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Streptococcus mutans GlnK protein: an unusual PII family member

M.E.G. Portugal, E.M. Souza, F.O. Pedrosa and E.M. Benelli

Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba, PR, Brasil

Abstract

Streptococcus mutans is a Gram-positive bacterium present in the oral cavity, and is considered to be one of the leading causes of dental caries. *S. mutans* has a *glnK* gene, which codes for a PII-like protein that is possibly involved in the integration of carbon, nitrogen and energy metabolism in several organisms. To characterize the GlnK protein of *S. mutans*, the *glnK* gene was amplified by PCR, and cloned into the expression vectors pET29a(+) and pET28b(+). The native GlnK-Sm was purified by anion exchange (Q-Sepharose) and affinity (Hi-Trap Heparin) chromatography. The GlnK-His-Sm protein was purified using a Hi-Trap Chelating-Ni^{2+} column. The molecular mass of the GlnK-His-Sm proteins was 85 kDa as determined by gel filtration, indicating that this protein is a hexamer in solution. The GlnK-His-Sm protein is not uridylylated by the *Escherichia coli* GlnD protein. The activities of the GlnK-Sm and GlnK-His-Sm proteins were assayed in *E. coli* constitutively expressing the *Klebsiella pneumoniae nifLA* operon. In *K. pneumoniae*, NifL inhibits NifA activity in the presence of high ammonium levels and the GlnK protein is required to reduce the inhibition of NifL in the presence of low ammonium levels. The GlnK-Sm protein was unable to reduce NifL inhibition of NifA protein. Surprisingly, the GlnK-His-Sm protein was able to partially reduce NifL inhibition of the NifA protein under nitrogen-limiting conditions, in a manner similar to the GlnK protein of *E. coli*. These results suggested that *S. mutans* GlnK is functionally different from *E. coli* PII proteins.

Key words: GlnK; *Streptococcus mutans*; Nitrogen metabolism

Introduction

In Gram-negative bacteria, the PII proteins are involved in the regulation of the nitrogen regulation (Ntr) system and glutamine synthetase (GS) activities (1). The Ntr system consists of the NtrB and NtrC proteins. In the presence of high ammonium concentrations NtrB interacts with a PII protein and dephosphorylates NtrC, which in this form is not able to induce the transcription of the genes related to the utilization of alternative nitrogen sources (2).

The activity of the GS protein, which converts glutamate to glutamine, is controlled by several mechanisms. One is the covalent modification catalyzed by the ATase (formerly GlnE) protein (3). The ATase protein interacts with the PII proteins under high and low ammonium levels, catalyzing the deadenylylation (activation) or adenylylation (inactivation) of the GS protein (4). The interaction of the PII protein with other proteins is also modulated by the binding of the effectors ATP, ADP and 2-oxoglutarate (2-OG) (5,6). In Gram-negative bacteria, the PII proteins are also regulated by uridylylation of the Try51 residue mediated by the GlnD protein under nitrogen-limiting conditions (7).

In nitrogen-fixing bacteria, the expression of nitrogen fixation genes is under the control of the NifA protein. In the enteric Gram-negative bacterium *Klebsiella pneumoniae,* the transcription of the genes that code for the nitrogenase enzyme (*nif* genes) is activated by NifA (8,9) coded by the *nifA* gene, which is co-transcribed with the *nifL* gene. The NifL protein interacts with and inhibits NifA in the presence of high ammonium or oxygen levels (10). When the ammonium levels are low, the GlnK protein, whether urydylylated or not, blocks the formation of the NifL-NifA complex and NifA becomes active.

The *glnK* gene is frequently co-transcribed with the *amtB* gene in proteobacteria and the transcription of this operon is activated by the phosphorylated form of NtrC. The *amtB* gene codes for a membrane protein involved in ammonium transport (11,12). When the ammonium levels are high, the free GlnK protein binds to the AmtB protein, blocking the passage of the ammonium ions (13).

The PII proteins were also identified in cyanobacteria. In *Synechococcus* sp PCC7942, the GlnK protein undergoes

Correspondence: E.M. Benelli, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Caixa Postal 19046, 81531-990 Curitiba, PR, Brasil. E-mail: benelli@ufpr.br

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reversible phosphorylation of Ser49 when the ATP and 2-OG levels are high. In the presence of high ammonium levels, GlnK is dephosphorylated by the PphA protein, a reaction dependent on Mg^{2+} or Mn^{2} ions and inhibited by PO_4^- (14).

