Role and regulation of glutamate dehydrogenase activity in *Bacteroides fragilis* bf1

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CONTENTS

ABSTRACT

Bacteroides fragilis is a gram-negative obligate anaerobe that exists normally in the human large intestine. It is, however, capable of causing a variety of infections once outside of this environment. Previous studies have suggested that the regulation of the ammonia assimilatory pathways in *B. fragilis* occurs in a manner distinct from that found in other gram-negative bacteria, with glutamate dehydrogenase (GDH) activity serving as the primary route of ammonia incorporation in both ammonia-limited and ammonia-excess environments. While these studies showed that *B. fragilis* produces two distinct GDH enzymes, one NAD(P)H-dependent (GdhA) and the other NADH-dependent (GdhB), their specific roles in nitrogen assimilation were not clearly resolved. In this dissertation, the physiological and molecular factors affecting the regulation of GDH activity in *B. fragilis* were examined, with a view to extending our understanding of nitrogen assimilation in this organism.

Physiological analysis revealed that activities of the two *B. fragilis* GDH enzymes were differently regulated in response to both the nitrogen source (ammonia or peptides) and availability in the growth medium. GdhA activity was present in *B. fragilis* cells during growth in both ammonia-limited and ammonia-excess environments. Its activity was, however, found to be greatest in ammonia-limited cultures, and was down-regulated when peptides replaced ammonia as the sole nitrogen source for growth. GdhB activity, by contrast, was up-regulated following growth with peptides, but not ammonia, as the sole nitrogen source. These findings, taken together, suggest that the two GDH enzymes fulfill distinct functions in nitrogen nutrition in *B. fragilis*.

The *gdh*B structural gene was cloned via heterologous complementation of an *E. coli* glutamate auxotroph. The gene was found to encode a deduced polypeptide of 445 amino acid residues with homology to previously studied GDH enzymes. Northern blot analysis showed that the *gdh*B gene is transcribed as a monocistronic 1.5 kb mRNA, and that the regulation of GdhB activity occurs at the level of transcription. The transcriptional regulation of the *gdh*B gene was further examined by primer extension analysis, and transcriptional fusions using a xylosidase reporter gene. The results demonstrated that *gdh*B gene expression occurs from an inducible promoter located upstream of the *gdh*B gene, with transcription being induced by the increased availability of peptides in the growth medium. Deletion

analysis of the *gdh*B promoter region resulted in the identification of several remote sequence-elements that may be involved in the transcriptional activation of this gene.

The *gdh*B gene was expressed in *E. coli*, and the recombinant enzyme (rGdhB) purified. Immunoblot analysis, using antiserum raised against the rGdhB enzyme, identified a single immunoreactive protein in cell free extracts obtained from *B. fragilis* grown with high concentrations of organic nitrogen. Experiments designed to establish whether the GdhB enzyme was regulated at the post-translational level, showed that the activity of the pre-existing enzyme was modulated by rapid inactivation in response to a sudden decrease in peptide availability in the growth medium. Analysis of fractionated *B. fragilis* cells demonstrated that GdhB activity was predominantly associated with the membrane fraction of the cell. The presence of GdhB at the *B. fragilis* cell-surface was further confirmed by immunogold labelling of *B. fragilis* cells, followed by electron microscopy. The cell-surface localisation of this enzyme may facilitate the utilisation of glutamate, derived from peptides incorporated from the external milieu.

ABBREVIATIONS

A	adenosine
aa	amino acids
Ap	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
bp	base pair(s)
C	cytosine
C-	carboxy-(terminal)
Cm	chloramphenicol
CFE	cell free extract
Da	Dalton
DIG	digoxygenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
FAD	flavin mononucleotide
G	guanosine
g	gram or standard gravitational acceleration, depending on context
ΔG	Gibbs free energy change
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
h	hour
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kilodalton
K_m	Michaelis constant
l	litre(s)
LB	Luria-Bertani medium
m	metre(s)

min	minute(s)
mRNA	messenger RNA
MM	minimal media
MW	molecular weight
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
n	nano
N-	amino-terminal
nt	nucleotide(s)
NCBI	National Centre for Biotechnology Information
nm	nanometres
OD_x	optical density at x nm
ORF	open reading frame
ori	origin of replication
p	plasmid
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PEP:PTS	PEP-dependent sugar phosphotransferase system
r	(superscript) resistant
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
T	thymidine
TAE	Tris-acetate-EDTA electrophoresis buffer
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
w/v	weight per volume
YT	yeast tryptone broth
α	alpha
β	beta
Δ	delta

λ	lambda
μ	micro
::	novel joint fusion

CHAPTER 1

GENERAL INTRODUCTION

Contents

1.1 Introduction to *Bacteroides fragilis*

The human colon harbours a large and diverse microbial population that forms part of the normal microflora. The colonic environment is characterised by its relative lack of oxygen and the microbial population is, accordingly, comprised mainly of anaerobic bacterial species (Moore *et al.*, 1978), one of the numerically predominant components of which are members of the genus *Bacteroides* (Macy and Probst, 1979; Sghir *et al.*, 2000; Suau *et al.*, 1999). The interest in these organisms is manifold, and stems from their involvement in various facets of the physiology of their host, their participation in anaerobic infections and their role in the transfer of antibiotic-resistant determinants among the colonic bacteria. The primary objectives of this research were to further characterise the primary enzymes of nitrogen assimilation in *Bacteroides fragilis*, to identify the genes encoding these proteins, and to analyse the regulatory mechanisms controlling their modulation in response to nitrogen source and availability. This introductory chapter, therefore, aims to provide the context within which this work was conducted, and covers several background topics, including the phylogeny, ecological niche and pathogenesis of the *Bacteroides*. In addition, our current understanding of bacterial nitrogen metabolism as developed from studies conducted primarily on the enteric bacteria will be discussed in relation to that found in *B. fragilis*.

1.1.1 Phylogeny of the *Bacteroides*

Since their original description, the classification and nomenclature of the *Bacteroides* has undergone a number of changes. Formerly, the genus was defined as encompassing a group of gram negative, obligately anaerobic, non spore forming, rod-shaped bacteria (Holdeman *et al.*, 1984). This grouping was, however, found to be quite variable since its rather broad definition allowed for many species with only scant similarity to be grouped together within the genus. This taxonomic diversity was reflected in the results of several studies, which revealed a number of differences in the biochemical and physiological characteristics of members of the genus (reviewed by Olsen, 1994). The genealogical heterogeneity amongst the *Bacteroides* was subsequently confirmed by the application of nucleic-acid based methods for bacterial classification, such as DNA-DNA hybridisation (Johnson and Ault, 1978), oligonucleotide cataloguing (Paster *et al.*, 1985) and RNA sequence analysis (Van den Eynde *et al.* 1989; Paster *et al.*, 1994). On this basis, the genus *Bacteroides* was reclassified to include only those saccharolytic, non-pigmenting species such as *B. fragilis* and related organisms, which are confined mainly to the colon (Shah and Collins, 1989). Species predominating in the oral cavity and rumen were reassigned to the genera *Prevotella* and

Porphyromonas, respectively (Shah and Collins, 1988; 1991). Notwithstanding their phylogenetic divergence, these latter two genera are more closely related to the *Bacteroides* than other taxa in the domain Bacteria, and together with several other genera form part of the family *Bacteroidaceae*. Along with the *Cytophaga* and *Flavobacteria,* the *Bacteroidaceae* comprise one of the ten major phyla in the bacterial line of descent (Paster *et al.*, 1985; Woese *et al.*, 1985). This particular phylum is thought to have branched off from the primary bacterial phylogenetic lineage early during the course of evolution and, as a result, contains members that are only distantly related to the majority of other gram negative organisms (Woese, 1987).

1.1.2 General characteristics of the *Bacteroides*

Currently, members belonging to the *Bacteroides* have been assigned based on the following characteristics: They are gram negative, obligately anaerobic, non-spore forming bacilli that possess a DNA G+C content of 39-48 mol%. *Bacteroides spp.* are saccharolytic and produce acetate and succinate as the major end products of glucose metabolism. They contain malate, glutamate, glucose-6-phosphate and 6 phosphogluconate dehydrogenases. Their major respiratory quinones are the menaquinones MK-10 or MK-11, or both. The *Bacteroides* produce sphingophospholipids, which constitute 40-70% of the total lipids, as well as 3-hydroxylated and non-hydroxylated fatty acids with a carbon chain length of 15 atoms. The latter consists predominantly of straight chain iso- and anteiso-methyl branched-chains. The presence of meso-diaminopimelic acid is typical of their cell wall peptidoglycan (Shah and Collins, 1989).

1.2 *Bacteroides* as commensal organisms

The colonic microflora is estimated to consist of ~400 hundred distinct bacterial species whose numbers range between 10^{11}-10^{12} organisms per g (wet weight) of large bowel contents (Moore *et al.*, 1988; Savage, 1977). Together, these organisms account for ~30% of the volume of the contents of the human colon. Since the colon is largely devoid of oxygen, nearly all of the colonic bacteria are strict anaerobes, with facultative anaerobes accounting for only 0.1-1% of the bacterial population. The *Bacteroides* are considered to be one of the numerically predominant genera in the colon, and comprise ~30% of the culturable bacterial species isolated from this environment (Macy and Probst, 1979; Marteau *et al.* 2001). The nutritional requirements of the *Bacteroides*, together with other intestinal microorganisms, are met through their utilisation of a wide range of dietary and host derived compounds that enter

the colon (Cummings, 1996; Cummings and Englyst, 1987). An estimated 10-60g of carbohydrate-containing, and 5–20g nitrogen-containing compounds are believed to enter the human colon per day, and the makeup of the microflora is, in part, determined by the ability of the different bacterial species to compete for these nutrients (Cummings and Macfarlane, 1997). The *Bacteroides* contain both saccharolytic and proteolytic species, and they are generally considered to make an important contribution to the utilisation of the organic compounds entering the human colon. In addition, their large numbers within the colon suggests that they are able to compete successfully with other bacterial species for the utilisation of these substrates.

1.2.1 Metabolism of carbohydrates

The majority of intestinal bacteria are saccharolytic and utilise carbohydrates that escape digestion in the upper gut as a source of carbon and energy (Salyers, 1979). Owing to the fact that the majority of simple sugars (mono- and disaccharides) ingested by the host are absorbed in the small intestine, they do not reach the colon in appreciable amounts. The *Bacteroides* are, nevertheless, capable of utilising these compounds when they are available (Blatch *et al.*, 1993; Hylemon *et al.*, 1977; Scholle *et al.*, 1990). By contrast, complex plant cell wall polysaccharides (eg. cellulose and xylan) and resistant starch (eg. amylose, amylopectin and pullunan) are impervious to hydrolysis by human digestive enzymes (Cummings, 1996). As such, they comprise the primary source of carbohydrates entering the colon, where they may serve as a fermentable carbon source for the growth of the colonic microorganisms (Englyst *et al.*, 1987; Salyers *et al.*, 1979). The *Bacteroides* are believed to contribute to polysaccharide digestion in the intestine through the production of, *inter alia,* amylase (Degnan, *et al.*, 1997a), pullunase (Smith and Salyers, 1989), xylanase (Whitehead and Hespell, 1990) and glycosidase (Berg *et al.*, 1980) activities. In addition, host-derived compounds such as mucin, muco-polysaccharides, chondroitin sulphate and hyaluronic acid can also act as substrates for microbial growth, and the *Bacteroides* produce several enzymes such as chondroitinases (Linn *et al.*, 1983), neuraminadase (Russo *et al.*, 1990) and glycosidases (Macfarlane and Gibson, 1991) that facilitate the digestion of the carbohydrate moieties of these compounds. The short chain fatty acids (SCFA), acetate, butyrate and propionate, are the major end products of carbohydrate fermentation in the colon (Cummings, 1984). These SCFAs are absorbed through the large intestine and subsequently used as an energy source by the host, where they may provide up to 540 $kcal.d^{-1}$, thus contributing significantly to the host's daily energy requirements (Mcneil, 1984).

The utilisation of carbohydrates by the *Bacteroides* has been most extensively studied in *Bacteroides thetaiotaomicron*. It has been shown that a number of proteins associated with polysaccharide utilisation in this species are cell associated, as opposed to extracellular (Anderson and Salyers, 1989; Kotarski *et al.*, 1985). For example, starch utilisation by *B. thetaiotaomicron* is mediated by an outer membrane receptor complex consisting of several proteins (Reeves *et al.*, 1997; Shipman *et al.*, 2000). In this organism, starch is thought to be initially bound to the cell surface and subsequently imported into the periplasm where hydrolysis into its constituent mono- or disaccharides is presumed to occur. This specific mechanism may be advantageous in the colonic ecosystem, since it allows for the seclusion of the end products of polysaccharide hydrolysis, which may otherwise be lost owing to diffusion or competition with other intestinal microbes.

The physiological mechanism by which the preferential uptake of carbohydrates by the *Bacteroides* occurs is not clear. The catabolite regulation of carbohydrate utilisation has, nevertheless, been demonstrated to occur in *B. ovatus* and *B. thetaiotaomicron* (Macfarlane *et al.*, 1990; Degnan and Macfarlane, 1995). In general, high levels of polysaccharidase activity are observed during growth with a fermentable carbohydrate source, such as starch (Degnan *et al.*, 1997b) or chondroitin sulphate (Salyers and Kotarski, 1980). During growth on simple sugars, however, the polysaccharidase activities are repressed, even in the presence of its particular substrate. This regulation is likely to differ from the mechanism of catabolite regulation found in many other bacteria though, since the *Bacteroides* do not possess cAMP and adenylate cyclase (Cotta *et al.*, 1994; Hylemon and Phibbs, 1974).

1.2.2 Metabolism of nitrogenous compounds

Besides contributing to the turnover of carbohydrates in the colon, the microflora play an integral role in the metabolism of nitrogenous substrates entering the large intestine (Fuller and Reeds, 1998; Wrong, 1988). The principal sources of nitrogen occurring in the gut are in the form of proteins and peptides, or ammonia (Cummings *et al.*, 1979; Summerskill and Wolpert, 1970). Both dietary and host derived proteins, such as albumin, collagen, elastin, and the pancreatic enzymes (trypsin and chymotrypsin) can potentially serves as substrates for the colonic bacteria. For proteins to be utilised, they must first be degraded to peptides or amino acids, a process that is facilitated by the action of bacterial proteases (Macfarlane *et al*, 1986). After the hydrolysis of proteins into shorter oligopeptides, they are hydrolysed by peptidases into smaller peptides and amino acids that can be taken up bacteria (Payne and

Smith, 1994). Peptides that have been assimilated are subsequently hydrolysed to yield free amino acids that can be incorporated either directly into microbial protein, or catabolised with the resulting formation of branched or short chain fatty acids and ammonia. The ammonia thus generated can be used for the resynthesis of amino acids intracellularly, or be secreted out of the cell. Significant amounts of ammonia are generated by bacteria growing on peptides and amino acids, and this is believed to account for the majority of ammonia occurring in the large intestine (Chacko and Cummings, 1988; Smith and Macfarlane, 1998). This ammonia can be subsequently utilised as a nitrogen source by other intestinal bacteria.

The *Bacteroides* are considered to be the predominant proteolytic bacteria in the large intestine, and are believed to play an important role in the degradation of proteins in the intestinal environment (Gibson *et al.*, 1989; Macfarlane *et al.*, 1986). *B. fragilis* produces at least three proteases, which posses specificity against several proteins such as casein, fibrinogen, trypsin and chymotrypsin (Chen *et al.*, 1995; Gibson and Macfarlane, 1988a, 1988b; Macfarlane and Macfarlane, 1991). Protease production by *B. fragilis* occurs constitutively. The levels of proteolytic activity are, however, dependent on growth conditions with the synthesis and secretion of proteolytic activity increasing several fold in response to nitrogen limitation (Gibson and Macfarlane, 1988a; Macfarlane *et al.*, 1992). Moreover, while studies of peptide utilisation by the colonic bacteria are limited, the *Bacteroides* have been reported to posses high dipeptidyl-peptidase activities, which is considered to be an important means of peptide catabolism in the human colon (Wallace and Mckain, 1997). Since nitrogen metabolism forms the main focus of this study, this topic is reviewed more extensively in later sections of this Chapter (Section 1.6).

1.2.3 Additional metabolic activities

Other than contributing to the fermentation of organic compounds in the large intestine, the colonic microflora also participate in a range of other metabolic activities that may impact upon the host. The intestinal microflora are considered to play an important role in the biotransformation of bile acids, as well as cholesterol and steroid hormones, that enter the colon (Baron and Hylemon, 1997). The primary bile acids synthesised by humans are cholic and chenodeoxycholic acids. These bile acids are secreted into the small intestinal lumen during digestion where they assist in the absorbtion and transport of lipids, from the small intestine, to the liver via the hepatic portal venous system. The vast majority of bile acids are recirculated in this manner. However, an estimated 5% escape reabsorbtion and enter the

colon where they are modified by the intestinal microflora. Approximately 20 different secondary bile acid metabolites can potentially be formed from the bacterial transformation of the primary bile acids via reactions that include deconjugation, dehydroxylation, dehydrogenation and epimerisation. The *Bacteroides* have been shown to express a number of enzymes, such as dehydrogenases, dehydroxylases and hydrolases, which are capable of modifying bile acids (Edenharder, 1984; Hylemon and Sherod, 1975; Kawamoto *et al.*, 1988; Stellwag and Hylemon, 1976). The ability to transform bile acids may be advantageous to the intestinal microbiota through their ability to provide growth substrates for these organisms, or due to the formation of compounds that inhibit the growth of competing bacteria (Baron and Hylemon, 1997). The deconjugation of the bile acids also benefits the host by allowing the bile acids to be reabsorbed and returned to the liver, where they begin their enterohepatic circulation again.

The *Bacteroides,* together with other members of the microflora, are also thought to benefit their host by preventing exogenous microorganisms, including potentially pathogenic organisms, from colonising the intestine (Van der Waaij *et al.*, 1983). The microflora are believed to contribute to colonisation resistance via four different mechanisms viz. competition for essential nutrients, competition for attachment sites, the creation of an inhibitory environment for the growth of other organisms, and the production of inhibitory compounds (Rolfe, 1997; Van de Waaij, 1987). The adherence of microorganisms to intestinal attachment sites is considered to be essential for the successful colonisation of the colon by the intestinal microflora, and transient organisms (Freter, 1978). The association of the intestinal microflora with the epithelial cells and mucous layer lining the intestine, however, impedes the ability of transient organisms to adhere to these surfaces, thus reducing the capacity of these organisms to colonise the intestinal tract and, therefore, cause disease. Many of the end products of bacterial metabolism, such as SCFAs and hydrogen sulphide, are also able to promote colonisation resistance by creating an adverse environment for the growth of exogenous organisms (Rolfe, 1997). SCFAs, which are formed as end products of carbohydrate fermentation, lower the pH and redox potential within the colon, factors that which have been shown to be inhibitory to growth of a wide range of microorganisms. In addition many of the intestinal microorganisms produce antibiotic-like compounds, such as bacteriocins, which inhibit the growth of potentially harmful microorganisms (Avelar *et al.* 1999; Portrait *et al*, 2000).

1.3 Pathogenicity of the *Bacteroides*

The *Bacteroides* are opportunistic pathogens and are able to cause infections if conditions suitable for their invasion, survival and colonisation exist (Duerden, 1994). These infections usually arise subsequent to the rupturing of the mucosal wall of the gastrointestinal tract, thereby allowing the colonic organisms to escape from the gut and to invade tissues in various body sites. Infections involving bacteria emanating from the colon are usually polymicrobial in nature, and consist of a mixture of obligately anaerobic species, or a combination of anaerobic and facultative organisms (Gorbach, 1982). In these cases, the bacteria typically develop a symbiotic relationship where they function synergistically in the infective process (Brook, 1994, Maclaren *et al.*, 1984). Since the peritoneal cavity, unlike the intestine, is highly oxygenated, the growth of anaerobes is initially limited. The initial stage of infection is, therefore, typified by the growth of facultative organisms, such as *E. coli,* which effect much of the early tissue destruction (Kelly, 1978; Verweij-Van Vught *et al.*, 1985). In addition, these organisms lower the oxygen potential of the infected area. As a result, these infected areas become oxygen deprived, allowing anaerobes such as the *Bacteroides* to become established, a process that culminates in these organisms taking over as the predominant members contributing to infection.

B. fragilis has been shown to be the *Bacteroides* species most commonly associated with diseases, which include bacteremia, abscess formation and soft tissue infections, and it is estimated to account for ~25% of all anaerobic bacteria isolated from clinical specimens (Patrick, 1993; Polk and Kasper, 1977). Considering that it is less numerous than other members of the genus present in the colon, it appears likely that *B. fragilis* produces a number of virulence factors which may account for its association with these infections. *B. fragilis* has the ability to produce a capsular polysaccharide, which is considered amongst the primary virulence determinants of this organism. The polysaccharide capsule is believed to contribute to virulence mainly through its ability to promote abscess formation (Finlay-Jones *et al.*, 1999; Onderdonk *et al.*, 1977). The polysaccharide capsule has been shown to have an unusual structure that consists of at least three separate high molecular weight polysaccharides termed, PSA, PSB and PSC (Coyne *et al.*, 2000). In animals, both PSA and PSB, in purified form, can induce intraabdominal abscesses formation. The presence of both positively and negatively charged groups on the repeating units of both PSA and PSB, has been shown to be essential for these particular polysaccharides to induce abscess formation (Tzianabos *et al.*, 1993). Analysis of *B. fragilis* mutants, defective in their ability to produce

either one of the three polysaccharides, have demonstrated that neither PSB or PSC alone is required to facilitate the formation of abscesses by *B. fragilis*. The synthesis of PSA, by contrast, has been demonstrated to be essential for this process, suggesting that it is the major virulence determinant in abscess formation by *B. fragilis* (Coyne *et al.*, 2001). In addition to promoting abscess formation, the polysaccharide capsule is thought to facilitate the adhesion of *B. fragilis* (Gibson *et al.*, 1998) as well as interfering with the phagocytic killing and clearance of bacterial cells (Onderdonk *et al.*, 1990). The adhesion of encapsulated *B. fragilis* to intestinal cells and mucous is also thought to be promoted by its expression of pili (Brook and Myhal, 1991), which may facilitate its entry into the peritoneal cavity relative to organisms found predominantly in the intestinal lumen.

B. fragilis is unusually aerotolerant, relative to other anaerobes, and can survive for extended periods of exposure to atmospheric oxygen (Tally *et al.*, 1975; 1977). Given that infection sites are aerobic during the initial stages of infection, this relative aerotolerance may contribute to virulence by allowing cells to survive exposure to oxygen until the redox potential of the site of infection has been reduced. Furthermore, the detoxification of reactive oxygen species that are generated by the host as part of its defence response may also be essential for its survival and proliferation. *B. fragilis* produces several enzymes that confer protection from reactive oxygen species. These include, amongst others, superoxide dismutase (Gregory, 1985), catalase (Rocha and Smith, 1995, 1996), and alkyl hydroperoxide reductase (Rocha and Smith, 1999).

The *Bacteroides* also produce a number of enzymatic activities that may be significant in the virulence of this organism. The production of neuraminidase (Russo *et al.*, 1990), chondroitinase (Linn *et al.*, 1993) and heparinase (Kim *et al.*, 1998) have been implicated in the pathogenesis of *Bacteroides* infection through their ability to degrade host structural components. The production of proteases has also been considered as potentially contributing to *B. fragilis* pathogenesis by degrading structural proteins, as well as those involved in local infection containment (Chen *et al.*, 1995; Gibson and Macfarlane, 1988a; McGregor *et al.*, 1986; Miyoshi and Shonoda, 2000). Iron transport proteins such as haemopexin and transferrin are also hydrolysed by *B. fragilis* (Rocha *et al.*, 2001). In addition, some *B. fragilis* strains have been demonstrated to be enterotoxigenic, a phenomenon that is believed to be associated with the occurrence of diarrhoeal disease in humans (Myers *et al.*, 1987). These particular strains produce a toxin, designated fragilysin. This is a zinc-dependent

metalloprotease that is thought to function by hydrolysing intercellular junction proteins. In so doing, the paracellular barrier between epithelial cells is disrupted, resulting in increased intestinal permeability and fluid loss (d' Abusco *et al.*, 2000; Obiso *et al.*, 1997).

1.4 Antibiotic resistance

The administration of appropriate antibacterial agents has been shown to be useful in the treatment of a number of infections caused by the *Bacteroides* (Falagas and Siakavellas, 2000). The *Bacteroides* have, however, been shown to possess resistance to a number of different antibiotics and the successful application of such treatment is, therefore, frequently limited. The basic mechanisms whereby anaerobic bacteria achieve resistance to antimicrobial agents can be divided into three classes: (1) prevention of access of the antimicrobial agent to the target site (2) alteration of the target site of antimicrobial action; (3) hydrolysis of the antimicrobial agent (Neu, 1995). Antibiotic resistance in the *Bacteroides* may be mediated by any one of the above mechanisms. The *Bacteroides* are universally resistant to the aminoglycoside antibiotics, and resistance to many other commonly used antibiotics such as the β-lactam antibiotics, tetracycline and the macrolide/lincosamide/ streptogramin (MLS) antibiotics, has risen over the last several decades (Garcia-Rodriguez *et al.*, 1995). Resistance to aminoglycoside antibiotics in the *Bacteroides* is due to their inability to transport these compounds, which prevents the drug from reaching its target site (Bryan *et al.*, 1979). Many *Bacteroides spp.* are resistant to β-lactam antibiotics, with resistance most frequently due to the enzymatic hydrolysis of these antibiotics (Aldridge *et al.*, 1983). Tetracycline resistance in the *Bacteroides* has been shown to be largely due to the presence of the *tet*Q gene, which encodes a protein that is able to modify the ribosomal target site of the tetracycline class of inhibitors (Nikolich *et al*, 1992). MLS resistance genes (*erm*F or *erm*FS), which confer resistance to clindamycin and erythromycin, have also been identified in the *Bacteroides* (Macrina and Smith, 1992). MLS resistance in these organisms is presumed to occur via the methylation of the ribosomal target site by an rRNA methylase encoded by the *erm* genes.

Antibiotic resistance in the *Bacteroides* is mediated by determinants that are carried on either plasmids, or chromosomally-located conjugative and mobilisable transposons (reviewed in Smith *et al.*, 1999). The dissemination of antibiotic resistance among the *Bacteroides* has, however, been shown to be largely associated with the transfer of conjugative transposons. These are large, self transmissible elements that are also capable of mobilising the transfer of

co-resident plasmids and mobilisable transposons. The majority of conjugative transposons carry *tet*Q, although some may also carry *erm*F (Salyers and Shoemaker, 1997). One interesting feature of these elements is that they display regulated transfer, with the frequency of their transfer, as well as that of mobilisable elements, increasing ten to a thousand-fold following treatment with low concentrations of tetracyline. This characteristic may be of particular importance in the human colon, where the transfer of resistance genes between members of the *Bacteroides,* and the *Bacteroides* and other genera, may contribute to the spread of antibiotic resistance (Shoemaker *et al.*, 2001).

1.5 Genetic manipulation of the *Bacteroides*

The use of genetic approaches for the analysis of the *Bacteroides* has, in the past, been limited due to the lack of a well-developed genetic system. However, significant progress in the field of *Bacteroides* genetics has been made in recent years, and the application of these genetic-based methodologies has allowed for a more detailed analysis of *Bacteroides* genes involved in metabolism and virulence (Salyers and Shoemaker, 1997; Salyers *et al.*, 2000). Although naturally occurring transformation systems have not been reported for any *Bacteroides spp.*, they can be rendered transformable using either chemical transformation and electroporation (Smith, 1985; 1995). Polyethylene glycol-facilitated transformation has been used for the transformation of *Bacteroides,* and while good transformation frequencies are attainable (~10^3 transformants/μg plasmid DNA), this protocol is restricted to only a number of *B. fragilis* strains (Smith, 1985). Electroporation has been used to obtain relatively high transformation frequencies (~ 10^6 transformants/μg plasmid DNA) for several *Bacteroides spp.* (Smith *et al.*, 1990). Unfortunately, the usefulness of both these protocols has been limited by the presence of specific restriction and modification systems present in *Bacteroides spp,* which appear to function at both the genus and species level (Macrina and Smith, 1992). As a result, the transformation of *Bacteroides spp.* with plasmid DNA isolated from *E. coli* results in transformation frequencies that correspond to only ~0.1% of that found for plasmids isolated from an isogenic source (Smith *et al.*, 1990; Thompson and Flint, 1989). An alternative and successfully applied technique for the introduction of foreign DNA is the conjugative mobilisation of plasmids to *Bacteroides spp.* (Guiney *et al.*, 1984). This technique relies on the presence of a broad host range IncP plasmids such as RK231 and R751, which serve to provide the conjugation functions in trans to a range of mobilisable *E. coli-Bacteroides* shuttle vectors (Salyers *et al.*, 2000). These shuttle vectors consist of both *E. coli* and *Bacteroides*

replicons, resistance marker genes for each host, and the transfer origin (oriT) for *E. coli*. This approach provides for the efficient mobilisation of plasmids from *E. coli* to *Bacteroides*, since it introduces single-stranded DNA, which is thought to be less susceptible to the action of restriction systems.

