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**The Cloning and Characterization  
of a *Butyrivibrio fibrisolvens* H17c  
*glnA* regulatory element  
in *E. coli*.**

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**In partial fulfillment of the requirements for the degree of**

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## Abstract

*Butyrivibrio fibrisolvens* H17C is an important anaerobic bacterium which occurs in the rumen of most ruminants. A key factor affecting the growth of this bacterium is the availability of nitrogen sources, particularly in the form of ammonia.

The aims of this study were to attempt the isolation of a gene/genes involved in the regulation of the *B. fibrisolvens* type III glutamine synthetase (GS), which is a key enzyme involved in ammonia assimilation in rumen bacteria. An existing *B. fibrisolvens* gene bank, as well as the *B. fibrisolvens glnA* gene cloned onto a low copy number plasmid, were used to generate a heterologous, *Escherichia coli*-based, two plasmid, *in trans* system. This system was used to isolate an *E. coli* clone which showed increased GS activity levels (2.5-fold) and a retarded growth rate phenotype in complete media.

A genetic element, pSAM1, was isolated from this clone and DNA hybridisation analysis confirmed that it originated from *B. fibrisolvens* chromosomal DNA. Deletion analysis of pSAM1 confirmed that the central portion of the pSAM1 insert DNA was required for the phenotype. The pSAM1 insert DNA could not be cloned onto a high copy number plasmid suggesting that overexpression of the genetic element might have a deleterious effect on *E. coli*.

Physiological analysis of the pSAM1 insert DNA revealed that it did not cause a similar elevation of GS activity levels or retarded growth phenotype for either the *E. coli* or *Bacteroides fragilis* GS's. However, the presence of pSAM1 did cause a 3-fold elevation of the *Klebsiella aerogenes* histidase (which occurs chromosomally in *E. coli* YMC11) indicating that pSAM1 affected the functioning of at least two genes involved in nitrogen metabolism. In addition, the presence of pSAM1 insert DNA in *E. coli* YMC11 carrying the *B. fibrisolvens glnA* gene on a compatible plasmid, *in trans*, also caused a two-fold elevation of GS mRNA levels indicating that the pSAM1 insert DNA could be affecting the GS activity at the transcriptional level.

Nucleotide sequencing revealed the presence of a truncated open reading frame (ORF1) of 321 base pairs which appeared to terminate in the vector to give rise to a protein of approximately 11.7 kDa. This value is in good agreement with the value of 11.75 kDa obtained for a protein produced from the pSAM1 insert DNA during *in vitro* transcription/translation and SDS-PAGE analysis. Analysis of the deduced amino acid sequence of ORF1 revealed the presence of an N-terminal signal sequence motif, similar to the signal sequence observed for other secreted lipoproteins. A search through the protein and nucleic acid databases yielded three proteins *viz.* the *B. subtilis* LytA, *E. coli* GlnH and the *E. coli* GlnS proteins which showed limited homology to the ORF1 protein.

The ORF1 protein shared the highest degree of homology with, and was most similar (in terms of physical characteristics) to the *B. subtilis* LytA protein.

Sequencing analysis did not allow the elucidation of the possible function of the ORF1 protein with any degree of certainty and further studies, possibly involving insertional inactivation of ORF1 in *B. fibrisolvans* will be required to determine the importance of this gene in nitrogen metabolism in *B. fibrisolvans*.

## ABBREVIATIONS

Ap	ampicillin
A600	absorbance at 600 nm
aa	amino acids
ATP	adenosine 5'-triphosphate
bp(s)	base pair(s)
BSA	bovine serum albumin
Cm	chloramphenicol
CsCl	cesium chloride
CMC	carboxymethyl cellulose
DNA	deoxyribonucleic acid
ds	double stranded
EDTA	ethylenediaminetetra-acetic acid
g	gram
GDH	glutamate dehydrogenase
GGT	γ-glutamyl transferase
hr	hour(s)
IPTG	isopropyl -D-thiogalactopyranoside
kan	kanamycin
kb	kilobases
LA	Luria agar
LB	Luria broth
M10	<i>B. fibrisolvens</i> non-rumen fluid medium
m	milli
MCS	multiple cloning site
mins	minutes
ml	millilitre
MM	(CHS) minimal medium
ng	nanograms
nm	nanometers
nt(s)	nucleotide(s)
°C	degrees Celcius

OD	optical density
ori	origin of replication
p	plasmid
r(superscript)	resistance
RNA	ribonucleic acid
s	seconds
Tc	tetracycline
UV	ultra violet
W	Watt
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D galactopyranoside
$\alpha$	alpha
$\beta$	beta
$\Delta$	delta
$\gamma$	gamma
$\mu$	micro
$\lambda$	lambda
p	pico
( )	plasmid carrier state
+(superscript)	presence of
-(superscript)	absence of

# Chapter 1

## Literature Review and Introduction

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## Chapter 1

### Literature Review and Introduction

#### 1.1 Importance of ruminants as a food source.

Ruminants are important sources of food in the form of beef and milk for human consumption. However, the ruminant also plays an important ecological role in providing the energy from primary producers (plants) to secondary and other consumers (animals and man) (Polan, 1987). The key to the ability of the ruminant to make use of potentially undigestible feed resources (for secondary and other consumers) lies in the rumen, the site of a complex, yet highly successful symbiosis. It is here that feedstuffs consumed by ruminants are initially exposed to fermentative activity before gastric and intestinal digestion. The crux of the symbiosis lies in the fact that the rumen provides a suitable niche in which rumen microorganisms degrade dietary polysaccharides and proteins to produce specific end products such as fatty acids. In turn, these fatty acids and microbial proteins provide nutrients for metabolism by the host animal. The ruminant animal thus provides a system for providing human food from materials that cannot be used directly by man. This places the ruminant in a central role in the total effective use of the world's nutrient resources (Phillips, 1981).

However, the anaerobic nature and other characteristics of the ruminal fermentation places an upper limit on the potential nutrient yield (Mackie and Kistner, 1985). Moreover, the growing world population (Phillips, 1981), and the fact that most of this population growth is occurring in countries that are experiencing food shortages, means that great strain will be placed on the limited food resources that do exist. Thus, the prospects for achieving either an adequate level of production of animal products in relation to overall world needs, or a reasonable balance in supply of animal products between the developing and developed countries are not encouraging (Phillips, 1981). A primary objective of research scientists working in the field of rumen function is, therefore, to advance animal production through better understanding of the mechanisms involved in digestion and metabolism (Mackie and Kistner, 1985).

## 1.2 The role of microorganisms in the rumen

The rumen ecosystem is an efficient protein production system utilizing energy derived from the digestion of plant polysaccharides such as cellulose, and nitrogen mainly derived from ammonia in the rumen to produce microbial proteins and volatile fatty acids (Hobson, 1988). These are then directly available to the ruminant for digestion and absorption. We will first consider how carbohydrate utilization is mediated in the rumen.

### 1.2.1 Carbohydrate utilization

Anaerobes are unable to oxidize food through hydration of carbon atoms and removal of the hydrogen to combine it with dioxygen, and, therefore, need to obtain energy by transferring hydrogen between carbon atoms. Carbohydrates represent the ideal substrate for this type of anaerobic oxidation, and probably evolved as an important food source because of this characteristic (Hungate, 1955).

Most polymeric carbohydrates (polysaccharides) entering the rumen can be considered as belonging to one of two general types: plant storage polysaccharides such as starch; and plant structural polysaccharides such as hemicellulose and cellulose (also termed fibre) (Hobson, 1988).

#### 1.2.1.1 Fibre utilization in rumen bacteria

The term "fibre" refers to the insoluble residue prepared from plant material (Chesson, 1986) and includes intact plant cell walls and their constituent polysaccharides.

Since fibre represents a significant portion of the diet of herbivores, their productivity is limited by their ability to consume or degrade the fibrous portion of the diet (Allen and Mertens, 1987). Polysaccharides extracted from intact cell walls are categorised into three groups: cellulose, hemicellulose and the pectic substances (Chesson and Forsberg, 1988). Several different bacterial genera and species have been identified as being involved in the digestion of fibre through the production of a variety of polysaccharide-degrading enzymes (Table 1.1).

The major cellulolytic bacteria include *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*.

**Table 1.1 The major bacteria involved in fibre degradation and the enzymes they produce.**

Bacterium	Substrate	Enzyme specificity	Reference
<i>R. albus</i>	cellulose	endoglucanase	Leatherwood (1965)
		cellobiosidase	Ohmiya <i>et al.</i> (1982)
		$\beta$ -glucosidase	Ohmiya <i>et al.</i> (1985)
<i>R. flavefaciens</i>	cellulose dextrins	cellobiose phosphorylase	Ayers (1958)
		exo- $\beta$ 1,4-glucanase	Gardner (1987)
<i>B. fibrisolvens</i>	cellulose	cellodextrinase	Wang and Thomson (1990)
		$\beta$ -glucosidase	Brown <i>et al.</i> (1993)
	xylan	endoglucanase	Rumbak (1990)
<i>C. longisporum</i>	cellulose	xylanase	Berger (1990)
		endo- $\beta$ -glucanase	Lin and Thomson (1990)
<i>F. succinogenes</i>	cellulose	endo- $\beta$ -glucanase	Mittendorf & Thomson (1990)
		$\beta$ -glucosidase	Groleau & Forsberg (1981)
	xylan	cellobiosidase	Huang & Forsberg (1987)
		cellodextrinase	
		four xylanases	Smith & Forsberg (1991)

Hemicellulose is a complex material and a combination of bacteria, fungi and protozoa are required to degrade this substrate (Chesson *et al.*, 1986). *F. succinogenes* has been identified as one of the foremost fibrolytic bacteria in the rumen and produces at least four xylanases (Williams *et al.*, 1984). *B. fibrisolvens*, which also produces high levels of xylanase activity (Lin and Thomson, 1990), occurs predominantly in ruminants surviving on poor quality, high fibre diets. The secretion of a xylanase enzyme complex in *B. fibrisolvens* is thought to allow this organism to degrade hemicellulose in the rumen.

## 1.2.2 Nitrogen metabolism in the rumen

The amino acid requirements of the ruminant are provided by microbial synthesis in the rumen and from dietary protein that is not degraded in the rumen but is intestinally digestible (bypass protein) (Mackie and Kistner, 1985). Amino acids are lost to the ruminant via three protein degradative processes viz. protein hydrolysis, peptide degradation and amino acid deamination. Enhancement of the portion that leaves the rumen (and is not degraded in the rumen) can lead to improvements in the quality and quantity of nutrients available to the host animal. Ammonia is thus a major source of nitrogen to ruminant bacteria (Hespell, 1966).

### 1.2.2.1 Protein degradation in the rumen.

The extent to which dietary proteins are degraded in the rumen, and the proportion which escapes degradation and is subsequently hydrolysed, are recognized as important factors in the nutrition of ruminants (Leng and Nolan, 1984). Proteolysis in the rumen results in losses of high quality dietary proteins that would otherwise be directly digested and absorbed in the small intestine of the ruminant animal (Black and Tribe, 1973). There is, therefore, considerable interest in suppressing microbial catabolism of proteins, peptides and amino acids in the rumen (Leng and Nolan, 1984). In the rumen, the process of protein degradation is composed of several different microbial processes that include protein hydrolysis, peptide degradation, amino acid deamination and fermentation of amino acid carbon skeletons.

#### i) Protein hydrolysis

According to the studies done by Fulghum and Moore (1963) and Prins *et al.* (1983), no common bacterial species actually uses protein as a major energy source. However, these studies did show that between 30 and 50% of the bacteria isolated from rumen fluid have proteolytic activity towards extracellular protein. Most species of rumen bacteria exhibit some proteolytic activity with the possible exception of the main cellulolytic bacteria such as *F. succinogenes*, *R. flavefaciens* and *R. albus*. The three main species of proteolytic bacteria are *Prevotella ruminicola*, *Ruminobacter amylophilus* and *B. fibrisolvans*. Studies by Russell *et al.* (1981) have also shown that *Streptococcus bovis* may be a potentially important proteolytic bacterium. Of the three predominant bacteria, both *B. fibrisolvans* and *P. ruminicola* can

grow in media where protein and not ammonia is the sole nitrogen source. In contrast, *R. amylophilus* requires ammonia as the principal nitrogen source and produces proteases in a medium lacking both proteins or amino acids (Blackburn, 1968). Cotta and Hespell (1986) suggested that this protease activity may exist more to break down structural protein within cereal particles rather than for fulfilling a nutritional function. General features of protease activity in the rumen bacteria are that it is loosely regulated (Blackburn, 1986), is mostly cell-bound and has a broad pH optimum around approximately pH 5.5-7.0 (Blackburn and Hobson, 1960) in rumen fluid. According to studies done by Wallace (1984), cysteine protease predominates in rumen fluid although serine protease, metalloprotease and aspartic protease activities are also present. *P. ruminicola* appeared to produce protease activity that was most similar to that of the rumen contents (Wallace and Brammall, 1985), while *R. amylophilus* and *B. fibrisolvans* exhibited predominantly serine protease activity (Wallace and Brammall, 1985, Cotta and Hespell, 1986). There have been studies (Prins *et al.*, 1983; Walter and Brammall, 1985) which provided evidence about the effect of animal diet on protease activity but work on these aspects is still at an early stage. It is not clear which criteria in terms of cell number or specific activity should be used to determine which of the three predominant protease producing bacteria are the most important. According to Wallace and Cotta (1988), *P. ruminicola* has the properties most similar to those of the rumen contents, is widely distributed and is, generally, the predominant proteolytic microorganism. Although *R. amylophilus* and *B. fibrisolvans* do not appear to have the characteristics consistent with the major part of the rumen activity, these organisms will still be important under certain dietary conditions.

## ii) Peptide metabolism and Amino acid metabolism

Hydrolysis of proteins by rumen microbial enzymes releases oligopeptides, which are then broken down to smaller peptides, and finally to amino acids (Russel *et al.*, 1983).

Studies by Wright (1967) and Prins *et al.* (1977) have established that peptides are used more rapidly by mixed rumen microorganisms than are the corresponding free amino acids. Small peptides are converted mainly to volatile fatty acids, while a higher proportion of the larger molecules are incorporated into bacterial protein (Wright and Hungate, 1967). According to Wallace and McKain (1989; 1991), peptide hydrolysis in the rumen follows a distinctive

pattern, characteristic of the peptide being broken down by a dipeptidyl aminopeptidase mechanism. Several species of bacteria, most notably, *Megasphaera elsdenii*, possessed this high dipeptidase activity, and current work in this area is aimed at finding the exact sites of the dipeptidase activity, the manner in which it is regulated and if it can be inhibited. It may then become possible to devise ways of slowing peptide breakdown in the rumen to the benefit of the host animal.

Metabolism of amino acids is the next stage in the metabolism of most of the constituents of dietary protein. Most amino acids are broken down to yield a variety of carbon skeletons and ammonia (reviewed by Wallace and Cotta, 1988). The carbon skeletons are metabolized to yield volatile fatty acids (reviewed by Blackburn, 1965; Allison, 1970) and ammonia, the main product of deamination that is then available for assimilation.

### 1.2.2.2 Ammonia assimilation in rumen bacteria.

Ammonia is an important source of N and is essential for the growth of most rumen microorganisms (Mackie and Kistner, 1985). A large proportion (60-90%, depending on the diet) of the daily nitrogen intake of the ruminant is converted to ammonia and from 50-70% of bacterial N can be derived from ammonia (Mathison and Milligan, 1971). A considerable amount of nitrogen is potentially recyclable between the rumen ammonia pool and microorganisms (Fig.1.1).

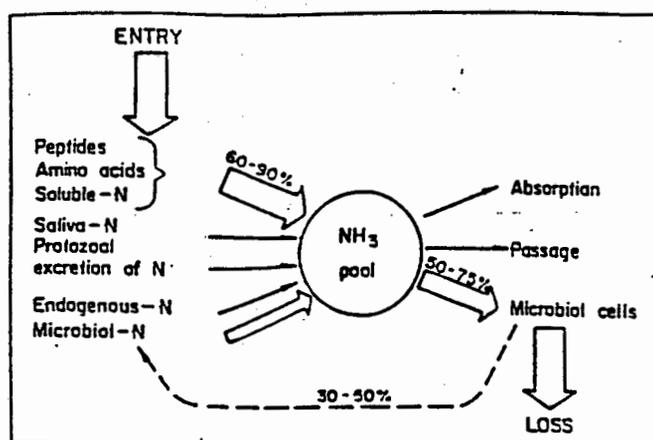


Fig. 1.1 Cycling of nitrogen through the ammonia pool of the rumen (after Leng and Nolan, 1984).

Several processes culminate in the production of ammonia and these include components soluble in the rumen liquid phase (peptides and amino acids), influx of nitrogen into the rumen via saliva, protozoan excretion of  $\text{NH}_3$  and the turnover of microbial protein and endogenously secreted protein (Leng and Nolan, 1984).

Ammonia is irreversibly lost from the rumen fluid by incorporation into microbial cells that pass out of the rumen, by absorption through the rumen epithelium, and in fluid passing out of the rumen. Bryant and Robinson (1962) found that 92% of ruminal bacterial isolates could use ammonia as the main source of N, while it was essential for the growth of 25% of the isolates tested. The latter are consistent with conditions found in the rumen micro-environment where little amino acid nitrogen is available for microbial growth due to rapid breakdown of amino acids to  $\text{NH}_3$ ,  $\text{CO}_2$  and volatile fatty acids (Mackie and Kistner, 1985).

Since ammonia is the major and often preferred source of nitrogen for most rumen microorganisms, enzymes for its assimilation are essential to the growth of most rumen microorganisms. According to Hespell (1984), the two most important routes by which ammonia may be assimilated are glutamate dehydrogenase (GDH) and the glutamine synthetase (GS) and glutamate synthase (GOGAT) dual enzyme system. (Discussed further in sections 1.3 and 1.4).

Which route then is the most important under the conditions found in the rumen?

Studies by Jenkinson (1979) of the growth of *B. amylophilus* under ammonia limited conditions suggested that GS activity was stimulated under these conditions whereas the main glutamate dehydrogenase activity (NADP-GDH) was repressed. No glutamate synthase (GOGAT) activity could be detected indicating that the GS-GOGAT system was not functional. These results implied that either the NADP-GDH was the principal uptake enzyme, or that GS was the first uptake enzyme but that a second aminotransferase activity different to GOGAT was present in *B. amylophilus* (Jenkinson, 1979). A similar stimulation of GS activity was noted by Smith *et al.*, (1980, 1981) and Hespell (1984) from studies on *Selenomonas ruminantium* under ammonia limited conditions and at high growth rate. However, unlike for *B. amylophilus*, a low GOGAT activity was discerned in *S. ruminantium* implying that the GS-GOGAT couple was functional at low ammonia concentrations. GOGAT was not induced by ammonia limitation while NADP-GDH activity was higher in glucose limited (ammonia -sufficient cultures), suggesting that it was the route used under these

conditions (Smith *et al.*, 1980; Hespell, 1984). Other less well studied species includes *S. bovis* (Griffith and Carlson, 1974) and *R. flavefaciens* (Pettipher and Latham, 1979) which were shown to use NADP-GDH as the main assimilatory pathway. These studies have been conducted using pure cultures and the mechanism of ammonia uptake has been implied from the enzymes present and by the way they are regulated (Wallace and Cotta, 1988). Further work requiring careful consideration of the conditions under which enzymes are measured and the use of  $^{15}\text{N}$  or  $^{13}\text{N}$  is required before any conclusions can be drawn about the mechanistics of these enzymes (Wallace and Cotta, 1988).

### **1.2.2.3 Bacterial biomass**

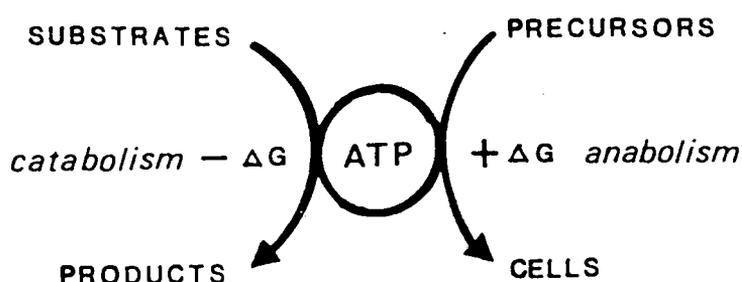
The term bacterial biomass refers to the fact that the rumen microbes not only produce forage degrading enzymes and the fermentation acids used by the host but that they also constitute the ruminant's supply of proteins and other essential nutrients (Hungate, 1966). Work at estimating the quality and quantity of microbial protein supplied to the ruminant is still at an early stage and remains one of the most difficult problems in rumen microbiology.

The above discussion has reviewed the variety of bacterial microorganisms in the rumen and the way in which they facilitate the workings of a very complex ecosystem. Polysaccharides (starch, xylans, pectins, cellulose) and proteins are broken down by various bacteria using various enzymes, while ammonia is assimilated and microbial protein is produced. The energy produced by the former catabolic processes is used to produce the microbial proteins ultimately assimilated by the ruminant host as bacterial biomass. Without oxygen, where and how do these microorganisms obtain their energy for growth, and what are the factors that limit the growth of these microorganisms? These questions will be addressed briefly in the following section.

### **1.2.3 Energy metabolism in ruminants.**

For catabolic (polysaccharide degrading) reactions to provide the energy for anabolic (protein producing) reactions a common energy currency is required (Fig.1.2). This common energy currency is provided by adenosine triphosphate (ATP), a phosphate ester compound that

allows the displacement of the mass action ratio from equilibrium allowing less energetically favoured reactions (such as anabolic reactions) to occur. All organisms require a continuous supply of this energy currency to maintain their structure, to grow and to multiply (Mackie and Kistner, 1985). There are two types of processes used to synthesize ATP. These are substrate level phosphorylation (SLP) and electron transfer linked phosphorylation processes (ETP) (Darnell *et al.*, 1990). SLP involves the chemical transformation of soluble substances in the cytosol by aqueous enzymes. For example, during glycolysis phosphorylation of various compounds ultimately yields ATP.



**Fig. 1.2** A schematic representing ATP-linked anabolic and catabolic reactions. Taken from Russel and Wallace, 1988.

ETP's are different types of processes that involve the generation and use of proton concentration gradients across the inner mitochondrial membrane (Darnell *et al.*, 1990) to yield ATP. Until recently, it was thought that most rumen anaerobes synthesized ATP exclusively by SLP. Hungate (1966) found that the majority of rumen bacteria which ferment a variety of carbohydrates were dependent on SLP for the major portion of ATP synthesized. However, it has been shown that many rumen bacteria also possess components of electron transfer chains that are essential for ATP synthesis (Mackie and Kistner, 1985). Dawson *et al.* (1979) found that *B. ruminicola*, *B. succinogenes* (reclassified as *F. succinogenes*; Montgomery *et al.*, 1988), and *B. fibrisolvens* could derive 33, 50, and 26% respectively of their total molar growth yield/mole of carbohydrate fermented from electron transport reactions. According to Mackie and Kistner (1985), these ETP mechanisms could have a profound influence on the overall energetic efficiency of the ruminal fermentation.

Four biochemical reactions which yield ATP via ETP are known to occur in ruminal bacteria. The most important of these is the reduction of  $\text{CO}_2$  to  $\text{CH}_4$ . At least three species of methanogens viz. *Methanobacter formicicum* (Bryant, 1965), *Methanobrevibacter ruminantium* (Smith and Hungate, 1958) and *Methanomicrobium mobile* (Paynter and Hungate, 1968) are known to occur in the ruminal ecosystem that obtain ATP in this way. Sulphate reducing bacteria (*Desulfovibrio* spp.) also occur in the rumen (Howard and Hungate, 1976) and it was shown that ATP is produced by an electron transfer chain and the reduction of  $\text{SO}_4^{2-}$  to  $\text{S}^{2-}$ . Nitrate reduction to nitrite has been reported in *Selenomonas ruminantium* and *Anaerovibrio lipolytica* (De Vries *et al.*, 1974), and the reduction of fumarate to succinate was shown in *Wolinella succinogenes* (Wolin *et al.*, 1961).

#### **i) Rumen microbial maintenance energy requirements**

The availability of protein to the host animal is affected by the efficiency with which the available energy sources and other nutrients in the rumen are used in the synthesis of microbial biomass (Mackie and Kistner, 1985). The following discussion regarding maintenance energy requirements has been reviewed by Hespell and Bryant (1979) and the main points from their paper affecting this discussion are presented below. An important factor affecting the yield of cells expressed as grams of dry mass per mole ATP expended is the maintenance energy requirement. This is defined as the net apparent or real diversion of energy and/or carbon from the growth limiting or energy - generating substrate to processes not resulting in an increase of cell mass. The maintenance energy requirement has been found to vary between micro-organisms and for a given species it is a function of the growth rate. The maintenance energy requirement also varies considerably depending on the growth conditions. The component of the maintenance energy that has the greatest effect on growth is energetic uncoupling. This uncoupling appears to be caused by the fact that most bacteria produce far more energy from catabolic processes such as carbohydrate degradation than is required for biosynthetic processes such as production of proteins. These studies have also shown that anaerobic bacteria in particular appear to have only coarse control over the rate of energy production. In contrast, ATP levels and ATP production from catabolic processes are subject to fine control mechanisms. Any condition, other than energy limitation, which restricts the growth rate of a population below its potential maximum value will have the

effect of impeding the rate of ATP utilization in biosynthetic processes and thereby decrease the rate of microbial cells per unit of energy substrate utilized. In the rumen ecosystem, the nature of the available **nitrogen sources** and their concentration appears to play a significant role in this respect (Mackie and Kistner, 1985).

At different times in the feeding cycle, ruminal organisms are subjected to periods of nutritional stress and starvation when concentrations of free sugars drop to very low levels and insoluble polymers are being degraded very slowly (Mackie and Kistner, 1985). The survival capacity of ruminal bacteria under these conditions is considerably less than that of other bacteria found in the soil or aquatic sediments (Mink and Hespell 1981 a, b). Results obtained by Hespell (1984) suggested that ruminal bacteria are unable to reduce their metabolism in an environment with low levels of readily available organic nutrients due to a lack of suitable genetic strategies to cope with these conditions. Further studies involving the measurement of the magnitude of the adenylate energy charge and the proton motive force are required before a proper understanding of the energy status of ruminal bacteria under different dietary conditions is achieved.

The above discussion has highlighted the broad diversity and the different survival strategies employed by the microorganisms in the rumen ecosystem. We now consider the characteristics and genetics of *B. fibrisolvens*, in greater depth.

### **1.3 Importance of *B. fibrisolvens* in the rumen**

#### **1.3.1 Introduction**

The genus *Butyrivibrio* is composed of gram-variable, strictly anaerobic bacteria that ferment a wide variety of carbohydrate substrates with the production of large amounts of butyrate (when glucose or maltose is fermented) (Bryant and Small, 1956). *Butyrivibrio* cells, as viewed by phase microscopy, appear to be short, curved rods that are between 1.5 and 6.0  $\mu\text{m}$  in length and 0.4 and 0.8  $\mu\text{m}$  in diameter. These cells may occur singly, or in short chains

or filaments of two to three cells that may or may not be helical.

Motility is rapid, vibrating and usually progressive, but often only a few cells in a culture show motility (Cheng and Costerton, 1977). It occurs by means of monotrichous flagellation, with the flagellum attached terminally or, less frequently, subterminally.

Members of this genus occur widely in nature and are among the most numerous bacteria isolated from the rumen of cows fed on a wide variety of rations (Roche *et al.*, 1973). They have been isolated from the rumens of sheep, cattle, zebu cattle, goats, Alaskan reindeer, Svalbard reindeer, and bison, as well as from faecal material obtained from horses, rabbits and humans (as reviewed by Berger *et al.*, 1990).

The cell wall structure of *Butyrivibrio* is particularly intriguing as it consists of elements that bear similarity to both Gram-positive and Gram-negative bacteria. This genus was initially described as Gram-negative due to the reaction obtained when the cells are stained by the standard Gram-staining technique, however it is now thought to be Gram-variable (Beveridge, 1990). The extreme thinness of these cell walls (12-18 nm) is thought to account for their inability to retain the Gram stain (Cheng and Costerton, 1977). Compounds characteristic of Gram-positive cell walls such as glycerol teichoic acid (Sharpe *et al.*, 1975) and lipoteichoic acids (Hewett *et al.*, 1976) have also been isolated from strains of *Butyrivibrio*.

The genus *Butyrivibrio* is composed of a number of strains that show great variability in terms of their phenotypic and physiological characteristics. Initially, all strains were classified as a single species, *B. fibrisolvans*, the name reflecting the importance of this organism in the digestion of the fibrous components of the food in ruminant rations (Bryant and Small, 1956). A number of strains subsequently isolated were assigned to this species. Numerous studies have attempted to reorganize the isolates into more uniform groups, illustrating the heterogeneity of this genus. A number of approaches have been used. Shane *et al.* (1969) placed cellulolytic strains into two groups based on their nutritional requirements and ability to utilize or produce lactate, acetate and formate and Hazlewood *et al.* (1976) used serological and immunochemical properties to differentiate between isolates. Studies by Stack (1988) and Manarelli *et al.* (1990) were based on the fact that most strains of *B. fibrisolvans* produce extracellular polysaccharides (EPSs) when grown on a defined medium. Differences in the EPSs compositional features of various isolates were used to assign isolates into different

groups (Stack, 1988; Manarelli *et al.*, 1990b). Several unusual sugars such as L-altrose, 4-O(1-carboxyethyl)-D-galactose and 4-O-(carboxyethyl)-L-rhamnose, only found in the EPS of *Butyrivibrio* strains were identified (Stack *et al.*, 1988; Manarelli *et al.*, 1990b). *B. fibrisolvens* strain H17C and 49 both contain an acidic sugar of the lactyl ether type and were therefore classified together as type IV B strains. More recent studies used the degree of DNA relatedness of *B. fibrisolvens* strains as the criterion for grouping these isolates (Manarelli, 1988; Manarelli *et al.*, 1990b). Again *B. fibrisolvens* strain H17C and 49 were grouped together and had a DNA relatedness of 96%. The great variability found for G+C content as well as for DNA hybridization values has led to the conclusion that the species *B. fibrisolvens* is composed of a number of distinct species and possibly several genera (Manarelli, 1988). A new species, *B. crossotus*, different from *B. fibrisolvens* in that it has lophotrichous flagella, produces no gas, and ferments only a few carbohydrates, was isolated from human rectal material (Moore *et al.*, 1976). The type strain has recently been shown to be closely related genetically to five strains of *B. fibrisolvens* isolated from the rumina of bison (Manarelli, 1990b). Cheng *et al.* (1989) characterized two strains, B-385-1 and 2-33, and suggest, due to their biochemical characteristics and cell wall structure, that these are strains of a different species of *Butyrivibrio*. These strains are large and have subpolar tufts of flagella and often replace *B. fibrisolvens* under acid conditions in the rumen (Bryant, 1984). They were originally not considered to belong to the *Butyrivibrio* genus (Hespell and Bryant, 1981).

