Expression and Purification of Glutamine Synthetase Cloned from Bacteroides fragilis

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A glutamine synthetase (GS) gene, glnA, from Bacteroides fragilis was cloned on a recombinant plasmid pJS139 which enabled Escherichia coli glnA deletion mutants to utilize (NH₄)₂SO₄ as a sole source of nitrogen. DNA homology was not detected between the B. fragilis glnA gene and the E. coli glnA gene. The cloned B. fragilis glnA gene was expressed from its own promoter and was subject to nitrogen repression in E. coli, but it was not able to activate histidase activity in an E. coli glnA ntrB ntrC deletion mutant containing the Klebsiella aerogenes hut operon. The GS produced by pJS139 in E. coli was purified: it had an apparent subunit M₉ of approximately 75000, which is larger than that of any other known bacterial GS. There was very slight antigenic cross-reactivity between antibodies to the purified cloned B. fragilis GS and the GS subunit of wild-type E. coli.

INTRODUCTION

The medically important obligate anaerobe Bacteroides fragilis is under investigation with regard to its genetic constitution, particularly the potential for the transfer of genetic material to the other microbial inhabitants of the mammalian gut. There has been some doubt regarding the possibility of expression of Bacteroides genes in other bacteria following a report by Guiney et al. (1984) which indicated that the ampicillin resistance gene from Escherichia coli was not expressed in B. fragilis and that the clindamycin resistance gene from the B. fragilis plasmid was not expressed in E. coli. However, the cloning and expression in E. coli of the fimbrial sub-unit protein, pilin protein, cellulase and chondroitin lyase genes from Bacteroides nodosus, Bacteroides succinogenes and Bacteroides thetaiotaomicron have been reported (Anderson et al., 1984; Crosby & Collier, 1984; Elleman & Hoyne, 1984; Guthrie et al., 1985).

Since B. fragilis is the anaerobic species most frequently isolated from human infections (Kasper et al., 1979) we investigated the expression of its genes in E. coli. Glutamine synthetase (GS) is a central enzyme in the pathways of nitrogen metabolism for many bacteria and as its presence in B. fragilis was expected a gene library prepared from B. fragilis strain BF-1 was screened for expression of GS.

In the Enterobacteriaceae the structural gene for GS, glnA, is regulated by the products of the ntrB and ntrC genes which are linked to the glnA gene, and by the product of the unlinked ntrA gene (Garcia et al., 1977; Kustu et al., 1979; Pahel & Tyler, 1979; McFarland et al., 1981; Magasanik, 1982). The ntr genes also regulate other operons involved in nitrogen metabolism.

GS from several bacterial genera has been purified utilizing the large size and heat resistance of the holoenzyme to separate it from other intracellular macromolecules (Streicher & Tyler, 1980; Bodasing et al., 1985). We report the use of polyethylene glycol (PEG) and column chromatography for the purification of the cloned GS and describe the production of specific antibodies in rabbits.

Abbreviation: GS, glutamine synthetase.

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them. The cells were collected by centrifugation, resuspended in 0.2 ml 0.15 M-NaCl and stored at -60°C.

The method of Smith et al. (1971) was used to permeabilize exponential-phase cells (2 ml) with 0.5 ml toluene to

nitrogen sources (Ntr+ phenotype) was determined by growth on minimal medium containing glutamine (0.5 mM)

sizes, and was then fractionated on a sucrose density gradient. DNA was precipitated from those fractions which

3-10 kb. Plasmid pEcoR251 was digested to completion with BglII, precipitated with ethanol and redissolved in TE buffer.

were shown by agarose gel electrophoresis to contain fragments of 3-10 kb. Plasmid pEcoR251 was digested to

EDTA buffer at pH 7.9 (TE buffer).

