GLUTAMINE SYNTHETASE IN *BACTEROIDES FRAGILIS*

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Abstract

Bacteroides fragilis is a gram-negative, non spore-forming, obligate anaerobe of the human intestinal microbiota. It is, however, an opportunistic pathogen and has been ranked as the most prevalent isolate in cases of anaerobic septicaemia. Similar to most bacteria, ammonium is assimilated in *B. fragilis* through the action of glutamine synthetase (GS). Glutamine is vital to nitrogen metabolism as it serves as a precursor to many secondary metabolites. GS enzymes are, therefore, vital to the growth of the organism and many prokaryotes are known to possess two or more isoforms of the enzyme. In addition, GS expression and regulation is usually tightly regulated in concert with the availability of nitrogen. Previous studies have identified a single GSIII encoding gene (*glnN*) in *B. fragilis*. In this dissertation, an additional ORF coding for a putative GSI enzyme in *B. fragilis* was identified, isolated and functionally characterized. A putative regulatory protein was also identified and its functional contribution to nitrogen metabolism was determined, in order to extend our understanding of nitrogen assimilation in *B. fragilis*.

In order to evaluate if glnN was the only functional GS encoding gene in *B. fragilis*, a mutant strain containing an insertional inactivation of the *glnN* gene, *B. fragilis* 638ΩglnN, was constructed. Physiological analyses demonstrated that *B. fragilis* 638ΩglnN was able to grow in minimal medium supplemented with elevated concentrations of ammonium chloride (NH_4Cl) as the only nitrogen source. Residual GS activity was also detected in *B. fragilis* 638ΩglnN grown in medium with both ammonium shortage and excess. These findings suggested that an additional gene in *B. fragilis*, may encode a functional GS, explaining the GS activity and growth of the bacterium in high ammonium-containing medium. Bioinformatic analyses lead to the identification of an ORF encoding a putative *glnA* gene in the *B. fragilis* genome. The gene was found to have a low amino acid identity to other characterized GSI enzymes from different bacteria, however, it contained three out of the five highly conserved GS regions. This gene was cloned into an *E. coli* glutamine auxotroph. Although RNA hybridization experiments showed that the gene was transcribed in *E. coli*, it failed to complement the glutamine auxotrophy of this strain.

Another ORF coding for a putative GS regulatory protein was also identified in *B. fragilis*. This gene was located directly downstream of *glnN* and was annotated as belonging to the

CRP/FNR family of transcriptional regulators. An insertional mutation of this gene was also generated to determine whether it played a functional role in nitrogen regulation. The regulatory mutant was phenotypically similar to the *glnN* mutant (*B. fragilis* 638ΩglnN) with regards to growth and GS activity under different ammonium conditions. These results appear to confirm the association of this gene with *glnN* in particular and nitrogen assimilation in general.

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CHAPTER 1

Literature Review

1.1 General Introduction

Bacteroides spp. are gram negative, rod-shaped bacteria which do not form spores. This genus belongs to a diverse group which includes the *Bacteroides-Flavobacterium-Cytophaga* subphylum (Gherna and Woese, 1992). As a class, bacteroides are amongst the most common culturable bacteria found in the intestinal microflora (Kuwahara *et al.*, 2004). These strictly-anaerobic colonizers live symbiotically with their host, assisting in fermentation of carbohydrates, production of essential nutrients such as Vitamin K, competition with micro-organisms for colonic resources, as well as biotransformation and enterohepatic circulation of bile acids (Hylemon *et al.*, 1977; Pomare *et al.*, 1985). However, when bacteroides are allowed to leave their colonic abode, they become severe opportunistic pathogens and have been found to be responsible for anaerobic septicaemia and the formation or aggravation of abscesses (Gibson *et al.*, 1998).

Infections caused by enteric bacteria usually occur in the following pattern: Following disruption of the intestinal wall and infiltration of the intestinal flora into a sterile body part, aerobic bacteria first engage in tissue destruction thereby reducing the redox potential of the tissues (first or acute stage of infection). Once enough oxygen has been depleted from the tissues, anaerobic growth is favoured and bacteroides proliferation occurs, eventually leading to their domination of the infection (second or chronic stage of infection) (Brook 2000).

Diagnosis and treatment of these infections can be intricate because of the slow growth of bacteroides and their resistance to many antibiotics (Brook 2002). In a 12-year study isolating anaerobic bacteria from clinical specimens, *Bacteroides* spp. accounted for 43% of anaerobic clinical isolates (Brook, 1988). When untreated, bacteroides infections used to be associated with high mortality rates of up to 50% (Brook 1988 & 2002). However, the mortality rate has greatly decreased due to early identification and administration of the proper antimicrobial treatment.

1.2 *Bacteroides fragilis*

Bacteroides fragilis is a notable member of the genus. Although *B. fragilis* comprises only 0.5-1 % of the *Bacteroides* spp. in the intestinal flora, it becomes the prevalent isolate in anaerobic septicaemia, accounting for 50 to 70% of the anaerobes found in human infections (Finegold and George, 1989; Brook and Frazier, 2000).

The prevalence of *B. fragilis* in clinical isolates can be attributed to several mechanisms, including the presence of a polysaccharide capsule, the activity of various proteolytic enzymes, and the fact that the bacteria are aerotolerant.

1.2.1 Virulence factors

a. Capsule

The primary virulence factor in *B. fragilis* infections is the formation of a polysaccharide capsule, which helps in promoting bacterial adhesion, abscess formation and impairs phagocytosis of the bacterial cells by macrophages (Onderdonk *et al.*, 1977; Rodloff *et al.*, 1986; Tzianabos, 2000). The formation of an abscess is actually a pathological response of the host immune system to the presence of *B. fragilis* capsular polysaccharides. An experimental model of intra-abdominal sepsis showed that *B. fragilis* alone caused abscesses while other *Bacteroides* spp. required the help of a synergistic aerobe (Onderdonk *et al.*, 1977). In effect, both heat-killed encapsulated *B. fragilis* and the purified capsule alone were able to induce intraperitoneal abscess formation. However, when the capsule was administered sub-cutaneously, it protected against further abscess formation during intraperitoneal infection (Tzianabos *et al.*, 1995). Unlike most bacterial polysaccharides which stimulated an antibody-mediated immune response, *B. fragilis* capsule stimulates this protection via a T-cell dependent immune response (Tzianabos *et al.*, 1995).

The capsule is made up of three distinct polysaccharides: polysaccharide A (PSA), PSB and PSC (Tzianabos *et al.*, 1993). Implantation of PSA and PSB in the peritoneal cavity of rats induced abscess formation. However, in a mutant unable to synthesize PSA, the abscess-inducing capability was greatly reduced, despite the synthesis of the other capsular polysaccharides (Coyne *et al.*, 2001). Consequently, it was proven that the capsular

polysaccharides did not contribute equally to abscess formation, but that PSA synthesis was crucial to initiate abscess formation (Coyne *et al.*, 2001) and that it was the main player in the cell-mediated immune response (Tzianabos *et al.*, 1995).

Tzianabos *et al.* (1993) showed that the capsular polysaccharides were arranged in repeating units, each of which had exposed positively and negatively charged side-chains (zwitterionic charge motifs). The capsule is similar in charge, but not structure, to the capsular polysaccharide of *Streptococcus pneumoniae* type 1. Remarkably, vaccination with *S. pneumoniae* polysaccharide also prevented intraperitoneal abscess formation subsequent to *B. fragilis* instillation, and this protection was not conferred if the charged groups were altered on the capsular vaccine (Tzianabos, 2000).

Recent studies on the newly sequenced *B. fragilis* genome, have revealed interesting discoveries. For example, Cerdeno-Tarraga *et al.* (2005) demonstrated that *B. fragilis* has a unique phase variation system composed of more than 30 enzymes involved in site-specific DNA inversions. DNA inversions are believed to control gene expression by on-off switching, mediating the construction of hybrid proteins, reorganising operons and by shuffling domains (Kuwahara *et al.*, 2004). The general belief concerning phase variation is that it enables proliferation of the bacteria even under stressful conditions, yet many of these phase variable genes showed no apparent function in stress survival (van der Woude, 2006). In *B. fragilis,* it was shown that antigenic variation of the polysaccharide capsular types occured. Patrick *et al.* (2003) showed that these specific inversions were related to *S. typhimurium* H flagellar antigen invertase (Hin) inversions. Furthermore, Kuwahara *et al.* (2004) reported that *B. fragilis* had the ability to evade the host immune response by employing the multiple DNA inversion systems. These systems could also be linked to the ability of *B. fragilis* to invade and colonize various tissues (Cerdeno-Tarraga *et al.*, 2005).

b. Catalytic activity

Another factor that may be significant to *B. fragilis* virulence is its production of a number of enzymes. Proteases produced by the bacteria degrade host structural proteins as well as various substances which help with infection containment (Gibson, 1988). Metalloproteases have emerged as important virulence factors in a number of diverse pathogenic organisms,

including bacteria and fungi, and *B. fragilis* enterotoxin (fragilysin) was shown to be a zinc-dependent metalloprotease with two distinct isoforms (Moncrief *et al.*, 1995). Enterotoxigenic strains of *B. fragilis,* associated with diarrheal disease, produced only one of the isoforms which changed the morphology of human epithelial cells in the intestine (Franco *et al.*, 1999). It was also discovered that proteases were regulated by the level of nitrogen available to the organism and that with excess nitrogen, protease activity was reduced (MacFarlane, 1992). Other enzymes such as neuraminidase and elastase also degrade host structural components during bacteroides infections (Berg, 1983). Neuraminidase has been suggested to be essential for *B. fragilis* growth in vivo, which may help in proliferation of the bacteria subsequent to infection (Godoy *et al.*, 1993). It was proposed that the ability of this enzyme to make sialic acid available as a carbon source may be what endorses growth in nutrient-limited conditions (Godoy *et al.*, 1993 and Byers *et al.*, 1996).

c. Aerotolerance

Although anaerobic, *B. fragilis* can survive for several days in an oxygenated environment without actively growing. This aerotolerance is believed to contribute to virulence, especially in the first (aerobic) stage of infection. The aerotolerance has also been suggested as resulting from the presence of the enzymes superoxide dismutase (SOD) and catalase (Kat) which inactivate toxic products of oxygen metabolism (Rocha *et al.*, 1995). In addition, an operon, appropriately named "*batI*" (*bacteroides* aerotolerance), has been reported to be involved in *B. fragilis* aerotolerance (Tang *et al.*, 1999). The *batI* mechanism is believed to act by generating or exporting reducing power equivalents, such as NADH, to the periplasm of *B. fragilis* cells (Tang *et al.*, 1999). In addition, during bacterial infections, bacteria are known to attach to phagocytes and activate oxidase leading to a respiratory burst producing reactive oxygen species such as hydrogen peroxide, superoxide and hydroxyl radicals (Yoneda *et al.*, 1989). Using two-dimensional gel electrophoresis techniques, Rocha *et al.* (1996) showed that *B. fragilis* synthesized 28 new proteins when shifted from an anaerobic to an oxygenated environment. They also showed that catalase (KatB) is a key enzyme needed for survival in the presence of hydrogen peroxide (Rocha *et al.*, 1996). A few years thereafter, another gene named *ahpCF* (alkyl hydroperoxide reductase) was also identified as playing a role in H_2O_2- scavenging (Rocha and Smith, 1999).

1.2.2 Antimicrobial Resistance

Yet another important factor in this organism's dominance is its resistance to a wide range of antimicrobial agents. *Bacteroides* spp. are inherently resistant to aminoglycosides such as streptomycin and gentamicin, since taking in these aminoglycosides requires energy from a nitrate- or oxygen- dependent electron transport system which appears to be absent in Bacteroides species (Bryan, 1979). These drugs are, therefore, not taken in and do not reach their targets. Most bacteroides are also resistant to β-lactam antibiotics because they produce enzymes (β-lactamases) that hydrolyse the antibiotic, such as *B. fragilis* metallo-β-Lactamase CcrA (Yang *et al.*, 1992).

In addition, many bacteroides display resistance to tetracycline and erythromycin (Cooper *et al.*, 1995). Of the three modes of tetracycline resistance (tetracycline modification, ribosome protection, tetracycline efflux), ribosome protection is the most common and it is found in various bacterial species (Nikolich, 1992). Many of the *Bacteroides* spp. antibiotic resistance genes can be found on plasmids or transposons. The *tet*Q gene, for example, is associated with conjugative transposon TC^r (tetracycline resistance) elements. These TC^r elements are located on the chromosome from which they excise and transfer themselves by conjugation to a recipient (Bass and Hecht, 2002).

A major contributor to antibiotic resistance in Gram-negative bacteria is the presence of multidrug efflux pumps (Ueda *et al.*, 2005). These pumps, made up of a periplasmic lipoprotein, an inner membrane transporter and an outer membrane channel, function by recognizing and expelling substances from the cell, without any build-up of the drugs in the periplasm (Zgurskaya and Nikaido, 2000). These efflux systems act on a broad range of antimicrobial agents such as biocides, detergents, dyes, organic solvents and antibiotics, among others (Poole, 2001). The work of Pumbwe *et al.* (2006) on the resistance nodulation division (RND) family of efflux pumps in *B. fragilis* confirmed the mutant selection window hypothesis. This hypothesis proposes that when antimicrobials are administered, selection of resistant bacterial mutants occur primarily in the window defined by the minimum inhibitory concentration (MIC) of the cells at the lower margin and at the higher margin, the mutant prevention concentration (drug concentration blocking the growth of the least vulnerable mutant) (Cui *et al.*, 2006). However, bacterial resistance to antimicrobials is dependent on a

synergy between active efflux pumps and the outer membrane barrier, as neither is fully effective without the other (Li *et al.*, 2000). The drug of choice used in treating *B. fragilis* infections is metronidazole (a 5-nitroimidazole derivative), which acts by causing DNA damage (Diniz, 2004). The mode of action of metronidazole occurs by a chemical reduction event of the nitro group, forming an active intermediate which oxidizes DNA, causing strand breaks (Edwards, 1993). In cases of resistance, three mechanisms have been identified: The first involves an inability to form the reduced, reactive intermediate (Cederbrant *et al.*, 1992). The second mechanism entails a decrease in intracellular buildup of the drug (or increased efflux) (Kui *et al.*, 2001) and the third mechanism involves the action of nitroimidazole resistance genes (*nim* genes) (Haggoud *et al.*, 1994).

1.2.3 Genetic manipulations

Members of the *Cytophaga-Flavobacterium-Bacteroides* phylum are as distinct from other Gram-negative enterics as they are from Gram-positive ones. As a result, genes from *bacteroides* are not frequently expressed in other Gram-negative organisms and vice-versa (Smith *et al.*, 1992). This explains why cross expression of genes between *Escherichia coli* (*E. coli*) and *Bacteroides* tends to be restricted. However, expression of bacteroides genes in *E. coli* generally occurs because of the fortuitous recognition of *E. coli*-like promoter sequences (Tancula *et al.*, 1992; Nikolich *et al.*, 1992).

Analysis of *B. fragilis* promoter regions for several genes has led to the finding of a unique promoter recognition sequence which, instead of the common hexameric sequences at -10 and -35 relative to the transcription initiation site, an octamer centered at -7 and a tetramer at -33 define the polymerase recognition sites for many *B. fragilis* genes (Bayley *et al.*, 2000). In addition, it has been found that certain insertion sequences (IS) can also act as promoters of *B. fragilis* genes. It was discovered that a rare resistance gene (*cfiA*) in *B. fragilis* can be found downstream of two insertion sequences (IS) (Podglajen *et al.*, 2001). It was further discovered that these two IS had inserted within 90-bp upstream of this gene in the same region of the chromosome, in all strains. This led Podglajen *et al.* (2001), to map the transcription start site of this gene using the 5'RACE system. In addition to the *B. fragilis* octameric motif, they discovered that this motif overlapped with a putative consensus σ^{70} promoter at the -10 position and the TTG triplet (-33) overlapped with the -35 position (Podglajen *et al.*, 2001). This led to the speculation that the range of consensus sequences

may enable *Bacteroides* IS elements with the means of providing mobile promoters to different bacterial species (Podglajen *et al.*, 2001). New genetic tools for gene disruption such as insertional mutagenesis, gene deletions, the use of transposons and gene silencing with RNA interference (RNAi), in combination with the availability of genome sequence data have greatly enhanced genetic analysis experiments (Diniz *et al.*, 2004; Shalel-Levanon *et al.*, 2005; Bass and Hecht, 2002; Tierney and Lamour, 2005, and http://www.sanger.ac.uk/Projects/B_fragilis/, respectively).

1.3 Metabolism

The human intestinal anaerobic microbiota is involved in breaking down carbohydrates which assists in digestion and provides energy for these saccharolytic anaerobes to propagate (Pomare *et al.*, 1985). The sources of these carbohydrates are the human diet content in the gut, host mucopolysaccharides associated with exfoliated epithelial cells, as well as gastric and colonic mucins (Macfarlane *et al.*, 1998). *Bacteroides thetaiotaomicron* has 163 paralogs of SusC and SusD (polysaccharide-binding proteins), which are believed to help their utilization of the dietary carbohydrates (Pumbwe *et al.*, 2006). Carbohydrate metabolism is also closely associated with nitrogen metabolism. Specifically, ammonia assimilation is immensely important since NH_3 is the preferred source of nitrogen for these saccharolytic bacteria. The emphasis of this work will be on glutamine synthetase, a key enzyme in nitrogen metabolism.

1.3.1 Nitrogen metabolism

Nitrogen is available to microorganisms in many different forms, amongst which are nitrate, ammonia, amino acids, and urea. Bacteria can use the nitrogen available extracellularly or they can synthesize nitrogen-containing compounds (Merrick and Edwards, 1995). The mechanisms which regulate cellular nitrogen metabolism seem to be of great importance to bacteria as we see major aspects of their biology linked to the nitrogen system. Sporulation in *Bacillus subtilis* and *Streptomycetes*, for example, is known to be triggered by nitrogen

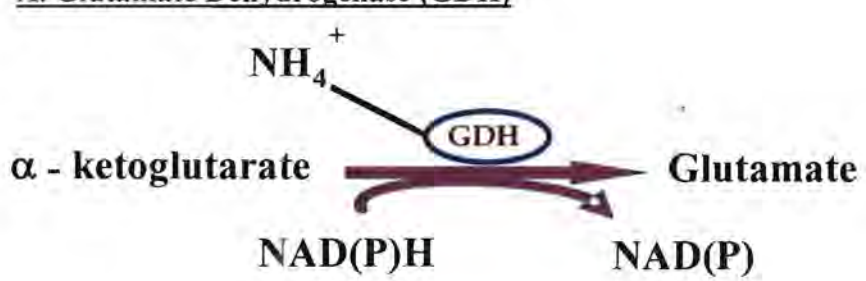
starvation, although our understanding of this mechanism still remains limited (Fisher, 1999). Similarly, it has been observed that synthesis of many secondary metabolites in *Streptomyces* is repressed by ammonia (Vigliotta *et al.*, 2005). Nitrogen metabolism could be of great importance to *B. fragilis* as it may be linked to this organism's pathogenicity through the regulation of proteolytic activity: Gibson *et al.* (1988) showed that reduced nitrogen conditions induce protease secretion in this organism.

1.3.2 Ammonia Assimilation Pathways

Bacteria in the gastrointestinal tract degrade amino acids, amines and purines. These reactions, together with the action of bacterial urease on urea, produce ammonia in the intestine. Detoxification of ammonia occurs in the liver but some of the ammonia is used in the production of glutamine from glutamate, via the action of glutamine synthetase, in the pathways which will be subsequently discussed (Pateman, 1969). Ammonia is customarily the preferred source of nitrogen for enteric bacteria because of its reduced form and consequent rapid assimilation. It was, thus, shown to support a higher bacterial growth rate than any other nitrogen source (Merrick and Edwards, 1995).

The incorporation of ammonia into macromolecules occurs via the synthesis of glutamine and glutamate. These serve as essential nitrogen donors for biosynthetic reactions (Merrick and Edwards, 1995). The enzymes responsible for these reactions are glutamine synthetase (GS), glutamate dehydrogenase (GDH) and glutamate synthase (ie. glutamine amide 2-oxoglutarate amino transferase, GOGAT). These enzymes act in two major pathways (Figure 1.1) with 2-oxoglutarate serving as the carbon skeleton for nitrogen incorporation in these pathways:

A. Glutamate Dehydrogenase (GDH)



B. GS-GOGAT (Glutamine Synthetase - Glutamine amide 2-oxoglutarate amino transferase)

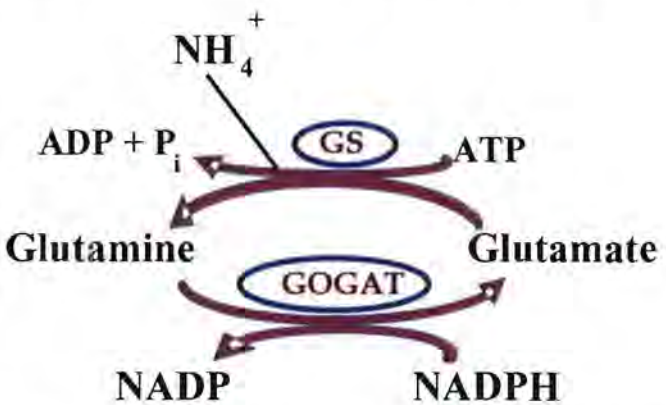


Figure 1.1 Ammonia assimilation pathways. (A) Pathway representing the reductive transfer of an amino group from ammonia to 2-oxo(keto)glutarate, catalysed by GDH. (B) Reactions involved in GS-GOGAT pathways. Pi = inorganic phosphate. (Adapted from Hu *et al.*, 1999)

The reductive amination of 2-oxoglutarate occurs in the GDH pathway, using NADP or NAD as a cofactor. The enzyme catalysing this reaction is GDH, with glutamate as the end product (Helling, 1998). The biosynthesis of 2-oxoglutarate in Bacteroidaceae occurs through reductive carboxylation of succinate rather than isocitrate as in *E.coli* (Allison *et al.*, 1979).

In the Enterobacteriaceae, the GS/GOGAT pathway involves two sequential steps: Initially, an amido group is reductively transferred from ammonia to glutamate thereby synthesizing glutamine. This reaction is energy-dependent, requiring the hydrolysis of a molecule of ATP. The enzyme catalysing this initial step is glutamine synthetase and the by-products of the reaction are ADP and inorganic phosphate (Merrick and Edwards, 1995). When *E. coli* cells are grown in nitrogen deficiency, GS levels are elevated and the GS/GOGAT reaction is favoured. In excess ammonia, however, low GS levels coupled to the high K_m of GDH for ammonia favours the GDH reaction (Helling, 1998).

The second part of this pathway involves the action of glutamate synthase which catalyses the reductive transfer of the amide group from glutamine to 2-oxoglutarate thereby forming two molecules of glutamate. NADPH or NADH is also used as a cofactor here (Bower and Zalkin, 1983).

In *Bacteroides* species, regulation of the ammonia assimilation enzymes occurs differently to other bacteria (Baggio and Morrison, 1996). In the *Bacteroides* spp., GDH enzymes can be subdivided further into anabolic or catabolic, depending on their cofactor specificity (NADP[H]- or NAD[H]- dependent, respectively). While the NADP[H]-dependent GDH (*gdhA*) of *B. fragilis* is induced by low ammonium in the growth medium, Abrahams and Abratt (1998) showed that the NADH-dependent GDH (*gdhB*) of *B. fragilis* is induced when high concentrations of peptides are present in the growth medium. It is worth noting that, in *B. fragilis*, the GS pathway is the only mechanism for glutamine synthesis and that there is, as yet, no evidence in the literature, of a gene coding for the GOGAT large subunit. The focus of this project is on the GS pathway, specifically on the glutamine synthetase enzyme.

1.4 Glutamine Synthetase

Glutamine synthetase is an enzyme which catalyses the production of glutamine. There are four families of GS enzymes: GSI, GSII, GSIII and GlnT (Merrick and Edwards, 1995).

GSI, the most common form of GS in prokaryotes, is encoded by the *glnA* gene. It is characterized by a dodecameric structure consisting of identical subunits, about 55kDa each, arranged in two superimposed hexagonal rings. GSII enzymes are octamers of identical subunits, each with a molecular weight of about 36kDa. Interestingly, GSII can be found in eukaryotes as well as prokaryotes (Merrick and Edwards 1995). The hypothesis for this phenomenon is that the gene originated in preprokaryotes (organisms prior to prokaryotes-eukaryote division) through gene duplication (Kumada *et al.*, 1993).

GSIII was first identified by Hill *et al.* (1983) in *B. fragilis* and has since also been identified in the following species from different taxonomic groups: *Synechococcus, Pseudanabaena sp., Butyrivibrio fibrisolvens, Synechocystis* and *Ruminococcus albus, Prevotella bryantii B4* (Sauer *et al.*, 2000; Crespo *et al.*, 1998; Goodman *et al.*, 1993; García-Domínguez *et al.*, 1997, Amaya *et al.*, 2005 and Wen *et al.*, 2003 respectively). The GSIII in *B. fragilis* was previously shown to have a subunit molecular weight (M_r) of about 82 kDa and to be hexameric in structure (Hill *et al.*, 1989). However, electron microscopy (EM) and single-particle reconstruction techniques have recently shown that this GSIII has a subunit

molecular weight of about 75 kDa and is composed of two superimposed hexagonal rings, forming a dodecameric structure, similar to *S. typhimurium* GSI enzyme (van Rooyen *et al.*, 2006). GSIII is encoded by the *glnN* gene, although the gene has been named *glnA* in some case in *B. fragilis* (Southern *et al.*, 1986 & 1987; Abratt *et al.*, 1983). However, it has most recently been described as *glnN* (van Rooyen *et al.*, 2006) which will be its nomenclature used in this study.

Van Rooyen *et al.* (2006) further studied the relationship between GSIII and the other GS enzymes, taking into account conserved regions, active site residues, an N-terminal motif and secondary structure packing interactions, among others. It was previously shown that the difficulty in obtaining a confident prediction of the structure of GSIII resided in the low sequence identity between GSIII and GSI (Pettersen, *et al.*, 2004). Using the above-mentioned methods and electron microscopy, van Rooyen *et al.* (2006) were able to provide the first GSIII structure, confirming the presence of the predicted barrel fold (site of the active site) as well as deriving the hypothesis that this enzyme can be found in two different states: an active hexamer or an inactive dodecamer.

The fourth GS enzyme has been identified in *Rhizobium leguminosarum* and *Rhizobium meliloti*. It is named GlnT and is an octamer, like GSII, with a subunit molecular mass of 47kDa. Despite their differences, GS enzymes have five conserved regions, which are associated with the GS active sites. García-Domínguez *et al.* (1997) have reported similar enzymatic properties, such as K_m and V_{max} values, for the GSI and GSIII enzymes of cyanobacterial species, while van Rooyen *et al.* (2006) have shown that two highly-conserved GSIII regions are also conserved in all GS enzymes.

1.5 Regulation of the nitrogen assimilation pathways

The regulation of enzymes in the two primary pathways mentioned above tends to be complex due to the importance of nitrogen assimilation for cellular metabolism. Cells have mechanisms to ensure maximum utilization of resources at a minimum energetic cost. Of the two pathways, the GS/GOGAT pathway seems to be more energetically demanding (with the hydrolysis of ATP) (Bruggeman *et al.*, 2005). Therefore, the reaction tends to be almost

irreversible. In addition, GS has a relatively high affinity for ammonia, making it effective in absorbing ammonia even under nitrogen scarcity (Miller, 1972).

The topic of nitrogen regulation has been extensively studied in a few model bacterial systems. In *B. fragilis*, however, a regulatory system or regulatory genes have yet to be identified. Below are a few examples of the main nitrogen regulatory systems in bacteria, followed by a review of the current knowledge regarding *B. fragilis*.

