THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 35, pp. 25097–25109, September 1, 2006

Regulation of Glutamine and Glutamate Metabolism by GlnR and GlnA in *Streptococcus pneumoniae*[*]

Received for publication, February 22, 2006, and in revised form, June 16, 2006 Published, JBC Papers in Press, June 20, 2006, DOI 10.1074/jbc.M601661200

Tomas G. Kloosterman[‡1], Wouter T. Hendriksen[§], Jetta J. E. Bijlsma[‡1], Hester J. Bootsma[¶1], Sacha A. F. T. van Hijum[‡], Jan Kok[‡], Peter W. M. Hermans[§¶], and Oscar P. Kuipers[‡2]

From the [‡]Department of Molecular Genetics, University of Groningen, Groningen Biomolecular Sciences and Biotechnology Institute, P.O. Box 14, 9750 AA Haren, The Netherlands, the [§]Department of Pediatrics, Erasmus Medical Center, Sophia Children's Hospital, 3000 DR Rotterdam, The Netherlands, and the [¶]Department of Pediatrics, Radboud University Medical Center, 6500 HB Nijmegen, The Netherlands

Several genes involved in nitrogen metabolism are known to contribute to the virulence of pathogenic bacteria. Here, we studied the function of the nitrogen regulatory protein GlnR in the Gram-positive human pathogen *Streptococcus pneumoniae*. We demonstrate that GlnR mediates transcriptional repression of genes involved in glutamine synthesis and uptake (*glnA* and *glnPQ*), glutamate synthesis (*gdhA*), and the gene encoding the pentose phosphate pathway enzyme Zwf, which forms an operon with *glnPQ*. Moreover, the expression of *gdhA* is also repressed by the pleiotropic regulator CodY. The GlnR-dependent regulation occurs through a conserved operator sequence and is responsive to the concentration of glutamate, glutamine, and ammonium in the growth medium. By means of *in vitro* binding studies and transcriptional analyses, we show that the regulatory function of GlnR is dependent on GlnA. Mutants of g*lnA* and *glnP* displayed significantly reduced adhesion to Detroit 562 human pharyngeal epithelial cells, suggesting a role for these genes in the colonization of the host by *S. pneumoniae*. Thus, our results provide a thorough insight into the regulation of glutamine and glutamate metabolism of *S. pneumoniae* mediated by both GlnR and GlnA.

Regulation of nitrogen metabolism in bacteria is closely connected with the intracellular levels of glutamine and glutamate, the main nitrogen donors in the cell. Glutamine is formed from glutamate and ammonium by glutamine synthetase (GlnA), which is a major way for the cell to assimilate ammonium. Glutamate can be formed either by glutamate dehydrogenase from 2-oxoglutarate and ammonium or by glutamate synthase, which converts glutamine and 2-oxoglutarate into two molecules of glutamate.

Several studies indicate that nitrogen metabolism, especially glutamine metabolism, is important for the virulence of various bacterial pathogens (1–3). Signature-tagged mutagenesis screens suggest that genes involved in glutamine metabolism, *glnQ* and *glnA*, are likely to play a role in the virulence of *S. pneumoniae* as well (4–6). However, so far, glutamine metabolism and the way in which it is regulated have not been studied in this human pathogen.

In the well characterized Gram-positive bacterium *Bacillus subtilis*, regulation of nitrogen metabolism is carried out mainly by CodY, GlnR, and TnrA (7). The latter two are members of the MerR family of regulators, and both recognize the same operator sequence: 5′-TGTNAN$_7$TNACA-3′. TnrA functions during growth on a poor nitrogen source (*e.g.* solely glutamate) when it activates or represses expression of various genes involved in nitrogen metabolism (8–12). GlnR represses its own operon *glnRA* (13), the *ureABC* operon (encoding urease) (14, 15), and *tnrA* (7) in the presence of a good nitrogen source, like glutamine.

Genetic experiments have shown that genes regulated by GlnR and TnrA are constitutively expressed in a mutant of *glnA* (8, 16–18). An explanation for this observation came with the discovery that *in vitro* DNA binding by TnrA is blocked by feedback-inhibited GlnA (19). Although it has been suggested that GlnA also controls the DNA binding activity of GlnR, this has never been shown. In fact, *B. subtilis* GlnR has a high affinity for DNA on its own (13).

B. subtilis CodY functions as a repressor of genes involved in nitrogen metabolism (20) but also of carbon and energy metabolism (21), motility (22), and competence development (23). In the lactic acid bacterium *Lactococcus lactis*, CodY represses genes of the proteolytic system and several amino acid transport and metabolism genes, among others *gltA* and *gltD*, which are involved in glutamate biosynthesis (24–26).

Analysis of the *S. pneumoniae* R6 (27) and TIGR4 (28) genomes revealed that they contain genes encoding orthologs of GlnR and CodY but not of TnrA. Furthermore, *Streptococcus pneumoniae* contains a putative ortholog of *glnA*, several predicted glutamine uptake systems, and a predicted biosynthetic glutamate dehydrogenase (27, 28). In contrast to *B. subtilis* and *L. lactis*, a gene encoding glutamate synthase is not present. This suggests that *S. pneumoniae* has various ways to secure sufficient cellular glutamine levels, either by uptake from the environment or by *de novo* synthesis.

In this study, we report on the important role of GlnR and GlnA in the regulation of glutamine and glutamate metabolism in *S. pneumoniae* and present indications for a role of GlnR targets in pneumococcal virulence.

[*] The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by Innovatie-gericht Onderzoeks Programma (IOP) Grant IGE03002.
[2] To whom correspondence should be addressed. Tel.: 31-50-363-2093; Fax: 31-50-363-2348; E-mail: o.p.kuipers@rug.nl.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions—Strains used in this study are listed in Table 1 and were stored in 10% glycerol at −80 °C. *S. pneumoniae* was grown essentially as described (29): on plates in a flame-pot, giving an elevated CO_2 concentration, or in liquid medium as standing cultures. *L. lactis* and *Escherichia coli* were grown as described previously (29). Kanamycin and tetracycline were used in concentrations of 500 and 2.5 μg/ml for *S. pneumoniae*, respectively. Ampicillin was used in a concentration of 100 μg/ml for *E. coli*. Chemically defined medium with a final pH of 6.4 was composed as described (29), except that sodium citrate was used in the buffer instead of ammonium citrate and that glutamine was omitted from the amino acid mixture. Glutamine, glutamate, and ammonium were added as specified under "Results." Induction of gene expression with nisin was performed as described, using a stock solution of nisaplin, containing 20 mg/ml nisin (29).

DNA Isolation and Manipulation—Primers used in this study are listed in Table 2. Primers were based on the genome sequence of strain *S. pneumoniae R6* (27). Unless otherwise indicated, chromosomal DNA of *S. pneumoniae* D39 was used as a template for PCR amplification. All DNA manipulations were done as described (29).

Construction of glnR, glnA, glnRA, zwf, and glnP Mutants of S. pneumoniae—The *glnR*-stop mutant (TK102) was constructed using plasmid pORI280 as follows. Primer glnR-stop 1 with two point mutations, leading to two premature stop codons at codon positions 20 and 21 in the *glnR* reading frame, was used in combination with primer glnR_R6–1 to PCR-amplify a fragment comprising the upstream part and the beginning of *glnR*. A second PCR product, comprising the rest of the *glnR* gene and part of the downstream sequence was produced with primers glnR-stop 2 and glnR-3. These PCR products were complementary by 20 bp covering the position of the stop codons and were used as a template in a PCR with primers glnR_R6-1 and glnR-3. The resulting product was cloned as an XbaI, BglII fragment in pORI280, giving plasmid pTK20. pTK20 was used to introduce the mutations into the chromosome of *S. pneumoniae* D39 as described (29), giving strain TK102. The mutations led to the disappearance of a HincII site, on the basis of which the proper mutant could be identified. The mutations were further verified by DNA sequencing.

The *glnA* deletion strain (TK103) was generated by allelic replacement mutagenesis, removing 1300 bp of the *glnA* open reading frame (ORF).[3] A PCR fragment, generated with primers Spec_pORI38-Fp and Spec_pORI38-Rp on the spectinomycin resistance gene from pORI38, was cloned into the HindIII site of pORI28 in the same orientation as the erythromycin gene on this vector, yielding pORI28spec1. Next, the 3′-flanking region of *glnA*, amplified with primer pair glnA_R6–3/glnA_R6–4 (886 bp), was cloned into the NcoI/BglII sites of pORI28spec1, giving pTK18. pTK18 was cut with NdeI/AatII, and a PCR fragment generated with primers glnA_R6–1/glnA_R6–2 (808 bp), which was digested with the same enzymes, was ligated to it. This ligation mixture was used to generate a PCR product with primers glnA_R6–1 and glnA_R6–4, which was transformed to *S. pneumoniae* D39. Spectinomycin-resistant clones were examined for the presence of the *glnA* deletion by PCR and Southern blotting. The *zwf* deletion mutant (TK107), removing 1416 bp of the *zwf* ORF, was constructed in a similar way as the *glnA* mutant, using primers G6PDH-4/G6PDH-5 (660 bp) and G6PDH-6/G6PDH-7 (610 bp).

To construct the *glnRA* mutant (TK104), the upstream part of *glnR*, amplified with primer pair glnR_R6–1/glnR_R6–2 (883 bp), was cloned into the XbaI/BamHI sites of pORI28spec1, giving pTK22. pTK22 was used together with pTK18, which contains the glnA_R6–3/glnA_R6–4 PCR product cloned into the NcoI/BglII sites of pORI28spec1, as a template in a PCR with primers glnR_R6–1 and glnA_R6–4. In this way, a PCR product was obtained containing the spectinomycin resistance gene flanked by the upstream and downstream sequence of *glnRA*. The resulting PCR product was transformed to D39. The deletion was confirmed by PCR and Southern blotting.

L. lactis 108 was used as the cloning host for plasmid pTK19. All other constructs were made in *E. coli* EC1000.

To construct the *glnP* deletion mutant (TK106), removing 2080 bp of the *glnP* ORF, a PCR fragment, generated with primer pair Ery-rev/Ery-for on the erythromycin resistance gene from pORI28, was fused to the flanking regions of *glnP*, which were PCR-amplified with primer pairs glnPKO-1/glnPKO-2 (628 bp) and glnPKO-3/glnPKO-4 (610 bp) by means of overlap extension PCR (30). The resulting PCR product was transformed to *S. pneumoniae* D39, and clones were checked for the presence of the mutation by PCR. In the same way, a deletion mutant of *gdhA*, removing 1311 bp of the *gdhA* ORF, was constructed in D39 using primer pairs gdhAKO-1/gdhAKO-2 (479 bp) and gdhAKO-3/gdhAKO-4 (498 bp).

Construction of capsuleless derivatives of D39 and its *glnA*, *glnR*, *glnRA*, and *glnP* mutants was done as described (31), using primers PE21 and FI4. Mutants were checked by PCR and appearance. In addition, they adhere several orders of magnitude better than the encapsulated mutants.

