Metabolic Studies of *Fusobacterium varium* Using NMR Spectroscopy

by

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ABSTRACT

A complex relationship exists between intestinal microorganisms and the human host; further knowledge of the microbial contributions is important to understand human health and disease. The metabolism of *F. varium*, a genome-sequenced constituent of the gut microbiota was investigated using NMR spectroscopy. The diverse amino acid requirements of *F. varium* were determined; while some amino acids were biosynthesized and utilized readily, others were required in the growth medium. The individual amino acid requirements were used to develop a chemically defined minimal medium for the determination of end products in lyophilized culture fluids by ^{1}H NMR spectroscopy. Six carboxylate fermentation products (acetate, butyrate and smaller amounts of succinate, propionate, formate and D-lactate) were detected in the exometabolome of *F. varium.* Glucose, glycerol and metabolic intermediates were identified as energy sources, and their addition to the defined growth medium led to variations in the composition of the exometabolome, attributed to the availability of substrates and the need to maintain redox balance through regeneration of reduced coenzymes. For example, the oxidative steps required for glucose catabolism resulted in higher concentrations of reduced products (butyrate and lactate), whereas propionate was formed exclusively from threonine. Substrate-product relationships were established using isotopically labeled substrates. The pathway of glutamate catabolism was dramatically influenced by the presence of coenzyme B_{12}, one of the B vitamins. Replacing the amino acid component of the defined medium resulted in *meso*-2,3-butanediol formation, a metabolite not previously identified in fusobacteria that may contribute to the pathogenesis of bowel disease.

LIST OF ABBREVIATIONS AND SYMBOLS USED

2-obu	2-oxobutyrate
δ	chemical shift
ADP	adenosine diphosphate
ATCC	American type culture collection
ATP	adenosine triphosphate
BHI	brain heart infusion
CDMM	chemically defined minimal medium/media
CE	capillary electrophoresis
CFU	colony forming unit
CoA	coenzyme A
Coenzyme B_{12}	5′-deoxyadenosylcobalamine
COSY	correlation spectroscopy
d	doublet
D1	delay time
DCA	dichloroacetic acid
dd	doublet of doublets
dm	doublet of multiplets
ddd	doublet of doublet of doublets
DNA	deoxyribonucleic acid
EC	enzyme commission
EMP	Embden-Meyerhoff-Parnas
ESI	electrospray ionization

GC	gas chromatography
glucose	D-glucose
GPT	glutamic-pyruvic transaminase
h	hour(s)
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum correlation
Hz	Hertz
Int. Std.	internal standard
LC	liquid chromatography
LDH	lactate dehydrogenase
MALDI	matrix assisted laser desorption/ionization
min	minute(s)
MHz	megaHertz
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	methyl *tert*-butyl ether
m/*z*	mass to charge ratio
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced form
NMR	nuclear magnetic resonance
OD	optical density
PCR	polymerase chain reaction
PLP	pyridoxal-phosphate

PYG	peptone yeast glucose
Rf	retention factor
RNA	ribonucleic acid
redox	reduction-oxidation
s	singlet
SCFA	short chain fatty acid
t	triplet
T_1	spin–lattice relaxation time
TA	tartaric acid
TLC	thin layer chromatography
TOF	time of flight
TYH	tryptone yeast extract heart infusion
UC	ulcerative colitis
UV	ultraviolet
v/v	volume per volume

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CHAPTER 1: INTRODUCTION

1.1 The Human Microbiome

Many microorganisms associated with the human body are found in the gastrointestinal tract, where anaerobic organisms outnumber aerobes by several orders of magnitude.[1] Contributions from the community of intestinal anaerobic bacteria to human physiology, health and disease are increasingly recognized.[2, 3]

The task of sequencing the genomes of all microorganisms living inside and on humans began in 2007.[4, 5] This project, known as the human microbiome project, is quite daunting, considering that the microbial population is estimated to be ten times greater than the number of human somatic and germ cells, and the number of genes in the microbiota is approximately 100 times the number of genes in the human genome.[6,7,8] The goals of the human microbiome project are to develop an understanding of the microbial contribution by determining the diversity of the microbiome, the factors that change the distribution or evolution of the microbiome, and whether changes in the microbiome affect human health and disease.[4,5]

Recent studies have revealed that infants begin to be colonized at birth and that the composition of the microbiota changes with illness and diet.[9,10, 11,12] The microbiota varies amongst individuals and different body habitats, yet it is stable over time within an individual.[13] The gastrointestinal microbiota of family members is similar, indicating an environmental role in developing the microbiota;[14] however, even genetically identical adult monozygotic twins each have their own unique set of microbial species in their gastrointestinal tracts.[15]

Within this context, an understanding of the interactions and metabolism of microorganisms within different microbial communities is needed to establish a connection with human health. In this thesis, the metabolism of *Fusobacterium varium,* one of the normal constituents of the intestinal microflora,[16,17,18] is investigated. *F. varium* is a Gram-negative, anaerobic bacterium and its genome sequence was determined as part of the human microbiome project.[19]

1.2 Effects of Gastrointestinal Tract Microflora

The importance of intestinal microorganisms has been demonstrated by studying mice grown under germ free environments, or gnotobioic mice, where the exact composition of the microbiota is known and selected.[20] Some contributions of microorganisms to growth and health were indicated by reduced vascularity, thinner muscle wall tissue, reduced digestive enzyme activity and greater susceptibility to enteric pathogens in the germ free mice.[21]

The intestinal microbiota prevents colonization of pathogens by secreting growth-inhibiting metabolites, competing for essential nutrients, and enhancing the immune response of the host by secreting immune mediators in response to antigens.[22,23] Overall, the microflora of the intestine aid the maturation of the immune system; it also responds to epithelial damage, affects energy balance and carries out biotransformations that human cells cannot.[8]

Surveying the microbiota of obese and non-obese people has revealed significant differences in the microbiomes between these two sets of people.[14] Within the microbiota of the obese people, the increased energy harvested from the ingested food was deposited

as adipose tissue.[24] In mice, however, a change to a high fat diet resulted in changes in the microbiota.[25] In the gut of both obese and non-obese mice fed the high fat diet there was a decrease in *Bacteroidetes* and an increase in both *Firmicutes* and *Proteobacteria* demonstrating that the high-fat diet, and not the obese state, mainly accounted for the observed changes in the gut microbiota.[26]

The composition of the microflora is variable, and changes in the natural composition are observed in certain diseases such as inflammatory bowel diseases,[27,28] colorectal cancer,[29] diabetes,[30] and patients with human immunodeficiency virus.[31] In addition to enhancing immune response,[32] the microbiota also influences brain development and behaviour, leading to anxiety or altered signaling pathways or neurotransmitter turnover. In mice this change in behaviour was attributed to the gut microbiota eliciting signals via the vagal nerve to the brain and modulation of neurotransmitters in the gut.[33]

Investigations of beneficial microorganisms could lead to new therapeutic strategies or could enhance the effects of pharmaceuticals, since these microorganisms may play a role in uptake and metabolism of certain xenobiotics.[34] Microorganisms in the gut also metabolize and produce different end products that enter into blood[35] and can affect other biological processes, such as drug metabolism. Different metabolic breakdown products could be produced, depending on which organisms are present in a microbiome. Metabolism of pharmaceuticals, such as acetaminophen,[36] the statin Simvastatin,[37] and Digoxin[38,39] has been affected by microorganisms.

Even short term exposure to antibiotics leads to a long term impact on the microbiota; differences in the microbiota of individuals were evident two to four years

after treatment,[40,41] impacting the beneficial gut microorganisms.[42] Mice show different metabolic profiles before and after treatment with the antibiotic vancomycin.[43] There was a reduction in the relative concentrations of fecal amino acids, and short chain fatty acids including acetate and butyrate. Antibiotic-mediated alterations of the microflora can allow pathogens to colonize.[44]

As an alternative therapy, the beneficial effects of the microbiota on the gastrointestinal tract have been developed into probiotics. Probiotics are live microorganisms administered to humans to alter the normal microflora, providing health benefits to the human host. Probiotics have been explored as a potential therapy for diseases, such as inflammatory bowel diseases, where the natural microflora has been altered.[45] Probiotic-induced alterations of the microflora in the intestines are thought to compete with more pathogenic bacteria and produce useful metabolites. Results collected from animal models show that probiotics may be useful to treat intestinal disorders, systemic immune/allergic conditions and metabolic syndromes. Commercially available probiotics typically consist of lactic acid bacteria and bifidobacteria.[28] Prebiotics is an alternative approach, in which certain oligosaccharides (such as inulin) are ingested to stimulate the growth, metabolic activity and output of microorganisms in the intestine.[46]

It also has been suggested that postbiotics, that is the metabolic products of bacterial metabolism, including short chain fatty acids, may be used as therapeutic tools to stimulate the adaptive immune system.[47] In order to use pro-, pre- or postbiotics, knowledge of the microorganism's metabolism must first be gained to learn how, the quantities of, and when the metabolic end products are made in order to optimize and understand the benefical effects to human health.

1.3 Anaerobic Microbial End Products

The mucosal surface of the large intestine allows for the absorption of beneficial metabolites produced by the microbiota and resists bacterial penetration. Nonetheless, products of microbial metabolism commonly affect the human host; metabolites can inhibit pathogen colonization[22] and are formed from the fermentation of undigested dietary components.[48] A comparison of germ-free and conventionally raised mice indicates that the microbiota affects metabolites found in blood, especially amino acid metabolites.[35]

Short chain fatty acids (SCFA), such as acetate, butyrate, formate, and propionate, are commonly produced by intestinal bacteria and are absorbed by the human host for use as energy sources in epithelial cells.[47] The SCFA butyrate, for example, appears to have both positive and negative effects. Some studies suggest that butyrate may cause ulcerations,[49] whereas others indicate that butyrate positively affects the colon by aiding in membrane synthesis, anti-inflammatory effects, sodium absorption and protection against colorectal cancer and causing apoptosis in cancer cells.[47,50,51] Acetate production in Bifidobacteria can provide protection against *E. coli* O157:H7 strains,[52] while both propionate and acetate also show anti-inflammatory[53] and chemopreventative properties.[54] As weak acids (pKa ~ 5), more than 90% of SCFAs exist in the anionic form in the colonic lumen.[55] Decreases in luminal pH can be influenced by fermentation of non digestible carbohydrates by gut bacteria, generating SCFAs as end products.[56]

1.3.1 Metabolic Footprinting

Using a metabolic approach to study microorganisms has the potential to give a more accurate picture of the actual physiological state of cells.[57] As an alternative to transcriptomics and proteomics, metabolomics has been used to study metabolism of organisms. Metabolomics is a comprehensive approach where all the metabolites in a biological system are identified and quantified to generate a metabolome.[58] The metabolome is composed of an endometabolome (the intracellular metabolites) and the exometabolome (the metabolites that are excreted out of the cell into growth medium).[59] Metabolomics has advantages over transcriptomics or proteomics; changes in cell physiological conditions lead to greater changes in the metabolome than in the transcriptome and proteome, since metabolism is closest to the activities of the cell.[60,61] The metabolome is the closest indicator to the cells phenotype.[62]

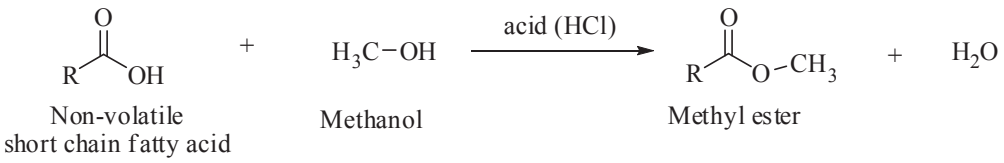
The metabolic footprint is a quantitative analysis of the metabolites excreted into culture fluid.[63] In anaerobic bacteria, the metabolic footprint consisting of carboxylic acid end products may be characteristic of the bacterium and/or the substrate catabolized. For example, branch chain carboxylic acids are derived from amino acids.[64] Studying the metabolic profile of a particular class of analytes, such as the short chain fatty acids, is used to investigate biochemical pathways.[65] Metabolomics also has been used to identify biomarkers for Crohn's disease,[66] liver and prostate cancers [67,68] and to identify pathogens.[69]

1.3.2 Determination of end products – chromatographic methods

Gas chromatography (GC) has been used previously to determine carboxylic acid end products and is commonly employed to identify anaerobic bacteria.[70] The GC profiles of accumulated fatty acids are characteristic of anaerobic organisms. Typically, pure cultures obtained from solid or liquid media,[71] are acidified and extracted with a solvent, such as diethyl ether or methyl *tert*-butyl ether.[72] These extractions are rarely 100% efficient and can lead to sample loss. To quantify the end products, the peak areas obtained from sample components can be compared to an internal standard, such as heptanoic acid, added before sample processing.[73]

Alternatively, headspace GC, where the vapour above heated broth cultures is sampled, can be used to detect volatile end products. Headspace GC does not require the extraction procedure described above, but only treatment of culture broth with sodium chloride and sulfuric acid. Typically, headspace samples were injected onto a column equipped with a flame ionization detector. A comparison indicated that headspace GC and traditional GC gave equivalent results.[74]

Non-volatile carboxylic acid end products, such as lactic and succinic acids, have been detected by GC after conversion to a methyl, ethyl or butyl ester (Scheme 1.1).[73] For example, butyl derivatives were formed by mixing lyophilized culture samples with chloroform, butanol and hydrochloric acid at pH $\leq$ 1, followed by heating at 80 ºC. The chloroform layer, which contained the butyl esters, was injected and analyzed using GC. Derivatized esters, however, elute close to the solvent peak, interfering with quantification and interpretation.[75] Although analysis times using a GC instrument are short, the need to extract and derivatize the end products can be time consuming.



Scheme 1.1. Methyl esterification of non-volatile short chain fatty acids (R = $CHOHCH_3$ or $CH_2CH_2CO_2H$).

Both volatile and non volatile end products can be determined simultaneously using high performance liquid chromatography (HPLC), with no need for esterification.[76] While the separation times were longer than those in GC, HPLC provided greater detection sensitivity for the non-volatile end products. HPLC analysis required only a single sample preparation step, in which short chain fatty acids were extracted and analyzed using an ion-exchange column. The carboxylic acid products were detected in the column effluent using ultraviolet (UV) absorption at 210 nm.[77] Alternatively, liquid chromatography (LC) coupled with mass spectrometry (MS) offered selectivity and sensitivity with detection limits of 10 ppm.[78]

Capillary electrophoresis (CE)[79] eliminated the extraction and derivatization steps used in GC and HPLC methods. After cultures were incubated in liquid broth, carboxylic acids in supernatants were separated by CE and detected by UV absorption at 220 nm. CE was able to detect and quantify end products down to concentration levels of 1 μmol/L.

1.4 Studying Metabolism with NMR Spectroscopy

NMR (nuclear magnetic resonance) methods are a non-destructive and non-invasive way of probing metabolism. A single NMR spectrum can provide quantitative and qualitative data on a broad range of metabolites produced by various metabolic pathways, even in the presence of complex biological matrices.[80] Using 1-D and 2-D NMR experiments, assignments of structures can be determined from several parameters, including chemical shift, coupling pattern and coupling constant data.[81] The moderate sensitivity of NMR spectroscopy is a disadvantage; typically 10 μM concentrations are at the very low end of sample concentrations detected with this method.[82] However, using low temperature probes[83,84] or using reduced detection volume NMR tubes has improved sensitivity.[85]

Previously, radiolabeled substrates (such as ^{14}C) have been used to detect the formation of end products in microorganisms.[86,87] Detecting radioactivity in the specific locations of the product provides direct evidence for the formation of the product from the radiolabeled substrate. With the advent of NMR techniques, the use of substrates labeled with ^{13}C isotopes has gained popularity, since NMR directly determines of the final position of the isotope in the end product.[85] Using labeled ^{13}C substrates, enrichment of the ^{13}C NMR signal and ^{13}C -^{13}C coupling patterns can provide evidence for the formation of the product from the substrate. The position of the label determined through chemical shift gives details on the metabolic processes occurring. Alternatively, ^{1}H NMR can be used indirectly to detect ^{13}C enrichment in the end products[88] via the ^{13}C-^{1}H coupling satellites.

The use of ^{13}C isotopic labeling in combination with NMR spectroscopy has been used for over 30 years to study bacterial metabolism.[89] By following the ^{13}C isotope, other products of metabolism, such as intermediates of pathways, have been discovered.[90] In microbial growth cultures supplemented with amino acids labeled with ^{13}C isotopes, the enrichments in final products were used to identify intermediates and final products in the metabolism of the labeled amino acid.[91]

Metabolic studies in *Fusobacterium* species have been conducted previously using a few ^{13}C labeled substrates and ^{13}C NMR spectroscopy. In *Fusobacterium nucleatum*, glutamate catabolism was investigated using L-[5-^{13}C]glutamate, L-[1-^{13}C]glutamate and L-[4-^{13}C]glutamate.[92] By using this series of labeled glutamate substrates, the hydroxyglutarate pathway was found as the predominant pathway used for the catabolism of glutamate by *F. nucleatum*. Also, a [1,2-$^{13}C_2$]acetate experiment in *F. nucleatum* showed the interconversion of acetate and butyrate. There was equal enrichment of the four carbons in butyrate, indicating that two contiguous carbons from acetate were incorporated into butyrate. In *F. varium*, cell resuspensions were used; the cells were resuspended in a phosphate-buffered saline solution and incubated with sodium [1-^{13}C]-butyrate (40%) and 10 mM L-glutamate. The ^{13}C enrichment detected in C-1 of both butyrate and acetate indicated that butyrate is not a static end product and can be metabolized to produce acetate in *F. varium,* when accompanied by another energy source such as L-glutamate.[93] Furthermore, administration of L-[U-$^{13}C_5$]glutamate to a cell resuspension led to ^{13}C enrichments at C1 and C2 of acetate and C1/C2 and C3/C4 of butyrate.[93] These results indicate that the methylaspartate pathway is functioning in *F. varium* (see Section 1.6.4.3 Glutamate). It is important to note that ^{13}C NMR

spectroscopy demonstrated that only 2 of the 4 butyrate carbons had ^{13}C enrichment and that four contiguous carbons with a ^{13}C enrichment were not detected. Four contiguous carbons would result in two doublet of doublets coupling patterns in the ^{13}C NMR spectra. This was not observed. In these experiments, isotopic labeling not only demonstrated substrate-product connections *via* a multistep sequence of enzyme-catalyzed reactions, but also distinguished between alternative metabolic pathways. The coupling patterns in the NMR spectra can give valuable information about how the ^{13}C enrichment is distributed in the end products.

1.5. *F. varium:* An Intestinal Bacterium

F. varium is a Gram-negative, obligate anaerobic bacillus.[94] *Fusobacterium* species commonly produce butyrate as a major metabolic end product formed from crotonyl-CoA, a terminal electron acceptor.[50] Other fusobacteria include *F. nucleatum*, commonly found in the mouth and associated with periodontal disease,[95] *F. necrophorum*, which has been related to conditions such as necrobacillosis and Lemierre's syndrome,[96] and *F. ulcerans*, which has been associated with tropical skin ulcers.[97] Very recently, *Fusobacterium* species, especially *F. nucleatum* have been associated with colorectal carcinoma.[98, 99] *F. varium*, however, was not detected in these genomic studies. *F. varium* is an obligate anaerobe and must be grown in the absence of oxygen, but can be manipulated in air, while other *Fusobacterium* strains (*F. nucleatum* and *F. necrophorum)* have variable aerotolerance which depend on isolation site and handling, indicating that *Fusobacterium* species are able to adapt to the environment.[100]

F. varium, an indigenous colonizer of the gastrointestinal tract, is a potential antagonist of the pathogen *Salmonella typhimurium*,[101] but also plays a role in conjunctivitis and intra-ocular infections.[97] While the culture supernatant of *F. varium* was found to cause ulcerative colitis (UC)-like lesions in mice,[49] it is uncertain whether the effect can be attributed to the organism or the butyrate present in the supernatants. *F. varium* has been found in patients with UC.[102] Patients treated with antibiotics targeting *F. varium* were found to benefit compared to the control group, although this study was done on a small number of patients (n = 20).[103] The investigators could not attribute this result completely to the elimination of *F. varium* since the antibiotics they used (amoxicillin, tetracycline, metronidazole) also affect the growth of many other bacterial species present in these patients.[104] *F. varium* can, but rarely does cause bacteremia.[105,106] *F. varium* also produces spermidine and putrescine, polyamines that are involved with cellular processes, such as RNA and DNA synthesis, but these polyamines also may promote cells to become malignant.[107]

Many of the relationships between humans and its microbiome are commensal and usually benign. These relationships are not necessarily mutualistic, where both organisms benefit. Some pathogenic bacteria can colonize many humans without symptoms;[108] alternatively, some commensal bacteria can cause disease in human hosts.[109] More knowledge of *F. varium* metabolism and physiology is needed to gain insight into the potential contribution to disease or the beneficial effects of *F. varium* in the human body.

1.6. Metabolism in *F. varium*

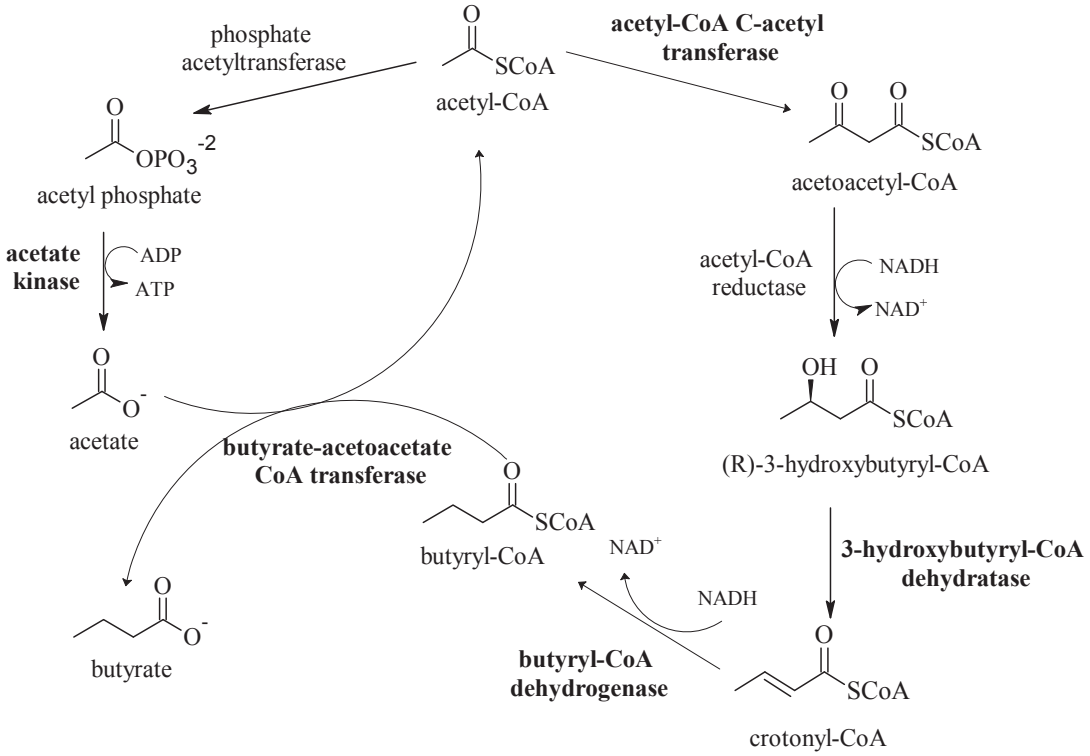
F. varium produces a number of short chain, primarily aliphatic carboxylic acids, including acetate, butyrate, formate, propionate, succinate and lactate,[110] and quantification of these metabolites has been used to distinguish *F. varium* from other fusobacteria. Concentrations of greater than 3 mM succinic acid and 6 mM lactic acid distinguish *F. varium* from other fusobacterial species when grown on trypticase-glucose yeast broth.[111]

1.6.1. Acetate-butyrate interconversion

Formation of acetate and butyrate, the major end products in *F. varium* metabolism, is linked to energy production and coenzyme regeneration via a core pathway (Scheme 1.2). In the acetate-butyrate pathway, acetate is formed when energy (ATP) is generated from acetyl-CoA. This is an especially significant reaction in anaerobic organisms lacking an electron transport chain to create ATP by oxidation of NADH. Alternatively, butyrate is formed from acetyl-CoA when the coenzyme NAD^+ is regenerated for use in the oxidative steps in the degradation of energy sources.

In *F. varium*, functioning of the acetate-butyrate pathway was demonstrated when the organism was grown on peptone medium supplemented with [1,2-$^{13}C_2$]acetate.[112] Using ^{13}C NMR spectroscopy, high isotopic enrichments were detected in butyrate, consistent with a net flow of carbon from acetate to butyrate. Moreover, the ^{13}C-^{13}C coupling pattern was consistent with the incorporation of two acetate units to form butyrate.[92,112] Subsequently, a proteomics study of *F. varium*[113] detected enzymes involved in the acetate-butyrate pathway, specifically, acetyl-CoA C-acetyltransferase, 2-

hydroxybutyryl-CoA dehydratase, butyryl-CoA dehydrogenase, the α and β subunits of an electron transfer flavoprotein accessory to butyryl-CoA dehydrogenase and butyrate-acetoacetate CoA-transferase (Scheme 1.2). Overall, the evidence available suggests that the acetate-butyrate pathway plays a key metabolic role in *F. varium*.



Scheme 1.2. Conversion of acetate to butyrate in *F. varium*. Enzymes identified by proteomics[113] study are shown in bold.

1.6.2. Energy sources

1.6.2.1. Glucose

Whether *F. varium* is able to ferment glucose for energy was investigated in a proteomics study.[113,114] When *F. varium* was grown on chemically defined minimal medium (CDMM) containing 1 mM amino acids and glucose (CDMM1/20 mM glucose), the glycolytic enzymes pyruvate kinase, enolase, glucose-6-phosphate isomerase, phosphoglycerate kinase, triosephosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase and fructose-bisphosphate aldolase, were identified using two dimensional gel electrophoresis and LC-MS/MS. Although previous studies report a lack of glucose fermentation,[115] the increased levels of seven glycolytic enzymes when glucose is supplied to the culture medium suggest that glucose is a major energy source for *F. varium*.

1.6.2.2. Amino acids

L-Amino acids are considered to be major energy sources for fusobacteria, including *F. varium*.[116,117] *F. varium* was found to utilize high levels of arginine, asparagine, cysteine, glutamate and serine and lower concentrations of aspartic acid, histidine, lysine and threonine with little metabolism of hydrophobic and aromatic amino acids.[116,117] While L-amino acids were utilized at a faster rate than the corresponding D-amino acids, D-glutamate, D-lysine and D-serine were efficiently utilized.[117] When L-histidine, D- and L-glutamate, D- and L-lysine and D- and L-serine were supplied to *F. varium* as sole energy sources, increased levels of enzymes in specific catabolic pathways were detected, indicating nutrient-induced changes in the proteome.[114]

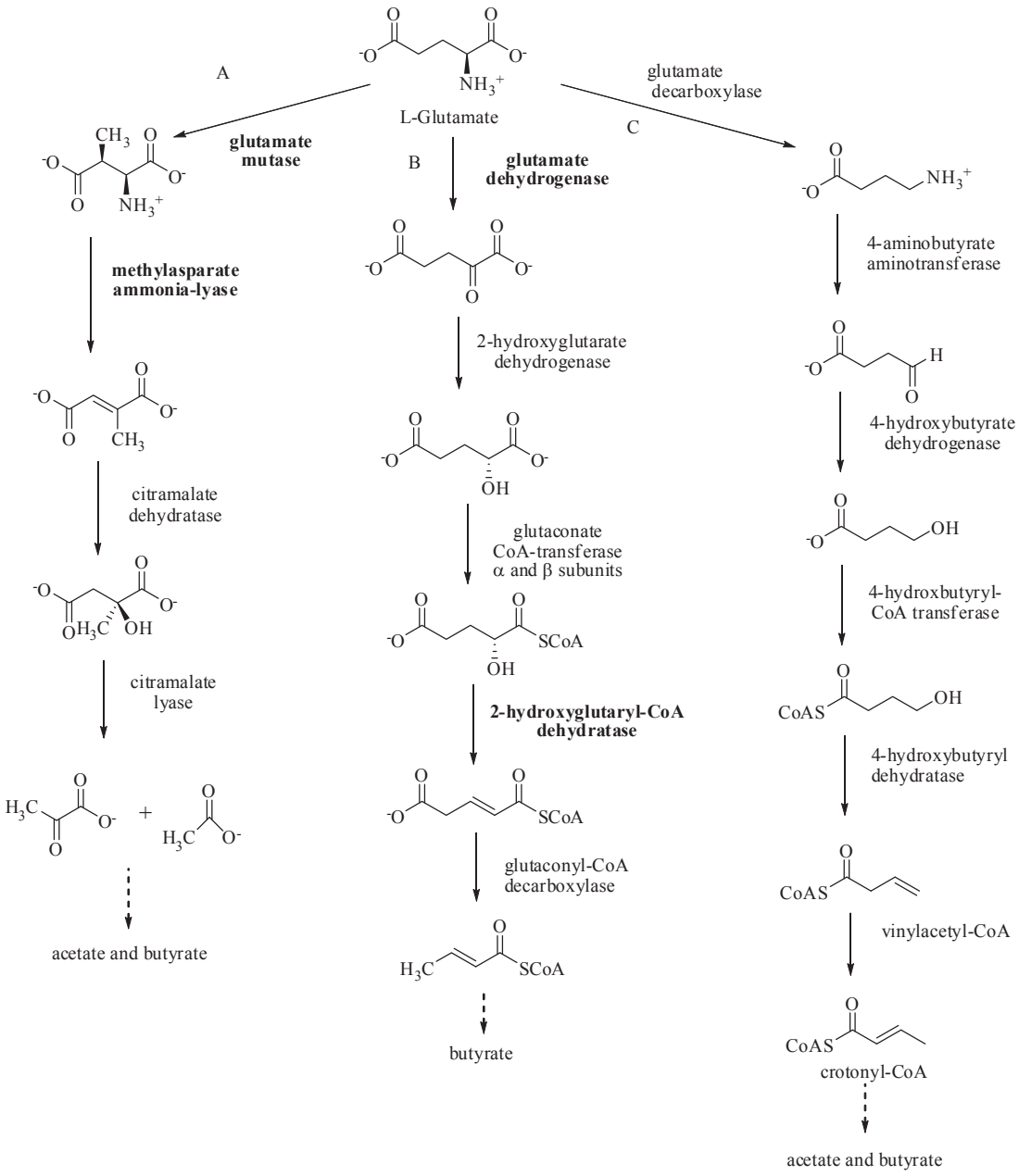
1.6.2.3. Glutamate

Five possible pathways for the catabolism of glutamate in anaerobic bacteria have been suggested.[118] Three of the pathways (*i.e.*, the aminobutyrate, methylaspartate and hydroxyglutarate pathways) (Scheme 1.3) have been proposed for glutamate catabolism in *F. varium*.[119] However, there is conflicting evidence. Initial studies using *F. varium* cultures grown with [1-^{14}C]glutamate showed a small amount of radioactive CO_2 being released.[119] The authors attributed this result to the aminobutyrate pathway (Scheme 1.3, Route C), although no further evidence exists for the presence of the aminobutyrate pathway in *F. varium*.

Previous work using *F. varium* grown in peptone medium supplemented with L-[4-^{13}C]glutamate[112] showed enrichment at C1 of acetate and equal enrichments at C1 and C3 of butyrate that are consistent with the cleavage of the carbon backbone of glutamate in the methylaspartate pathway. Catabolism of L-[4-^{13}C]glutamate by the hydroxyglutarate pathway would have placed label at C4 of butyrate, but no enrichment was detected at C4 of butyrate. Overall the labeling results are consistent with only the methylaspartate pathway for catabolism of glutamate (Scheme 1.3, Route A). No incorporation of label was obtained when L-[5-^{13}C]glutamate was added to *F. varium* on peptone medium, excluding the aminobutyrate pathway.[112] While the above experiments were performed using complex, undefined peptone medium, a similar conclusion was reached when L-[U-$^{13}C_5$]glutamate was catabolized by resuspended *F. varium* cells.[93]

The butyrate product was formed from acetate units and not directly from glutamate, as required for the hydroxybutyrate and aminobutyrate pathways.[118] In another study, label from [1-^{14}C]glutamate was incorporated preferentially into C1 of butyrate,

indicating the functioning of the hydroxyglutarate pathway in *F. varium* (Scheme 1.3, Route B).[120]

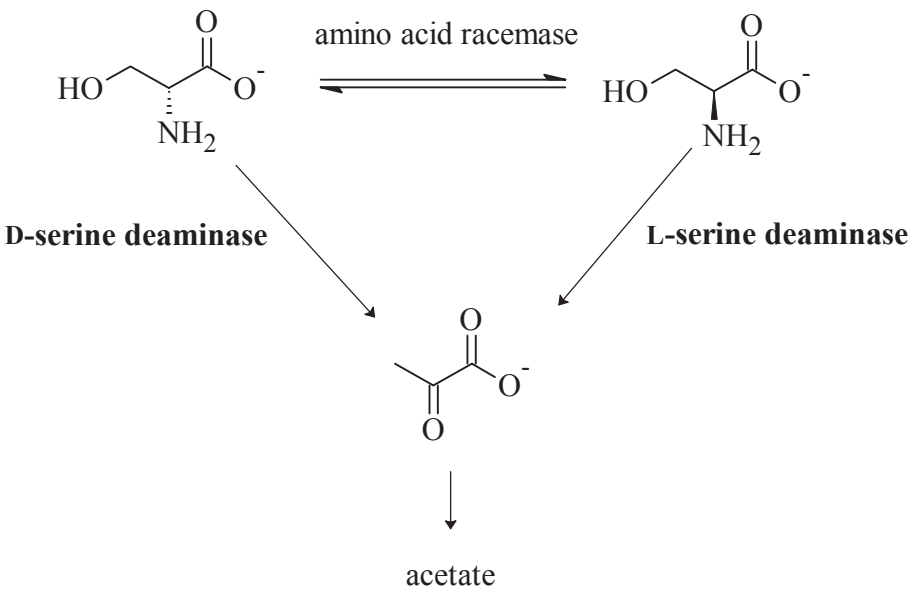


Scheme 1.3. Possible routes of glutamate catabolism in *F. varium*. (A) methylaspartate pathway; (B) hydroxyglutarate pathway; (C) aminobutyrate pathway. Enzymes found in the proteomics study[114] are given in bold.

β-Methylaspartase, (methylaspartate ammonia-lyase) the enzyme used in the second step in the methylaspartate pathway, has been purified from *F. varium* and characterized.[121] In the proteomics study,[114] methylaspartate ammonia lyase and glutamate mutase were detected as major proteins under all growth conditions, suggesting a major role for the methylaspartate pathway. However, two enzymes in the hydroxyglutarate pathway, NAD-specific glutamate dehydrogenase and 2-hydroxyglutaryl-CoA dehydratase, were detected when 40 mM L -glutamate was supplied to *F. varium* as the energy source in defined medium. Using polymerase chain reaction (PCR), genes for all enzymes in the hydroxyglutarate pathway were identified,[114] as well the genome sequencing studies show genes for both hydroxyglutarate and methylaspartate pathway enzymes,[19] suggesting that *F. varium* might have the capability to degrade glutamate by two chemically distinct pathways.

