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# The *Streptococcus mutans* GlnR protein exhibits an increased affinity for the *glnRA* operon promoter when bound to GlnK

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## Abstract

The control of nitrogen metabolism in pathogenic Gram-positive bacteria has been studied in a variety of species and is involved with the expression of virulence factors. To date, no data have been reported regarding nitrogen metabolism in the odontopathogenic species *Streptococcus mutans*. GlnR, which controls nitrogen assimilation in the related bacterial species, *Bacillus subtilis*, was assessed in *S. mutans* for its DNA and protein binding activity. Electrophoretic mobility shift assay of the *S. mutans* GlnR protein indicated that GlnR binds to promoter regions of the *glnRA* and *amtB-glnK* operons. Cross-linking and pull-down assays demonstrated that GlnR interacts with GlnK, a signal transduction protein that coordinates the regulation of nitrogen metabolism. Upon formation of this stable complex, GlnK enhances the affinity of GlnR for the *glnRA* operon promoter. These results support an involvement of GlnR in transcriptional regulation of nitrogen metabolism-related genes and indicate that GlnK relays information regarding ammonium availability to GlnR.

Key words: *Streptococcus mutans*; GlnR; Transcriptional regulator; GlnK; PII-type protein

## Introduction

Among odontopathogens, *Streptococcus mutans* is responsible for most of human dental decay (1). The primary *S. mutans* virulence factors associated with cariogenicity include adhesion, acidogenicity, and acid tolerance (2). The influence of nitrogen metabolism on virulence has been described for the Gram-positive bacteria *S. pyogenes*, *S. pneumoniae*, *Bacillus anthracis*, and *Staphylococcus aureus* (3-7). A recent study suggested a similar association between putative proteins of nitrogen metabolism and virulence in *S. mutans* (8).

Most bacteria regulate nitrogen uptake via AmtB, a well-conserved ammonium transport membrane protein (9). Intracellularly, nitrogen is assimilated by the glutamine synthetase/glutamate synthetase (GS/GOGAT) pathway or the glutamate dehydrogenase (GDH) pathway (9). The regulation of nitrogen metabolism is coordinated by the PII-type signal transduction proteins, GlnB and GlnK (formerly NrgB), which control the activities of enzymes, membrane transport proteins, and transcription factors (9,10). Although the GS/GOGAT assimilatory enzymes and PII signal transduction proteins are widely conserved in

Gram-positive bacteria, other modes of regulating nitrogen metabolism also exist (11).

The regulation of nitrogen metabolism in *S. mutans* is poorly understood. The best characterized Gram-positive bacterium is *Bacillus subtilis*, which is used as a model organism for low G+C Gram-positive bacteria. *B. subtilis* expresses two DNA-binding proteins of the MerR family, GlnR and TnrA, which cooperate in the transcriptional repression or activation of nitrogen metabolism genes (12,13). GlnR represses the *glnRA* and *ureABC* operons, which encode GlnR and glutamine synthetase and urea utilization enzymes, respectively, and the *tnrA* gene (14-16). TnrA activates the transcription of the *amtB-glnK* (ammonium transport), *ureABC*, *nasBC* and *nasDEF* (nitrate and nitrite utilization) operons and represses *glnRA* and *gltAB*, which encodes glutamate synthase (17-20). Feedback-inhibited GS regulates the activities of both proteins by stabilizing the GlnR-DNA complex and preventing TnrA from binding to DNA (21,22). A study of the *B. subtilis* GlnK protein identified a GlnK-TnrA interaction; however, the physiological significance of this interaction remains unknown (23).

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**Table 1.** Bacterial strains and plasmids.

Strains/plasmids	Genotype/phenotype	Reference
<i>Escherichia coli</i>		
BL21 AI	F <sup>-</sup> <i>ompT hsdS<sub>B</sub> (r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>) gal dcm araB::T7RNAP-tetA</i>	Invitrogen
<i>Streptococcus mutans</i>		
UA159	Wild-type strain, Bacteriocin-, BV+, Gramicidin+ (ATCC 700610)	24
Plasmids		
pET28R	Km <sup>R</sup> , <i>S. mutans glnR</i> gene cloned into pET28b+, used to express GlnR-(His) <sub>6</sub>	Present study
pET29R	Km <sup>R</sup> , <i>S. mutans glnR</i> gene cloned into pET29a+, used to express native GlnR	Present study

Studies of nitrogen metabolism in *S. mutans* may provide insight into the virulence mechanisms of this pathogen. In this report, we demonstrate that *S. mutans* GlnR binds to the promoter regions of *amtB-glnK* and *glnRA* operons. In addition, we show for the first time a stable interaction between the GlnR and GlnK proteins. GlnK within this complex enhances the affinity of GlnR for DNA.