As a result of their early evolutionary divergence, the *Bacteroides* appear to have developed a novel mechanism of genetic control (Smith *et al.*, 1992). This is manifested by the fact that many *Bacteroides* genes are not expressed in other gram-negative bacteria such as *E. coli,* and vice versa. Furthermore, in those instances in which *Bacteroides* genes have been cloned in *E. coli,* only low levels of gene expression has frequently been observed (Guthrie *et al.*, 1985; Russo *et al.*, 1990). The study of Bayley *et al.* (2000) demonstrated that *B. fragilis* possesses unconventional promoter motifs that differ from those found in *E. coli.* The promoter consensus sequence consists of the motifs TTTG and TANNTTTG that are centered at positions –33 and –7, respectively, relative to the transcriptional initiation site. The variation of these sequences from the *E. coli* promoter consensus, together with the fact that *Bacteroides* genes frequently lack an *E. coli*-like ribosome binding sites (Salyers and Shoemaker, 1997) may, in part, account for the reduced efficiency of expression of *Bacteroides* genes in a heterologous background.

1.6 General aspects of bacterial nitrogen metabolism

Nitrogen metabolism in bacteria has been extensively investigated, and this is reflected in the considerable amount of available literature pertaining to this particular subject. In preparing the following discussion, the general reviews of Schreier (1993), Merrick and Edwards (1995), Reitzer (1996a, 1996b) and Morrison and Mackie (1997) were, therefore, extensively consulted.

1.6.1 Nitrogen sources

Microorganisms encounter a wide range of nitrogenous compounds in their natural habitats. These include simple inorganic ions like nitrate, nitrite or ammonia and more complex organic compounds like urea, amino acids and nucleosides (Merrick and Edwards, 1995). When grown in the presence of multiple nitrogen sources, microbes preferentially utilise those compounds that result in the fastest bacterial growth rate and yields. Given that ammonia exists in a more reduced form than many other forms of inorganic nitrogen, it is assimilated relatively easily and directly into organic nitrogen compounds. Ammonia, therefore, serves as

the preferred source of nitrogen for the growth of the enteric, and many other bacteria, even in the presence of alternative nitrogen sources. In addition to ammonia, other good nitrogen sources include glutamate, glutamine and asparagine, which may serve as the preferred nitrogen sources for the growth of several bacterial species including *Bacillus subtilis, Corynebacterium callunae* and *Pseudomonas aeruginosa* (Atkinson and Fisher, 1991; Ertan, 1992; Craven and Montie, 1985)

Nitrogen regulation is a widespread phenomenon in microorganisms, whereby cells grown on the preferred nitrogen source, usually ammonia, repress the expression of a large number of genes that are required for the metabolism of alternate nitrogen sources (Reitzer, 1996a). The replacement of the preferred nitrogen source results in the activation of genes required for the transport and degradation of these alternate nitrogen sources. Since different microbes vary in their ability to utilise a particular nitrogen source, the regulatory systems controlling the preferential use of nitrogen compounds, together with the combination of pathways available, is usually dependent on the particular organism in question. The paradigm of microbial nitrogen metabolism has been derived primarily from studies conducted on gram negative enteric bacteria, and this will, therefore, be discussed as a well researched model against which other bacterial systems can be assessed.

1.6.2 The significance of glutamate and glutamine

In bacteria, most of the nitrogen required for the biosynthesis of macromolecules can be derived by secondary transfers from two central intermediates, glutamate and glutamine (Reitzer, 1996b). Amino and amide groups from these two amino acids are freely transferred to other carbon skeletons by transamination and transamidation reactions, and they can, therefore, serve as nitrogen donors for all other nitrogen-containing compounds in the cell. Glutamate serves as the amino group donor for the biosynthesis of approximately 88% cells nitrogenous requirements. It provides the α-amino groups of all the amino acids and approximately half of the nitrogen required for the synthesis of the purines, pyrimidines and imidazole ring. Glutamine, on the other hand, is an essential precursor for the biosynthesis of approximately 12% of the nitrogenous compounds in the cell, including amino sugars, p-aminobenzoate, purines, pyrimidines, histidine, tryptophan and asparagine and NAD.

1.6.3 Ammonia assimilation and the biosynthesis of glutamate and glutamine

In most bacteria, the incorporation of ammonia into organic compounds occurs via the biosynthesis of glutamate and glutamine. Both these amino acids are capable of being formed directly using ammonia as the donor group. Two major pathways facilitate the incorporation of ammonia into glutamate and glutamine. In the first of these pathways, ammonia is assimilated via the sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT). GS catalyses the formation of glutamine via the reductive amidation of glutamate in a reaction that is ATP dependent (Fig. 1.1). GS plays an integral role in ammonia assimilation, as it provides the sole route for the biosynthesis of glutamine in the cell. It is, therefore, an indispensable enzyme unless the growth medium is supplemented with glutamine. GOGAT catalyses the reductive transfer of the amide group of glutamine generated by GS, to the C(2) carbon of 2-oxoglutarate resulting in the formation of two molecules of glutamate. Since the amidation of glutamate is linked to ATP hydrolysis, the equilibrium of the reaction is strongly in favour of ammonia incorporation, making this an essentially irreversible pathway. The second pathway is catalysed by glutamate dehydrogenase (GDH), which catalyses the reversible, reductive amination of 2-oxoglutarate to glutamate using NADPH or NADH as a cofactor.

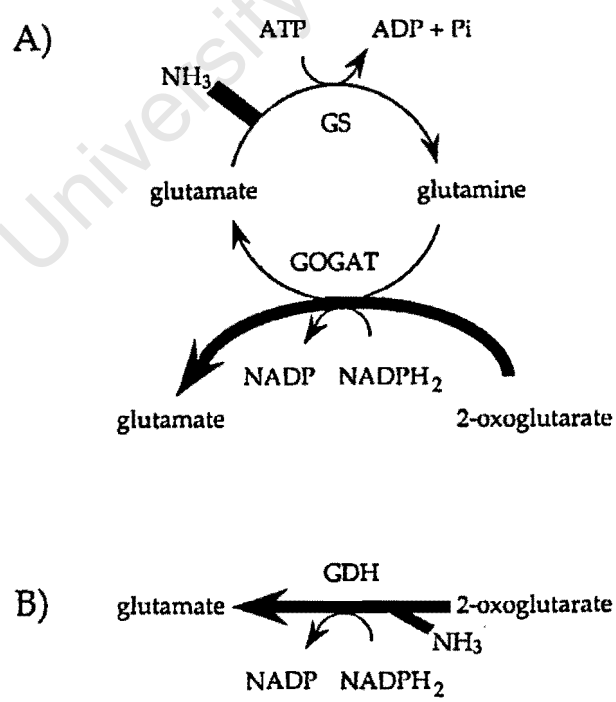


Figure 1.1. Pathways for ammonia assimilation. (A) GS/GOGAT cycle. (B) GDH pathway. From Ikeda *et al.* (1996).

As mentioned above, the cellular biosynthesis of glutamine occurs solely via the action of GS, and absolutely requires ammonia. If an environmental source of ammonia is lacking, it must be obtained via the catabolism of an organic nitrogen sources or by the reduction of atmospheric nitrogen. Glutamate, by contrast, can be formed via the conversion of several amino acids such as asparagine, arginine, histidine, and proline, or alternatively, by the transamination of 2-oxoglutarate (Reitzer, 1996b). When glutamine is the sole nitrogen source for growth, glutamate is formed via the action of GOGAT. In certain organisms, ammonia can also be produced via the action of glutaminases (L-glutamine amidohydrolase), which degrades glutamine to glutamate and ammonia (Reitzer, 1996a). In those instances where glutamate is a product of catabolism, ammonia is required solely for the biosynthesis of glutamine, thus obviating the need for the energy dependent biosynthesis of glutamate via the GOGAT pathway (Reitzer, 1996b). For nitrogen sources such as serine, however, which generate ammonia but not glutamate, GOGAT is required for glutamate biosynthesis.

Although the majority of microorganisms assimilate ammonia via the pathways outlined above, some bacterial species may assimilate ammonia via alternative routes, depending on their metabolic capabilities and the prevailing physiological conditions. Alanine dehydrogenase (ADH) is known to function in ammonia assimilation in some bacterial species, where it catalyses the reversible amination of pyruvate to form alanine. ADH has an assimilatory function in *Streptomyces clavuligerus* (Aharonowitz and Friedrich, 1980; Brana *et al.*, 1986) and in the *Norcadia mediterranei* (Mei and Jiao, 1988), where it may compensate for the lack of biosynthetic GDH activity. ADHs have also been identified in *Bacillus spp.* (Meers and Kjaergaard-Pedersen, 1972) and *Streptomyces coelicolor* (Fisher, 1989), where it contributes to alanine catabolism. Ammonia-dependent asparagine synthetase can also catalyse the amidation of aspartate to form asparagine (Reitzer, 1996a), and may play a role in ammonia assimilation in certain microorganisms, including some thermophillic clostridial strains (Bogdahn and Kleiner, 1986).

1.6.4 Physiological regulation of GS, GDH and GOGAT activities

The route of ammonia assimilation is generally dependent on the extracellular ammonia concentration. GDHs, in general, have relatively high K_m's for ammonia (>1 mM) and are, therefore, thought to be inefficient in assimilating ammonia under conditions of ammonia limitation. Under these conditions, ammonia is incorporated into glutamate via glutamine using the GS-GOGAT pathway. GS has a high affinity for ammonia (K_m ~ 0.2 mM) and is,

therefore, efficient in assimilating ammonia even under conditions of nitrogen limitation. The utilization of GS and GOGAT under these conditions allows the organism to scavenge ammonia, albeit at the expense of energy. Given that GDH-deficient strains have no discernible growth defect in energy rich media, it was initially considered dispensable for bacterial growth. *E. coli* strains impaired in GDH activity were, however, subsequently shown to be at a competitive disadvantage relative to wild type strains in an energy limited environment (Helling, 1994), indicating that GDH is important during energy limited growth where it would spare the need for the ATP-dependent biosynthesis of glutamate via the GS-GOGAT pathway.

It should, however, be noted that while the regulation of GDH and GS-GOGAT activities under different nitrogen concentrations outlined above holds true for the majority of microorganisms examined, several exceptions to this generality exist. In *Saccharomyces cerivisiae* (Folch *et al*, 1989) and *Neurospora crassa* (Lomnitz *et al.*, 1987), the GS-GOGAT pathway is used to incorporate ammonia under both nitrogen limiting or excess conditions, and in *A. nidulans*, the GDH and GS-GOGAT pathways presumably operate concurrently (Kusnan *et al.*, 1987). In *C. callunae* (Ertan, 1992) and *Brevibacterium flavum* (Tokichura *et al.*, 1984) GDH activities are not affected by the ammonia concentration in the growth medium. Further, some organisms possess GDHs with low K_ms for ammonia and can, therefore, assimilate ammonia even under ammonia-limiting conditions (Kanomori *et al.*, 1987). GDH activity in various streptococci have also been found to be elevated when grown under conditions of nitrogen limitation (Griffith and Carlsson, 1974). Pathway preference in these organisms may depend on the fermentation pathway used by the particular microorganism, and the generation of ATP and reducing equivalents (Kanamori *et al.*, 1989). For instance, the assimilation of ammonia through the GDH pathway, without the expenditure of energy, may be more advantageous to those organisms having low-energy yielding fermentation pathways.

The presence of both the GDH and GS-GOGAT pathways has been demonstrated in a diverse range of microorganisms. The individual contribution of each pathway to nitrogen assimilation depends on the prevailing nitrogen conditions, and can vary greatly between different organisms. Certain microorganisms are known to preferentially use only one of the two pathways for the incorporation of ammonia into carbon skeletons. Organisms such as *Bacillus subtilis (*Fisher and Sonnesheim, 1991), *Rhodopseudomonas acidiphila* (Herbert *et*

al., 1978), *Clostridium pasteurianum* (Dainty, 1972), *Rhizobium phaseoli* (Bravo and Mora, 1988) and many cyanobacteria (Carpenter *et al.*, 1992; Herrero *et al.*, 2001) exclusively utilise the GS-GOGAT pathway for ammonia assimilation under all nitrogen conditions examined. These organisms either lack GDH activities entirely, or specifically utilise GDH for the catabolism of glutamate. In contrast, GDH serves as the sole, or primary, ammonia assimilatory enzyme in some *Bacillus spp.* (Kanamori *et al.*, 1987) and streptococci (Griffith and Carlsson, 1974).

1.6.5 Control of nitrogen assimilatory pathways

1.6.5.1 The Ntr System

The expression of the nitrogen metabolic pathways in microorganisms is typically regulated in response to the nitrogen composition of the growth medium. In many instances, the presence of ammonia inhibits the synthesis of proteins involved in the utilisation of alternative nitrogen sources. If an environmental ammonia source is lacking, the synthesis of glutamine synthetase, together with a number of other proteins required for the transport and catabolism of alternative nitrogenous sources, is induced (Merrick and Edwards, 1995; Reitzer, 1996b). In enteric bacteria, this coordinate expression of nitrogen regulated genes is mediated by a global regulatory network, termed the Ntr system.

The primary components of the Ntr response are the nitrogen regulators, NtrB and NtrC, together with RNA polymerase complexed to σ^{54} (Stock *et al.*, 1989; Kustu *et al.*, 1989). The main role of the individual components of the Ntr system is to sense the nitrogen status of the cell and modulate the expression of target genes in response. The intracellular nitrogen status is sensed and transduced by a pair of proteins viz. the UTase (uridylyltranferase)/UR (uridylyl removing) and the PII signal-transduction protein (Magasanik, 1988). The overall uridylylation state of the PII protein controls the activity of the Ntr regulators, NtrC and NtrB, which are encoded by the *gln*L and *gln*G genes, respectively. NtrB and NtrC constitute a two component regulatory system, in which NtrB serves as the sensory histidine kinase, and NtrC as the phosphorylatable response regulator (Stock *et al.*, 1989). The phosphorylated form of NtrC is required to activate transcription of nitrogen-regulated genes by RNA polymerase and σ^{54} through binding to upstream activating sequences that function as transcriptional enhancers (Kustu *et al.*, 1991). The level of NtrC phosphorylation is, in turn, determined by the kinase/phosphatase activities of NtrB.

In enteric bacteria, external ammonia limitation is perceived as a decrease in the intracellular glutamine pool (Ikeda *et al.*, 1996). In the presence of excess nitrogen, the presence of glutamine stimulates the UR activity to remove UMP from uridylylated PII (PII-UMP), and prevents uridylylation of PII. In contrast, when cells are nitrogen limited, resulting in a diminished glutamine pool, UTase covalently modifies the PII protein via the addition of a UMP group to each subunit. In the non-uridylylated state (nitrogen excess), PII interacts with NtrB to stimulate the dephosphorylation and consequent inactivation of NtrC. In the uridylylated state (nitrogen limitation), however, PII does not interact with NtrB, which in response catalyses the phosphorylation of NtrC, thus enabling it to activate σ^{54}-dependent expression of the *gln*ALG operon (Ninfa and Magasanik, 1986). The resulting increase in the levels of GS enhances the ability of cells to use low concentrations of ammonia, while elevated levels of NtrC-phosphate results in increased expression of operons at σ^{54}-dependent promoters. Besides GS, other products of NtrC activated genes include glutamine-, arginine-, histidine- and ammonia-transporters, those involved in peptide transport and utilisation, as well as genes required for nitrogen fixation (Zimmer *et al.*, 2000).

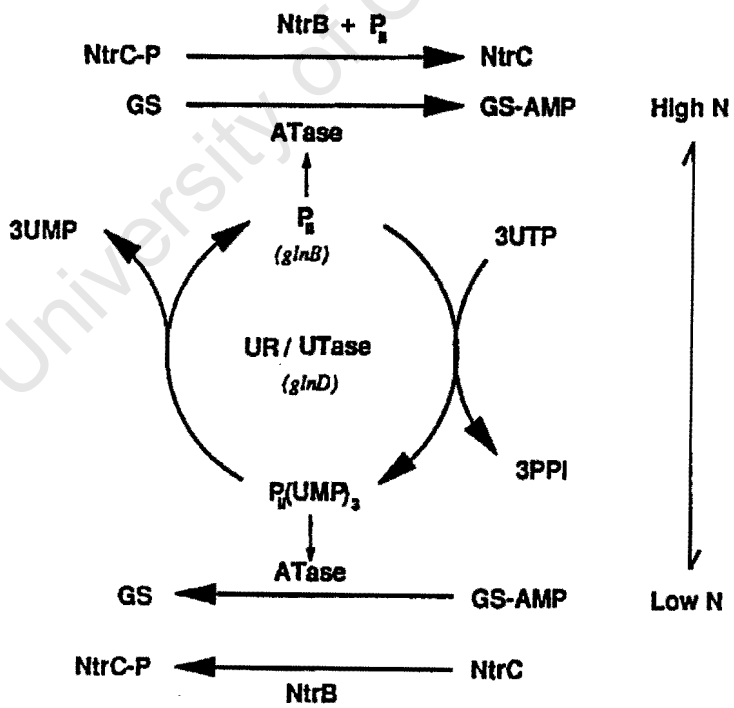


Figure 1.2. Regulation of the activities of GS and NtrC protein in response to nitrogen status of the cell. (From Merrick and Edwards, 1995)

1.6.5.2 The nac (nitrogen assimilation control) gene

The expression of a subset of nitrogen assimilatory genes in *E. coli* and *K. aerogenes* is controlled by the Nac (nitrogen assimilation control) protein, a transcriptional activator belonging to the LysR family of transcriptional regulators (Bender, 1991; Muse and Bender, 1998). The expression of the *nac* gene is σ^{54} dependent, and like many other nitrogen regulated genes, requires activation by NtrC. Under conditions of nitrogen limitation, the Nac protein activates the expression of several σ^{70} dependent operons, many of which encode proteins that are involved in the supply of ammonia or glutamate from alternative nitrogen source (Bender, 1991). These include those genes involved in histidine- (*hut*UH), proline- (*put*P) and urea- utilisation (*ure*DABCEFG). Nac represses the expression of the genes encoding GDH (*gdh*A), GOGAT (*glt*BD), and the *nac* gene itself (Feng *et al.*, 1995).

1.6.5.3 Transcriptional regulation of the *gln*ALG operon in *E. coli*

As mentioned above, nitrogen limitation induces the synthesis of GS in enteric bacteria. In *E. coli* the *glnA* structural gene forms an operon with the downstream genes, *gln*LG, encoding the NtrB and NtrC regulators. The *gln*A gene can be expressed from either one of two distinct promoters located upstream of the *gln*A structural gene (*gln*Ap1 or *gln*Ap2) (Pahel *et al.*, 1982; Reitzer and Magasanik, 1985). A third promoter, *gln*Lp, is located within the *gln*A-*gln*L intergenic region immediately upstream of the *gln*L gene (Ueno-Nishio *et al.*, 1984). Under conditions of nitrogen-excess, the two σ^{70}-dependent promoters, *gln*Ap1 and *gln*Lp, ensure basal levels of the three products of the *gln*ALG operon. The expression of the *gln*A gene from *gln*Ap1 is initiated during carbon-limited growth by a cAMP-CRP receptor protein complex, formed upstream of the *gln*A structural gene, and is terminated completely at a rho-independent terminator located downstream of the *gln*A gene. Transcription initiated from the *gln*Lp promoter, on the other hand, ensures that basal levels of *gln*L and *gln*G mRNA are maintained. The second promoter located upstream of *gln*A, *gln*Ap2, is recognised by RNA polymerase complexed with σ^{54}. During nitrogen-limited growth, NtrC binds to sequences in proximity to both the *gln*Ap1 and *gln*Lp promoters, thus interfering with the expression from these promoters. Under these conditions, NtrC activates expression from the stronger *gln*Ap2 promoter, leading to high levels of *gln*ALG transcription and the production of elevated levels of GS, as well as NtrB and NtrC, which are required to stimulate the expression of other Ntr-regulated genes (Reitzer, 1996b).

The Ntr system appears to function in the gram negative enteric bacteria, and *ntr* homologues have been identified in many other bacterial genera (Merrick and Edwards, 1995). However, nitrogen regulatory systems which are quite distinct from that found in enteric bacteria have been identified in many gram positive organisms and cyanobacterial *spp*.

1.6.5.4 Nitrogen regulation in Gram positive organisms

Unlike the enteric bacteria, *B. subtilis* utilises amino acids (glutamine, arginine and possibly others), rather than ammonia, as the preferred nitrogen source. Moreover, the expression of several nitrogen catabolic genes has been demonstrated to be regulated in response to metabolites other than ammonia (Atkinson and Fisher, 1991). Transcriptional regulation of the *gln*A gene, which forms part of the *gln*RA operon in *B. subtilis*, is negatively regulated by the global regulatory proteins, GlnR and TnrA, which belong to the MerR family of transcriptional regulators (Atkinson and Fisher, 1991; Wray *et al.*, 1996). TnrA positively regulates its own expression, and represses the expression of the *gln*RA operon as well as the gene encoding GOGAT in response to nitrogen limitation. GlnR, on the other hand, represses expression of *gln*RA and *tnr*A in cells grown with excess (preferred) nitrogen sources (Brown and Sonnesheim, 1996).

Although GlnR and TnrA differ in their C-terminal domains, their proposed DNA binding domains are virtually identical. Both proteins have been demonstrated to bind to similar DNA sequences ($TGTNAN_7TNACA$) under different physiological conditions, which may account for the cross regulation of genes observed in some instances (Fisher, 1999). Since not all nitrogen-regulated promoters are cross regulated by both proteins, additional regulatory factors must be required. A single, σA-dependent promoter is used to transcribe the *gln*RA operon under all growth conditions. During growth with excess nitrogen the GlnR protein represses *gln*RA expression by binding to two adjacent operators ($glnRA_{O2}$ and $glnRA_{O1}$) containing the GlnR/TnrA binding site, which overlaps the -35 region of the *gln*RA promoter (Brown and Sonnesheim, 1996). The upstream site, $glnRA_{O1}$, is a symmetrical inverted repeat sequence while the $glnRA_{O2}$ operator is only partially symmetrical. Under conditions of nitrogen limitation, TnrA negatively regulates *gln*RA expression by binding exclusively to the $glnRA_{O2}$ operator. The binding of GlnR to the *gln*A operator sequence requires GS (Schreier, 1993). Although the exact nature of this interaction is not known, the expression of all known nitrogen regulated genes has been shown to be constitutive in *B. subtilis gln*A

mutants, suggesting that GS may be involved in generating a global regulatory signal (Fisher, 1999).

Novel nitrogen control systems have also been identified in Gram positive bacteria other than *B. subtilis*. Nitrogen regulation in *Corynebacterium glutamicum* has been shown to be regulated by the AmtR protein, which represses the expression of several genes including those encoding the GOGAT, PII and UTase/UR proteins (Jakoby *et al.*, 2000; Beckers *et al.*, 2001). In *S. coelicolor*, *gln*A gene expression is regulated by the GlnR protein, a transcriptional regulator that shows homology to the VirG/OmpR family of activators, while in *C. acetobutylicum* its regulation is thought to be accomplished by the production of antisense RNA expressed from a nitrogen-regulated promoter located downstream of the *gln*A gene (Fierro-Monti *et al.*, 1992; Wray and Fisher, 1993).

1.6.5.5 Nitrogen regulation in cyanobacteria

Nitrogen sources commonly used by cyanobacteria include dinitrogen, nitrate, urea and ammonia. Ammonia is, however, assimilated in preference to other nitrogen sources (Herrero *et al.*, 2001). NtrB- and NtrC- like proteins have not been identified in cyanobacteria. Instead, nitrogen regulation in is mediated by NtcA, a member of the Crp/Fnr family of transcriptional activators (Vega-Palas *et al.*, 1992). In *Synechococcus spp.* NtcA positively regulates the expression of several genes involved in the assimilation of alternative nitrogen sources under conditions of ammonia limitation including those encoding glutamine synthetase and several proteins required for the uptake and reduction of nitrite and nitrate (Suzuki *et al*, 1995). NtcA also regulates its own expression from an NtcA dependent promoter (Herrero *et al.* 2001).

NtcA-regulated promoters have been shown to consist of an *E. coli* like –10 consensus sequence (TAN_3T) and an NtcA binding site overlapping and replacing the –35 binding site for RNA polymerase. The recognition sequence of NtcA consists of the palindromic consensus sequence $GTAN_8TAC$, and has been identified upstream of many of these nitrogen regulated genes (Luque *et al.*, 1994). The mechanism whereby NtcA perceives the nitrogen status of the cell is presently unknown, and no sensor protein that interacts with NtcA has been identified to date. In *Anabaena spp.*, a second nitrogen regulatory gene, NtcB, a LysR family protein, is additionally required for the expression of several genes involved in nitrate

assimilation (Frias *et al.*, 2000). The transcription of the *nir* operon requires the simultaneous binding of NtcA and NtcB to the *nir* promoter region, and NtcB is believed to augment NtcA dependent expression of the operon. The expression of the *ntc*B gene is regulated by NtcA.

1.7 Structure and regulation of enzymes of ammonia assimilation

Given that glutamate and glutamine are central to nitrogen metabolism, the activities of the nitrogen assimilatory enzymes are, in general, strictly controlled in response to both the type and concentration of nitrogen source present in the growth media. However, while the reactions catalysed by these enzymes are highly conserved in bacteria from diverse ecological niches, both their structure and regulatory mechanisms may vary considerably between different organisms. For the purpose of this thesis we will be focusing on the GDH pathway in relation to nitrogen metabolism in bacteria.

1.7.1 Glutamine synthetase (GS)

Three forms of GS enzymes may occur in prokaryotes. These are termed GSI, GSII and GSIII (Woods and Reid, 1993). GSI are the most common form of GS (GSI) found in bacteria, and have been found in the many enteric bacteria, as well as in *Vibrio*, *Streptomyces*, *Bacillus* and *Clostridium spp.* (Woods and Reid, 1993). In *E. coli*, the enzyme exists as a dodecamer with identical subunits of approximately 55 kDa that are arranged as two superimposed hexagonal rings. GSI enzymes can be subdivided according to whether, and how, the enzyme is modified in response to nitrogen availability. In enteric bacteria the GSI enzymes are subject to control of both synthesis (see Section 1.6.5.3) and via post-translational modification by adenylylation in response to nitrogen availability (Stadtman *et al.*, 1980). Adenylylation, occurs in response to an increase in nitrogen availability, and progressively inactivates the enzyme by addition of an AMP group to a tyrosine residue present in each of the enzymes subunits. Adenylylation of a subunit of GSI inactivates only that subunit, thereby allowing the enzyme to exist in a variety of activity states. In addition to regulation by adenylylation, the activity of GS is subject to feedback inhibition by nine end products of glutamine metabolism including serine, glycine, AMP and carbomyl phosphate. Partially adenylylated GS is also more sensitive to feedback inhibition than the non-adenylylated enzyme. The GS enzymes of archaea and the gram positive *Bacillus* and *Clostridium spp.*, do not display regulation by adenylylation (Woods and Reid, 1993)

GSII enzymes have been identified in a wide range of prokaryotes that includes *Rhizobia, Bradyrhizobia, Streptomyces and Frankia spp.* (Merrick and Edwards, 1995). These enzymes differ from GSI enzymes in that they exist as an octamer consisting of identical subunits with a molecular mass of approximately 36 kDa. The GSII subunit lacks the C-terminal portion of the GSI subunit, which includes the adenylylation site involved in post-translational control of activity (Woods and Reid, 1993). GSIII enzymes, by contrast, exist in a hexameric configuration and possess a subunit mass of 75 kDa. To date, this form of GS enzymes has been identified in several anaerobic microorganisms such as *Bacteroides fragilis* (Southern *et al.*, 1986), *Butyrivibrio fibrosolvens* (Goodman and Woods, 1993), as well as in several cyanobacterial *spp.* (Garcia-Dominguez *et al.*, 1997; Crespo *et al,* 1998). Although the GSII and GSIII enzymes are quite different from the GSI enzymes, several regions associated with the GS active site have been well conserved (Rawlings *et al.* 1987).