The PII proteins of Archaea show no evidence of covalent modification since they do not possess either Ser49 or Tyr51 (15). In these organisms, GlnK1 associates with and inhibits GlnA1 (a GS isoform), although high levels of 2-OG antagonize this inhibitory effect of GlnK1 (16).

In Gram-positive bacteria, the regulation of nitrogen metabolism varies. In high G+C organisms such as *Streptomyces coelicolor*, no gene similar to *ntrB* or *ntrC* was identified and the GlnK protein seems to be co-transcribed with the *amtB* and *glnD* genes forming the *amtBglnKglnD* operon. The expression of the *amtBglnKglnD* operon is positively controlled by the GlnR and GlnRII proteins when the nitrogen levels are low (17). Hesketh et al. (18) showed that in *S. coelicolor*, the GlnK protein is reversibly adenylylated at Tyr51 by the GlnD protein in the presence of low nitrogen levels. GlnK may also be irreversibly modified by the removal of the first three amino acid residues of its N-terminal end (18).

In *Bacillus subtilis*, a low G+C bacterium, the expression of the *amtBglnK* operon is activated by the TnrA protein. In this organism, in the presence of high ammonium levels, the GlnR protein represses the expression of the *glnRA* operon (19). The *B. subtilis* GlnK does not have uridylylation or phosphorylation sites conserved and this protein seems to be important for nitrate utilization (19). The *B. subtilis* GlnK protein is trimeric and binds ATP, whereas 2-OG is only weakly bound. In this organism, GlnK forms a tight complex with the membrane-bound ammonium transporter AmtB (formerly NrgA), from which it can be released by millimolar concentrations of ATP. The GlnK-AmtB complex also binds the TnrA protein (the major transcription factor of nitrogen metabolism). In the absence of ATP, TnrA is bound to the membrane-bound AmtB-GlnK complex, whereas in extracts from GlnK- or AmtB-deficient cells, TnrA is entirely cytoplasmic (19).

Streptococcus mutans is a bacterium found in the oral cavity, which is considered to be the leading cause of dental caries. This organism has developed several mechanisms of tooth colonization under extreme conditions (20) and is able to use a wide variety of fermenting carbohydrates, growing and surviving at low pH (20). In the oral cavity, the bacterial metabolism releases acid end products, which reduce the pH of the biofilm. When the pH is lower than 5.5, the enamel hydroxyapatite crystals of the tooth begin to demineralize, a process known as tooth decay (20).

A gene coding for a PII-like protein was identified in the *S. mutans* genome. This gene was called *glnK* since it forms an operon with a putative ammonium transporter (*glnKamtB*). The *glnK* gene codes for a protein of 113 amino acid residues, which lacks both the uridylylation and phosphorylation sites found in other GlnK proteins, suggesting novel regulatory properties. In the present study, the *S. mutans glnK* gene was cloned into expression vectors and the GlnK protein was purified in its native form and fused to a histidine tag. The results showed that this protein is probably a hexamer in solution. Due to this unusual oligomeric state, we tested if the *S. mutans* GlnK and GlnK-His proteins were able to regulate *K. pneumoniae* NifL activity using the *K. pneumoniae nifH* promoter as a probe.

Material and Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* strains were cultured at 37°C in Luria Bertani (LB) or nonfat dry milk medium (NFDM) (21) supplemented with antibiotics when necessary.

Cloning of the *S. mutans glnK* gene

The *S. mutans glnK* gene was PCR amplified using the primers glnKF (5'-GGAAGTCATATGAAAAAAATAG-3') and glnKR (5'-AAAGCTGGATCCTCTCTTTAG-3'), which included an *Nde*I and a *Bam*HI site (underlined), respectively. The PCR product was cloned into the pGEM-T vector (Promega Corporation, USA) and sub-cloned into the *Nde*I and *Bam*HI sites of the plasmids pET29a(+) and pET28b(+) (Novagen, EMD4 Biosciences, USA), originating pMEGP2 and pMEGP3, respectively. The construct was confirmed by sequencing. Plasmid pMEGP2 expresses the *S. mutans* GlnK protein in its native form (GlnK-Sm) and the pMEGP3 plasmid expresses the *S. mutans* GlnK protein with an N-terminal histidine tag (GlnK-His-Sm).