## Material and Methods

### Bacterial strains and growth conditions

*Escherichia coli* BL21-AI was grown at 37°C in Luria broth (LB) or Luria agar (LA) medium supplemented with kanamycin (50 µg/mL) as appropriate (Table 1).

### Cloning, expression and purification of recombinant GlnR

The *S. mutans* UA159 *glnR* gene was amplified by PCR with primers N-GlnR and C-GlnR (Table 2) using *S. mutans* chromosomal DNA as a template. PCR products were digested with *Nde*I and *Bam*HI and cloned into pET28b+ (Novagen, EMD4 Biosciences, USA) to produce the plasmid pET28R, which expresses GlnR-(His)<sub>6</sub>. The same digested PCR products were cloned into pET29b+ (Novagen, EMD4 Biosciences) to produce the plasmid pET29R, which expresses native GlnR (Table 1). These plasmids were then transformed into *E. coli* BL21-AI and transformants were selected on LA supplemented with kanamycin.

Bacteria transformed with pET29R were cultured on 1 L LB medium at 16°C for 16 h after induction with 0.2% arabinose. Cells were pelleted by centrifugation, resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 10% glycerol), and lysed by sonication. The soluble fraction of the cell extract was purified by ion exchange chromatography on a Q-Sepharose column (GE Healthcare Ltd., UK), and proteins were eluted with a NaCl gradient (0.2-1 M). Fractions containing GlnR were selected after SDS-PAGE visualization. These fractions were mixed and diluted in 50 mM Tris-HCl, pH 8.0, and 10% glycerol to a final concentration of 0.2 M NaCl and loaded onto a heparin column (GE Healthcare Ltd.). Proteins then were eluted through a second NaCl gradient (0.2-2 M).

**Table 2.** Primers used in the present study.

Primer	Sequence
N-GlnR	5' GAAAGGAGGAAACATATGAAAG 3'
C-GlnR	5' AAAAATTAAGGATCCACGTTACG 3'
PamtBfor	5' AGCTAAGCTTTAGAGCCCTAG 3'
PamtBrev	5' GATAATAAATGCTATAGATCC 3'
PglnRfor	5' GGCCATGAGTAATAAGACGGG 3'
PglnRrev	5' CCGCCATTGATCGTCAAGC 3'

Restriction enzyme cleavage sites are underlined.

GlnR fused to an N-terminal hexahistidyl tag was over-expressed in *E. coli* BL21-AI transformed with pET28R and grown on LA at 30°C. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 40 mM imidazole, 10% glycerol), and disrupted by sonication. The GlnR-(His)<sub>6</sub> protein was purified from the soluble cell fraction by chromatography on an immobilized nickel ion affinity HisTrap HP column (GE Healthcare Ltd.). Proteins were eluted with an imidazole gradient (40 µM to 1 M).

Protein purity was analyzed by 12% SDS-PAGE (25). Apparently homogeneous fractions were dialyzed in two steps: 1) against the equilibration buffer DA (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20% glycerol), and 2) against the buffer DB (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 20% glycerol). GlnR and GlnR-(His)<sub>6</sub> proteins were stored in buffer DB, and protein concentration was determined by the Bradford method (26). Protein concentrations were calculated as proteins in monomeric state.

*S. mutans* GlnK and GlnK-(His)<sub>6</sub> proteins were expressed and purified as described by Portugal et al. (27).

### Polyclonal antibody production

Three Wistar albino rats (140 ± 20 g) were immunized subcutaneously with 20 µg GlnR or GlnK, both in native form, in complete Freund's adjuvant. Three booster injections were given at 4-week intervals and rats were bled 4 weeks after the last injection. Whole blood was centrifuged

and the serum was used in the experiments. The antibody specificity was tested in dot blot experiments.