1.6.2.4. Serine and threonine

Separate deaminases are known for D-serine and L-serine.[122] In a proteomics study, L-serine deaminase was present under all growth conditions while the D-serine deaminase was detected only when D-serine was supplied to *F. varium* as an energy source.[114] The degradation of the serine stereoisomers is therefore catalyzed by separate D- and L-serine deaminases,[114] (EC 4.3.1.18 and EC 4.3.1.17, respectively). The catabolism of serine results in pyruvate (Scheme 1.4), which can be converted to acetate and lactate.



Scheme 1.4. Serine catabolism in *F. varium*. Enzymes found by proteomics are given in bold.[114]

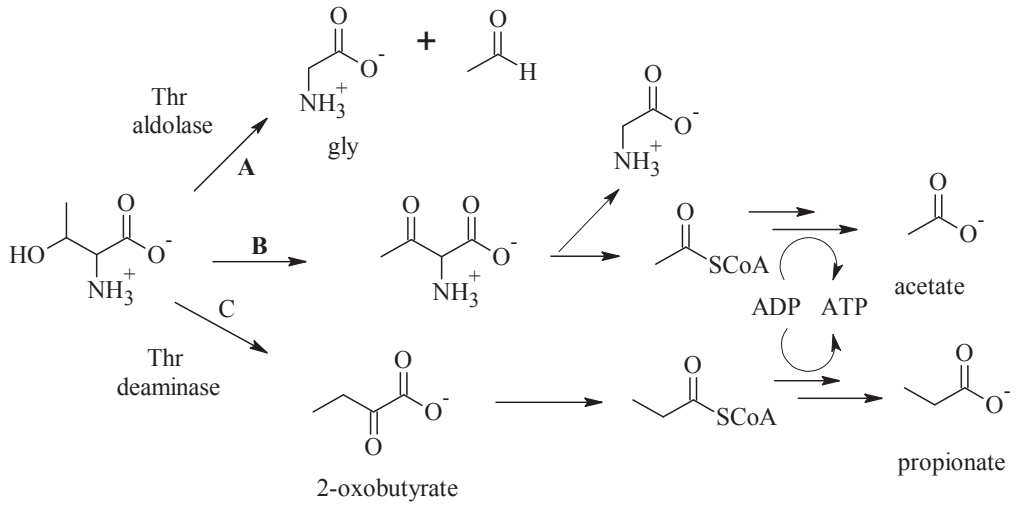
F. varium also utilizes the D stereoisomers of glutamate and lysine at rates similar to those for the corresponding L isomers.[117] Distinct multistep pathways have been suggested for the catabolism of D- and L-lysine in *Fusobacterium nucleatum*.[123] Alternatively, a racemase assists the conversion of D- to L-glutamate in *F. varium.*[124]

The efficient utilization of D- and DL-threonine on complex medium[125] suggests that threonine stereoisomers also can contribute to energy production in *F. varium*. While increased levels of propionate formation in threonine-supplemented culture medium is a standard classification test for *F. varium*,[70] the pathway or pathways utilized for the

degradation of L-threonine and its stereoisomers have not been established in *Fusobacterium* species.

Several pathways (Scheme 1.5) have been suggested for L-threonine catabolism in anaerobes,[126,127,128] facultative anaerobes[129] and rumen microorganisms.[130] Cleavage of threonine yielding either acetaldehyde and glycine or acetyl-CoA and glycine (after an oxidation step) (Scheme 1.5; Routes A and B) have been recognized in other bacteria. In the second pathway (Scheme 1.5; Route B), threonine is converted to 2-amino-3-ketobutyrate, which is converted to acetyl-CoA using 2-amino-3-ketobutyrate coenzyme A ligase. 2-Amino-3-ketobutyrate coenzyme A ligase has been purified from *E. coli.*[131] The non-oxidative conversion of threonine to propionate via 2-oxobutyrate (Scheme 1.5; Route C) has been documented in enterobacteria[132] and the four enzymes in the pathway have been characterized.[133]

Propionate is a distinctive end-product of the 2-oxobutyrate pathway; high levels of propionate were detected by gas chromatography when *F. varium* was cultured on undefined medium supplemented with either DL-threonine, D-threonine or 2-oxobutyrate,[125] but the accumulation of propionate was not linked directly to the substrates.



Scheme 1.5. Three pathways of threonine catabolism in anaerobic bacteria.

1.6.3. Defined media to study F. varium *metabolism*

Previously, a peptone medium has been used to study metabolism in *F. varium*.[117] This medium is neither defined nor uniform in composition. A defined medium is required to easily determine how energy, carbon and nitrogen sources can effect growth and metabolism. In a defined medium substrates are controlled and known, and the effects of additional energy or carbon sources can give insight into the catabolic processes present. In studies of *Clostridium difficile*,[134] for example, a minimal medium with varying amounts of amino acids and vitamins was developed. In that study, the requirement of amino acids provided insight into metabolic processes present, e.g. substituting adenine for threonine or glycine showed that these amino acids can be used for purine biosynthesis.[134] A chemically defined minimal medium has been developed to

study *F. varium*.[113] However, the individual amino acid requirements have not yet been determined.

1.7. Project Goals

The logical development of probiotics, prebiotics, postbiotics and antimicrobial agents requires a detailed understanding of the metabolic mechanisms present in intestinal microorganisms. This knowledge will contribute insights into the microbial contribution to human health and the conditions under which beneficial short chain carboxylic acids are produced. *F. varium* as a major producer of acetate and butyrate is of particular interest.[114] Determining energy sources and metabolic pathways present in intestinal tract organisms such as *F. varium* will be important for studying the survival of these organisms and determining how and under what conditions beneficial short chain fatty acids are produced. While some aspects of metabolism in *F. varium* have been investigated, the interaction of the major energy sources and the pathways for their catabolism are not yet fully understood.

The major goal of this project is to elucidate the major energy producing metabolic pathways in *F. varium.* ^{1}H NMR spectroscopy will be used to quantify the effects of individual energy sources on the metabolic footprint of *F. varium*. Links between end product formation and substrate catabolism will be made using isotopically labeled substrates and NMR determinations of isotopic incorporations into the metabolic end products. The utilization of pathway intermediates also will be investigated by ^{1}H NMR spectroscopy.

Amino acids as a group are essential components of a chemically defined minimal medium developed previously.[113] However, given the different rates of uptake of amino acids,[116, 117] it is possible that not all amino acids need to be supplied to *F. varium* at high concentrations. Determination of *F. varium's* requirements for individual amino acids will help to establish the potentially different roles played by different amino acids. Indications of amino acid – carboxylic acid product relationships will be further investigated using isotopically labeled substrates.

CHAPTER 2: DEVELOPMENT OF METHODS AND MEDIA TO CHARACTERIZE THE EXOMETABOLOME OF *F. VARIUM*

Short chain fatty acids are common end products of anaerobes. Acetate and butyrate typically are derived from carbohydrates and amino acids,[135,136] while degradation of specific amino acids (e.g., threonine, leucine and valine) leads to the accumulation of propionic and branched-chain carboxylic acids.[64] These carboxylate products are found in the exometabolome, the collection of metabolites excreted into the culture supernatant from the cell.[63] The exometabolome is easily determined through analysis of the culture supernatant. Unlike the endometabolome (metabolites in the cell), there is no need for extraction of intra cellular components, a method that can vary depending on the extraction method used.[137] For an accurate metabolic footprint, that is a quantitative analysis of the exometabolome,[63] the products to be quantified using ^{1}H NMR spectroscopy require a flat baseline surrounding their resonances. The product resonances must be resolved and require the removal of all interfering components affecting the integration of peaks to be quantified. Furthermore, for accurate quantitative NMR spectroscopy the delay time should be adjusted to ensure the complete relaxation of protons being quantified.

2.1. Identification of Metabolic Products

The ^{1}H NMR spectra of spent peptone,[117] CDMM2(10 mM glucose)[113] and CDMM*(10 mM glucose) culture media (Figure 2.1) showed the accumulation of six carboxylate end products. The resonances of acetate (δ 1.80), butyrate (δ 2.04, 1.44, 0.78), formate (δ 8.34), lactate (δ 4.01, 1.22), propionate (δ 2.06, 0.94) and succinate (δ

2.29) were assigned using chemical shift comparisons, the enhancement of signals upon the addition of a standard compound to spent culture medium, and correlation spectroscopy (COSY). The COSY correlations observed (Figure 2.2) were consistent with the assignment of butyrate, lactate and propionate resonances and also confirmed that the multiplet at 2.1 ppm corresponded to resonances for both butyrate and propionate.

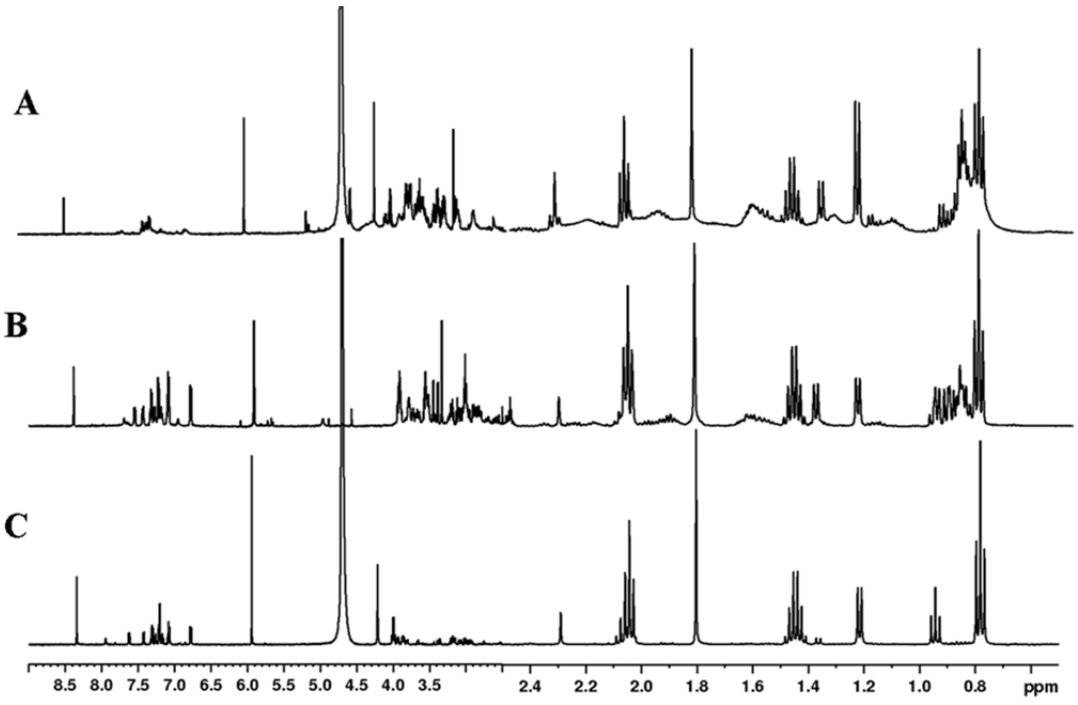


Figure 2.1. ^{1}H NMR spectra obtained using a lyophilized sample of the culture supernatant dissolved in D_2O after growing *F. varium* in (A) peptone medium; (B) CDMM2(10 mM glucose); and (C) CDMM*(10 mM glucose). Resonances were assigned to formate (δ 8.34), DCA (δ 6.01), TA (δ 4.28), lactate (δ 4.01, 1.22), succinate (δ 2.29) propionate (δ 2.06, 0.94), butyrate (δ 2.04, 1.44, 0.78), and acetate (δ 1.80).[138]
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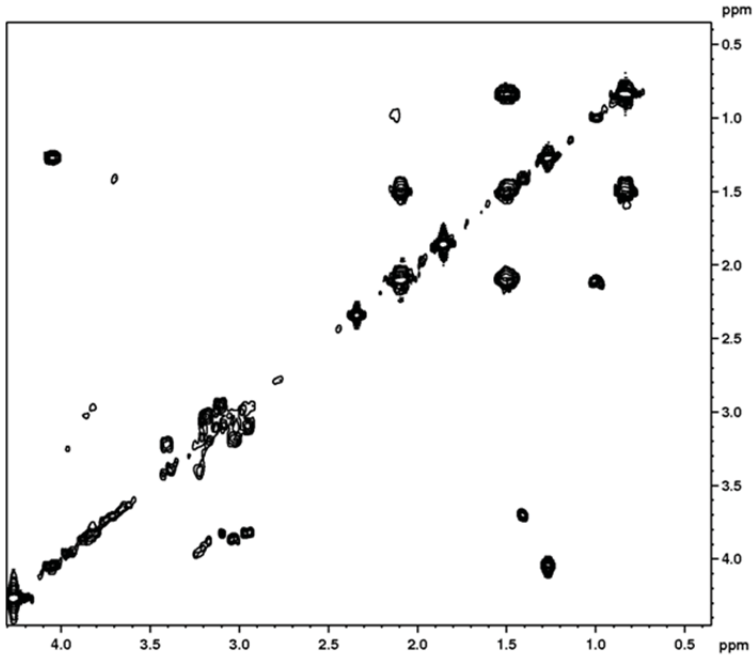
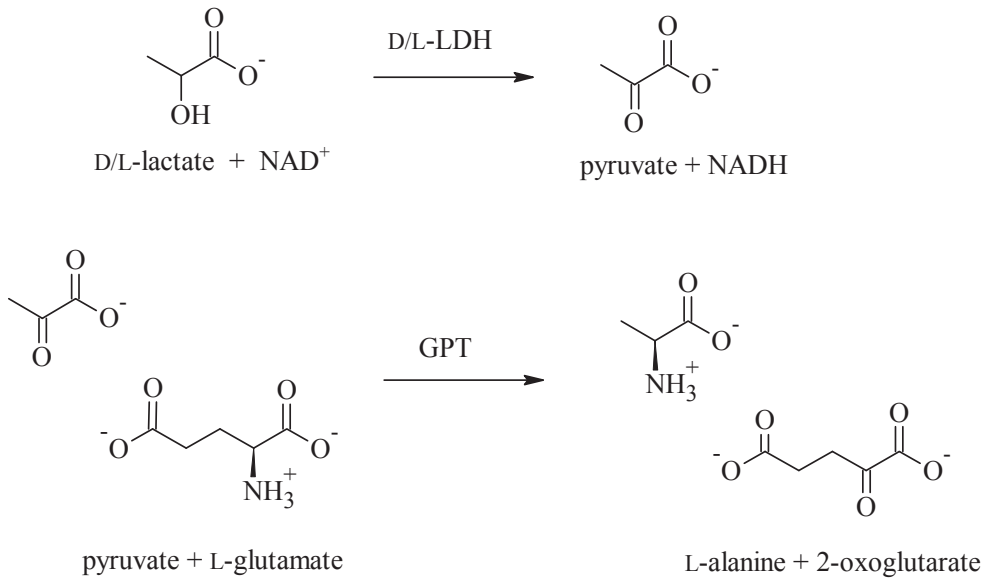


Figure 2.2. COSY spectrum of CDMM*(10 mM glucose).

2.1.1. Determination of Lactate Stereochemistry

Lactate has two possible stereoisomers, L-lactate and D-lactate. *F. varium* produces lactate as determined by ^{1}H NMR spectroscopy, but the stereochemistry has not been defined. The stereochemistry of the lactate product was determined enzymatically using lactate dehydrogenase (LDH). The oxidation of lactate to pyruvate is accompanied by the reduction of nicotinamide adenine dinucleotide coenzyme (*i.e.*, NAD^{+} to NADH). L-LDH which oxidizes L-lactate and D-LDH which oxidizes D-lactate have stringent substrate specificities and are commercially available. However, the equilibrium of the LDH catalyzed reaction lies towards lactate, with an equilibrium constant of 2.76 x 10^{-12}.[139] Thus utilization of the lactate reactant was accomplished by the addition of

glutamate pyruvate transaminase (GPT) and glutamate to convert pyruvate to alanine (Scheme 2.1) along with conversion of glutamate to 2-oxoglutarate.



Scheme 2.1. Coupled reactions used for determining lactate stereochemistry.

Using a modified enzymatic assay developed previously,[140, 141] lyophilized culture supernatant containing lactate (~2 mM), L- or D-LDH and GPT was incubated with NAD^+ in a glycylglycine-L-glutamate buffer. Progress of the reaction was monitored by measuring the absorbance of NADH at 340 nm and the distribution of end products was monitored using ^1H NMR spectroscopy. Incubation of the lyophilized culture supernatant with D-LDH was accompanied by a large absorbance change (0.57 to 1.73). The intensity of the lactate NMR resonances at δ 1.22 decreased while alanine resonances at δ 1.37 (Figure 2.3A) appeared. In the parallel incubation mixture, lyophilized sample with L-

LDH gave a small absorbance change (0.84 to 0.87). The ^{1}H NMR spectrum showed a product distribution similar to the control sample in which no enzymes were added to the incubation (c.f. Figure 2.3 B and C). The large absorbance change and decreased lactate peak when the lyophilized sample was incubated with D-LDH indicated that lactate produced by *F. varium* is D-lactate. D-Lactate is commonly produced by many metabolic reactions in anaerobic bacteria.[142] While the genome has genes encoding both D-LDH and L-LDH,[19] the accumulation of D-lactate indicates that mostly D-LDH is expressed under the growth conditions used.

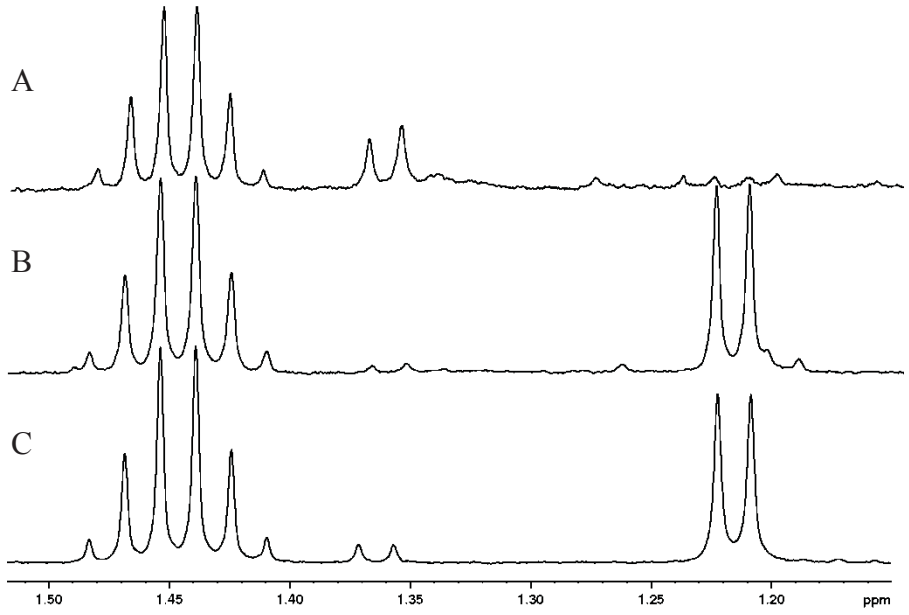


Figure 2.3. ^{1}H NMR spectra of lyophilized culture supernatants treated with LDH: (A) sample incubated with D-LDH; (B); sample incubated with L-LDH; and (C) control, no enzymes added. Resonances were assigned to (δ 1.44), alanine (δ 1.36) and lactate (δ 1.22).

2.2. Refinement of Defined Medium Composition

While abundant growth of *F. varium* was attained on the peptone[117] (OD_{660} 1.1 ± 0.2) and CDMM2(10 mM glucose)[113] (OD_{660} 1.2 ± 0.1) medium, residual medium components interfered with the quantitative determination of the carboxylate metabolites (Figure 2.1 A and B). Simplification of the chemically defined minimal medium (CDMM), therefore, was undertaken to provide suitable ^{1}H NMR spectra while maintaining nutrients to support abundant growth of *F. varium*.

Growth of *F. varium* was determined using OD_{660} measurements, damp cell mass, dry cell mass and number of colony forming units (CFU) (Table 2.1). Spent cultures (250 mL) after 16 h incubation with an OD_{660} of 0.48 had 60-30% less growth determined using damp cell, dry cell masses or colony counting when compared to spent cultures with an OD_{660} of 0.96. Overall, increased OD_{660} correlated with increased mass and numbers of CFU and was representative of growth. OD_{660} was chosen for measuring growth in all future experiments since it was quick, could be performed on cultures under anaerobic conditions directly in growth culture tubes with no additional sample preparation.

The amino acid component of the defined medium is essential to support growth of *F. varium*.[113] When all the amino acids were omitted from defined medium, there was no growth, while intermediate concentrations of amino acids gave intermediate levels of growth (e.g., OD_{660} 0.58 and 0.91 at 1 and 1.5 mM, respectively). But despite the dependence of amino acids for growth of *F. varium*, the ^{1}H NMR spectrum of spent CDMM2(10 mM glucose) (Figure 2.1B) showed, in addition to the prominent resonances of carboxylate end products, many resonances corresponding to unmetabolized amino

acids in the medium. Resonances at δ 7.8-6.8 corresponded to phenylalanine, tryptophan and tyrosine, and the α and side chain protons of alanine, arginine, glycine, isoleucine, leucine, methionine, proline and valine were found at δ 4.2-2.9. The aliphatic side-chain protons of alanine, arginine, isoleucine, leucine, methionine, proline and valine gave resonances at δ 2.7 – 0.4. Resonances were assigned using chemical shift comparisons and spiking experiments. These residual amino acids are consistent with the ability of *F. varium* to utilize a subset of the 20 protein amino acids. [116,117]

Table 2.1. Comparison of damp and dry cell masses, CFU and OD_{660}. The average and standard deviations of three separate growth experiments.

Sample	OD_{660} [b]	Damp cell mass (g) [c]	Dry cell mass (g) [d]	CFU [e]
CDMM* (10 mM glucose)	0.96	0.90	0.09 ± 0.02	1.9×10^8
CDMM*	0.48	0.43	0.03 ± 0.002	5.2×10^7
%[a]	50%	60%	33%	27%

[a]Percent calculated from CDMM* and CDMM*(10 mM glucose). [b]OD_{660} values were averaged from two culture tube growths. [c]Damp cell masses were determined from cell pellets after combining two culture tubes (250 mL culture total). [d]Dry cell masses were obtained by removing cell pellets with minimal amount of water and dried in oven, measurements taken at 24, 48 and 72 h, averaged and standard deviation reported. [e]CFU numbers were determined from colonies grown on brain heart infusion (BHI) agar plates (37 °C, 48 h) after inoculating plates with diluted (phosphate buffered saline solution) cells grown from CDMM*(10 mM glucose) and CDMM*.

To determine the requirements of individual amino acids, each amino acid was placed into a group based initially on their utilization, and groups were redefined based on their growth response. Concentrations of each group of amino acids were varied

individually from 0 – 2.0 or 2.5 mM in the presence and absence of 10 mM glucose, while keeping the concentrations of the other groups of amino acids constant. These experiments were performed in triplicate; each experiment had three replicate growth cultures, providing OD_{660} measurements as the average of nine independent readings. Group 1 consisted of amino acids that were incompletely utilized and provided signals in the ^1H NMR spectrum that interfered with the metabolic end product signals. This group included the amino acids alanine, arginine, glycine, isoleucine, leucine, methionine, proline, and valine. When the Group 1 amino acids were supplied at concentrations varying from 0 – 2.5 mM, maximum growth was reached at 0.2 mM and higher concentrations (Figure 2.4A). This trend was seen both in the presence and absence of glucose (Figure 2.4A). When one of the amino acids from Group 1 was supplied at 2 mM and the remaining Group 1 amino acids were kept at 0.2 mM, only the resonance(s) for the single amino acid supplied at the higher concentration was detected in spent culture medium. This demonstrated the overlapping resonances of isoleucine, leucine, valine (δ 0.90-1.05) and propionate methyl group (δ 0.94).

The aromatic amino acids phenylalanine, tryptophan and tyrosine (Group 2) also appeared to be underutilized by ^1H NMR spectroscopy. When the concentration of this group of amino acids was increased, from 0 to 0.2 mM, there was a rapid increase in growth; a slight, but reproducible, increase in growth once the concentrations of Group 2 amino acids were increased above 0.2 mM (Figure 2.4B). Taking into account the standard deviations, the lowest concentration that supported the greatest growth was about 1.5 mM (Figure 2.4B). The upper end of the concentration range was limited by the low solubility of these aromatic amino acids, particularly tyrosine. The resonances

corresponding to these amino acids were present in ^{1}H NMR spectra even at the reduced concentrations. The aromatic resonances, however, were located away from end products and did not interfere with quantification of the end products.

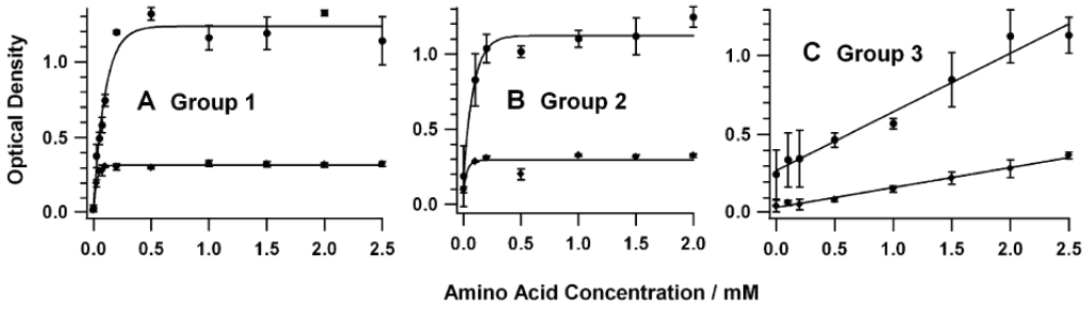


Figure 2.4. Growth of *F. varium* in response to varying the concentration of groups of amino acids on defined medium (■) and defined medium plus 10 mM glucose (●), averaged from nine different measurements. (A) Varying Group 1 amino acids while keeping Group 2 at 1.5 mM and Group 3 at 2.0 mM. (B) Varying Group 2 amino acids while keeping Group 1 at 0.2 mM and Group 3 at 2.0 mM. (C) Varying Group 3 amino acids while keeping Group 1 at 0.2 mM and Group 2 at 1.5 mM.[138] *Reproduced by permission of The Royal Society of Chemistry*

Signals for Group 3 amino acids, asparagine, aspartate, cysteine, glutamate, glutamine, histidine, lysine, serine and threonine did not appear in the ^{1}H NMR spectra of spent culture media. Group 3 amino acids produced a linear growth response in the absence and presence of 10 mM glucose when the amino acid concentration of this group was increased from 0 to 2.5 mM (Figure 2.4C).

The composition of the minimal medium was adjusted so the concentrations of Group 1, 2 and 3 amino acids were 0.2, 1.5 and 2.0 mM respectively, making a modified chemically defined minimal medium (CDMM*). CDMM*(10 mM glucose) supported abundant growth (OD_{660} 1.2 ± 0.1), similar to growth obtained on peptone or CDMM2(10 mM glucose). The ^{1}H NMR spectrum of spent CDMM*(10 mM glucose) medium (Figure 2.1C) had minimal residual amino acid resonances, particularly in the range of δ 2.4-0.7 (Figure 2.1C), and provided a smooth baseline for integrating the resonances of the carboxylate end products.

The growth response of Groups 1 and 2 amino acids compared to Group 3 differed considerably (Figure 2.4). Groups 1 and 2 had sharp increases in growth that leveled at higher amino acid concentrations, whereas Group 3 had a linear growth response. The different responses suggest different roles for these amino acids in the metabolism of *F. varium*. Group 1 and 2 amino acids were only needed at smaller concentrations, suggesting that these amino acids primarily serve as substrates for fundamental metabolic processes (e.g., protein biosynthesis), and are not metabolized for energy production. The utilization of Group 1 and 2 amino acids are consistent with previous results that show poor metabolism of hydrophobic and aromatic amino acids.[110,143,144] Conversely, the need for higher concentrations of Group 3 amino acids and the absence of resonances for these amino acids in the ^{1}H NMR spectra of spent culture medium indicate another metabolic role for Group 3 amino acids, in addition to serving as substrates for protein biosynthesis. Members for the Group 3 amino acids are regarded as energy sources, and the efficient utilization of glutamate, glutamine, histidine, lysine and serine by *F. varium* growing on peptone medium has been observed.[117] In defined medium lacking glucose, increasing

concentrations of Group 3 amino acids supported increased growth of *F. varium* (Figure 2.4C). When the defined medium was supplemented with glucose as the major energy source, Group 3 amino acids were needed to effectively derive energy from glucose, perhaps supporting the regeneration of NAD^+ needed for the oxidation of glucose. Further investigation of the individual requirements of amino acids will be discussed in Section 2.5.

2.3. Quantitative Determination of End Products

With a refined minimal medium that eliminated resonances interfering with the product resonances, it became possible to accurately quantify the metabolic end products using ^{1}H NMR spectroscopy. Eliminating unmetabolized amino acids ensured that resonances being quantified were the result of products of *F. varium* metabolism and not leftover components from the medium, leading to overestimation of the products. With the first criteria for quantitative NMR spectroscopy satisfied, the next step was to ensure the complete relaxation of protons being quantified through adjustment of the delay time.

The concentrations of end products in culture supernatants were calculated relative to the internal standards, dichloroacetic acid (DCA) and tartaric acid (TA). These standard acids were chosen since they would behave similarly as the carboxylic acid metabolic end products during sample processing and their singlet resonances at δ 6.01 and δ 4.28 are located away from the end product resonances. However, in initial experiments, use of the DCA integrated area led to unrealistically high concentrations of the carboxylic acid end products. Using the integrated area of the TA resonance, the concentration of formate was underestimated by approximately 20-30%. These

discrepancies were attributed to the incomplete relaxation of protons, because of the slower transfer of energy from the excited proton in DCA and formate.

2.3.1. Relaxation Time Determination

Proton spin-lattice relaxation times (T_1) can vary greatly and depend on the structure of molecule. The T_1 values of all the end products found in culture supernatants of *F. varium* and internal standards were determined for mixtures of standard compounds and end products in a lyophilized sample of the bacterial culture supernatant (Table 2.2).

Table 2.2. T_1 values for standard carboxylate ions and carboxylate end products in spent culture medium (determined at 250 MHz).

Carboxylate Ion	Chemical Shift δ (ppm)	Standard Sample T_1 (s)	Culture Sample T_1 (s)
Acetate	1.86 (s)	5.1 ± 0.4	2.5 ± 0.4
Butyrate	0.84 (t)	2.4 ± 0.1	1.9 ± 0.2
	1.51 (m)	1.1 ± 0.2	1.6 ± 0.1
	2.10 (t)	2.2 ± 0.1	ND
DCA	6.01 (s)	13.8 ± 0.5	5.5 ± 0.8
Formate	8.40 (s)	11.8 ± 0.7	6.2 ± 0.3
Lactate	1.28 (d)	0.9 ± 0.1	0.6 ± 0.1
	4.07 (q)	2.1 ± 0.5	ND
Propionate	1.00 (t)	4.7 ± 0.8	2.9 ± 0.4
	2.13 (q)	4.2 ± 0.8	ND
Succinate	2.35 (s)	1.9 ± 0.1	0.8 ± 0.1
TA	4.28 (s)	2.8 ± 0.1	0.5 ± 0.1

ND = not determined

The standard samples of DCA and formate had relaxation times greater than 10 s, while all the other end product standards had relaxation times less than 6 s (Table 2.2). The single protons in dichloroacetate and formate do not have surrounding protons to

help relaxation, resulting in longer T_1 relaxation times. The measured T_1 value for formate was 11.8 ± 0.7 s, in close agreement with the previously reported T_1 value for formate of 11.651 ± 0.002 s.[145] The T_1 values measured for compounds in the supernatants of spent cultures were significantly shorter, indicating that the medium plays a role in proton relaxation. The CDMM contains $MnSO_4$, a paramagnetic compound that helps protons relax. Similar T_1 relaxation times have been reported[146] for acetate (3.58 ± 0.71) and formate (6.59 ± 0.84) in human blood serum. In that instance, the shorter relaxation times were attributed to interactions between carboxylic acids and proteins.

To determine an appropriate delay time for quantitative ^{1}H NMR spectroscopy, a standard experiment was performed on four carboxylic acid sodium salts dissolved in D_2O. Delay times (D1) of 1, 5, 7 and 10 s were used while keeping all other parameters constant. Concentrations were calculated from the ^{1}H NMR spectra using either DCA or TA as an internal standard and reported as a percentage of the actual amounts dissolved (Table 2.3). In this experiment, the end product concentrations calculated were closer to actual concentrations using TA (Table 2.3B), with formate as an exception, while concentrations differed more significantly using DCA (Table 2.3A) as an internal standard. Increasing the D1 time decreased the differences between actual and calculated concentrations and similar results for the two internal standards were obtained at 10 s. Improved results using spent culture samples would be expected since carboxylate ion protons have lower T1 times in this environment (see Table 2.2). For all quantitative ^{1}H NMR analysis, a 10 s delay time was adopted to allow the majority of end products to relax within an experimental run time of 7 min per sample.

Table 2.3. Effects of delay time on quantitative analysis of end products, percentage compared to actual amount added. Internal standards DCA and TA were used to quantify carboxylate ions

.

Int. Std.				Percentage of actual amount			
DCA	**Substrate**	**δ (ppm)**	**Delay time:**	**1 s**	**5 s**	**7 s**	**10 s**
	Acetate	1.86 (s)		114.0	115.2	103.7	101.4
	Butyrate	1.51 (m)		122.0	119.8	108.1	104.5
	Formate	8.40 (s)		102.1	105.5	96.9	94.3
	Succinate	2.35 (s)		114.2	110.7	101.1	97.0
TA							
	Acetate	1.86 (s)		98.6	99.6	100.2	101.5
	Butyrate	1.51 (m)		105.6	103.6	104.5	104.6
	Formate	8.40 (s)		88.4	91.3	93.7	94.4
	Succinate	2.35 (s)		98.7	95.9	97.6	97.0

2.4. Method Reproducibility

With a medium and method to quantify end products of *F. varium* in place, it was necessary to ensure the method was reproducible. The reproducibility of the method was tested on three levels. First, using a standard sample, where known concentrations of the carboxylate products were added, end products were quantified using the method determined in Section 2.3. Typically, the concentrations calculated relative to each internal standard agreed with the actual value within one standard deviation (Table 2.4, Entry 1). Secondly, spectrometer variability was tested by taking five independent spectral acquisitions of the same sample solution (10 mg/0.75 mL D_2O) (Table 2.4, Entry 2); the variation was within one standard deviation. Finally, sample homogeneity was tested; five replicate 10 mg samples were taken from the same lyophilized residue (214 mg) (Table 2.4, Entry 3). Variability in the determined concentrations had small standard

deviations, indicating that the sample was homogeneous and that taking a small sample is representative of the large bulk sample. The variability of sampling and spectral acquisition was less than the variability between cultures (Table 2.4, Entry 4) and all quantitative data measurements for cultures were obtained by averaging at least three independent experiments. Due to the overlapping resonances for butyrate and propionate at 2.1 ppm, the resonances for butyrate at 1.44 ppm and propionate at 0.94 ppm were integrated to quantify those end products.