Construction of lacZ Fusions—Chromosomal transcriptional *lacZ* fusions were constructed with the integration plasmid pORI13 as described (29, 32). For *lacZ* fusions to *glnA*, *gdhA*, and *zwf*, 600–800-bp fragments of the 3′ ends of the genes were PCR-amplified using primer pairs R6_glnA-5/R6_glnA-6, R6_gdhA-4/R6_gdhA-5, and R6_G6PDH-1/R6_G6PDH-2, respectively. These fragments were digested and cloned into the XbaI/EcoRI sites of PORI13, giving pTK8, pTK10, and pTK11, respectively. The constructs were introduced into *S. pneumoniae* D39*nisRK*, and D39*nisRK* containing either the *glnA* (TK100) or *glnR* (TK105) mutation, and clones were checked by PCR. Analogously, pTK9 and pTK12 were constructed with primers R6_PglnP-1/R6_PglnP-2 and R6_arcA-3/R6_arcA-4. These plasmids were used to generate chromosomal *lacZ* fusions to the *glnP* and *arcA* promoters. The *glnQ-zwf* intergenic region was cloned into pORI13 using primers Pg6pdh-1/Pg6pdh-2, giving pTK21.

The *lacZ* fusions to the *gdhA* promoter were constructed in pPP2 with primer pair PgdhA-2/PgdhA-4, giving a PCR prod-

[3] The abbreviations used are: ORF, open reading frame; CDM, chemically defined medium; EMSA, electrophoretic mobility shift assay; H_6-GlnR and H_6-GlnA, His_6-tagged GlnR and GlnA, respectively.

TABLE 1

Strains and plasmids used in this study

Trmp^R, trimethoprim-resistance; Spec^R, spectinomycin-resistance; Em^R, erythromycin resistance; Tet^R, tetracycline resistance; Km^R, kanamycin resistance; Cm^R, chloramphenicol resistance.

Strain/plasmid	Description	Reference or source
S. pneumoniae		
D39	Serotype 2 strain, *cps2*	Ref. 50, laboratory of P. Hermans
D39*repA*	D39 Δ*bgaA*::*repA*; Trmp^R	Ref. 29
D39*nisRK*	D39 Δ*bgaA*::*nisRK*; Trmp^R	Ref. 29
TK100	D39*nisRK* Δ*glnA*; Spec^R	This work
TK102	D39 *glnR*-stop, contains two stop mutations in the start of *glnR*	This work
TK103	D39 Δ*glnA*; Spec^R	This work
TK104	D39 Δ*glnRA*; Spec^R	This work
TK105	TK102 Δ*bgaA*::*nisRK*; Trmp^R	This work
TK106	D39 Δ*glnP*; Em^R	This work
TK107	D39 Δ*zwf*; Spec^R	This work
TK108	D39 Δ*gdhA*; Em^R	This work
TK109	TK102 Δ*bgaA*::*repA*; Trmp^R	This work
WH101	D39 Δ*codY*; Trmp^R	Unpublished strain
TK108	TK102 Δ*codY*; Trmp^R	This work
TK109	TK106 Δ*bgaA*::*nisRK*; Trmp^R	This work
TK110	D39*nisRK glnA-lacZ*; Em^R	This work
TK111	D39*nisRK* P*glnP-lacZ*; Em^R	This work
TK112	D39*nisRK gdhA-lacZ*; Em^R	This work
TK113	D39*nisRK zwf-lacZ*; Em^R	This work
TK114	D39*nisRK* P*arcA-lacZ*; Em^R	This work
TK120	TK105 *glnA-lacZ*; Em^R	This work
TK121	TK105 P*glnP-lacZ*; Em^R	This work
TK122	TK105 *gdhA-lacZ*; Em^R	This work
TK123	TK105 *zwf-lacZ*; Em^R	This work
TK125	TK105 P*arcA-lacZ*; Em^R	This work
TK126	TK100 *glnA-lacZ*; Em^R	This work
TK127	TK100 P*glnP-lacZ*; Em^R	This work
TK129	TK100 *gdhA-lacZ*; Em^R	This work
TK130	TK100 *zwf-lacZ*; Em^R	This work
TK131	TK100 P*arcA-lacZ*; Em^R	This work
TK132	D39 Δ*bgaA*::P*gdhA*1-*lacZ*; Tet^R	This work
TK133	D39 Δ*bgaA*::P*gdhA*1-*lacZ*; Tet^R	This work
TK134	TK102 Δ*bgaA*::P*gdhA*2-*lacZ*; Tet^R	This work
TK135	TK102 Δ*bgaA*::P*gdhA*2-*lacZ*; Tet^R	This work
TK136	D39 Δ*cps*; Km^R capsuleless derivative	This work
TK137	TK102 Δ*cps*; Km^R capsuleless derivative	This work
TK138	TK103 Δ*cps*; Km^R capsuleless derivative	This work
TK139	TK104 Δ*cps*; Km^R capsuleless derivative	This work
TK140	TK106 Δ*cps*; Km^R capsuleless derivative	This work
L. lactis		
NZ9000	MG1363Δ*pepN*::*nisRK*	Ref. 33
LL108	MG1363 RepA$^+$ (multicopy); Cm^R	Ref. 51
E. coli		
EC1000	Km^R; MC1000 derivative carrying a single copy of the pWV01 *repA* gene in *glgB*	Ref. 52
Plasmids		
pORI13	Em^R; *ori*$^+$ *repA*$^-$; promoterless *lacZ*, for single-copy chromosomal *lacZ* fusions.	Ref. 32
pORI280	Em^R; *ori*$^+$ *repA*$^-$; deletion derivative of pWV01; constitutive *lacZ* expression from P32 promoter	Ref. 52
pORI28[a]	Em^R; *ori*$^+$ *repA*$^-$; deletion derivative of pWV01	Ref. 52
ORI38[a]	Spec^R; *ori*$^+$ *repA*$^-$; deletion derivative of pWV01	Ref. 52
pNZ8048	Cm^R; Nisin-inducible P*nisA*	Ref. 53
pNG8048E	Cm^R Em^R; Nisin-inducible P*nisA*, pNZ8048 derivative containing *em*^{R} gene to facilitate cloning	Laboratory collection
pPP2	Amp^R Tet^R; promoterless *lacZ*. For replacement of *bgaA* (*spr0565*) with promoter-*lacZ* fusions. Derivative of pPP1.	Kovacs *et al.*[b]
pORI28spec1[a]	pORI28 containing *spec*^{R} gene from pORI38	This work
pTK8	pORI13′*glnA-lacZ*	This work
pTK9	pORI13 P*glnP-lacZ*	This work
pTK10	pORI13′*gdhA-lacZ*	This work
pTK11	pORI13′*zwf-lacZ*	This work
pTK12	pORI13 P*arcA-lacZ*	This work
pTK13	pPP2 PgdhA-1-*lacZ*	This work
pTK14	pPP2 PgdhA-2-*lacZ*	This work
pTK15	pNG8048E carrying *His6-glnA* downstream of P*nisA*	This work
pTK16	pNG8048E carrying *His6-glnR* downstream of P*nisA*	This work
pTK17	pNG8048E carrying *glnPQ* downstream of P*nisA*	This work
pTK18	pORI28spec1 containing 886-bp downstream of *glnA*	This work
pTK19	pORI28spec1 Δ*zwf*	This work
pTK20	pORI280, containing *glnR* with stop mutations (K20(AAG) → stop(TAG) and L21(TTG) → stop (TAG))	This work
pTK21	pORI13 P*zwf-lacZ*	This work
pTK22	pORI28spec1 containing 883-bp upstream sequence of *glnR*	This work
pTK23	pNG8048E carrying *glnR* downstream of P*nisA*	This work

[a] Plasmid sequences are available on the World Wide Web at molgen.biol.rug.nl/publication/glnRAspn_data.

[b] M. Kovacs, A. Halfmann, I. Fedtke, M. Heintz, A. Peschel, W. Vollmer, R. Hakenbeck, and R. Brückner, submitted for publication.

TABLE 2
Oligonucleotide primers used in this study

Name	Nucleotide sequence (5′ to 3′); restriction enzyme sites underlined	Restriction site
Ery-for	GCATGCATCGATTAGATCTC	
Ery-rev	TAACGATTATGCCGATAACT	
Spec_pORI38-Fp	CCCCAAGCTTCTAATCAAAATAGTGAGGAGG	HindIII
Spec_pORI38-Rp	CCCCAAGCTTACTAAACGAAATAAACGC	HindIII
R6_glnA-5	TGCTCTAGACTTTCTTCTATATTAGTATTAGTAAAGGTC	XbaI
R6_glnA-6	CGGAATTCGGTATTGCTGGATCAGGTATGCAC	EcoRI
R6_PglnP-1	CGGAATTCCGAAAGTCACTTGTTTTATCC	EcoRI
R6_PglnP-2	TGCTCTAGAAGGACATCTGAATGTTCCAGC	XbaI
R6_gdhA-4	CGGAATTCCGATGAAAACGGTATCGACTTCG	EcoRI
R6_gdhA-5	TGCTCTAGACTTAAACAATACCTTGTGCAATCATAGC	XbaI
R6_G6PDH-1	CGGAATTCGCTCTGGTAAGATTGATGG	EcoRI
R6_G6PDH-2	TGCTCTAGATTTTTATTCTAAGCGACCATC	XbaI
R6_arcA-3	TGCTCTAGACCGAGAAGACCTGAATTGGATGTG	XbaI
R6_arcA-4	CGGAATTCGGTAGGCTCAGGATTTTTCTCC	EcoRI
Pg6pdh-1	CGGAATTCGAAAGAGTTCTTAGATAAGG	EcoRI
Pg6pdh-2	TGCTCTAGACGAAAATTGTAACAATAACC	XbaI
glnP-OX1	CGAGCCATCATGAAGAAAAAATTTCTAGCATTTTTGC	RcaI
glnP-OX2	TGCTCTAGAGTAGAAAAACTGCAAGGAAATCC	XbaI
glnR_R6–9	GCCATCATGAAGGAAAAAGAATTTCGCC	RcaI
glnR-9-his[a,b]	CGAGCCATCATG**AAACATCATCATCATCATCAT**AAGGAAAAAGAATTTCGCCG	RcaI
glnR_R6–10	GCTCTAGAGTTTTCTCCTTAATCTATGAC	XbaI
glnA-his[a]	GGCGGTCTCTCATG**CATCATCATCATCATCAT**CCAATCACAGCTGCAGATATTCG	BsaI
glnA_R6–8	GCTCTAGATGTGACTGTTTACCCACAG	XbaI
glnA_R6–1	GAATTCCATATGGGAAATAGTATCGGTGGAC	NdeI
glnA_R6–2	CGGCGACGTCGCAGCTGTGATTGGCATAAG	AatII
glnA_R6–3	CATGCCATGGGGAAATTGATAATTATTTAGACC	NcoI
glnA_R6–4	GAAGATCTCTAGACGTATCTATATACC	BglII
glnR_R6–1	TGCTCTAGAGGTGGTGACTCAGCTGCCGC	XbaI
glnR_R6–2	CGGGATCCCCTTCATTTACAATTTCCTTC	BamHI
glnR-stop 1[c]	*CGGTCTACT***A***CATAACACTG*CCGATAGGAAAAACAGCC	
glnR-stop 2[c]	*CAGTGTTATG***T***AGT***A***GACCG*ATCTATCGGCGCGTCAG	
glnR-3	GAAGATCTACTTCAAGTGTTGGGTCCCC	BglII
glnPKO-1	AAAAAGTGCCAAGCCTAGAC	
glnPKO-2[d]	**GAGATCTAATCGATGCATGC**TACCTAATGAGAAAATTGGG	
glnPKO-3[d]	**AGTTATCGGCATAATCGTTA**CGAAACGCTTAGAAAAGAGG	
glnPKO-4	TAATCATGGTCATGCCTTGC	
gdhAKO-1	GATCGATTTGCCCTGTTCTTG	
gdhAKO-2[d]	**GAGATCTAATCGATGCATGC**GCAGATGTCATATCGTTCTCC	
gdhAKO-3[d]	**AGTTATCGGCATAATCGTTA**CTATGATTGCACAAGGTATTG	
gdhAKO-4	AAGGAGTAGAGATGGCTATAG	
G6PDH-4	TGCTCTAGACCAACACTTCAACCTCTTCCC	XbaI
G6PDH-5	CGGGATCCCCGAAAATTGTAACAATAACC	BamHI
G6PDH-6	CATGCCATGGTCACCTATCGTCAAGATGGTCG	NcoI
G6PDH-7	GAAGATCTGGCCCACCGCTACAAACAAGC	BglII
PgdhA-2	TGCTCTAGATCTTTAGCAGATGTCATATC	XbaI
PgdhA-3	TGCTCTAGACATAAGACATTATAGCAG	XbaI
PgdhA-4	CGGAATTCGATATTTCCAAGAAAAACGTTCG	EcoRI
PE21	CTGGAACAACCATGACCTCCCTCG	
FI4	CGCTGAACTTTTGTAGTTGCTTGGTCAAC	
R6_glnP-1	CGGAATTCCATTTTTGAAGCTTGGAAGTC	Footprinting
R6_glnR-7	GCTCTAGACTGCCGATAGGAAAAACAGCC	Footprinting
R6_PglnR_FP	TTGTACGTGTTTGTGCGTG	Footprinting
R6_glnP-GFP1	CGGAATTCCATTTTTAGTCTCCTTTTCCG	Footprinting
PglnR-2	CGGAATTCCGATATTGATCGTATTCGTC	EMSA
PglnR-3	CGGAATTCATTATCAATTGACGTTTGTC	EMSA
PglnPQ-1	CGGAATTCGGCACTTTTTAATAGCAATTCAAG	EMSA
PglnPQ-2	TGCTCTAGAGGAATAAAATTAGCAAAAATGC	EMSA
PglnPQ-3	CGGAATTCGCGAAAAATATAACAATTTGCC	EMSA

