A Glutamine Transport Gene, glnQ, Is Required for Fibronectin Adherence and Virulence of Group B Streptococci

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Group B streptococci (GBS) are a leading cause of neonatal sepsis and meningitis. GBS adhere to fibronectin when it is attached to a solid phase. We isolated a Tn917 transposon mutant, COH1-GT1, which shows decreased adherence to fibronectin. COH1-GT1 also shows decreased adherence to and invasion of respiratory epithelial cells in vitro and decreased virulence in vivo. COH1-GT1 contains a Tn917 insertion in a homolog of glnQ, a gene from Escherichia coli which is required for glutamine transport and codes for a cytoplasmic ATP-binding cassette protein. To confirm that the decreased fibronectin adherence of COH1-GT1 was due to the mutation in glnQ, we constructed COH1-GT2, a strain with a nonpolar site-directed mutation in glnQ. COH1-GT2 showed decreased binding to fibronectin. We also demonstrated that complementation of glnQ in trans restored fibronectin adherence to COH1-GT1. COH1-GT1 shows decreased uptake of radiolabeled glutamine and is resistant to the toxic glutamine analog γ-1-glutamylhydrazide, demonstrating that the glnQ gene is required for glutamine transport in GBS. glnQ lacks a signal sequence and is a cytoplasmic protein in E. coli and thus is unlikely to act as a fibronectin adhesin. glnQ is transcribed in an operon with a putative glutamine permease gene, glnP, which has a novel predicted structure containing three distinct domains linked in a single gene. The first two domains are putative glutamine binding domains with homology to the E. coli periplasmic glutamine binding gene glnH. The third is a putative permease domain with homology to the E. coli glutamine permease gene glnP. RT-PCR analysis demonstrated that glnP and glnQ are contained within a single transcript. Transcription of scpB, encoding the only known fibronectin-binding adhesin of GBS, is unaffected. We speculate that glnQ may regulate expression of fibronectin adhesins by affecting cytoplasmic glutamine levels and that regulation may be posttranscriptional.

Group B streptococci (GBS) are a leading cause of neonatal sepsis and meningitis in neonates in the United States and western Europe (2) and are emerging pathogens in immunocompromised adults (8). Infection in newborns is preceded by maternal colonization in the rectum and/or vagina. Infection of the fetus may occur after ascent of the GBS to the amniotic cavity or through aspiration of vaginal contents during parturition. Following colonization of the infant, GBS disease usually manifests as pneumonia, suggesting that the primary portal of entry is the lung (29).

Adherence of bacterial pathogens to epithelial cells is an important virulence factor for many pathogens. Adherence of GBS to epithelial cells may allow GBS to persist in the rectum and vagina of the mother and the pulmonary epithelium of the infant in the face of host bulk flow defense mechanisms and may also play a role in the invasion of pulmonary epithelial cells (23). Putative receptors on epithelial cells to which GBS may adhere include fibronectin (28), laminin (24), and cyto-keratins (27). The adhesins expressed by GBS that mediate attachment to these host molecules are poorly defined. A laminin binding protein has been identified (24), although a role of this protein in virulence has yet to be defined. Streptococcal CSa peptidase has recently been shown to act as a fibronectin adhesin (3a). No genetic factors that mediate adherence of GBS to cyto-keratin have yet been identified.

In this study, we attempted to identify genes required for adherence to fibronectin by isolating Tn917 transposon mutants with decreased adherence to fibronectin. One mutant, COH1-GT1, showed a >90% decrease in adherence to fibronectin. Genetic analysis revealed that the gene mutated in COH1-GT1, glnQ, is a glutamine transport gene that is required both for adherence to fibronectin in vitro and for virulence in vivo.

MATERIALS AND METHODS

Bacterial strains and plasmids, epithelial cells, media, and growth conditions. The characteristics of the bacterial strains and plasmids used are described in Table 1. GBS were grown in Todd-Hewitt broth (THB) as previously described (26). E. coli strains were grown in Luria-Bertani medium. A549 respiratory epithelial cells were cultured as described previously (26). For γ-1-glutamylhydrazide (GGH) sensitivity testing, bacteria were grown on medium containing M9 salts, 1% glucose, 0.1% yeast extract, and 1.5% Bacto Agar, which had been autoclaved. Glucose and GGH were then added from stocks sterilized by filtration through a 0.2-μm-pore-size nitrocellulose filter. Strains containing plasmids were cultured in medium with erythromycin or chloramphenicol at 5 μg/ml.