Table 2.4. Method reproducibility: ^{1}H NMR spectra were acquired using a 10 s relaxation delay (D1) in the pulse sequence

			Concentration (mM)					
Entry	Sample	Int. Std	Acetate	Butyrate	Formate	Lactate	Propionate	Succinate
1	Standard solution	Actual	3.8	4.3	4.9	4.2	4.7	5.3
		DCA	4.1 ± 0.1	4.6 ± 0.3	4.7 ± 0.1	4.3 ± 0.2	4.9 ± 0.2	4.4 ± 0.1
		TA	3.9 ± 0.2	4.3 ± 0.2	4.7 ± 0.1	4.0 ± 0.1	4.6 ± 0.1	4.6 ± 0.2
2	CDMM* (10 mM glucose)[a]	TA	5.0 ± 0.1	11.2 ± 0.1	2.5 ± 0.1	1.7 ± 0.1	1.2 ± 0.1	0.5 ± 0.1
3	CDMM* (10 mM glucose)[b]	DCA	5.1 ± 0.1	11.7 ± 0.1	1.8 ± 0.2	1.9 ± 0.1	1.4 ± 0.1	0.5 ± 0.1
		TA	4.9 ± 0.1	11.3 ± 0.4	1.7 ± 0.1	1.8 ± 0.1	1.3 ± 0.2	0.5 ± 0.1
4	CDMM* (10 mM glucose)[c]	DCA	6.3 ± 1.5	12 ± 1	3 ± 2	2.4 ± 0.5	1.5 ± 0.2	2 ± 3

[a]Average (± standard deviation) of five independent spectral acquisitions on the same sample solution (10 mg/0.75 mL D_2O). [b]Average (± standard deviation) of five replicate 10 mg samples taken from the same lyophilized residue (214 mg). [c]Average of 4–6 replicate cultures

2.5. Amino Acid Requirements of *F. varium*

When all amino acids were omitted from defined medium, no growth of *F. varium* occurred, indicating an amino acid (or nitrogen) requirement. In the genome sequencing studies of the related organism *F. nucleatum*,[123] only a few genes encoding enzymes involved in the biosynthesis of amino acids were annotated. The absence of the corresponding genes in *F. varium* would suggest that *F. varium* would show a growth requirement for certain amino acids. In addition, the different concentrations of amino acids to support abundant growth of *F. varium* discovered when refining the CDMM indicated different roles for groups of amino acids.

To test the ability of *F. varium* to biosynthesize amino acids, a series of experiments was preformed using CDMM*(10 mM glucose) where each amino acid was individually omitted from the growth medium. The OD_{660} and accumulation of end products were determined. These experiments were preformed in triplicate, an average of 9 different OD_{660} readings. The results indicated four categories of growth responses towards amino acids; equivalent, good, moderate and poor growth compared to the control (CDMM*(10 mM glucose)). Elimination of alanine, asparagine, aspartate or proline from CDMM*(10 mM glucose) had little or no effect on growth (>75% of the control) (Table 2.5) and similar amounts of carboxylic acid end products were produced (Table 2.6).

Small effects on growth (OD 65-75% of the control) were observed when either glutamate, lysine, serine or threonine was eliminated from the growth medium (Table 2. 7). Moderate growth (OD 35-50% of the control) was achieved when cysteine, glutamine, glycine, histidine, phenylalanine, tryptophan or tyrosine were not included in

the medium (Table 2.8). Comparing the production of carboxylic acid end products to the control (CDMM*/10 mM glucose) indicated the production of similar concentrations of acetate, formate, lactate and succinate with smaller concentrations of butyrate and propionate were produced in both categories (Tables 2.9 and 2.10).

Table 2.5. Growth obtained when either alanine, asparagine, aspartate or proline was eliminated from CDMM*(10 mM glucose)

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Omitted	OD_{660}	% [a]
Asn	0.8 ± 0.3	79 ± 15
Ala	1.1 ± 0.2	138 ± 44
Asp	0.9 ± 0.3	89 ± 16
Pro	1.1 ± 0.2	140 ± 59
Average	1.0	112
Error	0.1	19[b]

[a]Percentages were calculated relative to the control sample, in which all amino acids were present (CDMM*(10 mM glucose)). [b]The uncertainty of the average is the square root of the sum of the standard deviation divided by the number of amino acids.

Table 2.6. End product accumulation when either alanine, asparagine, aspartate or proline was eliminated from CDMM*(10 mM glucose).

	End Products (mM)[a]						Residual
Omitted	Acetate	Butyrate	Formate	Lactate	Propionate	Succinate	Glucose
-	6.3 ± 1.5	12 ± 1	3 ± 2	2.4 ± 0.5	1.5 ± 0.2	2 ± 3	0
Asn	6.0 ± 1.9	6.9 ± 5.9	2.8 ± 1.6	2.1 ± 1.1	1.1 ± 0.9	0.3 ± 0.1	2.6 ± 3.4
Ala	4.1 ± 0.8	10.1 ± 3.8	1.4 ± 0.5	2.7 ± 0.8	1.4 ± 0.5	0.3 ± 0.1	0.4 ± 0.4
Asp	5.7 ± 2.2	6.1 ± 4.6	2.5 ± 2.0	1.6 ± 1.3	1.0 ± 0.9	0.4 ± 0.2	3.2 ± 4.2
Pro	4.2 ± 0.9	9.8 ± 1.3	1.8 ± 0.5	2.8 ± 1.4	1.1 ± 0.2	0.3 ± 0.1	0.3 ± 0.2
Average[b]	5.0	8.3	2.1	2.3	1.1	0.3	1.61
Error[c]	0.8	2.1	0.7	0.6	0.3	0.1	1.34

[a]End product concentrations are the average of at least three independent experiments ± standard deviation. [b]Average of the four amino acid experiments. [c]The uncertainty of the average is the square root of the sum of the standard deviation divided by the number of amino acids.

Table 2.7. Growth obtained when either glutamate, lysine, serine or threonine was eliminated from CDMM*(10 mM glucose).

Omitted	OD_{660}	%[a]
Glu	0.6 ± 0.2	66 ± 12
Lys	0.7 ± 0.2	76 ± 21
Ser	0.6 ± 0.3	71 ± 23
Thr	0.7 ± 0.2	68 ± 8
Average	0.7	70
Error[b]	0.1	9

[a]Percentages were calculated relative to the control sample, in which amino acids were present (CDMM*(10 mM glucose)). [b]The uncertainty of the average is the square root of the sum of the standard deviation divided by the number of amino acids.

Table 2.8. Growth obtained when either cysteine, glutamine, glycine, histidine, phenylalanine, tryptophan or tyrosine was eliminated from CDMM*(10 mM glucose).

Omitted	OD_{660}	%[a]
Cys	0.4 ± 0.2	47 ±18
Gln	0.5 ± 0.2	46 ± 18
His	0.4 ± 0.3	48 ± 22
Gly	0.4 ± 0.1	55 ± 14
Phe	0.5 ± 0.1	53 ± 10
Trp	0.4 ± 0.2	47 ± 25
Tyr	0.3 ± 0.2	34 ± 25
Average	0.4	41
Error	0.1	7[b]

[a]Percentages were calculated from the control sample, in which all amino acids were present (CDMM*(10 mM glucose)). [b]The uncertainty of the average is the square root of the sum of the standard deviation divided by the number of amino acids.

Table 2.9. End product accumulation when either glutamate, lysine, serine or threonine was eliminated from CDMM*(10 mM glucose).

	End Product (mM)[a]						Residual
Omitted	Acetate	Butyrate	Formate	Lactate	Propionate	Succinate	Glucose
-	6.3 ± 1.5	12 ± 1	3 ± 2	2.4 ± 0.5	1.5 ± 0.2	2 ± 3	0
Glu	4.6 ± 1.5	3.0 ± 3.1	3.9 ± 2.2	1.9 ± 1.3	0.5 ± 0.8	0.5 ± 0.5	4.5 ± 3.7
Lys	4.0 ± 1.1	3.4 ± 3.9	3.0 ± 1.6	1.2 ± 0.4	0.7 ± 0.9	0.6 ± 0.8	3.8 ± 2.9
Ser	5.1 ± 2.4	2.8 ± 3.4	3.2 ± 2.3	1.8 ± 1.7	0.6 ± 0.9	0.3 ± 0.3	5.3 ± 4.4
Thr	6.9 ± 2.2	4.5 ± 3.8	4.9 ± 2.7	2.0 ± 1.6	0.0 ± 0.0	0.5 ± 0.2	3.7 ± 3.5
Average[b]	6.0	3.5	2.9	1.4	0.5	0.4	4.3
Error[c]	0.6	0.9	0.6	0.4	0.2	0.1	1.8

[a]End product concentrations are the average of at least three independent experiments ± standard deviation. [b]Average of the four amino acid experiments. [c]The uncertainty of the average is the square root of the sum of the standard deviation divided by the number of amino acids.

Table 2.10. End product accumulation when either cysteine, glutamine, glycine, histidine, phenylalanine, tryptophan or tyrosine was eliminated from CDMM*(10 mM glucose).

	End Product (mM)						Residual
Omitted	Acetate	Butyrate	Formate	Lactate	Propionate	Succinate	Glucose
-	6.3 ± 1.5	12 ± 1	3 ± 2	2.4 ± 0.5	1.5 ± 0.2	2 ± 3	0
Cys	5.5 ± 3.1	0.7 ± 0.60	4.0 ± 1.7	2.6 ± 1.8	0.0 ± 0.1	0.3 ± 0.2	5.3 ± 4.5
Gln	5.7 ± 1.7	2.7 ± 1.8	2.6 ± 1.8	1.5 ± 1.3	0.3 ± 0.3	0.6 ± 0.5	6.9 ± 2.8
His	5.6 ± 2.1	1.3 ± 1.3	3.1 ± 2.1	1.8 ± 1.5	0.2 ± 0.3	0.3 ± 0.2	5.6 ± 3.9
Gly	7.2 ± 0.4	5.2 ± 1.9	1.5 ± 0.1	0.6 ± 1.1	0.5 ± 0.6	0.3 ± 0.2	5.1 ± 3.4
Phe	8.7 ± 2.7	5.1 ± 2.5	3.1 ± 2.3	0.7 ± 1.0	0.5 ± 0.5	0.3 ± 0.2	3.7 ± 2.7
Trp	7.2 ± 0.9	4.8 ± 2.2	2.1 ± 2.0	0.4 ± 0.7	0.8 ± 0.6	0.3 ± 0.3	3.8 ± 4.3
Tyr	8.2 ± 2.2	4.5 ± 4.4	1.7 ± 1.4	0.5 ± 1.0	0.8 ± 0.9	0.5 ± 0.6	6.3 ± 4.8
Average	6.0	3.5	2.9	1.4	0.5	0.4	4.6
Error	0.6	0.9	0.6	0.4	0.2	0.1	1.4

[a]End product concentrations are the average of at least three independent experiments ± standard deviation. [b]Average of the seven amino acid experiments. [c]The uncertainty of the average is the square root of the sum of the standard deviation divided by the number of amino acids.

Poor growth (OD < 35% of the control) and small amounts of end products were observed when any of arginine, isoleucine, leucine, methionine or valine were omitted from the growth medium (Tables 2.11). Succinate and propionate were not detected and large amounts of glucose (>5 mM) remained in the culture supernatant (Table 2.12).

Table 2.11. Growth obtained when any of arginine, isoleucine, leucine, methionine or valine was eliminated from CDMM*(10 mM glucose).

Omitted	OD_{660}	%[a]
Arg	0.1 ± 0.2	24 ±24
Ile	0.1 ± 0.03	8 ± 7
Met	0.1 ± 0.02	8± 5
Val	0.2 ± 0.3	25 ± 32
Leu	0.2 ± 0.3	32 ± 34
Average	0.2	20
Error	0.1	11[b]

[a]Percentages were calculated relative to the control sample, in which all amino acids were present (CDMM*(10 mM glucose)). [b]The uncertainty of the average is the square root of the sum of the standard deviation divided by the number of amino acids.

The pattern of the responses when different amino acids were eliminated from the growth medium indicated a correlation of growth with residual glucose and the accumulation of butyrate and lactate. In cultures showing poor growth (Table 2.12), butyrate and lactate production were low (< 0.8 mM) and residual glucose was high; increased butyrate (usually 1–5 mM) and lactate (0.5–2.4 mM) and decreased glucose, were present with good or moderate growth (Tables 2.9 and 2.10). The highest levels of butyrate (6–10 mM) and lactate (1.5–3 mM), and the least residual glucose, were seen

with the greatest growth (Table 2.6). The results illustrate correlations of growth with glucose utilization and the accumulation of reduced end products.

Table 2.12. End product accumulation when either arginine, isoleucine, leucine, methionine or valine was eliminated from CDMM*(10 mM glucose).

	End Products (mM)[a]						Residual
Omitted	Acetate	Butyrate	Formate	Lactate	Propionate	Succinate	Glucose
-	6.3 ± 1.5	12 ± 1	3 ± 2	2.4 ± 0.5	1.5 ± 0.2	2 ± 3	0
Arg	4.8 ± 2.4	0.4 ± 0.1	0.0 ± 0.0	0.6 ± 1.0	0	0	6.2 ± 2.3
Ile	2.2 ± 0.8	0.1 ± 0.1	0.1 ± 0.1	0.4 ± 0.7	0	0	5.6 ± 2.4
Met	2.4 ± 0.8	0.4 ± 0.2	0.1 ± 0.1	0.4 ± 0.8	0	0	8.0 ± 1.7
Val	2.6 1.9	0.3 ± 0.1	1.2 ± 2.3	1.8 ± 0.2	0	0	6.3 ± 2.9
Leu	2.9 ± 1.4	0.5 ± 0.5	1.7 ± 2.1	0.4 ± 0.9	0	0	7.6 ± 1.8
Average[b]	3.0	0.4	0.6	0.7	0	0	6.7
Error[c]	0.7	0.1	0.6	0.4	0	0	1.0

[a]End product concentrations are the average of at least three independent experiments ± standard deviation. [b]Average of the five average of each amino acid experiments. [c]The uncertainty of the average is the square root of the sum of the standard deviation divided by the number of amino acids.

Analysis of the genome of *F. varium*[19] suggests there are limited capabilities of *F. varium* to biosynthesize some amino acids. One or more genes from the biosynthetic pathways for arginine, isoleucine, leucine, methionine and valine, the amino acids that had poor growth when eliminated, were missing. This would indicate that the organism is unable to make these amino acids and must rely on the presence of these amino acids in the growth medium.

By contrast, genes from the biosynthetic pathways for alanine, asparagine, aspartate and proline were documented in the genome.[19] These amino acids were not

required to support abundant growth of the organism. As well, genes from glutamate, lysine, serine and threonine biosynthetic pathways were present in the genome. Good growth of the organism was observed when these amino acids were omitted from the growth medium. This demonstrates that *F. varium* can make these amino acids from other sources if they are not present in the growth environment. The ability to biosynthesize amino acids, while others must be supplied for growth (the essential amino acids) have been reported for humans and bacteria.[147] The growth experiments and genes present are consistent with one another, confirming that the organism is able to biosynthesize these amino acids, while other amino acids must be supplied in the growth medium. These metabolic studies complement genomic information and provide a more integrated understanding of amino acid metabolism in *F. varium.*

Amino acids can act as energy sources in *F. varium*[117] and many of the Group 3 amino acids (asparagine, aspartate, cysteine, glutamate, glutamine, histidine, lysine, serine and threonine) are regarded as energy sources.[117] As well, genes for the biosynthetic pathway enzymes for all of the Group 3 amino acids have been documented in the genome. Thus *F. varium* is able to synthesize required quantities of these amino acids and can afford to catabolize excess quantities for energy production, as indicated by the efficient degradation of Group 3 amino acids at high concentrations (2 mM).

Group 1 amino acids, based on their limited utilization, are most likely utilized for fundamental metabolic processes, such as protein biosynthesis, and are not used as energy sources. Biosynthetic pathway genes for several Group 1 amino acids (Arg, Ile, Leu, Met, Val) are not in the genome, in accord with the observed growth requirements of the organism.

The role of threonine and glutamate as energy sources and their metabolic pathways will be further demonstrated in the following chapters.

CHAPTER 3: ENERGY SOURCES AND THEIR EFFECTS ON THE EXOMETABOLOME

Chemoorganotrophic anaerobic bacteria derive energy from the oxidation of carbon-containing substrates.[148] The energy producing pathways in anaerobes also typically require oxidation steps, thereby generating reduced coenzymes. Without the use of oxygen as a terminal electron acceptor, anaerobes maintain redox balance by transferring electrons from reduced coenzymes to a variety of metabolic intermediates. The reduced products are often found in the exometabolome, while the oxidized coenzymes become available for further energy production through the oxidative degradation of carbon containing substrates.

The qualitative and quantitative method described in Chapter 2 was applied to determine the effects of possible energy sources on the exometabolome, including the determination of substrate-product relationships. In the human gastrointestinal tract, the availability of different substrates may influence the production of bacterial metabolites.[149,150,151] While SCFA metabolites generally are regarded to exert a beneficial effect on epithelial cells,[51] undesirable effects may arise from the production of other metabolites.

3.1. End Product Metabolism

In Chapter 2, six carboxylic acids were detected by ^{1}H NMR spectroscopy after growth of *F. varium* on CDMM*(10 mM glucose); acetate, butyrate, formate, lactate, propionate and succinate. To determine whether these end products were metabolically inert after they appear in culture fluid, *F. varium* was cultured on CDMM* supplemented

with each end product at 10 mM (Table 3.1). Glucose was not included in the medium to see if the end product could support growth of the organism. The OD_{660} value for the cultures ranged from 0.3 to 0.4, matching growth when no additional substrate was supplied (CDMM*, OD_{660} = 0.3). When compared to the control (CDMM*) cultures, increased accumulation of acetate and formate were evident when each end product was added, but overall, there was very little change in the end product distribution (Table 3.1). For each carboxylate product, the final concentration was ≥ 10 mM, corresponding to the sum of the initial amount and the expected production during the incubation period. Overall, very little metabolism was indicated by these results; however, the growth in each case was poor, suggesting a low level of metabolic activity.

Table 3.1. Growth and end product accumulation in 16-h *F. varium* CDMM* cultures supplemented with a carboxylic acid (10 mM).

Supplement	End Product (mM)[a]					
(10 mM)	Acetate	Butyrate	Formate	Lactate	Propionate	Succinate
none	8.6 ± 0.9	4.2 ± 0.5	0.7 ± 0.7	0.0	1.7 ± 0.1	0.4 ± 0.1
Acetate	**23.4 ± 3.4**	4.7 ± 0.8	3.6 ± 0.5	0.0	2.5 ± 0.4	1.1 ± 0.1
Butyrate	12.2 ± 1.9	**13.5 ± 0.8**	2.6 ± 2.3	0.0	2.4 ± 0.2	1.0 ± 0.6
Formate	11.7 ± 1.7	4.6 ± 0.5	**13.2 ± 0.5**	0.0	2.2 ± 0.1	1.1 ± 0.3
Lactate	12.7 ± 0.1	3.2 ± 0.7	4.4 ± 0.1	**10.5 ± 0.1**	2.1 ± 0.2	1.1 ± 0.1
Propionate	12.7 ± 0.2	3.8 ± 0.9	2.7 ±0.1	0.3 ± 0.2	**12.0 ± 0.2**	1.1 ± 0.2
Succinate	12.6 ± 0.4	3.3 ±0.6	4.4 ± 0.1	0.0	2.1 ± 0.1	**9.9 ± 0.5**

[a] Concentrations are reported as the average of at least three independent growth experiments ± standard deviations. Bold concentrations refer to product that is also supplemented.

When the same carboxylic acids were supplied to *F. varium* in CDMM*(10 mM glucose/10 mM substrate) cultures, there was greater growth (OD_{660} > 1.0) for each culture (Table 3.2). The final concentrations of formate, lactate and propionate were approximately 10 mM, indicating little or no metabolism of these end products. Otherwise, the end product distributions were similar to those in the control CDMM*(10 mM glucose) culture. The concentration of succinate was low, along with the concentrations of other end products. Higher concentrations of acetate and butyrate were found in the butyrate-supplemented and the acetate-supplemented cultures, respectively. This indicates that these end products are able to interconvert to one another, in good agreement with previous results that showed the interconversion of acetate to butyrate using peptone medium and resuspended cells.[93] The acetate and butyrate interconversion reflects the energy or redox needs of the organism; acetate is a product of ATP production, while butyrate is produced during NAD^+ regeneration (Scheme 1.2).

Similar results were collected using an *in vitro* model of human intestinal fermentation inoculated with standardized gastrointestinal microbiota.[152] The stable isotope metabolic flux analysis revealed that acetate was converted to acetyl-CoA while lactate was not converted to pyruvate. Oxidation of lactate to pyruvate requires the coenzyme NAD^+, increasing the need for regenerated coenzyme making this reaction unfavourable in *F. varium* especially in the presence of glucose, a preferred energy source that also requires oxidation.

Table 3.2. Growth and end product accumulation in 24-h *F. varium* CDMM*(10 mM glucose) cultures supplemented with a carboxylic acid (10 mM).

Substrate		End Products (mM)[b]					
(10 mM)	OD_{660}[a]	Acetate	Butyrate	Formate	Lactate	Propionate	Succinate
none	1.0 ± 0.1	7.1	11.7	4.9	3.7	1.5	0.8
Acetate	1.2 ± 0.1	**8.9**	18.5	1.7	1.7	1.7	0.6
Butyrate	1.1 ± 0.1	21.9	**16.5**	4.6	3.1	1.2	0.3
Formate	1.1 ± 0.1	5.3	13.4	**10.4**	2.7	1.3	0.3
Lactate	1.1 ± 0.1	5.7	12.0	1.2	**12.4**	1.2	0.4
Propionate	1.0 ± 0.2	9.3	8.5	0.5	2.0	**10.2**	0.5
Succinate	1.1 ± 0.2	4.6	7.6	1.2	0.9	0.9	**5.7**

[a]OD_{660} values are reported as the average of three growth experiments ± standard deviations. [b]End Products are from three culture tubes combined and averaged from one experiment. Bold numbers refer to product that is supplemented.

3.2. Metabolism of Acetate-Butyrate Pathway Intermediates

While catabolism of glucose is a source of acetyl-CoA, intermediates in the acetate-butyrate pathway (Scheme 1.2) also can be converted to acetyl-CoA and are possible energy sources. Intermediates along the acetate-butyrate pathway were supplied as carboxylic acids to CDMM* cultures of *F. varium* (Table 3.3) and growth and end product accumulation were determined at 16 h. Although conversion of the carboxylic acid form supplied to the CoA ester requires one mole of ATP per mole of acid, two moles of acetyl-CoA are formed upon cleavage of each substrate, allowing a net gain of ATP. Increased growth of *F. varium* was observed in the cultures containing acetoacetate, (*R*)-3-hydroxybutyrate and crotonate, but doubling the substrate concentration resulted in only slight increases in growth (Table 3.3).

Table 3.3. Growth after incubating cells with CDMM* and acetate-butyrate pathway intermediates at 10 and 20 mM.

Additional Substrate	OD_{660}[a]	
	10 mM	20 mM
none	0.30 ± 0.04	
Acetoacetate	0.4 ± 0.1	0.5 ± 0.2
(*R*)-3-Hydroxybutyrate	0.6 ± 0.1	0.6 ± 0.1
Crotonate	0.5 ± 0.1	0.6 ± 0.1

[a]OD_{660} values are reported as the average of three independent experiments ± standard deviations.

All substrates were utilized readily; after 16 h less than 1 mM of each was left over when the substrate was detected. Similar amounts of formate, lactate, propionate and succinate were produced by acetoacetate, crotonate and (*R*)-3-hydroxybutyrate supplemented cultures and the control CDMM* culture (Figure 3.1). Larger amounts of acetate and butyrate were produced, consistent with the direct metabolic connection shown in the acetate-butyrate pathway (Scheme 1.2).

Both acetate and butyrate were formed in the cultures supplemented with crotonate and (*R*)-3-hydroxybutyrate, indicating that both substrates underwent oxidation to acetate for energy production and reduction to butyrate to regenerate the NAD^+ coenzyme needed for energy production. To maintain redox balance, two moles of acetate would form for each mole of butyrate; the increased amounts of acetate and butyrate over the control culture (Figure 3.1) are consistent with this expected 2:1 ratio. Overall, one net ATP is generated for two moles of substrate.

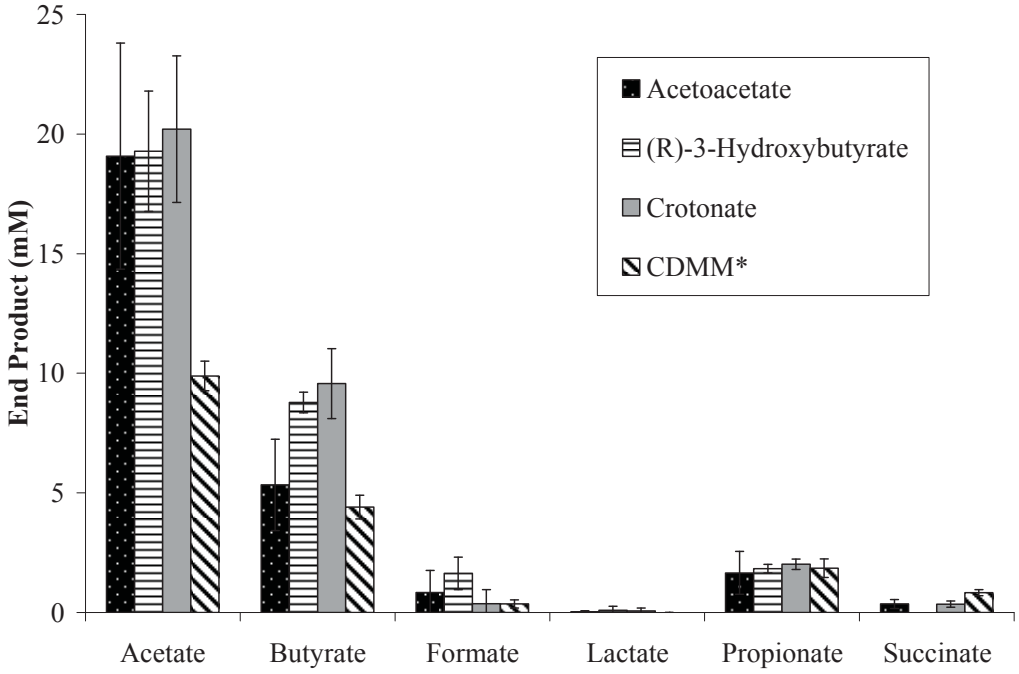


Figure 3.1. End product distribution after supplying intermediates along the acetate butyrate pathway as additional substrates at 10 mM to CDMM* cultures. Error bars represent the standard deviation obtained from three independent growth experiments.

Catabolism of acetoacetate to acetate involves neither oxidation nor reduction. The accumulation of butyrate was equivalent to that in the CDMM* control (Figure 3.1), indicating that coenzyme regeneration was not associated with acetoacetate degradation. One net mole of ATP is generated per mole of substrate.

3.3. Glucose, Glycerol and Pyruvate

Glucose, glycerol and pyruvate were tested as energy sources in *F. varium*, and their effect on end product distribution was determined. Each substrate produces acetyl-CoA, as an intermediate of the acetate-butyrate pathway (Scheme 1.2). Glucose, through

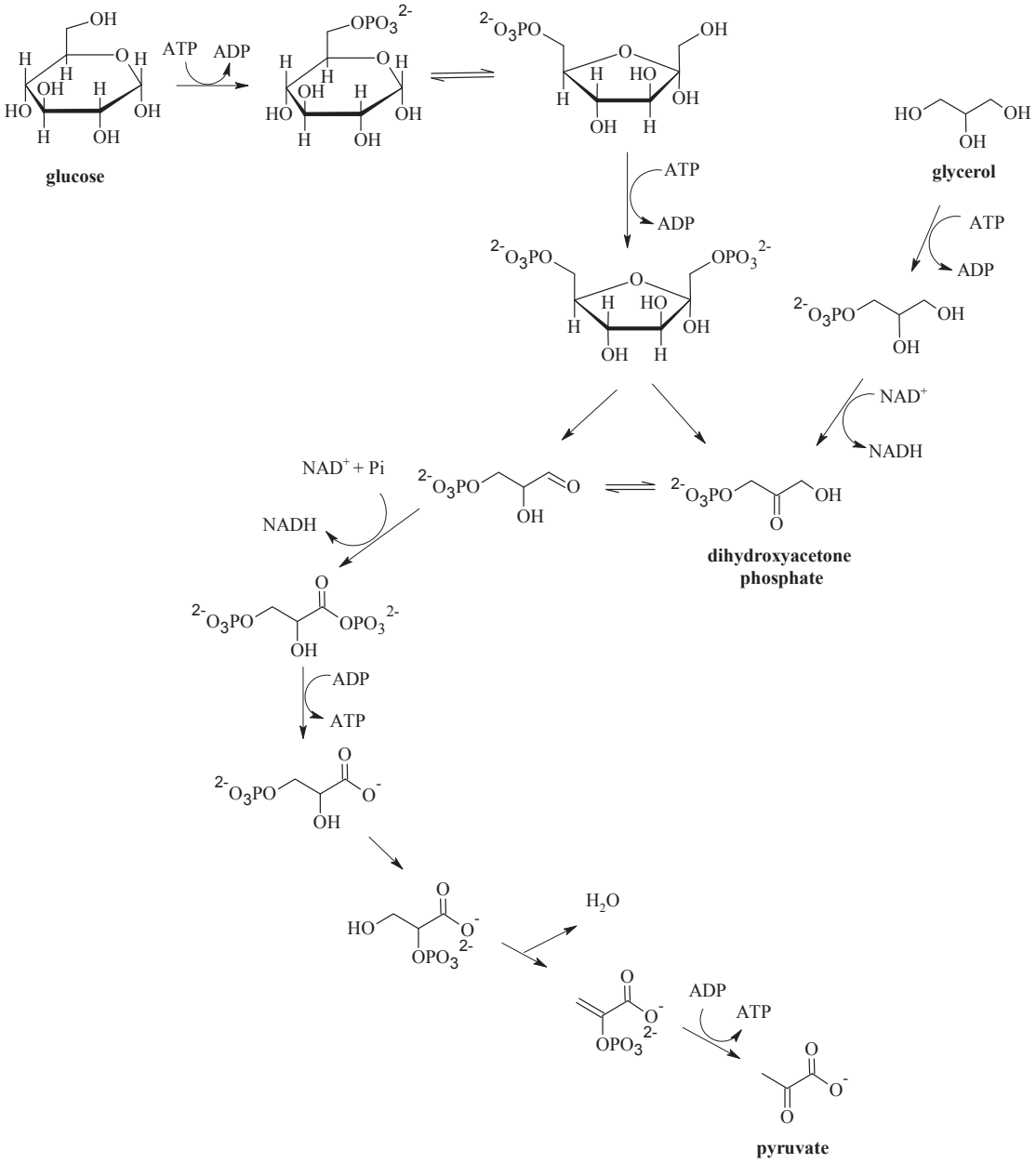
the Embden-Meyerhoff-Parnas (EMP) pathway[113] is converted to 2 pyruvate ions, generating 2 ATP molecules and 2 NADH molecules (Scheme 3.1). Glycerol is converted to glycerol-3-phosphate using glycerol kinase (E.C. 2.7.1.30), and then using glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8), glycerol-3-phosphate is converted to dihydroxyacetone-phosphate, an intermediate in the EMP pathway that subsequently forms pyruvate. Overall glycerol catabolism produces 2 NADH molecules and 1 ATP molecule (Scheme 3.1). Pyruvate can directly be converted to acetyl-CoA through two possible reactions (Scheme 3.2). All of the EMP enzymes and glycerol enzymes have been encoded in the *F. varium* genome.[19]

Very good growth, OD_{660} reaching 1.0, was obtained when glucose was supplied as a substrate, while glycerol and pyruvate led to smaller OD_{660} values, which were slightly above the control CDMM* culture that contained no additional substrate (Table 3.4). Small OD_{660} increases were observed when the substrate concentration was doubled.

Table 3.4. Growth after incubating cells for 16 h with CDMM* and glucose, glycerol or pyruvate at 10 and 20 mM.

	OD_{660}[a]	
Substrate	10 mM	20 mM
CDMM* (none)	0.30 ± 0.04	
D -Glucose	1.0 ± 0.1	1.2 ± 0.2
Glycerol	0.4 ± 0.1	0.5 ± 0.1
Pyruvate	0.4 ± 0.1	0.5 ± 0.1

[a]Average of three independent experiments ± standard deviation.



Scheme 3.1. Catabolism of glucose and glycerol to pyruvate in *F. varium*. Glycolysis produces two molecules of pyruvate, the second through the conversion of dihydroxyacetone to glyceraldehyde-3-phosphate. There is a net yield of 2 ATP molecules for glycolysis and 2 NADH molecules are produced. Glycerol catabolism produces 2 NADH molecules and 1 net ATP molecules.

Table 3.5. Amount of residual glucose, glycerol or pyruvate after 16 h in culture supernatants when added to CDMM*.

	Residual Substrate (mM) [a]	
Substrate	10 mM	20 mM
Glucose	2.1 ± 2.6	6.8 ± 6.0
Glycerol	2.7 ± 2.6	9.6 ± 4.0
Pyruvate	1.5 ± 2.6	0.4 ± 0.6

[a]Concentrations are reported on the average of at least three independent growth experiments ± standard deviations

At 10 mM, the substrates were mostly utilized, less than 3 mM of each substrate was detected by ^{1}H NMR analysis (Table 3.5). When the substrate was supplied at 20 mM, the residual glucose and glycerol in replicate cultures ranged from 1.4 to 13.3 mM and 5.0 to 14.7 mM respectively. Pyruvate was readily utilized even at the higher concentration. The end production distribution obtained with glucose, glycerol and pyruvate as substrates and that determined when no additional substrate was added (CDMM*), showed patterns unique to each culture condition (Figure 3.2), although each had similar concentrations of succinate and propionate. The highest concentrations of lactate and butyrate were produced in the glucose culture. Both products are formed as a consequence of coenzyme regeneration, enabling further oxidation of glucose and generation of ATP. Elevated amounts of butyrate were also present in the glycerol-supplemented culture, consistent with the need for coenzyme regeneration for the oxidative steps of glycerol catabolism.