1.5.1 Ntr

Control of the nitrogen assimilatory pathways can be mediated at the transcriptional level (by expression or hindering expression of certain genes) and the gene products can also be mediated post-translationally. It is believed that apart from the primary cellular nitrogen signal (glutamine), the cell senses and adapts to intracellular nitrogen and carbon reflected by the levels of glutamine and 2-oxoglutarate (Maheswaran and Forchhammer, 2003). This well-studied regulation in *E. coli* has been organized into a system called the nitrogen regulatory (*ntr*) system, which is reviewed below.

E. coli produces numerous proteins for the uptake and subsequent metabolism of nitrogenous compounds. The synthesis and activities of these proteins are tightly regulated in concert with the availability of their intracellular substrates (Merrick and Edwards 1995). The *E. coli glnA* is part of a complex, transcriptionally-regulated operon named *glnAntrBC*. The gene order is *glnA*, *ntrB* and *ntrC* (Figure 1.2A). The genes *ntrB* and *ntrC* encode an archetypal bacterial two-component regulatory system comprising the sensory histidine protein kinase (NtrB) and the phosphorylatable response regulator (NtrC), which is a DNA-binding protein (Chen *et al.*, 1995). The *glnA* gene is expressed from two promoters glnAp1 and glnAp2 (Figure 1.2A). When cells are growing in a good supply of nitrogen, *glnA* is expressed from promoter glnAp1, which is transcribed by RNA polymerase with the sigma factor σ70 (Hirschman *et al.*, 1985). Transcription from this promoter produces only basal levels of the transcripts.

Under nitrogen-limiting conditions, however, NtrC binds to sites that overlap glnAp1 thereby repressing expression from this promoter. At the same time, NtrC activates

transcription from glnAp2 (Reitzer and Magasanik, 1985). This transcription occurs at relatively higher levels and a proportion of the transcripts produced read through into *ntrBC* thus expressing *ntrB* and *ntrC*. The glnAp2 promoter is transcribed by RNA polymerase σ54 (Hirschman *et al.*, 1985). This polymerase binds the promoter, but cannot form the active open complex unless it is activated by phosphorylated-NtrC (Figure 1.2B).

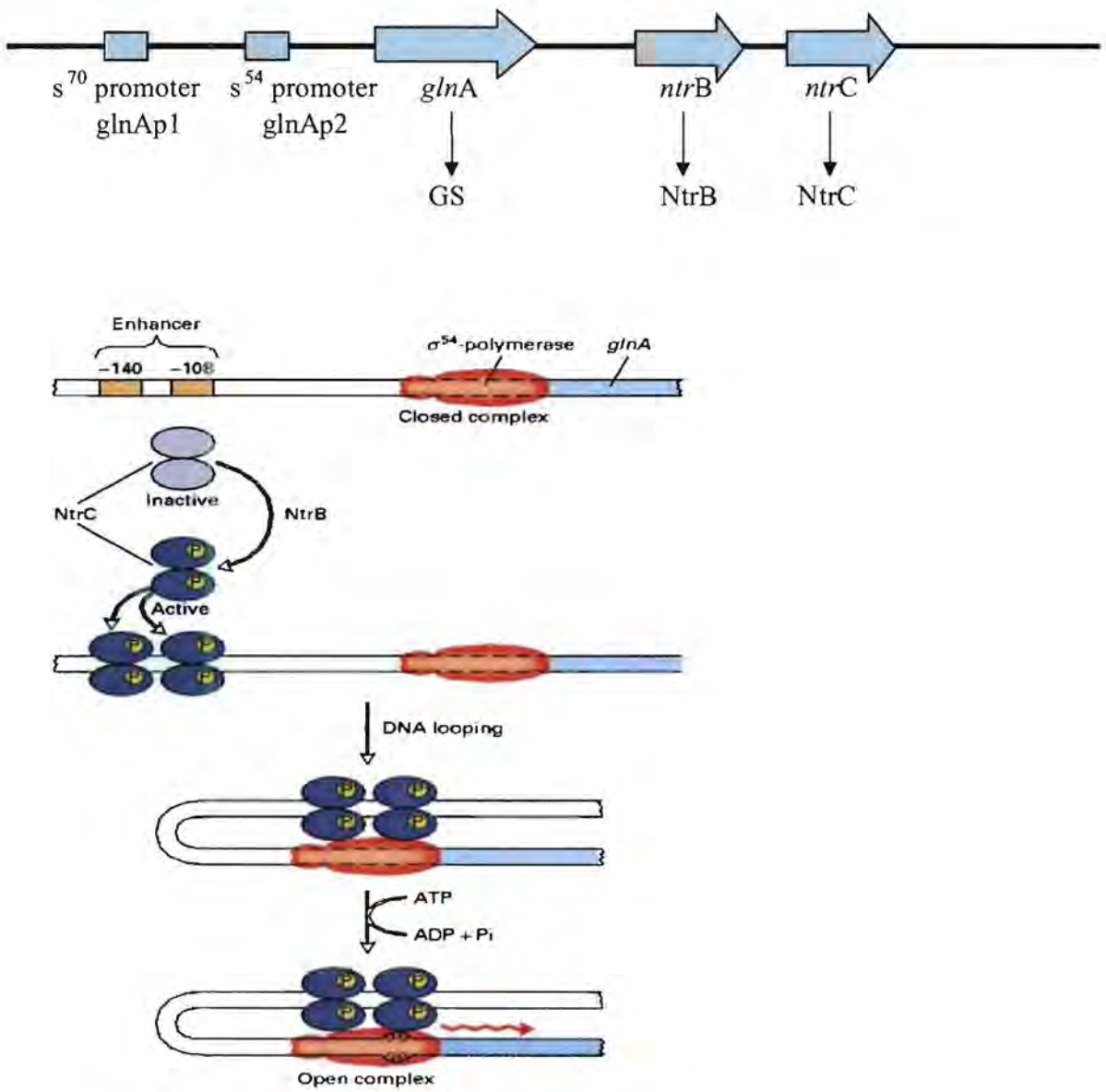


Figure 1.2 **(A)** Genetic organization of the *glnAntr*BC operon in the *E. coli* chromosome, showing genes and promoters arrangement. Blue arrows point in the direction of transcription. Arrows pointing downwards show the protein products of each gene **(B)** *glnA* transcription from s^{54}-polymerase showing the closed complex activation by (NtrB-induced phosphorylation of) NtrC (purple) in nitrogen limiting growth conditions. (Picture 'B' from Lodish *et al.*, 1999).

GSI enzymes can be subdivided further as to whether, and how, they are modified post-translationally (Merrick and Edwards, 1995). One form of post-translational modification occurs by adenylylation. This is the reversible covalent addition of AMP to the subunits of GSI, catalysed by Adenylyl-Transferase (ATase), thereby regulating the catalytic activity of GSI (Maheswaran and Forchhammer, 2003) (Figure 1.3). The components of the *ntr* system associated with post-translational modification are a bifunctional uridylyl-transferase/removing enzyme (UTase/UR) and a small signal-transduction protein called P_{II} (Maheswaran and Forchhammer, 2003) (Figure 1.3). UTase/UR is encoded by *glnD* and responds to cellular glutamine levels (Adler et al., 1975). When the latter is elevated (excess nitrogen), uridylylremovase activity dominates while nitrogen deficiency (low glutamine levels) induces uridylyltransferase activity (Maheswaran and Forchhammer, 2003).

Since the AMP group is added to each of the twelve subunits, GSI can be observed at many different activity states. This modification occurs via a cyclic cascade that comes about as a result of increasing the concentration of intracellular nitrogen (Merrick and Edwards, 1995).

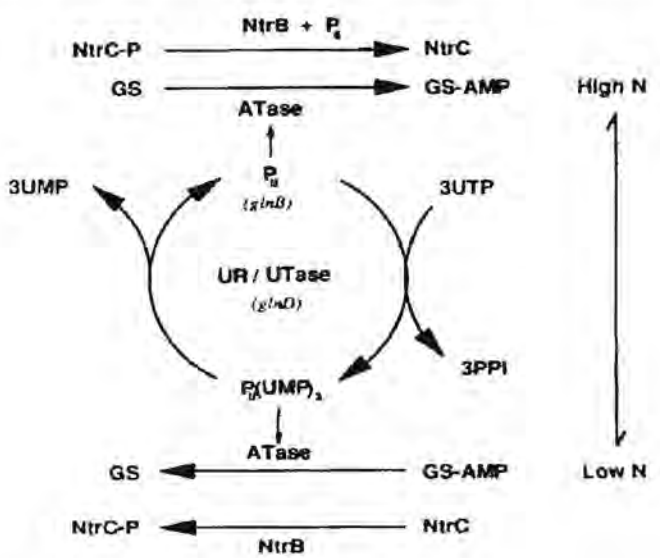


Figure 1.3 Cyclic Adenylylation of GS in *E. coli*. Adenylylation comes about when there is a high ratio of glutamine to 2-oxoglutarate (high N), leading to UR becoming more active than UTase. Deadenylylation occurs when the ratio of glutamine to 2-oxoglutarate is low (low N).
Symbols: GLN= glutamine; 2KG= 2oxo- or keto- glutarate. Picture from Merrick and Edwards, 1995.

A second form of GS post-translational modification is via ADP-ribosylation, also covalent in nature and also due to an increase in intracellular nitrogen. This phenomenon was observed *in vitro* for GSI in crude cell extracts of *Synechocystis* sp. PCC 6803 (Silman *et al.*, 1995) and for the GSIII of *Rhizobium meliloti* (Liu and Kahn, 1995), among others.

Studies on GS adenylylation have led to the observation that, in the presence of high levels of glutamine, GS is in a state of adenylylation by default. Under nitrogen-limited conditions, UTase covalently modifies the signalling protein P_{II} by the addition of a UMP group on each of the three subunits of the protein and the resultant uridylylated form of P_{II} promotes deadenylylation of GS by Adenylyl-Transferase (ATase) (Adler *et al.*, 1975) (Figure 1.3). This signalling molecule undergoes allosteric regulation by the binding of the effector molecule 2-oxoglutarate. Therefore, the adenylylation of eubacterial GS, as well as the control of cyanobacterial GS is dependent on the nitrogen-carbon balance of the cell, by integrating the nitrogen status (via uridylylation) and carbon metabolism (via 2-oxoglutarate) (García-Domínguez *et al.*, 1999).

In other (non-enteric) systems, mutational studies of the inactivation of *ntrC* showed that the NtrC-phenotype is characterized by failure to utilize nitrate as the sole nitrogen source, as is the case for *Azotobacter vinelandii* (Toukdarian and Kennedy, 1986). *R. leguminosarum* and *R. meliloti* are exceptions to these mutational characteristics. A second common function of NtrC is the activation of expression of *glnII*, the structural gene for GSII (Patriarca *et al.*, 1992).

In certain organisms, there is a striking difference between GSI and GSII expression in response to nitrogen: GSI levels remain relatively constant while GSII levels are substantially elevated under conditions of nitrogen scarcity (Merrick and Edwards 1995). This may suggest that GSII is more effective than GSI at very low nitrogen levels. In *R. meliloti*, *glnT* expression is strongly dependent on NtrC, indicating that *glnT* is also under the *ntr* regulon control. The *ntr* genes do not seem to play a major role in symbiotic nitrogen fixation and the symbiotic properties of *R. meliloti* and *B. japonicum* are not affected by *ntrC* mutations (de Bruijn *et al.*, 1989). There is no evidence of covalent adenylylation nor of any of the *ntr* genes being present in *B. fragilis* (Yamamoto *et al.*, 1984; 1987; Southern *et al.*, 1986; 1987).

1.5.2 nac

Another notable nitrogen control gene in enteric bacteria is the *nac* (nitrogen assimilation control) gene. The gene product, Nac, is a dimer with a subunit molecular weight of 32 kDa and it belongs to the LysR family of regulators (Schwacha and Bender, 1993). Nac expression is regulated by *NtrBC*. Therefore, it is only synthesized under nitrogen-limiting conditions. The exact need for Nac in a regulatory sense is not very clear, but it seems to provide a way of linking the expression of a number of genes that are dependent on σ70 with the nitrogen-dependent *ntr* system (Pomposiello and Bender, 1995). Although, the *nac* gene was initially discovered in *Klebsiella aerogenes* (Goss and Bender, 1995), a functional *nac* gene has also been found in *E. coli* (Muse and Bender, 1998). Interestingly, when Muse and Bender truncated the Nac protein by introducing stop codons in the open reading frame, they discovered that all of the known functions of Nac are determined by the 100 amino acids of the N-terminal region (1999).

1.5.3 ntcA

A third nitrogen control gene is *ntcA*, encoding a protein homologous to the cAMP receptor protein (CRP) family of bacterial transcription regulators. García-Domínguez *et al.* (2000) showed that in *Synechocystis* strain PCC 6803, the GSI structural gene (*glnA*) was regulated by NtcA. NtcA can act as activator and repressor (Jiang *et al.*, 1997). It acts as activator of *glnA, glnN, nirA* (first gene of nitrate assimilation operon), *ntcA* itself, as well as others (García-Domínguez *et al.*, 2000). Therefore, NtcA appears to be a global nitrogen regulator which acts as the NtrC of Cyanobacteria (Merrick and Edwards 1995). The *ntc*A gene has been identified in more than 11 cyanobacterial species and homologous genes have been sequenced from *Synechocystis* and *Anabaena* (Frías *et al.*, 1993; Ramasubramanian *et al.*, 1996).

Evidence has been presented that suggests that this control by NtcA occurs via an NtcA-like binding motif upstream of the transcription start point, such as is the case for *glnN* in *Synechococcus* (Sauer *et al.*, 2000). The NtcA binding site is meant to substitute for the *E. coli* σ^{70} consensus box located at position -35 relative to the transcription start site in all NtcA-regulated promoters (Herrero *et al.*, 2001). However, gel retardation experiments failed to show binding of NtcA to this sequence (Reyes et al., 1997). Therefore, it is

believed that this DNA sequence is a weak NtcA binding motif and that additional factors are required for strong binding (Sauer *et al.*, 2000). In this respect, P_{II} was proposed but is an unlikely candidate because the phenotype of a *glnB* mutant in *Cyanobacteria* is very distinct from that of an *ntc*A mutant. If P_{II} was indispensable for NtcA activation, the phenotypes of the mutants would have been similar. Therefore, it is believed that there must be some pathways that control nitrogen assimilation in *cyanobacteria*, following an unidentified primary nitrogen sensor (Merrick and Edwards 1995). Recent studies have revealed that a protein named pipX interacts with NtcA to induce *glnN* and other NtcA-induced genes (Espinosa *et al.*, 2007). In addition, two peptides, named inactivating factor 7 (IF7) and 17 (IF17), were shown to be responsible for GS inactivation by protein-protein interaction in cyanobacteria (García-Domínguez *et al.*, 1999). This mechanism is proven to be different to the adenylylation system in the complete inactivation of GS without any evidence of feedback inhibition and both peptides are needed for this inactivation (García-Domínguez *et al.*, 1999).

1.5.4 Nitrogen regulation in gram positive bacteria

Nitrogen regulation in gram-positive bacteria occurs differently to the systems described for gram-negative organisms. In *Bacillus subtilis*, for example, nitrogen metabolism is controlled by the GlnR and TnrA peptides. Activation of GS transcription by TnrA occurs during nitrogen limitation while repression of GS transcription by GlnR takes place in excess nitrogen (Fisher, 1999). The GS of *B. subtilis* is not known to be post-translationally modified (Hu *et al.*, 1999). Wray *et al.* (1996) observed that a *tnrA* mutant impaired expression of at least five nitrogen-regulated genes, which led to their conclusion that *B. subtilis* also possesses a global nitrogen regulatory system, the first depicted in a gram-positive organism.

It was discovered that the structure and function of GlnR in *Streptomyces* is different from the GlnR in *Bacillus* (Burkovski, 2003). While GlnR acts as a repressor of GS transcription in *B. subtilis,* functional complementation analysis of a glutamine autotrophic mutant in *S. coelicolor* has shown that GlnR is required for the transcription of *glnA* (Wray *et al.*, 1991). *Streptomyces* sp. generally have both GSI and GSII enzymes. Surprisingly, when *glnA* or *glnII* are disrupted in *S. coelicolor*, no glutamine auxotrophic phenotype is observed (Fink *et*

al., 2002). However, disruption of *glnR* leads to this phenotype. *S. coelicolor* GSI is regulated at the transcriptional and post-translational level. The transcription of *glnA* in *Clostridium acetobutylicum*, another Gram-positive bacterium, is also regulated by nitrogen, however, expression of antisense RNA from a downstream promoter was coupled to decreased GS activity (Fierro-Monti *et al.*, 1992)

1.6 Glutamine Biosynthesis in *Bacteroides fragilis*

Yamamoto *et al.* (1984) performed a series of experiments in which *B. fragilis* was supplemented with methionine sulfoximine (MSX), a GS inhibitor. This, however, failed to inhibit bacterial growth under nitrogen-limited growth conditions, leading the investigators to conclude that the GS-GOGAT pathway might not play a significant role in ammonia assimilation in *B. fragilis* and that the GDH pathway was the primary means of ammonia assimilation in *B. fragilis*. However, similar studies done with *Prevotella bryantii*, another Gram negative, obligate anaerobe which also has a GSIII, showed that the MSX concentration required to inhibit bacterial growth varies with respect to the nitrogen status (Wen *et al.*, 2003). Therefore, Wen *et al.* (2003) suggested that the reason Yamamoto *et al.* (1984) failed to see growth inhibition may have been due to an incomplete inhibition of GS activity because the MSX concentration used may not have been adequate to counter GS specific activity.

The *glnN* gene of *B. fragilis* has since been cloned, is able to complement a glutamine auxotrophic strain of *E. coli* and has been shown to be regulated transcriptionally (Abratt *et al.*, 1993). Furthermore, Southern *et al.* (1987) demonstrated using western hybridization that the amount of GS protein in *B. fragilis* varied according to nitrogen availability, showing an increase under low ammonia conditions. The conclusions of Yamamoto *et al.* (1984) may also have been partly due to their inability to detect GS activity in *B. fragilis* cell extracts using the γ–glutamyl transferase (GGT) assay. Southern *et al.* (1987) showed that the lack of GS activity in *B. fragilis* cell extracts was due to GS inactivation by the cell extracts. In the same experiment, GS inactivation in *E. coli* was very small relative to the GS of *B. fragilis*. Southern *et al.* (1987) concluded that the *E. coli* GS inactivation was due to feedback inhibition. However, the irreversible inactivation of *B. fragilis* GS by the cell

extracts remains a mystery but the involvement of an enzyme is unlikely since the cell extracts were boiled (Southern *et al.*, 1987).

No nitrogen regulatory system has, as yet, been identified in *B. fragilis* and there is no evidence of an *ntr* system of regulation nor of post-translational modification by adenylylation (Southern *et al.*, 1986; Southern *et al.*, 1987). There has also been no study to determine whether GSIII is the only enzyme responsible for glutamine biosynthesis in *B. fragilis*. .

1.7 Project aims

Glutamine synthetase is a key enzyme in nitrogen metabolism. The importance of this enzyme is evident by its intricate transcription and regulation control in most organisms. It is also not surprising that many prokaryotes possess multiple glutamine synthetase genes, encoding enzymes which have diverse roles under different growth conditions. In *B. fragilis,* GSIII is the sole glutamine synthetase identified to date. This GSIII enzyme has been well studied and characterized.

The aims of this project were, therefore:

- To identify additional GS orthologues in *B. fragilis* through genomic analysis, and to perform genetic and functional characterization of the possible candidates.
- To identify possible glutamine synthetase regulatory proteins and functionally determine their contribution to glutamine biosynthesis.

CHAPTER 2

CONSTRUCTION AND CHARACTERIZATION OF A *B. fragilis* MUTANT

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CHAPTER 2

Construction and characterization of a *B. fragilis glnN* mutant

2.1 Introduction

Glutamine synthetase (GS) is one of the key enzymes involved in nitrogen metabolism. It is responsible for the conversion of glutamate to glutamine, providing the nitrogen donor for the synthesis of amino acids and nucleic acids (Robinson *et al.*, 2001). GS enzymes are categorized into 4 types: GSI, GSII, GSIII, and glnT (see Chapter 1). Glutamine synthetase is the only enzyme able to manufacture glutamine and, therefore, its importance is universally evident. In *Streptococcus thermophilus* (a lactic acid bacterium), glutamine synthetase is required for growth in milk, regardless of the presence of glutamine as peptides and caseins in the milk (Monnet *et al.*, 2005). An unusual role for glutamine synthetase is seen in *Mycobacterium tuberculosis,* which requires the glutamine synthetase gene for virulence (Tullius *et al.*, 2003). In view of its importance, it is not surprising that many prokaryotes such as *Synechocystis, Ruminococcus albus* 8 and *Mycobacterium tuberculosis* possess multiple glutamine synthetase genes to help adapt to changing environments. (García-Domínguez *et al.*, 1997 ; Amaya *et al.*, 2005, and Harth *et al.*, 2005, respectively).

In the cyanobacterium *Synechocystis* sp. PCC6803, the *glnA* gene product (GSI) accounts for 97% of GS activity when the bacterium is grown in nitrate or ammonium, as nitrogen sources (Reyes and Florencio, 1994; Reyes *et al.*, 1997). However, when this cyanobacterium is grown in conditions of nitrogen shortage, *glnN* (GSIII) transcription is strongly stimulated and the total GS activity increases (Reyes *et al.*, 1997; García-Domínguez *et al.*, 1997). *Ruminococcus albus* 8 also possesses both GSI and GSIII enzymes. However, only the GSIII enzyme is active when expressed in *E. coli,* although both are transcribed (Amaya *et al.*, 2005). *Mycobacterium tuberculosis* possesses four glutamine synthetase encoding genes (Cole *et al.*, 1998). When each of these genes was expressed in a heterologous host, the gene products were all shown to have GS activity (Harth *et al.*, 2005). However, only one of these genes, *glnA1*, appears to be essential for the growth of *M. tuberculosis in vitro* (Tullius *et al.*, 2003).

To date, GSIII is the only GS enzyme known to be present in *Bacteroides fragilis*. The *glnN* gene (formerly called *glnA*) and its product have been extensively studied and well characterized (Abratt *et al.*, 1983; Hill *et al.*, 1983 & 1989; Southern *et al.*, 1986 & 1987; van Rooyen *et al.*, 2006). The prevalence of *Bacteroides fragilis* in intra-abdominal infections coupled to the importance of glutamine synthetase for growth and in some cases, virulence, have led to the hypothesis that *B. fragilis* may possess more than one GS orthologue.

Investigating the metabolic role of GS genes products can be done by examining the regulation of the expression of the gene as Reyes *et al.* (1997) did for the two GS genes products of *Synechocystis* sp. PCC 6803. An alternative approach is the creation of a mutant which enables a direct, phenotypic investigation of the gene function, especially when dealing with a gene which is required for viability, such as the *glnN*. There are various genetic tools available to manipulate *Bacteroides* spp., in order to investigate the roles of individual genes. These tools include the use of conjugation and vector systems. Random transposon mutagenesis with the *Bacteroides* transposon Tn*4351,* as well as insertional gene targeted mutagenesis or deletions by means of mating have been exploited to transfer cloned genes from *E. coli* to *Bacteroides* with the use of suicide vectors and mobilizing strains (Genco *et al.*, 1995; Lepine *et al.*, 1996).

It was decided that the approach used in this study to determine whether *glnN* is the only gene coding for glutamine synthetase in *B. fragilis*, would be to inactivate this gene by insertional mutagenesis using the Bacteroides suicide vector pGERM (Salyers *et al.*, 2000) and the *E. coli* S17 mobilizing strain (Simon *et al.*, 1983). In this system, insertional mutagenesis is achieved by constructing a plasmid in *E. coli*, containing the target DNA fragment, which cannot replicate in the *B. fragilis* recipient strain. Mating is then carried out between the *E. coli* donor containing the plasmid and the recipient. The selectable marker on the plasmid and failure of the plasmid to replicate allows the selection of transconjugants which have integrated the plasmid into the chromosome by homologous recombination between the cloned fragment and the corresponding region in the chromosome.

Inactivating a gene by inserting a large fragment of DNA within the coding region leads to translation of a non-functional protein. The effects caused by the lack of the gene product under study can then be observed functionally and phenotypically. The purpose of inactivating the functional *glnN* will be to determine if any growth occurs in the presence of

ammonia as the only nitrogen source. If that is the case, then the growth could be attributed to a second GS encoding gene. In addition, characterization of the *glnN* mutant will be carried out on two levels, in this study: Physiologically, by monitoring the growth pattern in the presence of different concentrations of nitrogen source, and enzymatically by performing GS assays.

2.2 Materials and methods

2.2.1 Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 2.1.

Table 2.1 Bacterial strains and plasmids

Strain or plasmid	Description	Source/ reference
B. fragilis 638R	Rif^r Em^s Gm^r	Privitera *et al.*, 1979
B. fragilis 638ΩglnN	Rif^r Em^r Gm^r *B. fragilis* mutant with a *glnN* disruption created by the suicide vector pGERM containing a PCR-generated 0.55-Kb internal fragment of *glnN*	This study
E. coli S17-1 λ *pir*	*recA thi pro hsdR⁻ M⁺* RP4::2-Tc::Mu::Km Tn7 lysogenized with λ *pir* phage Mobilizing strain for *Bacteroides* spp.; TMP^r	Simon *et al.*, 1983
E. coli YMC11	*thi, endA, hsr, araD, strA, rhaD,* Δ*lacU169 hutC*$_{Klebs}$ Δ(*glnG-glnA*)	Backman *et al.*, 1981
pJS139	(Ap^r, *glnA*$^+$) *B. fragilis glnN* gene in pEcoR251	Southern *et al.*, 1986
pGERM	pUC19 with 782 bp *HaeII* RK2oriT fragment cloned into *SapI* and 1.3 kb *ermG* cloned into *SspI*; Mobilized by IncPα plasmids. Insertional vector in *Bacteroides* spp. (Ap^r) Em^r Rep^-	Salyers *et al.*, 2000
pGERM-intGSIII	pGERM with an internal fragment of *glnN*	This study

Abbreviations:
Klebs denotes a gene in *Klebsiella aerogenes*
Rif, rifampicin; TMP, trimethoprim; Ap, ampicillin; Em, Erythromycin; Gm, Gentamicin; s, sensitive; r, resistant.

B. fragilis 638R was grown on supplemented Brain Heart Infusion (BHIS) broth or agar (Holdeman *et al.*, 1972) at 37 °C, in a Forma Scientific anaerobic chamber with an atmosphere of oxygen-free N_2 (85%), H_2 (5%) and CO_2 (10%). When required, the medium contained one or more of the following antibiotics: gentamicin, 200 µg/ml; rifampicin, 20 µg/ml. Growth studies for *B. fragilis* 638R and the *glnN* (GSIII) mutant were conducted in glucose minimal medium (Van Tassell and Wilkins, 1978) with ammonium chloride as the sole nitrogen source. High and low nitrogen conditions were generated with 50 mM and 0.5 mM NH_4Cl, respectively. When needed, 10 µg/ml erythromycin and 200 µg/ml gentamicin were used to select for insertion mutants. *E. coli* S17-1 was the donor in conjugation experiments. *E. coli* S17-1 was grown at 37 °C in Luria-Bertani (LB) agar or broth (Sambrook *et al.*, 1989). When necessary, ampicillin, 100 µg/ml was added to the medium for selection.

2.2.2 General recombinant DNA procedures

All DNA manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). Chromosomal DNA was isolated from *B. fragilis* using the method of Wehnert *et al.* (1990). Restriction enzymes were used according to the manufacturers' specifications (Fermentas; Roche Diagnostics). Agarose gel electrophoresis, ligations with T4 DNA ligase and transformation were carried out by standard procedures (Sambrook *et al.*, 1989). Competent *E. coli* S17-1 cells were prepared using the rubidium chloride method (Armitage *et al.*, 1988). Plasmid DNA was isolated using the alkali lysis method (Ish-Horowicz & Burke, 1981) or using the Qiaprep Spin Miniprep kit (Qiagen).