Selection of transposon mutants with decreased adherence to fibronectin. Tn917 mutants of COH1, a wild-type GBS clinical isolate from a neonate with sepsis, were isolated as previously described (10). Individual mutants were grown on Todd-Hewitt agar (THA) plates. The plates were swabbed and resuspended in THB, and the pool was diluted in THB to an optical density at 600 nm of 0.1 to 0.2. The culture was grown at 37°C to an optical density at 600 nm of 0.5 to 0.6. A 0.1-ml aliquot of bacteria was pelleted, washed three times with phosphate-buffered saline (PBS), resuspended in 5% bovine serum albumin (BSA fraction V; Sigma, St. Louis, Mo.), and then incubated on ice for 2 h. A 50-μl aliquot of this suspension was placed in a microtiter plate that had been coated with fibronectin at 10 μg/ml as previously described (28). The plate was centrifuged...
for 5 min at 4°C and 3,000 rpm 

for 5 min at 4°C and 3,000 rpm (1,800 × g) in a plate spinner (Jouan Instruments), and the nonadherent bacteria were recovered by shaking the plate on a Vortex Genie 2 (VWR Scientific, South Plainfield, N.J.) vortexer at level 2 for 10 s. Nonadherent bacteria were then plated in an adjacent well of the plate, and the supernatants were aspirated as described above. The plate was then centrifuged at 2,000 rpm (800 × g) for 10 min, and a determination was made that no putative terminators were present. Open reading frames were considered potentially hypoadhaptoric to fibronectin if binding was decreased by >50%. The adherence of these putative hypoadhaptoric clones was then confirmed as having fibronectin adherence in duplicate at a variety of fibronectin concentrations as described previously (28).

Adherence and invasion of AS49 epithelial cells. Adherence to AS49 cells was performed as described previously (26). Briefly, radiolabeled GBS were resuspended in 5% BSA in PBS and added to a 96-well plate containing confluent AS49 monolayers blocked with 5% BSA in PBS. The plate was centrifuged and washed as described above, adherent bacteria were removed with 2 N sodium hydroxide at 65°C for 20 min, and radioactivity was quantitated as described above.

Invasion assays were performed as described previously (23). Briefly, various dilutions of log-phase GBS were added to a 24-well plate containing confluent AS49 monolayers and centrifuged as described above. Bacteria were allowed to invade for 2 h at 37°C. The monolayers were washed with PBS, and adherent extracellular bacteria were killed with gentamicin (100 μg/ml) and penicillin (5 μg/ml) for 2 h at 37°C. AS49 cells were released with 0.1 ml of trypsin-EDTA (Sigma) for 10 min at 37°C and lysed with 0.4 ml of 0.025% Triton in water. The bacteria were quantitated by plating them on THA. Input CFU were quantitated by plating dilutions of the input on THA.

Nucleotide sequencing and DNA analysis. PCRs were performed under standard conditions with Taq polymerase (Promega Corp., Madison, Wis.), and the products were purified using the Qiagen (Valencia, Calif.) system. The nucleotide sequences of plasmids and purified PCR products were determined using fluorescent dye terminator sequencing reactions (Ready Reaction sequencing kit; Perkin-Elmer Cetus, Foster City, Calif.) according to the manufacturer's specifications and analyzed on an Applied Biosystems 373A automated sequencer. Both strands of each DNA template were sequenced using a series of reverse primers. Nucleotide sequences were analyzed using the Genetics Computer Group software and DNA Strider software. Homology searches were performed using the BLAST server at the National Center for Biotechnology Information (1).

Nucleotide sequence analysis of the Tn917 insertion site from COH1-GT1. Initial identification of the insertion site in COH1-GT1 was performed by sequencing PCR products generated by ligation PCR (20). Initial restriction mapping of the Tn917 insertion site by Southern blot analysis (data not shown) demonstrated that there was a HindIII site approximately 2 kb 5' of the Tn917 insertion and an EcoRI site approximately 2 kb 3' of the Tn917 insertion. Genomic COH1-GT1 DNA and plasmid DNA from pBluescript SK (+) (Stratagene) was digested with HindIII or EcoRI and ligated, and PCR was performed using the M13 primer (Stratagene) and externally oriented Tn917 primers. A primer from the 5' end of Tn917 (AATGTACAAATAACAGCGAA) was used with the HindIII-digested DNA, and a primer from the 3' end of Tn917 (TAG GCTTTGAAACATTGTT) was used with EcoRI-digested DNA. PCR products were sequenced using the above-mentioned primers. Using this preliminary sequence information, primers were synthesized from near the HindIII site 5' of the Tn917 insertion (GCTTACGCAATACGGAAGCCG) and from near the EcoRI site 3' of the Tn917 insertion (ATTCGTTCCATTCATTACGG CGACAAT). A 4-kb PCR product containing the Tn917 insertion region was generated using genomic wild-type COH1 DNA as a template. This was purified and cloned into pT7Blue (Novagen Inc., Madison Wis.), generating the plasmid pJAP8. The insert in this plasmid was sequenced as described above. Additional sequence 5' of the region cloned in pJAP8 was obtained by sequencing PCR products obtained using arbitrarily primed PCR (18). To eliminate the possibility that errors might have been introduced into the PCR product cloned into pJAP8, PCR products generated directly from genomic COH1 DNA were also sequenced as described above and gave identical results. Potential ribosome bind- ing sites were identified as having four out of seven of the sequence AGGAGGA, with at least two sequential Gs. Potential transcriptional terminators were identified as described by Lesnik et al. (15). A less rigorous search for potential terminators was also carried out by searching for stem-loop structures with a stem of at least 10 nucleotides. When no such loops were found, a determination was made that no putative terminators were present. Opening reading frames (ORFs) were defined as regions without a stop codon. Aside from certain genes (see Fig. 2), no ORF with a product of >30 amino acids was found.

