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Use of *glnQ* as a Counterselectable Marker for Creation of Allelic Exchange Mutations in Group B Streptococci

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Efficient allelic exchange mutagenesis in group B streptococci (GBS) has been hampered by the lack of a counterselectable marker system. Growth inhibition of GBS by the glutamine analog gamma-glutamyl hydrazide requires *glnQ*. We have used this phenomenon to create a counterselectable marker system for efficient selection of allelic exchange mutants in GBS.

Group B streptococci (GBS) are the leading cause of meningitis and sepsis in newborns in the United States and Western Europe (1). Recent advances have allowed for genetic manipulation of GBS, including the use of temperature-sensitive (TS) vectors for creation of allelic exchange mutations (17). However, creation of allelic exchange mutations in GBS is a laborious process that sometimes requires replica plating several thousand individual colonies (H. H. Yim, unpublished data). Creation of such mutations has been hampered by the lack of a counterselectable marker system for use with GBS.

We have recently discovered that wild-type GBS do not grow in the presence of the glutamine analog gamma-glutamyl hydrazide (GGH) and that this inhibition requires the glutamine transport gene *glnQ* (14). This finding led us to hypothesize that *glnQ*, when expressed on a plasmid in the background of a parent strain with a deletion in *glnQ*, could be used as a counterselectable marker. We now report that we have developed a counterselectable marker by using *glnQ* with GGH selection and have used this system to isolate mutant GBS strains that have the chromosomal capsular polysaccharide regulatory gene *cpsB* replaced by the chloramphenicol acetyltransferase (*cat*) gene.

Selection against *glnQ*-expressing GBS by GGH is robust. We previously isolated COH1-GT1, a Tn*917* transposon mutant derivative of a virulent GBS strain (COH1). COH1-GT1 is deficient in glutamine transport due to a Tn*917* insertion in *glnQ*, a homologue of a gene from *Escherichia coli* that is required for high-affinity, energy-dependent glutamine transport (14). We previously demonstrated that COH1-GT1 was resistant to GGH at 100 μg/ml when grown in a minimal medium containing M9 salts, 1% glucose, and 0.1% yeast extract (M9GYE), whereas the growth of COH1 was completely inhibited. We have subsequently demonstrated similar findings with inocula as high as 10^5 CFU/plate (data not shown). These results demonstrate that the *glnQ*-mediated sensitivity to GGH is sufficiently robust to allow for use of *glnQ* as a counterselectable marker.

Expression of *glnQ* in *trans* confers sensitivity to GGH in a *glnQ* mutant GBS strain. We hypothesized that the *glnQ* gene expressed on a plasmid could be used as a counterselectable marker when used in the background of a host lacking the *glnQ* gene, such as COH1-GT1. To test this hypothesis, we used pAG200, a complementation plasmid we had previously constructed which contains the *glnQ* gene expressed in the gram-positive shuttle vector pDC123. pAG200 is able to complement the glutamine transport defect of COH1-GT1 (14). We plated COH1-GT1 containing pAG200 onto M9GYE agar with 100 μg of GGH and 10 μg of chloramphenicol/ml to maintain the presence of the plasmid. The results are shown in Fig. 1. We confirmed that COH1-GT1 is able to grow in the presence of GGH (Fig. 1A). COH1-GT1 with pAG200 was able to grow on medium containing chloramphenicol (Fig. 1B) but not on medium containing both chloramphenicol and GGH (Fig. 1C). These results demonstrated that pAG200 confers sensitivity to GGH in a Δ*glnQ* background and thus acts as a counterselectable marker.

Creation of derivatives of GBS strains COH31rs and A909 with mutations in *glnQ*. We then sought to create Δ*glnQ* host strains from laboratory strains of GBS with high transformation efficiencies. Because COH1 has a very low transformation efficiency (~10^3 CFU/μg of DNA), much of the genetic manipulation of GBS has been performed on laboratory strains such as A909 and COH31rs that have much higher transformation efficiencies (~10^5 CFU/μg of DNA). We therefore undertook to create derivatives of these laboratory strains with site-directed mutations in *glnQ* that could then be used as hosts for a *glnQ* counterselectable marker system. We created site-directed mutations in *glnQ* in A909 and COH31rs by using pAG101, a plasmid containing the *glnQ* gene with an 80-bp internal fragment replaced with an erythromycin resistance gene (*erm*) in a TS background as previously described (14). The presence of the mutations was confirmed by PCR as described earlier. One isolate for each host was designated A909-DLS1 and COH31-DLS1, respectively, and used as the GGH-resistant host in subsequent experiments.

