. *Clostridium thermoautotrophicum* species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. Curr. Microbiol. **5**:255-260.

Wiegel, J., S.-U. Kuk, and G.W. Kohring. 1989. *Clostridium thermobutyricum* sp. nov., a moderate thermophile isolated from a cellulolytic culture, that produces butyrate as the major product. Int. J. Syst. Bacteriol. **39**:199-204.

Wiegel, J., and L.G. Ljungdahl. 1981. *Thermoanaerobacter ethanolicus* gen. nov., sp. nov., a new extreme thermophilic, anaerobic bacterium. Arch. Microbiol. **128**:343-348.

Wiegel, J., L.G. Ljungdahl, and J.R. Rawson. 1979. Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum*. J. Bacteriol. **139**:800-810.

Williams, D.K., and S.A. Martin. 1990. Xylose uptake by the ruminal bacterium *Selenomonas ruminantium*. Appl. Environ. Microbiol. **56**:1683-1688.

Willis, R.C., and C.E. Furlough. 1974. Purification and properties of a ribose binding protein from *Escherichia coli.* J. Biol. Chem. **249:**6926-6929.

Wilson, D.B. 1974. Source of energy for the *Escherichia coli* galactose transport systems induced by galactose. J. Bacteriol. **120:**866-871.

Winkler, H.H., and T.H. Wilson. 1966. The role of energy coupling in the transport of β-galactosides by *Escherichia coli.* J. Biol. Chem. **241:**2200-2211.

Winter, J., E. Braun, and H.P. Zabel. 1987. *Acetomicrobium faecalis* spec. nov., a strictly anaerobic bacterium from sewage sludge, producing ethanol from pentoses. Syst. Appl. Microbiol. **9:**71-76.

Winter, J., and G. Zellner. 1990. Thermophilic anaerobic degradation of carbohydrates - metabolic properties of microorganisms from the different phases. FEMS Microbiol. Rev. **75:**139-154

Wolin, M.J. 1963. Fructose-1,6-diphosphate requirement of streptococcal lactate dehydrogenases. Science **146:**775-777.

Wood, A.P., and D.P. Kelly. 1982. Mechanisms of sugar transport by *Thiobacillus* A2. Arch. Microbiol. **131:**160-164.

Wood, A.P., D.P. Kelly, and P.R. Norris. 1987. Autotrophic growth of four *Sulfolobus* strains on tetrathionate and the effects of organic nutrients. Arch. Microbiol. **146:**382-389.

Zeikus, J.G. 1979. Thermophilic bacteria: ecology, physiology and technology. Enzyme Microb. Technol. **1:**243-253.

Zeikus, J.G., A. Ben-Bassat, T.K. Ng, and R.J. Lamed. 1981. Thermophilic ethanol fermentations, p. 441-461. *In* A. Hollaender (ed.), Trends in the biology of fermentations for fuels and chemicals. Plenum Publishing Corporation, New York.

Zeikus, J. G., and V.G. Bowen. 1975. Fine structure of *Methanospirillum hungatii.* J. Bacteriol. **121:**373-380.

Zeikus, J.G., G. Fuchs, W. Kenealy, and R.K. Thauer. 1977. Oxidoreductases involved in cell carbon synthesis of *Methanobacterium thermoautotrophicum.* J. Bacteriol. **132:**604-613.

Zeikus, J.G., P.W. Hegge, and M.A. Anderson. 1979. *Thermoanaerobium brockii* gen. and sp. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch. Microbiol. **122:**41-48.

Zeikus, J.G., and T.K. Ng. 1982. Thermophilic saccharide fermentations. Annual reports in fermentation processes **5:**263-289.