Creation of COH1-GT2. COH1-GT2 was created by an allelic exchange that replaced glnQ with a glnQ gene containing an 80-bp deletion and an insertion of an erm gene that confers erythromycin resistance. The exchange was accomplished by creating the plasmid pAG101, which contains a mutated glnQ gene on a temperature-sensitive replicon. To create pAG101, the glnQ gene was replaced using a PCR with genomic wild-type COH1 DNA as a template. This was purified and cloned into pT7Blue (Novagen Inc., Madison Wis.), generating the plasmid pJAP8. The insert in this plasmid was sequenced as described above. Additional sequence 5' of the region cloned in pJAP8 was obtained by sequencing PCR products obtained using arbitrarily primed PCR (18). To eliminate the possibility that errors might have been introduced into the PCR product cloned into pJAP8, PCR products generated directly from genomic COH1 DNA were also sequenced as described above and gave identical results. Potential ribosome binding sites were identified as having four out of seven of the sequence AGGAGGA, with at least two sequential Gs. Potential transcriptional terminators were identified as described by Lesnik et al. (15). A less rigorous search for potential terminators was also carried out by searching for stem-loop structures with a stem of at least 10 nucleotides. When no such loops were found, a determination was made that no putative terminators were present. Opening reading frames (ORFs) were defined as regions without a stop codon. Aside from certain genes (see Fig. 2), no ORF with a product of >30 amino acids was found.
from each dilution was then used as a template in a PCR using the reverse primer purified. Individual colonies were picked and replica plated on erethromycin and chloramphenicol. A single clone which was resistant to erethromycin and sensitive to chloramphenicol was selected (COH1-GT2). The allelic exchange was confirmed by PCR using primers from outside the cloned region of pAG101 (sequences of primers, GTCAAATGGAAGCAAGTCGCA and CTG AAAGGCTTGTGGCAGAATT). COH1-GT2 gave the expected 1,923-bp PCR product (data not shown), indicating the presence of the ern gene within the glnQ gene.

Creation of pAG200. The glnQ gene was amplified using genomic COH1 DNA as a template and a forward primer containing a 5′ EcoRI adapter (GGAGATCTCAGCCCTGTTGACACATT) and a reverse primer containing a 5′ BgIII adapter (GGAGAATCTGAGGCGCTTTGGTGCAAAACATT) (the adapters are underlined). The PCR product was digested with EcoRI and BgIII, ligated into the EcoRI and BgIII sites of pDCl213, transformed into MC1061, and plated on Luria agar with 5 μg of chloramphenicol/ml and 5 μg of 5-bromo-4-chloro-3-indolyl phosphate/ml. White colonies were picked and screened by colony PCR using the above-mentioned primers. A single positive clone was designated pAG200, and the presence of the insert was confirmed by limited restriction mapping.

Quantification of cell-associated capsular polysaccharide. Mutanolysin digests of bacterial cells and quantitation of capsular polysaccharide by competitive inhibition enzyme-linked immunosorbent assay were performed as described by Chaffin et al. (5).

Growth in THB and plasma. Overnight cultures of GBS in THB were diluted 1:100 in either fresh THB, fetal bovine serum (catalog no. 10437-028; Gibco-BRL, Gaithersburg, Md.), or human plasma (catalog no. P0523; Sigma). Cultures were grown at 37°C in a shaking incubator, and aliquots were plated on THB at various dilutions to quantitate CFU per milliliter.

Glutamine transport assays. Glutamine transport was assayed by a modification of the method of Masters and Hong (17). One milliliter of exponential-phase cells grown in THB at 37°C to an optical density at 600 nm of 0.5 to 0.6 was centrifuged in a microcentrifuge for 1 min, washed by centrifugation three times in 0.1% SDS at 65°C, and allowed to bind to microtiter wells coated with various concentrations of fibronectin (Fn). The wells were washed, and bound bacteria were assayed as described in Materials and Methods. The results are presented as the percentage of the input counts per minute that adhered to the well (% Adherence). The error bars represent 95% confidence intervals for the mean. COH1; □, COH1-GT2.

In vivo virulence studies. For 50% lethal dose (LD50) studies, neonatal rat pups (0 to 24 h old) were inoculated intraperitoneally with 0.1 ml of each strain in PBS. Randomized groups of five pups were inoculated with serial log dilutions of mid-logarithmic-phase bacteria, and the LD50 was calculated after 72 h using the methods of Reed and Muench (21). A two-tailed t test was performed to compare LD50 results between strains. A chi-square test was performed to compare mortalities at inocula of 50 to 200 CFU/animal.

