# Limitations to Amino Acid Biosynthesis *de novo* in Ruminal Strains of *Prevotella* and *Butyrivibrio*



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**by**

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**Dedicated to my husband Isa Shariat**
**for his help, patience and understanding**

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# Declaration

This thesis contains no materials which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief no material previously published or written by another person , except where due reference is made in the text. I consent to this thesis, when deposited in the University library, being available for photocopying and loan.

Nafisseh Nili

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# List of Abbreviations

| | |
|---|---|
| A | absorbance |
| AdoMet | S-adenosylmethionine |
| ADP | adenosine 5'-diphosphate |
| ALA | 5-aminolevulinic acid |
| AlaDH | alanine dehydrogenase |
| approx. | approximately |
| AsS | asparagine synthetase |
| ATP | adenosine 5'-triphosphate |
| BSA | Bovine serum albumin |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphate |
| DER | Diglycidyl ether of polypropylene glycol |
| dGTP | deoxyguanosine triphosphate |
| DMAE | 2, Dimethylaminoethanol |
| DNA | deoxyribonucleic acid |
| dTTP | deoxythymidine triphosphate |
| e.g. | for example |
| EDTA | ethylenediaminetetraacetic acid |
| EPS | extracellular polysaccharide |
| ERL | Vinyl cyclohexane dioxide |
| *et al.* | et alia (and others) |
| g | gram |
| *g* | unit of gravitational field (acceleration of gravity) |
| GOGAT | glutamine 2-oxoglutarate amino transferase |
| GS | glutamine syntethase |
| h | hour(s) |
| i.e. | id est (that is) |

| | |
|---|---|
| ID | internal diameter |
| kDa | kilodalton |
| Km | Michaelis constant |
| l | litre(s) |
| M | molar |
| m | meter(s) |
| mg | milligram(s) |
| min | minute(s) |
| mM | millimolar |
| MW | molecular weight |
| n | number |
| $NAD^+$ | β-nicotinamide adenine dinucleotide |
| NADH | β-nicotinamide adenine dinucleotide (reduced form) |
| $NADP^+$ | β-nicotinamide adenine dinucleotide phosphate |
| NADPH | β-nicotinamide adenine dinucleotide phosphate (reduced form) |
| nm | nanometre |
| nmole | nanomole |
| NSA | Nonenyl succinic anhydride |
| NVFA | non-volatile fatty acid(s) |
| OAH | O-acetylhomoserine |
| p.s.i. | pounds per square inch |
| PCMB | *p*-chloromercuribenzoate |
| *per se.* | by or in itself; intrinsically |
| Pi | inorganic phosphate |
| pmole | picomole |
| PMSF | phenylmethylsulphuryl fluoride |
| PP. | pages |
| PVP | polyvinyl pyrolidine |
| RNA | ribonucleic acid |

| | |
|---|---|
| RO | reverse osmosis |
| SEM | scanning electron microscopy |
| TCA | tricarboxylic acid |
| TEM | transmission electron microscopy |
| TEMED | N, N, N', N'-Tetramethylethylenediamine |
| UV | ultraviolet |
| v/v | volume per volume |
| VFA | volatile fatty acid |
| VFA | volatile fatty acid(s) |
| w/v | weight per volume |
| µl | microlitre |
| °C | degree Centigrade (Celsius) |
| % | percent |
| / | per |
| < | less than |
| > | more than |

# Abstract

Peptides, amino acids and ammonia which arise as the end products of dietary protein breakdown act as nitrogen sources for rumen microorganisms. The nature of nitrogen sources and whether or not these are in adequate supply in the growth environment are the major factors that regulate the overall biosynthetic potential of cells. The aim of the work undertaken in my Ph.D project was to investigate nitrogen utilisation in some species of rumen bacteria with the object of understanding the role of ammonia versus exogenous amino acids in relation to microbial growth.

A fully defined medium was developed to determine the nitrogen requirements for several species of ruminal bacteria. Using this defined medium, nitrogen requirements of *Butyrivibrio fibrisolvens* strains H17c and E14, *Prevotella ruminicola* strains GA33 and P1, *Selenomonas ruminantium* strain S23 and *Streptococcus bovis* strains H24 and 2B were examined. All of the strains were able to grow on $NH_4Cl$ as a sole nitrogen source except *P. ruminicola* strain GA33 and *B. fibrisolvens* strain E14. Mixed amino acids and peptides were prepared from casamino acids and tryptone respectively by ligand exchange chromatography and used to examine the effect of amino acids and peptides on bacterial growth. In the medium containing $NH_4Cl$ plus mixed amino acids or peptides as nitrogen sources all bacterial strains including *P. ruminicola* strain GA33 and *B. fibrisolvens* strain E14 were able to grow. With mixed amino acids or peptides alone all bacterial strains grew, although to a lesser extent than when $NH_4Cl$ was also present. Growth on individual amino acids and enzyme assays indicated that *P. ruminicola* strain GA33 and *B. fibrisolvens* strain E14 are impaired in *de novo* biosynthesis of particular amino acids.

*P. ruminicola* strain GA33 grew in the presence of $NH_4Cl$ if an amino acid was also present, though methionine or cysteine did not produce this effect. Enzyme assays

revealed the absence of NADH- and NADPH-dependent glutamate dehydrogenases in this strain.

*B. fibrisolvens* strain E14 grew on $NH_4Cl$ only if methionine was present. No other amino acid, alone or in combination, was effective. Radiotracer experiments using $^{35}S$-methionine, $^{35}S$-cysteine and $^{14}C$-lysine followed by SDS-PAGE of bacterial cell-free extracts showed incorporation of labelled amino acids into newly synthesised bacterial protein. The requirement for methionine could be met by the addition of a methionine-containing dipeptide or S-adenosylmethionine (AdoMet) to the medium. Competition inhibition experiments using the dipeptide analogue 5-aminolevulinic acid showed that the strain did not hydrolyse the dipeptide extracellularly.

Enzyme studies were carried out to determine whether *B. fibrisolvens* strain E14 was defective in any particular reaction in methionine biosynthesis. Oligonucleotide primers were designed from published sequences of the *Escherichia coli* cobalamin-dependent methionine synthase (*metH*) and cobalamin-independent methionine synthase (*metE*) genes and the sequences were amplified from *E. coli* DNA by PCR. Chromosomal DNA from *B. fibrisolvens* strains was screened with the PCR products. There was only slight homology between *metE* genes from *E. coli* and *B. fibrisolvens* strains H17c and E14 and there was no homology between *metH* genes from *E. coli* and *B. fibrisolvens* strains. Cobalamin-dependent methionine synthase activity was not detectable either in strain E14 or the methionine independent strain H17c. Methionine biosynthesis from $^{14}C$-[β-C]-serine was studied in intact cells using an acid hydrolysate of bacteria and analysed by HPLC and paper chromatography. Strain H17C was shown to produce $^{14}C$-labelled methionine whereas strain E14 did not.

*B. fibrisolvens* strain E14 was tested for the presence of AdoMet synthetase gene and AdoMet synthetase activity to determine whether stimulation of growth by AdoMet was due to inability of the strain to synthesise AdoMet. Oligonucleotide primers were designed from published sequences of the AdoMet synthetase (*metK*) gene from *E. coli* and the sequence was amplified by PCR. *B. fibrisolvens* strains were screened with the PCR products. The AdoMet synthetase gene was detectable in Southern blots of DNA from *B. fibrisolvens* strain E14 and the methionine-independent strain H17c. AdoMet synthetase activity was also detectable in cell-free extracts of these strains.

AdoMet solutions were analysed by paper chromatography to determine whether growth of strain E14 on AdoMet was due to the presence of free methionine. No free methionine was detected but AdoMet was completely decomposed to homoserine and methylthioribose by autoclaving or incubating at 39°C. Radio tracer experiments using S-adenosyl-L-[$^{14}$C-methyl] methionine followed by SDS-PAGE showed radioactivity in newly synthesised bacterial protein. An acid hydrolysate of bacteria was analysed by HPLC and the radioactivity of each amino acid fraction was measured. Only methionine was labelled suggesting that strain E14 may produce methionine from methylthioribose. Similar results were obtained for *B. fibrisolvens* strain H17c.

During this work a variant of *B. fibrisolvens* strain E14 was discovered that appeared to differ in its ability to adhere to an agar plate. The original organism (S) appeared as circular colonies, more intensively pigmented and firmly attached to the plate so that they could not be removed without scraping the substratum. In contrast, the non-adherent variant (L) was loosely attached to the agar and could be readily removed by light washing. Variants S and L were colony purified and biochemical tests and enzymatic analysis were carried out to characterise variant L with respect to variant S. Scanning electron microscopy (SEM) showed exopolymers associated with S cells as

thin films bridging the space between S cells. In contrast, variant L was primarily devoid of exopolymer bridgings. Transmission electron microscopy (TEM) showed that variant S cells were surrounded by large quantities of an electron-dense residue of the ruthenium-red-reactive extracellular polysaccharide (EPS) whereas variant L was shown to be surrounded by only a small amount of the electron-dense EPS. Chromosomal DNA from variants S and L were digested with restriction enzymes, separated by electrophoresis on agarose gel and the DNA fragments from the two variants were compared. Soluble proteins were analysed by SDS-PAGE and the protein profile of the two variants were compared. No difference was observed between DNA fragments pattern and protein profile of the two variants. The nitrogen requirements of variants S and L were examined. The two variants were also tested for methionine synthase and AdoMet synthetase activities. Both variants had an absolute requirement for methionine or AdoMet for growth and this requirement could be replaced by the addition of a methionine-containing dipeptide to the medium. The results of enzyme assays were also similar for the two variants. EPS from variants S and L were isolated and measured. Variant S produced four times as much EPS as variant L. These results strongly suggest that the main difference between *B. fibrisolvens* strain E14 variants S and L is in the production of EPS but their utilisation of nitrogen is the same.

The results of the present study show that ammonia is the major nitrogen source for rumen bacteria and that most strains of the species examined are able to synthesise amino acids from ammonia even when exogenous amino acids are present in the growth medium. However, for the bacteria to grow on ammonia as a sole nitrogen source, they must be able to synthesise all 20 amino acids essential for protein biosynthesis. The present work revealed 2 strains of rumen bacteria that are impaired in *de novo* biosynthesis of particular amino acids. This is the first demonstration in rumen bacteria that the absolute requirement for amino acids is due to their inability to synthesise particular amino acids *de novo*. This finding is particularly important for strain GA33 as it is described as the type strain for *P. ruminicola.* The lack of glutamate

dehydrogenase activities in strain GA33 may make the strain a valuable tool for studying ammonia assimilation and for cloning the GDH gene by mutation complementation. The inability of strain E14 to synthesise methionine *de novo* is due to a block in the final steps of the methionine biosynthetic pathway. The strain may lack cobalamin-independent methionine synthase activity and/or has a defect in the folate pathway from tetrahydrofolate to $N^5$-methyltetrahydrofolate. Since a defect in the folate pathway is likely to be lethal, the more likely candidate is cobalamin-independent methionine synthase. Biosynthesis of methionine from methylthioribose by *B. fibrisolvens* strains suggests the presence of an alternative pathway of methionine biosynthesis different from the common pathway found in most bacterial species.

# CHAPTER 1

# CHAPTER 1



## Introduction and Review of the Literature

### 1.1 Introduction

Ammonia, amino acids and peptides which arise as the end products of protein degradation in the rumen (Chen *et al.*, 1987a) are also important substrates for protein biosynthesis by rumen microorganisms (Russell *et al.*, 1991). Since ruminant animals depend upon microbial protein as the major source of amino acids (Cotta and Russell, 1982), the effective utilisation of these nitrogen sources and the efficiency of microbial protein synthesis can have a significant impact upon nitrogen economy of the rumen ecosystem and hence animal production.

Results of studies on nitrogen requirements of rumen bacteria have shown that most ruminal bacteria use ammonia as the major nitrogen source but amino acids and/or peptides are either stimulatory for the growth or in some cases, essential (Bryant and Robinson, 1961; Wright 1967; Pittman and Bryant, 1964). The role of amino acids and peptides on the ruminal ecosystem has been studied from a number of perspectives, including their effects on microbial growth (Cotta and Russell, 1982; Argyle and Baldwin, 1989), the microbial peptidase activities (Wallace and McKain, 1989; 1991), their fermentation and production of ammonia and volatile fatty acids (Bladen *et al.*, 1961; Chen and Russell 1988; Chen and Russell, 1989b), and uptake and utilisation of specific amino acids and peptides (Wright, 1967; Scheifinger *et al.*, 1976; Cooper and Ling, 1985; Broderick *et al.*, 1988; Westlake and Mackie, 1990). Despite these and other studies, it is still unclear under what conditions a bacterium will utilise one particular nitrogen source in preference to others. More work has to be conducted to study the bacterial requirements for amino acids and peptides and to determine the role of these nitrogen sources in relation to microbial growth. Furthermore, the information

on amino acid metabolism in rumen bacteria remains surprisingly limited. A detailed understanding of amino acid and peptide utilisation and metabolism in rumen bacteria could lead to methods of controlling the overall process of ruminal protein utilisation.

## 1.2 Review of the literature

This review covers literature up to May 22nd, 1992, the date when experimental work for this project commenced. Reference to literature after that date, and up to the end of the project, are included in the subsequent discussions in the relevant chapters.

In the following sections I will consider, briefly: the rumen and its microbes, rumen fermentation, protein degradation, proteolytic ruminal microbes, the types of proteolytic activity in the rumen and aspects of bacterial nitrogen metabolism.

### 1.2.1 Conditions within the rumen

The rumen is a large muscular organ in which plant material is mixed with saliva and undergoes continuous fermentation (Wolin, 1981). The temperature of the rumen is usually maintained within the range of 38-41°C. A mean redox potential of -350 mV reflects the rapid utilisation of oxygen and the strong reducing medium (due to the production of $H_2S$). The rumen is usually well-buffered (pH 5.7-7.3) by the copious inflow of saliva in which the principal buffers are bicarbonate and the natural buffering capacity of plant material (Latham, 1980; Wolin, 1981; Yokoyama and Johnson, 1988). The presence of ammonia also acts to control the pH of the rumen (Wolin, 1981; Yokoyama and Johnson, 1988).

The dilution rate for rumen contents is influenced by the proportion and particle size of the roughage in the diet with long-form roughages which require extensive rumination stimulating a greater salivary flow than ground roughages. The length of time plant particles are retained in the rumen depends on their digestibility. Some poorly digested particles may be retained in the rumen for 4 to 5 days before being broken down to a

size sufficiently small to pass out of the rumen (Latham, 1980).

### 1.2.2 Rumen microorganisms

The rumen contains a complex flora of strictly anaerobic and facultative anaerobic microbes (Bryant, 1959; Hungate, 1966). The major microbial populations in the rumen are the bacteria, the protozoa and the fungi (Ørskov, 1982; Baker, 1985; Leng, 1985). Some microbes adhere tightly to the rumen epithelial lining, but most microbes are associated with particles in the rumen or float freely in ruminal fluid. The wall-adhering microorganisms hydrolyse urea and consume $O_2$ which diffuses through the rumen wall. The species present in this population are usually facultative anaerobes and appear to be independent of the substrates being fermented in the rumen. The solid-associated microbes are bound to feed particles in order to digest the insoluble polysaccharides, as well as the less soluble proteins. Since solid digesta are retained in the rumen for longer than the liquid fraction, solid-associated microbes have a distinct advantage in the competitive environment of the rumen (Morris and Cole, 1987). The rumen fluid or "free" organisms mainly utilise soluble carbohydrates and proteins (Cheng and Costerton, 1980; Ørskov, 1982; Owens and Zinn, 1988) although some are cellulolytic. The fermentative activity of rumen microorganisms varies according to factors such as diet, time after feeding and the rate of feed passage from the rumen (Hungate, 1975).

Of the three ruminal microbial groups (bacteria, protozoa and fungi), the bacteria are ubiquitous, and are numerically the most abundant with a biomass normally exceeding that of other microorganisms (Orpin *et al.*, 1988). Eight distinct groups of rumen bacteria have been recognised based on their utilisation of cellulose, hemicellulose, starch, sugars, intermediate acids, proteins, lipids and on methane production (Table 1.1). An expanded classification might also include pectin utilisers, ammonia producers and other ecological niches occupied by the various rumen bacteria. There

**Table 1.1 Major groups of rumen bacterial species**

| | |
|---|---|
| Cellulolytic Species | *Fibrobacter succinogenes*<br>*Ruminococcus flavefaciens*<br>*Ruminococcus albus*<br>*Butyrivibrio fibrisolvens* |
| Hemicellulolytic Species | *Butyrivibrio fibrisolvens*<br>*Prevotella ruminicola*<br>*Ruminococcus* sp. |
| Amylolytic Species | *Ruminobacter amylophilus*<br>*Streptococcus bovis*<br>*Succinimonas amylolytica*<br>*Prevotella ruminicola* |
| Methane-Producing Species | *Methanobrevibacter ruminantium*<br>*Methanobacterium formicium*<br>*Methanobacterium mobile* |
| Acid-Utilising Species | *Megasphaera elsdenii*<br>*Selenomonas ruminantium* |
| Proteolytic Species | *Ruminobacter amylophilus*<br>*Prevotella ruminicola*<br>*Butyrivibrio fibrisolvens*<br>*Streptococcus bovis* |
| Lipid-Utilising Species | *Anaerovibrio lipolytica*<br>*Butyrivibrio fibrisolvens*<br>*Treponema bryantii*<br>*Eubacterium* sp.<br>*Fusocillus* sp.<br>*Micrococcus* sp. |

is a considerable amount of overlap in assigning rumen bacteria to these groups, because most are capable of fermenting more than one substrate (Yokoyama and Johnson, 1988), resulting in microbial competition within the rumen.

### 1.2.3 Rumen fermentation

The ruminal microorganisms are in a symbiotic relationship with the animal, enabling it to degrade ingested food by fermentation prior to the digesta reaching the true stomach, or abomasum. This is one of the advantages of ruminants, because the products of the fermentation pass through the intestine for further digestion and absorption, in contrast to the situation in simple-stomached herbivores such as horses and rabbits (Hume and Warner, 1980; Wolin, 1981).

The most important aspect of rumen fermentation is degradation of plant fibres such as cellulose and hemicellulose. These fibres in animal's food are thought to be initially colonised by the cellulolytic bacteria, *Ruminococcus flavefaciens, Ruminococcus albus* and *Fibrobacter succinogenes*, and to some extent by cellulolytic *Butyrivibrio fibrisolvens* (Shane *et al.*, 1969; Bryant, 1973; Latham *et al.*, 1978; Czerkawski and Breckenridge, 1979; Cheng *et al.*, 1981; Cheng *et al.*, 1984). They break down the structural polysaccharide of the plant cell walls and release soluble carbohydrates which can be further fermented by other rumen bacteria. Other constituents of feed such as starch, proteins and lipids are also subject to microbial decomposition in the rumen (Wolin, 1981).

Microbial fermentation of foodstuffs results in a number of end products. The main products are volatile fatty acids (primarily acetic, propionic and butyric acids), occasionally lactic acid, ammonia and gasses such as methane and carbon dioxide. Most of these gases are eliminated from the rumen by belching, causing a loss of potential energy (Whitelaw *et al.*, 1970; Allison, 1978; Wolin, 1981). VFA can be used by rumen bacteria as carbon skeletons for the synthesis of amino acids

(Blackburn, 1965), but more importantly, they are absorbed through the rumen wall into the blood stream of the animal and serve as carbon and energy sources for the animal (Church, 1988). The quality and quantity of rumen fermentation products is therefore dependent on the types and activities of the rumen microorganisms. This, in turn, will have an enormous potential impact on animal performance and production.

### 1.2.4 Protein degradation in the rumen

A large proportion (50-90%) of dietary protein is degraded by rumen microorganisms (Figure 1.1) and the rate at which the different proteins can be hydrolysed controls the extent of their degradation before they pass out of the rumen (Cotta and Hespell, 1986a; Chen *et al.*, 1987a; Tamminga, 1979; Leng and Nolan, 1984; Mackie and Kistner, 1985). This has an important influence on the proportion of undegraded protein that is presented to the host animal postruminally. The amino acid composition of undegraded protein in the ruminant diet influences pattern and quantity of amino acids delivered to the small intestine for absorption and therefore plasma amino acids available for animal production (King *et al.*, 1990).

Protein degradation in the rumen is a composite of several microbial processes (Figure 1.2), including protein hydrolysis, peptide degradation, amino acid deamination and the fermentation of amino acid carbon skeletons (Chen *et al.*, 1987a; Cotta and Hespell, 1986b). Hydrolysis of proteins by rumen microbial enzymes releases oligopeptides, which are then broken down in turn to smaller peptides and finally to amino acids (Hoover and Stokes, 1991; Wallace and Cotta, 1988). The breakdown of peptides to amino acids by rumen microorganisms forms an integral part of the conversion of dietary protein to ammonia, a process which can destroy much of the nutritive value of dietary protein in ruminants (McKain *et al.*, 1990). Amino acids in turn are deaminated to ammonia, and the remaining carbon skeletons give rise to a variety of VFA products (Wallace and Cotta, 1988). The importance of amino aciddeamination within the rumen ecosystem may therefore be in the provision of

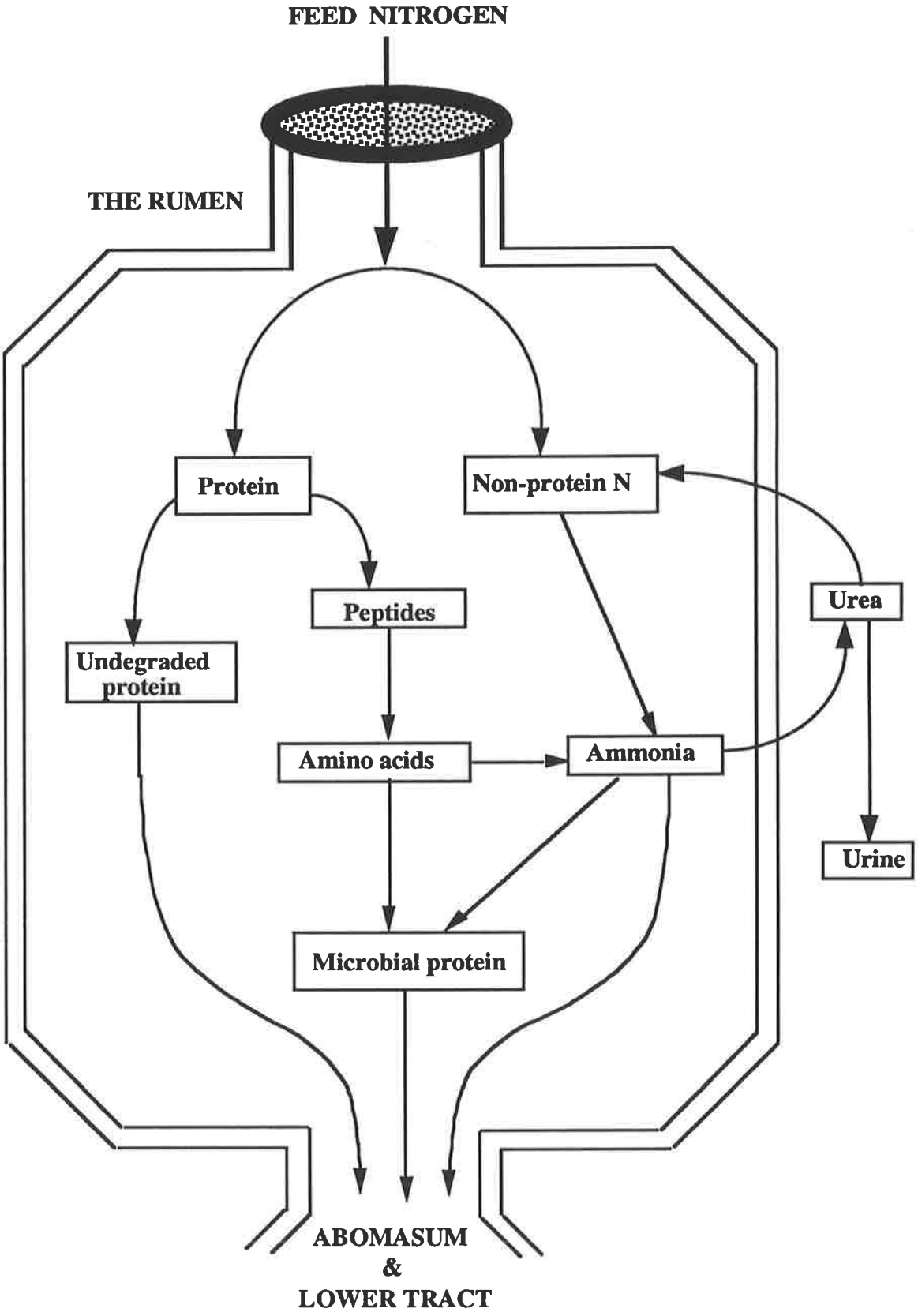



**Figure 1.1 A scheme for nitrogen utilisation by the ruminant animal**

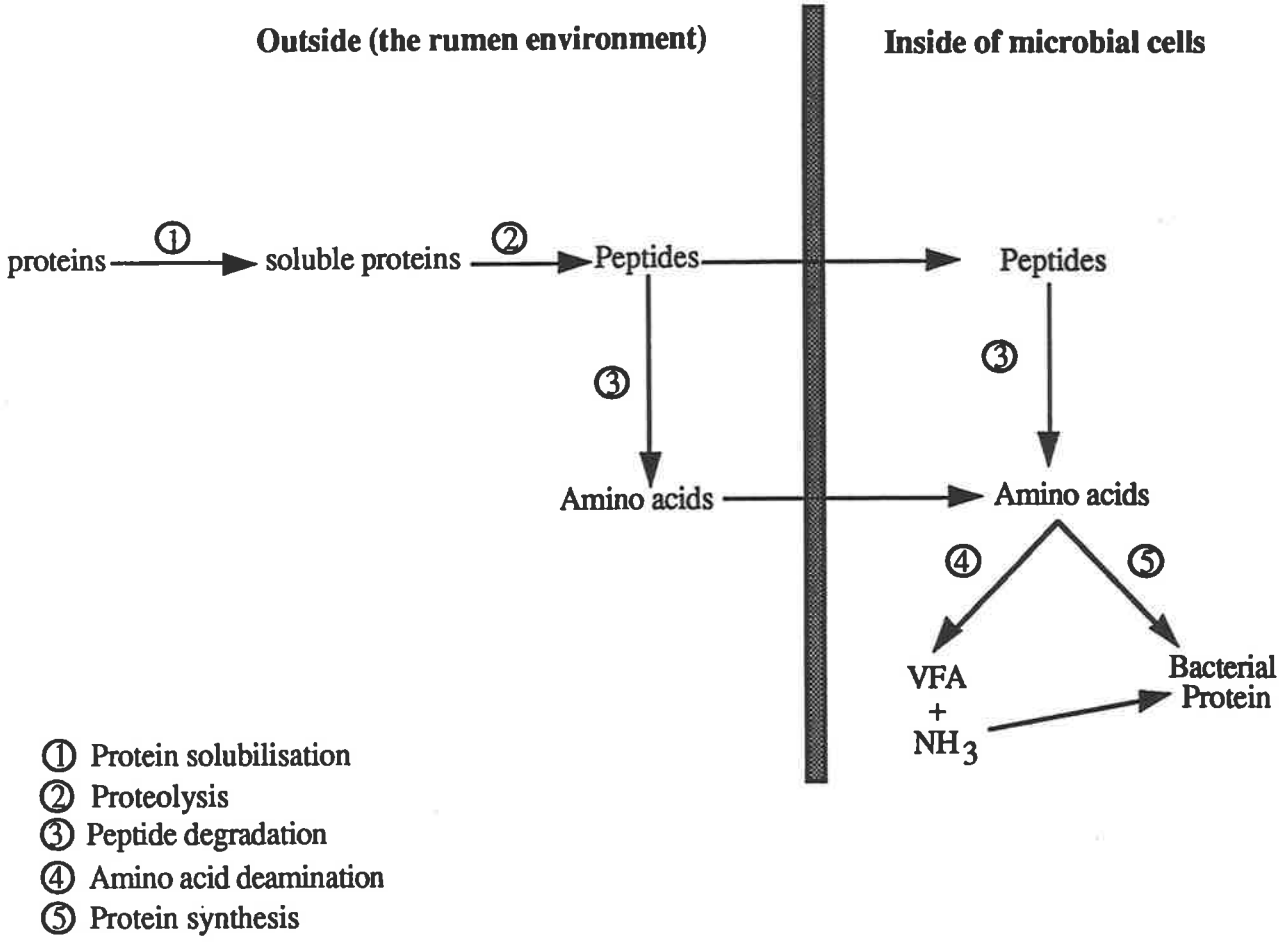



**Figure 1.2 A schematic representation of microbial protein degradation in the rumen**

ammonia and branched-chain VFA for rumen bacterial growth (Wallace and Cotta, 1988). The branched-chain VFA which are produced by deamination of branched-chain amino acids enhance the growth of many species of rumen microorganisms particularly cellulolytic bacteria (Allison and Bryant, 1958). Ammonia is the main nitrogen source for rumen bacteria (Bryant and Robinson, 1961; 1962; 1963) and it has been suggested to have a great influence on the rate of ruminal fermentation (Mehrez *et al.*, 1977). Hespell and Bryant, (1979) speculated that the enhancing effect of higher concentrations of ammonia on fermentation, dry matter digestibility, and cell yields may be indirect and not an effect on bacterial growth *per se*. For example higher $NH_3$ levels could lead to formation of ammonium bicarbonate and consequently prevent a decrease in pH which is caused by fermentation products such as VFA.

Protein degradation in the rumen can be considered as one of the most inefficient features of ruminant nutrition and wasteful to the animal (Wallace *et al.*, 1990). Ammonia is often produced in excess of microbial requirements for protein synthesis, and excess ammonia is absorbed into the portal blood through the rumen wall and eventually excreted as urea (Figure 1.1; Broderick and Wallace, 1988; Broderick and Craig, 1989; Russell, 1983). Under these conditions, it would be advantageous to limit degradation of dietary protein, provided this does not lead to a reduction in the microbial population and their activities (Smith, 1979). However, as ruminal microorganisms are the major source of protein available to the ruminant animal (Caldwell and Bryant, 1966; Cotta and Russell, 1982), proteolysis may not be wasteful if the resulting products are utilised to produce digestible microbial proteins of either the same or higher biological value than feed proteins (Fulghum and Moore, 1963; Tamminga, 1979).

Different protein supplements are broken down by mixed ruminal microorganisms at different rates, as are different pure proteins (Wallace *et al.*, 1987). Early workers proposed the general rule that protein breakdown in the rumen is proportional to

solubility, but subsequent research has shown that other properties are also important. For example, some soluble proteins are broken down more slowly than insoluble proteins, depending on the secondary and tertiary structure and the degree of disulphide bond formation (Wallace and Cotta, 1988). The soluble protein casein is rapidly hydrolysed by mixed rumen bacteria (Russell *et al.*, 1983), but bovine serum albumin is resistant to proteolytic attack due to the presence of 16 disulphide bonds which help stabilise its tertiary structure (Mahadevan *et al.*, 1980).

### 1.2.5 Proteolytic activity of rumen microorganisms

Proteolytic activity in the rumen contents is mostly associated with bacterial cells, whether free-living or attached to plant fibres (Wallace and Brammall, 1985; Cotta and Hespell, 1986a). Brock *et al.* (1982) found that about 75% of the proteolytic activity in rumen contents was associated with the particulate fraction. Upon fractionation, both bacterial and protozoal fractions showed proteolytic activity but the specific activity of the bacterial fraction was 6 to 10 times higher compared to that of the protozoal fraction. Protozoa are only likely to be important in the hydrolysis of particulate proteins as they have little activity towards soluble proteins (Nugent and Magan, 1981) but they play an important role in the engulfment of bacteria and particle matter and hence insoluble proteins (Coleman, 1979). Protozoa have higher activity against dipeptides whereas bacteria are more important in hydrolysing peptides containing three or more amino acids (McKain *et al.*, 1990). Using a dansylation procedure, Westlake and Mackie (1990) demonstrated that *S. bovis* possessed a wide range of extracellular peptidase activities against the pentapeptide Leu-Trp-Met-Arg-Phe. The rumen fungi also possess proteolytic activity (Wallace and Joblin, 1985; Wallace and Munro, 1986). Since fungal biomass is associated primarily with insoluble plant fibre, it might be expected that fibre would be the site at which evidence of protease activity would be found (Wallace and Cotta, 1988). However, the significance of fungal proteolytic activity within the rumen has not yet been established.

Proteolytic activity in the rumen is not confined to a single bacterium but is a property

possessed by many different bacteria that may also be active in the degradation of other feed constituents, mainly carbohydrates (Mackie, 1982). Between 30% and 50% of the bacteria isolated from rumen fluid display proteolytic activity towards extracellular protein (Fulghum and Moore, 1963; Wallace and Cotta, 1988). The predominant proteolytic bacteria will differ depending on diet (Mackie, 1982). However, under the variety of feeding conditions encountered by ruminants, the major proteolytic bacteria are thought to be *R. amylophilus, P. ruminicola, B. fibrisolvens* and *S. bovis* (Yokoyama and Johnson, 1988). Other species, probably of less significance are, *M. elsdenii, Clostridium* spp., *Eubacterium* spp. *Lachnospira multiparus, Succinivibrio dextrinosolvens* and the *Spirochaetes* (Allison, 1970).

Proteases have been studied in a few pure cultures of rumen bacteria. The proteolytic activity of *R. amylophilus* (Blackburn and Hullah, 1974) and *P. ruminicola* (Hazlewood and Edwards, 1981; Hazlewood *et al.*, 1981) is mostly cell-associated but is released by lysis of the cells during the stationary phase. Similarly, Westlake and Mackie (1990) found that proteolytic activity of *S. bovis* was largely cell-associated. In contrast to the activity of *R. amylophilus* and *P. ruminicola,* the proteolytic activity of *B. fibrisolvens* is extracellular, regardless of growth stage (Cotta and Hespell, 1986a). Using polyacrylamine gel electrophoresis (PAGE), Strydom *et al.* (1986) determined the number and approximate molecular weight (MW) of extracellular proteases produced by *B. fibrisolvens* strain H17c. They found that nine bands of protease activity with apparent molecular weights of approximately 101, 95, 87, 80, 76, 68, 63, 54 and 42 KDa were present in supernatants from exponential phase cultures. These data suggest that each species of rumen bacteria may possess several types of proteases and that individual strains may vary in protease production.

The proteolytic enzymes found in rumen contents are constitutive and seem to be stable and remain unchanged in stationary phase cultures (Hoover and Stokes, 1991). In general, rumen bacteria have a mixture of proteases based on the chemistry of their

active sites and the specificity of their hydrolytic activity (Brock *et al.*, 1982; Kopecny and Wallace, 1982; Wallace and Brammall, 1985). The predominant type of enzyme present in rumen contents, mixed rumen bacteria or extracted capsular material, is a cysteine-protease, sensitive to *p*-chloromercuribenzoate (PCMB). Other types of activity are also present. These include phenylmethylsulphonyl fluoride (PMSF)-sensitive serine proteases, metalloproteases and aspartic acid proteases (Wallace and Cotta, 1988). Aspartic acid proteases are of minor importance in rumen bacteria. In contrast, aspartic acid-specific activity is significant in rumen protozoa (Forsberg *et al.*, 1984). *Neocallimastix frontalis*, a predominant member of the anaerobic rumen fungi, has significant metalloprotease activity with trypsin-like specificity (Wallace and Joblin, 1985).

### 1.2.6 Approaches to improving protein utilisation in the rumen

Because so many rumen bacteria possess proteolytic activities and each ruminal bacterial strain may possess more than one type of protease, an approach to decrease these activities would be difficult. Research aimed at improving protein utilisation in ruminants has mainly focused on decreasing microbial degradation of dietary protein to increase outflow of undegraded protein to the lower digestive tract. This involves various treatments of dietary protein. Heat treatment can protect protein from microbial degradation (Ørskov, 1982). However, this treatment has the disadvantage of destroying some important amino acids such as lysine, cysteine, and tyrosine. In addition, heat treated protein tends to be less digestible in the abomasum (Bjarnason and Carpenter, 1970). Alternatively, protein can be coated with material such as whole blood, albumin, egg white or whey protein which is resistant to degradation (Ørskov, 1982). Another treatment involves the reaction of formaldehyde with protein which protects it from microbial degradation but is reversible in the acid conditions of the abomasum (Ferguson *et al.*, 1967). Protease inhibitors such as p-chloromercuribenzoate (PCMB) or phenylmethylsulphonyl fluoride (PMSF) may be included in the ruminant's diet to inhibit predominant proteases present in rumen

contents. It has also been proposed that the genes involves in the production of protease inhibitors can be cloned and introduced to rumen bacteria (Forsberg *et al.*, 1986). However, bacterial proteolytic capacity is necessary to some extent. For example, *Ruminobacter amylophilus* is unable to utilise the peptides and amino acids produced by its own protease (Hobson *et al.*, 1968) and probably has proteolytic capacity for gaining access to starch granules which are protected by proteinaceous coat (Cotta and Hespell, 1986b). A novel approach to solving the problem of protein supply to the ruminants could be to use genetically engineered plants containing proteins which are undegradable in the rumen (Higgins *et al.*, 1989). Ideally, this protein would not be degraded in the rumen and reach the lower tract for further digestion and absorption of resulting amino acids by the animal.

### 1.2.7 Importance of rumen microbes as a protein source

An important attribute of the ruminant digestive system is the synthetic ability of rumen microorganisms, rendering the adult ruminant independent of a dietary source of protein and the B-group of vitamins. Under most dietary conditions, rumen microorganisms are the major source of protein available to the ruminant animal, and the yield of microbial protein from the rumen controls the nitrogen status of the host (Cotta and Russell, 1982; Cotta and Hespell, 1986b; Wallace and Cotta, 1988). Periodic flushing of the rumen contents passes out a proportion of the microbial cells to the abomasum and small intestine where the cells are lysed and their protein is released. The microbial protein is subsequently hydrolysed by the host's enzymes and the resulting amino acids are absorbed by the animal (Hungate, 1966).

Although rumen microorganisms are thought to be able to synthesise all the amino acids required by the ruminant (Hungate, 1966; Sauer *et al.*, 1975), the amino acid composition of microbial protein leaving the rumen may not be ideal for maximum productivity (Buttery, 1976; Beever and Thomson, 1977). This is mainly due to the requirement for higher concentrations of some essential amino acids such as

methionine, threonine and lysine, which are required for wool and milk production (Barnett and Reid, 1961; Buttery, 1976, 1977; Owens and Bergen, 1983). One way to improve the balance and quantity of essential amino acids leaving the rumen is to genetically engineer several species of rumen microorganisms to synthesise specific proteins rich in the required amino acids. This would have a dual function of increasing both the quantity and quality of microbial protein produced in the rumen and so adjust the imbalance and amount of amino acid nitrogen reaching the animal (Brooker *et al.*, 1989). Another way is to feed the ruminant animals with the genetically engineered plants containing proteins which are rich in the required amino acids but are undegradable in the rumen.

### 1.2.8 Rumen nitrogen sources for microbial growth

There are protein- and non-protein nitrogen sources available for microbial growth in the rumen. Protein is not utilised by rumen microorganisms *per se*, but the ammonia, amino acids and peptides which arise as the end products of dietary protein breakdown act as nitrogen sources (Tamminga, 1979; Jenkinson *et al.*, 1979). Non-protein nitrogen sources including nucleic acids, purine and pyrimidine bases, amines, amides, alkaloids, choline, urea and nitrates, are mostly degraded to ammonia before being utilised by rumen microbes (Barnett and Reid, 1961; McAllan and Smith, 1973; Smith, 1975; Church, 1979; Patterson and Hespell, 1979; Nikolic *et al.*, 1980; Hobson and Wallace, 1982).

For many years, it was generally accepted that free amino acids are intermediate products in the breakdown of proteins by ruminal microorganisms (Yang and Russell, 1992). Nevertheless, peptides (but not amino acids) can accumulate, sometimes to an extent that significant amounts of nitrogen pass from the rumen in the form of peptides (Wallace *et al.*, 1990). Results of studies conducted both *in vivo* and *in vitro* have shown that the concentration of peptides can be as high as 1500 mg/litre of rumen fluid (Winter *et al.*, 1964; Russell *et al.*, 1983; Chen *et al.*, 1987a; 1987b).

In contrast to peptides, amino acids do not accumulate in significant concentrations in rumen fluid (<70 mg/litre), and only seven to eight amino acids are in detectable amounts (Wright and Hungate, 1967). The low concentration of amino acids occurring in ruminal fluid results from rapid deamination of free amino acids to keto acids and ammonia rather than from the rapid uptake of amino acids by microorganisms (Nolan and Leng, 1972; Wright, 1967).

Ammonia is the major component of soluble nitrogen within the rumen (Blackburn, 1965; Hespell and Bryant, 1979). It is produced from the degradation of dietary protein (Nolan and Leng, 1972), urea which enters the rumen via the saliva or diffuses across the rumen wall (Satter and Slyter, 1974), or other dietary non-protein sources such as nitrate (Schwartz *et al.*, 1991). The concentration of ammonia in rumen fluid varies widely ranging from 0.6 to 40 mM (Roffler and Satter, 1975). Satter and Slyter (1974) found that when the concentration of ammonia was lower than 3.5 mM, microbial growth decreased significantly. Levels of ammonia in dairy cows are reported to be 7-13.5 mM (Wohlt *et al.*, 1976). Thus, under adequate feeding regimens, prevailing ammonia concentrations should meet the animal's requirements for optimal ruminal bacterial growth.

### 1.2.9 Preferred nitrogen sources by rumen bacteria

The nature of nitrogen sources used for microbial growth and whether or not these sources are sufficient in the growth environment are the major factors that regulate overall biosynthetic potential of cells (Hespell and Bryant, 1979). Ammonia, amino acids and peptides are not only the inevitable products of protein degradation in the rumen, but are also important nitrogen sources for rumen microorganisms. Thus, it is important to establish which of these nitrogen sources is preferred by the predominant rumen bacteria.

Ammonia holds a central position in the growth of microorganisms on inorganic

sources of nitrogen ( Brown *et al.*, 1974). Ammonia is the main nitrogen source for 92% of ruminal bacterial isolates (Bryant and Robinson, 1961; 1962; 1963). Most of the rumen microorganisms can synthesise all the essential amino acids from ammonia even in the presence of preformed amino acids (Blackburn, 1965; Nolan and Leng, 1972; Bryant, 1974; Schmidt-Nielson, 1975). It is logical that it should be so since ammonia and not amino acids, is present in any quantity in the rumen (Blackburn, 1965; Bryant, 1974). In fact, the ability to synthesise cellular nitrogen compounds from ammonia is of considerable survival value in the ruminal environment which has provided the selection pressure for most species of ruminal bacteria (Bryant and Robinson, 1962).

The observation that most rumen bacterial strains are able to utilise ammonia as a sole nitrogen source has often been quoted without recognition that many rumen bacteria could also use amino acids and peptides as sole nitrogen sources and some have absolute requirements for these nitrogen sources (Argyle and Baldwin, 1989). Amino acids or peptides often stimulate microbial growth rates and yields over that which is obtained with ammonia as the sole nitrogen source (Cotta and Russell, 1982; Argyle and Baldwin, 1989) and they are more stimulatory in the absence of branched-chain VFA (Hespell and Bryant, 1979). *In vivo* studies have also shown that supplementation of low quality diets with protein increases microbial protein synthesis and thus protein flow from the rumen (Amos and Evens, 1976).

Despite the general belief that "the fate of amino acids in the rumen is predominantly to be broken down rather than to be assimilated into microbial protein" (Church, 1979; Hobson and Wallace, 1982), there is evidence for bacterial utilisation of amino acids. Using radiolabelling and dansylation techniques, Westlake and Mackie (1990) demonstrated that amino acid transport was much more predominant than peptide transport in *S. bovis*. Stevenson (1979) showed that *P. ruminicola* incorporated all amino acids except proline into cell protein. Scheifinger *et al.* (1967) found that

species of *Megasphaera, Streptococcus and Eubacterium* utilised a significant proportion of all 14 amino acids present in the medium but *Butyrivibrio* and *Selenomonas* were more selective. This implies that there is a preference for certain amino acids. Chen *et al.* (1987c) showed that hydrophobic amino acids were utilised more efficiently by mixed rumen bacteria than were hydrophilic amino acids. Chen and Russell (1988) showed that leucine, phenylalanine, serine and tyrosine were essential for growth of a monensin-sensitive ruminal *Peptostretococcus*. Gill and King (1958) reported that histidine, isoleucine, methionine, and lysine may be essential for the genus *Butyrivibrio* and Pittman and Bryant (1964) found that methionine was highly stimulatory or essential for many strains of *P. ruminicola.* Although these studies indicate the importance of amino acids as nitrogen sources, it is not clear under what conditions a bacterium has an absolute requirement for any particular amino acid(s). In addition, the biochemical nature of the stimulatory effect of amino acids on ruminal bacterial growth remains to be investigated.

For some rumen bacteria such as *P. ruminicola* (Pittman and Bryant, 1964; Pittman *et al.*, 1967) and *R. amylophilus* (Hullah and Blackburn, 1971) growth is dependent upon or is stimulated by the presence of peptides in the growth medium . In all such cases, the peptides have been shown to function as sources of specific amino acids which are otherwise in limited supply owing to inadequate uptake or rapid metabolism by cells (Pittman *et al.*,1967). Wright (1967), using $^{14}$C-peptides and -amino acids, showed that peptides were converted more efficiently to bacterial protein, but most of the corresponding free amino acids were fermented to volatile fatty acids. Hobson and Wallace (1982) speculate that rumen microorganisms may generally prefer oligopeptides over amino acids. This preference indicates that rumen bacteria may have a higher potential capacity to transport peptides compared with amino acids (Pittman and Bryant, 1964; Pittman *et al.*, 1967; Allison, 1970; Payne and Gilvarg, 1968; Hobson and Wallace, 1982). Alternatively, it is possible that peptide utilisation involves binding of the peptide to the bacterial cell-wall, followed by conversion by

cell-bound peptidases into its constituent amino acids which are then transported into the cell (Pittman *et al.*, 1967; Hobson and Wallace, 1982; Broderick *et al.*, 1988). Since mixed ruminal microorganisms metabolised longer peptides more rapidly than smaller ones, it was suggested that longer peptides may be hydrolysed by a peptidase closely associated with the bacterial cell wall and the resulting small peptides may be metabolised slowly and accumulate in the extracellular fluid (Wallace and McKain, 1989; Wallace *et al.*, 1990). When Payne and Bell (1979) incubated *E. coli* with peptides (composed of 2-6 amino acids), amino acids consistently appeared in the incubation medium. Since there was no evidence for extracellular or periplasmic peptidases, they concluded that this could result from a sequential process of peptide uptake, intracellular hydrolysis and amino acid exodus. However, another possibility is that of "coupled hydrolytic uptake", in which the hydrolysis of peptides (into amino acids) occurs as they are transported (Payne and Gilvarg, 1968; Snell, 1980). Thus, there are a few possibilities for bacterial peptide utilisation and the amino acids which arise from peptides are assimilated into cell protein or are excreted.

Concerning nitrogen preferences by rumen bacteria, ammonia is clearly the most abundant and important source of nitrogen in the rumen and is utilised by most rumen bacteria. Amino acids and peptides may be preferred nitrogen sources by some ruminal species *in vitro*, and required for maximal microbial growth in the rumen. It has also been shown that free amino acids are not readily utilised by mixed or pure cultures indicating that the cells preferentially synthesise amino acids *de novo* (Bryant and Robinson, 1963; Sauer *et al.*, 1975). However, resynthesis of amino acids from ammonia and catabolic products would appear to be an energetically wasteful process when amino acids are available (Stevenson, 1979). In the context of lack of detailed information on amino acid utilisation and metabolism in rumen bacteria and inconsistencies in this area of research in literature, it is important to study this aspect of ruminal nitrogen metabolism more extensively.

### 1.2.10 Bacterial cell envelope and transport systems

Every living cell must have the ability to acquire from its environments the substrates for energy production and biosynthesis, and release to its surroundings the metabolic waste products. In the case of the bacteria, this ability allows them to grow efficiently and compete in a diverse ecosystem such as the rumen.

A universal characteristic of cells is the membrane boundary that separates the interior cytoplasm from the exterior environment. The cytoplasmic membrane is composed of the lipid bilayer which provides a barrier to the free diffusion of solutes, but also contains specific carrier molecules or transport proteins (permeases) that permit the selective uptake and excretion of solutes (Chakrabarti and Deamer, 1992). Carrier proteins can be considered as membrane-bound enzymes. Instead of catalysing the conversion of a substrate to product, they mediate the vectorial reaction of solute transfer from one compartment to the other.

In addition to the cytoplasmic membrane, bacteria possess a cell wall which surrounds the cell and maintains cellular integrity. Bacterial walls are quite resilient to both mechanical or chemical breakdown. Only potent chemicals, such as sodium dodecyl sulphate or trichloroacetic acid, and a restricted number of enzymes, such as lysozyme and bacterial endopeptidases, accomplish their disruption (Beveridge, 1981). Gram-negative walls consist of a peptidoglycan layer and an outer membrane that contains lipopolysaccharide at its outer surface. Gram-positive walls lack the outer membrane but the peptidoglycan layer is usually much thicker (Beveridge, 1981; Russell *et al.*, 1990). Besides conferring strength and form to the bacterial cell, the wall must also control diffusion to a certain extent. It is the first structure encountered by the external milieu and, therefore, is the first to react to the extracellular environment (Beveridge, 1981). The outer membrane of Gram-negative bacteria functions as a molecular sieve through which high molecular weight substances cannot penetrate but low molecular weight compounds pass through porins which traverse the outer membrane and act as

channels (Russell *et al.*, 1990). However, the cytoplasmic membrane is responsible for the major screening of the cytoplasm from the environment, and determines to a large extent the entrance and exit of compounds to and from the cytoplasm. If the cell membrane was not semipermeable, it would be difficult for cells to retain the nutrients as these could diffuse out into the surrounding aqueous environment which is often hypotonic.

Transmembrane passage of hydrophobic (apolar) and small, uncharged compounds such as $CO_2$, $H_2O$, $O_2$, $N_2$ and $NH_3$ occurs by passive diffusion (Figure 1.3). Ions and large hydrophilic (polar) molecules (MW approx. 100 or more) do not easily pass through lipid bilayers and must be transported across cell membranes on carrier proteins (Figure 1.3; Kleiner, 1985; Russell *et al.*, 1990).

In simple passive transport, the solute always moves down its concentration gradient without the involvement of a carrier protein. Nevertheless, in some cases translocation of a solute may be facilitated by passing through a channel protein (Figure 1.3). Active transport, by contrast, can accumulate solute against extremely high gradients and this work may be driven by ion gradients or the hydrolysis of chemical bonds (Russell *et al.*, 1990). Bacterial active transport systems (Figure 1.3) can be classified into three categories with regard to their mechanism of energy coupling (Poolman, 1990; Mitchell, 1990). (1) Primary transport systems utilise chemical (e.g., ATP) or light energy to translocate a molecule across the cytoplasmic membrane. (2) Secondary transport systems utilise the electrochemical energy of a given solute (usually $H^+$ or $Na^+$) to transport another solute against its own concentration. The electrochemical energy inherent in the transmembrane difference in $H^+$ concentration (a proton gradient) is called proton-motive force. (3) Group translocation systems couple the translocation of a solute with the release of a modified solute at the other side of the membrane. Secondary transport systems, in turn, can be classified into three general categories in bacteria (Figure 1.3; Poolman, 1990; Mitchell, 1990). Symport or

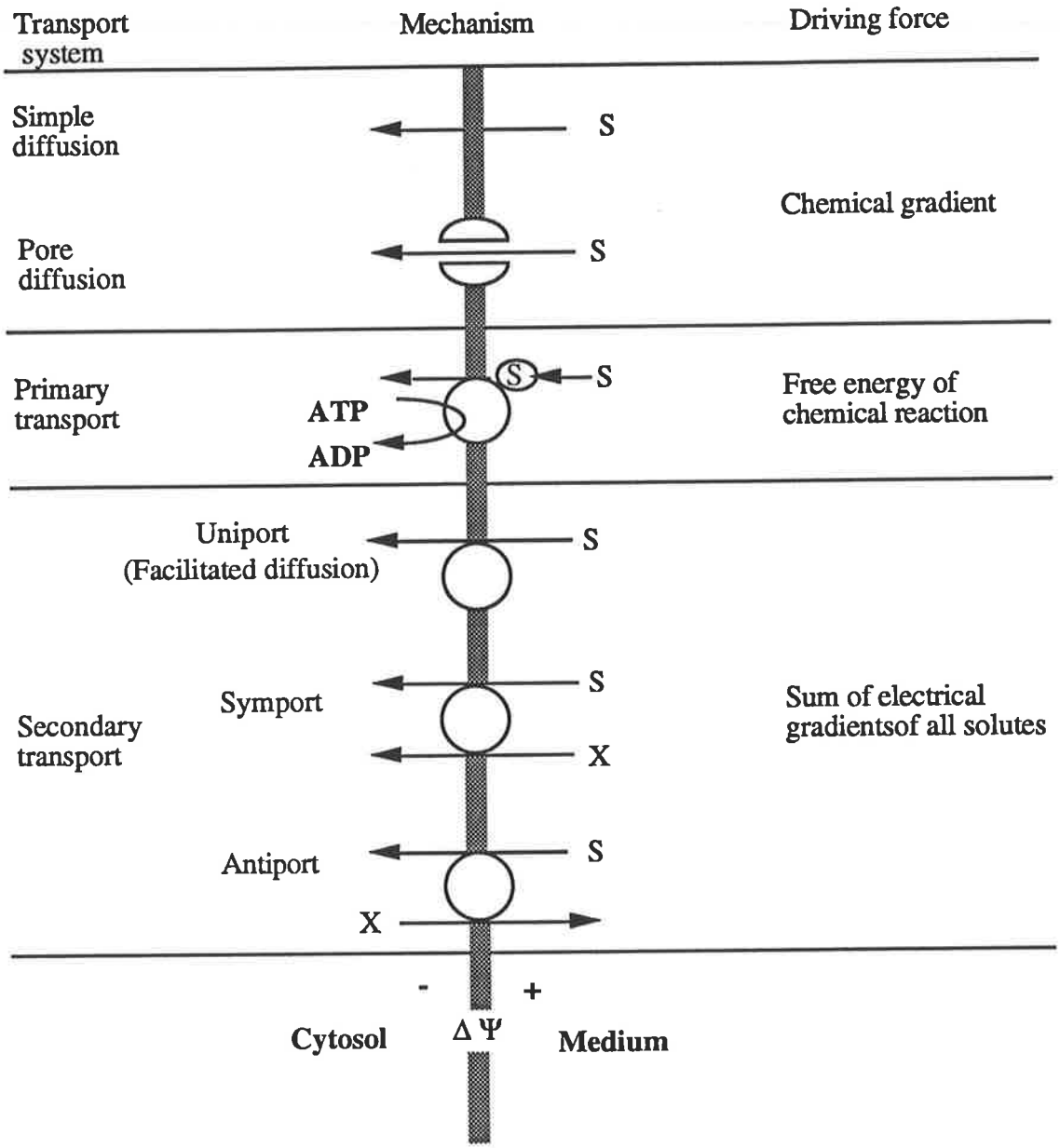



**Figure 1.3 Schematic diagram of solute transport systems in bacteria.**

cotransport system by which two solutes are translocated by a carrier protein in the same direction. Solute-cation symport transport has been found in bacteria. *E. coli* uses both $H^+$ and $Na^+$ as coupling ions for the uptake of sugars, amino acids, and other nutrients (Cairney *et al.*, 1984; Hama *et al.*, 1987; Reizer *et al.*, 1990; Kalman *et al.*, 1991). Antiport or countertransport refers to the coupled movement of two solutes in opposing directions. Antiport systems are generally used for the excretion of undesired solutes (products) from the cytoplasm (Konings *et al.*, 1992). When transport of a solute is carrier-dependent and the movement of the solute is independent of any coupling solute, the transport system is indicated as uniport.

As the starting point of a metabolic pathway, carrier-mediated transport of molecules is strictly regulated. In contrast, regulation of passive transport of a compound across a membrane is less direct, and if occurring at all in biological systems, is only possible by biologically variable parameters such as osmotic strength and temperature (Kleiner, 1985).

### 1.2.11 Ammonia transport systems in bacteria

The mechanism of ammonia uptake has a central position in bacterial protein synthesis. Ammonia exists in both charged and uncharged forms as the $NH_3$-$NH_4^+$ pair in a solution (Kleiner, 1985).

$$NH_3 + H^+ \rightleftharpoons NH_4^+ \qquad pKa = 9.25$$

Since ammonia, in its uncharged form ($NH_3$), can pass across cell membranes by passive diffusion (Kleiner, 1985; Russell *et al.*, 1990), for a long time it was believed that a specific transport system for the ionic form was not necessary (Kleiner, 1985). However, at physiological pH most of the ammonia (pKa = 9.25 at 24°C) would be present as $NH_4^+$, a polar species that is not permeable to the cell membrane (Russell *et al.*, 1990). $NH_4^+$ carrier systems have been reported in many species of bacteria

(Brown, 1980; Stewart, 1980). Kleiner (1985) reported more than 30 bacterial species possessing $NH_4^+$ carrier systems and stated that many more species may contain similar ammonia carriers under certain conditions. Thus, ammonia transport systems appear to be fairly common in bacteria and do not seem to be restricted to certain groups.

The exact mechanism of ammonia uptake is still not clear. Stevenson and Silver (1977), using methylammonium as an $NH_4^+$ analog, provided evidence for an energy-dependent $NH_4^+$ transport system in *E.coli.* Energy-dependent $NH_4^+$ transport has been inferred from dependence on an energy source, inhibition by inhibitors of energy metabolism and inhibition by compounds which decrease the proton motive force. In *Klebsiella pneumoniae*, under anaerobic conditions, when the proton motive force is exclusively generated by the translocating ATPase, inhibition of this enzyme also eliminates $NH_4^+$ transport. No effect was observed under aerobic conditions, where the proton motive force was generated by respiration in this organism (Kleiner, 1985). It has also been suggested that the maintenance of intracellular $NH_4^+$ pools by organisms living on $N_2$, $NO_3^-$ or organic nitrogen sources involves energy-dependent cyclic $NH_3/NH_4^+$ retention (Figure 1.4). These organisms produce $NH_3$ as a product of $N_2$ fixation, $NO_3^-$ reduction or degradation of organic nitrogenous compounds. Since the cellular membranes are so permeable to $NH_3$, it continuously diffuses out of the cell. In most cases under these conditions, organisms generally derepress the $NH_4^+$ carriers and supposedly retrieve the escaping $NH_3$ after protonation (Kleiner, 1985).