1.7.2 Glutamate synthase (GOGAT)

The presence of the GOGAT enzyme has been determined in a wide range of microorganisms. In *E. coli,* and many other bacterial species, the GOGAT enzyme consists of two dissimilar subunits, with molecular masses of approximately 53 and 135–175 kDa for the small (α) and large (β) subunits, respectively (Miller and Stadtman, 1972; Oliver *et al.*, 1987). The α-subunit contains the site of glutamine binding and hydrolysis as well as the site of actual glutamate synthesis. The β-subunit acts as a FAD-dependent NAD(P)H oxidoreductase, which serves to input electrons into the α subunit for reductive glutamate synthesis (Vanoni and Curti, 1999).

The structural genes encoding the large and small polypeptides are *gltB* and *glt*D, respectively. In *E. coli* these genes form an operon with a third gene, *glt*F (Castano *et al.*, 1992). Expression of the *glt*BD operon is not regulated by the global Ntr system (Reitzer, 1996b). The *glt*BD operon in *E. coli* is positively regulated by the leucine responsive protein (Lrp). Lrp appears to bind upstream of the *glt*BD operon and is thought to function as a leucine insensitive activator of GOGAT synthesis (Reitzer, 1996b). Carbon starvation also appears to repress GOGAT synthesis in *E. coli*, and a cAMP-CRP binding site overlapping the –35 region of the *glt*BD promoter has been identified. The role of the *glt*F in nitrogen regulation is unclear, since *E. coli glt*F deletion mutants display identical regulation of both GS and GOGAT activities in response to nitrogen source and availability, and possess similar growth rates to *E. coli* strains possessing functional copies of this gene (Goss *et al.*, 2001).

The second class of GOGAT enzyme is dependent on ferrodoxin as its electron donor, and is found in cyanobacteria (Navarro *et al.* 1995) and in the photosynthetic tissues of plants (Pajuelo *et al.*, 1997). Characterisation of these enzymes has revealed that they exist as homodimers composed of a single polypeptide chain that varies in size ranging from 125 to 180 kDa (Vanoni and Curti, 1999). The eukaryotic pyridine-dependent form of the enzyme constitutes the third class of GOGAT, and is composed of a high molecular weight polypeptide of approximately 200 kDa, which associates in a homotrimeric structure (Gregerson *et al.* 1993).

1.7.3 Glutamate Dehydrogenase (GDH)

1.7.3.1 General introduction

GDHs have been identified in a wide range of organisms, where they are responsible for either the anabolic function of glutamate biosynthesis or the catabolic function of glutamate utilization (Smith *et al.*, 1975). In the reductive amination reaction, GDH supplies glutamate required for the cellular biosynthesis of a variety of nitrogen containing compounds, while in the oxidative deamination reaction it produces a carbon (and energy) and nitrogen source, in the form of the TCA intermediate 2-oxoglutarate and ammonia, respectively. As a result, GDH occupies a pivotal role within central metabolism by providing a link between carbon and nitrogen metabolism.

The physiological role of GDH's as anabolic or catabolic enzymes is generally defined by their cofactor specificity. NADPH-specific enzymes usually catalyse the reductive biosynthesis of glutamate, while NADH dependent enzymes catalyse the oxidative degradation of glutamate. GDHs from prokaryotes and lower eukaryotes are usually found to be specific for either NADPH or NADH, while those present in certain bacterial species and higher eukaryotes posses dual-cofactor specificity [NAD(P)H-dependent GDHs], which may be indicative of an amphibolic role for these enzymes (Smith *et al.*, 1975).

1.7.3.2 Classes of GDH enzymes

The genes encoding GDH from numerous sources have been cloned and analysed at the molecular level, and a number of these enzymes have been purified to homogeneity. The comparison of the structural features of these enzymes has lead to the identification of several different classes of GDH enzymes, based upon their oligomeric structure and subunit size. The most widely distributed GDHs are hexameric in structure and consist of identical subunits with molecular masses that range in size from 48 kDa to 55 kDa (Baker *et al.*, 1992a; Smith *et al.*, 1975). Hexameric GDHs have been demonstrated to be structurally similar, irrespective of their cofactor specificity, and have been identified in many different bacterial species, fungi, algae and vertebrates (Smith *et al.*, 1975). The second class of GDHs is tetrameric enzymes that contain identical subunits of 115 kDa. Tetrameric GDH's appear to be less widely distributed in nature than hexameric GDHs, and to date these enzymes have only been identified only in lower eukaryotes such as *Neurospora crassa* (Veronese *et al.*, 1974) and *S. cerevisiae* (Hemmings, 1982). Until recently, GDH enzymes were believed to conform exclusively to two aforementioned classes. However, the GDH enzymes from *Streptomyces clavuligeris* (Minambrest *et al.*, 2000) and *P. aeruginosa* (Lu and Abdelal, 2001) have recently been shown to differ from previously described GDHs, in that they posses much larger subunits than those found in other GDH enzymes. The subunit sizes of the *S. clavuligerus* and *P. aeruginosa* GDH enzymes have been estimated as 183 and 182 kDa, respectively, and database comparisons identified genes encoding polypeptides with a high degree of homology in the genomes of several other bacterial species (Minambrest *et al.*, 2000; Lu and Abdelal, 2001). Nevertheless, contrasting results were presented as to the oligomeric structure of these enzymes. While the *S. clavuligeris* holoenzyme was reported to possess a hexameric configuration, the GDH enzyme from *P. aeruginosa* enzyme was shown to exist as a tetramer. Thus, while these enzymes certainly appear to differ from previously identified GDHs, their precise oligomeric structure awaits further clarification.

1.7.3.3 Structure of hexameric GDHs

The majority of hexameric GDH holoenzymes posses M_rs of approximately 300 kDa, with the subunits arranged in a symmetrical 3X2 configuration (Baker *et al.*, 1992a). Each of the identical polypeptide subunits consists of two structurally conserved domains that are separated by a deep, solvent exposed cleft (Baker *et al.*, 1992a; Britton *et al*, 1992). The first of these domains is comprised primarily of the N-terminal portion of the GDH polypeptide,

and is thought to be responsible for the symmetrical arrangement of the enzyme. The second domain, which is approximately two-thirds the size of the first, is formed largely from the C-terminal portion of the polypeptide, and contains the region responsible for dinucleotide binding. In general, the dinucleotide-binding domain of GDHs that use NADPH as a cofactor consists of the motif $GXGX_2AX_3AX_6G$, whereas NADH-dependent enzymes are characterised by the motif $GXGX_2GX_3GX_6G$ (Scrutton *et al.*, 1990). Several NADH-dependent GDHs have, however, been found to possess dinucleotide domains more commonly found in NADPH-dependent enzymes (Baker *et al.*, 1992b). In either event, the cofactors are thought to bind to the enzyme in an extended conformation, with the nicotinamide moiety located within the cleft located between the two domains (Britton *et al.*, 1992). Several conserved amino acid residues, including a glycine rich region together with three lysine residues, are located in this cleft and have been implicated in the specific binding of glutamate/2-oxoglutarate and the catalytic mechanism of the enzyme (Baker *et al.*, 1992a; Mcpherson *et al.*, 1988).

The comparison of the amino acid sequences of the GDH polypeptides from different organisms has revealed that 41 residues are strongly conserved, while about 100 residues are variable. The results of crystallographic studies, however, revealed that the contribution of these variable residues to enzyme structure is conserved (Teller *et al.* 1992). The greatest sequence similarity is found within the N-terminal half of the GDH polypeptide, which contains the regions implicated in substrate binding and catalytic activity. At the C-terminus the similarity is far lower, and the differences can be further used to distinguish non-vertebrate and vertebrate GDH enzymes. Vertebrate GDHs possess an additional 50 residues at the C-terminus of the enzyme, which is thought to form an antenna-like structure (Smith *et al.*, 2000). This region is thought to play a role in the communication between the different subunits, and may also contribute to the allosteric regulation in response to GTP, ADP and NAD(P)H displayed by vertebrate GDHs.

Hexameric GDH's display a strong degree of sequence conservation and are considered good models for evaluating evolutionary relationships, given their widespread occurrence and relatively slow mutation rate (1.8 amino acid substitutions per 100 residues per 10^8 years) (Wilson *et al.*, 1977). Based on sequence comparisons of hexameric GDH's from a diverse range of sources, Benechenhou-Lahfa *et al.* (1993) proposed that GDHs had diverged into two closely related, but distinct, families (Family I and II) prior to the divergence of bacteria,

archae, and eukaryotes. In general, enzymes belonging to Family I consists mainly of GDHs from bacteria and lower eukaryotes, while Family II enzymes consists largely of GDHs from archae and higher eukaryotes. Members of these two families are distinguishable by motifs unique to each family. While Family I enzymes are characterised by the presence of the amino acid motifs PSVNL, KFL(A/G)F(E/G)(E/Q) and R(P/T)EATC, enzymes belonging to Family II posses the sequences QG(F/I/Y)G(N/S)V and E(A/G)ANGP(T/L)T in their primary amino acid sequence.

1.7.4.4 Regulation of GDH activity

Given that GDH enzymes have been identified in a wide range of organisms, which frequently differ in their ability to utilise a particular nitrogen source, it is not entirely surprising to find that very different regulatory mechanisms have evolved to control their activities. Only a single copy of the gene has been found to occur in most of the organisms studied to date, although several exceptions to this generality exist. *E. coli* (Helling, 1990), *S. typhimurium* (Rosenfeld *et al.*, 1982), and *Corynebacterium glutamicum* (Bormann *et al.*, 1992), amongst others, posses only a single GDH with a specific requirement for NADPH as a cofactor. The main physiological role of these enzymes is considered to be biosynthetic, but given their low affinity for ammonia, it is unlikely that they function efficiently in ammonia assimilation, except when the environmental concentration of ammonia is high. The K_ms for ammonia of GDHs with a biosynthetic roles have been reported to be in the region of 1-3 mM (Helling, 1994). By contrast, several microorganisms, including *Bacillus subtilis* (Belitsky and Sonnesheim, 1998), *Clostridium botulinum* (Hammer and Johnson, 1988) and *Streptomyces clavuligerus* (Minambrest *et al.*, 2000), produce NADH-dependent GDH activity following growth with glutamate, or other organic nitrogen compounds that are easily converted to glutamate. In these instances, GDHs are considered to function catabolically where, depending on the organism and the prevailing growth conditions, glutamate may serve as a carbon, energy and/or nitrogen source. Certain microorganisms have also been found to possess two genetically distinct GDHs viz. an NADPH-dependent and NADH-dependent enzymes, which are presumed to function in the biosynthesis and catabolism of glutamate, respectively. In these organisms, the activity of the enzymes are frequently found to be regulated differently in response to the nitrogen source (inorganic vs. organic) available for growth. Usually an inverse relationship between the levels and activities of each enzyme is maintained ie. high levels of NADPH-dependent activity is typically associated with low

levels of NADH-dependent activity, and vice versa. Organisms known to possess two GDHs include *Pseudomonas aeruginosa* (Brown *et al.*, 1973), *Thiobacillus novellus* (LeJohn and McCrea, 1968) *and S. cerevisiae* (Hemmings, 1982).

1.7.4.4.1 Regulation of GDH activity in enteric bacteria

In the enteric bacteria such as *E. coli, S. typhimurium* and *K. aerogenes,* GDH activity is elevated under conditions of ammonia-excess, where it functions biosynthetically to supply the cell with glutamate (Reitzer, 1996b). GDH activity in these organisms is repressed by exogenous glutamate, or other glutamate generating nitrogen sources. While glutamate can serve as a sole nitrogen source for the growth of these organisms, GDH is not involved in its catabolism given that glutamate represses GDH synthesis, and *gdh*A mutants grow normally with this amino acid as a nitrogen source (Reitzer, 1996a).

Although nitrogen (ammonia) limitation strongly represses GDH synthesis in *K. aerogenes,* it has considerably less effect in *E. coli,* and none whatsoever in *S. typhimurium* (Bender, 1991; Muse and Bender, 1998). In *E. coli* GDH activity is transcriptionally regulated in response to the nitrogen source, with glutamate having been shown to reduce the expression of *gdh*A (Varrichio, 1969). The exact mechanism involved in this regulation has, however, not been elucidated. To date, there is little evidence for the regulation of GDH activity at the post-translational level, and generally, it has been found to be relatively insensitive to feedback inhibition. While the *E. coli* K12 GDH has been reported to be phosphorylated *in vitro* (Lin and Reeves, 1994), the effect(s) of this modification on enzymatic activity has not been reported. In addition, GDH synthesis has been demonstrated to be reduced by carbon limitation, presumably to prevent excessive removal of 2-oxoglutarate from the TCA during carbon limited growth (Reitzer, 1996b).

1.7.4.4.2 Regulation of GDH activity in *Pseudomonas spp.*

P. aeruginosa, together with most other pseudomonads, synthesise two GDHs, one NADPH-dependent and the other NADH-dependent (Brown *et al.*, 1973; Jansenn *et al.*, 1980). The NADPH-dependent GDH is produced in response to growth with ammonia as a nitrogen source, where it is presumed to play a role in the biosynthesis of glutamate. This is substantiated by the fact that *P. aeruginosa* strains lacking both GOGAT and NADPH-dependent GDH activities, but producing NADH-dependent GDH activity are glutamate auxotrophs (Joannou *et al.*, 1988). In the presence of organic nitrogen sources, biosynthetic

GDH activity is repressed and NADH-dependent GDH is produced (Jansenn *et al.*, 1980). Recent studies have shown that the NADH-dependent GDH is induced by arginine (Lu and Abdelal, 2001), an amino acid that serves as a good source of carbon and nitrogen for the growth of *P. aeruginosa*. The induction levels of NADH-GDH in *P. aeruginosa* was shown to be ninefold higher with arginine, as opposed to glutamate, when serving as the sole carbon and nitrogen source. In *P. aeruginosa,* the arginate-succinyl-tranferase (AST) pathway is responsible for the conversion of arginine to glutamate. Since glutamate is the end product of the AST pathway, exogenous arginine serves as an inducer of the enzymes of the AST pathway, in addition to NADH-dependent GDH activity required for the conversion of glutamate to 2-oxoglutarate and ammonia. Since cells grown with organic nitrogen require ammonia for the biosynthesis of glutamine, catabolic GDH activity is thought to provide ammonia for the production of glutamine during growth on a variety of organic nitrogen sources that a readily converted to glutamate. The NADH-dependent GDHs of *B. subtilis* (Belitsky and Sonnesheim, 1998) and *S. clavuligerus* (Minambrest *et al.*, 2000) have similarly been found to be induced in response to growth with arginine.

1.7.4.4.3 Regulation of GDH activity in *Prevotella spp.*

In those *Prevotella spp.* examined, both NADPH- and NADH-dependent have been identified (Wen and Morrison, 1996, 1997). In *Prevotella bryantii* B_14 these activities have been shown to be attributable to a single, dual-cofactor dependent GDH, whose activity has been shown to greatest following growth with limiting concentrations of ammonia. The structural gene (*gdh*A) encoding the NAD(P)H-GDH from *P. bryantii* has B_14 been cloned and sequenced, and GDH activity was shown to be controlled primarily at the level of transcription in response to ammonia availability (Wen and Morrison, 1996). NAD(P)H-dependent GDH activity in this organism is significantly reduced following growth with peptides, in the form of trypticase, as the sole nitrogen source. A similar down-regulation of NADPH-dependent GDH activity in response to growth with peptides has also been observed in *P. ruminicola* 23 and *P. brevis* GA33 (Wen and Morrison, 1997). In these two strains, NAD(P)H-dependent GDH activity appears to be additionally regulated by peptides at the level of enzyme activity, given that a substantial and rapid reduction in NADPH-dependent GDH activities was demonstrated to occur following the addition of peptides to ammonia-grown cultures. Interestingly, NADH-dependent GDH activities was shown to increase upon addition of peptides to the growth media, which in the case of *P. brevis* was shown to be due to the induction of a second, NADH-dependent GDH (Wen and Morrison, 1997).

1.8 Nitrogen assimilation in the *Bacteroides*

Nitrogen metabolism in *B. fragilis* has been shown to possess several unusual features when compared to the *Enterobacteriacae*. Ammonia is not replaced by amino acids as the sole source of nitrogen for growth (Varel and Bryant, 1974), while peptides can also be utilised (Macfarlane and Macfarlane, 1991). The initial studies of Yamamoto *et al.* (1984; 1987b) also suggested that the regulation of the nitrogen assimilatory pathways in the *Bacteroides* occurs in a manner distinct from that found in other gram negative bacteria. In *B. fragilis* (Yamamoto *et al.*, 1984) and related species (Baggio and Morrison, 1998; Yamamoto *et al.*, 1987c), the GDH-catalysed pathway appears to be the primary means of ammonia assimilation during growth with excess, and limiting concentrations of ammonia. Moreover, GDH specific activities have been demonstrated to increase by approximately ten-fold in response to growth in ammonia-limited environments (Baggio and Morrison, 1998; Yamamoto *et al*, 1984; 1987c). The activities of GS and GOGAT enzymes have been shown to be low in those *Bacteroides spp.* examined, and correspond to only ~1% of measurable GDH activities, even under conditions of nitrogen limitation (Yamomoto *et al.*, 1984, 1987c). Furthermore, the inhibition of GS activity with methionine sulfoximine (MSX) has been reported to have no observable effect on cell growth rate, or yield, of ammonia-limited *B. fragilis* cultures, despite having their GS specific activities reduced to ~15 % of that found in cells grown without MSX (Yamamoto, *et al.*, 1984). These findings, taken together, suggest that the GS-GOGAT pathway may not play a significant role in ammonia assimilation in the *Bacteroides*.

The gene encoding the *B. fragilis* GS (*gln*A) has previously been cloned and characterised (Southern *et al.*, 1986; Hill *et al.*, 1989), and shown to encode a novel GS (GSIII) that differs structurally from those found in many other prokaryotes (Southern *et al.*, 1987). GS activity is induced under low ammonia conditions, with *gln*A gene expression having been shown to be subject to transcriptional regulation in response to ammonia availability (Abratt *et al.*, 1993). This regulation was demonstrated to be dependent on the presence of two near perfect direct-repeat sequences, located in a 584 bp region upstream of the *gln*A gene. Unlike GS enzymes from other enteric bacteria, the *B. fragilis* GS is not subject to regulation by adenylylation, nor have homologues of NtrB or NtrC been identified (Southern *et al.*, 1987).

Two GDH enzymes have previously been identified in *B. fragilis* (Yamamoto *et al.*, 1984), one of which is NAD(P)H-dependent (GdhA), and the other NADH-dependent (GdhB). Physiological analysis revealed that GdhA activity was highest following growth with low exogenous concentrations of ammonia, while GdhB activity was thought to be partially induced at high ammonia concentrations (Yamomoto *et al.*, 1984). Characterisation of the former enzyme revealed that its activity is subject to regulation at the level of enzyme synthesis, as well as a reversible activation/inactivation mechanism controlled by the availability of ammonia (Yamamoto *et al*, 1987b). Immunochemical determination of cellular levels of GdhA using anti-GdhA antiserum revealed that it constitutes ~4% of the total cellular protein in cells grown with excess ammonia (> 10 mM NH_4Cl). However, when cells were grown with limiting concentrations of ammonia (1 mM NH_4Cl), the cellular levels of GdhA increased to ~8% of the total protein, suggesting that GdhA synthesis is stimulated by low ammonia concentrations (Yamamoto *et al.*, 1987b). In addition, GdhA activity was found to be rapidly inactivated upon the transfer of *B. fragilis* cells from an ammonia-limited (1 mM NH_4Cl) to ammonia excess (50 mM NH_4Cl) environment. Enzymatic activity was restored by transferring the cells to an ammonia limited environment, even in the presence of chloramphenicol, suggesting that the inactivation of enzyme activity is reversible (Yamamoto *et al.*, 1987b).

The precise mechanism whereby GdhA enzyme activity is regulated has not been elucidated. The reversible activation/inactivation mechanism does not, however, involve the phosphorylation of the enzyme (Yamamoto, 1987b). Furthermore, comparison of the purified active and inactive forms of the enzyme revealed no difference in its amino acid composition, subunit size or multimerisation state of the enzyme (Saito *et al.*, 1988). This led the authors to propose that the regulation of GdhA activity occurs via the modification of an active site residue present in the enzyme (Saito *et al.*, 1988). Support for this hypothesis has been provided by studies conducted on *B. thetaiotamicron,* which is similar to *B. fragilis* in possessing both NAD(P)H- and NADH-dependent GDH enzymes (Baggio and Morrison, 1998; Morrison, 2000). The structural gene (*gdh*A) encoding the *B. thetaiotaomicron* NAD(P)H-dependent enzyme has been cloned, and GdhA activity shown to be regulated by ammonia in a manner analogous to that found in *B. fragilis* (Baggio and Morrison, 1998). This gene required an additional gene product (GdhX), encoded by DNA sequences located downstream of the *gdh*A gene (or the sequence supplied in trans) for GdhA activity, but not transcription, in *E. coli* (Baggio and Morrison, 1998) The precise role of GdhX in regulating

GdhA activity is not known, but it was theorised that it may modulate GdhA enzymatic activity via the modification of the enzyme, or by facilitating the assembly of the polypeptide subunits into an active configuration.

1.9 Aims and objectives

Given their numerical predominance in the colon, the *Bacteroides* are likely to play a significant role in the turnover of nitrogenous compounds entering the human gut. Although much effort has been devoted to establishing an understanding of nitrogen metabolism in enteric bacteria, the process of nitrogen assimilation and regulation in the *Bacteroides* is less well understood. The potential involvement of proteases in the pathogenicity of the *Bacteroides* has further emphasised the need for analysis of the nitrogen metabolic pathways in these organisms. As outlined above, the physiological studies of Yamamoto *et al.* (1984; 1987b) revealed that ammonia assimilation in *B. fragilis* proceeds primarily via the GDH-catalysed pathway. To date, neither of its GDH enzymes have, however, been cloned or characterised at the molecular level, nor have the distinctive roles of the two enzymes in nitrogen assimilation been elucidated. The primary objectives of this study were, therefore, to examine the modulation of GDH activity in response to nitrogen source (inorganic vs. organic) and availability, so as to obtain a greater understanding of the role(s) of the individual GDH enzymes in the physiology of *B. fragilis*. In addition, the isolation and subsequent analysis of the genes encoding these enzymes, as well as their respective gene products, is likely to provide considerable insight into the mechanism(s) by which the modulation of GDH activity is achieved at the genetic and molecular levels. In view of the differences previously noted with regards to nitrogen assimilation in *B. fragilis*, research in this area may reveal the presence of regulatory systems different from that found in enteric, and other, bacteria.

CHAPTER 2

PHYSIOLOGICAL CHARACTERISATION OF THE GDH ENZYMES OF *B. fragilis* AND CLONING OF THE *gdh*B GENE

Contents

2.1 Summary

B. fragilis bf1 possesses both NAD(P)H-dependent (GdhA) and NADH-dependent GDH (GdhB) activities. A combination of enzyme assays and PAGE activity gels were used to assess the individual roles of these two enzymes in the physiology of *B. fragilis*. The activity of the GdhA was confirmed to be greatest in ammonia limited *B. fragilis* cultures, but was repressed by the inclusion of high concentrations of peptides in the growth media. GdhB activity, by contrast, was greatest following growth with high concentrations of peptides as the sole nitrogen source. No evidence for the regulation of GdhB activity by ammonia was found. The structural gene (*gdh*B) encoding the GdhB enzyme was isolated via heterologous complementation of an *E. coli* glutamate auxotroph. Attempts to clone the *gdh*A gene via functional complementation or DNA-DNA hybridisation were, however, unsuccessful. The complete nucleotide sequence of the cloned *gdh*B gene was determined, and an open reading frame of 1335 bp that is capable of encoding a polypeptide of 445 amino acids, with a predicted molecular mass of 48.3 kDa, was identified. The deduced amino acid sequence of GdhB polypeptides was found to exhibit significant similarity to the polypeptide subunits of Family I hexameric GDH enzymes from other bacterial species. Northern blot analysis revealed that the *gdh*B gene is monocistronic, and that the expression of GdhB activity is controlled at the level of transcription.

2.2 Introduction

B. fragilis has been shown to possess a novel mechanism of ammonia assimilation, in which the GDH-catalysed pathway is considered to play a preeminent role. Although two GDH enzymes have been identified in this organism, the unique contribution of these enzymes to nitrogen metabolism in *B. fragilis* has not been clearly resolved. In view of their physiological significance, the genes encoding GDH enzymes from various sources have been cloned via the functional complementation of *E. coli* glutamate auxotrophic mutants. To date, neither of the *gdh* genes from *B. fragilis* have been cloned, or characterised at the molecular level, in spite of their putatively pivotal role in nitrogen assimilation. There is evidence which suggests that the expression of *B. fragilis* genes in *E. coli* is frequently inefficient, presumably as a result of differences in their transcription and translation signals (Smith *et al.*, 1992). Notwithstanding, several *B. fragilis* genes involved in amino acid biosynthesis have been isolated via the functional complementation of *E. coli* mutants. The *B. fragilis leu*CDB genes involved in leucine biosynthesis have been cloned via *E. coli* mutant complementation (Sarker *et al.* 1995a, 1995b), and the *gln*A structural gene, which encodes the glutamine synthetase subunits, has been isolated using a similar approach (Southern *et al.*, 1986). This raised the possibility that genes encoding other nitrogen assimilatory enzymes could be isolated using a similar strategy.

In the current investigation, therefore, the modulation of GDH activity in response to nitrogen source and availability was examined so as to obtain a better understanding of the roles of the individual GDH enzymes in the physiology of *B. fragilis*. A further objective was to isolate and sequence the structural genes encoding these two enzymes, which would facilitate the analysis of the regulatory mechanisms involved in the control of GDH activity in this organism.

2.3 Materials and Methods

2.3.1 Bacterial strains, plasmids and culture conditions

B. fragilis bf1 was originally isolated from human faeces and described by Mossie *et al.* (1979). *B. fragilis* was grown routinely at 37°C in brain heart infusion (BHIS) broth (Difco) or agar (1.5%), supplemented according to Abratt *et al.* (1985). Cultures were grown in an anaerobic chamber (Forma Scientific) containing an atmosphere of N_2, CO_2 and H_2 (85:10:5 by volume). Nitrogen regulation studies in *B. fragilis* were conducted in glucose minimal medium (MM) broth (Varel and Bryant, 1974), modified as described by Abratt *et al.* (1993). The medium was supplemented with either 0.5, 5.0 or 50 mM NH_4Cl (low, medium and high ammonia, respectively), or 0.25% (w/v) or 1.25% (w/v) tryptone (low and high peptides, respectively). The construction of the *B. fragilis* gene library, using the suicide vector pEcoR251, has been described previously (Southern *et al.*, 1986). Plasmid pMT104, in which the *Eco*RI endonuclease gene of pEcoR251 is insertionally inactivated (Wehnert *et al.*, 1992), was used as a negative control for cloned GDH activity.

E. coli JM1O9 (Yanisch-Perron *et al.*, 1985) served as the cloning host for the introduction of recombinant plasmids. Screening for the *gdh* genes was carried out in *E. coli* MX3004 (*gdh*A *glt*D) (Castano *et al.*, 1992). *E. coli* strains were grown aerobically at 37°C in YT broth or agar (Sambrook *et al.*, 1989). Screening of the gene library was conducted using minimal medium A (MMA) (Sambrook *et al.*, 1989). Ampicillin (Ap) and chloramphenicol (Cm) were used at final concentrations of 100 $\mu g.ml^{-1}$ and 50 $\mu g.ml^{-1}$, respectively, in the medium when required.