2.2.3 Construction of plasmid pGERM-intGSIII

A 550 bp internal fragment of *glnN* (GSIII structural gene) was amplified by PCR, using primers GS3iF (5'-AATGGCCGACCTGATAGCC-3') and GS3iR (5'-AGCTGGCATACTTCGGTAGC-3'). Blunt ends were created on these amplicons, using T4 DNA polymerase (Fermentas) according to the manufacturer's instructions. The *Bacteroides* spp. suicide vector pGERM (described in Table 2.1) was digested with *Sma*I. Vector and insert DNA were ligated with T4 ligase, according to the manufacturer's instructions, to produce the pGERM-intGSIII construct, as shown in Fig. 2.1.

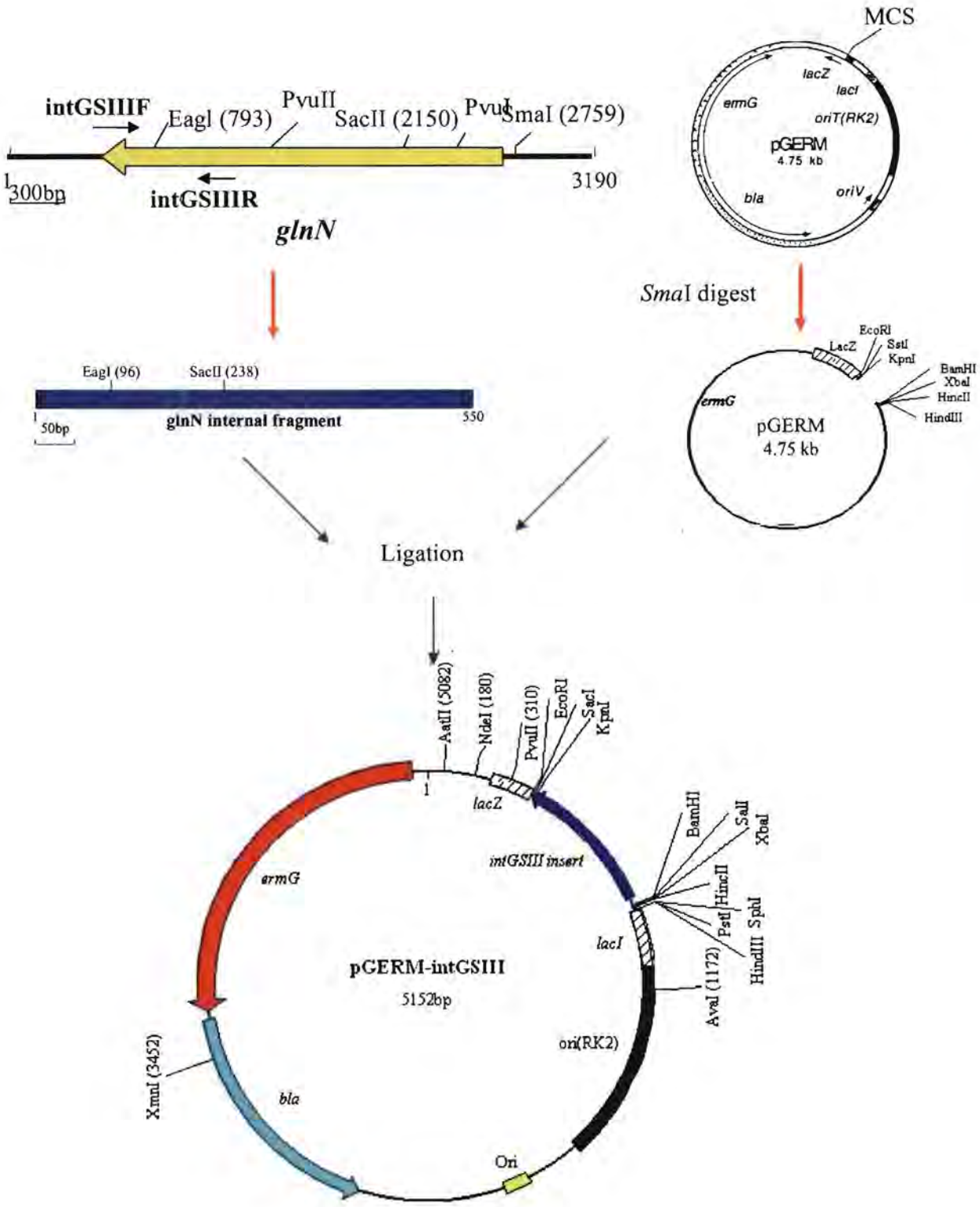

Fig. 2.1 Construction of plasmid pGERM-intGSIII. Physical map showing an internal fragment (dark blue bar) of the *glnN* gene (yellow arrow) from *B. fragilis* cloned into the *Sma*I site of pGERM. The horizontal arrows indicate position of primers used to amplify the internal fragment. MCS = multiple cloning site

2.2.4 Construction of *B. fragilis* 638ΩglnN

E. coli S17-1 was transformed with the above recombinant construct pGERM-intGSIII, using techniques described in section 2.2.2. *E coli* S17-1 containing the vector pGERM-intGSIII was used as the donor strain in a mating with *B. fragilis* 638R (the recipient). Standard filter mating techniques were used to transfer the plasmid, according to the procedure outlined by Shoemaker *et al.* (1989). Fresh broth subcultures of each strain, grown to an OD_{600} of 0.2, were mixed using a donor to recipient volume ratio of 1:5. Filters containing mating mixture were incubated on BHIS agar plates for 12-16 hours, aerobically, at 37°C. Following incubation, the cells were washed off the filters, incubated for 1 hour anaerobically and plated on BHIS agar containing 200 µg/ml gentamicin (to select against *E. coli*) and 10 µg/ml erythromycin to obtain transconjugants which acquired resistance through a chromosomal insertion of pGERM-intGSIII in the *glnN* gene (*B. fragilis* 638ΩglnN). The plates were subsequently incubated at 37°C anaerobically, for three days.

2.2.5 Southern blot analysis

Chromosomal DNA (10 µg) from *B. fragilis* 638R and *B. fragilis* 638ΩglnN was digested for 12-16 hours with *Hin*dIII and fractionated by electrophoresis on a 0.8 % (w/v) agarose gel. The DNA was then transferred to nitrocellulose paper and probed using standard Southern blot protocol (Sambrook *et al.*, 1989). The *glnN* internal fragment described in section 2.2.3 was used as a probe and labelled with Digoxigenin-11-dUTP (DIG) and exposed to X-ray film for visualization, according to manufacturer's instructions (Roche). As a positive control, 75 ng of plasmid pJS139 (containing the *glnN* gene- Table 2.1) was digested with *Xho*I and subjected to electrophoresis and transfer onto the same membrane as the genomic DNA.

2.2.6 DNA sequencing and analysis of *B. fragilis* 638ΩglnN

PCR was done using primers RealGSIIIF 5'-TGGGACGATTGGTCTGAGG-3' and RealGSIIIR 5'-GTTGACAGACGCTGGGATG-3' (which amplify the full-length *glnN* gene) in conjunction with M13F 5'-CGCCAGGGTTTTCCCAGTCACGAC-3' and M13R 5'-GAGCGGATAACAATTTCACACAGG-3' primers (sites in the pGERM vector) (Fig. 2.2A). The PCR products obtained for both the wild-type and mutant strains of *B. fragilis*

were purified and nucleotide sequences were determined using the M13 forward and reverse primers. Sequencing was done using the Big Dye terminator v3.1 Cycle sequencing kit (Applied Biosystems) run on a MegaBACE (MegaBACE 500; Amersham Biosciences), using the dideoxynucleotide chain termination chemistry (Sanger *et al.*, 1977).

2.2.7 Preparation of cell-free extracts

B. fragilis 638R and *B. fragilis* 638ΩglnN were grown anaerobically at 37°C, in glucose minimal medium broth with 0.5 mM (low) or 50 mM (high) ammonium chloride, as the sole nitrogen source. After six hours of growth, the cultures were harvested by centrifugation at 15 000 x g in a Beckman J2 – 21 centrifuge for 20 minutes at 4°C. The cell pellets were resuspended in 12.5 mM imidazole buffer. The resuspended cells were disrupted by sonication on ice with a Virsonic 50 (Virtis, USA) cell disrupter, using pulsed cycles of 30 seconds with 20 second intervals between the cycles, for a total time of 4 minutes. Cell debris were removed by centrifugation at 8000 x *g* for 10 min. The amount of total protein in the cell-free extract was determined using a commercially available diagnostic reagent based on Bradford's standard assay (Bio-Rad protein assay; Bradford, 1976).

2.2.8 Glutamine synthetase assays

The glutamine synthetase forward and transferase assays were done according to the method of Bender *et al.* (1977) modified so that the scale of the assays was reduced to microplate levels. These assays were based on measuring the formation of γ-glutamyl-hydroxamate spectrophotometrically at 540 nm in a microplate reader (Titertek Multiskan Plus MKII).

Forward assay mixture, prepared daily from stock solutions, contained: 94 mM imidazole (pH 7.15), 47 mM hydroxylamine-HCl, 168 mM monosodium L-glutamate, and 56 mM $MgCl_2$. The final pH was adjusted to 7.4 with KOH or HCl as required. Twenty microlitres of cell-free extract were added to the assay mix and equilibrated at 37°C for 5 min. The reaction was initiated by the addition of 16 µl of 120 mM ATP in a total reaction volume of 100 µl. The reaction was terminated after 25 minutes with the addition of 200 µl of "stop mix" (55 g $FeCl_3 \cdot 6H_2O$, 20 g trichloroacetic acid, 21 ml concentrated HCl and distilled water to 1 L).

The transferase assay mixture contained 25 mM L-α-glutamine, 0.27 mM $MnCl_2$, 18 mM hydroxylamine-HCl, 25 mM KH_2AsO_4, and 135 mM imidazole, pH 7.0. The reaction mixtures with the cell free extracts were equilibrated at 37°C for 5 min, and the reaction was initiated by adding 0.36 mM ADP (final concentration). The reaction was terminated after 15 min by the addition of "stop mix" as in the forward assay.

Reaction products were immediately mixed and centrifuged to remove precipitated protein and the absorbance measured at 540 nm, against a blank sample with no cell free extract. GS specific activity was defined as μmol of γ-glutamyl-hydroxamate formed per mg of protein per minute.

2.3 Results and Discussion

2.3.1 Construction and analysis of *B. fragilis* 638ΩglnN

A *glnN* gene disruption mutant of *B. fragilis* was constructed using a suicide vector (Fig. 2.2). The pGERM-intGSIII construct was made of a 550 bp internal fragment of *glnN* ligated into pGERM, previously digested with *Sma*I. Through a single recombination event between the internal fragment and the homologous region in the chromosomal *glnN* gene, the whole pGERM-intGSIII construct was integrated in the chromosomal *glnN*, thereby disrupting this gene. Because pGERM is a *Bacteroides* suicide vector, it does not replicate in *Bacteroides* and can only remain in succeeding generations if integrated in the chromosome. Transconjugants were then selected based on their acquired resistance to erythromycin due to the resistance marker gene in the construct. Analysis of transconjugant DNA was done by PCR (Fig 2.2B), sequencing and Southern blot hybridization (Fig. 2.3). Since pGERM-intGSIII is only present in the mutant, the vector-derived M13 primers have no complementary annealing sites in the wild-type *B. fragilis*. Therefore, PCR using primers RealGSIIIF and M13R displayed no band in the wild-type *B. fragilis* 638R but amplified a 750 bp fragment in *the glnN* mutant (Fig. 2.2B). Similarly, the combination of RealGSIIIR and M13F generated a 2.5 kb band from the mutant and none from the wild-type.

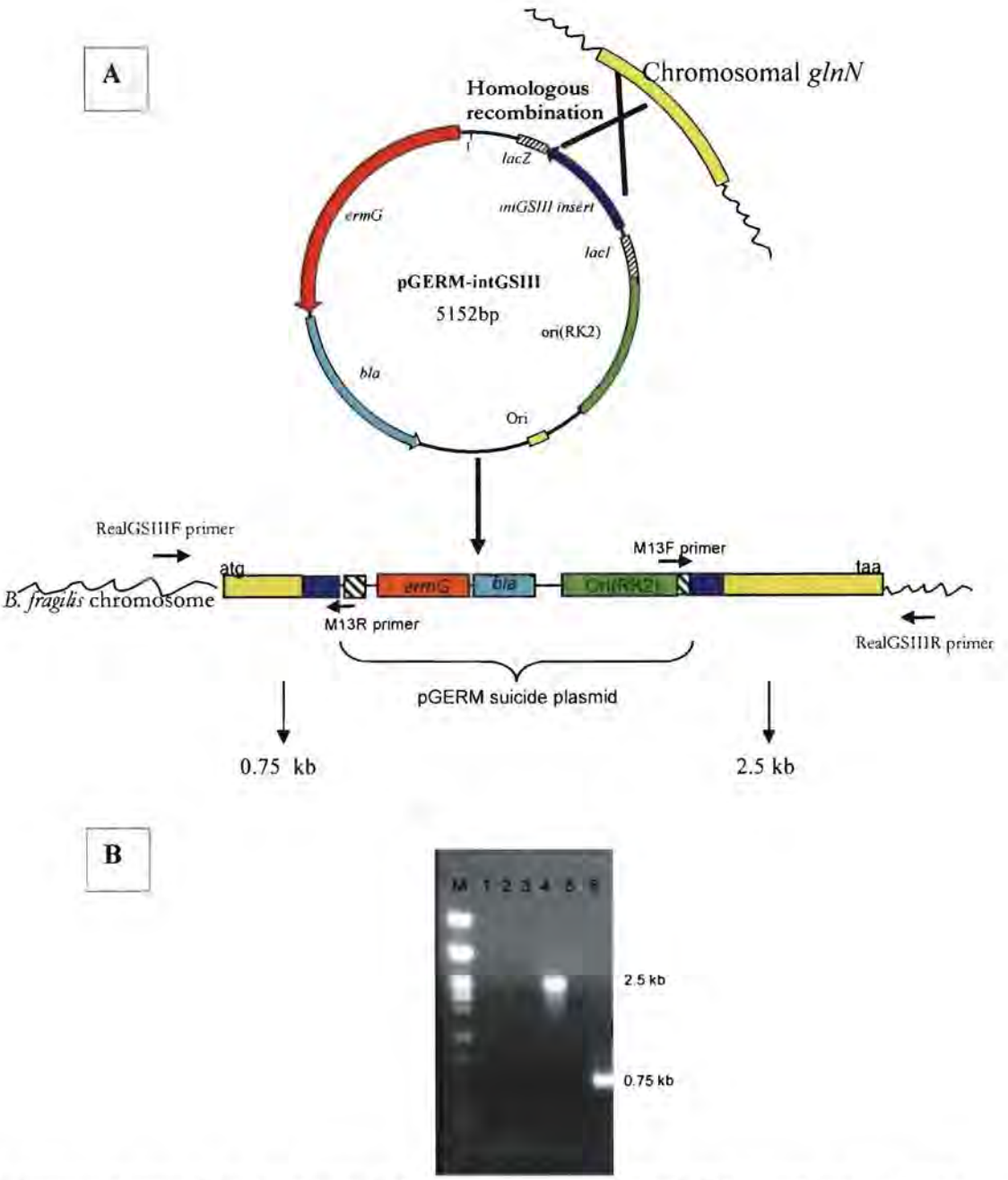

Fig. 2.2 Schematic diagram of the insertion-mediated mutagenesis. (A) The pGERM recombinant containing an internal DNA fragment of *glnN* (in blue) was integrated into the recipient by a single homologous recombination event (crossed black lines). Small arrows indicate positions and directions of primers. (B) PCR of the *B. fragilis glnN* mutant. PCR was done with chromosomal DNA from *B. fragilis* 638R and Erm^r transconjugants, using the indicated primer pairs: RealGSIIIF and M13R (primer set 1); RealGSIIIR and M13F (primer set 2), to detect the insertion. Lane labeled M contains lambda DNA digested with *PstI* as molecular weight marker; lanes 1 and 2 are controls with each primer set and no DNA to verify the purity of reactions; lane 3 is *B. fragilis* 638R DNA amplified with primer set 2; lane 4 is *B. fragilis* 638ΩglnN DNA with primer set 2; lane 5 is *B. fragilis* 638R DNA with primer set 1; lane 6 is *B. fragilis* 638ΩglnN DNA with primer set 1.
ermG, erythromycin-resistance marker for *Bacteroides* spp.; Ori(RK2), transfer origin from plasmid RK2; *bla*, gene encoding β-lactamase; Erm = Erythromycin.

DNA sequence analysis of the fragments amplified in the mutant revealed the insertion of the pGERM vector in the *glnN* gene, at the internal fragment region, resulting in a duplication of the internal fragment on either side of the vector as shown by the blue fragments in Fig. 2.2A. Southern hybridisation analysis confirmed the above results (Fig. 2.3). *B. fragilis* 638R and *B. fragilis* 638ΩglnN genomic DNA was digested to completion with *Hin*dIII, transferred to a nylon membrane and hybridized with a labeled probe made of the *glnN* internal fragment DNA.

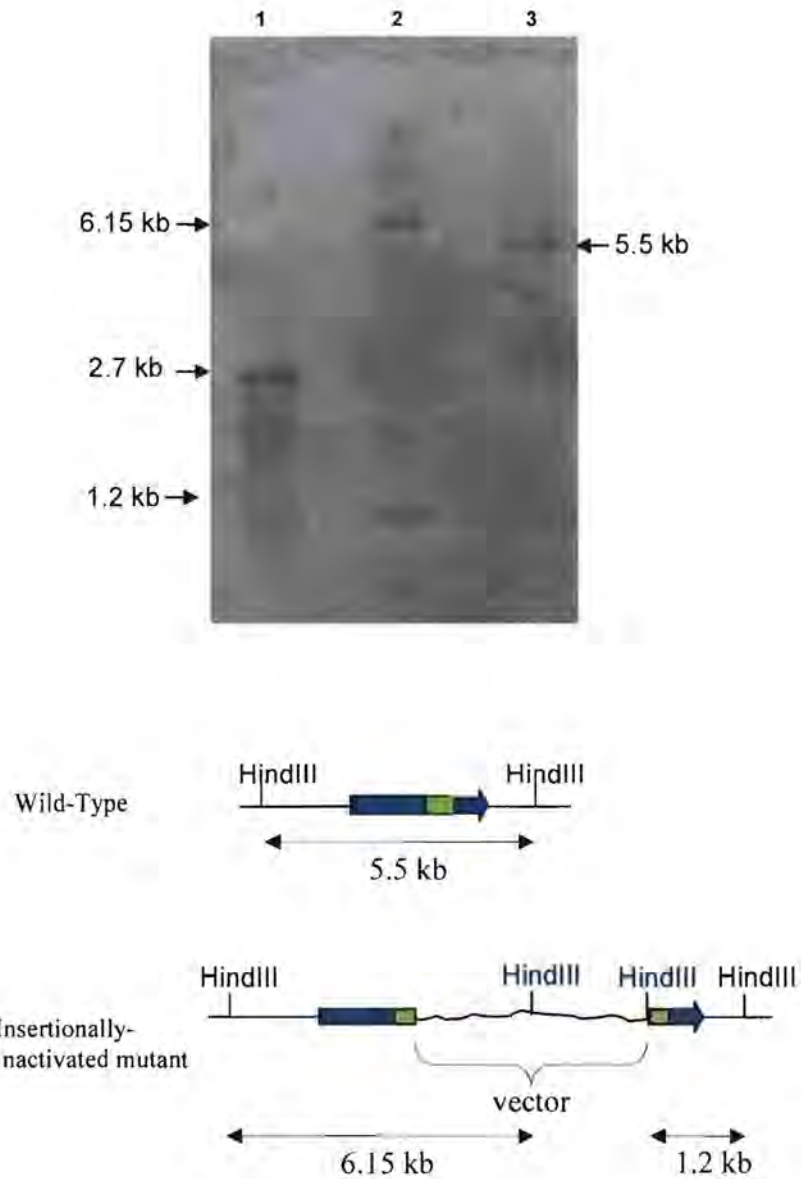

Fig. 2.3 Southern blot analysis of insertion in *B. fragilis* 638ΩglnN. Genomic DNA isolated from the parental *B. fragilis* 638R and *B. fragilis* 638ΩglnN was digested with *Hin*dIII, transferred to a nylon membrane and probed with a DIG-labeled 550-bp *glnN*-internal fragment. Lane 1, positive control (*glnN* gene in vector pJS139); lane 2, DNA purified from *B. fragilis* 638ΩglnN; lane 3, *B. fragilis* 638R DNA. ▬ = position of the internal fragment probe.

The inserted pGERM-intGSIII in *B. fragilis* 638ΩglnN introduced two additional *Hin*dIII sites, as well as the duplicated internal fragment, which resulted in the probe hybridizing twice (Fig. 2.3). Consequently, a single 5.5 kb band was observed in the wild-type *B. fragilis* but two bands (6.15 kb and 1.2 kb) were seen in the mutant (Fig. 2.3). A 2.7 kb fragment of pJS139 (containing the full *glnN* gene) was used as a positive hybridization control.

2.3.2 Growth studies of *B. fragilis* 638ΩglnN

Growth studies were carried out in glucose minimal medium with ammonium chloride as the sole nitrogen source. *B. fragilis* 638R and *B. fragilis* 638ΩglnN were streaked onto solid minimal medium containing 0.5 mM (low) or 50 mM (high) NH_4Cl. The results showed that *B. fragilis* 638ΩglnN failed to grow under low ammonia conditions (Fig. 2.4B) and grew poorly under high ammonia conditions, compared to the wild-type *B. fragilis* 638R (Fig 2.4A).

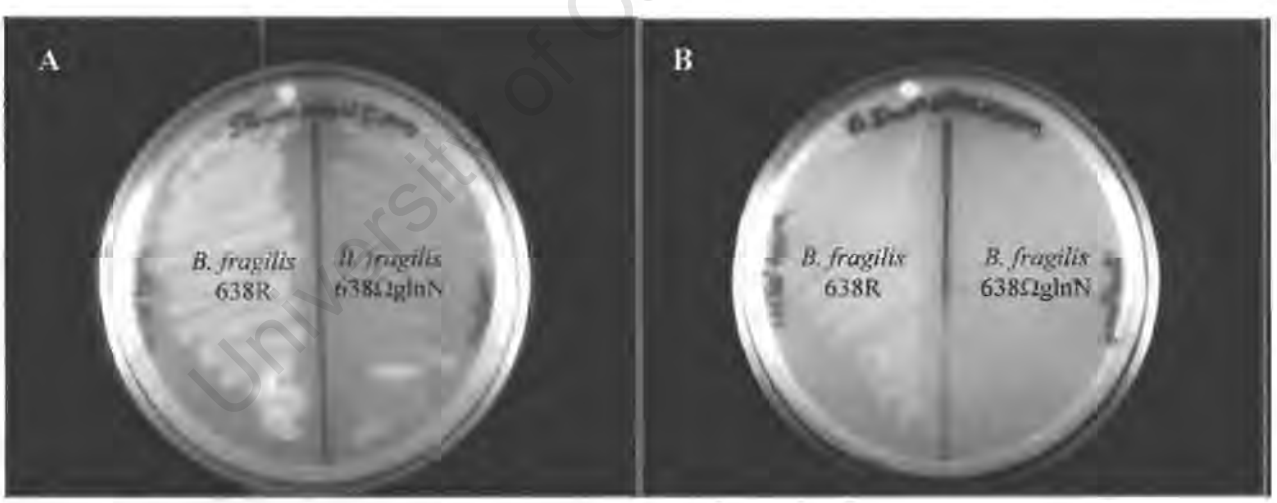

Fig. 2.4 *B. fragilis* 638R and *B. fragilis* 638ΩglnN grown on glucose minimal medium agar supplemented with (**A**) 50 mM (high) and (**B**) 0.5 mM (low) ammonium chloride.

Growth of *B. fragilis* 638R and *B. fragilis* 638ΩglnN in minimal medium broth revealed similar results to that observed on solid medium. Overnight cultures grown in complete medium were harvested and washed twice in the appropriate minimal medium before being inoculated into the growth media. The cultures were then incubated at 37°C under anaerobic conditions and growth was monitored at OD_{600} (Fig. 2.5).

After 6 hours of growth, the mutant reached an optical density of 0.33 under low NH_4Cl-containing media compared to the wild type whose growth was more than twice that of the mutant (Fig 2.5). This trend was maintained after 24 hours of growth, with the mutant reaching an OD_{600} of 0.35, compared to the wild-type OD_{600} of 0.7. Under high ammonia broth conditions, the mutant and the wild-type *B. fragilis* grew equally well initially (Fig 2.5). At the 24 hours time point, however, there was a significant difference between the two strains: The mutant gave an average OD_{600} of 1.0 versus 1.6 for the wild-type. The insertional-inactivation of the *glnN* gene which leads to the translation of a non-functional GSIII protein results in a lack of growth under low ammonia-containing medium.

These results strongly imply that the *glnN* product is the main contributor for growth under nitrogen-limiting conditions. Similarly, the *Synechocystis* strain PCC 6803, *glnN* is known to be strongly induced only under nitrogen-limiting conditions (Reyes *et al.*, 1997). The results obtained here are in agreement with previous published data showing up-regulation of GSIII, under nitrogen shortage, both transcriptionally (Abratt *et al.*, 1993) and at the protein level (Southern *et al.*, 1987). Interestingly, *B. fragilis* 638ΩglnN growth in conditions of ammonium sufficiency reveals that an unidentified gene is enabling glutamine biosynthesis and subsequently, the growth of the bacterium under these conditions.