There is little information on ammonia transport in rumen microorganisms. Is it active or passive, specific or non-specific (Smith, 1979; Russell and Hespell, 1981; Hespell 1984)? Russell and Strobel (1987) reported that ammonia gradients across the cell membranes were 1.8 to 15-fold in mixed rumen bacteria. This indicates that some of the bacteria may have active transport systems for ammonia. However, it is

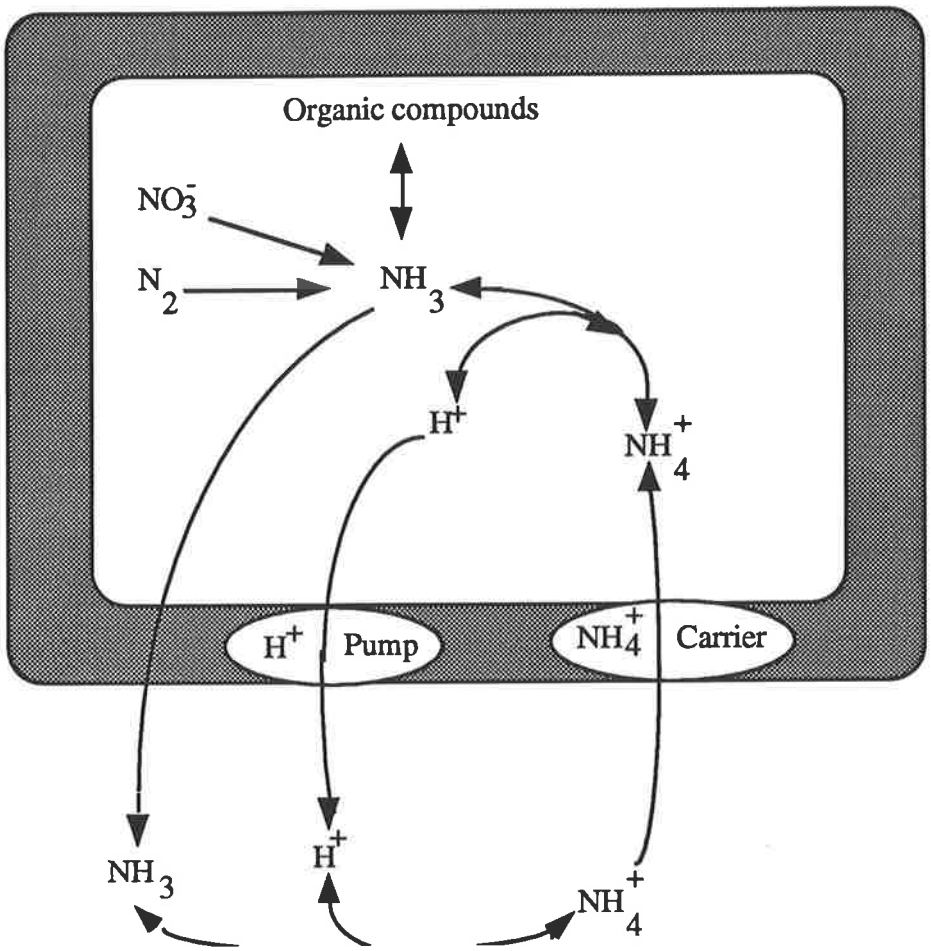



**Figure 1.4 Cyclic NH3/NH4+ retention**

improbable that the rate of ammonia transport limits the rate of ammonia assimilation into amino acids in rumen bacteria. What might be more important, at least in the interpretation of enzyme kinetic measurements from the study of ammonia assimilating enzymes, is whether ammonia accumulates within cells. Without this information, predicting likely transport mechanisms involved from the Km (ammonia) of different enzymes is hazardous (Wallace and Cotta, 1988).

### 1.2.12 Amino acid transport systems in bacteria

Amino acids are important transport solutes which are both taken up and excreted by the cell (Figure 1.5). If available, amino acids can be taken up by corresponding uptake systems, but they can also be excreted by many bacteria. Amino acids differ widely in the structure of their side chains and this affects their permeability across a cell membrane. Enteric bacteria have a multiplicity of amino acid transport systems. Firstly, different mechanisms for uptake exist, and secondly, more than one type of uptake system for any particular amino acid is frequently found in one organism (Chen *et al.*, 1987c). Kinetic examination of the transport of neutral and branched-chain amino acids in *Streptococcus cremoris* has led to the suggestion that specific transport systems exist for the following structurally related amino acids: L-alanine and glycine; L-serine and L-threonine; L-leucine, L-isoleucine and L-valine (Driessen *et al.*, 1987a; 1987b). *Lactococcus* (*Streptococcus*) *lactis* takes up lysine by an arginine-ornithine exchanger and a separate system which is proton-motive force driven (Driessen *et al.*, 1989). The arginine exchanger of *L. lactis* is a non-energy-requiring transport system for arginine uptake (Driessen and Konings, 1990).

Information on the transport of amino acids in rumen bacteria relates to a few organisms. Stevenson (1979), using competitive inhibition experiments, reported that six specific transport systems may occur for amino acids in *P. ruminicola* 23. One for leucine, isoleucine and valine; a second for histidine, lysine and arginine; a third for serine, glutamine and asparagine ; a fourth for phenylalanine, tryptophan and tyrosine;

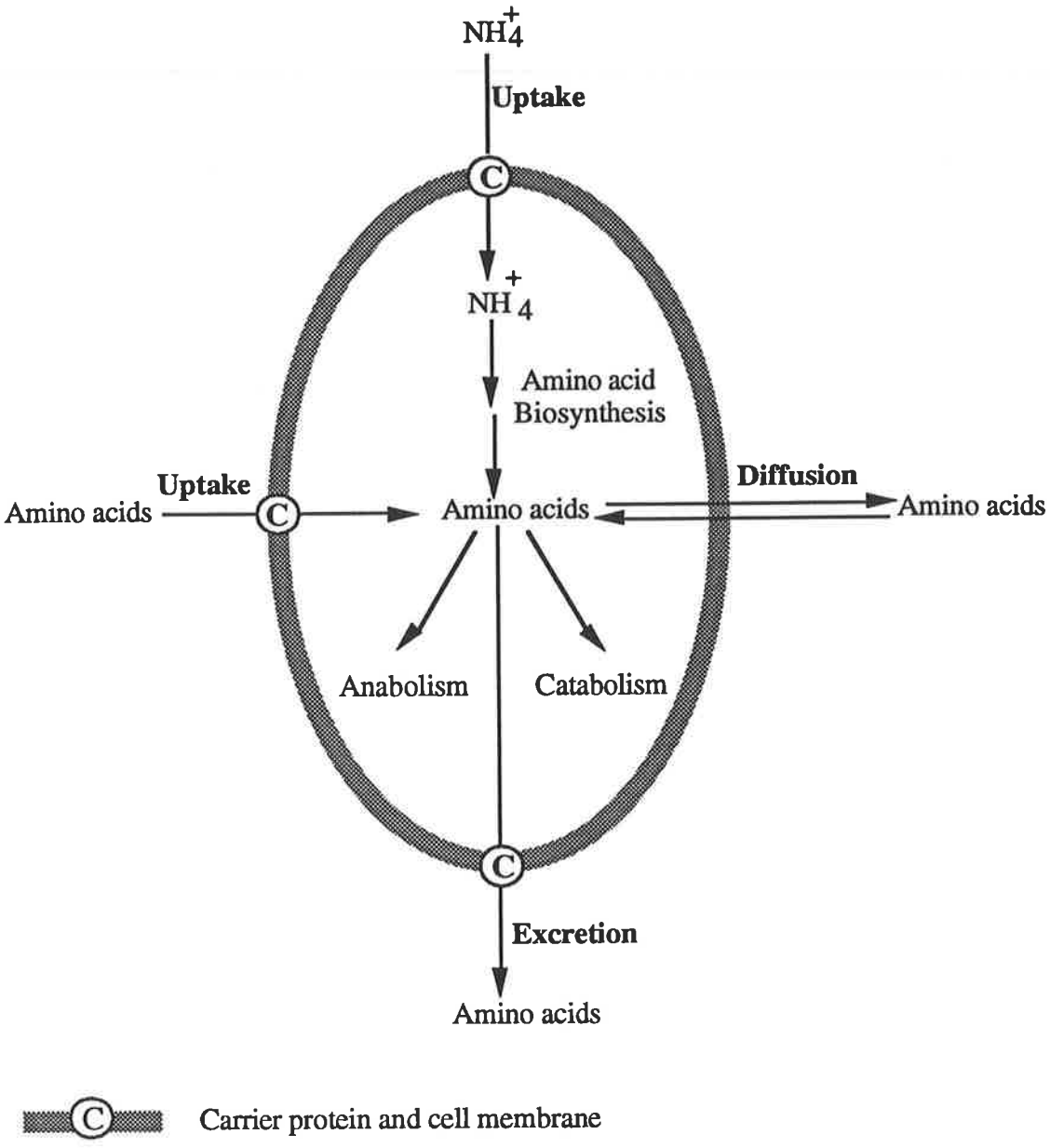



**Figure 1.5 Amino acid transport in bacteria**

a fifth for glutamate and aspartate; and a sixth for methionine alone. Since inhibition of L-leucine uptake by D-leucine was much less than inhibition by L-leucine itself, Stevenson (1979) suggested that these transport systems were specific for the L-isomers. Chen and Russell (1989a) have shown that ruminal *Peptostreptococcus* transports the branched-chain amino acids, leucine, isoleucine and valine by a common carrier which is dependent on $Na^+$ or $Li^+$. *S. bovis* possesses carrier proteins which can transport amino acids in symport with $Na^+$ (Russell *et al.*, 1988b). *S. ruminantium* also has a similar transport mechanism for amino acids (Strobel and Russell 1991). Van Kessel and Russell (1992) reported that in the monensin-sensitive *Peptostreptococcus* strain SR, the active transport of arginine could be driven by either an electric potential or a $Na^+$ gradient. Since arginine was a strong inhibitor of lysine transport and lysine was a weak inhibitor of arginine transport, Van Kessel and Russell (1992) concluded that the strain SR may have an arginine-lysine carrier which has a preference for arginine.

### 1.2.13 Peptide transport systems in bacteria

Peptide transport systems are widely distributed in bacteria (Chen *et al.*, 1987c). The ability to utilise peptides before they are split into amino acids is advantageous in the intensive competition for food in the rumen (Wright, 1967). The transport of some peptides can occur at a greater rate or to a greater extent than transport of the constituent amino acids. Chen *et al.* (1987c) reported that peptide nitrogen was taken up more than twice as fast as amino acid nitrogen by mixed rumen bacteria. There are bacteria which lack a transport system for a given amino acid but possess transport systems for peptides containing that amino acid. Pittman *et al.* (1967) reported that *P. ruminicola* was unable to take up free $^{14}C$-labeled proline or glutamic acid and took up very little $^{14}C$-valine but could take up $^{14}C$-proline-containing peptides and incorporate into trichloroacetic acid-insoluble cell material. It has been reported that uptake of a specific amino acid may be reduced by competition with other amino acids if they share the same transport system, whereas peptides which contain the same

amino acid, but which are not in competition with free amino acids in the medium, may be taken up rapidly (Kihara and Snell, 1955; Leach and Snell, 1960; Shelton and Nutter, 1964). Since the energy cost of transporting peptides and amino acids may be the same, the uptake of peptides by cells decreases the transport energy expenditure as an inverse function of the peptide length (Hespell and Bryant, 1979).

Three distinct systems for peptide transport, with overlapping substrate specificities, have been described in *Salmonella typhimurium* and *E. coli*. These systems are Opp, Tpp and Dpp for oligopeptide, tripeptide and dipeptide permeases respectively (Payne, 1983; Andrews *et al.*, 1986; Hiles *et al.*, 1987). It has been reported that peptide transport may be regulated by amino acids (Jamieson and Higgins, 1984; Andrews *et al.*, 1986; Andrews and Short, 1986). The Tpp-encoded peptide transport system of *S. typhimurium* is induced by leucine or anaerobiosis (Jamieson and Higgins, 1984), whereas, in *E. coli*, the Opp-encoded system rather than the Tpp-encoded system is induced by anaerobiosis, alanine, leucine, or peptides containing alanine or leucine (Andrews *et al.*, 1986; Andrews and Short, 1986).

Microorganisms can transport various peptides, but different species differ in the maximum peptide length they can transport (Pittman *et al.*, 1967) possibly because the cell wall acts as a molecular sieve preventing oligopeptides above a certain size from crossing the cell wall (Payne and Bell, 1979). Payne and Bell (1979), using a fluorescence-labelling procedure, have found that the upper size limit for transport of peptides in *E. coli* is a peptide of 5-amino acid residues. Pittman *et al.* (1967), using $^{14}$C-proline-containing peptides have shown that *P. ruminicola* transports large oligopeptides of molecular weights up to 2,000. However, the transport of peptides by bacteria that has been demonstrated using a radiolabelling technique is unable to distinguish between extracellular hydrolysis of labelled peptides into smaller peptides and/or amino acids, or rapid hydrolysis after transport and a possible efflux of labelled amino acids from the intracellular pool. Westlake and Mackie (1990), using a

dansylation method, demonstrated the transport of the pentapeptide Leu-Trp-Met-Arg-Phe in *S. bovis* suggesting the existence of mechanism for the transport of peptides up to 751 Da. They also observed that most of the pentapeptide was completely hydrolysed extracellularly into eight products after 10 min incubation. These results demonstrate limitations in the use of radiolabelling techniques in transport experiments and the necessity for considering possible extracellular hydrolysis of dipeptides, as was observed for *S. bovis* .

The composition of amino acid side chains has little influence on peptide uptake in *E. coli* (Broderick *et al.*, 1988) but in rumen bacteria the amino acid composition and sequence within the peptide is more important than chain length *per se* (Chen *et al.*, 1987c; Wallace *et al.*, 1990). Trialanine is taken up more rapidly than any other di- and tripeptides in mixed rumen bacteria (Broderick *et al.*, 1988). Chen *et al.* (1987c) found that mixed rumen bacteria utilised the alcohol insoluble portion of isopropanol-fractionated trypticase at twice the rate of the alcohol soluble material which contained high levels of hydrophobic amino acids, especially proline. Broderick *et al.* (1988) have also shown that proline-containing peptides are utilised at a lower rate than other peptides. Whether hydrophobic and hydrophilic peptides are transported by the same uptake system is still unclear (Chen *et al.*, 1987c). However, more work is required to determine how large oligopeptides of different compositions are utilised by species of rumen bacteria.

### 1.2.14 Ammonia assimilation in bacteria

Ammonia is an important source of nitrogen for many rumen bacteria (Blackburn, 1965; Leng and Nolan, 1984), and as such, enzymes of ammonia assimilation are vital for these microorganisms (Wallace and Cotta, 1988). Ammonia assimilation has been studied extensively in aerobic bacteria, especially in *E. coli*. The results of these studies have shown that glutamate dehydrogenase (Borghese and Wall, 1992; Bormann *et al.*, 1992; Ertan, 1992b) and the dual enzyme system, glutamine synthetase and

glutamate synthase (Tyler, 1978) are the two major pathways of ammonia assimilation. Glutamate dehydrogenase (GDH; Figure 1.6) is an important branch point between carbon and nitrogen metabolism since it catalyses the reductive amination of α-ketoglutarate to yield glutamate, as well as the oxidative deamination of glutamate (Khale *et al.*, 1992). In microorganisms, NAD-dependent GDH (E.C. 1.4.1.2) appears to serve a catabolic function, while the enzyme utilising NADPH (E.C. 1.4.1.4) serves primarily for the biosynthesis of glutamate (Tyler, 1978). Many bacteria possess NADPH-dependent rather than NAD-dependent GDH (Ertan, 1992a).

The second major pathway of ammonia assimilation (Figure 1.7) is mediated by coupled action of two enzymes. Glutamine synthetase (GS; E.C. 6.3.1.2) catalyses the ATP-dependent assimilation of ammonia to provide glutamine which serves as the nitrogen donor in the biosynthesis of many diverse metabolites (Jenkinson *et al.*, 1979). Glutamate synthase (GOGAT) is one of many enzymes that catalyse the transfer of the amide group of glutamine in various biosynthetic reactions. In this case, glutamine reacts with α-ketoglutarate to form two molecules of glutamate (Tyler, 1978). Most bacteria possess an NADPH-dependent GOGAT (E.C. 1.4.1.13) while some bacteria contain an NADH-dependent GOGAT (E.C. 1.4.1.14) and others possess a GOGAT enzyme that uses both of NADPH and NADH coenzymes (Ertan, 1992a). The end product of both GDH and GS/GOGAT pathways is L-glutamate (Rossi *et al.*, 1989) which is the central amino acid in nitrogen metabolism.

Most of the nitrogen assimilated via ammonia enters metabolism as the α-amino group of glutamate and thence the α-amino group of most other amino acids. Since most amino acids are synthesised by transamination from glutamate, bacteria such as *E.coli* are able to synthesise all 20 amino acids required for protein synthesis (Senior, 1975; Umbarger, 1978; Stryer, 1981). Transamination of glutamate to various other amino acids has also been demonstrated in cell extracts of rumen contents (Chalupa *et al.*, 1970; Tsubata and Hoshino, 1969) and individual ruminal bacterial species (Jenkinson

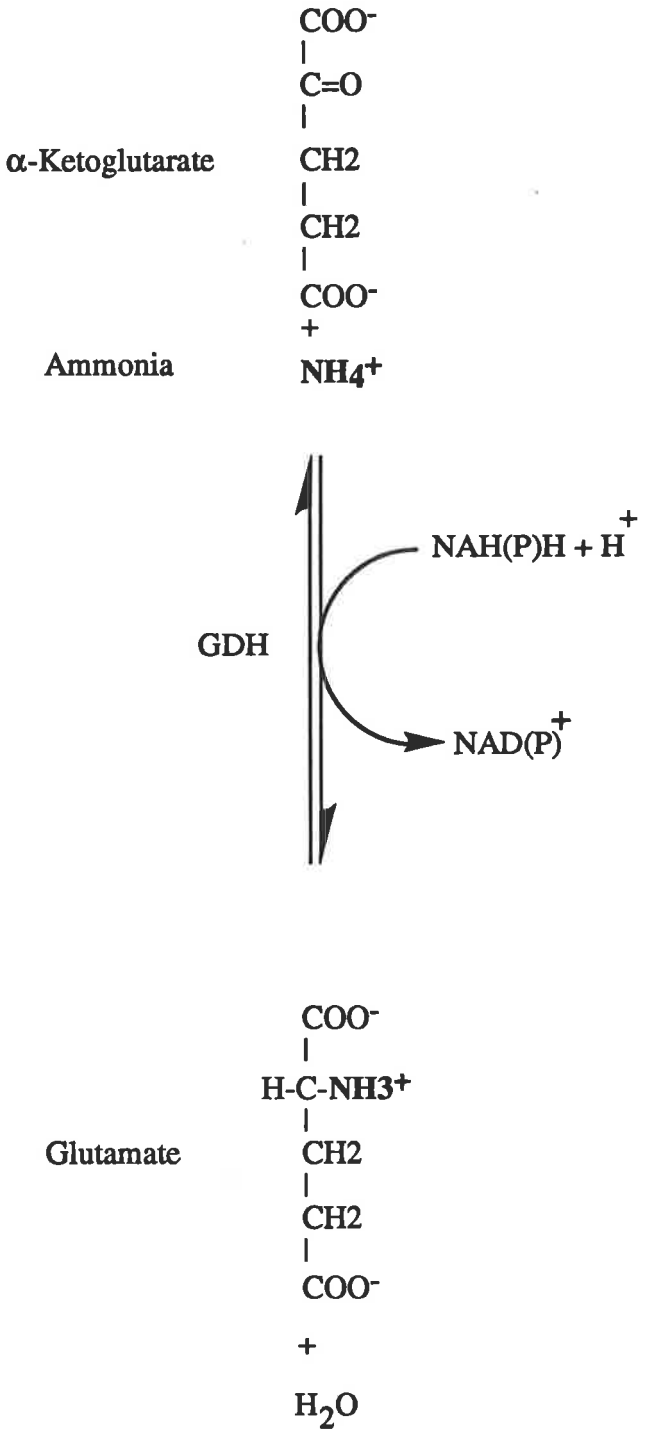



**Figure 1.6 Ammonia assimilation via GDH**

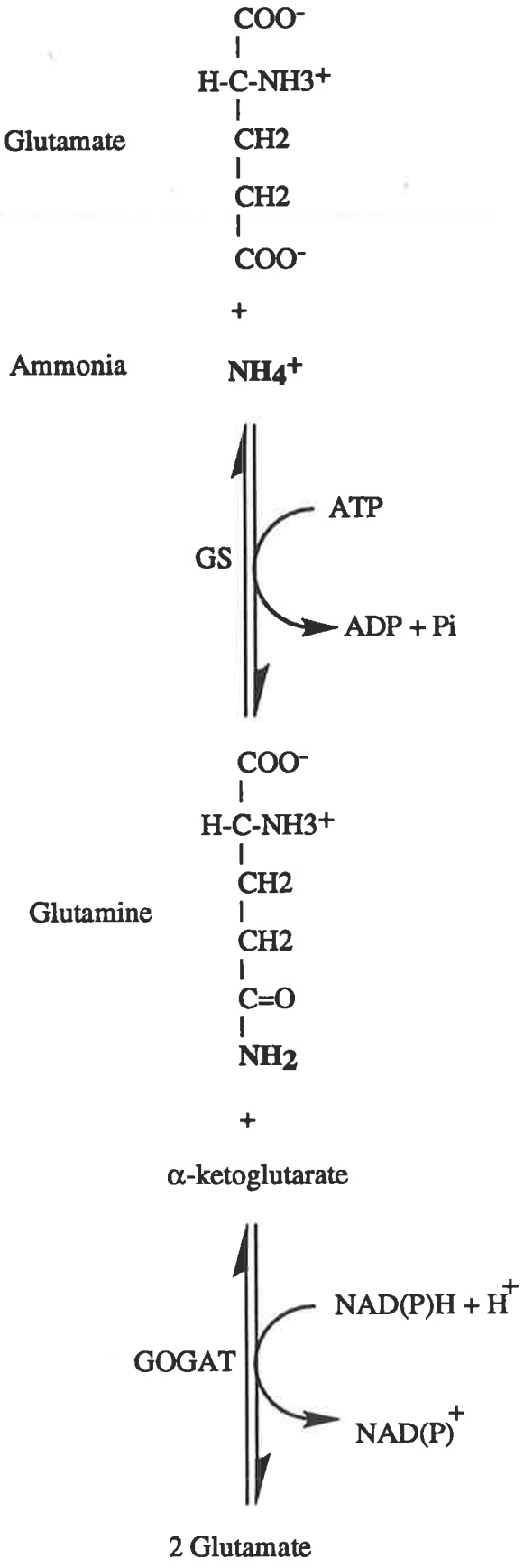



**Figure 1.7 Ammonia assimilation via GS/GOGAT**

*et al.*, 1979; Joyner and Baldwin, 1966; Kistner and Kotze, 1973). In most bacteria, both GDH and the GS/GOGAT pathways exist and the synthesis of the enzymes is regulated by a repression-depression mechanism driven by the amount and type of nitrogen source (Ertan, 1992b). GS is the key regulatory enzyme in nitrogen assimilation in many bacteria (Kremeckova *et al.*, 1992; Tyler, 1978). It appears that this enzyme interacts with DNA to activate transcription of certain genes (Tyler, 1978). GS itself is controlled by various mechanisms: feedback inhibition, post-translational modification by the adenylation of tyrosine residue in each subunit of the enzyme, activation and inhibition by divalent cations and nucleotides, and repression and depression by substrates and products of nitrogen metabolism (Kremeckova *et al.*, 1992). In aerobic microorganisms, maximum biosynthetic activity is obtained when the enzyme is completely unadenylated and decreases over a wide range as the degree of adenylation increases. Ammonia appears to cause a decrease in the level of both GS and GOGAT (Meers *et al.*, 1970; Dainty, 1972; Griffith and Carlsson, 1974; Tyler, 1978; Holmes *et al.*, 1989; Wallace, 1979; Ertan, 1992b). The GS in enteric bacteria grown in the presence of excess ammonia tends to be highly adenylated and consequently less biochemically active than that found in nitrogen limited cultures. The GDHs of most microorganisms have a relatively high Km for both α-ketoglutarate and ammonia (> 1mM), whereas GS has a much lower Km for ammonia (< 0.5 mM) than does GDH (Tyler, 1978; Ertan, 1992b). This suggests that GDH generally operates at high ammonia concentrations whereas the GS/GOGAT system operates under conditions of low ammonia concentrations and is thus able to scavenge ammonia at low concentrations (below 1 mM; Stadtman, 1973; Brown *et al.*, 1974; Brown, 1980). However, the level of activity of GOGAT has been found to be significantly lower than that of GDH and this may argue against the function of GOGAT in ammonia assimilation (Griffith and Carlsson, 1974; Ertan, 1992b).

Although GDH and GS/GOGAT are undoubtedly the most important ammonia-assimilatory pathways, a number of other ammonia-assmilatory enzymes exist, but are

not as important as either the GS/GOGAT or GDH (Tyler, 1978; Umbarger, 1978; Brown, 1980; Magasanik 1982). These enzymes can be alternatives when GDH or GS/GOGAT systems are lacking or inactive. One of these enzymes is alanine dehydrogenase (AlaDH: E.C. 1.4.1.1.) which catalyses the reductive amination of pyruvate to alanine in a reversible reaction utilising NADH or NADPH (Figure 1.8; Wiame *et al.*, 1962; Yoshida and Freese, 1965; Brown *et al.*, 1974; Caballero *et al.*, 1989). The main physiological function of the enzyme appears to be the conversion of L-alanine to pyruvate, which can be used as a carbon source. However, AlaDH has been detected in *Anabaena cylindrica*, *Cyanidium caldarium*, *Methanobacterium thermoautotrophicum*, *Rhodopseudomonas capsulata* and *Streptomyces clavuligerus* that lack GDH (Dunn and Klucas, 1973; Brown *et al.*, 1974; Kenealy *et al.*, 1982; Moreno-Vivián *et al.*, 1983; Brana *et al.*, 1986), suggesting a possible role in ammonia assimilation. The other ammonia-assimilating enzyme is asparagine synthetase (AsS: E.C.6.3.1.1), which catalyses the synthesis of L-asparagine from ammonia and aspartate, in a similar fashion to the way in which GS catalyses the synthesis of glutamine (Figure 1.9; Meister, 1974; Umbarger, 1978). Nevertheless, AsS enzyme has a minor role in ammonia assimilation. This is possibly because the reaction catalysed by this enzyme utilises two equivalents of ATP (forming AMP and pyrophosphate, which would be hydrolysed to Pi) compared to only one for GS (Meister, 1974; Umbarger, 1978; Magasanik, 1982).

Ammonia assimilatory enzymes similar to those of the enteric bacteria have been found in ruminal bacteria, although there are individual differences in their optimum assay conditions and apparent regulatory behaviour (Duncan *et al.*, 1992). The activities and kinetic properties of ammonia assimilating enzymes imply that GDH with its Km (ammonia) of 1.0-1.7 mM, is likely to be the principal ammonia assimilating enzyme in rumen bacteria (Wallace, 1979; Wallace and Cotta, 1988). However, it is possible that GDH may function over a wide physiological range of ammonia concentration. High levels of both NADH- and NADPH-dependent GDH activity have been observed

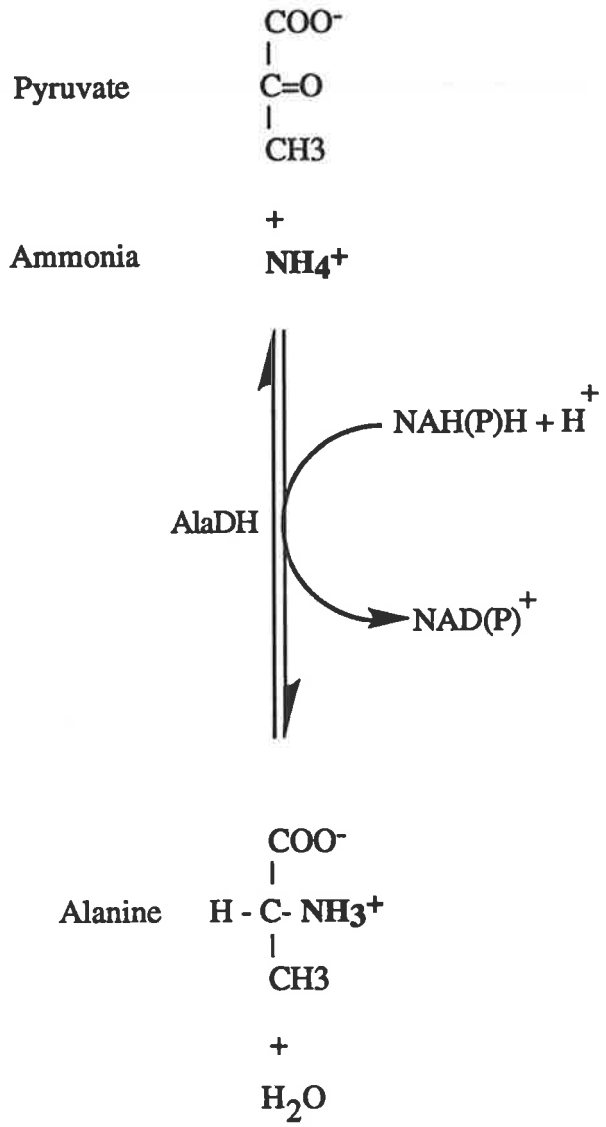



**Figure 1.8 Ammonia assimilation via AlaDH**

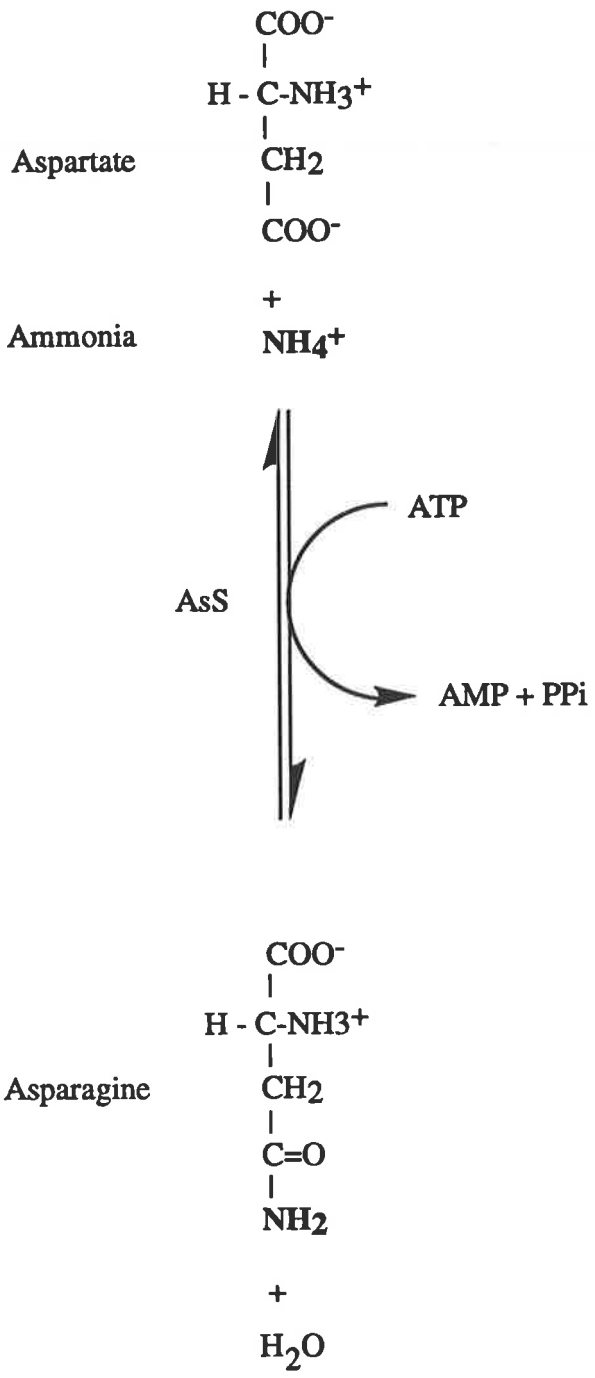



**Figure 1.9 Ammonia assimilation via AsS**

in rumen contents (Chalupa *et al.*, 1970) and in mixed rumen bacteria (Erfle *et al.*, 1977). The enzyme has also been detected in nine species of rumen bacteria which are considered representative of about 40% of the rumen microbial population (Jenkinson *et al.*, 1979). Either NADH- or NADPH-dependent GDH (but not both) were detectable in pure cultures of rumen bacteria including *P. ruminicola, B. fibrisolvens, R. flavefaciens, R. albus, F. succinogenes, S. bovis, S. ruminantium, R. amylophilus* and *M. elsdenii* (Burchall *et al.*, 1964; Joyner and Baldwin, 1966). Most ruminal bacteria possess NADPH-dependent GDH activity, whereas *R. albus* and *M. elsdenii* possess NADH-dependent GDH activity (Joyer and Baldwin, 1966). However, the presence of both NADH- and NADPH-dependent activities has been reported in *S. ruminantium* strain D (Smith *et al.*, 1980).

Although the results of studies in the field of rumen ammonia assimilation have indicated that GDH is the key enzyme for ammonia asimilation in rumen bacteria (Bhatia *et al.*, 1980; Blake *et al.*, 1983; Wallace and Cotta, 1988), other amino acid dehydrogenases, transaminases and synthetases are probably also important (Allison, 1969; Chalupa *et al.*, 1970; Chalupa, 1972). The discovery of GOGAT in non-rumen bacteria (Tempest *et al.*, 1970) led to an investigation of the effects of ammonia on the activities of GS, GOGAT, GDH, AsS, and aspartate and alanine aminotransferase in continuous cultures of mixed rumen bacteria (Erfle *et al.*, 1977). This work showed that the activity of GS was markedly induced at low ammonia concentrations, and that both NADH- and NADPH-dependent GDH activities were also present in the rumen. This work also demonstrated the presence of GOGAT activity in rumen microorganisms utilising both NADH and NADPH. When ammonia levels are less than 1 mM, the GS/GOGAT pathway of ammonia assimilation predominates in the rumen, whereas when ammonia levels are higher, the GDH pathway is more important (Hespell, 1984). Because ruminal ammonia concentrations are rarely, if ever, less than 0.7 mM, it is unlikely that the GS/GOGAT system is an important pathway of ammonia assimilation in rumen bacteria unless its Kms are different from aerobic organisms

(Russell *et al.*, 1990). However, GS activity has been demonstrated in *S. ruminantium* strain D (Smith *et al.*, 1980) and was unusual in that neither NADPH nor NADH acted as electron donors for the reaction. The enzyme used dithionite-reduced methyl viologen as the electron carrier system in the assay, suggesting that some unidentified low-electron carrier is required. In addition, GS in *S. ruminantium* is not subject to the same feedback inhibition as for enteric bacteria and low ammonia concentrations *per se* do not significantly increase GS activity except when values are less than 1 mM. High levels of GS activity may therefore be necessary in this species for adequate ammonia assimilation and glutamate formation under ammonia-limited conditions (Smith *et al.*, 1980; 1981). GOGAT has also been reported in *S. bovis* (Griffith and Carlsson, 1974). Erfle *et al.* (1977) have demonstrated AsS and aspartate aminotransferase activities in mixed rumen bacteria. Thus GS and AsS provide glutamate synthesising systems in rumen microorganisms. Since high levels of alanine was present in mixed rumen bacteria, Erfle *et al.* (1977) speculated that rumen bacteria may provide a means of trapping pyruvate and ammonia for subsequent utilisation by the host animal. This reaction is mediated by AlaDH, and also alanine aminotransferase which favours alanine accumulation (Erfle *et al.*, 1977).

In summary, the results of studies in mixed rumen bacteria, pure cultures of several species and *in vivo* have shown that rumen bacteria possess the type of ammonia-assimilating enzymes found in other environments, with the GDH and GS/GOGAT pathways predominating (Buttery, 1976; Baldwin and Denham, 1979; Harrison and McAllan, 1980; Russell and Hespell, 1981; Hobson and Wallace, 1982; Hespell 1984). Miflin and Lea (1976) have mentioned that the presence of GS and GOGAT does not prove that they are used *in vivo* as the major route of nitrogen assimilation. This may be relavant to rumen microbial studies, since, although these enzymes may not be important under the prevailing experimental conditions, they may be important under other conditions, or in some microenvironments. Russell and Hespell (1981) have pointed out that an interplay of ammonia assimilation pathways probably takes place at

various times after feeding and with various dietary changes. Thus it is possible that other ammonia-assimilating enzymes, such as AsS, AlaDH and other amino acid dehydrogenases may also play roles in ammonia assimilation in mixed flora of the rumen (Chalupa *et al.*, 1970; Chalupa, 1972; Buttery, 1976; Hobson and Wallace, 1982; Hespell, 1984).

### 1.2.15 Amino acid biosynthesis in bacteria

The pathways of amino acid amino acid biosynthesis in organisms have been determined by using four major methods, namely enzymological studies, radiolabelling, enzyme inhibitors and amino acid auxotrophs. A widely used approach to the elucidation of metabolic pathways is to use a isotopically labelled substrate or intermediate (e.g. $^{14}C$, $^{15}N$ or $^{18}O$) and study the appearance of isotope in the product (White *et al.*, 1973). The labelled product is isolated, purified and analysed for isotope incorporation. Another approach to determine the steps in metabolic pathways is to use metabolic inhibitors. Adding a specific enzyme inhibitor to a cell-free extract causes an accumulation of intermediates in the pathway prior to the point of inhibition. Studies with mutant strains of microorganisms have also discerned diverse, specific metabolic blocks, which have been of aid in delineating metabolic pathways and control metabolisms. Using these mutants is somewhat analogous to using metabolic inhibitors. Amino acid auxotrophs provide an approach for the identification of intermediate steps in amino acid biosynthetic pathways. These auxotrophs harbour gene mutations that often result in an inability to synthesise the enzyme in an active form. Such a defect leads to a block in the metabolic pathway at the point where the enzyme acts, and the enzyme's substrate accumulates. Mutations in biosynthetic pathways of amino acids are lethal, as amino acids are essential for protein biosynthesis. In microorganisms it is possible to manipulate the growth medium so that amino acids are provided. Thus, amino acid auxotrophs can be used for the elucidation of amino acid biosynthetic pathways in microorganisms.

Pathways of amino acid biosynthesis in bacteria have been reviewed by Umbarger (1978). According to this review, amino acids can be divided into five groups based on the carbon skeletons used for their synthesis:

I. The glutamate family consists of glutamate itself and those amino acids that drive all or most of their carbon chains from glutamate: glutamine, proline and arginine. Biosynthetic pathways to glutamate and glutamine are important in the assimilation of ammonia. The nitrogen assimilated by most bacteria from ammonia in the medium enters metabolic pathways as the amino group of glutamate or as the amide group of glutamine.

II. The serine family including serine and the two amino acids drived from it; cysteine and glycine. The pathway serves a far greater metabolic need than the mere synthesis of these three amino acids for protein. For example, most microorganisms form the methyl group of methionine from β-C of serine. The intact carbon chain of serine is incorporated directly into tryptophan and phospholipids, and the carbon chain of glycine is incorporated into purine and heme-containing compounds. Glycine provides a significant fraction of the total one-carbon pool needed for purine, thymine, methionine and histidine biosynthesis.

III. The aspartate family consisting of aspartate, asparagine, methionine, threonine, lysine and isoleucine. Since this family of amino acids is formed via a highly branched pathway, there are numerous branch points that are potential sites of regulation. Several patterns of control over this branching pathway have evolved in various organisms.

IV. The pyruvate family consisting of the three amino acids that drive the major portion of their carbon from pyruvate; alanine, valine and leucine. Isoleucine is also a member of the aspartate family but obtains two of its carbons from pyruvate. Lysine,

considered also as a member of the aspartate family, can actually derive half of its carbons from pyruvate.

V. The aromatic family including phenylalanine, tyrosine, tryptophan and histidine.

Since many ruminal microorganisms are able to grow on ammonia as a sole nitrogen source, *de novo* biosynthesis of amino acids from ammonia and available carbon sources must occur. In spite of the importance of ruminal amino acid biosynthesis, information on the biosynthesis of individual amino acids in rumen bacteria is limited and mainly qualitative.

### 1.2.16 Aims of the project

The information on nitrogen metabolism which has been obtained from experiments *in vivo* on ruminal contents directly, such as in fistulated animals, or mixed rumen bacteria *in vitro* may not be regarded as biochemically precise. These data are difficult to interpret as they represent the combined effects of many enzymes produced by different kinds of microorganisms. However, such studies have shown that most ruminal bacteria use ammonia as the major source of nitrogen and amino acids or peptides may be stimulatory or essential for others. Results of studies in pure cultures have also shown that ammonia is the main nitrogen source for rumen bacteria and other nitrogen sources such as amino acids and peptides are also important.

Nevertheless, the role of amino acids or peptides in relation to bacterial growth has not been investigated more extensively. It is not clear under what conditions a bacterium has an absolute requirement for amino acids or peptides for growth. No studies have been conducted to determine whether this requirement is due to possible limitations in ammonia assimilation or biosynthesis of a particular amino acid. There is evidence that ruminants need better supplies of methionine and lysine for growth and methionine and cysteine for production of wool. This may be due to limitations in

amino acid biosynthetic pathways in rumen microorganisms as microbial protein is generally the ruminant's principal source of amino acids. One way to overcome this limitation is to introduce DNA sequences which code for the most limiting amino acids into rumen bacteria. The other is to genetically engineer several species of rumen microorganisms to synthesise specific proteins rich in the essential amino acids. However, a thorough investigation of aspects of nitrogen utilisation in rumen bacteria is necessary before such genetic manipulations can be pursued. A better understanding of nitrogen utilisation can identify possible points at which ruminal bacterial nitrogen utilisation can be improved. A prerequisite for such studies is a chemically defined medium which allows as good growth as a rumen fluid medium, since otherwise stimulatory effects which may operate in the rumen could well be missed. Thus the ultimate aims of this thesis are:

1. To develop a completely defined medium for studying nitrogen requirements of some species of rumen bacteria.

2. To examine the utilisation of available nitrogen sources in the rumen by various ruminal bacterial species, with the object of understanding the role of ammonia versus exogenous amino acids and peptides in relation to microbial growth.

3. To investigate the biochemical limitations to ammonia utilisation.

4. To determine possible limitations in the enzymatic steps in the pathways of amino acid biosynthesis.

# CHAPTER 2

# CHAPTER 2

## A Defined Medium for Studying Nitrogen Requirements of Some Strains of Rumen Bacteria

### 2.1 Introduction

Peptides, amino acids and ammonia, the end products of dietary protein breakdown in the rumen, act as nitrogen sources for rumen microorganisms (Jenkinson *et al.*, 1979). Uptake and utilisation of these nitrogen sources is therefore central to the nitrogen economy of the rumen ecosystem and hence animal production.

Most ruminal bacteria have simple nitrogen requirements, and are known to be capable of utilising ammonia as the main if not the only nitrogen source even in the presence of amino acids and peptides (Bryant and Robinson 1962; Bryant and Robinson 1963; Hespell and Bryant 1979). Studies *in vivo*, using $^{15}NH_4^+$ indicate that 50 to 70% of microbial nitrogen can be derived from rumen ammonia (Pilgrim *et al.*, 1970; Mathison and Milligan, 1971; Nolan and Leng, 1972). However, there is evidence that amino acids and peptides are also important nitrogen sources for ruminal microorganisms. Nolan *et al.* (1976) demonstrated that in some circumstances approximately 60% of bacterial nitrogen requirements in the rumen can be supplied by amino acids and peptides. Other studies with pure cultures have shown that many ruminal bacteria have absolute requirements or are stimulated by the addition of amino acids or peptides to the growth medium (Hungate, 1966; Pittman and Bryant, 1964; Pittman *et al.*, 1967). Argyle and Baldwin (1989) demonstrated that availability of amino acids and peptides was an extremely important factor affecting mixed rumen bacterial growth. Ayers (1958) considered casein hydrolysate to be essential for growth of a strain of *Ruminococcus flavefaciens*, when grown in a medium containing ammonia. Hespell and Bryant (1981) have reported that most strains of *Butyrivibrio fibrisolvens* utilise

ammonia as the nitrogen source, but many strains also utilise mixtures of amino acids or complex nitrogen sources, such as casein hydrolysate or peptone. Some strains of *B. fibrisolvens*, *S. ruminantium*, *S. bovis* and *S. dextrinosolvens* exhibit a preference for utilising exogenous amino acids rather than synthesising them from ammonia and carbon sources (Bryant and Robinson, 1963). *P. ruminicola* subsp. *brevis* and *P. ruminicola* subsp. *ruminicola* have been shown to preferentially utilise peptides or ammonia over amino acids (Bryant and Robinson, 1963; Pittman and Bryant, 1964; Hobson and Wallace, 1982).

The effect of amino acids or peptides on ruminal bacterial growth has been studied from a number of perspectives. It has been reported that amino acids and peptides can contribute in a large way to the microbial nitrogen needs for growth (Hespell and Bryant, 1979) and thus can have a significant influence on overall efficiency of microbial protein synthesis in the rumen (Argyle and Baldwin, 1989; Hespell and Bryant, 1979). Cotta and Russell (1982) demonstrated that addition of amino acids increased the efficiency of utilising energy sources for the synthesis of microbial protein in pure cultures. Maeng and Baldwin (1976) found that addition of small amounts of amino acids to the whole rumen contents increased overall microbial cell yield by 36 to 62%. Hespell and Bryant (1979) reported that amino acid additions increased the observed $Y_{ATP}$ (grams dry weight cells formed per mole ATP expended). They speculated that the effect of amino acids on cell yields and $Y_{ATP}$ may not be due to a lower energy cost due to decreased amino acid biosynthesis, but most likely, that the amino acids decreased the degree of energy uncoupling (the relative degree to which ATP or other energy rich compounds are utilised by anabolic activities of the cell) with the rumen microbial population as a whole. Nevertheless, the effect obtained with amino acids may not always be due to these materials directly, but rather to ammonia and carbon skeletons produced from amino acid deamination (MacLeod and Murray, 1956; Dehority *et al.*, 1957; 1958). The deamination of specific amino acids is of special relevance to bacterial growth in the rumen. The most important of these is the

conversion of leucine, isoleucine and valine to isovalerate, 2-methylbutyrate and isobutyrate respectively. These branched-chain fatty acids are either required or highly stimulatory to the growth of many ruminal bacteria, particularly fibrolytic species (Bryant and Robinson, 1962, 1963). Despite these and other studies, the reason for absolute requirements for amino acids or peptides by some strains of rumen bacteria is not clear. More work is required to understand the biochemical nature of the stimulatory effects of amino acids or peptides on bacterial growth.

Although most rumen bacterial species are able to utilise ammonia as their main nitrogen source, ammonia is only one of the nitrogen sources available in the rumen and this may not always be the preferred nitrogen source for every microorganism in the rumen ecosystem (Bryant and Robinson, 1961, 1962, 1963; Allison, 1970; Russell, 1984). It is not clear under what conditions a bacterium will utilise one particular nitrogen source in preference to others. The role of amino acids or peptides in relation to growth of rumen bacteria has not been investigated more extensively. A detailed understanding of nitrogen utilisation in rumen bacteria could reveal ways to control the overall process of ruminal protein degradation.

To determine the nitrogen requirements of individual species of rumen bacteria, it is essential that bacterial growth is described in a medium of fully defined composition. To study the nutritional requirements of rumen bacteria has always been made difficult by the fact that these organisms require complex media for growth.

Various media containing rumen fluid have generally supported growth of large numbers of organisms and a greater variety of species than have media devoid of rumen fluid (Caldwell and Bryant, 1966). Since rumen fluid is a complex unidentified mixture of different compounds including proteins, peptides, amino acids and ammonia, a rumen fluid-containing medium is not suitable to study the nitrogen requirements of individual species of rumen bacteria.

For the description of nitrogen utilisation, few defined media have been described in which there has not been available at least some form of metabolisable nitrogen. Partially defined media, in which rumen fluid has been replaced by the addition of hemin, volatile fatty acids and yeast extract, have been developed and used for enumeration and isolation of rumen bacteria (Caldwell and Bryant, 1966), and for the definition of carbohydrate requirements (Leedle and Hespell, 1980) in bacterial cultures *in vitro*. However, yeast extract can also contain nitrogenous bases from degraded nucleic acids. Most functionally important rumen bacteria are obligate anaerobes and cannot initiate growth in culture unless the medium is poised at a sufficiently low redox potential (Jones and Pickard, 1980). Even if all $O_2$ is removed from a culture medium, addition of a reducing agent is necessary to achieve this low redox potential (Brock and O'Dea, 1977; Jones and Pickard, 1980). Cysteine hydrochloride has been used most extensively as an effective reducing agent (Bryant and Robinson, 1961; Hungate, 1969; Bryant and Robinson 1962; Gill and King, 1958), but it also provides a source of amino acid nitrogen for the bacteria and therefore it is a confounding factor in studies of nitrogen metabolism. Jones and Pickard (1980) described a medium in which titanium (III) citrate replaced cysteine as a reducing agent, but this was not effective for all bacterial species tested, especially *B. fibrisolvens*. Cotta and Hespell (1986a) described a defined medium to measure the proteolytic activity in various strains of *B. fibrisolvens* in response to differing nitrogen sources. However, the maximum growth achieved by the various strains of *B. fibrisolvens* in that medium was not described. I have attempted to use that medium to define the nitrogen requirements of strains of *B. fibrisolvens* and other rumen bacterial species, but under the conditions described by Cotta and Hespell (1986a), was not able to achieve bacterial growth comparable to that achieved in a rumen fluid-containing medium.

The aim of the experiments described in this chapter was to develop a completely defined medium in which to investigate the nitrogen requirements of some species of rumen bacteria. Using this defined medium, I have examined the nitrogen requirements

for strains of *B. fibrisolvens, P. ruminicola, S. ruminantium* and *S. bovis* species. *Bacteroides fragilis,* an anaerobic bacterium of the human lower intestine (Bryant, 1974), was also tested as a non-ruminal bacterium. *B. fibrisolvens, P. ruminicola* and *S. bovis* species were chosen in this study because they are the major proteolytic species of ruminal bacteria (Hazlewood and Edwards 1981; Russell *et al.*, 1981; Fulghum and Moore, 1963; Hazlewood *et al.*, 1983) and *P. ruminicola, S. bovis* and *S. ruminantium* species are known to be peptide (Chen *et al.*, 1987a) and amino acid utilisers (Cotta and Russell, 1982). Most importantly, these species are representatives of the predominant niches in the rumen, and often comprise significant proportions of the rumen microbial community (Hungate, 1966).

## 2.2 Materials and Methods

### 2.2.1 Bacterial strains

| Bacteria | Sources |
|---|---|
| *Butyrivibrio fibrisolvens* H17c | Hespell, R.B., Peoria, Illinois, USA. |
| *Butyrivibrio fibrisolvens* E14 | Orpin, C.G., Brisbane, Australia. |
| *Prevotella ruminicola* (brevis) GA33 | Hespell, R.B., Peoria, Illinois, USA. |
| *Prevotella ruminicola ruminicola* P1 | Brooker, J.D., Adelaide, Australia. |
| *Bacteroides fragilis* (strain unknown) | Brooker, J.D., Adelaide, Australia. |
| *Selenomonas ruminantium* S23 | Brooker, J.D., Adelaide, Australia. |
| *Streptococcus bovis* 2B | Gregg, K., Armidale, Australia. |
| *Streptococcus bovis* H24 | Gregg, K., Armidale, Australia. |

Stock cultures of bacteria were stored in a mixture of a rumen fluid medium and 80 % glycerol (final concentration, 20%) at -80°C.

### 2.2.2 Chemicals

Brain Heart Infusion (BHI) dehydrated media, casamino acids, and yeast extract were purchased from Difco Laboratories, Detroit, USA. Tryptone and agar were prepared from Oxoid Ltd., Basingstoke, Hampshire, England. L-cysteine, hemin (bovine) and vitamins (biotin, folic acid, *p*-amino benzoic acid, cyanocobalamin, calcium pantothenate, nicotinamide, riboflavin, thiamin hydrochloride, pyridoxamine and lipoic acid), blue dextran 2,000,000 and ninhydrin were prepared from Sigma Chemical Company St. Louis, USA. Resazurin, acetic acid, propionic acid, butyric acid, iso-butyric acid, n-valeric acid, iso-valeric acid and DL-α-methylbutyric acid were obtained from Aldrich Chemical Company, Milwaukee, USA. Sephadex G-25 (fine and medium) was purchased from Pharmacia LKB, Biotechnology AB Uppsala, Sweden. Chelex-100 resin (50-100 mesh; sodium form) was obtained from Bio-Rad Laboratories, Richmond, CA, USA.

All other chemicals, of the highest purity available, were obtained from the following sources: Ajax Chemicals, Sydney, Australia; B.D.H. Chemicals Australia Ltd., Boronia and Kilsyth, Australia.

### 2.2.3 Solutions and buffers

#### 2.2.3.1 Mineral solution I (Atlas and Parks, 1993)

*Ingredient*

$K_2HPO_4$ ..... 11.84 g

$K_2HPO_4$ was dissolved in reverse osmosis (RO) water to a final volume of 1 litre and the solution was stored at 4°C.

### 2.2.3.2 Mineral solution II (Atlas and Parks, 1993)

*Ingredients*

$KH_2PO_4$ ..... 7.08 g

NaCl ..... 1.78 g

$MgSO_4.7H_2O$ ..... 3.75 g

$MnCl_2.6H_2O$ ..... 0.20 g

$CoCl_2.6H_2O$ ..... 0.02 g

$Na_2SO_4$ ..... 8.30 g

$CaCl_2.2H_2O$ ..... 3.19 g

The ingredients were dissolved in RO water to a final volume of 1 litre and the solution was stored at 4°C. To prevent precipitation, $CaCl_2.2H_2O$ was dissolved in RO water separately and then added to the rest of the mineral solution II.

### 2.2.3.3 Trace element solution

Trace element solution was prepared as described by Gomez-Alarcon *et al.* (1982) except that $NiCl_2.6H_2O$ and $FeSO_4$ were added, and $Na_2SeO_3$ was omitted.

*Ingredients*

$ZnSO_4.7H_2O$ ..... 0.1 g

$H_3BO_3$ ..... 0.1 g

$Na_2MoO_4.2H_2O$ ..... 0.1 g

$FeSO_4$ ..... 0.1 g

$NiCl_2.6H_2O$ ..... 0.05 g

$CuSO_4.5H_2O$ ..... 0.05 g

$Al_2(SO_4)_3.12H_2O$ ..... 0.02 g

The ingredients were dissolved in RO water to a final volume of 1 litre and the solution

was stored at 4°C.

#### 2.2.3.4 Volatile fatty acid (VFA) mixture

The VFA mixture was prepared as described by Caldwell and Bryant (1966).

*Ingredients*

Acetic acid ..... 17.0 ml

Propionic acid ..... 6.0 ml

Butyric acid ..... 4.0 ml

Iso-butyric acid ..... 1.0 ml

n-valeric acid ..... 1.0 ml

Iso-valeric acid ..... 1.0 ml

DL-α-methylbutyric acid ..... 1.0 ml

The fatty acids were pipetted into a container that had a tight-fitting closure and the pH was adjusted to 7.0 using 10 M NaOH.

#### 2.2.3.5 Vitamin solution

The vitamin solution was slightly modified from that described by Cotta and Hespell (1986a).

*Ingredients*

Biotin ..... 8.3 mg

Folic acid ..... 8.3 mg

*p*-amino benzoic acid ..... 8.3 mg

Cyanocobalamin ..... 8.3 mg

Calcium pantothenate ..... 67.0 mg

Nicotinamide ..... 67.0 mg

Riboflavin ..... 67.0 mg

Thiamine hydrochloride ..... 67.0 mg

Pyridoxamine ..... 67.0 mg

Lipoic acid.....6.7 mg

Vitamins were dissolved in 1 litre of RO water and the pH was adjusted to 7.0. The solution was stored in aliquots of 10 ml at -20°C.

### 2.2.3.6 Hemin + 1,4-Naphthoquinone solution

The hemin + 1,4-naphthoquinone solution was prepared as described by Caldwell and Bryant (1966) and Gomez-Alarcon *et al.* (1982).

*Ingredients*

Hemin.....0.5 g

1,4 - Naphthoquinone.....0.1 g

Hemin and 1,4-naphthoquinone were dissolved in 10 ml of 1 M NaOH and then brought to a final volume of 1 litre with RO water.

### 2.2.3.7 Phosphate buffer 0.1 M, pH 6.8.

*Ingredients*

0.2 M $NaH_2PO_4$.....51.0 ml

0.2 M $Na_2HPO_4$.....49.0 ml

The pH was adjusted to 6.8 and the solution was diluted to a final volume of 200 ml and stored at 4°C.

### 2.2.3.8 Citrate buffer (0.5 M, pH 5.5)

*Ingredients*

1.0 M Citric acid.....16.0 ml

1.0 M Trisodium citrate.....34.0 ml

The pH was adjusted to 5.5 and the solution was diluted to a final volume of 100 ml

with RO water.

### 2.2.4 Fractionation of tryptone by gel filtration on Sephadex G-25

The method was as described by Phillips and Gibbs (1961). Dry Sephadex-G25 (70g; fine or medium) was suspended in RO water and allowed to settle at room temperature. Fine particles were removed by decantation. This was repeated three times and then the gel was resuspended in 0.1 M phosphate buffer pH 6.8 (section 2.2.3.7) and allowed to swell overnight at room temperature. A column (250 mm x 10 mm I.D.) was packed with a slurry of the gel under gravity and equilibrated with 3 bed volumes of 0.2 M ammonium hydroxide (eluent). The void volume of the column was measured using 0.1% (w/v) blue dextran as an exclusion material. Tryptone (200 mg) was dissolved in 2.5 ml of the eluent and applied onto the column. The column was eluted at the rate of 1 ml/min and the effluent from the column was collected in 1 ml fractions. The absorbance of eluted fractions was measured at 280 nm.

### 2.2.5 Fractionation of casamino acids and tryptone by ligand exchange chromatography on copper-chelex resin

The method was as described by Armstead and Ling (1991) except that a larger column (500 mm X 25 mm ID) and a larger amount of sample were used.

#### 2.2.5.1 Preparation of copper-chelex resin

Chelex-100 resin (300 g; 50-100 mesh; sodium form) was first washed with 1 M HCl and then added to 0.16 M cupric sulphate ($CuSO_4$. $5H_2O$) solution. The mixture was adjusted to pH 2.5 with 5 M NaOH and stirred for 16 h. The resultant copper-Chelex resin was washed with RO water until the supernatant was colourless and then it was adjusted to pH 9.5 with 20 M ammonium hydroxide solution.

#### 2.2.5.2 Separation of mixed amino acids from peptides

A column (500 mm X 25 mm ID) was packed with the copper-Chelex resin and washed

with 600 ml of 1 mM ammonium hydroxide solution. Casamino acids or tryptone (300 mg) were dissolved in 3.0 ml RO water and loaded onto the column. Each sample was eluted sequentially with 600 ml of 1mM and 1200 ml of 5M ammonium hydroxide solution at the rate of 1 ml/min. The effluent from the column was collected in 10.4 ml fractions. Effluent fractions were analysed spectrophotometrically at 280 nm. Column effluent fractions that corresponded to a peak of either amino acids or peptides were pooled.

To remove any residual copper, each of the pooled groups was evaporated to dryness under vacuum at 37°C in a rotary evaporator (BUCHI), dissolved in 5 ml RO water and loaded onto a column (250 mm x 10 mm I.D.) of Chelex-100 resin (20 g) which had already been equilibrated with RO water. The column was eluted with 100 ml of 10 mM ammonium hydroxide solution. The effluent was then concentrated under vacuum at 37°C in a rotary evaporator that also removed ammonia from the samples. The peptide samples were freeze dried and the resulting powder was stored at room temperature. Attempts to obtain amino acid samples as powder was unsuccessful because a sticky material was obtained which was difficult to detach from the container. The amino acid samples were therefore dissolved in RO water and the amino acid content of the resulting solution was measured as described in the following section. The amino acid solution was stored in 1 ml aliquots at -20°C.

### 2.2.6 Amino acid quantitation

The method was as described by Lee and Takahashi (1966). Samples (0.1 ml) were added to 1.9 ml of citrate buffer-ninhydrin solution-glycerol mixture that consisted of 0.2 ml of citrate buffer (section 2.2.3.8), 0.5 ml of 1.0% (w/v) ninhydrin solution (in 0.5 M citrate buffer, pH 5.5) and 1.2 ml of glycerol. The ninhydrin-citrate-glycerol mixture was prepared before each analysis. The samples were heated in a boiling water bath for 12 min, then cooled in a water bath. Within 1 h the absorbances were read at 570 nm against a reagent blank. Amino acid standards containing glycine were measured at the

same time.

### 2.2.7 Media preparation

#### 2.2.7.1 Brain Heart Infusion (BHI) Medium[a]

| *Ingredients* | per 100 ml |
|---|---|
| BHI powder | 3.70 g |
| L-Cysteine hydrochloride | 0.05 g |
| Clarified rumen fluid[b] | 20.0 ml |

*a*. The medium was brought to a volume of 100 ml with RO water, boiled, and placed in a Coy anaerobic hood (95% $CO_2$/5% $H_2$ atmosphere) for 2 h. The medium was then dispensed into Hungate tubes, sealed and autoclaved.