Nucleotide sequence accession numbers. The DNA sequences described below have been deposited in GenBank with the following accession numbers: glutamine transport operon region, AF372619; pJAP8, AY032976; pAG101, AF372620; and pAG200, AF372621.

RESULTS

Isolation of the fibronectin adherence mutant COH1-GT1. In order to define the genes required for GBS adherence to fibronectin, we undertook to identify mutant GBS with decreased adherence to fibronectin. We created a library of mutants of the wild-type GBS strain COH1 using the transposon Tn917. This library was then selected for mutants with decreased adherence to fibronectin by serial adherence to fibronectin-coated microtiter wells as described in Materials and Methods. Over 1,000 individual clones were then isolated and tested for adherence to fibronectin as described. Several clones were identified with decreased fibronectin adherence. Three clones showed a decrease of >90% in fibronectin adherence compared to the wild-type strain. Southern blot analysis revealed that these clones had identical transposon insertions (data not shown) and were presumably siblings. One of the clones was selected for further analysis and designated COH1-GT1. To confirm the decreased adherence of COH1-GT1 to fibronectin, the adherence of this mutant was compared to that of the wild type at several different fibronectin coating concentrations (Fig. 1). COH1-GT1 showed a >90% decrease in fibronectin adherence at all fibronectin coating concentrations tested.

Adherence and invasion of A549 epithelial cells by COH1-
We speculated that GBS adherence to respiratory epithelial cells might be mediated, in part, by adherence to fibronectin. If this were true, we speculated that expression of other known GBS virulence factors might be decreased. To test this hypothesis, we sequenced the DNA surrounding the Tn917 insertion site of GT1. Sequence analysis revealed that the Tn917 insertion site in COH1-GT1 is shown in Fig. 3. A small ORF (designated orfX) was found in COH1-GT1. ORF homologous to glnQ of E. coli, a glutamine transport gene in E. coli. A small ORF (designated orfX) 3' of glnQ and in the same orientation lacks a ribosome binding site, is preceded by a putative transcriptional terminator, and is unlikely to be expressed. Further 3' of orfX is a homolog of obg, a sporulation gene from Bacillus subtilis. Transcription of obg is in the opposite orientation and thus is presumably not directly affected.

**Virulence of COH1-GT1 in an intraperitoneal model in neonatal rats.** We hypothesized that fibronectin adherence and epithelial cell adherence and invasion may be important virulence determinants. With defects in these phenotypes, we hypothesized that COH1-GT1 would also show a defect in virulence in vivo. We compared the LD50 of COH1 and COH1-GT1 in an intraperitoneal-injection model in neonatal rats (Table 2). In three separate experiments, COH1-GT1 showed an increase in the LD50 of between 1 and 3 log units compared to COH1 (P = 0.077 by t test).

A comparison of mortality across all three experiments at an inoculum of 50 to 200 CFU/animal revealed a mortality of 9 of 13 animals with COH1 and 0 of 8 animals with COH1-GT1 (P < 0.01). These results confirm that COH1-GT1 has a defect in virulence in vivo.

**Virulence factor and protein expression by COH1-GT1.** We hypothesized that the defect in fibronectin adherence in COH1-GT1 might be related to a regulatory gene. If this were true, we speculated that expression of other known GBS virulence factors might be decreased. To test this hypothesis, we assayed hemolysin production, keratin binding, and capsule production, and these showed no significant changes (data not shown). These data suggested that the defect in COH1-GT1 is not due to a global virulence gene regulator.

**Sequence analysis of the Tn917 insertion site of GT1.** We sequenced the DNA surrounding the Tn917 insertion in COH1-GT1 as described in experimental procedures. A map of the Tn917 insertion site in COH1-GT1 is shown in Fig. 3. Sequence analysis revealed that the Tn917 insertion is in an ORF homologous to glnQ, a glutamine transport gene in E. coli. A small ORF (designated orfX) 3' of glnQ and in the same orientation lacks a ribosome binding site, is preceded by a putative transcriptional terminator, and is unlikely to be expressed. Further 3' of orfX is a homolog of obg, a sporulation gene from Bacillus subtilis. Transcription of obg is in the opposite orientation and thus is presumably not directly affected.

**TABLE 2. LD50 of COH1 and glnQ mutants in an intraperitoneal-injection model.**

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>COH1</th>
<th>COH1-GT1*</th>
<th>COH1-GT2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.08 x 10^2</td>
<td>1.37 x 10^2</td>
<td>ND&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>3.48 x 10^4</td>
<td>3.48 x 10^2</td>
<td>2.24 x 10^3</td>
</tr>
<tr>
<td>3</td>
<td>1.53 x 10^6</td>
<td>1.71 x 10^4</td>
<td>&gt;2.88 x 10^3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Various numbers of GBS were inoculated intraperitoneally into newborn rat pups. Mortality at 48 h was determined, and the LD50 was calculated as described in Materials and Methods.

<sup>b</sup> P = 0.077 compared to COH1.

<sup>c</sup> P = 0.084 compared to COH1-GT1.