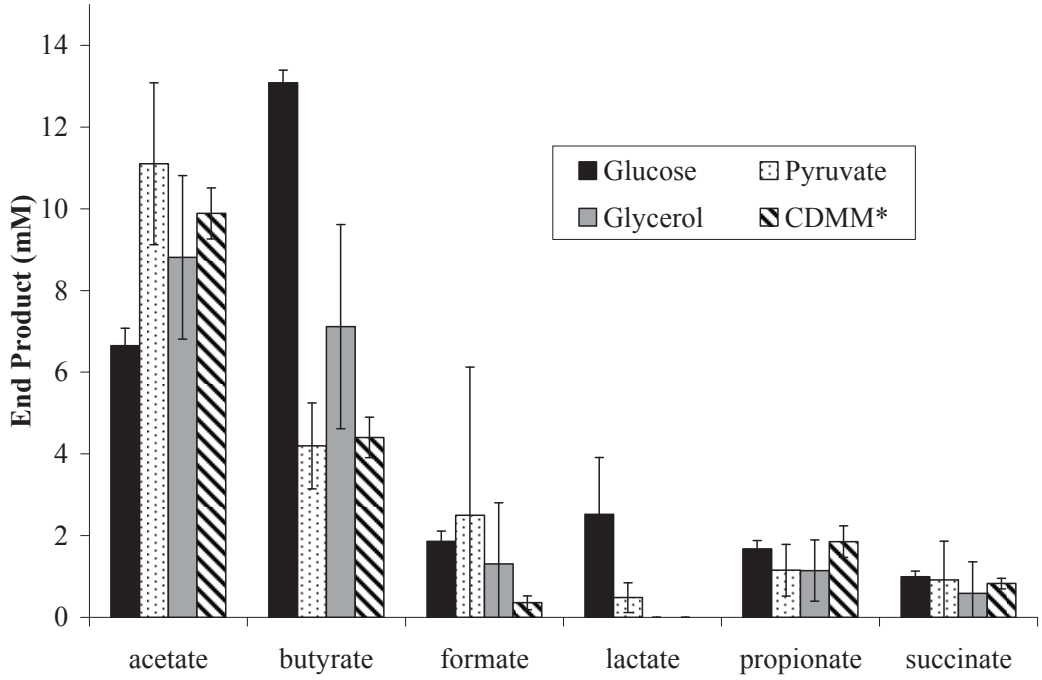
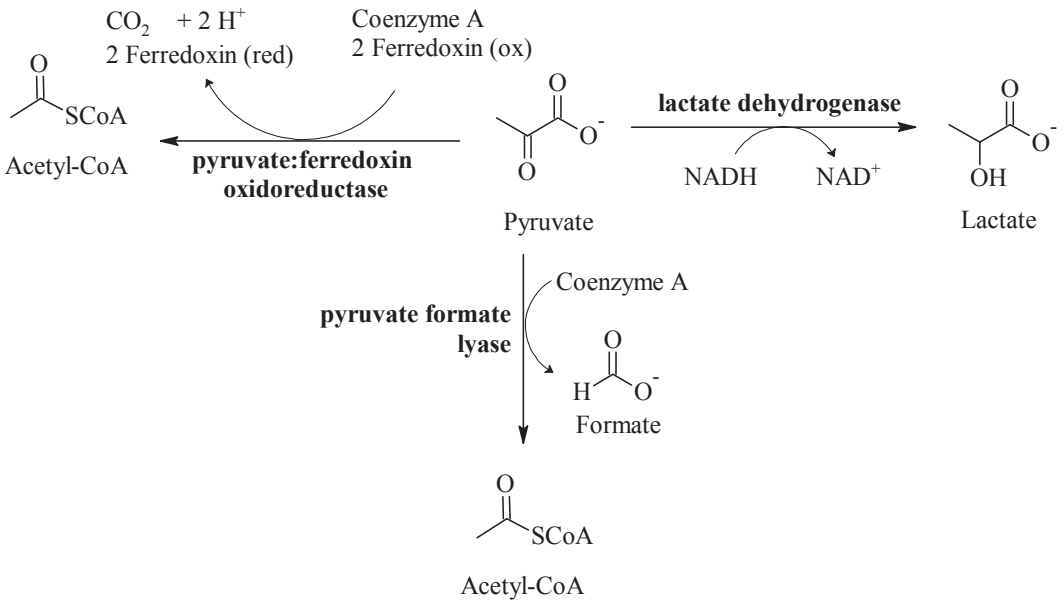


Figure 3.2. End product distribution at 16 h after growth of *F. varium* on CDMM* supplemented with either glucose, glycerol or pyruvate at 10 mM. The concentrations are reported as the average of three independent growth experiments and error bars represent the standard deviation.

In the pyruvate-supplemented culture, butyrate accumulation was identical to that in the CDMM* control, indicating that pyruvate is converted non-oxidatively to acetyl-CoA using pyruvate formate lyase (Scheme 3.2). Indeed, higher but variable levels of formate were observed in the cultures supplemented with pyruvate, but also in the cultures containing glucose and glycerol, metabolic precursors of pyruvate. In *Streptococcus bovis* the activity of pyruvate formate lyase was greatly influenced by pH and excess energy.[153] Based on the results of Figure 3.2, the pH or lack of excess energy could have caused pyruvate to be catabolized to acetyl-CoA and acetate to produce more

of pyruvate among the three pathways in Scheme 3.2 is unclear, but would be a topic for future investigation.



Scheme 3.2. Pathways for pyruvate catabolism. Enzymes encoded in *F. varium* genome in bold (pyruvate:ferredoxin oxidoreductase (E.C.1.2.7.1), pyruvate formate lyase (E.C. 2.3.1.54) and lactate dehydrogenase (1.1.1.27)).

In *Enterococcus faecalis*, grown under anaerobic conditions, the energy source (glucose or pyruvate) and culture pH greatly influenced the intracellular NADH/NAD^+ ratio.[154] At pH 7.0 and glucose as the energy source, the NADH/NAD^+ ratio was 0.91, while at pH 5.5 the ratio decreased to 0.62, compared to the ratio of only 0.33 at pH 5.5 with pyruvate as the energy source. For all growth experiments in this thesis, the pH was initially adjusted to pH 7.4, suggesting a redox state with a high NADH/NAD^+ ratio when glucose is the major substrate. Glucose, metabolized using oxidizing steps, results in

higher NADH/NAD^+ ratio, while pyruvate does not require as much NAD^+, and so has a smaller ratio. This ratio can also be observed with the distribution of end products; butyrate is the result of higher NADH/NAD^+ ratio, while increased acetate concentrations are obtained from smaller ratios.

3.3.1. Glucose substrate-product relationships

The increased growth and greater accumulation of end products when *F. varium* was grown in CDMM*(10 mM glucose) (Table 3.5 and Figure 3.2), indicates the conversion of glucose to end products. Identification of the end products produced from glucose was carried out by growing *F. varium* in CDMM*(10 mM D-[U-$^{13}C_6$]glucose). The ^1H NMR spectrum of the culture supernatant showed end product signals accompanied by ^{13}C satellites (Figure 3.3). Acetate (dd $^1J_{CH}$ = 127 Hz, $^2J_{CH}$ = 6 Hz), butyrate (dm $^1J_{CH}$ = 125, 127 and 127 Hz for C4, C3 and C2), formate (d $^1J_{CH}$ = 195 Hz) and lactate (dm $^1J_{CH}$ = 117 Hz for C3) all showed ^{13}C-^1H coupling, signifying ^{13}C enrichment and establishing a precursor-product relationship for glucose and these four carboxylic end products. Isotope enrichments in these end products established a precursor-product relationship with glucose to acetate, butyrate, formate and lactate.

In *F. varium*, glucose is degraded by the EMP pathway yielding pyruvate.[113] Pyruvate can be reduced to lactate, regenerating NAD^+, or pyruvate can undergo decarboxylation, yielding acetyl-CoA (Scheme 3.2). Acetyl-CoA can be used to generate ATP or regenerate NAD^+ upon conversion to butyrate via the acetate-butyrate pathway (Scheme 1.2)

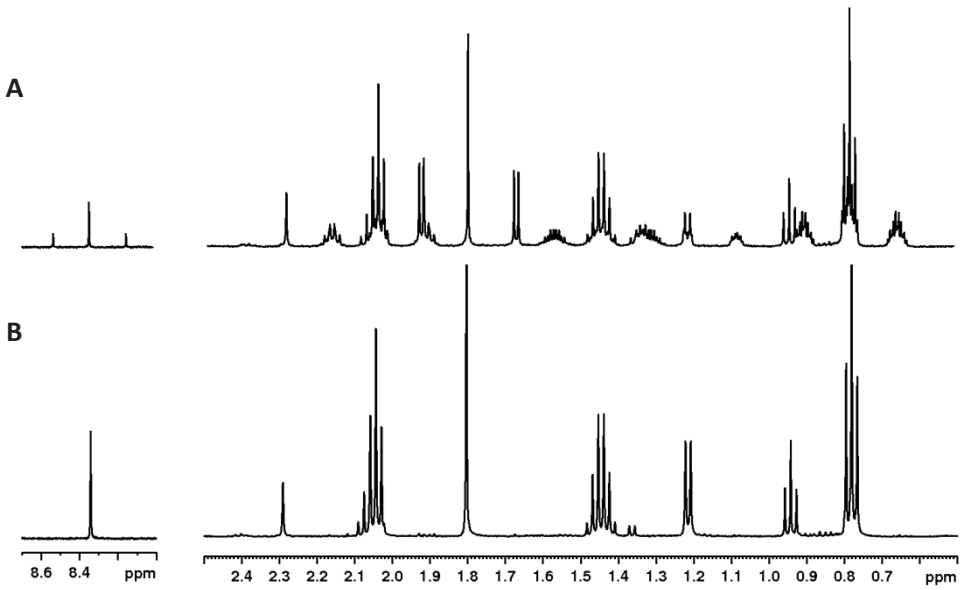


Figure 3.3. Partial ^{1}H NMR spectra after growth on CDMM* with (A) D-[U-$^{13}C_6$]glucose and (B) 10 mM glucose. Resonances in spectrum B correspond to formate (δ 8.34), succinate (δ 2.29), butyrate (δ 2.04, 1.44, 0.78), acetate (δ 1.80), lactate (δ 1.22), and propionate (δ 2.06, 0.94).

The ^{13}C NMR spectrum of the culture supernatant showed ^{13}C-^{13}C coupling patterns. The resonances for the terminal butyrate carbons appeared as doublets (δ 13.2 (J_{cc} = 34.2 Hz) and δ 184.2 (J_{cc} = 51.9 Hz)), whereas doublet (δ 39.6 (J_{cc} = 51.4 Hz)) and doublet of doublets (δ 39.6 (J_{cc} = 33.3 Hz and J_{cc} = 33.6 Hz)) patterns were observed for C2 (Figure 3.4). The dd pattern was observed because of the difference values for $J_{C1\text{-}C2}$ and $J_{C2\text{-}C3}$. Doublet (δ 19.3 (J_{cc} = 34.6 Hz)) and triplet (δ 19.3 (J_{cc} = 33.8 Hz)) patterns were apparent for C3 butyrate. The similar values for $J_{C2\text{-}C3}$ and $J_{C3\text{-}C4}$ led to the observed triplet pattern. The presence of the different coupling patterns at C2 and C3 demonstrate that butyrate is ^{13}C enriched in either two or four carbons (Figure 3.5). Enrichment in two

carbons gave the doublet coupling pattern, while four labeled carbons gave the doublet of doublets and triplet coupling patterns.

Butyrate in *F. varium* is formed by the combination of two acetyl-CoA units.[112] If two [$^{13}C_2$]acetyl-CoA join together, the resulting four-carbon chain would be enriched at each carbon. Alternatively, the combination of [$^{13}C_2$]acetyl-CoA and unlabelled acetyl-CoA would generate butyrate ^{13}C-enriched at only two of the four carbons (label on carbon 1 and 2 or 3 and 4). This is further evidence for the acetate-butyrate pathway functioning in conjunction with glucose catabolism and demonstrates NMR spectroscopy as a useful method to study metabolism, when combined with multiply labeled substrates that yield valuable information on substrate metabolism from coupling patterns.

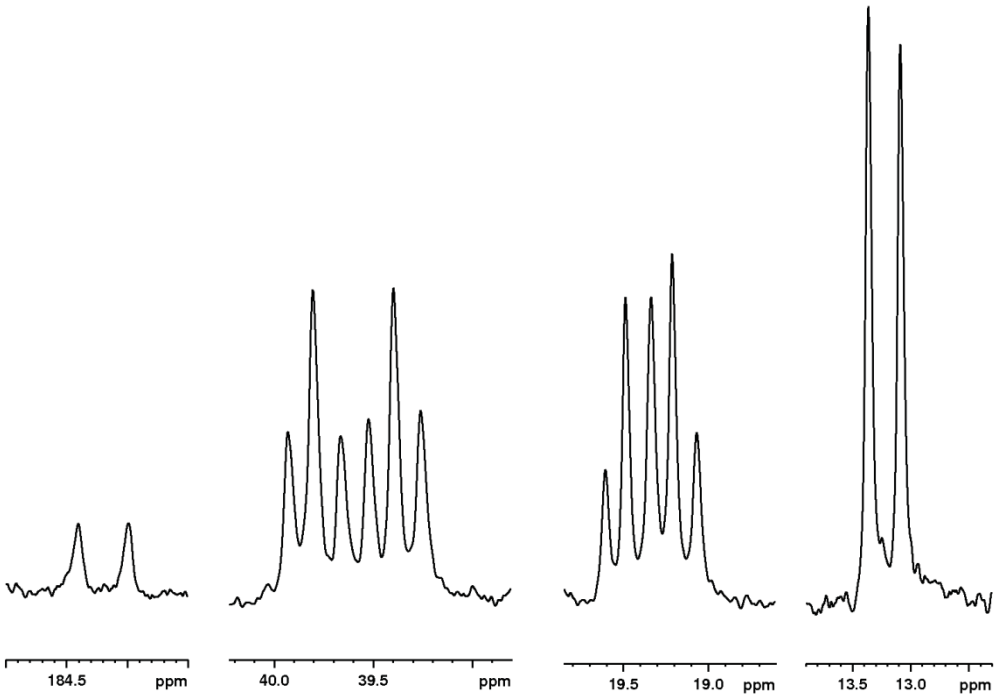


Figure 3.4. Partial ^{13}C NMR spectrum showing the coupling patterns of the C1-C4 butyrate resonances. The isotopically enriched butyrate was produced upon growth of *F. varium* on CDMM*(10 mM D-[U-$^{13}C_6$]glucose).

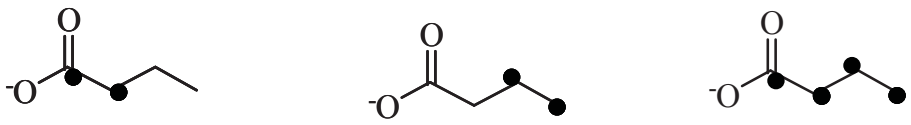


Figure 3.5. ^{13}C enrichment patterns in butyrate after growth on CDMM*(10 mM D-[U-$^{13}C_6$]glucose). A ^{13}C enrichment is indicated by ●.

The pathway to produce formate suggested in Section 3.3 has not yet been established. The genome[19] encodes a pyruvate formate lyase (E.C. 2.3.1.54), an enzyme which non-oxidatively uses pyruvate and coenzyme A as substrates, producing acetyl-CoA and formate as products. Using formate dehydrogenase enzyme (E.C. 1.2.1.2), formate could be further catabolized to CO_2 and H_2. This enzyme has not been annotated in the *F. varium* genome; however, the presence of this enzyme could explain the variability and large standard deviations obtained for formate concentrations, and the product CO_2 would not be detected by ^1H NMR spectroscopy. Preliminary results of *F. varium* grown on CDMM*(5 mM ^{13}C sodium bicarbonate/10 mM glucose) showed only a ^{13}C enrichment in formate (Figure 3.6). A ^{13}C-^1H coupling constant of 194 Hz was determined, consistent with the ^{13}C-^1H coupling constant determined from formate after supplying *F. varium* with [U-$^{13}C_6$]glucose. Formate could form from sodium bicarbonate, using the reverse direction of formate dehydrogenase. A purified enzyme, isolated from *Clostridium pasteurianum* was able to catalyze the reversible reaction of this enzyme using sodium bicarbonate as a source of CO_2 and dithiothreiol as a source of electrons.[155]

The ^{13}C enrichment from D-[U-$^{13}C_6$]glucose was not incorporated into propionate or succinate, indicating that these products are made from other sources in the growth media, *i.e.*, amino acids. Propionate production will be examined in Chapter 4. Furthermore, the acetate, butyrate, lactate or formate produced from D-[U-$^{13}C_6$]glucose had less than 100% ^{13}C enrichment. The dilution of ^{13}C label indicates that these end products also arise from other medium components (*i.e.*, amino acids), also serving as energy sources in *F. varium*.

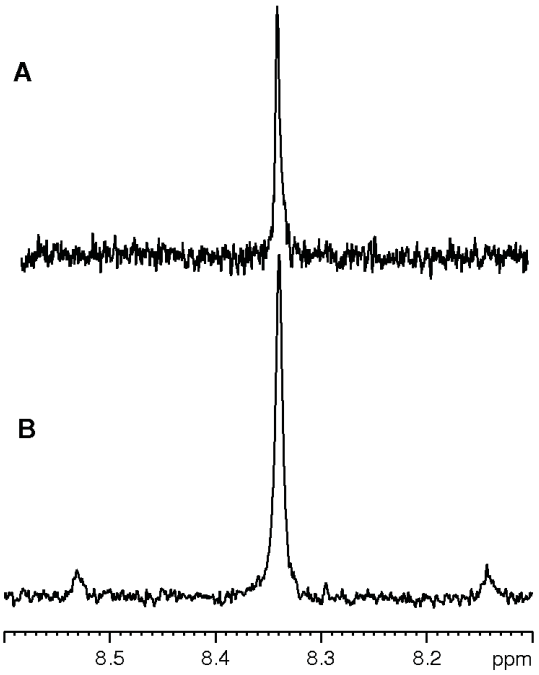


Figure 3.6. ^{1}H NMR spectra of formate, after growth on (A) CDMM*(10 mM glucose) or (B) CDMM*(5 mM ^{13}C sodium bicarbonate/10 mM glucose).

3.4. Identification of Energy Sources

The moderate growth observed when *F. varium* was cultured on CDMM* increased by a factor of 3-4 when glucose was added to CDMM* (Table 3.4). Similar additions of acetoactate, crotonate, (R)-3-hydroxybutyrate, glycerol and pyruvate led to smaller, but definite enhancements of growth. The end product distribution in culture supernatants was monitored using NMR spectroscopy. In each culture, the presence of a substrate led to the accumulation of larger quantities of end products (Figures 3.1 and 3.2). The distribution of the end products was influenced by the requirements for coenzyme regeneration. Substrates undergoing oxidation during their catabolism yielded larger quantities of butyrate and, in some cases, lactate. The accumulation of formate indicated that a nonoxidative conversion of pyruvate to acetyl-CoA was occurring. Generation of ATP from acetyl-CoA led to the accumulation of acetate, but acetyl-CoA was also converted to butyrate when coenzyme regeneration was required.

The increased growth was accompanied by substrate depletion (determined by ^{1}H NMR analysis of culture supernatants. At 10 mM, glucose was readily utilized (> 8 mM used) and at 20 mM, greater than 14 mM was used. All substrates, except glycerol at 20 mM, were utilized at least as well as glucose (Table 3.5).

Glucose has been reported as an energy source for *F. varium,* in the presence of amino acids.[113, 115] Glucose and the other substrates supported growth of *F. varium*. During the incubation, the substrates were utilized from the culture medium and were replaced by recognized end products. Substrates showing these three characteristics are energy sources for the bacterium. Therefore all substrates tested (acetoactate, crotonate, (*R*)-3-hydroxybutyrate, glucose, glycerol and pyruvate) are energy sources in *F. varium*.

3.5. Production of an Unanticipated Metabolite

In an attempt to study peptide utilization,[156] the amino acid component of CDMM* was replaced by bactopeptone at 1 mg/mL. Growth of *F. varium* in cultures containing 10 mM glucose, vitamins, salts and bactopeptone produced the expected carboxylate products, acetate, butyrate, formate, lactate, succinate, but resonances not previously found in the ^{1}H NMR spectra of CDMM*(10 mM glucose) were observed at δ 1.0, 2.5, and 3.6 (Figure 3.7). A COSY experiment showed correlations between the peaks at δ 1.02 and 3.6 and only correlations within the AB system at δ 2.5. The resonance at δ 2.5 was attributed to sodium citrate, a component of the salt solution developed previously for growth of *F. varium*.[113] Sodium citrate was supplied at 0.87 mM in the growth medium and a concentration of ~0.5 mM in the spent medium was determined from the ^{1}H NMR data, indicating that citrate is a residual medium component and not a fermentation product. Previous results[156] matched the resonances at δ 1.0 and 3.6 with a low molecular weight, neutral compound tentatively assigned as 2.3-butanediol.

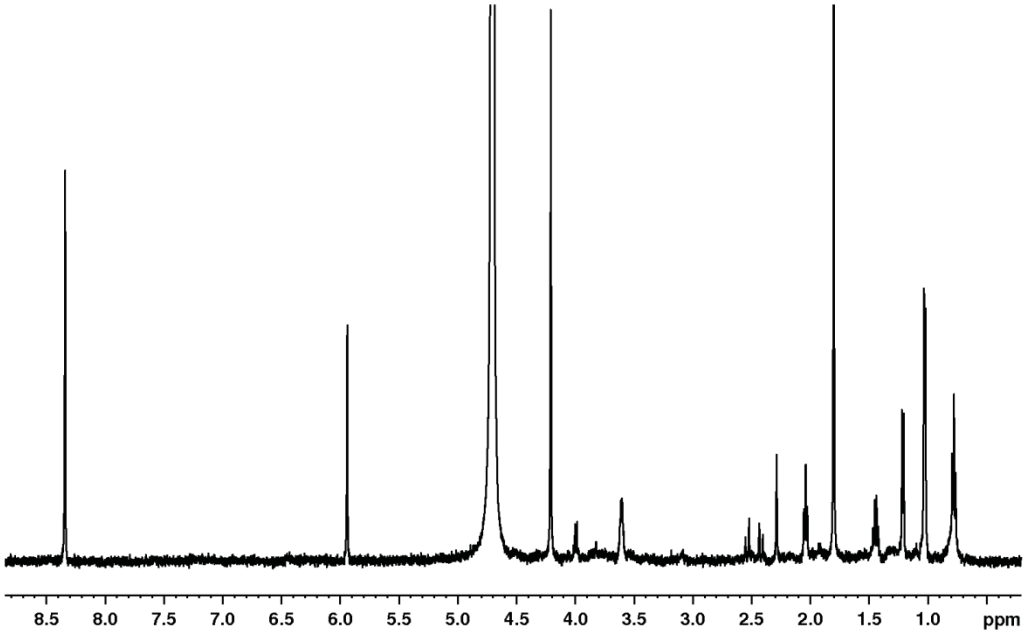
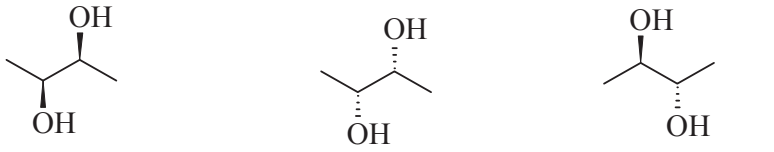


Figure 3.7. ^{1}H NMR spectrum of lyophilized 24-h culture supernatant after growth on bactopeptone, 10 mM glucose, salts and vitamins. Resonances for 2,3-butanediol were observed at δ 1.0 and δ 3.6, other resonances were assigned to formate (δ 8.34), DCA (δ 6.01), TA (δ 4.28), lactate (δ 4.01, 1.22), succinate (δ 2.29), butyrate (δ 2.04, 1.44, 0.78), and acetate (δ 1.80).

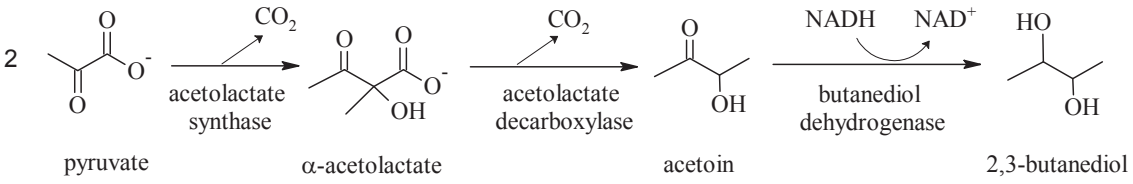
Butanediol is a well-known bacterial metabolite,[157, 158] typically produced as a mixture of two stereoisomers by enteric bacteria (*meso* and (2*S*,3*S*)) and *Bacillus* species (*meso* and (2*R*,3*R*)) (Figure 3.8).[157,159] In enteric bacteria, the best known producers, 2,3-butanediol is biosynthesized by the coupling of two molecules of pyruvate yielding α-acetolactate (Scheme 3.3); subsequent decarboxylation and reduction of the intermediate acetoin yields butanediol.[157, 159]



(2*S*,3*S*)-Butanediol (2*R*,3*R*)-Butanediol *meso*-2,3-butanediol

Figure 3.8. Stereoisomers of 2,3-butanediol.

In *F. varium*, pyruvate is formed as a product of ATP generation by catabolism of glucose *via* the EMP Pathway that also produces NADH (*i.e.*, glucose + 2 NAD^+ + 2 ADP → 2 pyruvate + 2 NADH + 2 ATP). Typically, NAD^+ is regenerated in *F. varium* by reduction of pyruvate to lactate and conversion of acetyl-CoA to butyrate (Sections 3.1-3.3). The formation of butanediol from pyruvate also serves to assist NAD^+ regeneration (Scheme 3.3); furthermore, fermentative production of the neutral diol avoids acidification of the culture medium.[160]



Scheme 3.3. Pathway of 2,3-butanediol production.

In the *F. varium* genome,[19] annotations for acetolactate synthase (E.C. 2.2.1.6), acetolactate decarboxylase (E.C. 4.1.1.5) and (S,S)-butanediol dehydrogenase (E.C.

1.1.1.76) or (R,R)-butanediol dehydrogenase (E.C. 1.1.1.4) were not evident, suggesting that butanediol production would not be predicted from known genetic information. Alternatively, butanediol formation may be catalyzed by glycerol dehydrogenase (E.C. 1.1.1.6), an enzyme encoded in the *F. varium* genome.[19] This enzyme from *Clostridium butyricum* accepts 2,3-butanediol as a substrate for oxidation by NAD^+,[161] but the activity using 2,3-butanediol is much less than that of the natural substrate glycerol. Purified glycerol dehydrogenase isolated from *Hansenula ofunaensis* showed better activity (50.0 U/mg) for the oxidation of 2,3-butanediol compared to glycerol (39.1 U/mg).[162] Catalysis of the reverse reaction (*i.e.*, the reduction of acetoin) might serve as a method to produce 2,3-butanediol in *F. varium*.

3.5.1. Isolation and structural assignment of meso-*2,3-butanediol*

To complete the identification of 2,3-butanediol and determine the stereochemistry a sample was isolated from the culture medium and purified. Various conditions for the isolation were explored. Culture supernatants were titrated to either pH 7.0 or 9.0 to place carboxylic acids in their ionized form to remain in the aqueous layer upon extraction with an organic solvent. Similar results were obtained at pH 7.0 and 9.0. Without the addition of sodium chloride, very little 2,3-butanediol was extracted from the culture medium. Upon extraction using ethyl acetate, 2,3-butanediol remained in the aqueous growth medium, but some carboxylic acid end products were extracted into the organic layer. On the other hand, extraction with diethyl ether showed 2,3-butanediol as the major component and only small amounts of acetate, butyrate, formate and succinate.

However, several extractions were needed; 2,3-butanediol was detected by ^{1}H NMR in the first four of five ether extracts.

2,3-Butanediol was extracted from 24-h culture supernatant (500 mL) at pH 7.0 after addition of sodium chloride, with four extractions using diethyl ether. The organic layers were combined and concentrated using rotary evaporation. Vacuum distillation at ambient temperature was used as a final step to isolate 2,3-butanediol (33 mg).

The ^{1}H and ^{13}C NMR spectra of the isolated product showed ^{1}H resonances centered at δ 1.02 and 3.60 (Figure 3.9A & B) and ^{13}C resonances at δ 70.96 and 16.66. By ^{1}H NMR spectroscopy, the isolated sample was 98% pure, showing only traces of acetate and butyrate (Figure 3.9A). Upon electrospray ionization mass spectrometry (ESI(+)MS), a major ion was observed at *m*/*z* 113.0577 ($[M + Na]^{+}$; calculated for $C_4H_{10}O_2Na$ 113.0573).

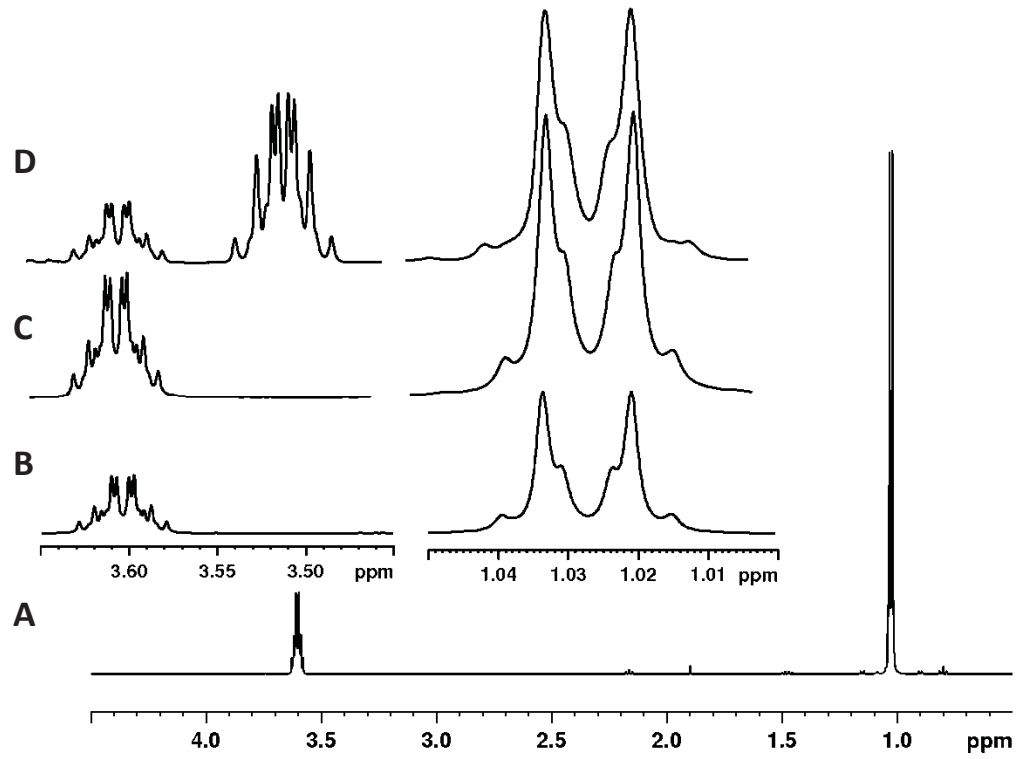


Figure 3.9. ^{1}H NMR spectra: (A) 2,3-butanediol produced by *F. varium* and isolated from culture supernatant. (B) Expanded view of (A). (C) Mixture of isolated butanediol and standard *meso*-2,3-butanediol. (D) Mixture of isolated butanediol and standard (2*R,*3*R)*-butanediol.

To determine stereochemistry, a portion (10 mg) of the purified 2,3-butanediol was mixed with either *meso*-2,3-butanediol or (2*R*,3*R*)-butanediol and ^{1}H and ^{13}C NMR spectra were acquired. The ^{1}H and ^{13}C NMR spectra of the mixture of sample and *meso*-2,3-butanediol were identical (compare Figure 3.9 B and C). The ^{1}H NMR spectrum of the sample-(2*R*,3*R*)-butanediol mixture showed distinct multiplets at 3.6 and 3.5 ppm and superimposed multiplets at 1.0 ppm (Figure 3.9D). The ^{13}C NMR spectrum showed four distinct resonances at 71.4, 71.0, 17.6 and 16.7 ppm. These results demonstrate that *meso*-2,3-butanediol is produced by *F. varium*.

3.5.2. Factors influencing meso-*2,3-butanediol production.*

Since pH can affect the production of 2,3-butanediol by other microorganisms,[163] the initial pH of growth medium was varied from 6.6 to 7.4. Similar growth (OD_{660} 0.7) and 2,3-butanediol production was observed at each pH, while the production of other end products was more variable (Table 3.6). A pH of 7.4 was used for future growth experiments since this provided good production of 2,3-butanediol and was consistent with other growth experiments performed in this thesis. Results were also consistent with production in *Klebsiella pneumoniae;* highest 2,3-butanediol production was achieved when the pH was not controlled, decreasing from pH 7.0 to 5.5 during fermentation.[164]

Table 3.6. Effects of initial pH on 2,3-butanediol production after growth on 10 mM glucose, vitamins, salts and 1 mg/mL bactopeptone, 24 h incubation.

		End Product (mM)						
start pH	end pH	Acetate	Butyrate	Formate	Lactate	Propionate	Succinate	2,3-butanediol
6.6	5.8	2.7	0	2.8	0.9	0	0.7	2.5
7.0	5.7	5.0	0.9	5.5	2.3	0	0.6	2.2
7.4	5.7	6.8	2.5	8.3	2.7	0	0.6	2.5

Bactopeptone was initially supplied at 1 mg/mL concentration. The effects of other concentrations on 2,3-butanediol production was examined (Table 3.7). Increasing amounts of bactopeptone resulted in slight increases in growth. Increases in some end products were also observed with increasing bactopeptone, but 2,3-butanediol production was not affected greatly. At greater than 1 mg/mL concentrations, unmetabolized medium components were evident in the ^{1}H NMR spectrum and interfered with the quantification of end products. Consequently, end product concentrations at the higher

bactopeptone concentrations may be over estimated. The insensitivity of 2,3-butanediol production to bactopeptone concentration suggests that bactopeptone is not serving as an energy source and is acting as a nitrogen source for protein biosynthesis. The initial 1 mg/mL bactopeptone was chosen as the optimum concentration since it provided good growth of *F. varium* producing 2,3-butanediol, and medium components were efficiently utilized.

Table 3.7. End product accumulation after 24 h incubation with varying amounts of bactopeptone added to media (vitamins, salts, and 10 mM glucose).

		mM of product					
Bactopeptone (mg/mL)	OD_{660}	acetate	butyrate	formate	lactate	succinate	2,3-butanediol
0.5	0.7	2.6	ND[a]	2.7	0.6	0.7	0.7
1	0.8	5.6	ND	5.1	1.5	1.7	1.2
2	0.8	7.1	ND	5.4	1.4	1.9	1.0
3	1.0	4.9	2.1	4.4	1.8	1.6	0.9

[a]ND = not detected; average of two independent experiments.

In cultures of *F. varium*, the OD_{660} values increased from 0 initially to 0.8 at the end of the 24 h incubation period. Decreasing amounts of glucose correlated with increasing amounts of acetate and butanediol (Table 3.8). The pH decreased to 5.9 at 12 h, and increased slightly to 6.5 at 24 h, perhaps due to decreases in the carboxylic acids formate, lactate and succinate. Overall, a 24 h incubation was required to reach the greatest amount of 2,3-butanediol production; this time was chosen for all future experiments on bactopeptone medium.

Increasing amounts of the energy source glucose had a significant effect on butanediol production (Table 3.9). Increasing amounts of all end products were observed, good growth (OD_{660} > 0.7) was achieved for all glucose concentrations.

Table 3.8. End product accumulation after varying incubation time (vitamins, salts, 1 mg/mL bactopeptone and 10 mM glucose).

Incubation time (h)	OD_{660}	residual Glucose (mM)	mM of product[a]					
			acetate	butyrate	formate	lactate	succinate	2,3-butanediol
0	0	10	ND[b]	ND	ND	ND	ND	ND
3	0.06	8.4	ND	ND	ND	ND	ND	ND
6	0.07	10	ND	ND	ND	ND	ND	ND
9	0.41	4.4	0.5	ND	1.4	ND	ND	0.1
12	0.77	0.8	2.5	ND	2.3	0.8	0.6	0.4
24	0.79	0	3.2	ND	1.3	0.2	0.4	1.0

[a]Representative data from three independent experiments: 5 mL culture samples were taken from total sample (40 mL), samples were removed with syringe through a septa to minimize culture's exposure to oxygen. [b]ND = not detected

Table 3.9. End product accumulation with varying amounts of glucose in the growth medium (vitamins, salts, and 1 mg/mL bactopeptone).