### 1.3.2 Enzymology of *B. fibrisolvens*

*Butyrivibrio* species appear to occur in ruminants that are subject to a wide range of very different, sometimes unfavourable physical conditions. The key to the ability of *B. fibrisolvens* to occur under these conditions lies in the wide diversity of substrates metabolized by this organism (Table 1.2).

**Table 1.2** Substrates fermented by *B. fibrisolvens* (after Stewart and Bryant, 1988).

Acid from:	
Starch	d
cellulose	+
xylan	+
pectin	d
maltose	d
cellobiose	d
sucrose	d
D-xylose	d
L-arabinose	+
glucose	+
fructose	+
galactose	+
mannose	+
lactose	d
Aesculin hydrolysis	d
H <sub>2</sub> S production	d
fermentation products:	F, B, A
Major	L, S
Minor/some strains	
Gas	H <sub>2</sub> CO <sub>2</sub>

Abbreviations: A = acetate, B = n-butyrate, F = formate, L = lactate, S=succinate, d = reaction varies between strains, + = positive reaction.

Previously, a large amount of information about the enzymology of *B. fibrisolvens* was generated by total enzyme activity studies involving crude enzyme extracts (Hobson *et al.*, 1988). Subsequently, the advent of recombinant DNA techniques has allowed the characterisation of a number of the individual enzymes involved in the degradation of the various substrates. The primary focus of these studies has been the characterization of those enzymes involved in the hydrolysis of the major plant polysaccharides.

### 1.3.2a Xylanases

*B. fibrisolvans* are of the most xylanolytic of rumen microorganisms (reviewed by Thomson, 1993) and have been found to be the most abundant fibrolytic species in ruminants subjected to adverse physical conditions. Studies by Coen and Dehority (1970) have shown that while a number of cellulolytic bacteria are capable of hydrolyzing a considerable amount of xylan, some of these bacteria are unable to utilize the resulting endproducts.

*B. fibrisolvans* eg. H17C and 49, however, is capable of growth on media that contains xylan as the only carbohydrate source (Dehority, 1966). More recent studies (Hespell *et al.*, 1987; Hespell and O'Bryan-Shah, 1988) has indicated that these strains possess the key enzymes required to degrade xylan. Studies by Lin and Thomson (1991) on *B. fibrisolvans* H17C cultures detected a multienzyme aggregate in the supernatant fluid that had predominantly xylanolytic activity, although cellulase activity was also detected.

An interesting feature of xylanase and carboxymethyl cellulases is that these enzymes are known to exhibit bifunctionality (Beguin, 1990; Gilkes *et al.*, 1991). Studies by Utt *et al.* (1991) revealed the presence of an unusual gene, *xykB*, from *B. fibrisolvans* GS113 that encoded both xylosidase and  $\alpha$ -L-arabinofuranosidase activities.

Rumbak (1991) proposed that factors such as the bifunctional nature of the xylosidase activity and the multienzyme xylanase activity could be crucial in optimizing the digestion of hemicelluloses by *B. fibrisolvans*. However, further research is required to determine the importance of these factors in the degradation of xylan.

### 1.3.2b Cellulases

It is known that *B. fibrisolvans* can be one of the most important cellulolytic rumen bacteria in domestic animals fed poor quality forage diets (Margherita and Hungate, 1963; Shane *et al.*, 1969; Orpin *et al.*, 1985), although it is generally accepted that *R. albus*, *R. flavefaciens* and *F. succinogenes* are more important in cellulose degradation under normal conditions. The combined activity of the following enzymes viz. endoglucanases, cellobiohydrolases and cellodextrinases are required to hydrolyse cellulose to glucose. Two endo- $\beta$ -1,4-glucanase genes have been cloned and expressed in *E.coli* (Berger *et al.*, 1989; Hazlewood *et al.*, 1990).

These enzymes have high activity towards  $\beta$ -1,4-glucans, but low activity towards xylan and cellobiosides. A cellodextrinase activity (*cedI*) and  $\beta$ -glucosidase activity (*bglA*) have also been cloned and sequenced from *B. fibrisolvens* H17C (Berger *et al.*, 1990; Lin *et al.*, 1990).

### 1.3.2c Pectinases

According to studies by Dehority (1966), *B. fibrisolvens* is one of the most important pectinolytic species in the rumen. Studies by Wojciechowitz *et al.* (1982) on *B. fibrisolvens* 718 revealed the presence of an extracellular pectinolytic enzyme. This enzyme is unusual as other pectinolytic species in the rumen produce mostly endopectinolytic enzymes. A pectin esterase (isolated from culture fluid) as well as an intracellular pectinolytic enzyme was detected in the same *B. fibrisolvens* strain.

### 1.3.2d Amylases

According to Dehority and Orpin (1988), *B. fibrisolvens* is the predominant organism in domestic animals fed a high roughage diet and one of the principle amylolytic bacteria in animals fed a high-concentrate diet. Studies by Rumbak (1991) on *B. fibrisolvens* H17C revealed the presence of an  $\alpha$ -amylase that showed high homology to other  $\alpha$ -amylases in three conserved regions. The *B. fibrisolvens* H17C  $\alpha$ -amylase degraded amylose, amylopectin and soluble starch with maltotriose as the major initial products of hydrolysis (Rumbak, 1991).

### 1.3.2e Other enzymes

#### i) Proteases

The distribution of proteolytic activity produced by strains 49 and H17C has been described (Cotta and Hespell, 1986; Strydom *et al.*, 1986). The characteristics of the protease activity in *B. fibrisolvens* H17C and 49 are very similar. They are both mainly extracellular, serine-like, and activity is positively correlated with growth rate. Studies by Strydom *et al.* (1986)

using SDS-PAGE analysis, allowed the detection of ten protease bands of varying molecular weights indicating that a number of different types of proteases could be present. Work on proteases in *B. fibrisolvens* is at an early stage and further work is required to identify the genes involved in protease production and expression.

## ii) Ammonia assimilatory enzymes

The glutamine synthetase gene from *B. fibrisolvens* H17C has been cloned and sequenced (Goodman and Woods, 1993). This gene was isolated by its ability to complement a *glnA*-deletion *E. coli* strain grown on minimal media. Work regarding ammonia assimilation as well as regulation of this process in this ruminant bacterium are at an early stage and requires further investigation. The latter aspect forms the major thrust of this study and is discussed in greater detail later.

The object of the following discussion will be to illustrate the role of regulation of GS in nitrogen metabolism.

### 1.4.1 Glutamine synthetase (GS)

The pathways of nitrogen metabolism may be divided into 2 classes; the pathways responsible for the uptake and assimilation of available nitrogen containing compounds and the biosynthetic pathways responsible for the production of nitrogen containing compounds in the cell (Tyler, 1978). In response to nitrogen deprivation, *E. coli* and other enteric bacteria respond by increasing the intracellular concentration of enzymes of both classes including; glutamine synthetase (GS), glutamate synthase (GOGAT), and a number of enzymes for the uptake and degradation of amino acids (Reitzer and Magasanik, 1985; Higgins, 1982).



**Table 1.3** Characteristics of GSI, GSII and GSIII.

GS class	Organism	Subunit arrangement	Subunit characteristics	Reference
GSI	<i>Vibrio</i> , <i>Enterobacteriaceae</i> , <i>Bacillus</i> , <i>Clostridium</i> .	dodecamers 2 discs of 6	444 aa	Bodasing <i>et al.</i> (1990) Streicher and Tyler (1980) Donohue and Bernlohr (1981) Usden <i>et al.</i> (1986)
GSII	<i>Rhizobium</i> , <i>Frankiaceae</i> , <i>Streptomyces</i> .	octamers 2 discs of 4	329 aa	Carlson and Chelm (1980) Darrow and Knotts (1977) Filser <i>et al.</i> (1986)
GSIII	<i>B. fragilis</i> , <i>B. fibrisolvens</i> , <i>Synechocystis</i> .	hexamer six subunits	729 aa	Southern <i>et al.</i> (1987) Goodman and Woods (1993) Reyes and Florencio (1994).

## 1.4.2 Regulation of GS enzymes

### 1.4.2.1 Regulation of GSI

#### a) Regulation of the Enterobacterial GSI

Regulation of the Enterobacterial GSI occurs at two levels; post translational modification i.e. adenylation, and at the transcriptional level that involves the action of a number of genes and their gene products viz. the *glnL*, *glnG*, *glnB*, *glnD* and *glnF* genes and gene products. The current model of Enterobacterial GSI regulation will be discussed below, followed by an analysis of the genes and gene products involved showing how the regulation is achieved.

#### i) Model of the Enterobacterial GSI regulation

A combination of genetic and mutational analysis of various *E. coli* mutant strains has allowed the elucidation of the following model of *glnA* gene regulation in the enteric bacteria (Magasanik and Neidhardt, 1987) (Fig. 1.4).

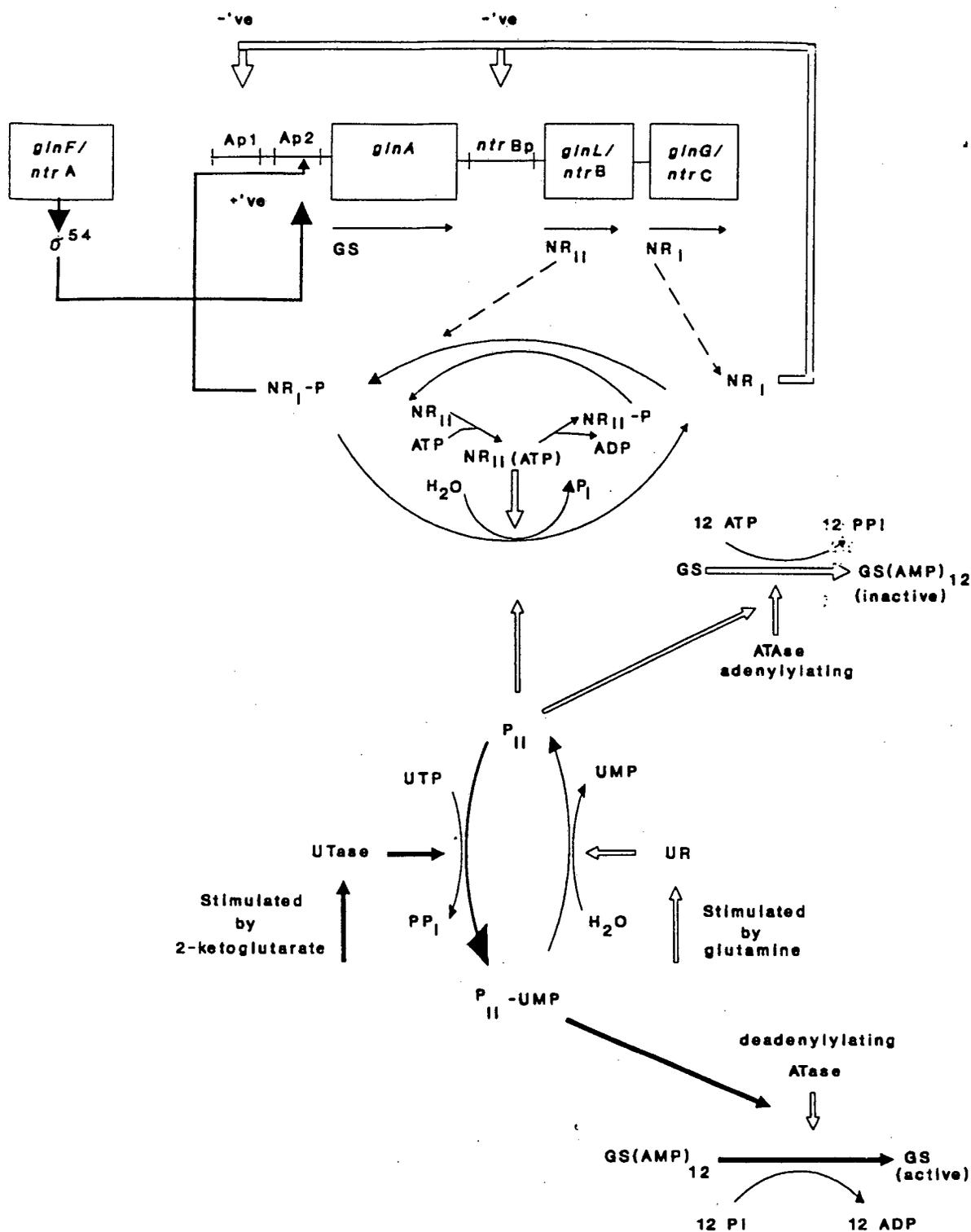
In the enteric bacteria, high nitrogen levels are associated with high glutamine and low 2-ketoglutarate levels. The opposite is true for conditions of nitrogen limitation. The key protein is PII that may be present in the nonuridylylated or uridylylated (PII-UMP) form.

The PII-UMP conformation is stimulated by high 2-ketoglutarate and low glutamine levels (low nitrogen), and is catalysed by the (uridylyltransferase) UTase enzyme. PII-UMP catalyzes the deadenylylation of GS to the active state. PII also facilitates the phosphorylation of a response regulatory protein NtrC by a corresponding sensory protein NtrB, thus allowing high level expression of GS from the *glnAp2* promoter under nitrogen-limiting conditions.

Other genes/operons involved in nitrogen metabolism such as the histidine and arginine transport systems are then also activated by the phosphorylated form of NtrC (NtrC-P) (Higgins and Ames, 1982). In some cases, however, the NtrC-P may indirectly affect the expression of an operon as is the case for the histidine utilisation system (*hut* system) in *K. aerogenes* (discussed in greater detail in Chapter 3) (Nieuwkoop *et al.*, 1988). In this case, NtrC-P is required for the production of a Lys-R type regulatory protein NAC (nitrogen assimilation control) (Henikoff *et al.*, 1988).

NAC, in turn, functions as an activator of the transcription of the GOGAT, *hut*, *put*, and urease operons and represses *gdh*, thus allowing assimilation of ammonium under conditions of nitrogen starvation. Under conditions of nitrogen-excess (and high glutamine levels), the PII nonuridylylated conformation is favoured (UTase catalyzes removal of the uridylyl group on PII) leading to adenylylation of GS (thus inactivating GS activity), the dephosphorylation of NtrC by NtrB, and as a consequence, low levels of expression of *glnALG* from *glnAp1*.

Regulation of transcription of the *glnA* gene as well as regulation of GS activity in the Enteric bacteria is therefore characterized by a bicyclic, cascade system in which two proteins viz. PII and the UT/UR enzyme play a central role in responding to changes in the nitrogen levels of the growth medium.



**Fig. 1.4** Regulation of the Enterobacterial GSI, showing the covalent modification of glutamine synthetase (GS), protein P<sub>II</sub> and NR<sub>I</sub>, the regulator of *glnA* transcription. UR/UTase, uridylyl-removing enzyme/uridylyltransferase; ATase, adenylyltransferase; conditions of high glutamine to 2-ketoglutarate (nitrogen-rich), open arrows; conditions of high 2-ketoglutarate to glutamine (nitrogen starvation), solid arrows (Woods and Reid, 1993).

## ii) The *glnALG* operon

The *glnALG* operon (also known as the *glnA ntrBC* operon) is a complex operon, the expression of which is regulated at three promoters (Fig. 1.4). Two of these promoters are located upstream of the *glnA* gene and are designated *glnAp1* and *glnAp2*. The third promoter is located between the *glnA* and *glnL* genes and is designated NtrBp (Miranda-Rios et al., 1987; Magasanik, 1988). Under excess nitrogen growth conditions (associated with high levels of glutamate and low levels of 2-ketoglutarate), promoters *glnAp1* and *glnAp2*, which are consensus sigma 70 promoters (Ausubel, 1984; Hunt and Magasanik, 1985) are repressed and give rise to only low levels of GS and of the products of the *ntrB* and *ntrC* genes viz. NtrB and NtrC, respectively (previously designated as proteins NR<sub>II</sub> and NR<sub>I</sub> respectively). NtrB (NR<sub>II</sub>), together with NtrC (NR<sub>I</sub>), forms part of a sensor (histidine kinase) -response regulator pair of signal transduction proteins involved in controlling enteric bacterial gene expression during changes in nitrogen levels in the growth media (McFarlane and Merrick, 1985). NtrB, the kinase component, is a 36-kDa, dimeric protein with a N-terminal, sensor domain and three highly conserved motifs in the kinase domains (Ronson *et al.*, 1987). It is a cytoplasmic protein and has no membrane anchoring domain. NtrB activity is modulated by PII (see below), which in the PII-UMP form signals nitrogen limitation and in the unmodified form signals nitrogen excess.

Thus, under nitrogen limiting conditions, NtrB catalyzes the phosphorylation of and activation of NtrC, the response regulator (Keener and Kustu, 1988; Ninfa and Magasanik, 1986).

NtrB, autophosphorylates itself on histidine residue 139 in an ATP-dependent reaction in which one subunit of the dimer binds ATP and phosphorylates the other subunit (Ninfa and Bennet, 1991). In contrast, PII in the nonuridylylated form interacts with NtrB during nitrogen limiting conditions that then dephosphorylates and inactivates NtrC. NtrC is a dimeric protein (subunit Mr of 55 kDa) which is characteristic of sigma 54-dependent activator proteins (Kustu *et al.*, 1989; Morett and Segovia, 1993). This protein has three distinct domains:

i) The N-terminal (domain approximately 12 kDa in size) is characteristic of response regulators and constitutes the domain with which NtrB interacts to phosphorylate a conserved aspartate residue (Asp-54). Only the phosphorylated form of NtrC is competent to activate transcription. Studies by Weiss *et al.* (1992) have shown that while phosphorylation stimulates DNA-binding, it is not required for the DNA-binding step but is needed to induce

oligomerization of NtrC dimers at upstream activator sites consisting of at least 2 NtrC-binding sites.

ii) The central domain (240 amino acids) contains a conserved nucleoside binding site and is considered to be the site of interaction with sigma 54 RNA polymerase to activate transcription.

iii) A typical DNA-binding, helix-turn-helix motif occurs in the C-terminal domain that mediates DNA-binding of the dimer. This motif allows recognition of enhancer upstream sequences, facilitating oligomerization of the protein to form a complex required for transcriptional activation and to tether NtrC at high local concentrations near the promoter thereby increasing its interaction with the sigma 54 (Drummond *et al.*, 1986; Reitzer and Magasanik, 1983).

As a consequence of its DNA-binding capabilities, NtrC can also function as a transcriptional repressor, and does function in this mode in the Enteric bacteria at both *glnAp1* and *glnLp* when cells are subject to nitrogen limitation and *glnALG* transcription is primarily from *glnAp2* (Reitzer and Magasanik, 1985; Ueno-Nishio *et al.*, 1983; Wei and Kustu., 1981). Interestingly, a cyclic AMP (cAMP) site was identified upstream of *glnAp1* and this promoter absolutely requires the presence of catabolite gene-activating protein (CAP) and cAMP for activation (Hirschman *et al.*, 1985). Under conditions of nitrogen limitation, high levels of transcription resulting in elevated GS levels occurs from *glnAp2*, a strong nitrogen regulated promoter (Hunt and Magasanik, 1985).

### iii) Sigma 54 (NtrA)

NtrA is an alternative sigma factor that changes the specificity of the core RNA polymerase so that it specifically binds nitrogen regulated promoters during nitrogen regulated growth. Sigma 54 promoters are characterized by an unusual spacing of the recognition elements, the dinucleotides GG and GC, at positions -24 and -12 from the transcription initiation site respectively. Studies by Buck and Cannon (1992) have shown that sigma 54 alone binds to promoter DNA and is also associated with core RNA polymerase, and is therefore responsible for many of the close contacts between RNA polymerase holoenzyme and promoter DNA.

#### iv) PII (GlnB)

Analysis of mutant forms of PII (Holtel and Merrick, 1988; Bueno and Magasanik, 1985) established that Tyr-51 was the site of uridylylation by the UT/UR enzyme (discussed below). Another important point that arose from these studies was that the uridylylation state of PII was crucial and affected the ability of PII to bind to NtrB. Studies by de Mel *et al.*, (1994) also showed that the PII protein can bind ATP, 2-ketoglutarate and glutamate that may indicate a key role for the PII protein in the monitoring of both the glutamate and 2-ketoglutarate levels under varying nitrogen conditions.

#### v) Uridylyltransferase (GlnD)

A key feature of the UTase activity of the GlnD protein is that the UTase activity is stimulated by 2-ketoglutarate and ATP and inhibited by glutamine (Magasanik and Neidhardt, 1987). Moreover, the availability of fixed nitrogen to the cell is reflected in the intracellular ratio of glutamine to 2-ketoglutarate such that sufficient ammonia levels are reflected in high glutamine/2-ketoglutarate levels whereas ammonia limitation would be expected to decrease this ratio (reviewed by Magasanik and Neidhardt, 1987). These observations have resulted in the formulation of the model (described above) in which the ratio of glutamine to 2-ketoglutarate determines the uridylylation state of PII.

#### vi) Adenylyltransferase

Adenylyltransferase (ATase) of the enteric bacteria has been purified and is a monomer with a molecular weight of 115,000 (reviewed by Neidhardt and Magasanik, 1987). ATase catalyzes the ATP-dependent addition of AMP to a subunit of GS with the release of PP<sub>i</sub>, thus leading to the partial inactivation of the GS enzyme itself. It can also catalyze the phosphate-dependent removal of AMP from each of the twelve subunits of glutamine synthetase, which would serve to restore GS activity (Ginsburg and Stadtman, 1973). The key to whether or not it adenylylates or de-adenylylates GS is dependent on the uridylylation state of PII; under conditions of nitrogen limitation, PII undergoes a conformational change induced by 2-ketoglutarate that allows formation of PII-UMP. This reaction is catalyzed by the

transferase activity of the UT/UR enzyme. PII-UMP in turn stimulates the deadenylylation reaction of ATase resulting in active GS. However, under conditions of nitrogen excess, UTase binds glutamine, inhibiting the uridylylation reaction, resulting in PII formation and ultimately in the adenylylation of GS by the ATase to yield inactive GS. Studies by Kustu *et al.* (1984) have indicated that adenylylation of GS plays an important role in preventing the depletion of cellular glutamate as well as ATP levels as excessive production of glutamine synthetase was found to cause a significant increase in generation time of the cells.

#### **b) Other mechanisms of regulating GSI activity**

It is important to note that not all GSI enzymes are regulated in the same manner as the Enterobacteriaceae. Different microorganisms appear to have evolved somewhat different mechanisms for regulating *glnA* gene expression and GS activity.

The above type of GSI control mechanisms do not appear to be functional in the cyanobacteria as well as in certain Gram-positive bacteria most notably *Clostridium*, *Bacillus subtilis* and *Streptomyces*.

#### **i) Regulation of the cyanobacterial GSI**

Ammonium assimilation in Cyanobacteria takes place mainly by the sequential action of GS and glutamate synthase (Merida *et al.*, 1991). The first GS enzyme to be identified was structurally similar to the GSI found in Enterobacteriaceae but was not regulated by adenylylation in response to the nitrogen source (Merida *et al.*, 1991). Instead the cyanobacterial GSI can be inactivated *in vivo* by ammonium addition through a new mechanism involving the binding of an inactivating factor. This factor, which is a phosphorylated protein, binds non-covalently to the GS under conditions in which ammonium is added to nitrate grown *Synechocystis* cells.

The gene encoding the GSI (*glnA* gene) of *Synechocystis* sp. PCC 6803 has been cloned and sequenced and showed high homology to other cyanobacterial *glnA* genes (Reyes and Florencio, 1995). These studies also illustrated for the first time in cyanobacteria, that *glnA* gene expression is regulated at the transcriptional level by the redox state of the cell. Studies by Reyes and Florencio (1994) have also indicated the presence of another GS gene (*glnN*) which is homologous to the GSIII isolated from *B. fragilis* and *B. fibrisolvens* (discussed below). Studies by Vegas-Palas *et al.* (1992) on pleiotropic *Synechococcus* strain PCC7942 mutants (unable to use inorganic nitrogen in a form other than ammonium) have resulted in the isolation of a gene termed *ntcA*, which belongs to the Crp family of bacterial transcriptional regulators. The Crp proteins are a family of DNA-binding proteins which function as transcriptional activators by binding to specific sites at promoter regions of CRP-controlled genes and allowing activation of transcription. The NtcA protein is a positive activator of the *glnA* and *nirA* genes in *Synechococcus* sp. and also activates transcription of the *ntcA* gene itself (reviewed by Merrick and Edwards, 1995). It functions by binding to target sequences in the *glnA*, *nirA* and *ntcA* promoters that consist of consensus GTAN<sub>8</sub>TAC sequences upstream of the transcription initiation site (Luque *et al.*, 1994). It may function in a similar manner to NtrC, which functions as a global nitrogen regulator in many other prokaryotes. The *ntcA* gene has been identified in 11 other species of cyanobacteria (Vegas-Palas *et al.*, 1992). Homologs of this gene have also been identified in *Synechocystis* strain PCC 6803 as well as in *Anabaena* strain PCC 7120 (Frias *et al.*, 1993). No primary sensory component that interacts with NtcA has as yet been identified.

## ii) Regulation of the *C. acetobutylicum* GSI

Regulation by an antisense RNA (AS-RNA) involves a simple, yet fundamental concept of molecular biology, that of base pairing of complementary nucleic acid strands. Studies by (Janssen *et al.*, 1990) identified two promoters, P1 and P2 upstream of the *C. acetobutylicum* *glnA* gene as well as a downstream promoter P3 (Fig.1.5a). The downstream promoter gave rise to an AS-RNA (Fig.1.5b) complementary to a 43 bp region of the 5' end of the *glnA* mRNA under conditions of nitrogen excess (Fierro-monti *et al.*, 1992). The AS-RNA binding region included the ribosome binding site as well as the *glnA* start codon and the formation of a RNA-RNA hybrid could be expected to interfere with translation of the *glnA* mRNA

under conditions of nitrogen excess since 1.6 fold more AS-RNA transcripts were produced than *glnA* mRNA transcripts. In contrast, under nitrogen-limiting conditions, 5-fold more *glnA* mRNA transcripts were produced than AS-RNA. Since high levels of AS-RNA are associated with low levels of *glnA* mRNA, and vice-versa, the characteristics of AS-RNA expression fitted with its role as an inhibitor of *glnA* gene expression. It should, however, be noted that promoters P1, P2 and P3 are all similar and are typical Gram-positive promoter sequences. Despite these similarities, they are differentially regulated by nitrogen. This data, therefore, supports the view that other as yet uncharacterized regulatory elements may also be involved in the *C. acetobutylicum* GSI regulation. In contrast to the Enterobacterial GSI, no adenylation site has been observed for the *C. acetobutylicum* GSI and no evidence for post translational modification has been shown (Usdin *et al.*, 1986).

### iii) Regulation of the *B. subtilis* GS

The GS enzyme from *Bacillus* species is very different to the GS enzyme found for the Enteric bacteria and is not subject to *ntr*-mediated regulation (Schreier *et al.*, 1989). Studies on the *B. subtilis* GS has indicated significant differences in terms of the amino acid composition, susceptibility to carboxypeptidase A digestion and immunochemical properties of these enzymes (Schreier, 1993). *In vitro* (Deuel and Stadtman, 1970) and *in vivo* studies (Schreier *et al.*, 1985) have shown that the *B. subtilis* GS enzyme is not subject to modification by adenylation and that other forms of post-translational control may largely be ruled out.

The fact that the *B. subtilis* GS is not regulated by covalent modification implies, therefore that differences in enzyme levels are solely due to control at the level of transcription (Strauch *et al.*, 1988; Schreier and Sonnenshein, 1986). This view is supported by studies in which the *glnA* mRNA levels were measured using the cloned gene as a probe, or from expression studies of the *glnRA* operon (Schreier *et al.*, 1989; Schreier *et al.*, 1985). These studies showed that the *glnA* mRNA levels are regulated 9-10 fold and vary with the nitrogen source in the same manner as does the GS activity.

There are two essential features of regulation of GS expression in *B. subtilis*;

a) GS has been implicated in its own regulation at the transcriptional level.

b) The interaction of GlnR with the *B. subtilis* GS that together forms part of the *glnRA* operon, negatively regulates transcription of the *glnA* gene.

These features are discussed below.

#### **a) Autoregulation of the *B. subtilis* GS**

Two experimental approaches have been used to establish the autoregulatory role of the *B. subtilis* GS viz. the characterization of *B. subtilis glnA* mutants, and studies of the effect of *glnA* mutations on *glnA* expression using *glnA-lacZ* fusions. Analysis of *B. subtilis glnA* mutant strains by Dean *et al.* (1977) revealed that these strains not only produced GS with altered catalytic activities, but that these mutants produced elevated levels of GS antigen. The results of these studies were confirmed and extended by the elegant studies of Schreier and Sonnenshein (1986) involving the use of *glnA-lacZ* fusions. Analysis of these fusions in a wild-type background revealed that  $\beta$ -galactosidase expression from the *glnA* promoter was regulated in the same manner as GS, with repression of both the *glnA* and reporter gene expression under conditions of nitrogen excess. This means that under conditions of nitrogen excess, *glnA* gene expression and hence production of GS as well as of  $\beta$ -galactosidase was repressed. However, mutations that altered GS activity or *glnA* deletion, which eliminated detectable GS activity or antigen, resulted in derepressed *lacZ* expression under nitrogen-excess conditions confirming the autoregulatory role of the *B. subtilis* GS.

More recent studies (Schreier and Sonnenshein, 1989) suggested that the active site of the *B. subtilis* GS may be involved in autoregulation of the GS.

## b) GlnR

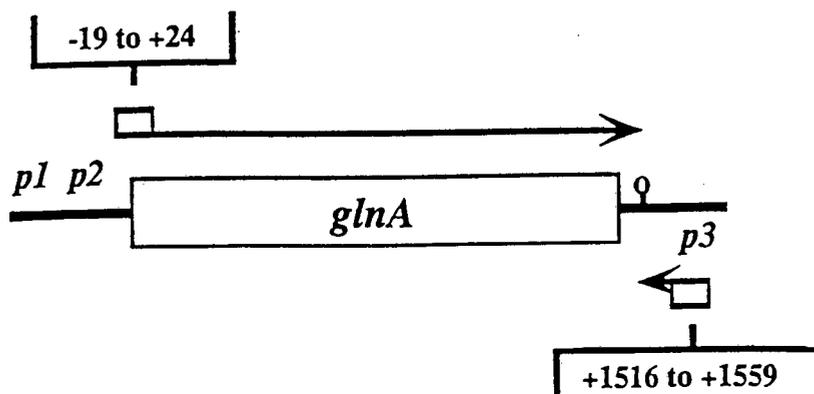
GlnR is a dimeric, 135 amino acid repressor protein that negatively regulates the *glnRA* operon under conditions of nitrogen excess (Schreier and Sonnenshein, 1989). In response to an as yet unidentified metabolic signal, the GlnR dimer binds to two operators viz. *glnRAo1* and *glnRAo2* (Fig. 1.6a & b), thus inhibiting transcription initiation of the *glnRA* operon under conditions of nitrogen excess.