2.3.2 Preparation of cell-free extracts

Bacterial cultures were grown to early stationary phase, as determined by optical density measurements over the time course of growth, and 100 ml harvested by centrifugation (10000 g for 10 min at 10°C). The cells were washed three times with 50 mM Tris-HCl (pH 8.0) and resuspended in 5 ml of the same buffer. Cells were disrupted by sonication at 95 W using 30 s bursts, for 5 min (VirSonic Digital 475 Cell Disruptor), centrifuged at 15000 g for 15 min at 4°C, and the crude cell-free extracts (CFE) used for GDH assays. For experiments to measure the activity of the enzyme under anaerobic conditions, cells were harvested under anaerobic conditions, washed with pre-reduced 50 mM Tris-HCl (pH 8.0) buffer, and disrupted using a

French pressure cell (Aminco; 32,000 psi). The disrupted cell material was collected in a nitrogen-filled tube, which was then centrifuged, and the CFE removed under anaerobic conditions. Assays were conducted under strict anaerobic conditions in the anaerobic chamber.

2.3.3 GDH enzyme assays

GDH activity was assayed spectrophotometrically (DU650 spectrophotometer, Beckman Industries) by following the decrease in absorbance at 340 nm during oxidation of NAD(P)H. The reactions were conducted at 25°C in 1.0 ml reaction mix containing 100 mM Tris-HCl (pH 8.0), 40 mM NH_4Cl, 5 mM 2-oxoglutarate and 0.15 mM NAD(P)H, and were initiated by the addition of 50 µl (75µg total protein) of cell free extract. When NADH was used as a cofactor, Tris-HCl (pH 7.4) was used. Enzyme activity was determined as the difference in oxidation rates of NAD(P)H in the presence and absence of substrate. Enzyme specific activities are expressed as units per milligram of protein in the cell free extracts, where one unit is defined as the amount of enzyme required to oxidise 1 µmol of cofactor per minute at 25°C. The protein concentrations of the cell free extracts were determined using the BioRad dye reagent with bovine serum albumin as a standard.

2.3.4 GDH zymograms

Cell free extracts (45 ug total protein) were subjected to discontinuous non-dissociating polyacrylamide gel electrophoresis using the buffer system of Laemmli (1970), modified by the omission of SDS. The acrylamide concentrations used were 7.5% and 3.5% in the stacking and resolving gels, respectively. Duplicate acrylamide gels were run, with an equivalent amount of protein being loaded in each lane. Following electrophoresis, the gels were incubated in staining solution consisting of 0.5 mM NADP or NAD, 20 mM L-glutamate, 0.3 $mg.ml^{-1}$ nitroblue tetrazolium, 0.05 $mg.ml^{-1}$ phenazine methosulphate, and 50 mM Tris-HCl (pH 8.0). GDH activity appeared as dark purple bands against a clear background.

2.3.5 Construction and screening of the recombinant DNA library

The construction of the *B. fragilis* genomic library has been previously described (Southern *et al.*, 1986). Recombinant plasmids were transformed into competent *E. coli* MX3004 cells. Transformants were screened for the expression of GDH and/ or GOGAT activity by plating

transformed cells onto MMA-agar plates containing Ap and Cm, and screening for glutamate prototrophy after 2 days incubation.

2.3.6 General recombinant DNA procedures.

All DNA modifications and manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). *B. fragilis* genomic DNA was prepared according to the method of Wehnert *et al.* (1992). Plasmid DNA was isolated by the alkali lysis method of Ish-Horowicz and Burke (1981) or with the High Pure Plasmid Purification Kit (Roche Diagnostics).

2.3.7 Southern hybridisation

Restriction endonuclease digested *B. fragilis* chromosomal and plasmid DNA was fractionated by electrophoresis in 0.8% agarose gels in Tris-Acetate-EDTA buffer. DNA was transferred to a nylon membrane (Hybond N^+; AmershamPharmacia Biotech) via the capillary alkali transfer procedure and fixed according to manufacturers instructions. DNA restriction fragments selected as probes were gel purified and random primed labelled with digoxigenin-11-UTP (DIG) using the Digoxigenin Labelling and Detection kit (Roche Diagnostics). Hybridisation with DIG labelled probes was performed overnight at 42°C using EasyHyb hybridisation buffer (Roche Diagnostics). This was followed by chemiluminescent detection with CSPD (Roche Diagnostics), according to manufacturers instructions.

2.3.8 PCR

To clone the *gdh*A gene, two oligonucleotide primers were designed based on the *B. thetaiotaomicron gdh*A sequence (Baggio and Morrison, 1998). These were: *gdh*A(F), 5'-TTC CCC AAC GAA CCG GAG TAT-3' (nucleotide positions 40-60) and *gdh*A(R) 5'-TAC AGA ATC TTA GCG TCT TGG AAT AC-3' (nucleotide positions 1230-1255). PCR amplification, using *B. fragilis* chromosomal as a template, was performed with *Taq* I polymerase (2.5U) in 100 µl reaction mixtures containing 100 ng of DNA, 100 pmol of each primer, 1.5 mM $MgCl_2$ and 200 µM deoxynucleotide triphosphates. The amplification cycles were as follows: 95°C for 5 min; 30 cycles of 95° C for 1 min; 50°C for 1 min and 72° C for 1 min; 72° C for 5 min. PCR products were purified using the High Pure PCR Purification Kit (Roche Diagnostics). The amplified product was treated with Klenow fragment to fill in recessed ends, and ligated into plasmid pBluescript SK(+) (Stratagene).

2.3.9 Construction of size selected gene libraries

Partial genomic libraries of *B. fragilis* bf1 were generated as follows: Genomic DNA was digested to completion with *Bam*HI, *Cla*I or *Hin*dIII restriction endonuclease and the resulting fragments fractionated via electrophoresis in 0.8% agarose in Tris-Acetate-EDTA buffer. Restriction fragments in the range of 2-5 kb were recovered using the Geneclean kit (Bio101), ligated into pSK(+) that had been digested with the appropriate endonuclease, and transformed into *E. coli* JM109.

2.3.10 Colony hybridisation

E. coli transformants that had been grown overnight on solid media were overlaid with Hybond N^+ membranes (AmershamPharmacia Biotech) to transfer colonies to the membrane filters. The membrane filters were processed as described by Sambrook *et al.* (1989). Hybridisation, using the DIG-labelled PCR fragment internal to the *gdh*A gene, and detection were performed as described for Southern hybridisation (Section 2.3.7)

2.2.11 Northern hybridisation

Total RNA was isolated from mid-exponential phase cultures (30 ml) of *B. fragilis* and *E. coli* MX3004 according to the method of Aiba *et al.* (1981). RNA was separated by electrophoresis in 1.5% denaturing formaldehyde gels according to Fourney *et al.* (1988). Equal amounts of RNA (15 µg) were loaded per lane and confirmed by the equal intensity of rRNA bands by ethidium bromide staining. The RNA was transferred by capillary action to a nylon membrane (Roche Diagnostics) and fixed according to manufacturers instructions. Hybridisation was performed at 42°C using a DIG-labelled, *gdh*B-specific probe in EasyHyb hybridisation buffer (Roche Diagnostics). This was followed by chemiluminescent detection with CSPD (Roche Diagnostics) according to manufacturers instructions. RNA molecular weight markers (0.25 - 9.5 kb) were obtained from USB.

2.3.12 Nucleotide sequencing and sequence analysis

Plasmid vector pBluescript KS(+) (Stratagene) was used to construct the subclones for DNA sequencing. Serial deletion of subclones was made by the *Exo*III nested-deletion method of Henikoff (1984). Nucleotide sequences were determined directly from plasmids by the fluorescent dideoxy chain termination method, using the Thermo-Sequenase Sequencing kit (USB) and 5'-fluorescently labelled universal forward and reverse primers. The sequencing

reaction products were analysed on the ALFexpress DNA sequencer (Pharmacia Biotech). The nucleotide sequence obtained was analysed using the University of Wisconsin Genetics computer Group DNA sequence analysis software (Devereux *et al.,* 1984) and the ClustalW (Thompson *et al.*, 1994), DNAman and GeneDoc software packages. The nucleotide sequence presented in this chapter was submitted to Genbank and assigned the accession number AAC26399. Additional GDH sequences used in this study, and their respective Genbank accession numbers are as follows: *Bacteroides thetaiotaomicron gdh*A (P94598), *Bacteroides thetaiotaomicron gdh*B (Morrison, M., personal communication), *Porphyromonas gingivalis gdh*B (AAA50985), *Prevotella bryantii gdh*A (AAB40142), *Escherichia coli gdh*A (AAG56747), *Pseudomonas aeruginosa gdh*A (AAGO7976), *Salmonella. typhimurium* (AAL20224), *Clostridium symbiosum gdh*B (CAA77805), *Corynebacterium glutamicum gdh*A (CAA51376). Additional sequence data for *B. fragilis* was obtained from the *B. fragilis* 9343 preliminary genome sequence produced by the Sanger Centre Sequencing group (www.sanger.ac.uk/projects/b_fragilis).

2.4 Results

2.4.1 GDH activity of *B. fragilis* bf1 under various nitrogen conditions.

Initially, all enzyme assays were conducted both aerobically and anaerobically. However, since GDH activity was not found to be affected by oxygen, all subsequent assays were performed aerobically. Cell free extracts of *B. fragilis* bf1 displayed both NADPH and NADH-dependent GDH activities. This finding is similar to previous studies conducted with *B. fragilis* ATCC 23745 by Yamamoto *et al.* (1984). The levels and ratios of these activities in *B. fragilis* bf1 varied according to the levels of inorganic or organic nitrogen supplied (Table 2.1). When ammonia was supplied as the sole nitrogen source, NADPH and NADH-dependent specific activities were highest for cultures grown in MM containing 0.5 mM NH_4Cl (1348U and 185U respectively), and the least for cultures grown with 50 mM NH_4Cl (291U and 80U respectively). Activities with NADPH exceeded those with NADH by approximately 8-fold in ammonia grown cultures, although this ratio decreased with increasing ammonia concentrations (Table 2.1). When *B. fragilis* was grown in complex medium (BHIS), NADPH-dependent GDH activity was markedly reduced relative to ammonia-grown cells, which was reflected in the ratio of NADH- to NADPH-dependent activities (Table 2.1).

Table 2.1. Comparison of GDH specific activities of *B. fragilis* Bf1 in response growth under different nitrogen conditions. One unit is defined as μmol cofactor oxidised min^{-1} (mg $protein^{-1}$). The values presented are the means of at least three different experiments ± standard deviation.

Nitrogen source	GDH specific activity (U)		
	NADPH	NADH	Ratio NADH:NADPH
NH_4Cl (O.5mM)	1348 ± 69	185 ± 4.6	0.137
NH_4Cl (5.0mM)	784 ± 22.2	120 ± 6.1	0.153
NH_4Cl (50mM)	291 ± 18.2	80 ± 5.0	0.27
BHI	107± 7.2	180 ± 16.0	1.68
Tryptone (0.25%)	406 ± 14.7	146 ± 7.2	0.35
Tryptone (1.25%)	85 ± 9.9	172 ± 12.3	2.02

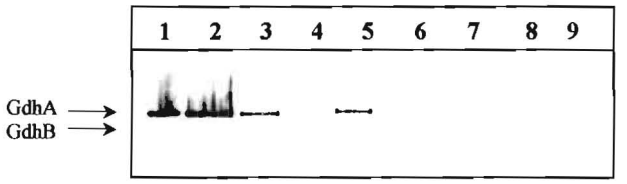
Since BHIS contains large quantities of organic nitrogen compounds, it was thought that these nutrients could be responsible for the observed modulation of GDH activities. *B. fragilis* is, however, unable to utilise amino acids as the sole nitrogen source, and the inclusion of glutamate in the growth media has been demonstrated to have no significant effect on cell growth or NADPH- or NADH-dependent GDH activities (Yamamoto *et al.*, 1984). To ascertain whether GDH activity was affected by peptides, *B. fragilis* was grown in MM supplemented with tryptone as a combination of peptides. Cells grown in the presence of low concentrations of tryptone possessed NADPH-dependent activity (406U) that was approximately three fold lower than that found in ammonia-limited cultures (Table 2.1), while NADH-dependent activity (146U) was marginally lower than that for cells grown in BHIS. Increasing the concentration of tryptone from 0.25% to 1.25%, however, resulted in a substantial decrease in NADPH-dependent GDH activity (406U vs. 85U respectively), while the NADH-dependent GDH activity was increased (146U and 172U).

2.4.2 GDH activity staining

The activities of the *B. fragilis* GDH enzymes were further examined using non-denaturing acrylamide gel electrophoresis and visualisation by activity staining. The two *B. fragilis* GDH enzymes were clearly distinguishable, both in their electrophoretic mobilities and

responses to the various cofactors and nitrogen sources supplied for growth (Fig. 2.1a, 2.1b; Lanes 1-6). Under low ammonia conditions (Lanes 1), the dual cofactor dependent GDH enzyme (GdhA) was most active, with relatively high levels of NADPH-dependent (Fig. 2a) and lower levels of the NADH-dependent (Fig. 2b) activity being detected. With increasing ammonia concentrations (5mM and 50mM, Lanes 2 and 3), the NAD(P)H-dependent GDH was progressively inactivated. No appreciable level of activity due to the NADH-dependent GDH (GdhB) was observed in response to growth with ammonia.

(a) NADPH



(b) NADH

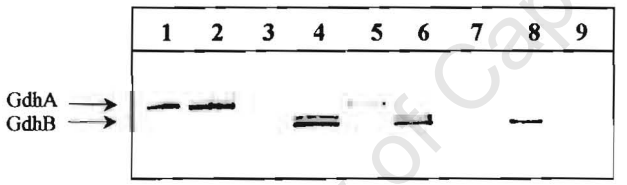


Figure 2.1. Activity staining of (a) NADPH- and (b) NADH-linked GDH activity on acrylamide gels. The extracts were prepared from *B. fragilis* cells grown under different nitrogen conditions and from *E. coli* MX3004 transformed with the cloned *gdh*B gene or a control plasmid, with 45 μg total protein run in each lane. *B. fragilis* cells were grown in 0.5 mM NH_4Cl (Lane 1), 5.0 mM NH_4Cl (Lane 2), 50 mM NH_4Cl (Lane 3), BHIS (Lane 4), 0.25% tryptone (Lane 5), or 1.25% tryptone (Lane 6). Lane 7: blank. *E. coli* MX3004(pGDH15) grown in YT broth (Lane 8). *E. coli* MX3004 (pMT104) grown in YT broth (Lane 9).

During growth of *B. fragilis* in BHIS activity of the GdhA enzyme was not detected, although high levels of NADH-dependent GdhB activity was observed (Fig. 2.1, Lanes 4). Under low-peptide conditions, the GdhA enzyme was active although its activity was slightly repressed relative to low ammonia conditions. No activity band corresponding to GdhB was present (Fig 2.1, Lanes 5). However, when high levels of peptides were included in the medium, GdhA activity was not detectable, while increased activity of GdhB was observed (Fig. 2.1, Lanes 6). Interestingly, when cells were grown in the presence of organic nitrogen (Fig. 2.1, Lanes 4 and 6), multiple bands possessing NADH-linked activity were observed.

2.4.3 Isolation of clones complementing the *gdh*A-*glt*D mutation in *E. coli*

For the identification of *B. fragilis* genes involved in ammonia assimilation, a plasmid library of *B. fragilis* genomic DNA (Southern *et al.*, 1986) was screened for its ability to restore the glutamate prototrophy of the glutamate auxotroph, *E. coli* MX3004. The plasmid library, constructed using pEcoR251, was transformed into *E. coli* MX3004 and the transformants plated on selective medium lacking glutamate (MMA-ampicillin agar plates). The growth of approximately 20 colonies was observed on the selective medium following an incubation period of 48 h. The glutamate prototrophy of these transformants was confirmed by restreaking on selective media. Plasmid DNA isolated from each of the transformants was also used to retransform *E. coli* MX3004, and the transformants replated on selective media. All of the transformants were simultaneously glutamate prototrophs and Ap^r, thus providing further confirmation of the conferred growth phenotype. Subsequent examination of the plasmids revealed that they all possessed similar DNA restriction fragment patterns (data not shown). Consequently, only one plasmid, designated pGDH15, which contains a 5.7 kb *Sau*IIIA DNA insert was selected for further analysis. A partial restriction map of the insert contained on this plasmid is shown in Fig. 2.2. To locate the gene(s) conferring glutamate prototrophy within this insert, transformants with insert deletions were examined for their ability to complement *E. coli* MX3004. This localised the gene(s) to an ~3.5 kb region encompassing a *Bgl*II and *Hin*dIII site contained on pGDH15.

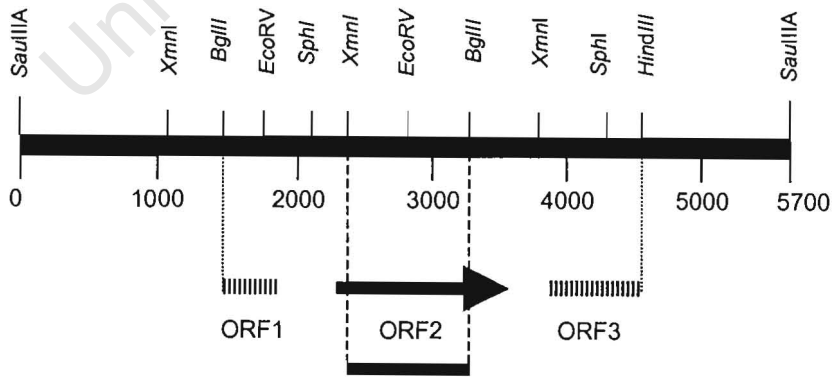


Figure 2.2. Partial restriction map of the *B. fragilis* bf1 DNA insert in pGDH15. The position and orientation of the *gdhB* gene, and the *Xmn*I–*Bgl*II fragment used as probe for Southern and Northern hybridisation analysis is indicated by the solid arrow and bar, respectively.

2.4.4 GDH activity of *E. coli* transformants

The activity of the cloned gene product was assayed in CFEs from *E. coli* MX3004 (pGDH15), and compared to that of *E. coli* MX3004 transformed with the control plasmid, pMT104. MX3004 carrying pGDH15 possessed high levels of NADH-dependent GDH activity (610 ± 32U), while no activity was found in the control system. The cloned gene product was also found to possess very low levels of NADPH-dependent activity (28 ± 6U). The activity of the cloned gene product in *E. coli* was confirmed to be NADH-specific by the appearance of a single band of equivalent mobility to the GdhB enzyme in cell lysates of *B. fragilis* (Fig. 2.1, Lane 8). No activity was detected in *E. coli* transformed with pMT104 (Fig. 2.1, Lane 9). These results indicated that cloned gene encodes the NADH-dependent GDH from *B. fragilis.*

2.4.5 Southern hybridisation analysis

Southern blot analysis was conducted to confirm that *B. fragilis* genomic DNA had indeed been cloned. A 966 bp *Xmn*I-*Bgl*II fragment, internal to the *gdh*B gene, was used as a probe. A single hybridisation signal, of equivalent size, was detected for both *B. fragilis* bf1 genomic DNA and pGDH15 digested with *Xmn*I and *Bgl*II (Fig. 2.3, Lane 1 and 3). No hybridisation signal was observed with *E. coli* DNA chromosomal DNA (Fig. 2.3, Lane 2), confirming that the insert DNA originated from *B. fragilis*.

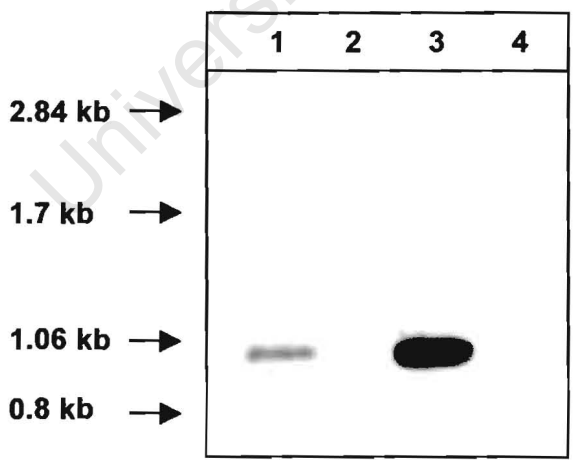


Figure 2.3. Southern blot analysis of genomic and plasmid DNA digested with *Bgl*II and *Xmn*I. Lane 1, *B. fragilis* bf1 genomic DNA; Lane 2, *E. coli* MX3004 genomic DNA, Lane 3, pGDH15; Lane 4, pMT104.

2.4.6 Nucleotide sequence analysis

In order to facilitate the sequencing of the *B. fragilis gdh*B gene and adjacent regions, appropriate subclones of pGDH15 were constructed. The 1.8 kb *Bgl*II and 1.6 kb *Bgl*II-*Hin*dIII fragments derived from pGDH15 were subcloned into pKS(+), to generate pGDH15-2 and pGDH15-3, respectively, and the nucleotide sequence of these plasmids determined, as described in the Material and Methods (Section 2.3.12). The combined nucleotide sequence of the *B. fragilis* DNA inserts of plasmids pGDH15-2 and pGDH15-3 spanned a 3489 bp region. Sequence analysis of this region revealed the presence of a complete open reading frame (ORF2), together with two incomplete ORFs (ORF1 and ORF3) (Fig. 2.2)

ORF2 is 1335 bp in size, and extends from a presumptive start codon (ATG, nucleotide position 301) to a stop codon (TGA, nucleotide position 1636) (Fig. 2.4). This ORF is capable of encoding a polypeptide of 445 amino acids with a molecular mass of 48.3 kDa, and it had homology to previously studied GDH proteins (Section 2.4.7). This indicates that ORF2 represents the *B. fragilis gdh*B structural gene. A second in frame ATG was detected further upstream (nucleotide position 256) that would yield a polypeptide 17 aa larger. The most probable initiation codon is, however, the former since the N-terminus of a polypeptide translated from position 301 shares homology with the N-termini of the majority of GDHs identified to date. The calculated G+C content of the *gdhB* gene was 46.5%, which corresponds well with the range of 39-48% determined for *B. fragilis* genomic DNA (Holdeman *et al.,* 1984).

The *gdh*B gene is not preceded by a conventional ribosome binding site (Shine and Dalgarno, 1974). Analysis of the region immediately upstream of the *gdh*B initiation codon resulted in the identification of sequences (AATG-N18-TATATTTG) similar to the *B. fragilis* promoter consensus sequences (Bayley *et al.,* 2000). In addition, several direct and inverse repeat sequences were identified in the 5' upstream region, which may function as potential binding sites for regulatory proteins controlling *gdh*B expression (Fig. 2.4). A region of dyad symmetry followed by a region rich in T residues was identified downstream of the stop codon. At the RNA level, this region would be capable of forming a stem-loop structure (ΔG = -21.4 kcal.mol^{-1}), similar to other prokaryotic rho-independent transcriptional terminators (Rosenberg and Court, 1979).

As mentioned above, the sequence revealed two additional, incomplete ORFs viz. ORF1 and ORF3, which are located in upstream and downstream regions of *gdh*B, respectively. ORF1 is oriented in the opposite direction to *gdh*B, with its putative initiation codon separated by 417 bp from the *gdh*B start codon. ORF3 starts 173 bp downstream of *gdh*B, and is oriented in the same direction as *gdh*B. To obtain additional information pertaining to the gene products encoded by these two ORFs, a BLAST search of the preliminary genomic sequence of *B. fragilis* 9343 (www.sanger.ac.uk/projects/b_fragilis), using the nucleotide sequence obtained in this study, was performed. The results showed that the *B. fragilis* bf1 and 9343 sequences corresponding to this region are highly similar, both in terms of their sequence (99.6% identity) and the chromosomal arrangement of genes contained therein. ORF3 was found to span 1161 bp and has a coding capacity of 387 amino acids with a predicted molecular mass of 42.6 kDa. Analysis of the deduced amino acid sequence revealed homology to aminopeptidases P, prolidases and methionine aminopeptidases, which together constitute the M24 family of metallopeptidases (Rawlings and Barret, 1995). The highest identities were found with aminopeptidases P from *Listeria monocytogenes* (25% identity), *Lactococcus pentosus* (24% identity), *E. coli* (23% identity), and *Streptococcus mutans* (22% identity). Aminopeptidases P catalyse the release of N-terminal amino acids from peptides that contain proline in the penultimate position. Whether the putative peptidase from *B. fragilis* possesses a similar specificity remains to be determined. Notwithstanding, the presence of a putative peptidase adjacent to *gdh*B may be significant, given the regulation of GdhB activity in response to the peptide availability in the growth medium.

ORF1 encompasses 2967 bp and encodes a protein of 989 amino acids with a predicted molecular mass of 113.5 kDa. A comparison of the predicted amino acid sequence of ORF1 with those of other proteins contained in the NCBI database did not reveal any overall homology to other protein sequences. Partial homologies with previously identified proteins were, however, evident at both the N- and C- terminal regions of this protein. The N-terminal region was found to possess two regions (aa 72-151 and 227-310) that exhibit significant homology with the N-terminal sensory domain of NtrC, a transcriptional regulator of nitrogen controlled genes. In addition, the C-terminal region (aa 459-801) was found to possess low, but significant homology to previously identified phosphoenolpyruvate synthases. At present, the function of this ORF is not known. The identification of regions similar to that found in NtrC proteins may, however, be indicative of an involvement for the ORF1 gene product in processes related to nitrogen metabolism.

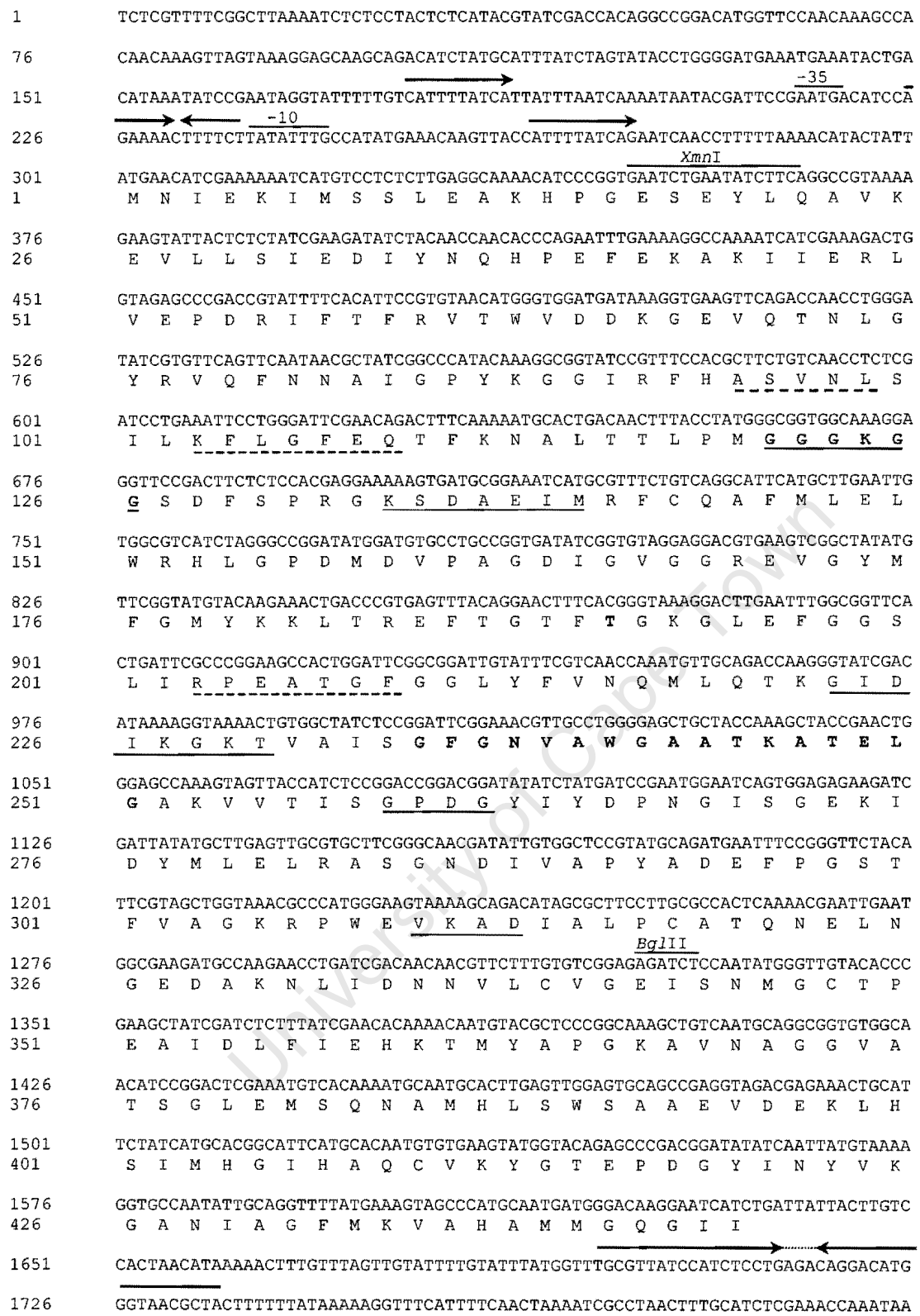


Figure 2.4. Nucleotide sequence of a 1.8 kb section of the pGDH15 insert DNA containing the *B. fragilis* Bf1 *gdhB* gene. In the upstream region, the putative promoter region is indicated. Arrows show the directions of the direct and inverse repeat sequences in the upstream and downstream regions. The deduced amino acid sequence of *gdhB* is depicted beneath the nucleotide sequence. The following features are shown: conserved Family I GDH regions, dashed underlined; GDH active site, bold underlined; NADPH binding site, bold; ATP / GTP binding site, underlined.