[a] His_6 tag in boldface type.
[b] Extra codon for lysine in italic type.
[c] Stop mutations are indicated in boldface type. Overlap of primers glnR-stop 1 and 2 is in italic type.
[d] Overlap with *emR* gene from pORI28 in boldface type.

uct comprising the full-length promoter (P*gdhA*-1) and primer pair PgdhA-3/PgdhA-4, resulting in a PCR product without the predicted GlnR operator (P*gdhA*-2), using *E. coli* EC1000 as the cloning host. The constructs were introduced in *S. pneumoniae* strains D39 and TK102.

Construction of Overexpression Constructs—The *glnR*, *glnA*, and *glnPQ* genes were PCR-amplified with primer pairs glnR-9-his/glnR_R6–10, glnA-his/glnA_R6–8 and glnP-OX1/glnP-OX2, respectively, and cloned into the NcoI/XbaI sites of pNG8048E, giving pTK16, pTK15, and pTK17. In addition, the native *glnR* gene was cloned into pNG8048E using primers glnR-_R6–9/glnR_R6–10, giving pTK23.

Purification of GlnR and GlnA—Overexpression of N-terminally His_6-tagged GlnR and GlnA (H_6-GlnR and H_6-GlnA) was achieved with the nisin-inducible system in strain *L. lactis* NZ9000 (33). Expression was induced with nisin in 1-liter cultures at an A_{600} of 0.6, using a 10^{-7} dilution (2 ng/ml) of nisaplin, which was prepared as described (29). After 2 h of induction, cells were harvested and resuspended in 10 ml buffer A (0.25 M NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 8, 10% glycerol, 1 mM β-mercaptoethanol) with 1 mg/ml lysozyme and one tablet of protease inhibitor mixture (Complete Mini; Roche Applied Science). After 20 min of incubation on ice, cells were disrupted by shaking five times for 1 min with 400 mg of glass

beads (75–150 μm; Fischer)/ml of cell suspension in a Biospec Mini-BeadBeater-8 (Biospec Products), and cell debris was removed by centrifugation. 1 ml of Ni^{2+}-nitrilotriacetic acid beads (Qiagen), pre-equilibrated in buffer A, was added to the cell lysate, and protein binding was allowed for 1 h at 4 °C with continuous gentle shaking. Beads were washed 10 times with buffer A containing 20 mM imidazole, after which H_6-GlnR and H_6-GlnA were eluted with buffer A containing 250 mM imidazole and subsequently with buffer A containing 350 mM imidazole. H_6-GlnA was dialyzed against a 2,000-fold excess of buffer B (20 mM Tris-HCl, pH 8.5, 10% glycerol, 1 mM β-mercaptoethanol) for 6 h at 4 °C. Since H_6-GlnR precipitated during dialysis, imidazole was removed by means of a PD-10 desalting column (Amersham Biosciences), using buffer C (20 mM Tris-HCl, pH 7.5, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol), in which way precipitation was not observed. Purified fractions contained >95% pure protein of the expected size with a concentration of between 0.2 and 1 mg protein/ml.

Electrophoretic Mobility Shift Assays (EMSAs) and DNAse I Footprinting—EMSAs were performed essentially as described previously (34). PCR products of P*glnR* with and without the predicted GlnR operator were made with primer pairs glnR_R6–2/PglnR-2 (P*glnR*-1, 146 bp) and glnR_R6–2/PglnR-3 (P*glnR*-2, 84 bp), respectively. In the same way, PCR products spanning P*glnP* were generated with primer pairs PglnPQ-1/PglnPQ-2 (P*glnP*-1, 190 bp) and PglnPQ-2/PglnPQ-3 (P*glnP*-2, 131 bp), respectively. The binding buffer was composed of 20 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 1 mM dithiothreitol, 8.7% (w/v) glycerol, 62.5 mM KCl, 25 μg/ml bovine serum albumin, 50 μg/ml poly(dI-dC), and 3000–5000 cpm of [γ-^{32}P]ATP-labeled PCR product. Glutamine, glutamate, ammonium, and purified H_6-GlnR and H_6-GlnA were added as specified under "Results." Reactions (20 μl) were incubated for 20 min at 25 °C, after which they were run on a 6% polyacrylamide gel for 75 min at 90 V.

DNAse I footprinting was done essentially as described (34). 150,000 cpm of [γ-^{32}P]ATP-labeled PCR products of the *glnR* and *glnP* promoters, made with primer pairs R6_PglnR_FP/R6_glnR-7 (244 bp) and R6_glnP-1/R6_glnP-GFP1 (235 bp), respectively, were used as probes in 40 μl of binding buffer (EMSA) containing 5 mM glutamine and purified H_6-GlnR and H_6-GlnA as specified under "Results."

Enzyme Assays—Cell-free extracts, used for the determination of glutamine synthetase (GlnA), biosynthetic glutamate dehydrogenase (GdhA), and glucose-6-phosphate dehydrogenase (Zwf) activity, were made from 1 or 2 ml of cells harvested in exponential phase of growth, which were resuspended in 250 μl of 20 mM Tris (pH 7.5) and disrupted by shaking for 1 min with 400 mg of glass beads (75–150 μm) in a Biospec Mini-BeadBeater-8. After the removal of cell debris by centrifugation, cell-free extracts were used in a concentration of one-tenth to one-twentieth of the total volume of the assay mixture. The A_{600} at which cells were harvested was used to calculate the enzyme activity per A_{600} unit. S.D. values were calculated from at least three independent replicate experiments. GlnA activity (transferase reaction) was determined as described (35). Biosynthetic glutamine synthetase activity of purified H_6-GlnA (ATP + L-glutamate → ADP + P_i + L-glutamine) was determined as described (36). Biosynthetic GdhA activity (2-oxoglutarate + NADPH + NH_4^+ → glutamate + $NADP^+$) was determined at 30 °C in a reaction mixture containing 50 mM Tris-HCl (pH 8.5), 35 mM 2-oxoglutarate, 80 mM NH_4Cl, and 0.3 mM 2′-NADPH by monitoring the decrease in absorption at 340 nm (A_{340}) caused by oxidation of NADPH. Catabolic GdhA activity was measured at 30 °C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 70 mM L-glutamate, and 0.5 mM $NADP^+$, by monitoring the increase in A_{340}. Activity of Zwf (glucose 6-phosphate + $NADP^+$ → gluconolactone-6-phosphate + NADPH) was measured in buffer containing 1 mM $NADP^+$, 2 mM glucose 6-phosphate, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 20 mM Tris-HCl (pH 8.0) by monitoring the increase in A_{340} at 30 °C. The activity of β-galactosidase was determined as described (37), except that cells were permeabilized with a final concentration of 0.06 mg/ml cetyltrimethyl ammonium bromide. During growth for β-galactosidase assays, no antibiotic selection was imposed.

Transcriptome Analysis Using S. pneumoniae DNA Microarrays—DNA microarray experiments were performed essentially as described (38). RNA was isolated from 50 ml of cells grown to midexponential phase of growth (A_{600} = 0.3) in M17 containing 0.25% glucose (GM17) containing 0.5 mg/ml glutamine (GM17Gln). Cells were harvested by centrifugation for 1 min at 10,000 rpm at room temperature. Cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C. Pellets were resuspended in 500 μl of 10 mM Tris-HCl, 1 mM EDTA ($T_{10}E_1$), pH 8.0, after which 50 μl of 10% SDS, 500 μl of phenol/chloroform, 500 mg of glass beads (75–150 μm), and 175 μl of Macaloid suspension (Bentone) were added.

Synthesis of cDNA and indirect Cy-3/Cy-5-dCTPs labeling of 15–20 μg of total RNA was performed with the CyScribe Post labeling kit (Amersham Biosciences) according to the supplier's instructions. Hybridization (16 h at 45 °C) of labeled cDNA was performed in Ambion Slidehyb #1 hybridization buffer (Ambion Europe) on superamine glass slides (Array-It; SMMBC), containing technical replicates of amplicons representing 2,087 ORFs of *S. pneumoniae* TIGR4 and 184 ORFs unique for *S. pneumoniae* R6. DNA microarrays were produced essentially as described (38, 39). Amplicon sequences are available on the World Wide Web at molgen.biol.rug.nl/publication/glnRAspn_data. Slides were scanned using a GeneTac LS IV confocal laser scanner (Genomics Solutions).