No evidence has been provided to indicate that GlnR is modified in response to nitrogen limitation (as is the case for the NtrC in the Enterobacteria under conditions of nitrogen limitation) and purified GlnR has been shown to bind the *glnRA* operon with high affinity. Although GlnR has been shown to cause derepressed expression of GSI under nitrogen-limited conditions, it has not been implicated in causing the derepressed expression of the asparaginase, aspartase, urease and aminobutyratepermease genes that are known to exhibit derepressed expression under these conditions (reviewed by Merrick and Edwards, 1995).

These observations indicate that global nitrogen control mechanisms distinct from those in Enteric bacteria are present in *B. subtilis*.

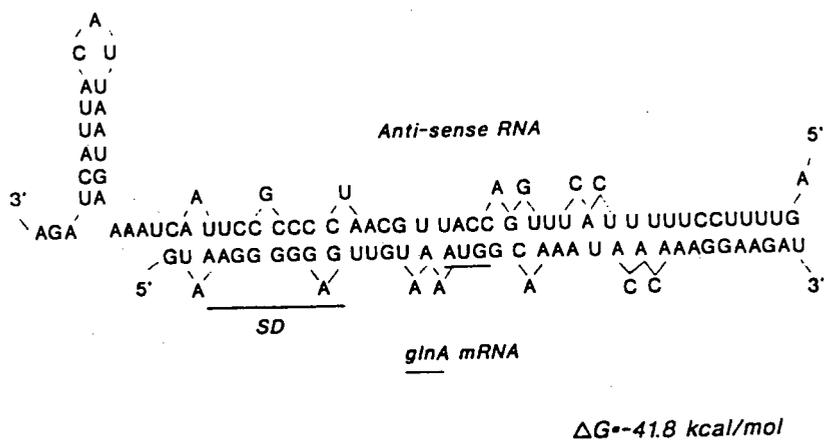
## iv) Regulation of the *Streptomyces* GSI

Studies on the regulation of *glnA* gene expression in *Streptomyces* are at an early stage and have indicated that GSI activity is regulated at both the transcriptional and posttranscriptional levels (reviewed by Hodgson, 1995). The selection of Gln<sup>-</sup> mutants in *S. coelicolor* has allowed the isolation of a gene, *glnR*, which is required for *glnA* transcription (Wray *et al.*, 1991). This gene encoding a 29 kDa protein, shows significant homology to a family of response regulator proteins that include OmpR and VirG. No sensor component has as yet been identified. *Streptomyces* species are interesting as they synthesize two forms of GS, GSI and GSII (Behrmann *et al.*, 1990; Kumada *et al.*, 1990). Other bacterial species eg. *Frankia* and *Bradyrhizobium* spp. also synthesize multiple forms of GS (isozymes). The regulatory mechanisms governing the expression of these enzymes is discussed below.



**Fig. 1.5a.** Structure of the *glnA* gene of *C. acetobutylicum*.

Orientation of the *glnA* transcript as directed from two promoters, p1 and p2, and a putative anti-*glnA* transcript expressed from promoter p3 from the opposite strand. Boxes represent complementary sequences. Schreier (1992).



**Fig. 1.5b.** Structure of the proposed RNA-RNA hybrid formed between the *glnA* mRNA and the antisense RNA, blocking the Shine-Dalgarno sequence and the AUG start codon. Fierro-Monti *et al.* (1992).



Recently, Shatters *et al.* (1993) reported the presence of a third GS in *R. meliloti* that is the product of the *glnT* gene. The authors have chosen to designate this gene as a GSIII type GS when it is totally dissimilar to the GS cloned from *B. fragilis* (discussed below). The GS gene isolated from *R. meliloti* will be referred to as the *glnT* gene or the GlnT protein (and not as the GSIII) in this review to avoid possible confusion with the *B. fragilis*, *B. fibrisolvans* and *Synechocystis* spp. GSIII genes that were isolated before the *R. meliloti glnT* gene and which are discussed below. The GlnT protein appears to be particularly abundant in *R. meliloti* mutants lacking GSI and GSII, has unusual kinetic properties, including a low affinity for ammonium and glutamate and no transferase activity (Shatters *et al.*, 1993). Analysis of the amino acid sequence of GlnT by Chiurrazzi *et al.* (1992) have shown that this protein can be distinguished from both the GSI and GSII families of GS proteins but that amino acids that are highly conserved in comparisons between GSI and GSII proteins are also found in GlnT. Interestingly, Liu and Kahn (1995) have reported that GlnT can be post-translationally modified *in vivo* at an Arg residue by ADP-ribosylation.  $^{32}\text{PO}_4$  attached to GlnT during bacterial growth could be removed by treatment with snake venom phosphodiesterase or by turkey erythrocyte ADP-ribosylarginine hydrolase. ADP-ribosylation was found to inhibit GlnT activity. These observations, therefore, indicate that post-translational control could play an important role in regulating the contribution of GlnT to cellular glutamine synthesis.

The most obvious difference between ammonia assimilation in the enteric bacteria and *R. meliloti* is that enteric bacteria have only one GS whereas *R. meliloti* has three GS enzymes. Further research will be required to elucidate the regulatory mechanisms that govern the expression of each of these enzymes in the cellular milieu.

#### 1.4.2.3) Regulation of GSIII

The GSIII protein, which is distinct from both the GSI and GSII proteins has been found in *B. fragilis*, *B. fibrisolvans* and comparatively recently was found to occur in *Synechocystis* species and other non  $\text{N}_2$ -fixing cyanobacteria. GSIII proteins have also been identified in other anaerobic rumen microorganisms i.e. *Ruminococcus flavefaciens* (Mackie, personal communication) as well as in *Prevotella ruminicola* (Morrison, personal communication). The GSIII protein discussed in this section should not be confused with the GSIII (product of the *glnT* gene) protein discussed above and known to occur in *R. meliloti*. The fact that the *R.*

*meliloti* GS has been designated GSIII when it is structurally non-homologous to the GSIII discussed in this section is indicative of the constant state of flux of the nomenclature of GS enzymes as new information is continually becoming available.

The *B. fragilis* GS has been characterized, and studies by Varel and Bryant (1974) have indicated the requirement of this obligately anaerobic bacterium for ammonia as a source of nitrogen for growth. Studies by Yamamoto *et al.* (1984) have also shown that GS activity is induced by low nitrogen levels in *B. fragilis* ATCC 23745 although work on other strains has been hampered by the specific inactivation of the GS after cell disruption (Yamamoto *et al.* 1984). Interestingly, Morrison (personal communication) also reported the inactivation of the *P. ruminicola* GSIII after cell disruption. Abratt *et al.*, (1993) have also shown, using reporter gene studies that an endoglucanase gene fused to the *B. fragilis glnA* promoter and carried on a *B. fragilis-E. coli* shuttle vector is regulated by nitrogen in *B. fragilis* at the level of transcription.

Transcriptional fusions using chloramphenicol acetyltransferase-*glnN* gene fusions as a reporter gene system has indicated that the GSIII *glnN* gene from *Synechocystis* sp. strain PCC 6803 is also regulated at the level of transcription. Furthermore, heterologous Southern hybridization analysis has also revealed the presence of the *glnN* gene in other cyanobacteria although it was not detected in the N<sub>2</sub>-fixing species (Reyes and Florencio, 1994; Dominguez *et al.*, 1997).

Studies by Southern *et al.* (1987) indicated that there was no evidence for adenylylation of the *B. fragilis* GS and hence no evidence for control of GSIII regulation at the post-translational level. However, the possibility that post-translational control could be occurring by a mechanism not involving adenylylation/de-adenylylation cannot be ruled out.

The above discussion has highlighted the fact that regulation of *glnA* gene expression in the enteric bacteria is certainly not the only way bacteria control the expression of this central enzyme of nitrogen metabolism. More information concerning the genetics of nitrogen metabolism of a number of different bacteria will lead to a better understanding of the various regulatory strategies bacteria employ to survive *in vivo*.

No information is available regarding the manner in which the GSIII gene is regulated in *B. fibrisolvens* and further work is required to unravel the nature of the regulatory mechanisms governing the expression of GSIII in this organism.

## **1.5 Genetic manipulation of rumen bacteria**

### **1.5.1 General considerations**

Both the potential and limitations involved in the genetic manipulation of rumen bacteria are great. To date, the major genetic engineering accomplishments have been associated with industrial microbiology where the engineered microorganisms are grown under carefully controlled conditions with antibiotics as selective agents (Russell and Wilson, 1988). However, in natural environments like the rumen, energy sources are usually limiting and competition is very intense (Patterson, 1989). Factors like substrate affinity, maintenance energy expenditure, resistance to toxic substances, attachment to solid surfaces and the ability to tolerate periods of nutrient starvation can be of critical importance to the survival of rumen bacteria (Russell and Wilson, 1988). These physiological factors are of little significance in industrial fermentations where the energy source is provided in excess and potential competitors are excluded.

Two major constraints for the establishment of a microbial species in the rumen are the **turnover time** in the rumen and **competition** with other organisms (Patterson, 1989). The microflora of the rumen, through a period of intense selection have adapted very well to their environment. Therefore, new genetically modified, non-rumen organisms introduced to the rumen may have little chance of surviving. Enumeration studies have shown that non-rumen bacteria such as *E. coli* does not compete well in the rumen microbial ecosystem (Russell and Wilson, 1988) and native rumen bacteria are, therefore, the organisms of choice for genetic manipulation of this ecosystem.

### **1.5.2 Genetic transfer systems**

A considerable amount of attention has been focused on the benefits to animal production that might result from the genetic manipulation of rumen bacteria. While the gains in productivity that could arise from potential improvements in the nutrition and health of ruminant animals

are readily apparent (Beard *et al.*, 1995), progress towards the attainment of these goals has been hampered by the lack of the necessary tools for genetic manipulation, such as stable vectors and efficient DNA delivery systems.

The two rumen bacterial species most frequently considered as recipients of new genetic material are the hemicellulolytic organisms *Prevotella ruminicola* (Thompson and Flint, 1989) and *B. fibrisolvens* (Ware *et al.*, 1992). Vectors developed for *P. ruminicola* are showing great promise but progress has been somewhat slower with *B. fibrisolvens*.

### 1.5.2 a) Genetic transfer systems of *B. fibrisolvens*

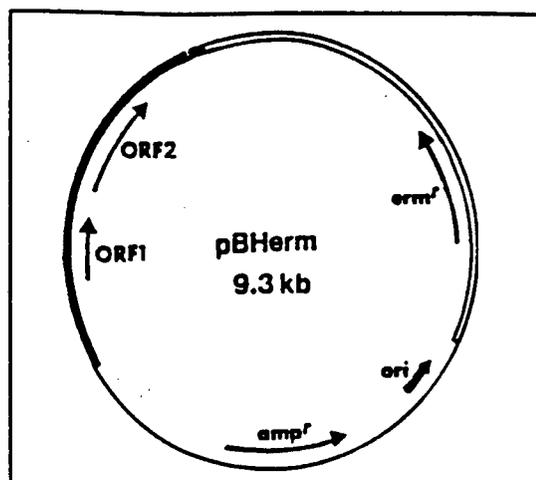
There have been reports of limited success in the transfer of DNA into *B. fibrisolvens*. Teather (1985) reported transfer of the broad-host-range plasmid RP4 from *E. coli* to *B. fibrisolvens* by conjugation, imparting ampicillin resistance to the recipient strain. Reports by Hazlewood and Teather (1988) have described the transfer of broad-host-range plasmid pRK248 by PEG treatment of spheroplasts. However, because of plasmid instability, these systems were not pursued. Studies by Ware *et al.* (1992) showed the transformation of *B. fibrisolvens* by electroporation with a recombinant plasmid. Plasmid maintenance over a number of generations was demonstrated using DNA hybridization analysis of probes homologous to regions of this plasmid; however, the plasmid lacked a suitable selectable genetic marker, which is essential for them to be employed as practical DNA vectors. Whitehead and Hespell (1991) have demonstrated transfer of the *Enterococcus faecalis* plasmid pAM $\beta$ 1 to *B. fibrisolvens* by conjugation, but the large size of pAM $\beta$ 1 (26.5 kb) makes it difficult to manipulate as a transformation vector. Subsequently, Whitehead (1992) introduced pBS42, an *E. coli*/*B. subtilis* shuttle vector, into *B. fibrisolvens* by electroporation. Although the feasibility of the system was demonstrated, transformation efficiency was low (25 transformants/  $\mu$ g DNA) and the use of this vector to transfer additional DNA has yet to be reported. For genetic engineering to be successful in these bacteria, suitable vectors, hosts, markers, and transfer systems are needed. The most promising vectors for ruminal bacteria are endogenous plasmids. A small cryptic plasmid pOM1 (2.8 kb) was isolated from *B. fibrisolvens* (Mann *et al.*, 1986) and ligated onto the *E. coli* vector, pBR325 to construct an *E. coli* -*B. fibrisolvens* shuttle vector. More recently, Beard *et al.*, (1995) described an *E.*

*coli* / *B. fibrisolvans* shuttle vector, pBHerm (Fig.1.7a) based on pUC118 and the native *B. fibrisolvans* plasmid pRJF1 which was maintained stably in both hosts without selection and could be efficiently transferred by electroporation ( $10^4$ - $10^5$  / $\mu$ g plasmid DNA). Sequencing and functional analysis of pRJF1 and pBHERm has allowed the determination of functional regions of these plasmids (ORF1 and ORF2, Fig. 1.7a). Using this data, Kobayashi *et al.*, (1995) were able to identify the minimal functional regions required for constructing an effective plasmid vector, culminating in the construction of an *E. coli*/*B. fibrisolvans* shuttle vector, pYK4 (Fig.1.7b). This plasmid (pYK4) is small enough in size to allow for maximum electroporation and ligation efficiency while at the same time retaining the essential features required for efficient replication in both *B. fibrisolvans* and *E. coli*.

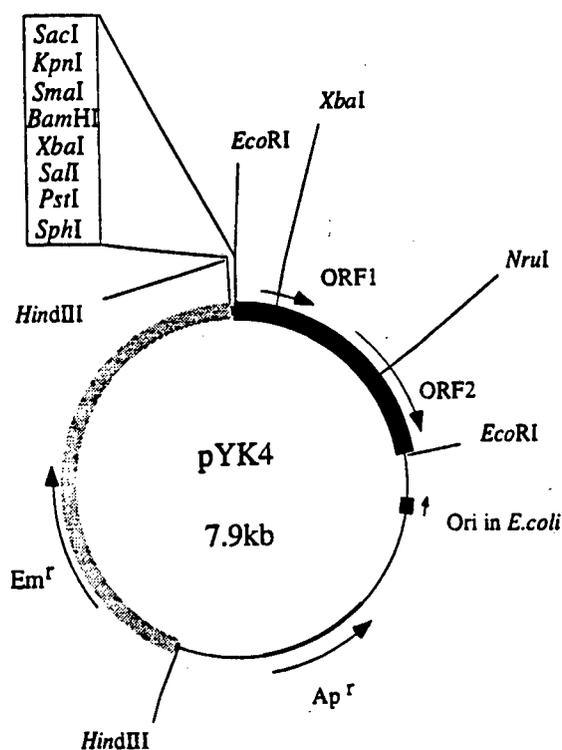
However, the problem of DNA restriction barriers between plasmids produced from various *B. fibrisolvans* strains to some extent limits the utility of this vector (Kobayashi *et al.*, 1995).

### 1.5.2b) Gene structure and expression in *B. fibrisolvans*

Despite the fact that the information available on the genetics of *B. fibrisolvans* and rumen bacteria is limited, a number of features can be deduced from the examination of cloned *B. fibrisolvans* genes (Rumbak, 1991). All the *B. fibrisolvans* genes cloned to date have been expressed in the host organism, *E. coli*, with most of these genes having been expressed from endogenous promoters (Rumbak *et al.*, 1991; Berger, *et al.*, 1990). Heterologous expression of these *B. fibrisolvans* genes imply that the DNA sequences responsible for the regulation of transcription and translation, for example promoter sequences, ribosomal binding sites, start and stop codons, must be compatible with the *E. coli* biosynthetic machinery. Moreover, the secretion of the enzymes encoded by the *B. fibrisolvans end1* gene (Berger *et al.*, 1989) into the periplasmic space implies that this gene product possesses a signal peptide recognized by the host protein export system. The product of the *ced1* gene has no recognizable signal sequence, but this cellulodextrinase is also secreted into the *E. coli* periplasm by an unknown mechanism (Berger *et al.*, 1989). The signal sequences of the *B. fibrisolvans* extracellular enzymes are not always recognized in *E. coli* and some expressed proteins such as the xylanases are accumulated in the cytoplasm (Manarelli *et al.*, 1990). Information on the



**Fig. 1.7a** Restriction map of the plasmid pBHerm. Narrow line - pUC118. Thick, filled lines - plasmid pRJF1; open line - DNA from pAM $\beta$ 1. Beard *et al.* (1995).



**Fig. 1.7b.** *E. coli* - *B. fibrisolvans* shuttle vector, pYK4. Thick bold lines - *B. fibrisolvans* pRJF1 sequences used in vector construction, thick grey lines - pAM $\beta$ 1; thin lines *E. coli* pUC118 vector DNA. Kobayashi *et al.* (1995).

regulation of gene expression and the secretion of proteins in *B. fibrisolvens* is not yet available and requires further investigation.

### **1.6 Aims of this study**

The aims of this study were to attempt the isolation of a gene/genes involved in the regulation of the *B. fibrisolvens glnA* gene from a previously established *B. fibrisolvens* H17C pEB1 gene bank (Rumbak, 1991). The *B. fibrisolvens glnA* gene has been cloned and sequenced (Goodman and Woods, 1993) and the gene was found to be expressed in *E. coli*. This information together with the availability of a pEB1 *B. fibrisolvens* H17C genebank allowed the design and implementation of a novel, *in trans*, two plasmid system for the screening of the genebank in a suitable *E. coli* GS mutant. This would allow for specific interaction between the *B. fibrisolvens glnA* gene or its gene product and any regulatory protein produced from the gene bank. Previously, this type of system was shown to be useful for the isolation of a putative *B. fragilis glnA* regulatory gene (Abratt and Woods, personal communication). Any GS regulatory genes thus isolated could be further characterized by sequencing and protein analysis. There is a lack of information concerning the regulation of gene expression in *B. fibrisolvens* as only structural and no regulatory genes have thus far been isolated. Isolation of nitrogen regulatory genes from *B. fibrisolvens*, specifically those involved in regulating the expression of the GS, would increase our knowledge of the manner in which the GS is regulated and provide potentially useful information about the mechanistics of gene expression in *B. fibrisolvens*. This will, ultimately, allow the manipulation of gene expression and facilitate improved growth and efficiency of the rumen ecosystem.

**Chapter 2**  
**Cloning and Characterization of pSAM1**

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## Chapter 2

### Cloning and Characterization of pSAM1

#### Summary

The *B. fibrisolvans* glutamine synthetase gene was subcloned into a low copy number plasmid pACYC184. This construct (pGS5) was transformed into an *E. coli glnALG* deletion strain (YMC11). *E. coli* YMC11 (pGS5) was then transformed with an existing *B. fibrisolvans* pEB1 gene bank to generate *in trans* two plasmid systems which were used to screen for the presence of a glutamine synthetase (GS) regulatory gene carried in the gene bank. A genetic element (pSAM1) was isolated which caused a 2.5- to 3-fold elevation of the GS activity levels in *E. coli* YMC11 (pGS5) cells relative to the basal levels. The plasmid, pSAM1 was characterized partially by restriction enzyme mapping, and DNA hybridization analysis confirmed the insert DNA to be *B. fibrisolvans* chromosomal DNA. Deletion analysis showed that the GS regulatory activity carried on pSAM1 was lost with the removal of the central 0.387 kb *EcoRV* fragment of the insert DNA of pSAM1.

Subcloning of the *B. fibrisolvans* insert DNA in two sections onto the high-copy-number plasmid pBluescript KS to yield pSAM3 and pSAM4 also resulted in the loss of elevated GS levels. *E. coli* YMC11 transformed with pSAM1 alone showed no growth retardation in complete media as compared with *E. coli* YMC11 transformed with a control plasmid, pCM1. No GS activity was observed in *E. coli* YMC11 transformed with pSAM1 alone indicating that the *B. fibrisolvans* insert did not code for another protein capable of GS activity.

#### 2.1 Introduction

The major, and often required, source of nitrogen for rumen bacteria is ammonia (Bryant and Robinson, 1962). For this reason, enzymes involved in ammonia assimilation are essential for the survival of most rumen microorganisms.

Studies by Hespell (1984) showed that the dual enzyme system, glutamine synthetase (GS) and glutamate synthase is an important route by which ammonia may be assimilated, while studies by Smith *et al.* (1981) illustrated the importance of the GS component in ammonia assimilation in the bacterial population of the rumen.

The *B. fibrisolvens glnA* gene has been cloned and sequenced (Goodman and Woods, 1993) and found to be homologous (40% amino acid homology) to a novel type III GS from *B. fragilis*. However, the lack of efficient yet stable, vector systems for transforming *B. fibrisolvens* H17c cells (Hespell & Whitehead, 1991), the absence of any characterized nitrogen metabolism mutants for *B. fibrisolvens* and the fact that no suitable nitrogen-limiting medium which supports the growth of *B. fibrisolvens* has as yet been found (Goodman & Woods, 1993) meant that studies on the regulation of GS in *B. fibrisolvens* were not feasible. For this reason, it was decided to attempt the isolation of *B. fibrisolvens* GS regulatory genes in a heterologous organism. A number of approaches could be used to isolate GS regulatory genes using *E. coli* as the heterologous host. One approach could be to use complementation analysis on defined media using heterologous *E. coli* mutants which lacked the necessary nitrogen regulatory genes. However, the unique structure of the *B. fibrisolvens* GS may indicate that the regulation of this gene in *B. fibrisolvens* is also novel thus limiting this approach. Another approach relies on a  $\beta$ -galactosidase reporter gene system (Reitzer & Magasanik, 1987). The promoter of the *B. fibrisolvens glnA* gene could be fused to a truncated *lacZ* gene to generate a *glnA - lacZ* gene fusion. Changes in the levels of the  $\beta$ -galactosidase fusion protein in the presence of a gene bank (*B. fibrisolvens* gene bank) and on suitable nitrogen selective media could then be used to identify putative regulatory genes. This approach could be used to identify genes which affected the expression of GS at the transcriptional level.

However, previous research on the *glnA* genes of a number of different microorganisms (reviewed by Woods & Reid, 1993; Reitzer & Magasanik, 1987; Kustu *et al.*, 1986) has shown that this gene appears to be regulated at the transcriptional, translational and post-translational levels. For this reason a different system which did not exclude any of these potential regulatory mechanisms was required to allow the isolation of such regulatory genes.

Previously, Abratt & Woods (personal communication) used an *in trans*, two - plasmid system which allowed the isolation of a putative GS regulatory gene from *Bacteroides fragilis*. This system permits the selection of regulatory proteins on the basis of a specific interaction between the cloned *glnA* gene or gene product and the homologous regulatory gene or gene product from the same organism carried on a compatible plasmid within a heterologous mutant host. Genes which affect the regulation of GS expression at transcriptional, translational and post-translational levels could be isolated. This experimental system was used in this study.

The *B. fibrisolvens glnA* gene was cloned onto the low copy number (18-22 copies/cell) plasmid, pACYC184 (Chang and Cohen, 1978) which has a p15A origin of replication (*ori*) and is compatible with plasmid pEB1 (Lin, 1990) which has the ColE1 *ori*. Transformation of this construct (pGS5) into an *E. coli glnALG* deletion strain YMC11 (Backman *et al.*, 1981) allowed the growth of these cells on minimal medium in which ammonium sulphate was the sole nitrogen source. Subsequently, these *E. coli* YMC11 (pGS5) transformants were co-transformed with a *B. fibrisolvens* gene library (Rumbak, 1991) carried on the compatible higher copy number (80 copies/cell) plasmid pEB1 (a positive selection, pEcoR251-derived *E. coli* - *B. subtilis* shuttle vector). *B. fibrisolvens* gene products which affected the regulation or functioning of the *B. fibrisolvens* GS *in trans* could then be identified by the ability or inability of *E. coli* YMC11 transformants to grow on minimal media with ammonium sulphate as the sole source of nitrogen.

The aims of this section of the study were to clone *B. fibrisolvens* GS regulatory genes using a novel, *in trans*, two- plasmid system, and the characterization of such GS regulatory genes.

## 2.2 Materials and Methods

Standard materials and methods are recorded and referenced in the Appendix.

### 2.2.1 Bacterial strains and plasmids used in this study

*B. fibrisolvens* H17c isolated by Dehority (1976) was originally obtained by Dr R.B. Hespell (Department of Animal Science, University of Illinois, Urbana) and maintained at -70°C on slopes of M10 medium (Appendix A) (Rumbak, 1990). *E. coli* strains and plasmids used in this study are indicated in Table 2.1.

### 2.2.2. Media and growth conditions

All media, buffers and growth conditions not described in the text are listed in Appendix A. *B. fibrisolvens* was grown at 37°C under stringent anaerobic conditions in a Forma Scientific anaerobic cabinet (Model 1024) with a gas mix of 5% hydrogen, 10% carbon dioxide and 85% nitrogen. The media on which it was grown was M10 non-rumen fluid medium (Appendix A) as described by Strydom *et al.* (1986). *E. coli* strains were grown in double strength yeast tryptone (2YT) broth or agar (2YTA) (1.5%) (Sambrook *et al.*, 1989) at 37°C supplemented with ampicillin (Ap, 100 µg/ml), tetracycline (Tc, 20 µg/ml) or chloramphenicol (Cm, 100 µg/ml) for the selection and maintenance of plasmids. Selection for growth on various nitrogen sources occurred on CSH minimal media agar plates (1.5%) containing either (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (15 mM) (MMN) or glutamine (15 mM) (MMG) as the sole nitrogen source, supplemented with the appropriate antibiotics. Growth of *E. coli* YMC11 (pGS5) clones transformed with the pEB1 gene bank was compared with *E. coli* (pGS5) clones transformed with control plasmids, pMT104 and pCM1.

### 2.2.3. Preparation and analysis of nucleic acids

Plasmid DNA was isolated according to the method of Ish-Horowicz and Burke (1981), and cloning and transformation were carried out as described by Sambrook *et al.* (1989). Restriction endonucleases were used according to the manufacturers' instructions. *E. coli* JM105 was used for transformation and preparation of recombinant DNA.

Table 2.1 Bacterial strains and plasmids

Strain	Relevant genotype	Reference or source
<i>E.coli</i>		
YMC10	} <i>glnA</i> <sup>+</sup> <i>ntxA</i> <sup>+</sup> <i>ntxB</i> <sup>+</sup> } <i>glnA</i> <sup>-</sup> <i>ntxA</i> <sup>-</sup> <i>ntxB</i> <sup>-</sup>	Backman <i>et al.</i> (1979)
YMC11		
HB101	<i>glnA</i> <sup>+</sup> <i>ntxA</i> <sup>+</sup> <i>ntxB</i> <sup>+</sup>	Boyer & Roulland-Dussoix (1969)
JM105	<i>glnA</i> <sup>+</sup> <i>ntxA</i> <sup>+</sup> <i>ntxB</i> <sup>+</sup>	Yannisch-Perron <i>et al.</i> (1985)
<i>B.fibrisolvens</i> H17c	wild type	Dehority (1976)
Plasmid	Description	Reference or Source
pEB1	Ap <sup>r</sup> <i>EcoRI</i> <sup>r</sup>	Lin (1990)
pCM1	pEB1 Ap <sup>r</sup> <i>EcoRI</i> <sup>r</sup>	Mittendorf (1994)
pSAM1	pEB1, Ap <sup>r</sup> <i>EcoRI</i> <sup>r</sup> containing putative GS regulator	} This work
pSAM2	pEB1, Ap <sup>r</sup> <i>EcoRI</i> <sup>r</sup> with <i>EcoRV</i> fragment deleted	
pSAM3	pKS Ap <sup>r</sup> <i>lacZ</i> with 0.8kb <i>EcoRI</i> - <i>PstI</i> fragment from pSAM1	
pSAM4	pKS Ap <sup>r</sup> <i>lacZ</i> with 0.4kb <i>PstI</i> - <i>SspI</i> fragment of pSAM1	
pACYC184	Cm <sup>r</sup> Tet <sup>r</sup>	Chang & Cohen (1978)
pGS4.δ16	pM13MB20 containing <i>B.fibrisolvens glnA</i> gene	Goodman & Woods (1993)
pGS5	<i>B.fibrisolvens</i> <i>glnA</i> gene subcloned from pGS4.δ16 onto pACYC184	This work
pGSR1	pEcoR251 containing <i>B.fragilis glnA</i> regulatory gene.	Supplied by Abratt (UCT)
pBluescript (pKS)	Ap <sup>r</sup> <i>lacZ</i> <i>lacI</i> <sup>h</sup>	Viera and Messing (1982)
pMT104	pEcoR251 Ap <sup>r</sup> <i>EcoRI</i> -	Wehnert <i>et al.</i> (1990)

Plasmid maps of pEB1 and pACYC184 are included in Appendix C.

*B. fibrisolvans* chromosomal DNA was isolated by the method of Marmur (1961). An overnight (16 h) culture (500 ml) was harvested by centrifugation (10 000g for 10 min), resuspended in 4ml Solution A (10 mM TRIS-HCL, pH 8.0; 25% w/v sucrose) containing 5 mg/ml lysozyme, and mixed gently at 37°C for 30 min. Two ml of ice-cold Solution B (0.5M EDTA, pH 8.0) were added and the mixture was kept on ice for 5 min. Four ml Solution C (10 mM TRIS-HCL, pH 7.5, 1 mM Na<sub>2</sub>EDTA, 2% w/v SDS) containing 5 mg/ml proteinase K were added. The mixture was kept at 20 °C for 10 min. CsCl (1g/ml) and EtBr (250 µg/ml) were added and the mixture was centrifuged at 15 000g for 30 min. All subsequent procedures were conducted as described by Maniatis *et al.* (1982). The pellet was resuspended in 0.5 ml of TE buffer.

Chromosomal DNA from *E. coli* HB101 was extracted using standard methods (Sambrook *et al.*, 1989).

The DNA concentration was determined by measuring the absorbance at 260 nm, where one absorbance unit is equivalent to 50 µg/ml DNA (Maniatis *et al.*, 1982).