### Electrophoretic mobility shift assay (EMSA)

The *S. mutans* promoter regions *PamtB-glnK* and *PglnRA* were amplified by PCR using primers *PamtBfor/PamtBrev* and *PglnRfor/PglnRrev*, respectively (Table 2). After amplification, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany). When indicated, probes were labeled with [ $\gamma^{32}\text{P}$ ]-ATP using T4 polynucleotide kinase (New England Biolabs, USA). For DNA binding experiments, reaction buffer (10 mM Tris, pH 8.0, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 8% [v/v] glycerol, 0.05% *N*-octyl glucoside, 100  $\mu\text{g}/\text{mL}$  BSA, and 50  $\mu\text{g}/\text{mL}$  poly[dA-dC]) was combined with DNA fragments corresponding to the *PglnRA* or *PamtB-glnK* region. Either unlabeled or radiolabeled fragments were used, as indicated. GlnR or GlnR-(His)<sub>6</sub> was then added at various concentrations, and the reactions were incubated at 30°C for 15 min. Samples were subjected to native 6.5% PAGE in TG buffer (50 mM Tris, 50 mM glycine, pH 8.0). Gels were stained with SYBR Green (Invitrogen, USA) when unlabeled DNA fragments were used. Radiolabeled DNA fragments in dried gels were visualized using a PhosphorImager (Molecular Dynamics, USA) system. Band intensities were analyzed using the LabWorks software (UVP, USA).

The effect of GlnK on the binding affinity of GlnR to *PglnRA* DNA was determined by adding various concentrations of native GlnK to the incubation buffer.

Approximated apparent dissociation constants ( $K_{0.5}$ ) were calculated by fitting the data to a double-reciprocal plot as described by Levitzki (28).  $K_{0.5}$  corresponds to the concentration of GlnR necessary for 50% of DNA binding sites to be occupied.

### His tag pull-down assays

GlnR/GlnK complex formation was studied *in vitro* using pull-down assays. The proteins GlnR and GlnK-(His)<sub>6</sub> were incubated in buffer A (10-mM Tris, pH 8.0, 5 mM MgCl<sub>2</sub>, 8% glycerol, 0.05% *N*-octyl glucoside, 80 mM imidazole) in the presence or absence of 15 nM DNA at room temperature. Ni<sup>2+</sup>-chelating Sepharose resin (50  $\mu\text{L}$ ; GE Healthcare Ltd.) was equilibrated in buffer A, and 5  $\mu\text{M}$  GlnK-(His)<sub>6</sub> was added. After 5 min, 2.5  $\mu\text{M}$  GlnR was added. The mixtures were washed three times with 300  $\mu\text{L}$  buffer A with or without DNA. Proteins were eluted with buffer B (10 mM Tris, pH 8.0, 5 mM MgCl<sub>2</sub>, 8% glycerol, 0.05% *N*-octyl glucoside, 1 M imidazole). As control, the resin was incubated in buffer A either with GlnK-His or GlnR proteins. Samples were analyzed by 10% Tricine-SDS-PAGE stained with colloidal Coomassie blue.

### Cross-linking experiments

The interaction of GlnR and GlnK was further analyzed via chemical cross-linking with glutaraldehyde. GlnK and

GlnR, both in native form, were incubated in HGNEED buffer (1 mM DTT, 100 mM NaCl, 0.2 M EDTA, 0.05% [v/v] NP-40, 10% glycerol, 25 mM HEPES, pH 8.0) at room temperature for 15 min. Glutaraldehyde was added to a final concentration of 0.1% (v/v) and the reactions were quenched after 7 min by the addition of 0.1 volume of 2 M Tris. Proteins were fractionated by 15% SDS-PAGE (25) and transferred to polyvinylidene difluoride membranes (Millipore, USA) (29). Membranes were probed with mouse antiserum against GlnK or GlnR, and proteins were visualized by chemiluminescence using horseradish peroxidase-labeled secondary antibodies and luminol (ECL Western Blotting System, GE Healthcare Ltd.).

In these experiments, the Prestained Protein Molecular Weight Marker (Fermentas, Canada) was used to verify the Western transfer efficiency and to estimate the protein weight through relative mobility (30).

All experiments were performed at least twice.