DNA Microarray Data Analysis—Slide images were analyzed using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). Processing and normalization (LOWESS spotpin-based) of slides was done with the in-house developed *MicroPrep* software as described (38, 40). DNA microarray data were obtained from three independent biological replicates hybridized to three glass slides, of which one was a dye swap. Expression ratios of mutant strain over the wild-type strain were calculated from the measurements of at least five spots. Differential expression tests were performed on expression ratios with a local copy of the Cyber-T implementation of a variant of the *t* test. False discovery rates were calculated as described (38). A gene was considered differentially expressed when p was <0.001 and false discovery rate was <0.05 and when at least five measurements were available. The DNA microarray data are

FIGURE 1. **Nucleotide sequences of the promoter regions of the indicated genes/operons of *S. pneumoniae* R6.** Predicted −35 and (extended) −10 core promoter regions are *underlined*. Putative GlnR operators are *boxed*. Translational starts are in *italic type*. The *numbers* indicate the base positions relative to the translational start. A predicted CodY operator in the *gdhA* promoter is *underlined* with a *dotted line*. Bases in P*glnR* and P*glnP* that are in *boldface type* were protected in the DNAse I footprinting analyses (Fig. 6*B*), and *vertical arrows below* the sequences indicate hypersensitive bases. The *horizontal arrows above* P*glnR* (P*glnR*-1 and P*glnR*-2), P*glnP* (P*glnP*-1 and P*glnP*-2) and P*gdhA* (P*gdhA*-1 and P*gdhA*-2) indicate the locations of the primers used to make the promoter truncations as used for Figs. 4*C* and 6, *A* and *B*.

available on the World Wide Web at molgen.biol.rug.nl/publication/glnRAspn_data. In addition, they have been deposited in the Gene Expression Omnibus GEO (available on the World Wide Web at www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE5088).

Reverse Transcription-PCR—RNA isolation and cDNA synthesis were performed as described above, except that aminoallyl-dUTP was replaced by dTTP during cDNA synthesis. To confirm the absence of DNA contamination, reactions were also carried out without reverse transcriptase. 100 ng of cDNA was used for each PCR, and after 20 amplification cycles (30 s, 95 °C; 30 s, 52 °C;, 60 s, 72 °C) with primers G6PDH4 and G6PDH5, reactions were analyzed on 1% agarose gels.

Adhesion Assays—Adhesion of pneumococci to epithelial cells was studied essentially as described previously (41). Briefly, the human pharyngeal cell line Detroit 562 (ATCC CCL-138) was cultured in RPMI 1640 without phenol red (Invitrogen) containing 1 mM sodium pyruvate and 10% fetal calf serum. Aliquots of bacteria, grown to midexponential phase in GM17 and stored until use at −80 °C, were thawed rapidly, har-

TABLE 3
Summary of transcriptome comparison of *S. pneumoniae* strains D39 *glnR*-stop and D39 Δ*glnA* with D39 wild type

Gene name[a]	Function[b]	glnR-stop[c]	*glnA*[d]
SP0092	ABC transporter, substrate-binding protein	**1.5** (3.1*e*−7)	NDE[e]
SP0237	Ribosomal protein L17	**1.5** (2.0*e*−8)	NDE
SP0295	Ribosomal protein S9	**1.6** (3.6*e*−9)	**1.6** (4.3*e*−5)
SP454	Hypothetical protein	**1.5** (2.2*e*−4)	NDE
glnR (*SP0501*)	Nitrogen regulatory protein	**5.0** (0.0)	**1.7** (3.8*e*−4)
glnA (*SP0502*)	Glutamine synthetase	**5.1** (0.0)	**−2.4** (5.3*e*−5)
SP0922	Carbon-nitrogen hydrolase family protein	NDE	**1.6** (2.0*e*−5)
SP0964	Dihydroorotate dehydrogenase	NDE	**1.8** (7.3*e*−6)
SP0965	Endo-β-*N*-acetylglucosaminidase	**−1.6** (2.4*e*−8)	NDE
SP1111	Hypothetical protein	NDE	**−3.7** (5.0*e*−4)
glnP (*SP1241*)	Glutamine ABC transport and substrate-binding protein	**4.3** (2.8*e*−15)	**2.8** (3.6*e*−12)
glnQ (*SP1242*)	Glutamine ABC transport and substrate-binding protein	**4.0** (0.0)	**2.5** (8.4*e*−11)
zwf (*SP1243*)	Glucose-6-phosphate dehydrogenase	**2.8** (4.7*e*−15)	**1.6** (7.3*e*−7)
SP1275	Carbamoyl phosphate synthase large subunit	**2.4** (1.6*e*−5)	**1.5** (7.9*e*−7)
SP1276	Carbamoyl phosphate synthase small chain	NDE	**1.6** (1.4*e*−8)
SP1277	Aspartate carbamoyltransferase	**1.8** (1.1*e*−4)	**1.8** (5.0*e*−7)
gdhA (*SP1306*)	NADPH-dependent biosynthetic glutamate dehydrogenase	**2.2** (6.9*e*−12)	**2.0** (1.0*e*−9)
SP1354	Ribosomal protein L7/L12	**1.8** (3.1*e*−9)	NDE
SP1936	Type II restriction-modification system regulatory protein	**1.6** (9.3*e*−6)	NDE
SP2055	Alcohol dehydrogenase, zinc-containing	**1.5** (2.2*e*−6)	NDE
SP2060	Pyrrolidone-carboxylate peptidase	NDE	**−1.7** (2.8*e*−5)
SP2063	LysM domain protein, authentic frameshift	**−1.9** (1.1*e*−11)	**−2.3** (6.5*e*−9)
arcA (*SP2148*)	Arginine deiminase	**1.6** (4.4*e*−4)	NDE

[a] Gene numbers refer to TIGR4 locus tags.
[b] TIGR annotation.
[c] Ratios >1.5 or <−1.5 (D39 *glnR*-stop compared with D39) in boldface type, *p* values in parentheses.
[d] Ratios >1.5 or <−1.5 (D39 Δ*glnA* compared with D39) in boldface type, *p* values in parentheses.
[e] NDE, not significantly differentially expressed.

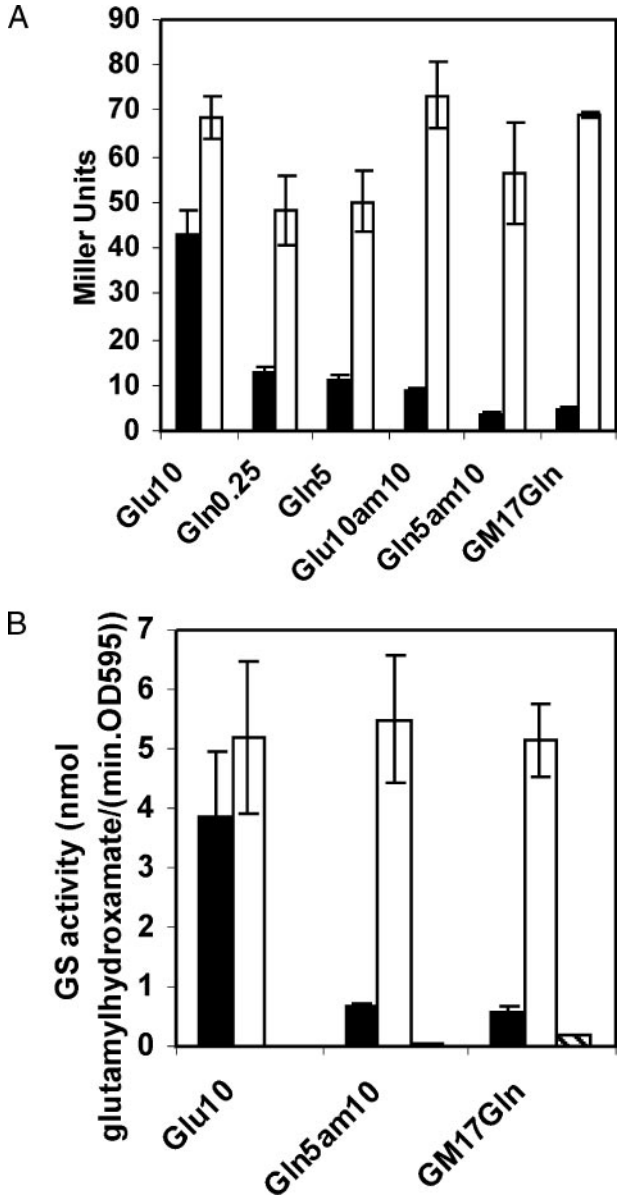

FIGURE 2. **Expression of *glnA* in *S. pneumoniae* D39 (*black bars*) and its *glnR* (*white bars*) and *glnA* (*diagonally hatched bars*) mutants in various media.** *A*, β-galactosidase activity in strains TK110 (D39*nisRK glnA-lacZ*) and TK120 (D39*nisRK glnR*-stop *glnA-lacZ*) in CDM supplemented with 10 mM glutamate (*glu10*), 0.25 mM glutamine (*Gln0.25*), 5 mM glutamine (*Gln5*), 10 mM glutamate and 10 mM NH_4Cl (*Glu10am10*), and 5 mM glutamine and 10 mM NH_4Cl (*Gln5am10*) and in GM17 with 0.5 mM glutamine (*GM17Gln*), which is indicated at the *x* axis. *B*, GlnA activity in strains D39, TK102 (D39 *glnR*-stop), and TK103 (D39 Δ*glnA*) in the media indicated on the *x* axis.

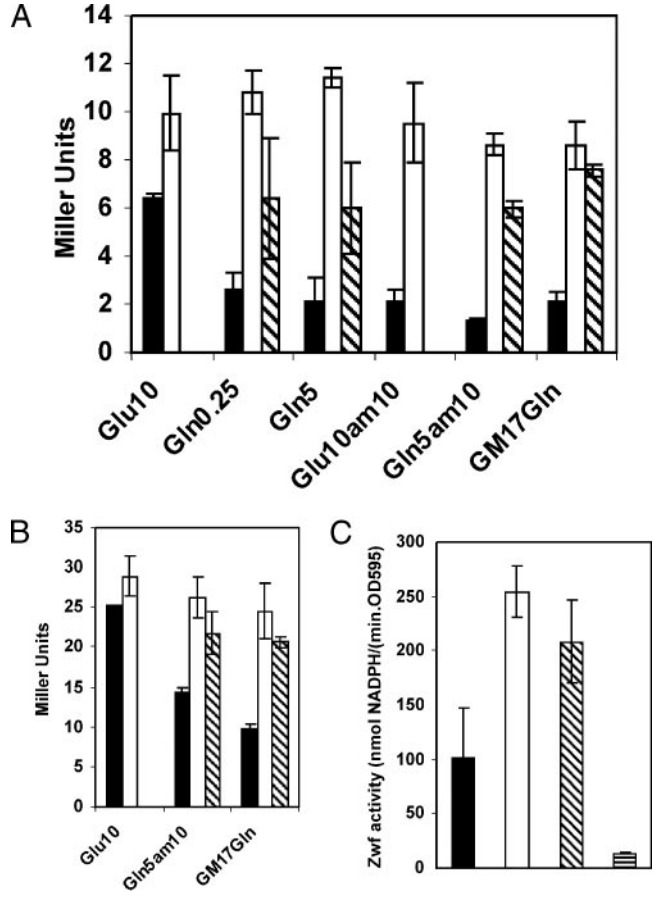

FIGURE 3. **Expression of *glnPQ* and *zwf* in *S. pneumoniae* D39 (*black bars*) and *glnR* (*white bars*) and *glnA* (*diagonally hatched bars*) mutants in various media.** *A*, β-galactosidase activity in strains TK111 (D39*nisRK* P*glnP-lacZ*), TK121 (D39*nisRK glnR*-stop P*glnP-lacZ*), and TK127 (D39*nisRK* Δ*glnA* P*glnP-lacZ*) in the media as indicated on the *x* axis (abbreviations as in Fig. 2). *B*, β-galactosidase activity in strains TK112 (D39*nisRK zwf-lacZ*), TK123 (D39*nisRK glnR*-stop *zwf-lacZ*), and TK130 (D39*nisRK* Δ*glnA zwf-lacZ*) in the media indicated on the *x* axis. *C*, Zwf activity in D39, TK102 (D39 *glnR*-stop), TK103 (D39 Δ*glnA*), and TK107 (D39 Δ*zwf*, *horizontally hatched bars*) in GM17Gln.