*b*. Rumen fluid was strained through four layers of cheese cloth and centrifuged (10,000 x g; 20 min; 4°C). Clarified rumen fluid was autoclaved and stored at 4°C until use.

#### 2.2.7.2 Rumen fluid (RF) medium

RF medium was prepared as described by Cotta and Hespell (1986a) with minor modifications (Table 2.1).

#### 2.2.7.3 Defined (NB) medium

NB medium was prepared as described in Table 2.1.

#### 2.2.7.4 Solid media

Solid media were prepared by adding 1.5% (w/v) agar to the media before autoclaving. The media were autoclaved for 15 min at 121°C and 15 psi. When the temperature of media decreased to 50°C plates were poured in a laminar floor hood and left with the lids off until the agar set. For preparation of anaerobic plates they were placed in a Coy anaerobic hood to allow the removal of $O_2$ from the solid media by diffusion.

**Table 2.1 Composition of RF and NB media**

| Components | Composition (% v/v)[a] | |
|---|---|---|
| | RF medium[b] | NB medium[c] |
| Mineral solution I | 6.0 | 6.0 |
| Mineral solution II | 6.0 | 6.0 |
| Trace elements | - | 0.5 |
| Hemin + 1,4-Naphthoquinone solution | - | 1.0 |
| Resazurin (0.1%) | 0.05 | 0.05 |
| Carbohydrate[d] | 0.5 g | 0.5 g |
| L-Cysteine -HCl | 0.05g | - |
| Sodium sulphide ($Na_2S.9\ H_2O$) solution[e] | - | 1.5 (3 mM) |
| Vitamin solution | - | 4.0 |
| Yeast extract | 0.1 g | - |
| $NH_4Cl$[f] | 0.374 g | 0.374 g (70 mM) |
| VFA mixture | - | 0.31 |
| An amino acid source | 0.15 | As required |
| Sodium carbonate 8% (w/v) | 5.0 | 5.0 |

*a.* unless otherwise indicated.

*b.* RF medium was brought to a final volume of 100 ml by adding RO water.

*c.* After addition of each component the solution was mixed well. The medium was brought to a final volume of 100 ml by adding RO water. The pH was adjusted to 9.4-9.5 using 10 M KOH.

*d.* Maltose was used in this work because it could be utilised by all of the bacteria under study and unlike glucose, was autoclavable in the medium.

*e.* Sodium sulphide solution (5% w/v) was prepared fresh weekly and stored at 4°C.

*f.* $NH_4Cl$ was dissolved in the medium as required.

### 2.2.8 Anaerobic conditions

The medium was boiled in a Schott bottle for 1 min and immediately transferred to a Coy anaerobic chamber containing a gas mixture of 95% carbon dioxide and 5% hydrogen. The medium was allowed to stand in the anaerobic chamber for 2 h with a loosened lid to facilitate the exchange of oxygen with $CO_2$. During this time the medium became anaerobic (the pink colour of resazurin disappeared), and the pH dropped to 6.7-6.8. The medium was dispensed into Hungate tubes, autoclaved at 121°C and 15 psi for 15 min, returned to the anaerobic chamber and shaken well to prevent the formation of a precipitate. All glassware and plasticware were held in the anaerobic chamber for at least two days prior to use.

### 2.2.9 Growth conditions

For anaerobic growth, batch cultures were grown in Hungate tubes (16 x 125 mm Pyrex® screw capped culture tubes, Corning Glass Works, Corning, USA) with butyl rubber septa inserted into the caps (Bellco Glass, Inc. Vineland, USA). Growth was monitored by the change in optical density at 600 nm directly in these tubes using the NOVASPEC spectrophotometer which was fitted with a special holding cage for these round tubes. Bacterial transfer from liquid to liquid was performed aseptically through the rubber septa of Hungate tubes using disposable 1 ml plastic syringes and 27 gauge hypodermic needles. This was carried out in the anaerobic chamber.

Cultures of all bacterial species were initiated from glycerol stocks held at -80°C (Figure 2.1). The cultures (10 µl) were streaked onto BHI medium (see section 2.4.2) plates and grown anaerobically at 39°C in a Coy anaerobic chamber (95% carbon dioxide and 5% hydrogen). Single colonies were inoculated into Hungate tubes containing NB medium supplemented with 70 mM $NH_4Cl$ and 0.15% casamino acids as nitrogen sources and 0.05% (w/v) cysteine as a reducing agent. The cultures were grown to $A_{600}$ of 0.6-0.8 and stored at 4°C as working stocks. A fresh bacterial culture was grown from the working stocks. This was used as an inoculum culture for nutritional

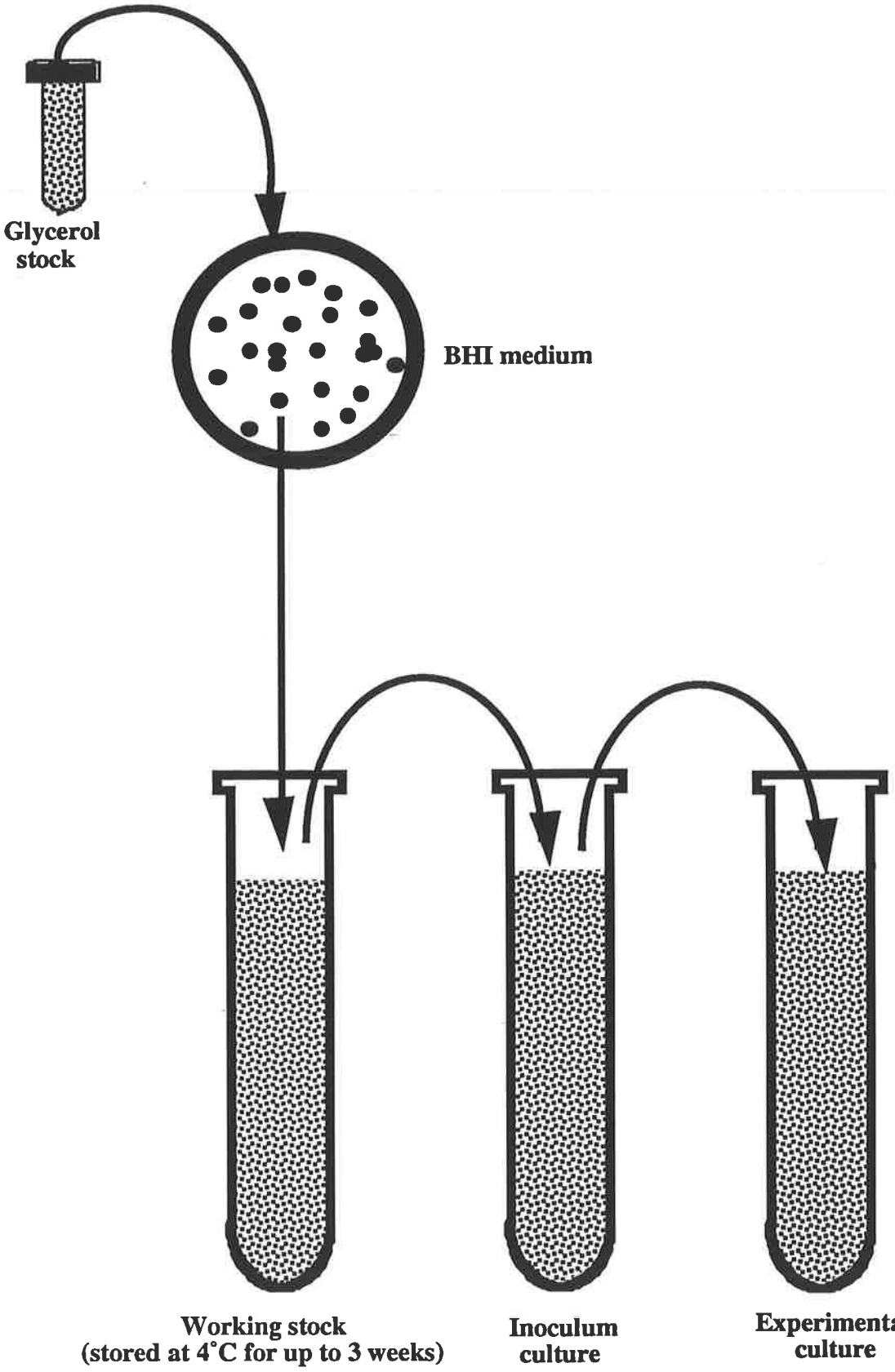



**Figure 2.1 Schematic representation of culture preparation.**

experiments. 0.1 ml from the inoculum culture was inoculated into 3x10 ml of experimental media in Hungate tubes. To ensure that there was no carry over of nitrogen from the inoculum culture, medium without any nitrogen was inoculated as a control. For growth rates, Hungate tube cultures were incubated at 39°C in a water bath situated beside a NOVASPEC spectrophotometer. Growth was monitored directly in Hungate tubes by the change in absorbance at 600 nm at time intervals shown in the figures for each strain. Maximum exposure time of cultures to temperatures less than 39°C was 5s.

## 2.3 Results

### 2.3.1 Preparation of mixed amino acids and peptides

Casamino acids (acid hydrolysate of casein) and tryptone (enzymatic digest of casein) are usually used as the source of mixed amino acid and peptides respectively. Nevertheless, these two sources are not pure because casamino acids also contain peptides and tryptone contains free amino acids (Chen and Russell, 1988).

To prepare amino acid-free peptides, gel filtration chromatography was used to fractionate tryptone according to molecular size. The results (Figure 2.2) show that amino acids were not well separated from peptides by this method. Armstead and Ling (1991) have described a chromatographic method using copper-chelex resin, capable of separating mixed amino acids from peptides in biological digests. The principle on which this method is based is that a transition metal is complexed with a cation exchange resin and the metallic ions can then shed their solvation shells to form stable complexes with amino acids. The metal-resin complex will continue to extract amines from very dilute solutions until coordinate valencies of the metal ions are saturated (Buist and O'Brien, 1967). Therefore, chromatography on copper-chelex resin was employed to prepare amino acid-free peptides and peptide-free amino acids from tryptone and casamino acids respectively. The results (Figure 2.3) show that amino

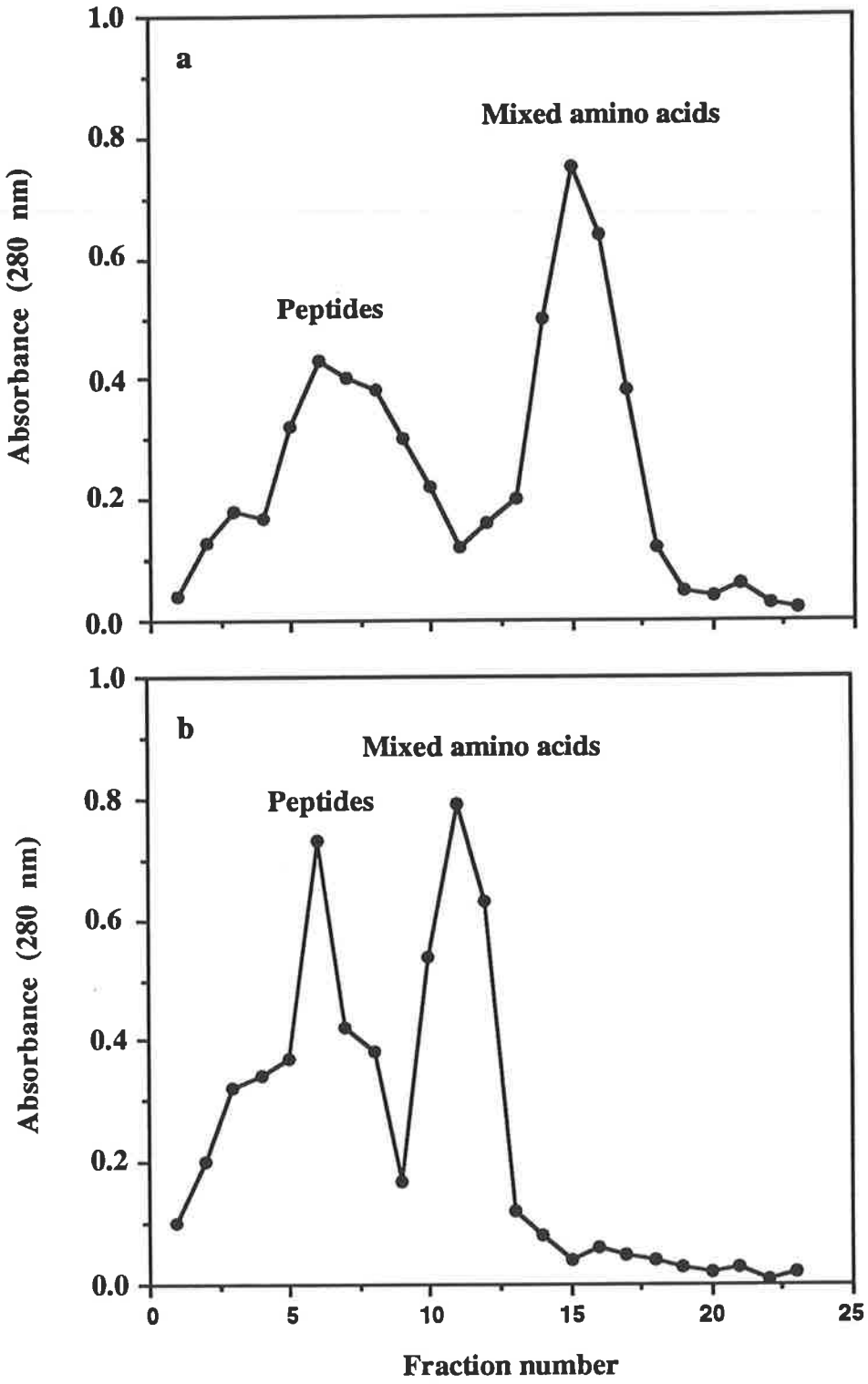



**Figure 2.2 Filtration diagram of tryptone on:**

a) Sephadex G-25 (fine)
b) Sephadex G-25 (medium)

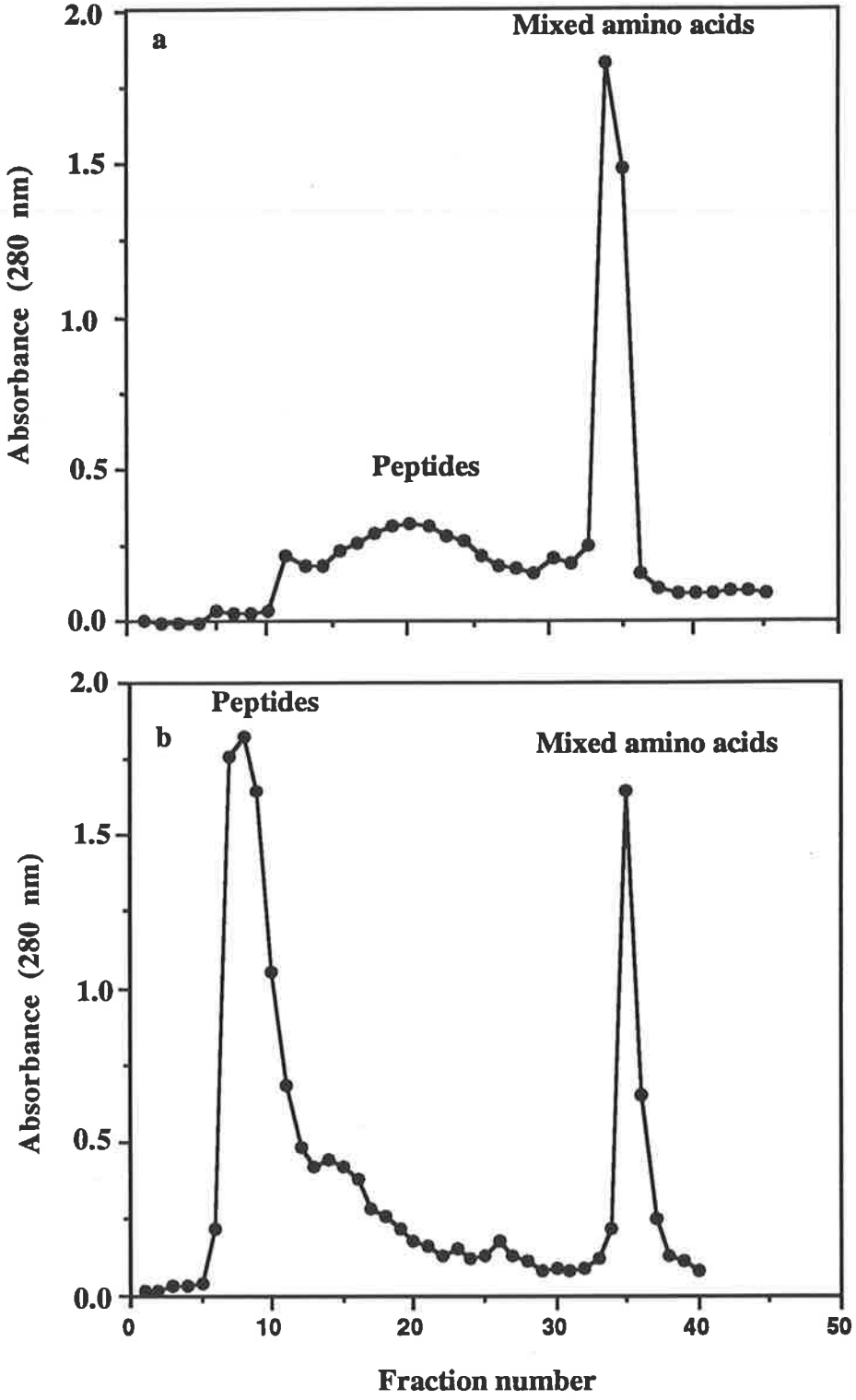



**Figure 2.3 Elution profile from a copper-chelex resin column of:**

a) Casamino acids
b) Tryptone

acids were well separated from peptides by this method. Casamino acids contained approximately 60% by weight of mixed amino acids with the remainder being peptides. The tryptone preparation was composed of 50% by weight peptides, the remainder being mixed amino acids.

### 2.3.2 Effect of increasing concentrations of $NH_4Cl$ on growth of *B. fibrisolvens* strains H17c and E14.

To determine the minimum concentration of $NH_4Cl$ required for maximum bacterial growth, NB medium containing 0.05% (w/v) cysteine as a reducing agent was tested with increasing concentrations of $NH_4Cl$ added as a sole nitrogen source. The results (Figure 2.4) show that 70 mM $NH_4Cl$ supports maximum growth of *B. fibrisolvens* strain H17c. *B. fibrisolvens* strain E14 did not grow in this medium at any concentration of $NH_4Cl$ tested (Figure 2.5). Argyle and Baldwin (1989) have previously reported that small amounts of amino acids would stimulate growth over that obtained with ammonia alone. Therefore, 0.2% (w/v) mixed amino acids or peptides were also added to the medium. This medium supported growth of strain E14 and the minimum concentration of $NH_4Cl$ for maximum bacterial growth was shown (Figure 2.5) to be 70 mM.

### 2.3.3 Effect of increasing concentrations of mixed amino acids or peptides on growth of *B. fibrisolvens* strain E14

To determine the minimum concentration of an amino acid source required for maximum growth of *B. fibrisolvens* strain E14, NB medium reduced with 0.05% (w/v) cysteine and containing 70 mM $NH_4Cl$ was tested with increasing concentrations of mixed amino acids or peptides. The results (Figure 2.6) show that a minimum concentration of 0.15% (w/v) mixed amino acids or peptides supported maximum bacterial growth.

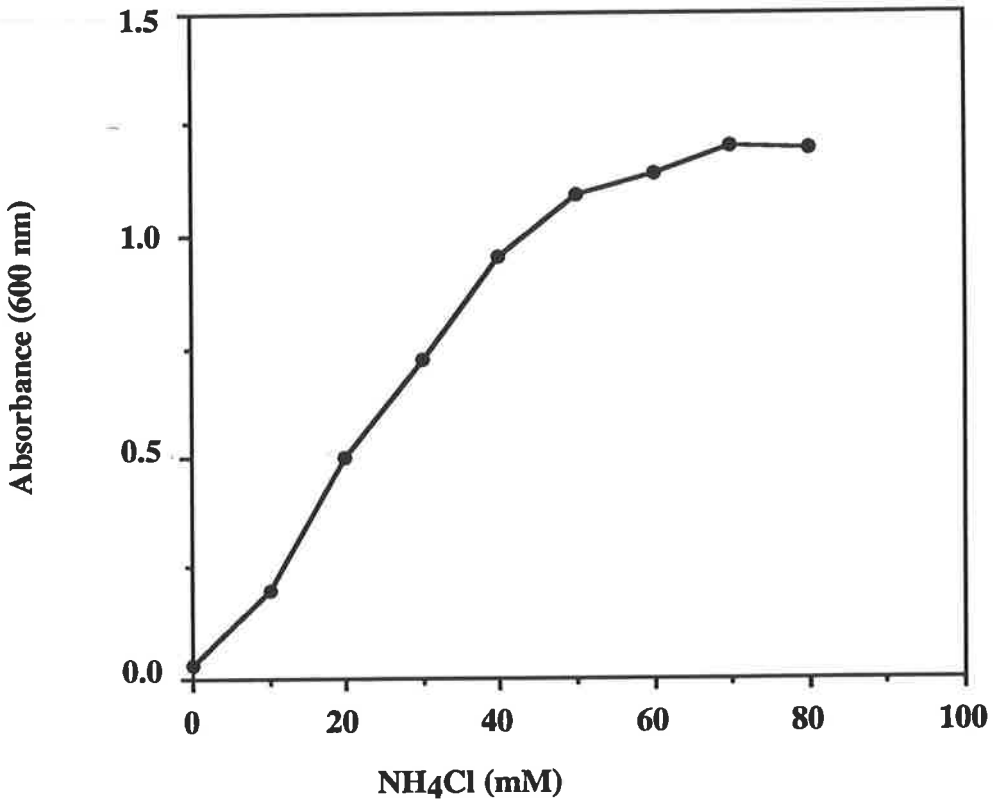



**Figure 2.4 Growth of *B. fibrisolvens* strain H17c in NB medium containing increasing concentrations of $NH_4Cl$.**

Cells were incubated anaerobically at 39°C and cell densities were measured at 28 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated medium blank. Absorbance values were the mean of triplicate assays, with variation between triplicates being less than 0.05.

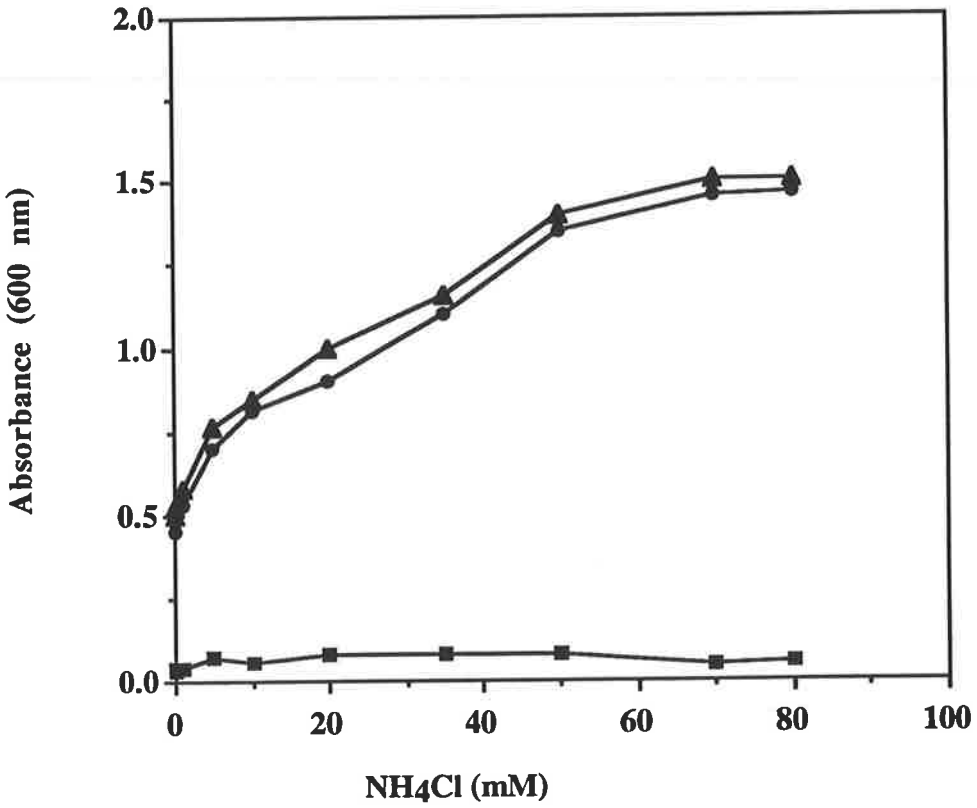



**Figure 2.5 Growth of *B. fibrisolvens* strain E14 in NB medium containing increasing concentrations of $NH_4Cl$ and supplemented with:**

—▲— peptides

—●— mixed amino acids

—■— no addition (control)

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h by a NOVASPEC spectrophotometer at 600 nm against an uninoculated medium blank. Absorbance values were the mean of triplicate assays, with variation between triplicates being less than 0.05.

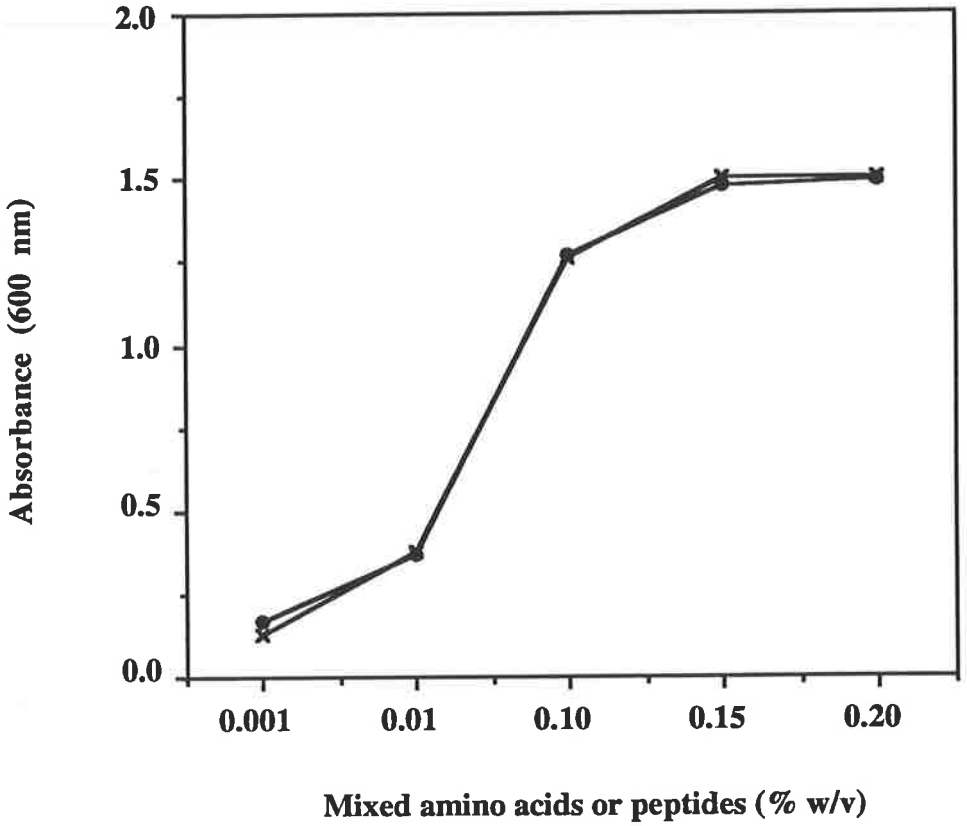



**Figure 2.6 Growth of *B. fibrisolvens* strain E14 in NB medium containing 70 mM $NH_4Cl$ and increasing concentrations of:**

—✖— mixed amino acids

—●— peptides

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated medium blank. Absorbance values were the mean of triplicate assays, with variation between triplicates being less than 0.05.

### 2.3.4 Effect of nitrogen-free reducing agents on bacterial growth in NB medium containing $NH_4Cl$ as a sole nitrogen source.

To select an effective nitrogen-free reducing agent, NB medium containing sodium sulphide, ascorbic acid or dithiothreitol was tested. The results show (Table 2.2) that strains of *Streptococcus* and *Selenomonas* would grow in the presence of 10 mM ascorbic acid or 1 mM dithiothreitol, but *Butyrivibrio*, *Prevotella* and *Bacteroides* species would not. In the presence of 1 mM sodium sulphide, all species grew on $NH_4Cl$ as a sole nitrogen source except *B. fibrisolvens* strain E14, and *P. ruminicola* strain GA33. *B. fibrisolvens* strain H17c and *B. fragilis* grew poorly in this medium but if the sodium sulphide concentration was increased to 3 mM, maximum growth was obtained. A higher concentration of sodium sulphide (4 mM) had an inhibitory effect on growth of *B. fibrisolvens* strain H17c and *B. fragilis* with $A_{600}$ of 0.8 and 1.65 respectively. A higher concentration of either ascorbic acid (30 mM) or dithiothreitol (3 mM) did not have any significant effect the bacterial growth (results are not shown). Neither *B. fibrisolvens* strain E14 nor *P. ruminicola* strain GA33 grew under any reducing condition when $NH_4Cl$ was present as the sole nitrogen source.

### 2.3.5 Utilisation of amino acids and peptides

Argyle and Baldwin (1989) have previously reported that small amounts of amino acids would stimulate bacterial growth over that obtained with ammonia alone. Mixed amino acids or peptides (0.15% w/v) were therefore added to NB medium containing $NH_4Cl$. This medium supported growth of all of the bacteria tested in the presence of either 1 mM or 3 mM sodium sulphide as a reducing agent (Table 2.2). To examine whether amino acids or peptides can act as sole nitrogen sources, growth of *P. ruminicola, B. fibrisolvens, S. ruminantium* and *S. bovis* was examined in NB medium containing 0.15% (w/v) mixed amino acids or peptides in the absence of $NH_4Cl$. The results (Figures 2.7 and 2.8) demonstrate that when the medium contained amino acids or peptides alone, all bacterial species grew, although to a lesser extent than shown in Table 2.2 when $NH_4Cl$ was also present. When the concentration of mixed amino

**Table 2.2 Effect of nitrogen-free reducing agents on bacterial growth[a] in NB medium containing $NH_4Cl$± 0.15% (w/v) mixed amino acids**

| | Ascorbic acid (10 mM) | | Dithiothreitol (1 mM) | | Sodium sulphide (mM) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | 1 | | 3 | |
| Bacteria | - aa[b] | + aa | - aa | + aa | - aa | + aa | - aa | + aa |
| *S. bovis* 2B | 1.14 | 1.20 | 1.11 | 1.18 | 1.25 | 1.30 | 1.40 | 1.42 |
| *S. bovis* H24 | 0.91 | 0.95 | 0.90 | 1.00 | 1.10 | 1.15 | 1.32 | 1.31 |
| *S. ruminantium* S23 | 1.25 | 1.22 | 1.28 | 1.23 | 1.21 | 1.21 | 1.23 | 1.24 |
| *P. ruminicola* GA33 | 0.08 | 0.45 | 0.07 | 0.48 | 0.10 | 0.82 | 0.06 | 0.85 |
| *P. ruminicola* P1 | 0.10 | 0.21 | 0.10 | 0.22 | 1.99 | 1.65 | 1.99 | 1.74 |
| *B. fibrisolvens* E14 | 0.16 | 0.20 | 0.16 | 0.22 | 0.16 | 1.45 | 0.16 | 1.46 |
| *B. fibrisolvens* H17c | 0.15 | 0.24 | 0.15 | 0.22 | 0.45 | 1.20 | 1.2 | 1.18 |
| *B. fragilis* | 0.14 | 0.30 | 0.14 | 0.33 | 1.30 | 1.99 | 1.99 | 1.99 |

*a.* Absorbance ($A_{600}$) values are the mean of duplicate assays, with variation between duplicates being less than 0.05.

*b.* Mixed amino acids

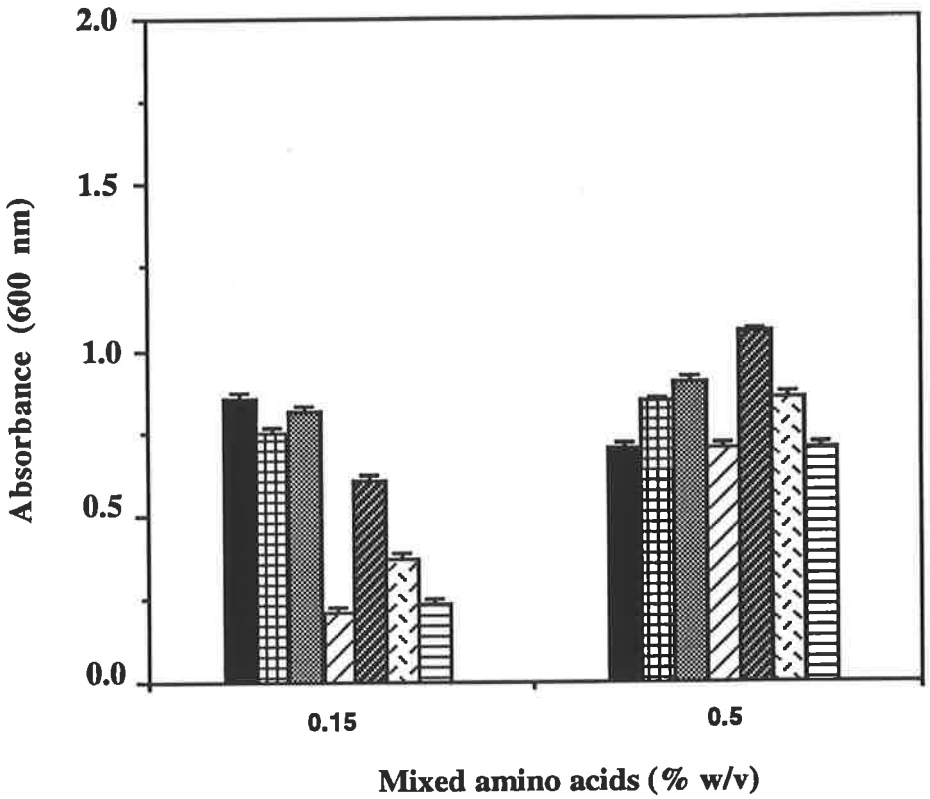



**Figure 2.7 Bacterial growth in NB medium containing mixed amino acids as sole nitrogen sources.**

- *S. bovis* strain 2B
- *S. bovis* strain H24
- *S. ruminantium* strain S23
- *P. ruminicola* strain GA33
- *P. ruminicola* strain P1
- *B. fibrisolvens* strain E14
- *B. fibrisolvens* strain H17c

Cells were incubated anaerobically at 39°C to achieve a maximum absorbance which was measured using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. The initial absorbances were < 0.02. Absorbance values are the mean of triplicate assays. Bars represent the standard error of the means.

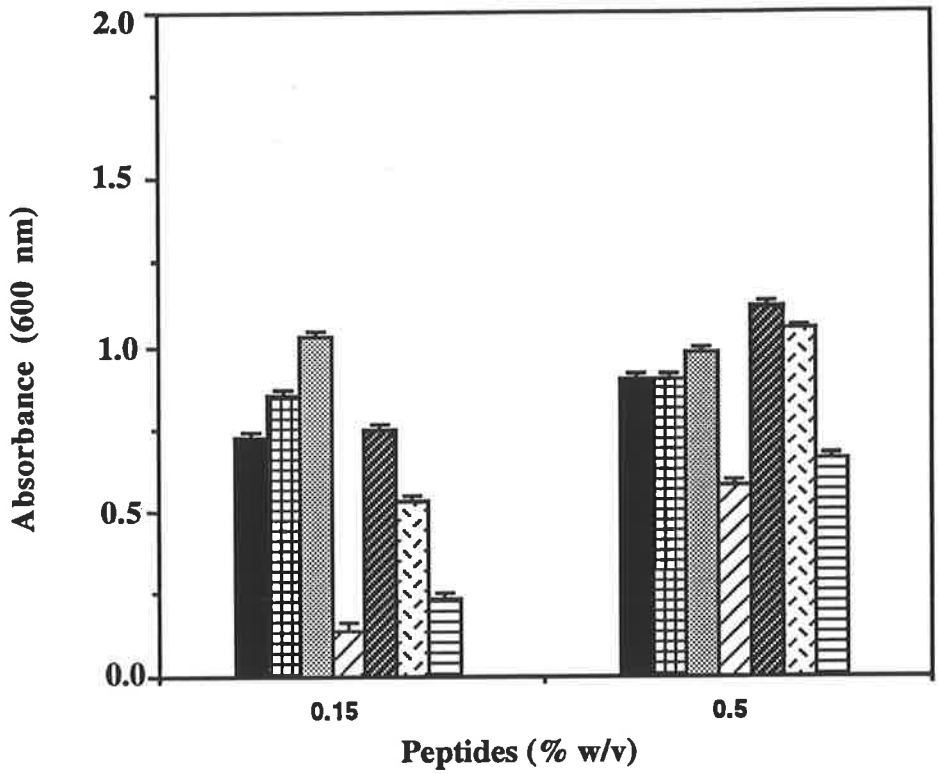



**Figure 2.8 Bacterial growth in NB medium containing peptides as sole nitrogen sources.**

- *S. bovis* strain 2B
- *S. bovis* strain H24
- *S. ruminantium* strain S23
- *P. ruminicola* strain GA33
- *P. ruminicola* strain P1
- *B. fibrisolvens* strain E14
- *B. fibrisolvens* strain H17c

Cells were incubated anaerobically at 39°C to achieve a maximum absorbance which was measured using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. The initial absorbances were < 0.02. Absorbance values are the mean of triplicate assays. Bars represent the standard error of the means.

acids or peptides was increased to 0.5% (w/v), increased growth was obtained for *P. ruminicola and B. fibrisolvens* although maximum growth achieved was still less than that shown in Table 2.2 when $NH_4Cl$ was also present. A higher concentration (0.5% w/v) of mixed amino acids or peptides had no significant effect on the growth of *S. ruminantium* and *S. bovis* (Figures 2.7 and 2.8).

### 2.3.6 Comparison of growth of *B. fibrisolvens* strains H17c and E14 in NB or RF medium.

Growth of *B. fibrisolvens* strains H17c and E14 was tested in RF and NB media reduced with 3 mM sodium sulphide in the presence and absence of an exogenous nitrogen source. The growth rate was similar in both media, although the lag period before exponential growth was several hours longer in NB medium. In the absence of a nitrogen source, no growth was obtained in NB medium, whereas rumen fluid medium continued to support growth (Figures 2.9 and 2.10).

### 2.3.7 Growth rates

To compare bacterial growth on different nitrogen sources, the growth rates of *B. fibrisolvens, P. ruminicola, S. bovis* and *S. ruminantium* were monitored in NB medium containing $NH_4Cl$ (70 mM), mixed amino acids (0.15% w/v), peptides (0.15% w/v) or a mixture of $NH_4Cl$ (70 mM) and mixed amino acids (0.15% w/v). *B. fibrisolvens* strain H17c attained an absorbance maximum of 1.2 at 28 h with $NH_4Cl$ either in the presence or absence of mixed amino acids. With mixed amino acids or peptides alone, the absorbance maximum attained was only 17% of that attained with $NH_4Cl$ as the sole nitrogen source (Figure 2.11). For *B. fibrisolvens* strain E14 no growth was observed with $NH_4Cl$ alone but an absorbance maximum of 1.5 was attained at 17 h with $NH_4Cl$ plus mixed amino acids . When mixed amino acids or peptides alone were used, the absorbance maximum was only 25-33% of that of when $NH_4Cl$ was also present (Figure 2.12).

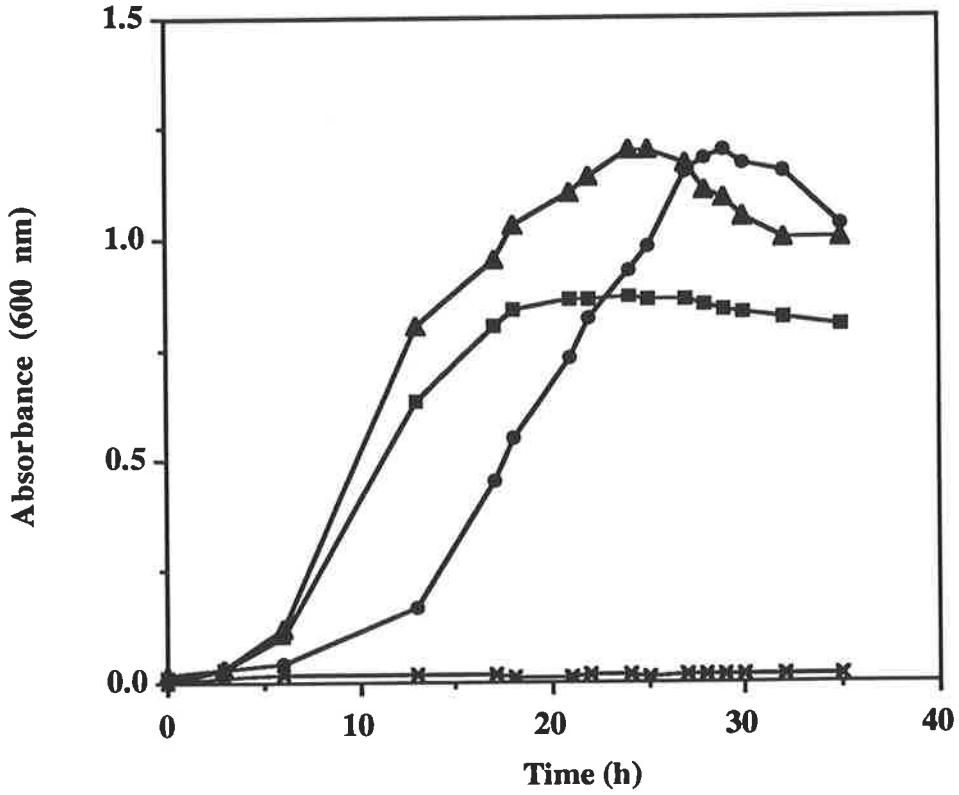



**Figure 2.9 Growth of *B. fibrisolvens* strain H17c in:**

—▲— RF medium containing 70 mM $NH_4Cl$

—■— RF medium without an exogenous nitrogen source

—●— NB medium containing 70 mM $NH_4Cl$

—✕— NB medium without an exogenous nitrogen source

Cells were incubated anaerobically at 39°C and cell densities were measured every 30 min using a NOVASPEC spectrophotometer at 600 nm against a medium blank. Absorbance values were the mean of triplicate assays, with variation between triplicates being less than 0.05.

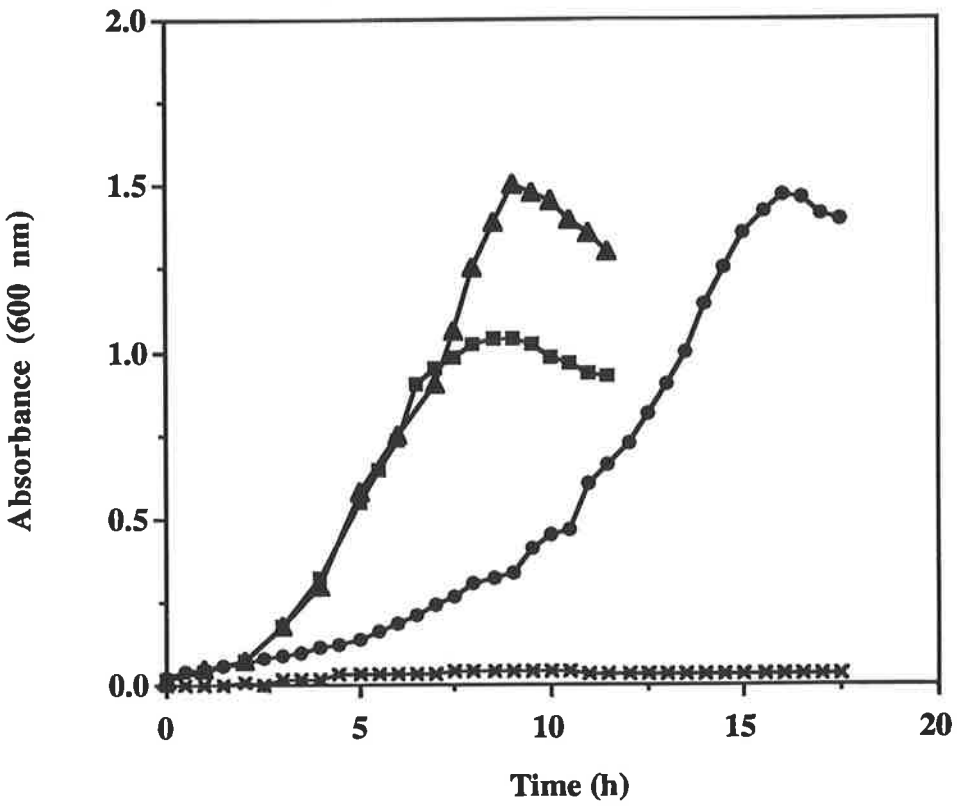



**Figure 2.10 Growth of *B. fibrisolvens* strain E14 in:**

- RF medium containing 70 mM $NH_4Cl$ plus 0.15% (w/v) mixed amino acids
- RF medium without an exogenous nitrogen source
- NB medium containing 70 mM $NH_4Cl$ plus 0.15% (w/v) mixed amino acids
- NB medium without an exogenous nitrogen source

Cells were incubated anaerobically at 39°C and cell densities were measured every 30 min using a NOVASPEC spectrophotometer at 600 nm against an uninoculated medium blank. Absorbance values were the mean of triplicate assays, with variation between triplicates being less than 0.05.

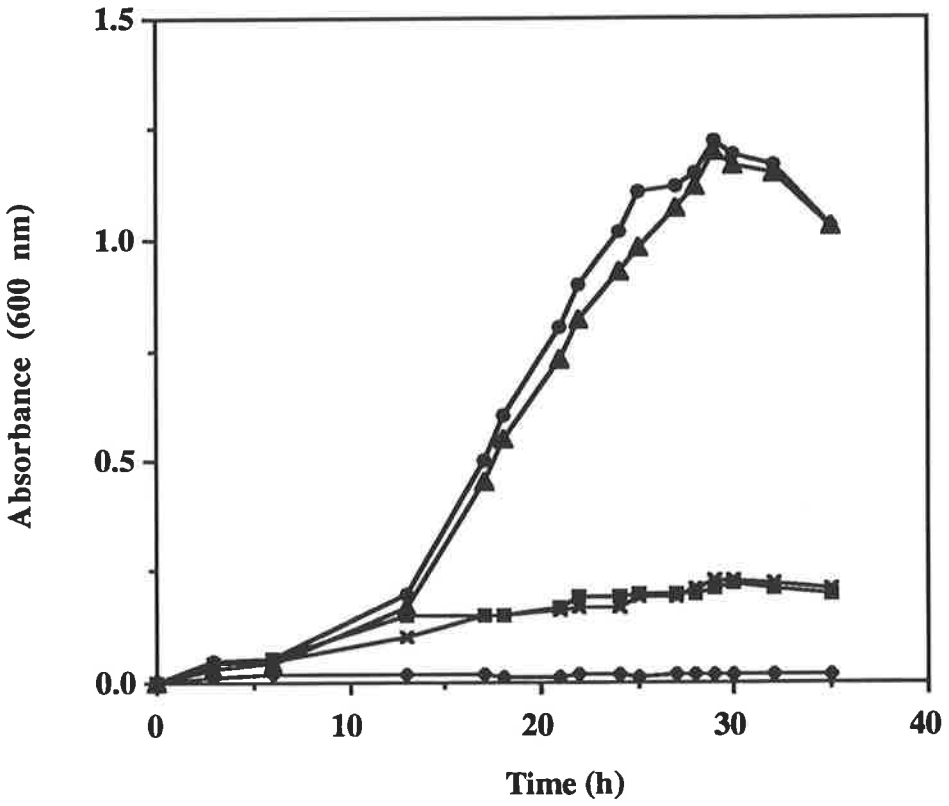



**Figure 2.11 Growth of *B. fibrisolvens* strain H17c in NB medium containing:**

—●— $NH_4Cl$ (70 mM) plus mixed amino acids (0.15% w/v)

—▲— $NH_4Cl$ (70 mM) alone

—■— mixed amino acids (0.15% w/v) alone

—✖— peptides (0.15% w/v) alone

—●— no addition (control)

Cell densities were measured using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values are the mean of triplicate assays, with variation between triplicates being less than 0.05.

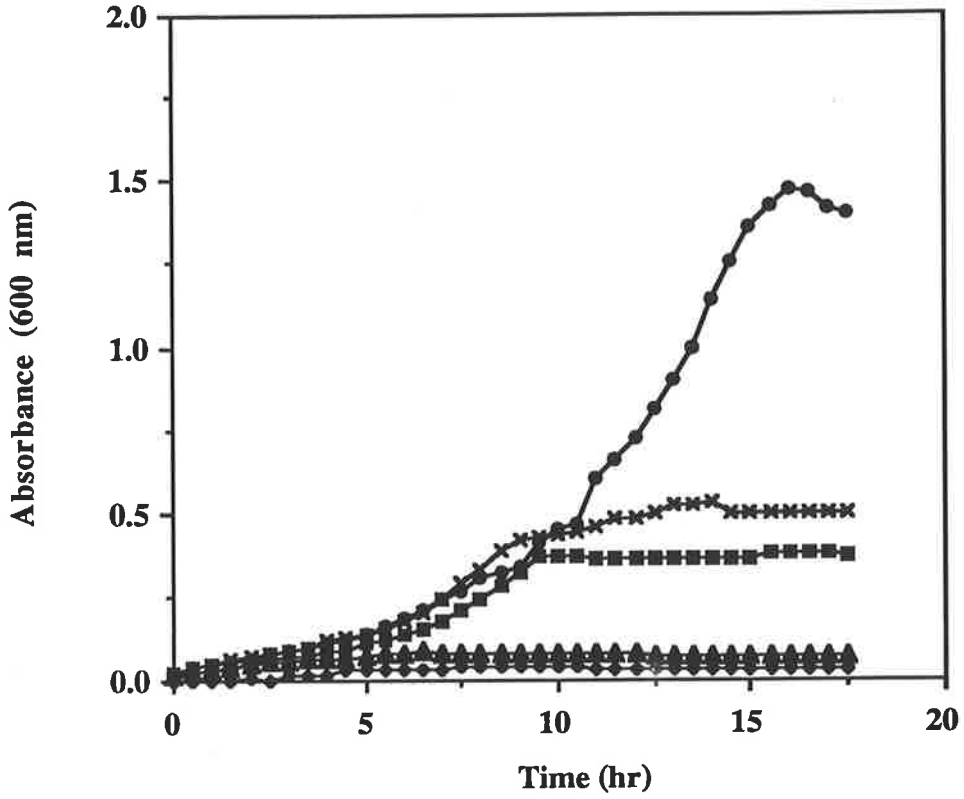



**Figure 2.12 Growth of *B. fibrisolvens* strain E14 in NB medium containing:**

- $NH_4Cl$ (70 mM) plus mixed amino acids (0.15% w/v)
- $NH_4Cl$ (70 mM) alone
- mixed amino acids (0.15 % w/v) alone
- peptides (0.15% w/v) alone
- no addition (control)

Cell densities were measured using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values are the mean of triplicate assays, with variation between triplicates being less than 0.05.

*P. ruminicola* strain P1 attained an absorbance maximum of 1.99 at 24 h with either $NH_4Cl$ alone or $NH_4Cl$ plus mixed amino acids (Figure 2.13). With mixed amino acids or peptides alone, the absorbance maximum was only 28-38% of that of $NH_4Cl$ (Figure 2.13). For *P. ruminicola* strain GA33 no growth was observed with $NH_4Cl$ alone but an absorbance maximum of 0.9 was attained at 12 h with $NH_4Cl$ plus mixed amino acids as nitrogen sources (Figure 2.14). When mixed amino acids or peptides alone were used, the absorbance maximum was only 26-23% of that of when $NH_4Cl$ was also present (Figure 2.14).

*S. bovis* strains 2B and H24 attained absorbance maxima of 1.2 and 1.4 respectively at 8 h with either $NH_4Cl$ alone or $NH_4Cl$ plus mixed amino acids. With mixed amino acids or peptides alone, the absorbance maximum was 63-73% of that of $NH_4Cl$ (Figures 2.15 and 2.16).

*S. ruminantium* strain S23 attained an absorbance maximum of 1.3 at 10 h with either $NH_4Cl$ alone or $NH_4Cl$ plus mixed amino acids. With mixed amino acids or peptides alone, the absorbance maximum was 61-77% of that of $NH_4Cl$ (Figures 2.17).

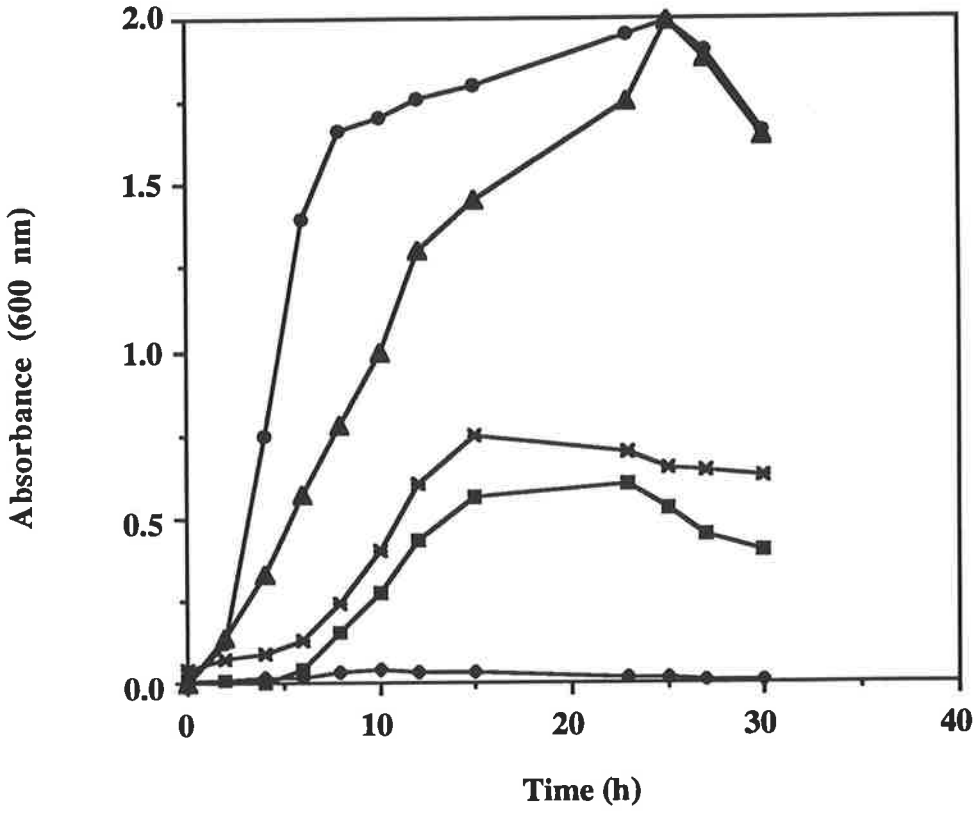



**Figure 2.13 Growth of *P. ruminicola* strain P1 in NB medium containing:**

—●— $NH_4Cl$ (70 mM) plus mixed amino acids (0.15% w/v)

—▲— $NH_4Cl$ (70 mM) alone

—■— mixed amino acids (0.15 % w/v) alone

—✖— peptides (0.15% w/v) alone

—●— no addition (control)

Cell densities were measured using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values are the mean of triplicate assays, with variation between triplicates being less than 0.05.

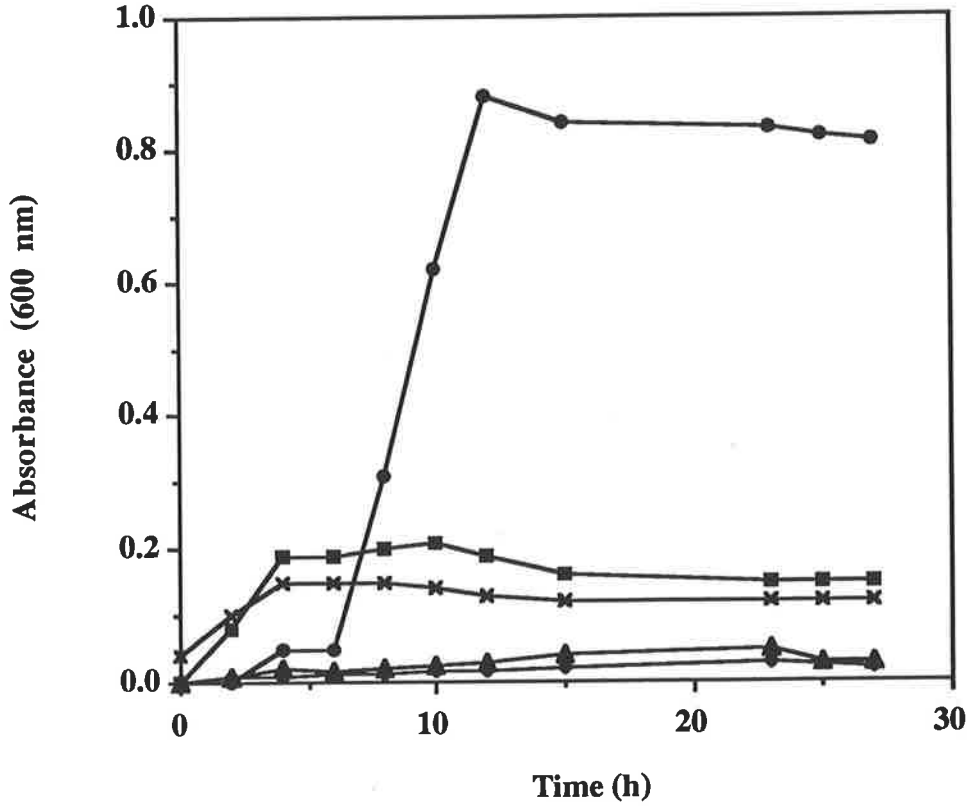



**Figure 2.14 Growth of *P. ruminicola* strain GA33 in NB medium containing:**

- $NH_4Cl$ (70 mM) plus mixed amino acids (0.15% w/v)
- $NH_4Cl$ (70 mM) alone
- mixed amino acids (0.15 % w/v) alone
- peptides (0.15% w/v) alone
- no addition (control)

Cell densities were measured using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values are the mean of triplicate assays, with variation between triplicates being less than 0.05.

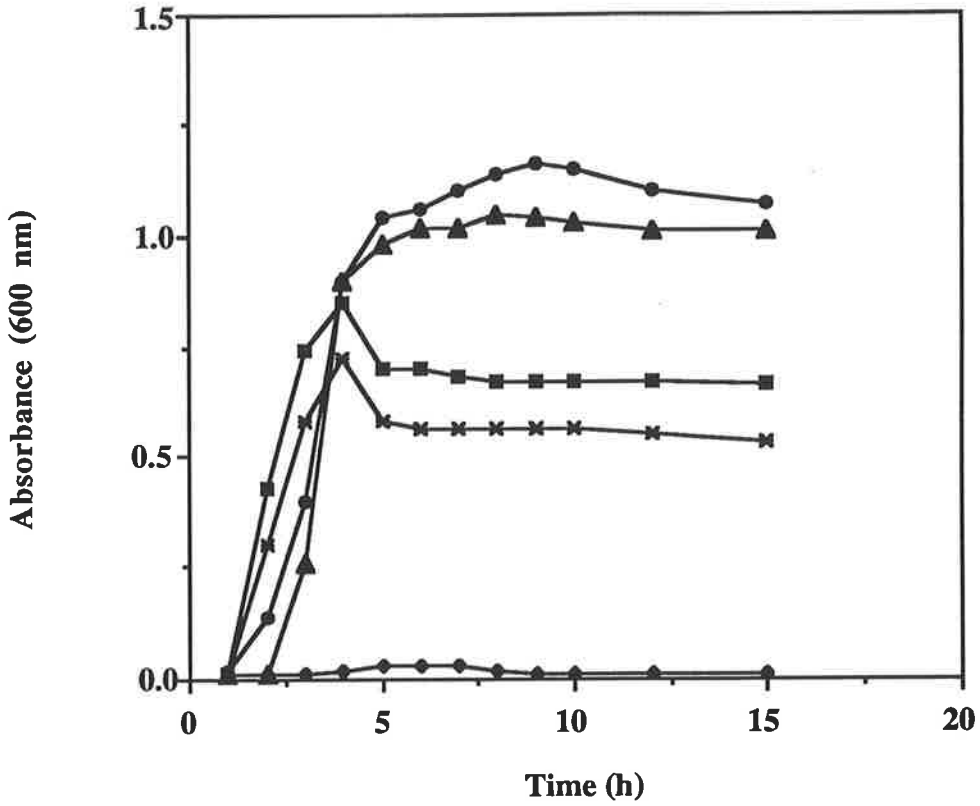



**Figure 2.15 Growth of *S. bovis* strain 2B in NB medium containing:**

—●— NH4Cl (70 mM) plus mixed amino acids 0.15% w/v)

—▲— $NH_4Cl$ (70 mM) alone

—■— mixed amino acids (0.15% w/v) alone

—✖— peptides (0.15% w/v) alone

—●— no addition (control)

Cell densities were measured using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values are the mean of triplicate assays, with variation between triplicates being less than 0.05.