## Results

### GlnR binds DNA corresponding to *glnK-amtB* and *glnRA* promoters *in vitro*

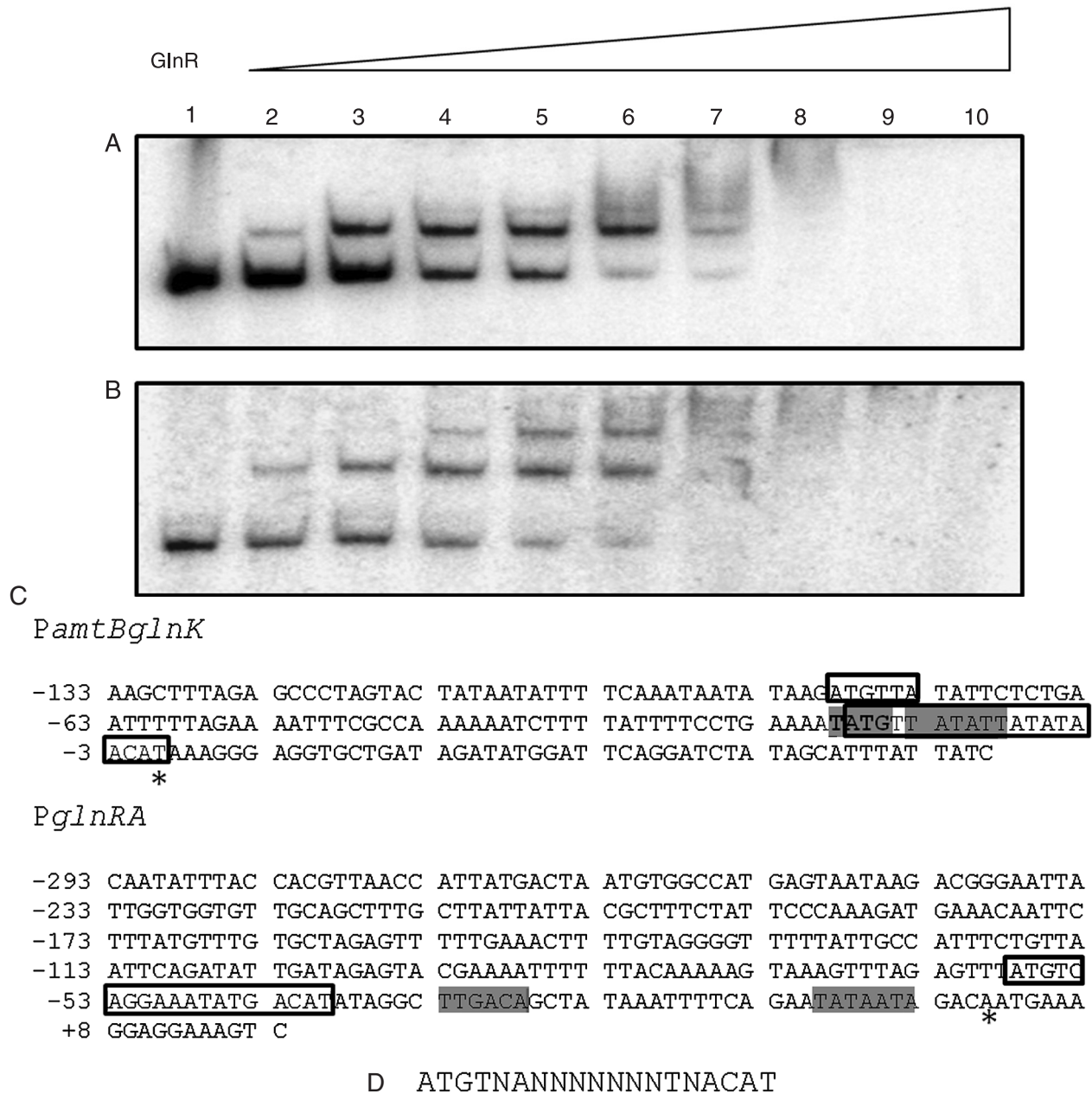
GlnR binding to *glnK-amtB* and *glnRA* promoter regions was examined by EMSA. The  $K_{0.5}$  values for *PglnRA* and *PamtB-glnK* were 50 and 24 nM, respectively (Figure 1A and B). The *PglnRA* DNA fragment used in this study includes a putative palindromic binding site (ATGTNAN<sub>7</sub>TNACAT) (8) at -58 bp relative to the possible transcriptional start point (Figure 1C). The DNA fragment with the *PamtB-glnK* region contains the putative GlnR binding site at -18 bp relative to the possible transcriptional start point and a 6-base conserved repeat at -79 bp (Figure 1C). The presence of two band shifts in GlnR concentrations over 50 nM suggests that these binding sites are recognized by GlnR *in vitro* (Figure 1B).