#### 2.2.4 GS assays.

Total GS activity was assayed using crude cell extracts by the  $\gamma$ -glutamyltransferase assay method of Bender *et al.* (1977). *E. coli* YMC11 clones containing pGS5 were transformed with pSAM1 (Fig.2.3), pSAM2 (Fig.2.3), pCM1 or pMT104 (Table 2.1). Plasmid pMT104, a derivative of pEcoR251 with an inactivated *EcoRI* gene (Wehnert *et al.*,1990) was initially used as a control for all pEcoR251 recombinant plasmids. However, pCM1 a derivative of pEB1 (Lin, 1990) with an inactivated *EcoRI* gene (Mittendorf, 1994) later became available and was used as a control for all subsequent assays. The cells were grown for 18h at 37°C in 2YT broth supplemented with Ap and Tc. The cultures were subinoculated (1/20 dilution) into prewarmed 2YT broth containing Ap and Tc and grown at 37°C with vigorous aeration. At various time intervals, the absorbance of the cultures was measured ( $A = 600\text{nm}$ ) using a Beckman Du 64 spectrophotometer, and 50 ml samples removed to be assayed for GS activity. The cell permeabilizer, *N*-cetyl-*N,N,N*- trimethylammonium bromide (CTAB) (Merck) was added to the sample to a final concentration of 0.01% (w/v) and incubation continued for 10 min at 37°C. All subsequent procedures were done at 4°C.

The cells were pelleted by centrifugation at 6 000 rpm for 10 min at 4°C. Subsequently, the cells were washed once with an equal volume of NaCl (0.8% w/v), harvested by centrifugation as before, and resuspended in 0.5 ml resuspension buffer (50 mM imidazole/HCl, 20 mM MgCl<sub>2</sub>, 0.1 mg/ml phenylmethylsulphonyl fluoride and 20 mM mercaptoethanol). Crude extracts were assayed immediately in triplicate. The mean and standard deviation of a number of experiments was calculated using a SHARP EL 532 statistical analysis package. Protein concentrations were determined by the dye-binding method of Bradford (1976). One unit of GS activity is defined as the number of  $\mu$ moles of  $\gamma$ -glutamylhydroxamate formed per min per mg of protein.

### **2.2.5. Subcloning of the *B. fibrisolvans* *glnA* gene onto the low copy number plasmid pACYC184**

The *B. fibrisolvans* glutamine synthetase gene present on the 5.1 kb NcoI-EcoRI fragment of pGS4.816 (Goodman and Woods, 1993) was subcloned into the *NcoI* - *EcoRI* restriction sites of pACYC184 using standard methods (Appendix B). The resulting recombinant plasmid was designated pGS5 (Fig.2.1). Transformants were selected for on 2YT plates containing tetracycline (Tc), assayed for GS activity and confirmed to be pGS5 by restriction endonuclease digestion.

### **2.2.6. Cloning of a putative *B. fibrisolvans* GS regulatory gene.**

Competent *E. coli* strain YMC11 was transformed with pGS5 (Dagert and Ehrlich, 1979) and *E. coli* YMC11 (pGS5) co-transformed with an existing *B. fibrisolvans* gene bank (Rumbak, 1991). Transformants were selected on 2YT plates containing both Tc (20  $\mu$ g/ml) and Ap (100  $\mu$ g/ml) and after 18 hours growth, colonies were replica plated onto minimal medium plates containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (15 mM) as the sole nitrogen source (MMN), minimal media plates containing glutamine (15 mM) as the sole nitrogen source (MMG) and onto 2YT plates. All media contained Ap and Tc. These plates were incubated for 24 hours at 37°C and then compared with master plates to identify colonies showing enhanced or reduced growth on the MMN plates but normal growth on MMG and 2YT plates. These colonies were assayed for GS activity.

### 2.2.7. Growth Curve Analysis

The growth curves of 4 clones which showed a reduced ability to grow on MMN but not on MMG were examined using 2YT broth since growth was limited on MMN. *E. coli* YMC11 (pGS5) clones 5, 7, 15 and 16 as well as clones containing a control plasmid (pCM1 or pMT104) were grown for 18 hours at 37°C. The cultures were then subinoculated (1/20 dilution) into prewarmed 2YT supplemented with Ap and Tc. Absorbance readings were taken at hourly intervals on a DU64 spectrophotometer (Beckman instruments) at 600 nm (A600 nm). Graphs of A600 nm vs time were then constructed for each of the clones (Fig.2.2).

### 2.2.8. Segregation of plasmids

In order to separate the Ap or Tc expressing plasmids contained in the isolates, the plasmid DNA from each of the isolates (5, 7, 15 and 16) was extracted using small scale DNA extraction procedures (minipreparations). The plasmid DNA of each isolate was retransformed into competent *E. coli* YMC11 and the resulting transformants were selected for on plates containing either Ap or Tc. Ap<sup>R</sup> or Tc<sup>R</sup> colonies were then tested for Tc<sup>R</sup> or Ap<sup>R</sup>, respectively to determine if both antibiotic resistance markers had been acquired. Colonies were isolated which had acquired only the Ap resistance marker (and hence only a plasmid from the genebank). These plasmids were then purified using large scale plasmid DNA extraction procedures (maxipreparations).

The pEB1 derived Ap<sup>R</sup> conferring plasmid obtained from *E. coli* YMC11 (pGS5) isolate 15 was called pSAM1.

### 2.2.9. Characterisation of pSAM1

#### 2.2.9.1. Restriction Endonuclease Analysis

The insert DNA of pSAM1 was partially mapped using a number of restriction endonucleases (RE) (Fig. 2.3). A linear restriction map was constructed by analysis of the restriction products of single and double digests with a range of RE. The sizes of fragments were estimated using the *Pst*I-digested lambda molecular weight marker.

### 2.2.9.2. Examination of the pSAM1 insert DNA for GS activity

*E. coli* YMC11 was rendered competent (Armitage, 1988) and transformed with pACYC184. Subsequently, competent *E. coli* YMC11 (pACYC184) was transformed with pCM1 or pSAM1 and examined using growth curve analysis and GS assays.

### 2.2.9.3. Localization of the active region of pSAM1 and subcloning of the *B.fibrisolvans* insert DNA.

The internal *EcoRV* fragment of pSAM1 was deleted by restriction endonuclease digestion and religation to generate pSAM2 (Sambrook *et al.* (1989) (Fig. 2.3).

The *EcoRI-SspI* 0.8kb fragment and the *PstI-SspI* 0.4kb fragment of pSAM1 were subcloned into the *EcoRI-EcoRV* and *PstI-EcoRV* sites (Fig.2.3) respectively of the high copy number plasmid pBluescript (pKS) (to generate pSAM3 and pSAM4 respectively) using standard techniques (Sambrook *et al.*, 1989). All constructs were tested for GS activity.

### 2.2.9.4. Origin of pSAM1 insert DNA

In order to confirm the origin of *B. fibrisolvans* H17c insert DNA of pSAM1 and the degree of relatedness of pSAM1 to the DNA of the other clones isolated as well as to pGSR1 (a *B.fragilis* gene), DNA hybridization was performed according to the method of Southern (1975).

All DNA was digested to completion with the appropriate restriction endonucleases. The DNA fragments were separated on a 0.8% agarose gel, transferred by capillary blotting onto a Hybond N+ nylon membrane (Amersham International, Amersham , UK) according to the manufacturer's instructions.

The internal *EcoRV* fragment of pSAM1 was obtained by restriction endonuclease digestion and gel electrophoresis, and purified using the GeneClean kit (BIO101 Inc.) according to the manufacturers instructions. This DNA fragment was non-radioactively labelled with digoxigenin using the random -priming method and used as a probe. DNA labelling and hybridization were according to the manufacturers instructions (Nonradioactive DNA labelling and Detection Kit (No. 1093657, Boehringer Mannheim (South Africa), Johannesburg) and detection of the hybridized probe was via chemiluminescence using the CSPD substrate (No.1357328, Boehringer Mannheim (SA)).

All practical details concerning nonradioactive hybridization are contained in the Appendix.

## 2.3. Results

### 2.3.1. Subcloning of the *B. fibrisolvens glnA* gene onto pACYC184 and determination of the optimum growth stage for assaying GS activity.

Restriction enzyme analysis using single and double digests was used to confirm the cloning of the *NcoI-EcoRI* 5.1 kb fragment from pGS4.816 into the low copy number plasmid pACYC184 (Fig.2.1) to yield pGS5. It had previously been established by Goodman and Woods (1993) that *B. fibrisolvens* GS activity was expressed in *E. coli*. The GS activity of *E. coli* YMC11 (pGS5) was monitored using the  $\gamma$ -glutamyltransferase assay to determine GS levels at early ( $A_{600}=0.75$ ), middle ( $A_{600}=1$ ) and late ( $A_{600}=2.1$ ) exponential phase.

**Table 2.2** GS activity of *E. coli* YMC11(pGS5) clones at 3 different growth stages assays.

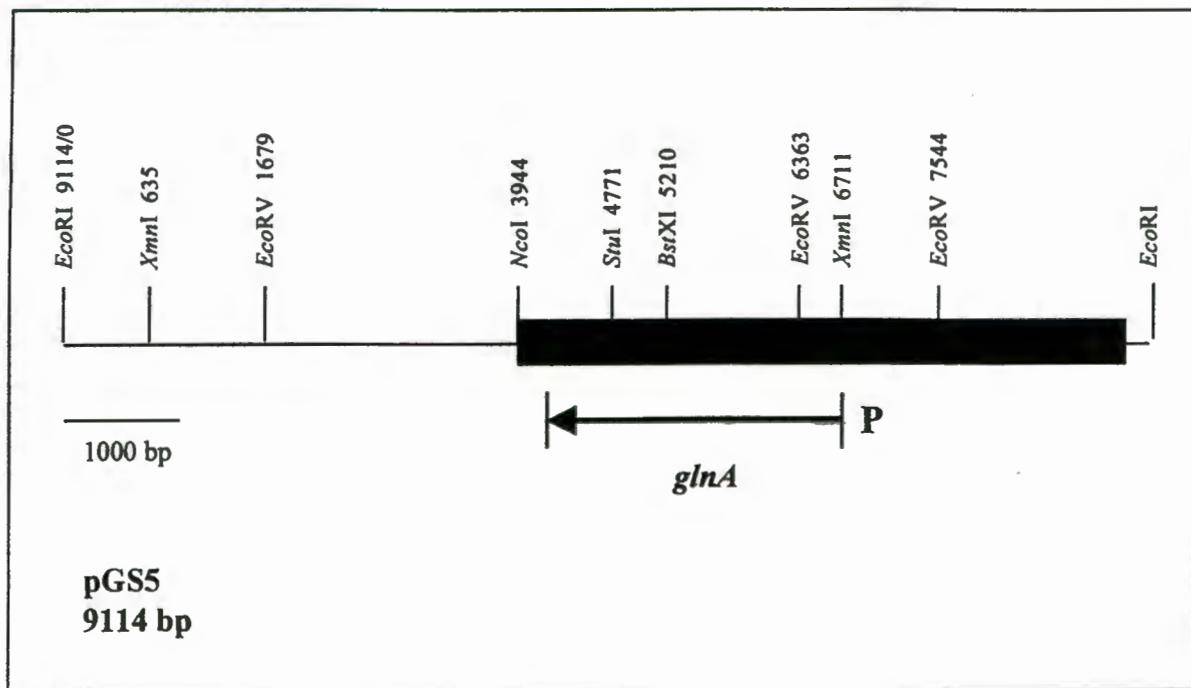
A600 nm	Units GS activity *
0.75	0.109 (0.02)
1	0.253 (0.033)
2.1	0.149 (0.005)

\* GS units,  $\gamma$ -glutamyltransferase  $\mu\text{M min}^{-1} \text{mg}^{-1}$  protein  
Average of 2 experiments shown with the standard deviation shown in brackets.

These results confirmed that the GS activity was present (Table 2.2) and that maximum GS levels ( $0.253 \pm 0.033$  units) were obtained at  $A_{600}$  nm equal to 1 at mid-exponential phase. All subsequent assays for GS activity in *E. coli* were therefore conducted at  $A_{600}$  nm of 1.

### 2.3.2 Cloning of a putative *B. fibrisolvens* GS regulatory gene

Transformation of the *B. fibrisolvens* pEB1 genomic library into *E. coli* YMC11 (pGS5) yielded 25 colonies which showed little or no growth on MMN media containing  $(\text{NH}_4)_2\text{SO}_4$  (15 mM) as the sole nitrogen source but which grew on MMG media supplemented with glutamine (15 mM) as well as on complete media. Of these 25 colonies, 4 showed pronounced growth inhibition on MMN and these were examined



**Fig 2.1** Partial restriction map of *B. fibrisolvans glnA* recombinant plasmid, pGS5. Thick arrow indicates the 2433 bp *glnA* gene. Filled block - insert DNA. Thin line - pACYC184 vector DNA. P - *glnA* promoter.

using growth curve analysis and GS assays. No colonies showing enhanced growth on MMN were isolated.

Analysis of these 4 colonies using GS assays yielded a single isolate (15) which showed a 2.5 to 3-fold elevation of GS activity (0.60±0.083 GS activity units) (Table 2.3) relative to the levels observed in *E. coli* YMC11 (pGS5) clones containing a control plasmid (pCM1, 0.253± 0.03 GS activity units). This was somewhat surprising as lower than basal GS activity levels were expected because of the slower growth rate on complete medium. The other isolates showed no marked difference as compared to the control levels of GS activity.

**Table 2.3.** GS levels of 4 isolates of *E. coli* YMC11 (pGS5) transformed with the pEB1 genebank.

<i>E. coli</i> YMC11 (pGS5) clones	Units GS activity*	Time taken (hr)
pCM1	0.25 (0.033)	4.5
15	0.60 (0.0833)	6.5
5	0.40 (0.069)	4.3
7	0.33 (0.025)	6.7
16	0.30 (0.02)	4.4

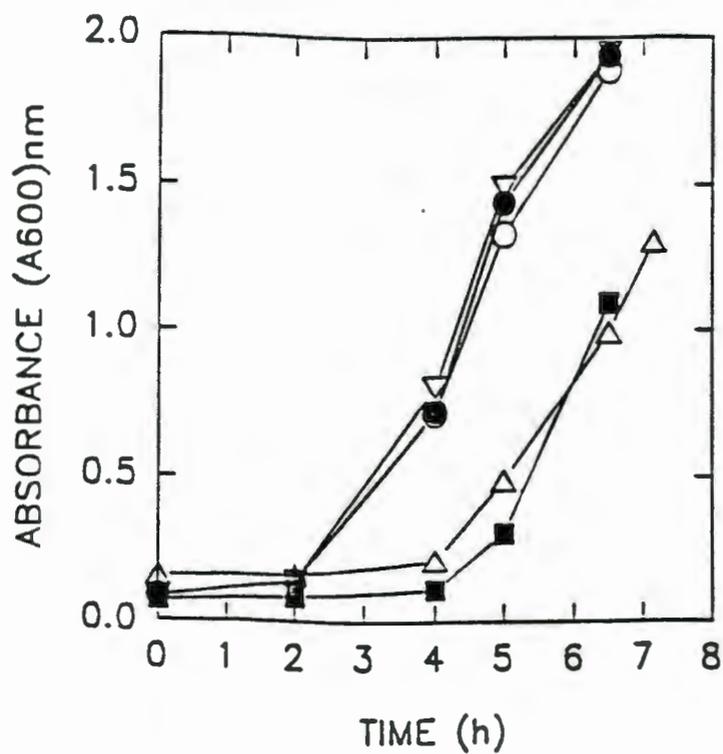
\* GS units,  $\gamma$  glutamyltransferase  $\mu\text{M min}^{-1} \text{mg}^{-1}$  protein

Average of 3 experiments shown (standard deviation in brackets).

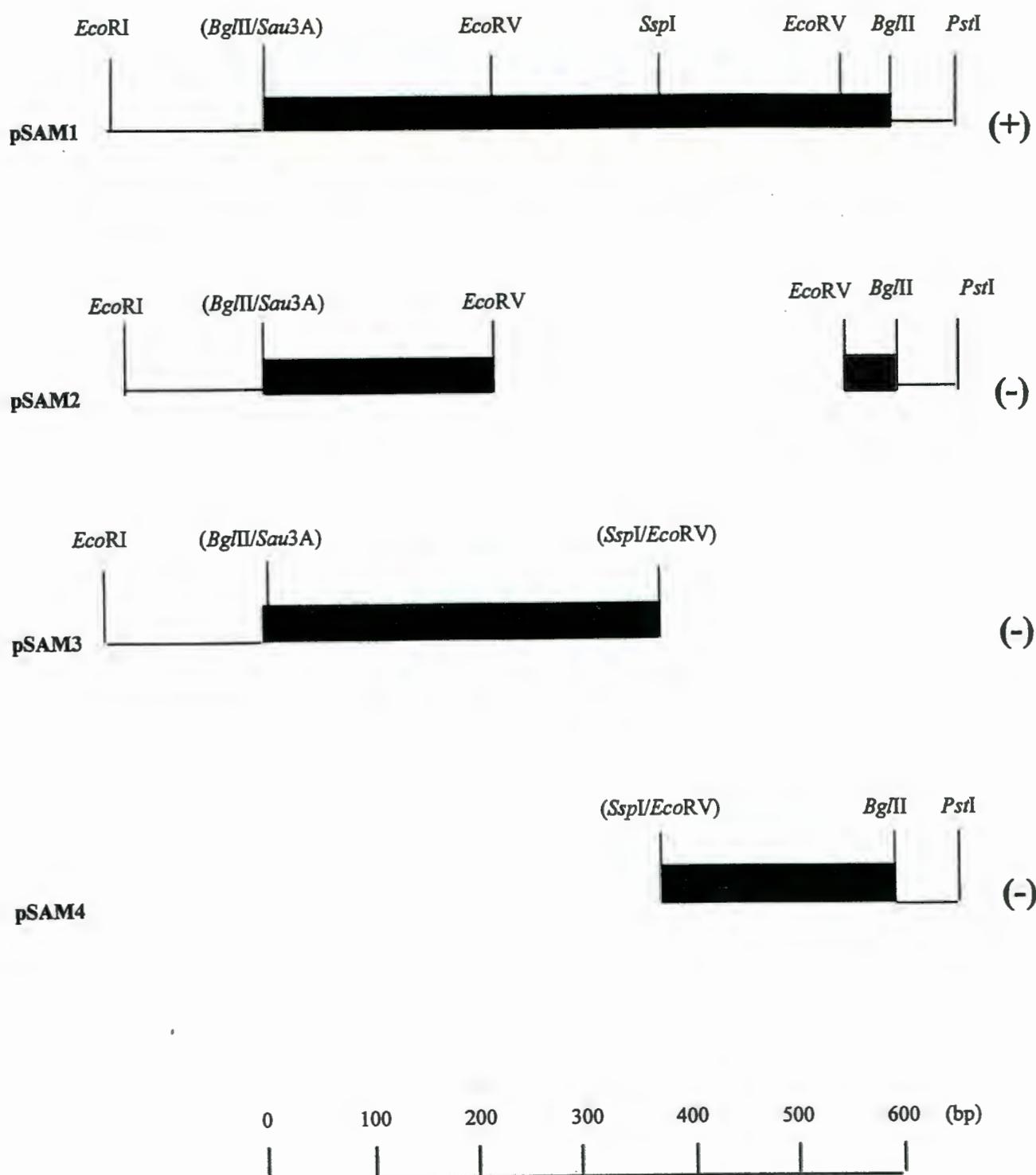
Isolate 15 also demonstrated a retarded growth rate in complete media relative to the growth of *E. coli* YMC11 (pGS5) containing the control plasmid, pCM1 (Fig.2.2).

Despite the fact that isolate 7 also showed a pronounced retardation of growth in 2YT relative to the control, pCM1, it did not show a concomitant elevation of GS activity levels as in the case of 15. Thus the growth retardation appeared to be unrelated to changes in the GS activity and isolate 7 was not examined further.

Segregation of the plasmids from isolate 15 to obtain the Ap<sup>R</sup> plasmid (and therefore the plasmid conferring the regulatory phenotype) yielded plasmid pSAM1.



**Fig. 2.2** Growth curve analysis of *E. coli* YMC11 (pGS5) cotransformed with a control plasmid, pCM1 (●) or with plasmids 5 (▽), 7 (Δ), 15 (■) and 16 (○). Cultures were grown in 2YT supplemented with Ap and Tc.



**Fig. 2.3** Restriction endonuclease maps of the pSAM1 recombinant plasmid and its deletion derivative, pSAM2. The restriction maps of pSAM3 and pSAM4, the subclones generated in KS are also shown. Filled blocks, *B. fibrisolvens* insert DNA; thin lines, pEB1 vector DNA. (+) indicates elevated GS activity levels. (-) indicates basal GS activity levels.

### 2.3.3. Characterization of pSAM1

#### 2.3.3.1. Restriction endonuclease analysis

Restriction enzyme analysis using single and double digests was used to obtain a partial restriction map of pSAM1 (Fig.2.3). This analysis established that a 0.58 kb insert was present and that the insert was cleaved by the *EcoRV* and *SspI* enzymes but not by any of the following enzymes:

*AccI*, *ApaI*, *BglI*, *BglII*, *ClaI*, *BstXI*, *BstYI*, *ClaI*, *HaeI*, *HindIII*, *HpaI*, *KpnI*, *NcoI*, *PstI*, *PvuI*, *PvuII*, *SacI*, *Sall*, *ScaI*, *StuI*, *StyI*, *XmnI*.

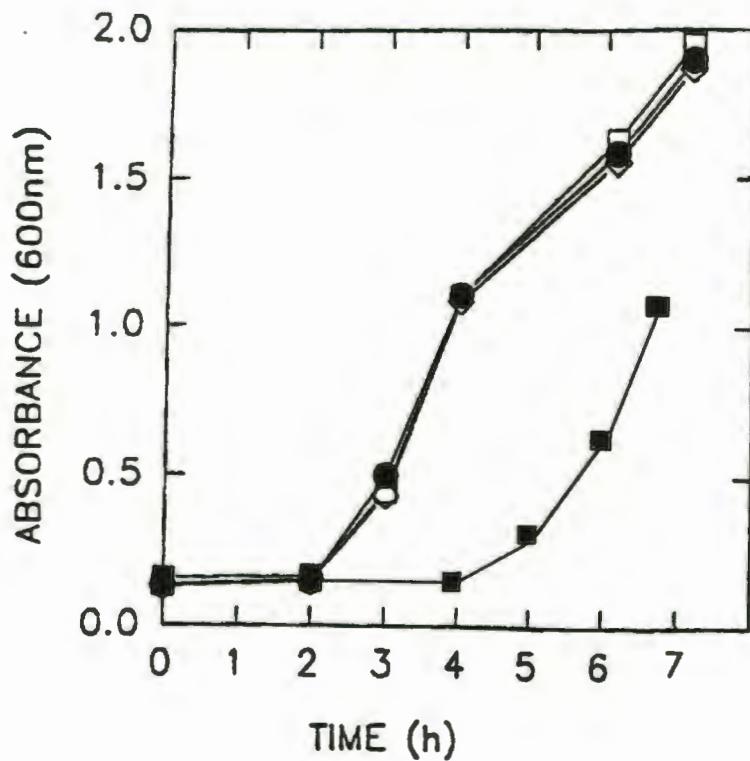
The presence of two *EcoRV* sites on the insert flanking a central 387 bp fragment was used to generate a deletion of pSAM1 designated as pSAM2 (Fig.2.3).

Two subclones viz. pSAM3 (*EcoRI-SspI* 0.8kb pSAM1 fragment) and pSAM4 (*PstI-SspI* 0.4 kb fragment pSAM1 fragment) were generated in pBluescript (Fig. 2.3)(Viera and Messing, 1982) for sequencing purposes and to determine if either of the subclones retained the phenotype.

#### 2.3.3.2. Effect of the cloned *B. fibrisolvens* DNA on *B. fibrisolvens* GS activity and cell growth in *E. coli*

*E. coli* YMC11(pGS5) clones retransformed with pSAM1 again showed a retardation of growth (Fig.2.4A) relative to *E. coli* YMC11 (pGS5) clones containing a control plasmid pCM1 (or pMT104).

This was no longer apparent with *E. coli* YMC11 (pGS5) clones transformed with the deletion plasmid, pSAM2 or with *E. coli* YMC11 transformed with pSAM1 alone. The retardation of growth was seen as a longer lag period of about one hour during early exponential phase of growth. Analysis of the *B. fibrisolvens* GS activity levels using  $\gamma$ -glutamyltransferase assays revealed that *E. coli* YMC11 (pGS5) clones containing pSAM1 showed a 2.5 to 3-fold elevation of GS levels relative to *E. coli* clones containing pCM1 (Table 2.4).



**Fig. 2.4A** Growth curve analysis of *E. coli* YMC11 (pGS5) co-transformed with a control plasmid, pCM1 (●) or with pSAM1 (■) and pSAM2 (□) compared with *E. coli* YMC11 (pACYC184) transformed with pSAM1 (∇). Cultures were grown in 2YT supplemented with Ap or Ap and Tc.

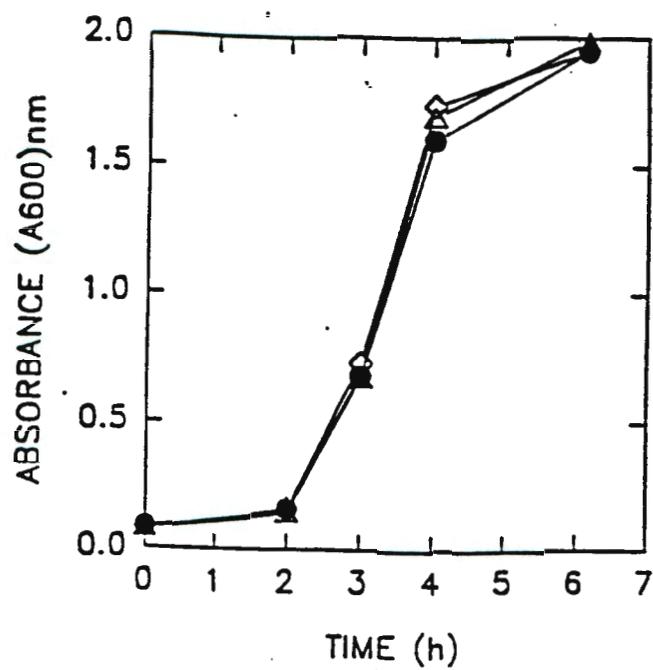
**Table 2.4** GS assays of *E. coli* YMC11 (pGS5) containing pSAM1, pSAM2, pSAM3 and pSAM4.

<i>E. coli</i> YMC11 (pGS5) clones	Units GS activity *
pCM1	0.25 (0.033)
pSAM1	0.60 (0.083)
pSAM2	0.29 (0.045)
pSAM3	0.27 (0.037)
pSAM4	0.30 (0.056)
pKS	0.30 (0.052)

\* GS units,  $\gamma$ -glutamyltransferase  $\mu\text{M min}^{-1} \text{mg}^{-1}$  protein

Average of 3 experiments (standard deviation shown in brackets).

GS values of 0.60 ( $\pm$  0.083) and 0.253 ( $\pm$  0.033) GS units were obtained for pSAM1 and pCM1 containing clones respectively. Deletion of a central 387 bp fragment of pSAM1 (to generate pSAM2) caused the loss of the retarded growth rate phenotype and the 2.5 to 3-fold elevation of GS activity levels. Repeated attempts to subclone the entire *B. fibrisolvens* pSAM1 insert DNA fragment onto the high copy number vector, pBluescript were unsuccessful (results not shown). The *B. fibrisolvens* pSAM1 insert DNA was, therefore, subcloned as two separate fragments onto pBluescript to generate pSAM3 and pSAM4 (Fig 2.3). *E. coli* YMC11 (pGS5) transformed with pSAM3 or pSAM4 no longer exhibited the elevated GS expression levels (Fig.2.4B; Table 2.4) and the retarded growth phenotype previously observed in the presence of pSAM1. No GS activity was observed for *E. coli* YMC11 (pACYC184) co-transformed with pSAM1.



**Fig. 2.4B** Growth curve analysis of *E. coli* YMC11 (pGS5) co-transformed with a control plasmid, pBluescript (●) or with pSAM3 (Δ) or pSAM4 (◇). Cultures were grown in 2YT supplemented with Ap and Tc.

### 2.3.3.3. Origin of DNA insert

Southern hybridization using the internal DIG-labelled 0.387kb *EcoRV* fragment as a probe confirmed that the insert carried on pSAM1 was *B. fibrisolvens* chromosomal DNA (Fig.2.5A & B).

Hybridization of the probe occurred to an approximately 0.4kb fragment of *B. fibrisolvens* chromosomal DNA (lane 5) and as expected to purified pSAM1 DNA (Lanes 3 and 4). Homology was not detected with *E. coli* HB101 chromosomal DNA (lane 6) or with *B. fragilis* chromosomal DNA (lane 7). Other clones which also showed a slight elevation of GS levels (Lanes 8, 9 & 10) (Table 2.3) also showed no homology to pSAM1.

No DNA homology was detected with a *B. fragilis* putative GS regulatory gene which had previously been cloned and characterized (Abratt and Woods, personally supplied).

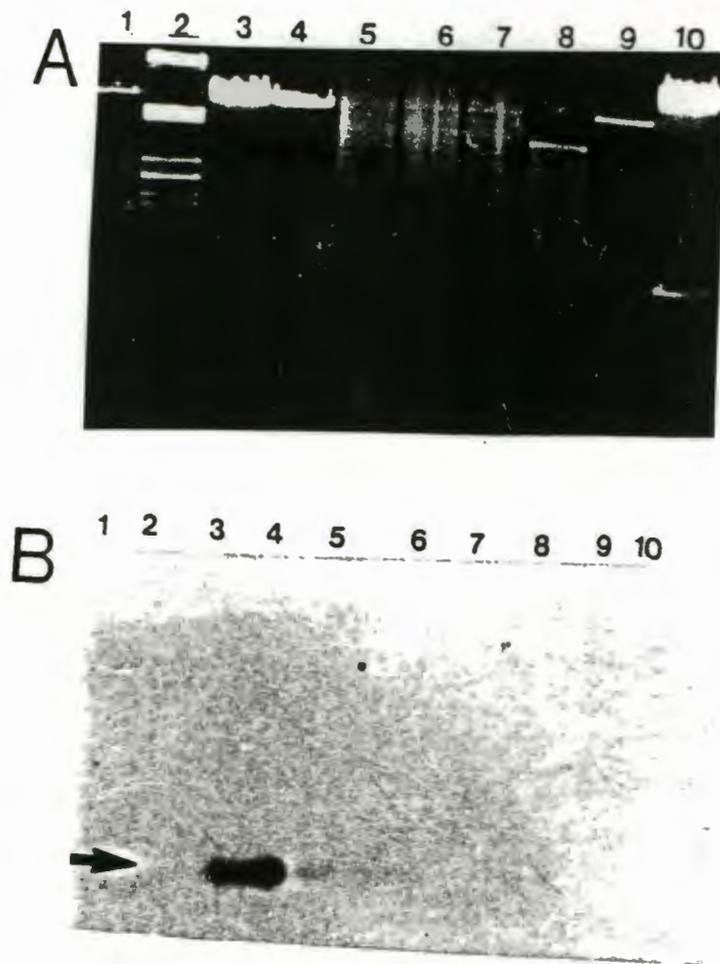
## 2.4 Discussion

GS activity assays and growth curve analysis were done on 50 ml samples of crude cell extract according to the method of Bender *et al.* (1977). However, a more recent assay method employed by Dominguez *et al.*(1977) for *in situ* assay of cyanobacterial GSIII allows stabilization of this GS by the presence of  $Mn^{2+}$  ions in the buffer and hence smaller, one ml sample volumes could have been used.