Generation of *cpsB* allelic exchange mutants. We then tested the utility of *glnQ* as a counterselectable marker in the creation of allelic exchange mutations in GBS by using the *cpsB* gene as a test case. *cpsB* is the second gene in the capsular

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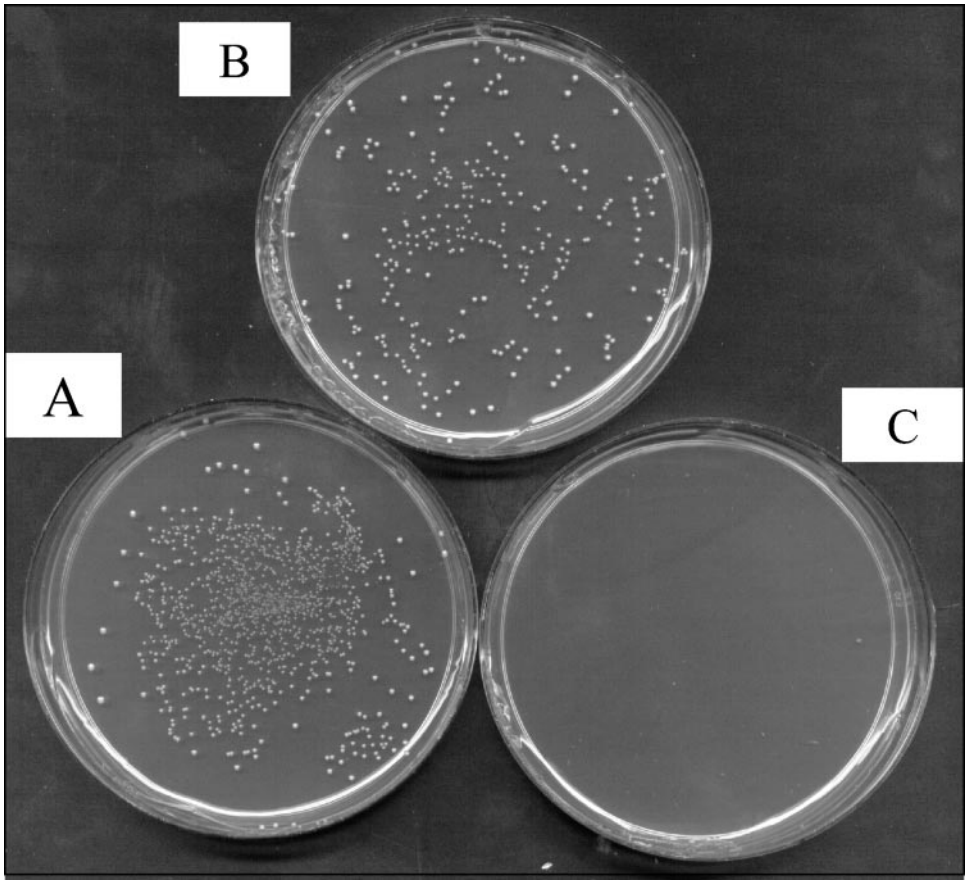

FIG. 1. pAG200 confers sensitivity to GGH in the background of COH1-GT1. (A) COH1-GT1, with GGH; (B) COH1-GT1:pAG200, with chloramphenicol; (C) COH1-GT1:pAG200, with chloramphenicol and GGH.

polysaccharide locus of GBS and appears to be involved in regulation of capsule expression (4). A previously described 3.0-kb EcoRI/EcoRV fragment from the capsule region of COH1 containing the *cpsB*, along with adjacent fragments of the *cpsA* and *cpsC* (2), was cloned into pHY304, a TS shuttle vector encoding erythromycin resistance, to create pHY306. An exact allelic replacement of the *cpsB* gene with the *cat* gene was then created by using an in vivo ligation. A DNA fragment containing the *cpsA* and *cpsC* gene fragments flanking *cpsB*, together with vector sequences, was created by PCR by using pHY306 as a template and outward-reading primers (Table 1, cpsA3′R and cpsC5′F) that were synthesized with a 5′ extension consisting of 20bp of the 5′ and 3′ ends of *cat*, respectively. The *cat* gene was amplified by using pDC123 as a template, and primers cat F and cat R (Table 1). Both PCR products were simultaneously electroporated into the *E. coli* strain MC1061 and transformants were selected on LA with 10 μg of chloramphenicol/ml. The integrity of one resulting clone was confirmed by limited restriction mapping, and designated pHY306.