vested by centrifugation, and resuspended to 1×10^7 colony-forming units/ml in RPMI 1640 medium without phenol red containing 1% fetal calf serum. Monolayers of Detroit 562 in 24-well tissue culture plates were washed twice with 1 ml of phosphate-buffered saline, after which 1 ml of bacterial suspension was allowed to adhere for 2 h at 37 °C in a 5% CO_2 atmosphere. Subsequently, nonadherent bacteria were removed by three washes with 1 ml of phosphate-buffered saline, and the epithelial cells were detached by treatment with 200 μl of 25% trypsin, 1 mM EDTA in phosphate-buffered saline. Detroit 562 cells were lysed by the addition of 800 μl of ice-cold 0.025% Triton X-100 in phosphate-buffered saline, and appropriate dilutions were plated on blood agar plates to count the number of adherent bacteria. This colony-forming unit count was first corrected mathematically to account for small differences in count in the initial inoculum, after which data were normalized so that the adhesion of the wild-type strain TK136 was expressed as 100%. Wild type and mutant pneumococci grew comparably in RMPI medium without Detroit 562 cells. All experiments were performed in triplicate and repeated at least three times. Significant differences between wild type and mutants were calculated by the Mann-Whitney *t* test ($p < 0.05$).

RESULTS

Prediction of Putative GlnR Operators in S. pneumoniae—B. subtilis GlnR is known to repress genes that contain two copies of the inverted repeat 5′-TGTNAN$_7$TNACA-3′ in their promoters (13, 15). This repeat is also present in the promoter regions of the *Lactobacillus rhamnosus* (42) and *Bacillus cereus* (43) *glnRA* operons. Since the GlnR binding box seems so well conserved between species, we screened the entire genome of *S. peumoniae* R6 for the presence of putative GlnR operators

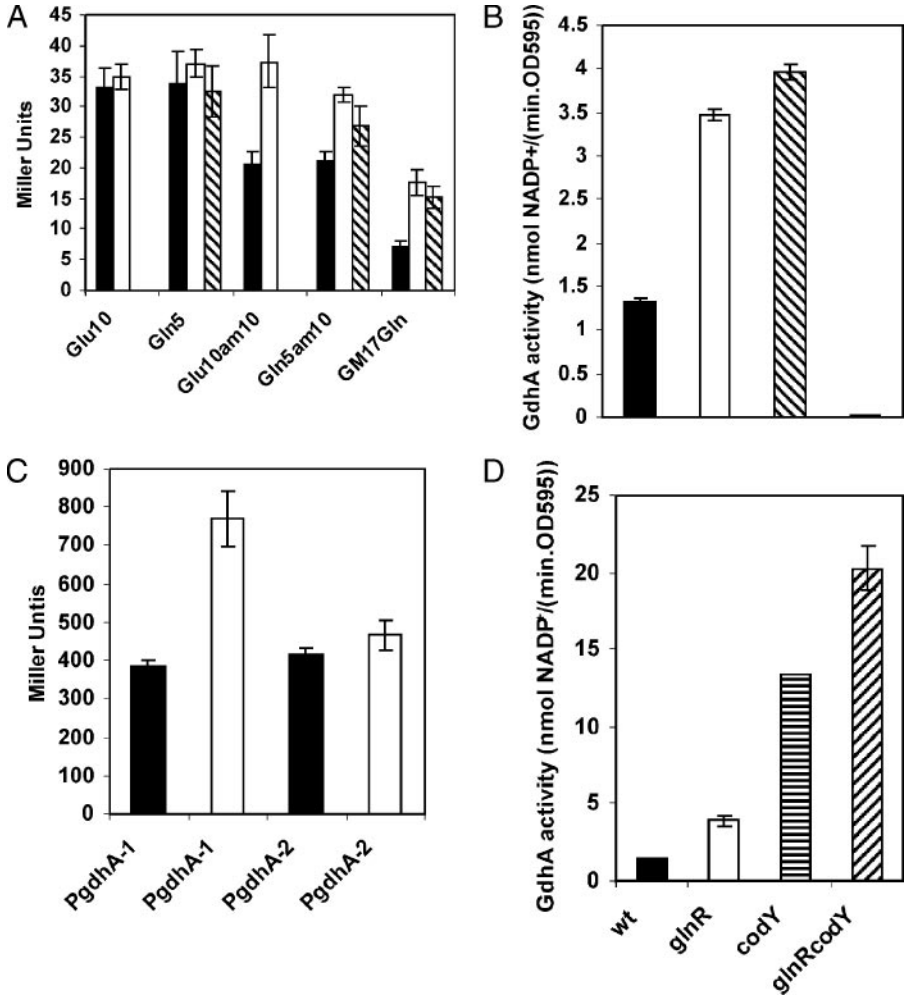

FIGURE 4. **Expression of *gdhA* in *S. pneumoniae* D39 and *glnR*, *glnA*, and *codY* mutant derivatives.** *A*, β-galactosidase activity in strains TK111 (D39*nisRK gdhA-lacZ*; *black bars*), TK121 (D39*nisRK glnR*-stop *gdhA-lacZ*; *white bars*), and TK127 (D39*nisRK* Δ*glnA gdhA-lacZ*; *hatched bars*) in the media indicated on the *x* axis (abbreviations as in Fig. 2). *B*, GdhA biosynthetic activity in D39 (*black bar*), TK102 (D39 *glnR*-stop, *white bar*), TK104 (D39 Δ*glnA*, *hatched bar*), and TK strains in GM17Gln. *C*, β-galactosidase activity in strains TK132 (D39 Δ*bgaA*::P*gdhA*-1-*lacZ*), TK133 (D39 Δ*bgaA* P*gdhA*-2-*lacZ*), TK134 (D39 *glnR*-stop Δ*bgaA*::P*gdhA*-1-*lacZ*), and TK135 (D39 *glnR*-stop Δ*bgaA*::P*gdhA*-2-*lacZ*) grown in GM17Gln. *Black bars*, D39 background; *white bars*, *glnR* mutant background. *PgdhA-1*, full-length *gdhA* promoter; *PgdhA-2*, *gdhA* promoter without the predicted GlnR operator. *D*, GdhA activity in D39, TK102 (D39 *glnR*-stop), WH101 (D39 Δ*codY*), and TK108 (D39 *glnR*-stop Δ*codY*) mutant strains grown in GM17Gln.

using Genome2D (44). Predicted operators with the highest similarity to the *B. subtilis* consensus sequence were present in the promoter regions of *glnR* (*spr0443*); *glnP* (*spr1120*), encoding a glutamine ABC transporter substrate-binding protein; *gdhA* (*spr1181*) encoding a NADP(H)-specific glutamate dehydrogenase; and *arcA* (*spr1955*, *spr1956*), encoding arginine deiminase (Fig. 1). Re-searching the R6 genome with a weight matrix built from these putative operators did not reveal additional putative GlnR operators.

The Regulon of GlnR and GlnA in S. pneumoniae—To investigate the role of GlnR in *S. pneumoniae* in more detail, we constructed a *glnR* mutant in strain D39. To preserve the *glnRA* operon structure, two consecutive stop codons were introduced in the beginning of the *glnR* ORF, specifying amino acids in the middle of the predicted helix-turn-helix DNA-binding motif.

DNA microarray analyses were performed of *S. pneumoniae* D39 wild-type and its isogenic *glnR* mutant grown in the nitrogen-rich medium GM17, supplemented with 0.5 mg/ml glutamine (GM17Gln). This amino acid is assumed to be a co-repressor of GlnR in *B. subtilis* (18), and we expected it to also induce repression of GlnR targets in *S. pneumoniae*. The operons/genes that were most highly up-regulated in the *glnR* mutant were *glnRA*, *glnPQ*, and *gdhA* (Table 3), all of which have a GlnR operator in their promoter regions (Fig. 1). The *arcA* gene, which also contains a putative GlnR operator in its promoter, was only weakly up-regulated. Remarkably, also *zwf*, encoding the key enzyme glucose-6-phosphate dehydrogenase of the pentose phosphate pathway, was up-regulated. This gene lies downstream of and in the same orientation as *glnPQ*.

To investigate the influence of GlnA on the expression of the identified GlnR targets, a comparison of the transcriptomes of wild-type D39 with its isogenic *glnA* mutant, grown in GM17Gln, was performed. This showed that *glnR*, *glnP*, *glnQ*, and *gdhA* were, like in the *glnR* mutant, also up-regulated in the *glnA* mutant (Table 3), indicating that GlnA is necessary for the functioning of GlnR.

Besides the genes mentioned above, *SP2063*, encoding a predicted LysM domain-containing protein, not directly involved in glutamine metabolism, was 2-fold down-regulated in both the *glnR* and the *glnA* mutant. Interestingly, two degenerate GlnR boxes (5′-TGTGACAGAGACCTAACA-3′ and 5′-TGTTATTAG-CGTCAACA-3′) are present in the promoter region of this gene.

In both the *glnR* and *glnA* mutant, genes predicted to encode proteins involved in pyrimidine metabolism (*SP1275*, *SP1276*, *SP1277*, and *SP0954*) were moderately up-regulated, which seems logical, since glutamine is a precursor of pyrimidine. However, since no GlnR operator could be identified upstream of any of these genes, the up-regulation is likely to be an indirect effect caused by altered intracellular glutamine/glutamate levels. Furthermore, a number of other genes of various functions were moderately up-regulated in either the *glnR* or the *glnA* mutant.