In this study, a *glnA<sup>-</sup> ntrA<sup>-</sup> ntrB<sup>-</sup>* *E. coli* strain (YMC11) transformed with the *B. fibrisolvens glnA* gene, and a pEB1 *B. fibrisolvens* gene bank was used to analyze the regulation of the *B. fibrisolvens glnA* gene expression in *E. coli* YMC11.

This *E. coli* strain is incapable of growth on media containing  $(NH_4)_2SO_4$  as the sole nitrogen source (MMN) unless a fully functional *glnA* gene is present or glutamine is provided in the growth medium (Backman *et al.*,1981). Transformation of this *glnA<sup>-</sup>* *E. coli* strain with the *B. fibrisolvens glnA* gene alleviates the auxotrophy and allows growth on MMN.

However, the addition of a putative regulatory gene *in trans* could cause increased or decreased glutamine synthetase gene expression for a positive or negative regulatory gene respectively. Thus the ability of *E. coli* YMC11 (pGS5) to grow on MMN would be affected by the degree of repression or activation of glutamine synthetase gene expression or activity caused by the putative regulatory gene.



**Fig. 2.5A** All samples were digested with *EcoRV* with the exception of the lambda marker.

Lane 1: pGSR1

Lane 2: lambda DNA digested with *PstI*.

Lane 3 & 4: 100 ng and 10 ng purified pSAM1.

Lane 5: *B. fibrisolvens* chromosomal DNA.

Lane 6: *B. fragilis* chromosomal DNA.

Lane 7: *E. coli* HB101 chromosomal DNA.

Lanes 8, 9, 10: Plasmid DNA from isolates 5, 7 and 16, respectively.

**Fig. 2.5B** Autoradiogram of a nylon membrane onto which the samples in Fig. 2.5A were transferred and probed with the 0.387 kb pSAM1 internal *EcoRV* fragment. Arrows and numbers indicate the size and position of the common band between the *B. fibrisolvens* chromosomal DNA and pSAM1.

The repression of *glnA* gene expression in *E. coli* YMC11 (pGS5) (and therefore heightened glutamine auxotrophy) by a putative regulatory gene would therefore be expected to cause decreased growth on MMN while enhancement of gene expression might be expected to cause increased growth on MMN.

However, analysis of the GS levels of *E. coli* YMC11 (pGS5) containing pSAM1, showed a 2.5 to 3-fold elevation of GS activity and a retarded growth rate in minimal and complete medium relative to the control.

The *B. fibrisolvens* insert DNA was involved in causing this elevation of GS activity levels as deletion of a central 387 bp *EcoRV* fragment resulted in the loss of the phenotype.

The insert DNA carried on pSAM1, on its own does not have GS activity and does not cause growth retardation of *E. coli* YMC11 suggesting that an interaction between the *B. fibrisolvens* GS and the insert DNA of pSAM1 causes the phenotype. The fact that the genetic element present on the pSAM1 *B. fibrisolvens* insert DNA could not be subcloned onto pKS (a high copy number plasmid) in its entirety suggests that a higher dosage of this genetic element may be lethal to the cell.

Previously, Abratt and Woods (personal communication) isolated a putative *B. fragilis* *glnA* regulatory gene which also caused an elevation of GS activity levels as well as a retarded growth phenotype in complete medium. Moreover, Goodman and Woods (1993) have shown that the *B. fibrisolvens* and *B. fragilis* *glnA* genes are homologous (40% amino acid homology). This raised the possibility that pGSR1 and pSAM1, which appeared to be functionally homologous, could be homologous at the DNA level. However, Southern hybridization analysis revealed that the pSAM1 insert DNA was not homologous to pGSR1 nor to any of the other clones isolated which also exhibited slightly elevated GS activity levels and similar growth retardation in complete media.

These results may be explained in a number of different ways.

i) It is possible that the pSAM1 *B. fibrisolvens* insert DNA could be causing an increase in the copy number of pGS5 leading to a higher number of copies of the *B. fibrisolvens* *glnA* gene being available for transcription by the *E. coli* transcriptional machinery. In the

absence of either the *E. coli glnL* and *glnG* regulatory genes or *B. fibrisolvans glnA* regulatory mechanisms, we might expect enhanced GS activity due to the presence of higher levels of GS protein. A possible mechanism for explaining the increased copy number of pGS5 is that the pSAM1 *B. fibrisolvans* insert DNA may code for a genetic element or protein which could be affecting the replication of pGS5. The increased burden on the cell due to the re-routing of essential cellular resources such as purines, pyrimidines, as well as amino acids to produce such copious amounts of pGS5 plasmid DNA as well as plasmid-encoded proteins, respectively, may be causing the slower-growth-rate phenotype observed in *E. coli* YMC11 cells containing the pSAM1 plasmid as well as pGS5 (Kurland and Dong, 1996).

ii) An alternative explanation is that the *B. fibrisolvans* insert DNA may code for a regulatory protein or regulatory element which functions directly or indirectly as an enhancer of *glnA* gene expression or enzyme activity. The product of the pSAM1 insert DNA may be binding directly to regions either upstream or downstream of the *B. fibrisolvans glnA* gene on pGS5 leading to enhancement of GS activity levels. Alternatively, it may be facilitating the binding of another as yet unidentified gene product with the same final result. However, subcloning of the *B. fibrisolvans glnA* gene removed much of the region upstream of this gene in pGS4, and as a consequence this region could not be implicated in causing the elevated GS activity levels. A 2.3 kb fragment of the downstream region of this gene was still present on pGS5, although the potential role of this region in causing the elevated GS activity levels was not investigated in this study.

iii) Blockage of systems involved in the uptake of ammonia or an essential amino acid such as glutamine in the surrounding medium by a protein or regulatory element on pSAM1 could also account for the observed phenotypes. This could be detected as an ammonia or glutamine shortage (even in complex media) by the *E. coli* signal transduction systems involved in detecting ammonia or glutamine levels in the environment. As a consequence, *E. coli* mechanisms allowing for the elevation of the heterologous *B. fibrisolvans* GS activity to compensate for the "apparent" lack of ammonia or glutamine may become functional. The retarded growth of *E. coli* YMC11 (pGS5) transformed with pSAM1 could result from overexpression of the GS leading to a depletion of glutamate (through incorporation of glutamate into glutamine by GS) thus

resulting in depletion of amino acid and pyrimidine pools (Kustu *et al.*, 1981).

Another way of assessing the effect of pSAM1 on the *B. fibrisolvens* GSIII would be to clone the *glnA* gene downstream of a strong promoter. We could then examine the effect of pSAM1 on this pGS plasmid. This experimental system would allow us to determine to what degree the pSAM1 insert DNA affects GS activity. A difficulty which may arise, however, is that the retarded growth phenotype associated with the presence of pSAM1 in *E. coli* YMC11 may prevent the growth of a strain carrying pSAM1 and the pGS plasmid. However, as Florencio (personal communication ) points out, *E. coli* strains lacking *glnA* which have been complemented with *glnA* from high copy number gene libraries can support this higher GS activity (Magasanik, 1987). Whether the *E. coli* system used in this study is capable of growth in the presence of such elevated GS activity levels is therefore questioned and represents the subject of future investigations.

In order to address the questions raised by these possibilities, further physiological analysis as well as protein and nucleotide sequencing studies were required and these are discussed in Chapters 3 and 4 respectively.

## Chapter 3

### Physiological analysis of pSAM1

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## Chapter 3

### Physiological analysis of pSAM1

#### Summary

The specificity of interaction between pSAM1 and *B. fibrisolvens* GS activity was examined using growth curve analysis and GS assays. The *B. fibrisolvens* insert DNA did not cause any change in basal GS activity levels of *E. coli* YMC10 and *B. fragilis glnA* genes nor was any retardation in growth observed for *E. coli* YMC11 co-transformed with the *B. fragilis glnA* gene and pSAM1. The interaction between the *B. fibrisolvens* GS and pSAM1 insert DNA, therefore appears to be specific for the *B. fibrisolvens* GS.

The effect of pSAM1 on the copy number of pEglA184 was also investigated using cellodextrinase assays. No differences could be detected between the levels of cellodextrinase observed in the presence or without pSAM1 in *E. coli* YMC11 (pEglA184) suggesting that pSAM1 insert DNA did not increase the copy number of a pACYC184-derived plasmid *in trans*.

The presence of pSAM1 insert DNA caused a 3-fold elevation of histidase levels in *E. coli* YMC11 compared with *E. coli* YMC11 containing pCM1, a control plasmid. This phenomenon was no longer observed when *E. coli* YMC11 was transformed with pSAM2, a deletion derivative of pSAM1. The presence of pSAM1 in *E. coli* YMC11 (pGS5) also caused a 2-fold elevation of *B. fibrisolvens glnA* mRNA levels concomitantly with the 2.5- to 3-fold elevation of GS activity levels.

#### 3.1 Introduction

The aim of this section of the study was to determine the level at which the pSAM1 insert DNA interacted with the *B. fibrisolvens* GS to cause the elevated GS activity levels and retarded growth phenotype. Several possible mechanisms were proposed in section 2.4. These are addressed below.

##### 3.1.1 Increase in copy number of pGS5

The elevated GS activity could be due to increased copy number of pGS5 and therefore an increase in the copies of the *B. fibrisolvens glnA* gene and its product, GS. The pSAM1 insert DNA may code for a protein or genetic element that affects the frequency of DNA replication initiation of pGS5 leading to an increased copy number of this plasmid. Previously, Thomas

*et al.*(1984) reported the presence of genetic elements, *copA* and *copB* that controlled the copy number of a broad host range plasmid RK2 plasmid. These genetic elements appeared to affect the copy number of pRK2 *in trans*. However, the mechanism of action of these genetic elements remains to be elucidated.

### 3.1.2 Increased transcription of the *B. fibrisolvens glnA* gene

An alternate mode of action of the pSAM1 gene product could be to increase the levels of transcription of the *glnA* gene.

The pSAM1 insert DNA may code for a *trans*-acting regulatory protein that could bind to a particular DNA site (*cis*-acting site eg. an operator site) or assist another protein to be able to do so, leading to increased transcription of the *B. fibrisolvens glnA* gene.

In *E. coli*, *Klebsiella aerogenes* and *Salmonella typhimurium* the *ntrA*, *ntrB* and *ntrC* genes are known to affect the regulation of *glnA* gene expression at the level of transcription (see Chapter 1).

The synthesis of many enzymes capable of supplying the cells with ammonia can also be increased by nitrogen deficiency (Magasanik, 1982). These enzymes, such as histidase, are subject to nitrogen regulation and exhibit the Ntr<sup>+</sup> phenotype. The activation of histidase expression requires neither CAP nor cAMP (Prival and Magasanik, 1971). In *K. aerogenes*, histidine can serve as a sole nitrogen or carbon source when it is enzymatically degraded. The histidine utilisation genes (*hut* genes) (Fig. 3.1) are arranged in two tandem operons, which encode the *hut(M)*, *IGC*, *(P)U* and *H* genes. HutIGU and H are four enzymes in the degradative pathway while HutC encodes a repressor that regulates both operons.

*hut(M)* and *hut(P)* are sequences that control the expression of the left and right operons (Nieuwkoop *et al.*, 1988). High level expression of the *hut* operons requires inactivation of the repressor HutC, and also positive activation of the promoter regions. During carbon or energy limitation, the CAP-cAMP complex functions as the activating signal (Nieuwkoop and Bender, 1988). Interestingly, the *hut* genes are not directly controlled by NtrC as Nieuwkoop *et al.* (1988) found that the *hutUH* operon initiated from a  $\sigma_{70}$ -specific promoter and not a  $\sigma_{54}$ -specific promoter implying an indirect effect of the *ntr*-system on the *hut* genes. Earlier studies (reviewed by Magasanik, 1982) had indicated that mutations in genes affecting GS activity also affected histidase activity in the same way. More recent studies (Henikoff *et al.*, 1988; Weiss *et al.*, 1992; Bender, 1991) have indicated the requirement for the NAC protein

(as discussed in Chapter 1) for activation of the *hut* genes verifying the indirect role of NtrC. Since expression of histidase is a convenient test for the Ntr phenotype, much of the work with *E. coli* has been done with strains such as *E. coli* YMC10 and YMC11 into which a *hut* operon with a defective *Klebsiella hutC* gene had been transduced (Tuli *et al.*, 1982).

Since the pSAM1 insert DNA causes an elevation of the *B. fibrisolvans* GS activity levels and a retardation of growth in complete media (2YT), it was important to establish the effect of pSAM1 insert DNA on the mRNA levels of the *B. fibrisolvans glnA* gene as well as on the histidase levels of the *Klebsiella hut* genes in *E. coli* YMC11.

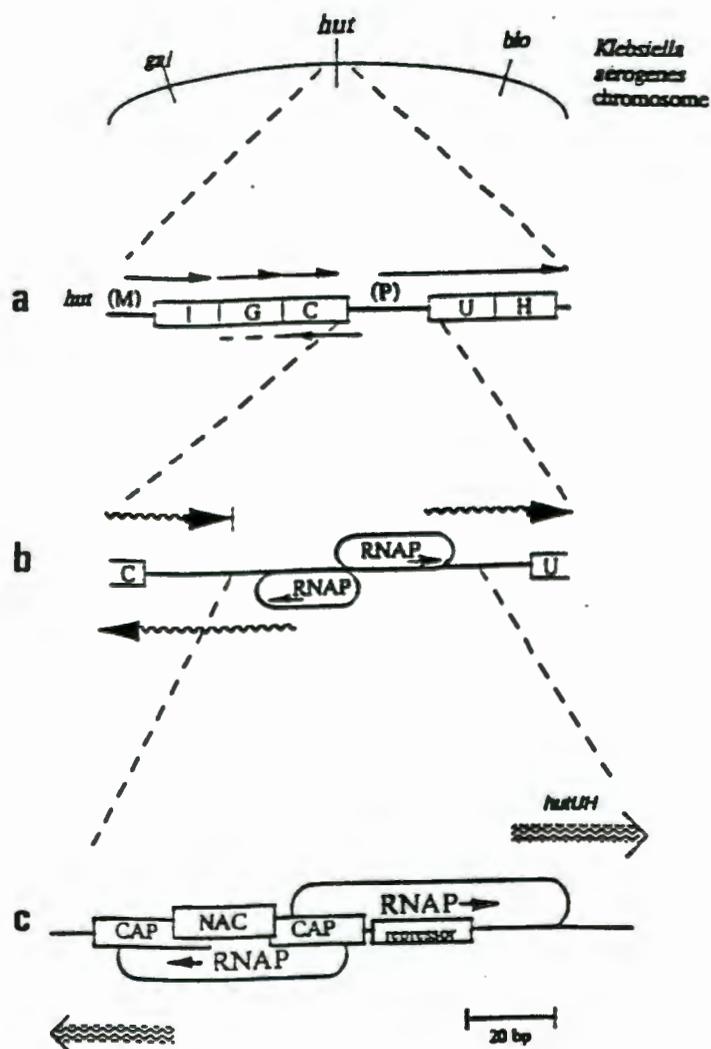
In Chapter 2 it was established that the presence of both the *B. fibrisolvans glnA* gene and the pSAM1 insert DNA was required for the elevation of GS activity levels and retarded growth phenotype. It was therefore necessary to determine if this interaction was specific for the *B. fibrisolvans* GS or if it could be extended to other glutamine synthetase genes such as those of *E. coli* and *B. fragilis*.

## 3.2 Materials and Methods

Standard materials and methods are recorded and referenced in the Appendix.

### 3.2.1 Bacterial strains and plasmids used

*E. coli* strains YMC10 and YMC11 carrying the appropriate plasmids, were used to prepare cell extracts for glutamine synthetase (GS), histidase and cellodextrinase assays. *E. coli* JM105 was used for the extraction and preparation of large quantities of purified recombinant DNA molecules. The genotypes of these *E. coli* strains and the details of the plasmids used are indicated in Table 2.1 in Chapter 2. Plasmid pHZ117 is a pEcoR251 derived, Ap<sup>R</sup> plasmid containing the *Clostridium acetobutylicum* endoglucanase gene (Zappe *et al.*, 1988), plasmid pACYC-GS is a pACYC184 derived, chloramphenicol resistant plasmid containing the *B. fragilis* GS (supplied by Abratt) and plasmid pMT104 is a pEcoR251 derived, Ap<sup>R</sup> plasmid with a stuffer fragment disrupting the EcoRI gene (Wehnert *et al.*, 1990).



**Fig. 3.1** (a) Location of *hut* genes of the *K. aerogenes* *hut* operon. The transcriptional units are indicated by arrows.

b) The direction of transcription of the *HuUH* operon, and an overlapping promoter of unknown function and reading in the opposite direction are shown.

c) The location of the known binding sites within the *hutUH* promoter region are shown. (Nieuwkoop and Bender, 1988).

### 3.2.2 Media, buffers and growth conditions

All media buffers and solutions not described in the text are listed in the Appendix. Growth conditions for *E. coli* were described in Chapter 2.

### 3.2.3 GS assays and growth curve analysis

Total GS activity was assayed using crude cell extracts according to the method outlined in section 2.2.4.

The following strains were transformed with either pSAM1 or pCM1 and assayed for GS activity and growth rate; *E. coli* YMC10 (pACYC184), *E. coli* YMC11 (pGS5), and *E. coli* YMC11 (pACYC-GS). Protein concentrations were determined according to the method of Bradford (1976). One unit of GS activity is defined as the number of  $\mu$ moles of  $\gamma$ -glutamylhydroxamate formed per min per mg of protein. Growth of the *E. coli* cultures was analysed according to the method outlined in section 2.2.7.

### 3.2.4 Determination of the effect of pSAM1 on the copy number of pEglA184.

#### 3.2.4.1 Subcloning of the *Clostridium acetobutylicum* $\beta$ 1-4 endoglucanase onto pACYC184

Plasmid pHZ117 (Zappe *et al.*, 1988) containing a *C. acetobutylicum* endoglucanase gene, which also showed cellodextrinase activity, was digested with *Pst*I and blunt-ended using DNA polymerase I (Klenow fragment). The DNA was then digested with *Eco*RI and the 1.4 kb fragment containing the endoglucanase gene was gel-purified using low melting point agarose (Seaplaque) (1%). This fragment was then ligated into the *Eco*RI-*Sca*I sites of pACYC184 (Chang and Cohen, 1978) using T4 DNA ligase (Appendix) to generate pEglA184.

This construct was transformed into competent *E. coli* JM105 cells (Armstrong *et al.*, 1984) and transformants were selected for on 2YT plates (Fig. 3.3) containing medium-viscosity CMC (0.5% w/v) (Sigma no. C4888) and Tc (20  $\mu$ g/ml). Detection of CMCase activity was by means of the Congo Red staining method (Zappe *et al.*, 1988). Partial restriction enzyme mapping confirmed the subcloning of the *C. acetobutylicum* endoglucanase gene into pACYC184.

### 3.2.4.2 p-Nitrophenyl cellobioside assay

Cell extracts of *E. coli* YMC11 (pEglA184) cotransformed with either pSAM1 or pCM1 were prepared as described in section 2.2.4. The cells were washed once in phosphate-citrate (P-C) buffer (Appendix) as specified in section 2.2.4 and resuspended in 1ml of PC buffer.

The cells were disrupted by sonication (one minute bursts of sonication with 30s rest on ice) using a Soniprep 150 sonicator (MSE LTD). The samples were then centrifuged at 14 000 rpm in an Eppendorf microfuge for 15 min at 4°C. Samples were assayed immediately for p-Nitrophenyl cellobiosidase (pNPC) activity.

Samples (250  $\mu$ l) were mixed in a 1:1 ratio with pNPC stock solution (Appendix). The mixture was incubated at 50°C for 30 min. Five hundred  $\mu$ l of 14% Na<sub>2</sub>CO<sub>3</sub> and 1ml dH<sub>2</sub>O was added to the mixture which was centrifuged at 14 000 rpm for 5 min to remove the flocculant precipitate. The pNPC activity was determined spectrophotometrically at OD 405 nm using a Du-64 spectrophotometer (Beckman Instruments). The blank sample contained P-C buffer instead of sample extract. One unit of activity was defined as the amount of enzyme that liberated 1  $\mu$ mole p-Nitrophenyl equivalents in 1 min and specific activity was defined as units/mg protein (Deshpande *et al.*, 1984). Proteins were determined by the dye-binding method of Bradford (1976).

### 3.2.5 Histidase assays

#### 3.2.5.1 Preparation of cell extracts

Competent *E. coli* YMC11 (Armstrong *et al.*, 1984) was transformed with plasmids pCM1, pSAM1 or pSAM2. Transformants were selected on 2YT containing Ap. These *E. coli* cultures were inoculated into 25 ml 2YT containing Ap and grown at 37°C with vigorous aeration. *E. coli* cell extracts were prepared as described in section 2.2.4. The cells were well mixed with 0.5 ml toluene to permeabilize them. The cells were collected by centrifugation at 6000 rpm for 10 min, resuspended in 0.2 ml 0.15M NaCl and assayed immediately.

Histidase activity was assayed using the method of Smith *et al.* (1971). A sample (20  $\mu$ l) was mixed with 100  $\mu$ l 1M diethanolamine/HCl buffer (pH 9.4), 10  $\mu$ l commercial 0.5 M reduced glutathione (Sigma) in 0.1 M potassium phosphate buffer (pH 7.4), and 550  $\mu$ l distilled H<sub>2</sub>O. After 5 min incubation at 37°C, 100  $\mu$ l 0.1 M histidine was added and mixed. Incubation at 37°C was then continued for 15 min. The reaction was stopped by the addition of 1 ml saturated sodium tetraborate solution. The absorbance at 277 nm (A<sub>277</sub>) was determined and

the specific activity was expressed as A277 (mg protein)<sup>-1</sup> (arbitrary units). Proteins were determined according to the method of Bradford (1976).

### 3.2.6 RNA analysis

#### 3.2.6.1 Preparation of *E. coli* YMC11 for RNA extraction and GS assays

*E. coli* YMC11 (pGS5) cell extracts containing either pSAM1 or pCM1 and *E. coli* YMC11 were prepared as described previously (section 2.2.4)

Half of the samples were assayed for GS activity as outlined in section 2.2.4 in Chapter 2. The other samples were used for total RNA extractions by a modification of the method of Aiba *et al.* (1981). All solutions were prepared using Millipore water which had been autoclaved three times and was used exclusively for RNA work. Freshly autoclaved tips and eppendorfs were always used. *E. coli* growing in culture (25 ml) was poured into an SS34 centrifuge tube and the cells harvested at 5000 rpm for 10 min at 4°C in a JA-20 rotor. The bacterial pellets were then resuspended in 125 µl of ice-cold 0.3 M sucrose, 0.01 M sodium acetate (pH 4.5) and transferred to a 1.5 ml eppendorf for storage at -20°C. The cell pellets were then thawed and 125 µl 0.01 M sodium acetate (pH 4.5), 2% SDS was then added. This suspension was then heated in a 65°C water bath for 1.5 min, following which 250 µl hot phenol (heated to 65°C) was added. After being vortexed briefly, the samples were heated to 65°C for 3 min and cooled at -70°C for 45 sec followed by centrifugation in an Eppendorf microfuge at 14 000 rpm for 10 min. The upper aqueous layer was removed and subjected to a further two cycles of hot phenol extraction. The RNA was precipitated by the addition of 3M sodium acetate (10% of volume) and 96% ethanol (2 volumes). After being kept on ice for 10 min, samples were centrifuged at 4°C for 10 min and the pellets washed with 70% ethanol. The pellets were then partially dried and dissolved in 180 µl RNase free water. To remove any DNA contaminants, 20 µl 10X DNase buffer (Appendix) was added as well as 30u RNase-free DNase (Boehringer Mannheim). DNA was allowed to digest for one hour at room temperature (to prevent the action of RNases at 37°C). The DNase was inactivated by the addition of 20 µl 250 mM EDTA followed by extraction with phenol (10% of volume) and 1 volume of chloroform-isoamyl alcohol (24:1). Ethanol precipitation was then performed on the pellets that were then resuspended in 100 µl of RNA storage buffer.

### 3.2.6.2 RNA concentration and quality determination

RNA samples were diluted 1/20 in sterile, RNase free MilliQ water and the quantity of RNA present was measured by determining the absorbance peaks obtained between 220 and 310 nm on a Beckman Du-64 spectrophotometer (Beckman instruments) and on a Genequant (Promega Scientific) system specifically designed for measuring nucleic acid concentrations. Concentration of RNA present was calculated using the relationship  $A_{260\text{ nm}} = 1$  (for 40  $\mu\text{g/ml}$  RNA). The quality of the RNA was determined by agarose gel electrophoresis.

Gels containing formaldehyde were prepared in a fume hood according to the method of Sambrook *et al.* (1989). Gels were stained with EtBr for 30 min after electrophoresis. Gel trays, combs and tanks were rendered RNase-free by treatment with hydrogen peroxide. Electrophoresis took place at 30 V for 6-7 hours.

### 3.2.6.3 Preparation of pGS5 DNA probe

Large scale plasmid extraction and purification allowed the isolation of purified pGS5 plasmid DNA from *E. coli* JM105. This plasmid was then digested with *EcoRI* (Sambrook *et al.*, 1989) to linearize the plasmid.

The plasmid was then labelled by random primed incorporation of digoxigenin-labelled dUTP according to the manufacturers instructions (Boehringer Mannheim).

### 3.2.6.4 Dotblotting and Hybridization

Amersham Hybond N+ membranes were used and the manufacturers instructions were followed for pre-treatment of the RNA samples. A commercial vacuum dotblotting apparatus (Omeg Scientific) was used to blot the samples (5, 10 and 20  $\mu\text{g}$  quantities) onto the membrane according to the method of Sambrook *et al.* (1989). Prehybridisation, hybridisation and washing conditions were according to the manufacturers instructions (Boehringer Mannheim). Agfa curix films were used for autoradiography. The intensities of the dots produced on an X-ray autoradiograph were then measured using a GS300 Transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco). The data was analyzed using the HSPI Windows GS365W (version 3.01) electrophoresis Data reduction system (Hoefer Scientific Instruments) which allowed the intensity of the RNA dots to be quantitated via Gaussian integration analysis.

### 3.3 Results

#### 3.3.1. Analysis of the specificity of the pSAM1 *B. fibrisolvens* insert DNA for the *B. fibrisolvens* GS

No growth retardation or elevation of *B. fragilis* GS activity levels was observed when *E. coli* YMC11 (pACYC-GS) was transformed with pSAM1. GS values of 0.14 (+ 0.02) units and 0.17 (+ 0.004) units were obtained for *E. coli* YMC11 (pACYC-GS) containing pSAM1 or pMT104 respectively (Table 3.1; Fig. 3.2). *E. coli* YMC10 also showed no elevation of GS activity levels and no retardation of growth in the presence of pSAM1 *in trans*, the GS levels being 0.10 (+ 0.020) units and 0.097 (+ 0.009) units for the experimental and control samples, respectively.

**Table 3.1** GS assays of *E. coli* YMC10 transformed with pSAM1 or pCM1 (a control plasmid) and pACYC184, *E. coli* YMC11 (pGS5) transformed with pCM1 or pSAM1 and *E. coli* YMC11 (pACYC-GS) transformed with pSAM1 or pCM1.

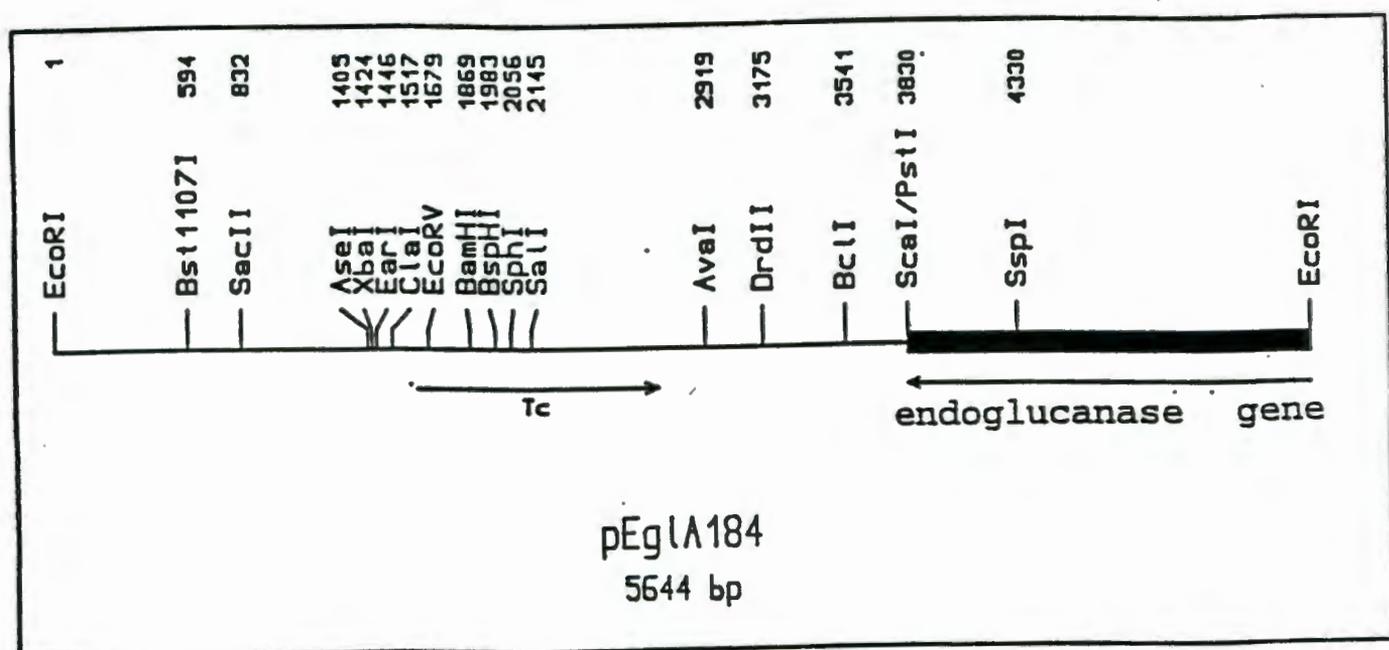
<i>E. coli</i> strain	Units GS activity *
YMC11 (pACYC-GS)(pSAM1)	0.14 (0.02)
YMC11 (pACYC-GS)(pMT104)	0.17 (0.004)
YMC11 (pGS5)(pSAM1)	0.60 (0.083)
YMC11 (pGS5)(pCM1)	0.25 (0.033)
YMC10 (pACYC184)(pSAM1)	0.10 (0.020)
YMC10 (pACYC184) (pCM1)	0.10 (0.009)

\*GS units,  $\gamma$ -glutamyltransferase  $\mu\text{moles min}^{-1} \text{mg}^{-1}$  protein

Mean of 5 experiments (standard deviation in brackets)

### 3.3.2 Determination of the effect of the pSAM1 insert DNA on the copy number of pEglA184.

The endoglucanase gene of *Clostridium acetobutylicum* (Zappe *et al.*, 1988) was subcloned onto the low copy number plasmid pACYC184 (Chang and Cohen, 1978). Restriction endonuclease digests verified the subcloning (Fig.3.3) of the entire *C. acetobutylicum* endoglucanase gene into pACYC184.