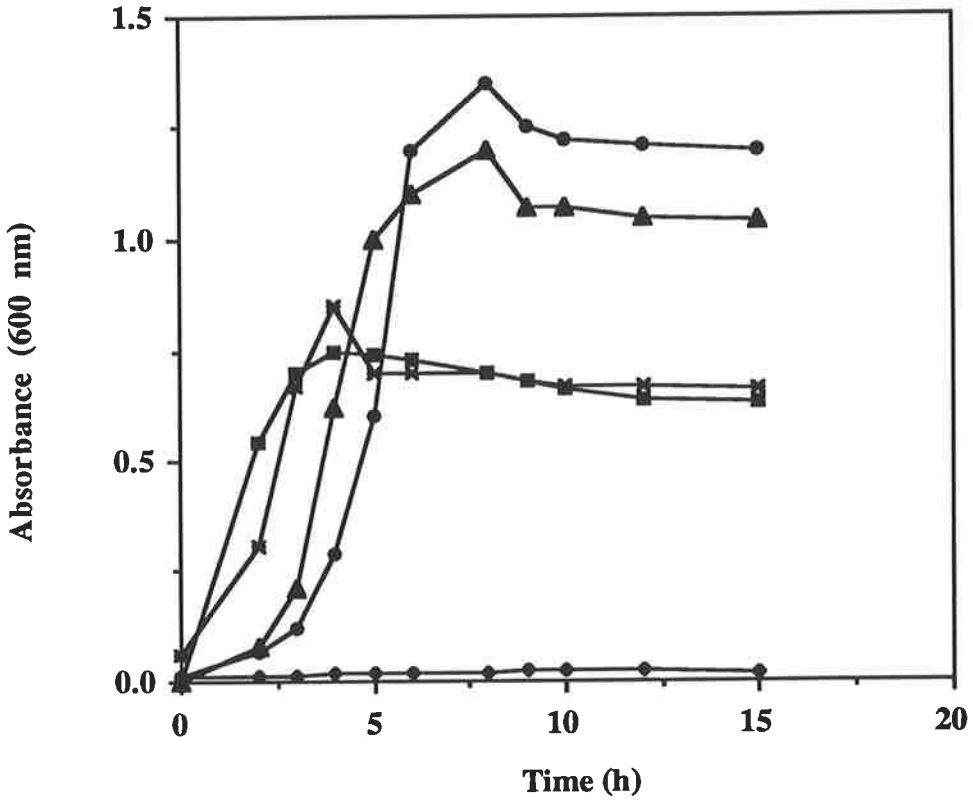



**Figure 2.16 Growth of *S. bovis* strain H24 in NB medium containing:**

—●— NH4Cl (70 mM) plus mixed amino acids 0.15% w/v)

—▲— $NH_4Cl$ (70 mM) alone

—■— mixed amino acids (0.15% w/v) alone

—✖— peptides (0.15% w/v) alone

—●— no addition (control)

Cell densities were measured using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values are the mean of triplicate assays, with variation between triplicates being less than 0.05.

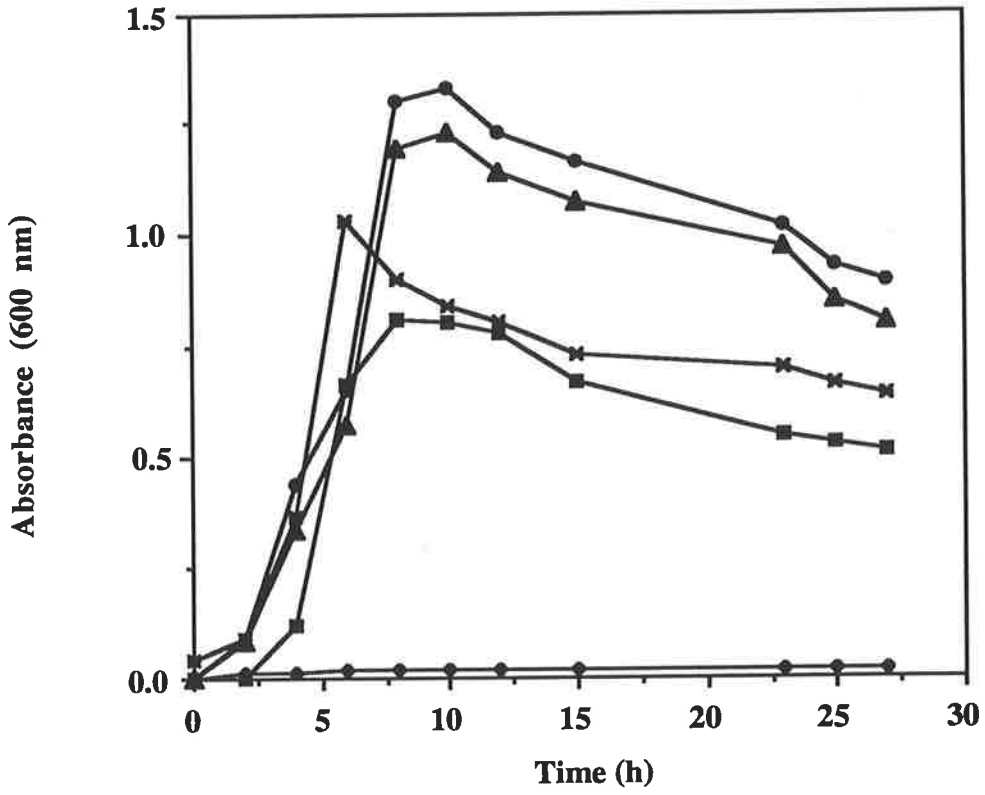



**Figure 2.17 Growth of *S. ruminantium* strain S23 in NB medium containing:**

—●— NH4Cl (70 mM) plus mixed amino acids 0.15% w/v)

—▲— $NH_4Cl$ (70 mM) alone

—■— mixed amino acids (0.15% w/v) alone

—✖— peptides (0.15% w/v) alone

—●— no addition (control)

Cell densities were measured using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values are the mean of triplicate assays, with variation between triplicates being less than 0.05.

## 2.4 Discussion

My goal was to prepare a defined medium that would be physiologically optimal for rumen bacteria and experimentally useful for studying bacterial nitrogen utilisation and metabolism. Specifically a medium with the following characteristics was desired: (1) the concentration of each of the major nutrients should be independently adjustable; (2) should be nitrogen-free and completely anaerobic; (3) the medium should support growth up to cell densities that are useful for biochemical measurements; (4) the medium should permit absorbance measurements; (5) should give reproducible growth rates.

NB medium described here meets all these specifications. It is carbohydrate-free and nitrogen-free and can be used to define nitrogen or fermentable carbon sources that will support bacterial growth. The medium will support bacterial growth to a cell density comparable to that obtained in rumen fluid medium (Figure 2.9 and 2.10). In the absence of an exogenous nitrogen source no growth was obtained in this defined (NB) medium whereas significant growth still occurred in rumen fluid medium. Although the medium was developed for studying the nitrogen requirements of rumen bacteria, it can also be used for studying other nutrient requirements as they are easily adjustable in the medium. Nevertheless, a disadvantage of NB medium is that it is not suitable for determining of sulphur requirements of rumen bacteria. Ascorbic acid, a non-sulphur reducing agent, was shown to be an ineffective reducing agent. Brock and O'Dea (1977) proposed the use of amorphous ferrous sulphide as a reducing agent where soluble sulphide is not desirable. Jones and Pickard (1980) used titanium (III) citrate as a non-sulphur reducing agent. More work is required to examine these reducing agents in NB medium.

$(NH_4)_2SO_4$ is usually included in the mineral solutions to supply media with nitrogen and sulphur sources (Caldwell and Bryant, 1966). Since the aim of developing a

defined medium in this study was to determine the nitrogen requirements of rumen bacteria, it was necessary to omit any source of nitrogen from mineral solutions. Therefore, $Na_2SO_4$ was added to mineral solution II in place of $(NH_4)_2SO_4$ to supply the medium with sulphur. $NH_4Cl$ was used as a source of nitrogen. Although ammonia has been recognised as a major nitrogen source for rumen bacteria (Bryant and Robinson, 1961; Allison *et al.*, 1962; Bryant and Robinson, 1963), there is considerable disagreement concerning the optimal concentration. Satter and Slyter (1974) indicated that approximately 3.5 mM ammonia was needed for optimal microbial protein production in continuous fermentors charged with rumen contents. Mehrez *et al.* (1977) reported that the minimum ammonia concentration for a maximal rate of rumen fermentation was about 14 mM. Wallace (1979) found that an increased ruminal ammonia concentration (from 6.1 to 13.4 mM) increased the size of the microbial flora and its hydrolytic activity. He therefore concluded that higher ammonia concentrations may be required for effective ammonia assimilation. In contrast, Schaefer *et al.* (1980) reported that pure cultures of rumen bacteria had affinity constants ranging from 6 to 50 μM for ammonia. However, mean $NH_4^+$ concentrations ranges from 2 to 40 mM of rumen fluid and increases as the CP (crude protein) in the ration dry matter increases (Satter and Slyter, 1974; Roffler and Satter, 1975). The results of the present study show that 70 mM $NH_4Cl$ supports maximum bacterial growth. It is not clear whether this concentration of $NH_4Cl$ is utilised by cells or it has an indirect effect on bacterial growth. For example higher concentrations of $NH_4^+$ may be required for full expression of the enzymes of ammonia assimilation. There is evidence that glutamate dehydrogenase, the major enzyme of ammonia assimilation generally operates at high ammonia concentrations (Tyler, 1978; Ertan, 1992b). It is also possible that a higher level of $NH_4^+$ increases the buffering capacity of the medium. These possibilities can be investigated by experiments using $^{15}NH_4^+$ and assaying enzymes of ammonia assimilation in the presence of differing concentrations of $NH_4Cl$.

An important factor which has a significant effect on bacterial growth is pH. Caldwell and Bryant (1966), adjusted the pH of their media to 6.5 before gassing and autoclaving. However, gassing with $CO_2$ decreases the pH of media, possibly resulting in reduced bacterial growth. In the present work the pH of the medium was adjusted to 9.4-9.5 before boiling and transferring to the anaerobic chamber. When the medium became saturated with $CO_2$ the pH dropped to 6.7-6.8 which is in the optimum range for rumen bacteria. This pH was maintained during bacterial growth by the buffer systems, $H_2PO_4^-/HPO_4^{-2}$ and $HCO_3^-/CO_3^{-2}$ present in NB medium.

To maintain anaerobic conditions, 3 mM sodium sulphide was found to be effective for the bacteria tested (Table 2.2). This result indicates that with 3 mM sodium sulphide a low redox potential is achieved which is sufficient to support growth of strictly anaerobic bacteria. With a higher concentration (4 mM) of sodium sulphide growth of *B. fibrisolvens* strain H17c and *B. fragilis* decreased. This may have been due to a possible toxic effect of higher concentrations of sodium sulphide as suggested by Brock and O'Dea (1977). Ascorbic acid and dithiothreitol only supported growth of the facultative bacteria tested, suggesting that those reducing agents did not efficiently reduce the medium. This is in agreement with previous studies that ascorbic acid and dithiothreitol did not support growth of *P. ruminicola* strains 23 and GA33 (Pittman and Bryant, 1964; Jones and Pickard, 1980).

Earlier anaerobic techniques (Bryant, 1972) involved boiling media and then gassing with $O_2$-free $CO_2$. $O_2$-free $CO_2$ was prepared by passing $CO_2$ through a column of reduced copper heated at 350°C. I have attempted to use this technique for preparation of NB medium but a completely anaerobic liquid medium was not obtained. This may have been due to the fact that gassing was performed in an aerobic atmosphere and, in the absence of a powerful reducing agent such as cysteine, the medium may not become completely anaerobic. Procedures described in this work show the preparation of completely anaerobic liquid media without the use of a strong reducing agent such as

cysteine. In addition, the process is quite convenient and simple as extra equipment such as a gassing system and copper column are not required.

The results reported in the present chapter show that NB medium will support growth of *S. bovis* strains 2B and H24, *S. ruminantium* strain S23, *P. ruminicola* strains GA33 and P1 and *B. fibrisolvens* strains E14 and H17c. The medium will also support growth of the non-ruminal bacterium *B. fragilis*. *B. fibrisolvens* strain H17c, *S. bovis* strains 2B and H24, *P. ruminicola* strain P1 and *S. ruminantium* strain S23 had the least complex nitrogen requirements of the bacteria tested as they were able to grow on $NH_4Cl$ as the sole nitrogen source (Table 2.2). This suggests that these bacteria are able to synthesise cellular nitrogen compounds from ammonia. However, the present data are in conflict with the report by Strydom *et al.* (1986) that *B. fibrisolvens* strain H17c could not grow in a minimal medium containing $(NH_4)_2SO_4$ as a sole nitrogen source. This latter result could have been due to inadequate levels of sodium sulphide reducing agent and therefore an incomplete anaerobic environment since I have shown here that the amino acid requirement in this strain is relaxed by increasing the sodium sulphide concentration (Table 2.2).

*B. fibrisolvens* strain E14 and *P. ruminicola* strain GA33 showed an absolute requirement for amino acids or peptides for growth (Table 2.2). $NH_4Cl$ was still required because in its absence, a maximum $A_{600}$ was not achieved even when the total concentration of amino acids was increased to 0.5% (w/v). These results confirm the report by Bryant and Robinson (1962) that growth of *P. ruminicola* strain GA33 was stimulated by casein hydrolysate. However, the requirement for amino acids or peptides by *B. fibrisolvens* strain E14 has not been previously reported. MacLeod and Murray (1956) and Dehority *et al.* (1957; 1958) suggest that amino acids are stimulatory on ruminal bacterial growth because they are converted to VFA which enhance the growth of many ruminal bacteria. This however may not be the reason for the promotion of growth of *B. fibrisolvens* strain E14 and *P.ruminicola* strain GA33 by

amino acids or peptides since NB medium contains adequate levels of VFA. Further studies on the biochemical basis for these growth limitations are reported in the following chapters.

It has been previously reported that amino acids are not able to serve as the sole nitrogen source for growth of *P. ruminicola* (Pittman and Bryant, 1964), *Bacteroides fragilis* spp. *fragilis* (Varel and Bryant, 1974) and *Bacteroides amylophilus* (Hullah and Blackburn, 1971). Similarly, the data reported here indicate that none of the bacterial strains tested were able to utilise amino acids and peptides as sole nitrogen sources. The bacteria grew only to a limited extent compared with growth in medium containing $NH_4Cl$ (Figures 2.11 to 2.17) even when the amino acid or peptide concentration was increased to 0.5% w/v (Figures 2.7 and 2.8). It is well known that amino acids can be deaminated by some ruminal bacteria, yielding VFA and ammonia (Nolan and Leng, 1972; Wright, 1967; Wallace and Cotta, 1988). Therefore, it is possible that in these experiments, amino acids and peptides are not utilised as such, but deaminated and ammonia is utilised by the cells. However, since it is shown here that addition of 70 mM $NH_4Cl$ promotes bacterial growth (Figures 2.11 to 2.17), it is likely that this requirement cannot be adequately met by deamination of 0.5% (w/v) amino acids which is equivalent to approximately 4.5 mM ammonia nitrogen. This is supported by the results shown in Figures 2.4 and 2.5 that low levels of $NH_4Cl$ were not sufficient to support maximum growth of *B. fibrisolvens* strains E14 and H17c. It is also conceivable that the bacteria utilise amino acids as such, but maximum growth is not achieved due to depletion of a particular amino acid(s). Another possibility is that the presence of $NH_4Cl$ in the medium is required for amino acid uptake. Stevenson (1979) has reported that omission of $(NH_4)_2SO_4$ from the culture medium not only resulted in a slowing, and finally cessation of growth, but also an inhibition of amino acid uptake. Jahns (1994) also found that $NH_4^+$ stimulated glutamine uptake in *Bacillus pasteurii*. However, the results shown in Figures. 2.11 to 2.17 suggest that ammonia is not an absolute requirement for amino acid uptake but may be stimulatory for these bacteria.

When the amino acid or peptide concentration was increased to 0.5% (w/v), growth of *P. ruminicola* and *B. fibrisolvens* strains was increased, but a higher concentration of amino acids or peptides had no significant effect on growth of *S. bovis* and *S. ruminantium* strains. A possible explanation for these growth differences is that the latter may be more sensitive to possible inhibitory effect(s) of higher levels of VFA which may have accumulated in the medium as a result of deamination and degradation of high concentrations of amino acids or peptides. It has been previously reported that increasing amounts of VFA, especially acetate, inhibited amino acid uptake and bacterial growth (Stewart, 1975; Stevenson, 1979). Stevenson (1979) suggested that acetate may act either directly on the membrane and on amino acid transport processes, or through a general effect on the growth of the cell.

The present results together with previous studies (Phillipson *et al.*, 1959; Bryant and Robinson, 1961; Al-Rabbat and Baldwin, 1971; and Satter and Slyter, 1974) suggest that ammonia is the main nitrogen source for rumen bacteria and that organisms are able to synthesise amino acids from ammonia even when exogenous amino acids are present in the growth medium. However, amino acids, when available, may also be utilised by bacteria for immediate use in protein biosynthesis. The ability to utilise ammonia as the main nitrogen source appears to be an advantage for rumen microorganisms, since the rumen almost always contains relatively large amounts of ammonia (Blackburn, 1965; Bryant, 1974) and low levels of amino acids (Wright and Hungate, 1967). However, although ammonia is the main nitrogen source for ruminal bacteria, there are variations in ruminal bacterial nitrogen requirements (Bryant and Robinson, 1961, 1962, 1963; Hungate, 1966; Russell and Hespell, 1981). The absolute requirement for amino acids or peptides by *B. fibrisolvens* strain E14 and *P. ruminicola* strain GA33 are examples of these differences.

# CHAPTER 3

# CHAPTER 3

## Nitrogen Utilisation in *Prevotella ruminicola* Strain GA33

### 3.1 Introduction

In the previous chapter I have reported that *Prevotella ruminicola* strain GA33 is unable to grow on $NH_4Cl$ unless mixed amino acids or peptides are added to the medium. Strain GA33, the type strain for *P. ruminicola* subsp. *brevis*, was isolated from the bovine rumen by Bryant *et* al. (1958) and characterised by Bryant and Robinson (1962). Since the isolation of this strain it has been shown that casein hydrolysate was required for its growth on ammonia (Bryant *et al.*, 1958) but no further work had been conducted to study this requirement. It is therefore important to study this limitation in more detail especially because strain GA33 is the type strain for *P. ruminicola* and other strains of the genus *P. ruminicola* supposedly relate to this strain.

*P. ruminicola* (previously called *Bacteroides ruminicola*) represents a high proportion of the rumen bacterial population under most dietary regimes (Bryant, 1959; Hungate, 1966; Howlett *et al.*, 1976; Russell, 1984; Van Gylswyk, 1990). It has also been isolated from the intestinal contents of chickens (Holdeman *et al.*, 1984). The species is subdivided into two subspecies based on their requirement for hemin. Strains of *P. ruminicola* subsp. *brevis* are able to synthesise hemin, whereas strains of *P. ruminicola* subsp. *ruminicola* require hemin for growth (Bryant *et al.*, 1958).

Strains of *P. ruminicola* can represent 6 to 19 % of carbohydrate fermenters in the rumen (Joyner and Baldwin, 1966; Howlett *et al.*, 1976). They utilise a wide range of sugars (Holdeman *et al.*, 1984) and are capable of fermenting hemicellulose (Williams and Withers, 1985), but not cellulose. The species is also one of the major proteolytic rumen bacteria (Hazlewood *et al.*, 1981; Wallace and Brammall, 1985) and can grow in

a medium containing protein as the sole nitrogen source (Hazlewood and Nugent, 1978; Wallace and Brammall, 1985). However, proteolytic strains of *P. ruminicola* cannot be isolated from all animals (Hazlewood *et al.*, 1983).

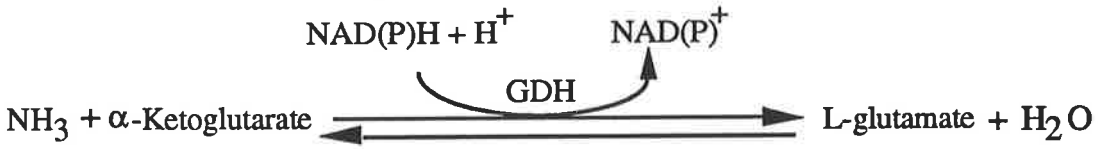
Ammonia is the favoured nitrogen source for *P. ruminicola* (Hazlewood *et al.*, 1981) but peptides are also important nitrogen sources (Munn *et al.*, 1983; Russell, 1983). Most studies on peptide uptake and utilisation in rumen bacteria have been conducted on strains of this species. Pittman and Bryant (1964) showed that peptides could be utilised as a nitrogen source in place of ammonia, whereas free amino acids would not serve as the nitrogen source for growth. They therefore concluded that the reason for poor amino acid utilisation was lack of amino acid transport systems. Stevenson (1979) questioned this conclusion by studying amino acid uptake systems in *P. ruminicola* strain 23. She found that amino acid uptake occurred in this strain. Since a high rate of uptake occurred only in fresh medium, Stevenson concluded that inability of *P. ruminicola* to use free amino acids as a nitrogen source may not simply be due to the lack of a mechanism to transport these compounds into the cell, but it may be the result of non-specific inhibitors present in the spent media.

Pittman *et al.* (1967) reported that oligopeptides of molecular weights up to 2,000 (about 16 amino acids) can be taken up by cells, hydrolysed during or after uptake and the liberated amino acids are apparently used directly for growth. However, Russell *et al.* (1991) discuss this point and mention that extracellular peptidases may have hydrolysed the larger peptides to a length that is more likely to pass through the cytoplasmic membrane. When Wallace and McKain (1991) screened 14 species of predominant rumen bacteria for peptidase activity they found that *P. ruminicola* was the most important species in peptide breakdown and had a peptidase activity similar in specificity to the main activity present in rumen fluid.

The results reported in the previous chapter of this thesis showed that *P. ruminicola*

strain GA33 is not able to grow on $NH_4Cl$ as a sole nitrogen source. Ammonia uptake may not be a problem, since it has been shown in the previous chapter (Figure 2.14) that the strain did not attain an absorbance maximum on mixed amino acids unless $NH_4Cl$ was added. This shows that strain GA33 is able to transport ammonia and utilise it, but lack of growth on ammonia as a sole nitrogen source may either be due to a limitation in ammonia assimilation or a block in the biosynthetic pathway of one or more amino acids.

In order to grow in a simple salts medium in which ammonia provides the sole source of utilisable nitrogen, microorganisms must possess some mechanism for ammonia assimilation. In many bacteria this requirement can be met solely by glutamate dehydrogenase (Meers and Tempest, 1970) by which α-ketoglutarate is aminated reductively to form L-glutamate (Borghese and Wall, 1992; Börmann *et al.*, 1992; Ertan, 1992b).




L-glutamate occupies a central position in amino acid metabolism. Most of the nitrogen assimilated via ammonia enters metabolism as the α-amino group of glutamate and thence the α-amino group of all other amino acids by transamination from glutamate. Therefore, bacteria such as *E. coli* are able to assimilate ammonia and synthesise all 20 amino acids required for protein synthesis (Senior, 1975; Umbarger, 1978; Stryer, 1981).

In the experiments described here, I have examined the growth response of *P. ruminicola* GA33 to individual amino acids to determine whether there is an inability to synthesise a certain amino acid. The results obtained from these growth experiments led to assaying NADH- and NADPH-dependent glutamate dehydrogenases to determine

whether the inability of strain GA33 to grow on $NH_4Cl$ is due to an inherent defect in ammonia assimilation.

## 3.2 Materials and Methods

### 3.2.1 Chemicals

L-amino acids, α-ketoglutarate, Tris-HCl, EDTA, NADH, NADPH, bovine serum albumin (BSA) and azaserine were obtained from Sigma Chemical Company, St. Louis, USA. Coomassie Brilliant Blue was obtained from Bio-Rad Laboratories, Richmond, CA., USA. 2-Mercaptoethanol was purchased from BDH Chemicals Ltd., Poole, England.

All other chemicals, of the highest purity available, were obtained from the following sources: Ajax Chemicals, Sydney, Australia; B.D.H. Chemicals Australia Ltd., Boronia and Kilsyth, Australia.

### 3.2.2 Buffers and solutions

All solutions including buffers which were used in enzyme assays were made using nano pure water. All other buffers and solutions were prepared using RO water.

#### 3.2.2.1 CE buffer pH 10.0 (Dean *et al.*, 1989)

| *Ingredients* | mM |
|---|---|
| Sodium carbonate | 50.0 |
| EDTA | 25.0 |

#### 3.2.2.2 Extraction buffer pH 7.0 (Werner Schmidt and Werner Schmidt, 1987).

| *Ingredients* | mM |
|---|---|
| Triethanolamine (TEA) | 50.0 |
| EDTA | 2.0 |

### 3.2.2.3 Coomassie Brilliant Blue solution

*Ingredients*

Coomassie Brilliant Blue.....100 mg

Ethanol, 95 %..... 50 ml

Phosphoric acid 85 %.....100 ml

Coomassie Brilliant Blue R-250 was dissolved in ethanol and then phosphoric acid was added. The solution was brought to a final volume of 1 litre with RO water, filtered through Whatman No. 1 filter paper and stored at 4°C.

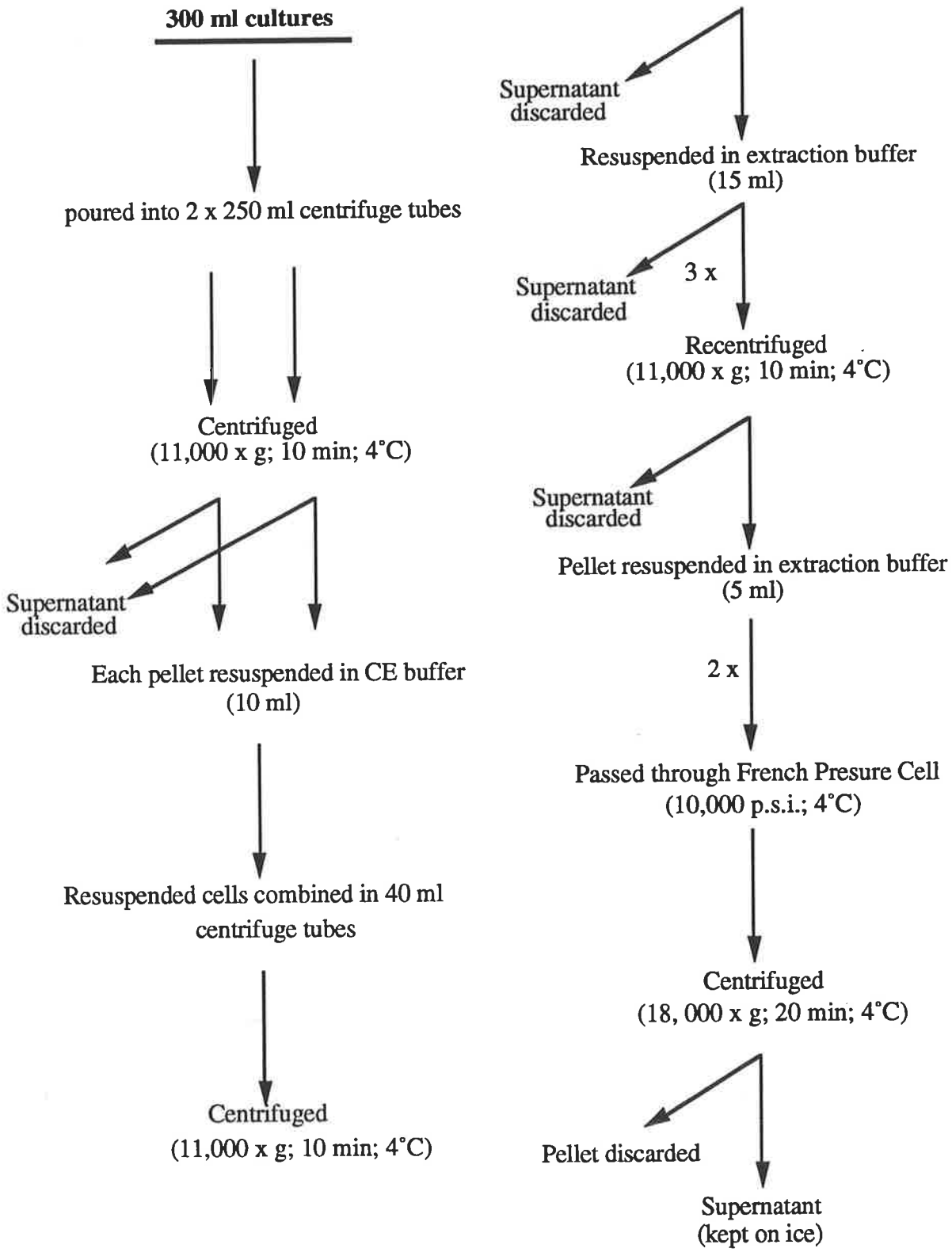
### 3.2.3 Culture conditions

NB medium was prepared as described in chapter 2. The growth conditions were as outlined in section 2.2.9. Data from growth curves determined during experiments reported in chapter 2 (Figures 2.13 and 2.14) were used as a guide to the growth rates for the cultures and were referred to when determining the maximum absorbances or to harvest cells for enzyme assays. For preparation of cell extracts for enzyme assays, batch cultures were grown in NB medium containing $NH_4Cl$ and 0.15% (w/v) casamino acids in 150 ml serum bottles sealed with butyl rubber septa (Pierce Chemical Company, Rockford, USA). Growth in these cultures was followed with a parallel Hungate tube culture (10 ml) and measurement of the absorbance at 600 nm.

### 3.2.4 Cell lysis using a French Pressure Cell and preparation of cell-free extracts for enzyme assays

Cultures were harvested at the log phase of growth and disrupted by the procedures outlined in Figure 3.1. The cultures were decanted into 250 ml screw-capped polyallomer centrifuge tubes (Beckman Instruments, Inc., Palo Alto, USA) and centrifuged (11,000 x g; 10 min; 4°C) in a Beckman centrifuge (J2-HS, USA). Each pellet was suspended in CE buffer, combined and transferred into a 40 ml screw-capped

**Figure 3.1 Culture harvesting and cell lysis using a French Pressure Cell**



polyallomer centrifuge tube. Cells were washed at least twice with CE buffer to remove extracellular polysaccharide material and then washed at least three times with extraction buffer and suspended in 5 ml of the same buffer. Cell suspensions were passed twice through a French Pressure Cell (10,000 p.s.i.; 4°C) to disrupt the cells and the resulting suspension was centrifuged (18,000 x g; 20 min; 4°C) to remove cell debris. Supernatants (cell-free extracts) were stored on ice if they were to be assayed on the same day, or at 4°C overnight otherwise.

### 3.2.5 Protein quantitation

The method was as described by Bradford (1976). Samples were poured into microcentrifuge tubes and brought to a volume of 100 µl with 0.15 M NaCl. Standard tubes containing 5, 10, 15 and 20 µl of a 0.5 mg/ml BSA solution were adjusted to 100 µl with 0.15 M NaCl and run at the same time. Blank tubes contained 100 µl of 0.15 M NaCl. 1 ml Coomassie Brilliant Blue solution was added to each tube, vortexed and allowed to stand at room temperature for 2 min. The absorbances were read at 595 nm against the blank. The concentration of proteins in the samples was determined from the standard curve prepared by plotting the absorbances of standards versus the concentration of BSA. This value was used to calculate the specific activity of each enzyme, expressed per mg of protein in the cell-free extract.

### 3.2.6 GDH assay

NADH- and NADPH-dependent glutamate dehydrogenases (GDH) were determined by a spectrophotometric method modified from Werner Schmidt and Werner Schmidt (1987) from the rate of oxidation of NADH or NADPH. Optimal enzyme assay conditions were determined in preliminary assays with cell-free extracts from *P. ruminicola* strain P1. Except for NADH and NADPH, all the assay components listed in Table 3.1 were transferred to two 1 cm path length cuvettes and mixed well. One of these cuvettes was placed in the reference beam of the spectrophotometer and the other in the sample beam. After zeroing the spectrophotometer (Shimadzu UV-160A

**Table 3.1 Components of glutamate dehydrogenase (GDH) assay mixture[a].**

| Volume (µl) | Components[b] | Final Concentration (mM) |
|---|---|---|
| 600 | 100 mM Ammonium acetate | 20.0 |
| 300 | 10 mM α-Ketoglutarate[c] | 1.0 |
| 75 | 10 mM NADH[c] or NADPH[c] | 0.25 |
| 240 | 100 mM Azaserine[d] | 8.0 |
| 1785 - x | Extraction buffer | |
| x | Cell-free extract (20 µg protein) | |

*a*. Total volume of the assay mixture was 3 ml.

*b*. All components were prepared as stock solutions prior to the experiment, unless stated otherwise.

*c*. Neutralised with 2.0 M NaOH.

*d*. Azaserine was added to inhibit glutamate synthesis via glutamine-oxoglutarate aminotransferase (Ertan, 1992b).

UV/VIS Recording Spectrophotometer) with this reaction mixture, the sample cuvette was removed, NADH or NADPH was added, mixed well and incubated at 37°C. The $A_{340}$ of the sample cuvette was read each minute for 5 min ($\Delta A1/\Delta t$). A control without ammonium acetate was run for each set of samples to detect any non-specific oxidation of NADH or NADPH ($\Delta A2/\Delta t$). Boiled cell-free extract was assayed as a negative control. Under the assay conditions employed, the decrease in absorbance was linear with respect to incubation time and the amount of protein. Specific activity is expressed as nmoles NADH or NADPH oxidised $min^{-1}$. mg $protein^{-1}$. Results are the mean ± the standard error of the mean for the triplicate assays.

### 3.2.7 Calculation of GDH activities (Bergmeyer, 1978)

The mean values of $\Delta A2/\Delta t$ were subtracted from the mean values of $\Delta A1/\Delta t$ to correct for non-specific NADH and NADPH oxidation. This difference, $\Delta A/\Delta t$, was used to calculate GDH activities.

$$\text{Specific catalytic activity} = \frac{\Delta A \times V \times 1000}{\varepsilon \times d \times \Delta t \times v \times c}$$

$$= \mu\text{mole NAD(P)H. min.}^{-1}\ \text{mg protein}^{-1} = \text{U. mg}^{-1}$$

$\Delta A$ Absorbance change

V Assay volume, l

ε Absorption coefficient, 0.63 $mmol^{-1}$ x $mm^{-1}$

d Light path, mm

$\Delta t$ Interval time, min.

v Volume of cell-free extract used in assay, l

c Protein concentration mg. $l^{-1}$

## 3.3 Results

### 3.3.1 Effect of individual amino acids on growth of *Prevotella ruminicola* strain GA33.

The data reported in chapter 2 indicated that *P. ruminicola* strain GA33 was unable to grow in NB medium containing $NH_4Cl$ unless the medium was supplemented with mixed amino acids or peptides. To examine this growth limitation in more detail, growth of strain GA33 was tested in NB medium containing 70 mM $NH_4Cl$ plus 0.01% (w/v) of each individual amino acid. The results (Figure 3.2) show that any amino acid other than cysteine or methionine was able to promote growth. None of the amino acids could support bacterial growth in the absence of $NH_4Cl$. It has been previously reported (Pittman and Bryant, 1964; Jones and Pickard, 1980) that methionine and cysteine stimulated growth of *P. ruminicola* strain GA33 in a defined medium containing ammonium sulphate. Growth of this strain was therefore tested in NB medium containing $NH_4Cl$ and higher concentrations (0.05 or 0.1% w/v) of methionine or cysteine. Neither methionine nor cysteine promoted the growth at any concentration tested.

### 3.3.2 Glutamate biosynthesis in *P. ruminicola* strains P1 and GA33.

*P. ruminicola* strain GA33 cannot grow in NB medium containing $NH_4Cl$ unless any amino acid except cysteine or methionine is also present. This suggests that the strain may be defective in *de novo* glutamate biosynthesis via GDH, but is able to produce glutamate from most other amino acids by transamination with α-ketoglutarate. To examine this possibility, strain GA33 was tested for NADPH- and NADH- specific GDH activities. Extracts of *P. ruminicola* strain P1 were tested as positive controls to ensure that the assay was working. The results (Table 3.2) show GDH activities in extracts from strain P1 but neither NADH nor NADPH-dependent GDH activities were detectable in extracts of *P. ruminicola* strain GA33.

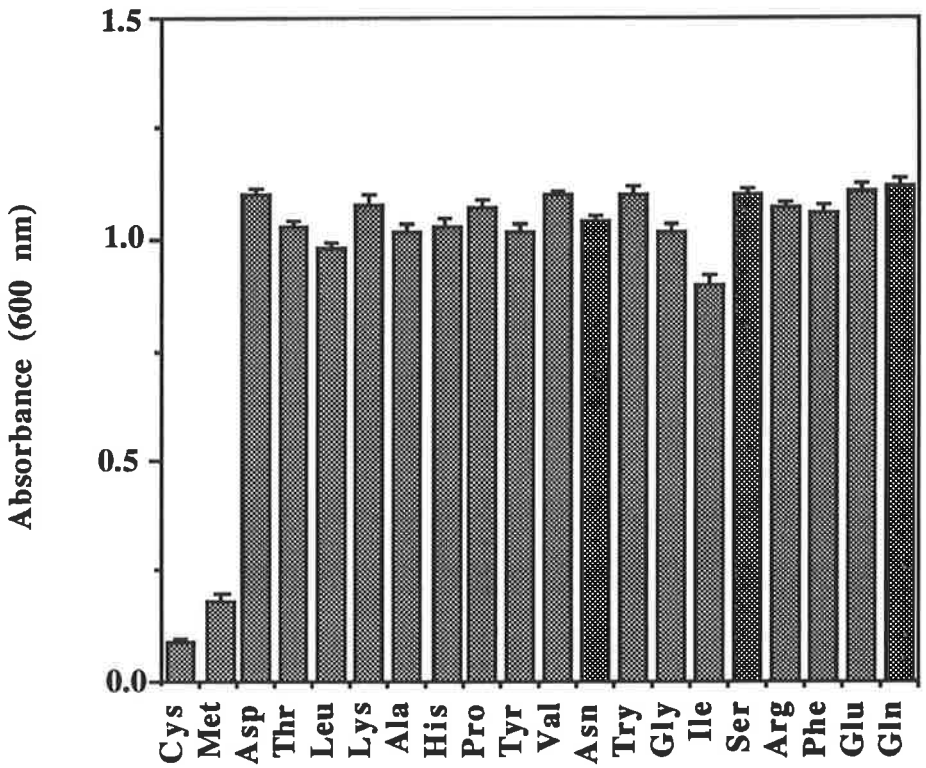



**Figure 3.2 Effect of individual amino acids on growth of *P. ruminicola* strain GA33 in NB medium containing $NH_4Cl$.**

Cells were incubated anaerobically at 39°C and cell densities were measured at 12 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. The initial absorbances were < 0.02. Absorbance values are the mean of triplicate assays. Bars represent standard error of the means.

**Table 3.2 NADH- and NADPH-dependent Glutamate Dehydrogenase activities in *P. ruminicola* strains P1 and GA33.**

| *P. ruminicola* | GDH specific activities[a] | |
|---|---|---|
| | NADH-dependent | NADPH-dependent |
| strain P1 | 20.0 ± 2.1 | 52.0 ± 2.6 |
| strain GA33 | 0.00 | 0.00 |

*a.* Specific activities expressed as nmoles NAD(P)H. $min^{-1}$. mg $protein^{-1}$ and are the mean ± standard error of the mean from triplicate assays.

## 3.4 Discussion

The results presented here show that any amino acid except methionine and cysteine could promote growth of *P. ruminicola* strain GA33. Jones and Pickard (1980) reported that growth of several strains including *P. ruminicola* strain GA33 was stimulated by cysteine when titanium citrate was used as a reducing agent. However, the time required to achieve maximal growth was inversely proportional to the cysteine concentration and the maximum $A_{600}$ achieved was only 0.238. These results are therefore probably due to the reducing capacity of cysteine rather than its use as a nitrogen source since I have shown here that even in the presence of higher concentrations of cysteine no growth was observed for this strain. The present results also show that *P. ruminicola* strain GA33 did not grow on methionine at any concentration tested. This contradicts the report by Pittman and Bryant (1964) that methionine replaced the casein hydrolysate requirements for this strain.

Different reasons for the lack of growth of *P. ruminicola* strain GA33 on cysteine or methionine can be envisaged. Cysteine and methionine may not be taken up by the cells due to lack of specific transport systems as suggested by Pittman and Bryant (1964). The strain may possess specific transport mechanisms, but the cells may be unable to translocate cysteine or methionine due to the presence of inhibitory factors present in the growth medium (ie acetate) as suggested by Stevenson (1979). Finally it is conceivable that strain GA33 is able to transport cysteine and methionine but lacks specific aminotransferases for which these amino acids act as amino group donors to generate glutamate from α-ketoglutarate. These aminotransferases have been reported in *E. coli* (Sokatch, 1969) but there is no evidence that they are present in rumen bacteria.

Complementation by any amino acid except methionine or cysteine suggests that *P. ruminicola* strain GA33 may be defective in the biosynthesis of L-glutamate *de novo*.

This is the likely explanation since glutamate occupies a central position in the biosynthesis of most bacterial amino acids and, in the absence of *de novo* synthesis from ammonia, can be generated by transamination between α-ketoglutarate and most other amino acids. This is confirmed by the results of growth experiments reported in the previous chapter (Figures 2.7 and 2.8) demonstrating that a higher concentration of mixed amino acids or peptides (in the absence of $NH_4Cl$) increased growth of strain GA33. Enzyme assays also confirmed this conclusion by demonstrating the absence of NADH- and NADPH-dependent GDH activities in this strain. Fincham (1950) previously reported mutant strains of *Neurospora crassa* which had a nutritional requirement for α-amino acids for growth. Since these mutants lacked glutamate dehydrogenase, Fincham (1951a; 1951b) concluded that promotion of growth by different α-amino acids is due to the presence of a range of transaminases in *N. crassa.* Transaminases have also been reported in cell extracts of rumen contents (Chalupa *et al.*, 1970; Tsubata and Hoshino, 1969) and individual ruminal bacterial species (Jenkinson *et al.*, 1979; Joyner and Baldwin, 1966; Kistner and Kotze, 1973).

GDH was tested in the cell-free extract of *P. ruminicola* strain P1 as a positive control. Both NADH- and NADPH-dependent GDH activities were detectable in this strain and the level of GDH activities were similar to that reported in *S. ruminantium* strain D by Smith *et al.* (1980). The presence of both NADH- and NADPH-dependent GDH activities in *P. ruminicola* strain P1 is interesting since it has been reported that many bacteria such as *E. coli*, *B. subtilis*, *A. aerogenes* and *B. licheniformis* contain only one type of GDH with a specific requirement for NADPH (Brown *et al.*, 1974). Similarly, it has been reported that either NADH- or NADPH-dependent GDH (but not both) were detectable in pure cultures of rumen bacteria including *P. ruminicola, B. fibrisolvens, R. flavefaciens, R. albus, F. succinogenes, S. bovis, S. ruminantium, R. amylophilus* and *M. elsdenii* (Burchall *et al.*, 1964; Joyner and Baldwin, 1966), whereas both activities were found in mixed rumen bacterial samples (Palmquist and Baldwin, 1966; Chalupa *et al.*, 1970; Wallace, 1979). Nevertheless, the presence of

both NADH- and NADPH-dependent activities has been reported in *Hydrogenomonas* H16 (Krämer, 1970) and *S. ruminantium* strain D (Smith *et al.*, 1980). Smith *et al.* (1980) have suggested that the dual specificity in *S. ruminantium* strain D might involve use of GDH for regenerating NAD from NADH produced by glycolysis. Since the highest content of NADPH-dependent GDH and the lowest content of NADH-dependent GDH was found in cultures grown in the presence of an excess ammonia, Krämer (1970) suggested that the NADPH-dependent enzyme had a predominantly biosynthetic function whereas the NADH-dependent GDH was predominantly catabolic. Smith *et al* (1980) found that the ratio of NADH/NADPH GDH activities was fairly constant, being between 0.25 to 0.35 in extracts prepared from *S. ruminantium* strain D grown under a variety of conditions. Similarly, the results reported in the present chapter show that *P. ruminicola* strain P1 possesses a lower NADH-dependent GDH specific activity, with a NADH/NADPH specific activity ratio of 0.38. The higher NADPH-GDH specific activity in strain P1 may be due to the high concentration of $NH_4Cl$ (70 mM) present in the culture medium which would induce the production of the NADPH-dependent enzyme. However, more work is required to determine whether the NADPH/NADH GDH specific activity ratio in *P. ruminicola* strain P1 changes under a variety of growth conditions.

*P. ruminicola* strain GA33 did not grow on any individual amino acid (0.01% w/v) in the absence of $NH_4Cl$. Since addition of 70 mM $NH_4Cl$ promoted bacterial growth (Figure 2.14), the only possible explanation for the lack of growth in the absence of $NH_4Cl$ is that an individual amino acid at a concentration of 0.01% w/v (about 0.83 mM nitrogen) does not provide the cells with sufficient nitrogen for growth. Therefore, *P. ruminicola* strain GA33 can utilise ammonia as a nitrogen source but requires amino acid supplementation. In order to utilise ammonia, strain GA33 must possess some mechanism for ammonia assimilation. In bacteria lacking GDH, an alternative ammonia assimilatory pathway such as alanine dehydrogenase, GS/GOGAT or asparagine synthetase is essential. As mentioned in section 1.2.14

alanine dehydrogenase appears to catalyse the conversion of L-alanine to pyruvate, which can be used as a carbon source. It has also been reported that the enzyme has a high Km for ammonia (Wiame *et al.*, 1962; Yoshida and Freese, 1965). Meers and Kjaergaard-Pedersen (1972) found that the enzyme from *B. licheniformis* had a Km of 300 mM for ammonia. Since strain GA33 does not grow on $NH_4Cl$ as a sole nitrogen source, the strain may also lack AlaDH or, if the enzyme is present, it may be unable to assimilate ammonia at a concentration of 70 mM. The GS/GOGAT pathway and asparagine synthetase would require glutamate and aspartate respectively as a substrate which may be provided in the medium or is produced by some other reactions. Furthermore, both pathways are energy (ATP) demanding processes (section 1.2.14) thus their activities may be restricted in an anaerobic environment where ATP production is limited. The microbial GS/GOGAT pathway generally predominates at ammonia concentrations below 1 mM but declines in activity above this value (Meers *et al.*, 1970; Hespell, 1984). Therefore, the GS/GOGAT pathway may not be active when strain GA33 is grown in the presence of 70 mM $NH_4Cl$. Patterson and Hespell (1985) showed that GS activity was high in *S. dextrinosolvens* grown in continuous culture under ammonia limitation, but the addition of a high level (15 mM) of $NH_4Cl$ resulted in a rapid loss of GS activity. In contrast, Meers *et al.* (1970) found that *Erwinia carotovora*, which totally lacks a glutamate dehydrogenase synthesised GOGAT constitutively. Further study is clearly necessary to establish the pathway of ammonia assimilation in *P. ruminicola* strain GA33.

The lack of GDH activity and consequently the inability to synthesise L-glutamate *de novo* by *P. ruminicola* strain GA33 is an important finding since GA33 is the type strain for the species. It is not clear whether this phenotype is a characteristic of the original strain or whether it was generated by prolonged passage in the laboratory. However, it has been known since its isolation (Bryant *et al.*, 1958) that casein hydrolysate was required for growth of the organism on ammonia, but no further work has been conducted in detail to reveal the reason for this limitation. This is the first

demonstration that the inability of strain GA33 to grow on ammonia as a sole nitrogen source is due to the lack of GDH activity in this strain.

Differences in nitrogen requirements and enzyme activities between *P. ruminicola* strains P1 and GA33 in itself is not surprising and probably reflects the genetic diversity in *P. ruminicola* that has been reported by Mannarelli *et al.* (1991). Since virtually any amino acid can complement the defect in *P. ruminicola* strain GA33, it probably does not represent a significant impediment to competitive growth in the rumen. Nevertheless, the strain may be a valuable tool for studying ammonia assimilation and for cloning the GDH gene by mutation complementation.

# CHAPTER 4

# CHAPTER 4

## Nitrogen Utilisation in *Butyrivibrio fibrisolvens* Strain E14

### 4.1 Introduction

The report in chapter 2 of this thesis shows that *B. fibrisolvens* strain E14 is unable to grow on $NH_4Cl$ unless mixed amino acids or peptides are also provided in the medium. The experiments reported in this chapter were therefore carried out to examine this growth limitation in more detail.

*Butyrivibrio fibrisolvens* strain E14 was isolated from the rumen of high-arctic Svalbard reindeer by Orpin *et al.* (1985). Svalbard reindeer survives in one of the most inhospitate natural environments in the world. At Svalbard there is no daylight for almost 3 months in winter and there is an equally long period of continuous daylight in summer. Plant growth is restricted to about 2 months in summer. In winter most of the range is covered by snow, and unlike other high-arctic regions, at Svalbard the ambient temperature often rises above freezing, even in midwinter. Such episodes of warm weather followed by subzero temperatures produce a crust of solid ice which severely limits the access of the animals to the already poor winter range (Orpin *et al.*, 1985). To survive under such conditions it is imperative that the animals digest the poor-quality, fibrous plants which are available in winter as well as to make maximum use of the summer forage rich in seed heads (Orpin *et al.*, 1985; Mathiesen *et al.*, 1987). Orpin *et al.* (1985) have characterised the dominant rumen bacteria in these animals and have shown that a highly specialised rumen microflora, which is particularly effective in fiber digestion may contribute to this end. Cellulolytic strains of *B. fibrisolvens* with a mean population density of $(20.1 \pm 14.1) \times 10^7$ in summer and $(6.6 \pm 2.8) \times 10^7$ in winter were principal cellulolytic organisms representing 65 and 52% of the cellulolytic

population in summer and winter respectively. *B. fibrisolvens* strain E14 is one of these cellulolytic organisms which is also able to digest xylan, starch and protein (Orpin *et al.*, 1985). This is unusual as *B. fibrisolvens* does not usually form a significant proportion of the cellulolytic population in domestic ruminants (Hungate, 1966). These characteristics make strain E14 an interesting organism to study.

Nitrogen requirements of *B. fibrisolvens* strain E14 have not been studied previously. However, the results of the experiments reported in chapter 2 of this thesis show that the strain has an absolute requirement for amino acids or peptides for growth. Therefore, this strain offers an opportunity to investigate the biochemical nature of the stimulatory effect of these nitrogen sources on bacterial growth.

In the experiments reported in this chapter I have examined the growth response of strain E14 to mixed amino acids and peptides as carbon sources. Radiotracer experiments using $^{35}$S-methionine, $^{35}$S-cysteine or $^{14}$C-lysine followed by SDS-PAGE were also carried out to determine whether exogenous amino acids were incorporated into newly synthesised bacterial protein. The absolute requirement of strain E14 for amino acids may be due to a block in the biosynthetic pathway for a particular amino acid. Growth response of the strain was therefore tested to individual amino acids.

Since peptides also promote growth of *B. fibrisolvens* strain E14, it was worthwhile to determine the nutritional value of these nitrogen sources. Therefore, bacterial growth was examined on the dipeptide alanyl-methionine. There are different routes by which a bacterium can utilise an exogenous peptide. The intact peptide can be hydrolysed on the outside of the cell, after which the liberated free amino acids are taken up by specific amino acid transport systems. Alternatively, a peptide can be transported by a specific peptide transport system into the cell, where it is subsequently hydrolysed by intracellular peptidases. It is also possible that both peptide transport and hydrolysis are

functions associated with the cytoplasmic membrane (Verheul *et al.*, 1995). One way to distinguish between these possibilities is to perform competition inhibition experiments using peptide analogues. 5-Amino levulinic acid (ALA) resembles the dipeptide glycyl-glycine but does not contain a peptide bond and therefore is not hydrolysed by peptidases (Elliott, 1993). It has been shown that ALA is a substrate for the dipeptide permeases (Dpp) of *Salmonella typhimurium* (Elliott, 1993) and *E. coli* (Verkamp *et al.*, 1993). Recently, Verheul *et al.* (1995) have used the dipeptide analogue ALA to study dipeptide utilisation in *Listeria monocytogenes.* To distinguish the route by which *B. fibrisolvens* strain E14 utilises dipeptides, the effect of ALA was tested on bacterial growth in the presence of alanyl-methionine.

## 4.2 Materials and Methods

### 4.2.1 Chemicals

L-amino acids, alanyl-methionine, 5-aminolevulinic acid (ALA), α-methyl-DL-methionine, L-methionine, S-adenosylmethionine (chloride form), EDTA, Tris (hydroxymethyl) aminomethane hydrochloride, sodium dodecyl sulphate (SDS), acrylamide and N,N'-methylenebisacrylamide were obtained from Sigma Chemical Company, St. Louis, USA. $^{35}$S-methionine, $^{35}$S-cysteine, $^{14}$C-lysine and Scintillation liquid (BCS) were purchased from Amersham, IL, USA. Bromophenol blue was from Ajax Chemicals, Sydney, Australia. Coomassie Brilliant Blue G-250 and N, N, N', N'-Tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Laboratories, Richmond, CA., USA.

All other chemicals, of the highest purity available, were obtained from the following sources: Ajax Chemicals, Sydney, Australia; B.D.H. Chemicals Australia Ltd., Boronia and Kilsyth, Australia.

### 4.2.2 Buffers and solutions

#### 4.2.2.1 2 x PAGE loading buffer

*Ingredients*

| | |
|---|---|
| Tris.HCl, pH 6.8 | 100 mM |
| 2-mercaptoethanol | 200 mM |
| Bromophenol blue | 0.2% (w/v) |
| Glycerol | 20.0% (v/v) |
| SDS | 4.0% (w/v) |

#### 4.2.2.2 SDS-PAGE running buffer

*Ingredients*

| | |
|---|---|
| Tris.HCl, pH 8.3 | 25 mM |
| Glycine | 250 mM |
| SDS | 0.1% (w/v) |

#### 4.2.2.3 TE buffer (Mannarelli, 1988)

*Ingredients*

| | |
|---|---|
| Tris.HCl, pH 8.0 | 50.0 mM |
| EDTA | 20.0 mM |

#### 4.2.2.4 SDS-PAGE minigel solutions

| *Ingredients* | Resolving gel (ml/10 ml) | Stacking gel (ml/4 ml) |
|---|---|---|
| $H_2O$ | 4.0 | 2.7 |
| Acrylamide mix[a] | 3.3 | 0.67 |
| Tris.HCl[b] buffer | 2.5 | 0.5 |
| SDS (10w/v) | 0.1 | 0.04 |
| Ammonium persulphate[c] (10% w/v) | 0.1 | 0.04 |

| | | |
|---|---|---|
| TEMED | 0.004 | 0.004 |

*a*. Acrylamide mixture contained 29% (w/v) acrylamide and 1% (w/v) N,N'-methylenebisacrylamide.

*b*. 1.5 M Tris.HCl pH 8.0 and 1.0 M Tris.HCl pH 6.8 were used for preparation of resolving and stacking gels respectively.

*c*. Freshly prepared each time.

#### 4.2.2.5 Coomassie Brilliant Blue solution

*Ingredients*

| | |
|---|---|
| Coomassie Brilliant Blue | 250 mg |
| Methanol, 50% | 90 ml |
| Glacial acetic acid | 10 ml |

Coomassie Brilliant Blue was dissolved in the mixture of methanol and glacial acetic acid. The solution was filtered through a Whatman No.1 filter paper and stored at room temperature.

### 4.2.3 Growth conditions

NB medium was prepared as described in chapter 2. Alanyl-methionine and ALA (neutralised with 2 M NaOH) were added to NB medium as required from filter-sterilised stock solutions. The growth conditions were as outlined in section 2.2.10. For growth experiments, data from growth rates determined during the experiment reported in chapter 2 (Figure 2.12) were used as a guide to the growth rate for the cultures and were referred to when determining the maximum absorbance. For radiotracer experiments, bacteria were grown in 10 ml of NB medium containing 70 mM $NH_4Cl$ plus 0.15% (w/v) casamino acids and 0.1 μCi/ml of $^{35}$S-methionine, $^{35}$S-cysteine or $^{14}$C-lysine.

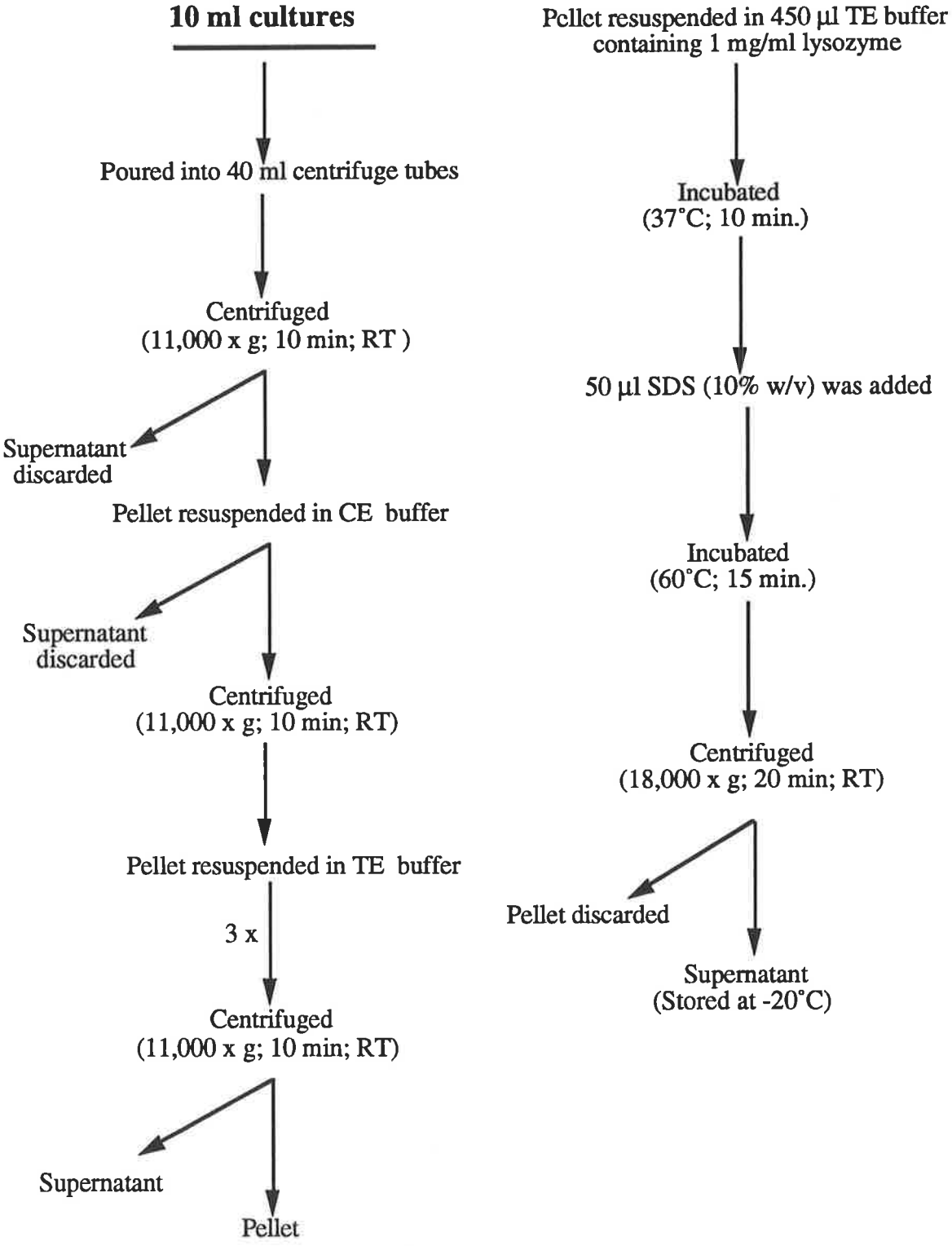
### 4.2.4 Cell lysis using lysozyme/SDS and preparation of cell-free extracts

Cultures were harvested at late log to stationary phase and lysed by the procedures outlined in Figure 4.1. The method was as described by Mannarelli (1988) except that CE buffer (section 3.2.2.1) was used as a wash buffer. Cultures were decanted into 40 ml screw-capped polyallomer centrifuge tubes (Beckman Instruments, Inc., Palo Alto, USA) and centrifuged in a Beckman centrifuge (J 2-HS, USA). Pellets were washed once with CE buffer to remove extracellular polysaccharide material from the cells and then washed at least three times with TE buffer. Cell pellets were suspended in 450 µl TE buffer containing 1 mg/ml lysozyme and the suspension was incubated at 37°C for 10 min. Cells were lysed by adding 50 µl of SDS solution (10% w/v) and incubating at 60°C for 15 min. The resulting suspension was centrifuged (18,000 x g; 20 min; 4°C) to remove cell debris. Supernatants (cell-free extracts) were stored at -20°C until use.