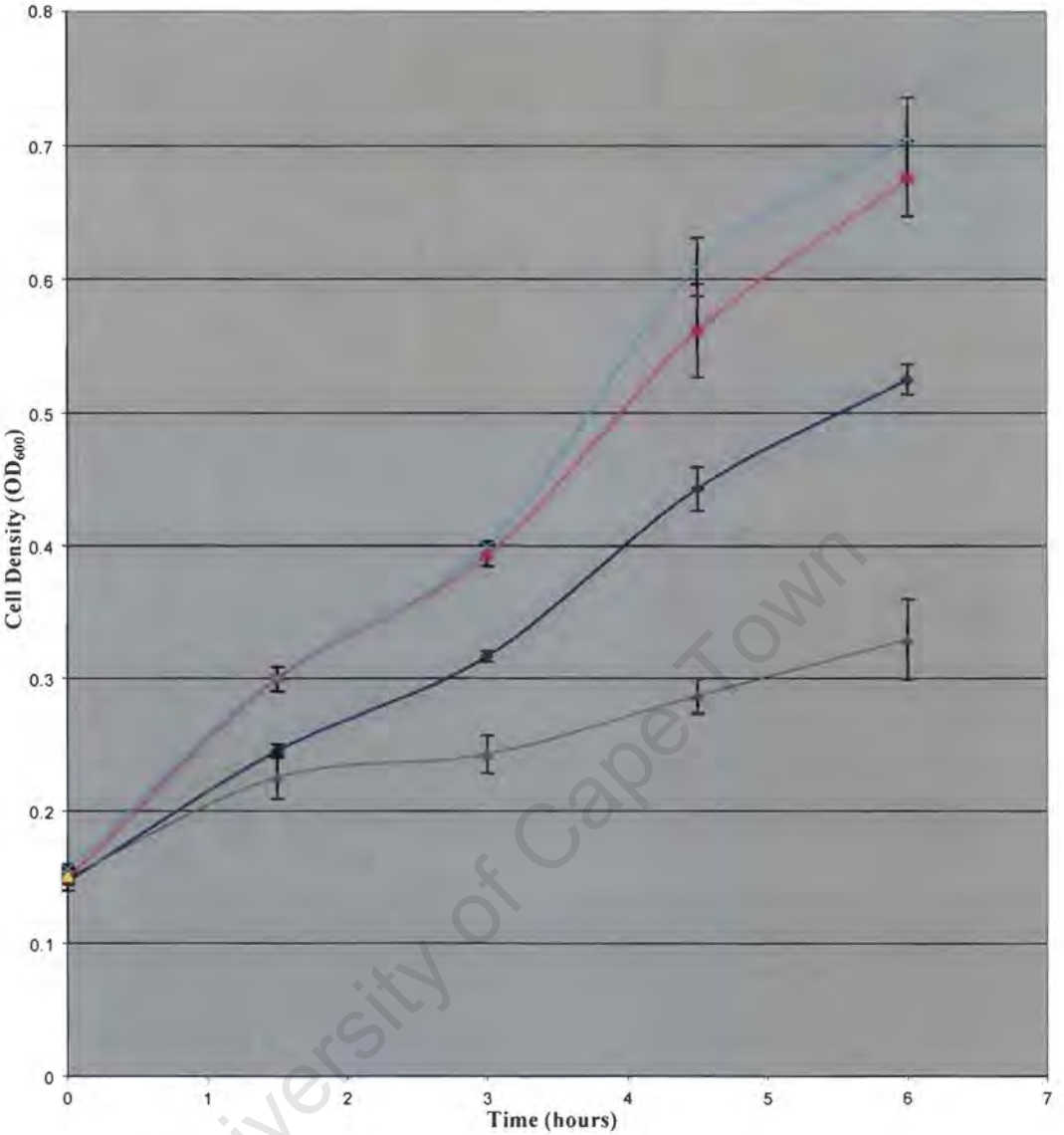

Fig. 2.5 Growth of *B. fragilis* 638ΩglnN in glucose minimal medium supplemented with 0.5 mM (low) and 50 mM (high) ammonium chloride as the sole nitrogen source. Cultures were incubated anaerobically at 37°C. (◆), *B. fragilis* 638R (0.5 mM); (X), *B. fragilis* 638R (50 mM); (▲), *B. fragilis* 638 ΩglnN (0.5mM) ; (■), *B. fragilis* 638 ΩglnN (50 mM).

2.3.3 Comparison of the GS enzymatic activity of *B. fragilis* 638R and *B. fragilis* 638ΩglnN

The glutamine synthetase forward and transferase assays were carried out using crude extracts from *B. fragilis* 638R and *B. fragilis* 638ΩglnN strains grown for 6 hours under high or low nitrogen conditions, in order to determine whether GSIII is the only glutamine synthetase present in *B. fragilis*. Figure 2.6 shows the commonly used GS assays with the related reactions.

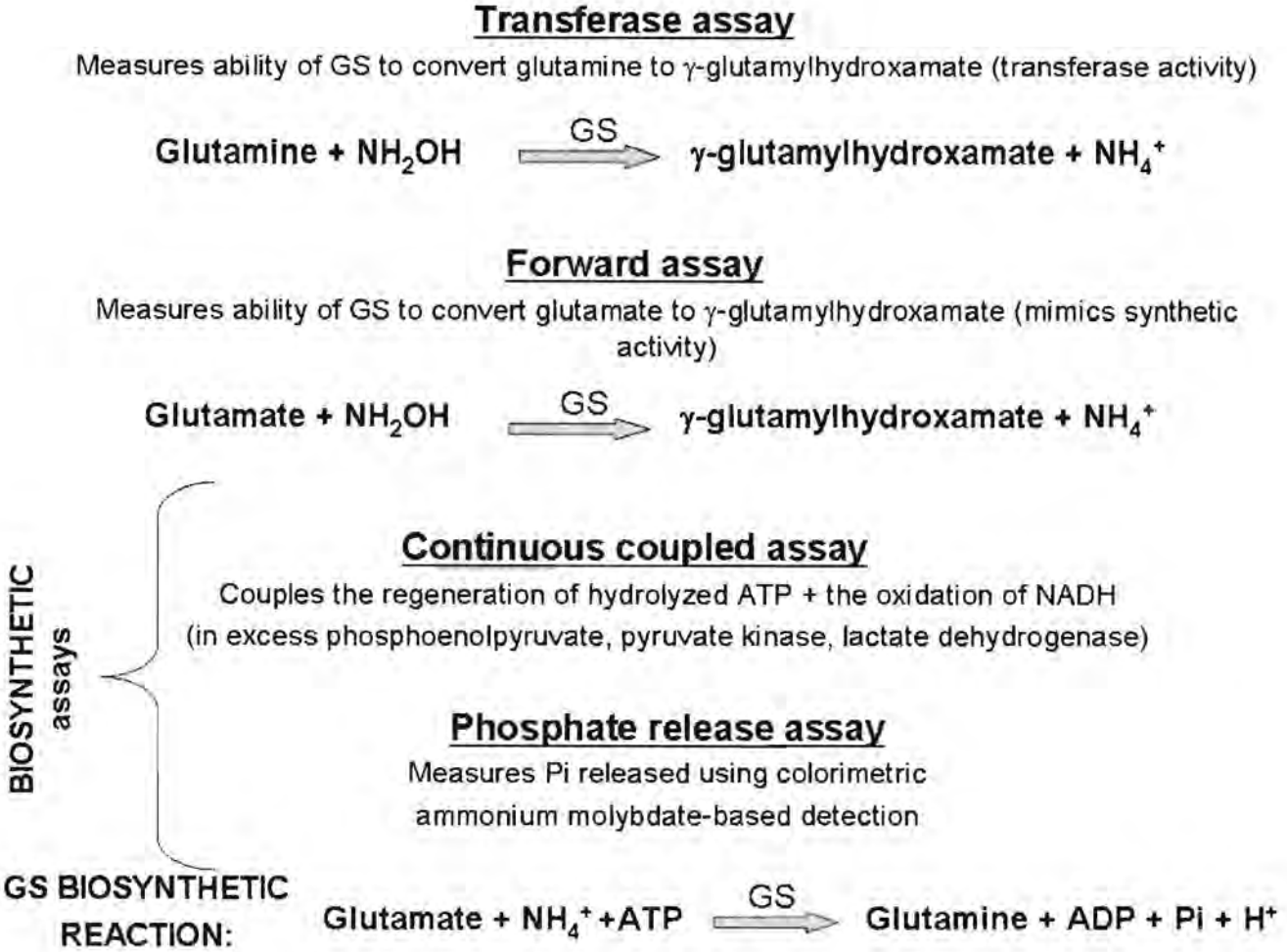

Fig. 2.6 Glutamine synthetase assays commonly used. Information compiled from Bender *et al.*, 1977; Robinson *et al.*, 2001; Amaya *et al.*, 2005 ; Gawronski and Benson, 2003 ; Shapiro *et al.*, 1967 ; Atkins *et al.*, 1993 and Webb, 1992.

The literature reveals that most bacterial GS enzymes are assayed with the forward and/or the transferase assays due to their accuracy, sensitivity and the relative ease of the assays. The forward and transferase assays were, therefore, used in this study. The assays were carried out on cells harvested after 6 hours and 24 hours of growth, with similar results. The transferase assay failed to give a detectable OD reading for both *B. fragilis* 638R and *B. fragilis* 638ΩglnN. Crude extract obtained from *E. coli* YMC11 (pJS139), which contains the *B. fragilis glnN* gene, was used as a positive control. The failure to detect GS activity in *B. fragilis* using this assay had previously been reported by Yamamoto *et al.* (1984). It is believed that an unidentified constituent of the cell extracts inhibits GS activity (Southern *et al.*, 1987).

The Forward assay, however, gave reproducible readings with the following trend: The highest GS activity was detectable in the wild-type strain grown under high ammonia conditions, which was two-fold higher than its growth under low ammonia (Fig. 2.7). The mutant had constant GS activity levels in all conditions, which was less than the level of the wild-type under low ammonia conditions (Fig 2.7). These results indicate that the inactivation of *glnN* in *B. fragilis* reduces the total amount of GS activity, however, there is some residual GS activity present. The results also indicate that although *glnN* is necessary for growth in nitrogen-limiting conditions, it also seems to contribute to a large portion of the total GS activity in *B. fragilis* when the bacterium is grown in nitrogen excess. Although the western blot results by Southern *et al.* (1987) showed high GS protein levels under low nitrogen, the protein may not necessarily be active.

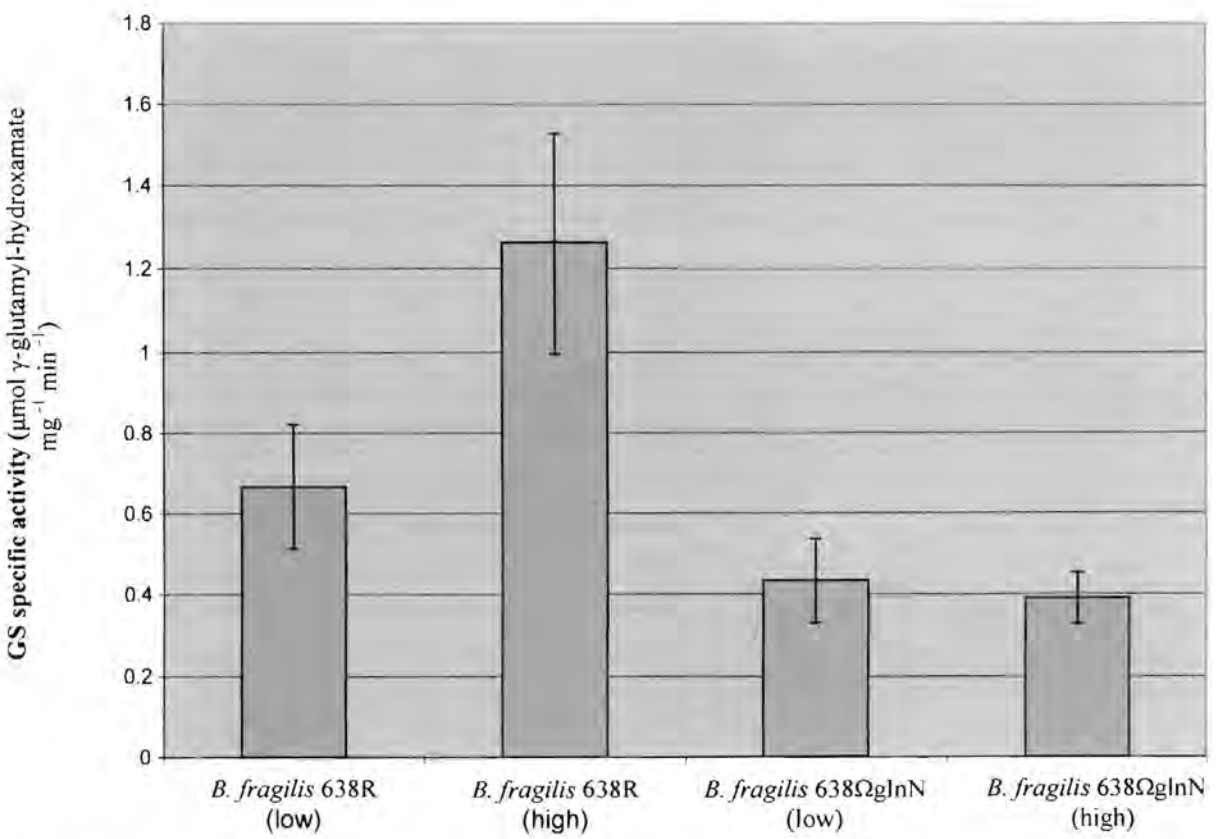

Fig 2.7 Glutamine synthetase activity of WT and *glnN* mutant. Activity was determined at 37°C using the forward assay. Cell free extract was obtained from cells grown for 6 hours in glucose minimal medium supplemented with either 0.5 mM (low) or 50 mM (high) ammonium chloride. These results are the means and standards deviations of at least three separate experiments.

2.4 Conclusions

The creation of *B. fragilis* 638ΩglnN is described here. This mutant was created with the aim of inactivating the functional *glnN* gene in order to establish if it is the only gene coding for glutamine synthetase in *B. fragilis.* Glutamine synthetase is a central enzyme in nitrogen metabolism, being the only enzyme able to produce glutamine. Since glutamine acts as the precursor for the production of many secondary metabolites, the lack of a functional GS-encoding gene generally results in a failure of the bacterium to proliferate under certain conditions, such as the growth of *Streptococcus thermophilus* (a lactic acid bacterium) in milk (Monnet *et al.*, 2005).

Inactivating *B. fragilis glnN* revealed a lack of growth in conditions of nitrogen shortage, as seen from the plates and growth curves (Fig. 2.4B & Fig. 2.5). However, in excess nitrogen, the mutant clearly displayed some growth although the growth was visibly reduced, compared to the wild-type (Fig. 2.4A & Fig. 2.5). Ammonium chloride was the nitrogen source used in these experiments because ammonia is the preferred nitrogen source for enteric bacteria (Merrick and Edwards, 1995). The growth in high ammonium conditions was interesting as it revealed that the mutant was able to synthesize glutamine in the absence of the *glnN* gene product.

Further characterization of *B. fragilis* 638ΩglnN with regards to GS enzymatic activity was also done. Because glutamine synthetase functions as one of the main enzymes for the assimilation of ammonia, its synthesis and activity is expected to be strictly regulated in response to the available environmental nitrogen. Southern *et al.* (1987) showed, using western blots on crude extract from *B. fragilis* grown under different nitrogen conditions, that GSIII production in *B. fragilis* was regulated by nitrogen. They specifically showed up-regulation under nitrogen-limiting conditions (Southern *et al.*, 1987). Likewise, the work reported here confirms that GSIII is vital for growth under nitrogen-limiting conditions, as observed by the lack of growth of *B. fragilis* 638ΩglnN under these conditions. However, higher total GS activity was observed, using the GS forward assay, when *B. fragilis* was grown in high nitrogen conditions (Fig 2.7). The fact that this elevated activity was absent in the mutant grown under the same conditions, led to the conclusion that GSIII contributes to the higher GS activity observed.

Initial efforts were made to harvest cells for the assays at the mid-exponential phase of growth, however, many factors made such an attempt extremely difficult. Some of the constraints were the relative slow growth of *B. fragilis*, the vast difference in growth between wild-type and mutant strains, as well as the difference in growth even within each strain in different concentrations of nitrogen. For this reason, the cells were harvested at two time points (6 hours and 24 hours), which gave the same GS activity pattern.

Future work should be directed at the creation of a complemented strain of *B. fragilis* 638ΩglnN, by cloning the full-length, functional *glnN* into *B. fragilis* 638ΩglnN. The phenotype of the complemented strain should be identical to the wild-type phenotype concerning growth and GS enzymic activity. Initial attempts were made at creating the *glnN* complemented strain of *B. fragilis* 638ΩglnN, however, time contraints did not allow for the successful completion of this strain. Additional future work could also be directed at analyzing relative *glnN* expression in the wild type, using real time quantitative PCR (RT-qPCR), which would help to detect the correlation between the increase in activity seen at the protein level and the expression of the gene. This would determine whether the observed changes in enzyme activity were due to transcriptional and/or post-translational regulation.

In addition, since the *B. fragilis* 638ΩglnN mutant was generated by introducing the pGERM suicide plasmid into the *B. fragilis* genome, the recovery of the original gene through a loss of the construct is a possibility. This mutant reversion may result in a mixed strain having residual GS activity. Selection using the plasmid-borne antibiotic resistance may not be helpful in the case where the pGERM vector may have randomly recombined elsewhere in the genome. Therefore, in order to monitor new colonies for the proper construct, PCR reactions using a primer external to the disrupted gene together with the M13 primer docked in the vector, should give a specific amplicon for the mutant and none for strains that have lost the construct. Similarly, colony PCR done with two primers flanking the gene of interest should give an amplicon the size of the gene for the wild-type and either no amplicons or a very large fragment (depending on the polymerase and PCR conditions used) where the construct is intact.

Alternatively, antibodies may be raised against the GS of interest which would allow localization of the protein in western blotting (immunoblotting). Antibodies may also be helpful in determining whether the residual activity observed belongs to this GS and not another enzyme, by testing the active fractions during GS purification.

The results presented at this juncture suggest that *B. fragilis* may possess an additional gene coding for a glutamine synthetase enzyme. With the use of the recently annotated genome of *B. fragilis,* a genomic analysis and search of the open reading frames (ORFs) of this organism was, therefore, conducted with the aim of finding a gene fitting the profile (Chapter3).

CHAPTER 3

IDENTIFICATION, CLONING AND CHARACTERIZATION OF A PUTATIVE *B. FRAGILIS* GLUTAMINE SYNTHETASE TYPE I GENE.

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CHAPTER 3

Identification, Cloning and Characterization of a putative *B. fragilis* Glutamine Synthetase type I gene.

3.1 Introduction

The recently annotated genome of *B. fragilis* has allowed for the rapid and efficient study of the genetic make-up of this opportunistic pathogen. The published genome of this organism made it possible to undertake a global search of the possible nitrogen metabolic genes in this organism, with an emphasis on genes that could be encoding glutamine synthetase enzymes. The objective of the search was to identify genes of interest which would then be subjected to bioinformatic analysis in order to determine their nucleotide sequences, homology to other genes, conserved domains, deduced protein sequences and predicted biochemical functions. The candidates of interest would be experimented on further, to unravel the exact biological function of these genes in the cellular context. These experiments would include, but not be limited to, complementary cloning of the genes for functional characterization.

A *glnN* gene, encoding a GSIII protein, had previously been identified in *B. fragilis* and its product well-characterised (Hill *et al.*, 1989, Southern *et al.*, 1987; van Rooyen, *et al.*, 2006). Inactivation of the *B. fragilis glnN,* reported in this work (Chapter 2), resulted in reduced ability of the *B. fragilis* mutant to grow in ammonium deficiency. However, this same mutant exhibited growth in excess ammonium chloride as the only nitrogen source, which led to the hypothesis that the presence of another GS-encoding gene in *B. fragilis* was responsible for glutamine biosynthesis and the growth of the bacterium under these conditions. Analysis of the *B. fragilis* genome sequence revealed an ORF, annotated as Bf2343, encoding a putative GSI enzyme. This putative gene may be a likely candidate coding for a second GS orthologue and it may help to further elucidate the mechanisms by which *B. fragilis* incorporates ammonia.

It is not unusual for a bacterium to carry more than one of the glutamine synthetase isoforms as can be observed in *Synechocystis* sp PCC6803 (Reyes *et al.*, 1994 and García-Domínguez *et al.*, 1997), *Ruminococcus albus* 8 (Amaya *et al.*, 2005) and *Streptomyces hygroscopicus* (Kumada *et al.*, 1990). The presence of multiple isoforms is usually to the advantage of the bacterium as it adapts to diverse environmental conditions, where resources can change

dramatically and certain nutrients can become scarce. In the above-mentioned *Synechocystis*, for example, *glnA* is constitutively expressed while *glnN* is only active under nitrogen deficiency (Reyes *et al.*, 1997). Another bacterium carrying many GS isoforms is *Mycobacterium tuberculosis*, which possesses a *glnA1* gene similar to *E. coli glnA* in its transcriptional and post-translational regulation, as well as *glnA2, glnA3* and *glnA4* (Harth *et al.*, 2005). It was also shown that the *glnA1* gene is required for virulence in *M. tuberculosis* (Tullius *et al.*, 2003).

The experimental approach that allowed for the discovery of *B. fragilis glnN* involved the cloning, expression and characterization of the gene in a heterologous *E. coli* system (Southern *et al.*, 1986). It was decided that a similar approach would be used in this study to investigate the functionality and metabolic contribution of the putative GSI-encoding gene in *B. fragilis*. The full length gene, including its upstream region to include any native promoter, was cloned onto a vector which includes an *E. coli* recognised promoter, to ensure expression of the gene. An *E. coli glnA*$^-$ mutant was used to test for functionality by the gene's ability to complement the glutamine auxotrophy, thereby allowing the *E. coli* strain to use ammonia as the sole nitrogen source and therefore grow on minimal medium. Glutamine synthetase assays were also performed to determine the activity of the cloned gene.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are described in Table 3.1.

Table 3.1 Bacterial strains and plasmids

Strain or plasmid	Description	Source/ reference
B. fragilis 638R	Em^s Gm^r	Privitera *et al.*, 1979
E. coli DH5α	*F' endA1 supE44 thi-1 hsdR17*(rK^- mK^+) *recA1 gyrA relA1 Δ(lacIZYA-argF)U169 deoR* (φ *80 dLacδ(lacZ)M15)*	Gibco-BRL
E. coli YMC10	*thi, endA, hsr, araD, strA, rhaD, ΔlacU169, hutC*$_{Klebs}$	Backman *et al.*, 1981
E. coli YMC11	*thi, endA, hsr, araD, strA, rhaD, ΔlacU169 hutC*$_{Klebs}$ *Δ(glnG-glnA)*	Backman *et al.*, 1981
pGEMT-Easy	TA cloning vector	Promega
pGEMT2343	PGEMT-Easy with full-length putative *glnA* gene	This study
pBluescriptSK$^+$ (pSK)	Ap^r	Stratagene
pJS139	(Ap^r, *glnA*$^+$) *B. fragilis glnN* gene in pEcoR251	Southern *et al.*, 1986

Abbreviations:
Klebs denotes a gene in *Klebsiella aerogenes*
TMP, trimethoprim; Ap, ampicillin; Em, Erythromycin; Gm, Gentamicin; s, sensitive; r, resistant

B. fragilis was grown as described in section 2.2.1. All *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) agar or broth (Sambrook *et al.*, 1989). When necessary, ampicillin (100 μg/ml) was added to the medium for selection. *E. coli* YMC11 was grown on CSH minimal medium (Davis *et al.*, 1980) containing 20 mM NH_4Cl as a nitrogen source. Different nitrogen conditions were created by the addition of 0.25 mM (low) or 50 mM (high) NH_4Cl to the media.

3.2.2 Bioinformatic analysis

The sequences of *B. fragilis* NCTC9343 (http://www.ncbi.nlm.nih.gov/) along with *B. fragilis* 638R assembly (http://www.sanger.ac.uk/Projects/B_fragilis/) were analysed. Open Reading Frames (ORFs) encoding putative glutamine synthetase enzymes were identified. Nucleotide and predicted amino acid sequences were determined and compared to those in the database using the NCBI Blast search (http://www.ncbi.nlm.nih.gov/BLAST) program. Multiple sequence alignments were done using the DNAssist software package (Version 2.2), for comparison to the GSIII orthologue and other biochemically characterised GS enzymes.

3.2.3 General recombinant DNA techniques

All DNA manipulations and molecular cloning techniques used are described in section 2.2.2.

3.2.4 PCR amplification, cloning and sequencing of the putative GSI encoding gene from *B. fragilis*

The *B. fragilis* 638R sequence lodged on the Sanger Centre database (http://www.sanger.ac.uk/Projects/B_fragilis) was used to design oligonucleotide primers specific to the gene encoded by ORF2343 (Table 3.2). The full length gene, together with 250 bp upstream and 258 bp downstream flanking regions, was amplified by PCR using primers BF2343F and BF2343R. PCR amplification was performed with *Taq* polymerase (1.5 U) in 50 µl reactions containing 25 pmol of each primer, 2 mM $MgCl_2$, 125 µM deoxynucleotide triphophates and 89 ng of DNA. Thirty amplification cycles were performed with an initial denaturation step at 95 °C for 5 minutes and a final elongation step at 72 °C for 5 minutes. Each cycle contained a denaturing (95 °C), annealing (54 °C) and extension (72 °C) step of 30, 45 and 60 seconds, respectively.

Table 3.2 Sequences of oligonucleotide primers

Primer	Sequence
BF2343F	5'-TCTACGAATCCGCGGAGTCTG-3'
BF2343R	5'-CGAAAGGAGAATTCCGTCACG-3'
LastR	5'-AATTTGTTCACCAGTGCCAGC-3'
ProF	5'-TAATGAGTCCCAACCGTTTGG-3'
ProR	5'-ATCCTCGGAAATTACATAATATTCC-3'
F27	5'-AGAGTTTGATCMTGGCTCAG-3'
R5	5'-ACGGMTACCTTGTTACGACTT-3'

The PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. This DNA fragment was then directly cloned into the pGEM-T Easy vector (Table 3.1), to create plasmid pGEMT2343, and transformed into competent *E.coli* DH5α cells (Sambrook *et al.*, 1989). Single white colonies were randomly chosen and subjected to colony PCR (Plourde-owobi *et al.*, 2005), using primers BF2343F and BF2343R (Table 3.2), to screen for plasmids containing the full-length gene insert. Plasmid was extracted from positive colonies and subjected to restriction enzyme analysis. Constructs giving the correct, predicted band pattern were sequenced. Nucleotide sequencing was performed using primers BF2343F, BF2343R and LastR, which is an internal primer to the gene (1.47 kb from the start codon). Sequencing was carried out using the Big Dye terminator v3.1 Cycle sequencing kit (Applied Biosystems) and the samples were run on a MegaBACE (MegaBACE 500; Amersham Biosciences), using the dideoxynucleotide chain termination chemistry (Sanger *et al.*, 1977). The Chromas sequence analysis package was used to assemble and analyse the sequences (www.technelysium.com.au/chromasPro.html). Multiple sequence alignment, sequence identity and analysis were performed with the DNAman software package (Version 4.13, Lynnon Biosoft, Canada).

3.2.5 Transformation and complementation of *E.coli* YMC11

E. coli YMC11 (Table 3.1) was transformed with pGEMT2343. Following growth on LB agar with ampicillin, these transformants were tested for the plasmid's ability to complement the glutamine auxotrophy of *E. coli* YMC11 by plating these colonies onto CSH minimal medium with 0.25 mM or 50 mM NH_4Cl. *E. coli* YMC11 (pJS139) was used as a positive control while *E. coli* YMC11(pSK) was used as negative control.

3.2.6 Transcriptional expression of *glnA*.

Total RNA was extracted, using the method of Aiba *et al.* (1981), from mid-exponential phase cultures of *E.coli* YMC11 (pGEMT2343) grown in glucose minimal medium with differing ammonium chloride concentrations. RNA extracted from *E. coli* YMC11 transformed with pSK was used as a control. Total RNA was also extracted from *B. fragilis*

638R and *B. fragilis* 638ΩglnN grown for 6 hours in glucose minimal medium with 0.25 mM (low) or 50 mM (high) ammonium chloride, as the sole nitrogen source. The RNA samples were checked for integrity on 1.5 % denaturing agarose gel containing 5 % formaldehyde and quantified by spectrophotometric methods. Dot blot analysis was then performed as follows: 3 µg of total RNA in a 3 µl volume was spotted onto a nylon membrane (Hybond N^+; Amersham). The membrane was crosslinked using UV light at 100 µJ/cm^2 with a Hoefer UVC500 Crosslinker (Amersham Biosciences). A DNA probe was prepared by amplification of an internal fragment of ORF2343 using specific primers ProF and ProR (Table 3.2). This probe was labelled by the DNA random priming digoxigenin (DIG) method (Roche). The *E. coli* and *B. fragilis* 16S rRNA gene probes were generated using the universal primers F27 and R5 (http://www.mcb.uct.ac.za/Sequencing%20Service%20web/index_files/Page903.htm) and labelled as described. The membranes were hybridized with probes at 50°C overnight in the Dig Easy Hyb solution (Roche), followed by washes at 68°C, and chemiluminescent detection was done by exposure to X-ray films.