Chromosomal transcriptional *lacZ* fusions were used to confirm that in both the *glnR* and the *glnA* mutant, expression of *glnA*, *glnP*, *zwf*, and *gdhA* was derepressed (Figs. 2*A*, 3, *A* and *B*, and 4*A*). In addition, enzymatic activity assays

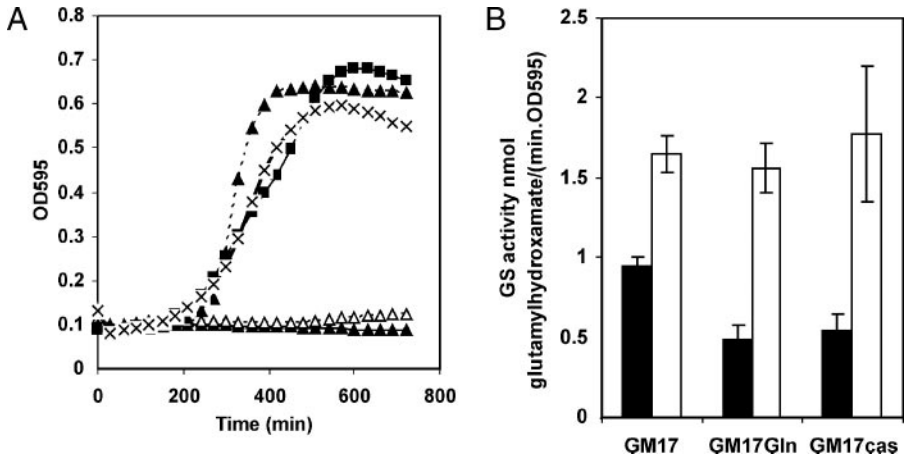

FIGURE 5. **GlnPQ is the main glutamine/glutamate ABC transporter in *S. pneumoniae* D39.** *A*, growth of D39 (*squares*), TK106 (D39 Δ*glnP*; *white triangles*), and TK106 containing plasmid pTK17 (*dotted line*, nisin (2 ng/ml)-induced *glnPQ* expression; *solid line*, without nisin) in CDM with 1 mM glutamine. *Crossed dotted line*, growth of TK106 in CDM with a 10 mM concentration of the dipeptide Gly-Gln. *B*, GlnA activity in strains D39 (*black bars*) and TK106 (*white bars*), grown in GM17, GM17Gln, and GM17 with 2% casitone (*GM17cas*).

showed that the observed effects on transcription corresponded with altered activities of GlnA, GdhA, and Zwf in the *glnR* and *glnA* mutants (Figs. 2*B*, 3*C*, and 4*B*). No strong effect of the *glnR* or the *glnA* mutation on the expression of a chromosomal P*arcA-lacZ* transcriptional fusion was observed in a range of different media (data not shown).

Nitrogen Source-dependent Regulation of glnRA and glnPQ by GlnR— To address the role of GlnA substrates in the observed GlnR-dependent regulation in *S. pneumoniae*, expression of the chromosomal *glnA-lacZ* and *glnP-lacZ* fusions was measured in a chemically defined medium (CDM), to which glutamine, glutamate, and ammonium were added in varying amounts (Fig. 2*A*). In CDM with only glutamate, *glnA* expression was similar in the wild type and the *glnR* mutant. In contrast, glutamine led to repression of *glnA* expression in the wild type already at a relatively low concentration (0.25 mM). A higher glutamine concentration did not lead to stronger repression in the wild type. However, when besides glutamine ammonium was included, *glnA* expression could be further repressed. The combination of glutamate and ammonium also gave rise to repression of *glnA* expression in the wild-type. None of the above combinations caused repression of *glnA* expression in the *glnR* mutant. GlnA enzymatic activity is regulated in the same way (Fig. 2*B*).

Regulation of *glnP* in response to glutamate, glutamine, and ammonium is very similar to that of *glnA* (Fig. 3*A*). Derepression of *glnP* expression is also seen in the *glnA* mutant, albeit to a somewhat lower extent than in the *glnR* mutant. This could indicate that, in the absence of GlnA, GlnR is still able to exert a weak repressive effect on the expression of *glnP*.

Regulation of zwf by GlnR and GlnA Occurs via the glnP Promoter—The DNA microarray results showed that *zwf*, which lies downstream of *glnPQ*, is also up-regulated in the *glnR* and *glnA* mutants. Reverse transcription-PCR demonstrated that *zwf* lies on the same transcript as *glnPQ* and thus forms an operon with these genes (data not shown). To examine whether *zwf* is only transcribed from P*glnP* or also from a possible promoter in the *glnQ-zwf* intergenic region, the latter was cloned upstream of *lacZ* in pORI13 and introduced in the RepA$^+$ strain D39*repA* (29) and its *glnR* mutant. Promoter activity was present in this fragment (~5 Miller units), which was not dependent on GlnR (data not shown). Regulation of *zwf* by GlnR and GlnA was similar to but weaker than regulation of *glnPQ* (Fig. 3, *B* and *C*), which can be explained by the presence of the second promoter upstream of *zwf*. Thus, expression of *zwf* initiates from two promoters, a GlnR-dependent promoter upstream of *glnP* and a second promoter in the *glnQ-zwf* intergenic region.

Regulation of gdhA by GlnR and CodY—Despite the fully conserved GlnR operator in the *gdhA* promoter, regulation of *gdhA* by GlnR and GlnA in GM17Gln and CDM was weaker than regulation of *glnPQ* and *glnRA* (Fig. 4, *A* and *B*). However, expression of an ectopic *lacZ* fusion to the full-length *gdhA* promoter (P*gdhA*-1) and to a truncated version without the GlnR box (P*gdhA*-2; see also Fig. 1) showed that deletion of the predicted GlnR operator abolished the GlnR-dependent repression of P*gdhA* (Fig. 4*C*), demonstrating that the predicted GlnR operator in the *gdhA* promoter is functional.

Interestingly, in the *S. pneumoniae* R6 genome, putative CodY operator sequences are present in the promoter regions of, among others, *gdhA* and *zwf* (24). To examine whether CodY regulates these genes in *S. pneumoniae*, the activity of the corresponding enzymes was measured in a *codY* deletion mutant. No effect of the *codY* deletion was seen on the activity of Zwf in GM17Gln (data not shown), but activity of GdhA was strongly increased in the *codY* mutant (Fig. 4*D*). In a *glnRcodY* double mutant, GdhA activity was even higher than in the *codY* mutant, indicating that GlnR and CodY independently repress *gdhA* in *S. pneumoniae* (Fig. 4*D*).

glnPQ Encodes the Main Glutamine/Glutamate Transport Operon in S. pneumoniae—Of the genes encoding predicted glutamine transporters in the R6 genome (27), *glnPQ* were the only ones found to be regulated by GlnR. To investigate the role of *glnPQ* in glutamine metabolism, a deletion of *glnP*, encoding the permease component of the GlnPQ ABC transporter, was constructed in D39. Whereas *S. pneumoniae* D39 is able to grow in CDM containing glutamine (Fig. 5*A*) or glutamate (29), but not in their absence, the *glnP* mutant was not able to grow in CDM with either glutamine (Fig. 5*A*) or glutamate (data not shown). This phenotype could be complemented by in *trans* expression of *glnPQ* from a nisin-inducible promoter (Fig. 5*A*). Moreover, the addition of the dipeptide Gly-Gln to the CDM also rescued growth of the *glnP* mutant (Fig. 5*A*), whereas this was not the case with the dipeptide Phe-Gly (data not shown). These data indicate that *glnPQ* encode the only actively expressed glutamine and glutamate uptake system in *S. pneumoniae* under these conditions.

GlnA activity was increased in the *glnP* mutant in GM17 (Fig. 5*B*), although to a lower extent as in the *glnR* mutant (Fig. 2*B*). To investigate whether regulation of *glnA* is affected in the *glnP*

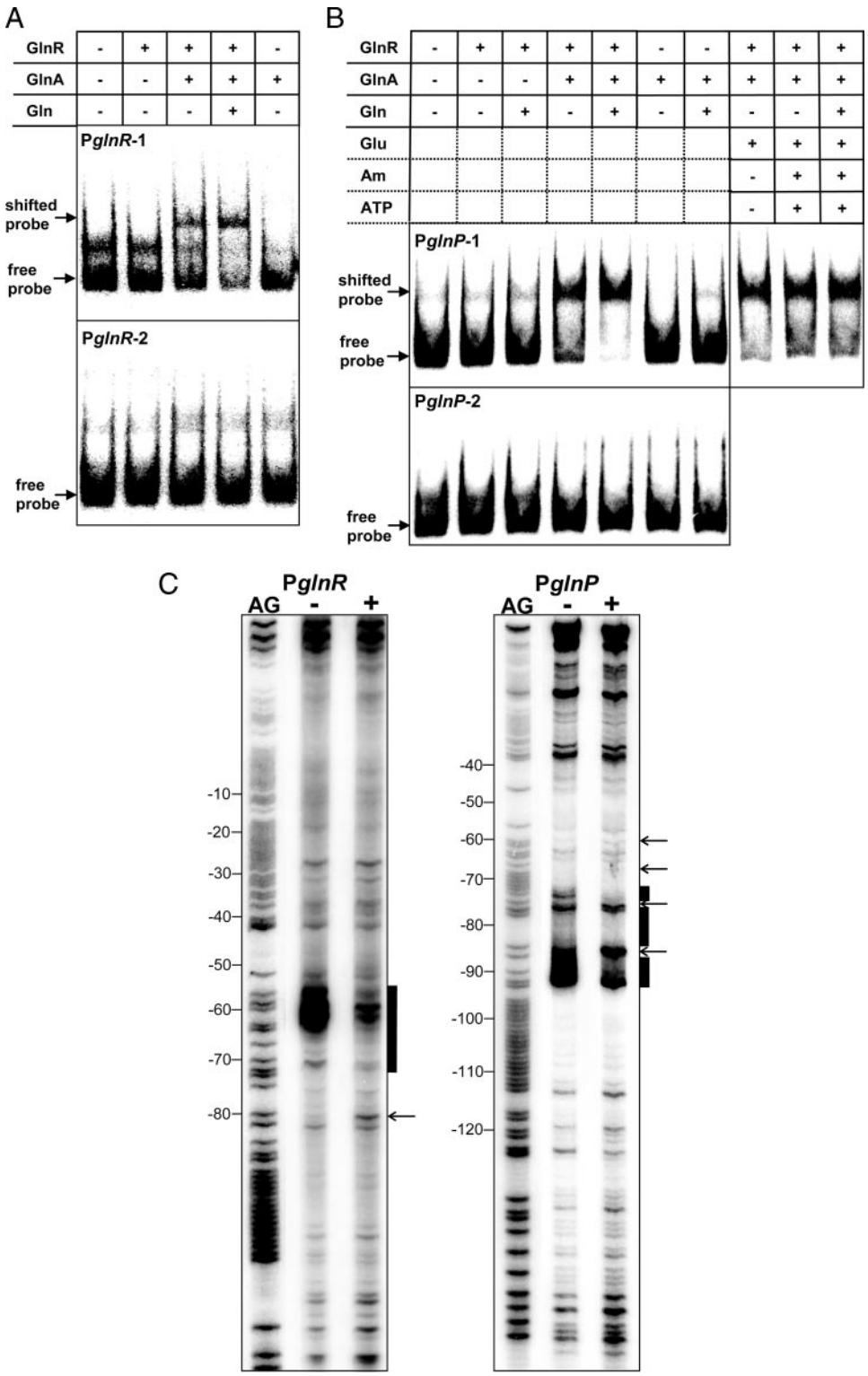

FIGURE 6. ***In vitro* interaction of GlnR with the *glnR* and *glnP* promoters.** *A* and *B*, EMSA of binding of GlnR to the *glnR* (P*glnR*-1) and *glnP* (P*glnP*-1) promoter regions and to truncated *glnR* (P*glnR*-2) and *glnP* (P*glnP*-2) promoters, lacking their respective GlnR operators. H_6-GlnR and H_6-GlnA were added as indicated *above* the *panels* in concentrations of 400 nM (monomer*) and 1.5 μM (monomer*), respectively, and glutamine (*Gln*), glutamate (*Glu*), ammonium (*Am*), and ATP were all added at 5 mM. The higher band seen for the free P*glnR* probe is probably single-stranded DNA (34). *C*, DNAse I footprint of P*glnR* and P*glnP* in the absence (−) and presence of 400 nM H_6-GlnR monomer* and 1.5 μM H_6-GlnA monomer*. Glutamine was present in all reactions in a concentration of 5 mM. Protected regions are indicated by *black bars*. The *arrows* indicate hypersensitive sites. *Numbers* on the *left* indicate bp positions relative to the translational starts of *glnP* and *glnR*. *AG*, Maxam-Gilbert sequence ladder. *, GlnR is probably active as a dimer (13, 15, 48), and GlnA is probably active as a dodecamer (49).