Glucose (mM)	End Products (mM)[a]					
	acetate	butyrate	formate	lactate	succinate	2,3-butanediol
5	3.7 ± 1.5	0.1 ± 0.2	2.4 ± 1.2	0.5 ± 0.5	0.4 ±0.3	0.4 ± 0.3
10	7.7 ± 1.1	0.3 ± 0.5	7.3 ± 1.3	2.1 ± 0.6	1.2 ± 0.7	1.9 ± 0.6
15	4.3 ± 1.0	1.1 ± 1.5	7.1± 2.1	1.9 ± 1.0	1.4 ± 0.4	2.9 ± 1.2
20	4.5 ± 1.0	1.7 ± 0.9	7.6 ± 0.9	3.0 ± 0.9	1.5 ± 0.5	3.5 ± 1.3

[a]Concentrations are reported on the average of three independent growth experiments ± standard deviations. 24 h incubation

3.5.3. Significance of 2,3-butanediol production in F. varium

The correlation between increasing glucose concentrations and increased production of 2,3-butanediol (Table 3.9) indicate 2,3-butanediol's role as an electron acceptor to maintain redox balance within the organism. Similar to the production of lactate or butyrate, conversion of pyruvate to 2,3-butanediol regenerates NAD^+ (Scheme 3.3), which is needed for the catabolism of glucose (Section 3.3). Increasing amounts of glucose would require more coenzyme regeneration, which in the presence of bactopeptone appears to be achieved primarily through the production of lactate and 2,3-butanediol. Additionally, 2,3-butanediol is a neutral compound and can prevent acidification of cultures, as noted in the 24 h time course experiment (Table 3.8). The pH decreased then increased at 12 to 24 h along with an increase in 2,3-butanediol production (Table 3.8). In *Paenibacillus polymyxa*, the addition of acetic acid induced the biosynthesis of 2,3-butanediol.[165] The formation of acidic end products by *F. varium* could also induce the formation of *meso*-2,3-butanediol.

Previously,[152] after incubation of [U-^{13}C]glucose with standardized gastrointestinal microbiota, the HSQC NMR spectrum showed a signal corresponding to C1 and C4 of 2,3-butanediol, linking 2,3-butanediol's production to glucose metabolism by the microbiota.[152] However, no other literature references have been reported that associated 2,3-butanediol to gut microbiota. On the other hand, as discussed in Sections 1.2 and 1.3, the microbiota play an important role in human health and disease the microbial end products may affect gut health or contribute to disease. A recent report,[166] suggested that the many unidentified small molecules may have important biological functions, such as chemical signals. The microbial contribution to diseases such as obesity and cancer of

current interest, and studies of small molecules produced by the microbiota may elucidate some health effects and disease causing mechanisms

Only a few investigations have been carried out to examine the biological effects of 2,3-butanediol. In one study, supplying 2,3-butanediol to rats with the lipopolysaccharide from *Serratia marcescens* resulted in the inhibition of neutrophils and decreased production of pro-inflammatory cytokines and cytokine gene expression.[167] This suggested that 2,3-butanediol may assist bacteria from being eliminated by the host immune system. In *Vibrio cholerae*, the biotype that produces 2,3-butanediol also inhibited pro-inflammatory biomarkers in cultured human intestinal epithelial cell lines, again suggesting the 2,3-butanediol may serve as an immune modulator.[168] In both these studies however, the stereochemistry of 2,3-butanediol was not given.

The production of 2,3-butanediol by *F. varium* may have health implications because of *F. varium's* presence in the human gut. Over the past decade, *F. varium* has been associated with ulcerative colitis[102] and most recently, *Fusobacterium* species have been associated with human colorectal cancer.[98, 99] By using 2,3-butanediol to decrease activities of the innate immune system, fusobacteria could avoid elimination by the host's immune system, allowing the bacteria to survive and exert potentially harmful effects on the host. Overall, the discovery and study of small molecules produced by microorganisms may give further insight into human health and disease and may provide a link between the presence of a microorganism in a disease state and the pathogenesis of disease.

CHAPTER 4: THREONINE METABOLISM

As observed in Chapter 2, good growth was obtained when *F. varium* was cultured on defined growth medium lacking threonine (Table 2.7); also biosynthetic genes for threonine are annotated in the genome of *F. varium*[19] indicating that the bacterium devotes cellular energy for the biosynthesis of this amino acid. Threonine, a Group 3 amino acid (Section 2.2), has not yet been examined as an energy source in *F. varium*. Published characteristics of *F. varium* indicate that when threonine is added to the growth medium (TYH broth) propionate is produced and a direct conversion of threonine to propionate is assumed.[125] While the presumed conversion of threonine to propionate has been commonly used to classify/identify clinical isolates of *F. varium,*[70] the utilization of the threonine stereoisomers, the enzymes and metabolic pathways employed have not been examined in detail.

Addition of substrate to growth medium has been shown in the previous chapter to influence end product accumulation. In this chapter, the preparation of cell extracts is described and used to provide complementary information to that obtained by *in vivo* methods of determining metabolism. The induction of enzymes by the addition of a substrate to the growth medium and information about substrate metabolism pathway intermediates is presented.

4.1. L-Threonine Utilization and Propionate Formation

To further examine this substrate-product relationship, the utilization of L-threonine by *F. varium* was investigated. The concentration of L-threonine was varied at concentrations of 0 to 15 mM in CDMM*(10 mM glucose) (Table 4.1) and incubation

times of 24 to 72 h. The accumulation of end products and residual threonine were determined by ^{1}H NMR analysis of lyophilized culture supernatants. Growth at each threonine concentration and incubation time was very similar, with OD_{660} values ranging from 0.9 to 1.2. At initial concentrations of 2, 5 and 10 mM, threonine was utilized completely within 24 h. At 15 mM, the amount of residual threonine in replicate cultures varied, ranging from 0.2-2.6 mM (10 mM glucose). When no threonine was supplied, acetate and formate concentrations were higher; butyrate was lower and no propionate accumulated, in agreement with a previous result (Table 2.9). Also, with CDMM*(0 mM L-threonine/10 mM glucose) growth was on the lower end of the range (OD_{660} 0.9).

For L-threonine supplied at 2 - 15 mM (Table 4.1) very similar amounts of acetate, butyrate, formate and succinate accumulated, but the amount of lactate and propionate increased with increasing initial threonine concentrations. Product accumulation was similar for 24, 48 and 72 h incubation times, so a 24 h incubation time was used for all future experiments.

Utilization of L-threonine was also examined on CDMM* (Table 4.2). In the absence of glucose, decreased growth of *F. varium* was obtained; OD_{660} values ranged from 0.3 to 0.5, increasing slightly with increasing L-threonine concentrations. The pattern of end product accumulation differed from the glucose experiment. No lactate was produced and smaller concentrations of butyrate, larger amounts of acetate and similar amounts of propionate and succinate were observed. As seen in the previous experiment, residual threonine, at concentrations ranging from 0-1.6 mM was only detected at the highest initial concentration of L-threonine (15 mM).

Table 4.1. End product accumulation after 24-, 48- and 72-h incubations of *F. varium* on CDMM*(10 mM glucose) containing varying initial concentrations of L-threonine.

L-Threonine (mM)	End Product (mM)[a]						residual threonine (mM) [a]
	acetate	butyrate	formate	lactate	propionate	succinate	
24 h incubation:							
0	8.2 ± 0.8	9.3 ± 2.8	5.2 ± 3.0	2.3 ± 0.5	0	0.9 ± 0.2	-
2	6.5 ± 0.4	12.8 ± 0.4	1.8 ± 0.2	2.5 ± 1.2	1.6 ± 0.2	1.0 ± 0.1	0
5	6.4 ± 0.3	12.8 ± 1.3	1.8 ± 0.3	4.3 ± 1.2	3.1 ± 0.6	0.8 ± 0.1	0
10	6.7 ± 0.4	12.8 ± 0.9	1.6 ± 0.5	6.2 ± 0.7	6.2 ± 0.7	0.6 ± 0.1	0
15	6.7 ± 0.8	12.5 ± 0.8	1.9 ± 0.3	7.0 ± 1.3	8.1 ± 0.8	0.5 ± 0.1	1.6 ± 1.1
48 h incubation:							
2	7.1 ± 1.2	14.7 ± 1.3	1.8 ± 0.2	4.2 ± 3.5	1.6 ± 0.2	1.1 ± 0.1	0
5	7.0 ± 0.7	14.2 ± 0.9	1.8 ± 0.3	4.4 ± 0.6	3.4 ± 0.3	0.6 ± 0.1	0
10	6.0 ± 1.3	11.7 ± 2.3	1.7 ± 0.6	5.2 ± 1.5	5.6 ± 1.3	0.5 ± 0.1	0
15	8.1 ± 1.8	15.2 ± 3.0	1.8 ± 0.6	5.5 ± 2.2	10.7 ± 1.8	0.6 ± 0.2	1.4 ± 0.9
72 h incubation:							
2	6.8 ± 0.6	11.0 ± 4.8	4.3 ± 3.0	3.2 ± 0.8	2.0 ± 0.9	1.1 ± 0.3	0
5	7.1 ± 0.6	14.1 ± 1.0	2.3 ± 0.3	5.3 ± 0.6	3.5 ± 0.3	1.2 ± 0.3	0
10	7.0 ± 0.3	13.0 ± 0.7	2.1 ± 0.2	6.5 ± 0.4	6.3 ± 0.4	0.7 ± 0.1	0
15	7.5 ± 0.7	13.7 ± 1.3	1.9 ± 0.5	7.1 ± 0.9	8.1 ± 3.0	0.7	1.1 ± 1.0

[a] Concentrations are reported as the average of at least three independent growth experiments ± standard deviations.

Table 4.2. End product accumulation at 24 h after incubation of *F. varium* on CDMM* supplemented with L-threonine (0 – 15 mM).

L-Threonine (mM)	End Product (mM)[a]						residual threonine (mM) [a]
	acetate	butyrate	formate	lactate	propionate	succinate	
0	9.0 ± 1.7	3.7 ± 0.8	0.3 ± 0.1	0	0	0.2 ± 0.2	0
2	9.7 ± 0.5	4.3 ± 0.4	0.3 ± 0.1	0	1.8 ± 0.3	0.8 ± 0.1	0
5	10.1 ± 0.7	5.0 ± 0.5	0.2 ± 0.1	0	4.2 ± 0.4	0.8 ± 0.1	0
10	9.8 ± 0.4	5.3 ± 0.3	0.3 ± 0.1	0	7.6 ± 0.6	1.0 ± 0.1	0
15	9.2 ± 0.2	5.3 ± 0.4	0.3 ± 0.1	0	10.8 ± 1.1	1.1 ± 0.1	0.6 ± 0.8

[a]Concentrations are reported as the average of at least three independent growth experiments ± standard deviation.

In the presence of glucose (Table 4.1), increasing amounts of threonine led to increased amounts of lactate and propionate. However, no lactate was produced in the absence of glucose (*i.e.*, CDMM*; Table 4.2). As shown in Chapter 3, ^{13}C enrichment was detected in lactate after growth of *F. varium* in CDMM*(10 mM D-[U-$^{13}C_6$]glucose) demonstrating a precursor-product relationship between glucose and lactate. A direct metabolic connection between lactate and threonine is unlikely, but the correlation of propionate accumulation with the increase in initial L-threonine concentrations required further investigation (Tables 4.1 and 4.2) to determine whether a direct metabolic connection exists between L-threonine and propionate.

To investigate the possible threonine-propionate precursor-product relationship, *F. varium* was cultured on CDMM*(5 mM L-[U-$^{13}C_4$,^{15}N]threonine/10 mM glucose). The ^{1}H NMR spectrum of the lyophilized 24-h culture supernatant showed resonances for five carboxylate end-products (acetate, butyrate, formate, lactate, propionate and succinate). The lactate resonance at δ 1.21 (d, J = 6.7 Hz) (Figure 4.1A) showed no coupling to ^{13}C, consistent with the lack of a direct precursor-product relationship between L-threonine and lactate. The propionate resonances, however, appeared as doublets of multiplets centered at δ 2.04 and 0.94 (Figure 4.1A). The separation of the multiplets was consistent with one-bond C-H coupling ($^{1}J_{C2\text{-}H}$ = 126.9 Hz and $^{1}J_{C3\text{-}H}$ = 126.4 Hz). Corresponding ^{1}H-^{12}C resonances were not detected. The ^{13}C NMR spectrum (Figure 4.1B) showed only ^{13}C-^{13}C coupled resonances (*i.e.*, δ 10.2 (d), 30.7 (dd), 185.0 (d): $^{1}J_{C1\text{-}C2}$ = 34.0 Hz; $^{1}J_{C2\text{-}C3}$ = 51.2 Hz) at chemical shifts corresponding to carbons in propionate.[169] The doublet of doublets pattern of the C2 resonance demonstrates [$^{13}C_3$]propionate as the predominant product and the transfer of three contiguous carbons from L-[U-$^{13}C_4$,^{15}N]threonine.

Together, the NMR results establish threonine as the sole source of propionate when *F. varium* is cultured on defined medium (*i.e.*, CDMM*(10 mM glucose)).

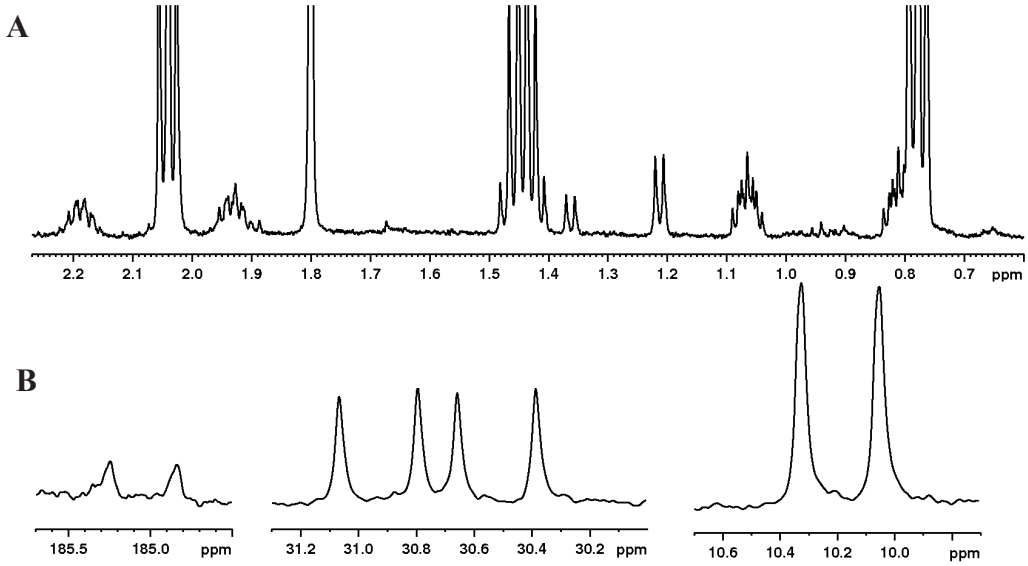


Figure 4.1. Partial NMR spectra of lyophilized culture supernatant (in D_2O) after 24 h incubation with CDMM*(5 mM L-[U-$^{13}C_4$,^{15}N]Thr/10 mM glucose). (A) ^{1}H NMR spectrum and (B)^{13}C NMR spectrum.

4.2. 2-Oxobutyrate Utilization and Propionate Formation

The incorporation of three contiguous carbons from L-threonine into propionate is consistent with the 2-oxobutyrate pathway (Scheme 1.5, Route C).[133, 170] *F. varium* was incubated from 24-72 h with the postulated pathway intermediate 2-oxobutyrate (2-15 mM) in CDMM*(0 mM L-Thr/10 mM glucose) (Table 4.3). L-Threonine was removed from CDMM* so accumulations of propionate could only be attributed to 2-oxobutyrate present in the growth medium.

Table 4.3. End product accumulation after 24-, 48- and 72-h incubations of *F. varium* on CDMM*(10 mM glucose) containing varying initial concentrations of 2-oxobutyrate.

2-Oxobutyrate (mM)	End Products (mM)[a]						Residual 2-oxobutyrate (mM) [a]
	acetate	butyrate	formate	lactate	propionate	succinate	
24 h incubation:							
2	7.4 ± 0.8	11.9 ± 0.8	1.6 ± 0.7	2.0 ± 0.7	1.3 ± 0.2	0.6 ± 0.1	0
5	7.5 ± 1.0	11.7 ± 2.1	2.8 ± 2.5	2.3 ± 0.5	3.0 ± 0.4	0.6 ± 0.2	0
10	9.3 ± 1.3	6.6 ± 3.1	6.1 ± 6.3	2.4 ± 1.2	6.4 ± 1.0	0.8 ± 0.5	0.6 ± 1.3
15	8.1 ± 1.8	3.7 ± 1.3	5.9 ± 6.6	2.4 ± 1.3	8.5 ± 1.4	0.7 ± 0.6	1.3 ± 1.5
48 h incubation:							
2	7.8 ± 0.1	12.8 ± 1.7	2.5 ± 1.9	2.5 ± 0.3	1.7 ± 0.3	0.7 ± 0.2	0
5	7.4 ± 1.2	13.0 ± 3.0	3.5 ± 2.3	3.7 ± 0.5	3.5 ± 0.3	0.8 ± 0.1	0
10	9.4 ± 1.1	11.9 ± 6.6	3.9 ± 5.3	3.8 ± 1.8	7.1 ± 1.1	0.8 ± 0.4	0.7 ± 1.4
15	7.9 ± 1.5	9.5 ± 6.3	3.1 ± 5.1	4.3 ± 2.0	9.1 ± 1.6	0.5 ± 0.5	0.5 ± 1.2
72 h incubation:							
2	7.4 ± 0.3	11.9 ± 1.9	3.3 ± 2.2	2.4 ± 0.9	1.2 ± 0.2	0.7 ± 0.1	0
5	7.5 ± 1.5	14.3 ± 3.0	2.8 ± 1.6	3.9 ± 1.0	2.8 ± 1.2	0.7 ± 0.3	0
10	8.1 ± 0.8	11.2 ± 4.4	4.8 ± 4.5	4.2 ± 0.4	6.4 ± 0.4	1.0 ± 0.4	0
15	9.2 ± 0.5	7.7 ± 3.5	8.8 ± 3.5	5.5 ± 0.7	8.8 ± 0.5	1.4 ± 0.3	0

[a]concentrations are reported as the average of at least three independent growth experiments ± standard deviation.

After 24 h incubation, low concentrations (2-5 mM) of 2-oxobutyrate resulted in product accumulation (Table 4.3) that was very similar to that obtained with CDMM*(2-5 mM L-Thr/10 mM glucose) and was accompanied by similar growth, OD_{660} averages of 0.9 and 1.0. At higher concentrations (10-15 mM) of 2-oxobutyrate, however, less butyrate and higher amounts of formate were obtained. Formate accumulation, however, showed large culture-to-culture variations. The smaller amounts of butyrate were consistent with less growth; the average OD_{660} values were 0.7 at 10 mM 2-oxobutyrate and 0.6 at 15 mM 2-oxobutyrate. The butyrate concentrations were higher at 48 h and 72

h for these 2-oxobutyrate concentrations, and the average OD_{660} values were above 0.8 for each 2-oxobutyrate concentration. Propionate concentrations increased with increasing initial concentrations of 2-oxobutyrate and reached levels similar to those determined in the L-threonine experiments (compare Tables 4.1 and 4.3)

The utilization of 2-oxobutyrate in the absence of glucose (*i.e.*, CDMM*(0 mM L-Thr)), was also determined (Table 4.4). Similar amounts of growth were achieved (OD_{660} average values ranged from 0.2 to 0.3) when compared to the L-threonine experiments (Section 4.1). There was a slight decrease in growth with increasing concentrations of 2-oxobutyrate, but this did not affect propionate production; slightly higher concentrations of propionate were produced when compared to increasing threonine concentrations (Figure 4.2), the effect was greatest with no glucose (Figure 4.2B)

Table 4.4. End product accumulation at 24 h after incubation of *F. varium* on CDMM*(0 mM L-Thr) supplemented with 2-oxobutyrate (2 – 15 mM).

2-Oxobutyrate (mM)	End Product (mM)[a]						Residual 2-oxobutyrate (mM)[a]
	acetate	butyrate	formate	lactate	propionate	succinate	
2	9.7 ± 0.4	3.9 ± 0.3	0.1 ± 0.1	0	1.5 ± 0.3	0.3 ± 0.1	0
5	8.4 ± 2.1	3.6 ± 1.0	0.3 ± 0.5	0	3.1 ± 0.9	0.4 ± 0.3	0
10	5.6 ± 1.5	2.2 ± 0.9	1.5 ± 1.9	0	6.5 ± 0.7	0.6 ± 0.7	2.8 ± 2.0
15	6.1 ± 0.7	2.1 ± 1.5	2.1 ± 2.3	0	9.9 0.7	0.9 ± 0.9	5.8 ± 5.4

[a]Concentrations are reported as the average of at least three independent growth experiments ± standard deviation

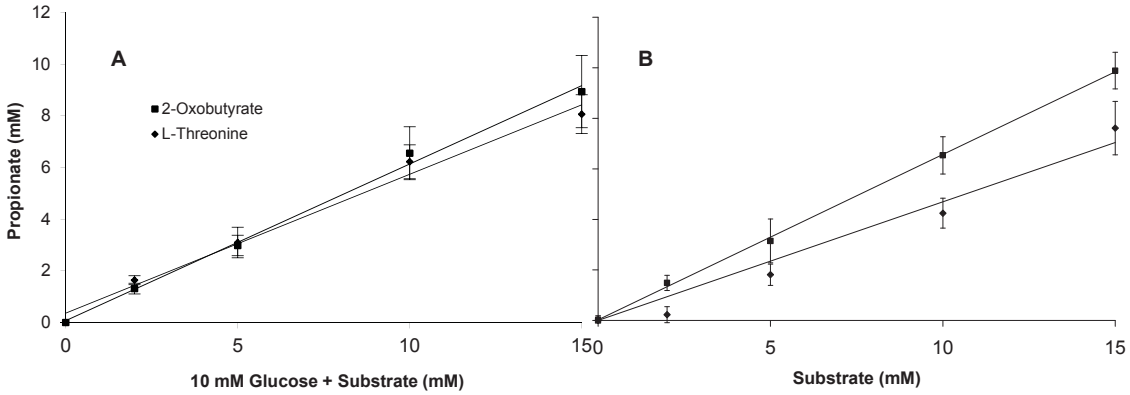


Figure 4.2. Propionate accumulation in CDMM* with increasing amounts of L-threonine (♦) and 2-oxobutyrate (■), grown in (A) CDMM*(10 mM glucose) and (B) CDMM*.

4.2.1. Substrate Preference: Competition Experiments with 2-Oxobutyrate and L-Threonine

In an experiment, where both L-threonine and 2-oxobutyrate (2-obu) were included in the defined culture medium (*i.e.*, CDMM*(15 mM L-Thr/15 mM 2-obu/10 mM D-glucose)), 13.0 mM of propionate accumulated while some L-threonine (12.3 mM) and 2-oxobutyrate (2.3 mM) remained unmetabolized. The possible utilization of both substrates was further explored in a second competition experiment using ^{13}C and ^{15}N enriched L-threonine (CDMM*(5 mM L-[U-$^{13}C_4$,^{15}N]Thr/5 mM 2-obu/10 mM D-glucose)). By ^{1}H NMR analysis (Figure 4.3), both 2-oxobutyrate and L-[U-$^{13}C_4$,^{15}N]threonine were utilized completely, [$^{13}C_3$]propionate (3.5 mM) and unlabelled propionate (3.8 mM) were detected in the 24-h culture supernatant. As seen previously (Figure 4.1) propionate was the only ^{13}C enriched product detected and the formation of both labeled propionate from L-[U-$^{13}C_4$,^{15}N]threonine and unlabelled propionate from 2-oxobutyrate demonstrated co-metabolism of these substrates. The greater utilization of 2-oxobutyrate and the greater proportion of propionate derived from 2-oxobutyrate,

indicated that 2-oxobutyrate is metabolized more efficiently than L-threonine, a result consistent with 2-oxobutyrate as a more advanced precursor of propionate.

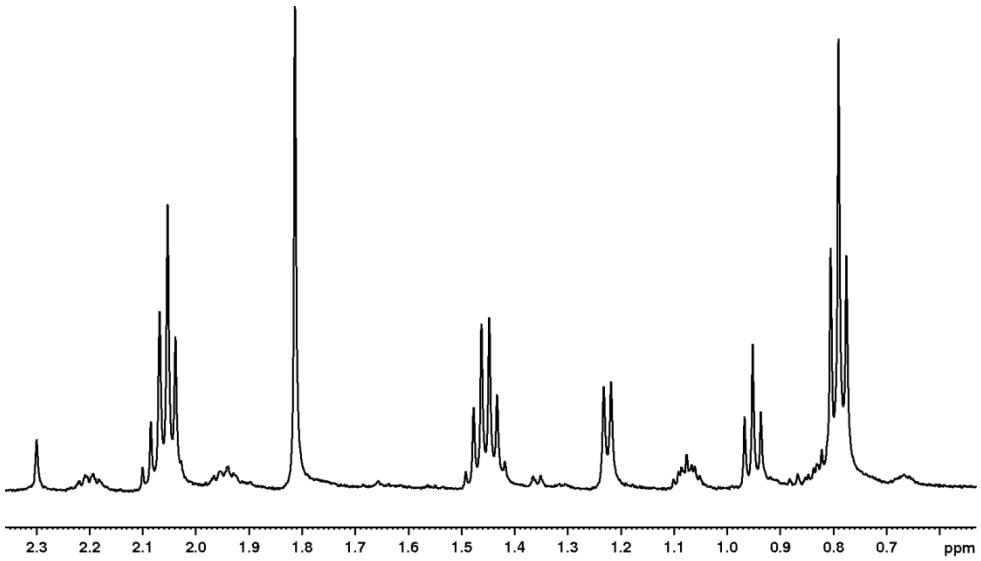


Figure 4.3. Partial ^{1}H NMR spectrum of 24-h culture supernatant after incubation of *F. varium* with 5 mM L-[$^{13}C_4$,^{15}N]Thr and 5 mM 2-oxobutyrate.). Resonances were assigned to succinate (δ 2.29) propionate (δ 2.06, 0.94), butyrate (δ 2.04, 1.44, 0.78), acetate (δ 1.80) and lactate (δ 1.22).

While accounting for unmetabolized substrates in each experiment, the accumulated propionate was always less than the initial amount of L-threonine (Tables 4.1 and 4.2) or 2-oxobutyrate (Tables 4.3 and 4.4). Thus, the conversion of L-threonine and 2-oxobutyrate to propionate was not quantitative, unlike previous results on undefined medium,[125] even after 72 h incubation (Tables 4.1 and 4.3). The propionate accumulated accounted for only 60% of the initial 2-oxobutyrate (Figure 4.2). This suggests that threonine was also utilized for another purpose.

4.3. Threonine Degradation in Cell Extracts

Cell extracts were prepared from cells grown for 8.5 h on CDMM*(10 mM glucose) and 10 mM of an additional substrate. Centrifuged cell pellets were washed twice to reduce contributions from the growth medium. A crude cell extract was created by sonicating cells resuspended in a potassium phosphate-buffer (pH 7.4) with 2 mM of dithiothreitol. Cells extracts prepared from fresh cells and cells frozen overnight showed similar conversions of substrate to product (threonine to propionate). It was necessary to keep the cells under nitrogen gas during sonication and to purge incubation tubes with nitrogen. Sonicating the cells in air and not purging tubes resulted in low (< 5%) conversion of threonine to products. Over sonication was also detrimental. Comparing cells before and after 2 min of sonication (15 s intervals) under the microscope revealed that 2 min was sufficient to disrupt cells; only very small particles with no visible cells were present. After typically 4 h incubation, methanol was added to denature proteins and the mixture was rotovapped to dryness. The residues were analyzed by NMR spectroscopy.

4.3.1. Co-Factor Requirements of Cell Extracts

The effects of adding co-factors to cell extracts were tested to see if their addition would result in an increase in substrate conversion. Pyridoxal phosphate (PLP) and adenosine diphosphate (ADP), shown previously to enhance degradation of threonine,[171,172] were added together and independently to cell extracts with L-threonine. Similar high conversions were observed in extracts containing both PLP and ADP and PLP alone and (Table 4.5, Entries 1 and 2) and no additional of co-factors (Table 4.5,

Entries 2 and 4). Adding ADP resulted in a slightly smaller conversion of threonine to products (Table 4.5, Entry 3). No conversion was observed when the cell extract was eliminated and L-threonine was incubated with buffer components K_2HPO_4, dithiothreitol and pyridoxal phosphate (Table 4.5, Entry 5), indicating the cell extract is required for the conversion of threonine. Given that addition of co-factors did not improve the conversion of substrate to product in cell extracts, none were added in future experiments with cell extracts.

Table 4.5. Co-factor requirements for incubation of L-threonine (10 mM) in crude cell free extracts

Entry	Extract (mL)[a]	ADP (mM)	PLP (mM)	% conversion[b]
1	1	1	1	98
2	1	-	1	99
3	1	1	1	90
4	1	-	-	99
5	-	-	1	0

[a]Cell Extracts were prepared from 8.5-h cells incubated in CDMM*(10 mM L-threonine 10 mM glucose). [b]% conversions obtained by comparing the areas threonine and propionate in the ^{1}H NMR spectrum of lyophilized products determined after 4 h incubation.

Dilution of cell extract with buffer resulted in little change to the conversion of L-threonine to products (Table 4.6) except at the most dilute cell extract, which resulted in 81% conversion. At larger dilutions, there was incomplete conversion of threonine to propionate, as seen by the formation of pathway intermediate 2-oxobutyrate when 0.1 and 0.25 mL of cell extract was used. The cell extract volume was kept at 1 mL, ensuring

good conversion of substrate to product. At all dilutions, the ^{1}H NMR spectra were free of interfering components that might have affected quantification of the end products.

Table 4.6. Effects of dilution on conversion of 10 mM L-threonine in crude cell extracts.

Cell Extract (mL)[a]	% Conversion[b]	% 2-oxobutyrate[c]	% propionate[d]	% glycine[e]
0.1	81	67	5	9
0.25	98	55	32	11
0.5	98	ND[f]	79	19
1	97	ND	76	21

[a]Cell extract was prepared from cells grown for 8.5 h in CDMM*(10 mM glucose/10 mM L-threonine). [b]% conversion obtained by comparing peak areas of L-threonine and products, conversion of L-threonine to propionate/2obu/glycine was determined after 4 h incubation time. [c]Conversion of L-threonine to 2-oxobutyrate. [d]Conversion of L-threonine to propionate. [e]Conversion of L-threonine to glycine, formation of glycine will be described in section 4.3.2. [f]ND = not detected

4.3.2. Degradation of L-[$^{13}C_4$,^{15}N]Threonine

When L-[$^{13}C_4$,^{15}N]threonine was incubated with crude cell extract prepared from cells grown with CDMM*(10 mM L-threonine /10 mM glucose), ^{1}H NMR resonances coupled to ^{13}C were assigned to propionate (δ 2.0, $^{1}J_{CH}$ = 125 Hz) and 2-oxobutyrate (δ 2.5, $^{1}J_{CH}$ = 125 Hz). In the ^{13}C NMR spectrum, the resonances of propionate (δ 10.2 (d, $^{1}J_{CC}$ = 34.0 Hz), δ 30.7 (dd, $^{1}J_{CC}$ = 34.1 Hz and 51.2 Hz), δ 185.0 (d, $^{1}J_{CC}$ = 51.1 Hz)) and 2-oxobutyrate (Figure 4.4B) (δ 6.7 (d, $^{1}J_{CC}$ = 34.9 Hz), δ 32.7 (ddd, $^{1}J_{CC}$ = 35.2 Hz and 38.1 Hz $^{2}J_{CC}$ = 12.9 Hz), δ 171.0 (dd, $^{1}J_{CC}$ = 61.8 Hz, $^{2}J_{CC}$ = 13.1 Hz), δ 208.3 (dd, $^{1}J_{CC}$ = 61.7 Hz and 38.3 Hz)) showed one-bond carbon-carbon coupling and the longer

range coupling between carbons of 2-oxobutyrate demonstrate the formation of 2-oxo-[$^{13}C_4$]butyrate and incorporation of the complete carbon skeleton of threonine.

The two other coupled resonances in the ^{13}C NMR spectrum (Figure 4.4A) were attributed to [$^{13}C_2$,^{15}N]glycine (δ 41.46 (dd, $^1J_{CC}$ = 53.5 Hz, $^1J_{CN}$ = 6.3 Hz), δ 172.51 (d, $^1J_{CC}$ = 53.0 Hz)). The values of the coupling constants are consistent with previously measured values,[173] and a coupled peak ($^1J_{CH}$ = 138 Hz), centered around δ 3.44 in the ^1H NMR spectrum, was attributed to glycine. Acetaldehyde, the other cleavage product, is possibly lost upon lyophilization prior to NMR analysis

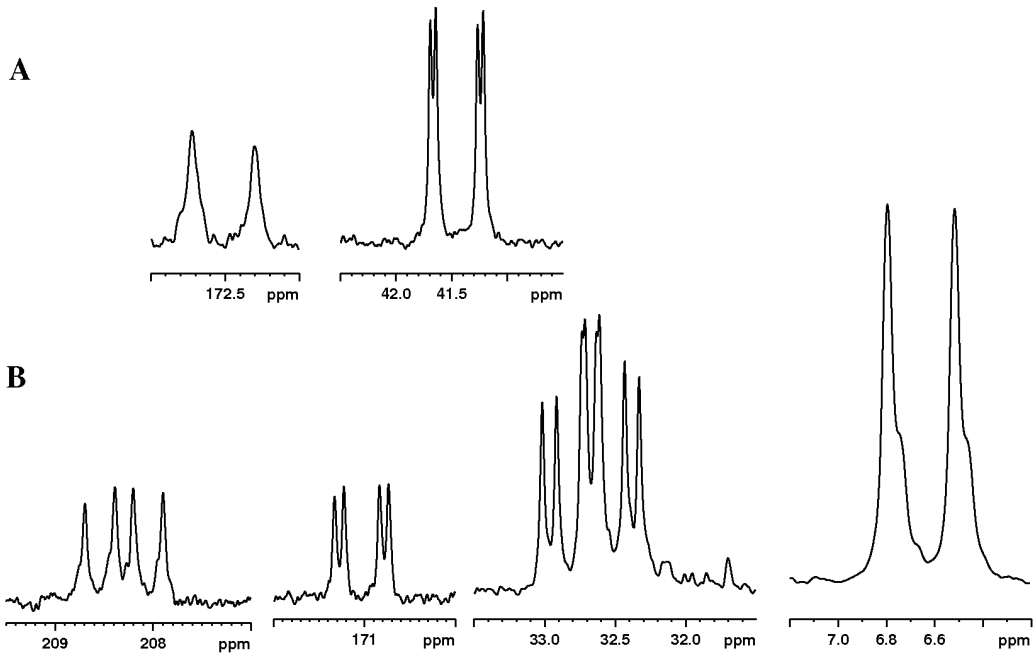


Figure 4.4. Partial ^{13}C NMR spectrum of cell extract (grown using CDMM*(10 mM L-Thr/10 mM glucose) after incubation with 10 mM L-[$^{13}C_4$,^{15}N]Thr. Resonances grouped for (A) [1,2-$^{13}C_2$,^{15}N]glycine and (B) 2-oxo-[$^{13}C_4$]butyrate.