3.2.7 Preparation of cell-free extracts

E. coli YMC11 (pGEMT2343) was grown at 37°C, with aeration, in LB broth. The cells were grown until late exponential phase, harvested by centrifugation at 13 000 x g in a Beckman J2 – 21 centrifuge for 20 minutes at 4°C. The cell pellets were resuspended in 12.5 mM imidazole buffer. The resuspended cells were disrupted by sonication on ice, with a Virsonic 50 (Virtis, USA), using pulsed cycles of 30 seconds with 20 second intervals between the cycles, for a total time of 4 minutes. Cell debris was removed by centrifugation at 8000 x *g* for 10 min. The amount of total protein in the cell-free extract was determined using a commercially available diagnostic reagent based on Bradford's standard assay (Bio-Rad protein assay; Bradford, 1976).

3.2.8 Glutamine synthetase assays

The glutamine synthetase forward and transferase assays were done as described in section 2.2.8.

3.3 Results and Discussion

3.3.1 Identification and sequence analysis of a putative glutamine synthetase-encoding gene in *B. fragilis*

Analysis of the *B. fragilis* genome revealed an ORF, annotated as locus BF2343 (GenBank accession no. YP_211965), encoding a putative glutamine synthetase enzyme. This gene sequence was 1502 bp long with 501 deduced amino acid residues. A sequence resembling the unique consensus promoter structure of many *Bacteroides* genes (Bayley *et al.*, 2000) was identified as **TA**TC**TTTG** at the -7 region but no **TTTG** motif was identified -33 relative to the transcription initiation site. The sequence also showed that the gene had conserved glutamine synthetase beta-grasp and catalytic domains (Fig. 3.1). Database search revealed that this ORF shared a high level of identity (88%) with *Bacteroides thetaiotaomicron* VPI-5482 putative glutamine synthetase I, at the amino acid level, however, this gene has not yet been functionally characterized. In contrast, an amino acid alignment to biochemically characterized glutamine synthetases gave only 27.27 % identity to *Synechocystis sp.* PCC 6803 GSI and 20.06 % to the GSI belonging to *Ruminococcus albus* 8. Analysis of the flanking ORFs revealed a conserved putative DNA binding hypothetical protein directly upstream of *B. fragilis* Bf2343 and a putative fosmidomycin resistance protein downstream. Neither of these ORFs seems to be linked to nitrogen metabolism.


```
1       MMNQELLMSP NRLVTFLQKP AAEFTKADII NYIQQNEIRM VNFMYPAADG RLKTLNFVIN
61      NASYLDAILT CGERVDGSSL FPFIEAGSSD LYVIPRFRTA FVDPFAEIPT LVMLCSFFNK
121     DGEPLESSPE YTLHKACKAF TDVTGMEFQA MGELEYYVIS EDDGLFPATD QRGYHESGPY
181     AKFNDFRTQC MSYIAQTGGQ IKYGHSEVGN FMLDGKVYEQ NEIEFLPVNA ENAADQLMIA
241     KWVIRNLAYQ YGYDITFAPK ITVGKAGSGL HIHMRMMKDG QNQMLKDGVL SDTARKAIAG
301     MMQLAPSITA FGNTNPTSYF RLVPHQEAPT NVCWGDRNRS VLVRVPLGWS AQTDMCALAN
361     PLESDSNYDT TQKQTVEMRS PDGSANLYQL LAGLAVACRH GFEIENALAI AEQTYVNVNI
421     HQKENADKLK ALAQLPDSCA ASADCLQKQR TVFEQYNVFS PAMIDGIISR LRSYNDATLR
481     KDIQDKPEEM LALVNKFFHC G*
```

Fig 3.1 Deduced amino acid sequence of locus Bf2343, showing the position and length of the C-terminal catalytic domain (Gln-synt_N in blue) and the N-terminal beta-grasp domain (Gln-synt_C in red). Accession number: YP_211965 (picture from NCBI website, query sequence: gi|60681821|ref|YP_211965.1|)

Comparison of the deduced amino acid sequence of the putative GSI with the amino acid sequences of the GSI enzymes belonging to *Salmonella typhimurium* and *Escherichia coli* revealed low identities- 28.5% and 29% ID to *S. typhimurium* and *E. coli*, respectively. However, the five highly conserved regions of GS enzymes, corresponding to the critical α/β barrel fold that forms the active site, showed significant conservation of regions 1, 3 and 4 but not regions 2 and 5 in the putative GSI (Fig. 3.2). The conserved regions 1, 3 and 4 represent the N-terminal latch, the ATP-binding domain and the glutamate binding domain, respectively (van Rooyen *et al.*, 2006). In contrast, regions 2 and 5 are not very well conserved in the putative GSI of *B. fragilis*. It is believed that region 2 may contribute to the second site for ammonium binding while region 5 appears to participate in hydrophobic stacking of residues (van Rooyen *et al.*, 2006). The tryptophan residue of highly-conserved region I, involved in completing the active site formed between adjacent subunits, tends to be substituted for phenylalanine in the GS belonging to Gram-positive bacteria such as *Streptomyces coelicolor, Mycobacterium tuberculosis, Corynebacterium glutamicum* and others (Peng *et al.*, 2006). Interestingly, the putative GSI of *B. fragilis* has a phenylalanine residue instead of tryptophan in that position (Fig. 3.2). This observation may be significant in relation to the evolution of *B. fragilis*.

It was also observed that the two histidine residues at positions 4 and 12 in *E. coli* are not conserved in the *B. fragilis* putative GSI (Fig. 3.2). These histidine residues correspond to distinct metal binding sites in the *E. coli* GS, which are responsible for the "stacking" effect of this GS leading to their forming hollow tubes in the presence of divalent metal ions (Schurke *et al.*, 1999). There is, therefore, no reason to believe that this putative GSI in *B. fragilis* would exhibit the same phenotype. Similarly, the tyrosine residue (Tyr-397) which is the site of adenylylation of *E. coli* GS is not conserved in the putative GSI of *B. fragilis*, neither are the amino acids flanking this region (Fig. 3.2). Since this region is highly conserved in all GS enzymes which undergo regulation via adenylylation, it may be deduced that the putative GSI of *B. fragilis* is probably not regulated by adenylylation.

In addition to the five conserved GS regions, Crespo *et al.* (1998) found 4 regions which are conserved in all GSIII enzymes. Van Rooyen *et al.* (2006) further showed that two of these regions were also conserved in every GS families. Of the two regions, only one seemed to

be conserved in the putative GSI of *B. fragilis,* the region corresponding to *S. typhimurium* residues 317-335 (Fig. 3.2). Some key features of this region are the E327 flap and R321 responsible for the coordination of the carboxylate group of glutamate (Eisenberg *et al.*, 2000).

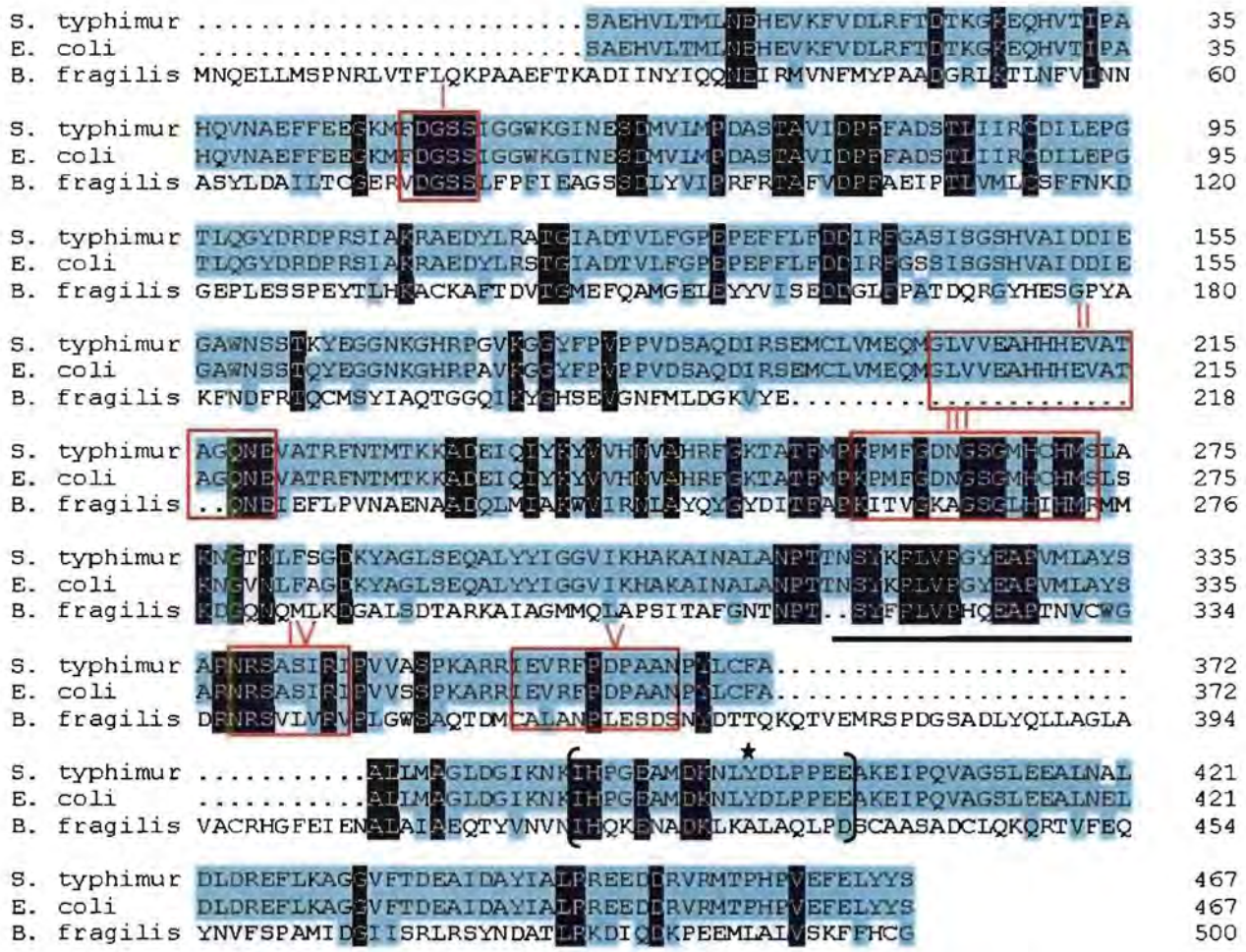

Fig. 3.2 Alignment of the amino acid sequences of GSI proteins from *S. typhimurium* (P06201), *E. coli* K12 (P0A9C5) and *B. fragilis* (YP_211965). Accession numbers in parentheses. Similar amino acids are highlighted in blue while amino acids which are identical in all three sequences are highlighted in black. Areas boxed in red and numbered with roman numerals represent the five conserved GS regions which make up the active site. Area in brackets marks the amino acids flanking the adenylylation site (Tyr-397 loop) depicted by a star. The additional conserved region (residues 317-335) is underligned.

3.3.2 Isolation, cloning and characterisation of putative *B. fragilis glnA* in *E.coli*

The putative *glnA,* including 250bp and 258bp upstream and downstream sequences, respectively, was isolated by PCR from *B. fragilis* 638R genomic DNA. The 2014 bp PCR amplicons were cloned into the pGEMT-Easy vector, transformed into *E. coli* DH5α and

colony PCR was done on white clones to confirm the correct-size insert (Fig. 3.3). Positive recombinant clones were selected and the plasmids extracted were subsequently confirmed for the presence and orientation of the inserted gene fragment by various restriction endonuclease digestions (Fig. 3.4). The correct construct was then sequenced with primers Bf2343F, Bf2343R and LastR (Table 3.2). Primer Bf2343F resides upstream of the gene while BF2343R lies downstream of the gene. An additional primer, LastR, was designed internal to the gene (1.47 kb from start codon) in order to sequence the middle section of the gene. No PCR error was identified from the sequences, when compared to the published sequence of this gene from the Sanger database (http://www.sanger.ac.uk/Projects/B_fragilis/).

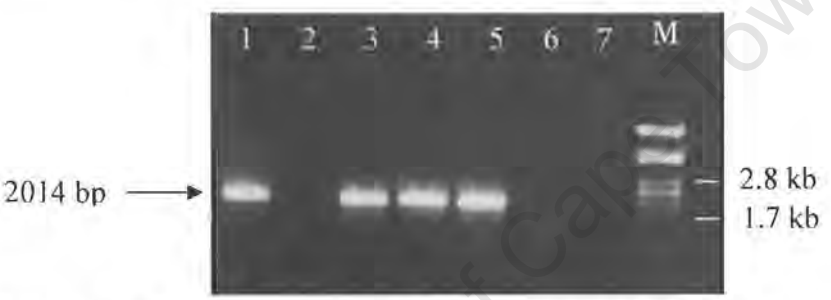

Fig. 3.3 Colony PCR of the putative *glnA*. Amplification of the full length *glnA* gene (+ flanking regions) overexpressed in several different *E. coli* DH5α colonies. Lanes 1, 3, 4 and 5 are positive for the gene insert. Lanes 2 and 6 are negative. Lane 7 = negative control (PCR mix with no DNA). M= λDNA digested with *Pst*I.

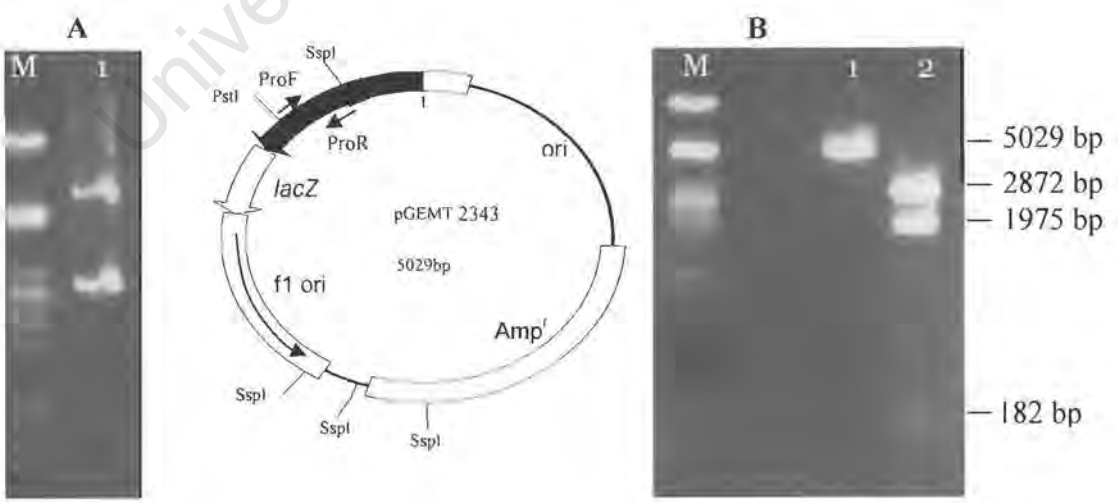

Fig 3.4 Restriction analysis of pGEMT2343. Conventional agarose (0.8%) gel electrophoresis of (**A**) Undigested plasmid DNA construct pGEMT2343 and (**B**) the restriction enzyme digest of pGEMT2343 with *Pst*I (lane1) and *Ssp*I (lane 2). M= λDNA digested with *Pst*I. Arrows indicate position of primers, ProF and ProR, used to generate internal fragment of *glnA* gene for transcription studies. (■), *glnA*.

To confirm that the pGEMT2343 clone encoded a functional glutamine synthetase gene, its ability to complement a *glnA*⁻ mutant strain of *E. coli* (*E. coli* YMC11) which is defective in the glutamine synthetase structural gene and regulatory regions, was investigated. *E. coli* YMC11 transformed with pGEMT2343 was plated on minimal medium with ammonia as the sole nitrogen source. *E. coli* YMC11 (pGEMT2343) failed to grow on this medium indicating a failure to complement the GS deficiency and thus restore glutamine production in the *E. coli* mutant (Fig. 3.5). Using high (50 mM) or low (0.25 mM) concentrations of NH_4Cl did not alter the viability of YMC11 (pGEMT2343). *E. coli* YMC11 (pJS139), carrying the *B. fragilis* functional GSIII, was used as a positive control. This strain grew better on minimal medium supplemented with 50 mM than 0.25mM NH_4Cl (Fig 3.5A and B).

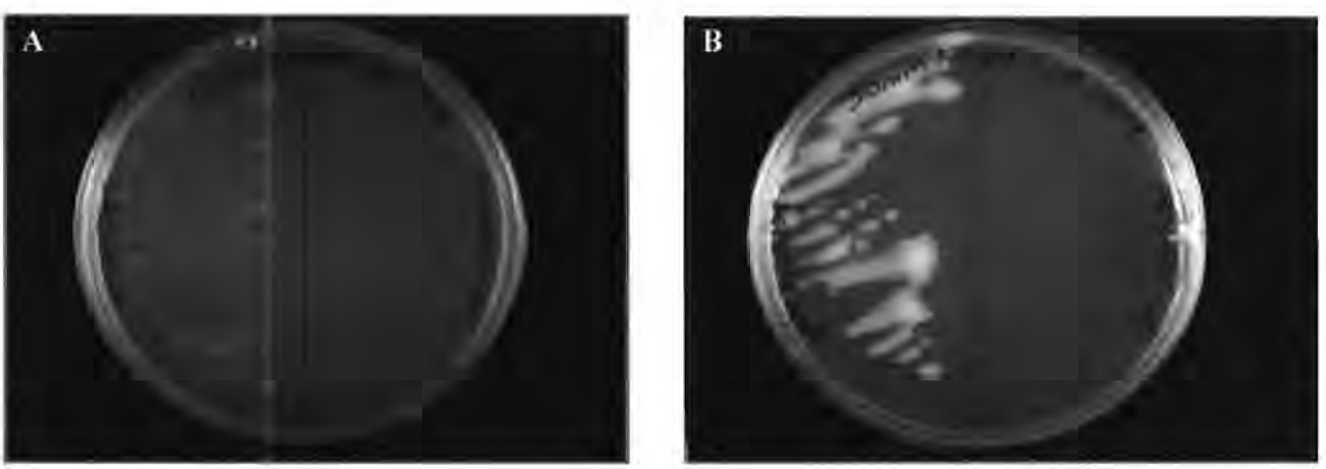

Fig. 3.5 Growth of *E.coli* clones on minimal medium containing ammonia. *E. coli* YMC11 (pGEMT2343) [cloned putative GSI] on right hand side of plates; *E. coli* YMCII (pJS139) on left hand side (**A**) plated on minimal medium with 0.25 mM NH_4Cl and Amp. (**B**) plated on minimal medium with 50 mM NH_4Cl with Amp. *E. coli* YMC11 (pJS139) was used as a positive control.

3.3.3 Transcriptional expression of the cloned putative *glnA* in *E. coli* YMC11

In order to determine whether the cloned putative GSI was being transcribed in *E. coli* YMC11, equal concentrations of total RNA were extracted from the experimental and control strains (Fig. 3.6A), and dot blot analysis was performed using a 422-bp internal fragment of the gene as probe (Fig 3.4). The presence of a transcript was evident in the *E. coli* recombinant containing the cloned gene, while *E. coli* YMC11 transformed with the vector pSK showed no signal (negative control) (Fig. 3.6B). An internal control using the

E. coli 16S rRNA gene probe was used to verify that equal amounts of total RNA had been loaded. As previously mentioned, unique promoter recognition sequences have been found in *Bacteroides*, however, bacteroides genes are usually expressed in *E. coli* due to the presence of fortuitous *E. coli* promoters (Bayley *et al.*, 2000). The pGEMT-Easy vector used in this cloning experiment has a multiple cloning site which is flanked by promoters recognised by SP6 and T7 RNA polymerases. Although 250 bp of the upstream flanking region of this gene was cloned with it to include the promoter, the gene was also cloned in the same orientation as the *lacZ* gene so that transcription could occur from the SP6 promoter in the vector.

When designing primers for the probe, a region in the putative GSI sequence was chosen that had low identity to the *B. fragilis* GSIII (39 % in non-continuous bases). Cross-hybridization of the probe to GSIII (pJS139) was, nonetheless, observed in *E. coli* (pJS139) RNA (Fig. 3.6B, lane 1). However, no hybridization to *E. coli* RNA alone was seen. The results obtained in lane 3, therefore, could confidently be ascribed to the presence of the *glnA* transcript in *E. coli* (pGEMT2343). For quantitative experiments where background signals from cross-hybridization of the probe need to be minimized, northern blots may be more advantageous due to the ability to identify the transcript sizes.

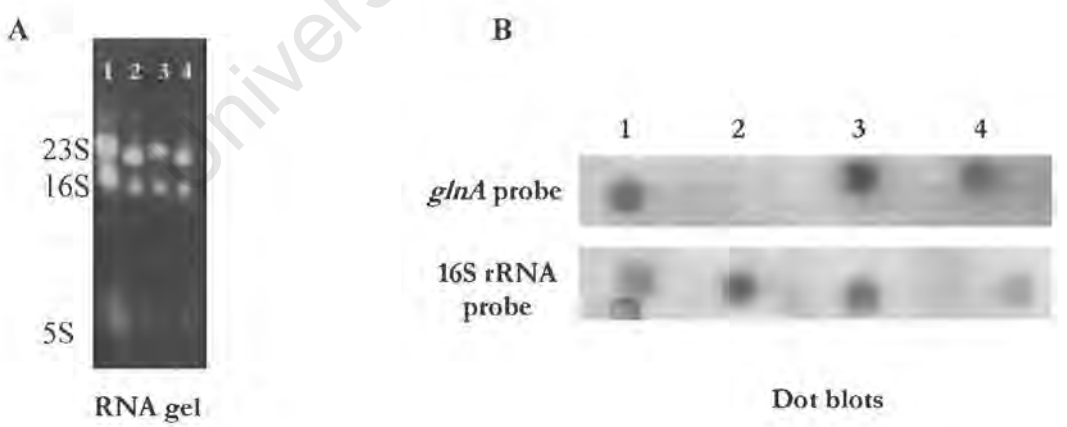

Fig. 3.6 Transcriptional analysis of Bf2343 in *E. coli* YMC11. A; Agarose gel confirming the concentration and integrity of total RNA extracted. B; RNA Dot-blot hybridisation of *E. coli* clones against the *B. fragilis glnA* gene probe or the *E. coli* 16S rRNA gene probe. 1 = *E. coli* YMC11 (pJS139) Positive control, 2 = *E. coli* YMC11 Negative control, 3 = *E. coli* YMC11 (pGEMT2343) in 0.25 mM ammonium, 4 = *E. coli* YMC11 (pGEMT2343) 50 mM ammonium.

3.3.4 Transcription of *glnA* in *B. fragilis* 638ΩglnN.

Transcriptional studies were also conducted in the wild-type *B. fragilis* and in the *B. fragilis glnN* mutant created as described in Chapter 2. This was done in order to determine whether the transcription of the putative *glnA* gene was regulated by the levels of ammonia present in the growth media, or by the presence or absence of a functional *glnN* gene product.

B. fragilis 638R WT and *B. fragilis* 638ΩglnN strains were grown to mid-logarithmic phase, in minimal medium containing different nitrogen concentrations. Total RNA was isolated as described in section 3.2.6. RNA concentrations were measured and to ensure the quality and quantity of the RNA, 10 μg of each sample was electrophoresed in a 1.5% formaldehyde-agarose gel, stained with ethidum bromide and visualized on a UV transilluminator (Fig 3.7A). RNA was hybridized to nitrocellulose membrane, as described in section 3.2.6, and probed with a *glnA* probe (Fig 3.7B). No difference in *glnA* RNA levels could be detected between *B. fragilis* 638R and the *glnN* mutant in either low or high ammonia conditions. This suggests that the BF2343 gene product is not regulated by ammonia levels under the conditions tested and that it is not affected by *glnN* inactivation.

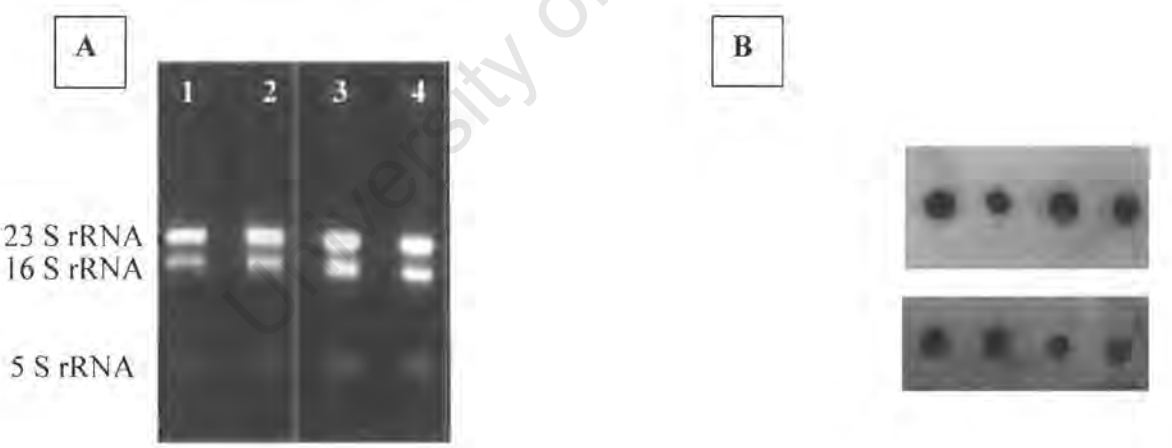

Fig 3.7 RNA Dot blot hybridization. RNA from *B. fragilis* 638R and the *glnN* mutant grown in 0.5 mM (low) and 50 mM (high) NH_4Cl was isolated. (A) Total RNA (10 μg) was run on a denaturing agarose gel. (B) The same amount of RNA was blotted on nitrocellulose paper and hybridized with the respective probes. (1), WT in low NH_4Cl; (2), WT in high NH_4Cl; (3), *glnN* mutant in low NH_4Cl; (4), *glnN* mutant in high NH_4Cl. The *B. fragilis* 16S rRNA gene product was used as an internal control. The *glnA* probe was generated by DIG-labeling an internal fragment of *glnA*. WT, wild-type.

3.3.5 Enzymatic activity of the cloned putative *glnA*

To establish if the transcribed recombinant putative GSI gene had any activity in *E. coli* YMC11, the GS transferase and forward assays were carried out, as detailed in section 2.2.8. The transferase assay revealed no detectable GS activity from the recombinant putative GSI (Fig. 3.8). The forward assay registered very little activity from the same recombinant protein (Fig. 3.8). *E. coli* YMC11 (pJS139) carrying the *glnN* gene showed significant activity as determined by both assays.