### 4.2.5 SDS-Polyacrylamine gel electrophoresis (PAGE)

The method was as described by Sambrook *et al.* (1989). Vertical gels (Bio-Rad Laboratories Ltd., Watford) containing a 10% acrylamide resolving gel and a 5% acrylamide stacking gel were cast. Cell-free extracts were prepared (section 4.2.4) and protein concentration of the extracts was determined as described in section 3.2.5. One volume of the cell extract was mixed with one volume of 2 x gel-loading buffer and boiled for 3 min. Samples containing 15 µg protein were run on 7.3 cm x 8.0 cm x 0.75 mm gels at 20 mA through the stacking gel and 30 mA through the separating gel. Gels were stained for one h in Coomassie Blue solution, destained in methanol-acetic acid-water (30: 10: 60, by volume) and dried onto Whatman 3MM filter papers using a gel drier (Bio-Rad Laboratories Ltd., Watford) at 60°C for 20 min. Dried gels were exposed to x-ray film (Fuji medical X-ray film) for 3-4 weeks. The films were developed by an automatic developer (Agfa, CURIX 60).

**Figure 4.1 Culture harvesting and cell lysis using SDS and lysozyme**



### 4.2.6 Measurement of the radioactivity in intact cells and TCA precipitable material

Bacteria were grown on labelled amino acids as described in section 4.2.3. Cultures were harvested at late log to stationary phase and washed once with CE buffer (section 3.2.2.1) and at least three times with TE buffer (section 4.2.2.3). Cells were then suspended in 500 µl of TE buffer. For measurement of radioactivity in intact cells, 100 µl of each cell suspension was filtered on a Whatman GF/C filter paper and the filter was then washed with 5 ml of TE buffer. Radioactivity on the filters was measured in a Beckman LS-3801 liquid scintillation spectrophotometer, using BCS as a counting fluid.

For measurement of radioactivity of TCA precipitable material, 100 µl of each cell suspension was lysed using lysozyme and SDS as outlined in section 4.2.4. 200 µl of RO water and 2 ml of ice-cold TCA solution (10% w/v) were added to each cell extract, mixed well and kept on ice for 20 min. The samples were then boiled for 15 min and kept on ice for 10 min before filtering. The samples were filtered on Whatman GF/C filter papers and the filters were then washed with 5 ml of ice-cold TCA solution (10% w/v) and 5 ml of 95% (v/v) ethanol. Radioactivity on filters was measured in a Beckman LS-3801 liquid scintillation spectrophotometer, using BCS as a counting fluid.

$$\text{\% Incorporation of labelled amino acids into TCA precipitable material} = \frac{\text{cpm of TCA precipitable material}}{\text{cpm of intact cells}} \times 100$$

## 4.3 Results

### 4.3.1 Use of mixed amino acids or peptides as carbon sources

Argyle and Baldwin (1989) suggest that amino acids can be fermented as an energy source by rumen bacteria. To define whether *B. fibrisolvens* strain E14 is able to utilise

mixed amino acids or peptides as energy sources, bacterial growth was tested in NB medium devoid of a carbon source and containing 70 mM $NH_4Cl$ plus 0.5% (w/v) mixed amino acids or peptides. The same media containing maltose were also tested as controls. The results (Figure 4.2) show that neither mixed amino acids nor peptides could support bacterial growth in the absence of maltose. A higher concentration of mixed amino acids or peptides (2.0 % w/v) was also tested but no growth was observed even when cultures were incubated for 2 days (results not shown).

### 4.3.2 Effect of individual amino acids on growth of *B. fibrisolvens* strain E14

The data reported in chapter 2 indicate that *B. fibrisolvens* strain E14 is unable to grow on $NH_4Cl$ unless mixed amino acids or peptides are also present. To examine whether this growth limitation is due to a requirement for any particular amino acid, bacterial growth was tested in NB medium containing 70 mM $NH_4Cl$ plus 0.01% (w/v) of each individual amino acid. The results (Figure 4.3) show that only methionine was able to promote growth to an $A_{600}$ of 1.5. No other amino acid, alone or in combination, was effective. The minimum effective concentration of methionine needed to achieve the maximum growth response was shown to be 0.2 mM (0.003% w/v) (Figure 4.4). In the absence of $NH_4Cl$, methionine did not support growth at any concentration tested (Figure 4.4).

### 4.3.3 Comparison of growth rate of *B. fibrisolvens* strain E14 on mixed amino acids and methionine

To compare growth of *B. fibrisolvens* strain E14 on mixed amino acids or methionine, NB medium containing 70 mM $NH_4Cl$ was supplemented with mixed amino acids or methionine and the bacterial growth was monitored every 30 min. The results (Figure 4.5) show that the growth rate on methionine was similar to that obtained on mixed amino acids.

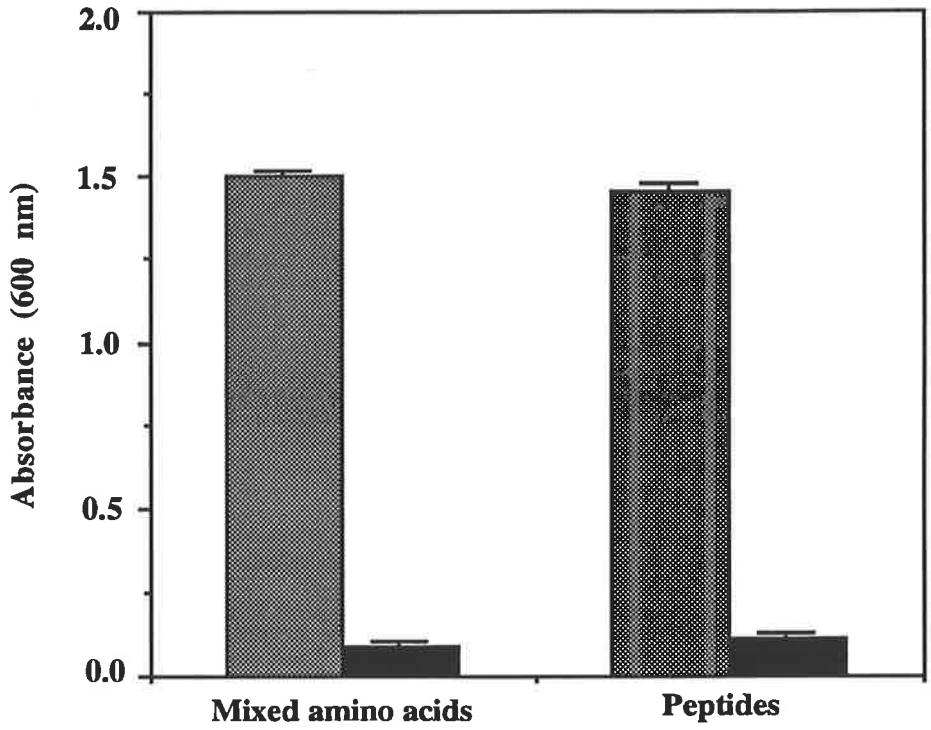



**Figure 4.2 Growth of *B. fibrisolvens* strain E14 in NB medium containing 70 mM $NH_4Cl$ plus 0.5% (w/v) mixed amino acids or peptides:**

▒ With maltose

■ without maltose

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. The initial absorbances were < 0.02. Absorbance values are the mean of triplicate assays. Bars represent standard error of the means.

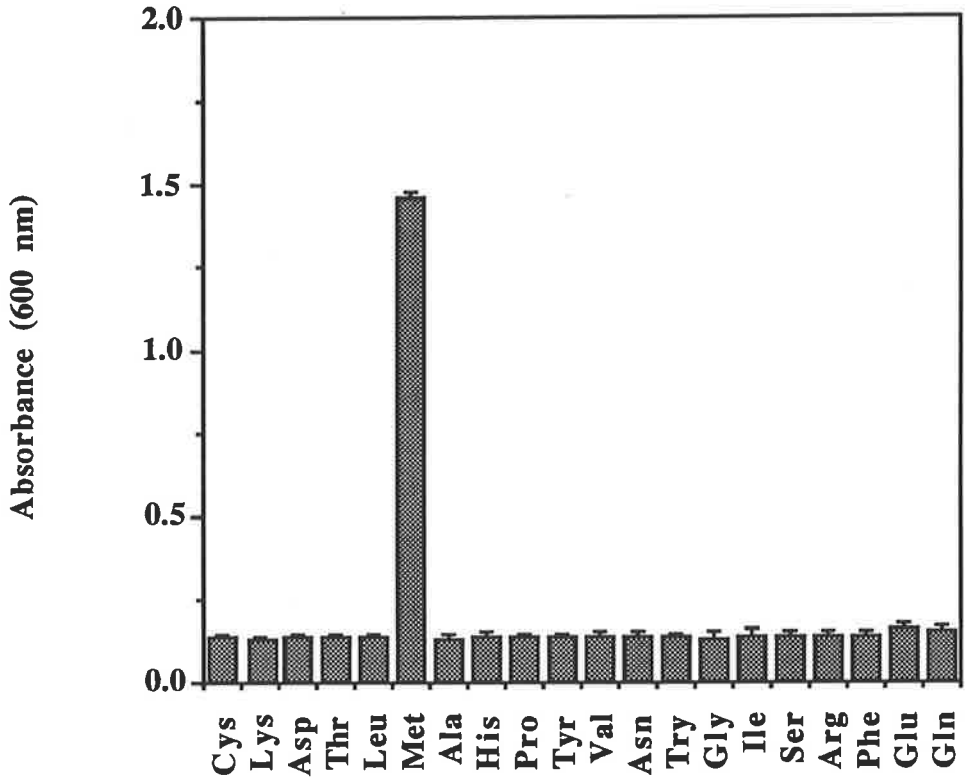



**Figure 4.3 Growth of *B. fibrisolvens* strain E14 on individual amino acids (0.01% w/v) in the presence of 70 mM $NH_4Cl$.**

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. The initial absorbances were < 0.02. Absorbance values are the mean of triplicate assays. Bars represent standard error of the means.

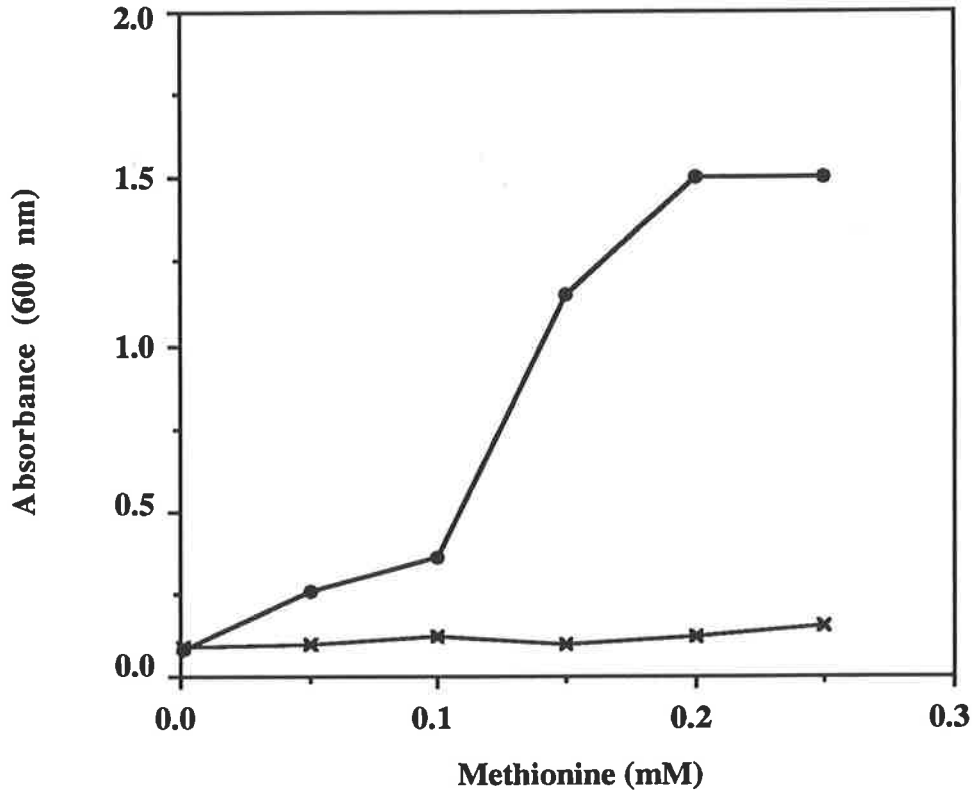



**Figure 4.4 Effect of increasing concentrations of methionine on growth of *B. fibrisolvens* strain E14 in NB medium:**

—●— With 70 mM $NH_4Cl$

—✖— Without $NH_4Cl$

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values represent the mean of triplicate assays, with variation between triplicates being less than 0.05.

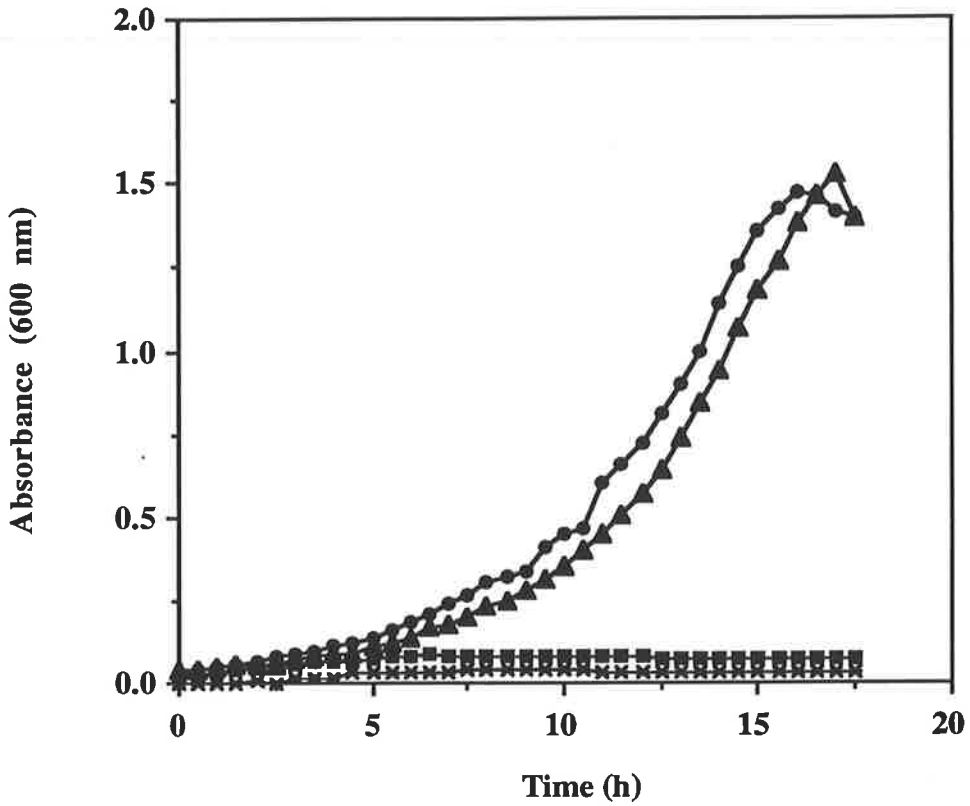



**Figure 4.5 Growth of *B. fibrisolvens* strain E14 in NB medium containing:**

—●— 0.15% (w/v) mixed amino acids plus 70 mM $NH_4Cl$

—▲— 0.2 mM methionine plus 70 mM $NH_4Cl$

—■— 70 mM $NH_4Cl$ alone

—✖— no addition (control)

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values represent the mean of triplicate assays, with variation between triplicates being less than 0.05.

### 4.3.4 Growth of *B. fibrisolvens* strain E14 on S-adenosylmethionine

To determine whether S-adenosylmethionine (AdoMet) could replace the requirement for methionine in strain E14, bacterial growth was tested in NB medium containing filter sterilised or autoclaved AdoMet (0.01 % w/v). These media enhanced bacterial growth to an $A_{600}$ of 0.75 but an absorbance maximum compared to that obtained on 0.2 mM methionine was not achieved. Higher concentrations of AdoMet were therefore tested. The results (Figure 4.6) show that the minimum effective concentration of AdoMet (filter sterilised or autoclaved) needed to achieve an $A_{600}$ of 1.5 was 0.6 mM.

### 4.3.5 Incorporation of amino acids into bacterial protein

SDS-PAGE was carried out to determine whether exogenous amino acids were incorporated into bacterial protein by *B. fibrisolvens* strain E14 and the non-growth limited strain H17c. The results (Figure 4.7) show incorporation of $^{35}$S-methionine, $^{35}$S-cysteine and $^{14}$C-lysine into newly synthesised bacterial protein. Incorporation of each labelled amino acid into TCA precipitable material was similar for the two strains (Table 4.1). Approximately 60% of cysteine and methionine, and greater than 90% of $^{14}$C-lysine was incorporated into TCA precipitable material.

### 4.3.6 Growth of *B. fibrisolvens* strain E14 on the dipeptide alanyl-methionine

The data in chapter 2 (Figure 2.5) showed that peptide supplementation could promote growth of *B. fibrisolvens* strain E14 on $NH_4Cl$. Since methionine replaced this requirement, the bacterium may obtain methionine from methionine-containing peptides. To examine this hypothesis, growth of strain E14 was tested in NB medium containing 70 mM $NH_4Cl$ plus 0.2 mM alanyl-methionine. The strain grew in this medium to an $A_{600}$ of 1.5. In the absence of $NH_4Cl$, 0.2 mM alanyl-methionine did not support growth. NB medium containing 70 mM NH4Cl plus 0.2 mM alanyl-alanine was tested as a control. This medium did not support bacterial growth.

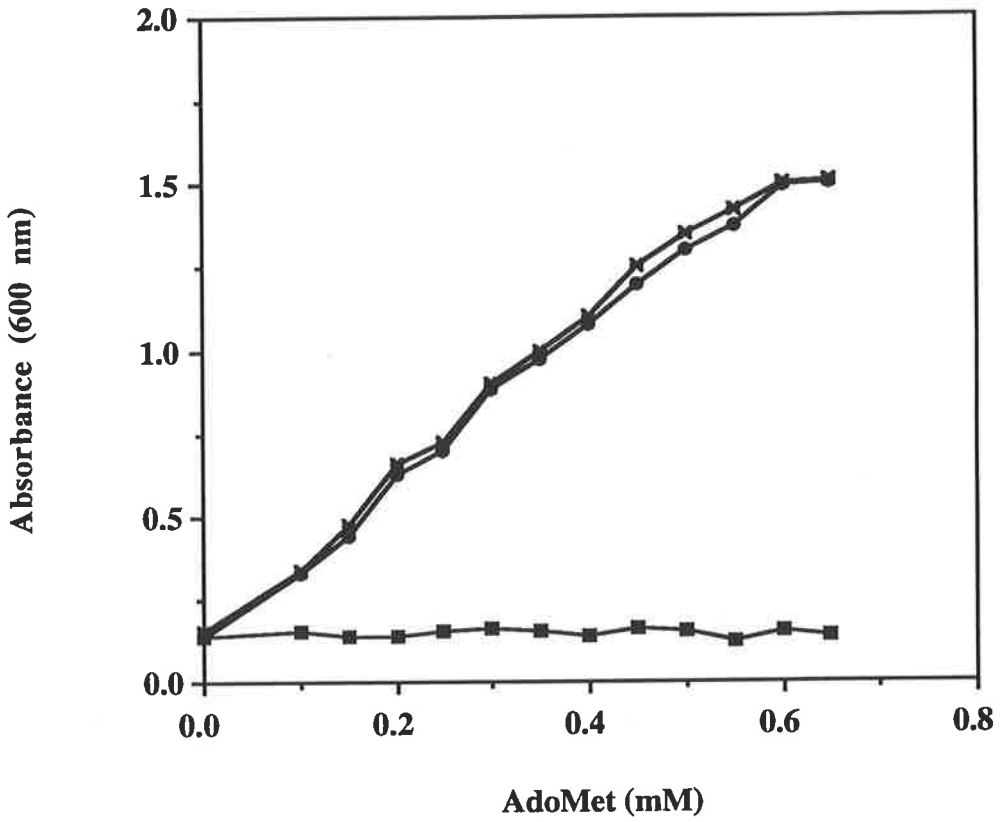



**Figure 4.6 Growth of *B. fibrisolvens* strain E14 in NB medium containing $NH_4Cl$ plus increasing concentrations of:**

—●— filter sterilised AdoMet
—✖— autoclaved AdoMet
—■— no addition (control)

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values represent the mean of triplicate assays, with variation between triplicates being less than 0.05.

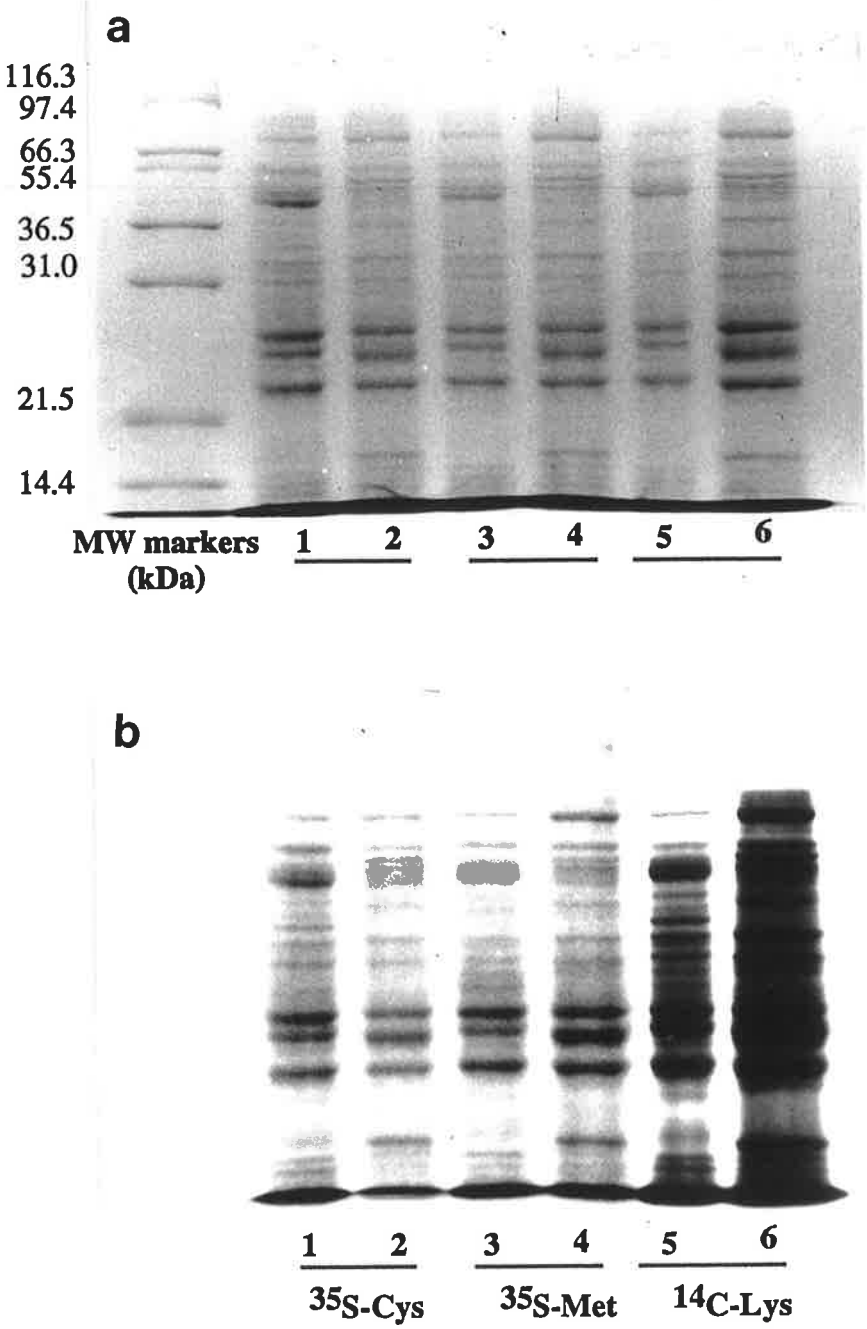



**Figure 4.7 Incorporation of $^{35}$S-cysteine, $^{35}$S-methionine and $^{14}$C-lysine into newly synthesised bacterial protein**

a) SDS-PAGE after staining with Coomassie Blue and drying. b) autoradiography of the dried gel. Lanes 1, 3, and 5; *B. fibrisolvens* strain E14. Lanes 2, 4, 6; *B. fibrisolvens* strain H17c.

**Table 4.1 Incorporation of labelled amino acids into the TCA precipitable material in *B. fibrisolvens* strains H17c and E14.**

| Labelled amino acids | Incorporation of labelled amino acids into TCA precipitable material (%)[a] | |
|---|---|---|
| | Strain E14 | Strain H17c |
| $^{35}$S-Methionine | 58.2 | 55.2 |
| $^{35}$S-Cysteine | 57.5 | 56.8 |
| $^{14}$C-Lysine | 90.2 | 92.3 |

*a*. Incorporation of labelled amino acids as a per cent (%) of total radioactivity taken up by cells.

### 4.3.7 Effect of the methionine analogue α-methyl-DL-methionine on growth of *B. fibrisolvens* strains E14 and H17c

Smith (1971) reported that uptake of methionine in *Salmonella typhimurium* is significantly reduced by the presence of the methionine analogue α-methyl-DL-methionine at a concentration of 1000 times greater than that of methionine. The effect of increasing concentrations of α-methyl-DL-methionine was therefore tested on growth of *B. fibrisolvens* strains E14 and H17c in NB medium containing 70 mM $NH_4Cl$ plus 0.2 mM L-methionine. The results (Figure 4.8) show that α-methyl-DL-methionine inhibited growth of strain E14 completely at 200 mM but had no significant effect on growth of strain H17c at any concentration tested.

To determine whether the methionine analogue α-methyl-DL-methionine was able to inhibit growth of strain E14 on dipeptides, bacterial growth was tested in NB medium containing 70 mM $NH_4Cl$, 0.2 mM alanyl-methionine and increasing concentrations of α-methyl-DL-methionine. The results (Figure 4.8) show that α-methyl-DL-methionine had no significant effect on growth of strain E14 on the dipeptide alanyl-methionine at any concentration tested.

### 4.3.8 Effect of the dipeptide analogue 5-aminolevulinic acid (ALA) on growth of *B. fibrisolvens* strain E14 in NB medium containing methionine or alanyl-methionine

The effect of ALA on bacterial growth was examined in NB medium containing 70 mM $NH_4Cl$ plus 0.2 mM alanyl-methionine or L-methionine. The results (Figure 4.9) show that in NB medium containing methionine, ALA had no significant effect on bacterial growth at any concentration tested whereas, in the medium containing the dipeptide alanyl-methionine bacterial growth was almost completely inhibited by the presence of 50 mM ALA.

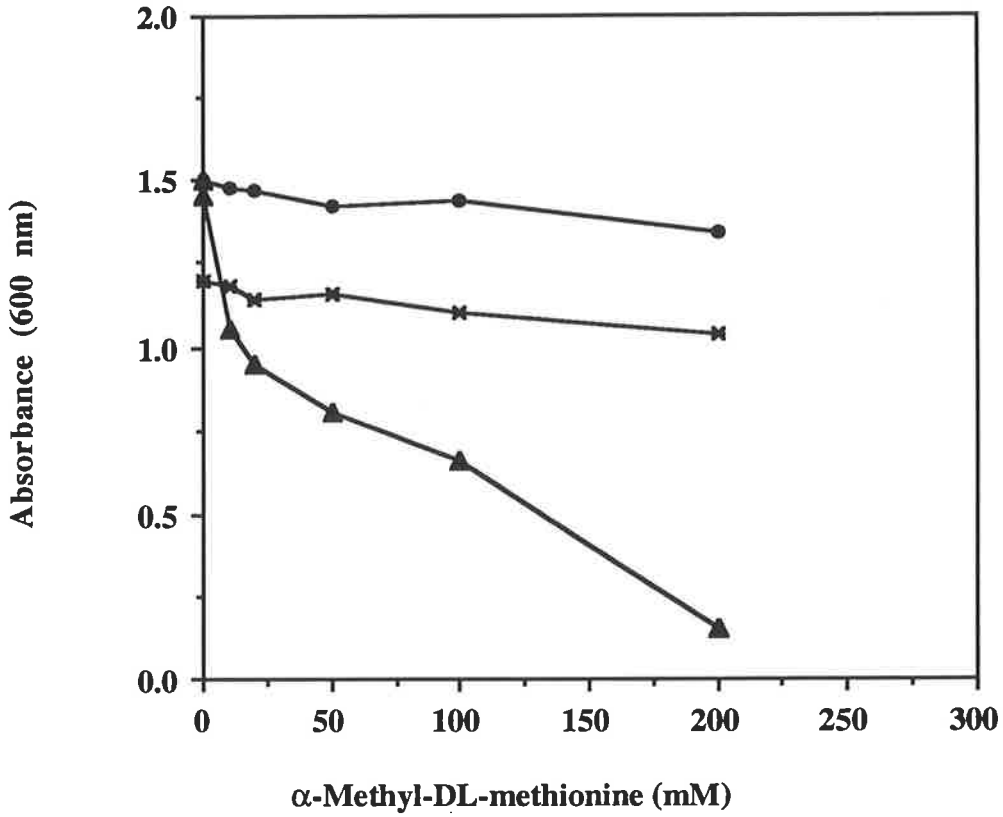



**Figure 4.8 Effect of increasing concentrations of α-methyl-DL-methionine on growth of *B. fibrisolvens* strain:**

—●— E14 in the presence of 70 mM $NH_4Cl$ plus 0.2 mM alanyl-methionine.

—✖— H17c in the presence of 70 mM $NH_4Cl$ plus 0.2 mM methionine.

—▲— E14 in the presence of 70 mM $NH_4Cl$ plus 0.2 mM Methionine.

Cells were incubated anaerobically at 39°C and cell densities of strains E14 and H17c were measured at 17 h and 28 h respectively using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values represent the mean of triplicate assays, with variation between triplicates being less than 0.05.

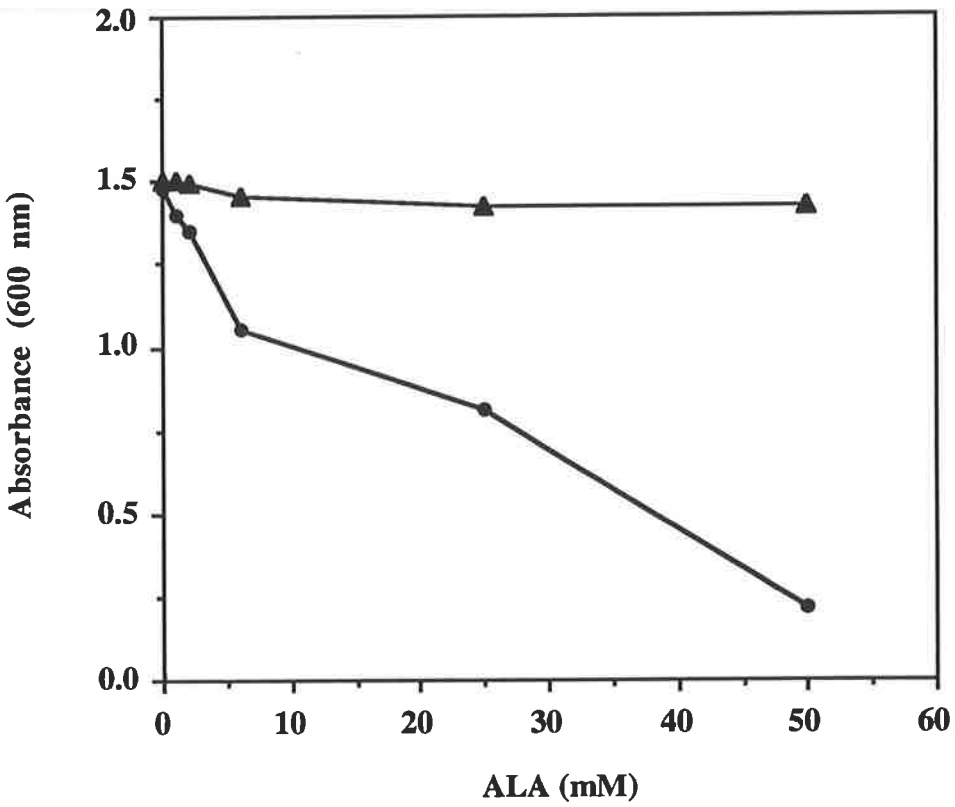



**Figure 4.9 Effect of the dipeptide analogue ALA on growth of *B. fibrisolvens* strain E14 in NB medium containing 70 mM $NH_4Cl$ plus 0.2 mM:**

—▲— methionine

—●— alanyl-methionine

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values represent the mean of triplicate assays, with variation between triplicates being less than 0.05.

### 4.3.9 Effect of increasing concentrations of alanyl-methionine on growth of *B. fibrisolvens* strain E14 in the presence of ALA.

To determine at what concentration alanyl-methionine is able to overcome the inhibitory effect of ALA, bacterial growth was tested in NB medium containing 70 mM $NH_4Cl$ plus 50 mM ALA and increasing concentrations of alanyl-methionine. The results (Figure 4.10) show that 2.0 mM alanyl-methionine was required to overcome the inhibitory effect of 50 mM ALA and supported bacterial growth to an $A_{600}$ of 1.5.

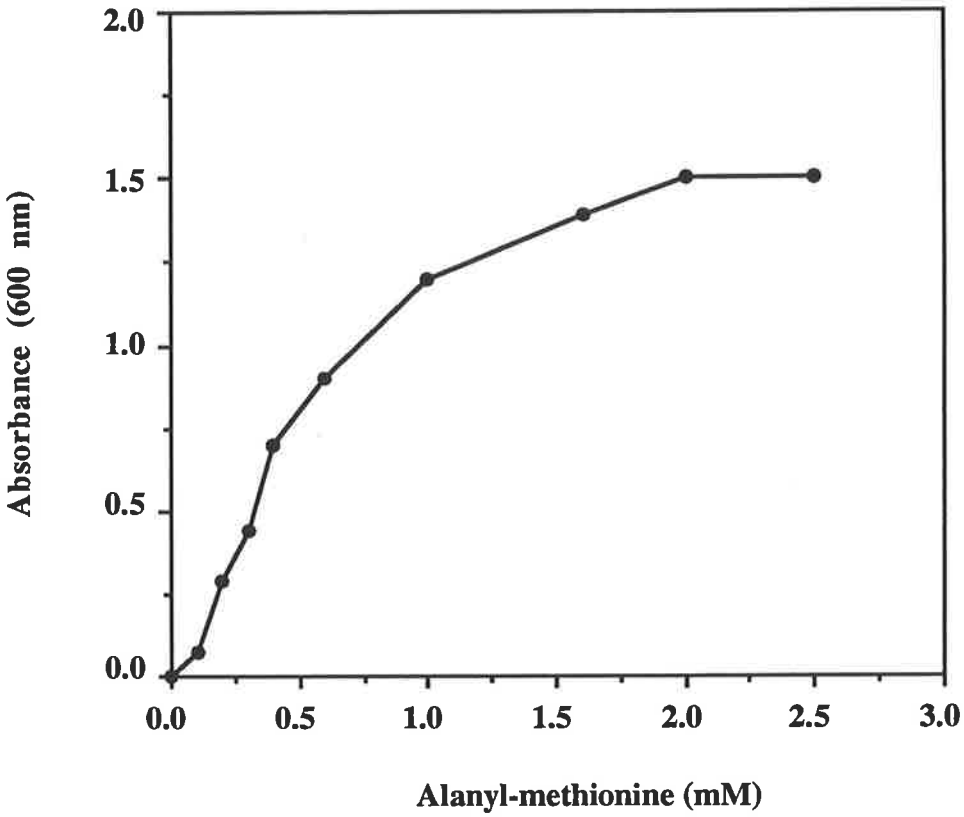



**Figure 4.10 Effect of increasing concentrations of alanyl-methionine on growth of *B. fibrisolvens* strain E14 in the presence of 50 mM ALA.**

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values represent the mean of triplicate assays, with variation between triplicates being less than 0.05.

## 4.4 Discussion

In addition to nitrogen sources, free amino acids and peptides can be utilised as energy sources (Tamminga, 1979; Russell *et al.*, 1983; Russell, 1984). In the unique environment of the rumen, there exist bacteria that are able to utilise amino acids and/or peptides as energy and nitrogen sources (Chen and Russell, 1988; Russell *et al.*, 1988a; Chen and Russell, 1989b). Russell *et al.* (1983) have reported that *M. elsdenii* is able to grow with amino acids and peptides both as nitrogen and energy sources. Russell (1984) has also shown that *P. ruminicola* is able to utilise carbon skeletons of amino acids with the same efficiency as it utilises glucose, but the rate of amino acid fermentation is not fast enough to meet the bacterial energy requirements. The results reported here show (Figure 4.2) that *B. fibrisolvens* strain E14 is unable to utilise amino acids or peptides as sole energy sources, because in the absence of a carbohydrate source no growth was observed even when the amino acid or peptide concentration was increased to 2% (w/v) and cultures were incubated for longer than 17 h. A possible explanation is that the limiting factor may be the rate at which cells can ferment amino acids or peptides. Thus, the energy yield from amino acid fermentation alone may not be sufficient to be used as energy source by strain E14. This is in agreement with previous reports that most rumen bacteria are unable to utilise amino acids and peptides as energy sources in the absence of carbohydrates because the energy yield from amino acid fermentation is poor (Russell and Hespell, 1981; Russell *et al.*, 1983; Russell, 1983, 1984; Mackie and Bryant, 1990).

Lack of growth of strain E14 in the absence of a carbohydrate source suggests that amino acid fermentation may not occur, but amino acids may be utilised as such for protein synthesis. This is confirmed by the results obtained from radiotracer experiments (Figure 4.7), since SDS-PAGE and measurement of radioactivity in TCA precipitable material (Table 4.1) showed that labelled amino acids were incorporated directly into newly synthesised bacterial protein in *B. fibrisolvens* strain E14 and the

methionine-independent strain H17c. All of the labelled amino acids tested were incorporated into bacterial protein regardless of being growth-limiting or not, suggesting that *B. fibrisolvens* strains are able to utilise preformed amino acids for immediate use in protein synthesis. This result supports the statement made by Stevenson (1979) that resynthesis of amino acids from ammonia and catabolic products appears to be an energetically wasteful process when amino acids are available.

Tests with individual amino acids showed that *B. fibrisolvens* strain E14 has an absolute requirement for methionine for growth. $NH_4Cl$ was still required since in its absence no growth was observed at any concentration of methionine tested. These results suggest that strain E14 is able to synthesise all essential amino acids from ammonia and catabolic products, except methionine. This conclusion is supported by the results (Figure 4.5) that bacterial growth rates were similar on mixed amino acids or methionine. Therefore, it can be concluded that the main nitrogen source for strain E14 is ammonia and the absolute requirement for methionine may be due to a loss of activity of one or more enzymes in the pathway of methionine biosynthesis.

AdoMet was able to replace the requirement for methionine for growth, although a concentration of 3 times that of methionine was required. This result suggests that strain E14 may have an abnormality in AdoMet biosynthesis. To examine the growth requirement for methionine or AdoMet in more detail, further work has been conducted and has been reported in the following chapter.

Since uptake and utilisation of methionine is vital for *B. fibrisolvens* strain E14, the bacterium must be able to scavenge this amino acid from its growth environment. Stevenson (1979) have reported that *P. ruminicola* subsp. *ruminicola* strain 23 possesses six transport systems for amino acids. Of these, methionine alone has a specific transport system. Smith (1971) has reported that methionine uptake in *S. typhimurium* is significantly reduced by the presence of the methionine analogue α-

methyl-DL-methionine at a concentration of 1000 times greater than that of methionine. The results of competition inhibition experiments reported here show that the methionine analogue α-methyl-DL-methionine inhibited growth of strain E14 on methionine (Figure 4.8). This result suggests that α-methyl-DL-methionine may compete with methionine for binding to a transport molecule such as methionine permease and prevent methionine uptake. This conclusion is supported by the result that α-methyl-DL-methionine did not inhibit growth of strain E14 when the dipeptide alanyl-methionine was present as a methionine source (Figure 4.8). For strain H17c which was tested as a control, α-methyl-DL-methionine had no significant effect on growth (Figure 4.8). A possible explanation is that inhibition of methionine uptake by α-methyl-DL-methionine does not inhibit growth of strain H17c as the strain is able to synthesise methionine *de novo*, and as such, is independent of exogenous methionine.

The results reported here show that the methionine-containing dipeptide alanyl-methionine can replace the requirement for methionine but the non-methionine dipeptide alanyl-alanine can not. These findings suggest that strain E14 is able to obtain methionine from methionine-containing peptides. As mentioned in section 4.1, peptides can be either hydrolysed extracellularly, and the liberated amino acids are taken up by amino acid transport systems (Verheul *et al.*, 1995), or they can be transported into the bacterial cell, where they are hydrolysed intracellularly prior to utilisation of the amino acids (Payne, 1980; Snell, 1980; Verheul *et al.*, 1995). It is also possible that both peptide transport and extracellular hydrolysis are involved in peptide utilisation (Verheul *et al.*, 1995). In the presence of 50 mM ALA, growth of *B. fibrisolvens* strain E14 was inhibited when alanyl-methionine was added to the medium as the source of the essential amino acid methionine (Figure 4.9). A possible explanation is that ALA competes with the alanyl-methionine for uptake and consequently the cells are not able to obtain methionine. Since ALA had no effect on uptake and utilisation of methionine (Figure 4.9), it is likely that the dipeptide was transported intact and hydrolysed intracellularly or hydrolysed as it was transported into

the cell. This is supported by the demonstration that the methionine analogue α-methyl-DL-methionine had no effect on bacterial growth when alanyl-methionine was present in the medium (Figure 4.8). This conclusion is supported by the report of Wallace and McKain (1991) that *B. fibrisolvens* did not produce any extracellular peptidase activity against dipeptide substrates.

The requirement for high concentrations (50 mM) of ALA to inhibit the transport of 0.2 mM alanyl-methionine and at least 2.0 mM alanyl-methionine to overcome this inhibitory effect shows that ALA has a low affinity for a dipeptide transport system in strain E14. Since bacterial growth was not inhibited by ALA when methionine was present (Figure 4.9) or by α-methyl-DL-methionine when alanyl-methionine was present (Figure 4.8), it appears that the strain E14 may have separate transport systems for amino acids and dipeptides.

Kreig and Holt (1984) suggest that *B. fibrisolvens* is able to grow with ammonia as its only nitrogen source, however they indicate that there is some variability between strains. Mannarelli (1988) supports this premise and suggests that the strains presently assigned to the species *B. fibrisolvens* comprise a genetically heterogeneous group of bacteria. Thus the difference in nitrogen requirements between *B. fibrisolvens* strains H17c and E14 reported here is not surprising and probably reflects the genetic diversity in *B. fibrisolvens*. Another possibility is that this requirement in strain E14 is due to a mutation that may have occurred during continual passaging in pure culture. However, the absolute requirement of *B. fibrisolvens* strain E14 for methionine may be a growth-limiting feature *in vivo*. Since ruminal free methionine is low (Salter *et al.*, 1979), a requirement for methionine may reduce its competitiveness in the rumen and prevent this strain achieving a high population density. These questions may be addressed in fermenter trials of mixed cultures *in vitro*.

# CHAPTER 5

# CHAPTER 5

## Biosynthesis of Methionine and S-Adenosylmethionine in *Butyrivibrio fibrisolvens* Strains E14 and H17c

### 5.1 Introduction

In the previous chapter I have reported that *B. fibrisolvens* strain E14 has an absolute requirement for methionine or S-adenosylmethionine (AdoMet) for growth. This limitation may be due to the inability of the strain to synthesise methionine, AdoMet or both.

#### 5.1.1 Methionine biosynthesis

Methionine was discovered in 1922 by Muller. This sulphur-containing amino acid is unique in that it is required not only for incorporation into protein, but also for initiation of protein biosynthesis. It is a precursor of S-adenosylmethionine the universal methyl donor, and of the polyamine spermidine. Methionine is also involved more directly in the synthesis of other substances; for example it is the source of both the methyl group and the sulphur atom of the thiazole component of thiamine (Smith, 1971; Old *et al.*, 1991).

Results of isotope competition studies with *E.coli* have shown that methionine is a member of the aspartate family of amino acids which also includes lysine and threonine (Sokatch, 1969). In terms of building blocks, methionine is the most expensive amino acid to synthesise (Old *et al.*, 1991). Two alterative pathways have been proposed to account for sulphur incorporation into methionine in plants and microorganisms (Kerr, 1971). One (transsulphuration) is that sulphur is initially incorporated into cysteine and then transferred to homocysteine via cystathionine (Figure 5.1). The other (direct

sulphydrylation) is that methylmercaptan, attaches directly to a 4-carbon acceptor to form methionine.

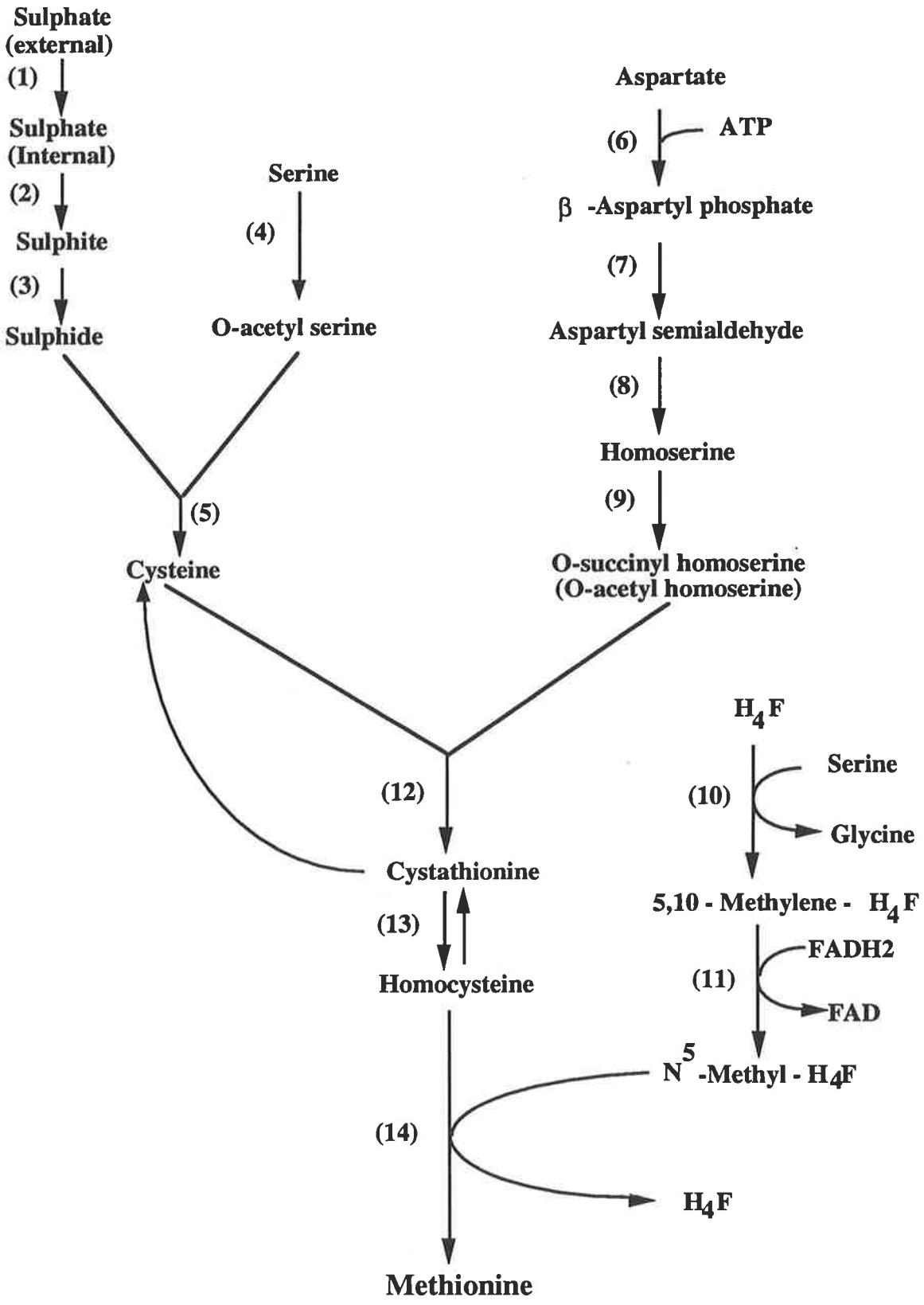
### 5.1.1.1 Transsulphuration pathway of methionine biosynthesis (common pathway)

The transsulphuration pathway of methionine biosynthesis in the Enterobacteriaceae is quite complex and highly branched with both divergent and convergent pathways (Saint-Girson, 1988). The methionine biosynthetic genes (met genes) are all repressed by the addition of methionine to the growth medium. The level of repression varies for the different met genes. These differences can be explained by the fact that the met genes are not arranged in a single operon, but scattered throughout the respective chromosomes (Old *et al.*, 1991). The transsulphuration pathway of methionine biosynthesis can be divided into four steps (Figure 5.1) as follows:

#### 5.1.1.1.1 The synthesis of cysteine from sulphate (Cysteine biosynthetic pathway)

Unlike animals, microorganisms and plants contain a cysteine biosynthetic pathway (Kredich and Tomkins, 1966; Satio *et al.*, 1993). The pathway is branched and convergent with sulphate uptake and reduction comprising one arm (Figure 5.1, reactions 1 to 3) and formation of O-acetyl-L-serine the other (Figure 5.1, reaction 4; Peck, 1961; Cherest and Surdin-Kerjan, 1992). Uptake of sulphate is mediated by a specific permease (Smith, 1971). Both *E. coli* and *Salmonella typhimurium* have an efficient sulphate permease which also transports thiosulphate (Dreyfuss, 1964). The activity of the sulphate transport system of *E. coli* is subject to inhibition by cysteine (Smith, 1971). O-acetyl serine is synthesised from acetyl coenzyme A and serine by the enzyme serine acetyl transferase (E.C. 2.3.1.30). Cysteine is a potent inhibitor of this enzyme. Thus, cysteine regulates its pathway through feedback inhibition of its carbon skeleton precursor (Kredich and Tomkins, 1966). The final reaction to produce cysteine is that between sulphide and O-acetyl-serine (Figure 5.1, reaction 5). This convergent step is catalysed by O-acetylserine sulphydrylase (cysteine synthase; E.C. 4.2.99.8) (Byrne *et al.*, 1988). Cysteine can also be synthesised by serine sulphydrylase

**Figure 5.1 The pathway of methionine biosynthesis (transsulphuration).**



from free serine and hydrogen sulphide (Sokath, 1969).


However, in *E. coli* and *S. typhimurium*, as well as in plants and other microorganisms, O-acetylserine, rather than serine, is the immediate precursor of the carbon moiety of cysteine (Kredich and Tomkins, 1966).

#### 5.1.1.1.2 The synthesis of O-succinyl homoserine from aspartate

One arm of the pathway of methionine biosynthesis is production of O-succinyl homoserine from aspartate (Figure 5.1, reactions 6 to 9). The conversion of aspartate and ATP to β-aspartylphosphate is catalysed by aspartokinase (E.C. 2.7.2.4). Aspartate semialdehyde dehydrogenase (E.C. 1.20.2.11) catalyses the reversible, substrate-dependent reduction of β-aspartylphosphate to aspartate semialdehyde (Saint-Girons *et al.*, 1988). The reduction of aspartate semialdehyde to homoserine is catalysed by homoserine dehydrogenase (E. C. 1.1.1.3). Homoserine is the common precursor of threonine and isoleucine as well as methionine (Soda, 1987). O-succinyl homoserine, the first specific precursor of methionine, is produced by acylation of homoserine with succinyl CoA by homoserine transsuccinylase (succinyl-CoA: L-homoserine O-succinyltransferase; E.C. 2.3.1.46). The activity of homoserine-O-transsuccinylase is subject to feedback inhibition by methionine and its derivative, AdoMet, either separately or, more efficiently, in combination (Smith, 1971; Saint-Girson, 1988).

#### 5.1.1.1.3 The synthesis of homocysteine

Homocysteine is the immediate precursor of methionine. It is synthesised in enteric bacteria through transsulphuration by two reactions (Figure 5.1, reactions 12 and 13). In the first reaction the succinyl group of O-succinyl homoserine is replaced by cysteine in a reaction catalysed by cystathionine-γ-synthetase (E.C. 4.2.99.9) to give thioether cystathionine. In the second reaction cystathionine is cleaved by cystathionine β-lyase (β-cystathionase) (E.C. 4.4.1.8) and homocysteine is produced

(Smith, 1971). The synthesis of cystathionine by cystathionine γ-synthetase and its subsequent cleavage by β-cystathionase is the major route of homocysteine synthesis in bacteria (Smith, 1971; Yamagata, 1980).

#### 5.1.1.1.4 Methylation of homocysteine

Methylation of homocysteine represents the terminal step in methionine biosynthesis (Figure 5.1, reaction 14) and involves a crossroads in the methionine and folate pathways (Banerjee and Matthews, 1990; Old *et al.*, 1991). In the folate pathway (Figure 5.1, reactions 10 and 11) serine serves as the donor of 1-carbon to form L-$N^5$-methyltetrahydrofolate from tetrahydrofolate (Hatch *et al.*, 1961). The pathway comprises two reactions (Old *et al.*, 1991). The first reaction involves the conversion of serine and tetrahydrofolate to glycine and 5,10-methylenetetrahydrofolate (Figure 5.1, reaction 10) and the reaction is catalysed by serine transhydroxymethylase (E.C. 2.1.2.1). In the second reaction, 5,10-methylenetetrahydrofolate is reduced to 5-methylteterahydrofolate (Figure 5.1, reaction 11) by the catalytic activity of 5,10-methylenetetrahydrofolate reductase (E.C. 1.7.99.5).

Methionine synthase catalyses the transfer of methyl group from $N^5$-methyltetrahydrofolate to homocysteine to form methionine and tetrahydrofolate (Figure 5.1, reaction 14; Figure 5.2). The enzyme is so-called long-phase enzyme, because it has an increased activity in the period of logarithmic growth of rapidly proliferating cell populations and low activity during steady-state growth (Sauer, 1987). Two forms of methionine synthase exist in nature. One is cobalamin-dependent methionine synthase which contains a cobalamin prosthetic group. The other is cobalamin-independent methionine synthase.

Cobalamin-dependent methionine synthase (EC 2.1.1.13) is the product of the *metH* gene. The enzyme from *E.coli* is an unusually large, monomeric protein with a mass of

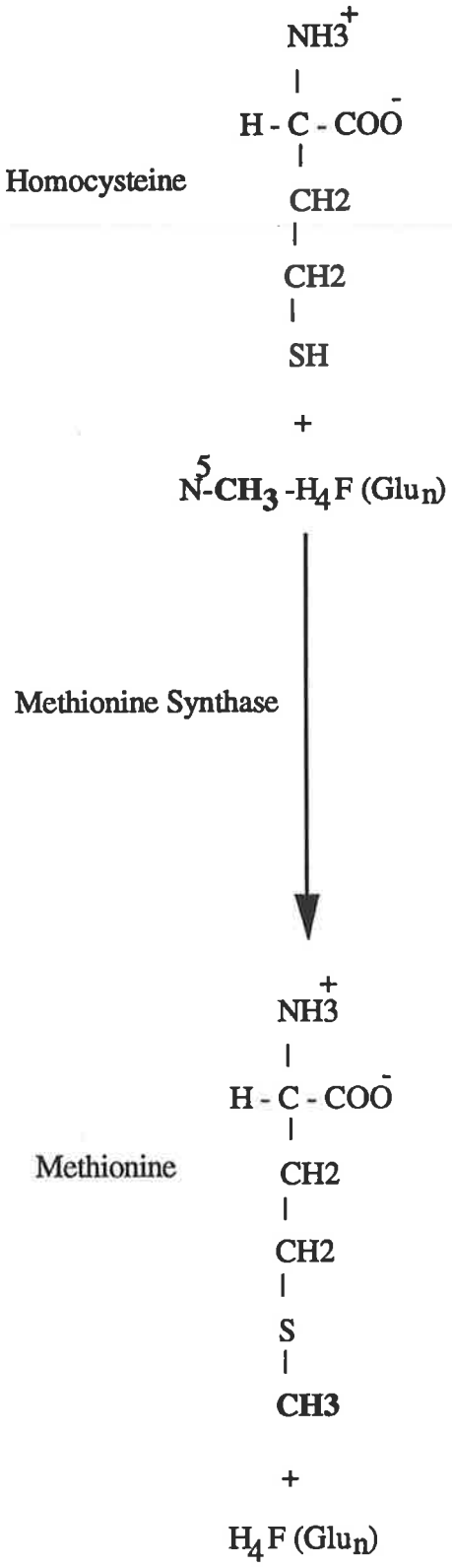



**Figure 5.2 The methylation of homocysteine to methionine**

136.1 kDa. Although the apoenzyme is synthesised in the absence of vitamin $B_{12}$, the enzyme is only active when it is noncovalently bound to a cobalamin prosthetic group. The enzyme uses this prosthetic group to catalyse a methyl group transfer from $N^5$-methyltetrahydrofolate ($CH_3$-$H_4$folate) to homocysteine, and this is achieved indirectly as the sum of the following two half-reactions.

$$\text{E. cob(I)alamin} + CH_3\text{-}H_4\text{folate} \longrightarrow \text{E. Methylcobalamin} + H_4\text{folate} \quad (1)$$

$$\text{E. Methylcobalamin} + \text{Homocysteine} \longrightarrow \text{Methionine} + \text{E.cob(I)alamin} \quad (2)$$

Cob(I)alamin is one of the most potent nucleophiles known, and is essential for the function of the cobalamin-dependent enzyme. It acts as a nucleophile in the displacement of the methyl group from $N^5$-methyltetrahydrofolate (González *et al.*, 1992). The highly reactive, enzyme-bound cob(I)alamin nucleophile removes the methyl group from $CH_3$-$H_4$folate (reaction 1), and cob(I)alamin then acts as the leaving group and homocysteine accepts the methyl group to form methionine (reaction 2; Taylor and Hanna, 1970). Under *in vitro* assay conditions, the reaction catalysed by the cobalamin-dependent enzyme displays an absolute requirement for catalytic amounts of AdoMet and a reducing system (Foster *et al.*, 1964a). In studies of coupled reduction/methylation reactions, Banerjee *et al.* (1990a) elucidated the absolute requirement for AdoMet as a methyl donor *in vitro* activation systems. The role of this cofactor is to convert the catalytically inactive cob(II)alamin to the catalytically active methyl-cob(I)alamin (Figure 5.3).