<sup>d</sup> ND, not determined.
by the transposon insertion. Upstream of the \( \text{glnQ} \) homolog is a long ORF with segments homologous to both \( \text{glnP} \), a glutamine permease gene from \( E. \text{coli} \), and \( \text{glnH} \), a periplasmic glutamine binding protein gene from \( E. \text{coli} \). Upstream of this gene, which we designated \( \text{glnP} \), are putative \(-17\) and \(-35\) promoter sequences. We have sequenced an additional 400 bp upstream of \( \text{glnP} \), and there do not appear to be additional genes in this region. Overall, the sequence analysis suggested that the effect of the Tn917 insertion in \( \text{COH1-GT1} \) was due to interruption of \( \text{glnQ} \), and it did not appear to be due to polar effects on genes \( 3' \) of \( \text{glnQ} \).

**COH1-GT1 is defective in glutamine transport.** We tested the hypothesis that \( \text{glnQ} \) is a glutamine transport gene by two different methods. First, we compared the abilities of \( \text{COH1} \) and \( \text{COH1-GT1} \) to transport radiolabeled glutamine (Table 3). \( \text{COH1-GT1} \) showed a 94% decrease in its ability to transport glutamine, confirming that \( \text{glnQ} \) is a glutamine transport gene. Secondly, we tested the ability of \( \text{COH1-GT1} \) to grow in the presence of the toxic glutamine analog GGH. Strains which have a decreased ability to transport glutamine are relatively resistant to GGH (31). The parental strain showed no growth in minimal medium containing GGH, while \( \text{COH1-GT1} \) was not inhibited by GGH (Table 4). Neither strain was sensitive to GGH in THB, which is rich in amino acids. These combined results demonstrate that \( \text{glnQ} \) is a glutamine transport gene in GBS.

We also considered the possibility that \( \text{COH1-GT1} \) might have a growth defect based upon the strain’s decreased ability to transport glutamine. We felt this was unlikely because GBS are not auxotrophic for glutamine (30) and are able to synthesize glutamine from glutamate (25). \( \text{COH1} \) and \( \text{COH1-GT1} \) showed identical growth in THB, in human plasma, and in fetal bovine serum, indicating that under these in vitro conditions, glutamine transport is not required for optimal growth by GBS.

We then attempted to define specific conditions under which the decrease in glutamine transport by \( \text{COH1-GT1} \) would result in decreased growth. These experiments were complicated by the fact that GBS are not auxotrophic for glutamine and are auxotrophic for multiple other amino acids (30). Attempts to grow GBS in the presence of glucose as a carbon source and glutamine as a sole nitrogen source were unsuccessful (data not shown), as one would expect from the multiple amino acid requirements of GBS. We then used a chemically defined medium developed for GBS that contains glucose as a carbon source and all of the amino acids for which GBS are auxotrophic (30). No difference in growth was seen between \( \text{COH1} \) and \( \text{COH1-GT1} \) either in the absence or presence of glutamine using this chemically defined medium (data not shown). Similarly, the growth rates of the mutant and the wild type were similar in medium containing M9 salts, 1% glucose as a carbon source, and limiting amounts of yeast extract (0.1 and 0.01%), both in the presence and absence of 100 \( \mu \text{g} \) of glutamine/ml (data not shown). Thus, GBS do not appear to require glutamine transport for optimal growth under any of the conditions that we tested in vitro. These results indicate that GBS are able to grow optimally on endogenously synthesized glutamine under a wide variety of conditions.

**Inactivation of \( \text{glnQ} \) by cassette insertion.** We considered the possibility that the decreased fibronectin adherence and virulence of \( \text{COH1-GT1} \) was unrelated to the Tn917 insertion in \( \text{glnQ} \). To address this concern, we created a site-directed mutant in \( \text{glnQ} \) as described in Materials and Methods. Briefly, the \( \text{glnQ} \) gene was cloned into a temperature-sensitive vector, pVE6007. An 80-bp deletion in \( \text{glnQ} \) was generated via inverse PCR, and an erythromycin resistance gene was inserted into this deletion to generate the plasmid pAG101. This construct was then transformed into GBS, and glutamine transport mutants were selected on GGH agar as described in Materials and Methods. Individual clones were isolated, and those that were resistant to erythromycin and sensitive to chloramphenicol were selected for further analysis. The allelic exchange was confirmed by PCR (data not shown). One clone (\( \text{COH1-GT2} \)) was selected for further analysis.

We confirmed that \( \text{COH1-GT2} \) had a defect in glutamine transport by using both glutamine transport and GGH sensitivity assays. As expected, \( \text{COH1-GT2} \) showed a marked decrease in glutamine transport (Table 3) and was resistant to GGH (Table 4), as observed with \( \text{COH1-GT1} \).