**Fig. 3.3** Partial restriction enzyme map of pEglA184. The arrows indicate the direction of transcription of the Tc and the *C. acetobutylicum* endoglucanase genes. Thin line -plasmid pACYC184.

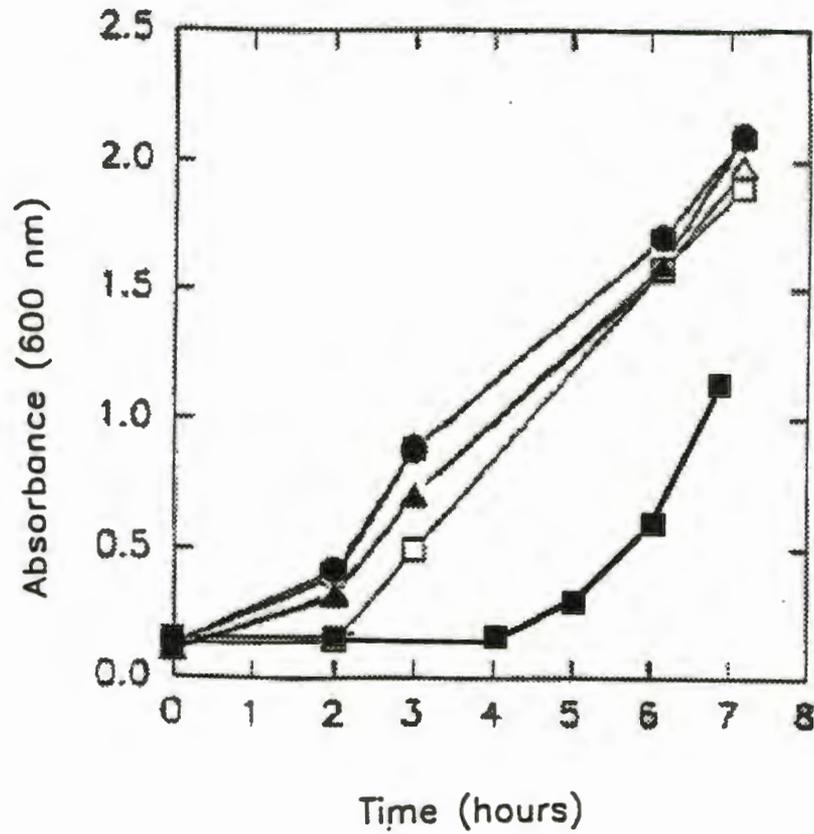
Thick line - *C. acetobutylicum* endoglucanase gene.

The functionality and presence of the endoglucanase gene was confirmed by the detection of halos after growth of *E. coli* JM105 on 2YT plates (Fig. 3.4) (1.5% agar) containing 0.5% carboxymethylcellulose (CMC) and Congo Red staining.

The effect of pSAM1 on the copy number of pEglA184 in *E. coli* YMC11 was then examined using the p-Nitrophenol- $\beta$ -D-cellobioside (pNPC) hydrolysis assays. One unit of activity is defined as the amount of enzyme that liberated 1  $\mu$ mole p-Nitrophenyl equivalents in 1 min and specific activity was defined as units/mg protein (Deshpande *et al.*, 1984). Equivalent amounts of cellodextrinase activity was obtained for both the control (pCM1/pEglA184) and experimental (pSAM1/pEglA184) samples viz. 0.11 (+0.004) and 0.14 (+0.004) units, respectively. (The mean of 3 experiments is shown with standard deviation in brackets).



**Fig. 3.4** Yeast tryptone agar plate containing 0.5% CMC and Tc showing the halos formed by *E. coli* JM105 transformed with pEglA184.



**Fig.3.2** Growth curve analysis of the *E. coli* strains carrying the *E. coli*, *B. fragilis* and *B. fibrisolvens glnA* genes co-transformed with pSAM1 or control plasmids.

Samples were grown in 2YT supplemented with Ap and Tc.

- E. coli* YMC10 (pACYC184)(pSAM1) -●
- E. coli* YMC10 (pACYC184)(pCM1) -○
- E. coli* YMC11 (pGS5)(pSAM1) -■
- E. coli* YMC11 (pGS5)(pCM1) -□
- E. coli* YMC11 (pACYC-GS)(pSAM1) -▲
- E. coli* YMC11 (pACYC-GS)(pMT104) -△

### 3.3.3 Analysis of the effect of pSAM1 on the Histidase levels of *E. coli* YMC11.

The aim of this section of the work was to determine if pSAM1 insert DNA affected the histidase levels (Table 3.3) of the *Klebsiella aerogenes hut* operon in *E. coli*.

**Table 3.2** Histidase activity of *E. coli* YMC11 transformed with pCM1, pSAM1 or pSAM2.

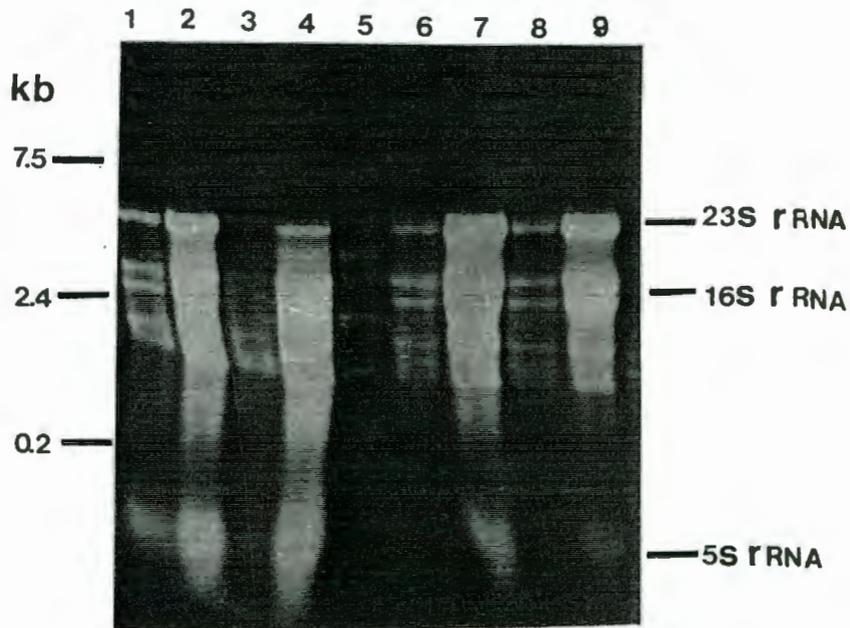
<i>E. coli</i> YMC11	Histidase activity *
YMC11	6.4 (0.08)
YMC11 (pSAM1)	19.1 (1.31)
YMC11 (pCM1)	7.5 (0.45)
YMC11 (pSAM2)	5.1 (0.94)

\* Histidase activity is defined as the  $A_{277} \cdot 15 \text{ min}^{-1} \text{ mg}^{-1}$  protein  
Mean of 3 experiments (standard deviation in brackets).

The histidase activity reported here for *E. coli* YMC11 (6.4  $\pm$  0.075 units) is in good agreement with the histidase activities found by Southern *et al.*, (1986) who obtained a value of 6.35 units for this strain under high nitrogen conditions (0.15 mM glutamine, 15 mM glutamate). The histidase activities obtained for *E. coli* YMC11 (pSAM2) and *E. coli* YMC11 (pCM1) were 5.1 ( $\pm$  0.94) units and 7.5( $\pm$  0.453) units respectively and were approximately equivalent to the basal levels obtained for *E. coli* YMC11. However, *E. coli* YMC11(pSAM1) showed histidase activity of 19.125 ( $\pm$  1.312) units, 2.5- and 3-fold in excess of the values found for *E. coli* YMC11 (pCM1) and *E. coli* YMC11, respectively.

### 3.3.4 Analysis of the effect of pSAM1 on the levels of the *B. fibrisolvens* GS mRNA levels using RNA dotblotting analysis with plasmid pGS5 as probe.

The aim of these experiments was to determine the effect of the pSAM1 insert DNA on the levels of *B. fibrisolvens* GS mRNA levels in *E. coli* YMC11 (pGS5). Total RNA extracted from *E. coli* YMC11, *E. coli* YMC11 (pGS5)(pCM1) and *E. coli* YMC11 (pGS5)(pSAM1) was electrophoresed on formaldehyde gels to test the quality of the RNA extracted (Fig.3.5) The levels of *B. fibrisolvens* GS mRNA as determined by RNA dotblotting analysis (and the determination of the intensity of these dots using a densitometer) were compared (Table 3.3,



**Fig. 3.5** Electrophoretic analysis of total RNA from *E. coli* YMC11.

Lanes 1, 2, - *E. coli* YMC11 total RNA

Lanes 3, 4, - *E. coli* YMC11 (pGS5) total RNA

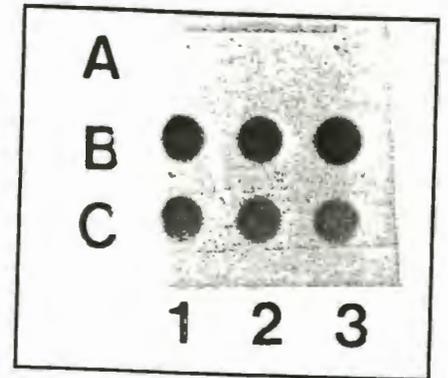
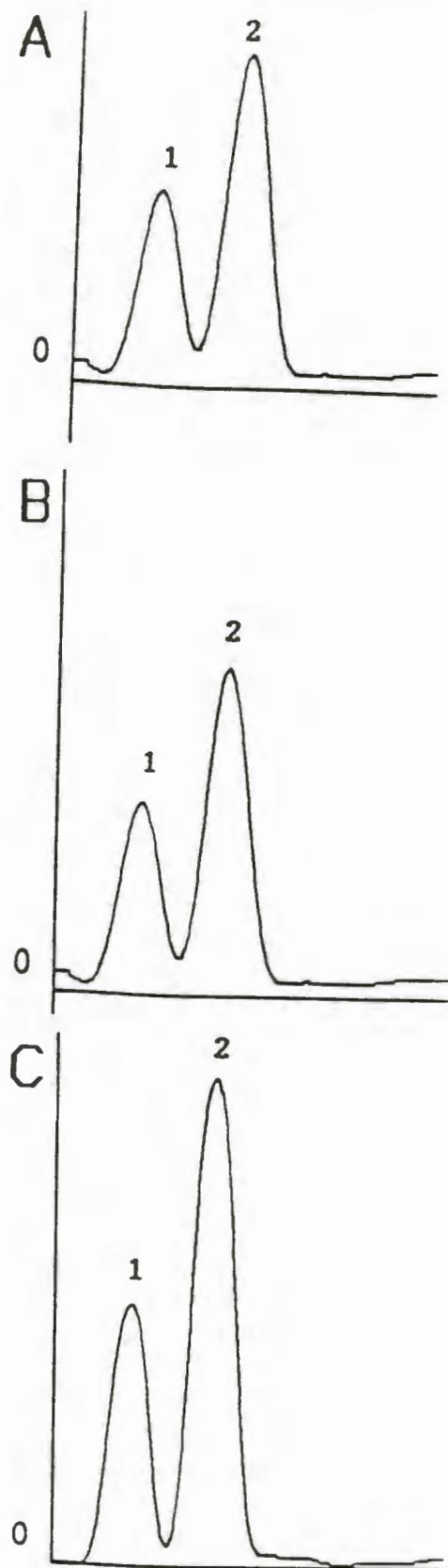
Lane 5 - RNA molecular weight markers

Lane 6, 7, - *E. coli*. YMC11 (pGS5)(pSAM1)

Lane 8, 9, - *E. coli*. YMC11 (pGS5)(pCM1)

Lanes 1, 3, 6 and 8 contain 5  $\mu$ g total RNA.

Lanes 2, 4, 7 and 9 contains 10  $\mu$ g total RNA.



**Fig. 3.6D** A1, A2, A3 - 5, 10, 20  $\mu\text{g}$  *E. coli*  
 YMC11 total RNA.  
 B1, B2, B3 - 5, 10, 20  $\mu\text{g}$  *E. coli*  
 YMC11 (pGS5)(pSAM1) total RNA.  
 C1, C2, C3 - 5, 10, 20  $\mu\text{g}$  *E. coli*  
 YMC11 (pGS5)(pCM1) total RNA.

**Fig. 3.6 A, B and C.** Densitometer scans of the intensity of the dots produced on Agfa curix film illustrated as the area under a gaussian peak for the 5  $\mu\text{g}$  (A), 10  $\mu\text{g}$  (B) and 20  $\mu\text{g}$  (C) samples of total RNA.

1, *E. coli* YMC11 (pGS5)(pCM1); 2, *E. coli* YMC11 (pGS5)(pSAM1)

**Table 3.4** GS mRNA levels obtained using dotblotting analysis for each of the 5, 10 and 20  $\mu\text{g}$  concentrations of total mRNA used.

Total RNA concentration	% Absorbance *		ratio **
	pCM1	pSAM1	
5 $\mu\text{g}$	34	66.0	1.9
10 $\mu\text{g}$	38	61.8	1.6
20 $\mu\text{g}$	36	64.0	1.7

\* % Absorbance (arbitrary units) of *E. coli* (pGS5) transformed with pSAM1 or pCM1 is proportional to the intensity of the dot relative to a clear area on the autoradiograph that was used to calibrate the densitometer. The intensity of the dot is proportional to the amount of mRNA produced for each concentration of total RNA used.

\*\* (pSAM1/pCM1)

No signal was obtained on the autoradiograph for each of the 5, 10 and 20  $\mu\text{g}$  concentrations of *E. coli* YMC11 total RNA.

*E. coli* YMC11 (pGS5) transformed with pSAM1 showed an approximately 2-fold elevation of *glnA* mRNA levels for each concentration of total RNA used relative to the *B. fibrisolvens* *glnA* mRNA levels obtained for *E. coli* YMC11 (pGS5) transformed with a control plasmid, pCM1. Induction ratios of 1.9, 1.6 and 1.7 were obtained for each of the 5, 10 and 20  $\mu\text{g}$  concentrations of total mRNA used.

### 3.4 Discussion

In Chapter 2 it was established that the presence of the pSAM1 insert DNA caused a 2.5-3-fold elevation of GS activity levels coupled with a retardation in the growth rate. The fact that it was the insert DNA and not an artefact of the two plasmid, *in trans* system was

established by the fact that the pSAM1 insert DNA did not by itself cause retardation of growth when transformed into *E. coli* YMC11. In addition, a deletion derivative of pSAM1, pSAM2, which had a central region of the *B. fibrisolvans* insert DNA deleted did not reproduce the elevated GS activity levels or the retarded growth phenotype. Subcloning of the pSAM1 insert DNA to generate pSAM3 and pSAM4 also did not reproduce these phenotypes indicating that the entire insert was required for full activity.

The pSAM1 insert DNA did not possess any intrinsic GS activity and could therefore not cause increased GS activity levels in this way. Copy number studies done on a pACYC184-derived vector in *E. coli* YMC11 carrying a *C. acetobutylicum* endoglucanase showed that the levels of cellodextrinase activity produced both in the presence and absence of pSAM1 were approximately equal. These results, therefore, indicated that the increased GS activity observed in the presence of pSAM1 was not due to the increased copy number of the pACYC184-derived vector bearing the *B. fibrisolvans glnA* gene.

In the light of the above results it was therefore interesting to find that the presence of pSAM1 caused a 2-fold elevation of the mRNA levels of the *B. fibrisolvans* GS relative to control levels. These results indicated that the pSAM1 insert DNA causes increased transcription of the *B. fibrisolvans* GS, although the increase in *glnA* mRNA levels was not precisely the same as the elevated GS activity levels (2.5 to 3-fold) previously noted. This difference in GS activity levels as opposed to *glnA* mRNA levels may be explained as being due to regulation at another level. Post-translational modification of the *B. fibrisolvans* GS might be occurring, although Southern *et al.*(1987) showed that the *B. fragilis* GS (which shares 40% amino acid homology with the *B. fibrisolvans* GS) did not appear to be regulated by adenylation. An alternative type of post-translational modification involving ADP-ribosylation has been noted for the GSIII of *Rhizobium meliloti* (Liu and Kahn, 1994). However, it is not known if this type of post-translational modification system occurs in *E. coli*. The fact that higher *B. fibrisolvans* GS mRNA levels were obtained in the presence of the pSAM1 insert DNA suggests that it may code for a regulatory protein that enhances the transcription of the *glnA* gene. It is not known whether it acts directly by binding to a particular site at or near the *B. fibrisolvans glnA* gene and allows increased transcription of this gene or whether it acts indirectly to enhance the transcriptional activation activity of another component of the system.

Previously, Kustu *et al.*(1979) had shown that the expression of the *hut* operon of *Klebsiella*

*aerogenes* is controlled by the *ntrB* and *ntrC* genes. In addition, Magasanik (1982) also reported that these *ntr* genes caused regulation of another set of genes involved in nitrogen metabolism viz. the histidine utilization genes. It was, therefore, important to examine whether the pSAM1 insert DNA could also function in a similar manner. The presence of pSAM1 insert DNA did cause a 3-fold elevation of histidase levels of *E. coli* YMC11 clones containing pSAM1 relative to clones which contained a control plasmid, pCM1. These results are interesting as they point to the fact that the pSAM1 insert DNA affected the regulation of another set of genes involved in nitrogen metabolism, viz. the *hut* genes of *K. aerogenes*. The fact that a deletion derivative of pSAM1 viz. pSAM2 did not show any elevation of histidase levels beyond those reported for *E. coli* clones containing control plasmid pCM1 is further evidence that the elevated histidase levels were due to the presence of the pSAM1 insert DNA and not an artefact of the two plasmid system.

The results obtained in this Chapter and in Chapter 2 strongly indicated that the *B. fibrisolvens* pSAM1 insert DNA had a regulatory effect on the *B. fibrisolvens* GS activity levels. Further investigation of the pSAM1 *B. fibrisolvens* insert DNA by protein analysis and nucleotide sequencing was thus undertaken.

**Chapter 4**  
**Protein and nucleotide analysis of the pSAM1**  
***B. fibrisolvens* insert DNA**

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## Chapter 4

### Protein and nucleotide analysis of the pSAM1 *B. fibrisolvens* insert DNA

#### Summary

*In vitro* transcription/translation analysis and SDS-PAGE established that a 11.75 kDa protein was produced from pSAM1 but not from the control plasmid pEB1. Sequencing of pSAM1 insert DNA and the subclones pSAM3 and pSAM4 established that a truncated ORF of 315 bp was present. This ORF was found to terminate in the pEcoR251 vector with the addition of 2 amino acids and a stop codon (9 bp). The calculated Mr of this ORF (allowing for the 9 additional base pairs of the vector) was 11.70 kDa which is in good agreement with the Mr (11.75 kDa) established from SDS-PAGE analysis.

Analysis of the nucleic acid sequence of the ORF using the MOTIF programme on Genetics Computer Group (GCG) suggested the presence of a potential signal sequence at the amino terminus of the 105 amino acid protein. The nucleic acid sequence and deduced amino acid sequences were compared to nucleic acid and protein sequences of existing proteins using the BLAST and FASTA packages on GCG. The homology of 3 genes identified by the BLAST program to be homologous to the *B. fibrisolvens* putative regulatory gene viz. the *B. subtilis* *lytA* gene, the *glnS* and the *glnH* genes was examined using the GCG BESTFIT package.

## 4.1 Introduction

Protein and nucleotide sequence analysis are invaluable tools in the characterisation of genes and gene products isolated from a variety of organisms. The power of this analysis lies in the comparison of the newly isolated gene with previously sequenced genes (because of the almost universal nature of the genetic code) allowing for an assessment of the degree of relatedness (homology) of the new gene to those previously characterized. This analysis may also reveal intimate details concerning the possible function of the newly isolated protein. Although there is the likelihood that a novel gene may be isolated, this is an accepted approach used for characterisation of newly isolated gene products and, was, therefore, used in this study.

At this juncture, it is important to note that very few *B. fibrisolvens* genes have been cloned and sequenced (as compared to the large number cloned and sequenced in for eg. *E. coli*) and only a single gene involved in nitrogen metabolism viz. the GS gene (Goodman and Woods, 1993) used in this study has been cloned and sequenced. This GS gene is very different to the GS genes previously isolated from *E. coli* and other Enterobacteria, and it is not implausible that the regulatory mechanisms governing the functioning of this gene may be different. Protein and sequence analysis of the *B. fibrisolvens* pSAM1 insert DNA was therefore required to evaluate these possibilities.

## 4.2 Materials and Methods

All additional details of materials and methods are contained in the Appendix (as specified below). Bacterial strains and plasmids were the same as those used in Chapter 2 (Table 2.1).

### 4.2.2 DNA preparation

DNA for nucleotide sequencing and general use was prepared according to Ish-Horowicz and Burke (1981). Large scale pure plasmid DNA preparations required for sequencing were prepared using the Nucleobond<sup>R</sup> kit according to the manufacturer's instructions.

### 4.2.3. Cell-free coupled transcription-translation system

The Promega procaryotic DNA-directed translation kit (No. 14500) was used according to the manufacturer's instructions. Plasmid pSAM1 and a control plasmid, pEB1 (Lin, 1990) was used to correlate protein expression with the elevated GS activity and mRNA levels and the

retarded growth phenotype.

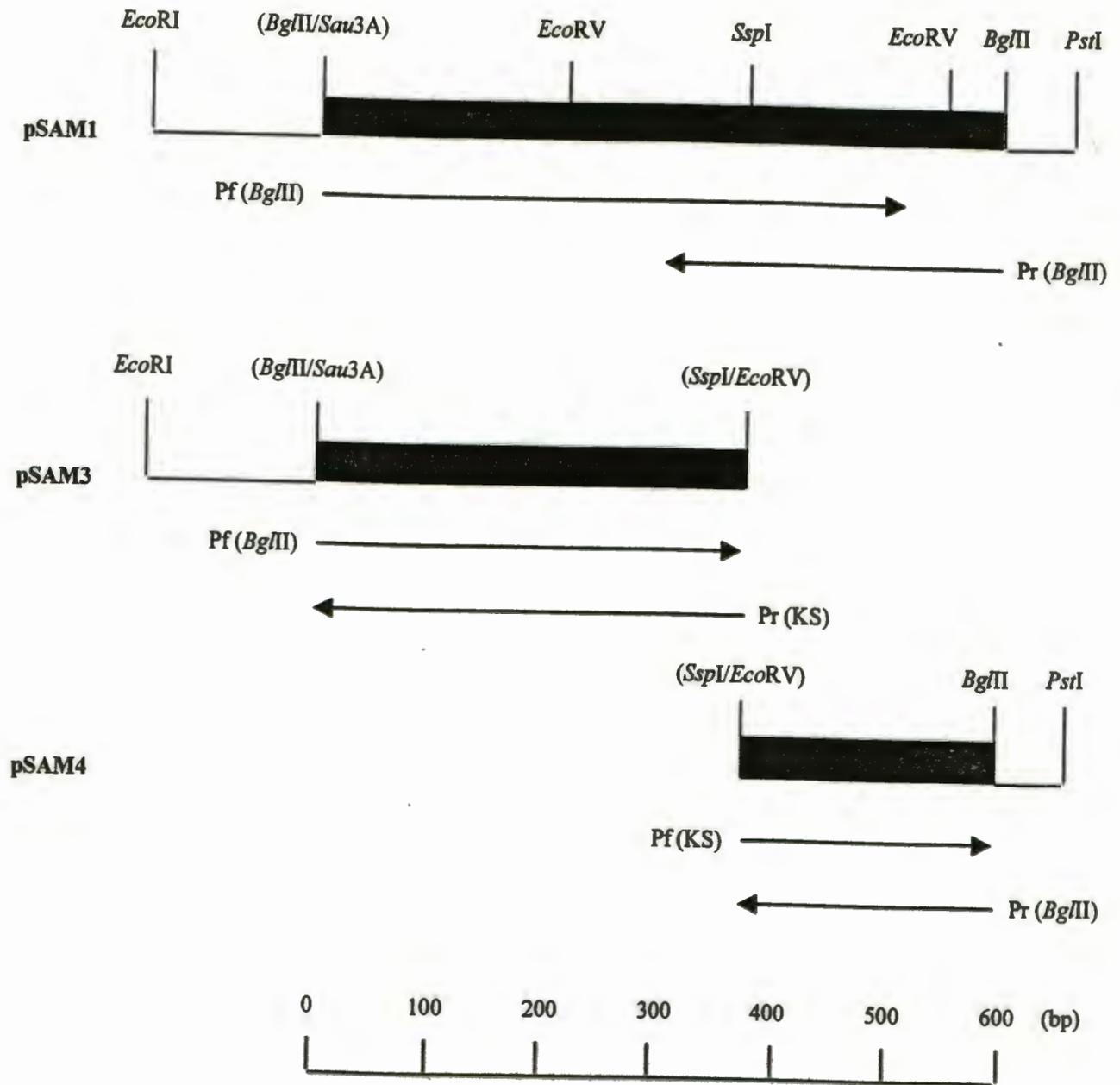
[<sup>35</sup>S]-methionine labelled proteins were separated on a discontinuous SDS-PAGE (15% acrylogel) system (Laemmli, 1970) and viewed by autoradiography (Appendix).

#### 4.2.4 Nucleotide sequencing

The nucleotide sequence of both strands of the *B. fibrisolvens* H17C insert DNA of pSAM1 and the subclones pSAM3 and pSAM4 were determined by an adaptation of the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977).

The Sequenase<sup>R</sup> Version 2.0 DNA sequencing kit was used (No. 70700, United States Biochemical, Cleveland, Ohio, USA) which incorporates the Sequenase<sup>R</sup> enzyme originally described by Tabor and Richardson (1987). Sequencing the pSAM1 insert DNA involved the use of the pEcoR251 *Bgl*III forward (5'-TTGGGAAAAGAGGAGA-3') and reverse primers (3'-GGTGAAAGGAATGCAGGAAAAG-5') flanking the *Bgl*III insertion site. The location of a *Ssp*I site at approximately 228 bp then facilitated the subcloning of the pSAM1 insert DNA onto pBluescript (KS) (refer Chapter 2). The entire insert sequence was subcloned by cleavage of the approximately 0.9 kb *Eco*RI-*Ssp*I and approximately 0.4 kb *Ssp*I-*Pst*I fragments of pSAM1 into the *Eco*RI-*Eco*RV and *Pst*I-*Eco*RV sites of pBluescript, respectively. The use of an Alfexpress (Pharmacia) automated sequencer to sequence the subclones pSAM3 and pSAM4 (Fig.4.1) then allowed the determination of the entire pSAM1 insert DNA sequence in both the forward and reverse directions using the pBluescript universal primers and labelled with Cy<sup>5</sup>.

The sequencing data was analyzed using the sequence analysis programs CODONPREFERENCE, COMPOSITION, ISOELECTRIC and PEPLOT of the Wisconsin University Genetics Computer Group Inc. (Devereux *et al.*, 1984) on a VAX-6000-330 computer. The Genbank, EMBL, SWISS-protein and PIR databases were searched directly for DNA and amino acid homology. Databases were also accessed using the BLAST network service (Basic local alignment search tool) through the NCBI (National Centre for Biotechnology Information), Maryland, USA, (Altschul *et al.*, 1990). The centre was accessed via electronic mail to the NCBI BLAST E-Mail Server (blast@ncbi.nlm.nih.gov). The optimal alignment of each of the *B. subtilis* *lytA*, *E. coli* *glnH* and *glnS* genes to the *B. fibrisolvens* ORF1 gene was determined using the BESTFIT program on GCG.



**Fig. 4.1** Restriction maps of pSAM3 and pSAM4. Single line, pEB1 vector DNA; *Pf (BgIII)* and *Pr (BgIII)*, forward and reverse primers flanking the pEcoR251 *BgIII* site, respectively. *Pf (KS)* and *Pr (KS)*, forward and reverse primers flanking the multiple cloning site of KS. Arrows indicate extent and direction of sequencing.

## 4.3 Results

### 4.3.1 Protein analysis of the pSAM1 insert DNA

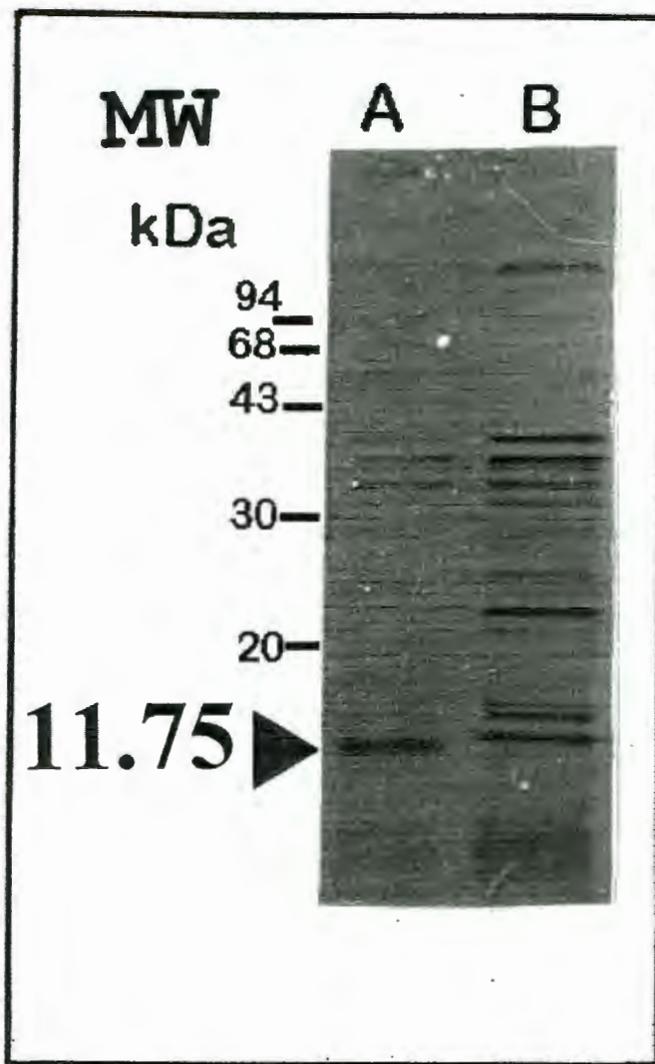
*In vitro* transcription/translation analysis and SDS-PAGE gel electrophoresis was used to determine whether the pSAM1 *B. fibrisolvens* insert DNA produced unique protein products (Fig. 4.2). The proteins produced by pEB1 (Lane B), which did not contain any insert were used as a control (Lane A). A 11.75 kDa protein was produced from the pSAM1 insert DNA but not from the control pEB1 plasmid. The presence of the 11.75 kDa protein in the experimental sample and not in the control sample appeared to indicate that the pSAM1 *B. fibrisolvens* insert DNA coded for a protein product involved in causing the elevated GS activity levels. However, the possibility that the protein produced is a fusion protein which terminated in the vector could not be ruled out. Moreover, the presence of an open reading frame on the pSAM1 insert DNA still had to be established. Sequencing analysis was, therefore, carried out.