Construction of the B. fragilis genomic library and selection of the GS gene. B. fragilis DNA was digested with

Preparation of DNA. Plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowitz & Burke (1981); B. fragilis chromosomal DNA was prepared as follows. Bacterial cells collected from 100 ml of a late-exponential-phase culture (about 10^9 cells ml^-1) were suspended in 5 ml 0.01 M-Tris/HCl buffer (pH 7.8) containing 10 mM-EDTA and 80 mM-glucose. Streptomyces griseus protease (Merck) (10 mg) and 100 mg SDS were added and gently mixed to dissolve. The resulting cell lysate was extracted with a phenol/chloroform/amyl alcohol (50:49:1 by vol.) mixture (Marmur, 1961), to remove proteins and lipids, until the supernatant was clear. After precipitation with an equal volume of 2-propanol, the pellet was redissolved in 5 ml 10 mM-Tris/HCl, 1 mM-EDTA buffer at pH 7.9 (TE buffer).

Bacterial strains and plasmids. B. fragilis strain BF-1 was described by Mossie et al. (1979). The plasmid pEcoR251 was a gift from M. M. Zabeau, Plant Genetic Systems, Ghent, Belgium. This positive selection vector contains the E. coli EcoRI gene under the control of the λ rightward promoter, the ampicillin (Ap) resistance gene and the pBR322 origin of replication. It was derived from the pCL plasmids described by Zabeau & Stanley (1982). The EcoRI gene product, expressed at high levels by the λ promoter on pEcoR251, is lethal unless insertionally inactivated or regulated by plasmid pC1857, which contains a temperature-sensitive λ repressor gene (Remaut et al., 1983). The EcoRI gene has a single BglII restriction enzyme site which can be used for the insertion of DNA digested with BglII, BamHI or Sau3AI. The E. coli strains used are described in Table 1.

B. fragilis was grown under anaerobic conditions in a complex medium which contained (g l^-1) Difco tryptic soy broth (24), Difco yeast extract (10), glucose (1) and L-cysteine hydrochloride (0.5) (Southern et al., 1984). The E. coli strains used are described in Table 1.

METHODS

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**Cloned B. fragilis glutamine synthetase gene**

Cloning of the B. fragilis GS gene in E. coli

A library of B. fragilis DNA was established in E. coli HB101 by insertional inactivation of the EcoR1 gene of pEcoR251. Plasmid DNA prepared from pools of clones (about 10000) containing B. fragilis DNA was used to transform the E. coli YMC-11 GlnA⁻ NtrB⁻ NtrC⁻ deletion strain. An E. coli YMC-11 GlnA⁺ transformant was isolated on minimal medium. The transformant contained a recombinant pEcoR251 plasmid.

The presence of a GS structural gene on this plasmid was confirmed by retransformation of E. coli YMC-11 GlnA⁻ NtrB⁻ NtrC⁻ and E. coli ET8051 GlnA⁻ NtrB⁻ NtrC⁻ deletion strains. After transformation approximately equal numbers of colonies were obtained on minimal medium (GlnA⁺) and Luria medium containing Ap (Ap⁺). This recombinant plasmid was designated pJS139. Digestion of pJS139 with Psrl indicated that an approximately 8 kb DNA fragment was inserted into pEcoR251 (Fig. 1).

**RESULTS**

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Fig. 1. Partial restriction and deletion map of pJS139. 1, Scale (kb); 2, pJS139 AprGlnA+; 3, pEcoR251 Apr; 4, B. fragilis fragment; 5, pJS139.1 Ap'GlnA- (XhoI deletion); 6, pJS139.2 Ap'GlnA+ (StuI deletion).

Fig. 2. Hybridization of 32P-labelled pJS139 with B. fragilis and E. coli DNA. (a) Ethidium-bromide-stained agarose gel electrophoresis of restriction digests of plasmid and chromosomal DNA: lane 1, pJS139-XhoI; 2, λ-PstI; 3, E. coli HB101-XhoI; 4, B. fragilis BF-1-XhoI; 5, λ-BstEII. (b) Autoradiograph of a Southern blot of the gel in (a): lanes 1 and 2, 24 h exposure; lanes 3, 4 and 5, 48 h exposure. The hybridization probe was 32P-labelled pJS139, to which 32P-labelled λ DNA was added as an internal reference.