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**TABLE 3. Glutamine transport by \( \text{COH1} \) and \( \text{glnQ} \) mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>mmol of glutamine/10⁷ CFU</th>
</tr>
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<tbody>
<tr>
<td>( \text{COH1} )</td>
<td>33.38</td>
</tr>
<tr>
<td>( \text{COH1-GT1} )</td>
<td>1.13</td>
</tr>
<tr>
<td>( \text{COH1-GT2} )</td>
<td>1.90</td>
</tr>
</tbody>
</table>

*Log-phase GBS were exposed for 5 min to L-[G-3H]glutamine in the presence of M9 salts with 1% glucose. The bacteria were washed, and the uptake of glutamine was determined by scintillation counting as described in Materials and Methods. The results are presented as nanomoles of glutamine taken up per 10⁷ CFU.*

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**TABLE 4. Growth of \( \text{COH1} \) and \( \text{COH1-GT1} \) in the presence and absence of GGH**

<table>
<thead>
<tr>
<th>Medium</th>
<th>( \text{COH1} )</th>
<th>( \text{COH1-GT1} )</th>
<th>( \text{COH1-GT2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>THA</td>
<td>Sensitivity to GGH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimal medium</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>
| Growth was assessed after 24 h. Strains were considered resistant (R) if growth rates were equivalent on media with and without GGH and sensitive (S) if no growth was seen only in the presence of GGH.
We then tested the ability of COH1-GT2 to adhere to fibronectin in vitro and to cause lethal disease in neonatal rat pups in vivo. COH1-GT2 demonstrated defects in fibronectin adherence (Fig. 4) and virulence (Table 2) similar to those of COH1-GT1. This result confirmed that these phenotypes were due to the insertion of Tn917 into glnQ.

A polar effect of the Tn917 insertion on expression of orfX is not responsible for the phenotype of COH1-GT1. In general, Tn917 mutations exert polar effects on expression of downstream genes. orfX is the only potential ORF downstream of the Tn917 insertion in COH1-GT1. Nucleotide sequence analysis made this unlikely for two reasons. First, orfX lacks an obvious ribosome binding site. Second, there is a stem-loop structure immediately 3’ of glnQ that has many features of a transcriptional terminator. Nevertheless, we considered the possibility that the Tn917 insertion in COH1-GT1 was affecting fibronectin adherence through a polar effect on orfX. We demonstrated that this was not the case by two lines of evidence.

First, we tested the ability of the glnQ gene to complement the fibronectin adherence defect in COH1-GT1 in trans. A glnQ expression vector (pAG200) was created by replacing the phoZ gene of the shuttle expression vector pDC123 with the glnQ gene. pAG200 was then transformed into COH1-GT1. To confirm that pAG200 complemented the glnQ defect in COH1-GT1, we tested the ability of pAG200 to restore glutamine transport to COH1-GT1. COH1-GT1/pAG200 demonstrated normal levels of glutamine transport and sensitivity to GGH (data not shown). These results indicated that pAG200 produced a functional glnQ gene product. We then tested the ability of the cloned glnQ gene to complement the mutation and restore fibronectin adherence. The adherence of COH1-GT1/pAG200 to fibronectin was identical to that of COH1, demonstrating that the fibronectin adherence defect of COH1-GT1 could be complemented by the glnQ gene (Fig. 4). Complementation with vector alone (COH1-GT1/pDC123) did not restore fibronectin adherence (data not shown).

Second, RNA analyses using both Northern blotting and RT-PCR were performed. These results demonstrated a low level of transcription of orfX which was unaffected by the Tn917 insertion in COH1-GT1 or the emr gene insertion in COH1-GT2 (data not shown). Thus, there did not appear to be polar effects of transcription on orfX in either of the glnQ mutant strains.

Together, these results confirm that the phenotype of COH1-GT1 is due to the mutation in the glnQ gene and also demonstrate that this phenotype is not due to a polar effect on transcription of orfX.

Analysis of the transcription of glnQ, glnP, and scpB. Nucleotide sequence analysis of the glnQ region demonstrated that the glnQ and glnP genes were not separated by a transcriptional terminator and in fact overlapped by a single nucleotide. These results indicated that glnQ and glnP were cotranscribed as part of an operon. To test this hypothesis, we performed RT-PCR using a reverse primer from the glnQ gene and a forward primer from the glnP gene. cDNA from the parental COH1 strain was generated using RT and a reverse primer from the glnQ gene. A negative control reaction was performed in parallel using identical reagents in the absence of RT. Various amounts of cDNA were added in a 10-fold dilution series, and PCR was performed as described above. As shown in Fig. 5, when 1 or 0.1 μl of cDNA was used as a template, the expected 1,565-bp product was observed, while 0.01 μl of cDNA gave no product. One microliter of the no-RT control reaction gave no product, indicating that the products seen were not due to DNA contamination. These results demonstrate that glnQ and glnP are cotranscribed.