The labeling results described in the previous paragraph demonstrate the conversion of L-threonine to glycine, probably catalyzed by threonine aldolase (E.C. 4.1.2.5) (Scheme 1.5) an enzyme commonly found in other Gram-negative bacteria.[174] Earlier in this thesis (Section 2.5), moderate growth of *F. varium* on CDMM*(0 mM Gly/10 mM glucose) demonstrated that glycine is not a growth requirement and is made from other sources, most likely threonine or serine. Incubation of L-threonine with cell extracts prepared from cells grown on CDMM*(0 mM glycine/10 mM glucose) gave similar distributions of glycine and propionate, indicating that elevated levels of threonine aldolase were not present under these culture conditions. Presumably only small amounts of glycine are needed to satisfy growth requirements.

When cell extracts prepared from cells grown on CDMM*(10 mM L-Thr/10 mM glucose) were incubated with ^{13}C glycine and acetaldehyde, no threonine was detected under these conditions. Catalysis of the reverse reaction by threonine aldolase is unfavourable.

In *E. coli*, low substrate specificity allows threonine aldolase to convert phenylserine to benzaldehyde and glycine.[175] Incubation of cell extract prepared from cells grown in CDMM*(10 mM L-threonine/10 mM glucose) with DL-3-phenylserine and DL-*threo*-β-phenylserine led to the detection of glycine by ^{1}H NMR. Benzaldehyde, the other product expected by aldolase catalyzed cleavage, would be pumped away during lyophilization. Conversions of 51% (DL-3-phenylserine) and 61% (DL-*threo*-β-phenylserine) indicated a possible broad substrate specificity for the threonine aldolase in *F. varium*.

4.3.3. Product Accumulation Over Time

The conversion of 10 mM L-threonine to propionate, 2-oxobutyrate and glycine in cell extract was monitored over time (Figure 4.5). The formation of 2-oxobutyrate peaked at 0.5 h when threonine was mostly depleted, but the accumulation of propionate continued until the 2-oxobutyrate also was depleted. These observations are consistent with the conversion of L-threonine to 2-oxobutyrate and the subsequent conversion of 2-oxobutyrate to propionate. The slower rate of glycine accumulation also correlates with L-threonine depletion.

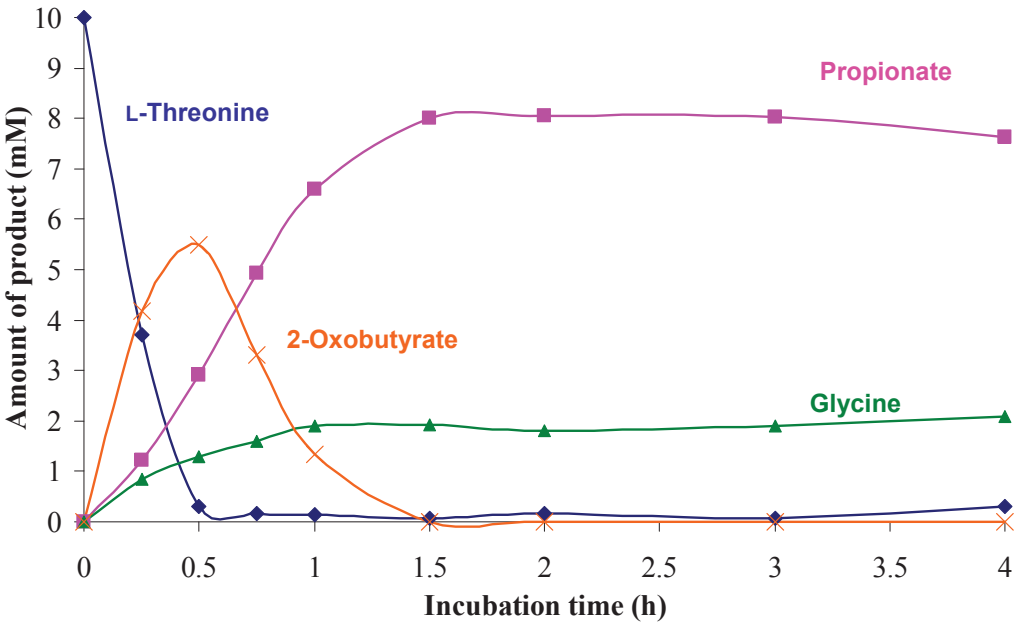


Figure 4.5. Substrate depletion and product accumulation (L-threonine (♦), 2-oxobutyrate (x), propionate (■), glycine (▲)) over time in cell extracts incubated with 10 mM L-threonine. Concentrations were calculated by assuming that the sum of the concentrations of threonine and the three products remained constant and equal to the initial threonine concentration. Cell extracts were prepared from cells grown in CDMM*(10 mM L-threonine/10 mM glucose).

4.4. Cleavage of 2-Oxobutyrate and Propionate Production

In *E. coli* and *Salmonella typhimurium*, the conversion of 2-oxobutyrate into propionyl-CoA and formate is catalyzed by 2-oxobutyrate formate lyase (E.C. 2.3.1.-). Propionyl-CoA is then phosphorylated using phosphotransacetylase (E.C. 2.3.1.8) and propionate is produced using propionate kinase (E.C. 2.7.2.15), while generating ATP for energy.[133] The degradative pathway for serine (pyruvate formate-lyase, acetate kinase, phosphotransacetylase), involves similar reactions, and intermediates of threonine catabolism are substrates for enzymes in the serine pathway.[132]

Previously,[125] *F. varium* cultures supplemented with 2-oxobutyrate (10 mM) produced large amounts of propionate (9.5 mM), and 2-hydroxybutyrate (2 mM) as well as acetate and butyrate; however, no pathways were elucidated and there was no mention of other carboxylate products, such as formate.

If the catabolism of 2-oxobutyrate in *F. varium* is catalyzed by pyruvate formate lyase (E.C. 2.3.1.54), which is encoded in the genome,[19] formate and propionate would form as major end products. In an experiment using CDMM*(15 mM 2-oxobutyrate/10 mM glucose), varying amounts of formate (0.2-13.3 mM) produced in replicate cultures correlated with growth (OD_{660}) (Table 4.3). However, a similar relationship was not observed using CDMM*(0-15 mM 2-oxobutyrate). Furthermore, ^{13}C-enriched formate would be expected as a product if L-[U-$^{13}C_4$,^{15}N]threonine was catabolized in *F. varium* by a pathway containing pyruvate formate lyase. However, formate formed under those growth conditions, did not show ^{13}C enrichment.

Alternatively pyruvate:ferredoxin oxidoreductase (E.C. 1.2.7.1) is encoded in the *F. varium* genome,[19] and has a broad substrate specificity, accepting both pyruvate and 2-

oxobutyrate.[176,177] Using this enzyme, 2-oxobutyrate can be converted to propionyl-CoA in the presence of coenzyme A, ferredoxin and NAD^+. Finally, the genome has encoded propionate CoA-transferase (E.C. 2.8.3.1), an enzyme, which in the presence of acetate, converts propionyl-CoA to propionate and also produces acetyl-CoA, an intermediate of the acetate-butyrate pathway (Scheme 1.2). The production of acetyl-CoA from threonine in this pathway is equivalent to generating ATP making threonine catabolism a route for energy (ATP) production in *F. varium*.

4.5. L-Threonine and 2-Oxobutyrate as Energy Sources

Increasing L-threonine concentrations led to a greater accumulation of propionate and lactate when grown in the presence of glucose. Only at high concentrations (15 mM) was threonine left over in the growth media, showing it is readily utilized by *F. varium* and converted to a carboxylate end product (Tables 4.1 and 4.2). OD_{660} readings indicate that growth increases with increasing threonine concentrations in both CDMM*(0-15 mM L-Thr) and CDMM*(0-15 mM L-Thr/10 mM D-glucose). Overall, L-threonine was readily utilized and converted to a carboxylate end product while stimulating growth of *F. varium*. Thus, L-threonine fulfills the criteria in Chapter 3 and functions as an energy source in *F. varium*.

2-Oxobutyrate also led to an increase in the accumulation of propionate in culture supernatants, but growth decreased with increasing 2-oxobutyrate concentrations. However, at all 2-oxobutyrate concentrations, growth was higher than that obtained when no additional energy source was supplied in CDMM*. At higher concentrations (CDMM*/10 or 15 mM 2-obu), there was on average, 2.8 and 5.8 mM of residual 2-

oxobutyrate (Table 4.4), indicating that most of the substrate was metabolized. When glucose was present in the growth medium, (CDMM*(2-15 mM 2-oxobutyrate /10 mM D-glucose)) growth was observed and less 2-oxobutyrate was leftover at the higher initial concentrations of 10 mM (0.6 mM of residual 2-oxobutyrate) and 15 mM (1.3 mM residual 2-oxobutyrate) (Table 4.3), indicating more complete metabolism of 2-oxobutyrate with glucose as a co-substrate. Given increased growth, accumulation of propionate and ready utilization, 2-oxobutyrate can also be classified as an energy source in *F. varium*.

4.6. Utilization of D- and DL-*allo*-Threonine

The utilization of D- amino acids by *F. varium* has been noted in previous investigations.[117] On complex peptone medium the D-isomers of glutamate, serine and lysine at 10 mM were utilized within 42 h.[117] Similarly, *F. varium* utilized D-threonine (10 mM) and DL-threonine (20 mM) from complex biotrypticase medium.[125] In this investigation, good growth ($OD_{660} \geq 1.0$) on CDMM*(0 mM L-Thr/10mM glucose) containing 10 mM of either D-, DL- or DL- *allo*-threonine was observed (Table 4.7) along with utilization of the threonine stereoisomers. Although L-threonine was omitted from CDMM*, the accumulation of propionate and the other carboxylate end-products when D- and DL-threonine were present was similar to the concentrations determined in the previous L-threonine experiments (Table 4.1), indicating the conversion of D-threonine to propionate. D-Threonine, however, was not used as readily as L-threonine; about 23% of the initial D substrate remained. When DL-*allo*-threonine was supplied, smaller amounts of propionate accumulated (2.4 mM) and more than half of the initial DL-*allo*-threonine

remained unutilized, indicating degradation of only one of the *allo*-threonine stereoisomers, or a preference towards one stereoisomer.

Table 4.7. End product accumulation after 24 h incubation in culture supernatants with threonine analogs grown with CDMM*(0 mM L-Thr/10 mM glucose).

Substrate (10 mM)	End Product (mM)[a]						Residual threonine (mM)[a]
	acetate	butyrate	Formate	lactate	propionate	succinate	
DL-threonine	6.3 ± 0.5	12.0 ± 0.8	2.0 ± 0.3	5.0 ± 0.4	6.4 ± 0.9	0.6 ± 0.1	0.9 ± 0.9
D-threonine	7.8 ± 0.2	11.8 ± 0.8	2.7 ± 1.1	2.2 ± 1.9	4.7 ± 1.3	0.9 ± 0.3	2.3 ± 0.6
DL-*allo*-threonine	7.4 ± 1.3	11.5 ± 2.0	2.0 ± 0.7	2.2 ± 0.5	2.4 ± 0.6	0.6 ± 0.1	5.6 ± 0.9
L-threonine	6.7 ± 0.4	12.8 ±0.9	1.6 ± 0.5	6.2 ± 0.7	6.2 ± 0.7	0.5 ± 0.1	0

[a]Concentrations are reported as the average of at least three independent growth experiments ± standard deviation

In a competition experiment, *F. varium* was grown on defined growth medium with 5 mM L-[U-$^{13}C_4$,^{15}N]threonine and 5 mM D-threonine (unlabelled) (*i.e.*, CDMM*(5 mM L-[U-$^{13}C_4$,^{15}N]Thr/5 mM D-Thr/10 mM D-glucose)). The ^{13}C NMR spectrum of the lyophilized supernatant was similar to that in Figure 4.1B, demonstrating the formation of [$^{13}C_3$]propionate. On the other hand, the presence of ^{13}C coupled and uncoupled propionate resonances in the ^{1}H NMR spectrum demonstrated the formation of a mixture of labeled (2.8 mM) and unlabeled propionate (4.0 mM). Under these culture conditions, threonine is the only source of propionate (Section 4.1); thus D-threonine must be the

source of the unlabelled propionate. The slightly larger accumulation of unlabelled propionate suggests that *F. varium* exhibits a slight preference for utilizing D-threonine.

In a second competition experiment, growth of *F. varium* in a mixture of 5 mM L-[U-$^{13}C_4$,^{15}N]threonine and 5 mM DL-*allo*-threonine (unlabelled) (*i.e*. CDMM*(5 mM L-[U-$^{13}C_4$,^{15}N]Thr/5 mM DL-*allo*-Thr/10 mM D-glucose)), produced approximately, equal amounts of labeled (3.4 mM) and unlabelled (3.7 mM) propionate. The formation of similar amounts of labeled and unlabelled propionate suggest no preference between L- and DL-*allo*-threonine for propionate production. The high concentration of unlabelled propionate suggest that both the D and L isomers of *allo*-threonine were used to produce propionate.

4.7. Stereochemical Preferences of Threonine Deaminase, Serine Deaminase and Threonine Aldolase

The metabolism of DL-*allo*-threonine and D-threonine in whole cells indicates that these stereoisomers are substrates for at least one enzyme in *F. varium*. It is possible that separate D/L- threonine deaminases are present or just one deaminase acts on both D- and L-threonine. Studies also suggest that serine deaminase is able to use threonine as a substrate,[178] so it was also examined as a possible catalyst for threonine catabolism.

To distinguish among the threonine/serine deaminase/aldolase possibilities, enzyme activities were determined in crude cell extracts prepared from cells grown with CDMM*(10 mM glucose) and either D-serine, L-serine, D-threonine, or L-threonine at 10 mM. In order to distinguish enzymes induced for the catabolism of an amino acid substrate, enzyme activities were determined in extracts prepared from cells grown in

CDMM*(10 mM glucose) with the omission of either serine or threonine. Cells were grown up to produce ~1 g of damp cell mass (8.5 h incubation). Enzyme activities were determined by incubating crude cell extract with an amino acid substrate, either D-serine, L-serine, D-threonine, DL-*allo*-threonine or L-threonine, and product formation was determined using ^{1}H NMR spectroscopy. Deaminase activity was indicated by the formation of 2-oxobutyrate and propionate from threonine or pyruvate and acetate from serine, while aldolase activity was indicated by the formation of glycine.

Extracts prepared from cells grown on CDMM*(10 mM glucose) lacking either L-threonine or L-serine contained low levels of deaminase activity (towards L-threonine, L-serine and D-serine) and aldolase activity (towards L-threonine and DL-*allo*-threonine) (Tables 4.8 and 4.9). The L-serine deaminase activity present in cells grown in the absence of serine was consistent with a previous suggestion of a constitutive low level production.[114] Activity levels were not raised when 2 mM L-serine was supplied to the growing cells (Table 4.8) but higher concentrations of L-serine or L-threonine in the growth medium led to high levels of L-serine deaminase activity. The latter result indicates that high levels of substrate can induce enzyme expression. In *Clostridium propionicum*, addition of L-threonine to growth medium increased L-threonine deaminase activity, while the addition of L-serine did not increase L-serine activity.[179] The increase in serine activity from 0.1 to 10 (Table 4.9) resulted after L-serine was added to the growth medium, indicating that in *F. varium* that both deaminases are induced by the addition of substrates.

Table 4.8. Product formed in cell extracts prepared from cells (~1 g/L) grown in CDMM*(10 mM glucose) with or without 10 mM threonine.

Substrate supplied with cell extract (10 mM)	10 mM L-Thr[a]		10 mM D-Thr[b]		0 Thr[c]	
	Deaminase Product (mM)	Aldolase Product (mM)	Deaminase Product (mM)	Aldolase Product (mM)	Deaminase Product (mM)	Aldolase Product (mM)
L-Threonine	6.5	3.1	2.0	1.2	0.5	0.8
D-Threonine	0.7	0	0.6	0	0	0
DL-*allo*-Threonine	0.9	2.9	0.3	0.7	0	0.3
L-Serine	10.0	0	0.6	0	0.2	0
D-Serine	2.3	0	1.0	0	0.2	0

[a]Cell extract prepared from cells grown in CDMM*(10 mM L-threonine/10 mM glucose). [b]Cell extract prepared from cells grown in CDMM*(0 mM L-threonine/10 mM D-threonine/10 mM glucose). [c]Cell extract prepared from cells grown in CDMM*(0 mM L-threonine/10 mM glucose). Results are the average of two independent experiments.

Table 4.9. Product formed in cell extracts prepared from cells (~1 g/L) grown in CDMM*(10 mM glucose) with or without 10 mM serine.

Substrate supplied with cell extract	10 mM L-Ser[a]		10 mM D-Ser[b]		0 mM Ser[c]	
	Deaminase Product (mM)	Aldolase Product (mM)	Deaminase Product (mM)	Aldolase Product (mM)	Deaminase Product (mM)	Glycine Product (mM)
L-Threonine	6.2	2.5	1.0	3.0	0.3	0.8
D-Threonine	4.8	0.5	8.5	0.3	0	0
DL-*allo*-Threonine	2.0	4.6	1.0	1.6	0	0.5
L-Serine	10.0	0	10.0	0	0.1	0
D-Serine	10.0	0	10.0	0	0.1	0

[a]Cell extract prepared from cells grown in CDMM*(10 mM L-serine/10 mM glucose). [b]Cell extract prepared from cells grown in CDMM*(0 mM L-serine/10 mM D-serine/10 mM glucose). [c]Cell extract prepared from cells grown in CDMM*(0 mM L-serine/10 mM glucose). Results are the average of two independent experiments.

When L-threonine was present in the growth medium, the cell extract showed elevated levels of deaminase activity towards L-threonine and L-serine (Table 4.8), consistent with substrate-induced expression of enzymes in *F. varium*. On the other hand, extracts prepared from cells grown with D-threonine, showed little (or no) elevation of deaminase activity. High levels of deaminase activity towards all amino acid substrates were found in the extract prepared from cells grown in the presence of L-serine (Table 4.9). Except for L-threonine deaminase, similar deaminase activities were induced by D-serine.

The induction of D-serine deaminase activity by both L- and D-serine is consistent with the previous proteomics result.[114] The simultaneous induction of D-threonine deaminase activity and the low levels of D-threonine deaminase activity observed under the other experimental conditions, suggests that D-threonine is a substrate for D-serine deaminase and that a separate D-threonine deaminase is not present in *F. varium*. On the other hand, L-threonine deaminase activity was low and L-serine deaminase activity was high in cells grown with D-serine. Separate enzymes are therefore likely to be produced for the deamination of L-threonine and L-serine. Lower levels of DL-*allo*-threonine deaminase activity were found whenever deaminase activity was induced by the other substrates. The levels, however, correlated with L-threonine deamination, suggesting that DL-*allo*-threonine is a substrate for L-threonine deaminase. However, catalysis of DL-*allo*-threonine deamination by serine deaminases cannot be ruled out from the results in Tables 4.8 and 4.9.

Increased aldolase activity towards L-threonine and DL-*allo*-threonine was detected when cells were grown with either L-threonine, L-serine or D-serine. Given the

broad substrate specificity of threonine aldolases isolated from other bacteria,[180] it is likely that both L-threonine and DL-*allo*-threonine are substrates for the same enzyme. By contrast, no aldolase activity was observed in cell extracts when serine was provided as a substrate.

The enzymes deduced from the above results are consistent with annotations in the *F. varium* genome.[19] Genome sequences corresponding to threonine aldolase (low specificity, catalyzing the conversion of L-threonine or L-*allo*-threonine), threonine dehydratase, serine racemase, D-serine dehydratase, and L-serine ammonia-lyase have been identified. Stereochemistry given where indicated in the genome. D-Serine deaminase (E.C. 4.3.1.18), L-serine deaminase (E.C. 4.3.1.17), L- threonine deaminase (E.C. 4.3.1.19), have several synonyms including ammonia-lyases and dehydratases.[181]

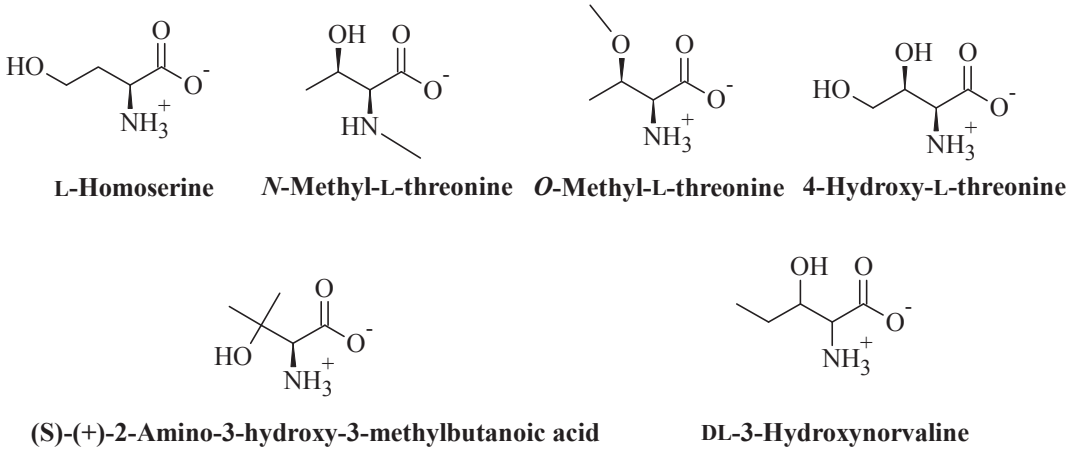
4.8. Metabolism of Threonine Analogs

The substrate specificity of serine deaminase and threonine aldolase was probed in whole cells and in some experiments, cell extracts, by supplying analogs of threonine (Figure 4.6) for metabolism by *F. varium*.

Defined medium (CDMM*(0 Thr/10 mM glucose)) supplemented with 10 mM L-homoserine supported abundant growth of *F. varium* (OD_{660} 1.1 ± 0.1), but poor utilization of L-homoserine (7.8 ± 2.1 mM unmetabolized) was observed. Only small accumulations of propionate (0.2 ± 0.3 mM) were detected after 24 h, whereas the accumulation of other carboxylic acid end products was similar to those found when L-threonine was supplied to growth media. Addition of 10 mM 4-hydroxythreonine inhibited growth of *F. varium* (OD_{660} < 0.1) and production of end products.

Growth of *F. varium* was poor (OD_{660} < 0.2) on defined medium CDMM*(0 threonine/10 glucose) containing either 10 mM of (*S*)-(+)-2-amino-3-hydroxy-3-methylbutanoic acid or DL-3-hydroxynorvaline and carboxylic acid end product accumulation was negligible. When these threonine analogs were supplied to crude cell extracts prepared from CDMM*(10 mM L-threonine/10 mM glucose), 10 mM of residual substrate was present and no end product was detected.

Good growth was obtained when either *N*-methyl-L-threonine or *O*-methyl-L-threonine (OD_{660} > 0.8) was present in the medium (CDMM*(0 mM threonine/10 mM glucose). Small amounts of acetate, butyrate, formate and lactate end products and 8.0 - 10.0 mM of the threonine analog were detected in spent culture medium. In crude cell extract incubations, only the supplied substrate was detected in ^{1}H NMR spectra.



L-Homoserine ***N*-Methyl-L-threonine** ***O*-Methyl-L-threonine** **4-Hydroxy-L-threonine**

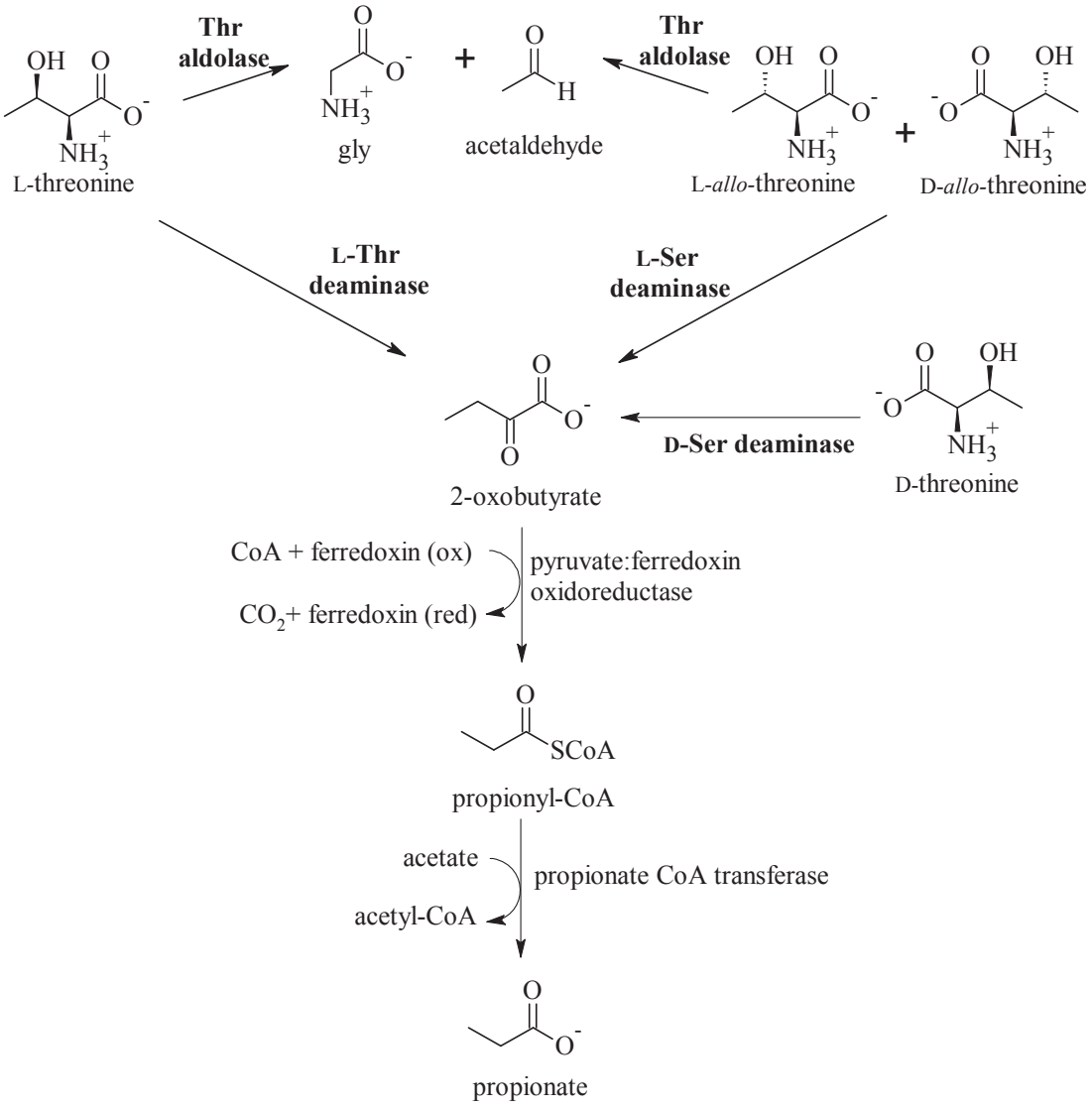
(S)-(+)-2-Amino-3-hydroxy-3-methylbutanoic acid **DL-3-Hydroxynorvaline**

Figure 4.6. Threonine analogs tested as alternative substrates or metabolic inhibitors.

While the analogs only differed from threonine by the addition of a methyl group (*N*-methyl-L-threonine, *O*-methyl-L-threonine, (S)-(+)-2-amino-3-hydroxy-3-methylbutanoic acid, DL-3-hydroxynorvaline), or the addition/location of a hydroxyl group (L-homoserine, 4-hydroxythreonine), none served as substrates for enzymes in *F. varium*. Three compounds (4-hydroxythreonine, (S)-(+)-2-amino-3-hydroxy-3-methylbutanoic acid and DL-3-hydroxynorvaline), however, inhibited growth of *F. varium* when supplied at high concentrations. Whether these compounds are effective growth inhibitors at lower concentrations and the mechanism of the inhibition are interesting points for further investigation.

4.9. Summary of Threonine Catabolism

The hypothesis of threonine being a source of propionate was tested by eliminating L-threonine from CDMM*. Knowing the exact composition of the medium, this amino acid was easily removed with small effects on growth. Removal of L-threonine resulted in no propionate being produced, supporting the threonine-propionate substrate-product relationship. Adding L-threonine to the growth medium showed an increase in propionate production, however, using a quantitative analysis, the ratio of threonine to propionate was not 1 to 1. These results allowed for the exploration of other possible pathways of threonine catabolism, leading to the determination of the enzymes used for the catabolism of threonine stereoisomers summarized in Scheme 4.1. Overall, the results demonstrate the benefits of using a defined medium where the exact composition is known and a quantitative method to explore metabolism in microorganisms.



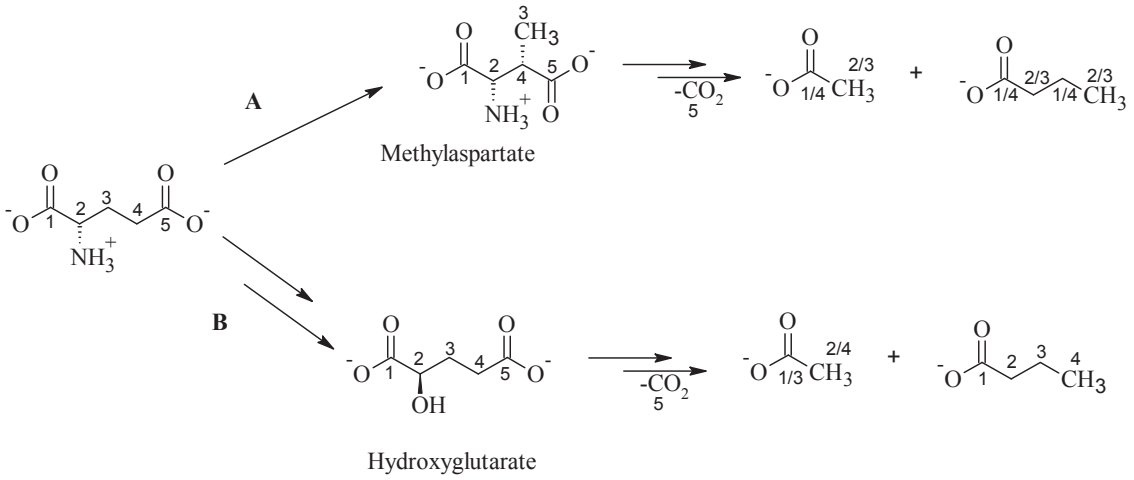
Scheme 4.1. Summary of threonine catabolism in *F. varium*. Enzymes that use various threonine stereoisomers determined in this chapter are given in bold.

CHAPTER 5: PATHWAYS OF GLUTAMATE CATABOLISM

Catabolism of glutamate by either the aminobutyrate, hydroxyglutarate or methylaspartate pathway creates distinctive patterns for the distribution of C1-C5 of glutamate in CO_2, acetate and butyrate, the common end products for each pathway (Scheme 1.3 and Scheme 5.1).[92, 120, 124] In the 4-aminobutyrate and hydroxyglutarate pathways, crotonyl-CoA and subsequently butyrate are formed from four carbons of glutamate (C2-C5 and C1-C4, respectively). The methylaspartate pathway, however, forms acetate and acetyl-CoA by cleaving the C2-C3 bond in glutamate; butyrate is then formed from two acetyl units via the acetate-butyrate pathway (Scheme 1.2) transferring C1-C4 of glutamate equally into two positions of butyrate (Scheme 5.1). In previous *F. varium* experiments,[92] label from L-[5-^{13}C]glutamate was not incorporated into the carboxylate end-products, in accord with the hydroxyglutarate and methylaspartate pathways in which C5 is lost as CO_2 (Scheme 5.1). This labeling result is inconsistent with glutamate catabolism via the 4-aminobutyrate pathway that delivers C5 of glutamate into C1 of butyrate. Other than one report,[119] no experimental evidence has been found in support of the 4-aminobutyrate pathway in *F. varium*.

On undefined medium (peptone), catabolism of L-[4-^{13}C]glutamate by *F. varium* enriched C1 of acetate and gave equal enrichments at C1 and C3 of butyrate, results that are consistent with the methylaspartate pathway.[112] In a proteomics investigation,[114] *F. varium* was cultured on CDMM1, a defined medium in which all amino acids were supplied at 1 mM (except tyrosine at 0.3 mM). The proteomics study detected the expression of two enzymes in the hydroxyglutarate pathway (NAD-specific glutamate dehydrogenase and 2-hydroxyglutaryl-CoA dehydratase), and genes for all enzymes in

the hydroxyglutarate pathway were identified using PCR. Finally, a much earlier study[120] cultured *F. varium* on undefined basal PYG medium containing DL-[1-^{14}C]glutamic acid. The ^{14}C label was detected at C1 of butyrate, consistent with the hydroxyglutarate pathway. The isotopic enrichment was located by chemical degradation of butyrate. After the chemical decarboxylation of butyrate, label was detected in CO_2 and only a small amount (~ 3 %) was recovered in propionic acid, the other degradation product. Glutamate used in this experiment was a racemic mixture. However, supplying D-[3-^{13}C]glutamate in peptone to *F. varium* produced acetate with enrichment at C2, and butyrate equally enriched at C2 and C4, results consistent with the methylaspartate pathway.[112] Most likely, D-[3-^{13}C]glutamate was converted to L-glutamate using glutamate mutase, which then followed the methylaspartate pathway.



Scheme 5.1. Catabolism of glutamate by the (A) methylaspartate and (B) hydroxyglutarate pathways. Numbers referring to the carbon atoms in glutamate show the distribution of glutamate carbons in acetate and butyrate.

Overall, there is evidence to suggest that both the hydroxyglutarate and methylaspartate pathways are present in *F. varium*, the conditions under which each pathway is used remain unclear.[114] In this chapter, glutamate catabolism was investigated using L-[4-^{13}C]glutamate and *F. varium* cultured on the defined medium (CDMM1)[114] used in the previous studies that showed evidence of the hydroxyglutarate pathway. A label at C4 of glutamate leads to distinctive labeling patterns in acetate and butyrate formed by the methylaspartate and hydroxyglutarate pathways (Scheme 5.1), allowing the relative contributions of each pathway to be assessed.

5.1. Growth and Utilization of Glutamate

Growth of *F. varium* on CDMM1(40 mM L-glutamate)[114] inoculated with cells grown in CDMM1(20 mM glucose), reached OD_{660} of 0.5 ± 0.01 (average of 3 cultures). Quantification of the end products indicated 10.3 mM of butyrate, 21.3 mM of acetate and 31.8 mM of residual L-glutamate. No formate, lactate or succinate was detected, but propionate was present at 2 mM, a value higher than 1 mM, the concentration of L-threonine supplied in the initial growth medium, but could be overestimated due to the presence of Group 1 amino acids supplied at 1 mM in CDMM1 (see Chapters 2 and 4). This indicated that catabolism of glutamate produces only acetate and butyrate.