Characterization of the recombinant plasmid pJS139

The restriction map of pJS139 was obtained by complete single or double digestions with restriction endonucleases (Fig. 1). The location of the DNA region determining the GlnA+ phenotype was determined by the isolation of pJS139 deletion plasmids. The GS gene was located within a 4.2 kb fragment of the 8 kb insert, which was close to the junction of the B. fragilis DNA insert and the pEcoR251 DNA. Excision of a 2.7 kb fragment of DNA from this region using XhoI abolished the GlnA+ phenotype (pJS139.1), while excision of a 4.0 kb plasmid fragment bounded by StuI restriction sites (pJS139.2) did not cause the loss of the GlnA+ phenotype.

The origin of the 8 kb insert in pJS139 was determined by Southern blotting and DNA hybridization between B. fragilis chromosomal DNA and 32P-labelled pJS139 (Fig. 2).
Cloned B. fragilis glutamine synthetase gene

Table 2. Relative levels of GS and histidase activity

Strains were grown in CSH glucose minimal medium (Miller, 1972) with 15 mM-glutamate as nitrogen source and then diluted in minimal medium supplemented as follows: H, 15 mM-glutamate, 15 mM-glutamine; L, 15 mM-glutamate, 0.15 mM-glutamine; N, 1 g (NH₄)₂SO₄ l⁻¹ in CSH minimal medium; F, nitrogen-free CSH minimal medium. Samples were assayed after 3 h incubation at 37 °C in shake flasks. Histidase activity is defined in Methods. GS activity is expressed as pmol γ-glutamyl hydroxamate formed min⁻¹ (mg protein)⁻¹. SEM values were 5–10% of the values in the Table.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>GS activity</th>
<th>Histidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td>YMC-10 GlnA⁺ Ntr⁺</td>
<td>0.08</td>
<td>1.24</td>
<td>1.1</td>
</tr>
<tr>
<td>YMC-11 glnA ntr</td>
<td>0.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>YMC-11 pJS139 GlnA⁺ ntr</td>
<td>0.05</td>
<td>1.25</td>
<td>4.15</td>
</tr>
</tbody>
</table>

Plasmid pJS139 has two XhoI fragments of approximately 9.3 and 2.7 kb. The 2.7 kb fragment is internal to the putative B. fragilis glnA insert, but is adjacent to the junction between the vector and insert DNA. Therefore ³²P-labelled pJS139 was hybridized to both B. fragilis chromosomal DNA and pJS139 digested with XhoI: a positive 2.7 kb hybridization signal was detected in both digests (Fig. 2a, lanes 2 and 5). A second band of hybridization of approximately 10 kb was also observed. No DNA hybridization was detected between ³²P-labelled pJS139 and a XhoI digest of chromosomal DNA from the E. coli HBlOl glnA+ strain.

Regulation of the cloned GS activity

GS activity was readily detectable in E. coli YMC-11 containing pJS139 and E. coli YMC-10 grown under nitrogen-limiting conditions. While no GS activity was detected in extracts of E. coli YMC-11 or ET8051 grown under any conditions (Table 2).

GS activity in E. coli YMC-11 containing pJS139 was partly repressed by ammonium salts but was fully repressed by glutamate and glutamine. GS activity of the E. coli YMC-10 wild-type strain was partly repressed by glutamate and ammonium salts but was fully repressed by glutamine (Table 2).

In E. coli and Salmonella typhimurium the ntrB and ntrC genes, which are closely linked to the GS gene, regulate not only the GS gene but also a high-affinity glutamine and a high-affinity arginine transport system (Kustu et al., 1979). These ntr genes also activate the histidine utilization operon (Magasanik, 1982).