### 4.3.2 Analysis of the nucleotide sequence of pSAM1

The nucleotide sequence of the entire 0.581 kb insert DNA fragment was determined as described in the methods (Section 4.2). A truncated ORF (ORF1) was identified which started at position 267 and terminated in the vector (Fig. 4.3) This ORF did not contain an inframe translational stop codon and the protein was presumably terminated by a stop codon within the vector DNA. A likely inframe translational stop codon within the vector was identified 9 bp downstream of the vector-insert junction. The GCG TESTCODE, FRAMES and CODONPREFERENCE programs all verified that an ORF (ORF1) was present (Fig. 4.4).

The calculated Mr of a protein produced from a 321 bp (including the 9 bp of vector sequence) sequence is 11.70 kDa which is nearly identical to the Mr of 11.75 kDa obtained using SDS-PAGE analysis.

A putative ribosome binding site for ORF1 consisting of an ATG initiation codon and a strong Shine-Dalgarno complementarity (Shine and Dalgarno, 1974) containing five G-C bp situated 4 bp upstream of the initiation codon was identified in the insert sequence (Table 4.5).



**Fig. 4.2** *In vitro* transcription/translation and SDS-PAGE analysis of the proteins produced from *E. coli* YMC11 (pSAM1) (Lane A) and *E. coli* YMC11 (pEB1) (Lane B).

MW - molecular weight in kilodaltons

kDa - kilodaltons



**Table 4.5** Potential ribosome binding sites of the *B. fibrisolvans end1* and *ced1* genes (Berger, 1991) and the *glgB* and *amyA* genes (Rumbak, 1991) compared to the potential ribosome binding site of ORF1. The Shine-Dalgarno regions and start codons are underlined.

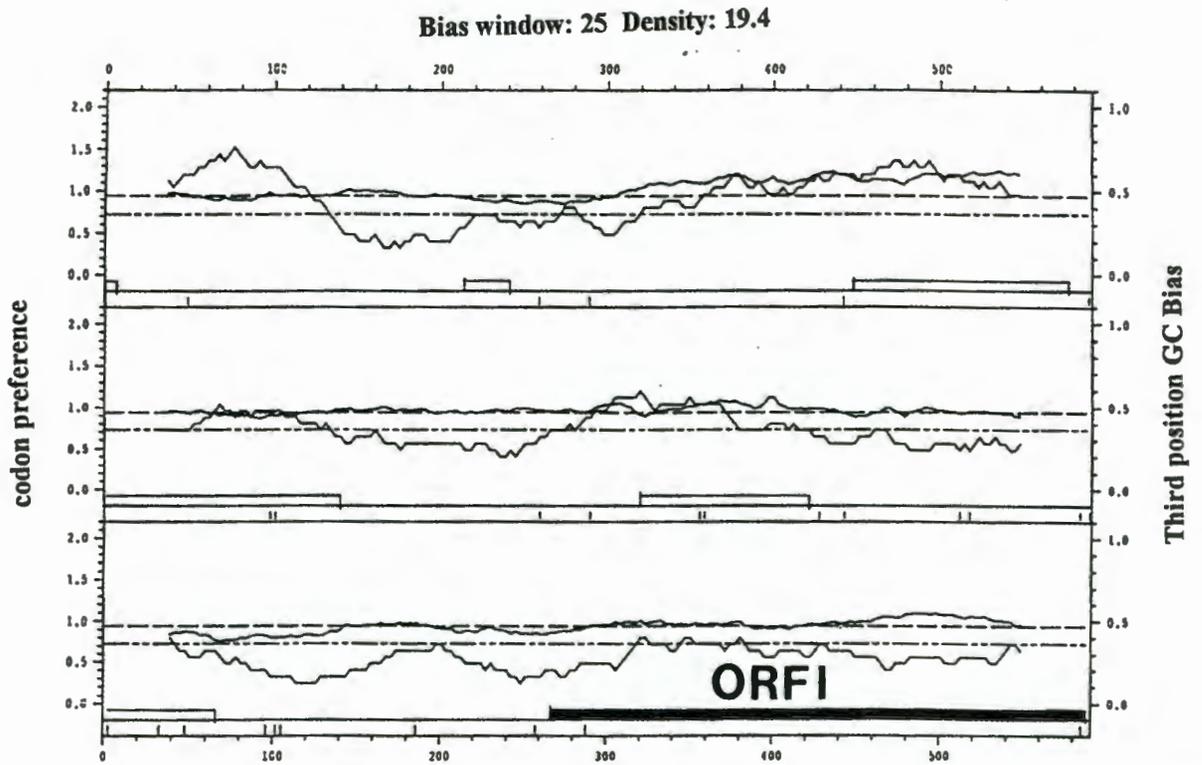
gene	Potential ribosome binding site	Start
ORF1	5' <u>G G G G G</u> T T T T	<u>A T G</u> 3'
<i>end1</i>	5' <u>G G A G G</u> T G C T T A A G <u>A T G</u> 3'	
<i>ced1</i>	5' <u>G G A G G G</u> C T T A C A <u>G T G</u> 3'	
<i>glgB</i>	5' <u>G A G G G G G</u> T T A T T T <u>A T G</u> 3'	
<i>amyA</i>	5' <u>G G A G G</u> T T G T <u>A T G</u> 3'	

The ORF1 (including the vector sequence) encoded a protein of 107 amino acids with a calculated Mr of 11,7. The (G+C) content of the nucleotides within the ORF1 (37.4%) was slightly higher than the (G+C) content of the nucleotides in the regulatory region upstream of the gene (34.4%). It has been reported that the average (G+C) ratio for the entire *B. fibrisolvans* genome was 42% (Manerelli, 1988). The (G+C) ratio for ORF1, therefore, appears to reflect a lower value than is expected for *B. fibrisolvans* genes, perhaps because the gene is truncated.

Two direct repeats (10 bp) (Fig.4.3) and an inverted repeat (Energy of  $\Delta G = -10$  KJ/mol) were identified using the REPEAT and SQUIGGLES GCG programs respectively. Studies by Bohannon and Sonnenshein (1989) on the regulation of glutamate biosynthesis in *B. subtilis* have illustrated the importance of DNA direct repeats in the positive regulation of the *gltC* gene. These authors showed that these sites function as binding sites for the *gltC* protein.

#### 4.3.2.1 Putative promoter sequences

Two putative promoter sequences were identified in the sequence upstream of the ATG start codon of ORF1 (Fig. 4.3). The region upstream of ORF1, between 133 and 108 base pairs from the ATG start site contained a putative promoter sequence (P1) which consisted of a TTGAAT -35 region and a TATATA -10 region separated by 20 nucleotides (Fig. 4.3). A second putative promoter sequence (P2) occurred between 44 and 69 nucleotides from the ATG start site just upstream of a putative ribosome binding site (Fig.4.3). These putative promoter sequences were compared to both typical *E. coli* promoters, *B. subtilis* vegetative promoter regions and to putative promoter consensus sequences established for *B. fibrisolvans* promoter sequences (Fig. 4.5).



**Fig. 4.4** Open reading frames and codon preference plot of the 0.581 kb *B. fibrisolvens* insert DNA sequence. This plot was compiled by the GCG program CODONPREFERENCE using a codon preference table based on previously sequenced *B. fibrisolvens* genes.

		Promoter sequence		
		-35	Spacer (bp)	-10
<i>B. subtilis</i>		<b>TTG</b> <u>AC</u> Ca (17)		<b>TAt</b> <u>AA</u> T
<i>E. coli</i>		<b>TTG</b> <u>AC</u> g (17)		<b>TAt</b> <u>AA</u> T
<i>amyA</i>		<b>TTG</b> <u>AC</u> g (17)		<b>TAt</b> <u>AA</u> T
<i>ced1</i>		<b>TTG</b> <u>AA</u> Aa (20)		<b>TAt</b> <u>A</u> TA
<i>eglA</i>		<b>TTG</b> <u>AC</u> Ca (15)		<b>AA</b> t <u>AA</u> T
<i>B. f</i> consensus		<b>TTG</b> <u>A</u> Ca		<b>TAt</b> <u>AA</u> T
ORF1	P1	<b>TTG</b> GCT (20)		<b>TAt</b> <u>A</u> CT
	P2	<b>TTG</b> <u>AA</u> T (20)		<b>TAt</b> <u>A</u> TA

**Fig. 4.5** *B. fibrisolvens* ORF1 putative promoter sequences P1 and P2 as compared with the *E. coli* (reviewed by Matthews and Van Holde, 1990) and *B. subtilis* (Haldenwang, 1995) consensus promoter sequences. Putative promoters of other *B. fibrisolvens* genes viz. *amyA* (Rumbak, 1991), *ced1* and *eglA* genes (Berger, 1990) are also indicated. The *B. fibrisolvens* consensus sequence was obtained from the alignment of all the existing *B. fibrisolvens* promoter sequences and the determination of those nucleic acid residues which were most conserved. Highly conserved bases (>75%) are indicated in bold type; moderately conserved bases (>50%) are underlined and weakly conserved bases (>41%) are lower case letters (after Matthews and Van Holde, 1990).

The consensus sequence obtained for the promoters of the *B. fibrisolvens* genes examined above was identical to the consensus sequence for the -10 and -35 regions of Gram-positive *B. subtilis* promoters and to the consensus sequence established for *E. coli* promoters. Both P1 and P2 showed high homology to the *B. fibrisolvens* consensus sequence and to the *B. subtilis* promoter consensus sequences. Primer extension analysis will be required to locate the transcriptional start sites and verify the existence of a promoter sequence in these regions. No NtrC binding sites (Reitzer *et al.*, 1989) nor evidence for the presence of  $\sigma^{54}$  controlled nitrogen regulated promoters (Reitzer and Magasanik, 1985) was found in the region upstream of the putative initiation codon of ORF1.

Previously (Chapter 2), it was shown that neither of the two subclones (pSAM3 and pSAM4) could restore the elevated GS activity levels observed for pSAM1. Sequence analysis confirmed that cleavage at the *SspI* site would most likely result in the separation of the

putative promoter region upstream of the ATG initiation codon (on pSAM3) from the rest of ORF1 thus preventing initiation of transcription by RNA polymerase.

#### **4.3.2.2 Analysis of the deduced amino acid products of the pSAM1 *B. fibrisolvens* putative regulator sequence**

Analysis of the deduced amino acid (aa) sequence of the protein produced from the pSAM1 insert DNA (321 bp) revealed many interesting properties. The protein consisted of 107 aa, appeared to be highly acidic (pI = 3.75) and had a deduced total charge of -18. The protein appeared to have a highly hydrophobic region in the amino terminal end (Fig.4.6) which was identified using the GCG PLOTSTRUCTURE program.

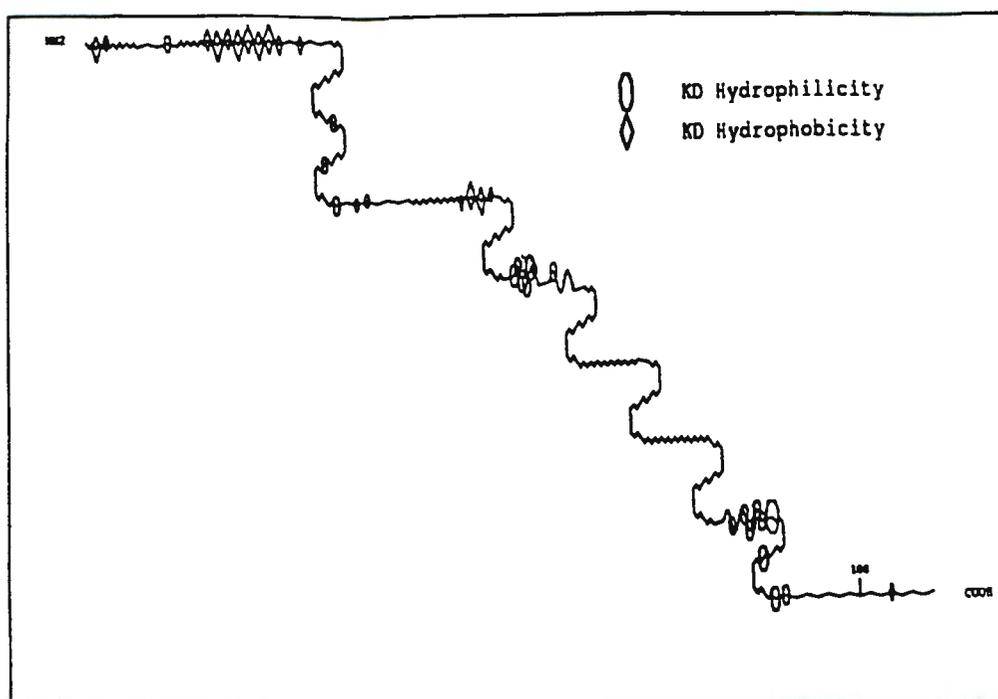
#### **4.3.2.3 Putative signal sequence**

A possible signal sequence and lipoprotein membrane attachment site was identified in the amino terminus of the ORF1 deduced amino acid sequence using the GCG programme, MOTIF. The ORF1 putative signal sequence was compared with signal sequences identified for other bacterial lipoproteins (Fig. 4.7).

The prototypical lipoprotein signal sequence contains a cluster of positive charges in the NH<sub>2</sub>-terminal region, followed by hydrophobic residues and a polar C-terminal processing site (Duffaud *et al.*, 1988). In addition, Hayashi and Wu (1990) have proposed a Leu-Ala-Gly-Cys consensus sequence for the polar C-terminal processing site. For the deduced N-terminal aa sequence of ORF1, all these features are found with a positive net charge at the NH<sub>2</sub> terminus, a hydrophobic stretch at the centre and GC conserved residues at the predicted polar C-terminal processing site.

#### **4.3.2.4 Amino acid sequence comparison with other genes**

The deduced amino sequence was compared to other amino acid sequences using the BLAST network service (Basic local alignment search tool) through the NCBI (National Centre for Biotechnology information), Maryland, USA, (Altschul *et al.*, 1990). A search of the Genbank, EMBL, SWISS-protein and PIR databases using the deduced amino acid sequence of the truncated ORF revealed some degree of homology to the *B. subtilis* *lytA* protein (Lazarevic, 1992), the *B. stearothermophilus* glutamine-binding protein (Wu and Welker, 1991) (*glnH* gene) and the *E. coli* glutaminyl tRNA synthetase (*glnS*) (Yamao *et al.*, 1982).



**Fig. 4.6** Two-dimensional structural representation of the secondary structure of the deduced amino acid sequence of ORF1.

The structure was compiled using the PLOTSTRUCTURE program on GCG.

	+++	
B. fib ORF1	MYILSKRRGLT <b>LSILLSICSISG</b>	 <u>CS</u> SQD
	+ +	
E. coliLP28	MKLTTHHLRT <b>GAALLLAGILLAG</b>	<u>CD</u> QSSS
	+ ++	
E. coliTraT	MKMKKLMM <b>VALVSSTLALSG</b>	<u>CG</u> AMST
	+	
K. pneuPUL	MLRYTC <b>NALFLGSLILLSG</b>	<u>CD</u> NSS
	+ + ++	
B. licPEN	MKLWFSTLKLK <b>KAAVLLFSCVALAG</b>	<u>CAN</u> NQ
	+	
N. gonOMP	MKAYLALIS <b>AAVIGLAA</b>	<u>CS</u> QEP 

**Fig. 4.7.** Comparison of the putative signal sequence of ORF1 with other bacterial lipoprotein signal sequences. Bfib ORF1 - putative *B. fibrisolvens* ORF1 deduced amino acid signal sequence (this work); *E.coli*LP28 - lipoprotein 28 ((Yu *et al.*, 1986); *E.coli*TraT - TraT protein (Perumal and Minkley., 1984); K.neuPUL - *Klebsiella pneumoniae* pullulanase; B.licPEN - *Bacillus licheniformis* penicillinase; N.gonOMP - *Neisseria gonorrhoea* outer membrane protein (sequence data obtained from Hayashi and Wu, 1990). The symbol + denotes basic residues in the hydrophilic leader regions and the hydrophobic residues are indicated in **boldface**. The sequence is shown up to the cleavage site or the predicted cleavage site indicated by the arrows. Conserved C-terminal cleavage site residues are underlined.

Since the *B. stearothersophilus glnH* gene product is homologous to the *E.coli argT* and *hisJ* gene products (reviewed by Magasanik, 1987), these were also aligned with the *B. fibrisolvens* ORF1 deduced amino acid sequence. Alignment of each of these proteins to ORF1 allowed the assessment of the degree of similarity and identity (Table 4.1).

Only limited amino acid homology in short regions to a number of proteins was found.

The highest degree of amino acid homology viz. 32.1% identity and 56.2% similarity over the entire gene was found to the *B. subtilis lytA* gene product (Lazarevic, 1992).

#### 4.4 Discussion

The *B. fibrisolvens* pSAM1 insert DNA was characterized by protein analysis and nucleotide sequencing. Protein analysis revealed the presence of an 11.75 kDa protein which occurred in experimental but not in control lanes during SDS-PAGE. Nucleotide sequencing revealed the presence of a truncated 321 bp ORF which appeared to terminate in the vector with the addition of 9 bp (2 amino acids and a stop codon). The Mr of the protein produced from such an ORF is 11.70 kDa and is in good agreement with the 11.75 kDa established by SDS-PAGE. Interestingly, a putative signal sequence was identified at the N-terminus of the putative regulator using the GCG MOTIF programme.

Many signal peptides are cleaved immediately after an A-X-A sequence (Pearlman and Halvorsen, 1983) and this holds true for the *B. subtilis* endoglucanase and other *B. subtilis* genes (Zappe *et al.*, 1988). However, signal sequences of lipoproteins are cleaved by lipoprotein specific peptidase II at a site involving a Leu-Ala-Gly-Cys- amino acid consensus sequence (Duffaud *et al.*, 1988). The cysteine residue is crucial as it forms the attachment point for S-[propane-2',3'diol]-3-thioamino propionic acid to which two fatty acid residues are linked by two ester linkages and one fatty acid residue by an amide linkage in the case of the major lipoprotein (Yamaguchi *et al.*, 1988). Although a cleavage site for ORF1 was not determined, a predicted cleavage site could be at Gly-Cys as indicated (Fig. 4.8) which is similar to the cleavage site found for a number of other lipoproteins (Mattar *et al.*, 1994). No other functional motifs such as helix-turn-helix DNA-binding motifs could be identified in the ORF, nor were any potential NtrC-binding sites identified in the regulatory region upstream of the putative ATG initiation codon.

A search through the nucleic acid and protein sequence databases revealed sequences with limited similarity to the truncated ORF. These are discussed below with reference to assigning a possible function to the *B. fibrisolvens* ORF1 protein which may explain how this protein causes the elevated GS mRNA levels and the corresponding elevation of GS activity levels.

The protein which showed the highest degree of homology to ORF1 was the *B. subtilis* LytA protein viz. 32.1% identity and 56.2% similarity.

This protein forms part of the *lytRABC* divergon involved in the production of *N*-acetylmuramoyl - *L* - alanine amidase, one of the two major *B. subtilis* autolysins (Lazarevic

*et al.*, (1992). The LytA protein shares a number of deduced amino acid properties with the ORF1 protein. These include the small size, the signal sequence, as well as the highly acidic nature of both proteins (Table 4.2).

**Table 4.1** Similarity and identity between the *B. fibrisolvens* ORF1 protein and related proteins.

Gene product	% Identity *	Total aa.	Reference
B. f ORF1	100 (100)	105	This work
B. s LytA	32.1 (56.2)	102	Lazarevic <i>et al.</i> (1992)
B. st GlnH	25.7 (44.3)	263	Wu and Welker (1991)
E. c GlnH	15.8 (43.6)	249	Higgins <i>et al.</i> (1982)
N. g HisJ	24.7 (57.4)	269	Ames (1984)
S. t HisJ	17.8 (53.3)	261	Ames (1984)
S. t ArgT	19.2 (46.5)	261	Ames (1984)
E. c ArgT	21.2 (48.5)	146	Ames (1984)
E. c GlnS	28.4 (47.7)	557	Yamao <i>et al.</i> (1982)
S. p AmiA	20.0 (69)	471	Alloing <i>et al.</i> (1990)

B. f: *Butyrivibrio fibrisolvens*

E. c: *E. coli*

S. t: *Salmonella typhimurium*

B. s: *Bacillus subtilis*

S. p: *Streptococcus pneumoniae*

\* % similarity indicated in brackets; aa, amino acids.

Although the function of the LytA protein has as yet not been determined, its sequence strongly suggests that it is a lipoprotein (Lazarevic *et al.*, 1992) and that it is anchored to the cytoplasmic membrane via the N-terminal glyceride-modified cysteinyl residue, and localised on its outer surface. By analogy with the PrsA lipoprotein (Kontinen *et al.*, 1991), Lazarevic *et al.* (1992) suggested that it may play a role in the secretion of the *lytB* and *lytC* gene products.

Two other proteins viz. the *E. coli* GlnS protein (28.4% identity) and the *B. stearothermophilus* GlnH protein (25.7% identity) also showed limited homology to the *B. fibrisolvens* ORF1 protein. The truncated nature of ORF1 does, however, make it difficult to achieve optimal alignment with these larger proteins.

**Table 4.2. Features of amino acid products deduced from the nucleotide sequences of *lytA* and *ORF1*.**

Property	LytA *	ORF1**
<u>Uncleaved</u>		
No. of amino acids	102	105
Molecular mass (kDa)	11.2	11.75
<u>* Mature</u>		
No. of amino acids	86	82
Molecular mass (kDa)	9.4	9.14
Isoelectric point	3.9	3.75
Nett Charge	-13	-18

\* Lazarevic *et al.* (1992); \*\* This work.

The results obtained in this chapter have indicated that a truncated, yet functional protein (the ORF1 protein) with an N-terminal signal sequence has been isolated from *B. fibrisolvans*. The functionality of this signal sequence could be tested by inframe fusion of the N-terminal portion of the *B. fibrisolvans* ORF1 protein to the alkaline phosphatase gene carried on *TnphoA* (Manoil and Beckwith, 1985) which requires a signal peptide for export and alkaline phosphatase activity.

While analysis of the protein and nucleic acid sequence of the ORF1 protein has provided some interesting information about the physical structure of the protein, it has not provided any certain information concerning the possible function of the ORF1 protein. While some degree of similarity was noted between the *B. fibrisolvans* ORF1 protein and the *B. stearothermophilus* GlnH and *E. coli* GlnS proteins, the *B. subtilis* LytA protein shared the highest degree of nucleic and amino acid homology with the *B. fibrisolvans* ORF1 protein as well as many of its physical properties.

This information together with the physiological analysis of the effects of this protein on *E. coli* YMC11 (pGS5) is evaluated in the next Chapter with reference to providing a possible model for the mode of action of the ORF1 protein.

## Chapter 5

### General Conclusions

Ammonia is a major and often essential source of nitrogen for the growth of most rumen microorganisms (Mackie and Kistner, 1985). The highly competitive rumen environment, in which N-resources may quickly become limiting, means that enzymes involved in ammonia assimilation are crucial to the survival of most rumen microorganisms (Hespell, 1984). Consequently, enzymes such as GS, GOGAT and GDH would be expected to play a central role in the acquisition of N in the form of ammonia. Previously, the *B. fibrisolvans* GS was cloned and sequenced and found to belong to a structurally novel group of enzymes termed GSIII (Goodman and Woods, 1993). Despite the novel structural features of the *B. fibrisolvans* GS, it was still functionally similar to other GS's as it complemented an *E. coli glnA*<sup>-</sup> strain, YMC11 when this strain was grown on minimal media with ammonia as the sole nitrogen source.

In this study a heterologous, *E. coli*, two plasmid system was used to screen for the presence of *B. fibrisolvans glnA* regulatory genes using a previously constructed pEB1 genebank. This system was used for several reasons. Firstly, the plasmid and genetic systems of *E. coli* are well characterized and are routinely used for the isolation of genes from other organisms eg. *B. fibrisolvans* (Pouwels *et al.*, 1987). In contrast, there is a lack of information concerning the plasmid and genetic systems of *B. fibrisolvans*. Secondly, several well -characterized nitrogen regulatory mutants exist in *E. coli* (Magasanik, 1982). No such nitrogen regulatory mutants have yet been identified for *B. fibrisolvans*. Finally, *E. coli* is much easier to cultivate on the media required for nitrogen regulatory studies than anaerobic microorganisms such as *B. fibrisolvans* that require special growth media and anoxic conditions for cultivation (Goodman and Woods, 1993). However, the heterologous system also has its disadvantages as opposed to working with a gene of interest in the host organism. A fundamental problem is that of assigning a particular function to a specific gene. A heterologous gene may exhibit functional characteristics totally different from the actual function of the gene in the host organism. Sequence homology of genes cloned from different organisms may provide information regarding the function of a particular gene but the possibility also exists that an apparently novel gene/genes may be isolated as in this study.

The use of the heterologous system has, to some extent, been successful, in that it allowed for the isolation of a gene (*ORF1*) which caused a 2.5 to 3-fold elevation of *B. fibrisolvans* GS activity levels. The presence of *ORF1* caused an elevation of GS activity levels under conditions of growth in which levels of the *E. coli* GS is normally depressed due to the presence of the cell in nitrogen-rich media. It also caused a 3-fold elevation of the *K. aerogenes* histidase level in *E. coli* known to fluctuate with the glutamine synthetase levels in *E. coli* (Magasanik, 1982). The level of histidase in *E. coli* was previously used as a convenient test for the *ntr* phenotype mediated by the NtrB and NtrC proteins (Magasanik, 1982; Magasanik and Neidhardt, 1987). Thus, the *ORF1* protein may be influencing the functioning of two key systems involved in nitrogen metabolism in *E. coli* viz. the *K. aerogenes hut* system and the *B. fibrisolvans* GS.

The presence of *ORF1* also caused a twofold elevation of the *B. fibrisolvans glnA* gene mRNA levels suggesting transcriptional enhancement of this gene. This may be due to a direct effect of the *ORF1* protein on the *B. fibrisolvans glnA* gene, or via an effector molecule. All attempts to clone the entire pSAM1 *B. fibrisolvans* insert DNA onto a high copy number plasmid, were unsuccessful. However, it was possible to clone the insert as two separate fragments that no longer caused elevated GS activity levels and the increased-lag time-phenotype. These results seemed to suggest that the presence of the *ORF1* protein in high copy number could be deleterious to the cell.

The nucleic acid and deduced amino acid sequence of this protein were, therefore, used to attempt to assess its function.

*ORF1* coded for an 11.7 kDa, C-terminally truncated vector fusion protein that appeared to have a signal sequence characteristic of secreted lipoproteins. This protein also appeared to be structurally novel as it showed no homology to any previously characterized nitrogen regulatory proteins. It did, however, show limited homology to the *B. subtilis* LytA, and to a lesser extent to the *B. stearrowthermophilus* GlnH and *E. coli* GlnS proteins. No glutamine binding domains nor any DNA-binding domains that have previously been found for other regulatory proteins could be identified from the deduced amino acid sequence of the *ORF1* gene. The presence of a glutamine binding motif for the *B. stearrowthermophilus* GlnH (involved in glutamine uptake) and *E. coli* GlnS (involved in acylation of the glutaminyl tRNA with glutamine) proteins are crucial to the functioning of these proteins and the absence of such a motif in the *ORF1* protein appears to rule out the possibility that the *B. fibrisolvans* *ORF1* protein could be functioning

similarly to these proteins. The ORF1 protein causes the above-mentioned phenomena in *E. coli*, but is structurally novel thus limiting the informativity of the nucleic acid sequencing approach. The sequence homology to the LytA protein, does, however, require further consideration. If the ORF1 protein has functional homology to LytA, it may be functioning as a regulator of the *E. coli* general amidase activity (Parquet *et al.*, 1983), perhaps in a similar fashion as is suggested by Lazarevic *et al.* (1992) for the *B. subtilis* LytA protein.

Amidases are a group of proteins that allow prokaryotes to use a class of nitrogen containing compounds known as acetamides (Soubrier *et al.*, 1992). Studies by Herbert and Macfarlane (1980) on *Rhodopseudomonas acidophila* grown on glutamine and asparagine, have shown that all the nitrogen requirements of this organism could be satisfied by transamination reactions mediated by these amidases. Studies by Calderon and Mora (1989) on *Neurospora crassa* have also shown that glutamine could be produced by an alternate ammonia assimilatory pathway to the classical pathway that involves GS activity. This pathway involves the action of an amidase and glutamate dehydrogenase, and a glutamine cycle in which glutamine is continually being regenerated.

If there is enhancement of amidase levels through the overexpression of ORF1, this may lead to a modification of the nitrogen source in *E. coli* that could in turn affect the *B. fibrisolvens* GS activity levels. The reduced growth rate observed in *E. coli* YMC11 (pGS5) may then be due to the metabolic load of expressing excess large polypeptide (the *B. fibrisolvens* GS) and from imbalances in the intracellular amino acid pools.

The work presented here has raised several questions concerning the mode of action of the *B. fibrisolvens* ORF1 protein that need to be addressed in future studies. The following represent some research directions that may be pursued.

The effect of ORF1 on the amidase activity of *E. coli* YMC11 containing the *B. fibrisolvens* GS requires examination. This could be done by determining the levels of amidase activity for this strain both in the presence and absence of ORF1.

Studies on the uptake of glutamine and other amino acids in this *E. coli* strain are also required to investigate the possibility that the *B. fibrisolvens* ORF1 protein could be functioning as a

transporter of glutamine (or other amino acids) into the cell. These studies could also indicate whether the ORF1 protein is affecting the functioning of the *E. coli* transport systems as mutations in the *ntrC* gene (which regulates these systems in the enteric bacteria) are known to have diverse consequences that may include the phenotype observed in this study (reviewed by Magasanik and Neidhardt, 1987).

An alternative approach would be the cloning and nucleotide sequencing of the entire ORF1 gene. This would involve the screening of a genebank of *B. fibrisolvens* chromosomal DNA using DIG-labelled ORF1 DNA to detect the clones carrying the remaining C-terminal region of the ORF1 gene. However, this approach may have limited value as sequence analysis of the N-terminal fragment yielded scant information about the function of the ORF1 protein (Chapter 4).

A more informative approach could be to use the truncated ORF1 gene on a suicide vector to do site directed mutagenesis of the gene in *B. fibrisolvens*.