mutant, the effect of casitone as the nitrogen source in the medium was tested. Casitone, an enzymatic (pancreatic) digest of casein, consists of casein-derived peptides and contains no free glutamine and only a very low level of free glutamate (available on the World Wide Web at www.bd.com/ds/technicalCenter/misc/bionutrientmanual.pdf). Growth of the *glnP* mutant in CDM containing 2% casitone as the only nitrogen source was the same as that of the wild-type strain (data not shown), indicating that the uptake of peptides can bypass the inability to take up glutamine and glutamate. The addition of casitone, like glutamine, to GM17 resulted in an ~2-fold reduced GlnA activity in the wild-type strain, but not in the *glnP* mutant (Fig. 5*B*). Thus, besides GlnA, also GlnPQ appear to be necessary for efficient repression by GlnR.

Binding of GlnR to PglnP and PglnR Is GlnA-dependent—The transcriptional data presented above show that the activity of GlnR is dependent on GlnA. Therefore, we investigated whether GlnA is required for the binding of GlnR to the *glnR* and *glnP* promoters *in vitro*. For this we used a His-tagged variant of each protein (H_6-GlnA and H_6-GlnR). Nisin-induced expression of H_6-GlnA in strain TK100 restored growth in CDM with glutamate and no glutamine (data not shown). Nisin-induced expression of H_6-GlnR in the *glnR* mutant (TK105) led to 5-fold lower GlnA activity in CDM with 5 mM glutamine and 10 mM ammonium (data not shown). In CDM with 10 mM glutamate, the level of repression was still 4-fold, although the effect was weaker at low nisin concentration. With wild-type GlnR, the repressive effect was also 5-fold in CDM with 5 mM glutamine and 10 mM ammonium, but only 2-fold in CDM with 10 mM glutamate. These data indicate that H_6-GlnA and H_6-GlnR are functional, although the latter seems to respond in a less sensitive way to its assumed co-repressor glutamine.

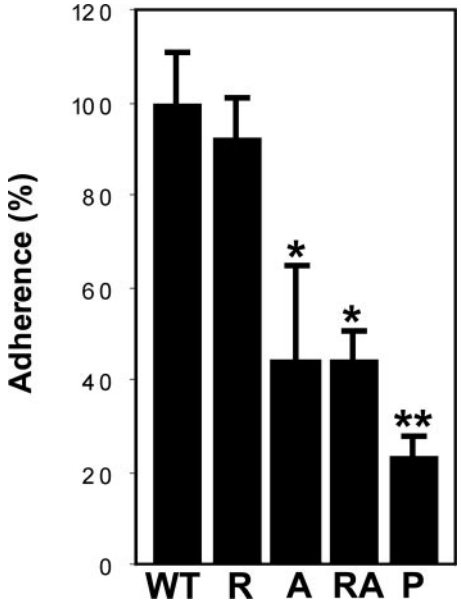

FIGURE 7. **Adhesion of *S. pneumoniae* strains TK136 (D39 Δ*cps*) (*WT*), TK137 (D39 *glnR*-stop Δ*cps*) (*R*), TK138 (D39 Δ*glnA* Δ*cps*) (*A*), TK139 (D39 Δ*glnRA* Δ*cps*) (*RA*), and TK140 (D39 Δ*glnP* Δ*cps*) (*P*) to Detroit 562 human pharyngeal epithelial cells.** Adhesion is given as a percentage relative to TK136. *, $p = 0.0011$; **, $p < 0.0001$.

H_6-GlnR alone at the concentration shown (400 nM) did not bind to P*glnR* and P*glnP* (Fig. 6, *A* and *B*), also not in the presence of glutamine. However, in the presence of H_6-GlnA, binding of H_6-GlnR to P*glnR* and P*glnP* was observed (Fig. 6, *A* and *B*). At a 5–10 times higher H_6-GlnR concentration (2–4 μM), a shifted band at the same position could be observed in the absence of H_6-GlnA (data not shown), which corresponds with the transcriptional data, showing some weak repression by GlnR in the *glnA* mutant (Table 3, Fig. 3*A*). No binding of H_6-GlnR was seen with the controls, the promoters without their GlnR operators (Fig. 6, *A* and *B*).

DNase I footprinting showed that, in the presence of H_6-GlnA, H_6-GlnR specifically reduces DNAse I sensitivity of the predicted GlnR operator in P*glnP* (Figs. 1 and 6*C*). Remarkably, the protected region in P*glnR* only partially overlapped with the predicted GlnR operator (Figs. 1 and 6*C*), suggesting that GlnR binds in a different manner to this promoter than to P*glnP*.

In contrast to what would be expected from the expression data, GlnA-dependent binding of GlnR to P*glnP* and P*glnR* was only weakly stimulated by the addition of glutamine (Fig. 6, *A* and *B*). This could be explained by the observation mentioned above, that H_6-GlnR seems to be less sensitive to glutamine than the native protein. The addition of the other GlnA substrates glutamate, ammonium, ATP, and AMP alone or in combination did not alter the observed GlnA dependence of the GlnR-DNA interaction at P*glnP* (Fig. 6*A*). Thus, although GlnA is required for the binding of GlnR to the GlnR operators in the *glnP* and *glnR* promoters, this effect was not modulated by GlnA substrates.

GlnA and GlnP Contribute to Adhesion to Pharyngeal Epithelial Cells—The crucial first step of pneumococcal virulence is the colonization of the nasopharynx. Therefore, we tested the ability of *glnR*, *glnA*, *glnRA*, and *glnP* mutants to adhere to the human pharyngeal epithelial cell line Detroit 562. These mutants were created in the capsuleless background strain D39 Δ*cps*, since unencapsulated strains tend to show higher levels of adhesion (45) (data not shown). The *glnP*, *glnA*, and *glnRA* mutants displayed a significantly decreased adhesion to the pharyngeal epithelial cells compared with the capsuleless wild-type strain (Fig. 7). Thus, both GlnP and GlnA could play a role in colonization of the nasopharynx. However, since most pneumococcal isolates are encapsulated, the actual contribution of these two proteins to virulence remains uncertain.

DISCUSSION

In this study, we characterized the regulation of glutamine and glutamate metabolism mediated by GlnR and GlnA in the human pathogen *S. pneumoniae*. Previously, GlnR-dependent regulation of nitrogen metabolism has been thoroughly studied in *B. subtilis*. The only target that *B. subtilis* GlnR shares with GlnR from *S. pneumoniae* is *glnRA* (13). In addition, *B. subtilis* GlnR is a repressor of the *ureABC* operon (7, 14) and *tnrA* (7), which are absent in *S. pneumoniae* (27). Another difference is that whereas GlnR is a repressor of *glnPQ* and *gdhA* in *S. pneumoniae*, in *B. subtilis* the catabolic glutamate dehydrogenase gene *rocG* is regulated by CcpA, RocR, and AhrC (46), and the *glnQH* glutamine transport operon is activated by TnrA (11).

We found that also expression of *zwf*, encoding a putative glucose-6-phosphate dehydrogenase, is regulated by GlnR. This enzyme catalyzes the first reaction in the pentose phosphate pathway, which provides the cell with NADPH and ribose 5-phosphate, a building block of nucleic acids. Since glutamine is a precursor for the synthesis of nucleotides as well, it might be advantageous for *S. pneumoniae* to coordinate *zwf* expression with glutamine metabolism.

Our data suggest that the regulation by *S. pneumoniae* GlnR depends on a conserved inverted repeat. The *B. subtilis* GlnR targets contain two copies of the same inverted repeat in their promoter regions (13, 15). *S. pneumoniae* GlnR resembles *B. subtilis* TnrA in this respect, since TnrA activates or represses promoters containing only one copy of this repeat (11).

The distance between the GlnR operator and the −35 box in P*glnR* is 7 bp, and for P*glnP* it is 16 bp. GlnR boxes are also present at a distance of 5–7 bp from the −35 in the *glnR* promoters of the *S. pneumoniae* relatives *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Streptococcus mutans*. Moreover, GlnR operators are located in the *glnP* promoters of *S. pyogenes* and *S. agalactiae*, which, like in *S. pneumoniae*, have a 16-bp spacing with the −35 sequence. Since the spacing in the *glnP* promoters is 9–10 bp longer than in the respective *glnR* promoters, regulation by GlnR via these operators might be helix side-dependent in these organisms.

The GlnR operator in P*gdhA* confers a less pronounced GlnR-dependent effect than the GlnR operators in P*glnP* and P*glnR*, although the inverted repeat is perfectly conserved. The same accounts for the GlnR operator in P*arcA*. In P*glnR* and P*glnP*, there is a stretch of A nucleotides immediately upstream of the repeat and a stretch of T nucleotides between the two half-sites. These stretches might explain the more efficient transcriptional repression of P*glnP* and P*glnR* than of P*arcA* and P*gdhA*,

since AT-rich stretches on these positions of *B. subtilis* P*nrgAB* enhance TnrA-dependent transcriptional activation (47).

Both GlnR and CodY function as a repressor of *gdhA* in *S. pneumoniae*, of which CodY seems to be the more important regulator. Furthermore, both regulators control *gdhA* transcription independently of each other, which is in agreement with the location of their operators, that for CodY lying upstream of the −35 and the GlnR operator downstream of the −10 in P*gdhA*. In *B. subtilis*, CodY controls the cellular nutritional and energy status (20, 21). Although GdhA is obviously connected to glutamine metabolism, the observation that *gdhA* expression is, next to GlnR, also regulated by CodY in *S. pneumoniae*, might indicate that GdhA is an important control point of the cellular nutritional status in this bacterium.

We show that GlnR DNA binding is dependent on GlnA, in contrast to the situation in *B. subtilis*, where GlnR alone binds with high affinity to its target promoters in the absence of any effectors (13). Since a high concentration of GlnR alone led to a shifted band at the same position as in the presence of GlnA, it is unlikely that GlnR and GlnA bind as a complex to the DNA. It might be that GlnA induces a conformational change or multimerization of GlnR, which increases its DNA binding affinity. Next to GlnA, also GlnP seems important for activity of *S. pneumoniae* GlnR. Although GlnR and GlnA alone were sufficient for *in vitro* binding to the *glnP* and *glnR* promoters, it could be that *in vivo* both GlnPQ and GlnA are needed for optimal activity of GlnR.