The ability of the various E. coli strains to grow on minimal medium containing low concentrations of arginine and glutamine was determined: while the ET8051 and YMC-11 glnA deletion strains were unable to grow on these media, when these strains contained the recombinant plasmid pJS139 very weak growth was obtained. In comparison, the YMC-10 glnA⁺ ntrB⁺ ntrC⁺ strain grew well under these conditions.

The E. coli strains YMC-10 and YMC-11 carry a Klebsiella aerogenes hut operon (Tuli et al., 1982) that has a hutC mutation resulting in high basal levels of histidase. The results in Table 2 show that this enzyme level was increased in the YMC-10 strain by the ntrB and ntrC coregulation of the hut operon (Tuli et al., 1982) in low concentrations of glutamine, but that this was not observed with the YMC-11 glnA ntrB ntrC or the YCM-11(pJS139) glnA+ strains. Increased levels of histidase were observed with all strains when cells were suspended in nitrogen-free minimal medium.

The β-lactamase activities of E. coli YMC-11(pJS139) grown in minimal medium with and without glutamine (15 mM) were similar (35.1 and 37.8 units mg⁻¹ respectively).

Purification of the cloned GS gene product

The specific activity of GS increased during the purification which resulted in a 280-fold purification of the enzyme (Table 3). GS activity was not precipitated by 4% (w/v) PEG under
Fig. 3. SDS-PAGE of cell extracts and purified, cloned GS from E. coli cells. The gel contained 10% (w/v) polyacrylamide and was 0.5 mm thick. After electrophoresis as described in Methods it was stained with Coomassie brilliant blue. The samples were reduced and treated with SDS to remove secondary and tertiary structures. Lane 1, M, markers; lane 2, E. coli YMC-10 cell lysate (20 μg); lane 3, E. coli YMC-11 cell lysate (10 μg); lane 4, E. coli YMC-11(pJS139) cell lysate (20 μg); lane 5, peak GS activity fraction from Sephacryl S-1000 (~1 μg).

Table 3. Purification of the cloned B. fragilis GS enzyme from E. coli YMC-11(pJS139)

Total GS activity was determined by the γ-glutamyl transferase assay. The details of the purification and assay procedures are described in Methods. GS activity is expressed as μmol γ-glutamyl hydroxamate min⁻¹. Fraction 1 and fraction 5 correspond to lanes 3 and 5 of Fig. 3.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml)</th>
<th>Total protein (mg)</th>
<th>Total GS activity (per mg protein)</th>
<th>Specific GS activity (per mg protein)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ultrasonic extract</td>
<td>25</td>
<td>175</td>
<td>62.5</td>
<td>0.36</td>
<td>1</td>
</tr>
<tr>
<td>2. 4% PEG supernatant</td>
<td>25</td>
<td>152.5</td>
<td>59.4</td>
<td>0.39</td>
<td>1.09</td>
</tr>
<tr>
<td>3. 6% PEG supernatant</td>
<td>25</td>
<td>131</td>
<td>10.2</td>
<td>0.08</td>
<td>0.22</td>
</tr>
<tr>
<td>4. 6% PEG precipitate</td>
<td>1.5</td>
<td>36</td>
<td>133.9</td>
<td>3.72</td>
<td>10.41</td>
</tr>
<tr>
<td>5. S-1000 peak</td>
<td>1.0</td>
<td>0.7</td>
<td>71</td>
<td>101.4</td>
<td>284.3</td>
</tr>
</tbody>
</table>

these conditions but was precipitated by 6% (w/v) PEG. It was necessary to increase the pH of the solution in which the 6% PEG pellet was resuspended to pH 8.0 to dissolve the pellet. An increase in total GS activity following 6% PEG precipitation was observed.

SDS-PAGE of cell lysates showed an increase in the M, 75–90000 protein content of crude cell extracts of E. coli YMC-11(pJS139), but not in extracts from E. coli YMC-10 wild-type or YMC-11 GlnA⁻ strains. This is the protein which is purified after Sephacryl chromatography (Fig. 3). Electrophoresis in 8% SDS-PAGE showed the apparent M, of this polypeptide to be approximately 75 000.