The highly unfavourable reduction of cob(II)alamin to cob(I)alamin ($E^{\circ\prime}$ = -526 mV vs the standard hydrogen electrode) can be coupled to the highly favourable methylation by the methylsulphonium cation of AdoMet. Since AdoMet is generated from methionine and ATP, remethylation of homocysteine with this agent would be energetically very costly and biosynthetically futile. Instead, AdoMet is a

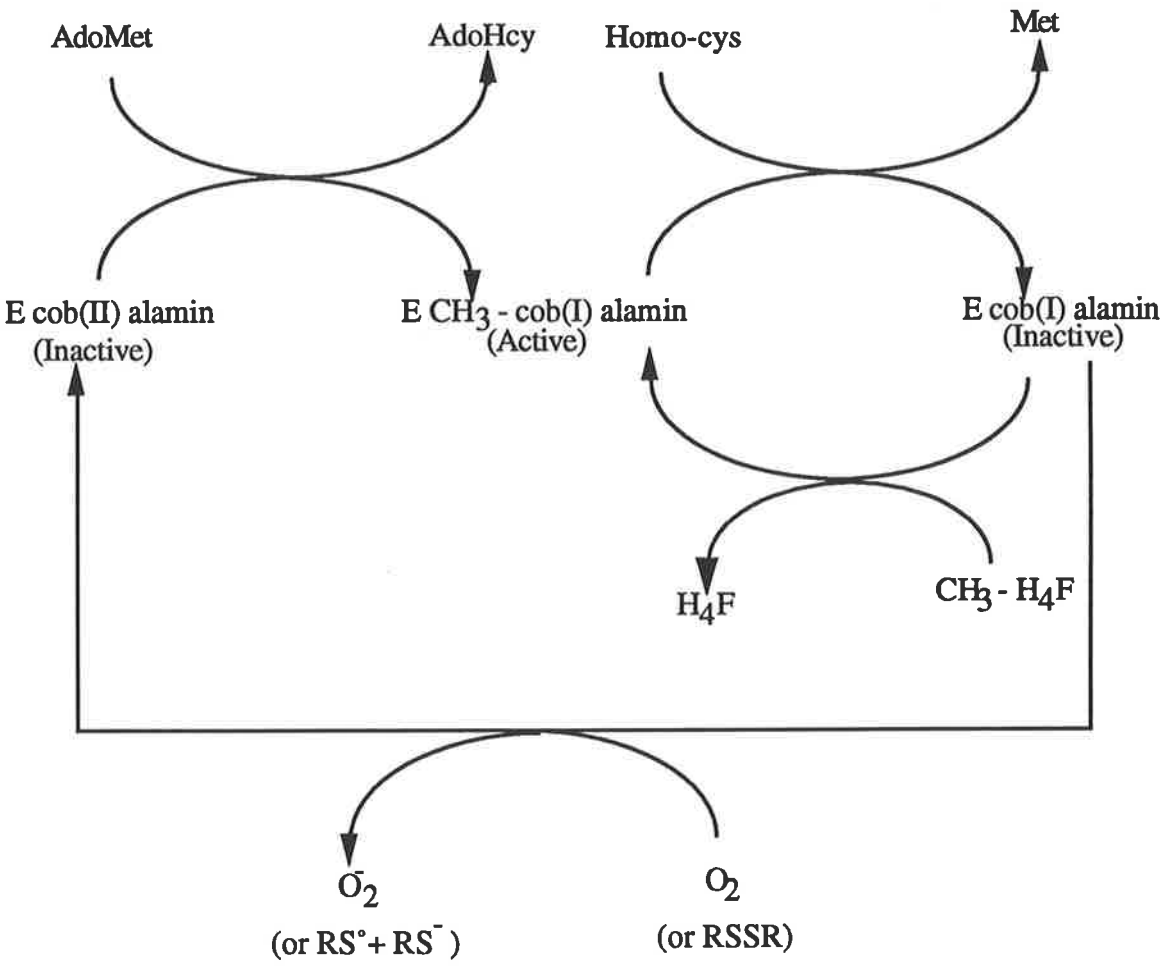



**Figure 5.3 Interconversion of forms of cobalamin-dependent methionine synthase**

thermodynamically activating methyl donor for methionine synthase and repeated turnovers with $CH_3$-H4folate as the methyl donor should occur following a single reductive methylation by AdoMet. However, in the absence of AdoMet, the production of methionine ceases over the course of several minutes. The implication is that the cob(I)alamin is becoming oxidised to co(II)alamin during the normal course of turnover, and in the absence of AdoMet the enzyme cannot be reductively activated back to the turnover-competent methylcobalamin form (Banerjee and Matthews, 1990; Banerjee *et al.*, 1990b). The methyl group of methylcobalamin is transferred to homocysteine and cob(I)alamin is produced. The highly reactive cob(I)alamin is rapidly remethylated by $CH_3$-H4folate. This latter process accounts for the bulk of remethylation but when catalysis is intercepted by an oxidant and enzyme-bound cob(I)alamin is converted to co(II)alamin, reactivation by remethylation with AdoMet is required (Banerjee *et al.*, 1990b).

Cobalamin-independent methionine synthase (EC 2.1.1.14) is the product of the *metE* gene. The enzyme was first purified 15-fold by Foster *et al.* (1961). The *E. coli* enzyme has absolute requirements for Pi and $Mg^{+2}$ for activity. The *metE* enzyme is unique among folate-dependent enzymes in its absolute requirement for a triglutamate form of $N^5$-methyltetrahydrofolate as a methyl donor (Whitfield and Weissbach, 1968; Whitfield *et al.*, 1970). In *E. coli*, a monoglutamate form of $N^5$-methyltetrahydrofolate cannot replace the triglutamate folate derivative as methyl donor. The specificity exhibited by the *E. coli metE* enzyme would appear to be universal as the cobalamin-independent transmethylase from different organisms are all unable to use the monoglutamate form of $N^5$-methyltetrahydrofolate. Unlike the *metH* enzyme there is no evidence for the formation of a methylated *metE* enzyme. Instead it would appear that the $N^5$-methyltetrahydrofolate (triglutamate form) is stoichiometrically bound to the enzyme at the catalytic site (Old *et al.*, 1991). González *et al.* (1992) discovered that there is an extremely reactive thiol group in the *metE* enzyme from *E. coli* which was shown to be cysteine 726. They speculated that the thiol group of this reactive

residue possibility functions as an intermediate methyl acceptor in catalysis, analogous to the role of cobalamin in the reaction catalysed by *metH*.

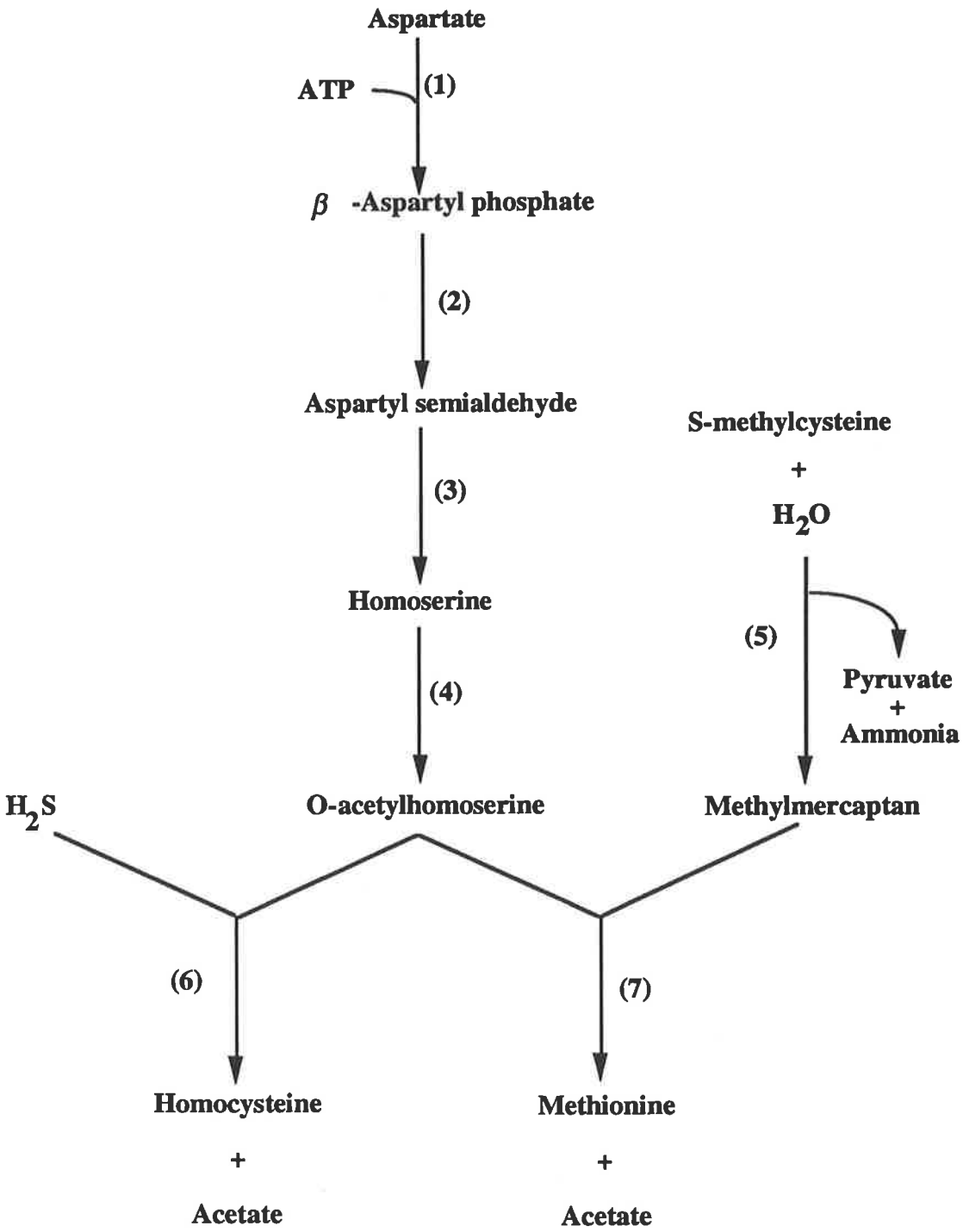
Generally, organisms that are able to synthesise or take up cobalamin utilise only the cobalamin-dependent enzyme (e.g. mammals) while those which lack this ability contain the cobalamin-independent transmethylase (e.g. plants, fungi and certain prokaryotes) (Flavin, 1975). Enterobacteria such as *E. coli*, *S. typhimurium* and *Aerobacter aerogenes* are unusual in that they contain both methylating systems (Foster *et al.*, 1961, 1964b; Morningstar and Kisliuk, 1965). Studies with mutants led Foster *et al.* (1964b) to the conclusion that the cobalamin-independent system was the primary one for synthesis of methionine, and that the cobalamin-dependent system was a secondary system which was evoked either by provision of cobalamin or mutation resulting in loss of the primary system.

#### 5.1.1.2 Direct sulphydrylation pathway of methionine biosynthesis

The direct sulphydrylation pathway of methionine biosynthesis (Figure 5.4) provides an alternative pathway for the synthesis of methionine from methylmercaptan without going through cysteine, cystathionine and homocysteine as intermediates (Kerr, 1971; Yamagata and Takeshima, 1976). O-acetylhomoserine (OAH) sulphydrylase (EC 4.2.99.10) catalyses the synthesis of methionine directly from O-acetylhomoserine and methylmercaptan ($CH_3SH$; Figure 5.4, reaction 7). OAH sulphydrylase also catalyses the synthesis of homocysteine using hydrogen sulphide as sulphydryl donor (Figure 5.4, reaction 6; Kerr, 1971; Yamagata, 1976).

Therefore, OAH sulphydrylase can function as the enzyme of homocysteine or methionine synthesis depending on metabolic condition of the cell (Yamagata, 1989). Methylmercaptan can be produced from S-methylcysteine by γ-cystathionase (Figure 5.4, reaction 5). Neither methylcysteine nor methylmercaptan is an intermediate in the "common" pathway of methionine formation from sulphate (Moore and Thompson,

**Figure 5.4 Direct sulphydrylation pathway of methionine biosynthesis**



1967). Since there is no mechanism for synthesis of S-methylcysteine or methylmercaptan which does not require prior synthesis of methionine, *de novo* biosynthesis of methionine is not likely through this route (Kerr, 1971). However, under conditions when methionine biosynthesis is inadequate to meet the needs of cells, methionine may be formed from methylcysteine through the intervention of methylcysteine lyase and O-acetylhomoserine sulphydrylase (Smith and Thompson, 1969).

### 5.1.2 Biosynthesis of S-adenosylmethionine

S-adenosylmethionine (AdoMet) was first discovered in 1953 by Cantoni. It is a high energy, positively charged, sulphonium compound (Figure 5.5) which is energetically capable of donating its methyl group, or following decarboxylation, its polyamine (aminopropyl) group to appropriate acceptors (Stoner and Eisenberg, 1975).

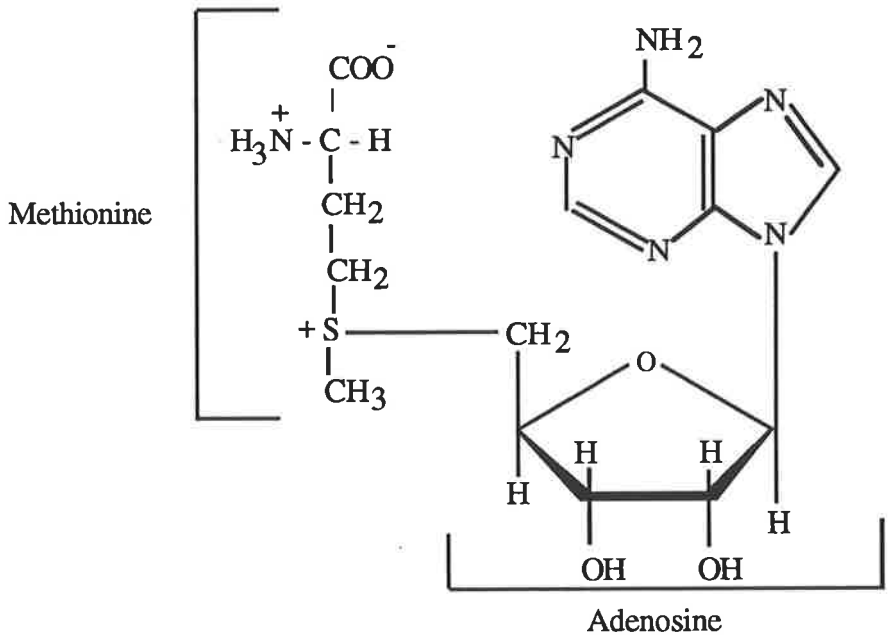



**Figure 5.5 The structure of S-adenosylmethionine**

AdoMet functions in diverse biological reactions such as the synthesis of the polyamines, spermine and spermidine, the conversion of unsaturated fatty acids to cyclopropane fatty acids, and in biosynthesis of methionine, methylmethionine and methylene tetrahydrofolate (Satishchandran *et al.*, 1993). Unlike methionine itself, AdoMet is characterised by its ability to function as a methyl donor for numerous enzymatic transmethylation reactions (Cantoni, 1953; Tabor and Tabor, 1976). Since transmethylation involves modification of DNA, RNA, proteins, lipids and polysaccharides as well as numerous other metabolites, one can hardly overestimate the importance of AdoMet to cellular metabolism (Kotb and Kredich, 1985).

S-adenosylmethionine synthetase (ATP:L-methionine S-adenosyltransferase, EC 2.5.1.6) catalyses the enzymatic synthesis of AdoMet from L-methionine and ATP (Cantoni, 1953). The reaction occurs in animals, plants and microbial systems and involves the transfer of the adenosyl moiety of ATP to one pair of free electrons of the sulphur atom of L-methionine.

$$\text{Methionine + ATP} \xrightarrow{Mg^{+2},\ K^{+1}} \text{AdoMet + PPi + Pi}$$

The reaction is unusual in several respects. In the first place, it represents the only example of the utilisation of the energy of the phosphate bond of ATP for the generation of an energy-rich sulphonium compound. Next the three phosphates of ATP are cleaved in an oriented nonrandom fashion to generate pyrophosphate and inorganic phosphate; pyrophosphate originates from the $\alpha$ and $\beta$ phosphate of ATP and inorganic phosphate from $\gamma$ phosphate (Chiang and Cantoni, 1977).

*B. fibrisolvens* strain E14 is absolutely dependent on the presence of methionine or AdoMet for growth in the presence of $NH_4Cl$. This suggests that there may be a block in the biosynthetic pathway of methionine and/or a limitation in AdoMet biosynthesis. The experiments reported in the present chapter were therefore carried out to

investigate these possibilities.

The growth response of strain E14 was tested with intermediates of the methionine biosynthetic pathway. In most systems, serine is utilisecl as the donor of 1-carbon units for synthesis of the methyl group of methionine (Figure 5.1, reactions 10, 11 and 14; Hatch *et al.*, 1961), but formaladehyde and betaine have also been reported to act as 1-carbon donors in methionine biosynthesis (Hatch *et al.*, 1961; Finkelstein, 1990). Homocysteine is required as a methyl group acceptor (Figure 5.1, reaction 14) in all systems (Hatch *et al.*, 1961). Therefore, the effect of serine, betaine and formaldehyde was examined on growth of strain E14 in NB medium containing $NH_4Cl$ plus L-homocysteine. The effect of dl, L-$N^5$-methyl-$H_4$folate-$Glu_1$, the immediate methyl group donor (Figure 5.1, reaction 14) in a system catalysed by cobalamin-dependent methionine synthase was also tested.

The strain was also tested for cobalamin-dependent methionine synthase activity. Old *et al.* (1990) have reported that the N-terminal third of the cobalamin-dependent methionine synthase (*metH*) genes from *E. coli* K-12 and *S. typhimurium* LT2 are 92% identical. To examine whether there is homology between *metH* genes from *E.coli* and *B. fibrisolvens*, oligonucleotide primers were designed from published sequences of the *E. coli metH* gene, the sequences were amplified by the polymerase chain reaction (PCR) and *B. fibrsolvens* strains were screened with the PCR product.

Unfortunately I was unable to examine strain E14 for cobalamin-independent methionine synthase activity because the triglutamate form of $N^5$-(methyl-$^{14}$C)-methyltetrahydrofolate was not commercially available. Another approach was to perform intact cell methionine biosynthesis using a radiolabelled precursor of $N^5$-methyltetrahydrofolate. Serine is utilised as the donor of 1-carbon in most systems to form L-$N^5$-methyltetrahydrofolate from tetrahydrofolate (Figure 5.1, reactions 10, 11 and 14; Hatch *et al.*, 1961). Therefore, cells were grown on $^{14}$C-[β-C]-serine to

determine whether they were able to synthesise the triglutamate form of $N^5$-[methyl-$^{14}$C]-methyltetrahydrofolate and use it as a specific substrate for cobalamin-independent methionine synthase to produce $^{14}$C-Methionine. HPLC and paper chromatography of an acid hydrolysate of bacterial cells were performed and radioactivity of amino acid fractions was measured. To examine whether there is homology between the *metE* genes from *E.coli* and *B. fibrisolvens*, oligonucleotide primers were designed from the published sequences of the *E. coli metE* gene, the sequences were amplified by PCR and *B. fibrsolvens* strains were screened with the PCR product.

Since the requirement for methionine by *B. fibrisolvens* strain E14 can be replaced by AdoMet (section 4.3.4), the strain may lack AdoMet synthetase activity. Two AdoMet synthetases have been found in *E. coli*. One is the product of the *metK* gene and is essential for growth in minimal media (Markham *et al.*, 1980). The second enzyme is the product of the metX gene and is essential for growth in rich media (Satishchandran *et al.*, 1993). Extracts of *B. fibrisolvens* strain E14 grown in a rich (BHI) or minimal (NB) medium were therefore tested for AdoMet activities. Comparison of known AdoMet synthetase sequences among different species have shown that these proteins have a high degree of similarity and the enzyme has been exceptionally well conserved through evolution (Thomas and Surdin-Kerjan, 1991; Larsson and Rasmuson-Lestader, 1994). To determine whether there is homology between the AdoMet synthetase gene (*metK*) from *E.coli* and *B. fibrisolvens*, oligonucleotide primers were designed from published sequences of the *E.coli metK* gene, the sequence was amplified by PCR and strains of *B. fibrisolvens* were screened with the PCR product.

To determine whether the methyl group of AdoMet was incorporated into newly synthesised bacterial protein, radiotracer experiments using S-adenosyl-L-[$^{14}$C-methyl] methionine followed by SDS-PAGE of bacterial protein were carried out. HPLC analysis of acid hydrolysates of bacterial cells was also performed to determine

whether the methyl group of AdoMet was incorporated into methionine.

## 5.2 Materials and Methods

### 5.2.1 Chemicals

$N^5$-Methyltetrahydrofolate, L-homocysteine, betaine, homoserine, O-succinyl homoserine, (±) L-$N^5$-methyltetrahydrofolate (monoglutamate form, barium salt), cystathionine, dATP, dCTP, dGTP, dTTP, phenylmethylsulphonyl fluoride (PMSF), pentachlorophenol, Dowex 1-$Cl^-$ (200-400 mesh), phenol, 8-hydroxyquinoline, ethidium bromide, and agarose were obtained from Sigma Chemical Company, St. Louis, MO, USA. Bis (3, 5, 5-trimethylhexyl) phthalate was obtained from Fluka Chemika-Biochemika, Switzerland. Cellulose phosphate cation exchange paper P 81 was purchased from Whatman, England. Ficoll 400 was obtained from Pharmacia LKB, Biotechnology AB Uppsala, Sweden.

(±) L-$N^5$-[methyl-$^{14}$C]-methyltetrahydrofolate (monoglutamate form, barium salt), L-[$^{14}$C-methyl]-methionine, S-adenosyl-L-[$^{14}$C-methyl]-methionine and L-[β-$^{14}$C]-serine were obtained from Amersham IL, USA.

Restriction enzymes were obtained from Boehringer Mannheim, Australia. Oligonucleotides were synthesised by the DNA and Protein Chemistry Unit, Waite Institute, Adelaide University.

All other chemicals, of the highest purity available, were obtained from the following sources: Ajax Chemicals, Sydney, Australia; B.D.H. Chemicals Australia Ltd., Boronia and Kilsyth, Australia.

### 5.2.2 Buffers and solutions

#### 5.2.2.1 Extraction buffer for preparation of cell-free extracts for cobalamin-dependent methionine synthase assay

| *Ingredients* | mM |
|---|---|
| Tris.HCl buffer (pH 7.0) | 50.0 |
| Mercaptoethanol | 2.0 |
| PMSF | 1.0 |

PMSF was added to the buffer before use from the stock solution (400 mM in ethanol and stored at -20°C).

#### 5.2.2.2 Extraction buffer for preparation of cell-free extracts for cobalamin-independent methionine synthase assay

| *Ingredients* | mM |
|---|---|
| Phosphate buffer (pH 7.2) | 20.0 |
| Dithiothreitol | 200.0 |
| PMSF | 1.0 |

Phosphate buffer was prepared according to Gomori (1989). PMSF was added to the buffer before use from the stock solution (400 mM in ethanol and stored at -20°C).

#### 5.2.2.3 Extraction buffer for preparation of cell-free extracts for AdoMet synthetase assay

| *Ingredients* | |
|---|---|
| Tris.HCl (pH 7.0) | 125.0 mM |
| 2-Mercaptoethanol | 10.0 mM |
| PMSF | 1.0 mM |
| Glycerol | 10 % (w/v) |

PMSF was added to the buffer before use from the stock solution (400 mM in ethanol and stored at -20°C).

#### 5.2.2.4 Buffers for HPLC

##### 5.2.2.4.1 Buffer A

*Ingredients*

Trisodium citrate, $2H_2O$ ..... 19.6 g

Pentachlorophenol 5.0 mg/ml ethanol ..... 30.0 µl

The ingredients were dissolved in 1 litre of ammonia-free RO water and the pH was adjusted to 3.2 with concentrated HCl. The solution was filtered through Millipore Durapore filter HVLP0700 and stored under nitrogen until use.

##### 5.2.2.4.2 Buffer B

*Ingredients*

Trisodium citrate, $2H_2O$ ..... 19.6 g

Pentachlorophenol 5.0 mg/ml in ethanol ..... 30 µl

NaCl ..... 58.4 g

The ingredients were dissolved in 1 litre of ammonia-free RO water and the pH was adjusted to 6.2 with concentrated HCl. The solution was filtered through Millipore Durapore filter HVLP0700 and stored under nitrogen until use.

#### 5.2.2.5 Ninhydrin solution

*Ingredients*

Ninhydrin ..... 0.1 g

n-butanol ..... 90.0 ml

RO water ..... 10.0 ml

#### 5.2.2.6 Phenol

Phenol was melted at 68°C and 8-hydroxyquinoline was added to a concentration of 0.1%. The phenol was equilibrated with an equal volume of 1.0 M Tris.HCl pH 8.0 overnight with stirring. The upper aqueous phase was removed and an equal volume of 0.1 M Tris.HCl pH 8.0 was added and equilibrated until the pH of the aqueous phase was 7.6. The equilibrated phenol was stored under the 0.1 M Tris.HCl buffer at 4°C.

#### 5.2.2.7 50 x TAE buffer

| *Ingredients* | Per litre |
|---|---|
| Tris base (Trizma) | 242.0 g |
| Glacial acetic acid | 57.1 ml |
| EDTA (0.5 M, pH 8.0) | 100.0 ml |

#### 5.2.2.8 6 x Agarose gel loading buffer

| *Ingredients* | Per 10 ml |
|---|---|
| Bromophenol blue | 0.025 |
| Xylene cyanol | 0.025 |
| Glycerol | 3.0 ml |

#### 5.2.2.9 10 x PCR buffer

| *Ingredients* | mM |
|---|---|
| Tris-HCl (pH 8.3) | 100.0 |
| KCl | 500.0 |
| $MgCl_2$ | 15.0 |

#### 5.2.2.10 dNTP mixture

| *Ingredients* | mM |
|---|---|
| dATP | 1.25 |
| dCTP | 1.25 |

dGTP.....1.25

dTTP.....1.25

#### 5.2.2.11 20 x SSC

| *Ingredients* | g/l |
|---|---|
| Sodium chloride | 175.3 |
| Sodium citrate | 88.2 |

The solution was adjusted to pH 7.0 with 10 M NaOH.

#### 5.2.2.12 100 x Denhardts solution

| *Ingredients* | g/100 ml |
|---|---|
| Ficoll 400 | 2.0 |
| Polyvinyl pyrolidine (PVP) | 2.0 |
| Bovine serum albumin (BSA) | 2.0 |

#### 5.2.2.13 Prehybridisation solution

| *Ingredients* | Per 100 ml |
|---|---|
| 20 x SSC | 30.0 ml |
| 100 x Denhardts | 5.0 ml |
| Sodium pyrophosphate | 0.05 g |
| SDS (10 % w/v) | 5.0 ml |
| Salmon sperm DNA (10 mg/ml) | 0.1 ml |

Salmon sperm DNA was boiled and added to the solution before use.

#### 5.2.2.14 Hybridisation solution

| *Ingredients* | Per 100 ml |
|---|---|
| 20 x SSC | 30 ml |

100 x Denhardts.....1ml

Sodium pyrophosphate.....0.05 g

### 5.2.3 Media and growth conditions

#### 5.2.3.1 Media and growth conditions for *B. fibrisolvens*

NB and BHI media for growth of *B. fibrisolvens* were prepared as described in chapter 2. The growth conditions were as outlined in section 2.2.9. Data from growth curves determined during experiments reported in chapter 2 (Figures. 2.11 and 2.12) were used as a guide to the growth rate for the cultures and were referred to when determining the maximum absorbances or to harvest cells for enzyme assays. For preparation of cell extracts for enzyme assays, batch cultures were grown in 150 ml serum bottles sealed with butyl rubber septa (Pierce Chemical Company, Rockford, USA). Growth in these cultures was followed with a parallel Hungate tube culture (10 ml) and measurement of the optical density at 600 nm.

#### 5.2.3.2 Media and growth conditions for *E. coli*

##### 5.2.3.2.1 Luria Broth (LB) medium

| *Ingredients* | g/100 ml |
|---|---|
| Tryptone | 1.0 |
| Yeast Extract | 0.5 |
| NaCl | 1.0 |

The medium was brought to a final volume of 100 ml, covered and autoclaved.

##### 5.2.3.2.2 Defined medium

The medium was prepared as described by Hatch *et al.* (1961) except that NaCl, yeast extract and $CaCl_2$ were also added.

| *Ingredients* | g/100 ml |
|---|---|
| $K_2HPO_4$ | 0.7 |
| $KH_2PO_4$ | 0.3 |
| Sodium citrate | 0.045 |
| $MgSO_4$ | 0.005 |
| NaCl | 0.05 |
| DL-aspartic acid | 0.1 |
| Yeast extract | 0.01 |
| $CaCl_2$ | 0.015 |
| Glucose[a] | 0.50 |

*a*. Glucose was autoclaved separately and then added to the autoclaved medium.

#### 5.2.3.2.3 Culture conditions

Cultures of *E. coli* HB101 were initiated from glycerol stocks held at -80°C. The cultures were streaked onto LB medium plates and grown aerobically at 37°C. Single colonies were inoculated aseptically and aerobically into Erlenmeyer flasks containing liquid medium. Cultures were incubated at 37°C with shaking at 220 rpm in an orbital shaker.

### 5.2.4 Preparation of samples for enzyme assays

Cultures were harvested at mid log of growth and cell-free extracts were prepared by passing the samples through a French pressure cell as outlined in section 3.2.4. The extraction buffers for preparing cell-free extracts for assaying cobalamin-dependent methionine synthase, cobalamin-independent methionine synthase and AdoMet synthetase specific activities were as outlined in sections 5.2.2.1 and 5.2.2.2, 5.2.2.3 respectively. Protein concentration of cell-free extracts were measured by the method of Bradford (1976) using Coomassie Brilliant Blue G-250, with BSA as standard (section 3.2.5). This value was used to calculate the specific activity of each enzyme,

expressed per mg of protein in the cell-free extract.

### 5.2.5 Preparation of cellulose phosphate cation exchange paper P 81

The method was as described by Chou and Lombardini (1972). The cation exchange paper was cut as 3-cm diameter disks. The paper disks were converted to the hydrogen form by immersion in 4% acetic acid for 20 min and then washed with water 5 times by decantation. The paper discs were separated and dried before use.

### 5.2.6 Assay of cobalamin-dependent methionine synthase

The method was modified from Taylor and Weissbach (1971). The conversion of L-homocysteine to L-[$^{14}$C-methyl] methionine was carried out in the system designated as the reaction mixture in Table 5.1. The reaction was started by addition of cell-free extracts and the reaction mixture was immediately flushed with nitrogen and overlayered with 50 µl of di "isononyl" phthalate as recommended by Garras *et al.* (1991). The incubation was carried out at 37°C in the dark for 20 min. The reaction was terminated by the addition of 0.8 ml ice-cold water and the entire diluted mixture was immediately applied onto a 0.5 x 3.0 cm column of Dowex 1-Cl$^-$ (200-400 mesh) equilibrated with water. The column was then washed with 2 x 1.0 ml of water to remove L-[$^{14}$C-methyl]-methionine from untreated L-N$^5$-[methyl-$^{14}$C]-methyltetrahydrofolate which remains adsorbed to the resin. The total column effluent (3.0 ml) was collected in a scintillation vial, and the amount of radioactivity was measured in a Beckman LS-3801 liquid scintillation spectrophotometer, using BCS as a counting fluid. Controls in which cell-free extracts were replaced with boiled cell-free extracts were run with each set of assays to check for background levels of radioactivity. Radioactivity of a radioactive standard containing 10 µl of 10 mM (±) L-N$^5$-[methyl-$^{14}$C]-methylH4-folate (2 Ci/mole) was also measured. Under the assay conditions employed, the amount of methionine formed was linear with respect to the incubation time and the amount of protein.

**Table 5.1 Ingredients of cobalamin-dependent methionine synthase assay mixture**[a]

| Volume (µl) | Ingredients | Final concentration (mM) |
|---|---|---|
| 50 | 8 mM L-homocysteine[b] | 2.00 |
| 10 | 10 mM (±) L-$N^5$-[methyl-$^{14}$C]-methyl$H_4$-folate[c] (2 Ci/mole) | 0.50 |
| 10 | 1 mM Vitamin $B_{12}$ | 0.05 |
| 10 | 1 mM S-adenosylmethionine | 0.25 |
| 8 | 5 M 2-mercaptoethanol | 0.20 |
| 112-x | Extraction buffer | |
| x | Cell-free extract (100 µg protein) | |

*a*. Total volume of the assay mixture was 0.2 ml.

*b*. The solution was neutralised with 2.0 M NaOH.

*c*. (±) L-$N^5$-[methyl-$^{14}$C]-methyltetrahydrofolate was dissolved in 10 mM ascorbic acid and stored as 200 µl aliquots under nitrogen at -20°C until use.

### 5.2.7 Assay of cobalamin-independent methionine synthase

The method was as described by González *et al.* (1992) except that monoglutamate form of $N^5$-methyltetrahydrofolate was used. The assay was carried out in the system designated as the reaction mixture in Table 5.2. The assay mixture lacking $N^5$-methyltetrahydrofolate was incubated at 37°C for 2 min, and the reaction was initiated by the addition of $N^5$-methyltetrahydrofolate. After incubation at 37°C for 15 min, the reaction was terminated by the addition of 0.95 ml of ice-cold water and the entire diluted mixture was immediately applied onto a 0.5 x 3.0 cm column of Dowex 1-$Cl^-$ (200-400 mesh) equilibrated with water. The column was then washed with 2 x 1.0 ml of water to remove L-[$^{14}$C-methyl] methionine from unreacted L-$N^5$-[methyl-$^{14}$C]-methyltetrahydrofolate which remained adsorbed to the resin. The total column effluent liquid (3.0 ml) was collected in a scintillation vial, and the amount of radioactivity was measured in a Beckman LS-3801 liquid scintillation spectrophotometer, using BCS as a counting fluid. Controls in which cell-free extracts were replaced with boiled cell-free extracts were run with each set of assays to check for background levels of radioactivity. Radioactivity of a radioactive standard containing 3.3 µl of 1.0 mM 10 mM (±) L-$N^5$-[methyl-$^{14}$C]-methylH4-folate (2 Ci/mole) was also measured.

### 5.2.8 Assay of S-adenosyl methionine synthetase

The method was modified from Markham *et al.* (1980). The optimal enzyme assay procedure described in this section was determined in preliminary assays with the cell-free extracts of *B. fibrisolvens* strain H17c grown in NB medium containing 70 mM $NH_4Cl$. The conversion of L-methionine to S-adenosylmethionine was carried out in the system designated as the reaction mixture in Table 5.3. Reactions were initiated by addition of cell-free extracts. The samples were incubated at 37°C for 20 min. The reactions were terminated by addition of 20 µl of 2 M $HClO_4$ in an ice bath as recommended by Okada *et al.* (1981). The resulting precipitate was removed by centrifugation in a bench top centrifuge at low speed for 5 min. A 100 µl aliquot of

**Table 5.2 Ingredients of cobalamin-independent methionine synthase assay mixture[a]**

| Volume (µl) | Ingredients | Final concentration (mM) |
|---|---|---|
| 5 | 20 mM L-homocysteine[b] | 2.00 |
| 3.3 | 1.0 mM (±) L-$N^5$-[methyl-$^{14}$C]-methyl$H_4$-folate[c] (2 Ci/mole) | 0.066 |
| 5 | 1 mM $MgSO_4$ | 0.10 |
| 36.7-x | Extraction buffer | |
| x | Cell-free extract (100 µg protein) | |

*a*. Total volume of the assay mixture was 50 µl.

*b*. The solution was neutralised with 2.0 M NaOH.

*c*. (±) L-$N^5$-[methyl-$^{14}$C]-methyltetrahydrofolate was dissolved in 10 mM ascorbic acid and stored as 200 µl aliquots under nitrogen at -20°C until use.

**Table 5.3 Ingredients of AdoMet synthetase assay mixture[a]**

| Volume (µl) | Ingredients | Final concentration (mM) |
|---|---|---|
| 5.0 | 2.0 M KCl | 100.0 |
| 5.0 | 0.2 M $MgCl_2$ | 10.0 |
| 10.0 | 10 mM L-[methyl-$^{14}$C] Methionine (2 Ci/mole) | 1.0 |
| 5.0 | 0.2 M ATP | 10.0 |
| 5.0 | 0.16 M Glutathione[b] (reduced form) | 8.0 |
| 70.0 - x | Extraction buffer | |
| x | Cell-free extract (100 µg protein) | |

*a*. total volume of the assay mixture was 0.1 ml.

*b*. Glutathione was added as suggested by Pajares *et al.* (1992).

each supernatant was applied on a Whatman P 81 cellulose paper disk (prepared as described in section 5.2.5) previously placed on pins inserted into a cork ring, and dried under an infrared lamp. The dry papers were then washed on an Ace Buchner funnel, with fibrous glass fit (Aldrich Chemical Company) by suction filtration with 500 ml water to remove the unreacted L-[$^{14}$C-methyl]-methionine from S-adenosyl-L-[$^{14}$C-methyl]-methionine which remains adsorbed to the cellulose phosphate paper. Controls in which cell-free extracts were replaced with boiled cell-free extracts were run with each set of assays to check for background levels of radioactivity. The paper discs were then placed in scintillation vials and counted under 5 ml of BCS scintillation liquid in a Beckman LS-3801 liquid scintillation spectrophotometer. Radioactivity of a radioactive standard containing 10 µl of 10 mM L-[methyl-$^{14}$C] Methionine (2 Ci/mole) was also measured. Under the assay conditions employed, the amount of AdoMet formed was linear with respect to the incubation time and the amount of protein.

### 5.2.9 Calculation of enzyme specific activities

The specific activities of methionine synthase and AdoMet synthetase were calculated according to the following equations (Wolf *et al.*, 1983):

$$\text{Catalytic activity} = \frac{C_n \times V}{\eta \times 2.22 \times 10^{12} \times 10^{-9} \times X_0 \times v \times \Delta t} = \text{nmol product} \cdot \text{min}^{-1}$$

$$\text{Specific activity} = \frac{\text{Catalytic activity}}{\text{mg protein in assay mixture}} = \text{nmol product} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$$

| | |
|---|---|
| $C_n$ | net counting rate (cpm) of the radioactive product |
| $\eta$ | counting efficiency |
| $X_0$ | specific radioactivity (Ci/mol) of the substrate |
| $\Delta t$ | reaction time (min) |

$2.22 \times 10^{12}$ factor for conversion from decay rate (dpm) to Ci

V volume of incubation mixture (l)

v volume taken for scintillation counting (l)

### 5.2.10 Paper chromatography of amino acids

The method was as described by Long (1961). In a 100-ml measuring cylinder, n-butanol-acetic acid-water (4: 1: 5, by volume) were mixed and let stand until the two phases were separated. The upper layer was separated and used as the solvent. Samples (4 µl) were spotted 2-3 cm away from each other on a line placed a short distance, 2-3 cm, from the edge of the paper (Whatman No.1). Ascending paper chromatography was carried out and the paper was dried using a hair drier in a fume hood. To reveal the colourless spots, the chromatogram was sprayed with ninhydrin solution (section 5.2.2.5). The paper was then dried by a hair drier under a fume hood and heated in an oven for about 3 min at 105°C. RF values were calculated according to the following equation.

$$RF = \frac{\text{Movement of zone from origin}}{\text{Movement of advancing liquid front from origin}}$$

For radiotracer experiments, the spot corresponding to the amino acid of interest was cut, transfered into a scintillation vial and the radioactivity was measured in a Beckman LS-3801 liquid scintillation spectrophotometer, using BCS as a counting fluid

### 5.2.11 SDS-Polyacrylamide gel electrophoresis

Cultures were harvested at late log to stationary phase and cell-free extracts were prepared as outlined in section 4.2.4. SDS-PAGE was carried out as described in section 4.2.5. Dried gels were exposed to X-ray film (Fuji medical X-ray film) for 4 weeks and the films were developed by an automatic developer (Agfa, CURIX 60).

### 5.2.12 HPLC analysis of amino acids in newly synthesised bacterial protein

Cultures were grown in NB medium containing 70 mM $NH_4Cl$ plus 0.6 mM AdoMet and supplemented with L-[β-$^{14}$C]-serine (0.1 μCi/ml) or S-adenosyl-L-[$^{14}$C-methyl]-methionine (0.1 μCi/ml). Cold AdoMet was added to the medium to support growth of strain E14. Cells were harvested at late log to stationary phase and washed with RO water at least four times. The samples were preoxidised with performic acid to prevent degradation of methionine which otherwise occurs during acid hydrolysis. The preoxidation was carried out in 90% v/v formic acid in hydrogen peroxide for 1 h at room temperature. Hydrobromic acid (20% v/v) was added to the solution and the samples were kept (left) at room temperature for 4 h. The samples were then evaporated to dryness and acid hydrolysed in 6 M HCl at 110°C for 24 h. 40 μl of each sample was loaded onto a HPLC (Waters, Millipore) cation exchange column. The column (56°C) was run with buffer A (section 5.2.2.4.1) for 5 min and then buffer B (section 5.2.2.4.2) was used for amino acid separation. Fractions of 1 ml were collected by a fraction collector (FRAC-100, Pharmacia) at a rate of 0.4 ml/min. Each fraction was transferred to a scintillation vial, and the radioactivity was measured in a Beckman LS-3801 liquid scintillation spectrophotometer, using BCS as a counting fluid.

### 5.2.13 DNA isolation (Maniatis *et al.*, 1982)

Cell-free extracts were prepared from overnight cultures in BHI medium as outlined in section 4.2.4. A 0.5 volume of phenol was added to the cell extract and mixed by inversion and then 0.5 volume of chloroform was added and mixed as before. The sample was centrifuged at 10,000 x g for 5 min and the upper layer was transferred to a clean tube using cut off tips. Phenol chloroform extractions were repeated until the upper aqueous phase was clear. A final extraction with an equal volume of chloroform was then carried out and the aqueous phase was removed to a fresh tube. 2 volumes of 100% ice-cold ethanol was added to this solution and cooled to -20°C for 1 h to precipitate DNA. The precipitated DNA was recovered by centrifugation at 4°C, for 10

min at 10,000 x g. The supernatant was tipped off and the DNA pellet was washed with 70% ethanol. After recentrifugation the supernatant was removed and the pellet dried in a vacuum drier. DNA was dissolved in sterile nano pure water and stored in aliquots at -20°C until use.

### 5.2.14 Restriction digestion

Restriction endonuclease digests were carried out using the buffers supplied by the manufacturer. Digests were carried out in 1.5 ml microcentrifuge tubes which contained 0.5 to 1.0 µg of DNA dissolved in 2 µl of TE buffer, 6.5 µl of nanopure water, 1 µl of the supplied 10 x buffer, and 0.5 µl (~2.5-5.0 units) of restriction endonuclease. The reaction was set up on ice, adding the restriction enzyme last. The contents of the tubes were mixed by tapping the bottom of the tubes and the liquid was returned to the bottom of the tube by a short spin in a microcentrifuge. The reaction then proceeded at 37°C overnight. 2 µl of 6 x gel loading buffer was added and the restriction fragments were analysed by agarose gel electrophoresis.

### 5.2.15 Agarose gel electrophoresis

0.8% agarose gels were prepared by melting the appropriate amount of agarose in 1 x TAE buffer. The molten agarose was poured into gel trays sealed with masking tape containing well-forming combs of appropriate size. When the gel had solidified the masking tape and comb were removed and the gel submerged in 1 x TAE buffer in a gel electrophoresis chamber (Pharmacia). DNA samples which had already been mixed with gel loading buffer were loaded into the wells using a P20 micropipette (Gilson, France). The gel was run at 7.5 V/cm and the progress of the electrophoresis monitored by the migration of the indicator dyes, bromophenol blue and xylene cyanol. The gels were run until the bromophenol blue marker almost reached end of the gel. The gel was removed from the electrophoresis apparatus and stained in a 0.5 µg/ml solution of ethidium bromide, destained in RO water and examined on a UV-transilluminator. Ethidium bromide-stained gels were photographed under UV illumination using a

Polaroid Land Camera.

### 5.2.16 Designing of oligonucleotide primers

PCR primers (Table 5.4) were designed from conserved regions of published sequences. The oligonucleotide primers for the cobalamin-dependent methionine synthase gene were designed from conserved sequences of the *E. coli metH* gene (Banerjee *et al.*, 1989). The sequence between deoxynucleotides 1 to 30 was chosen for the forward primer and the sequence between deoxynucleotides 2962 to 2991 for the reverse primer. The oligonucleotide primers for cobalamin-independent methionine synthase gene were designed from conserved sequences of the *E. coli metE* gene (González *et al.*, 1992). The sequence between deoxynucleotides 37 to 66 was chosen for the forward primer and the sequence between deoxynucleotides 1087 to 1116 for the reverse primer. The oligonucleotide primers for the AdoMet synthetase gene were designed from the *E. coli metK* gene (Markham *et al.*, 1984). The sequence between deoxynucleotides 13 to 42 was chosen for the forward primer and the sequence between deoxynucleotide 1006 to 1035 for the reverse primer.

### 5.2.17 Polymerase chain reaction

One colony of *E. coli* HB101 grown on LB agar was suspended in 100 µl of sterile nano pure water and boiled for 10 min. This was used as a source of template DNA in the PCR reaction. The reaction mixture contained:

*Ingredients*

10 x PCR buffer ..... 2.5 µl

dNTP mixture ..... 4.0 µl

*Taq* DNA polymerase ..... 1.0 Unit

Forward primer ..... 25 pmoles

Reverse primer ..... 25 pmoles

Template DNA ..... 2 µl

**Table 5.4 Sequence of oligonucleotide primers for the *metH*, *metE* and *metK* genes**

| Genes | Forward primer | Reverse primer |
|---|---|---|
| *metH*[a] | 5'ATG GGC ACC ATG ATC CAG AGT TAT CGA CTG3' | 5'GCC ACG CGG ATT CAG CGT TTT CTC GGC GCT3' |
| *metE*[b] | 5'GTT GGC CTG CGT CGC GAG CTG AAA AAA GCG3' | 5'TGC CAG AGC TGC CGT GTC ACC ACT GTT CAC3' |
| *metK*[c] | 5'CTT TTT ACG TCC GAG TCC GTC TCT GAG GGC3' | 5'CAG CAT CTG AAT CAG ACC GAT TGG CAG GTC3' |

*a.* cobalamin-dependent methionine synthase gene

*b.* cobalamin-independent methionine synthase gene

*c.* AdoMet synthetase gene

The mixture was brought to a final volume of 25 µl with sterile nanopure water. The final concentration of $MgCl_2$ was 1.5 mM which was the optimum concentration for PCR amplification of *metK*, *metE* and *metH* genes. The reaction mixture in 0.5 ml microcentrifuge tubes were overlaid with 25 µl of mineral oil. DNA samples were amplified using a Perkin-Elmer Cetus PCR machine. The optimised amplification conditions for each gene were as listed in Table 5.5, except that for the first cycle, melting time was set for 5 min to ensure complete denaturation. The amplification was carried out up to 40 cycles. A final step of 10 min at 72°C was included to ensure the complete extension of the primers. At the end of the amplification reaction, samples were left at 4°C until needed. PCR samples were then pipetted away from the mineral oil, placed in clean microcentrifuge tubes and stored at -20°C until required. PCR samples (10 µl) were then analysed by gel electrophoresis in 1.0% agarose in TAE buffer. The appropriate size band was then cut out of the gel, being careful to get as little as possible of the surrounding gel. The cut band was placed into a plastic bag, sealed and stored at -20°C until frozen. The fragment was pushed into a corner and squeezed. the liquid portion was pipetted out into a microcentrifuge tube and TE buffer was added to a final volume of 100 µl. DNA was isolated from this solution as described in section 5.2.13 and used as a probe.

### 5.2.18 Oligolabelling of DNA probes

The PCR products prepared in section 5.2.17 were labelled by a nick translation system (Promega). 25 µl of DNA solution in nanopure water containing about 100 ng DNA were added into a microcentrifuge tube containing 5 µl nick translation buffer (10x) and 10 µl of deoxynucleotide mixture (dATP, dGTP and dTTP, 0.3 mM each). This was mixed and 5 µl of $^{32}$P-dCTP (50 µCi) plus 5 µl of the enzyme mix (DNA polymerase I, 1U/µl and DNAase I, 0.2 ng/µl) were added and the reaction was incubated at 15°C for 1 h. The reaction was stopped by adding 5 µl of 0.25 M EDTA pH 8.0. The tube was then placed in a boiling rack and boiled for 5 min before being added to the hybridisation mixture.

**Table 5.5 Amplification conditions**

| | Melting | | Annealing | | Polymerase reaction | |
|---|---|---|---|---|---|---|
| Genes | Temperature[a] (°C) | Time (Min) | Temperature (°C) | Time (Min) | Temperature (°C) | Time (Min) |
| *metH*[b] | 96.0 | 0.5 | 65.0 | 0.5 | 72.0 | 2.0 |
| *metE*[c] | 96.0 | 0.5 | 70.0 | 0.5 | 72.0 | 2.0 |
| *metK*[d] | 94.0 | 1.0 | 60.0 | 2.0 | 72.0 | 1.5 |

*a.* Melting temperature was calculated according to $T_m = (C+G)4 + (A+T)2$

*b.* Cobalamin-dependent methionine synthase gene

*c.* Cobalamin-independent methionine synthase gene

*d.* AdoMet synthetase gene

### 5.2.19 Southern transfer

DNA fragments were transferred from agarose gel to nylon membranes by Southern transfer as described by Maniatis *et al.* (1982). The gel was soaked in 0.25 M HCl for 10-15 min (to partially hydrolyse the DNA) until the bromophenol blue bands turned yellow, then rinsed in RO water. The transfer was then set up in a glass baking dish. Two layers of sponges were placed on the bottom of the dish. The gel was placed on Whatman 3MM filter paper wick (wet in 0.4 M NaOH) and any air bubbles were smoothed out. The gel was overlaid with a piece of nylon membrane (Biotrace, Schleicher and Schuell Inc, Keene, NH, USA) which had been soaked in RO water taking care not to trap bubbles underneath. The membrane was overlaid with 3 layers of filter papers wet in RO water followed by several layers of dry filter papers and a stack of dry paper towels. A weight was placed on the top of the stack and 0.4 M NaOH was used as the transfer solution. After overnight transfer the stack was disassembled and the nylon membrane was washed in 2 x SSC.

### 5.2.20 Prehybridisation and hybridisation of membranes

Following Southern transfer the nylon membranes were washed in 2 x SSC, placed between two layers of mesh and transferred into a hybridisation bottle (Hybaid) containing 10 ml prehybridisation solution. The membranes were prehybridised for 6 h at 40°C. The prehybridisation solution was discarded and 10 ml of hybridisation solution containing the boiled, labelled probe was added to the bottle. Hybridisation was carried out overnight at 40°C. After hybridisation the membranes were washed with 3 changes of 6 x SSC/0.1 %(w/v) SDS at 37°C for 30 min each. The optimal stringencies described in this section were determined by testing different stringencies for hybridisation and washing of the membranes. The washed membranes were sealed in a plastic bag and autoradiographed. Autoradiography was carried out by placing Kodak X-ray film directly over the membrane in an autoradiography cassette inside a dark room, sealing the cassette and exposing at -80°C. The exposure time depended on the radioactivity of the filter. The autoradiograms were developed using an automatic

developer (Agfa, CURIX 60).

## 5.3 Results

### 5.3.1 Effect of intermediates of methionine biosynthesis on growth of *B. fibrisolvens* strain E14

To determine whether *B. fibrisolvens* strain E14 has an abnormality in the pathway of methionine biosynthesis, bacterial growth was tested in NB medium containing 70 mM $NH_4Cl$ plus 0.01% (w/v) of various intermediates of methionine biosynthesis. The results (Table 5.6) show that there was no significant growth in any supplemented media. Bacterial growth was also tested on homocysteine (0.01% w/v) plus 0.01 % (w/v) of serine, betaine, formaldehyde and L-N5-methyl-H4folate-$Glu_1$. No growth was observed on these supplements. Higher concentrations of each intermediate alone or in combination were also tested but no growth was observed (Table 5.6).

### 5.3.2 Methionine biosynthesis in *B. fibrisolvens*

Since homocysteine alone or in combination with other relevant intermediates could not replace methionine for growth of strain E14, the strain may not be able to methylate homocysteine to produce methionine. To examine this possibility, the strain was tested for methionine synthase activities.

#### 5.3.2.1 Cobalamin-dependent methionine synthase activity in *B. fibrisolvens*

Extracts of *B. fibrisolvens* strains E14 and H17c grown in NB medium containing 70 mM $NH_4Cl$ plus 0.6 mM AdoMet were tested for cobalamin-dependent methionine synthase activity. AdoMet was added to the medium to support growth of strain E14. Extracts of *B. fibrisolvens* strain H17c were tested as positive controls to ensure that the assay was working. Cobalamin-dependent methionine synthase activity was not detectable either in strain H17c or strain E14 under the assay conditions explained in section 5.2.6. Different assay conditions including various pH,

**Table 5.6 Growth[a] of *B. fibrisolvens* strain E14 in media supplemented with intermediates of the methionine biosynthetic pathway**

| Supplements 0.01 % (w/v) | Absorbance maximum (600 nm) |
|---|---|
| L-homoserine | 0.14 |
| O-succinyl homoserine | 0.13 |
| Cystathionine | 0.15 |
| dl, L-$N^5$-methyl-$H_4$-folate | 0.14 |
| L-homocysteiene | 0.15 |
| L-homocysteiene + dl, L-$N^5$-methyl-$H_4$-folate | 0.14 |
| L-homocysteine + Serine | 0.15 |
| L-homocysteine + Betaine | 0.13 |
| L-homocysteine + Formaldehyde | 0.14 |

*a*. Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values represent the mean of triplicate assays, with variation between triplicates being less than 0.05.

temperatures and substrate concentrations were employed, but no activity was observed for either strain E14 or strain H17c. It has been reported that methionine and AdoMet repress the enzymes of methionine biosynthetic pathway (Old *et al.*, 1991). Therefore, extracts of strain H17c grown in NB medium containing $NH_4Cl$ alone were tested both aerobically and anaerobically. No activity was detectable in these extracts. Since *B. fibrisolvens* is characterised as a proteolytic species, 1 mM PMSF was added to the cell suspensions before preparation of cell-free extracts to inhibit protease activity and thus to protect the enzymes. To ensure that PMSF did not inhibit the enzyme activity, extracts without PMSF were also tested. No activity was detectable in these extracts. Cell-free extracts (with PMSF) of *E. coli* HB101 grown in a defined medium (Section 5.2.3.2.2) were tested as positive controls to ensure that the assay was working. Cobalamin-dependent methionine synthase specific activity was $2.05 \pm 0.08$ nmol. $mg^{-1}$. $min^{-1}$ in this organism. The result is the mean $\pm$ the standard error of the mean for triplicate assays.

#### 5.3.2.2 Cobalamin-independent methionine synthase activity in *B. fibrisolvens*

To measure cobalamin-independent methionine synthase activity *in vitro*, the triglutamate form of L-$N^5$-[methyl-$^{14}C$]-methyltetrahydrofolate has been reported to be essential as a methyl group donor (Whitfield and Weissbach, 1968; Whitfield *et al.*, 1970; Old *et al.*, 1991). However, this compound is not commercially available. Therefore, the activity was tested in the presence of monoglutamate form of L-$N^5$-[methyl-$^{14}C$]-methyltetrahydrofolate. No activity was observed either for strain E14 or strain H17c.

Since cobalamin-independent methionine synthase does not require vitamin $B_{12}$ for activity (Flavin, 1975), growth of strain E14 was tested in NB medium devoid of vitamin $B_{12}$ and containing 70 mM $NH_4Cl$ and 0.6 mM AdoMet. This medium supported bacterial growth.

#### 5.3.2.3 Methionine biosynthesis in intact cells using L-[β-$^{14}$C]-serine

Methionine biosynthesis in intact cells was examined using L-[β-$^{14}$C]-serine to determine whether cells were able to synthesise the triglutamate form of L-N$^{5}$-methyltetrahydrofolate and to use the latter as a methyl group donor in methionine biosynthesis. HPLC analysis of an acid hydrolysate of the cells followed by measurement of radioactivity of each fraction showed that strain H17c produced labelled methionine (Figure 5.6b) whereas strain E14 did not (Figure 5.7b). However, Figures 5.6a and 5.7a show that the methionine peak migrates very close to the peak corresponding to serine. To make sure that the radioactivity observed in the methionine fraction in strain H17c was not due to carry over from labelled serine fraction, the samples were also analysed by paper chromatography followed by measurement of radioactivity of the spots corresponding to serine and methionine. Serine and methionine migrated with RF values of 0.25 and 0.48 respectively. The methionine spot was labelled in an acid hydrolysate of strain H17c whereas no radioactivity was detected from strain E14.

### 5.3.3 AdoMet biosynthesis in *B. fibrisolvens*

To determine whether the requirement for AdoMet in strain E14 was due to the lack of AdoMet synthetase activity, the strain was tested for this enzyme. Extracts of *B. fibrisolvens* strain H17c were tested as positive controls to ensure that the assay was working. The results (Table 5.7) show that both strains possessed AdoMet synthetase activity, although, the specific activity for strain E14 was less than that of strain H17c. There was no significant difference between the specific activities of each strain grown in NB or BHI medium. For *B. fibrisolvens* strain H17c, addition of methionine to the growth medium decreased AdoMet synthetase specific activity compared to that obtained when the bacteria were grown on $NH_4Cl$ alone.

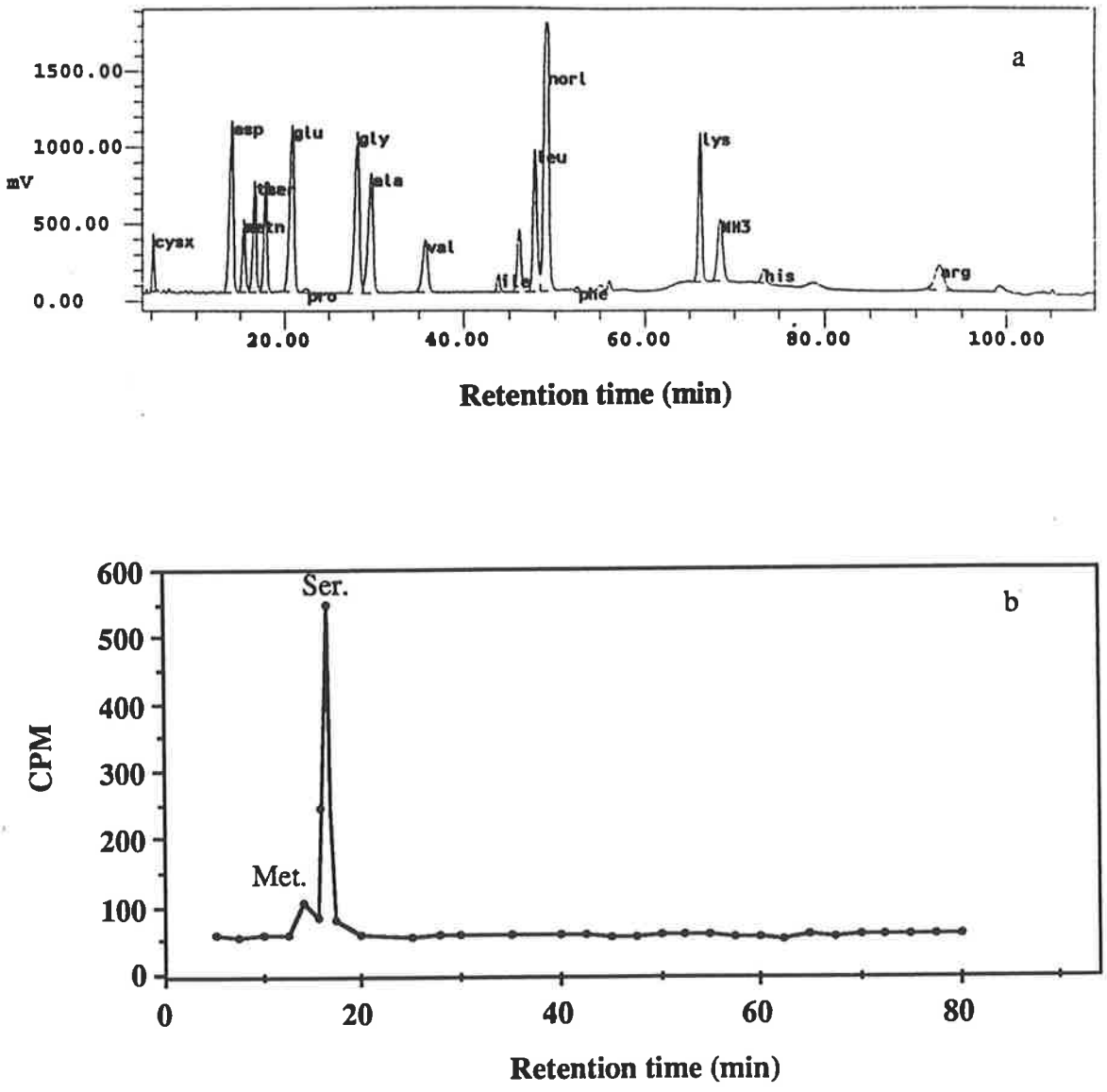



**Figure 5.6 Analysis of amino acid constituents of cellular protein of *B. fibrisolvens* strain H17c by HPLC**

Cultures were grown on L-(β-$^{14}$C)-serine and cells were acid hydrolysed. The samples were analysed by HPLC and the radioactivity of each fraction was measured by liquid scintillation spectroscopy. a) The elution profile of the column. b) The radioactivity associated with each fraction.