2.4 7 Analysis of the deduced GdhB amino acid sequence

A comparison of the predicted GdhB amino acid sequence with those contained in the genetic databases indicated that it shares significant identity with known GDH polypeptides (Fig. 2.5). Several particularly well conserved regions have been identified for GDH proteins, and these were discussed in Section 1.7.3.3. It is evident from multiple sequence alignments of GDH proteins that these regions have been conserved in the deduced *B. fragilis* GdhB polypeptide (Fig. 2.4). The most highly conserved regions include: (1) GGGKGG (aa 121-126) in which the critical lysine residue (aa 124) in the glycine-rich region is thought to be involved with the binding of glutamate/2-oxoglutarate and the catalytic activity of the enzyme (Baker *et al.*, 1992a); (2) the NADPH dinucleotide-binding motif, consisting of GFGNVAWGAATKATELG located between residues 235– 51. A distinctive feature of the primary sequence of the *B. fragilis* GdhB was the presence of a sequence motif, consisting of the amino acid residues GIDIKGKT (aa 223-230), GPDG (aa 259-263), and VKAD (aa 310-313), which corresponds to conserved regions of ATP/GTP-binding domains found to occur in a wide range of proteins. No leader sequence was evident from the deduced N-terminal polypeptide sequence, but the last 18 C-terminal amino acids were strongly hydrophobic in character.

Analysis of the overall amino sequence identity revealed the highest similarities to the GDH enzymes from other gram negative anaerobes, including the *B. thetaiotaomicron* GdhB (87% identity), *P. gingivalis* GdhB (72% identity), *B. thetaiotaomicron* GdhA (68% identity) and *P. bryantii* GdhA (66% identity). Lower identities were observed with GDHs from the enteric bacteria such as *S. typhimurium* and *E. coli* (57% and 56% identity, respectively). The deduced GdhB polypeptide also possesses regions similar to motifs characteristically found in Family I hexameric GDHs viz. PSVNL (aa 104-108), KFL(A/G)FEQ (aa 111-117) and RPEATGF (aa 212-217) (Benachenhou-Lahfa *et al.*, 1993).

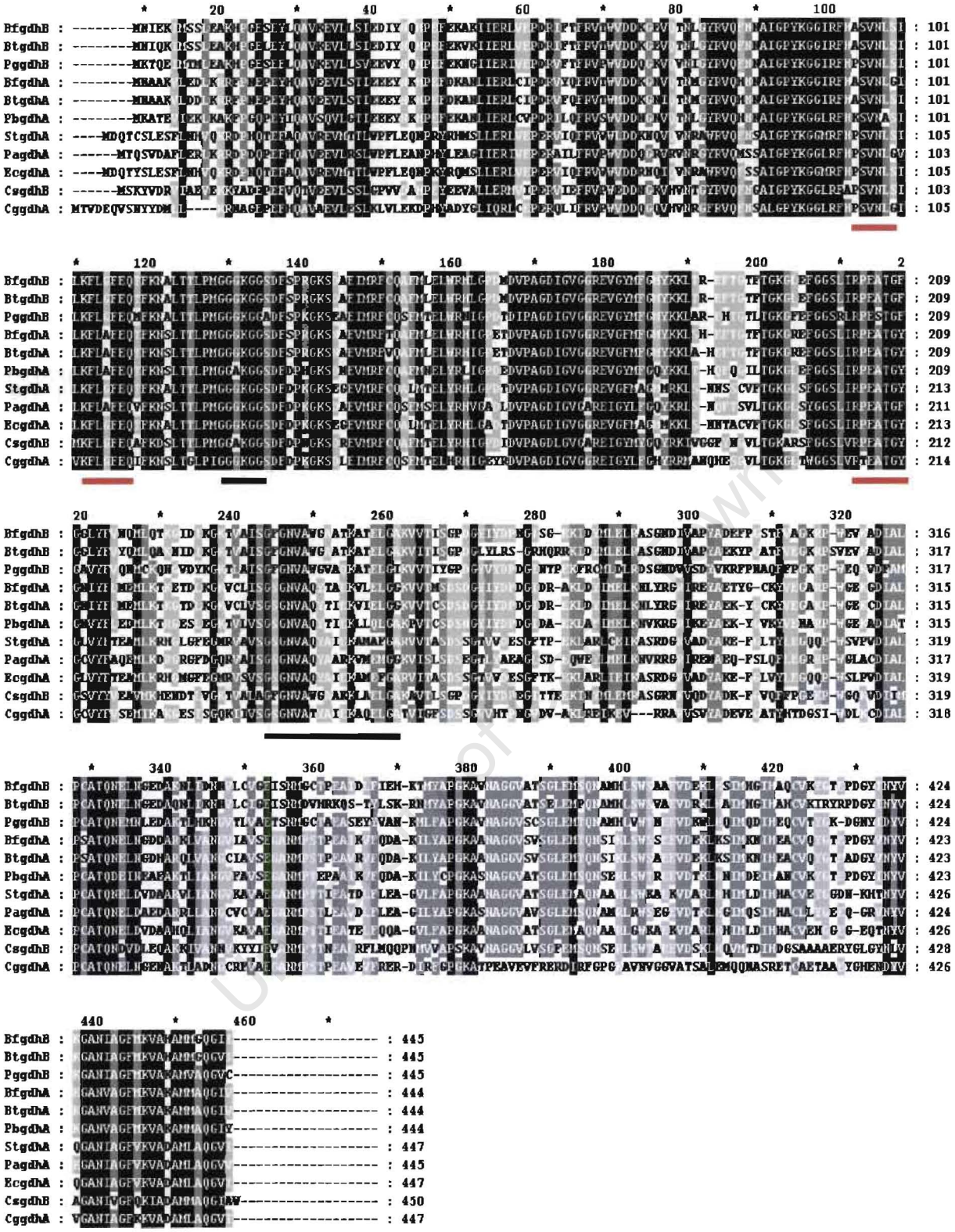


Figure 2.5. Multiple amino acid sequence alignment *B. fragilis* GdhB polypeptide with that of various other GDH enzymes. The origin of these proteins are as follows: BfgdhB: *B. fragilis gdh*B; BtgdhB: *B. thetaiotamicron gdh*B; PggdhB: *P.gingivalis*; BfgdhA: *B. fragilis gdh*A; BtgdhA: *B. thetaiotamicron gdh*A PbgdhA: *P. bryantii*; EcgdhA: *E.coli*; StgdhA: *S. typhimurium;* PagdhA: *P. aeruginosa*; CsgdhB: *C. symbiosum*; CggdhA: *C. glutamicum*. Amino acid residues considered important for glutamate binding and catalytic activity, and for NAD(P)H binding are underlined in black. Amino acids residues characteristic of Family I GDH sequences are underlined in red.

2.4.8 Northern hybridization analysis

To investigate the transcriptional regulation of the *gdh*B gene, Northern blot analysis of total RNA obtained *B. fragilis* cells grown under different conditions was performed. The *gdh*B-specific probe (969 bp *Xmn*I-*Bgl*II fragment from pGDH15) hybridised strongly with RNA isolated from *B. fragilis* cells grown in BHIS broth (Fig. 2.6, Lane 4). Only basal levels of *gdh*B gene expression were, however, observed when using RNA prepared from ammonia-grown *B. fragilis* cells (Fig. 2.6, Lanes 1-3). RNA prepared from *E. coli* MX3004 transformed with pGDH15 hybridised to the probe (Fig. 2.6, Lane 6), whereas *E. coli* MX3004 carrying the control plasmid pMT104 showed no hybridisation signal (Fig. 2.6, Lane 5). The *gdh*B transcript was estimated to be ~1500 nucleotides in size, which is in good agreement with the predicted size of the transcription product that extends from the putative promoter region upstream of the *gdh*B gene, to the inverted repeat sequence of the putative transcription terminator. This result is consistent with *gdh*B being expressed in a monocistronic fashion.

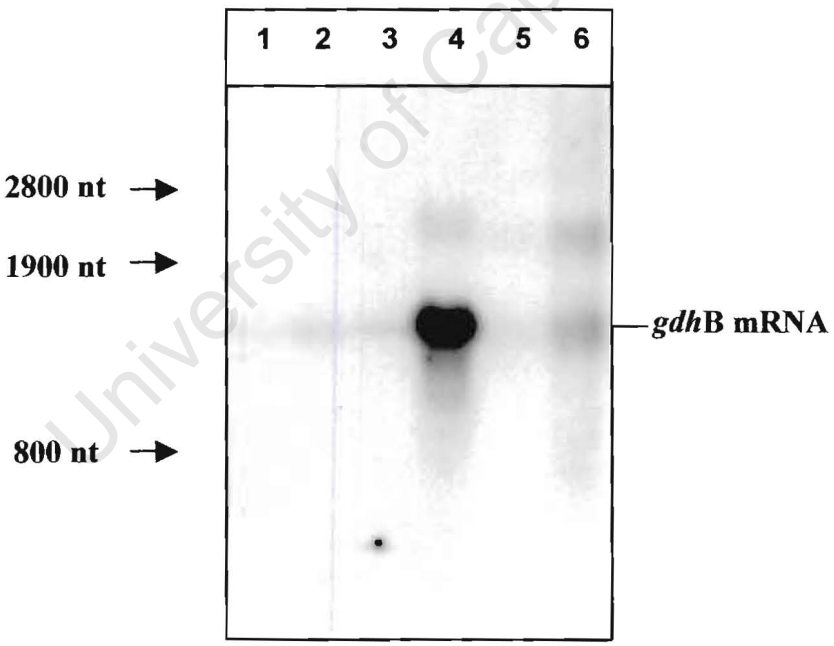


Figure 2.6. Northern blot analysis of *gdh*B expression in *B. fragilis* bf1 and *E. coli* MX3004. RNA was probed with the internal *Xmn*I-*Bgl*II fragment of the *gdhB* gene. Lanes: *B. fragilis* cells were grown in 0.5 mM NH_4Cl (Lane 1), 5.0 mM NH_4Cl (Lane 2), 50 mM NH_4Cl (Lane 3) or BHIS (Lane 4). Lane 5, *E. coli* MX3004 (pMT104); Lane 6, *E. coli* MX3004 (pGDH15). The positions of the reference markers are indicated.

2.4.9. PCR amplification

Attempts to clone the structural gene encoding the *B. fragilis* GdhA via functional complementation of *E. coli* MX3004 were unsuccessful. This suggests that the *gdh*A gene is not expressed in *E. coli,* or alternatively, that the GdhA enzyme is regulated in a manner or possesses biochemical characteristics that do not allow for complementation in the recombinant background. An alternative strategy was, therefore, designed to isolate the *B. fragilis gdh*A gene. Two oligonucleotide primers based on the *B. thetaiotaomicron gdh*A gene sequence (nt 40-60 and 1230-1255) were designed, and PCR with the primers resulted in the successful amplification of an approximately 1.0 kb fragment. The amplification product was subsequently cloned into pSK(+), and its nucleotide sequence determined from both strands. Nucleotide sequence comparison of this sequence with the corresponding region of the *B. thetaiotaomicron gdh*A gene revealed 94% identity, confirming that the PCR product encoded part of the *B. fragilis* GdhA protein.

2.4.10 Colony hybridisation

To isolate the complete *gdh*A structural gene, partial genebanks were prepared from *Bam*HI, *Cla*I or *Hin*dIII digested genomic DNA, as described in Materials and Methods (Section 2.3.9). Southern blot analysis of *B. fragilis* genomic DNA restricted with these enzymes, and probed with the DIG-labelled PCR product indicated that the *gdh*A gene was located on fragments of approximately 3 kb (*Cla*I and *Hind*III) and 4.5 kb (*Bam*HI) in size (Fig. 2.7). Two hybridisation signals were, however, observed for genomic DNA restricted with *Eco*RI, indicating the presence of an *Eco*RI restriction site within the *B. fragilis gdh*A structural gene.

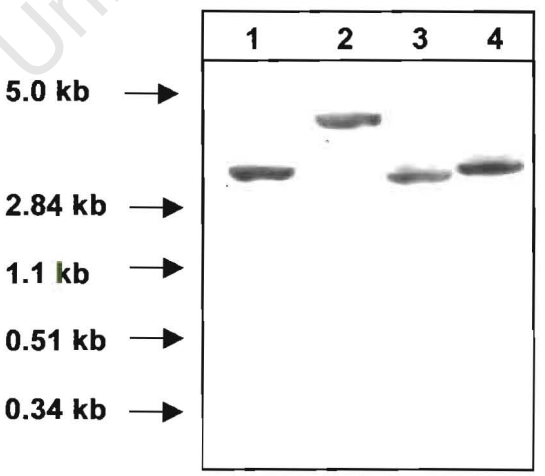


Figure 2.7. Southern hybridisation analysis of *B. fragilis* genomic hybridised with the 1024 bp probe from pGDH15. Genomic DNA digested with Lane 1, *Hin*dIII; Lane 2, *Bam*HI; Lane 3, *Eco*RI; and Lane 4, *Cla*I.

The partial gene libraries constructed were transformed into *E. coli* JM109 and screened by colony hybridisation, using the DIG-labelled PCR fragment as a probe. The screening of ~2000 colonies, however, failed to yield any hybridising clones.

A BLAST search of the preliminary genomic sequence of *B. fragilis* 9343, with the partial nucleotide sequence of the *B. fragilis* bf1 *gdh*A gene obtained here, however, resulted in the identification of a single candidate with a perfect homology to the input sequence, thus permitting the analysis of the genetic locus containing the *B. fragilis* 9343 *gdh*A structural gene. The nucleotide sequence of a 1600 bp region, containing the *gdh*A structural gene and adjacent regions, retrieved from the *B. fragilis* genome project is shown in Fig. 2.8. Computer analysis showed that the *gdh*A coding region is 1332 bp long and encodes 444 amino acids with a predicted molecular mass of 48.8 kDa, which is in good agreement with the subunit size of the majority of hexameric GDH enzymes studied to date. Sequence homology searches with the predicted GdhA amino acid sequence confirmed the similarity of this protein to previously identified GDH polypeptides. The *B. fragilis* GdhA displays a significant level of homology to the corresponding GdhA polypeptides from *B. thetaiotaomicron* and *P. bryantii* (94 and 76% identity, respectively), while lower levels of homology with the *B. fragilis* and *B. thetaiotaomicron* GdhB subunits (69% and 63%, respectively) was observed. The predicted *B. fragilis* GdhA amino acid sequence contains regions similar to the three motifs characteristic of Family I hexameric GDH enzymes, as well as the amino acid residues considered essential for glutamate binding and catalysis, and for NADPH binding (Fig. 2.8).

In the region immediately upstream of the *gdh*A gene, a sequence (CTTG-N19-TACTTTTG) resembling the *B. fragilis* promoter consensus sequence was evident (Fig. 2.8). In addition, several repeat sequences, distinct from those found upstream of the *gdh*B gene, were identified in the region of the putative -35 sequence, which could potentially serve as binding sites for regulators. The *gdh*A gene was followed by an inverted repeat sequence characteristic of rho-independent transcription terminators (Rosenberg and Court, 1979).

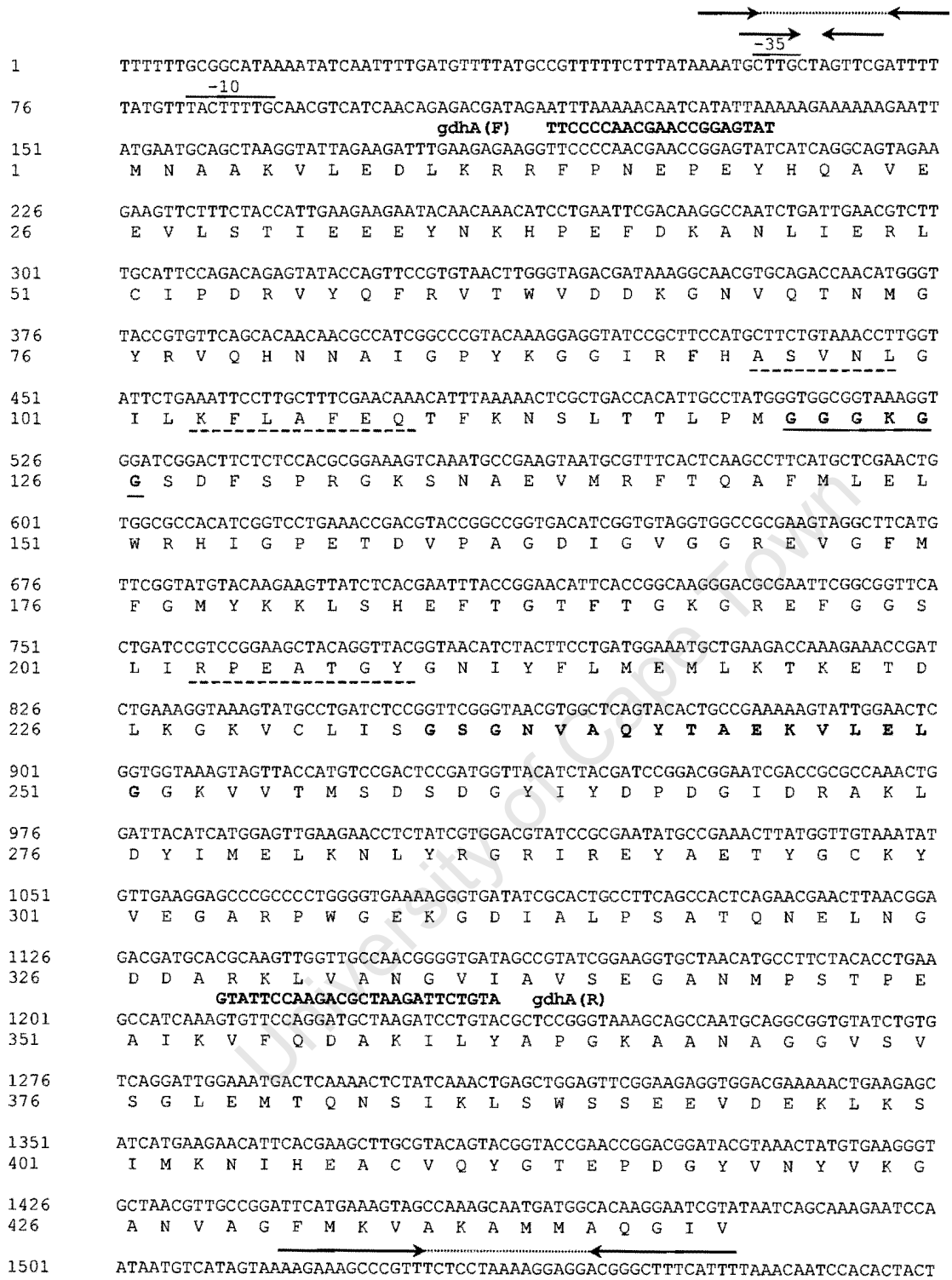


Figure 2.8. Nucleotide sequence of the *B. fragilis* 9343 *gdh*A gene. The sequence of the oligonucleotide primers used for PCR amplification of the internal fragment of the *B. fragilis* bf1 *gdh*A is depicted in boldface. The nucleotide sequence obtained from sequencing of the PCR fragment amplified is positioned between nucleotides 212 and 1209. In the upstream region, the putative promoter region is indicated. Arrows show the directions of the direct, indirect and inverse repeat sequences in the upstream and downstream regions. The deduced amino acid sequence of *gdh*A is depicted beneath the nucleotide sequence. The following features are shown: conserved hexameric Family I GDH regions, bold and dashed underlined; glutamate binding site, bold underlined; NADPH binding site, boldface type.

2.5 Discussion

The presence of two GDH enzymes in *B. fragilis* was first reported by Yamamoto *et al* (1984) who proposed that the NAD(P)H-dependent GDH (GdhA) was responsible for ammonia assimilation primarily under conditions of low ammonia, and that the NADH-specific GDH (GdhB) was induced when ammonia was in excess. In the present study, *B. fragilis* bf1 was found to possess both the GdhA and GdhB enzymes, and their activities were demonstrated to be differently regulated in response to the type (inorganic vs organic nitrogen) and concentration of nitrogen source provided. GdhA was most active when ammonia was provided as the sole nitrogen source. In agreement with previous studies, its activity was found to be maximal at low exogenous concentrations of ammonia, and decreased as the levels of ammonia was increased. Thus, while the regulation of GdhA in response to ammonia concentration differs from that found many in other gram negative bacteria, its presence as the sole GDH in ammonia-grown cultures of *B. fragilis* is consistent with the premise that its role is primarily that of ammonia assimilation and the biosynthesis of glutamate (Yamamoto *et al*, 1984; 1987b). In support of this notion, the substitution of ammonia with peptides as the sole nitrogen source resulted in a dramatic reduction of GdhA activity. The peptide-dependent repression of GdhA activity was more pronounced at high concentrations, suggesting that this phenomenon occurs in a concentration–dependent manner. The pattern of regulation of the *B. fragilis* GdhA in response to nitrogen source demonstrated here is analogous to that found in the ruminal *Prevotella* species, such as *P. bryantii* B_14 and *P. ruminicola*, where a marked inhibition of biosynthetic GDH activity is observed in response to the replacement of ammonia with peptides as the cellular nitrogen source (Wen and Morrison 1996, 1997).

By contrast, GdhB activity was only present when peptides were included in the growth media as the sole nitrogen source. In the studies conducted by Yamamoto *et al.* (1984), a lower NADPH to NADH-dependent activity ratio in high as compared to low ammonia was noted, and this was interpreted as evidence for induction of GdhB under high ammonia conditions. Although a similar trend was observed here, no evidence for the induction of GdhB activity by ammonia was found. The results of Northern hybridization analysis indicated that the same basal levels of *gdhB* mRNA were produced under all ammonia conditions examined. The observed difference in ratios for ammonia grown cultures may therefore be a consequence of basal constitutive levels of the GdhB enzyme being maintained

throughout, with a repression of the dual cofactor GdhA enzyme occurring with increasing ammonia concentrations.

Several bacterial species have been shown to produce NADH-dependent GDHs following growth with preformed amino acids, where they are presumed to function in the oxidative degradation of glutamate. The observation that GdhB is subject to physiological activation by peptides suggests that it may perform such a role in *B. fragilis*. There is currently, however, very little information regarding the mechanism of peptide uptake and utilisation in the *Bacteroides*. Although a similar induction of NADH-dependent GDH activity has been reported in *B. thetaiotaomicron* (Morrison, 2000) and *P. brevis* (Wen and Morrison, 1997), the molecular mechanisms, and metabolic pathways involved have not been defined. Several parallels can, however, be drawn from studies conducted on the closely related oral anaerobe, *P. gingivalis*, which possesses a single NADH-dependent GDH (Joe *et al.*, 1993; Shah and Gharbia, 1995). This organism possesses a peptide-dependent metabolism, and has been shown to preferentially utilise glutamate/glutamine and aspartate/asparagine containing peptides present in tryptone (Takahashi *et al.*, 2000; Takahashi and Sato, 2001). Moreover, the catabolism of glutamate arising from the hydrolysis of glutamate-containing peptides by dipeptidases was demonstrated to occur via the GDH pathway. Although the precise nature, and composition, of the peptides regulating GdhB (and GdhA) activity have not been defined, it is conceivable that a similar mechanism of glutamate catabolism may exist in *B. fragilis*. Further studies are required to address this issue.

The structural gene encoding GdhB was cloned and sequenced, and transcriptional analysis indicated that the *gdh*B gene is expressed as a single cistron. The inability to clone the structural gene encoding GdhA in *E. coli* using either of the approaches used in this study is not completely clear. Similar difficulties in the cloning of the *gdh*B gene from *C. symbiosum* have, however, been reported elsewhere (Teller *et al.*, 1992), presumably due to the toxic effects of regions adjacent to the *gdh* gene on the *E. coli* host. A search of the unfinished *B. fragilis* 9343 genomic sequence, however, yielded the entire *gdh*A gene, thus allowing analysis of the structural gene and flanking regions. Analysis of the regions upstream of both *gdh* genes revealed the presence of several sequence elements, including promoter-like and repeat sequences, which may represent DNA recognition sites for regulatory proteins involved in the regulation of *gdh* gene expression in response to nitrogen source and/or

availability. The potential role of these elements in the transcriptional regulation of *gdh*B expression is examined in Chapter 3.

As anticipated, the predicted GdhA and GdhB polypeptides shared extensive homology to GDHs from other gram negative anaerobes. Analysis of the deduced amino acid sequence of the *B. fragilis* GdhB revealed the presence of an ATP/GTP binding motif, lacking in most of the other bacterial GDHs examined to date. While the bovine GDH has been shown to be subject to complex regulation in response to allosteric effectors, including the purine nucleotides ATP and GTP (Smith *et al.*, 2001), no such regulation by these compounds have been reported for bacterial hexameric GDHs. Although similar motifs have been shown to be present in the GDH enzyme from *P. bryantii* (Wen and Morrison, 1996), the physiological significance of these in the regulation of GDH activity remains unclear. Of particular interest, however, was the presence of multiple bands possessing NADH-dependent activity following activity staining, which may reflect a modification of the GdhB enzyme at the post-translational level in response to factor(s) other than those examined in this study. The identification of only a single activity band corresponding to the cloned GdhB protein in cell free extracts from *E. coli* MX3004 (pGDH15) suggests that the factor(s) responsible for such regulation are not present in the recombinant host. The potential involvement of a post-translational mechanism in regulating GdhB activity is further investigated in the Chapter 4.

CHAPTER 3

TRANSCRIPTIONAL REGULATION OF THE *B. fragilis gdh*B GENE

Contents

3.1 Summary

The transcriptional regulation of the *B. fragilis gdh*B gene was examined. Primer extension analysis was used to map the *gdh*B transcription start sites in cells grown with 1.25% or 0.25% tryptone (high- and low- peptides, respectively). Two transcription start sites were mapped to nucleotides positioned 50 and 52 bp upstream of the *gdh*B initiation codon, under each of the growth conditions examined. Sequences that correspond closely to the consensus promoter described for *B. fragilis* were identified immediately upstream of the transcription start sites. The expression of *gdh*B gene in *B. fragilis* was examined using a *gdh*B'::β-xylosidase gene transcriptional fusion, and promoter activity measured by assaying the levels of β-xylosidase reporter gene activity. A region encompassing 313 bp upstream and 169 bp downstream of the *gdh*B the translational start site, respectively, was found to be sufficient for promoter activation and regulation in *B. fragilis*. Expression of the *gdh*B gene was found to be highest following growth in high-peptide medium, and decreased approximately 10-fold when cells were grown in low-peptide medium. Deletion analysis of the region upstream of the *gdh*B promoter revealed that sequences located between 313 and 139 bp upstream of the translation initiation codon are essential for *gdh*B promoter activity.

3. 2 Introduction

The isolation and molecular analysis of the *B. fragilis gdh*B gene was described in Chapter 2. These studies established that GdhB activity was highest when *B. fragilis* was grown with high levels of organic nitrogen, either in BHIS broth or in minimal medium containing 1.25% tryptone as the sole source of nitrogen. Northern blot analysis of *gdh*B expression revealed that the gene is expressed as a single cistron, and suggested that the regulation of GdhB activity occurs primarily at the level of transcription in response to the nitrogen source supplied for growth. Analysis of the DNA sequence upstream of the *gdh*B gene resulted in the identification of several sequence elements potentially involved in the expression and regulation of *gdh*B expression. In this study, *gdh*B'::β-xylosidase transcriptional fusions were used to define the minimal promoter requirements for the expression of the *gdh*B gene, and to determine the significance of the abovementioned sequence elements in the regulation of GdhB activity. Such an analysis may serve to provide useful information pertaining to nitrogen-regulated promoters in this organism.