Our results and previous STM screens (4–6) implicate a role for both GlnP and GlnA in pneumococcal adhesion to human pharyngeal cells, which is a prerequisite to invade the host. Previously, GlnQ was shown to be required for adhesion of *S. pyogenes* to fibronectin and epithelial cells of the respiratory tract (2). However, it remains to be investigated whether the effect of GlnPQ on adhesion by *S. pneumoniae* and *S. pyogenes* is caused by a general effect of distorted glutamine metabolism (*e.g.* on the cell surface composition) or if GlnPQ are directly involved. We are currently analyzing *glnR*, *glnA*, *glnP*, and *gdhA* mutant strains in several *in vivo* mouse models to gain more insight into the role of glutamate and glutamine metabolism during infection by *S. pneumoniae*.

Acknowledgments—We thank Chris den Hengst for help with the DNA binding studies and Anne de Jong for help with the DNA microarray studies. We thank Dr. R. Brückner for the generous gift of pPP2 and Dr. D. Morrison for the generous gift of CSP-1.

REFERENCES

1. Klose, K. E., and Mekalanos, J. J. (1997) *Infect. Immun.* **65,** 587–596
2. Tamura, G. S., Nittayajarn, A., and Schoentag, D. L. (2002) *Infect. Immun.* **70,** 2877–2885
3. Tullius, M. V., Harth, G., and Horwitz, M. A. (2003) *Infect. Immun.* **71,** 3927–3936
4. Hava, D. L., and Camilli, A. (2002) *Mol. Microbiol.* **45,** 1389–1406
5. Polissi, A., Pontiggia, A., Feger, G., Altieri, M., Mottl, H., Ferrari, L., and Simon, D. (1998) *Infect. Immun.* **66,** 5620–5629
6. Lau, G. W., Haataja, S., Lonetto, M., Kensit, S. E., Marra, A., Bryant, A. P., McDevitt, D., Morrison, D. A., and Holden, D. W. (2001) *Mol. Microbiol.* **40,** 555–571
7. Fisher, S. H. (1999) *Mol. Microbiol.* **32,** 223–232
8. Wray, L. V., Jr., Ferson, A. E., Rohrer, K., and Fisher, S. H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 8841–8845
9. Nakano, M. M., Hoffmann, T., Zhu, Y., and Jahn, D. (1998) *J. Bacteriol.* **180,** 5344–5350
10. Schultz, A. C., Nygaard, P., and Saxild, H. H. (2001) *J. Bacteriol.* **183,** 3293–3302
11. Yoshida, K., Yamaguchi, H., Kinehara, M., Ohki, Y. H., Nakaura, Y., and Fujita, Y. (2003) *Mol. Microbiol.* **49,** 157–165
12. Belitsky, B. R., Wray, L. V., Jr., Fisher, S. H., Bohannon, D. E., and Sonenshein, A. L. (2000) *J. Bacteriol.* **182,** 5939–5947
13. Brown, S. W., and Sonenshein, A. L. (1996) *J. Bacteriol.* **178,** 2450–2454
14. Wray, L. V., Jr., Ferson, A. E., and Fisher, S. H. (1997) *J. Bacteriol.* **179,** 5494–5501
15. Brandenburg, J. L., Wray, L. V., Jr., Beier, L., Jarmer, H., Saxild, H. H., and Fisher, S. H. (2002) *J. Bacteriol.* **184,** 6060–6064
16. Schreier, H. J., and Sonenshein, A. L. (1986) *J. Bacteriol.* **167,** 35–43
17. Schreier, H. J., Fisher, S. H., and Sonenshein, A. L. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 3375–3379
18. Schreier, H. J., Brown, S. W., Hirschi, K. D., Nomellini, J. F., and Sonenshein, A. L. (1989) *J. Mol. Biol.* **210,** 51–63
19. Wray, L. V., Jr., Zalieckas, J. M., and Fisher, S. H. (2001) *Cell* **107,** 427–435
20. Shivers, R. P., and Sonenshein, A. L. (2005) *Mol. Microbiol.* **56,** 1549–1559
21. Kim, H. J., Kim, S. I., Ratnayake-Lecamwasam, M., Tachikawa, K., Sonenshein, A. L., and Strauch, M. (2003) *J. Bacteriol.* **185,** 1672–1680
22. Bergara, F., Ibarra, C., Iwamasa, J., Patarroyo, J. C., Aguilera, R., and Marquez-Magana, L. M. (2003) *J. Bacteriol.* **185,** 3118–3126
23. Molle, V., Nakaura, Y., Shivers, R. P., Yamaguchi, H., Losick, R., Fujita, Y., and Sonenshein, A. L. (2003) *J. Bacteriol.* **185,** 1911–1922
24. den Hengst, C. D., van Hijum, S. A. F. T., Geurts, J. M., Nauta, A., Kok, J., and Kuipers, O. P. (2005) *J. Biol. Chem.* **280,** 34332–34342
25. Guedon, E., Sperandio, B., Pons, N., Ehrlich, S. D., and Renault, P. (2005) *Microbiology* **151,** 3895–3909
26. Guedon, E., Serror, P., Ehrlich, S. D., Renault, P., and Delorme, C. (2001) *Mol. Microbiol.* **40,** 1227–1239
27. Hoskins, J., Alborn, W. E., Jr., Arnold, J., Blaszczak, L. C., Burgett, S., DeHoff, B. S., Estrem, S. T., Fritz, L., Fu, D. J., Fuller, W., Geringer, C., Gilmour, R., Glass, J. S., Khoja, H., Kraft, A. R., Lagace, R. E., LeBlanc, D. J., Lee, L. N., Lefkowitz, E. J., Lu, J., Matsushima, P., McAhren, S. M., McHenney, M., McLeaster, K., Mundy, C. W., Nicas, T. I., Norris, F. H., O'Gara, M., Peery, R. B., Robertson, G. T., Rockey, P., Sun, P. M., Winkler, M. E., Yang, Y., Young-Bellido, M., Zhao, G., Zook, C. A., Baltz, R. H., Jaskunas, S. R., Rosteck, P. R., Jr., Skatrud, P. L., and Glass, J. I. (2001) *J. Bacteriol.* **183,** 5709–5717
28. Tettelin, H., Nelson, K. E., Paulsen, I. T., Eisen, J. A., Read, T. D., Peterson, S., Heidelberg, J., DeBoy, R. T., Haft, D. H., Dodson, R. J., Durkin, A. S., Gwinn, M., Kolonay, J. F., Nelson, W. C., Peterson, J. D., Umayam, L. A., White, O., Salzberg, S. L., Lewis, M. R., Radune, D., Holtzapple, E., Khouri, H., Wolf, A. M., Utterback, T. R., Hansen, C. L., McDonald, L. A., Feldblyum, T. V., Angiuoli, S., Dickinson, T., Hickey, E. K., Holt, I. E., Loftus, B. J., Yang, F., Smith, H. O., Venter, J. C., Dougherty, B. A., Morrison, D. A., Hollingshead, S. K., and Fraser, C. M. (2001) *Science* **293,** 498–506
29. Kloosterman, T. G., Bijlsma, J. J. E., Kok, J., and Kuipers, O. P. (2006) *Microbiology* **152,** 351–359
30. Song, J. H., Ko, K. S., Lee, J. Y., Baek, J. Y., Oh, W. S., Yoon, H. S., Jeong, J. Y., and Chun, J. (2005) *Mol. Cells* **19,** 365–374
31. Pearce, B. J., Iannelli, F., and Pozzi, G. (2002) *Res. Microbiol.* **153,** 243–247
32. Sanders, J. W., Venema, G., Kok, J., and Leenhouts, K. (1998) *Mol. Gen. Genet.* **257,** 681–685
33. Kuipers, O. P., Ruyter, P. G., Kleerebezem, M., and Vos, W. M. (1998) *J. Biotechnol.* **64,** 15–21
34. den Hengst, C. D., Curley, P., Larsen, R., Buist, G., Nauta, A., van Sinderen, D., Kuipers, O. P., and Kok, J. (2005) *J. Bacteriol.* **187,** 512–521
35. Fisher, S. H., and Sonenshein, A. L. (1984) *J. Bacteriol.* **157,** 612–621
36. Ebner, E., Gancedo, C., and Holzer, H. (1970) *Methods Enzymol.* **17,** 922–927
37. Israelsen, H., Madsen, S. M., Vrang, A., Hansen, E. B., and Johansen, E. (1995) *Appl. Environ. Microbiol.* **61,** 2540–2547

38. van Hijum, S. A. F. T., de Jong, A., Baerends, R. J. S., Karsens, H. A., Kramer, N. E., Larsen, R., den Hengst, C. D., Albers, C. J., Kok, J., and Kuipers, O. P. (2005) *BMC Genomics* **6,** 77
39. Kuipers, O. P., de Jong, A., Baerends, R. J. S., van Hijum, S. A. F. T., Zomer, A. L., Karsens, H. A., den Hengst, C. D., Kramer, N. E., Buist, G., and Kok, J. (2002) *Antonie Leeuwenhoek* **82,** 113–122
40. van Hijum, S. A. F. T., Garcia de la Nava, J., Trelles, O., Kok, J., and Kuipers, O. P. (2003) *Appl. Bioinformatics* **2,** 241–244
41. Kharat, A. S., and Tomasz, A. (2003) *Infect. Immun.* **71,** 2758–2765
42. Varmanen, P., Savijoki, K., Avall, S., Palva, A., and Tynkkynen, S. (2000) *J. Bacteriol.* **182,** 146–154
43. Nakano, Y., and Kimura, K. (1991) *J. Biochem.* (*Tokyo*) **109,** 223–228
44. Baerends, R. J. S., Smits, W. K., de Jong, A., Hamoen, L. W., Kok, J., and Kuipers, O. P. (2004) *Genome Biol.* **5,** R37
45. Paterson, G. K., and Mitchell, T. J. (2006) *Microbes Infect.* **8,** 145–153
46. Belitsky, B. R., Kim, H. J., and Sonenshein, A. L. (2004) *J. Bacteriol.* **186,** 3392–3398
47. Wray, L. V., Jr., Zalieckas, J. M., Ferson, A. E., and Fisher, S. H. (1998) *J. Bacteriol.* **180,** 2943–2949
48. Gutowski, J. C., and Schreier, H. J. (1992) *J. Bacteriol.* **174,** 671–681
49. Almassy, R. J., Janson, C. A., Hamlin, R., Xuong, N. H., and Eisenberg, D. (1986) *Nature* **323,** 304–309
50. Avery, O. T., Macleod, C. M., and McCarty, M. (1944) *Mol. Med.* **1,** 344–365
51. Leenhouts, K., Bolhuis, A., Venema, G., and Kok, J. (1998) *Appl. Microbiol. Biotechnol.* **49,** 417–423
52. Leenhouts, K., Buist, G., Bolhuis, A., ten, B. A., Kiel, J., Mierau, I., Dabrowska, M., Venema, G., and Kok, J. (1996) *Mol. Gen. Genet.* **253,** 217–224
53. de Ruyter, P. G., Kuipers, O. P., and de Vos, W. M. (1996) *Appl. Environ. Microbiol.* **62,** 3662–3667