Purification and biochemical characterization of the *S. mutans* GlnK protein

Purification of GlnK-Sm and GlnK-His-Sm proteins. The plasmid pMEGP2 was introduced by electroporation into *E. coli* BL21Codon-Plus and three transformant colonies were inoculated into 5 mL LB medium supplemented with kanamycin (50 µg/mL), tetracycline (10 µg/mL), and chloramphenicol (30 µg/mL), and incubated at 125 rpm for 8 h at 37°C. These cultures were re-inoculated at 1:1000 dilution in 3 L LB medium, supplemented with 50 µg/mL kanamycin and incubated at 37°C until the culture reached an absorbance at 600 nm of 0.06. Protein expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture was incubated at 37°C for 3 h. The cells were harvested by centrifugation at 12,000 *g* for 10 min, resuspended in sonication buffer containing 1 mM phenylmethanesulfonylfluoride (PMSF) and frozen at -70°C. Upon thawing, lysozyme (50 µg/mL) was added and the cells were incubated at 4°C for 30 min. The cells were then diluted five times in sonication buffer without glycerol and sonicated five times for 25 s each at 1-min intervals. The lysed cells were treated with 2% streptomycin sulfate,

under shaking at 4°C for 30 min. The soluble fraction was collected by centrifugation at 26,900 *g* and 4°C for 30 min, and applied to a Q-Sepharose column equilibrated with buffer A (50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM EDTA, 10% glycerol) and connected to a Fast Performance Liquid Chromatography system (FPLC, GE Healthcare UK Ltd., UK). The column was washed with buffer A (2 mL/min) and the protein was eluted with an NaCl gradient ranging from 50 mM (buffer A) to 1.0 M in buffer A. The fractions were analyzed by SDS-PAGE (15%) and pooled according to their concentration and purity.

The pooled fractions were diluted in buffer I (50 mM Tris-HCl, pH 8, 10% glycerol) in order to reduce the salt concentration and to remove the EDTA, and applied to the Hi-Trap Heparin column (GE Healthcare), equilibrated with buffer E (50 mM Tris-HCl, pH 8, 50 mM NaCl, 10% glycerol), and connected to the FPLC system. The column was washed with buffer E (2 mL/min) and the protein was eluted with an NaCl gradient (50 to 2.0 mM) in buffer H (50 mM Tris-HCl, pH 8, 2 M NaCl, 10% glycerol). The fractions were analyzed and pooled as described before.

The GlnK-His-Sm protein was overexpressed in *E. coli* BL21 AI using the same procedure, except that protein expression was induced with 0.2% L-arabinose for 4 h at 37°C. To purify GlnK-His-Sm, cells were resuspended in buffer F (20 mM sodium phosphate, pH 7.8, 500 mM NaCl, 10% glycerol) and lysed as before. After this step, 1 mM PMSF was added and the broken cells were treated with 2% streptomycin sulfate under shaking at 4°C for 30 min. After centrifugation at 26,900 *g* at 4°C for 30 min, the soluble fraction was loaded onto a 5-mL Hi-Trap Chelating-Ni^{2+} column (GE Healthcare) equilibrated with buffer F and connected to an FPLC system. The GlnK-His-Sm protein was eluted with an imidazole gradient (0 to 1 M) in buffer F. The fractions containing the GlnK-His-Sm were dialyzed for 12 h against buffer D (50 mM Tris-HCl, pH 8, 50 mM NaCl, 1 mM EDTA, 50% glycerol). All the purification steps were carried out at 4°C and the protein was stored at -20°C.

Determination of protein concentration. The protein concentration in the cell extracts and of the purified protein was determined by the Bradford procedure (22). Bovine serum albumin was used as the standard.

Determination of molecular mass and purity of S. mutans GlnK proteins. The molecular mass of GlnK-His-Sm was determined by gel filtration on a Superdex 200 HR10/30 column (GE Healthcare) connected to an ÄKTA system (GE Healthcare). The MWND500 kit for molecular weights (Sigma-Aldrich Co., USA) was used to obtain a standard curve. Protein homogeneity was determined by densitometric analysis of SDS-PAGE (15%) stained with