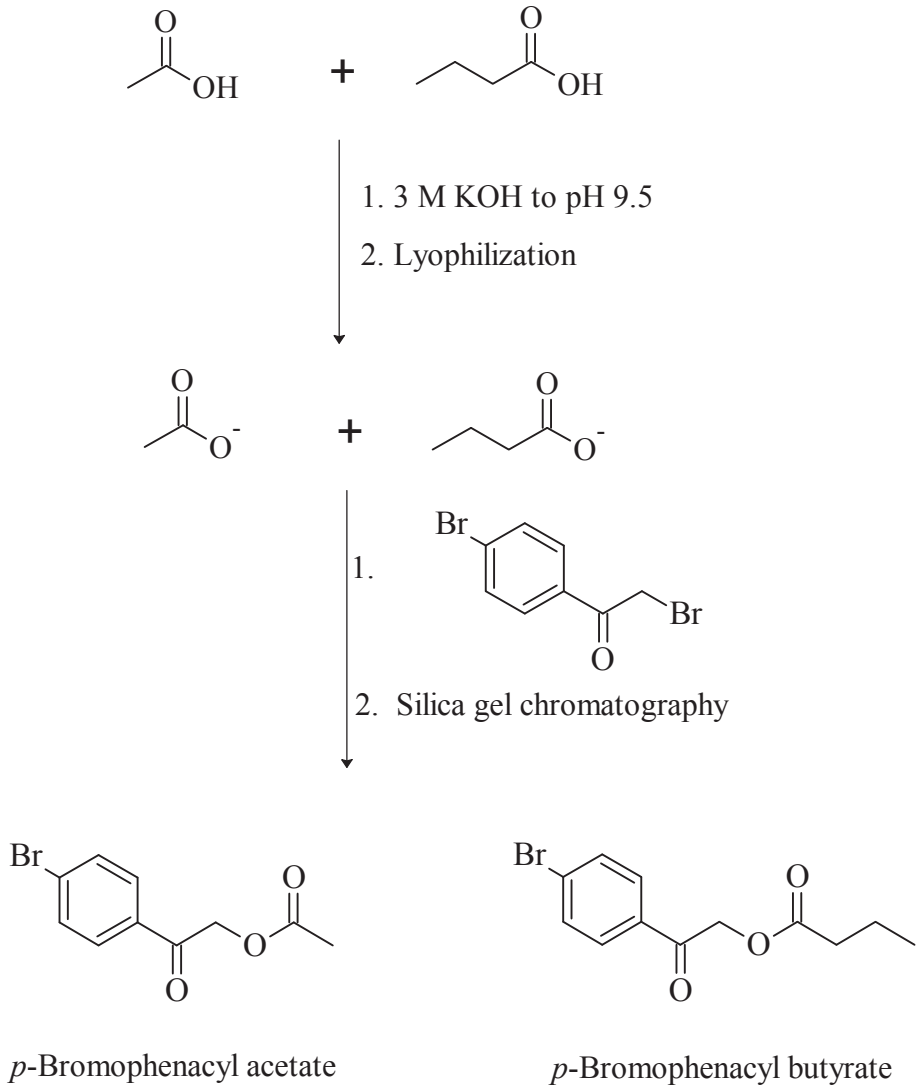
During growth of *F. varium*, approximately 10 mM of L-glutamate was utilized. Glutamate catabolism, therefore, accounts for only about half the accumulated acetate and butyrate. In Chapter 2, the efficient utilization of nine amino acids by *F. varium* was elucidated. The collective catabolism of these other amino acid substrates would account for the remaining acetate and butyrate production.

The moderate growth of *F. varium* on CDMM1(40 mM L-glutamate), the utilization of glutamate and the likely conversion of glutamate to metabolic end products reinforce glutamate as an energy source for *F. varium*.[113,119]

These growth conditions and L-glutamate ^{13}C enriched at carbon four were used to determine the pathway(s) of glutamate catabolism. The end products acetate and butyrate were derivatized as *p*-bromophenacyl esters, to allow ^{13}C enrichments in the carboxylic acids to be determined relative to the ^{13}C at natural abundance in the carbons of the derivatizing reagent (Scheme 5.2).[92]

5.2. Pathway Determination: L-[4-^{13}C]Glutamate and CDMM1

F. varium was grown for 24 h in defined medium (CDMM1(16 mM L-[4-^{13}C]glutamate/24 mM L-glutamate)). Only 40% of the supplied glutamate had a ^{13}C enrichment, this was to dilute the ^{13}C enrichment through the possible recombination of two acetate units to butyrate. After incubation, the culture supernatant was titrated to pH 9.5 using potassium hydroxide and the sample was freeze dried. The freeze dried powder was refluxed with *p*-bromophenacyl bromide, and dicyclohexano-18-crown-6 in acetonitrile. The reaction was monitored using thin layer chromatography (TLC); the acetate and butyrate derivatives were separated using silica gel flash chromatography (Scheme 5.2). The ^{13}C NMR spectra showed ^{13}C enrichment in both carbons of acetate and all four carbons of butyrate (Table 5.1, Expt 1) (Figures 5.1 A and 5.2A). The enrichment in all carbons indicated that more than one pathway of glutamate catabolism was used by *F. varium* during growth in CDMM1 (Scheme 5.1). Approximately 70% of the label was located at C4 of butyrate, demonstrating a major contribution by the



Scheme 5.2. Procedure for the derivatization of acetate and butyrate as *p*-bromophenacyl esters.

hydroxyglutarate pathway (Scheme 5.1, Route B). During catabolism of L-[4-^{13}C]glutamate, crotonyl-CoA, an intermediate in the hydroxyglutarate and core acetate–butyrate pathways, also would become enriched at C4. Cleavage of [4-^{13}C]crotonyl-CoA to [2-^{13}C]acetyl-CoA, and subsequent resynthesis of crotonyl-CoA from [2-^{13}C]acetyl-

CoA units, would place smaller ^{13}C enrichments at C2 of acetate and butyrate, and a minor contribution of ^{13}C at C4 of butyrate. The labeling at C1 of acetate and C1/C3 of butyrate are consistent with catabolism of a small fraction of L-[4-^{13}C]glutamate by the methylaspartate pathway (Scheme 5.1, Route A).

Table 5.1. Isotopic enrichments in acetic and butyric acids derived from L-[4-^{13}C]glutamate. Lyophilized products were derivatized, separated and analyzed by ^{13}C NMR spectroscopy. The standard deviation calculated from the natural abundance signals contributed by the derivatization reagent is given in parentheses

		^{13}C enrichment (%) (^{13}C NMR analysis)							
		Acetate			Butyrate				
Expt #	Addition to CDMM1 (1 μM)[a]	C-1	C-2		C-1	C-2	C-3	C-4	
1	-	8.4	8.6	(0.2)	3.7	2.6	3.4	21.1	(0.1)
2	Coenzyme B_{12}	19.1	0.2	(0.05)	16.1	–0.1	16.6	1.7	(0.1)
3	$CoCl_2$	18.2	0.2	(0.05)	15.4	–0.1	15.4	1.7	(0.1)

[a]Cells were grown in CDMM1((16 mM L-[4-^{13}C]glutamate/24 mM L-glutamate) for 24 h (37 ºC).

In the previous proteomics experiment,[114] growth on CDMM1(40 mM L-glutamate) resulted in the expression in glutamate mutase and methylaspartase, enzymes in the methylaspartate pathway. The above results showed, however, that this pathway was not functioning under these conditions. The defined medium, CDMM1 only contains calcium-D-pantothenate and folate as vitamins.[113] Glutamate mutase (E.C. 5.4.99.1), an enzyme from the methylaspartate pathway (Scheme 5.1, Route A), requires coenzyme B_{12}.[182] The absence of this coenzyme, or cobalt ion, the essential metal in coenzyme B_{12}, could reduce the functionality of this pathway. To test this hypothesis, *F. varium* was

grown on CDMM1(16 mM L-[4-^{13}C]glutamate/24 mM L-glutamate) supplemented with either cobalt ion (cobalt (II) chloride) or coenzyme B_{12}. The culture supernatants were freeze dried and *p*-bromophenacyl ester derivatives were made from the carboxylic acids. The ^{13}C NMR spectra of the products isolated from both experiments showed similar patterns of ^{13}C enrichment (Table 5.1, Expts 2 and 3) (Figures 5.1 B, C and 5.2 B, C). High ^{13}C enrichment were observed at C1 of acetate and C1/C3 of butyrate. The positions of these high isotopic incorporations and the equal enrichment at two positions in butyrate were consistent with the methylaspartate pathway (Scheme 5.1, Route A). The small enrichment at C4 of butyrate shows only a minor participation of the hydroxyglutarate pathway when coenzyme B_{12} is available.

Glutamate catabolism using the aminobutyrate pathway would place a ^{13}C enrichment from L-[4-^{13}C]glutamate on to C2 of butyrate and acetate. In all conditions tested, C2 of butyrate was not ^{13}C enriched, while the ^{13}C enrichment found on C2 of acetate in Experiment 1 (Table 5.1) could be explained from the interconversion of acetate and butyrate. The labeling results confirm the previous results of the lack of the aminobutyrate pathway in *F. varium* to catabolize glutamate.[92]

The similar ^{13}C enrichments obtained when either coenzyme B_{12} or $CoCl_2$ was added to (CDMM1(16 mM L-[4-^{13}C]glutamate/24 mM L-glutamate) indicate that *F. varium* is able to biosynthesize coenzyme B_{12} when cobalt ion is available. In both cases, the methylaspartate pathway, which requires coenzyme B_{12} to function, was favoured over the hydroxyglutarate pathway. The biosynthesis of coenzyme B_{12} is common in bacteria such as *Pseudomonas denitrificans, Salmonella typhimurium* or *E. coli*.[183,184,185] The genome of *F. varium*[19] does have encoded several enzymes for the biosynthesis of

coenzyme B_{12} including alpha-ribazole-5'-phosphate phosphatase (E.C. 3.1.3.73), an enzyme which catalyzes the final step of coenzyme B_{12} biosynthesis.

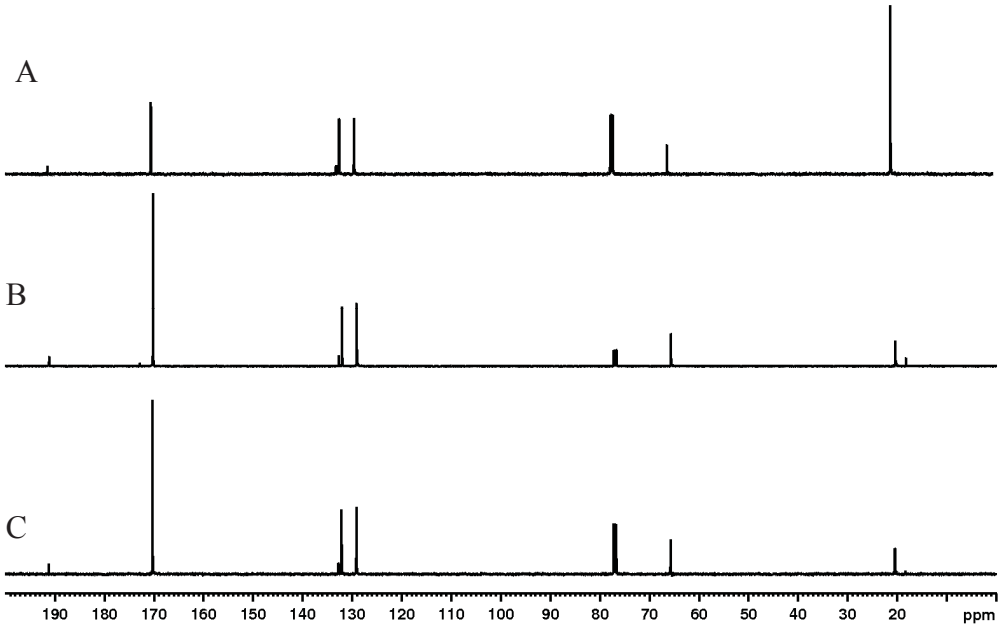


Figure 5.1. ^{13}C NMR spectra of *p*-bromophenacyl acetate isolated after culturing *F. varium* on CDMM1((16 mM L-[4-^{13}C]glutamate/24 mM L-glutamate) supplemented with (A) no additions, (B) 1 μM Coenzyme B_{12} (C) 1 μM $CoCl_2$. C1 of acetate at δ170.7 and C2 at δ 20.8; remaining peaks from derivatizing agent or $CDCl_3$.

The results of the L-[4-^{13}C]glutamate experiments demonstrate that the medium can greatly influence the pathway used to metabolize substrates. In the presence of cobalt ion or coenzyme B_{12}, glutamate catabolism by the methylaspartate pathway was favoured over the hydroxyglutarate pathway. It was believed previously that the pathways of

glutamate catabolism was species specific;[186] however, it now appears that for *F. varium* that it's medium dependent.

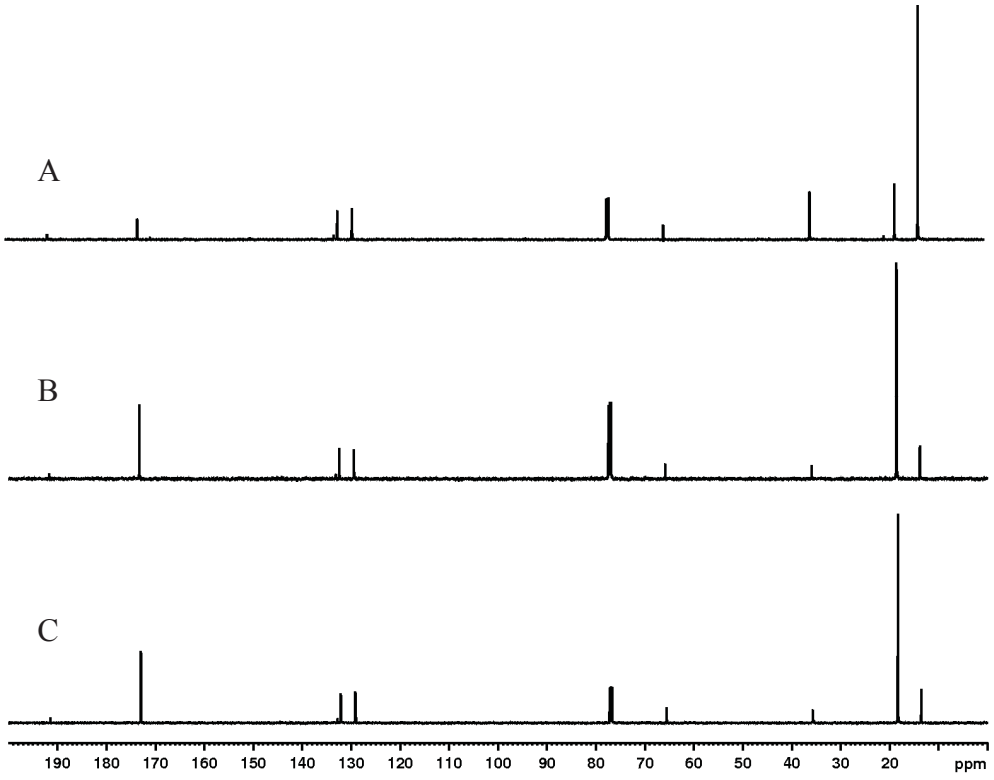


Figure 5.2. ^{13}C NMR spectra of *p*-bromophenacyl butyrate isolated after culturing *F. varium* on CDMM1((16 mM L-[4-^{13}C]glutamate/24 mM L-glutamate) supplemented with (A) no additions, (B) 1 μM Coenzyme B_{12} (C) 1 μM $CoCl_2$. C1 of butyrate at δ 173.3, C2 at δ 35.9, C3 at δ 18.6 and C4 at δ 13.9; remaining peaks from derivatizing agent or $CDCl_3$.

Why two separate pathways for the catabolism of L-glutamate exist in *F. varium* remains unclear, as well as the control parameters for the expression for the enzymes in each of these pathways. The hydroxyglutarate pathway yields crotonyl-CoA, which can, through the acetate-butyrate pathway, be converted to acetate generating ATP for energy.

However, conversion of crotonyl-CoA to acetyl-CoA requires oxidation of crotonyl-CoA. Alternatively, in the methylaspartate pathway, acetate and pyruvate are produced without the need for NADH/NAD^+ redox steps. Pyruvate can be converted to acetyl-CoA, which can be used to generate ATP or to regenerate NAD^+ upon conversion to butyrate. The methylaspartate pathway provides a more efficient route to generate ATP from glutamate. However, the biosynthesis of coenzyme B_{12} requires about 30 enzyme-catalyzed steps.[187] Loss of the ability of *F. varium* to biosynthesize coenzyme B_{12}[182] or the lack of cobalt could be the reason for the presence of an alternative pathway for catabolism of glutamate, a major energy source in *F. varium*.[117,119]

CHAPTER 6: EXPERIMENTAL

6.1. Chemicals

D-[U-$^{13}C_6$]glucose and L-[$^{13}C_4$,^{15}N]threonine were purchased from Cambridge Isotope Laboratories (Andover, MA). Yeast extract and BHI agar purchased from Fluka Biochemika (Buchs, Switzerland). Bactopeptone and peptones from DIFCO laboratories (Detroit, MI). DL-*allo*-Threonine was supplied by Acros Organics (New Jersey), and *N*-methyl-L-threonine and *O*-methyl-L-threonine were provided by CHEM-IMPEX International (Wood dale, IL). All other chemicals were obtained from Sigma-Aldrich (Oakville, ON).

6.2. Media

BHI Agar: Agar plates were prepared from autoclaved (20 min, 121 ºC) brain heart infusion (BHI) agar (52.0 g/L; Fluka, Buchs, Switzerland).

Peptone medium:[117] Peptone medium was made by dissolving in deionized water: proteose peptone (5.0 g/L); trypticase peptone (5.0 g/L); yeast extract (5.0 g/L); D-glucose (5.0 g/L); NaCl (5.0 g/L) and L-cysteine HCl (0.8 g/L). The solution was titrated to pH 7.4 using 5 M NaOH and autoclaved (20 min, 120 ºC).

CDMM1 and CDMM2: Chemically defined minimal medium containing amino acids at 1 mM (CDMM1) and 2 mM (CDMM2) as described.[113]

Basal Medium: NaCl (5.00 g/L) and KH_2PO_4 (2.72 g/L) were dissolved in water and adjusted to pH 7.4 using 5 M NaOH.

CDMM*: Modified chemically defined minimal medium consisted of the salts (4.54 mM $(NH_4)_2SO_4$, 0.135 M $CaCl_2$, 0.0592 mM $MnSO_4$, 1.45 mM $MgSO_4$, 0.870 mM sodium citrate, 0.0360 mM $FeSO_4$, and 0.0615 μM Na_2MoO_4), and vitamins (9.19 μM

calcium D-pantothenate and 1.13 μM folic acid) used in CDMM1 and CDMM2,[113] 0.2 mM Group 1 amino acids (alanine, arginine, isoleucine, leucine, glycine, methionine, proline and valine), 1.5 mM Group 2 (tyrosine, tryptophan and phenylalanine) and 2 mM Group 3 amino acids (aspartate, asparagine, cysteine, glutamine, glutamate, histidine, lysine, serine, threonine). Concentrated stock solutions of salts, vitamins and amino acids were prepared in basal medium. The solutions of amino acids were readjusted to pH 7.4 using NaOH as needed. The solutions of vitamins, amino acids were filter sterilized (0.20 μm filters) upon addition to autoclaved (20 min, 120 ºC) basal medium and salt solution.

Growth Media: Typically, growth media were prepared by the addition of an energy source (e.g. glucose, glycerol, pyruvate, acetoacetate, (*R*)-3-hydroxybutyrate, crotonate, threonine, serine, 2-oxobutyrate) or end product (acetate, butyrate, formate, lactate, propionate, succinate) dissolved in basal medium at pH 7.4. The composition of the media is described by the abbreviation for the medium following in parenthesis by omissions and additions of medium components. For example, CDMM*(0 Thr/10 mM glucose) indicates modified chemically defined minimal medium prepared without threonine and supplemented with 10 mM glucose.

6.3. Microorganism

A revived lyophilized culture of *Fusobacterium varium* (ATCC 8501) was maintained by biweekly transfers to a fresh BHI agar plate. The freshly inoculated BHI agar plate was incubated at 37 ºC for 24 h and stored at 4 ºC. All bacterial transfers were performed in air; agar and liquid cultures were incubated and stored in anaerobic jars containing a palladium catalyst (Oxoid Canada, Nepean, ON). Anaerobic conditions

were established by evacuating air three times and pressurizing with $N_2/H_2/CO_2$ (80:10:10 v/v/v). Centrifugation to collect cells from liquid cultures was performed at 8000 *g* for 10 min at 20 ºC.

6.4. Growth of *F. varium*

Peptone medium (10 mL) was inoculated with a colony picked from a BHI agar plate. After incubation, under anaerobic conditions at 37 ºC for 16 h (unless stated otherwise), the cells were collected and washed (2 x 5 mL basal medium) by centrifugation. Washed cells were resuspended in basal medium (10 mL) and used to inoculate (2% v/v) growth media (5 mL) in 10 mL culture tubes, each fitted with a septum pierced with a narrow bore (23 gauge) syringe needle to allow exchange of gases during the establishment of anaerobic conditions and to minimize exposure to oxygen during optical density measurements. The cultures were incubated under anaerobic conditions at 37 ºC. Optical densities at 660 nm (OD_{660}) (Agilent 8453 Vis-UV Spectrophotometer) were measured before and after incubation using water as a blank. Unless indicated otherwise, after 16 h, the cultures were centrifuged and the pellets discarded. Dichloroacetic acid (15 mg/15 mL) and tartaric acid (10 mg/15 mL) were added to the culture supernatants; the supernatants were titrated to pH 9.5 using 5 M NaOH and lyophilized.

6.5. ^{1}H and ^{13}C NMR Analysis

The lyophilized residues were dissolved in D_2O (10 mg/0.75 mL). ^{1}H NMR spectra were acquired (typically 32 scans) at 500 MHz on a Bruker AVANCE 500 MHz

Spectrometer typically using a relaxation delay (D1) time of 10 s and an acquisition time of 3.25 s. The data acquired were processed using 1D settings, multiplied by an exponential apodization with line broadening of 0.3 Hz prior to Fourier transformation. The baseline was corrected manually with a polynomial function. Peaks were phase adjusted manually. Chemical shifts were referenced to residual water in D_2O. The amount of end product was calculated by comparing integrated peak areas to those of the known amounts of the internal standards, dichloroacetic acid (δ 6.03 (s)) or tartaric acid (δ 4.28 (s)). ^{13}C NMR (proton decoupled) spectra were acquired at 125 MHz on a Bruker AVANCE 500 MHz spectrometer with a D1 time of 1 s, acquisition time of 0.8 s. Peaks were phased manually.

6.6. T_1 Relaxation Time Determination

T_1 times were determined on a standard sample of carboxylate ions, where the carboxylic acid was dissolved in D_2O and T_1 time was measured and on carboxylate end products from culture samples, where ~20 mg of lyophilized cell supernatants were dissolved in D_2O and T_1 times were measured. T_1 times were determined using a Bruker/Tecmag AC-250 spectrometer, T_1 proton inversion recovery experiments were performed. T_1 proton 2D sequences were used (h90 = 9 ms and h180 = 18 ms), three scans and an acquisition time of 3.08 s. A total of 10 2D data points were collected using tau (τ) values of 0.1, 0.5, 1, 1.5, 2, 4, 8, 10, 25 and 50 s with a 20 s last delay for relaxation times of less than 5 s, and tau (τ) values of 0.5, 2, 4, 5, 8, 10, 15, 20, 30 and 60 s with a 50 s last delay for relaxation times greater than 5 s. Spectra were processed using 1D settings; exponential apodization was applied with a baseline correction, a zero fill of

1, line broadening of 0.2 Hz, and Fourier transformation with system phasing. Using NTNMR data analysis, T_1 values were calculated from the intensity (I) measured at each tau value, the maximum intensity (I_0) and the equation: $I = I_0(1–2e^{-\tau/T1})$.

6.7. Determination of Lactate Stereochemistry

The catalytic activity of lactate dehydrogenase (LDH) and glutamic-pyruvic transaminase (GPT) enzymes were determined using a series of standard assays[188] by incubating a known amount of substrate and enzyme, the absorbance change over time was measured to calculate the units of active enzyme.

A lyophilized sample of spent culture medium (3.0 mg) and 10.0 μmol NAD^+ were dissolved in glycylglycine buffer (50 mM glycylglycine, 40 mM L-glutamate, pH 10.0). The enzymes GPT (100 μL; 10 U in 1.0 mL of nanopure water) and either D- or L-LDH (100 μL; 80 or 120 U in 1.0 mL of nanopure water) were added and the mixture was incubated (25 ºC, 3 h). The absorbance change at 340 nm was monitored. After incubation, the solution was frozen and freeze dried. ^{1}H NMR spectra were obtained using a 1 s delay time and 512 scans.

6.8. Preparation of Cell Extracts

Peptone (20 mL) was inoculated with 2 loopfuls of *F. varium* cells, grown at 37 °C for 24 h on BHI agar. Cells grown in peptone were collected by centrifugation (3 x 8000 *g*, 10 min, 20 °C), washed three times with basal medium (10 mL) and resuspended. Resuspended cells in basal medium (20 mL) were used to inoculate 1 L of CDMM*(10 mM glucose) supplemented with 10 mM threonine or serine. After 8.5 h incubation (37

°C), the cells (~ 1 g, damp cell mass) were harvested by centrifugation in tubes purged with nitrogen gas (8200 *g*, 20 min, 20 °C). The pelleted cells were washed twice with potassium phosphate buffer (50 mM, pH 7.4) containing 2 mM of dithiothreitol and the cells were stored at -20 °C overnight. Thawed cells were resuspended in 5 mL of potassium phosphate buffer (50 mM , pH 7.4) containing 2 mM of dithiothreitol and sonicated for 2 min in 15 s intervals, while cooling in ice-sodium chloride bath and purging with nitrogen gas. The sonicated suspension was centrifuged (10000 *g*, 20 min, 4 °C); the supernatant was used as crude cell free extract.

6.8.1. Substrate-product conversions in crude cell extract

Crude cell free extract (1 mL) containing 10 mM substrate was incubated (37 °C, 4 h) and purged with nitrogen gas. The reaction was stopped with 7 mL of methanol, to precipitate the proteins; the solution was rotary evaporated to dryness. The solid residue (~ 20 mg) was dissolved in 0.75 mL D_2O and the solution was filtered through a cotton plug in a Pasteur pipette prior to NMR analysis. (^{1}H NMR: 500 MHz, 64 scans, 1 s delay, ^{13}C NMR: 125 MHz, 512 scans, 1 s delay).

6.9. Growth on Bactopeptone

F. varium grown on peptone medium (24 h) and was used to inoculate (2% v/v) cultures (5 mL) containing salts and vitamins solutions, 10 mM glucose, bactopeptone (1 mg/mL), dissolved in basal medium, pH 7.4 (pH adjusted using 5 M NaOH as required). Cultures were incubated at 37 ºC for 24 h and centrifuged (8000 *g*, 10 min, 20 °C). DCA

(10 mg) was added to the supernatant; pH was adjusted to 9.5 and the solution lyophilized.

6.9.1. Isolation and extraction of meso-*2,3-butanediol*

F. varium, after 24 h growth on peptone medium (10 mL, washed and resuspended in basal medium) was used to inoculate 500 mL culture media containing 10 mM glucose, salts, vitamins and 0.5 g bactopeptone dissolved in basal medium (pH 7.4)). After 24 h incubation, the culture was centrifuged (8000*g* for 10 min at 20 ºC) and the pellet discarded. A portion (5 mL) of the supernatant was removed; internal standard was added (10 mg DCA); the pH was adjusted to 9.5 using 5 M NaOH, and the mixture was freeze dried for quantitative NMR analysis. NaCl (95 g) was dissolved in the remaining 495 mL supernatant; the solution was titrated to pH 7.0 using 5 M NaOH and extracted with diethyl ether (4 x 100 mL). Combined organic layers were dried (anhydrous $MgSO_4$) and rotary evaporated. The liquid residue was vacuum distilled at ambient temperature, and the collection tube was cooled using liquid nitrogen. The liquid distillate (0.0330 g) was dissolved in 0.75 mL of D_2O for ^1H NMR (500 MHz, 1 s delay, 32 scans) and ^{13}C NMR (125 MHz, 1 s delay, 625 scans) analysis. The isolated butanediol was 98% pure by ^1H NMR. Spiking experiments were preformed by mixing ~10 mg of the purified 2,3-butanediol sample with ~10 mg of pure compound, either *meso*-2,3-butanediol or (2*R*,3*R*)-(-)-2,3-butanediol. The mass spectrum obtained from ESI, positive ion mode (Bruker microTOF instrument) showed a peak at *m/z* 113.0577, consistent with the sodium ion adduct with butanediol.

6.10. Glutamate CDMM1 Experiments

CDMM1(20 mM glucose) (5 mL) was inoculated with a colony picked from a BHI agar plate. After incubation, under anaerobic conditions at 37 ºC for 16 h, a portion of the cell suspension (0.2 mL) was used to inoculate 20 mL of growth medium (20 mL CDMM1(24 mM L-glutamate and 16 mM L-[4-^{13}C]-glutamate)). In replicate experiments, the growth medium also contained either cobalt (II) chloride (1 μM) or coenzyme B_{12} (1 μM; 5′-deoxyadenosylcobalamine). Cultures were incubated for 24 h at 37 ºC. Cells were collected using centrifugation (8000*g*, 10 min, 20 °C) and the pellets were discarded. The supernatant was titrated to pH 9.5 using 3 M KOH and freeze dried.

6.10.1. Derivatization of acetate and butyrate

The lyophilized residue was combined with 4-bromophenacyl bromide (0.40 g) and dicyclohexano-18-crown-6 (30 mg) and refluxed in acetonitrile (35 mL). The reaction mixture was monitored by TLC (silica gel, dichloromethane) for the formation of 4-bromophenacyl acetate (Rf 0.4) and 4-bromophenacyl butyrate (Rf 0.7). After approximately 9 h, the reaction mixture was filtered through sintered glass and the filter was washed with acetonitrile (4 x 10 mL); the filtrate was rotary evaporated. The residual oil was dissolved in dichloromethane (2 mL) and mixed with approximately 1 g of silica gel. The mixture was added to the top of a flash chromatograph column containing 15 g of silica gel (dried overnight at 100 °C). The column was eluted with dichloromethane:cyclohexane, concentrations ranging from 30 to 60% dichloromethane (v/v). Fractions (20 mL) were collected and analyzed by TLC; those visibly containing

only acetate or butyrate 4-bromophenacyl esters were pooled separately and evaporated *in vacuo*.

6.10.2. Determination of isotopic enrichments

The isolated 4-bromophenacyl esters and reference samples were dissolved in $CDCl_3$ (~20 mg) for analysis by ^{1}H (500 MHz, 1s delay, 32 scans) and ^{13}C (125 MHz, ~500-2000 scans, 1 s delay) NMR spectroscopy. The ^{13}C enrichment was calculated[92] for each carbon atom by normalizing peak intensities to the average peak intensity of the five carbons in the phenacyl moiety contributed by the derivatization agent. The enrichment factor was determined by dividing the normalized intensity for each peak in spectrum of the labeled compound by the normalized intensity for the corresponding peak in the reference sample. Percentage enrichments were calculated as followed: 1.1(enrichment factor) – 1.1.

CHAPTER 7: CONCLUSIONS

The experimental results described in Chapters 2-5 of this thesis revealed several metabolic characteristics of *F. varium*, an anaerobic, intestinal bacterium. Modifications of a chemically defined minimal medium provided a growth medium and enabled direct analysis of lyophilized culture supernatants using ^{1}H NMR spectroscopy. The ^{1}H NMR spectra clearly showed the accumulation of six carboxylate products (acetate, butyrate, formate, D-lactate, propionate and succinate) and changes in their distribution in response to variations of the composition of the growth medium. By monitoring growth and utilization of amino acids, arginine, isoleucine, leucine, methionine and valine were identified as required substrates to support the growth of *F. varium*.

Substrate-product relationships were determined using isotopically labeled substrates. The incorporation of label from D-[U-$^{13}C_6$]glucose established substrate-product relationships for glucose and acetate, butyrate, lactate and formate while the L-[$^{13}C_4$,^{15}N]threonine experiment demonstrated that threonine was the sole source of propionate on defined medium. 2-Oxobutyrate was identified as an intermediate in the threonine-propionate pathway, and threonine aldolase also catalyzed the cleavage of threonine, contributing to threonine catabolism. The metabolic pathways and enzymes used in *F. varium* to catabolize D- and DL-*allo*-threonine were established using cell extracts.

End product accumulation was influenced by the composition of the defined medium, particularly the energy source. Acetoacetate, crotonate, (*R*)-3-hydroxybutyrate, glucose, glycerol and pyruvate were identified as energy sources in *F. varium*.

Catabolism of the energy source led to intermediates of the acetate-butyrate pathway, a major route for energy (ATP) generation and coenzyme regeneration. Depending on the oxidative requirements for degrading the energy source, variable amounts of acetate and butyrate accumulated.

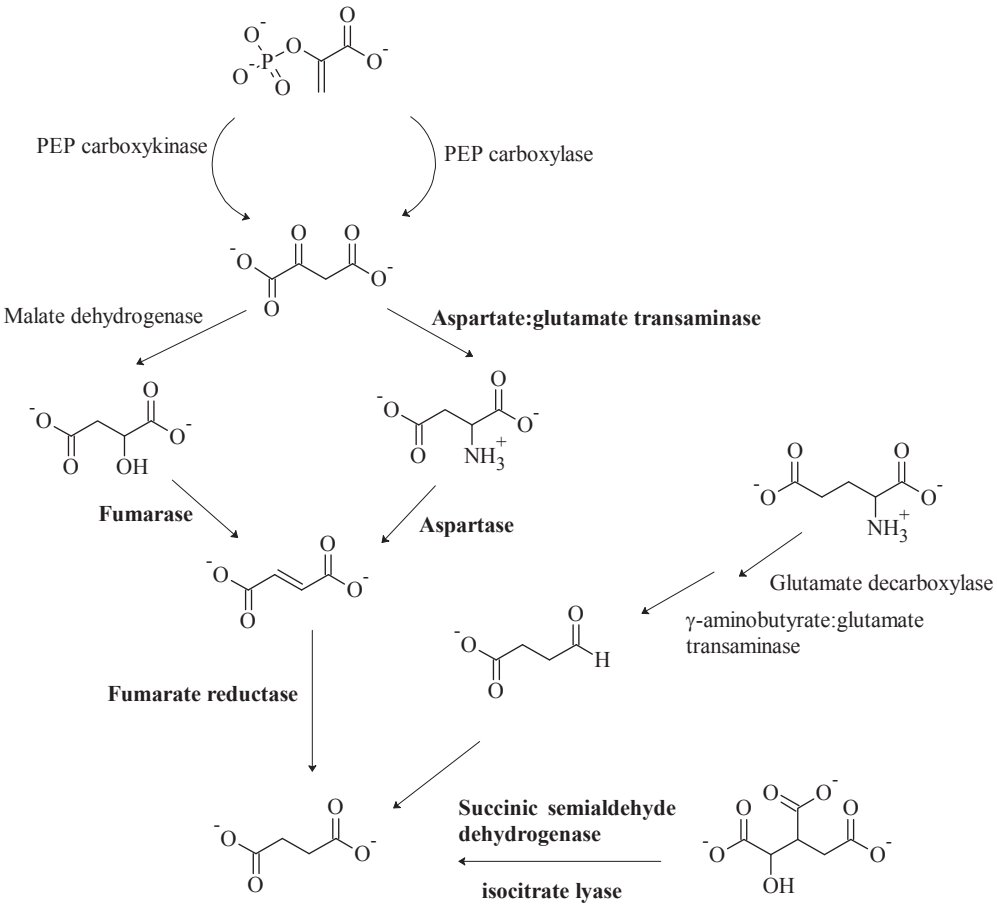
Metabolic pathways in *F. varium* were also influenced by the medium composition. Replacing the amino acid component of CDMM* with bactopeptone resulted in the production of *meso*-2,3-butanediol, a metabolite not previously reported for *F. varium* and a possible link between *F. varium* and bowel disease. The pathway of glutamate catabolism was influenced by the presence of coenzyme B_{12}. In minimal medium without coenzyme B_{12}, the hydroxyglutarate pathway was favoured, while the addition of coenzyme B_{12} or cobalt ion resulted in the methylaspartate pathway being used. The production of butanediol was not predicted by the genome annotations and the defined medium was required to understand when each pathway functioned for the catabolism of glutamate. Thus the experimental approach and the metabolic results complement the genomic data available for *F. varium* and contribute to understanding metabolism in a member of the human microbiome.