A kanamycin resistance cassette and the *glnQ* gene were then added to create pDLS104. The *kan* gene from pCIV2 was amplified with primers kanF and kanR containing NotI sites (Table 1), digested with NotI, and ligated into pBluescript II SK(+) (Stratagene, La Jolla, Calif.) to create pDLS101. The insert from pHY306 was released with EcoRV and EcoRI, and cloned into pDLS101 digested with EcoRI and HincII to create pDLS102. The *glnQ* gene from pAG200 was then cloned into pDLS102 by using XhoI and KpnI to create pDLS103. The entire insert from pDLS103 containing *glnQ*, *kan*, and the allelic exchange construct from pHY306 was cloned into pHY304 by using SacII and ClaI to create pDLS104.

To create allelic exchange mutations in *cpsB*, pDLS104 was transformed into COH31-DLS1 and A909-DLS1. Transformed strains were grown overnight in Todd-Hewitt broth with 500 μg of kanamycin/ml at 30°C, diluted 1:100, and grown overnight at 37°C in the absence of antibiotic selection to cure the plasmid. Approximately 10^6 CFU were then subjected to GGH selection in the presence of chloramphenicol on M9GYE. Individual colonies were then replica plated onto Todd-Hewitt broth with 10 μg of chloramphenicol and 500 μg of kanamycin/ml. A total of 31 of 42 (74%) of the COH31-DLS1 derivatives and 22 of 31 (71%) of the A909-DLS1 derivatives gave the expected antibiotic phenotype (chloramphenicol resistant, kanamycin sensitive), indicating that they had lost the plasmid *kan* marker but had retained the *cat* gene.

A single clone in each background was then designated COH31-DLS2 and A909-DLS2, respectively, and tested for the presence of the allelic exchange mutation by Southern blot analysis. The *cat* gene probe was created by PCR by using pDC123 DNA as a template and the catF and catR primers. The *cpsB* probe was created by PCR with genomic COH1

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence[a]	Source or reference
Strains		
COH1	Wild-type III GBS strain	15
COH1-GT1	COH1, Tn*917 glnQ*/Em^r	14
COH31rs	Wild-type III GBS strain	12
COH31rs-DLS1	COH31rs, *glnQ*::*erm*	This study
COH31rs-DLS2	COH31rs *glnQ*::*erm cpsB*::*cat*	This study
A909	Wild-type Ia GBS strain	5
A909-DLS1	A909, *glnQ*::*erm*	This study
A909-DLS2	A909, *glnQ*::*erm cpsB*::*cat*	This study
Plasmids		
pVE6007	Cm^r temperature-sensitive shuttle vector; 3.4 kb; *ori*(Ts)	8
pCER1000	Em^r; *erm* cloned into pUC8	11
pDC123	Cm^r *phoZ*; blue-white screening shuttle expression vector	3
pAG101	Cm^r Em^r; pVE6007 with partial 739-bp *glnQ* with an 80-bp deletion and an insertion of 868-bp *erm* from pCER1000 via HindIII ligation	14
pAG200	Cm^r; pDC123, *cat glnQ*	14
pHY304	Em^r; derivative of pVE6007, lacZα/multiple cloning site of pBluescript	10
pHY306	Em^r Cm^r; pHY304 with *cat* flanked by 365 bp of *cpsA* and 573 bp of *cpsC*	This study
pDLS104	Cm^r Km^r Em^r; *kan* from pCIV2 and *glnQ* from pAG200 cloned into pHY306	This study
pCIV2	Km^r/ΩKm-2	9
Primers		
kanF	ATAAGAATGCGGCCGCTAGATTTTAATGCG	
kanR	ATAAGAATGCGGCCGCATGGACGATACAAA	
cpsAF	ATGACGGTTAATACCAATACCCA	
cpsCR	CCTGCACCAACAATAAACCCGATTAG	
glnQF1	GGCTTAATCGATGGCAGAATTAAAAATTGATGTCCA	
glnQR1	AACTGAATTCAGGAAGTCTTGGAGACGTGGG	
glnQF2	GTCAAATGGAAGCAAGTCGCA	
glnQR2	CTGAAAGCCCTTGTGCAACATT	
glnPF	GTCAAATGGAAGCAAGTCGCA	
glnQ3′R	CTGAAAGCCCTTGTGCAACATT	
cpsBF	TCGACATCACATAGAAGAAAAGG	
cpsBR	ATGGCGGTCTAACATCAAGG	
catF	GACAAGCTTAGCAGACAAGTAAGCCTCCTA	
catR	GCAGCGCTCTCATATTATAAAAGCCAGTC	
cpsA3′R	CCTAATGACTGGCTTTTAGATTATCTGTAGAGATTTCGGTAA	
cpsC5′F	TCAATTTTATTAAAGTTCATAATTTTTGATTTTGCATTAGATA	

[a] Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Km^r, kanamycin resistant.