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid	Genotype/phenotype	Source/reference
S. mutans		
UA159	Wild-type strain, Bacteriocin-, BV+ Gramicidin+ (ATCC 700610)	33
E. coli		
YMC10	*endA1 thi-1 hsdR17 supE44*Δ*lacU169* [Kan*r*- √ *(nifH'-'lacZ*)]	34
DH10B	F-*mrcA Φ*Δ80d*lacZ* ΔM 15 Δ*lac*X74 *endA1 rec*A1 *deo*R Δ(*ara, leu*)7697 *ara*D139 *galU galK nupG rpsL* λ-	21
RB9065(λDE3)	*glnB2306 glnD99*::Tn*10*, TcR (T7 polymerase promoter)	35
UNF3435	*end*A1 *thi-1 hsdR17 supE44*Δ*lacU169* [Kan*r-Φ(nifH'-'lacZ*)] Δ*glnB2306* Δ*glnK*	31
NCM1686	*glnD99*::Tn*10* [Kanr -*Φ*(*nifH'-'lacZ*)]	29
BL21 AI	F-ompT hsdSB(rB-mB-) gal dcm araB::T7RNAP-tetA	Invitrogen, USA
pGEM-T	Amp^R, A/T cloning vector	Promega, USA
pET29a(+)	Km^R, pT7	Novagen, USA
pET28b(+)	Km^R, pT7	Novagen, USA
pTZ18R	Amp^R, cloning and sequencing multifunctional, p*lac*	36
pCC46	Cm^R, *K. pneumoniae nifLA* cloned into pHSG575, p*lac*	37
pWVH149	Amp^R, *E. coli glnK* cloned into pBluescript-II KS+, p*lac*	11
pDK601	Amp^R, *E. coli glnB* cloned into pUC18 p*lac*	38
pMEGP1	Amp^R, *S. mutans glnK* cloned into pGEM-T p*lac*	Present study
pMEGP2	Km^R, *S. mutans glnK* cloned into pET29a(+) pT7. It expresses native Sm GlnK	Present study
pMEGP3	Km^R, *S. mutans glnK* cloned into pET28b(+) pT7. It expresses Sm GlnK-His	Present study
pMEGP4	Amp^R, *S. mutans glnK* cloned into pTZ18R, p*lac*	Present study
pMEGP5	Amp^R, *S. mutans glnK-His* cloned into pTZ18R, p*lac*	Present study

Coomassie blue R.

Uridylylation assay. The *E. coli* GlnD protein was purified as described by Benelli (23). The uridylylation reaction contained 2 µM GlnK-His-Sm or *Herbaspirillum seropedicae* GlnB proteins, 0.1 µM *E. coli* GlnD protein, 1 mM UTP, 1 mM ATP, 10 mM 2-OG, 6 µL uridylylation buffer (50 mM Tris-HCl, pH 7.6, 100 mM KCl and 10 mM $MgCl_2$) in a final volume of 30 µL. The reactions were incubated at 30°C for 30 min and 10 µL nondenaturating sample buffer was then added to and analyzed on a nondenaturating polyacrylamide gel (24). The gels were stained with silver (25).

Effect of *S. mutans* GlnK protein on NifA activity

To functionally characterize the *S. mutans* GlnK protein, plasmid pCC46 (*K. pneumoniae nifLA* operon expressed from the *lac* promoter) was introduced by electroporation in the *E. coli* YMC10 (wild-type), UNF3435 (*glnB*$^-$ *glnK*$^-$) and NCM1686 (*glnD*$^-$) strains. Next, pMEGP4 (*S. mutans glnK*), pMEGP5 (*S. mutans glnK-His*), pDK601 (*E. coli glnB*), pWVH149 (*E. coli glnK*) plasmids, and the pTZ18R vector were also introduced by electroporation in each of the *E. coli*-transformed strains. The β-galactosidase assays were carried as described (26).

Results and Discussion

Uridylylation assays of the *S. mutans* GlnK-His protein

The GlnK-Sm and GlnK-His-Sm proteins obtained were 92 and 98% homogeneous, respectively, on the basis of densitometric analysis of silver-stained SDS-PAGE gel.

In *E. coli*, the GlnD protein uridylylates the GlnB and GlnK proteins at the Tyr51 residue when the glutamine/2-OG ratio is low, thereby transmitting the prevailing nitrogen levels to the receptor proteins (11).

The GlnK-Sm protein does not possess the Tyr51 residue, but two tyrosine residues are present at positions 43 and 46, both located at the T-loop. A structural model of the GlnK-Sm protein constructed using the 3D-JIGSAW program (27) indicates that the Tyr43 and Tyr46 residues are on the surface of the protein (Figure 1). To determine if these GlnK-Sm residues could be uridylylated, we performed uridylylation assays using the *E. coli* GlnD protein. Upon uridylylation, PII proteins show a native electrophoretic migration shift, which depends on the number of uridylylated monomers. Figure 2 shows that both GlnK-Sm and the GlnK-His-Sm proteins are not uridylylated by the *E. coli* GlnD protein under the conditions tested.