The disruption in the glnQ gene may also have had indirect effects on the transcription of the glnQ operon by altering cytoplasmic glutamine levels. Because glnP is a membrane protein, we considered the possibility that glnP is a fibronectin

![FIG. 4. Adherence to immobilized fibronectin by COH1, COH1-GT1, COH1-GT2, and COH1-GT1 plus pAG200. GBS strains were labeled with [4,5-3H]leucine and allowed to bind to microtiter wells coated with various concentrations of fibronectin (Fn). The wells were washed, and bound bacteria were assayed as described in the text. The results are presented as the percentage of the input counts per minute that adhered to the well (% Adherence). The error bars represent 95% confidence intervals for the mean. ■, COH1; □, COH1-GT1; ▲, COH1-GT2;△, COH1-GT1 plus pAG101.

![FIG. 5. Analysis of the transcription of glnQ and glnP by RT-PCR. cDNA was produced from COH1 RNA using RT and a reverse gene-specific primer from glnQ. A negative control reaction was performed without RT. PCR was then performed using 1, 0.1, and 0.01 μl of cDNA as templates and the reverse glnQ primer and a forward primer from glnP. The PCR products were analyzed by agarose gel electrophoresis. The templates used were as follows: lane A, 1 μl of cDNA; lane B, 0.1 μl of cDNA; lane C, 0.01 μl of cDNA; lane D, negative control (1 μl of no-RT control reaction mixture); lane E, positive control (10 ng of COH1 DNA). The no-template control gave no products (data not shown).](http://iai.asm.org)
adhesin. We hypothesized that disruption of glutamine transport might result in downregulation of expression of glnP. To test this hypothesis, we performed Northern blot analysis to assess levels of transcription of glnP. Total RNAs from COH1 and COH1-GT1 were separated by agarose gel electrophoresis and analyzed in a Northern blot using digoxigenin-labeled DNA probes. Lanes 1, COH1 RNA; lanes 2, COH1-GT1 RNA; lanes 3, COH1-GT2 RNA.

FIG. 6. Northern blot analysis of transcription of scpB and glnP. Total RNAs from COH1 and COH1-GT1 were separated by agarose gel electrophoresis and analyzed in a Northern blot using digoxigenin-labeled DNA probes. Lanes 1, COH1 RNA; lanes 2, COH1-GT1 RNA; lanes 3, COH1-GT2 RNA.

SCPB glnP

It has recently been demonstrated that ScpB, the GBS C5a peptidase, also acts as a fibronectin adhesin (3a). We hypothesized that transcription of scpB might be altered in COH1-GT1 or COH1-GT2. To test this hypothesis, we performed a Northern blot analysis comparing RNAs isolated from COH1, COH1-GT1, and COH1-GT2 and probed with scpB (Fig. 6). There was no decrease seen in the levels of scpB transcript in COH1-GT1 or COH1-GT2. A semiquantitative RT-PCR analysis of transcription of scpB also showed no difference in transcription (data not shown). Thus, glnQ does not appear to regulate fibronectin adherence through effects on the transcription of scpB.

**DISCUSSION**

The findings described in this study demonstrated that the glutamine transport gene glnQ was required for fibronectin binding in GBS. However, the mechanisms by which glnQ exerts its effects are unclear.

It does not appear that glnQ could act directly as an adhesin gene. Adhesin genes must code for proteins that are exposed on the surface of the organism. The glnQ homolog in *E. coli* is a cytoplasmic protein that hydrolyzes ATP to provide energy for glutamine transport. The GBS glnQ gene identified in this study is likely to serve a similar function and lacks a signal sequence.

The fibronectin adherence defect in COH1-GT1 can be complemented by glnQ in *trans*, demonstrating that the phenotype of COH1-GT1 is due to the glnQ gene and not to polar effects or unrelated mutations elsewhere in the chromosome.

Nucleotide sequence analysis of the glnQ region revealed that immediately S′ of glnQ is glnP. RT-PCR analysis revealed that glnP is transcribed in an operon with glnQ. Analysis of the glnP gene reveals that it encodes a putative signal sequence and consists of three distinct domains. The first two domains are homologous to the *E. coli* gene glnH, a soluble periplasmic glutamine binding protein, which acts as a shuttle to transport glutamine from the outer membrane to the inner membrane. The third domain is homologous to the *E. coli* gene glnP, a glutamine permease that contains a number of transmembrane domains that create the pore through which glutamine is transported. Protein homology searches using the BLAST program revealed that this fusion of two glutamine binding proteins to the permease is also found in *Lactococcus lactis* and in *Streptococcus pyogenes, Streptococcus pneumoniae, and Streptococcus mutans*. It appears that these streptococci have anchored the glutamine substrate binding protein by creating a transcriptional fusion of two substrate binding domains to the membrane-anchored glutamine permease domain.

Recently, a phage display library selected on fibronectin was used to identify C5a peptidase (ScpB) as a potential fibronectin adhesin, and subsequently it was demonstrated that ScpB is a fibronectin adhesin of GBS (3a). We hypothesized that glnQ mutations might act by reducing the transcription of scpB. However, RNA analysis by both Northern blotting and RT-PCR demonstrated that transcription of scpB is unaffected by the mutation in glnQ. Thus, it does not appear that glnQ mutations act by transcriptionally regulating the expression of scpB. We speculate that glnQ may alter the expression of ScpB by altering the translation or increasing the turnover of ScpB. Alternatively, glnQ may alter the transcription of as-yet-unidentified fibronectin adhesins or may act to increase expression of factors which mask ScpB and prevent fibronectin binding to these proteins. We are in the process of testing these possibilities.