DNA as a template, and the cpsBF and cpsBR primers. Both probes were labeled with digoxigenin by using the DIG Chem-Link labeling and detection set (Roche Applied Science, Indianapolis, Ind.) with HindIII-digested chromosomal DNA from COH31-DLS2 and A909-DLS2 according to the manufacturer's protocol. The results are shown in Fig. 2. The *cpsB* region probe (left lanes) gave the expected 1.1-kb band for strains COH31rs (lane 1) and A909 (lane 3). No band hybridizing to the *cpsB* gene was seen for COH31-DLS2 (lane 2) or A909-DLS2 (lane 4), indicating that, as expected, the *cpsB* gene is not contained in these strains. A *cat* gene probe gave the expected 3.1-kb band for both COH31-DLS2 (lane 2) and A909-DLS2 (lane 4), confirming the presence of the expected allelic exchange mutation. Overall, these results demonstrate that an allelic exchange can be rapidly and efficiently performed in GBS with GGH as a counterselectable marker.

GGH counterselection could conceivably be used for a wide variety of organisms. In theory, GGH counterselection could be used with any organism that requires an intact glutamine transport gene for sensitivity to GGH. This phenomenon has been described for the *glnQ* gene of *Bacillus stearothermophilus* (16), *Rhodobacter capsulatus* (18), *Rhodobacter sphaeroides* (6), and the GNP1 gene of *Saccharomyces cerevisiae* (19).

In addition, *glnQ* homologues are found in wide variety of organisms, and the feasibility of GGH counterselection can be easily ascertained by comparing the sensitivity of wild-type and *glnQ* mutant strains to GGH. Testing for GGH sensitivity is generally done under nitrogen-limiting conditions that result in

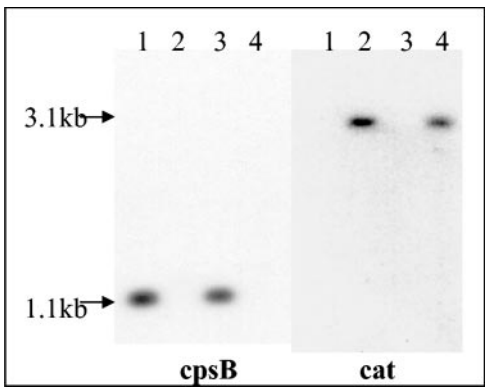

FIG. 2. Southern blot analysis of *cpsB* and *cat* genes of allelic exchange mutants. Genomic GBS DNA was digested with HindIII, and Southern blot analysis was carried out as described with probes specific for *cpsB* (left lanes) and *cat* (right lanes). Lanes: 1, COH31rs DLS1; 2, COH31rs DLS2; 3, A909 DLS1; 4, A909 DLS2.

the induction of glutamine transport genes. Thus, one requirement for efficient GGH selection is the ability of the host organism to grow in nitrogen-poor minimal medium. Thus, GGH selection may not be applicable to bacteria that require complex media for growth.

A counterselectable marker system for gram-positive bacteria with *rpsL* has been described for *S. pneumoniae*. This system was used to create gene replacements without introduction of antibiotic resistance markers; we speculate that similar gene replacements could be introduced into GBS by using GGH counterselection.

One drawback of both the *glnQ* and *rpsL* systems is that both the *glnQ* mutations we describe (14) and *rpsL* mutations in other hosts (7, 13) can create fitness defects in vivo. Thus, mutants created by using these systems may not be optimal for use in some applications, such as virulence testing in vivo. Hydrazides of several amino acids have been used to demonstrate that particular genes are required for transport of particular amino acids. This suggests that other amino acid transport genes, together with their cognate amino acid hydrazides, could be used to create counterselectable marker systems. We speculate that such systems might not impose fitness constraints that complicate the interpretation of data from these mutants obtained by using animal models of infection.

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