Molecular mass determination of the GlnK-His-Sm protein

All the PII-like proteins studied are homotrimers (1,14). The molecular mass of *S. mutans* GlnK-His protein was shown to be 85 kDa by gel filtration chromatography, indicating that *S. mutans* GlnK is a hexamer in solution (Figure 3). In *Methanococcus maripaludis* there are two PII-like proteins, $NifI_1$ and $NifI_2$, which form a heterohexameric complex of 85 kDa and have another oligomeric state in the presence of 2-OG (145 kDa) (28). Sequence comparison of PII-like proteins showed that *S. mutans* GlnK forms a cluster with *Lactobacillus lactis* and *Streptococcus thermophilus* and this cluster is close to the *M. maripaludis* NifI cluster (data not shown), suggesting functional similarities among these proteins.

The GlnK-Sm protein does not reduce the NifL inhibition of the NifA protein

In *K. pneumoniae*, the transcription of the *nif* genes

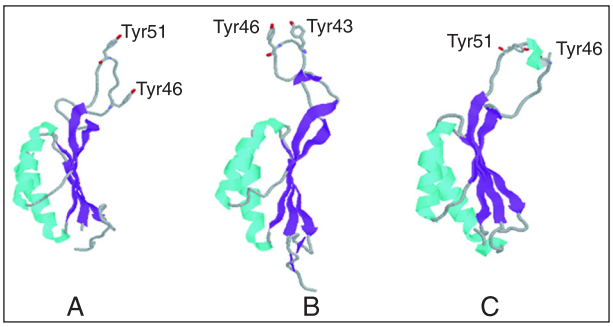

Figure 1. Monomeric structure of the PII family proteins: *Escherichia coli* GlnB (A), *Streptococcus mutans* GlnK model (B) and *E. coli* GlnK (C). The structures of the *E. coli* GlnB and GlnK proteins were obtained from the Protein Data Bank (ID: 2pii and 1GlK, respectively) and the structural model of the *S. mutans* GlnK protein was obtained using the 3D-JIGSAW program (24). The structural models were designed using the RasMol program (39). The *S. mutans* GlnK presents a Tyr43 and Tyr46 residue on the protein surfaces as the residues Tyr46 and Tyr51 from *E. coli* GlnB and GlnK.

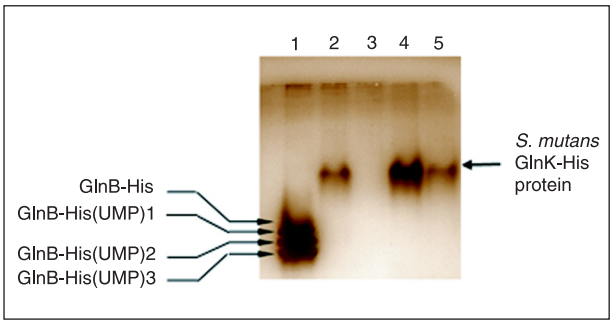

Figure 2. Uridylylation of *Streptococcus mutans* GlnK by *Escherichia coli* GlnD. Reactions were carried out as described in Material and Methods and loaded on non-denaturing polyacrylamide gel. The proteins were silver-stained. *Lane 1*, Uridylylation reaction of *Herbaspirillum seropedicae* GlnB-His protein (2 µM); *lane 2*, uridylylation reaction of GlnK-His-Sm (2 µM); *lane 3*, uridylylation reaction in the absence of GlnK-His-Sm protein; *lane 4*, uridylylation reaction of GlnK-His-Sm (2 µM) in the absence of GlnD protein; *lane 5*, uridylylation reaction of GlnK-His-Sm (2 µM) without UTP. UMP = uridine monophosphate.

(structural genes that code for the nitrogenase enzyme) is dependent on NifA protein activity, which is controlled by the NifL protein. In the presence of high ammonium and/or oxygen concentrations, NifL interacts with NifA, blocking its activating effect on *nif* gene transcription. When the levels of ammonium and oxygen are low, the GlnK protein dissociates the NifL-NifA complex, allowing NifA to induce *nif* gene transcription (29-32). To study the effect of the GlnK-Sm and GlnK-His-Sm proteins on the *K. pneumoniae* NifL-NifA system, the *S. mutans glnK* and *glnK-His* genes were cloned into the pTZ18R vector, originating the plasmids pMEGP4 (*glnK* gene) and pMEGP5 (*glnK-His* gene). These plasmids were introduced by electroporation into the *E. coli* YMC10 (wild-type), UNF3435 (*glnB*$^-$ *glnK*$^-$) and NCM1686 (*glnD*$^-$) strains. These *E. coli* strains contain the *nifH::lacZ* fusion and were previously transformed with the pCC46 plasmid, which carries the *nifLA* operon of *K. pneumoniae* under the control of the *lac* promoter. Thus, the activity of the NifA protein can be evaluated by the expression level of the *nifH* promoter, which is quantified by β-galactosidase assays (26). The pMEGP4 (*S. mutans glnK*), pMEGP5 (*S. mutans glnK-His*), pDK601 (*E. coli glnB*), pWVH149 (*E. coli glnK*) plasmids, and the pTZ18R vector were then introduced by electroporation into each one of the *E. coli* strains containing pCC46. The *E. coli* GlnB (pDK601) and GlnK (pWVH149) proteins were used as controls since the effects of these proteins on the *K. pneumoniae* NifL-NifA system have already been described (26,30).