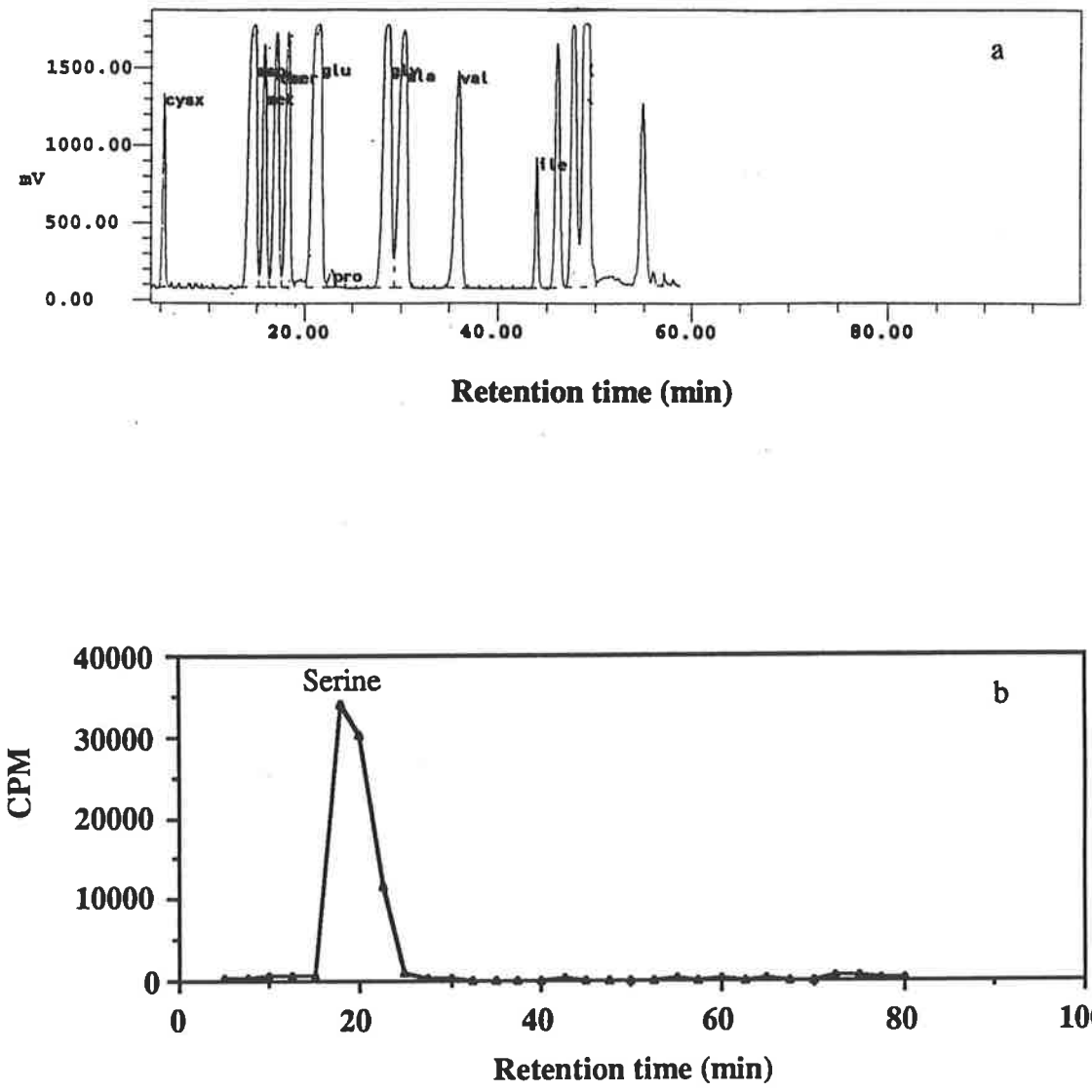



**Figure 5.7 Analysis of amino acid constituents of cellular protein of *B. fibrisolvens* strain E14 by HPLC**

Cultures were grown on L-(β-$^{14}$C)-serine and cells were acid hydrolysed. The samples were analysed by HPLC and the radioactivity of each fraction was measured by liquid scintillation spectroscopy. a) The elution profile of the column. b) The radioactivity associated with each fraction.

**Table 5.7 S-adenosylmethionine synthase specific activities in *B. fibrisolvens* strains E14 and H17c**

| *Butyrivibrio fibrisolvens* | AdoMet synthetase specific activity[a] | | |
|---|---|---|---|
| | NB medium[b] | NB medium[c] plus Met | BHI medium[d] |
| strain H17c | 33.0 ± 2.0 | 21.0 ± 1.8 | 20.0 ± 1.9 |
| strain E14 | - | 12.0 ± 1.7 | 11.0 ± 2.0 |

*a.* Expressed as pmol AdoMet. $min^{-1}$. mg $protein^{-1}$

*b.* Cells were grown in NB medium containing 70 mM $NH_4Cl$ alone

*c.* Cells were grown in NB medium containing 70 mM $NH_4Cl$ plus 0.2 mM methionine

*d.* Cells were grown in BHI medium

### 5.3.4 Homology between the *metH*, *metE* and *metK* genes from *E. coli* and *B. fibrisolvens*

The results of enzyme assays (section 5.3.2.1) showed the lack of cobalamin-dependent methionine synthase activity in *B. fibrisolvens* strains H17 and E14. To determine whether there is homology between the cobalamin-dependent methionine synthase (*metH*) gene from *E. coli* and *B. fibrisolvens* , oligonucleotide primers were designed from published sequences of the *E. coli metH* gene, the sequences were amplified by PCR and *B. fibrisolvens* strains H17c and E14, variants S and L (these variants are introduced in chapter 6 of this thesis) were screened with the PCR product. The same experiments were also carried out for cobalamin-independent methionine synthase (*metE*) and AdoMet synthetase (*metK*). The results show that there was no homology between the *metH* gene from *E. coli* HB101 and *B. fibrisolvens* strains (Figure 5.8). Little homology was observed between the *metE* gene from *E. coli* and *B. fibrisolvens* (Figure 5.9). For *metK* gene there was homology between the gene from *E. coli* and *B. fibrisolvens* (Figure 5.10).

### 5.3.5 Paper chromatography of AdoMet solutions

The results of AdoMet synthetase assay show (section 5.3.3) that *B. fibrisolvens* strain E14 is able to synthesise AdoMet. Therefore, bacterial growth on AdoMet may be due to the presence of free methionine in AdoMet solutions. To examine this possibility, AdoMet solutions (0.6 mM ) in NB medium were analysed by paper chromatography. No free methionine was observed in the solutions but AdoMet was completely decomposed by autoclaving or incubating at 39°C (Figure 5.11). One of the products of AdoMet decomposition was shown to be homoserine but the remainder did not react with ninhydrin.

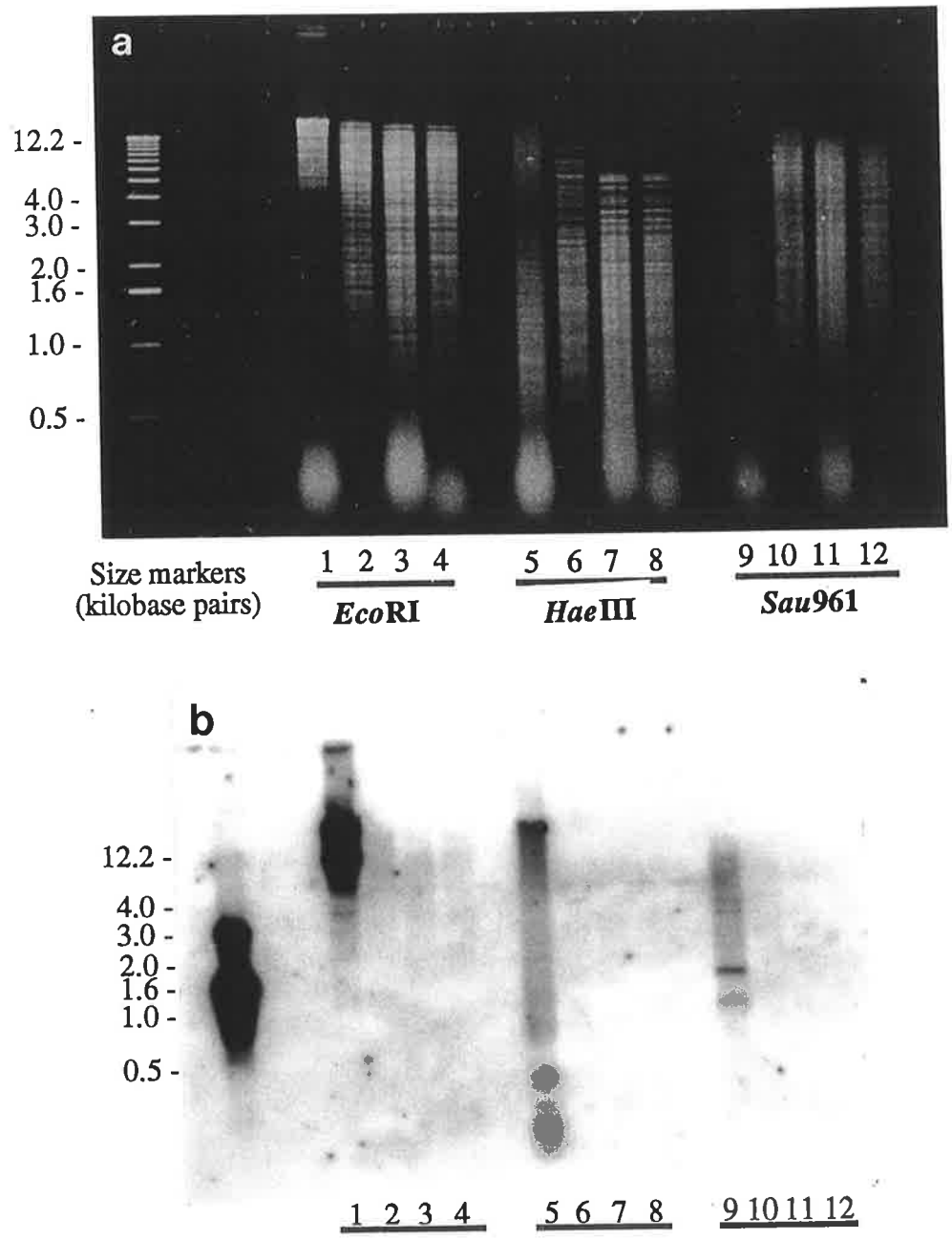



**Figure 5.8 Southern blot of chromosomal DNA from *B. fibrisolvens* strains H17c and E14 (variants L and S) probed with the *metH* gene from *E. coli* HB101**

a) Restriction digest of chromosomal DNA. *E.coli* HB101 (lanes 1, 5 and 9); *B. fibrisolvens* H17c (lanes 2, 6 and 10); *B. fibrisolvens* E14, L (lanes 3, 7 and 11); *B. fibrisolvens* E14, S (lanes 4, 8 and 12). b) Autoradiograph of DNA from panel a, Southern blotted to a nylon membrane and hybridised with $^{32}$P-labelled PCR product from the *E. coli* HB101 *metH* gene. The PCR product is on the left.

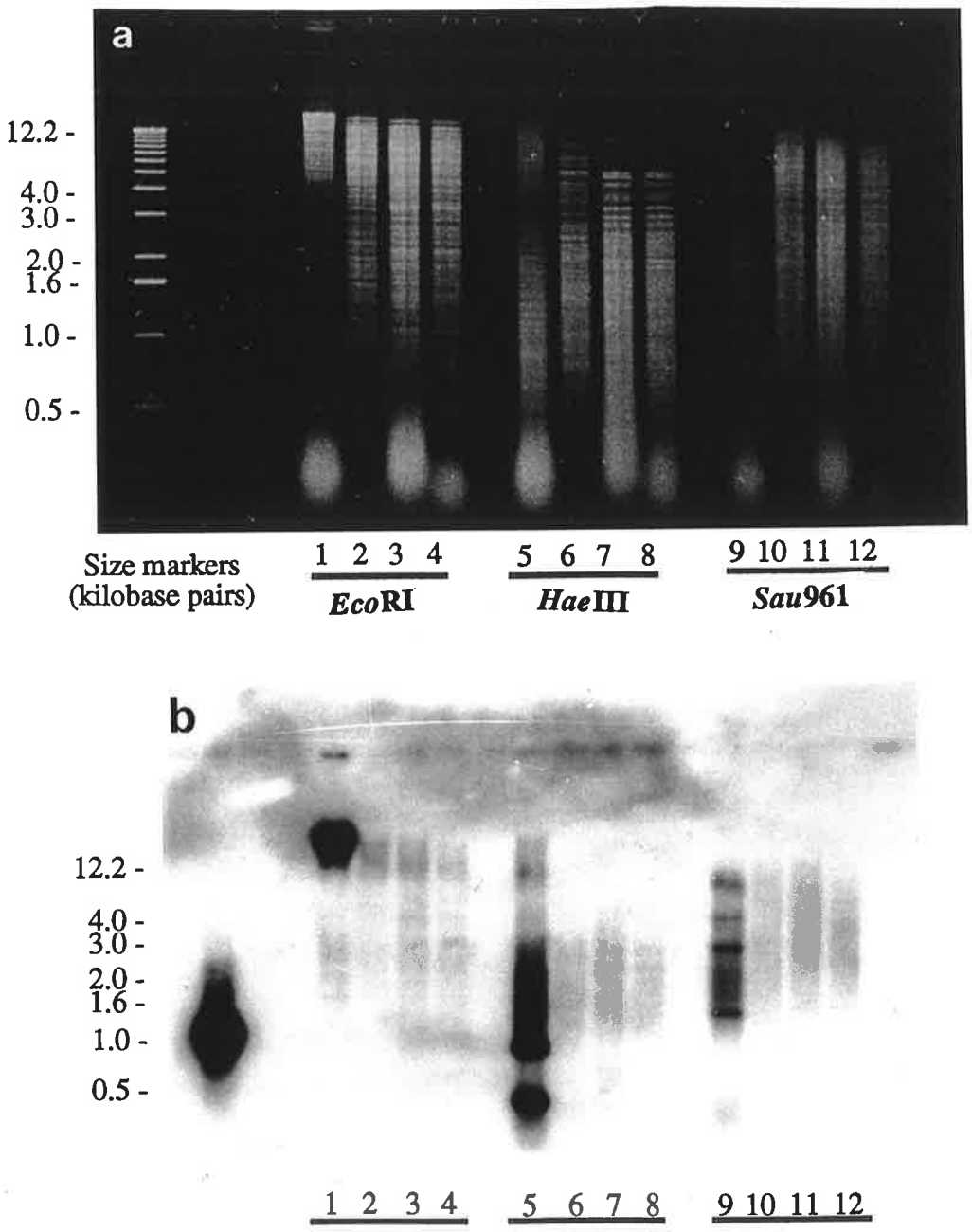



**Figure 5.9 Southern blot of chromosomal DNA from *B. fibrisolvens* strains H17c and E14 (variants L and S) probed with the *metE* gene from *E. coli* HB101**

a) Restriction digest of chromosomal DNA. *E.coli* HB101 (lanes 1, 5 and 9); *B. fibrisolvens* H17c (lanes 2, 6 and 10); *B. fibrisolvens* E14, L (lanes 3, 7 and 11); *B. fibrisolvens* E14, S (lanes 4, 8 and 12). b) Autoradiograph of DNA from panel a, Southern blotted to a nylon membrane and hybridised with $^{32}$P-labelled PCR product from the *E. coli* HB101 *metE* gene. The PCR product is on the left.

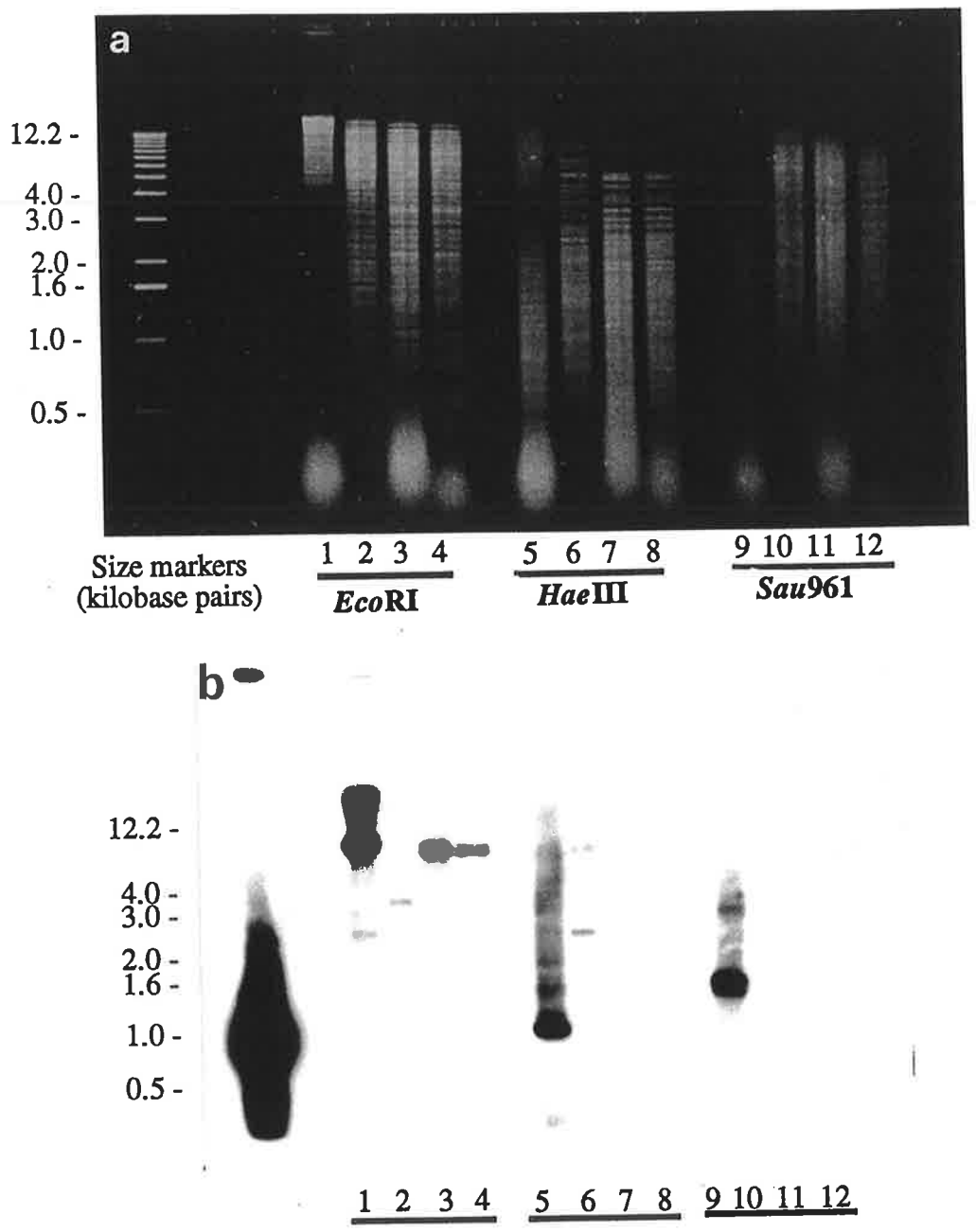



**Figure 5.10 Southern blot of chromosomal DNA from *B. fibrisolvens* strains H17c and E14 (variants L and S) probed with the *metK* gene from *E. coli* HB101**

(a) Restriction digest of chromosomal DNA. *E.coli* HB101 (lanes 1, 5 and 9); *B. fibrisolvens* H17c (lanes 2, 6 and 10); *B. fibrisolvens* E14, L (lanes 3, 7 and 11); *B. fibrisolvens* E14, S (lanes 4, 8 and 12). b) Autoradiograph of DNA from panel a, Southern blotted to a nylon membrane and hybridised with $^{32}$P-labelled PCR product from the *E. coli* HB101 *metK* gene. The PCR product is on the left.

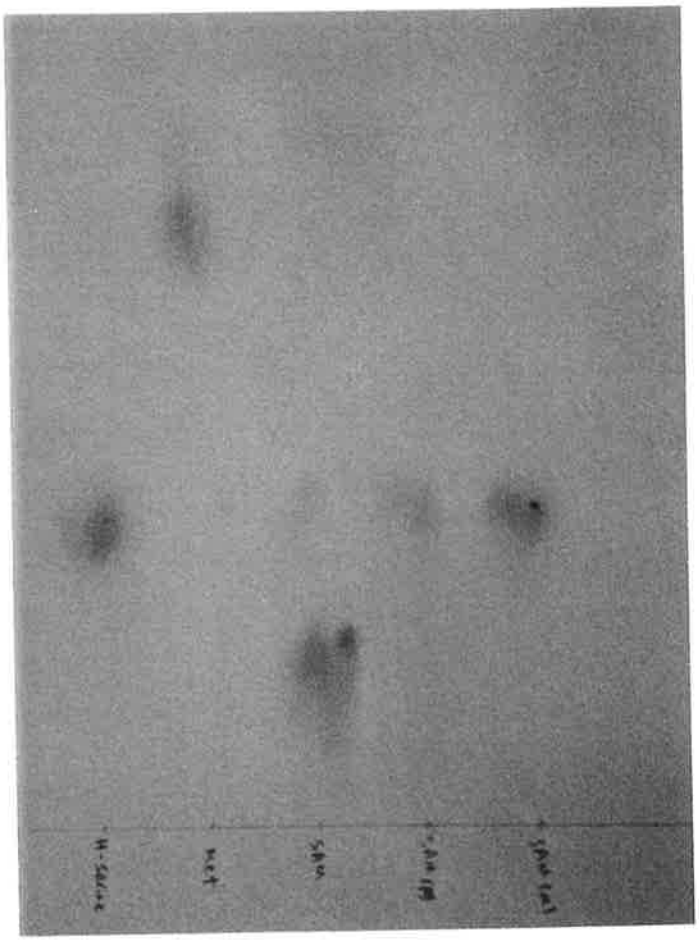



**Figure 5.11 Paper chromatogram of homoserine, methionine and AdoMet solutions stained with ninhydrin:**

Lane 1. Homoserine in NB medium (no treatment).

Lane 2. Methionine solution in NB medium (no treatment).

Lane 3. AdoMet solution in NB medium (no treatment).

Lane 4. Filter sterilised AdoMet solution in NB medium, incubated at 39°C for 2 h.

Lane 5. Autoclaved AdoMet solution in NB medium, incubated at 39°C for 2 h.

### 5.3.6 Incorporation of ($^{14}$C-methyl) S-adenosylmethionine into bacterial protein

To determine whether the methyl group of a decomposition product of AdoMet (section 5.3.5) was incorporated into bacterial protein, *B. fibrisolvens* strains E14 and H17c were grown in $NH_4Cl$-containing NB medium supplemented with S-adenosyl-L-[$^{14}$C-methyl] methionine. SDS-PAGE of cell-free extracts showed (Figure 5.12) radioactivity in the newly synthesised bacterial protein .

### 5.3.7 Methionine biosynthesis in intact cells using S-adenosyl-L-[$^{14}$C-methyl] methionine

Methionine biosynthesis in intact cells using S-adenosyl-L-[$^{14}$C-methyl] methionine was carried out to determine whether cells were able to utilise a decomposition product of AdoMet to produce methionine. HPLC analysis of an acid hydrolysate of strains H17c (Figure 5.13) and E14 (Figure 5.14) followed by measurement of the radioactivity of each fraction showed that only methionine was labelled. More that 90% of the total of recovered radioactivity was incorporated into methionine in both strains (Table 5.8).

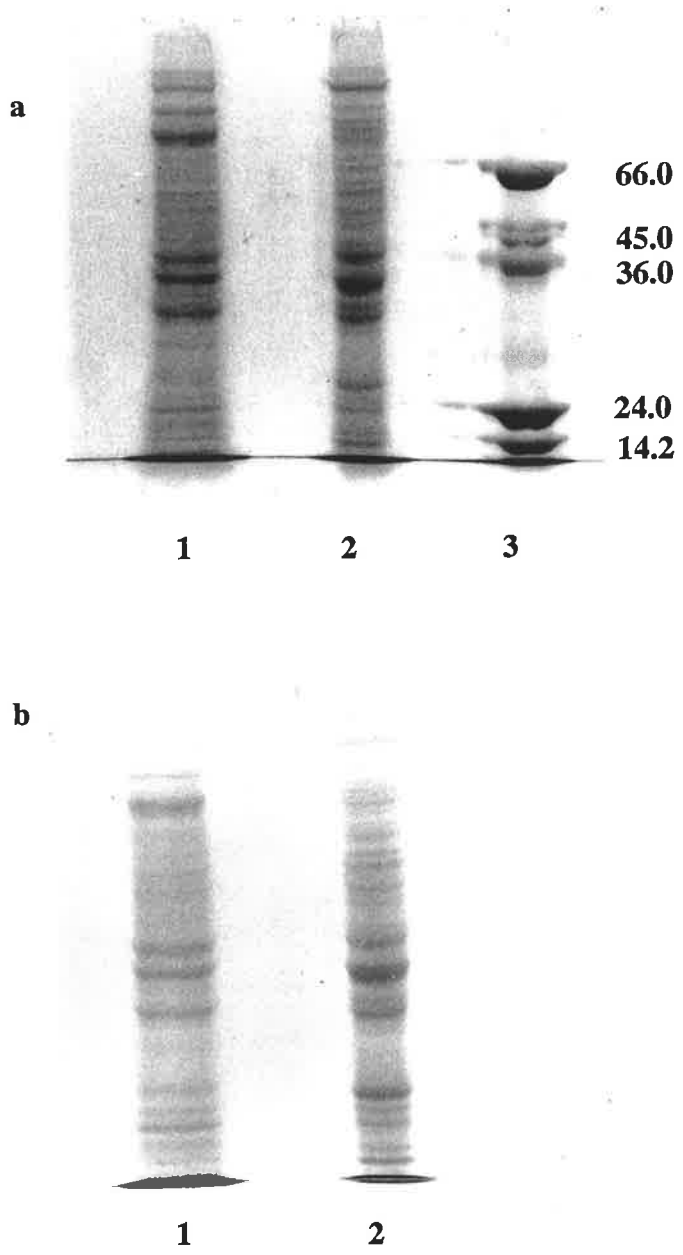



**Figure 5.12 SDS-PAGE of cell-free extracts of *B. fibrisolvens* strains E14 and H17c grown on S-adenosyl-L-[$^{14}$C-methyl] methionine.**

a) SDS-PAGE after staining with Coomassie Blue and drying. Lane 1, *B. fibrisolvens* strain E14; Lane 2, *B. fibrisolvens* strain H17c; lane 3, molecular weight markers (kDa). b) autoradiography of the dried gel. Lane 1, *B. fibrisolvens* strain E14; Lane 2, *B. fibrisolvens* strain H17c.

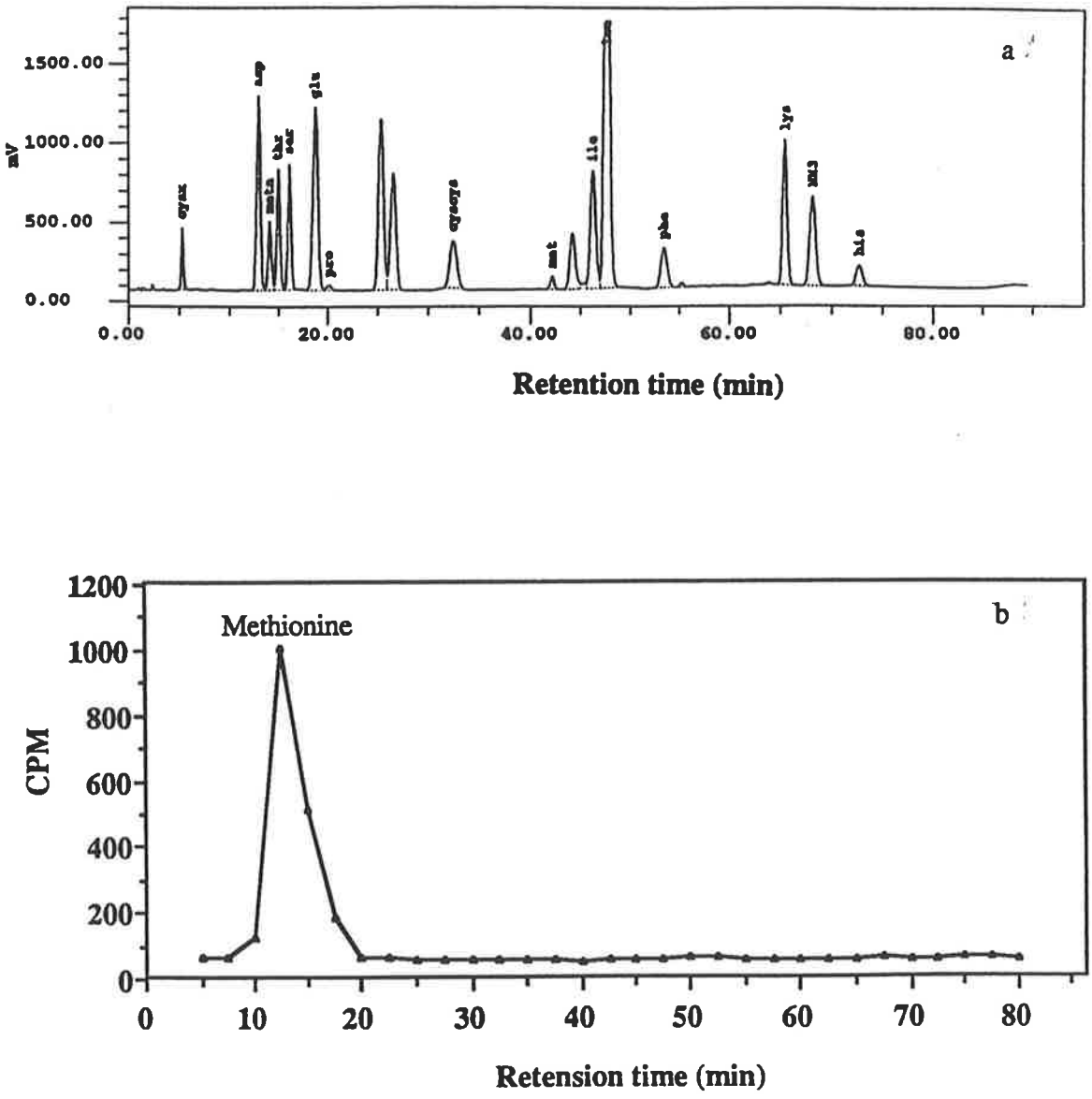



**Figure 5.13 Analysis of amino acid composition of cellular protein of *B. fibrisolvens* strain H17c by HPLC**

Cultures were grown on S-adenosyl-L-[$^{14}$C-methyl] methionine and cells were acid hydrolysed. The samples were analysed by HPLC. The radioactivity of each fraction was measured by liquid scintillation spectroscopy. a) The elution profile of the column. b) The radioactivity associated with each fraction.

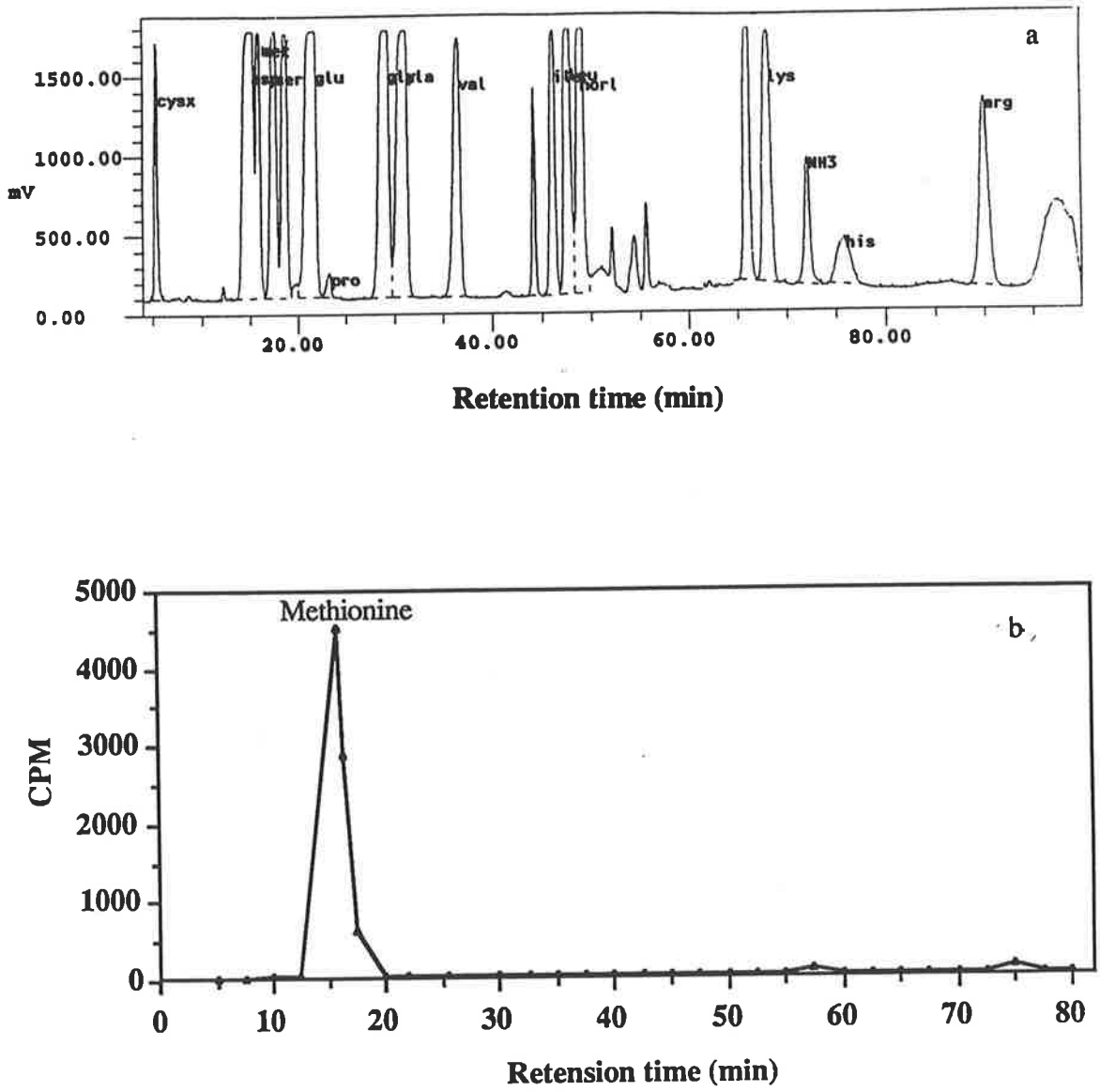



**Figure 5.14 Analysis of amino acid composition of cellular protein of *B. fibrisolvens* strain E14 by HPLC**

Cultures were grown on S-adenosyl-L-[$^{14}$C-methyl] methionine and cells were acid hydrolysed. The samples were analysed by HPLC. The radioactivity of each fraction was measured by liquid scintillation spectroscopy. a) The elution profile of the column. b) The radioactivity associated with each fraction.

**Table 5.8 Incorporation of the methyl group of S-adenosyl-L-[$^{14}$C-methyl] methionine into methionine**

| *B. fibrisolvens* | Radioactivity (cpm) | | Recovery of radioactivity in methionine (%)[a] |
|---|---|---|---|
| | Total lysate | Met. fraction | |
| strain E14 | 7560 | 7426 | 98 |
| strain H17c | 1975 | 1805 | 91 |

Cells were grown on S-adenosyl-L-[$^{14}$C-methyl] methionine and acid hydrolysed. The hydrolysate was analysed by HPLC and the radioactivity of methionine fraction was measured.

*a*. Incorporation of the methyl group of S-adenosyl-L-[methyl-$^{14}$C] methionine into methionine as a per cent (%) of total radioactivity taken up by cells.

## 5.4 Discussion

Tests with intermediates of the methionine biosynthetic pathway showed (Table 5.1) that *B. fibrisolvens* strain E14 might have an abnormality in the last step of the pathway from homocysteine to methionine (Figure 5.1, reaction 14). Uptake of homocysteine is not probably a limiting factor as the results of radiotracer experiments reported in section 4.3.5 of this thesis showed that structurally similar molecules such as cysteine and methionine were readily incorporated into newly synthesised bacterial protein. However, to provide direct evidence for this, it would be necessary to test strain E14 for uptake of radiolabelled homocysteine. This trial was not possible as labelled homocysteine was not commercially available.

DNA-DNA hybridisation did not show (Figure 5.8) homology between the cobalamin-dependent methionine synthase (*metH*) gene from *E. coli* and *B. fibrisolvens.* No conclusion can be drawn from this result as a specific probe was not available. The results of enzyme assays showed the lack of cobalamin-dependent methionine synthase activity in both strain E14 and the methionine-independent strain H17c. This is an interesting finding as it supports the speculation by Drummond *et al.* (1993) that obligate anaerobes may not possess cobalamin-dependent methionine synthase. Drummond *et al.* (1993) separated cobalamin-dependent methionine synthase from *E. coli* into fragments by limited proteolysis with trypsin. They found that the carboxy-terminal domain had all of the requirements required for AdoMet binding and was responsible for remethylation of the enzyme by reductive methylation. The amino-terminal domain which was obtained from cleavage of the methylated holoenzyme retained the ability to catalyse methylation of homocysteine by $CH_3$-$H_4$folate, and this activity was independent of AdoMet. In contrast, the amino-terminal domain with bound cob(II)alamin was inactive in the absence of AdoMet. Since the chances of cob(I)alamin interception by oxidation would be greatly diminished in an anaerobic environment, Drummond *et al.* (1993) speculated that cobalamin-dependent methionine

synthases from obligate anaerobes may resemble the amino-terminal fragment of the *E. coli* enzyme and the activity of these enzymes may be independent of AdoMet as described in section 5.1.1.1.4 (AdoMet is required for activity of cobalamin-dependent methionine synthase in aerobic organisms). Although the results presented here show that *B. fibrisolvens* strains E14 and H17c lack the cobalamin-dependent methionine synthase (*metH* enzyme), more anaerobic bacteria should be tested to determine whether the lack of the *metH* enzyme activity is a general phenomenon.

It has been reported that bacteria that lack cobalamin-dependent methionine synthase contain the cobalamin-independent form of the enzyme (Flavin, 1975). Therefore, cobalamin-independent methionine synthase may generally be present in *B. fibrisolvens* but strain E14 may lack this enzyme. Unfortunately I was unable to test *B. fibrisolvens* strains for cobalamin-independent methionine synthase, as a triglutamate form of L-$N^5$-[methyl-$^{14}$C]-methyltetrahydrofolate, the specific substrate for this enzyme was not commercially available. However, the assay was performed with a monoglutamate form of L-$N^5$-methyltetrahydrofolate but no activity was observed in either strain H17c or E14. This may suggest that, like other organisms (Old *et al.*, 1991), cobalamin-independent methionine synthase from *B. fibrisolvens* has absolute requirement for a triglutamate form of L-$N^5$-methyltetrahydrofolate as a methyl group donor. The results of DNA-DNA hybridisation using the cobalamin-independent methionine synthase (*metE*) gene from *E. coli* HB101 as a probe showed (Figure 5.9) slight homology between the gene from *E. coli* HB101 and *B. fibrisolvens* strains H17c and E14 (L and S). This result may indicate the presence of the *metE* gene in *B. fibrisolvens* strains. However, the homology was not sufficient enough to use the PCR product as a probe for Northern blot.

The results of methionine biosynthesis in intact cells using $^{14}$C-[β-C]-serine show that the peak corresponding to serine in strain E14 (Figure 5.7b) is larger than that of strain H17c (Figure 5.6b). This may have been due to the more rapid growth of strain E14

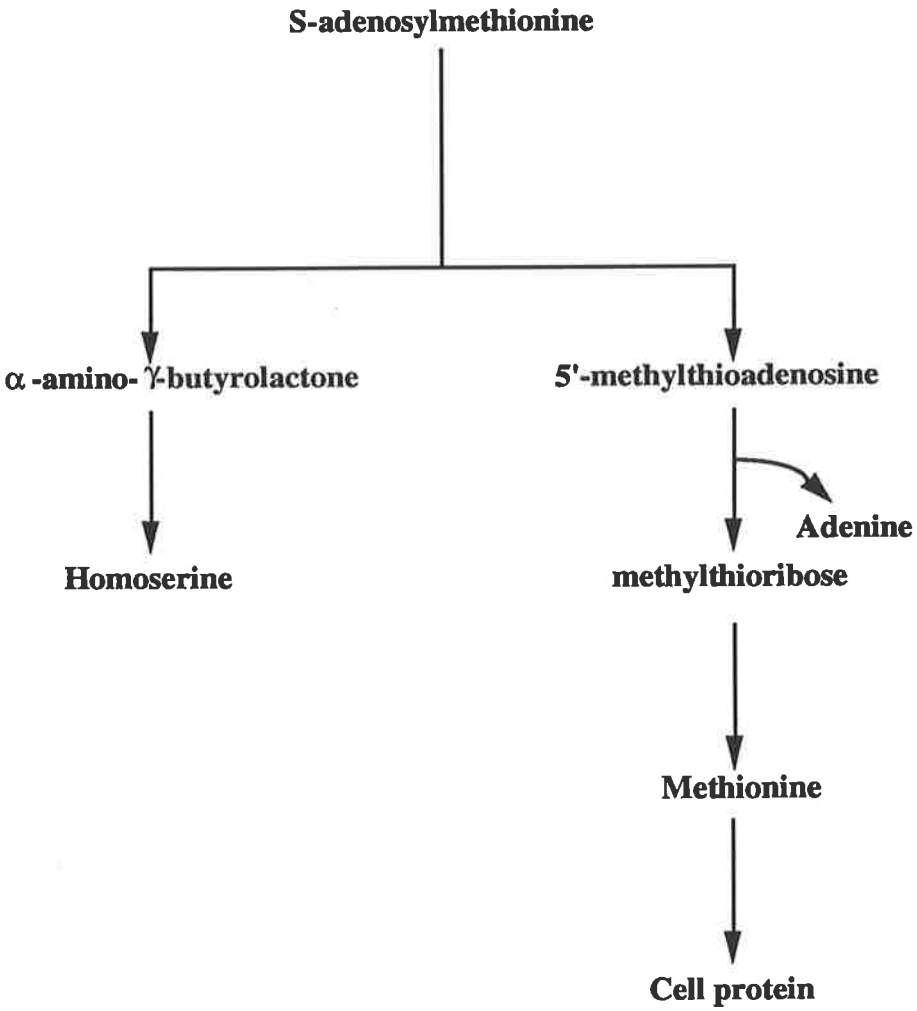
compared to H17c, and consequently the incorporation of a larger amount of $^{14}$C-[β-C]-serine into cell protein in strain E14. However, these results show that the methionine-independent strain H17c was able to incorporate of $^{14}$C-[β-C]-serine into methionine (Figure 5.6b) whereas strain E14 was not (Figure 5.7b). Uptake of serine was not a limiting factor for strain E14 as Figure 5.7b shows the incorporation of $^{14}$C-[β-C]-serine into bacterial protein. The lack of incorporation of $^{14}$C-[β-C]-serine into methionine in strain E14 confirms the results of the growth experiments using intermediates of the methionine biosynthetic pathway and shows that the strain is unable to methylate homocysteine to synthesise methionine. However, this result does not specify which enzyme is lacking in strain E14, as serine transhydroxymethylase, 5,10-methylenetetrahydrofolate reductase and methionine synthase (Figure 5.1, reactions 10, 11 and 14) are all involved in the incorporation of β-C of serine into the methyl group of methionine.

Homology between the *metK* gene from *E. coli* HB101 and *B. fibrisolvens* strains E14 and H17c shows (Figure 5.10) the presence of the AdoMet synthetase gene in *B. fibrisolvens* strains H17c and E14 (variants L and S). This result supports the reports by Thomas and Surdin-Kerjan (1991) and Larsson and Rasmuson-Lestander (1994) that AdoMet synthetase has been exceptionally well conserved through evolution. AdoMet synthetase activity was detectable in *B. fibrisolvens* strains E14 and H17c (Table 5.7) and the level of activities were similar to that reported in crude extracts of *E. coli* (Holloway *et al.*, 1970) and rat liver (Cabrero *et al.*, 1987). This result suggests that promotion of growth of strain E14 by AdoMet is not due to the lack of AdoMet biosynthesis in this strain. Assay of AdoMet synthatase in cell-free extracts of *B. fibrisolvens* strains H17c and E14 grown in rich or minimal medium showed activity in both extracts. This suggests that *B. fibrisolvens* strains may either possess both *metK* and metX genes or possess only one of the genes which is expressed in both media. It has been reported that AdoMet synthetase is inducible by methionine in yeast (Pigg *et al.*, 1964). In contrast, the results reported here show that AdoMet synthetase activity

decreased in strain H17c if methionine was added to the growth medium. Similarly, Holloway *et al.* (1970) found that AdoMet synthetase is repressed by the addition of methionine to the growth medium in *E. coli*. They therefore concluded that addition of methionine to the growth medium causes repression rather than induction of AdoMet synthetase. Since all methionine biosynthetic genes are reported to be repressed by the presence of methionine in the growth medium, Smith (1971) and Holloway *et al.* (1970) suggested that AdoMet synthetase may share some regulatory elements with that of the methionine biosynthetic enzymes.

Promotion of growth of strain E14 by AdoMet was not due to the presence of free methionine, as the results of paper chromatography showed (Figure 5.11) that methionine was not removed from AdoMet. Wu *et al.* (1983) have previously reported that AdoMet is hydrolysed by a mechanically complicated route to homoserine and 5'-methylthioadenosine which is subsequently broken down to methylthioribose (Figure 5.15). These data suggest that growth of strain E14 on AdoMet is not due to the utilisation of AdoMet *per se* but from methylthioribose which can arise as the end product of AdoMet breakdown and can be utilised by the cells to synthesise methionine. This conclusion is supported by the results of radiotracer experiments using AdoMet labelled at the methyl group. SDS-PAGE of cell-free extracts showed (Figure 5.12) that newly synthesised bacterial protein was labelled. HPLC analysis of acid hydrolysates of the bacteria showed (Figures 5.13 and 5.14) that the label was associated exclusively with methionine. There is evidence for the existence of a pathway for the incorporation of some parts of 5'-methylthioadenosine (the precursor of methylthioribose) into the methionine pool of certain organisms. Sugimoto *et al.* (1976) have reported that the methylthio group of 5'-methylthioadenosine accumulated as cellular methionine in *Ochromonas malhamensis*. Schlenk and Ehninger (1964) reported that the radiolabelled methyl group and the sulphur atom of 5'-methylthioadenosine was incorporated into AdoMet in *Candida utilis*. Shapiro and

**Figure 5.15 AdoMet decomposition and production of methionine from methylthioribose**



Schlenk (1980) showed that when *C. utilis* was grown with 5'-methylthio-[adenosine-U-$^{14}$C]-adenosine, S-adenosylmethionine which was synthesised by the cells was labelled in the carbon chain of the amino acid moiety. This suggests that the ribose carbons of 5'-methylthioadenosine are incorporated into methionine by some mechanism. 5'-Methylthioadenosine is also a natural metabolite derived from S-adenosylmethionine as a stoichiometric by-product of polyamine (spermine and spermidine) biosynthesis in microorganisms (Tabor *et al.*, 1958) and animal tissues (Backlund and Smith, 1981). Therefore, the synthesis of methionine from 5'-methylthioadenosine provides the cell with a means of removing 5'-methylthioadenosine by conserving it as methionine. Incorporation of the methyl group of S-adenosyl-L-[methyl-$^{14}$C] methionine into methionine suggests that *B. fibrisolvens* strains E14 and H17c possess an alternative pathway different from the common methionine biosynthetic pathway for the synthesis of methionine from a metabolite of AdoMet. This pathway may be particularly important for strain E14 as it enables the organism to remove methylthioribose by salvaging it as methionine.

The results reported in this chapter show that *B. fibrisolvens* strain E14 is unable to synthesise methionine *de novo* from ammonia, carbon sources and sulphur but is able to synthesise methionine from a metabolite of AdoMet. However, the main question is yet not fully answered. In the light of the evidence provided here, it is only possible to say that strain E14 may lack cobalamin-independent methionine synthase (Figure 5.1, reaction 14) and/or has a defect in the folate pathway (Figure 5.1, reactions 10 and 11). Since a defect in the folate pathway is likely to be lethal, the more likely candidate is cobalamin-independent methionine synthase. Growth of strain E14 in the absence of vitamin $B_{12}$ and presence of AdoMet may support this conclusion. However, the conversion of AdoMet to methionine may occur and this may be via a different pathway. Biosynthesis of methionine from methylthioribose, a metabolite of AdoMet is an interesting finding and indicates that an alternative pathway of methionine biosynthesis is present in these *B. fibrisolvens* strains H17c and E14. However, *de*

*novo* biosynthesis of methionine is not likely through this route, as there is no mechanism for AdoMet which does not require prior biosynthesis of methionine. More strains of *B. fibrisolvens* should be tested to determine whether biosynthesis of methionine from methylthioribose is a general phenomenon in the species. It is not clear whether the inability to synthesise methionine *de novo* by strain E14 is a characteristic of the original strain or whether it was generated by prolonged passage in the laboratory. However, this finding may support the previous reports that the extent to which methionine is synthesised in the rumen may be variable depending on the bacteria (Gawthorne and Nader, 1976; Salter *et al.*, 1979; Mathers and Miller, 1980).

# CHAPTER 6

# CHAPTER 6

## Characterisation of a Non-Adhering Variant of *Butyrivibrio fibrisolvens* Strain E14

### 6.1 Introduction

During my studies of nitrogen utilisation in *B. fibrisolvens* strain E14, a variant was discovered that appeared to differ from the wild type organism in its ability to adhere to NB agar. It is possible that adherence to substrate is important in the nitrogen economy of the organism.

The adherence of microorganisms to surfaces is a primary step in substratum colonisation. Subsequent cell growth and biosynthesis of extracellular polysaccharide (EPS) leads to the formation of a biofilm (Cheng *et al.*, 1989). EPS can form tight cell-associated (integral) capsules or dispersed (peripheral) slime matrices which can slough into the aqueous phase. Integral EPS is thought to be adhesive, allowing attachment to surfaces and exploitation of solid nutrients. Peripheral EPS may release attached bacteria from the surface when nutrients are depleted (Sutherland, 1980; Hermansson and Marshall, 1985; Whitfield, 1988; Leigh and Coplin, 1992). For some bacteria, fimbriae (pili) are involved in primary adhesion, and secretion of EPS assists attachment to surfaces (Hultgren *et al.*, 1993). EPS forms a hydrated matrix, often containing secreted proteins, and comprises the biofilm in which multiple layers of bacterial cells become embedded. Many different species of the family Enterobacteriaceae have been shown to possess a range of fimbriae which facilitate adhesion to epithelial cells of the gastrointestinal and urogenital tracts. These fimbriae, of which there are several classes, are rod-like heteropolymeric fibres that bind carbohydrate moieties on epithelial cells (Doig and Trust, 1994). In the enteric bacteria, interactions between fimbriae and epithelial cell surfaces have been thoroughly investigated with regard to pathogenic processes and some enteric *E. coli* can be

converted from commensal to pathogenic forms by the expression of fimbriae (Hultgren *et al.*, 1993). However, the precise role of fimbriae in other genera remains uncertain. The ability of enteric bacteria to produce a morphologically and antigenically diverse range of fimbriae with varying receptor specificities indicates that individual strains may have evolved to express distinct attachment moieties under different environmental conditions.

Numerous microorganisms produce and secret carbohydrate polymers or EPS. Such polymers vary considerably in their chemical structure. They can be either homopolymers or heteropolymers and may carry a variety of noncarbohydrate substituents (Sutherland, 1980; Leigh and Coplin, 1992). Some components such as D-glucose, D-mannose, D-galactose and D-glucuronic acid occur very frequently. Others such as L-rhamnose or L-fucose are slightly less common. D-Mannuronic acid and L-glucuronic acid are rare (Sutherland, 1980). EPS biosynthesis in *B. fibrisolvens* has been described previously (Ha *et al.*, 1991) and has been shown to comprise unusual sugars such as L-altrose, 4-*O*-(1-carboxyethyl)-D-galactose and 4-*O*-(1-carboxyethyl)-L-rhamnose. The unusual composition of *B. fibrisolvens* EPS suggests that it may have unique functional properties. However, these properties are unknown.

The precise role played by EPS is dependent on the natural environment of the microorganism and its ability to produce EPS is a direct and logical response to the selective pressures in the natural environment (Whitfield, 1988). EPS enable free-living bacteria to adhere to sister cells and to inert or tissue substrata that provide a constantly renewed supply of organic nutrients (Costerton and Irvin, 1981; Leigh and Coplin, 1992). Starch-digesting bacteria in the rumen adhere to granules of starch so specifically that the best way to separate such cells from the mixed bacterial population of the rumen is to recover the starch grains from the rumen fluid (Costerton *et al.*, 1978). In the rumen, bacterial attachment occurs on at least two distinct types of surfaces, the rumen epithelium and solid plant material. Therefore, bacterial-substratum

interactions may involve additional factors to those associated with gastrointestinal tract. The structural barriers that are interposed between rumen microbes and their substrates are often very complex and resistant to digestion. These may consist of layers of cellulose, lignin and hemicellulose in plant cell walls or protein coats surrounding starch grains. Adhesion may therefore be especially important where the digestion of a complex substrate such as a plant cell wall or cellulose is involved, since a number of enzymes must act in combination (Morris and Cole, 1987). Direct examinations and *in vitro* studies of ruminal microorganisms have clearly shown that bacteria must attach to cellulose substrates in order to promote fibre digestion (Kudo *et al.*, 1987). Attachment is therefore a precondition and trigger for digestion in the rumen. Digestive microbial consortia then form within adherent biofilms on the surfaces of plant material. Successful rumen microorganisms combine adhesive and degradative capacities so that they can digest away complex plant cell walls or protein coats to gain access to nutrients. Gong and Forsberg (1989) have isolated adherence defective mutants of *F. succinogenes* that retain their full complement of cellulase enzymes (β, 1-4 endoglucanase, cellobiosidase, cellobiase) but are unable to adhere to, or digest, cellulose. Even where the levels of cellulases were elevated, cellulose digestion did not occur in the absence of adherence. These results clearly demonstrate the importance of adherence. EPS may also have a protective role. The ability of a microorganism to surround itself in a highly hydrated EPS layer may provide it with protection against desiccation and predation by protozoans. The presence of a gelled polysaccharide layer around the cell may also have significant effects on the diffusion properties, both into and out from the cell (Whitfield, 1988).

The experiments reported in the present chapter were carried out to purify the non-adherent variant of *B. fibrisolvens* strain E14 and characterise it with respect to the original organism. Bacterial growth experiments, biochemical tests and enzymatic analysis were carried out. Since the strain has been characterised as a cellulolytic organism (Orpin *et al.*, 1985), β 1-4 endoglucanase activity of the two variants was also

tested on carboxymethylcellulose (CMC) agar plates. EPS from the two variants were isolated and measured. Electron microscopy (scanning and transmission) was performed. Chromosomal DNA from the variants S and L were digested with restriction enzymes, separated by agarose gel electrophoresis and the DNA fragments from the two variants were compared. Soluble proteins were analysed by SDS-PAGE and the protein profile of the two variants were compared. Since variant L was discovered during the studying of nitrogen utilisation and metabolism in the original organism (S), all experiments reported in chapter 4 and 5 of this thesis were repeated for the two variants.

## 6.2 Materials and Methods

### 6.2.1 Chemicals

Paraformaldehyde, lactic acid, succinic acid, carboxymethyl cellulose (sodium salt) and phenol were prepared from Sigma Chemical Company, USA. VFA were purchased from Aldrich Chemical Company, Milwaukee, USA. Ruthenium red, glutaraldehyde, Spurr's resin (obtained as a kit) and osmium tetroxide were purchased from Probing Structure, Queensland, Australia. Rapid ID32 A system was purchased from Bio Merieux SA, France.

All other chemicals, of the highest purity available, were obtained from the following sources: Ajax Chemicals, Sydney, Australia; B.D.H. Chemicals Australia Ltd., Boronia and Kilsyth, Australia.

### 6.2.2 Buffers and solutions

#### 6.2.2.1 Phosphate-Buffered Saline (PBS) pH 7.6 (Smibert and Krieg, 1994)

| *Ingredients* | g/l |
|---|---|
| $Na_2HPO_4$, anhydrous | 1.236 |

$NaH_2PO_4 . H_2O$ ..... 0.18

NaCl ..... 8.50

#### 6.2.2.2 Fixative solution

*Ingredients*

Paraformaldehyde ..... 4.0 g

Glutaraldehyde ..... 1.25 g

PBS plus 4% (w/v) sucrose ..... 100 ml

Paraformaldehyde powder was dissolved in sucrose-containing PBS buffer by heating to 60-70°C and stirring. One to three drops of 1 M NaOH were added with stirring until the solution became clear. The solution was cooled and glutaraldehyde which had already been dissolved in PBS buffer was added and the solution was brought to a final volume of 100 ml with PBS buffer.

#### 6.2.2.3 Spurr's resin

*Ingredients*

ERL (Vinyl cyclohexane dioxide) ..... 10.0 g

NSA (Nonenyl succinic anhydride) ..... 26.0 g

DER (Diglycidyl ether of polypropylene glycol) ..... 5.0 g

DMAE (2, Dimethylaminoethanol) ..... 0.2 ml

#### 6.2.2.4 Volatile fatty acid (VFA) standard solution

| *Ingredients* | ml |
|---|---|
| Acetic acid | 0.057 |
| Propionic acid | 0.075 |
| Isobutyric acid | 0.092 |
| n-Butyric acid | 0.091 |
| Isovaleric acid | 0.109 |

| | |
|---|---|
| Valeric acid | 0.109 |
| Caproic acid | 0.126 |

The components were added and brought to a final volume of 100 ml with RO water.

#### 6.2.2.5 Non-volatile fatty acid (NVFA) standard solution

*Ingredients*

| | |
|---|---|
| Lactic acid (85 %) | 0.084 ml |
| Succinic acid(1 M) | 0.060 g |

The components were added and brought to a final volume of 100 ml with RO water.