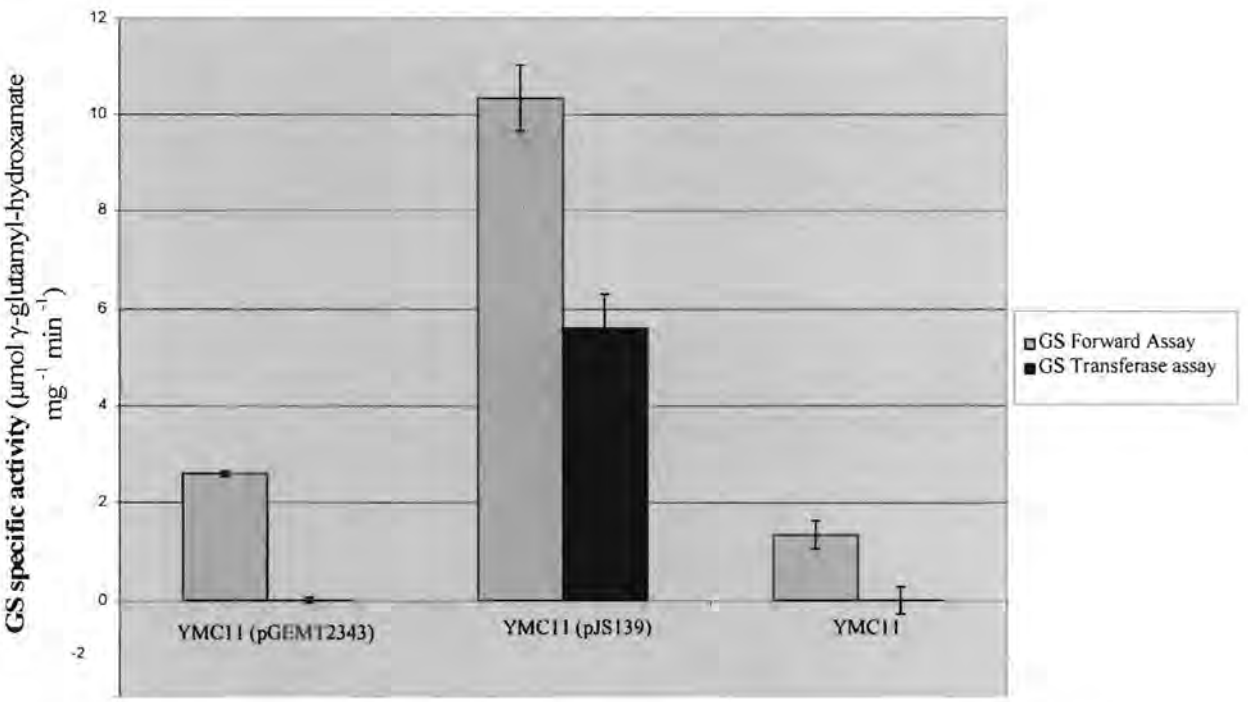

Fig. 3.8 Comparison of the activities of *E. coli* YMC11 (pJS139) [GSIII] and *E. coli* YMC11 (pGEMT2343) [GSI] using the transferase assay (conversion of α-glutamine to γ-glutamylhydroxamate) and the GS Forward assay (conversion of α-glutamate to γ-glutamylhydroxamate). The results are the means and standards deviations of at least three separate experiments.

Failure of the cloned Bf2343 to complement GS deficiency in *E. coli* and to show activity in the enzyme assays indicated that, although the putative *glnA* gene was transcribed in *E. coli*, it either does not encode a GSI enzyme, or it may be producing a non-functional protein which may need to undergo post-translational modifications in order to be functional.

3.4 Conclusions

A search of the *B. fragilis* genome was undertaken to identify potential genes encoding glutamine synthetase enzymes. This search was done in order to identify the gene whose enzyme is responsible for the growth and GS activity observed in the *B. fragilis glnN* mutant constructed and characterized in this work (Chapter 2). The discovery of an additional gene coding for a glutamine synthetase enzyme in *B. fragilis* would also be of interest because of the implications to the overall understanding of the nitrogen metabolism of *B. fragilis*.

An ORF (Bf2343) coding for a putative GSI enzyme was identified, however, an amino acid alignment of this gene to other characterized GSI genes revealed little homology. For example, the putative *B. fragilis* GSI showed only 20 % identity to *Ruminococcus albus* 8 GSI and 27 % to the GSI of *Synechocystis sp.* PCC 6803 at the amino acid level. When BF2343 was overexpressed in *E. coli*, it was unable to complement glutamine synthesis in the *E. coli* auxotrophic mutant. Amaya *et al.* (2005) similarly showed that, of the two GS present in *Ruminococcus albus 8,* GSIII is active in *E. coli* but GSI, although transcribed, was not active. In addition, the GS assays in their study failed to show any significant activity conferred by the cloned GSI. Similarly, the cloned *B. fragilis* putative *glnA* failed to show GS activity.

The lack of activity combined with the failure of the gene to complement glutamine biosynthesis under high or low ammonium, demonstrated that this gene lacked significant functionality in *E. coli*. Due to the complex nature of proteins, glutamine synthetase being no exception, the results obtained in the heterologous *E. coli* cannot be interpreted as indicating that Bf2343 does not function as a GS in *B. fragilis*. *E. coli* YMC11 was used to express the putative *glnA*, specifically because this strain has a deletion in its own *glnA* gene. However, the genes expressing the regulatory components *ntrB* and *ntrC* are also deleted in *E. coli* YMC11. The *E. coli* GS is known to be regulated post-translationally by the reversible covalent addition of AMP to each subunit of GS, a process also known as adenylylation (Maheswaran and Forchhammer, 2003). The components of the *ntr* genes linked to this post-translational modification are the uridylyl- transferase/removing enzyme (UTase/UR) and the *glnB* gene product: a small signal transducing protein called P_{II} which

senses the carbon and nitrogen status of the cell (Forchhammer and Tandeau de Marsac, 1995). In *B. fragilis,* however, none of the *ntr* genes seem to be present and there is no evidence of post-translational modification via adenylylation (Yamamoto *et al.,* 1984 & 1987; Southern *et al.,* 1986 & 1987).

In addition, it was shown in the bioinformatic analysis of Bf2343 conducted in this work, that the tyrosine residue responsible for adenylylation was not conserved in the putative *glnA* gene (Fig. 3.2). This suggested that adenylylation is not likely to be the mechanism by which the protein is regulated. Nevertheless, there are numerous and complex nitrogen regulatory systems existing and although no system has yet been described in *Bacteroides*, the possibility of one being present cannot be ruled out. Therefore, despite the care taken to express this gene from an *E.coli*-recognised promoter, the translated protein may still require some external trigger such as a unique cofactor or some form of post-translational modification in order to be fully active. This could be one reason for the lack of functionality seen in this putative GSI enzyme.

Another possibility for the lack of functionality seen in the *glnA* of *B. fragilis* may be due to the use of the wrong substrates. Ammonium chloride was chosen as the nitrogen source in these experiments because ammonia was proven to be the preferred source of nitrogen for enteric bacteria as it sustains a higher bacterial growth rate than any other nitrogen source, due to its reduced form and therefore, rapid assimilation (Merrick and Edwards, 1995). Therefore, experimenting with different nitrogen sources may reveal that the putative GSI enzyme of *B. fragilis* might be active in a different nitrogen source.

A further possibility for the lack of functionality of this enzyme may be related to the low homology to other GS enzymes and the lack of preservation of the highly-conserved regions 2 and 5 as deduced from the amino acid alignment of the putative GSI to other characterized GSI enzymes (Fig 3.3). If this is the case, it may simply mean that this enzyme is not functional at all and that it is merely a vestigial gene from some evolutionary ancestor.

It was previously observed that *B. fragilis* 638ΩglnN (Chapter 2), the mutant defective in *glnN*, still had residual GS activity when grown in ammonia (Fig 2.7). The above-mentioned possible reasons for the apparent lack of functionality of this putative *glnA*, result in an

inability to completely eliminate this gene as the candidate responsible for the GS activity observed. Therefore, further experiments need to be conducted to derive more conclusive answers. One suggestion would be the inactivation of Bf2343 in *B. fragilis*, as well as the creation of a *glnA*$^{-}$ *glnN*$^{-}$ double mutant. A Bf2343 mutant would help in determining whether the residual GS activity can be attributed to the gene product of this ORF. The double mutant would reveal the presence of other GS encoding genes if the mutant can grow in minimal medium with ammonium as the only nitrogen source, as this would imply that some other GS enzyme is allowing for glutamine biosynthesis. In addition, the search for other GS candidates can be conducted using proteomics or micro-array techniques by selecting for nitrogen responsive proteins or genes, respectively.

The quest to elucidate the types, functions and capacities of glutamine synthetase genes and their relevant enzymes in *B. fragilis* would not be complete without an understanding of the regulation of nitrogen metabolism in general and of these enzymes in particular. The fact that no regulatory system has yet been described for this organism made this endeavor even more significant. The work described in the following chapter attempted to shed some light on a possible nitrogen regulatory mechanism in *B. fragilis*.

CHAPTER 4

IDENTIFICATION OF A PUTATIVE NITROGEN REGULATOR IN *B. FRAGILIS* 638R

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CHAPTER 4

Identification of a putative nitrogen regulator in *B. fragilis* 638R

4.1 Introduction

Nitrogen metabolism in prokaryotes is a complex process involving the incorporation of available nitrogen from the environment as well as the production of nitrogen-containing compounds (Merrick and Edwards, 1995). It is no surprise then, that the regulation of glutamine synthetase (one of the main enzymes in ammonia incorporation) is highly specialized and well characterized in most organisms:

In most enterobacteria, for example, GS regulation takes place through the concerted action of transcriptional regulation from multiple promoters (Merrick and Edwards, 1995) and an adenylylation-deadenylylation system to alter the enzyme activity (Stock *et al.*, 1989). Sensing of external nitrogen, in these species, usually occurs through the internal shortage of glutamine (Jiang *et al.*, 1998). In addition, the adenylylation-deadenylylation involves a trimeric PII-like signal protein and includes cumulative feedback inhibition by seven different glutamine metabolism end products (Stadtman, 2001).

In *Bacillus subtilis,* a gram-positive, spore-forming bacterium, GS is regulated by the products of *glnR* and *ntrA* (Hu *et al.*, 1999). Activation of GS transcription in *B. subtilis* occurs through the action of TnrA only during nitrogen limitation while repression of GS transcription by GlnR takes place in excess nitrogen (Fisher, 1999). Unlike the regulation of *E. coli* GS activity by adenylylation, *B. subtilis* GS is subject to feedback inhibition, which regulates the activities of GlnR and TnrA (Fisher and Wray, 2006).

In *cyanobacterium* spp, such as *Anabaena* sp. strain PCC 7120 and *Synechococcus* sp. strain 7942, regulation of GS occurs via selective transcription from different promoters which are regulated based on the nitrogen composition of the growth media (Valladares *et al.*, 2004). In these cyanobacteria, sensing of nitrogen occurs by assessing the internal 2-oxoglutarate level (Muro-Pastor *et al.*, 2001). *Cyanobacterium* spp. possess a global nitrogen regulatory transcription factor named NtcA, belonging to the CAP (catabolite activator protein)- family of bacterial transcriptional regulators. The Crp-Fnr regulators, of which NtcA is a member,

are named after the first members of this family identified in *E. coli*: the cyclic AMP receptor protein-CRP or catabolite activator protein-CAP and the fumarate and nitrate reductase regulator-Fnr (Korner *et al.*, 2003). In *Synechococcus*, 2-oxoglutarate levels, the cellular signal indicative of the level of ammonium present, are signaled to NtcA which then regulates expression of nitrogen-dependent genes, such as *glnA* (Tanigawa *et al.*, 2002).

NtcA, which is a DNA binding protein, directly binds to the promoters of nitrogen regulated genes at the palindromic sequence $GTAN_8TAC$ (Luque *et al.*, 1994). When ammonium is absent, NtcA induces the transcription of genes which are required for the assimilation of alternative sources of nitrogen (Su *et al.*, 2005). It is interesting that in *Synechocystis sp.* PCC 6803, although *glnN* is regulated by the nitrogen levels and an NtcA binding site has been identified upstream of this gene, yet mobility shift assays have shown that *E. coli*-expressed NtcA does not binds to this site (Reyes *et al.*, 1997). In *Pseudanabaena sp.* PCC 6903, another *cyanobacterium* spp., NtcA does bind to the promoter region of *glnN* (Crespo *et al.*, 1998). It is suggested that this is due to the lack of a *glnA* gene in this bacterium, in other words, it is an adaptation that enables NtcA to control the only available GS structural gene (Crespo *et al.*, 1998).

In *Synechococcus* PCC 7942, *glnN* is indirectly regulated by NtcA, via a regulatory factor called PipX (Espinosa *et al.*, 2006). Under conditions of nitrogen shortage (or high 2-oxoglutarate levels), PipX was shown to activate NtcA-dependent promoters *glnB* (P_{II}) and *glnN* (GSIII) (Espinosa *et al.*, 2006). The phenotype of a *Synechococcus* $PipX^-$ mutant ressembled that of a less severe $NtcA^-$ mutant, which led to the conclusion that PipX must play an auxillary role to NtcA (Espinosa *et al.*, 2007). In addition, the *Synechococcus* $PipX^-$ mutant difficulty at assimilating nitrate compared to the WT strain (or the complete inability of $NtcA^-$ mutant to assimilate nitrate) suggested that PipX is needed for the rapid adaptation to a lack of ammonium (Espinosa *et al.*, 2007).

In *B. fragilis,* there is, as yet, no evidence of a global system of nitrogen regulation. Investigations were carried out by Southern *et al.* (1986) to determine whether *B. fragilis* possessed an *E. coli*-like regulatory system. Both *E.coli* strains YMC-10 (wild type) and YMC-11 ($glnA^-ntr^-$) carry a *Klebsiella aerogenes hut* operon which has a *hut*C mutation that results in high basal levels of histidase. It was shown that histidase levels increase in YMC-

10 by the regulatory actions of NtrB and NtrC on the *hut* operon under low glutamine concentrations. However, this was not observed with either *E. coli* YMC-11 *glnA⁻ntrB⁻ntrC⁻* nor *E. coli* YMC-11 (pJS139) (*E. coli* YMC11 transformed with a plasmid containing the *B. fragilis glnN* insertion) (Southern *et al.*, 1986). Therefore, the cloned *B. fragilis glnA* gene product failed to activate histidase kinase activity in an *E. coli glnA ntrB ntrC* deletion mutant (Southern *et al.*, 1986). However, these results could simply mean that the cloned fragment only carried GSIII and not the specific *B. fragilis* genes responsible for regulation.

In addition, Southern *et al.* (1987) showed that the activity of the cloned GS from *B. fragilis* was not inhibited by AMP (adenylylation). They also demonstrated that the *B. fragilis glnA* gene cloned into an *E. coli glnA⁻* deletion mutant was repressed by glutamate while the *glnA* of *E.coli* in the WT strain was expressed in the presence of glutamate (Southern *et al.*, 1986). Therefore, it was suggested that some nitrogen regulatory activities located on the vector they used (pJS139) was the cause of this regulation but these activities were not directly analogous to the NtrB and NtrC system. This evidence that the nitrogen regulation in *B. fragilis* is atypical of the enteric bacterial paradigms, combined with the knowledge that the *Synechococcus* GSIII structural gene (*glnN*) is regulated by NtcA, has led to the hypothesis that the GSIII of *B. fragilis* could also be regulated at the genetic level by an NtcA-like protein.

An ORF in the *B. fragilis* genome, locus Bf0954, was located directly downstream of *glnN*. This ORF encodes a putative FNR-type family transcriptional regulator. The position and putative identity of this ORF renders it a possible candidate as a nitrogen regulator, one resembling the *NtcA* family of regulators. As a result, it was decided to study the possible function of this gene in nitrogen regulation by inactivating the gene in *B. fragilis* and observing the behavior of this mutant with regards to its growth and GS activity under different ammonium conditions.

4.2 Materials and Methods

4.2.1 Bacterial strains, plasmids and growth conditions.

All *B. fragilis* and *E. coli* strains were grown as described in section 2.2.1 and 3.2.1. Plasmids used are listed and described in Tables 2.1 and 3.1.

4.2.2 Bioinformatic analysis

The sequences of *B. fragilis* NCTC9343 (http://www.ncbi.nlm.nih.gov/) and *B. fragilis* 638R assembly (http://www.sanger.ac.uk/Projects/B_fragilis/) were used for analyze locus Bf0955. Nucleotide and predicted amino acid sequences were determined and compared to those in the database using the NCBI Blast search (http://www.ncbi.nlm.nih.gov/BLAST) program. Multiple sequence alignments were done using the DNAman software package (Version 4.13, Lynnon Biosoft, Canada).

4.2.3 DNA manipulations and DNA sequencing

All DNA manipulations and molecular cloning techniques used are described in section 2.2.2 and 3.2.4. Primers used are detailed in table 4.1

Table 4.1 Sequences of oligonucleotide primers

Primer	Sequence
RegF	5'-CATCCCAGCGTCTGTC-3'
RegR	5'-CGGTTGATGAGGCTGTC-3'
NtcAF	5'-GCAGAGATGTGGGCTCC-3'
NtcAR	5'-CGTCGATGGTGATCAGTCG-3'

4.2.4 Construction of a putative regulator single cross-over insertion *B. fragilis* mutant

A single cross-over insertion mutation in the putative regulator of *B. fragilis* was obtained by inactivating the gene using methods described in section 2.2.3. A 570 bp internal fragment of the putative regulator was amplified from *B. fragilis* genomic DNA by PCR, using the primers NtcAF and NtcAR. This internal fragment was ligated into the *Bacteroides* suicide vector- pGERM and transformed into *E. coli* S17-1 cells. This construct was mobilized from *E. coli* S17-1 into *B. fragilis* 638R by biparental mating using a donor to recipient ratio of 1:5. The new *B. fragilis* exconjugants, carrying the insertion mutation, were selected anaerobically on BHIS agar plates containing 10 µg/ml erythromycin and 200 µg/ml gentamicin. The insertional mutation was verified by sequencing using the M13 primers (section 2.2.6). Sequencing was carried out as detailed in section 2.2.6.

4.2.5 Transcriptional analysis of the putative regulator mutant.

These procedures were described in section 3.2.6.

4.2.6 Enzymatic activity of the putative regulator mutant.

These procedures were described in sections 2.2.7 – 2.2.8.

4.3 Results and Discussion

4.3.1 Identification and sequence analysis of a putative nitrogen regulator in *B. fragilis*

Analysis of the *B. fragilis* genome revealed an ORF, annotated as locus BF0954, encoding a putative regulator belonging to the FNR-family of transcriptional regulators. This gene sequence was 702 bp long with 234 deduced amino acid residues. The CRP/FNR superfamily of DNA-binding proteins have a characteristic helix-turn-helix motif close to their C-terminal ends, which enables them to interact with DNA (Benoff *et al.*, 2002). A

blast search of the putative regulator in *B. fragilis* predicted a possible helix-turn-helix motif at amino acids 182-203 (Fig. 4.1). A comparison of the amino acid sequences of this putative regulator to the *E. coli* CRP, FNR and *Synechococcus* NtcA reveal that it only has 16.85%, 6.08% and 17.19% identity, respectively. An alignment of these sequences indicated that the conserved regions in CRP and FNR are not very well conserved in this putative regulator, however, 3 out of the five conserved glycine residues flanking β-strands of the β-roll in CRP are conserved in the putative regulator (Fig. 4.1) (Anjum *et al.*, 2000).

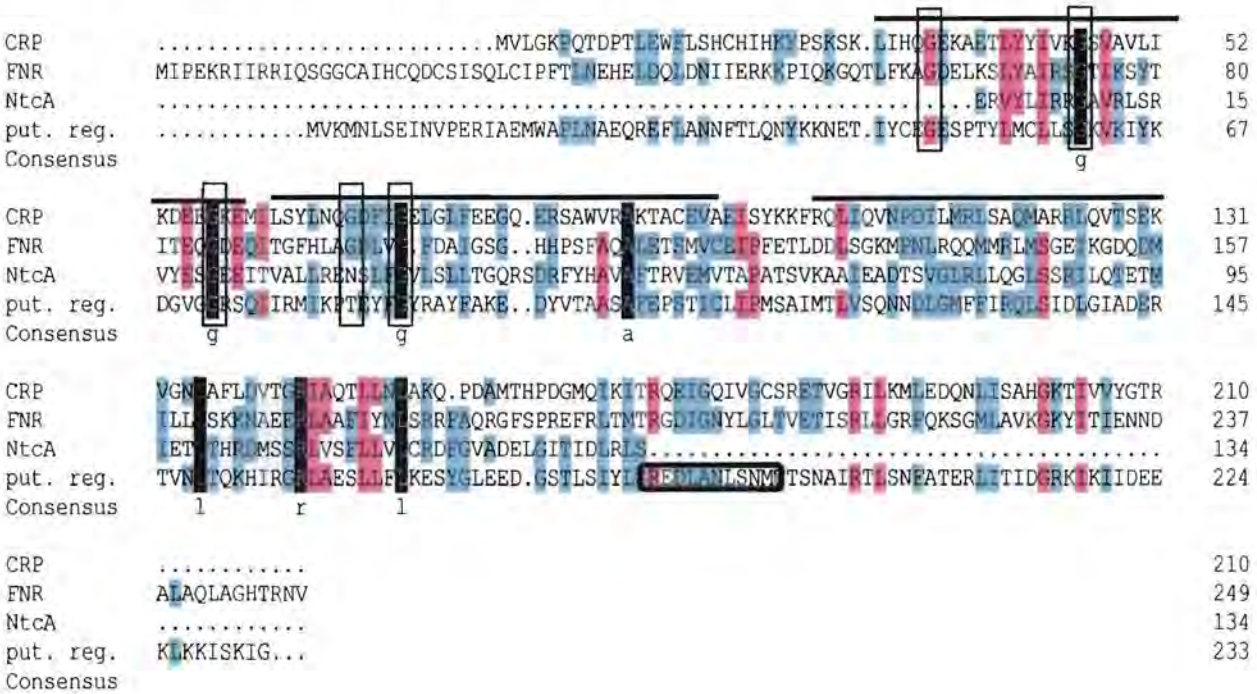

Fig 4.1 Multiple sequence alignment of *E. coli* CRP, *E. coli* FNR, *Synechococcus* NtcA and *B. fragilis* putative regulator. Amino acid residues similar in all sequences are shaded black, those similar in at least three sequences are shaded pink and the residues found in common between only two of the sequences are shown in blue. Regions corresponding to highly conserved sequences in the CRP sequence (according to Staden, 1982) are depicted by black solid lines above the sequence. Conserved glycine residues flanking β-strands are boxed. The predicted DNA-binding region is shown in a circle.

The nitrogen regulator of cyanobacteria, NtcA, is known to have a consensus binding site composed of a highly conserved palindromic sequence ($GTAN_8TAC$) (Luque *et al.*, 1994). This sequence can be identified in the promoter regions of all genes regulated by NtcA (Figure 4.2), including *ntcA* itself since the latter is also regulated by its own gene product (Herrero *et al.*, 2004). The NtcA binding site is meant to substitute for the *E. coli* σ^{70} consensus box located at position -35 relative to the transcription start site in all NtcA-

regulated promoters (Herrero *et al.*, 2001). In Cyanobacteria, the typical -10 pribnow-like box of *E. coli* σ^{70} consensus promoter (TAN_3T) is usually present, resulting in the following sequence: $\mathbf{GTAN_8TAC}N_{22}\mathbf{TAN_3T}$ (Fig 4.2). However, no NtcA consensus sequence was identified upstream of the putative regulator nor the *glnA* and *glnN* genes in *B. fragilis*.

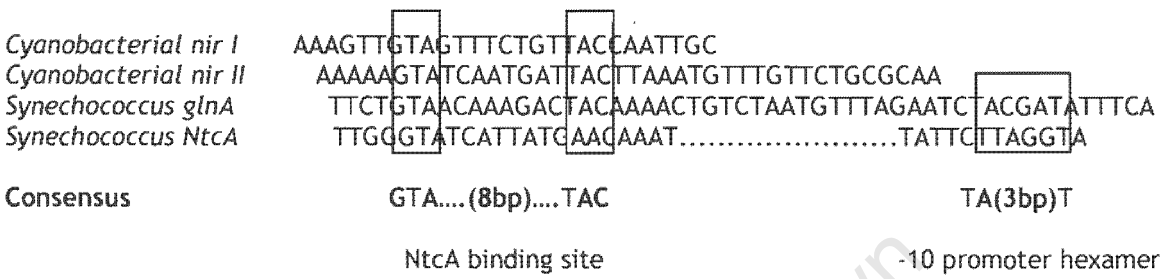

Fig 4.2 Palindromic sequence of NtcA in the promoter region of genes regulated by NtcA in different species. The *nir* gene is the gene encoding nitrite reductase, *glnA* encodes glutamine synthetase. I and II differentiate between the two NtcA binding sequences for the *nir* gene, with I being proximal and II being more distal to the nir gene. Consensus NtcA binding site and -10 hexamer sequences are boxed.

4.3.2 Construction and analysis of targeted insertional mutation of a putative regulator of nitrogen metabolism in *B. fragilis*

A gene disruption of locus Bf0954 of *B. fragilis* was constructed, as described in section 4.2.4. Analysis of the transconjugant DNA was done by sequencing and PCR (Fig 4.3). PCR using primers RegF and the vector-borne M13R primer revealed no band in the wild-type *B. fragilis* 638R but amplified a 880bp fragment in the regulator mutant (Fig. 4.3). Similarly, the combination of RegR and M13F generated a 720bp band from the mutant and none from the wild-type (Fig 4.3). When both primers external to the gene (RegF and RegR) were used, a 1 kb fragment was amplified in the wild-type (Fig 4.3). No fragment is observed from the mutant using the same primer set because of the plasmid insertion in the gene which increases its size from 1 kb to 5.6 kb, therefore, PCR under these conditions (Taq polymerase and 1-minute elongations) could not amplify this large fragment (Fig 4.3). These results together confirmed the insertion in the putative regulator gene of the mutant. In other words, this gene has been inactivated in a way that will result in the translation of a non-functional protein.

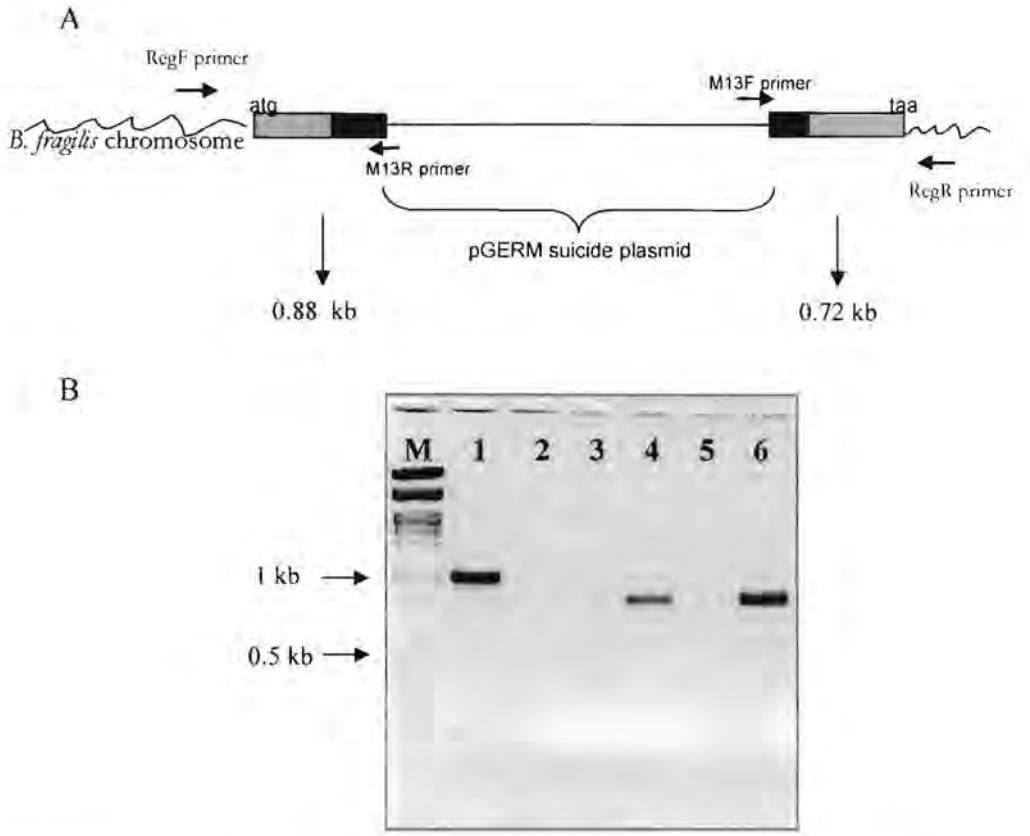

Fig 4.3 Analysis of the *B. fragilis* putative regulator mutant. A. Diagram of the insertion of pGERM into the putative regulator. B. PCR products. Chromosomal DNA from *B. fragilis* 638R and Erm^r transconjugants was extracted and used as a template in PCR reactions used to detect the insertion with the following primer pairs: RegF and RegR (primer set 1); RegR and M13F (primer set 2) and RegF and M13R (primer set 3). Lane labeled M contains lambda DNA digested with *PstI* as molecular weight marker; lanes 1 and 2 are PCR of *B. fragilis* WT and mutant, respectively, with primer set 1; lanes 3 and 4 are PCR of *B. fragilis* WT and mutant, respectively, with primer set 2; lanes 5 and 6 are PCR of *B. fragilis* WT and mutant, respectively, with primer set 3; Erm^r = Erythromycin resistance. (▒), putative regulator coding region; (■), internal fragment.