As expected, the *nifH* promoter was not activated in the *glnB*$^-$ *glnK*$^-$ background. The NCM1686 (*glnD*$^-$) strain showed slightly higher a β-galactosidade activity than that of the UNF3435 (*glnB*$^-$ *glnK*$^-$) strain. In the *glnD*$^-$ strain, the NtrB protein phosphatase activity is stimulated and therefore the NtrC protein is constitutively dephosphorylated and unable to activate expression of the *glnK* gene (1). The *E. coli* GlnK protein restored the NifA activity in the UNF3435 (*glnB*$^-$ *glnK*$^-$) and NCM1686 (*glnD*$^-$) strains. In a similar manner, high GlnB levels also reduced NifL inhibition of NifA in the UNF3435 (*glnB*$^-$ *glnK*$^-$) and NCM1686 (*glnD*$^-$) strains. Surprisingly, expression of *S. mutans* GlnK protein (GlnK-Sm) caused a dramatic reduction of NifA activity in the wild-type *E. coli* strain (Figure 4) under derepressing conditions, suggesting that GlnK-Sm may stabilize the NifL-NifA complex, preventing its dissociation. It is possible that GlnK-Sm interacts with *E. coli* GlnB and GlnK proteins, forming non-functional hetero-oligomers, thus titrating the active form of GlnK. Also, expression of the GlnK-Sm protein in the UNF3435 (*glnB*$^-$ *glnK*$^-$) strain did not restore NifA activity (Figure 4).

Bonatto et al. (26) showed that the activation of the NifA protein of *K. pneumoniae* by the *H. seropedicae* GlnB protein requires the Tyr51 residue in the T-loop to reduce the NifL-dependent inhibition of NifA. Furthermore, both the uridylylated and the ununidylylated forms are equally effective. These investigators also demonstrated that, although the *H. seropedicae* GlnB Y51F mutant can partially restore NifA activity in the UNF3435 (*glnB*$^-$ *glnK*$^-$) and NCM1686 (*glnD*$^-$) strains, this protein was not able to block the ef-

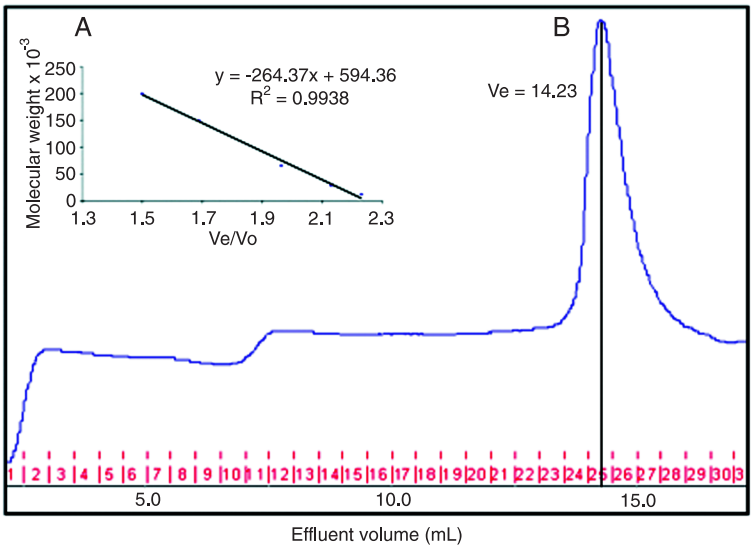

Figure 3. Determination of the molecular weight of GlnK-Sm. *A*, Calibration curve performed in Superdex 200. *B*, Gel filtration chromatography elution profile of GlnK-Sm. Column bed dimensions were 10 x 30 mm; bed volume was 24 mL; average particle size was 13 µm; load volume was 200 µL; flow rate was 0.4 mL/min.

fect of the *H. seropedicae* GlnB and GlnK proteins, as observed with the GlnK-Sm protein. Thus, the negative effect of GlnK-Sm may be due to the absence of the Tyr51 residue in this protein. Also, heterologous PII proteins may form heterotrimeric structures (11), raising the possibility that GlnK-Sm may associate with endogenous *E. coli* PII proteins rendering them incapable of relieving the NifL inhibition on NifA.