#### 6.2.2.6 Carboxymethyl cellulose (CMC) solution (Béguin, 1983)

| *Ingredients* | g/100 ml |
|---|---|
| CMC (sodium salt) | 1.0 |
| $K_2HPO_4$ | 0.87 |
| Citric acid | 0.25 |

$K_2HPO_4$ and citric acid were dissolved in RO water and the pH was adjusted to 6.3. CMC was dissolved in this solution.

### 6.2.3 Media and growth conditions

RF and NB media were prepared as described in chapter 2. The growth conditions were as described in section 2.2.9. Solid media were prepared as described in section 2.2.7.4. CMC agar plates were prepared by adding the CMC solution (10% v/v) to RF medium (section 2.2.7.2).

### 6.2.4 VFA analysis

VFA analysis was carried out according to Holdeman *et al.* (1977). 1 ml of the spent

medium from each culture of variants S and L was pipetted into 10 ml test tubes. As a standard control, 1 ml of VFA standard solution was also prepared. To each tube, 0.2 ml of 50% $H_2SO_4$, 0.4 g NaCl and 1 ml of ethyl ether were added. The tubes were capped, mixed by inversion 20 times and centrifuged at 1500 x g for 5 min to break the ether-culture emulsion. 1 µl of the ether layer (top) was injected into a Hewlett-Packard gas chromatograph (N0. 5710A) fitted with a packed column (FFAP on Chromosorb-G) using a 5 µl microsyringe. The detector was a flame ionisation system.

Column conditions: carrier gas: $N_2$; 30 ml/min; temperature: 140°C (isothermal); injector temperature 200°C; detector temperature 200°C.

### 6.2.5 NVFA analysis

The method was as described by Holdeman *et al.* (1977). 1 ml of the spent medium from each culture of variants S and L was pipetted into 10 ml tubes. As a standard control, 1 ml of NVFA standard solution was also prepared. 0.4 ml of 50% $H_2SO_4$ and 2.0 ml of methanol were added and tubes capped and mixed by inversion. Tubes were heated in a 55°C water bath for 30 min. 1 ml of RO water and 0.5 ml of chloroform was added followed by mixing and centrifuging at 1500 x g for 5 min to break the emulsion. 1 µl of the ether layer (top) was injected into a Hewlett-Packard gas chromatograph (N0. 5710A) fitted with a packed column (FFAP on Chromosorb-G) using a 5 µl microsyringe. The detector was a flame ionisation system. The column conditions was as described for VFA analysis.

### 6.2.6 Determination of capsules

The method was as described by Duguid (1951). A loopful of Indian ink was placed on a clean glass slide and mixed with a loopful of an overnight culture. A glass coverslip was placed on the mixture and using several thicknesses of absorbant paper it was pressed down, until the ink was sepia colour beneath the cover glass. The cells were then checked for capsular polysaccharides with high-dry and oil-immersion lens

systems under an Olympus BH-2 light microscope (Olympus Optical Co., Tokyo, Japan).

### 6.2.7 Electron microscopy

For scanning electron microscopy (SEM), cells were grown on RF medium agar plates and harvested from the agar surface using RO water by a bent glass rod. The samples were centrifuged (11,000 x g; 10 min; 4°C) and the pellet was gently resuspended in the fixative solution and left overnight at 4°C (Karnovsky, 1965). The cell suspension was centrifuged as before and the cells were washed gently twice (30 min each) in PBS buffer containing 4% (w/v) sucrose. The pellets were suspended in 1% (w/v) osmium tetroxide solution in RO water and left at room temperature for 2 h. The cells were further centrifuged and dehydrated through a graded series of ethanol solutions (70, 90, 95, 100 %), 2 changes of 30 min in each and an extra change of 1 h in 100 % ethanol. The bacteria were spotted onto aluminium SEM stubs and allowed to dry overnight before coating with gold-palladium-carbon and examined using a Phillips XL20 Scanning Electron Microscope operated at 10 kV.

For transmission electron microscopy (TEM), cells were prepared as described for SEM, except that cells were fixed in the presence of ruthenium red by the method of Luft, (1971) as described by Cheng and Costerton (1977). The concentration of ruthenium red was 0.15% (w/v) in the fixative solution and 0.05% (w/v) in osmium tetroxide and the dehydration solutions. The stepwise infiltration was carried out with 50% (w/v) Spurr's resin in ethanol for 8 h (overnight) and three changes with 100 % spurr's resin (8 h each). The cells were then embedded in fresh 100 % Spurr's resin at 60°C for 24 h. Embeded preparations were cut with a diamond knife in ultrathin sections using a Reichert Ultracut E microtome and mounted on celloidin-coated 400-mesh copper grids. The preparations were examined using a Phillips CM100 electron microscope at an accelerating voltage of 60 kV, and images were recorded on Kodak No. 4489 electron microscope films.

### 6.2.8 Quantitation of EPS

Overnight cultures grown on RF medium agar were harvested using a minimal amount of 0.15 M sodium acetate solution pH 3.0 by a bent glass rod. The suspension was then vortexed vigorously for 5 min and centrifuged at 11,000 x g for 10 min. The supernatant was removed and the cells were washed twice in the sodium acetate solution, vortexed and centrifuged as before. The pellet was kept on ice until used for determinating cell number and weight. All supernatant fluids were combined and recentrifuged at 11,000 x g to remove any remaining cells. EPS was measured in the supernatant as described by Daniels *et al.* (1994). Samples were pipetted into thick-walled pyrex tubes and adjusted to 0.5 ml with RO water. A reagent blank was prepared by using 0.5 ml of distilled water. A standard curve was prepared by using 0.5 ml solutions containing 50 to 500 μg of glucose. 0.5 ml of phenol solution (5% w/v) was added to each tube and mixed rapidly and thoroughly. Concentrated sulphuric acid (2.5 ml) was added, mixed rapidly and let stand at room temperature for 25 min. The absorbance of each tube was read against the blank at 488 nm. The concentration of extracted EPS in the samples was determined from the standard curve prepared by plotting the absorbances of standard solutions versus the concentration of glucose.

### 6.2.9 Determination of total cell number and weight

Each cell pellet obtained in section 6.2.8 was suspended in a known volume of water and cell number was counted using a haemocytometer. For determining cell weight, a known volume of each cell suspension was centrifuged and the pellet was dried under vacuum at 60°C and then weighed.

### 6.2.10 β 1-4 endoglucanase activity

Cultures were streaked onto CMC agar plates and incubated anaerobically at 39°C for 24 h. β-glucan hydrolysis was visualised as described by Teather and Wood (1982). The agar medium was flooded with 0.1% (w/v) Congo red solution for 15 min. The Congo red solution was then poured off, and plates were further treated by flooding

with 1 M NaCl for 15 min. The visualised zones of hydrolysis were stabilised by flooding the agar with 5% (v/v) acetic acid.

### 6.2.11 Growth tests and biochemical analysis

Growth tests and biochemical analysis were carried out according to Holdeman *et al.* (1977). Enzymes produced by the variants S and L were analysed using API rapid ID32 A system according to the manufacturer's instructions.

### 6.2.12 SDS-Polyacrylamide gel electrophoresis

Bacteria were grown in RF medium (section 2.2.7.2). Cultures were harvested at late log to stationary phase and cell-free extracts were prepared as outlined in section 4.2.4. SDS-PAGE was carried out as described in section 4.2.5.

### 6.2.13 Restriction digests

Chromosomal DNA from the two variants was isolated as described in section 5.2.13, and digested to completion with the restriction enzymes, *Eco*RI and *Hae*III as described in section 5.2.14. The restriction fragments were separated by 0.8% agarose gel electrophoresis as described in section 5.2.15..

## 6.3 Results

### 6.3.1 Morphological characteristics of *B. fibrisolvens* strain E14, variants S and L

Upon growth of *B. fibrisolvens* strain E14 on NB medium agar plates containing 70 mM $NH_4Cl$ plus 0.15% (w/v) casamino acids, a variant that appeared to differ from the original organism in its ability to adhere to the agar was observed.

The wild type adherent organism (S) appeared as circular colonies, more intensely pigmented and firmly attached to the plate so that could not be removed without scraping the substratum. These colonies were extremely sticky during the active growth

phase but were released from agar if the plates were incubated for a longer period of time. In contrast, the non-adherent variant (L) was loosely attached to the agar at all stages of growth and could be readily removed by light washing with water or buffer. This is shown in Figure 6.1 when the cells were tested for endoglucanase activity on CMC agar plates. The staining and washing processes washed off L variant colonies from agar whereas S colonies remained attached. The cells in one colony of the adherent organism (S) stuck together so tightly that the dispersion of the cells was very difficult and depending on the stage of growth sometimes impossible. In an overnight broth culture, S cells aggregated at the bottom of the tube, whereas L cells formed a homogeneous mixture (Figure 6.2). Variants S and L were colony purified and maintained as separate cultures.

### 6.3.2 Biochemical characteristics of *B. fibrisolvens* strain E14, variants S and L

Variants S and L were compared biochemically. The results (Tables 6.1 and 6.2) show that variants S and L were identical based on fermentation of substrates, production of volatile and non-volatile fatty acids and the content of various enzymes. GLC profiles of the spent media showed that both variants produce butyric acid and lactic acid. All of the experiments reported in chapter 4 and 5 of this thesis were repeated for the two variants. The results of these experiments showed that variants S and L had identical nitrogen requirements. Orpin *et al.* (1985) have reported that *B. fibrisolvens* strain E14 is a cellulolytic organism. The two variants were therefore tested for β 1-4 endoglucanase activity. The results (Figure 6.1) show that both variants possess similar levels of endoglucanase activity.

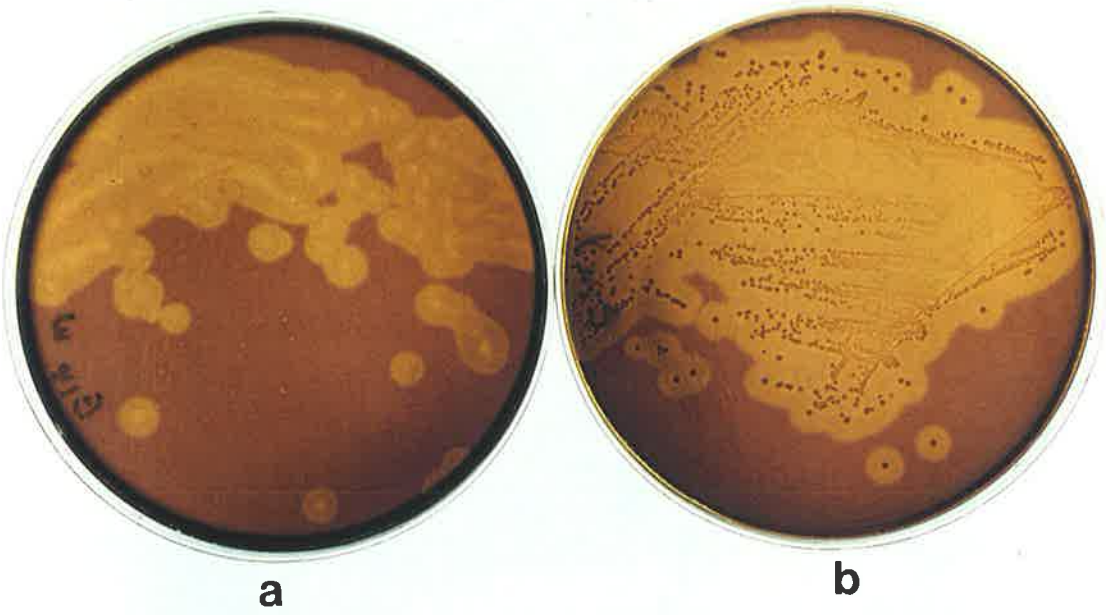



**Figure 6.1 Cultures of *B. fibrisolvens* strain E14, variants S and L on CMC agar plates**

Cells were streaked onto CMC agar plates and incubated anaerobically at 39°C for 24 hours. Plates were stained with 0.1% (w/v) Congo red solution, destained with 1 M NaCl and stabilised with 5% acetic acid.

a) Variant L

b) Variant S

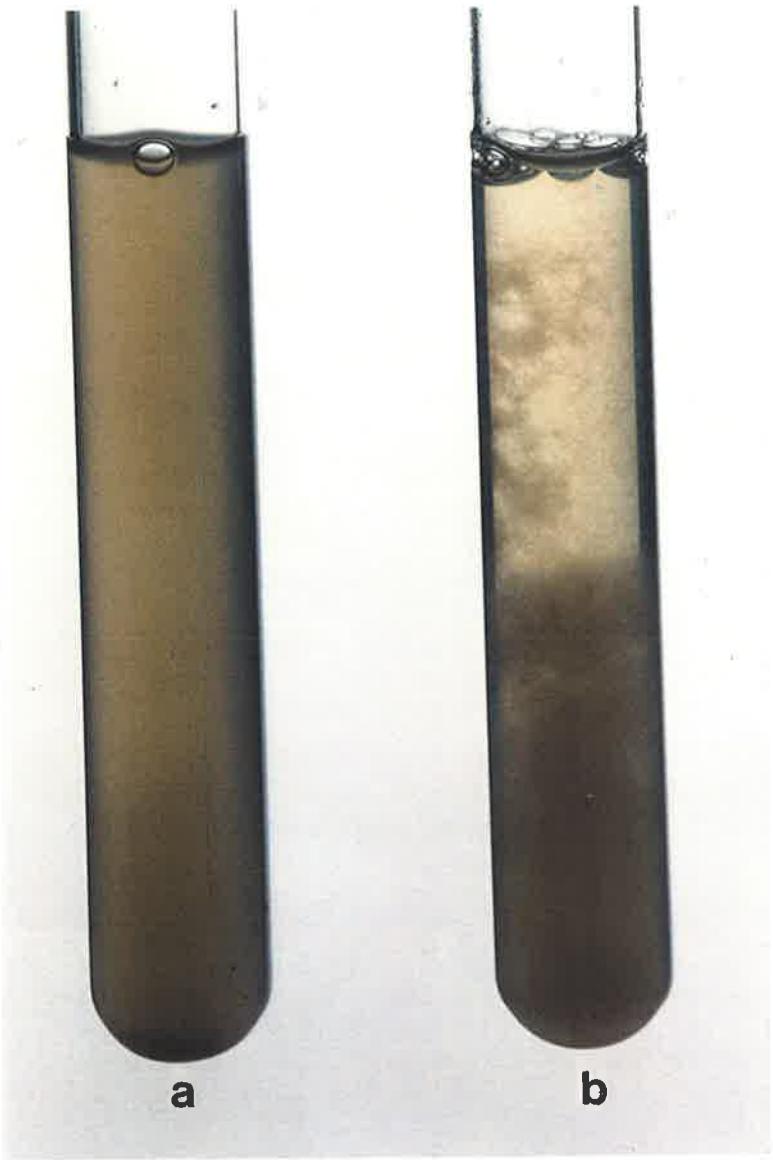



**Figure 6.2 Growth of *B. fibrisolvens* strain E14, variants S and L, in liquid media.** Cells were grown in NB medium containing 70 mM $NH_4Cl$ plus 0.15% (w/v) casamino acids and incubated at 39°C for 17 hours.

a) Variant L

b) Variant S

**Table 6.1 Characterisation of *B. fibrisolvens* strain E14, variants S and L (Holdeman *et al.*, 1977).**

| Growth tests and biochemical analysis | *Butyrivibrio fibrisolvens* strain E14 | |
|---|---|---|
| | Variant S | Variant L |
| Arabinose | + | + |
| Cellobiose | + | + |
| Fructose | + | + |
| Glucose | + | + |
| Inositol | - | - |
| Lactose | + | + |
| Maltose | + | + |
| Mannitol | - | - |
| Raffinose | + | + |
| Rhamnose | + | + |
| Ribose | - | - |
| Sorbitol | - | - |
| Starch | + | + |
| Sucrose | + | + |
| Xylose | + | + |
| Gelatin | + | + |
| Aesculin | + | + |
| Melibiose | + | + |
| Salicin | + | + |
| Trehalose | - | - |
| Catalase | - | - |
| Butyric acid production | + | + |
| Lactic acid production | + | + |
| Reduction of nitrates | - | - |
| Indole production | - | - |

**Table 6.2 The enzyme content of of *B. fibrisolvens* strain E14, variants S and L as determined by the API rapid ID32 A system.**

| Enzymatic activity | *Butyrivibrio fibrisolvens* strain E14 | |
|---|---|---|
| | Variant S | Variant L |
| Urease | - | - |
| Arginine dihydrolase | - | - |
| α-Galactosidase | + | + |
| β-Galactosidase | + | + |
| β-Galactosidase 6 phosphate | - | - |
| α-Glucosidase | - | - |
| β-Glucosidase | + | + |
| α-Arabinosidase | + | + |
| β-Glucuronidase | - | - |
| β-N-Acetyl-glucosaminidase | - | - |
| Glutamic acid decarboxylase | - | - |
| α-Fucosidase | - | - |
| Phosphatase Alkaline | - | - |
| Arginine arylamidase | - | - |
| Proline arylamidase | - | - |
| Phenylalanine arylamidase | - | - |
| Leucine arylamidase | - | - |
| Tyrosine arylamidase | - | - |
| Alanine arylamidase | - | - |
| Glycine arylamidase | - | - |
| Leucyl glycine arylamidase | - | - |

### 6.3.3 Comparison between growth rates of *B. fibrisolvens* strain E14, variants S and L

To determine whether there is any difference between growth rates of the two variants, bacterial growth was monitored in NB medium containing $NH_4Cl$ plus 0.2 mM methionine. The results (Figure 6.3) show that there was no significant difference between growth rates of the two variants in this medium.

### 6.3.4 Comparison of protein profiles of*B. fibrisolvens* strain E14 variants S and L

To compare the protein profiles of variants S and L, cell-free extracts of each organism were analysed by SDS-PAGE. *B. fibrisolvens* strain H17c was also tested as a control. The results (Figure 6.4) show that both variants have identical pattern of stained bands which are different from those of H17c.

### 6.3.5 Comparison of the restriction digestion patterns of DNA from *B. fibrisolvens* strain E14 , variants S and L

To compare the restriction digestion patterns from the two variants, chromosomal DNA was digested to completion and separated by agarose gel electrophoresis. *B. fibrisolvens* strain H17c was used as a control. The results (Figure 6.5) show that the pattern of digested DNA from the two variants are identical. These patterns are different from those of strain H17c.

### 6.3.6 EPS production

To determine whether differences in the adherent behaviour of variants S and L is due to different amounts of EPS synthesised, EPS produced by each variant was measured. The results show that variants S and L produced $14.45 \pm 0.01$ and $3.42 \pm 0.009$ mg EPS/mg dry cells respectively. One mg of cells comprises approximately $5 \times 10^8$ cells. These results are expressed as the mean $\pm$ the standard error of the mean for the triplicate assays.

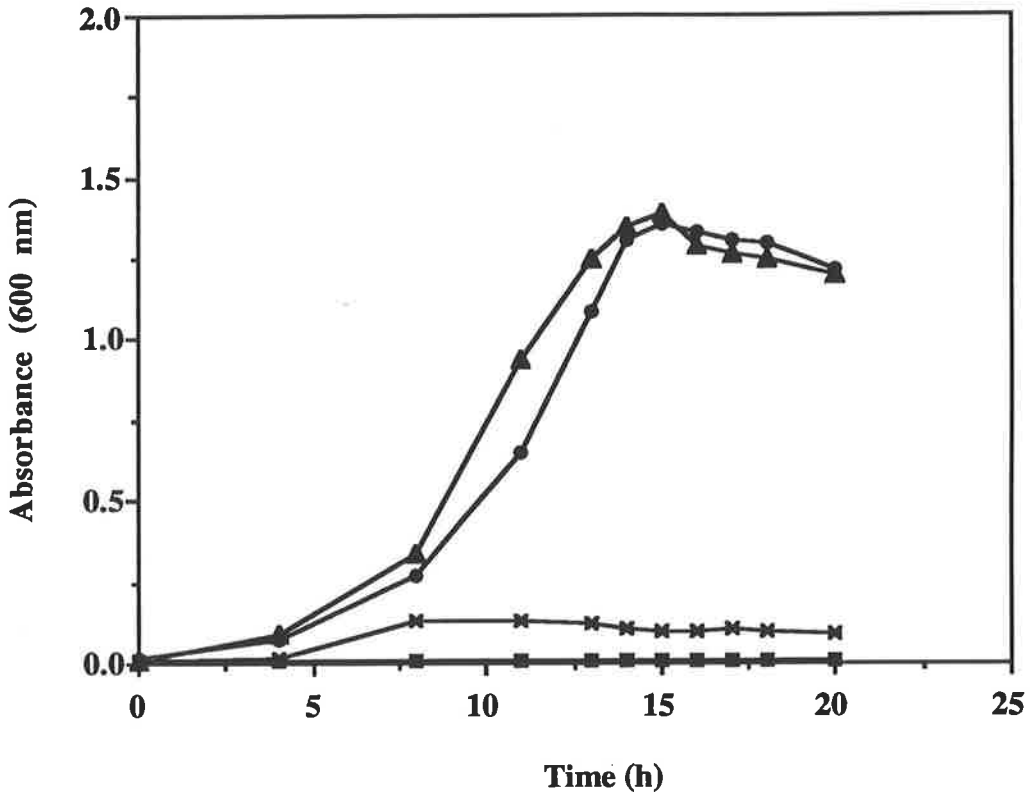



**Figure 6.3 Growth of *B. fibrisolvens* strain E14, variants S and L:**

—▲— variant L on 70 mM $NH_4Cl$ plus 0.2 mM methionine

—●— variant S on 70 mM $NH_4Cl$ plus 0.2 mM methionine

—✖— variant L without an exogenous nitrogen source (control)

—■— variant S without an exogenous nitrogen source (control)

Cells were incubated anaerobically at 39°C and cell densities were measured at 17 h using a NOVASPEC spectrophotometer at 600 nm against an uninoculated NB medium blank. Absorbance values represent the mean of triplicate assays, with variation between triplicates being less than 0.05.

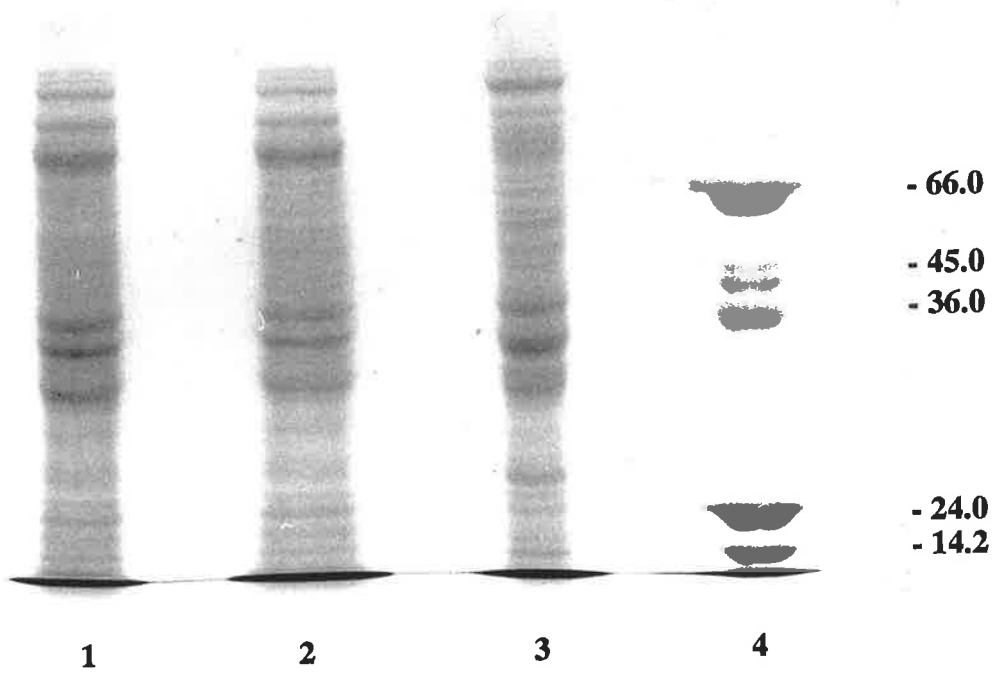



**Figure 6.4 Protein profile of *B. fibrisolvens* strain E14, variants S and L and strain H17c**

Cell-free extracts were analysed by SDS-PAGE, stained with Coomassie blue and dried on Whatman paper 3 MM. Lanes 1 and 2 are *B. fibrisolvens* strain E14, variants L and S respectively. Lane 3 is *B. fibrisolvens* strain H17c. Lane 4 molecular weight markers (kDa).

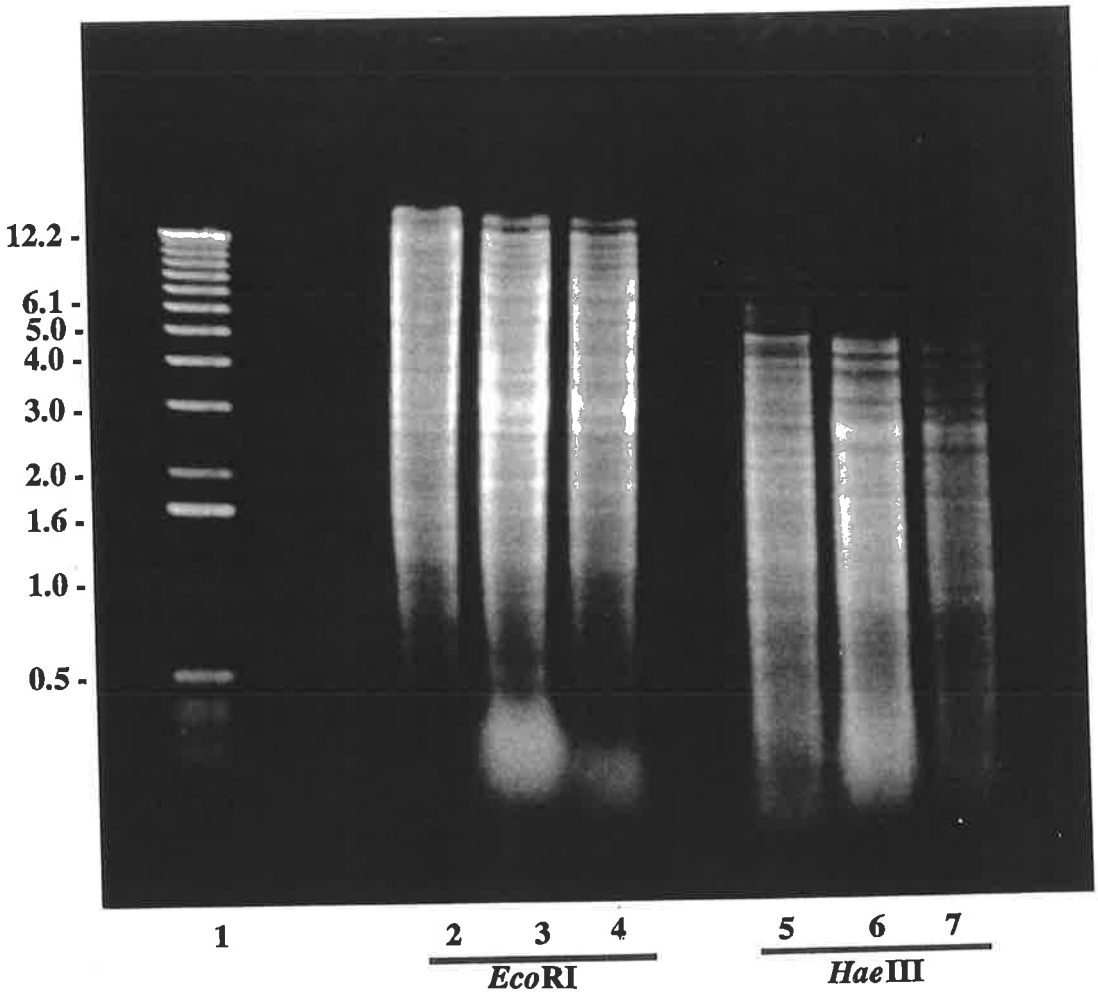



**Figure 6.5 Profiles of restriction digests of chromosomal DNA from *B. fibrisolvens* strain H17c and E14, variants S and L**

Bacterial DNA was digested with the restriction enzymes and separated by electrophoresis on 0.8% agarose gel.

Lane 1, DNA size markers indicated in kilobase pairs

lanes 2 and 5, strain H17c

lanes 3 and 6, strain E14, variant S

lanes 4 and 7, strain E14, variant L

### 6.3.7 Microscopy

Light microscopy of Gram stained preparations showed that variants S and L are rod-shaped and Gram negative. Light microscopy of Indian ink preparations did not show any capsular polysaccharides for either the adherent (S) or the non-adherent (L) variant. SEM (Figure 6.6) show exopolymers associated with S as a thin film bridging the space between cells. In contrast, variant L occurred primarily devoid of exopolymer bridgings (Figure 6.7).

TEM (Figure 6.8) show that variant S cells were surrounded by large quantities of an electron-dense residue of the ruthenium-red-reactive EPS that had been produced by condensation during dehydration of the extensive glycocalyxes that surrounded these cells. In contrast, variant L was shown to be surrounded by only a small amount of the electron-dense EPS (Figure 6.9).

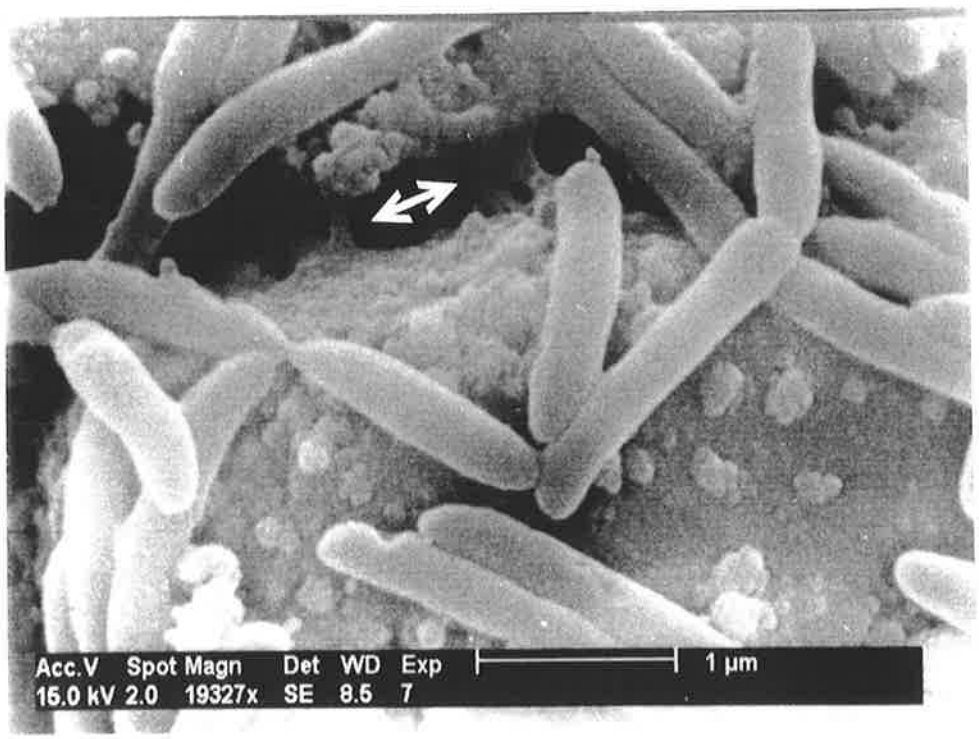



**Figure 6.6 Scanning electron microscopy of *B. fibrisolvens* strain E14, variant S**

Note exopolymers (mucoid EPS) bridging the spaces between cells (arrows) and exopolysaccharide matrix.

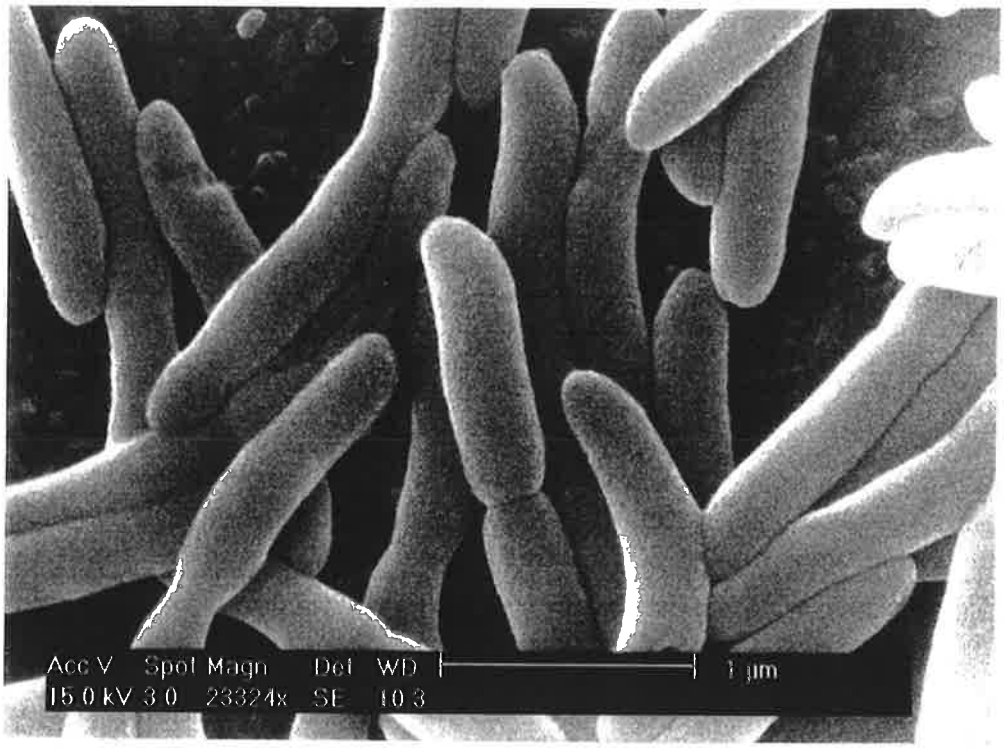



**Figure 6.7 Scanning electron microscopy of *B. fibrisolvens* strain E14, variant L**

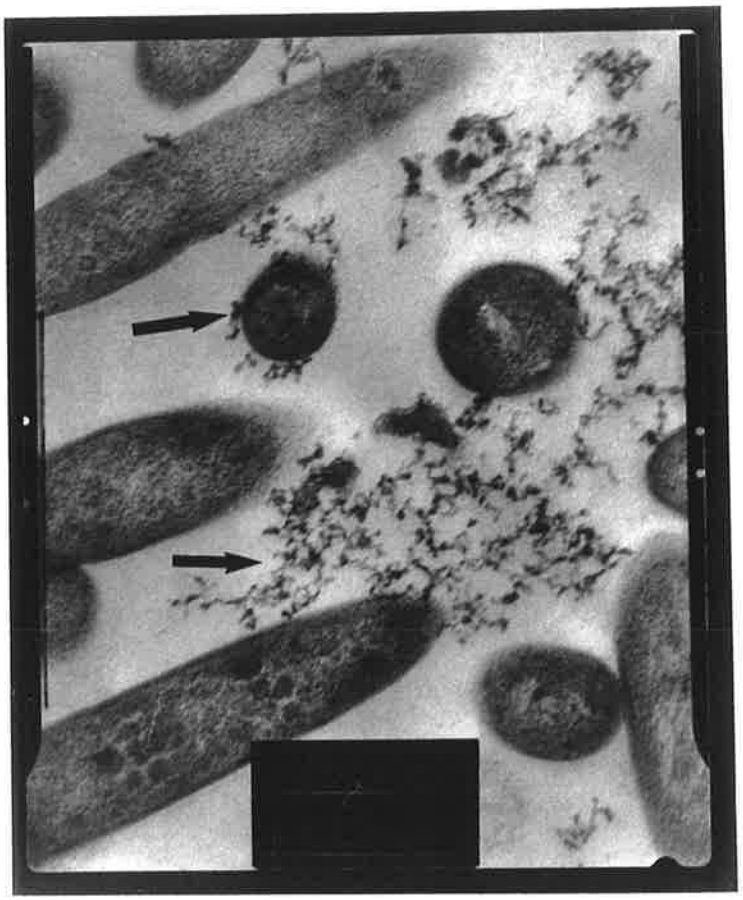



**Figure 6.8 Transmission electron microscopy of sections of *B. fibrisolvens* strain E14, variant S stained with ruthenium red**

Note extracellular polysaccharide matrix (arrows) around the cells.

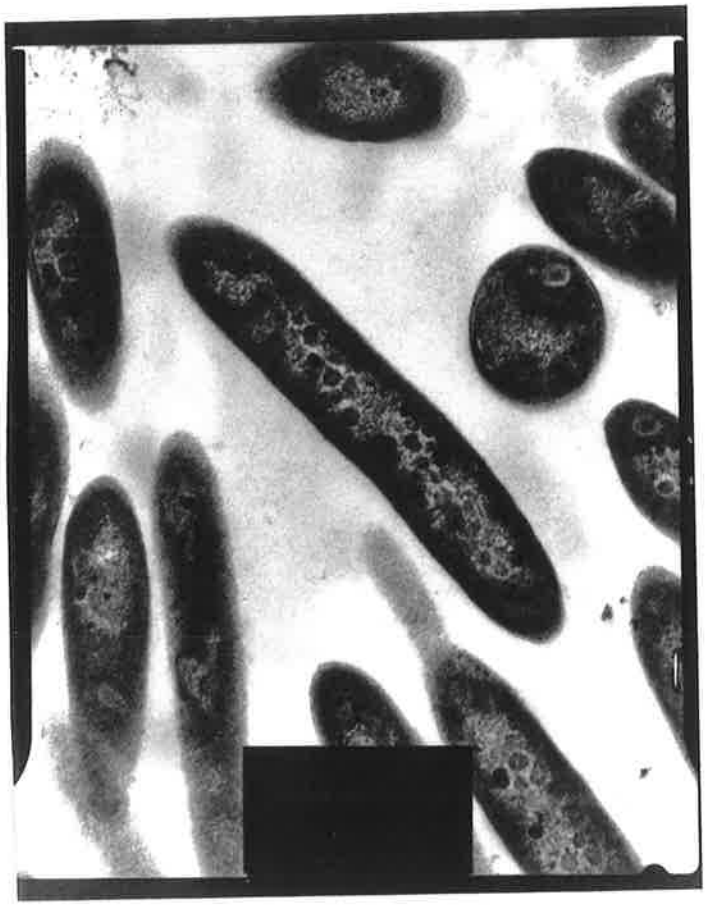



**Figure 6.9 Transmission electron microscopy of sections of *B. fibrisolvens* strain E14, variant L stained with ruthenium red**

## 6.4 Discussion

The results of nutritional and biochemical experiments, comparison of the patterns of chromosomal DNA digests and the bacterial protein profiles suggest that variants S and L are two phenotypes of *B. fibrisolvens* strain E14. However, the adherent variant (S) produced more than 4 times as much EPS as the non-adherent variant (L). These results strongly suggest that the main difference between variants S and L is in the production of EPS. This conclusion is confirmed by SEM where it was shown that variant S produced a large quantity of mucoid extracellular polymers bridging the space between cells (Figure 6.6) whereas variant L did not (Figure 6.7). TEM also showed a large quantity of extracellular polymers surrounded variant S cells (Figure 6.8).

It is thought that capsular EPS is adhesive (Hermansson and Marshall, 1985), and slime EPS may release attached bacteria from the surface of a substrate. Indian ink stained smears did not show the presence of a classical capsular glycocalyx in variant S suggesting that the EPS produced by the organism may be of the slime form. Nevertheless, variant S is able to attach to solid media whereas variant L is not. This suggests that EPS could precondition the bacterium for attachment and may enhance the process by maintaining juxtaposition of the bacterial cells. Similarly, it has been reported that in the marine prokaryote, *Hyphomonas,* EPS alone appears to be the primary adhesion and $EPS^-$ variants do not attach to surfaces (Quintero and Weiner, 1995). The greater amount of EPS produced by variant S compared to that produced by variant L may explain the sticky nature of variant S. Holzwarth and Ogletree (1979) have suggested that adhesion behaviour of bacteria may be due to the chemical changes in EPS. These chemical changes may be due to differences in the acyl and ketal groups rather than carbohydrate structures. It has also been suggested that protein and lipid components may be present and involved in stickiness (Fletcher, 1980). However, the present work does not provide evidence to explain adhesion behaviour of variant S in more detail. Further investigation is required to determine whether there are structural

differences in the exopolymers produced by the two variants.

Variant S strongly attaches to the agar during exponential phase of growth but releases from the agar if it is incubated for a longer period of time. An explanation for this phenomenon is that the cells produce EPS to be able to attach to nutrients during the growth phase and release from the agar once nutrients are depleted. Wrangstadh *et al.* (1990) have reported a similar phenomenon for marine bacteria. Hermansson and Marshall (1985) and Wrangstadh *et al.* (1990) have suggested that EPS may be responsible both for attachment to surfaces and the exploitation of surface-associated nutrients and detachment of the cells from the surface once nutrients are depleted, thereby allowing for colonisation of new surfaces. Montgomery and Kirchman (1974) have demonstrated that in the marine environment, *Vibrio harveyi* produces specific chitin-binding proteins in the presence of chitin, giving the bacterium a selective advantage over other bacteria in that nutrient-poor environment. Starvation-induced changes in EPS production have also been observed in marine bacteria (Wrangstadh *et al.*, 1990). Cornish *et al.* (1988) has reported the isolation of a non-adherent variant of *Agrobacterium radiobacter* by growing the parent organism under prolonged glucose limitation. It has also been reported that many EPS-producing bacteria synthesise polysaccharides at progressively lower rates during prolonged growth in continuous culture or following repeated subcultures (Cornish *et al.*, 1988). Therefore, it is possible that the non-adherent variant of *B. fibrisolvens* strain E14 has been generated by prolonged passage in the laboratory.

Since *B. fibrisolvens* strain E14 variants S and L differ in their production of EPS, they are clearly useful experimental models for studying biochemical and genetic aspects of EPS production and cell attachment in this species. The change in attachment characteristics of variant L may be associated with molecular mechanisms that mediate attachment to substrates *in vivo*. Since adhesion is the first specific event in the digestion of cell wall material (Cheng *et al.*, 1984), these two variants are useful

experimental models for studying fibre digestion and the genetics of adhesion to fibre in *B. fibrisolvens*.

Whitfield (1988) has suggested that EPS has significant effects on diffusion properties of the cells. Therefore, it is also interesting to study the effect of EPS on uptake of nutrients by the two variants.

Much attention has been focussed on the adhesion of rumen bacteria to plant surfaces from the standpoint of fibre degradation but not from protein degradation. The microbial adhesion to surfaces is an ecological adaptation and may be an important factor in protein degradation. When Cotta and Hespell (1986a) attempted to purify protease activity from a culture fluid of *B. fibrisolvens* strain 49, they found that a substantial amount of high molecular weight polysaccharide material was associated with the protein. The nature of the association between the proteases and these polysaccharide material was not explored. However, this may suggest that EPS is a significant factor in proteolysis. Therefore, variants L and S are useful tools for studying the effect of EPS and bacterial adhesion on protein degradation. This may lead to manipulation of the kinetics of protein degradation in the rumen.

# CHAPTER 7

# CHAPTER 7

## General Discussion and Future Studies

Proteolytic and deaminative activity within the rumen results in the production of ammonia, amino acids and peptides. Utilisation and metabolism of these products of protein degradation are therefore central to the nitrogen economy of the rumen ecosystem, and the ruminant animal. Although some aspects of protein degradation by ruminal microorganisms is known, surprisingly little is known about the role of amino acids and peptides in relation to bacterial growth. There is substantial indirect evidence that amino acids and peptides are important nitrogen sources for rumen bacteria. However, many researchers still contend that the role of amino acids and peptides within the rumen is "uncertain" (Armstead and Ling, 1993). Additional research is needed to assess ruminal bacterial ammonia, amino acid and peptide utilisation in more detail. It is only when these processes are better understood that the manipulation of rumen bacteria can be undertaken to improve nitrogen utilisation in ruminants.

The role of ammonia, amino acids and peptides in relation to ruminal bacterial growth has been studied in this work. This area of research is littered with inconsistencies in the literature. These inconsistencies may have been largely caused by the unavailability of a completely defined medium and suitable substrates. Commercially available acid hydrolysates and enzymatic digests of proteins have been usually used as amino acid and peptide sources. However, these are unsuitable sources of amino acids and peptides as they contain high proportions of peptides and free amino acids respectively.

In the work described in chapter 2, peptide-free amino acids and amino acid-free peptides were prepared from commercially available sources and a completely defined medium was developed. The development of the defined medium was essential for this study as without this the work reported in this thesis was not possible. The medium was

not only used for studying the nitrogen requirements of 7 strains of rumen bacteria and a non-ruminal bacterium, but also for identifying limitations in amino acid biosynthetic pathways, preparing of cultures grown under controlled condition (e.g. for enzyme assays), and performing competition inhibition experiments.

Using this defined medium the nitrogen requirements of *Streptococcus bovis* strains 2B and H24, *Selenomonas ruminantium* S23, *Prevotella ruminicola* strains GA33 and P1 and *Butyrivibrio fibrisolvens* strains E14 and H17c were established. It was found that ammonia is the main nitrogen source for all bacterial strains tested. However, *P. ruminicola* strain GA33 and *B. fibrisolvens* strain E14 are not able to utilise ammonia unless mixed amino acids or peptides are also provided. *B. fibrisolvens* strain H17c is able to grow on ammonia only if reducing conditions are enhanced by the addition of 3 mM $Na_2S$. Mixed amino acids and peptides are not as effective nitrogen sources as ammonia. Therefore, the hypothesis that ammonia is the main nitrogen source for rumen bacteria appears to be quite sound.

The hypothesis of the experiments described in chapter 3 was that the inability of *P. ruminicola* strain GA33 to grow on ammonia as a sole nitrogen source may be due to limitations in the biosynthetic pathway of one or more amino acids. Growth experiments using individual amino acids indicated that the strain was able to grow on ammonia when the medium was supplemented with any individual amino acid except cysteine and methionine. It was found that the growth limitation was due to the lack of NADH - and NADPH-dependent GDH activities in this strain.

Despite considerable knowledge concerning ammonia concentration in the rumen under different dietary conditions and the enzymes involved in ammonia assimilation in mixed rumen bacteria and pure isolates, information on factors influencing ammonia assimilation and glutamate formation in rumen bacteria is limited. The recent biotechnology has mostly focused on genetic manipulation of rumen bacteria to

improve fibre degradation but there are few reports concerning the purification and subsequent biochemical and genetic characterisation of the key enzymes involved in ammonia assimilation (Duncan *et al.*, 1992). Therefore, a study of the biochemistry and genetics of ammonia assimilation is essential. This would involve establishing the regulatory mechanisms for the ammonia assimilatory enzymes and investigating the genes involved in the formation and regulation of the key enzymes in the pathways of ammonia assimilation. These studies may lead to ways to increase ammonia assimilation by genetic manipulation and thus increase bacterial protein biosynthesis. GDH with its high Km for ammonia appears to be the most important enzyme in ammonia assimilation in rumen bacteria. Lack of GDH activity in *P. ruminicola* strain GA33 makes the strain a useful tool for studying the biochemical and genetic aspects of ammonia assimilation. Complementation experiments using DNA from *P. ruminicola* strain P1 which was found in the present study to possess both NADH- and NADPH-dependent activities can be performed to clone the GDH genes of strain P1 and to study the genetics of ammonia assimilation via GDH. The results of these studies may be used to increase ammonia assimilation in rumen bacteria. More fixation of ammonia nitrogen into bacterial cells will assist in developing animal production systems which are suitable both environmentally and economically.

*P. ruminicola* strain GA33 is the type strain for *P. ruminicola* subsp. brevis (Bryant *et al.*, 1958). The lack of GDH in strain GA33 raises the question of whether this strain is appropriate as a type strain for *P. ruminicola* species ? Previous studies used traditional identification and characterisation techniques which have tended to group phenotypically similar organisms together. Recent studies have indicated that the subgroups of *P. ruminicola* are not closely related and there are differences between them (Holdeman *et al.*, 1984; Begbie and Stewart, 1984). This may suggest that what was previously thought to be a single bacterial entity may in fact be a collection of species. Techniques of molecular biology such as DNA-DNA hybridisation and PCR technology can be used for identification and classification of these bacteria. These

techniques may be used to choose a more appropriate strain of *P. ruminicola* as a type strain for the species.

In Chapters 4 and 5, the absolute requirement of *B. fibrisolvens* strain E14 for mixed amino acids or peptides was investigated. Growth experiments using individual amino acids indicated that only methionine is able to replace the requirement for mixed amino acids or peptides. SDS-PAGE of the cell-free extracts obtained from the cultures grown on $^{35}$S-methionine showed that methionine is incorporated into cell protein. It was concluded that the limitation in the availability of methionine is due to the inability of the strain to synthesise methionine *de novo*. The pathway of methionine biosynthesis is a complex pathway consisting of 14 reactions (Figure 5.1). In addition, some reactions may be catalysed by more than one enzyme. In order to find step(s) in the pathway in which strain E14 is limited, all of the reactions involved in the pathway should be tested. In the present work growth experiments using intermediates of the methionine biosynthetic pathway were performed. Since homocysteine alone or in combination with other appropriate intermediates of the methionine biosynthetic pathway was not able to promote bacterial growth, it was concluded that the defect is associated with one or more enzymes in the final steps of the pathway. However, lack of bacterial growth on a substrate may be either due to the inability of the strain to transport that substrate into the cells or to the lack of a specific enzyme to metabolise that substrate. To distinguish between these possibilities radiolabelled substrates are required. Unfortunately, most radiolabelled intermediates of the methionine biosynthetic pathway are not commercially available. Enzyme assays indicated the lack of cobalamin-dependent methionine synthase in strain E14 and the methionine-independent strain H17c. Since neither strain E14 nor H17c express cobalamin-dependent methionine synthase activity, this is unlikely to be the difference between the two strains. Therefore, this implies that strain H17c differs from strain E14 in that it possesses cobalamin-independent methionine synthase activity whereas strain E14 does not. Assay of cobalamin-independent methionine synthase was not possible as the

triglutamate form of $N^5$-methyltetrahydrofolate, the specific substrate for the enzyme is not commercially available. Southern blotting using probes prepared from the *E. coli* cobalamin-independent methionine synthase (*metE*) gene may indicate the presence or absence of this gene in strain E14. Northern blotting could be carried out to examine the expression of this gene. However, little homology was observed between the *E. coli* *metE* gene and DNA from *B. fibrisolvens* strains E14 and H17c. Another approach was to investigate methionine biosynthesis in intact cells using L-[$\beta$-$^{14}$C]-serine. Strain E14 was not able to utilise serine as a 1-C donor to synthesise methionine. Although these experiments confirm that the defect in strain E14 is in the final steps of the methionine biosynthetic pathway, it is not clear which enzyme is lacking. The strain may lack cobalamin-independent methionine synthase (Figure 5.1, reaction 14) and/or has a defect in the folate pathway from tetrahydrofolate to $N^5$-methyltetrahydrofolate (Figure 5.1, reactions 10, 11). Since a defect in the folate biosynthetic pathway is likely to be lethal, the more likely candidate is cobalamin-independent methionine synthase.

In animal tissues the availability of methionine in the diet is necessary for normal growth and development and it is a precursor of cystathionine and cysteine (Finkelstein, 1990). Methionine has also been recognised as the primary factor that limits wool growth in sheep (D'Andrea *et al.*, 1989), as these animals can convert excess methionine to cysteine in the skin for incorporation into wool (Pisulewski and Buttery 1985). There is evidence that administration of methionine to the ruminant diet affects animal production and growth. Clark and Petersen (1988) have reported that compared with urea alone, methionine addition increased *in vitro* fermentation rate and *in vivo* apparent dry matter digestion rate of mature range grasses, yielding more positive heifer weight changes. Since the infusion of a combination of lysine and methionine into the abomasum of lactating dairy cattle increased milk protein, Schwab *et al.* (1976) concluded that methionine and lysine are the most limiting amino acids. Cronjé *et al.* (1992) have also found that infusion of methionine increased whole-body protein synthesis in merino cross-bred wethers. Since ruminants depend on microbial protein, a

limitation in ruminal microbial amino acid biosynthesis can have a significant impact on animal production. Thus, promotion of animal growth and production by methionine may be at least partly due to the inability of other strains of rumen bacteria to synthesise methionine *de novo*. Further work is required to investigate this possibility.

The dipeptide alanyl-methionine is able to replace the requirement for methionine in strain E14 whereas alanyl-alanine is not. Thus, strain E14 can obtain the essential amino acid methionine from dipeptides. Competition inhibition experiments using 5-aminolevulinic acid as a dipeptide analogue showed that the dipeptide alanyl-methionine is not hydrolysed extracellularly but is either transported intact and hydrolysed intracellularly or hydrolysed as it is transported into the cells. A mixture of peptides of different sizes present in the enzymatic hydrolysate of casein is also able to promote growth of strain E14 on $NH_4Cl$ (Figure 2.5). This suggests that longer peptides can also replace the requirement for methionine. However, it is not clear whether these peptides are hydrolysed extracellularly and the liberated methionine is utilised by the cells or whether they can be transported into the cells, where they are hydrolysed intracellularly prior to utilisation of methionine. If the latter occurs, what is the upper size limit for a peptide to be transported into the cells in this strain ? These questions my be answered by competition inhibition experiments using methionine-containing oligopeptides of different sizes and oligopeptides devoid of methionine as the oligopeptide competitors. If the oligopeptides are hydrolysed extracellularly, there will be no competition between them for transport as the cells will grow on the liberated methionine. If the oligopeptides are not hydrolysed extracellularly, bacterial growth may be inhibited because of competition for uptake between methionine-containing peptides and oligopeptides devoid of methionine. Transport of peptides can also be demonstrated using the traditional radiolabelling techniques. However, radiolabelling techniques may give misleading results as they are unable to distinguish between extracellular hydrolysis of peptides into smaller peptides and/or amino acids, or rapid

hydrolysis after transport and a possible efflux of labelled amino acids from an intracellular pool. Another approach is to use a dansylation method which allows a direct measurement of the uptake of any peptide (Westlake and Mackie, 1990). This method involves reacting peptides left in the media or accumulated intracellular peptides with dansyl chloride, the dansyl derivatives being separated by TLC and their amounts estimated from the intensities of the fluorescent spots. Using fluoresence methods, any exodus of cleaved amino acid or peptide residues is readily apparent (Payne, 1983).

S-adenosylmethionine (AdoMet) can also replace the requirement for methionine in *B. fibrisolvens* strain E14. Since the AdoMet synthetase gene (*metK*) and activity were detectable in strain E14, growth stimulation by AdoMet is not due to the lack AdoMet biosynthesis in this strain. Interestingly, promotion of growth of strain E14 by AdoMet is possibly due to the ability of the strain to synthesise methionine from methylthioribose, a metabolite of AdoMet. This suggests that an alternative methionine biosynthetic pathway similar to that previously reported in *Ochromonas malhamensis* (Sugimoto *et al.*, 1976) and *Candida utilis* (Schlenk and Ehninger, 1964; Shapiro and Schlenk, 1980) may be present in strain E14. Methionine-independent strain H17c was also able to synthesise methionine from methylthioribose. Therefore, this ability may be more common irrespective of whether the bacterium is able to synthesise methionine *de novo* via the common methionine biosynthetic pathway or not. More strains of rumen bacteria should be tested to confirm this possibility.

Previous studies indicate that, compared with *E. coli*, most strains of rumen bacteria are very inefficient in incorporating the carbon of exogenous amino acids during growth in media containing ammonia (Pittman and Bryant, 1964). However, recent studies by Armstead and Ling (1993) and Ling and Armstead (1995) show the potential importance of both amino acids and peptides. Ling and Armstead (1995) have indicated that amino acids can supply up to 62%, and peptides, 43% of the nitrogen

requirements of rumen bacteria. Similarly, the present study shows that both *B. fibrisolvens* strain E14 and the methionine-independent strain H17c are able to utilise preformed amino acids. SDS-PAGE of cultures grown on labelled amino acids showed that strain E14 is able to incorporate the limiting amino acid methionine and the non-limiting amino acids cysteine and lysine into bacterial protein. Stimulation of growth of *P. ruminicola* strain GA33 with virtually any amino acid also indicates that this strain is able to utilise amino acids. These findings show the vital role of amino acids in relation to bacterial growth in strains of rumen bacteria impaired in *de novo* biosynthesis of particular amino acids.

Most of studies on amino acid metabolism have focused on the deamination of amino acids in the rumen. Therefore, the importance of amino acids as nitrogen sources is underestimated. It has long been recognised that some rumen bacteria have absolute requirements or are stimulated by the addition of amino acids or peptides to the growth medium (Pittman and Bryant, 1964; Hungate, 1966; Pittman *et al.*, 1967). Nevertheless, no detailed study has explored this limitation. This is apparently the first report that clearly shows that the absolute requirement of some strains of rumen bacteria for amino acids or peptides is due to the inability of these bacteria to synthesise particular amino acid(s) *de novo*.

It has been reported here that growth stimulation of *P. ruminicola* strain GA33 and *B. fibrisolvens* strain E14 by mixed amino acids or peptides is due to the supply of specific growth limiting amino acids which are not synthesised by the cells. The finding that 2 strains from 7 strains tested are impaired in *de novo* biosynthesis of particular amino acids does not clearly have statistical value. More strains of rumen bacteria should be tested to determine whether limitations in *de novo* biosynthesis of amino acids is a common phenomenon in rumen bacteria. It is not clear whether specific environmental conditions lead to the introduction of mutations in amino acid biosynthetic pathways in these bacteria or it was generated by prolonged passage in the laboratory. It is possible

that these bacteria have lost the ability to synthesise particular amino acid(s) due to the availability of amino acids and peptides in the rumen, especially when the energy cost of biosynthesis of some amino acids such as methionine may be high for an anaerobic bacterium. Another possibility is that the lack *de novo* biosynthesis of particular amino acid(s) is a genotypic characteristic of these bacteria. If so, this criteria can be used as a marker for studying the genetic diversity in rumen bacteria. However, these interesting strains of rumen bacteria can be used to investigate amino acid biosynthesis in rumen bacteria. For example, complementation experiments using DNA from a closely related strain can be employed to produce genetic maps of these bacteria and study the genetics of amino acid biosynthesis in rumen bacteria. Information on amino acid biosynthesis in rumen bacteria may be then used to improve nitrogen utilisation in ruminants.

An interesting outcome from this study is the discovery of a non-adherent variant of *B. fibrisolvens* strain E14 (chapter 6). The larger amount of extracellular polysaccharide (EPS) produced by the adherent variant S compared to that produced by the non-adherent variant L may explain the adhesion behaviour of variant S. However, further studies are clearly needed to determine whether there are structural differences in the exopolymers produced by the two variants that are responsible for the difference in their adhesion behaviour and whether adhesion has any impact on proteolysis or substrate utilisation *in vivo*. Identification of the adherent and non-adherent variants of strain E14 is the first demonstration in *B. fibrisolvens*. These variants are of particular importance, as they provide an opportunity to study adhesion in this species, particularly at the genetic level. They can also be used to study the biochemistry and genetics of EPS biosynthesis. The results of these studies will provide a detailed knowledge of the organisation of genes involved in EPS biosynthesis in *B. fibrisolvens* and an understanding of how the composition of the bacterial microenvironment controls adhesion.

By mutation complementation, it is possible to apply the tools of classical microbial

genetics to elucidate the genetic control of bacterial adhesion to fibre in *B. fibrisolvens*. Variants S and L can also be used to elucidate the genetic control of bacterial adhesion to the ruminal epithelium in *B. fibrisolvens*. It is not known whether adhesion to the ruminal epithelium is a precondition for attachment to fibre or performs some other function in the ecology of rumen organisms. Information on the biochemistry and genetics of adherence in *B. fibrisolvens* can also be used in continuing studies to enhance fibre digestion and reduce protein degradation in the rumen. For example, it is possible that improved attachment of an organism to fibre may be more effective in enhancing cellulolysis than introducing additional cellulase genes. These variants may be used to determine whether adhesion enhances proteolysis. If so, the genes involved in EPS production can be knocked out in the predominant proteolytic rumen bacteria and thus reduce bacterial proteolysis in the rumen.

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