### GlnR interacts with GlnK *in vitro*

The oligomeric states of GlnR and GlnK and complex formation between these proteins were studied using chemical cross-linking with glutaraldehyde (Figure 2A and B). GlnR existed as a dimer independently of the presence of *glnRA* promoter DNA (Figure 2A, lanes 2 and 3). After chemical cross-linking, GlnK protein samples were visualized as five bands with different migration characteristics (Figure 2B, lane 5). Although this result may be explained as a cross-linking artifact, it is also possible that GlnK exists as five different oligomers under the tested conditions. The observed bands possibly correspond to many oligomeric forms of GlnK. GlnR interacts directly with different putative GlnK oligomers (Figure 2A, lane 7).

Complex formation between native GlnR and GlnK-(His)<sub>6</sub> was examined using pull-down assays in the absence or presence of *glnRA* promoter DNA (Figure 2C and D). GlnR was found to stably interact with GlnK in the absence





**Figure 1.** GlnR-(His)<sub>6</sub> binding to DNA. **A**, Increasing quantities of GlnR-(His)<sub>6</sub> were incubated in the presence of 0.3 nM of a radio-labeled *glnRA* promoter DNA fragment (311 bp). Lane 1 = no GlnR; lanes 2-10 = 10, 25, 50, 10, 250, and 500 nM, 1, 2.5, and 5 μM GlnR, respectively. **B**, Increasing quantities of GlnR-(His)<sub>6</sub> were incubated in the presence of 0.6 nM of an *amtB-glnK* promoter DNA fragment (174 bp). Lane 1 = no GlnR; lanes 2-10 = 10, 25, 50, 10, 250, and 500 nM, 1, 2.5, and 5 μM GlnR, respectively. **C**, DNA sequence of *amtB-glnK* and *glnRA* promoter regions. The putative GlnR binding sites (8) are boxed, the possible transcription start points are indicated by asterisks and possible -10 and -35 elements are presented in gray boxes. These are the sequences of DNA fragments used in electrophoretic mobility shift assay (EMSA) experiments. **D**, GlnR binding site consensus sequence with 6-base inverted repeats spaced by 7 nt (8).

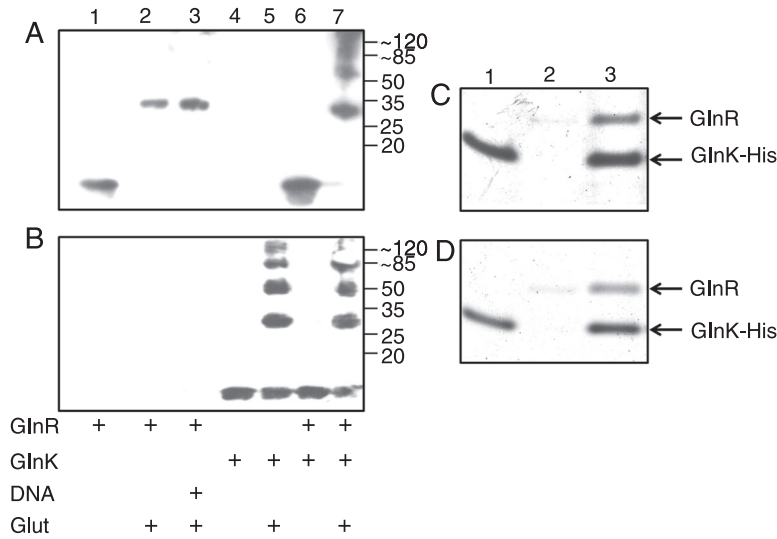
of DNA or in the presence of 15 nM *glnRA* promoter DNA (Figure 2), implying that DNA is not required for GlnR-GlnK complex formation.