Expression of the GlnK-Sm protein in the NCM1686 (*glnD*$^-$) strain leads to increased β-galactosidase activity when compared to the same strain with the vector. It is possible that this residual activity is due to activation of the Ntr system from *E. coli* leading to expression of the *glnK* gene and thus partial reduction of NifA activity.

Effect of the GlnK-His-Sm protein on the *K. pneumoniae* NifL-NifA complex

The GlnK-His-Sm protein had an opposite effect on the relief of NifA inhibition by NifL when compared to GlnK-Sm (Figure 4). In the presence of the GlnK-His-Sm protein, the NifA activity is similar to that observed in the wild-type strain (YMC10) carrying the pTZ18R vector, although the tight ammonium regulation was partially lost. It is possible that the His tag causes structural changes that could modify the affinity and activity of the GlnK-His-Sm protein. Accordingly, the GlnK-His-Sm protein is able to restore NifA activity in the UNF3435 (*glnB*$^-$ *glnK*$^-$) strain, again with partial loss of ammonium regulation, when compared with the YMC10 (wild-type) strain. Finally, NifA activity was also restored in the NCM1686 (*glnD*$^-$) strain expressing GlnK-His-Sm protein. Previously, Benelli (23) showed that the behavior of the *H. seropedicae* GlnB and GlnB-His proteins during the uridylylation reaction of the *E. coli* GlnD protein was substantially different and attributed these differences to distinct protein structures. These results suggest that the His tag peptide, which consists of 20 amino acids linked to the N-terminal end of the GlnK-Sm protein, changed the protein structure and its capacity to affect NifL.

Structural analysis of GlnK-Sm

The similarity dendrogram of PII proteins shows that *S. mutans* GlnK protein clusters separately from those of *E. coli* and *K. pneumoniae* GlnK (data not shown). Although GlnK-Sm presented 50 and 41% identity to *E. coli* GlnB and GlnK, respectively, the *S. mutans* protein was called GlnK since it forms an operon with the putative ammonium transport (*amtBglnK*). Our results showed that GlnK-Sm is not able to complement the *E. coli glnBglnK*-double mutant, suggesting functional differences between these proteins. By aligning *E. coli* and *S. mutans* GlnK we observed that the majority of non-conserved amino acid substitutions occurred on the surface and interface between monomers (Figure 5). Comparing *S. mutants* GlnK and *E. coli* GlnK protein models, 10 non-conserved substitutions were found on the top, 4 on the lateral, and 7 on the bottom surfaces of *S. mutants* GlnK. Two additional changes were found in the central cavity. When compared with *E. coli* GlnB, 10 non-conserved substitutions were on the top, 3 on the lateral, and 8 on the bottom surfaces of *S. mutants* GlnK. The majority of the substitutions were observed on the top