We considered the possibility that GlnP was a fibronectin adhesin and that the mutation in glnQ resulted in altered expression of GlnP. Consistent with this hypothesis, GlnP was also identified as a possible fibronectin adhesin from a phage display library selected on fibronectin, but a role for GlnP as a fibronectin adhesin has not been confirmed. However, as for scpB, transcription of glnP does not appear to be altered in COH1-GT1. It is possible that regulation of GlnP expression is posttranscriptional or that GlnP is not a fibronectin adhesin. We are currently performing experiments to test these possibilities.

Alterations in glutamine transport may result in a decrease in cytoplasmic glutamine levels. Numerous previous studies have demonstrated that gene expression may be regulated through alterations in cytoplasmic amino acid levels in general...
(3, 7) and glutamine levels in particular (4, 9, 11). In E. coli, nitrogen metabolism is directly controlled through phosphorylation of regulatory proteins in response to cytoplasmic glutamine levels. We speculate that cytoplasmic glutamine levels are altered in COH1-GT1 and that these altered glutamine levels are responsible for the decrease in fibronectin adherence.

In support of this possibility, amino acid metabolism in general appears to play a role in fibronectin adherence. We have isolated two other GBS mutants with decreased fibronectin adherence and with mutations in an oligopeptide peptidase gene (pepN) and an oligopeptide permease gene (data not shown). Furthermore, the adherence of COH1-GT1 is increased when it is grown in a very nitrogen-rich chemically defined medium (data not shown). These data suggest that glutamine transport alters fibronectin adherence more profoundly under conditions of relative amino acid starvation. We hypothesize that this may be due to high cytoplasmic glutamine levels when levels of other amino acids in the growth medium are high. Alternatively, levels of amino acids other than glutamine may also play a role in regulating the level of expression of fibronectin adhesins.

COH1-GT1 and COH1-GT2 also show a defect in virulence in vivo, with an LD50 1 to 4 log units higher than for the wild-type bacteria. These findings demonstrate that glnQ is required for virulence. The glnO gene has also been identified as a virulence gene in S. pneumoniae in two different animal models (14, 19). The mechanism of this decrease in virulence has not been elucidated. We considered a number of possibilities.

It is possible that the mutation in glnQ creates a nutritional defect that does not allow for growth of GBS in vivo. Because the growth of COH1-GT1 in plasma is unaffected, this growth defect would have to occur in a niche other than free in the blood, possibly while the bacteria are intracellular or on an epithelial surface. We feel that COH1-GT1 is unlikely to have decreased virulence because of a nutritional defect for a number of reasons. First, GBS are not auxotrophic for glutamine (30) and are able to synthesize glutamine from glutamate (25). Second, a glutamine transport mutant in Salmonella did not appear to show a defect in survival either extracellularly, intracellularly, or in vivo unless glutamine synthesis was also eliminated (13). Third, the growth rates of COH1-GT1 and COH1 are identical in vitro under a wide variety of conditions, including THB and plasma and a wide variety of minimal media both in the presence and absence of glutamine. We hypothesize that the virulence defect is related to alterations in expression of other virulence factors.

Decreased virulence in an intraperitoneal model suggests that COH1-GT1 may be defective in avoiding host defenses. Expression of capsular polysaccharide, which plays an important role in GBS evasion of host defenses, appears to be unaffected. A fibronectin adhesin was required for virulence of S. pneumoniae after intraperitoneal injection, suggesting that fibronectin adherence may play a role in evading host defenses (14). We plan to investigate the ability of COH1-GT1 to avoid phagocytosis and to survive in whole blood. We also plan to examine the expression of identified factors that may be important in avoiding host defenses, including C5a peptidase.

Toxin production can also play a role in virulence. The virulence defect is not due to a decrease in production of the GBS hemolysin or cytolsin. However, expression of other GBS toxins may be affected.

We also compared the surface protein profiles of COH1 and COH1-GT1. The patterns on SDS-polyacrylamide gel electrophoresis were very similar, although a single polypeptide was identified which showed an approximately 50% decrease in expression in COH1-GT1 compared to that in COH1 (data not shown). We are attempting to identify this band, as well as to identify other polypeptides with altered expression, by two-dimensional gel analysis.

Little is known about the genetic basis for the regulation of virulence factor gene expression in GBS. Capsular polysaccharide production is affected by the growth phase; however, the mechanisms by which this regulation occurs are unknown. No global virulence regulatory genes, such as mga in group A streptococci, have yet been described. Recently, using signature tag mutagenesis, a number of genes required for virulence were identified with homology to regulatory genes in other bacteria (12). However, whether these genes are regulating virulence gene expression by GBS has yet to be confirmed. We are currently designing experiments to test whether glnQ acts to regulate the expression of ScpB and other virulence factors.

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