3.3 Materials and Methods

3.3.1 Bacterial strains, growth conditions and plasmids.

B. fragilis bf1 was grown in supplemented brain heart infusion (BHIS) broth (Abratt *et al.*, 1985) in an anaerobic chamber (Forma Scientific) containing an atmosphere of 5% H_2, 10% CO_2 and 85% N_2. For nitrogen regulation studies, *B. fragilis* bf1 was grown in glucose minimal medium (MM) (Varel and Bryant, 1972), modified as described by Abratt *et al* (1992), and supplemented with 0.25% tryptone (low-peptides) or 1.25% tryptone (high-peptides) as the sole source of nitrogen. Gentamicin or erythromycin were added to the growth medium at 200 $\mu g.ml^{-1}$ and 10 $\mu g.ml^{-1}$, respectively, when required. Cloning, subcloning and mating experiments involving *E. coli* were conducted with strains JM109 or HB101, which were grown in Luria-Bertani (LB) broth or agar (Sambrook *et al.*, 1989). Plasmids were selected for by the incorporation of ampicillin (100 $\mu g.ml^{-1}$), spectinomycin (50 $\mu g.ml^{-1}$) or tetracycline (10 $\mu g.ml^{-1}$) in the growth medium.

3.3.2 General recombinant DNA procedures.

All DNA modifications and manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). *B. fragilis* genomic DNA was prepared according to the method of Wehnert *et al.* (1992). Plasmid DNA was isolated by the alkali lysis method of Ish-Horowicz and Burke (1981) or with the High Pure Plasmid Purification Kit (Roche Diagnostics).

3.3.3 RNA extraction and primer extension reactions

RNA was extracted from mid-exponential phase *B. fragilis* bf1 cultures according to the method of Aiba *et al* (1981). Potential transcription start sites of the *gdh*B gene were identified by automated fluorescent primer extension analysis using a Cy5-labelled oligonucleotide primer (*gdh*B-R1, Table 3.1), which is complementary to the nucleotides 148 to 169 bp downstream of the *gdh*B initiation codon. RNA samples (100 µg) were ethanol precipitated, resuspended in 100 µl of RNA hybridisation buffer (40 mM PIPES, pH 6.4; 1 mM EDTA, 400 mM NaCl, 80% deionised formamide), and added to 5 pmol labelled primer. The nucleic acids were denatured at 95°C for 10 min, and annealed overnight at 42°C. After ethanol precipitation, the annealed RNA was resuspended in 20 µl RTB (1X AMV Reverse Transcriptase buffer (Promega); 0.5 mM dNTP's, 40 U RNazin (Roche Diagnostics); 2 µg Actinomycin D) and the primer extended on the mRNA template at 42°C for 2 hours using 20 units of AMV Reverse transcriptase (Promega). The reaction was terminated by the addition of 1 µl 0.5 M EDTA (pH 8.0). The RNA was degraded with RNase A (10 µg) for 30 minutes at 37°. After ethanol precipitation, the cDNA was resuspended in 5 µl Tris-EDTA buffer (pH 8.0), to which 8 µl of sequencing stop buffer was added. The primer extension products were analysed using the ALFexpress automated DNA sequencer (Pharmacia Biotech), with analysis of the sequencing reaction products, obtained using the same primer, performed in parallel.

3.3.4 Construction of *gdh*B'::XA transcriptional fusions

PCR was used to amplify various fragments of the *gdh*B promoter region using *B. fragilis* chromosomal DNA as a template. The forward primers were tagged with *Sph*I sites to facilitate subsequent cloning (Table 3.1) and, excluding the 5' restriction site, annealed 411 bp (primer GdhB-F1), 313 bp (primer GdhB-F2), 139 bp (primer GdhB-F3), 45 bp (primer GdhB-F4) and 4 bp (primer GdhB-F5) upstream of the *gdh*B initiation codon, respectively.

The forward primers were used in combination with a standard reverse primer, GdhB-R1, located within the *gdh*B coding sequence.

Table 3.1 Oligonucleotide primers

Primer[a]	Sequence[b] (5'→3')	Position (bp)[c]
Forward		
GdhB-F1 (pGDX411)	ATTTGCTGAGCATGCGCTTCCG	-427 to -405
Gdhb-F2 (pGDX313)	TATCCATGCATGCACAGATTG	-326 to -306
GdhB-F3 (pGDX139)	ATACTGACATAAGCATGCGAAT	-157 to -136
GdhB-F4 (pGDX45)	TATATTTGCCGCATGCAACAAG	-61 to -40
GdhB-F5 (pGDX4)	CCTTTTTAAAGCATGCTATTATG	-20 to +3
Reverse		
GdhB-R1	AAATACGGTCGGGCCCTACC	+148 to +169

a. Plasmids containing the DNA fragments amplified by the corresponding forward primers are shown in parenthesis

b. The underlined sequences of the primers denote *Sph*I restriction sites

c. Numbering corresponds to the sequence presented in Figure 3.2 (c)

PCR amplification was performed in 100 µl reaction mixtures containing 100 ng of DNA, 100 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates and 2.5U Expand High Fidelity DNA polymerase (Roche Diagnostics). The amplification cycles were as follows: 95°C for 5 min; 30 cycles of 95° C for 1 min; 50°C for 1 min and 72° C for 1 min; 72° C for 5 min. PCR products were purified using the High Pure PCR Purification Kit (Roche Diagnostics). The DNA fragments amplified by PCR were blunt-ended and phosphorylated, and inserted into the *Sma*I site of the reporter gene vector, pXA1 (Whitehead, 1997) to generate a fusion between the *gdh*B promoter-containing regions and the promoterless xylosidase/arabonisidase (XA) gene (Fig. 3.1). Restriction digestion was used to verify that the promoter fragments were in the same orientation as the XA gene, and their sequence confirmed by sequencing. For analysis in *B. fragilis*, the *cat* fusion vector pFD395 (pFD325TT; Smith *et al*, 1992) was digested with *Eco*RI to remove the promoterless chloramphenicol resistance (*cat*) gene, and religated to generate pFD395ΔEcoRI. After digestion with *Xba*I, the linearised plasmids containing the *gdh*B'::XA fusions were treated with Klenow fragment to fill in recessed ends, and then digested with *Sph*I. The fragments carrying the *gdh*B'::XA fusions were subsequently cloned into the *Sph*I/*Sma*I site of pFD395ΔEcoRI for analysis of promoter activity.

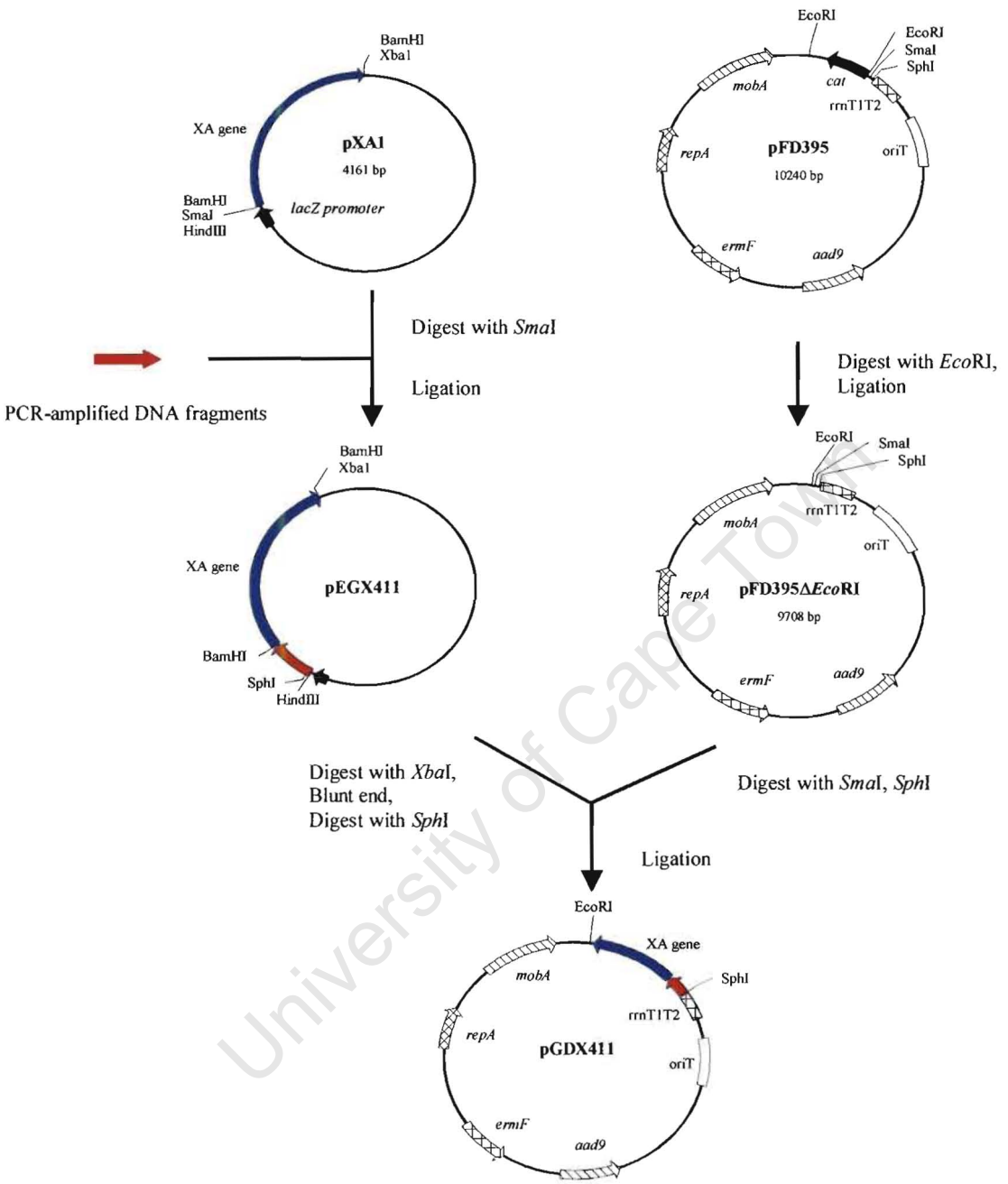


Figure 3.1. Construction of *gdh*B'::XA transcriptional fusion plasmids pGDX4-411. The DNA fragments containing regions upstream of the *gdh*B promoter and first 169 bp of the coding region are depicted by the red arrow. The promoterless XA gene is shown as a blue arrow. The relevant restriction enzyme sites are shown.

3.3.5 Introduction of the *gdh*B'::XA constructs into *B. fragilis* bf1

Plasmids were introduced into *B. fragilis* by the conjugal transfer from *E. coli* to *B. fragilis* via a triparental mating procedure (Shoemaker *et al.*, 1986). *E. coli* donor strains bearing the plasmid to be mobilised and *E. coli* HB101(RK231) were cultured aerobically in LB broth to an OD_{600} of 0.2, while *B. fragilis* bf1 was grown anaerobically in BHIS to an OD_{600} of 0.2. The cells were mixed at a ratio of 1:1:5 and harvested by centrifugation. The cell suspension was suspended in 0.2 ml BHIS and spotted onto sterile 0.45 μM nitrocelluose filters (HAWP025; Millipore) that had been placed on BHIS-agar plates. The mating was performed aerobically for 16 h at 37°C. The bacteria were washed from the surface of the membrane filters by vortexing in 3 ml of BHIS and incubated at room temperature for 1 hour. Transconjugant colonies were selected for by anaerobic growth on BHIS-agar plates containing gentamicin (200 $\mu g.ml^{-1}$) and erythromycin (10 $\mu g.ml^{-1}$).

3.3.6 Preparation of cell free extracts

For the xylosidase assays, *B. fragilis* cultures were grown overnight (16 h) in MM containing 0.25% or 1.25% tryptone. These cultures were used to inoculate fresh MM, and bacterial cultures were grown to late exponential phase. Cells were harvested by centrifugation (10000 g for 10 min at 4°C), washed with ice-cold 100 mM potassium phosphate buffer (pH 7.4) and resuspended in 5 ml of the same buffer. Cells were disrupted by sonication at 95 W using 30 s bursts, for 5 min (VirSonic Digital 475 Cell Disruptor, Virtis), centrifuged at 15000 g for 15 min at 4°C, and the cell-free extracts (CFE) obtained used for xylosidase assays.

3.3.7 Determination of xylosidase activities

Expression of the XA gene under control of the *gdh*B promoter fragments was measured by a spectrophotometric assay with p-nitrophenyl-β-D-xylopyranoside as a substrate. The assays were conducted at 37°C in a 0.5 ml reaction mix containing 100 mM sodium phosphate buffer (pH 6.8), 1 mM dithiothreitol and 1 mM p-nitrophenyl-β-D-xylopyranoside. The reaction was terminated by the addition of 0.5 ml 2% sodium carbonate. One unit of β-xylosidase activity is defined as the amount of enzyme that catalyses the release 1 μmol of p-nitrophenol per min at 37°C, measured at 405 nm. The protein concentrations of the cell free extracts were determined using the BioRad dye reagent with bovine serum albumin as a standard.

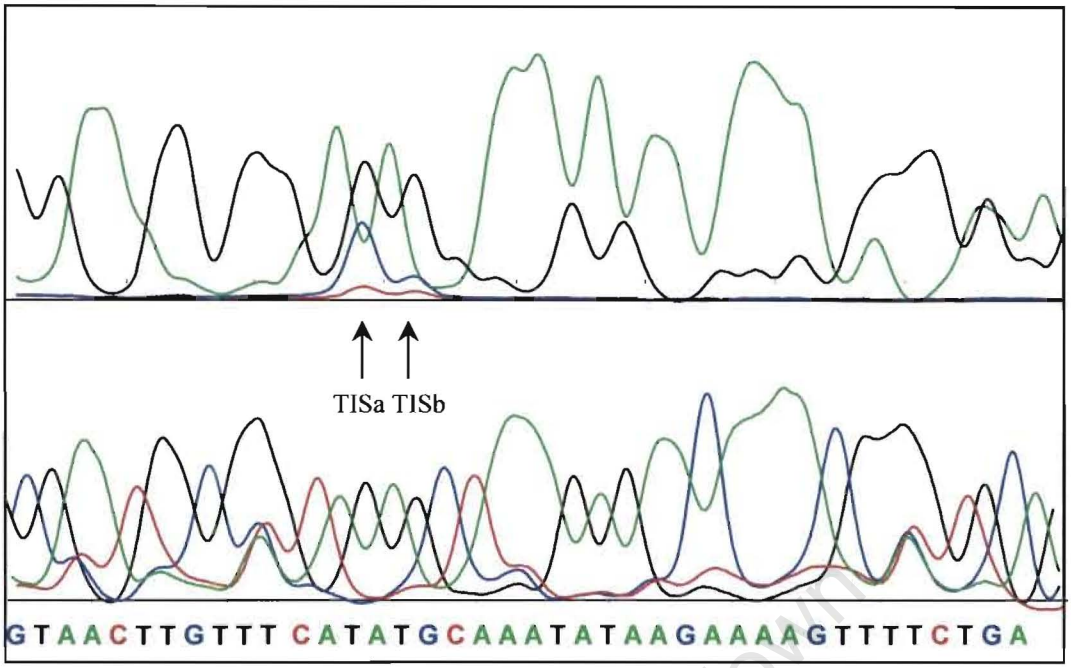
3.4 Results

3.4.1 Primer extension analysis

Primer extension analysis was performed to identify a nitrogen-regulated promoter directing *gdh*B gene expression. Total RNA extracted from *B. fragilis,* grown in MM supplemented with either high- or low- peptides, was primed with a Cy5-labelled oligonucleotide primer complementary to the coding strand of the *gdh*B gene. As shown in Fig. 3.2 (a), two primer extension products, which correspond to transcription being initiated at A residues positioned 50 (TISa) and 52 (TISb) bp upstream of the *gdh*B ATG start codon, were identified under each of the growth conditions examined, with the majority of transcripts appearing to be initiated from TISa. Furthermore, the primer extension signals were greater for RNA obtained from cells grown with high- as opposed to low- peptides. Analysis of the DNA sequence immediately upstream of the transcriptional initiation sites revealed the presence of motifs (AATG-N18-TATATTTG), whose sequence and spacing correspond closely to the consensus regions of *B. fragilis* promoters (Bayley *et al.*, 2000). An additional feature of the upstream region is the presence of several direct and inverse repeat sequence elements [Fig. 3.2 (c)], which may be involved in the regulation of *gdh*B expression. Nitrogen regulation of the *gdh*A gene in some enteric bacteria is mediated by the Nac protein, which represses *gdh*A transcription in response to nitrogen limitation (Bender, 1991; Muse and Bender, 1998). However, no homology between the repeat sequences upstream of the *B. fragilis gdh*B gene and the Nac binding consensus sequence was found.

(a)

(b)