Analysis of a mutant generated in this manner could provide valuable information concerning the role of the ORF1 protein in N-metabolism and more specifically in *glnA* gene regulation in *B. fibrisolvens*.

The aim of this study was to isolate *B. fibrisolvens* GS regulatory genes from an existing *B. fibrisolvens* gene bank using a heterologous *E. coli* system, and the characterisation of any such genes. This approach was at least partially successful in that a gene (the ORF1 gene) which affected GS activity levels was isolated although protein and nucleotide sequence analysis did not provide enough information to be able to determine the function of the ORF1 protein with any certainty.

The lack of suitable plasmid vectors for *B. fibrisolvens* and the fact that details regarding the genetics of this ruminal microorganism are only now emerging, ultimately limited the scope of this study to analysis of this gene in a heterologous organism. However, the advent of more advanced cloning systems, coupled with advances in the technology available to rumen molecular microbiologists, will, eventually, lead to a clearer picture of the molecular genetics of this ubiquitous rumen microorganism.

**Appendix A**  
**Media, Buffers and solutions**

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## Appendix A

### Media, buffers and solutions

All media, buffers and solutions were sterilized by autoclaving at 121°C for for 30 min unless otherwise indicated. Heat labile substances were sterilized by filtration through 0.22  $\mu$ m membrane filters (Millipore). Polyacrylamide gel solutions and buffers are given in Appendix B.

#### A1.1 *B. fibrisolvens* non-rumen fluid medium (M10)

Ingredient	Amount/l
Glucose	10.0 g
Cellobiose	0.5 g
Mineral solution 1 <sup>a</sup>	30.0 ml
Mineral solution 2 <sup>b</sup>	38.0 ml
Cysteine HCl (5% w/v)	10.0 ml
Na <sub>2</sub> CO <sub>3</sub>	50.0 ml
Resazurin (0.1% w/v)	1.0 ml
Trypticase	2.0 g
Yeast extract	0.5 g
Volatile fatty acids <sup>c</sup>	3.1 ml
Hemin <sup>d</sup>	10.0 ml
Distilled water	850.0 ml

<sup>a</sup>Mineral solution 1 contained/l:

K<sub>2</sub>HPO<sub>4</sub> 6.0 g

<sup>b</sup>Mineral solution 2 contained/l:

KH<sub>2</sub>PO<sub>4</sub> 6.0 g  
 NaCl 12.0 g  
 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 6.0 g  
 CaCl<sub>2</sub>·2H<sub>2</sub>O 1.6 g  
 MgSO<sub>4</sub>·7H<sub>2</sub>O 2.5 g

<sup>c</sup>Volatile fatty acid solution contained:

acetic acid 17 ml  
 propionic acid 6 ml  
 butyric acid 4 ml  
 isobutyric acid 1 ml  
 n-valeric acid 1 ml  
 iso-valeric acid 1 ml  
 2-methylbutyric acid 1 ml

Solution is stable at 4°C.

All the ingredients were added and the pH adjusted to 6.8 prior to autoclaving.

<sup>d</sup> Hemin solution:

KOH	0.28 g
ethanol (95%)	25.0 ml
hemin	0.10 g
distilled water	75.0 ml

Solution is stable at 4°C.

#### A1.2 Luria-Bertani medium (LB)

Bacto tryptone	5 g
yeast extract	5 g
NACl	5 g
Distilled water	1000 ml

Solid media (LA) contained 1.5% (w/v) agar.

#### A1.3 2YT medium

Bacto tryptone	16 g
yeast extract	10 g
NACl	5 g
Distilled water	1000 ml

For selection of pKS recombinant selection, IPTG (0.1 ml) and X-Gal (0.8 ml) were added to 250 ml agar before pouring the plates.

#### A1.4 *E.coli* minimal medium (CHS Minimal medium)

##### Solution 1

Agar (Oxoid No.1)	15 g
Distilled H <sub>2</sub> O	600 ml

##### Minimal salts solution (5X)

KH <sub>2</sub> PO <sub>4</sub>	52.5 g
K <sub>2</sub> HP <sub>4</sub>	22.5 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	45.0 g
Na-citrate	2.5 g
Distilled H <sub>2</sub> O	to 1000 ml

This solution was stored over chloroform at 4°C.

##### Solution 2

Minimal salts solution (5X)	200 ml
Distilled H <sub>2</sub> O	200 ml

The agar and salt solutions are autoclaved separately, cooled to 50°C, combined, and then the remainder of the constituents added.

Glucose (20 %, w/v)	10 ml
MgSO <sub>4</sub> (20 %, w /v)	1 ml
Vitamin B1 (0.5 %, w/v)	1 ml

## **A2 Media additives**

Media were cooled to 50°C before addition of antibiotics, XGal, XP or IPTG.

### **A2.1 Antibiotics**

Antibiotic stock solutions were as follows:

Ampicillin	100mg/ml water
Chloramphenicol	20 mg/ml ethanol (96%)
Tetracycline	15 mg/ml ethanol (50%)

All antibiotics were filter sterilized and stored at -20°C, except for Tc which was always made fresh.

### **A2.2 IPTG (isopropyl-β-D-thio-galactopyranoside)**

IPTG (100 mM)	23.4 mg
Distilled water	1 ml

The solution was stored as aliquots at -70°C.

### **A2.3 X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside)**

X-Gal (2% w/v)	0.2 g
Dimethylformamide	10 ml

The solution was stored at -70°C.

## **A.3 Buffers and solutions.**

### **A3.1 Bradford solution**

Coomassie Brilliant Blue (G250)	100 mg
Ethanol (95%)	50 ml

Dissolve, then add 100 ml phosphoric acid (85 %). Dilute to final volume of 1 l.

Filter through Whatman GF/C filter paper. Store in dark bottle.

**A3.2 DNA sample loading solutions**

For agarose gels: Sucrose (8%, w/v)  
 Bromophenol blue (0.04%, w/v)  
 EDTA (10 mM)

For acrylamide gels: Formamide (99%, v/v)  
 Bromophenol blue (0.01%, w/v)  
 xylene cyanol (0.01%, w/v)

**A3.3 Ethidium Bromide solution.**

(2.7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide). A solution of 10 mg/ml in distilled water was made and stored in a dark bottle.

**A3.4 Isopropanol (salt saturated).**

Isopropanol was saturated with aqueous 5 M NaCl, 10 mM Tris- HCl and 1 mM EDTA, pH 8.

**A3.5 Phenol (TE saturated)**

Phenol (200 g, Merck) was melted at 65°C and 0.3 g of 8-hydroxyquinoline was added. The phenol was extracted 3 times with TE (10X) or until the the pH of the aqueous phase was approximately pH 7.6. The phenol was stored under TE (1X) at -20°C.

**A3.6 Sodium acetate (3M, pH 5.2)**

Sodium acetate.3H<sub>2</sub>O 4.08 g  
 Distilled water to 10 ml  
 Adjust pH with glacial acetic acid. Autoclave.

**A3.7 SSC (20X)**

NaCl (3M)  
 Sodium citrate (0.3M)  
 Distilled water  
 Adjust pH to 7 with NAOH (10N). Autoclave.

**A3.8 Tris-acetate buffer (50X).**

Tris base 242 g  
 Acetic acid 57.1 ml  
 EDTA (0.5M pH 8.0) 100 ml  
 Distilled water to 1000 ml

**A.3.9 CTAB/NaCl solution**

NaCl 4.1 g  
 CTAB 10 g  
 Distilled water 80 ml  
 Heat at 65°C to dissolve. Make up to 100 ml.

**A4. Buffers for the  $\tau$ -glutamyl transferase assay.**Stock solutions:

1 M Imidazole buffer	6.808 g/ 100 ml
0.8 M Hydroxylamine-HCl	5.560 g/ 100 ml
0.1 M MnCl <sub>2</sub>	1.979 g/ 100 ml
0.28 M Potassium arsenate	5.040 g/ 100 ml
0.2 M glutamine	0.292 g/ 10 ml
0.1 M ADP (sodium salt)	0.017 g/ ml

Resuspension buffer:

The buffer was made according to the following table and stored at 4°C.

Stock solution	per 200 ml	Final conc.
1 M Imidazole pH7.15	4 ml	20 mM
1 M MgCl <sub>2</sub> . 6H <sub>2</sub> O	2 ml	10 mM
14 M $\beta$ -mercaptoethanol	28 $\mu$ l	2 mM

Assay reaction buffer (5X)

A fresh assay reaction mixture was prepared using the following stock solutions:

Stock solution	per 20 ml	Final conc.
1 M Imidazole	3.71 ml	185 mM
0.8 M Hydroxylamine	0.61 ml	24.5 mM
0.1 M MnCl <sub>2</sub>	74 $\mu$ l	0.37 mM
0.28 Potassium arsenate	2.47 ml	35 mM
0.28 M ADP (sodium salt)	247 $\mu$ l	3.5 mM
Distilled water	12.9 ml	-

Termination reaction solution:

FeCl <sub>3</sub> . 6H <sub>2</sub> O	55 g
Trichloroacetic acid (TCA)	20 g
HCl (concentrated)	21 ml
Distilled water	to 1000 ml

**A5 Solutions for DNA hybridization analysis**Buffer 1

NaCl	150 mM
Maleic Acid	100 mM

Adjust to pH 7.5 with solid or concentrated NAOH. Autoclaved.

Buffer 2

1% blocking reagent (Boehringer Mannheim), diluted in buffer 1.

Buffer 3

Tris-HCl pH 9.5	100 mM
NaCl	100 mM
MgCl <sub>2</sub>	50 mM

Standard Hybridisation solution

SSC	5X
N-lauroylsarcosine	0.1%
SDS	0.02%
Blocking reagent	1%

2X Washing solution

2XSSC  
0.1% SDS

0.1X Washing solution

0.1XSSC  
0.1% SDS

**A6 Solutions required for Northern Dotblotting analysis**DNase Buffer

NaAc, pH 4.5	200 mM
MgCl <sub>2</sub>	100 mM
NaCl	100 mM

10XMOPS

Morpholinopropanesulphonic acid	200 mM
Sodium acetate	50 mM
EDTA pH 7.0, autoclaved.	10 mM

5X Formaldehyde Gel-running buffer

MOPS, pH 7.0	0.1 M
sodium acetate	40 mM
EDTA, pH 8.0	5 mM

Formaldehyde gel loading buffer

Glycerol	50%
EDTA pH 8.0	1 mM
bromophenol blue	0.25%
xylene cyanol FF	0.25%

**Appendix B**  
**General Techniques**

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## Appendix B

### General Techniques

#### B.1 Standard Methods

##### B.1.1 Small scale isolation of plasmid DNA (miniprep)

Plasmid was isolated from a 5 ml overnight culture (LB + Ap, 100 µg/ml) as described by Ish-Horowitz and Burke (1981). Cells from a 1.5 ml sample of the culture were harvested by centrifugation in an Eppendorf microfuge tube for 1 min. The pellet was resuspended in 200 µl Solution 1 (50 mM glucose; 25 mM Tris-HCl, pH 8.0), incubated for 5 min at room temperature, and then 400 µl of Solution II (0.2 M NaOH, 1 % (w/v) SDS) was added. The sample was vortexed briefly and placed on ice for 5 min, before the addition of 150 µl ice-cold Solution III (5 M KOAc, pH 4.8). The sample was gently mixed, and, after 5 min on ice, cellular debris and denatured chromosomal DNA were pelleted by centrifugation for 5 min. The supernatant (450 µl) was removed to a fresh tube and sedimented by centrifugation with two volumes of 95% ethanol for 5 min. The pellet was resuspended in TE (150 µl), sodium acetate (15 µl, 3 M, pH 4.8) and two volumes 95% ethanol were added and the sample held at -70°C for 5 min. The DNA was pelleted by centrifugation for 15 min, washed with 70% ethanol, dried and resuspended in 50 µl TE buffer.

##### B.1.2 Large scale isolation of plasmid DNA (maxiprep)

A 200 ml culture was grown overnight at 37°C in the presence of the appropriate antibiotic. The cells were harvested by centrifugation at 6 000 *g* for 5 min and then resuspended in 4 ml Solution I. After 5 min at room temperature 8 ml Solution II was added, and the mixture was kept on ice for 5 min, before the addition of 6 ml ice cold Solution III. After a further 5 min on ice the cellular debris was removed by centrifugation at 12 000 *g* for 10 min. An equal volume of isopropanol was added to the supernatant and the DNA was precipitated by centrifugation at 27 000 *g* for 15 min. The pellet was washed with 70% ethanol and resuspended in 4.2 ml TE buffer, and purified by isopycnic CsCl-EtBr ultracentrifugation (Maniatis et al. 1982). The plasmid

preparation was prepared for ultracentrifugation by the addition of CSCl (1 mg/ml) and EtBr (0.5 ml of a 10 mg/ml stock). The solution was centrifuged at 27 000 g for 15 min to precipitate any remaining protein debris. The refractive index of the supernatant was adjusted to 1.395, the sample sealed in Beckman Quickseal ultracentrifuge tubes and centrifuged for 12 h at 55 000 rpm at 15°C in a Beckman Vti 65.21 rotor. The plasmid DNA band was visualized by long wave UV light (350 nm), and removed in the smallest volume possible. The EtBr was removed by extraction (3 times) with equal volumes of NaCl-saturated isopropanol. The DNA was precipitated from the CSCl solution by the addition of two volumes of water followed by an equal volume of isopropanol, and centrifugation in an Eppendorf microfuge for 15 min. The pellet was resuspended in 200  $\mu$ l TE buffer and the concentration was determined spectrophotometrically by measuring the absorbance of 10  $\mu$ l (diluted in TE) between 220 and 310 nm. The concentration was determined by using the relationship  $A_{260} = 1$  for 50  $\mu$ g/ml double-stranded DNA.

#### **B.1.3 Extraction of chromosomal DNA from *B. fibrisolvens***

The method described by Strydom et al. (1986), a modification of the method of Marmur (1961) was used (described in Chapter 2).

#### **B.1.4 Restriction endonuclease digestion**

Restriction digests were carried out as described by Maniatis et al. (1982), using restriction buffers salt requirements of the particular enzyme as specified according to the manufacturers instructions (Boehringer Mannheim). Digestion volumes were routinely 20  $\mu$ l containing 300-500 ng DNA and one unit of restriction enzyme. Digestions were incubated at recommended temperatures for 1 h. For electrophoretic analysis, the digestions were terminated by the addition of 5  $\mu$ l DNA loading solution (Appendix A) to the 20  $\mu$ l digestions. If the digestion products were to be ligated, or filled in before ligation, they were purified by a phenol-ether extraction.

### **B 1.5 Agarose gel electrophoresis**

Agarose gel electrophoresis was carried out using a horizontal submerged gel system. Tris-acetate (Appendix A) were used routinely. Sigma type II agarose was used at varying concentrations (0.5%-1.2%). The amount of DNA loaded/lane varied with the sizes and number of fragments, but usually about 300 ng of plasmid DNA was used. The gels were electrophoresed at 2 V/cm for 16 h. Gels were stained in electrophoresis buffer containing EtBr (0.5 µg/ml) for 15-30 min. DNA bands were visualised using a 254 nm transilluminator. A 310 nm transilluminator was used if the DNA was to be recovered from the gel.

Gels were photographed using a Polaroid CU-5 Land camera fitted with a red filter and a fixed focal length attachment. Polaroid type 667 film (ASA 3 000) was used with an exposure time of 1-2 sec at f4.7. If a negative was required then a Polaroid type 665 film (ASA 64) with an exposure of 120-140 sec at f4.7 was used.

### **B 1.6 DNA ligation reactions**

DNA ligation reactions were of two basic types: recircularization of plasmids for the isolation of deletion clones (use low DNA concentrations, 1 pmole DNA/ml) and recombination reactions, for example in subcloning (use 5 pmole DNA/ml). DNA concentration was calculated using the formula  $1\text{pmole} = (0.662 \times \text{kb})\mu\text{g}$ . Vector and insert DNA were added to the ligation reactions at a molar ratio of 1:2. Ligation reactions containing DNA, ligation buffer (Boehringer Mannheim) and water to the required volume were performed in sterile centrifuge tubes. Sticky-end ligations were performed at room temperature for 3 h or at 15°C overnight using 0.1-0.25 U of ligase whereas blunt-end ligations were performed at room temperature for 3-20 h using 20-100X more ligase.

### **B 1.6 Subcloning protocol**

The rapid subcloning protocol of Struhl (1985) was used. DNA fragments were separated by electrophoresis through low melting point (LMP) agarose (1%) (Seaplaque<sup>R</sup>) in TAE buffer (50mM pH 8.2, no EDTA, no EtBr). Gels were stained after electrophoresis, viewed at 310 nm UV light, and the bands excised using sterile

scalpel blades in as small a volume as possible. The gel fragments were melted at 70°C and added in the required ratio of insert to vector. Ligations were incubated at room temperature for 3 h and used to transform *E. coli* competent cells.

### **B 1.7 Preparation of competent cells.**

Competent cells were prepared according to the method of Dagert and Ehrlich (1979) or according to the rubidium chloride method of Armitage (1988).

### **B.1.8 Nucleotide sequencing**

**Primer annealing reaction.** The supercoiled DNA (6-10  $\mu$ g, in TE buffer) was diluted to a final volume of 20  $\mu$ l in distilled water. Alkaline denaturation in 0.2 N NaOH (5 min at room temperature) was followed by the addition of 5  $\mu$ l of 3 M sodium acetate (pH 5.2), 25  $\mu$ l of distilled water and 150  $\mu$ l of ethanol. This mixture was chilled to -70°C, centrifuged at 4°C for 20 min in a microfuge and washed with 200  $\mu$ l of ethanol (70%). The DNA pellet was dried and resuspended in a final volume of 10  $\mu$ l of sequencing buffer (40 mM Tris-HCl, pH 7.5; 20 mM MgCl<sub>2</sub>; 50 mM NaCl) and 12 ng of primer. This mixture was annealed for 15 min at 40°C immediately prior to sequencing.

**Sequencing reactions.** DNA sequencing was done by the dideoxy chain termination method of Sanger *et al.* (1977) according to the protocol of Tabor and Richardson (1987), using T7 DNA polymerase and a "Sequanase" sequencing kit supplied by the US Biochemical Corporation, Cleveland, Ohio. The DNA chain was radiolabelled with [ $\alpha$ -<sup>35</sup>S] dATP (1200 Ci/mmol; Amersham).

**Gel electrophoresis and autoradiography.** The sequencing reactions were analyzed on standard 6% denaturing acrylamide urea sequencing gels. The composition and running conditions of the gels were as described in the Amersham M13 Sequencing Handbook. After electrophoresis the gels (0.2mm thick) were dried onto Whatman No. 3 filter paper using a Dual Temperature Slab Gel Dryer (Model 1125B; Hoefer Scientific Instruments, San

Francisco). Gels containing  $^{35}\text{S}$ -labelled DNA were placed under XAR-5 autoradiographic film and exposed for 1-2 days. The autoradiographs were developed using Kodak GBX X-ray developer and fixer.

### **B 1.9 DNA/RNA alkali blotting procedure**

DNA fragments resolved by agarose gel electrophoresis were transferred to a Hybond N+ hybridization membrane (Amersham) by the protocol of Reed and Mann (1985). After electrophoresis the gel was rinsed in 2 volumes of HCl (0.25 M) for 20 min at room temperature with gentle agitation, followed by a brief rinse in distilled water. The gel was then placed onto 2 sheets of Whatman 3 MM filter paper (wetted with 0.4 N NaOH, and placed on top of a small platform in a plastic box, so that the filter paper formed a wick), and was flooded with 50-100 ml of 0.4 N NaOH. A sheet of Hybond N+ (prewetted with water) was placed on top of the gel, and any air bubbles were removed. Three sheets of Whatman 3 MM filter paper, wetted in 0.4 N NaOH, were laid onto the membrane, followed by a 4 cm thick layer of absorbant paper. A light weight was placed on top of this, and left overnight. After transfer, the membrane was rinsed briefly with gentle agitation in 2 x SSC (Appendix A). The membrane was then ready for hybridization or could be wrapped in Saran wrap and stored at 4°C.

### **B 1.10 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE procedure. Discontinuous SDS-PAGE was done according to the method of Laemmli (1970), using a Hoefer SE600 vertical slab electrophoresis unit (Hoefer Scientific Instruments, San Fransisco, CA, USA). The 1.5 mm thick gel spacers were used. The resolving gel was prepared and degassed before pouring. Water was layered on the gel to promote a sharp interface. After the gel had polymerized (30 min), the water was removed by rinsing with the stacking gel buffer, and the stacking gel was cast. Samples were prepared in sample treatment buffer (Appendix A) and placed in a boiling waterbath for 2 min before being loaded onto the gel. Electrophoresis was continued at 35 mA (constant

current)/gel until the dye front migrated to the end of the gel (four to five hours).

After electrophoresis, the gels were stained in Page C-90 staining solution with gentle agitation, destained and dried.

The acrylamide gels (15%) were prepared as follows:

Solution	Resolving gel	Stacking gel
Acrylamide solution	18.75 ml	2.66 ml
1.125 M Tris-HCl pH 8.8	8.35 ml	-
0.375 M Tris-HCl pH 6.8	-	-
10% SDS	0.25 ml	0.125 ml
Distilled water	18.75 ml	5.55 ml
Ammonium persulphate	0.25 ml	0.3 ml
TEMED	25 $\mu$ l	20 $\mu$ l

#### **SDS-PAGE solutions:**

##### Acrylamide solution

Acrylamide	40.0 g
Bis-acrylamide	0.2 g
Distilled water	to 100 ml

Filter through Whatman's paper (No.1) and stored in a dark bottle at 4°C.

##### Ammonium persulphate (10% w/v)

A fresh solution was made immediately before use.

##### Reservoir buffer (10x)

Tris base	8 g
Glycine	30.0 g
SDS (0.1% w/v)	0.2 g
Distilled water	to 2000 ml

The pH should be approximately 8.5.

##### SDS (10%)

SDS	50 g
Distilled water	to 500 ml

Page C-90 staining solution

Page C-90 (0.1% w/v)	1 g
Phosphoric acid	final 3%
Distilled water	1000 ml

The solution was stirred vigorously to dissolve the dye and then filtered through Whatman's paper (No. 1).

Destain solution

Acetic acid (7%)	70 ml
Distilled water	1000 ml

**B 1.11 Southern Hybridisation analysis using DIG-labelled probes**

Nylon hybond N+ membranes were probed using DIG-labelled probes strictly according to the manufacturers instructions (Boehringer-Mannheim). Detection of the DIG-labelled probe was done by chemiluminescent detection with CSPD<sup>R</sup> exactly according to the procedure outlined in the "Boehringer Mannheim DIG system user's guide" (1994).

**B 1.12 The gamma-glutamyl transferase assay (GGT).**

The method of Bender *et al* (1977) was used. Cells were prepared as described in the text (Chapter 2) and resuspended in 0.5 ml resuspension buffer (Appendix A). Cells were disrupted using CTAB/NaCl method (refer Appendix A), and the cell extract was placed on ice until used. Samples (0.1 ml) were added to 0.4 ml GGT-reaction buffer (Appendix A), and equilibrated at 37°C for 5 min. The reaction was initiated by the addition of 50  $\mu$ l glutamine (0.2 M). After 15 min at 37°C, the reaction was stopped by the addition of 1 ml of "stop mix" (Appendix A) and the absorbance determined spectrophotometrically at 540 nm. A standard curve was constructed using L-glutamine acid gamma-monohydroxamate (GAMH; Sigma G-2253) as a substrate, gave an absorbance at 540 nm of 0.755 for 1 micromole of glutamyl hydroxamate. GS activity was expressed as  $\mu$ moles glutamyl hydroxamate produced per min per mg protein.

**B.1.13 Determination of protein concentrations**

Protein concentrations were determined by the method of Bradford (1976). Assays were performed in triplicate using disposable cuvettes. The reaction contained protein solution (0.1 ml) and 2 ml Bradford solution (Appendix A). The mixture was left for 5 min at room temperature and the optical density monitored at OD<sub>595</sub>. Protein concentrations were calculated using a standard curve (BSA fraction V; 10-200 µg/ml). Protein samples were diluted such that OD<sub>595</sub> did not exceed 0.8.

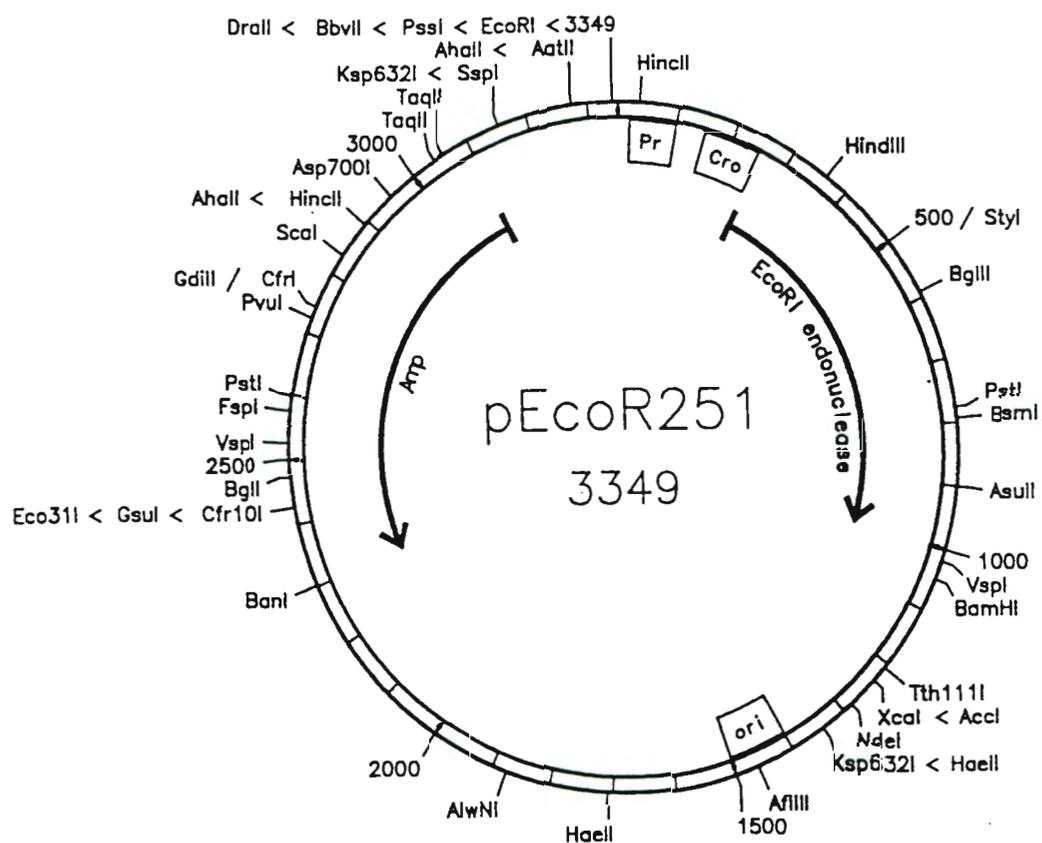
## Appendix C

**One and three-letter codes used for amino acids.**

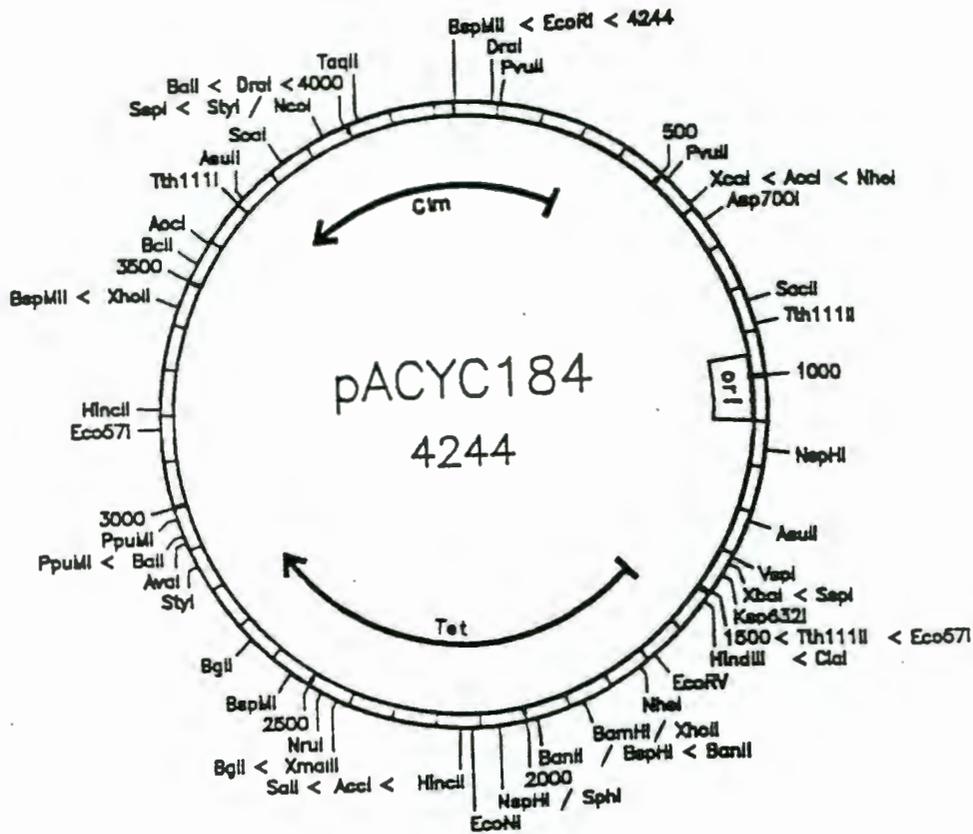
Amino acid	Codes		Code	Amino acid
Alanine	Ala	A	A	Alanine
Arginine	Arg	R	C	Cysteine
Asparagine	Asn	N	D	Aspartic acid
Aspartic acid	Asp	D	E	Glutamic acid
Cysteine	Cys	C	F	Phenylalanine
Glutamine	Gln	Q	G	Glycine
Glutamic acid	Glu	E	H	Histidine
Glycine	Gly	G	I	Isoleucine
Histidine	His	H	K	Lysine
Isoleucine	Ile	I	L	Leucine
Leucine	Leu	L	M	Methionine
Lysine	Lys	K	N	Asparagine
Methionine	Met	M	P	Proline
Phenylalanine	Phe	F	Q	Glutamine
Proline	Pro	P	R	Arginine
Serine	Ser	S	S	Serine
Threonine	Thr	T	T	Threonine
Tryptophan	Trp	W	V	Valine
Tyrosine	Tyr	Y	W	Tryptophan
Valine	Val	V	Y	Tyrosine







A restriction map of **pEcoR251** (Zabeau and Stanley, 1982). This is a positive selection vector using insertional inactivation of the lethal *EcoRI* gene.



A restriction map of pACYC184 (4244 bp, Chang and Cohen, 1978). This is a low to intermediate copy number plasmid compatible with ColE1- or pMB1-derived plasmids. Cloning sites for inactivation are *Bam*HI, *Eco*RI and *Sal*I.

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