4.3.3 Growth studies of putative regulator mutant.

Growth studies were carried out on *B. fragilis* 638R and the putative regulator mutant in glucose minimal medium with ammonium chloride as the sole nitrogen source, as detailed in section 2.3.2. High and low ammonium conditions were achieved by using 50 mM and 0.25 mM NH_4Cl, respectively. Figure 4.4 revealed that this putative regulator mutant generally grew slower than the wild-type *B. fragilis* in all growth conditions. This difference in growth was even more evident under low nitrogen conditions where the wild-type grew twice as fast as the mutant. This trend was maintained after 24 hours of growth, with the mutant reaching on OD_{600} of 0.38, compared to the wild-type OD_{600} of 0.69. In this respect,

the regulatory mutant growth pattern was similar to the *B. fragilis glnN* mutant (Fig 2.5). In high ammonium, the mutant averaged an OD_{600} of 1.76 at 24 hours compared to the wild-type average OD_{600} of 2.04.

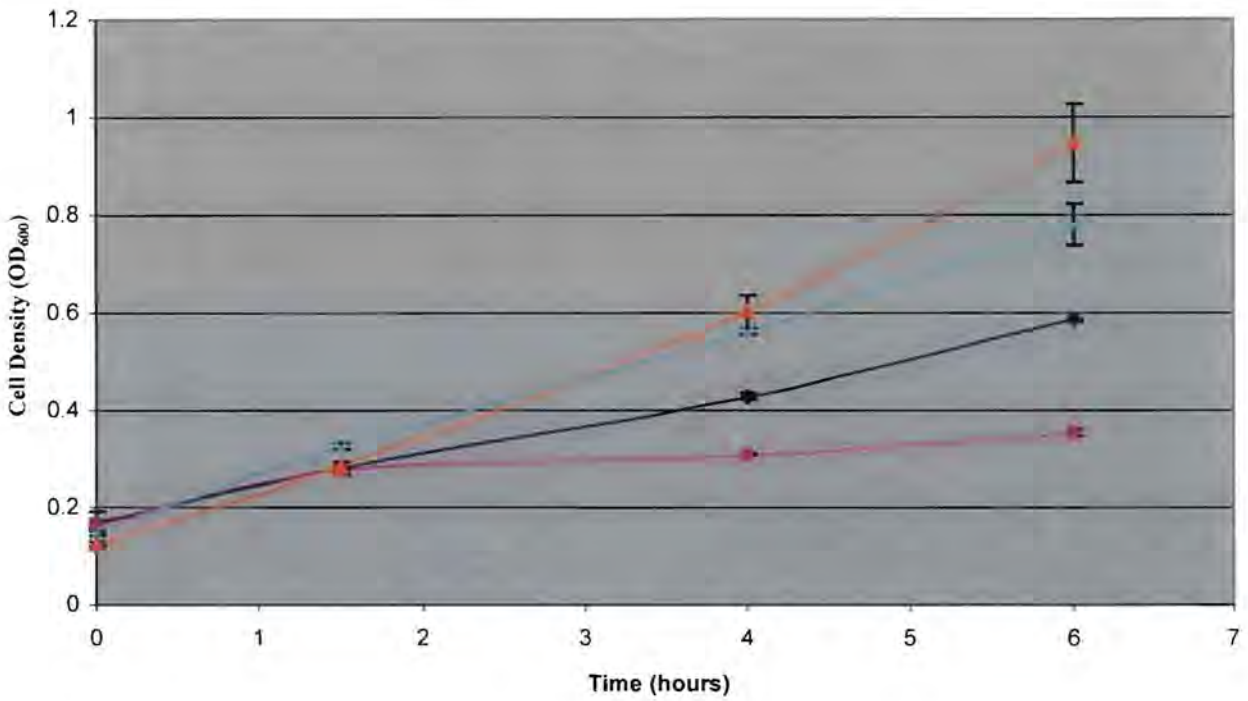

Fig. 4.4 Growth of *B. fragilis* putative regulator mutant in glucose minimal medium supplemented with 0.25 mM (low) and 50 mM (high) ammonium chloride as the sole nitrogen source. Overnight cultures grown in rich medium were harvested and washed twice in the appropriate minimal medium before inoculating in the growth media and incubating at 37°C under anaerobic conditions. Growth was monitored over the indicated time course using a spectrophotometer at 600nm. Results are the means and standards deviations of at least three separate experiments. WT=Wild type. (▲), WT in 50 mM NH_4Cl; (◆), WT in 0.25 mM NH_4Cl; (✕), Mutant in 50 mM NH_4Cl; (■), Mutant in 0.25 mM NH_4Cl.

4.3.4 Comparison of the GS enzymatic activity of *B. fragilis* 638R and the putative regulator mutant.

The glutamine synthetase forward assay was carried out using crude extracts from *B. fragilis* 638R and putative regulator mutant strains grown for 6 hours in glucose minimal medium with 0.25 mM (low) or 50 mM (high) ammonium chloride, as the sole nitrogen source (Fig. 4.5). Procedures to obtain crude extract and assay preparations are detailed in sections 2.2.7-2.2.8.

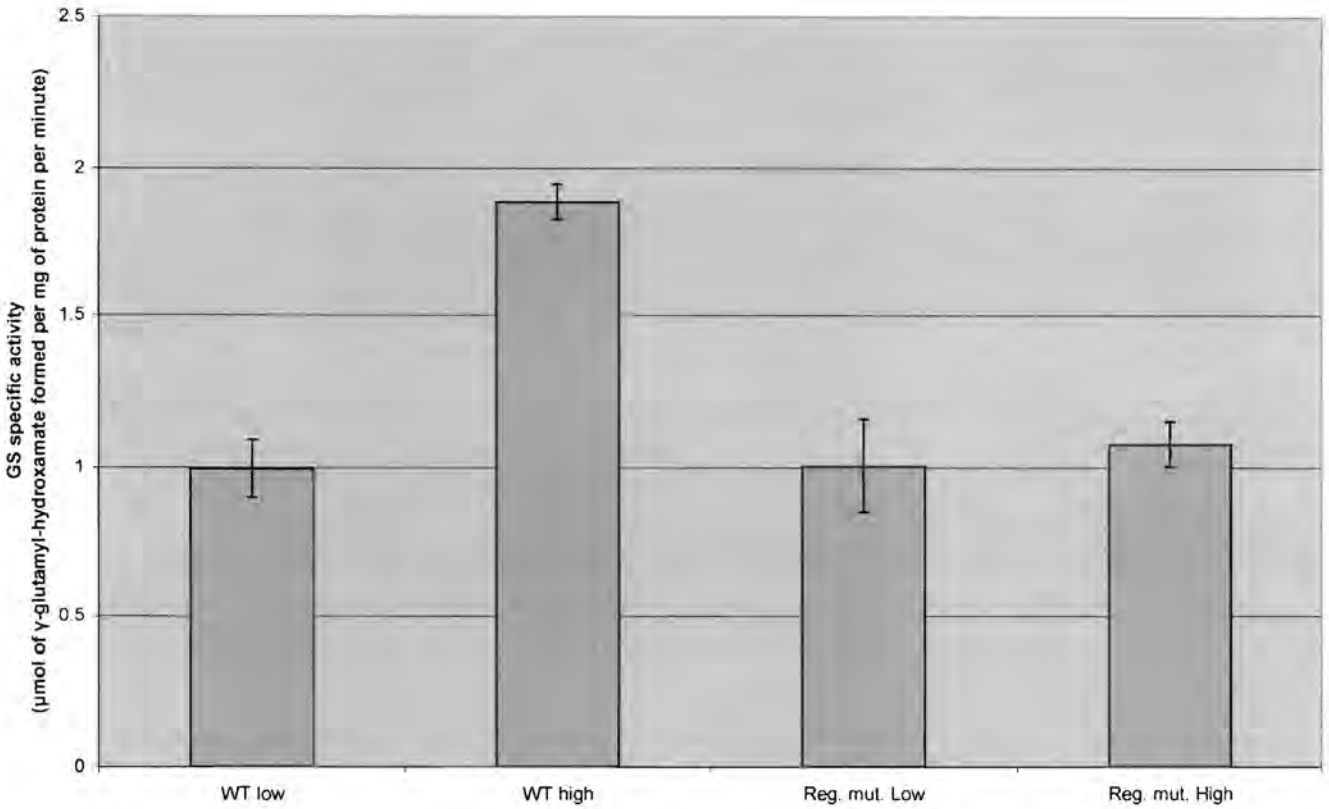

Fig 4.5 Glutamine synthetase activity of WT and putative regulator mutant. Activity was determined at 37°C using the forward assay. Cell free extract was obtained from cells grown in glucose minimal medium supplemented with either 0.25 mM (low) or 50 mM (high) ammonium chloride. This assay was repeated at least three times with the same trend.

The forward assay revealed that there was an almost two-fold increase of GS activity in *B. fragilis* 638R wild-type grown in high ammonium as opposed to low ammonium. When the putative regulator was inactivated, this increase of GS activity in high ammonium did not occur. Therefore, whichever GS is responsible for the increase in GS activity in high ammonium growth conditions, it was not induced when the putative regulator was inactivated. Interestingly, these results are similar to what was observed with the *glnN* mutant (Figure 2.7). Therefore, it can be concluded that this putative regulator may be involved in the regulation of *glnN* because inactivating the regulator leads to the same phenotype as a *glnN* mutant with regards to growth and GS specific activity.

4.4 Conclusions

Identification of a putative regulator of the Crp/Fnr-family of transcriptional regulators in *B. fragilis,* encoded by an ORF which lies directly downstream of *glnN,* led to the hypothesis

that this gene might encode a nitrogen regulator similar to the Cyanobacterial NtcA. An insertional inactivation of this putative regulator is reported here. The resulting mutant displayed the same reduced growth pattern (Fig. 4.4) seen in the *glnN* mutant (Fig. 2.5). Similarly, the GS forward assay revealed similar residual GS activity in the regulator mutant when grown in high or low nitrogen conditions, and a lack of increased GS activity in high ammonium (Fig. 4.5) as observed in the *glnN* mutant (Fig. 2.7). Therefore, if this regulator is involved in modulating *B. fragilis glnN* gene expression, inactivating this gene in *B. fragilis* ought to have a similar phenotype to a *B. fragilis glnN* mutant. This was, in fact, observed. The decrease in growth and GS activity observed in the mutant, led to the conclusion that the protein possibly acts as a positive regulator. The majority of CRP/FNR regulators work as positive transcriptional activators, however, some have been identified which have repressor functions (Korner *et al.*, 2003).

Another reason for the observed phenotype may be due to possible co-transcription of the GS and the putative regulator. If this is the case, then inactivation of the regulator alone may lead to changes in transcript as well as mRNA stability for *glnN*, thereby giving the same phenotype as a *glnN* mutant.

It was interesting to see that NtcA-binding sites could not be found in the promoter regions of either *glnA* or *glnN*. Aldehni *et al.* (2003) have shown that, under conditions of nitrogen deficiency, regulation by NtcA requires the P_{II} signal transduction protein. A search of the published *B. fragilis* genome, however, showed no evidence of P_{II} or its structural gene in *B. fragilis* (this study). Therefore, it would not be surprising if the modes of action of this putative regulator are different from NtcA. This is the first evidence of a protein with potential nitrogen regulatory activity in *B. fragilis*.

Since the mechanisms of action of this regulator are not yet clarified, additional experiments need to be conducted for further understanding its role. Crp/Fnr regulators are DNA-binding proteins and a possible helix-turn-helix domain via which binding to DNA occurs was identified in this regulator. Further studies should involve purification of the protein, and performance of DNA binding studies and electrophoretic mobility shift assays to determine whether the putative regulator gene product actually binds to a DNA region upstream of *glnA, glnN* and itself, and consequently determine what the binding site is. Quantitative RT-

PCR could also help in determining the various nitrogen conditions under which this gene is induced.

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General Conclusions

Glutamine is vital in nitrogen metabolism. It serves as a precursor to many secondary metabolites, providing nitrogen for the synthesis of nitrogen-containing compounds (Reitzer, 2003). Glutamine synthetase (GS) is the only enzyme known to produce glutamine in cells, it catalyzes the ATP-dependent reductive transfer of an amido group from ammonium to glutamate, to produce glutamine. Therefore, GS enzymes are generally tightly regulated transcriptionally as well as translationally, in response to the availability of nitrogen in the growth medium. In addition, many prokaryotes possess two or more isoforms of the enzyme, which have different roles in under different growth conditions. To date, a single GS encoding gene (*glnN*) has been identified in the opportunistic pathogen, *Bacteroides fragilis*. The aims of this project were to identify additional GS orthologues in *B. fragilis* and characterize any candidates genetically as well as functionally. An additional aim was to identify possible regulatory proteins and to determine their functional contribution to glutamine biosynthesis.

The construction of *B. fragilis* 638ΩglnN, a mutant strain in which the *glnN* gene has been inactivated by an insertion in its coding region, is reported here. The *glnN* gene codes for glutamine synthetase III (GSIII). Due to the importance of glutamine in nitrogen metabolism, inactivating the gene coding for GS usually results in a lack of viability of the bacterium due to a lack of glutamine synthesis. The ability of *B. fragilis* 638ΩglnN to grow in minimal medium with ammonium as the only nitrogen source was investigated. It was observed that the mutant grew very poorly when the ammonium concentration was limiting, however, the mutant grew well in high ammonium levels (Figs 2.4 and 2.5). An assay of GS activity in *B. fragilis* 638ΩglnN confirmed that the mutant had some residual GS activity, although there was no change in GS activity level in the mutant under high or low ammonium growth conditions (Fig. 2.7). Although these results established that *glnN* is essential for growth in nitrogen deficiency, the growth of this mutant in high ammonium concentrations led to the conclusion that either an unknown additional pathway for glutamine biosynthesis exists or a gene (other than *glnN*) might be responsible for glutamine synthesis.

A search for Open Reading Frames (ORFs) coding for possible GS candidates was conducted using the *B. fragilis* 638R genome lodged at the Sanger Institute (http://www.sanger.ac.uk/Projects/B_fragilis/). Locus Bf2343 was identified, which encodes a putative glutamine synthetase I (GSI). Although this putative *glnA* was only 20-30% similar to other characterized *glnA* genes, the deduced amino acid sequence of this gene revealed that three out of the five highly conserved GS regions pertaining to the active site, were preserved in this gene (Fig. 3.2). Of the two recently-identified, additional regions conserved in all GS enzymes (van Rooyen *et al.*, 2006), one seems to be conserved in this gene. In addition, all GS enzymes which undergo post-translational modification by adenylylation possess a tyrosine residue (Tyr-397) associated with the adenylylation site of these enzymes. This tyrosine residue was not found in the putative GSI of *B. fragilis* (Fig. 3.2), therefore, this gene product is not expected to be modified by adenylylation.

BF2343 coding for the putative *glnA* was cloned in the pGEMT vector (pGEMT-2343) (Fig. 3.4) and expressed in *E. coli* YMC11 (*glnA*$^-$*ntr*$^-$), a strain defective in the *glnA* gene and regulatory components associated with this gene. When expressed in *E. coli* YMC11, this gene failed to complement glutamine auxotrophy, as seen by the inability of YMC11 (pGEMT2343) to grow on minimal medium with ammonium as the sole nitrogen source (Fig. 3.5). Molecular and biochemical analyses were conducted on the recombinant protein, which revealed that although the gene was transcribed in *E. coli* (Fig. 3.6), the recombinant putative GSI did not display significant GS activity in the *E. coli* clone (Fig. 3.8). In *B. fragilis, glnA* was also transcribed and no variation in transcript levels could be detected in *B. fragilis* grown in either low- or high- ammonium containing minimal medium (Fig. 3.7). In addition, RNA hybridization studies in *B. fragilis* 638ΩglnN revealed that the inactivation of *glnN* did not seem to affect the levels of transcripts of the putative *glnA* (Fig. 3.7). The reasons for the inactivity of the cloned putative *glnA* could that the protein requires some post-translational modification, that the wrong substrates were tested here, or it could simply be that this ORF does not encode a GS enzyme. In order to prove conclusively whether it is a functional GS, it would be essential to create a *glnA* mutant.

The presence of an additional GS in *B. fragilis* was of further interest because of a trend observed in *Rhizobiaceae* and *Synechocystis* sp. Strain PCC 6803, among others (Carlson *et*

al., 1987). In both *Rhizobiaceae* and *Synechocystis* sp. PCC 6803, *glnA* is constitutively expressed whereas *glnN* (*Synechocystis*) and *glnII* (*Rhizobiaceae*) undergoes strict regulation based on the nitrogen availability (Reyes *et al.*, 1997). The pattern seemed to be consistent with the findings reported here, as it is observed that in *B. fragilis* there appears to be a constitutive expression of the putative *glnA*, seen by the lack of change in transcript levels (Fig. 3.7) as well as the same amount of residual GS activity (Fig. 2.7), regardless of the growth conditions. Conversely, the *glnN* of *B. fragilis* is regulated transcriptionally (Abratt *et al.*, 1993) and results in increased GSIII protein (Southern *et al.*, 1987).

Studies conducted by Southern *et al.* showed, using western hybridizations, that GS levels in *B. fragilis* varied according to nitrogen availability (Southern *et al.*, 1987). Abratt *et al.* (1993), using a reporter gene vector made of a promoterless gene fused to the *glnA* (now *glnN*) promoter region, further showed that the regulation occurred at the level of transcription. Both of these reports demonstrated increased transcription (Abratt *et al.*, 1993) and protein levels (Southern *et al.*, 1987) of GSIII, under low nitrogen. The creation of *B. fragilis* 638ΩglnN, reported here, showed no induction of GS activity under high ammonia conditions in this *glnN* mutant, compared to the wild-type *B. fragilis* which displayed twice as much GS activity in high ammonia (Fig 2.7). This implies that the GSIII of *B. fragilis* is also involved in contributing GS activity under high ammonia although there is no evidence of a greater amount of protein present as shown by Southern *et al.* (1987). It is possible that some post-translational modification mechanism could be activating the available GSIII when the cells are subjected to high nitrogen conditions thus explaining the increase in activity. This hypothesis could be tested by doing some activation/inactivation studies on the purified protein, in parallel with western hybridization. An interesting discovery was made by van Rooyen *et al.* (2006), showing evidence of two structural forms of GSIII in *B. fragilis*; the hexameric form believed to be a less active intermediate to the most active dodecamer.

No regulatory system or genes involved in nitrogen regulation have been identified in *Bacteroides* species prior to this study. It was previously shown that the GSIII enzyme of *B. fragilis* is not adenylylated. The GDH enzyme of *Bacteroides thetaiotaomicron* also does not shows evidence of adenylylation (Baggio and Morrison, 1996). In addition, none of the *ntr* genes have been found in *Bacteroides* genomes. The work reported in this thesis has

identified a putative regulator located directly downstream of *glnN* (GSIII structural gene) and belonging to the CRP/FNR family of transcriptional regulators. This gene is believed to be a possible candidate for a nitrogen regulator because of its close proximity to *glnN* and its membership to the same family as NtcA, the cyanobacterial nitrogen regulator. This hypothesis was tested by inactivating the gene and conducting physiological studies to determine the effects of the mutation on *B. fragilis* grown in various nitrogen environments. The regulatory mutant revealed similar growth pattern (Fig. 4.4) as well as GS enzymatic activity (Fig. 4.5) as the *glnN* mutant (Figs. 2.5 and 2.7). These results indicate that this gene may, directly or indirectly, act as a positive regulator for *glnN,* therefore supporting the hypothesis that this gene could be a nitrogen regulator.

Despite the family-association with NtcA and the predicted helix-turn-helix motif associated with the DNA binding domain (Fig. 4.1) found in this putative regulator, the gene does not appear to be an *ntc*A homologue because no NtcA-binding motifs could be detected in the promoter regions of *glnN* or the regulator itself. Future research directions should be aimed at elucidating the exact role and mode of action of this regulator, as well as the genes it regulates. The use of 2D gels and quantitative rt-PCR would help to visualize changes in protein and transcript levels, respectively under different nitrogen growth conditions or when one or more of the genes under study is inactivated. DNA binding studies should also be performed to determine whether this putative regulator binds to regions upstream of the nitrogen-regulated genes.

The *Mycobacterium tuberculosis glnA1* mutant was shown to be avirulent (Tullius *et al.*, 2003). For this reason, it would be interesting to test the pathogenicity of the different *B. fragilis* mutants constructed in this study. The results obtained could assist in furthering our understanding of the pathways of nitrogen assimilation and possibly help in finding novel drug targets for this opportunistic pathogen.

References

Abrahams, G. L., Abratt, V. R. 1998. The NADH-dependent glutamate dehydrogenase enzyme of *Bacteroides fragilis* Bf1 is induced by peptides in the growth medium. *Microbiol.* **144**: 1659-1667.

Abratt, V. R., Zappe, H., Woods, D. R. 1993. A reporter gene vector to investigate the regulation of glutamine synthetase in *Bacteroides fragilis* Bf1. *J Gen Microbiol.* **39**(1):59-65.

Adler, S.P., Purich, D., Stadtman, E.R. 1975. Cascade control of *Escherichia coli* glutamine synthetase. Properties of the PII regulatory protein and the uridylyltransferase-uridylyl-removing enzyme. *J Biol Chem.* **250**(16):6264-6272

Aiba, H., Adhya, S. and de Crombrugghe, B. 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *J Biol chem.* **256:** 11905-11910

Aldehni, M., Sauer, J., Spielhaupter, C., Schmid, R., Forchhammer, K. 2003. Signal Transduction Protein PII Is Required for NtcA-Regulated Gene Expression during Nitrogen Deprivation in the Cyanobacterium *Synechococcus elongatus* Strain PCC 7942. *J. Bacteriol.* **185**: 2582-2591

Allison, M. J., Robinson, I. M. & Baetz, A. L. 1979. Synthesis of α-ketoglutarate by reductive carboxylation of succinate in *Veillonella*, *Selenomonas*, and *Bacteriodes* species. *J Bacteriol* **140**: 980–986

Amaya, K. R., Kocherginskaya, S. A., Mackie, R. I., Cann, I. K. O. 2005. Biochemical and Mutational Analysis of Glutamine Synthetase Type III from the Rumen Anaerobe *Ruminococcus albus* 8. *J Bacteriol* **187:** 7481-7491

Anjum, M. F., Green, J. and Guest, J. R. 2000. YeiL, the third member of the CRP-FNR family in Escherichia coli. *Microbiol.* **146**: 3157-3170

Armitage, P., R. Walden, and Draper, J. 1988. Vectors for the transformation of plant cells using agrobacterium. *In* Plant genetic transformation and gene expression, a laboratory manual. Draper, J.R. Scott, R., P. Armitage and W. Walden. Blackwell Scientific Publications Ltd., Oxford, United Kingdom.

Atkins, W.M., Cader, B. M., Hemmingsen, J., Villafranca, J.J. 1993. Time-resolved fluorescence and computational studies of adenylylated glutamine synthetase: analysis of intersubunit interactions. *Protein Sci.* **2**: 800-813.

Backman, K., Chen, Y.M., Maganasik, B. 1981. Physical and genetic characterization of the glnA-glnG region of the *Escherichia coli* chromosome. *Proc Natl Acad Sci. USA* **78**(6): 3743-3747

Baggio, L., Morrison, M. 1996. The NAD(P)H-utilizing glutamate dehydrogenase of *Bacteroides thetaiotaomicron* belongs to enzyme family I, and its activity is affected

by trans-acting gene(s) positioned downstream of gdhA. *J Bacteriol.*; **178**(24):7212–7220

Bass, K. A., Hecht, D. W. 2002. Isolation and Characterization of cLV25, a Bacteroides fragilis Chromosomal Transfer Factor Resembling Multiple Bacteroides sp. Mobilizable Transposons. *J Bacteriol.* **184**: 1895-1904

Bayley, D. P., Rocha, E. R., and Smith J. 2000 Analysis of cepA and other *Bacteroides fragilis* genes reveals a unique promoter structure. *FEMS Microbiol lett.* **193**(1): 149-154

Bender, R. A., Janssen, K. A., Resnick, A. D., Blumenberg, M., Foor, F. And Maganasik B. 1977. Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*. *J Bacteriol.* **129**: 1001-1009

Benoff, B., Yang, H., Lawson, C.L., Parkinson, G., Liu, J., Blatter, E., Ebright, Y.W., Berman, H.M., Ebright, R.H. Structural basis of transcription activation: the CAP-alpha CTD-DNA complex. *Science* **297**: 1562-1566

Berg, J. O., Lindqvist, L., Andersson, G., Nord, C. E. 1983. Neuraminidase in *Bacteroides fragilis*. *Appl Environ Microbiol.* **46**(1):75–80.

Bower, S, Zalkin, H. 1983. Chemical modification and ligand binding studies with *Escherichia coli* glutamate synthase. *Biochem.* **22**(7):1613–1620.

Bradford, M. M. 1976 A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal Biochem.* **72:** 248-254

Brook, I. 1988. Recovery of Anaerobic Bacteria from Clinical Specimens in 12 Years at Two Military Hospitals. *J clinical Microbiol.*: 1181-1188

Brook, I., and Frazier, E. H. 2000. Aerobic and anaerobic microbiology in intra-abdominal infections associated with diverticulitis. *J Med Microbiol.* **49:**827-830.

Brook, I. 2002. Clinical review: Bacteremia caused by anaerobic bacteria in children. *Crit Care.* **6**(3): 205–211.

Bruggeman, F.J., Boogerd, F.C., Westerhoff, H.V. 2005. The multifarious short-term regulation of ammonia assimilation of *Escherichia coli*: dissection using an in silico replica. *FEBS J* **272**:1965

Bryan, L. E., Kowland, S.K. and Van Den Elyen, H.M. 1979. Mechanism of aminoglycoside antibiotic resistance in anaerobic bacteria: *Clostridium perfringens* and *Bacteroides fragilis*. *Antimicrob Agents Chemother.* **15**:7-13.

Burkovski, A. 2003. Ammonium assimilation and nitrogen control in *Corynebacterium glutamicum* and its relatives: an example for new regulatory mechanisms in actinomycetes. *FEMS Microbiol Rev.* **27**:617-628

Byers, H. L., Homer, K. A., Beighton, D. 1996. Sialic acid utilisation by viridans streptococci. *Adv. Exp. Med. and Biol.* **418:** 713-6

Carlson, T. A., Martin, G. B. and Chelm, B. K. 1987. Differential transcription of the two glutamine synthetase genes of *Bradyrhizobium japonicum*. *J. Bacteriol.* **169:**5861–5866.

Cederbrant, G., Kahlmeter, G., Ljungh, A. 1992. Proposed mechanism for metronidazole resistance in *Helicobacter pylori*. *J Antimicrob Chemother.* **29**: 115–20.

Cerdeno-Tarraga, A.M., Patrick, S., Crossman, L.C., Blakely, G., Abratt, V., Lennard, N., Poxton, I., Duerden, B., Harris, B., Quail, M.A., Barron, A., Clark, L., Corton, C., Doggett, J., Holden, M.T., Larke, N., Line, A., Lord, A., Norbertczak, H., Ormond, D., Price, C., Rabbinowitsch, E., Woodward, J., Barrell, B. and Parkhill, J. 2005. Extensive DNA inversions in the *B. fragilis* genome control variable gene expression. *Science.* **307:** 1463–1465.