(c)

```
-529 TAAAAGCATCATAAGGATTAGCTATCAGTAAGACGTTAAAAATTCGCCTTGTCATCAGGTTAGCAAACTGGGTATCTTTA -450
-449 AAGTAGAGCTGGTTTAATTTAAATTTGCTGAGCATGGGCTTCCCGTAGTTTTAAAATTCAACAGACAAAAGTCGCTGTTT -370
-369 TTATTGGGAAAAACAAGAAAAAACAATTCACTTTATTGCTGACTATCCATATATGCACAGATTGCTTTCTCGTTTTCGGC -290
-289 TTAAAATCTCTCCTACTCTCATACGTATCGACCACAAGGCCGGACATGGTTCCAACAAAGCCACAACAAAGTTAGTAAAG -210
-209 GAGCAAGCAGACATCTATGCATTTATCATGTATAACCTGGGGATGAAAATGAAATACTGACATAAATATCCGAATAGGTA -130
                                                  -35          -10    * *
-129 TTTTTGTCATTTTATCATTATTTAATCAAAATAATACGCCATTCCGAATGACATCAGAAAACTTTTCTTATATTTGCATA -50
                                                     +1
-49  TGAAACAAGTTACATTTTATCAGAATCAACCTTTTTAAAACATACTATTATGAACATCGAAAAAATCATGTCCTCTCTTG +31
                                                     -1
+32  AGGCAAAACATCCCGGTGAATCTGAATATCTTCAGGCCGTAAAAGAAGTATTACTCTCTATCGAAGATATCTACAACCAA+111
+112 CACCCAGAATTTGAAAAGGCCAAAATCATCGAAAGACTGGTAGGGCCCGACCGTATTT -169
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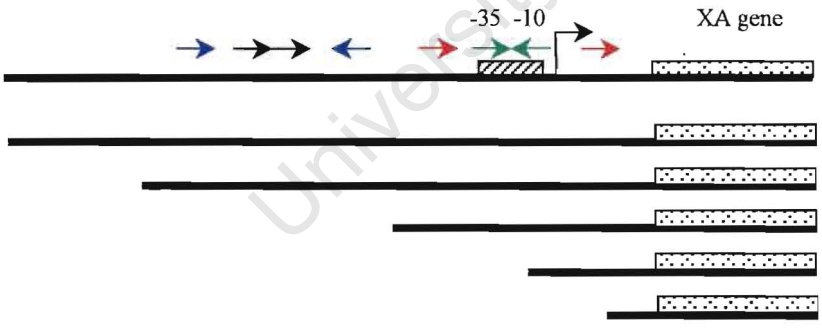
Figure 3.2. Mapping of the transcription initiation site of the *B. fragilis gdh*B gene by primer extension analysis. (a) Primer extension products obtained using RNA isolated from *B. fragilis* grown in MM containing high- (blue curve) or low peptides (red curve). A and T sequencing reactions (green and black curves, respectively) were used as tracking lanes. The positions of the transcription initiation sites (TISa and TISb) are indicated by arrows. (b) The DNA sequencing flourogram corresponding to the region analysed. The same Cy^5 labelled primer was used as a primer for both sequencing and primer extension reactions. (c) Nucleotide sequence of the *gdh*B regulatory region. The start codon of the *gdh*B gene is boxed. The putative transcription initiation sites are depicted by asterisks. Putative -35 and -10 promoter regions are underlined. The direct and indirect repeat sequences are indicated by arrows.

3.4.2 Reporter gene analysis

To define the minimal *gdh*B promoter requirements, a series of transcriptional fusions between putative promoter regions and a promoterless β-xylosidase reporter gene were constructed as described in the Materials and Methods (Section 3.2.4), and the resulting plasmids introduced into *B. fragilis* bf1 by conjugation. The reporter gene activity was quantified in cell free extracts as a measure of β-xylosidase gene expression under the control of the *gdh*B promoter. The initial fusion constructed, pGDX411, contained a 580 bp fragment that extended 411 bp upstream and 169 bp downstream, of the *gdh*B translation start codon, respectively. The maximal level of reporter gene activity in *B. fragilis* (pGDX411) was observed in cells grown in high-peptide medium (4.2U). This was reduced approximately 10-fold (0.4U) following growth in low-peptide medium. Control *B. fragilis* cells harbouring constructs containing only the promoterless xylosidase gene exhibited negligible activity following growth in both high-, and low-peptide media. These results confirmed that the *gdh*B promoter is regulated by the external peptide concentration, and that all the information required for expression and regulation are located on the promoter region contained on pGDX411.



	XA activity % tryptone	
	1.25	0.25
pGDX411	4.2±.0.3	0.4±0.03
pGDX313	3.8±0.2	0.4±0.04
pGDX139	ND	ND
pGDX45	ND	ND
pGDX4	ND	ND

Figure 3.3. Xylosidase activities in *B. fragilis* carrying transcriptional fusions. The bold line represents the relative lengths of DNA fragments carrying the promoter studied. The location of the direct and indirect repeats is indicated by arrows and the putative -35 and -10 are shown. XA activities of *B. fragilis* grown in MM supplemented with 0.25% or 1.25% tryptone are shown as mean ± standard deviation. The values presented are the means of at least three different experiments. ND: not detected

To further localise the 5' boundary required for promoter activity, deletion analysis of the 411 bp *gdh*B promoter region contained on pGDX411 was performed. The promoter regions present in the various deletions constructs and the corresponding changes in xylosidase activity are shown in Fig. 3.3. Within experimental error, pGDX313, which had a 98 bp region deleted from the 5' end of the promoter fragment contained on pGDX411, exhibited similar xylosidase activities to pGDX411 in cells grown under both high- and low- peptide conditions. This suggests that the fragment contained on pGDX313 carries all the *gdh*B promoter regulatory elements, and that sequences upstream of position -313 play no apparent role. The removal of a further 174 bp of the *gdh*B promoter region (pGDX139), however, caused a complete loss of xylosidase activity following growth in both high- and low- peptide medium. A similar loss of xylosidase activities was observed for larger deletions contained on plasmids pGDX45 and pGDX4 (Fig. 3.3). These findings indicate that an extended promoter region, which includes specific sequences located between positions -139 and -313, [Fig. 3.2 (c)], is required for expression of the *gdh*B gene in *B. fragilis*.

3.4 Discussion

To obtain a greater understanding of the regulatory mechanisms controlling GdhB activity, the transcriptional regulation of the *gdh*B gene was examined. Transcription of the *gdh*B gene was found to be specifically initiated both 50 and 52 bp upstream of the *gdh*B initiation codon. The nucleotide sequence of the predicted -10 and -35 elements (AATG-N18-TATATTTG), ascribed on the basis of the primer extension data, possesses significant homology with the *B. fragilis* promoter consensus sequence (-35; TTTG and -10; TANNTTTG) described by Bayley *et al.* (2000). Previous findings have shown that GdhB activity is induced following growth with high concentrations of peptides, and suggested that the regulation of its activity is achieved at the level of transcription (Chapter 2). In the current investigation, a high level of reporter gene activity was measured in *B. fragilis* cells grown under high-peptide conditions, with approximately 10-fold less induction observed in cells grown under low-peptide conditions. These results confirm that GdhB activity is transcriptionally regulated in response to growth with organic nitrogen in the form of peptides, and that *gdh*B expression is induced by the increased availability of these nutrients.

Deletion analysis of the *gdh*B promoter identified nucleotide sequences important for promoter function. Sequences extending beyond position -313 seem to play no role in the regulation of promoter activity, since promoter fragments spanning 411 bp (pGDX411) and 313 bp (pGDX313) upstream of the ATG start codon were found to promote expression of xylosidase activity with similar efficiency. The deletion of sequences between -313 and -139, by contrast, eliminated promoter activity. It, therefore, appears likely that specific sequences residing within this region function in the regulation of *gdh*B gene expression, perhaps by acting as cis-acting elements that serve as binding sites for proteins that stimulate transcription initiation. The complete lack of reporter gene activity in *B. fragilis* strains harbouring pGDH139 was nevertheless surprising, considering that the promoter fragment present in this fusion contains a considerable amount of DNA upstream of *gdh*B transcriptional initiation site (-88 relative to TISa). In gram-negative bacteria, the transcriptional activation of a number of nitrogen-regulated genes is controlled by the NtrC and NifA proteins (Merrick and Edwards, 1995). It is well known that these regulators promote transcription activation by interacting with specific upstream activating sequences, which are frequently located 100-200 bp upstream of its cognate promoter (Kustu *et al.*, 1991; Merrick, 1993). Inspection of the DNA sequences upstream of *gdh*B did not, however, reveal the presence of sequences with strong homology to the binding sites of these, or other, nitrogen regulatory proteins. Interestingly though, a 8 bp direct repeat sequence, which is flanked on either side by a 8 bp indirect repeat with a spacing of 23 bp (5'TAGCTATC-N23-CAACAAAG-N3-CAACAAAG-N23-CTATCGAT3'), was identified between positions -268 and -188 [Fig. 3.2 (c)]. At this point, the significance of these, or other sequences contained within this region is not known, and a more detailed analysis is required to establish more precisely the contribution of these sequence(s) in the regulation of *gdh*B expression.

CHAPTER 4

PURIFICATION, LOCALISATION AND REGULATION OF THE GdhB PROTEIN

Contents

4.1 Summary

The *B. fragilis* GdhB protein was expressed in *E. coli* from the recombinant plasmid, pGDH15-1, carrying the *gdhB* gene. The recombinant protein was purified to electrophoretic homogeneity, and the characteristics of the recombinant enzyme (rGdhB) determined. The temperature and pH activity optima were 38°C and 8.0, respectively, and rGdhB enzyme activity was inhibited approximately 2-fold by the presence of divalent salts ($CaCl_2$, $MgCl_2$). The presence of monovalent salts (NaCl and KCl) or metabolites (ATP, AMP, or GTP) did not significantly affect rGdhB activity. The enzyme characteristics of the recombinant GDH were, however, found to differ to some extent from those obtained for purified native GdhB (Lee, Chi-Lei, 2000). Western blot analysis, using antiserum raised against the rGdhB protein, revealed that a single immunoreactive protein was present in cell free extracts obtained from *B. fragilis* grown in BHIS, or minimal media supplemented with 1.25%, but not 0.25% tryptone. Cellular localisation studies using cell fractions of *B. fragilis* grown under inducing conditions indicated that ~79% of GdhB activity was present in the membrane fraction. Immunogold labelling and electron microscopy confirmed that the GdhB enzyme is located at the surface of *B. fragilis* cells. The regulation of GdhB activity was examined at the protein level and evidence of post-translational regulation of the protein in response to peptides, but not ammonia, was found.

4.2 Introduction

While the GDH enzymes from *B. fragilis* appear to possess many structural similarities to those found in other bacterial species, significant differences in their regulatory properties have been shown to exist. Prior characterisation of the *B. fragilis* GdhA enzyme revealed that its activity is subject to regulation at the level of enzyme synthesis, as well as by reversible activation of enzyme activity in response to the extracellular ammonia concentration (Yamamoto *et al.*, 1987b). The exact mechanism by which this regulation is achieved has, however, not been established (Saito *et al.*, 1988). The results presented in previous chapters revealed that GdhB activity is up-regulated in response to growth with high concentrations of peptides. Northern blot analysis suggested that regulation of its activity occurred primarily at the transcriptional level, although additional mechanisms were not excluded as potentially playing a role in the regulation of enzymatic activity. The present experiments were, therefore, undertaken to determine whether GdhB activity was additionally regulated at the post-translational level.

Further, in the majority of published reports, GDHs have been described as cytoplasmic enzymes where they are typically involved in intermediary metabolism (Smith *et al*, 1975). Joe *et al.* (1994), however, reported that the NADH-dependent GDH of *P. gingivalis* is localised to the surface of the bacterial cell. Despite its lack of a conventional N-terminal signal sequence, the GDH from this organism was theorised to be associated with the cell membrane via a hydrophobic region located at the C-terminus of the protein. A comparison of the *B. fragilis* GdhB with its counterpart in *P. gingivalis* revealed that a high degree of amino acid similarity between the two polypeptides exists, which extended to the C-terminal region believed to be involved in its cell surface location. This, together with the close evolutionary relationship between these two organisms, raised the possibility that the GdhB enzyme might be similarly located in *B. fragilis*. To further investigate this matter, the cellular distribution of the GdhB protein in *B. fragilis* was determined.

4.3 Materials and Methods

4.3.1 Bacterial strains, plasmids and growth conditions.

B. fragilis bf1 was grown routinely at 37°C in brain heart infusion (BHIS) broth or agar (1.5%), supplemented according to Abratt *et al.*, (1985). Anaerobic conditions for the growth of *B. fragilis* were achieved in an anaerobic chamber (Forma Scientific) containing a gas mixture of 5% H_2, 10% CO_2 and 85% N_2. Nitrogen regulation studies in *B. fragilis* were conducted in glucose minimal medium (MM) (Varel and Bryant, 1974), modified as described by Abratt *et al.* (1992). The medium was supplemented with either 1.25% (w/v) or 0.25% (w/v) tryptone (high and low peptides, respectively) as a nitrogen source, as specified.

E. coli strains were grown at 37°C in yeast tryptone (YT) broth or agar (Sambrook *et al.*, 1989). For expression of the *gdh*B gene, a 2242 bp *Nsi*I–*Sal*I fragment from pGDH15 was cloned into pSK(+), thereby generating pGDH15-1. The cloned insert encompassed a region extending from 188-bp upstream to 719 bp downstream of the *gdh*B structural gene. *E. coli* MX3004 (*gdh*A *glt*D) (Castano *et al.*, 1992) was used for expression of the cloned *gdh*B gene. Ampicillin (Ap) and chloramphenicol (Cm) were added to the medium at concentrations of 100 $\mu g.ml^{-1}$ and 50 $\mu g.ml^{-1}$, respectively, when required.

4.3.2 General recombinant DNA procedures

All DNA modifications and manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). Plasmid DNA extraction from *E. coli* was performed according to the alkaline method of Isch-Horowitz and Burke (1981), or with the High Pure Plasmid Purification Kit (Roche Diagnostics).

4.3.3 Protein Purification.

In order to purify the recombinant GdhB enzyme of *B. fragilis,* the *gdhB* gene was expressed in the glutamate auxotroph *E. coli* MX3004. *E. coli* MX3004 (pGDH15-1) was grown overnight in 8 L YT broth, harvested by centrifugation (10000 g for 10 min at 4°C), washed with an equal volume of 50 mM Tris HCl (pH 8.0), and resuspended in 400 ml 50 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$ and 20 $ug.ml^{-1}$ each of RNase A and DNase I. The cells were disrupted by sonication (Virsonic Digital 475 Cell Disrupter) at 95W for 10 min (30 s bursts with 30 s cooling intervals) and centrifuged (5000 g for 15 min at 4°C) to remove

cell debris. The crude cell extract was used in subsequent purification steps. All procedures were conducted at 4°C unless otherwise stated. The cell extract was subjected to ammonium sulphate precipitation. The precipitate obtained between 45 and 70 % ammonium sulphate saturation was dissolved in 50 mM Tris-HCl (pH 8.0, 50 ml) and dialyzed against the same buffer. The solution was applied to a DEAE-cellulose anion exchange column (Whatman), which had been equilibrated with 50 mM Tris HCl (pH 8.0). The column was washed with buffer to remove unbound proteins. The column was then eluted with a linear NaCl gradient (0 – 0.2 M) in 50 mM Tris-HCl (pH 8.0) and 5 ml fractions were collected, which were subsequently assayed for NADH-dependent GDH activity. Fractions displaying GDH activity were pooled and concentrated by ultrafiltration using a membrane with a molecular weight exclusion limit of 10 kDa (Amicon). The concentrated enzyme was then applied to a Reactive Red-agarose column (Sigma) that had been equilibrated with 50 mM Tris HCl (pH 8.0). The enzyme was eluted with a linear NaCl gradient (0-0.3 M) and 2 ml fractions were collected. The fractions were assayed for NADH-dependent GDH activity and the active fractions pooled.

4.3.4 GDH enzyme assays

GDH activity was assayed spectrophotometrically (DU650 spectrophotometer, Beckman) by determining the decrease in absorbance at 340 nm during oxidation of NADPH or NADH. The reactions were conducted at 25°C in 1.0 ml reaction mix containing 100 mM Tris-HCl (pH 8.0), 40 mM NH_4Cl, 5 mM α-ketoglutarate and 0.15 mM NAD(P)H. In experiments to determine the temperature and pH optima of the purified enzyme, the temperature was varied between 25 - 45°C, and the buffer pH between 6.5 and 9.5. To determine the effect of cations on enzyme activity, monovalent (NaCl or KCl) or divalent ($MgCl_2$ or $CaCl_2$) salts were added at a final concentration of 100 mM. Alternatively, ATP, AMP and GTP were added at a final concentration of 1 mM. Enzyme activity was determined as the difference in oxidation rates of NADH or NADPH in the presence and absence of substrates. Enzyme specific activities are expressed as units.mg^{-1} protein, where one unit is defined as the amount of enzyme required to oxidise 1 μmol of cofactor per minute. The protein concentration was determined using the BioRad dye reagent with bovine serum albumin as a standard.

Assays on whole cell GdhB activity were carried out using a modification of the method of Gil-Navarro *et al.* (1997). *B. fragilis* cells were grown to mid log phase (OD_{600} ~0.6) in MM supplemented with 1.25% tryptone, harvested by centrifugation (10000 g for 10 min) and washed with an equal volume of 100 mM Tris-HCl (pH 8.0). Washed cells were incubated with or without glutamate (5 mM) in the presence of NAD (0.15 mM) in the assay buffer (100 mM Tris-HCl, pH 8.0). The reaction mixtures were allowed to incubate for 1 h at room temperature, following which the cells were removed by centrifugation (10000 g for 10 min). The supernatants were analysed for the presence of NADH by determining the absorbance at 340 nm with a Beckman spectrophotometer. Activity was expressed as μmol NADH generated per unit OD cells used during the assay.

4.3.5 Preparation of antiserum

Antibodies against the recombinant GdhB protein were prepared by injecting a New Zealand white rabbit with 1 ml (1 $mg.ml^{-1}$) of protein, mixed with Freunds incomplete adjuvant. Injections were administered intradermally for three weeks. Blood samples were taken weekly after administration of the third antigen injection. These were allowed to clot, and centrifuged (2000 g for 10 minutes) to obtain the serum fraction. Serum samples were sealed in sterilized bottles, and either frozen at –20°C for long term storage, or kept at 4°C for immediate use.

4.3.6 Polyacrylamide gel electrophoresis

Protein samples were separated by SDS-PAGE using the discontinuous buffer system of Laemmli (1970). Acrylamide concentrations for SDS-PAGE were 3.5% and 10% in the stacking and resolving gels, respectively. Pharmacia low molecular weight markers were used as molecular weight standards. Non-denaturing PAGE was performed in the same manner as SDS-PAGE, modified by the omission of SDS and β-mercaptoethanol, and acrylamide concentrations were 3.5 and 7.5% in the stacking and resolving gels, respectively. Gels were either fixed and stained with Coomassie blue, followed by destaining, or the proteins transferred to nitrocellulose for immunoblotting.

4.3.7 Immunoblotting

Following non-denaturing PAGE, proteins were transferred to a nitrocellulose membrane with a semi-dry electrophoretic transfer apparatus (Omeg Scientific). Transfer was carried out at

250 mA for 1 h in transfer buffer (25 mM Tris-HCl, pH 7.5/10% methanol). Membranes were blocked with 1% BSA in Tris-buffered saline (TBS), pH 7.5, for 30 minutes and then placed in primary antibody (1:100 dilution) in TBS for 1 h. The membrane was washed three times for 5 min with TBS and then processed for the detection of antigen-antibody complexes with alkaline phosphatase conjugated secondary antibodies according to manufacturers instructions (Sigma).

4.3.8 Cellular fractionation

In order to determine the cellular localisation of the *B. fragilis* GdhB, bacterial cells were grown overnight in MM supplemented with 1.25% tryptone. Preparation of the cytoplasmic and membrane fractions was performed as described by McCarthy *et al.* (1985). Extracellular and periplasmic fractions were prepared using the method of Huang and Forsberg (1987). Briefly, overnight cultures were harvested by centrifugation (10000 g for 10 min) and the supernatant retained as the extracellular fraction. Cell pellets were washed twice with 100 ml PBS. The cells were subsequently resuspended in 50 ml of 25% sucrose/1 mM EDTA, shaken at room temperature for 10 min, and harvested by centrifugation (20000 g for 20 min). Cells were then rapidly resuspended in 20 ml of cold sterile water and shaken gently for 10 min at 4°C. The suspension was centrifuged at 30000 g for 20 minutes, and the supernatant retained as the periplasmic fraction. Cell pellets were resuspended in 10 ml PBS and disrupted by passage through a French pressure cell at 200000 kPa (SIM, Aminco). Cell debris was removed by centrifugation (17500 g for 15 min) and the supernatant subjected to ultracentrifugation (110000 g for 2.5 h). The supernatant was retained as the cytoplasmic fraction and the pellet retained as the membrane fraction. Since suitable marker enzymes for use in the *Bacteroides* are not currently available, these were not included in the present study.

4.3.9 Immunogold labelling

B. fragilis cells, grown overnight in MM supplemented 1.25% tryptone, were harvested and resuspended in phosphate buffered saline (PBS), pH 7.4. The cells were washed twice with an equal volume of buffer and fixed in 1% formaldehyde in PBS for 1 h. The cells were resuspended in low-melting point agarose [1% (w/v)], placed into polypropylene capsules, and allowed to solidify. The agarose embedded cells were impregnated with 2.3 M sucrose and plunge frozen at -180°C using a Leica KF80 cryofixation apparatus. Thin sections (90

nm) were cut at –110°C on a Reichert Ultramicrotome Ultracutter, and placed on 200-mesh nickel grids covered with Formvar. Cell labelling was performed by floating the grids successively on the surface of drops of 20 mM glycine in PBS, 1% BSA-PBS, and anti-rGDH antiserum diluted with 1% BSA-PBS. The grids were washed thoroughly with PBS between treatment with each solution. The grids were then floated on goat anti-rabbit IgG colloidal-gold bead conjugate, diluted with 1% BSA-PBS, and washed with H_20. Cells were negatively stained with 2% uranyl acetate in methylcellulose and viewed using a Zeiss EM109 electron microscope.

4.3.10 Nitrogen "shock" experiments

B. fragilis cells were grown to late exponential phase in MM supplemented with 1.25% tryptone to obtain high levels of fully active GdhB activity. The cells were then exposed to a rapid reduction of the peptide concentration by the addition of pre-reduced MM, or MM supplemented with NH_4Cl, to give a final concentration of 0.25% tryptone or 0.25% tryptone/5 mM NH_4Cl, respectively. Control cultures were treated with an equivalent volume of MM supplemented with 1.25% tryptone. Cells were harvested at 5 and 20 min post-shock, washed three times with 50 mM Tris- HCl (pH 8.0) and resuspended in 5 ml of the same buffer. Cells were disrupted by sonication (Virsonic Digital 475 Cell Disrupter) at 95W using five 30 s bursts with 30 s cooling intervals, and the cell debris removed by centrifugation at 15000 g for 15 min at 4°C. The cell free extracts (CFE) obtained were used for enzyme assays and PAGE activity staining.

4.3.11 GDH zymograms

Cell free extracts (75 μg) were analyzed using non-denaturing PAGE and activity staining. Following non-denaturing PAGE (Section 4.3.6), gels were incubated in staining solution containing 0.5 mM NAD or NADP, 20 mM L-glutamate, 0.3 $mg.ml^{-1}$ nitroblue tetrazolium, 0.05 $mg.ml^{-1}$ phenazine methosulphate and 50 mM Tris-HCl (pH 8.0). GDH activity appeared as dark purple bands against a clear background.

4.4 Results

4.4.1 Purification of the recombinant glutamate dehydrogenase

In order to purify the NADH-dependent GDH enzyme from *B. fragilis,* the *gdh*B gene was expressed in *E. coli* MX3004, and the recombinant protein (rGdhB) purified as described in the Materials and Methods (Section 4.2.3). SDS-PAGE analysis of the purified protein revealed the presence of a single protein band following staining with Coomassie brilliant blue R-250 (Fig 4.1), with an apparent molecular mass of approximately 48 kDa, which is in good agreement with the predicted monomeric subunit size of the GdhB protein (Chapter 2.4.6).

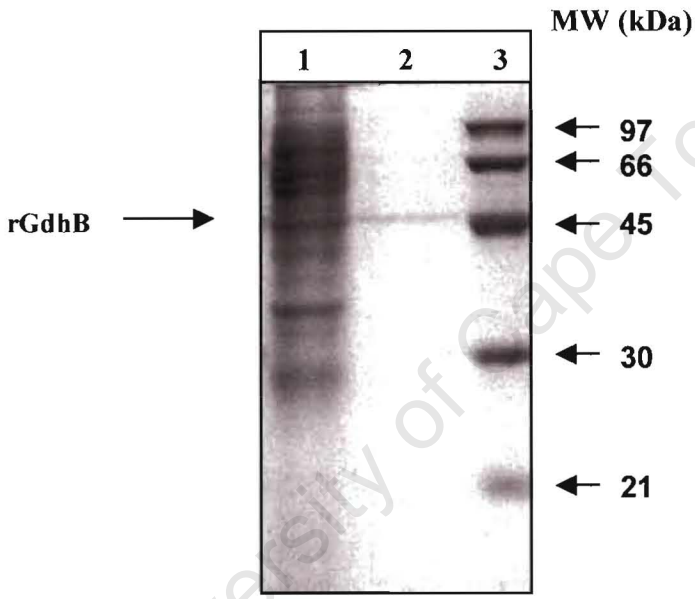


Figure 4.1. SDS-PAGE analysis of the purified rGdhB protein. Lane 1, CFE from *E. coli* MX3004 (pGDH15-1); Lane 2, purified rGdhB protein; Lane 3, molecular weight markers. Numbers on the right indicate the molecular mass in kilodaltons.

4.4.2 Activity of the purified rGdhB under various conditions

The specific activity of the purified recombinant enzyme was calculated to be 2810 (+/- 133) units.mg^{-1} protein, under standard assay conditions (Table 4.1). The recombinant protein was found to have temperature and pH optima of 38°C and 8.0 respectively. To evaluate the effect of salts on the enzymatic activity of the rGdhB protein, the monovalent salts NaCl and KCl, or the divalent salts $CaCl_2$ and $MgCl_2$ was added to the standard assay mixture. The presence of either NaCl or KCl resulted in a relatively minor reduction in rGdhB activity (~ -14% and -

8%, respectively). By contrast, the presence of either $CaCl_2$ or $MgCl_2$ resulted in a marked reduction of enzymatic activity (~ -58 and -52%, respectively) (Table 4.1). Since the GdhB protein was found to possess a putative GTP/ATP binding domain (Chapter 2.3.6), the effect of the metabolites ATP, AMP, and GTP on rGdhB activity was examined. These compounds were, however, found not to significantly affect the activity of the recombinant enzyme, at the concentration used in this study (Table 4.1).

Table 4.1. Effects of monovalent cations, divalent cations and nucleotide cofactors on the specific activity of the purified rGdhB enzyme.

Compound added[a]	**NADH-dependent GDH activity (U)** [b] **± (SD)** [c]	**% Activity** [d]
No addition	2810 ± 133	100
NaCl	2413 ± 98	85.8
KCl	2586 ± 102	92.02
$MgCl_2$	1189 ± 79	42.31
$CaCl_2$	1353 ± 107	48.15
ATP	2668 ± 146	94.94
AMP	2711 ± 88	96.47
GTP	2699 ± 84	96.06

a. Salts (100 mM final concentration), or ATP, AMP and GTP (1 mM final concentration) were added to the standard reaction mixture and the rGdhB enzyme activity assayed as described in the text.

b. U, Specific activity expressed as units.mg^{-1} protein.

c. SD, Standard deviation calculated on a minimum of three experiments.

d. % Activity of GdhB calculated relative to the standard enzyme reaction conditions (100%).

4.4.3 Localisation of the GdhB enzyme

Localisation studies were used to identify the cellular distribution of the GdhB enzyme in *B. fragilis*. Cell fractions were obtained from *B. fragilis* cells grown in MM supplemented with 1.25% tryptone, and the NADH-dependent GDH activity of the individual fractions determined. The results showed that the majority of GDH activity was associated with the membrane fraction, while lower levels of GDH activity were found to partition with the cytoplasmic, periplasmic and extracellular fractions (Table 4.2).