**GlnK enhances DNA-binding activity of GlnR *in vitro***

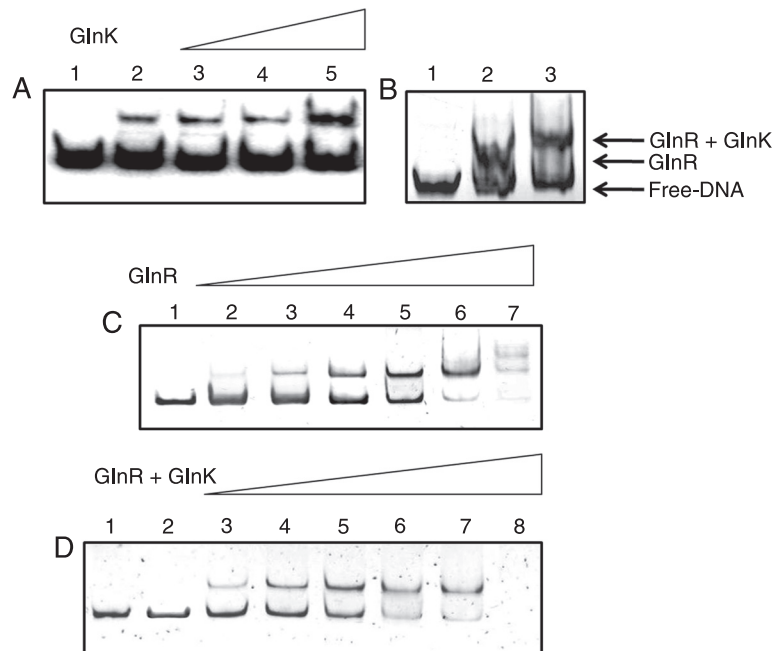
The effect of GlnK on the ability of GlnR to bind *glnRA* promoter DNA was determined using EMSA (Figure 3). Various concentrations of GlnK were added to 50 nM GlnR (protomer). In the presence of 150 nM GlnK (protomer), which corresponds to a 6-fold excess over dimeric GlnR

(25 nM), binding of GlnR to DNA was increased 5-fold as estimated by band densitometry (Figure 3A, lane 5). This result suggests that two GlnK trimers or, possibly, the hexameric form of GlnK increases the affinity of the GlnR dimer for DNA.

The EMSA analyses detected the formation of a stable ternary complex between GlnR, GlnK and DNA and confirmed the results of the pull-down experiments in the presence of DNA. Long-term EMSA indicated that the ternary complex exhibited a considerably lower mobility than the



**Figure 2.** Formation of the GlnR-GlnK complex *in vitro*. *A* and *B*, Western blots of GlnR and GlnK cross-linking experiments. Cross-linking reactions containing the indicated components were separated by 15% SDS-PAGE and evaluated by Western blot analysis with anti-GlnR (*A*) or anti-GlnK (*B*) antiserum. Native GlnR, native GlnK, *glnRA* promoter DNA, and glutaraldehyde (Glut) were used at concentrations of 2.5, 2.5, and 0.4 μM, and 0.1% (v/v), respectively. *C* and *D*, His-tag pull-down assay. Resin was loaded with 5 μM GlnK-(His)<sub>6</sub> (12.1 kDa; *C* and *D*, lanes 1 and 3) and 2.5 μM GlnR (14.3 kDa; *C* and *D*, lanes 2 and 3). DNA fragments containing *glnRA* promoter DNA (15 nM) were added to the reaction mixture (*D*) Tricine-SDS 10% gel electrophoresis of proteins eluted from the resin. Arrows indicate the identified proteins.



**Figure 3.** The influence of GlnK on GlnR binding to DNA. *A*, Native GlnR (50 nM promoter) was incubated with 0.3 nM of a radiolabeled 311-bp *glnRA* promoter DNA fragment, and increasing quantities of native GlnK protomer. Lane 1 = no GlnR; lane 2 = GlnR; lanes 3-5 = GlnR and 25 nM GlnK; GlnR and 75 nM GlnK, and GlnR and 150 nM GlnK, respectively. *B*, Native GlnR (50 nM) was incubated with 6 nM of the *glnRA* promoter DNA fragment and 25 nM native GlnK, and electrophoresis was performed for twice the duration of the other gels described in this study. Lane 1 = no GlnR; lane 2 = GlnR; lane 3 = GlnR and GlnK. *C*, Increasing quantities of native GlnR were incubated in the presence of 3 nM *glnRA* promoter DNA fragments. Lane 1 = no GlnR; lanes 2-7 = 10, 25, 50, 10, 250, and 500 nM GlnR, respectively. *D*, Increasing quantities of native GlnR and native GlnK were incubated in the presence of 3 nM *glnRA* promoter DNA fragments. Lane 1 = no proteins; lane 2 = 600 nM GlnK; Lanes 3-8 = 10 nM GlnR and 30 nM GlnK; 20 nM GlnR and 60 nM GlnK; 50 nM GlnR and 150 nM GlnK; 100 nM GlnR and 300 nM GlnK; 200 nM GlnR and 600 nM GlnK, and 500 nM GlnR and 1.5 μM GlnK, respectively.