Chen, P., and Reitzer, L. J. 1995. Active contribution of two domains to cooperative DNA binding of the enhancer-binding protein nitrogen regulator I (NtrC) of *Escherichia coli*: stimulation by phosphorylation and the binding of ATP. *J Bacteriol.* **177**:2490-2496

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E. 3rd, Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M. A., Rajandream, M. A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Taylor, K., Whitehead, S., Barrell, B. G. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393:** 537–544

Coyne, M.J., Tzianabos, A.O., Mallory, B.C., Carey, V.J., Kasper, D.L., and Comstock, L.E. 2001. Polysaccharide Biosynthesis Locus Required for Virulence of *Bacteroides fragilis*. *Infect Immun.* **69**(7): 4342–4350.

Crespo, J. L., García-Domínguez, M., Florencio, F.J. 1998. Nitrogen control of the *glnN* gene that codes for GS type III, the only glutamine synthetase in the cyanobacterium *Pseudanabaena* sp. PCC 6903. *Mol Microbiol.* **30**: 1101-1112.

Cui, J., Liu, Y., Wang, R., Tong, W., Drlica, K., Zhao, X. 2006. The mutant selection window in rabbits infected with *Staphylococcus aureus*. *J Infect Dis.* **194:** 1601-1608

Davis, R. W., Botstein, D. and Roth, J. R. 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

de Bruijn, F. J., Rossbach, S., Schneider, M., Ratet, P., Messmer, S., Szeto, W. W., Ausubel, F. M. and Schell, J. 1989. *Rhizobium meliloti* 1021 has three

differentially regulated loci involved in glutamine biosynthesis, none of which is essential for symbiotic nitrogen fixation. *J Bacteriol.* **171:**1673–1682.

Diniz, C. G., Farias, L. M., Carvalho, M. A. R., Rocha, E. R., Smith, C. J. 2004. Differential gene expression in a *Bacteroides fragilis* metronidazole-resistant mutant. *J Antimicrob Chemother* **54:** 100-108

Edwards, D. I. 1993. Nitroimidazole drugs: action and resistance mechanism. I. Mechanism of action. *J Antimicrob Chemother.* **31**:9–20

Eisenberg, D., Gill, H. S., Pfluegl, G. M. and Rotstein, S. H. 2000. Structure-function relationships of glutamine synthetases *Biochim Biophys Acta.* **1477**: 122-145.

Espinosa, J., Forchhammer, K., Burillo, S., Contreras, A. 2006. Interaction network in cyanobacterial nitrogen regulation: PipX, a protein that interacts in a 2-oxoglutarate dependent manner with PII and NtcA. *Mol. Microbiol.* **61:** (2), 457–469.

Espinosa, J., Forchhammer, K., Contreras, A. 2007. Role of the *Synechococcus* PCC 7942 nitrogen regulator protein PipX in NtcA-controlled processes. *Microbiol.* **153**: 711-718

Franco, A. A., R. K. Cheng, G.-T. Chung, S. Wu, H.-B. Oh, and C. L. Sears. 1999. Molecular evolution of the pathogenicity island of enterotoxigenic *Bacteroides fragilis. J Bacteriol.* **181:**6623-6633

Fierro-Monti, I. P., Reid, S. J., and Woods, D. R. 1992. Differential expression of a *Clostridium acetobutylicum* antisense RNA: implications for regulation of glutamine synthetase. *J Bacteriol.* **174**: 7642-7647.

Finegold, S. M., and W. L. George. 1989. Anaerobic infections in humans. Academic Press, San Diego, CA.

Fink, D., Weissschuh, N., Reuther, J., Wohlleben, W., Engels, A. 2002. Two transcriptional regulators GlnR and GlnRII are involved in regulation of nitrogen metabolism in Streptomyces coelicolor A3(2). *Mol Microbiol.* **46:** 331–347.

Fisher, S. H. 1999. Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la difference! *Mol Microbiol.* **32**: 223-232.

Fisher, S. H., Wray, L. V. Jr. 2006. Feedback-Resistant Mutations in *Bacillus subtilis* Glutamine Synthetase are clustered in the active site. *J. Bacteriol.* **188**: 5966-5974

Forchhammer, K., and Tandeau de Marsac, N. 1995. Functional analysis of the phosphoprotein P_{II} (*glnB* gene product) in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol.* **177:**2033-2040

Frías, J. E., Mérida, A., Herrero, A., Martín-Nieto, J., Flores, E. 1993. General distribution of the nitrogen control gene *ntcA* in cyanobacteria. *J Bacteriol.* **175**(17):5710–5713.

García-Domínguez, M., Reyes, J.C. & Florencio F.J. 1997 Purification and characterization of a new type of glutamine synthetase from cyanobacteria, *Eur J Biochem.* **244**: 258-264.

García-Domínguez, M., Reyes, J.C. & Florencio F.J. 1999 Glutamine synthetase inactivation by protein-protein interaction, *Proc Natl Acad Sci. USA* **96**: 7161-7166.

García-Domínguez, M., Reyes, J.C., Florencio, F.J. 2000. NtcA represses transcription of *gifA* and *gifB*, genes that encode inhibitors of glutamine synthetase type I from *Synechocystis* sp. PCC 6803. *Mol Microbiol.* **35:**1192-1201.

Gawronski, J. D., Benson, D.R. 2004. Microtiter assay for glutamine synthetase biosynthetic activity using inorganic phosphate detection. *Anal Biochem.* **327**: 114-118.

Genco, C. A., Schifferle, R. E., Njoroge, T., Forng, R. Y., Cutler, C. W. 1995. Resistance of a Tn4351-generated polysaccharide mutant of *Porphyromonas gingivalis* to polymorphonuclear leukocyte killing. *Infect Immun.* **63**(2):393–401

Gherna, R., and C. R. Woese. 1992. A partial phylogenetic analysis of the flavobacter-bacteroides" phylum: basis for taxonomic restructuring. *Syst Appl Microbiol.* **15:**513–521.

Gibson, S. A. W., and Macfarlane, G. T. 1988. Studies on the proteolytic activity of *Bacteroides fragilis. J Gen Microbiol.* **134:**19–27.

Godoy, V. G., Dallas, M. M., Russo, T. A., Malamy, M. H. 1993. A role for*Bacteroides fragilis* neuraminidase in bacterial growth in two model systems. *Infect Immun.* **61**:4415–4426.

Goodman, H.J.K. and D.R. Woods. 1993. Cloning and nucleotide sequence of the *Butyrivibrio fibrisolvens* gene encoding a type III glutamine synthetase. *J Gen Microbiol.* **139:** 1487-1493.

Goss, T. J., and R. A. Bender. 1995. The nitrogen assimilation control protein, NAC, is a DNA binding transcription activator in *K. aerogenes. J Bacteriol.* **177**:3546-3555

Haggoud, A., Reysset, G., Azeddoug, H., and Sebald, M. 1994. Nucleotide sequence analysis of two 5-nitroimidazole resistance determinants from Bacteroides strains and of a new insertion sequence upstream of the two genes. *Antimicrob. Agents Chemother.* **38**: 1047-1051.

Harth, G., Maslesa-Galic, S., Tullius, M. V. & Horwitz, M. A. 2005. All four *Mycobacterium tuberculosis glnA* genes encode glutamine synthetase activities but only GlnA1 is abundantly expressed and essential for bacterial homeostasis. *Mol Microbiol* **58**, 1157–1172.

Helling, R. B. 1998 Pathway choice in glutamate synthesis in *Escherichia coli*. *J Bacteriol.* **180:** 4571-4575.

Herrero, A., Muro-Pastor, A. M., Flores, E. 2001. Nitrogen Control in Cyanobacteria. *J Bacteriol.* **183**: 411-425

Hill, R. T., Parker, J. R., Goodman, H. J., Jones, D. T. and Woods, D. R. 1989. Molecular analysis of a novel glutamine synthetase of the anaerobe Bacteroides fragilis. *J Gen Microbiol.* **135**: 3271-3279.

Hirschman, J., Wong, P.K., Sei, K., Keener, J., Kustu, S. 1985. Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription in vitro: evidence that the ntrA product is a sigma factor. *Proc Natl Acad Sci* USA. **82**(22):7525–7529

Holdeman, L. V., and W. E. C. Moore (ed.). 1972. Anaerobe Laboratory Manual. Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg.

Hu, P., Leighton, T., Ishkhanova, G. and Kustu, S. 1999. Sensing of nitrogen limitation by *Bacillus subtilis*: Comparison to Enteric Bacteria. *J Bacteriol.* **181**: 5042-5050.

Hylemon, P. B., Young, J. L., Roadcap, R. F. and Phibbs, P. V. J. 1977. Uptake and incorporation of glucose and mannose by whole cells of *Bacteroides thetaiotaomicron*. *Appl Environ Microbiol.* **34:**488-494

Ish-Horowicz, D., Burke, J. F. 1981. Rapid and efficient cosmid cloning. *Nucleic Acids Res.* **9**(13): 2989–2998.

Jiang, F., Mannervik, B., and Bergman, B. 1997. Evidence for redox regulation of the transcription factor NtcA, acting both as an activator and a repressor, in the cyanobacterium *Anabaena* PCC 7120. *Biochem J.* **327:** 513–517

Jiang, P., Peliska, J.A., and Ninfa, A.J. 1998. Enzymological characterization of the signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and its interaction with the PII protein. *Biochem.* **37**: 12782–12794.

Korner, H., Sofia, H. J., Zumft, W. G. 2003. Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs *FEMS Microbiol. Rev* **27**: 559–592

Kui, J., Jianzhong, Z., Guozong, P. 2001. Mechanism of metronidazole resistance in *Helicobacter pylori Chinese J of Digest Dis.* **2** (2); 95–99

Kumada, Y., Takano, E., Nagaoka, K. & Thompson, C. J. 1990. *Streptomyces hygroscopicus* has two glutamine synthetase genes. *J Bacteriol* **172**: 5343-5351

Kumada, Y., Benson, D. R., Hillemann, D., Hosted, T. J, Rochefort, D. A., Thompson, C. J., Wohlleben, W., and Taten, Y. 1993. Evolution of the Glutamine Synthetase

Gene, One of the Oldest Existing and Functioning Genes. *Proc Natl Acad Sci.* **90**:3009-3013

Kuwahara, T., Yamashita, A., Hirakawa, H., Nakayama, H., Toh, H., Okada, N., Kuhara, S., Hattori, M., Hayashi, T., and Ohnishi, Y. 2004. Genomic analysis of *Bacteroides fragilis* reveals extensive DNA inversions regulating cell surface adaptation. *PNAS.* **101**: 14919 - 14924.

Lepine, G., Ellen, R. P. and Progulske-Fox, A. (1996). Construction and Preliminary characterization of three hemagglutinin mutants of *Porphyromonas gingivalis. Infect Immun.* **64:** (4) 1467-1472

Li, X. Z., and Poole, K. 2001. Mutational analysis of the OprM outer membrane component of the MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa. J Bacteriol.* **183:** 12–27

Liu, Y., Kahn, M. L. 1995. ADP-ribosylation of *Rhizobium meliloti* glutamine synthetase III in vivo. *J Biol Chem.* **270**:1624-1628.

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C. A., Krieger, M., Scott, M. P., Zipursky, S. L. and Darnell, J. 1999. *Molecular Cell Biology* (4th Ed.). New York, NY: W. H. Freeman & Co.

Luque, I., Flores, E., Herrero, A. 1994. Molecular mechanisms for the operation of nitrogen control in cyanobacteria. *EMBO J* **13**: 2862–2869

Macfarlane, G.T., MacFarlane, S., Gibson, G.R. 1992. Synthesis and release of proteases by *Bacteroides fragilis. Curr Microbiol.* **24**: 55–59

Macfarlane, S., Quigley, M. E., Hopkins, M., J., Newton, D. F., and Macfarlane, G. T. 1998 . Polysaccharide degradation by human intestinal bacteria during growth under multi-substrate limiting conditions in a three-stage continuous culture system. *FEMS Microbiol Ecol.* **26:**231-243.

Maheswaran, M. and Forchhammer, K. 2003. Carbon-source-dependent nitrogen regulation in *Escherichia coli* is mediated through glutamine-dependent GlnB signalling. *Microbiol.* **149**: 2163-2172.

Merrick, M. J. and Edwards R. A. 1995. Nitrogen control in bacteria. *Microbiol Revs.* **59:** 604-622.

Miller, R. E., Stadtman, E. R. 1972. Glutamate synthase from *Escherichia coli*: An iron-sulfide flavoprotein. *J Biol Chem.* **247**(22):7407–7419

Moncrief J. S., Obiso Jr., R., Barroso, L.A., Kling, J.J., Wright, R.L., Van Tassell, R.L., Lyerly, D.M., AND Wilkins, T.D. 1995. The enterotoxin of *Bacteroides fragilis* is a metalloprotease. *Inf & Imm.* **63**(1): 175-181

Monnet, C., Mora, D., Corrieu, G. 2005. Glutamine synthesis is essential for growth of *Streptococcus thermophilus* in milk and is linked to urea catabolism. *Appl and Environ Microbiol.* **71**:3376-3378

Muro-Pastor, M. I., Reyes, J. C., and Florencio, F. J. 2001. Cyanobacteria Perceive Nitrogen Status by Sensing Intracellular 2-Oxoglutarate Levels. *J Biol Chem.* **276**(41):38320-38328

Muse, W. B., and Bender, R. A. 1998. The *nac* (Nitrogen Assimilation Control) Gene from Escherichia coli. *J Bacteriol.* **180**: 1166-1173

Muse, W. B., and R. A. Bender. 1999. The amino-terminal 100 residues of the nitrogen assimilation control protein (NAC) encode all known properties of NAC from *Klebsiella aerogenes* and *Escherichia coli. J Bacteriol.* **181**:934-940

Nikolich, M. P., Shoemaker, N. B., and Salyers, A. A. 1992. A Bacteroides tetracycline resistance gene represents a new class of ribosome protection tetracycline resistance. *Antimicrob Agents Chemother.* **36**:1005-1012.

Onderdonk, A. B., Kasper, D. L., Cisneros, R. L. and Bartlett, J. G. 1977. The capsular polysaccharide of *Bacteroides fragilis* as a virulence factor: Comparision of the pathogenic potential of encapsulated and unencapsulated strains. *J Infect Dis.* **136**:82-89

Pateman, J. A. Regulation of synthesis of glutamate dehydrogenase and glutamine synthetase in micro-organisms. *Biochem J.* **115**(4):769–775

Patriarca, E. J., Chiurazzi, M., Manco, G., Riccio, A., Lamberti, A., De Paolis, A., Rossi, M., Defez, R. & Iaccarino, M. 1992. Activation of the *Rhizobium leguminosarum glnII* gene by NtrC is dependent on upstream DNA sequences. *Mol Gen Genet.* **234**: 337-345

Peng, W-T., Wang, J., Wu, T., Huang, J-Q., Chiao, J-S., Zhao, G-P. 2006. Bacterial type I glutamine synthetase of the Rifamycin SV producing *Actinomycete amycolatopsis mediterranei* U32, is the only enzyme responsible for glutamine synthesis under physiological conditions. *Acta Biochim Biophys Sin.* **38** (12): 821-830

Pettersen, E. F., Goddard, T. D. Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. 2004. UCSF Chimera: a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25:**1605-1612.

Plourde-Owobi, L., Seguin, D., Baudin, M.-A., Moste, C., Rokbi, B. 2005. Molecular Characterization of Clostridium tetani Strains by Pulsed-Field Gel Electrophoresis and Colony PCR. *Appl Environ Microbiol.* **71**: 5604-5606

Podglajen, I., Breuil, J., Rohaut, A., Monsempes, C. & Collatz, E. 2001. Multiple mobile promoter regions for the rare carbapenem resistance gene of *Bacteroides fragilis*. *J. Bacteriol.* **183:** 3531–3535

Pomare, E. W., Branch, W. J., Cummings, J. H. 1985. Carbohydrate fermentation in the human colon and its relation to acetate concentration in venous blood. *J Clin Invest.* **75**:1448-1454

Pomposiello, P. J. and Bender, R. A. 1995. Activation of the *Escherichia coli lacZ* promoter by the *Klebsiella aerogenes* nitrogen assimilation control protein (NAC), a LysR family transcription factor. *J Bacteriol.* **177:** 4820-4824

Poole, K. 2001. Multidrug resistance in gram-negative bacteria. *Curr Opin Microbiol.* **4**:500-508.

Privitera, G., Dublanchet, A., Sebald, M. 1979. Transfer of multiple antibiotic resistance between subspecies of *Bacteroides fragilis*. *J Infect Dis.* **139**: 97-101.

Ramasubramanian, T. S., Wei, T. F., Oldham A. K., Golden, J. W. 1996. Transcription of the *Anabaena* sp. strain PCC 7120 *ntcA* gene: multiple transcripts and NtcA binding. *J Bacteriol.* **178**(3):922–926

Reitzer L. J., Magasanik B. 1985. Expression of *glnA* in *Escherichia coli* is regulated at tandem promoters. *Proc Natl Acad Sci* USA. **82**(7):1979–1983

Reitzer L. 2003. Nitrogen assimilation and global regulation in *Escherichia coli*. *Annu Rev Microbiol.* **57**: 155-176.

Reyes, J. C., and Florencio F. J. 1994. A new type of glutamine synthetase in cyanobacteria: the protein encoded by the *glnN* gene supports nitrogen assimilation in Synechocystis sp. strain PCC 6803. *J Bacteriol.* **176**:1260–1267.

Reyes, J. C., Muro-Pastor, M. I., Florencio, F. J. 1997. Transcription of glutamine synthetase genes (*glnA* and *glnN*) from the cyanobacterium *Synechocystis* sp. strain PCC 6803 is differently regulated in response to nitrogen availability. *J Bact* **179**: 2678–2689 (1997).

Robinson, P., Neelon, K., Schreier, H.J., Roberts, M., F. 2001. Beta-glutamate as a substrate for glutamine synthetase. *Appl Environ Microbiol.* **67**(10): 4458-63

Rocha, E. R., and Smith, C. J.. 1995. Biochemical and genetic analyses of a catalase from the anaerobic bacterium *Bacteroides fragilis*. *J Bacteriol.* **177**(11): 3111–3119

Rocha, E. R., Selby, T., Coleman, J. P. and Smith, C. J. 1996. The oxidative stress response in an anaerobe, *Bacteroides fragilis*: a role for catalase in protection against hydrogen peroxide. *J Bacteriol.* **178:**6895-6903

Rocha, E. R., and C. J. Smith. 1999 . Role of the alkyl hydroperoxide reductase (*ahpCF*) gene in oxidative stress defense of the obligate anaerobe *Bacteroides fragilis*. *J Bacteriol.* **181:**5701-5710.

Rodloff, A. C., Becker, J., Blanchard, D., Klein, T. W., Hahn, H., Friedman, H. 1986. Inhibition of macrophage phagocytosis by *Bacteroides fragilis* in vivo and in vitro. *Infect Immun.* **52**: 488-492

Salyers, A. A., Bonheyo G., and Shoemaker, N. B. 2000. Starting a new genetic system: lessons from Bacteroides. *Methods.* **20**:35–46.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sanger, F., Nicklen, S. and Coulson, S. 1977. DNA sequencing with chainterminating inhibitors. *Proc Natl Acad Sci USA.* **74**: 5463 -- 5467.

Sauer, J., Dirmeier, U., Forchhammer, K. 2000. The Synechococcus Strain PCC 7942 *glnN* Product (Glutamine Synthetase III) Helps Recovery from Prolonged Nitrogen Chlorosis. *J Bacteriol.* **182**: 5615-5619

Schurke, P., Freeman, J., Dabrowski, M. J. and Atkins, W. M. 1999. Metal-dependent self-assembly of protein tubes from *E. coli* glutamine synthetase: Cu^{2+} EPR studies of the ligation and stoichiometry of intermolecular metal binding sites. *J Biol Chem.* **274**: 27963-27968

Schwacha, A., and Bender, R. A. 1993. The *nac* (nitrogen assimilation control) gene from *Klebsiella aerogenes*. *J Bacteriol* **175**:2107-2115

Shapiro, B.M., Kingdon, H.S., Stadtman, E.R. 1967. Regulation of glutamine synthetase. VII. Adenylyl glutamine synthetase: a new form of the enzyme with altered regulatory and kinetic properties. *Proc Natl Acad Sci U S A.* **58**(2): 642–649.

Shoemaker, N. B., Barber, R. D., and Salyers, A. A. 1989. Cloning and characterization of a *Bacteroides* conjugal tetracycline-erythromycin resistance element by using a shuttle cosmid vector. *J Bacteriol.* **171:** 1294-1302

Silman, N. J., Carr, N. G. and Mann, N. H. 1995. ADP-ribosylation of glutamine synthetase in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J of Bacteriol. **177**; 3527-3533

Simon, R., Priefer, U. and A. Pühler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Tech. **1**:784-791

Smith, C. J., M. B. Rogers, and M. L. McKee. 1992. Heterologous gene expression in *Bacteroides fragilis*. *Plasmid* **27**:141-154.

Southern J. A., Parker J. R., and Woods D. R. 1986. Expression and purification of glutamine synthetase cloned from *Bacteroides fragilis*. *J Gen Microbiol.* **132**(10):2827-35.

Southern, J. A., Parker, J. R., and Woods, D. R. 1987 Novel structure properties and inactivation of glutamine synthetase cloned from *Bacteroides fragilis*. *J Gen Microbiol.* **133**: 2437-2446.

Staden, R. 1982. An interactive graphics package for comparing and aligning nucleic acid and amino acid sequences. *Nucleic Acid Res* **10**, 2951-2961.

Stadtman, E. R. (2001) The story of glutamine synthetase regulation. *J Biol Chem.*; **276**, 44357–44364

Stock J. B., Ninfa A. J., Stock A. M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol Rev.*; **53**(4):450–490.

Su, Z., Olman, V., Mao, F., Xu, Y. 2005. Comparative genomics analysis of NtcA regulons in cyanobacteria: regulation of nitrogen assimilation and its coupling to photosynthesis. *Nucleic Acids Res.* **33**: 5156–5171

Tancula, E., Feldhaus, M. J., Bedzyk, L. A. and Salyers, A. A. 1992. Location and characterization of genes involved in binding of starch to the surface of *Bacteroides thetaiotaomicron*. *J Bacteriol.* **174**: 5609-5616.

Tang, Y. P., Dallas, M. M., and Malamy, M. H. 1999. Characterization of the Batl (Bacteroides aerotolerance) operon in Bacteroides fragilis: isolation of a *B. fragilis* mutant with reduced aerotolerance and impaired growth in in vivo model systems. *Mol Microbiol.* **32**(1):139-49

Tanigawa, R., Shirokane, M., Maeda, S.-i., Omata, T., Tanaka, K., Takahashi, H. 2002. Transcriptional activation of NtcA-dependent promoters of Synechococcus sp. PCC 7942 by 2-oxoglutarate invitro. *Proc Natl Acad Sci USA.* **99**: 4251-4255

Tierney, M.B. and Lamour, K.H. 2005. An Introduction to Reverse Genetic Tools for Investigating Gene Function. *The Plant Health Instructor.* DOI: 10.1094/PHI-A-2005-1025-01.

Toukdarian, A. & Kennedy, C. 1986. Regulation of nitrogen metabolism in *Azotobacter vinelandii*: isolation of *ntr* and *glnA* genes and construction of *ntr* mutants. *EMBO J.* **5**: 399–407

Tullius, M. V., Harth, G. and Horwitz, M. A. 2003. Glutamine synthetase *GlnA*1 is essential for growth of Mycobacterium tuberculosis in human THP-1 macrophages and guinea pigs. *Inf and Imm.* **71** (7): 3927-3936

Tzianabos, A. O., Onderdonk, A. B., Rosner, B., Cisneros, R. L. and Kasper, D. L. 1993. Structural features of polysaccharides that induce intra abdominal abscesses. *Science* **262**:416-419.

Tzianabos, A. O., Kasper D. L., Cisneros, R. L., Smith, R. S. and Onderdonk, A. B. 1995. Polysaccharide-mediated protection against abscess formation in experimental intra-abdominal sepsis. *J Clin Invest.* **96**(6): 2727–2731.

Tzianabos, A. O. (2000). Polysaccharide Immunomodulators as Therapeutic Agents: Structural Aspects and Biologic Function. *Clin Microbiol Rev.* **13:** 523-533

Ueda, O., Wexler, H. M., Hirai, K., Shibata, Y., Yoshimura, F., Fujimura, S. 2005. Sixteen Homologs of the Mex-Type Multidrug Resistance Efflux Pump in *Bacteroides fragilis*. *Antimicrob Agents Chemother.* **49**: 2807-2815

Valladares, A., Muro-Pastor, A. M., Herrero, A., Flores, E. 2004. The NtcA-Dependent P1 Promoter Is Utilized for glnA Expression in N2-Fixing Heterocysts of Anabaena sp. Strain PCC 7120. *J Bacteriol.* **186**: 7337-7343

van der Woude, M. W. 2006. Re-examining the role and random nature of phase variation. *FEMS Microbiol Lett.* **254**: 190-197

van Rooyen, J. M., Abratt, V. R., Sewell, B. T. 2006. Three-dimensional structure of a type III glutamine synthetase by single-particle reconstruction. *J Mol Biol.* **361**(4): 796-810

Van Tassell, R. L. and Wilkins, T. D. 1978. Isolation of auxotrophs of *Bacteroides fragilis*. *Can J Microbiol.* **24**: 1619-1621.

Vigliotta, G., Tredici, S. M., Damiano, F., Montinaro, M. R., Pulimeno, R., di Summa, R., Massardo, D. R., Gnoni, G. V. and Alifano, P. 2005. Natural merodiploidy involving duplicated *rpoB* alleles affects secondary metabolism in a producer actinomycete. *Mol Microbiol.* **55**:396-412

Webb, M. R. 1992. A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. *Proc Natl Acad Sci USA.* **89;** 4884-4887.

Wehnert, G. U., Abratt, V. R., Goodman, H. J. K. and Woods, D. R. 1990. Cloning of *Bacteroides fragilis* plasmid genes affecting metronidazole resistance and ultraviolet survival in *Escherichia coli*. *Plasmid* **23**:155-158

Wen, Z. T., Peng L., and Morrison M. 2003. The glutamine synthetase of *Prevotella bryantii* B(1)4 is a family III enzyme (GlnN) and glutamine supports growth of mutants lacking glutamate dehydrogenase activity. *FEMS Microbiol Lett.* **229**(1):15-21.

Wray, L.V.,Jr, Ferson, A. E., Rohrer, K. and Fisher, S. H. 1996. TnrA, a transcriptional factor required for global nitrogen regulation in *Bacillus subtilis*. *Proc Natl Acad Sci. (USA)* **93**: 8841-8845.

Wray, L Atkinson, M., Fisher, S. H. Identification and cloning of the *glnR* locus, which is required for transcription of the *glnA* gene in *Streptomyces coelicolor* A3(2). *J Bacteriol.* **173**: 7351-7360

Yang, Y., Rasmussen, B. A. and Bush, K. 1992. Biochemical characterization of the metallo-β-lactamase CcrA from *Bacteriodes fragilis* TAL-3636. *Antimicrob Agents Chemother.* **36:**1155-1158

Yamamoto, I., Abe, A., Saito, H. and Ishimoto, M. 1984. The pathway of ammonia assimilation in *Bacteroides fragilis. J Gen Appl Microbiol.* **30:**499–508.

Yamamoto I., Saito H., and Ishimoto M. 1987. Regulation of synthesis and reversible inactivation in vivo of dual coenzyme-specific glutamate dehydrogenase in *Bacteroides fragilis. J Gen Microbiol.* **133**: 2773-2780.

Zgurskaya, H.I. and Nikaido, H. 2000. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol. Microbiol.* **37**: 219–225