Table 4.2. Percentage GDH specific activity in different cellular fractions of *B. fragilis*

Cellular fraction	% GDH activity
Cytoplasm	4.6
Periplasm	4.9
Membrane	79.7
Extracellular	10.6

Further, enzyme assays performed on whole *B. fragilis* cells, grown in MM supplemented with 1.25% tryptone, showed that 109 +/- 11 µmol NADH was generated per unit OD cells per h in the presence glutamate. Far lower levels of reduced cofactor were formed by intact cells in the absence of glutamate as a substrate (31 +/- 7 NADH per unit OD cells per h). These results, taken together, suggest that an enzymatically active form of GdhB is found at the cell surface of *B. fragilis* cells.

4.4.4 Immunolocalisation of GdhB

To determine the specificity of antibodies generated against the purified rGdhB protein, antiserum was used to examine the immunoreactivity of proteins produced by *B. fragilis* and *E. coli* cells. Western blot analysis revealed the presence of a single immunoreactive protein in cell free extracts obtained from *E. coli* MX3004 (pGDH15), but not from *E. coli* MX3004 (pMT104) (Fig. 4.2, Lanes 1 and 2). When the anti-rGdhB antiserum was used to probe cell free extracts obtained from *B. fragilis,* a single hybridising protein that comigrated with the rGdhB was detected in cells that were grown in BHI or MM supplemented with 1.25% tryptone (Fig 4.2, Lane 3 and 5). No immunoreactive proteins were, however, observed in cell free extracts obtained from cells grown in MM supplemented with 0.25% peptides (Fig 4.2, Lane 4), conditions under which GdhA has previously been shown to be active. This observation indicated that antiserum generated against rGdhB does not cross react with the *B. fragilis* GdhA enzyme.

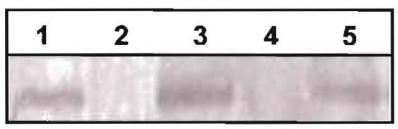
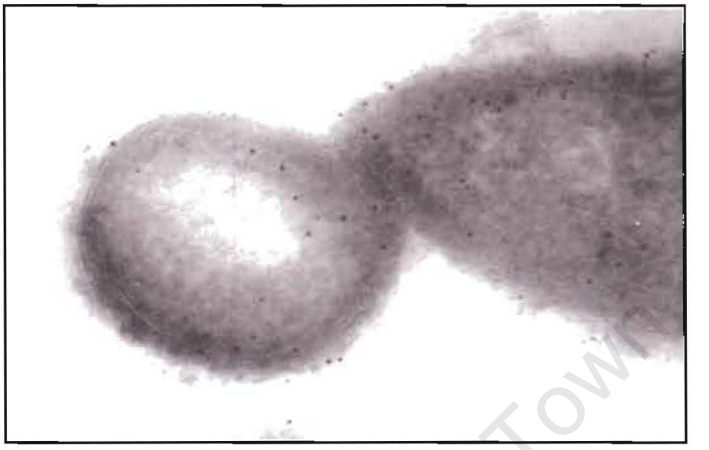


Figure 4.2. Immunoblot analysis with the antisera raised against the rGdhB. Lane 1, *E. coli* MX3004 (pGDH15); 2, *E. coli* MX3004 (pMT104); *B. fragilis* grown in: 3, BHIS; 4, MM + 0.25% tryptone; 5, MM + 1.25% tryptone.

In order to confirm the cell-surface localisation of the GdhB enzyme, immunogold labelling of ultrathin sections of *B. fragilis* cells was performed. Electron micrographs of immunogold-labelled *B. fragilis* cells, grown in MM supplemented with 1.25% tryptone, revealed that the majority of immunoreactive protein was localised along the surface of the bacterial cell [Fig. 4.3 (a) and 4.3 (b)]

(a)



(b)

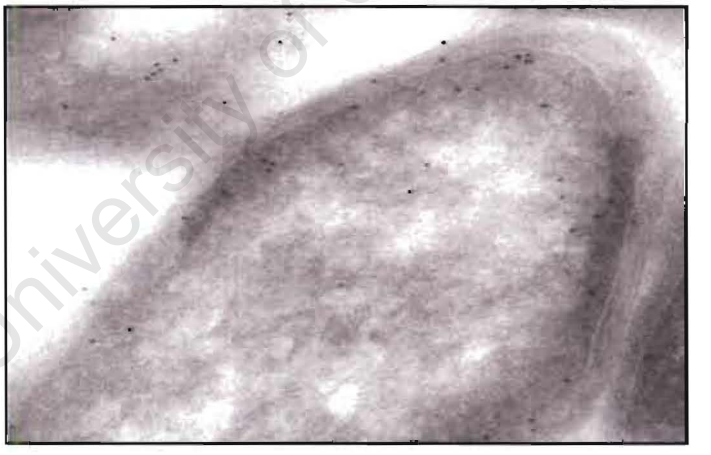


Figure 4.3: (a) and (b) Immunogold bead labelling of GdhB on the surface of *B. fragilis*. The cells were grown under high peptide conditions, sectioned using an ultramicrotome, and incubated with anti-rGDH antiserum followed by goat anti-rabbit IgG colloidal gold bead conjugate. The cells were negatively stained with uranyl acetate and viewed by electron microscopy. 1.25 cm = 100 nm.

4.4.5 Regulation of GdhB enzyme activity

In order to ascertain whether the GdhB enzyme was regulated at the post-translational level, *B. fragilis* cells were grown in MM supplemented with 1.25% tryptone (high peptide medium) to obtain high levels of fully active GdhB, and then shocked via the addition of prereduced MM to give a tryptone concentration of 0.25% (low peptide medium). An equivalent amount of prereduced MM, with a tryptone concentration of 1.25%, was added to control cultures. A rapid loss of GdhB activity was observed after reducing of the peptide concentration of the growth medium, with an approximately 3-fold decrease in NADH-dependent GDH activity occurring after 5 min post-shock (55U vs. 187U) (Table 4.3; Fig. 4.4, Lanes 3 and 4, respectively). The inclusion of 5 mM NH_4Cl in the low peptide medium appeared to have no additional effect on the modulation of GdhB activity (60U vs. 55U) (Table 4.3; Fig. 4.4, Lanes 5 and 6). The control cells, resuspended in fresh high peptide medium, maintained a similar level of GdhB activity as the overnight culture after 5 min incubation, although a slight decrease was observed after 20 min (Table 4.3; Fig. 4.4, Lanes 1 and 2, respectively). An increase in the levels of NAD(P)H-dependent GdhA activity was detected after 5 min incubation in the low peptide medium (118U vs. 84U), with a further increase being observed after 20 min (183U) (Table 4.3; Fig. 4.4, lanes 3 and 4). The activation of GdhA appeared to be further enhanced by the inclusion of 5 mM NH_4Cl in the low peptide medium (169U vs. 118U) (Table 4.3; Fig. 4.4, Lanes 5 and 6, respectively).

Table 4.3. Effect of low peptide shock and ammonia on the activity of glutamate dehydrogenase in the presence of various cofactors.

	GDH Specific activity with indicated cofactor ($U^a \pm SD$)[b]					
Growth media	**1.25 % tryptone**		**0.25 % tryptone**		**0.25 % tryptone + 5.0 mM NH_4Cl**	
Time	**NADPH**	**NADH**	**NADPH**	**NADH**	**NADPH**	**NADH**
0 min	91 ± 3.3	202 ± 5.1	-	-	-	-
5 min	84 ± 3.6	187 ± 7.7	118 ± 6.1	55 ± 5.1	169 ± 4.3	60 ± 3.7
20 min	69 ± 3.7	140 ± 3.7	183 ± 8.3	67 ± 4.2	191 ± 6.4	71 ± 5.4

a U: Specific activity expressed as units.mg^{-1} protein. The values presented are the means of at least three indepependent experiments.

b SD: Standard deviation.

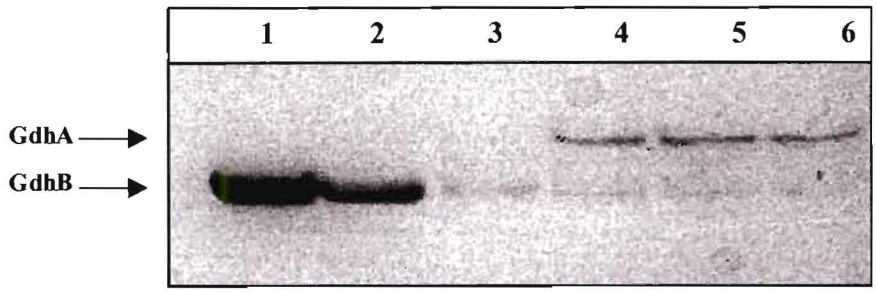


Figure 4.4. Regulation of GDH activity by peptides and ammonia. Non-denaturing PAGE and activity staining, using NAD as a cofactor, was used to visualise GDH activity in *B. fragilis* cell free extracts obtained from cells grown under high peptide conditions (1.25% tryptone), which were then exposed to low peptide conditions (0.25% tryptone), in the presence or absence of ammonia (5 mM NH_4Cl). Lanes: 1 and 2, cells were kept at 1.25% peptides for 5 and 20 min, respectively; 3 and 4, peptide concentration was reduced to 0.25% for 5 and 20 min respectively; 5 and 6, nitrogen concentration was reduced to 0.25% peptides/5 mM ammonia for 5 and 20 min, respectively. Arrows indicate the positions of the GdhA and GdhB enzymes.

4.5 Discussion

In this study, the *B. fragilis* GdhB was expressed in *E. coli,* and the purified enzyme characterised with regards to its response to monovalent and divalent salts, and purine nucleotides. Previous studies conducted on the purified NAD(P)H dependent enzyme from *B. fragilis* (Yamamoto *et al.*, 1987a) indicated that its activity was influenced by the presence of monovalent salts, with NAD-dependent activity inhibited by approximately 50% by 100 mM NaCl, while NADP-dependent activity was stimulated by 18%. Furthermore, its activity was not significantly affected by the presence of purine nucleotides. In this study, the presence of monovalent and divalent salts was found to inhibit the activity of the rGdhB enzyme, with divalent cations having the greater inhibitory effect. The native GdhB enzyme has recently been purified from *B. fragilis*, and shown to possess a subunit size of 49.22 kDa, as determined by MALDI-TOF mass spectrometric analysis (Lee, Chi-Lei, 2000). The apparent K_m values for substrates were determined to be 17 mM for NH_4^+, 0.37 mM for 2-oxoglutarate, and 0.054 mM for NADH in the amination reaction, which are within the range of values established for other GDHs of prokaryotic origin. Several differences in the characteristics of the native enzyme were, however, found when compared to that of the purified rGdhB protein. The inhibitory effect of mono- and di- valent salts on native enzyme activity was shown to be far more pronounced than that found for the rGdhB enzyme. The addition of NaCl and KCl was found to reduce GdhB activity to 65.6% and 61.9% of that found under the

standard assay conditions, respectively, whereas $MgCl_2$ and $CaCl_2$ reduced enzymatic activity to 21.7% and 15.0%, respectively. Furthermore, whereas the purine nucleotides were found to have a relatively minor effect on the activity of the rGdhB protein, native enzyme activity was reduced to 71.8% and 64.5% upon addition of ATP and GTP, respectively. These differences may be reflective of differences in the processing of the GdhB enzyme in the recombinant and native host organisms. At present, the basis for the observed regulation of GdhB activity is not clear. It is, however, worthwhile to note that similar decreases in NADH-dependent GDH activities in response to monovalent salts (0.2 M KCl) have been observed in those *Prevotella spp.* examined (Wen and Morrison, 1997). A more detailed analysis of the properties of the *B. fragilis* GDH enzymes is, however, needed to determine the physiological significance of these findings.

Previous studies have shown that the regulation of GDH activity in various microorganisms, including *E. coli, K. aerogenes, and S. typhimurium,* occurs primarily at the level of enzyme synthesis (Reizer, 1996b). The activity of the *B. fragilis* GdhA has, however, been found to be additionally controlled at the post-translational level in response to the ammonia concentration in the growth medium (Yamamoto *et al.*, 1987b). It was, therefore, considered possible that the activity of GdhB might be regulated via modulation of enzyme activity, in addition to that of enzyme synthesis. In the present study, the activity of the GdhA enzyme was found to increase in response to a decrease in the level of peptides present in the growth medium. The increase in GdhA activity was greater after 20 min than 5 min, suggesting that it may involve a mechanism besides, or in addition to, the activation of existing protein. The inclusion of ammonia in the low peptide medium, however, effected a far greater, and more rapid, increase in GdhA activity. Furthermore, increases in GdhA activity were found to be accompanied by a rapid decrease in GdhB activity, suggesting that the existing enzyme is inactivated in response to the decreased availability of peptides. No additional decrease in GdhB activity was, however, observed when 5 mM ammonia was included in the low peptide medium. Based upon these properties, it appears that GdhB activity is regulated by peptides at the post-translational level, in addition to the previously demonstrated transcriptional regulation (Chapters 2 and 3). Such a control system may enable *B. fragilis* cells to adapt to sudden changes in nitrogen availability, and may serve to protect them from metabolic imbalances that may arise following changes in environmental nitrogen availability. Whether the observed post-translational regulation of GdhB activity occurs via the covalent modification of the enzyme, or by an alternative mechanism, remains to be determined. In

this regard, it has been demonstrated that the catabolic GDHs of *S. cerevisiae* and *C. utilis* are selectively degraded when cells grown with glutamate are transferred to ammonia containing medium (Hemmings, 1980; 1982).

A combination of immunogold labelling, whole cell assays and cell fractionation analysis indicated that the *B. fragilis* GdhB was associated with the surface of the bacterial cell. It has previously been reported that the GDHs of *P. gingivalis* (Joe *et al.*, 1994) and *Streptococcus suis* (Okwumabua *et al.*, 2001) are cell surface associated. While the localisation of the *B. fragilis* GdhB is therefore not unique, its presence along the cell surface is still unusual for this group of enzymes. The GDH enzymes from the two aforementioned organisms are similar to the *B. fragilis* GdhB, in that they do not appear to possess an N-terminal leader sequence. This suggests that the targeting of these proteins to the cell surface may occur via a sec-independent pathway, as described for several other prokaryotic proteins (von Heijne, 1994). However, details regarding the association of GdhB with the cell surface remains to be determined. At present, the physiological advantage of possessing a surface associated GDH is not clear. However, a number of cell-surface associated proteases and peptidases have been identified in *P. gingivalis* (Banbula *et al.*, 1999; Grenier and Mcbride, 1987; Travis *et al.*, 1997). The generation of peptides along the cell surface of this organism is considered to be advantageous, given that other microorganisms in the periodontal area are also capable of utilising these substrates. It is, therefore, possible that the presence of GDH at the cell surface in these organisms facilitates the utilisation of amino acids made available by the action of peptidase enzymes. Further studies on the physiological role of the enzyme are required to confirm this theory.

CHAPTER 5

GENERAL CONCLUSIONS

The physiology and regulation of the ammonia assimilatory pathways in *B. fragilis* has previously been examined by Yamamoto *et al.* (1984, 1987b). Based on these initial studies, it was concluded that ammonia assimilation in *B. fragilis* proceeds primarily via the glutamate dehydrogenase-catalysed pathway, during growth in both ammonia-limiting and excess environments. Whilst it was shown that *B. fragilis* expresses two distinct GDH enzymes, the specific roles of these enzymes in nitrogen assimilation were not clearly resolved. The work presented in the current study was, therefore, designed to focus on the role and regulation of these enzymes in the physiology *B. fragilis*, with a view to extending our understanding of the process of nitrogen assimilation in this organism.

The findings presented in this investigation confirmed that *B. fragilis* bf1 contains two GDH enzymes, which are specific for either NAD(P)H (GdhA), or NADH (GdhB) as a cofactor. At the physiological level, these two enzymes were shown to differ from each other with regards to their regulation in response to the nitrogen source (ammonia or peptides) and availability. Growth experiments revealed that GdhA activity was present during growth in both ammonia-limited and ammonia-replete environments. In contrast to the situation found in most other prokaryotes examined to date, however, its activity was found to maximal during growth with limiting concentrations of ammonia. GdhB activity, on the other hand, was induced in response to growth with increasing concentrations of peptides, conditions under which GdhA activity was repressed. These findings differ from those of Yamamoto *et al.* (1984), with regards to GdhB, where it was proposed that this enzyme functions in ammonia assimilation in ammonia-excess environments. Our findings suggest that the two GDH enzymes fulfill distinct physiological functions in the metabolism of *B. fragilis*, with their regulation occurring in a manner that is consistent with a role for GdhA and GdhB in glutamate biosynthesis and degradation, respectively. No evidence for the induction of GdhB activity by ammonia was found at either the physiological or the molecular level, which would appear to exclude a role for this enzyme in glutamate biosynthesis. Furthermore, the control of GdhB activity was shown to occur relative to the availability of exogenous peptides, at both the level of *gdh*B gene expression and regulation of enzyme activity. The data from Northern blot analyses and reporter gene studies suggested that this regulation occurs primarily at the

level of transcription. The rapid inactivation of GdhB upon transfer of pregrown cells from high- to low- peptide conditions, however, suggests that GdhB activity is further controlled at the level of enzyme activity. This could be similar to the regulation of GDH activity found in other organisms that possess two GDH enzymes, where the physiological inactivation of catabolic GdhB activity is required following a change in nutritional conditions (Smith *et al.*, 1975; Hemmings and Sims, 1977). This usually occurs in response to those conditions where biosynthetic GDH activity is up-regulated, thus avoiding the occurrence a potentially futile cycle of glutamate biosynthesis and degradation.

Glutamate has previously been demonstrated to have no effect on GDH activities of ammonia grown cultures of *B. fragilis* (Yamamoto *et al.*, 1984). This is in line with previous studies, which have shown that *B. fragilis* is unable to utilise this, and other free amino acids, as a sole source of nitrogen (Varel and Bryant, 1974). Moreover, there is evidence that the colonic bacteria prefer to assimilate and ferment organic nitrogen in the form of peptides, as opposed to amino acids (Smith and Macfarlane, 1998). It is therefore, reasonable, to assume that GdhB contributes to the catabolism of glutamate arising from the hydrolysis of glutamate-containing peptides found in tryptone. Such a scheme of glutamate utilisation has been shown to exist in *P. gingivalis,* which is capable of metabolising glutamate when supplied in peptide, but not free amino acid form (Takahashi and Sato, 2001). In this organism, glutamate is degraded via the NADH-dependent GDH-catalysed pathway subsequent to the hydrolysis of glutamate-containing peptides (Takahashi *et al.*, 2000). Given the close evolutionary relationship between these two organisms, it is possible that GdhB performs a similar role in *B. fragilis.* Furthermore, both the *P. gingivalis* GDH (Joe *et al.*, 1994) and *B. fragilis* GdhB (this study) enzymes, together with the *S. suis* catabolic GDH (Okwumabua *et al.*, 2001), have been shown to be cell surface associated. The physiological advantage of such an arrangement is, at present, not clear. *P. gingivalis* has, however, been shown to possesses a number of proteases and peptidases that are cell surface associated, an adaptation that is thought to reflect its nutritional requirements for peptides (Travis *et al.* 1997). The presence of a surface-associated GDH in these organisms may, therefore, facilitate the utilisation of amino acids derived from peptides incorporated from the external milieu.

In the present study, the structural gene encoding the NADH-dependent GDH from *B. fragilis* was cloned. DNA sequence and comparative analysis of the cloned *gdh*B gene, and of adjacent ORFs using sequence data obtained from the *B. fragilis* 9343 preliminary genome

sequence, allowed us to characterise the *gdh*B gene in the context of its neighbouring ones on the chromosome. This resulted in the identification of an ORF, located immediately downstream of the *gdh*B gene, which exhibits DNA and amino acid sequence homology to previously identified bacterial aminopeptidases. Given the regulation of GdhB activity by peptides, it is tempting to speculate that this enzyme functions in providing amino acids, including glutamate, from peptides during growth with organic nitrogen sources. Although the nutritional factors regulating the expression of this gene have not been determined, it seems reasonable to assume that genes encoding peptide degradative and transport pathways are coordinately regulated in *B. fragilis*. *B. fragilis* has previously been shown to produce several proteases, whose synthesis and secretion was demonstrated to be subject to nitrogen regulation (Gibson and Macfarlane, 1988a; Macfarlane *et al.*, 1992). It has been suggested that these proteases may contribute to the pathogenesis of this organism. Several recent studies have suggested that GDH activity may be related to the virulence of certain microorganisms. Okwumabua *et al.* (2001) reported that virulent strains of *S. suis* could be distinguished from moderately virulent or avirulent strains, based on differences in GDH profiles following non-denaturing gel electrophoresis and activity staining. Further, it has been shown that virulent, proteolytic strains of *Clostridium botulinum* possess unusually high levels of catabolic GDH activity, relative to avirulent or moderately virulent, nonproteolytic strains, and it was proposed that GDH activity was important in proteolytic strains of this microorganism to generate 2-oxoglutarate as a substrate for transamination reactions (Hammer and Johnson, 1988). The regulation of the *B. fragilis* GdhB by organic nitrogen may, therefore, be linked to protease activity and the breakdown of proteins *in vivo*, and hence to pathogenesis.

A short distance upstream (417 bp) of the *gdh*B gene, an ORF encoding a 989 amino acid protein was identified. No overall homology of this ORF was found to other known sequences contained in the genetic databases. The deduced polypeptide was, however, shown to be composed of two domains: (i) a N-terminal domain, which possesses two regions displaying homology to the N-terminal sensory domain of NtrC and (ii) a C-terminal domain, which displays homology to previously identified phosphoenolpyruvate synthases (PPS). Interestingly, several recent studies have resulted in the identification of an novel group of enzymes, termed Enzyme I^{Ntr} (EI^{Ntr}), in a number of bacterial species (Reizer *et al.*, 1996; Rabus *et al.*, 1999). These enzymes are homologues of the Enzyme I (EI) proteins, members of the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PEP:PTS), an

elaborate protein system involved in the phosphorylation and transport of various sugars into the cell (Postma *et al.*, 1993). The EI^{Ntr} proteins differ from the EI proteins in that they posses an additional N-terminal extension, which has homology to the N-terminal sensory domain of NifA. Like NtrC, NifA regulates the transcriptional activation of various genes in response to the nitrogen status of the cell. It has been proposed that, in *E. coli,* EI^{Ntr} plays a role in regulating the activity of the IIA^{Ntr} and Npr proteins, which are homologues of the PEP:PTS enzymes IIA and Hpr, respectively (Rabus *et al.*, 1999). These two proteins, in turn, have been implicated in the activation of the σ^{54}-dependent expression of genes involved in organic nitrogen utilisation (Powell *et al.*, 1995). Further, in *Bradyrhizobium japonicum*, mutants disrupted in the *pts*P gene that encodes the EI^{Ntr} enzyme have been demonstrated to be defective in their ability to transport proline containing oligopeptides (King and O' Brian, 2001). This finding led the authors to propose that the EI^{Ntr}, and related proteins, may have a general role relating to the transport of nitrogen-containing compounds into the cell. It is, therefore, interesting to note that EI proteins are homologous to PPSs and, together with pyruvate phosphate dikinases (PPDK), form part of a larger family (the EI-PPS-PPDK family) (Reizer *et al.*, 1993). This, together with the modular structure of the protein encoded by ORF1, may be indicative of a function relating to the utilisation of organic nitrogen sources for this protein in *B. fragilis*.

The work presented in this thesis provides an overview of the function and regulation of the glutamate dehydrogenase enzymes by *B. fragilis*, which may serve as the basis for future studies. The findings suggest that the individual GDH enzymes fulfill distinct physiological functions in the growth of this organism. However, additional studies, involving the construction and analysis of *B. fragilis* mutants impaired in GdhA and/or GdhB activities are required to confirm these findings. In the case of GdhA, this would require the additional cloning of the structural gene encoding this enzyme. Such an approach should also be useful in establishing the relative importance of the GDH and the GS-GOGAT pathways in ammonia assimilation in *B. fragilis*. At present, the significance of the latter pathway in ammonia assimilation and glutamate biosynthesis in the *Bacteroides* is unclear, given that the levels of GOGAT activity in these organisms are either low or undetectable (Yamamoto *et al.*, 1984, 1987c; Baggio and Morrison, 1998), nor have any *glt*BD homologues been identified in these organisms. The further cloning of the gene coding for GdhA will be also useful for the study of the regulation of its activity in *B. fragilis*.

As mentioned previously, GdhB activity was found to be modulated in response to the availability of peptides at the level of enzyme synthesis, in addition to control of its enzymatic activity. It would be of interest to establish how this regulation is achieved at the genetic and biochemical levels, as well as the nature of the effectors responsible for the regulation GdhB (and GdhA) activity. Given the heterogeneous amino acid composition of peptides contained in tryptone, the precise nature of these effectors was not readily apparent in this study. Although glutamate-containing peptides seem to be a likely candidate, further studies are required to determine whether this is indeed the case. This could be accomplished by examining the effect of peptides of defined sequence on the modulation of GdhB activity in *B. fragilis*. Nevertheless, since *gdh*B gene expression was found to be regulated in response to nutritional conditions, it is probable that gene expression involves specific DNA binding factors. In this study, deletion analysis of the *gdh*B promoter region revealed that the induction of *gdh*B transcription was dependent on the presence of an extended *gdh*B promoter region. While several potential cis-acting elements were identified within the *gdh*B upstream region, no obvious homology between these and consensus binding sequences of known nitrogen regulatory proteins was identified. The use of the *gdh*B'::XA reporter gene vectors developed in this study could be used to define the *gdh*B promoter regions more precisely, which could then serve as a basis for specific site directed mutagenesis or deletions to determine the critical features of the promoter elements involved in the expression of *gdh*B gene. The identification of the mechanisms involved in the regulation of *gdh* activity will provide a better understanding of the roles of these enzymes in the nitrogen assimilatory pathways of *B. fragilis*.

APPENDIX A

BACTERIAL STRAINS USED IN THIS STUDY

Strain	Genotype	Reference
Escherichia coli		
JM109	F'*tra*D36 *pro*A^{+}B^{+} *lac*I^{q} Δ(*lac*Z)M15/ Δ(*lac-pro*AB) *gln*V44 *e*14- *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi hsd*R17	Yanisch-Perron *et al.* (1985)
HB101	F^{-} Δ(*gpt-pro*A)62 *leu*B6 *gln*V44 *ara*14 *gal*K2 *lac*Y1 Δ(*mrc*C-*mrr*) *rps*L20 (Str^{r}) *xyl*-5 *mtl*1 *rec*A13	Sambrook *et al.* (1989)
MX3004	*thi gdh*1 *pro* (*lac*U169) *hut*C *glt*D236::*MudII* (Cm^{r})	Castano *et al.* (1992)
Bacteroides fragilis		
B. fragilis bf1	wild type	Mossie *et al.* (1979)

APPENDIX B

PLASMID VECTORS

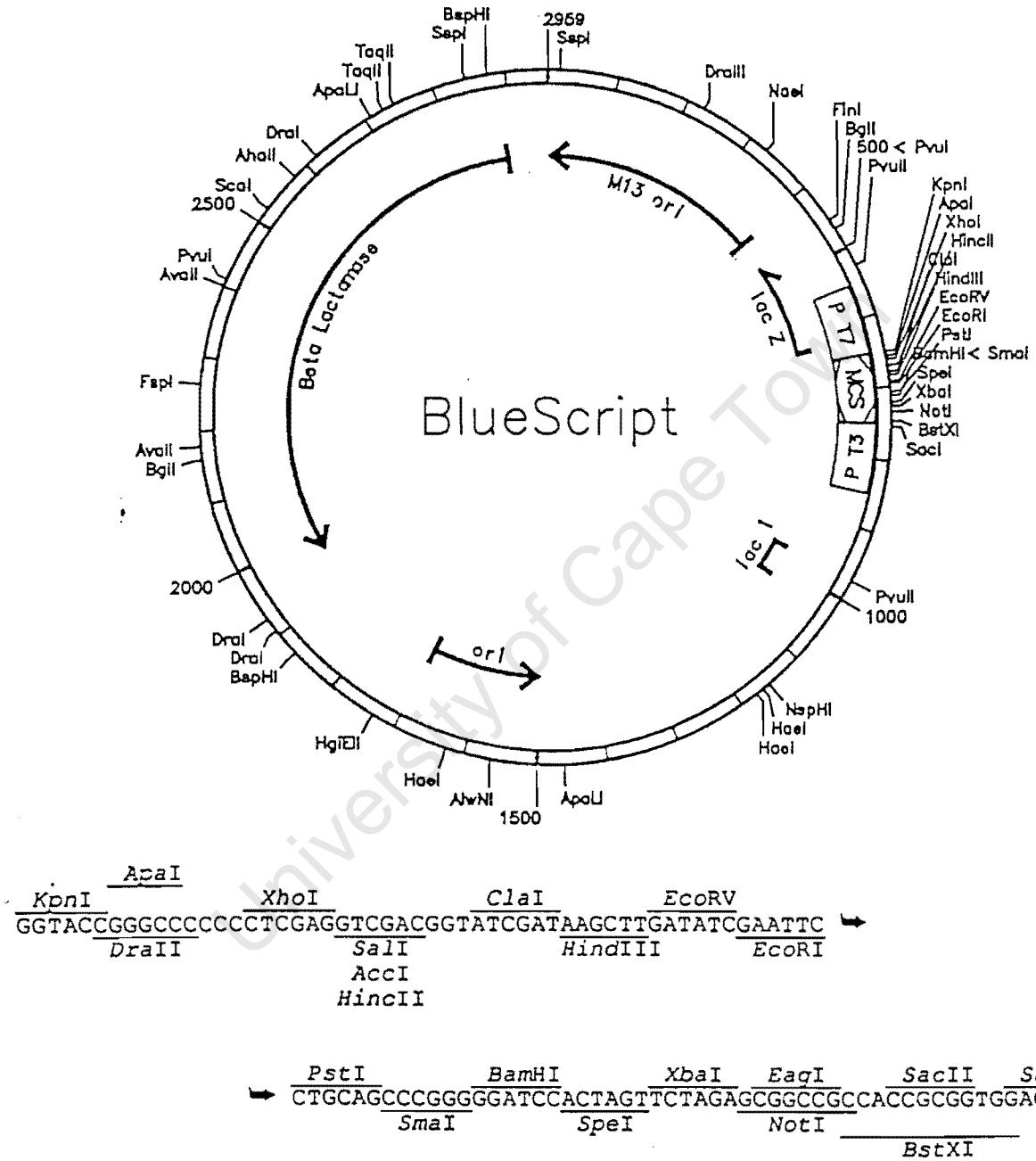


Fig. B1. Restriction map of pBluescript SK (2959 bp; Stratagene, San Diego, CA). The nucleotide sequence of the multiple cloning site is shown below the circular plasmid map. pBluescript KS(+) differs from pBluescript SK in the orientation of the multiple cloning site polylinker.

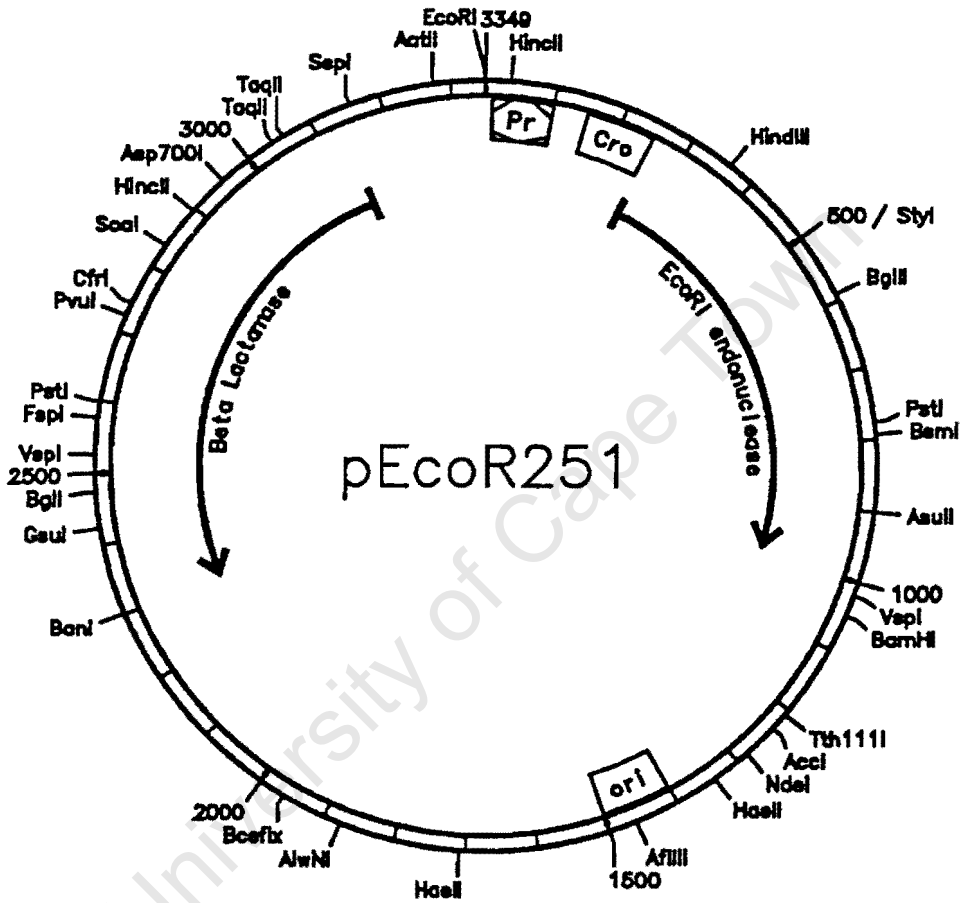


Fig. B2. Restriction map of pEcoR251 (Zabeau and Stanley, 1982)

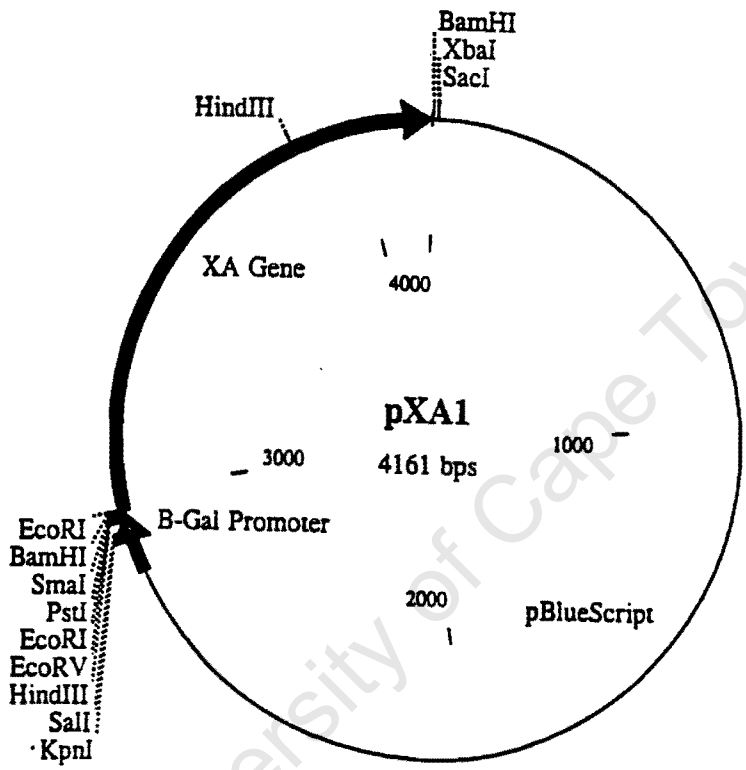


Fig. B3. Restriction map of pXA1 (Whitehead, 1997)

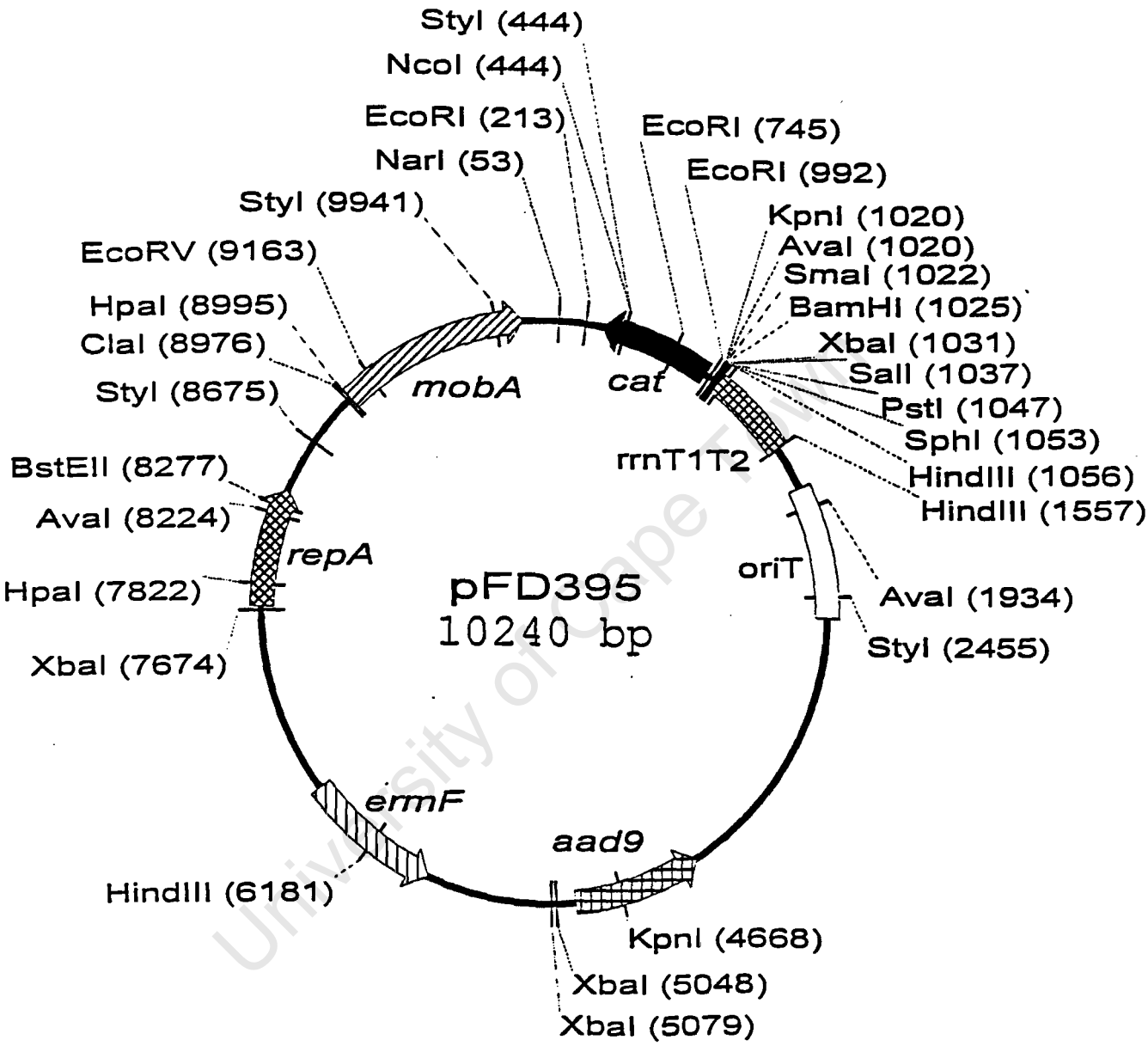


Fig. B4. Restriction map of pFD395 (pFD325TT; Smith *et al.*, 1992).

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