7.1. Future Work

As described in Chapter 3, the route of formate production has not yet been determined. The production of formate was extremely variable; large standard deviations were associated with measured formate concentrations. Although a substrate-product relationship was shown for glucose and formate, formate was not entirely ^{13}C enriched and therefore derived from other sources in the medium, such as CO_2. Repeating the

experiment with ^{13}C enriched sodium bicarbonate, without the presence of CO_2 as the $N_2/H_2/CO_2$ gas mixture used in the anaerobic environment may help to show the contribution of CO_2 to formate. Also, H_2 capture may be used to investigate the production of H_2 when/if formate is converted to CO_2 and H_2 using formate dehydrogenase or a similar enzyme.

Succinate production has also not yet been examined in detail. There are several sources of succinate in microorganisms. Succinate in *Bacteroides fragilis*, a gut anaerobe, is formed from phosophoenolpyruvate, producing oxaloacetate, which is reduced to malate, followed by dehydration to fumarate and then reduction making succinate.[189] In *E. coli*, three pathways have been discovered to produce succinate (Scheme 7.1) using phosphoneolpyruvate, glutamate or isocitrate as substrates.[190] Two pathways for generating fumarate have also been determined. Using ^{13}C or ^{2}H enriched substrates for aspartate, (iso)citrate or fumarate may help to elucidate some pathways present in *F. varium*. Several of the enzymes along these metabolic pathways have been identified in *F. varium* genome.[19]



Scheme 7.1. Production of succinate in *E. coli.* Enzymes identified in *F. varium* genome are shown in bold.[19]

REFERENCES

(1) Neish, A. S. *Gastroenterology* **2009***, 136*, 65-80.

(2) Sekirov, I.; Russell, S. L.; Antunes, L. C. M.; Finlay, B. B. *Physiol. Rev.* **2010***, 90*, 859.

(3) Ha, E. M. *Biomol. Ther.* **2011***, 19*, 155-173.

(4) Turnbaugh, P. J.; Ley, R. E.; Hamady, M.; Fraser-Liggett, C. M.; Knight, R.; Gordon, J. I. *Nature* **2007***, 449*, 804.

(5) Peterson, J.; Garges, S.; Giovanni, M.; McInnes, P.; Wang, L.; Schloss, J. A.; Bonazzi, V.; McEwen, J. E.; Wetterstrand, K. A.; Deal, C.; Baker, C. C.; Di Francesco, V.; Howcroft, T. K.; Karp, R. W.; Lunsford, R. D.; Wellington, C. R.; Belachew, T.; Wright, M.; Giblin, C.; David, H.; Mills, M.; Salomon, R.; Mullins, C.; Akolkar, B.; Begg, L.; Davis, C.; Grandison, L.; Humble, M.; Khalsa, J.; Little, A. R.; Peavy, H.; Pontzer, C.; Portnoy, M.; Sayre, M. H.; Starke-Reed, P.; Zakhari, S.; Read, J.; Watson, B.; Guyer, M. *Genome Res.* **2009***, 19*, 2317.

(6) Savage, D. C. *Annu. Rev. Microbiol.* **1977***, 31*, 107.

(7) Bäckhed, F.; Ley, R. E.; Sonnenburg, J. L.; Peterson, D. A.; Gordon, J. I. *Science* **2005***, 307*, 1915.

(8) Gill, S. R.; Pop, M.; DeBoy, R. T.; Eckburg, P. B.; Turnbaugh, P. J.; Samuel, B. S.; Gordon, J. I.; Relman, D. A.; Fraser-Liggett, C. M.; Nelson, K. E. *Science* **2006***, 312*, 1355.

(9) Koenig, J. E.; Spor, A.; Scalfone, N.; Fricker, A. D.; Stombaugh, J.; Knight, R.; Angenent, L. T.; Ley, R. E. *Proc. Natl. Acad. Sci. U. S. A.* **2011***, 108*, 4578.

(10) Dominguez-Bello, M. G.; Blaser, M. J.; Ley, R. E.; Knight, R. *Gastroenterology* **2011***, 140*, 1713.

(11) Hopkins, M. J.; Sharp, R.; Macfarlane, G. T. *Dig. Liver Dis.* **2002***, 34*, S12.

(12) Palmer, C.; Bik, E. M.; DiGiulio, D. B.; Relman, D. A.; Brown, P. O. *PLoS Biol.* **2007***, 5*, 1556.

(13) Costello, E.; Lauber, C. L.; Hamady, M.; Fierer, N.; Gordon, J. I.; Knight, R. *Science* **2009***, 326*, 1694.

(14) Turnbaugh, P. J.; Hamady, M.; Yatsunenko, T.; Cantarel, B. L.; Duncan, A.; Ley, R. E.; Sogin, M. L.; Jones, W. J.; Roe, B. A.; Affourtit, J. P.; Egholm, M.; Henrissat, B.; Heath, A. C.; Knight, R.; Gordon, J. I. *Nature* **2009***, 457*, 480.

(15) Turnbaugh, P.; Quince, C.; Faith, J.; McHardy, A.; Yatsunenko, T.; Niazi, F.; Affourtit, J.; Egholm, M.; Henrissat, B.; Knight, R.; Gordon, J. I. *Proc. Natl. Acad. Sci. U. S. A.* **2010***, 107*, 7503.

(16) Moore WEC, Holdeman LV & Kelley RW (1984) Genus II. Fusobacterium Knorr 1922, 4AL. In Bergey's Manual of Systematic Bacteriology (Krieg NR & Holt JG eds)pp 631–6351 Williams & Wilkins, Baltimore, MD .

(17) Kirby, B. D.; George, W. L.; Sutter, V. L.; Citron, D. M.; Finegold, S. M. *Rev. Infect. Dis.* **1980***, 2*, 914.

(18) Wang, M.; Ahrne, S.; Jeppsson, B.; Molin, G. *FEMS Microbiol. Ecol.* **2005***, 54*, 219.

(19) http://www.ncbi.nlm.nih.gov/; Identification Number: ACIE00000000 .

(20) Nell, S.; Suerbaum, S.; Josenhans, C. *Nat. Rev. Microbiol.* **2010***, 8*, 564.

(21) O'Hara, A. M.; Shanahan, F. *EMBO Rep.* **2006***, 7*, 688.

(22) Stecher, B.; Hardt, W. D. *Curr. Opin. Microbiol.* **2011***, 14*, 82.

(23) Mazmanian, S. K.; Liu, C. H.; Tzianabos, A. O.; Kasper, D. L. *Cell* **2005***, 122*, 107.

(24) Turnbaugh, P. J.; Ley, R. E.; Mahowald, M. A.; Magrini, V.; Mardis, E. R.; Gordon, J. I. *Nature* **2006***, 444*, 1027-31.

(25) Turnbaugh, P. J.; Bäckhed, F.; Fulton, L.; Gordon, J. I. *Cell Host Microbe* **2008***, 3*, 213.

(26) Hildebrandt, M. A.; Hoffmann, C.; Sherrill–Mix, S. A.; Keilbaugh, S. A.; Hamady, M.; Chen, Y. Y.; Knight, R.; Ahima, R. S.; Bushman, F.; Wu, G. D. *Gastroenterology* **2009***, 137*, 1716.

(27) Sartor, R. B. *Gastroenterology* **2004***, 126*, 1620.

(28) Macfarlane, S.; Steed, H.; Macfarlane, G. T. *Crit. Rev. Clin. Lab. Sci.* **2009***, 46*, 25.

(29) Scanlan, P. D.; Shanahan, F.; Clune, Y.; Collins, J. K.; O'Sullivan, G. C.; O'Riordan, M.; Holmes, E.; Wang, Y. L.; Marchesi, J. R. *Environ. Microbiol.* **2008***, 10*, 789.

(30) Larsen, N.; Vogensen, F. K.; van den Berg, F. W. J.; Nielsen, D. S.; Andreasen, A. S.; Pedersen, B. K.; Abu Al-Soud, W.; Sorensen, S. J.; Hansen, L. H.; Jakobsen, M. *PLoS ONE* **2010***, 5*, e9085.

(31) Gori, A.; Tincati, C.; Rizzardini, G.; Torti, C.; Quirino, T.; Haarman, M.; Ben Amor, K.; van Schaik, J.; Vriesema, A.; Knol, J.; Marchetti, G.; Welling, G.; Clerici, M. *J. Clin. Microbiol.* **2008***, 46*, 757.

(32) Round, J. L.; Mazmanian, S. K. *Nat. Rev. Immunol.* **2009***, 9*, 313.

(33) Heijtz, R. D.; Wang, S. G.; Anuar, F.; Qian, Y.; Bjorkholm, B.; Samuelsson, A.; Hibberd, M. L.; Forssberg, H.; Pettersson, S. *Proc. Natl. Acad. Sci. U. S. A.* **2011***, 108*, 3047.

(34) Bjorkholm, B.; Bok, C. M.; Lundin, A.; Rafter, J.; Hibberd, M. L.; Pettersson, S. *PLoS ONE* **2009***, 4*, e6958.

(35) Wikoff, W. R.; Anfora, A. T.; Liu, J.; Schultz, P. G.; Lesley, S. A.; Peters, E. C.; Siuzdak, G. *Proc. Natl. Acad. Sci. U. S. A.* **2009***, 106*, 3698.

(36) Clayton, T. A.; Baker, D.; Lindon, J. C.; Everett, J. R.; Nicholson, J. K. *Proc. Natl. Acad. Sci. U. S. A.* **2009***, 106*, 14728.

(37) Aura, A.; Mattila, I.; Hytylinen, T.; Gopalacharyulu, P.; Bounsaythip, C.; Orešič, M.; Oksman Caldentey, K. *Mol. Biosyst.* **2011***, 7*, 437.

(38) Goldin, B. R. *Ann. Med.* **1990***, 22*, 43.

(39) Lindenbaum, J.; Rund, D. G.; Butler, V. P.; Tseeng, D.; Saha, J. R. *N. Engl. J. Med.* **1981***, 305*, 789.

(40) Jernberg, C.; Lofmark, S.; Edlund, C.; Jansson, J. K. *The ISME journal* **2007***, 1*, 56.

(41) Jakobsson, H. E.; Jernberg, C.; Andersson, A. F.; Sjolund-Karlsson, M.; Jansson, J. K.; Engstrand, L. *PLoS ONE* **2010***, 5*, e9836.

(42) Dethlefsen, L.; Relman, D. A. *Proc. Natl. Acad. Sci. U. S. A.* **2011***, 108*, 4554.

(43) Yap, I. K. S.; Li, J. V.; Saric, J.; Martin, F. P.; Davies, H.; Wang, Y.; Wilson, I. D.; Nicholson, J. K.; Utzinger, J.; Marchesi, J. R.; Holmes, E. *J. Proteome. Res.* **2008***, 7*, 3718.

(44) Croswell, A.; Amir, E.; Teggatz, P.; Barman, M.; Salzman, N. H. *Infect. Immun.* **2009***, 77*, 2741.

(45) Sartor, R. B. *Gastroenterology* **2008***, 134*, 577.

(46) Gibson, G. R.; Roberfroid, M. B. *J. Nutr.* **1995***, 125*, 1401.

(47) Hamer, H. M.; Jonkers, D.; Venema, K.; Vanhoutvin, S.; Troost, F. J.; Brummer, R. J. *Aliment. Pharmacol. Ther.* **2008***, 27*, 104.

(48) Bernalier-Donadille, A. *Gastroenterol. Clin. Biol.* **2010***, 34*, S16.

(49) Ohkusa, T.; Okayasu, I.; Ogihara, T.; Morita, K.; Ogawa, M.; Sato, N. *Gut* **2003***, 52*, 79-83.

(50) Pryde, S. E.; Duncan, S. H.; Hold, G. L.; Stewart, C. S.; Flint, H. J. *FEMS Microbiol. Lett.* **2002***, 217*, 133.

(51) Kanauchi, O.; Matsumoto, Y.; Matsumura, M.; Fukuoka, M.; Bamba, T. *Curr. Pharm. Des.* **2005***, 11*, 1047.

(52) Fukuda, S.; Toh, H.; Hase, K.; Oshima, K.; Nakanishi, Y.; Yoshimura, K.; Tobe, T.; Clarke, J. M.; Topping, D.; Suzuki, T.; Taylor, T. D.; Itoh, K.; Kikuchi, J.; Morita, H.; Hattori, M.; Ohno, H. *Nature* **2011***, 469*, 543.

(53) Tedelind, S.; Westberg, F.; Kjerrulf, M.; Vidal, A. *World J. Gastroenterol.* **2007***, 13*, 2826.

(54) Al-Lahham, S. H.; Peppelenbosch, M. P.; Roelofsen, H.; Vonk, R. J.; Venema, K. *B. B. Acta.* **2010***, 1801*, 1175.

(55) Velzquez, O. C.; Lederer, H. M.; Rombeau, J. L. *Adv. Exp. Med. Biol.* **1997***, 427*, 123.

(56) Bown, R. L.; Gibson, J. A.; Sladen, G. E.; Hicks, B.; Dawson, A. M. *Gut* **1974***, 15*, 999.

(57) Hollywood, K.; Brison, D.; Goodacre, R. *Proteomics* **2006***, 6*, 4716.

(58) Fiehn, O. *Plant Mol. Biol.* **2002***, 48*, 155.

(59) Nielsen, J.; Oliver, S. *Trends Biotechnol.* **2005***, 23*, 544-546.

(60) Oliver, S. G.; Winson, M. K.; Kell, D. B.; Baganz, F. *Trends Biotechnol.* **1998***, 16*, 373.

(61) Kell, D. B.; Brown, M.; Davey, H. M.; Dunn, W. B.; Spasic, I.; Oliver, S. G. *Nat. Rev. Microbiol.* **2005***, 3*, 557.

(62) Sariyar-Akbulut, B.; Salman-Dilgimen, A.; Ceylan, S.; Perk, S.; Denizci, A. A.; Kazan, D. *Arch. Microbiol.* **2008***, 189*, 19.

(63) Mashego, M. R.; Rumbold, K.; De Mey, M.; Vandamme, E.; Soetaert, W.; Heijnen, J. J. *Biotechnol. Lett.* **2006***, 29*, 1.

(64) Sanceda, N. G.; Suzuki, E.; Kurata, T. *Amino Acids* **2003***, 24*, 81.

(65) Fiehn, O. *Comp. Funct. Genomics* **2001***, 2*, 155.

(66) Jansson, J.; Willing, B.; Lucio, M.; Fekete, A.; Dicksved, J.; Halfvarson, J.; Tysk, C.; Schmitt-Kopplin, P. *PLoS ONE* **2009***, 4*, e6386.

(67) Paris, D.; Melck, D.; Stocchero, M.; D'Apolito, O.; Calemma, R.; Castello, G.; Izzo, F.; Palmieri, G.; Corso, G.; Motta, A. *Metabolomics* **2010***, 6*, 405.

(68) Jordan, K. W.; Cheng, L. L. *Expert Rev. Proteomic.* **2007***, 4*, 389.

(69) Bundy, J. G.; Willey, T. L.; Castell, R. S.; Ellar, D. J.; Brindle, K. M. *FEMS Microbiol. Lett.* **2005***, 242*, 127.

(70) Holdeman, L. V.; Cato, E. P.; and Moore, W.E.C. (1977) Anaerobe Laboratory Manual, Anaerobe Laboratory, 4th edn. Blacksburg: Virginia Polytechnic Institute and State University .

(71) Wiggins, R. J.; Wilks, M.; Tabaqchali, S. *J. Clin. Pathol.* **1985***, 38*, 933.

(72) Thomann, W. R.; Hill, G. B. *J. Clin. Microbiol.* **1986***, 23*, 392.

(73) Salanitro, J. P.; Muirhead, P. A. *Appl. Microbiol.* **1975***, 29*, 374-381.

(74) Cundy, K. V.; Willard, K. E.; Valeri, L. J.; Shanholtzer, C. J.; Singh, J.; Peterson, L. R. *J. Clin. Microbiol.* **1991***, 29*, 260-263.

(75) Rizzo, A. F. *J. Clin. Microbiol.* **1980***, 11*, 418-421.

(76) Guerrant, G. O.; Lambert, M. A.; Moss, C. W. *J. Clin. Microbiol.* **1982***, 16*, 355-360.

(77) Ehrlich, G. G.; EM; Goerlitz, D. F.; Bourell, J. H.; Eisen, G. V.; Godsy, E. M. *Appl. Environ. Microbiol.* **1981***, 42*, 878-885.

(78) Gamoh, K.; Saitoh, H.; Wada, H. *Rapid Commun. Mass Spectrom.* **2003***, 17*, 685-689.

(79) Arellano, M.; ElKaddouri, S.; Roques, C.; Couderc, F.; Puig, P. *J. Chromatogr.* **1997***, 781*, 497-501.

(80) Fan, T. W. M.; Lane, A. N. *Prog Nucl Magn Reson Spectrosc* **2008***, 52*, 69-117.

(81) Szyperski, T. *Eur. J. Biochem.* **1995***, 232*, 433-448.

(82) Pan, Z.; Raftery, D. *Anal. Bioanal. Chem.* **2007***, 387*, 525-527.

(83) Dear, G. J.; Roberts, A. D.; Beaumont, C.; North, S. E. *J. Chromatogr. B* **2008***, 876*, 182-190.

(84) Kovacs, H.; Moskau, D.; Spraul, M. *Prog. Nucl. Magn. Reson. Spectrosc.* **2005***, 46*, 131-155.

(85) Grivet, J.; Delort, A. *Prog Nucl Magn Reson Spectrosc* **2009***, 54*, 1-53.

(86) Speckman, R.; Collins, E. *Appl. Microbiol.* **1973***, 26*, 744-746.

(87) Bergman, E. N.; Reid, R. S.; Murray, M. G.; Brockway, J. M.; Whitelaw, F. G. *Biochem. J.* **1965***, 97*, 53.

(88) Schneider, B. *Prog Nucl Magn Reson Spectrosc* **2007***, 51*, 155-198.

(89) Eakin, R. T.; Morgan, L. O.; Gregg, C. T.; Matwiyof, N. A. *FEBS Lett.* **1972***, 28*, 259-264.

(90) Jeffrey, F. M. H.; Rajagopal, A.; Malloy, C. R.; Sherry, A. D. *Trends Biochem. Sci.* **1991***, 16*, 5-10.

(91) Vederas, J. C. *Nat. Prod. Rep.* **1987***, 4*, 277-337.

(92) White, R. L.; Ramezani, M.; Gharbia, S. E.; Seth, R.; Doherty-Kirby, A. L. *Biotechnol. Appl. Biochem.* **1995***, 22*, 385-396.

(93) F. S. Lee, MSc Thesis, Dalhousie University, 2000 .

(94) George, W. L.; Kirby, B. D.; Sutter, V. L.; Citron, D. M.; Finegold, S. M. *Rev. Infect. Dis.* **1981***, 3*, 599.

(95) Roberts, G. L. *Br. J. Biomed. Sci.* **2000***, 57*, 156-162.

(96) Riordan, T. *Clin. Microbiol. Rev.* **2007***, 20*, 622.

(97) Bennett, K. W.; Eley, A. *J. Med. Microbiol.* **1993***, 39*, 246-254.

(98) Kostic, A. D.; Gevers, D.; Pedamallu, C. S.; Michaud, M.; Duke, F.; Earl, A. M.; Ojesina, A. I.; Jung, J.; Bass, A. J.; Tabernero, J.; Baselga, J.; Liu, C.; Shivdasani, R. A.; Ogino, S.; Birren, B. W.; Huttenhower, C.; Garrett, W. S.; Meyerson,M. 2011 Genome Res. doi:10.1101/gr.126573.111. .

(99) Castellarin, M. W.,R.L.; Freeman, J. D.; Dreolini , L.; Krzywinski, M.; Strauss, J.; Barnes, R.; Watson, P.; Allen-Vercoe, E.; Moore, R. A.; Holt, R.A. (2011) Genome Res. doi:10.1101/gr.126516.111 .

(100) Farias, F. F.; Lima, F. L.; Carvalho, M. A. R.; Nicoli, J. R.; Farias, L. M. *Anaerobe* **2001***, 7*, 271.

(101) Coleman, M. E.; Dreesen, D. W.; Wiegert, R. G. *Appl. Environ. Microbiol.* **1996***, 62*, 3632.

(102) Minami, M.; Ando, T.; Okamoto, A.; Sasaki, N.; Ohkura, T.; Torii, K.; Hasegawa, T.; Ohta, M.; Goto, H. *FEMS Immunol. Med. Microbiol.* **2009***, 56*, 67.

(103) Ohkusa, T.; Nomura, T.; Terai, T.; Miwa, H.; Kobayashi, O.; Hojo, M.; Takei, Y.; Ogihara, T.; Hirai, S.; Okayasu, I.; Sato, N. *Scand. J. Gastroenterol.* **2005***, 40*, 1334-1342.

(104) Ohkusa, T.; Kato, K.; Terao, S.; Chiba, T.; Mabe, K.; Murakami, K.; Mizokami, Y.; Sugiyama, T.; Yanaka, A.; Takeuchi, Y.; Yamato, S.; Yokoyama, T.; Okayasu, I.; Watanabe, S.; Tajiri, H.; Sato, N. *Am. J. Gastroenterol.* **2010***, 105*, 1820.

(105) Legaria, M. C.; Lumelsky, G.; Rodriguez, V.; Rosetti, S. *J. Clin. Microbiol.* **2005***, 43*, 4293.

(106) Bourgault, A. M.; Lamothe, F.; Dolce, P.; SaintJean, L.; SaintAntoine, P. *Clin. Infect. Dis.* **1997***, 25*, S181.

(107) Noack, J.; Dongowski, G.; Hartmann, L.; Blaut, M. *J. Nutr.* **2000***, 130*, 1225.

(108) Falkow, S. *Cell* **2006***, 124*, 699-702.

(109) Casadevall, A.; Pirofski, L. *Infect. Immun.* **2000***, 68*, 6511-6518.

(110) Jackins, H. C.; Barker, H. A. *J. Bacteriol.* **1951***, 61*, 101.

(111) Carlier, J. P. *Lett. Appl. Microbiol.* **1990***, 11*, 133-136.

(112) M. Ramezani, PhD Thesis, Dalhousie University, 1996. .

(113) Potrykus, J.; Mahaney, B.; White, R. L.; Bearne, S. L. *Proteomics* **2007***, 7*, 1839-1853.

(114) Potrykus, J.; White, R. L.; Bearne, S. L. *Proteomics* **2008***, 8*, 2691-2703.

(115) Gharbia, S. E.; Shah, H. N. *Curr. Microbiol.* **1988***, 17*, 229-234.

(116) Gharbia, S. E.; Shah, H. N. *Curr. Microbiol.* **1989***, 18*, 189-193.

(117) Ramezani, M.; MacIntosh, S. E.; White, R. L. *Amino Acids* **1999***, 17*, 185-193.

(118) Buckel, W. *Appl. Microbiol. Biotechnol.* **2001***, 57*, 263-273.

(119) Gharbia, S. E.; Shah, H. N. *J. Gen. Microbiol.* **1991***, 137*, 1201-1206.

(120) Foglesong, M. A.; Cruden, D. L.; Markovetz, A. J. *J. Bacteriol.* **1984***, 158*, 474.

(121) Bearne, S. L.; White, R. L.; McDonnell, J. E.; Bahrami, S.; Gronlund, J. *Mol. Cell. Biochem.* **2001***, 221*, 117-126.

(122) Pardee, A. B.; Prestidge, L. S. *J. Bacteriol.* **1955***, 70*, 667.

(123) Kapatral, V.; Ivanova, N.; Anderson, I.; Reznik, G.; Bhattacharyya, A.; Gardner, W. L.; Mikhailova, N.; Lapidus, A.; Larsen, N.; D'Souza, M.; Walunas, T.; Haselkorn, R.; Overbeek, R.; Kyrpides, N. *Genome Res.* **2003***, 13*, 1180-1189.

(124) Ramezani, M.; Resmer, K. L.; White, R. L. *FEBS J.* **2011***, 278*, 2540-2551.

(125) Carlier, J. P.; Henry, C.; Lorin, V.; Rouffignat, K. *Lett. Appl. Microbiol.* **1997***, 25*, 371-374.

(126) McInerney, M.J. Anaerobic Hydrolysis and Fermentation of Fats and Proteins. In Zehnder, A.J., Editor, Biology of Anaerobic Microorgansims, 1988, pp 373-415. .

(127) Barker, H. A. *Annu. Rev. Biochem.* **1981***, 50*, 23-40.

(128) Doelle, H.W. Bacterial Metabolism, 2nd Edition, Academic Press, New York, 1975, p 654 .

(129) McFall, E.; Newman, E.B. Amino Acids as Carbon Sources. In Neidhardt, F.C. Escherichia coli and Salmonella Cellular and Molecular Biology, 2nd Edition, 1996, ASM Press, Washington, D.C., pp 358-379 .

(130) Or-Rashid, M. M.; Onodera, R.; Wadud, S.; Oshiro, S.; Okada, T. *Amino Acids* **2001***, 21*, 383-391.

(131) Marcus, J.; Dekker, E. *J. Bacteriol.* **1993***, 175*, 6505-6511.

(132) Sawers, G. *Arch. Microbiol.* **1998***, 171*, 1-5.

(133) Simanshu, D. K.; Chittori, S.; Savithri, H. S.; Murthy, M. R. N. *J. Biosci.* **2007***, 32*, 1195-1206.

(134) Karasawa, T.; Ikoma, S.; Yamakawa, K.; Nakamura, S. *Microbiology* **1995***, 141*, 371-375.

(135) Scheppach, W.; Weiler, F. *Curr. Opin. Clin. Nutr.* **2004***, 7*, 563-567.

(136) Blachier, F.; Mariotti, F.; Huneau, J. F.; Tome, D. *Amino Acids* **2007***, 33*, 547-562.

(137) Winder, C. L.; Dunn, W. B.; Schuler, S.; Broadhurst, D.; Jarvis, R.; Stephens, G. M.; Goodacre, R. *Anal. Chem.* **2008***, 80*, 2939-2948.

(138) Resmer, K. L.; White, R. L. *Mol. Biosyst.* **2011***, 7*, 2220-2227.

(139) Hakala, M. T.; Glaid, A. J.; Schwert, G. W. *J. Biol. Chem.* **1956***, 221*, 191.

(140) Talasniemi, J. P.; Pennanen, S.; Savolainen, H.; Niskanen, L.; Llesivuori, J. *Clin. Biochem.* **2008***, 41*, 1099.

(141) Boehringer Mannheim / R-Biopharm. UV-method of d- and l-lactic acid in foodstuffs and other materials. Cat.No. 11 112 821 035. .

(142) Kim, J.; Hetzel, M.; Boiangiu, C. D.; Buckel, W. *FEMS Microbiol. Rev.* **2004***, 28*, 455.

(143) Rogers, A. H.; Zilm, P. S.; Gully, N. J.; Pfennig, A. L.; Marsh, P. D. *Oral Microbiol. Immunol.* **1991***, 6*, 250-255.

(144) Gharbia, S. E.; Shah, H. N.; Welch, S. G. *Curr. Microbiol.* **1989***, 19*, 231-235.

(145) Berregi, I.; del Campo, G.; Caracena, R.; Miranda, J. I. *Talanta* **2007***, 72*, 1049.

(146) Kriat, M.; Confortgouny, S.; Viondury, J.; Sciaky, M.; Viout, P.; Cozzone, P. J. *NMR Biomed* **1992***, 5*, 179-184.

(147) Gale, E. F.; Folkes, J. P. *Biochem. J.* **1953***, 53*, 483.

(148) Thauer, R. K.; Jungermann, K.; Decker, K. *Bacteriol. Rev.* **1977***, 41*, 100-180.

(149) Vogt, J. A.; Pencharz, P. B.; Wolever, T. M. S. *Am. J. Clin. Nutr.* **2004***, 80*, 89-94.

(150) Mortensen, P. B.; Holtug, G. K.; Rasmussen, H. S. *J. Nutr.* **1988***, 118*, 321-325.

(151) Wang, X.; Gibson, G. R. *J. Appl. Bacteriol.* **1993***, 75*, 373-380.

(152) de Graaf, A. A.; Maathuis, A.; de Waard, P.; Deutz, N. E. P.; Dijkema, C.; de Vos, W. M.; Venema, K. *NMR Biomed.* **2010***, 23*, 2-12.

(153) Asanuma, N.; Hino, T. *Appl. Environ. Microbiol.* **2000***, 66*, 3773.

(154) Snoep, J. L.; Joost, M.,; de Mattos, T.; Neijssel, O. M. *FEMS Microbiol. Lett.* **1991***, 81*, 63.

(155) Liu, C. L.; Mortenson, L. E. *J. Bacteriol.* **1984***, 159*, 375-380.

(156) Moussa, NT, BSc Thesis, Dalhousie University, 2011 .

(157) Ji, X. J.; Huang, H.; Ouyang, P. K. *Biotechnol. Adv.* **2011***, 29*, 351-364.

(158) Syu, M. J. *Appl. Microbiol. Biotechnol.* **2001***, 55*, 10-18.

(159) Celińska, E.; Grajek, W. *Biotechnol. Adv.* **2009***, 27*, 715-725.

(160) Xiao, Z.; Xu, P. *Crit. Rev. Microbiol.* **2007***, 33*, 127-140.

(161) Malaoui, H.; Marczak, R. *J. Appl. Microbiol.* **2001***, 90*, 1006.

(162) Yamada-Onodera, K.; Nakajima, A.; Tani, Y. *J. Biosci. Bioeng.* **2006***, 102*, 545-551.

(163) Garg, S. K.; Jain, A. *BioresoureTechnol.* **1995***, 51*, 103.

(164) Biebl, H.; Zeng, A. P.; Menzel, K.; Deckwer, W. D. *Appl. Microbiol. Biotechnol.* **1998***, 50*, 24-29.

(165) Nakashimada, Y.; Marwoto, B.; Kashiwamura, T.; Kakizono, T.; Nishio, N. *J. Biosci. Bioeng.* **2000***, 90*, 661-664.

(166) Antunes, L. C. M.; Davies, J. E.; Finlay, B.B. F1000 Biol Reports 2011, 3:4 (doi: 10.3410/B3-4) .

(167) Hsieh, S. C.; Lu, C. C.; Horng, Y. T.; Soo, P. C.; Chang, Y. L.; Tsai, Y. H.; Lin, C. S.; Lai, H. C. *Microb. Infect.* **2007***, 9*, 1402-1409.

(168) Bari, W.; Yoon-Jae, S.; Sang, S. S. *Infect. Immun.* **2011***, 79*, 3149-3158.

(169) Winter, S. M.; Weber, G. L.; Gooley, P. R.; Mackenzie, N. E.; Sipes, I. G. *Drug Metab. Disposition* **1992***, 20*, 665-672.

(170) Gottschalk, G. Bacterial Metabolism, 2nd edn. Springer, Berlin Heidelberg New York 1986. .

(171) Nakazawa, A.; Hayaishi, O. *J. Biol. Chem.* **1967***, 242*, 1146.

(172) Tokushige, M.; Whiteley, H. R.; Hayaishi, O. *Biochem. Biophys. Res. Commun.* **1963***, 13*, 380.

(173) White, R. L.; Smith, K. C.; DeMarco, A. C. *Can. J. Chem.* **1994***, 72*, 1645-1655.

(174) Bell, S. C.; Turner, J. M. *Biochem. J.* **1977***, 166*, 209-216.

(175) Liu, J.; Dairi, T.; Itoh, N.; Kataoka, M.; Shimizu, S. *Eur. J. Biochem.* **1998***, 255*, 220-226.

(176) Husain, A.; Jeelani, G.; Sato, D.; Ali, V.; Nozaki, T. *Mol. Biochem. Parasitol.* **2010***, 170*, 100-104.

(177) Meinecke, B.; Bertram, J.; Gottschalk, G. *Arch. Microbiol.* **1989***, 152*, 244.

(178) Hofmeister, A. E. M.; Berger, S.; Buckel, W. *Eur. J. Biochem.* **1992***, 205*, 743-749.

(179) Hofmeister, A. E. M.; Grabowski, R.; Linder, D.; Buckel, W. *Eur. J. Biochem.* **1993***, 215*, 341-349.

(180) Liu, J. Q.; Dairi, T.; Itoh, N.; Kataoka, M.; Shimizu, S.; Yamada, H. *J. Mol. Catal. B. Enzym.* **2000***, 10*, 107.

(181) Boyce, S.; Tipton, K. F. 2005. Enzyme Classification and Nomenclature. eLS. .

(182) Zelder, O.; Beatrix, B.; Leutbecher, U.; Buckel, W. *Eur. J. Biochem.* **1994***, 226*, 577-585.

(183) Cameron, B.; Briggs, K.; Pridmore, S.; Brefort, G.; Crouzet, J. *J. Bacteriol.* **1989***, 171*, 547-557.

(184) Roth, J. R.; Lawrence, J.; Bobik, T.; Lawrence, J. G.; Bobik, T. A. *Annu. Rev. Microbiol.* **1996***, 50*, 137-181.

(185) Lawrence, J.; Roth, J. *J. Bacteriol.* **1995***, 177*, 6371-6380.

(186) Buckel, W.; Barker, H. A. *J. Bacteriol.* **1974***, 117*, 1248.

(187) Raux, E.; Schubert, H.; Warren, M. *CMLS, Cell Mol Life Sci* **2000***, 57*, 1880-1893.

(188) H. U. Bergmeyer, E. Bernt, In Methods of Enzymatic Analysis, H. U. Bergmeyer, ed. Volume 2; Academic Press: New York, 1974; p 574. .

(189) Macy, J. M.; Ljungdahl, L.; Gottschalk, G. *J. Bacteriol.* **1978***, 134*, 84-91.

(190) VanderWerf, M. J.; Guettler, M. V.; Jain, M. K.; Zeikus, J. G. *Arch. Microbiol.* **1997***, 167*, 332-342.

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