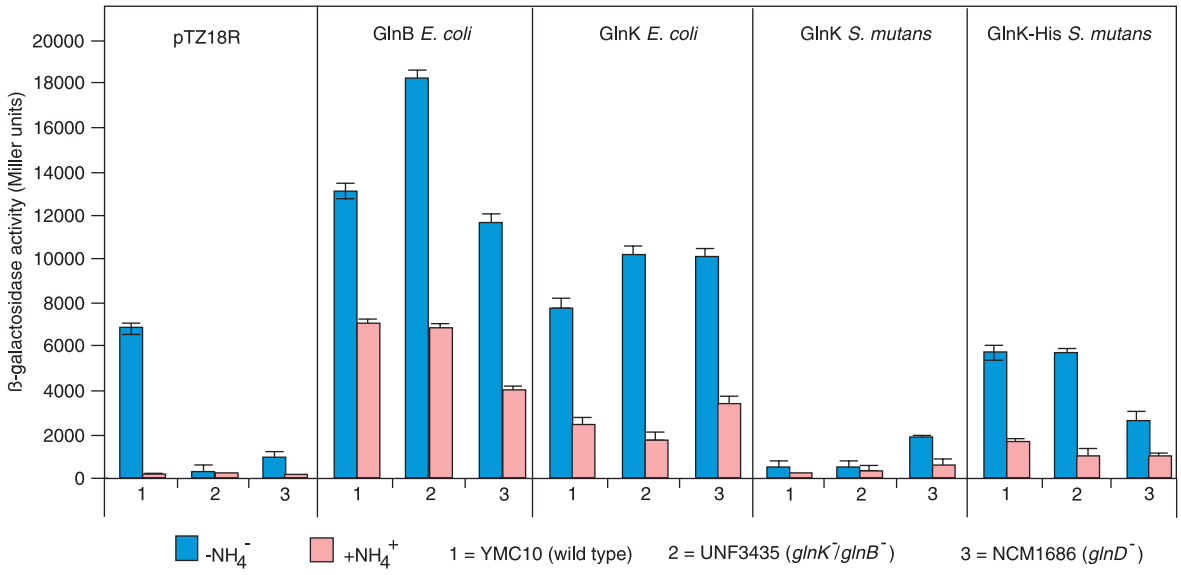

Figure 4. Effect of *Streptococcus mutans* GlnK protein on *Klebsiella pneumoniae* NifA activity. All *Escherichia coli* strains carry a (*nifH::lacZ*) fusion and plasmid pCC46, which contains the *nifLA* operon of *K. pneumoniae*. Strains were grown in the absence or presence of ammonium chloride (20 mM), under anaerobic conditions. β-galactosidase activity is reported in Miller units. Results are the means of at least three experiments with standard deviations of 15%.

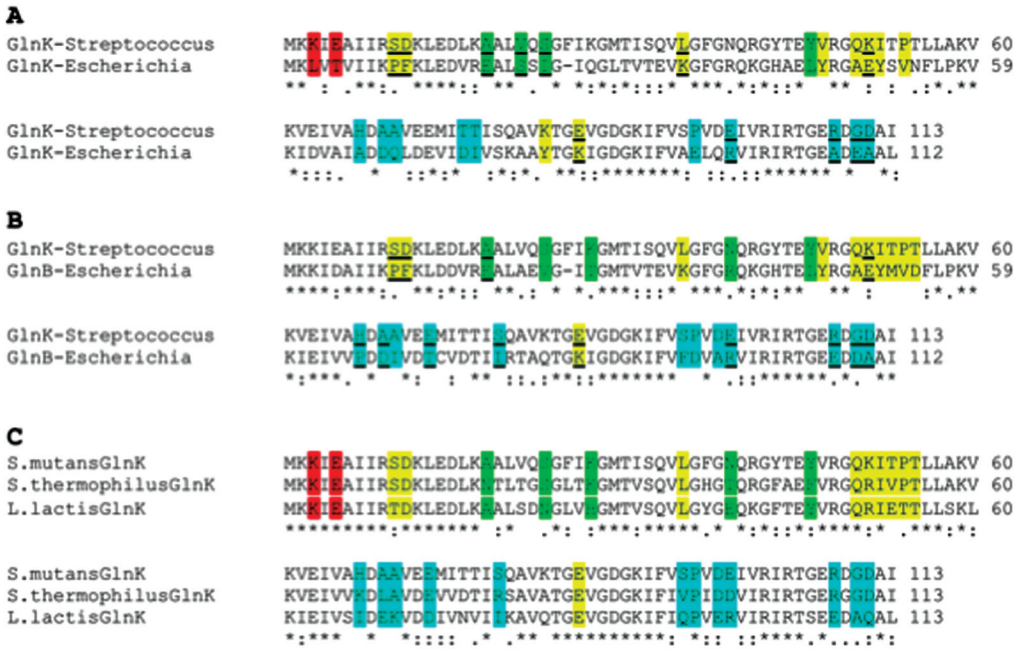

Figure 5. Alignments of the *Streptococcus mutans* GlnK and other organisms PII-like proteins. *A*, Alignment of *S. mutans* GlnK and *Escherichia coli* GlnK. *B*, Alignment of *S. mutans* GlnK and *E. coli* GlnB. *C*, Alignment of *Streptococcus mutans* GlnK, *S. thermophilus* GlnK and *Lactobacillus lactis* GlnK. The protein sequences were obtained from NCBI and the alignments were performed with Clustal X (40). Yellow = residues on the top surface; green = residues on the side surface; blue = residues on the bottom surface; red = residue towards the central cavity of PII proteins. The underlined residues indicate the change in the polarity of the protein.

surface of GlnK, and these substitutions render the *S. mutans* GlnK surface more electronegative than *E. coli* GlnK, an effect that probably leads to changes in the interaction with the proteins involved in nitrogen metabolism.

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