Rabbit antibodies to this protein gave a single band in an Ouchterlony plate and showed homology to a single protein in a crude extract of the E. coli YMC-11(pJS139) strain, but no cross-reaction with cell extracts from E. coli YMC-10 GlnA⁺ or YMC-11 GlnA⁻ was observed.
Cloned B. fragilis glutamine synthetase gene

Western blot analysis showed a weak cross-reactivity between the antiserum prepared against the purified cloned B. fragilis GS and a polypeptide of the same M, as E. coli GS.

DISCUSSION

A cloned B. fragilis glnA gene was shown to function in E. coli GlnA− deletion mutants: this is the first report of the expression of a gene from the medically important, obligate anaerobe, B. fragilis in E. coli.

The origin of the glnA gene was confirmed by DNA hybridization. The gene directed the synthesis in E. coli glutamine auxotrophs of a B. fragilis GS which was enzymically active.

The cloned GS gene appeared to be expressed from a promoter contained within the inserted B. fragilis DNA segment of pJS139 as it is unlikely that a plasmid promoter would be regulated by nitrogen levels. In addition, E. coli YMC-11(pJS139) containing pC1857 Kan' and λ-repressor(ts), produced the same levels of GS per mg of protein when incubated in nitrogen-free minimal medium at 23 °C and 39 °C, which indicated that the cloned B. fragilis glnA gene was not expressed from the rightward λ-promoter present on the vector.

The purified B. fragilis GS subunit had an apparent M, of approximately 75000. Assuming that the enzyme is the typical dodecamer, then the particle M, for the undissociated GS would be approximately 900000, a value larger than that reported for other bacteria. E. coli, Bacillus subtilis, Rhizobium japonicum and Vibrio alginolyticus have subunits with M, values of 50000, 56000, 60000 and 60000 respectively (Stadtman & Ginsberg, 1974; Deuel et al., 1970; Bhandari et al., 1983; Bodasing et al., 1985).

DNA hybridization experiments indicated that there was less than 50% DNA homology between the B. fragilis glnA gene and the E. coli glnA gene. Although no cross-reactivity was detected by the Ouchterlony immunoprecipitation, Western blotting detected weak cross-reactivity between antiserum raised to the purified cloned B. fragilis GS and E. coli GS. Lack of DNA homology but antigenic cross-reactivity was reported for the glnA gene cloned from the Gram-negative chemolithotroph Thiobacillus ferrooxidans and the E. coli glnA gene and GS (Barros et al., 1985, 1986). These observations extend the findings that GS enzymes from Gram-negative bacteria are antigenically related (Tronick et al., 1973).

The B. fragilis glnA gene was subject to nitrogen repression in E. coli. The regulation by nitrogen affected the glnA gene and was not due to an increase in plasmid copy number as a result of growth in the different media. The levels of another plasmid gene product, β-lactamase, produced by E. coli YMC-11(pJS139) and assayed after growth on the different nitrogen media, did not show any variation [35–38 U (mg protein−1)] in the samples assayed.

The regulation of the cloned B. fragilis glnA gene differed from the regulation of the E. coli glnA gene in the wild-type strain: the cloned B. fragilis glnA gene was repressed by glutamate whereas the E. coli glnA gene was expressed in the presence of glutamate. Since the glnA gene on pJS139 was regulated by nitrogen it is suggested that some nitrogen regulatory activities are located on pJS139 but that these activities are not directly analogous to the ntrB and ntrC system of the enteric bacteria.

In comparison with the E. coli wild-type strain the cloned B. fragilis DNA fragment only enabled weak growth of the E. coli glnA ntrB ntrC deletion mutant on media containing arginine or low concentrations of glutamate as sole sources of nitrogen and it was not able to activate the Klebsiella hut operon in E. coli YMC-11.

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REFERENCES


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