GlnR-DNA complex (Figure 3B, lane 3).

The effect of GlnK on the affinity of GlnR binding to *glnRA* promoter DNA was also examined by EMSA (Figure 3C and D). GlnK did not bind DNA fragments under these conditions (Figure 3D, lane 2). In the presence of a 6-fold excess of GlnK protomer relative to the GlnR dimer, the  $K_{0.5}$  was 13 nM (Figure 3D). When only GlnR was present in the reaction mixture, the  $K_{0.5}$  was 50 nM (Figures 1A and 3C). These results indicate that GlnK increases GlnR-DNA binding activity by a factor of approximately 4.

## Discussion

We report here the cloning, expression, and purification of the *S. mutans* GlnR protein. Our results demonstrate that *S. mutans* GlnR binds to *glnRA* promoter DNA, as described for *B. subtilis* GlnR (12), and that *S. mutans* GlnR binds the *amtB-glnK* promoter. These results agree with the *in vivo* results obtained by Chen et al. (8), which showed that *S. mutans* GlnR regulates its own transcription and the transcription of the *amtB-glnK* operon.

The present study describes for the first time an interaction between *S. mutans* GlnK and GlnR *in vitro* (Figure 2). EMSA analyses identified stable GlnR-GlnK-DNA complexes (Figure 3B). In *B. subtilis*, GlnK binds to the TnrA protein, which is functionally and structurally analogous to GlnR (23). However, no studies have examined the modulation of TnrA or GlnR activity by GlnK.

Compared to GlnR alone, *S. mutans* GlnR bound to GlnK has a 4-fold higher affinity for *glnRA* promoter DNA (Figure 3C and D), suggesting that GlnK enhances GlnR-mediated transcriptional repression of the *glnRA* promoter.

Interactions between GlnK and various transcription factors have been described for the Gram-positive *Corynebacterium glutamicum*. In this microorganism, adenylylated GlnK interacts with AmtR, a transcriptional repressor, leading to de-repression of genes involved in the control of

nitrogen metabolism (31). In cyanobacteria, GlnK regulates gene expression by binding to NtcA and PipX proteins. NtcA is a global transcriptional activator, and PipX binds to and enhances the activity of NtcA during nitrogen starvation. Under nitrogen excess, GlnK interacts with PipX to impair NtcA activation (32-34).

Almost all PII-type proteins are trimeric; however, gel filtration chromatography results suggest that the GlnK protein of *S. mutans* has the molecular weight of a hexamer in solution (27). Supporting this, an *S. mutans* GlnK structure deposited in the Protein Data Bank (PDB ID: 3L7P) is described as a biological assembly of six domains (Fan X-X, Wang K-T, Su X-D; Protein Data Bank, 2011). Our results suggest that a 6-fold excess of GlnK protomer increases the affinity of the GlnR dimer for *glnRA* operon promoter DNA. This implies that two trimers of GlnK protein, or possibly its hexameric form, are responsible for the increase in GlnR DNA-binding activity (Figure 3A and D).

GlnR-DNA binding was confirmed *in vitro* for the *amtB-glnK* and *glnRA* promoters. The *in vitro* formation of a stable complex between GlnR and GlnK is described here for the first time. Once bound to GlnK, GlnR exhibits a higher affinity for *glnRA* promoter DNA. Information regarding nitrogen availability is likely to be transmitted to GlnR via GlnK binding in *S. mutans*.

Future *in vivo* studies should permit the determination of the physiological conditions in which the GlnR-GlnK interaction occurs.

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