

Cloning, expression and characterization of glucokinase gene involved in the glucose-6-phosphate formation in *Staphylococcus aureus*

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Abstract:

Glucose-6-phosphate (G-6-P) formation in *Staphylococcus aureus* is catalysed by glucokinase (glkA) gene under high glucose concentration leading to upregulation of various pathogenic factors; therefore the present study is aimed in the cloning and characterization of glk A gene from *S. aureus* ATCC12600. The glk A gene was cloned in the Sma I site of pQE 30, sequenced (Accession number: JN645812) and expressed in *E. coli* DH5 α . The recombinant glk A expressed from the resultant glk A 1 clone was purified using nickel metal chelate chromatography, the pure enzyme gave single band in SDS-PAGE with molecular weight of 33kDa. The rglk A showed very high affinity to glucose K_m 5.1 \pm 0.06mM with Hill coefficient of 1.66 \pm 0.032mM. Analysis of glucokinase sequence of *S. aureus* showed presence of typical ATP binding site and ROK motif CNCGRSGCIE. Sequentially and phylogenetically *S. aureus* glk A exhibited low identity with other bacterial glk A and 21% homology with human glucokinase (GCK). Functionally, *S. aureus* glk A showed higher rate of G-6-P formation compared to human GCK which may have profound role in the pathogenesis.

Keywords: glk A, pQE 30, ROK, K_m .

Background:

Staphylococcus aureus is an important nosocomial and community-acquired pathogen and its infections are known to be a leading cause of bacteremia and are associated with severe morbidity and mortality in hospitalized infections [1]. In *S. aureus* 85% of Glucose is catabolised through EMP pathway and the very first step of the formation of G-6-P is carried out in two pathways one through phosphotransferase (PTS) which usually functions under low glucose concentration and the second PTS independent system catalysed by glucose permease (glk U) and glucokinase (glk A) which functions mostly under high glucose concentration [2]. This G-6-P plays critical role in the pathogen for energy generation in catabolic reactions for the synthesis of all the intermediates for its very survival [3].

Recent findings of cytoplasmic glucokinase is detected in both Gram positive and Gram negative bacteria has 315-321 residues and a monomeric mass of 33-35 kDa, K_m values of glucokinase varied from 0.3- 0.8mM for glucose and 0.4-4mM for ATP substrates in both Gram positive and Gram negative bacteria [4]. This enzyme showed greater affinity for glucose than with either mannose or galactose and showed detectable activity with fructose in Gram positive bacteria, while this enzyme showed no affinity for fructose in Gram negative bacteria [5]. Glk A in Gram positive bacteria is composed of higher percentage of alpha-helix compared to β -strand or β -turns and the enzyme exhibited both structural and functional differences with human glucokinase. Glucokinases of bacteria are divided in to 2 groups (1) Glucokinases that belongs to repressor/open

reading frames of unknown function/sugar kinases (ROK) family which is characterized by the presence of CXCGX (2)GCXE motifs, and (2) Glucokinases without ROK motifs. However, in Archaea two types of glucokinases are found one ATP-dependent glucokinase and the other ATP-dependent glucokinase having ROK motifs [6]. In multidrug resistant and vancomycin resistant strains of *S. aureus* increased cell wall

biosynthesis and high reductive conditions are the characteristic features where G-6-P formation in such strains may be playing a critical role in drug resistance and pathogenesis [7]. Therefore glk A may be playing a pivotal role, hence the present study is focused on the cloning, expression and characterization of glucokinase from *Staphylococcus aureus* ATCC12600.

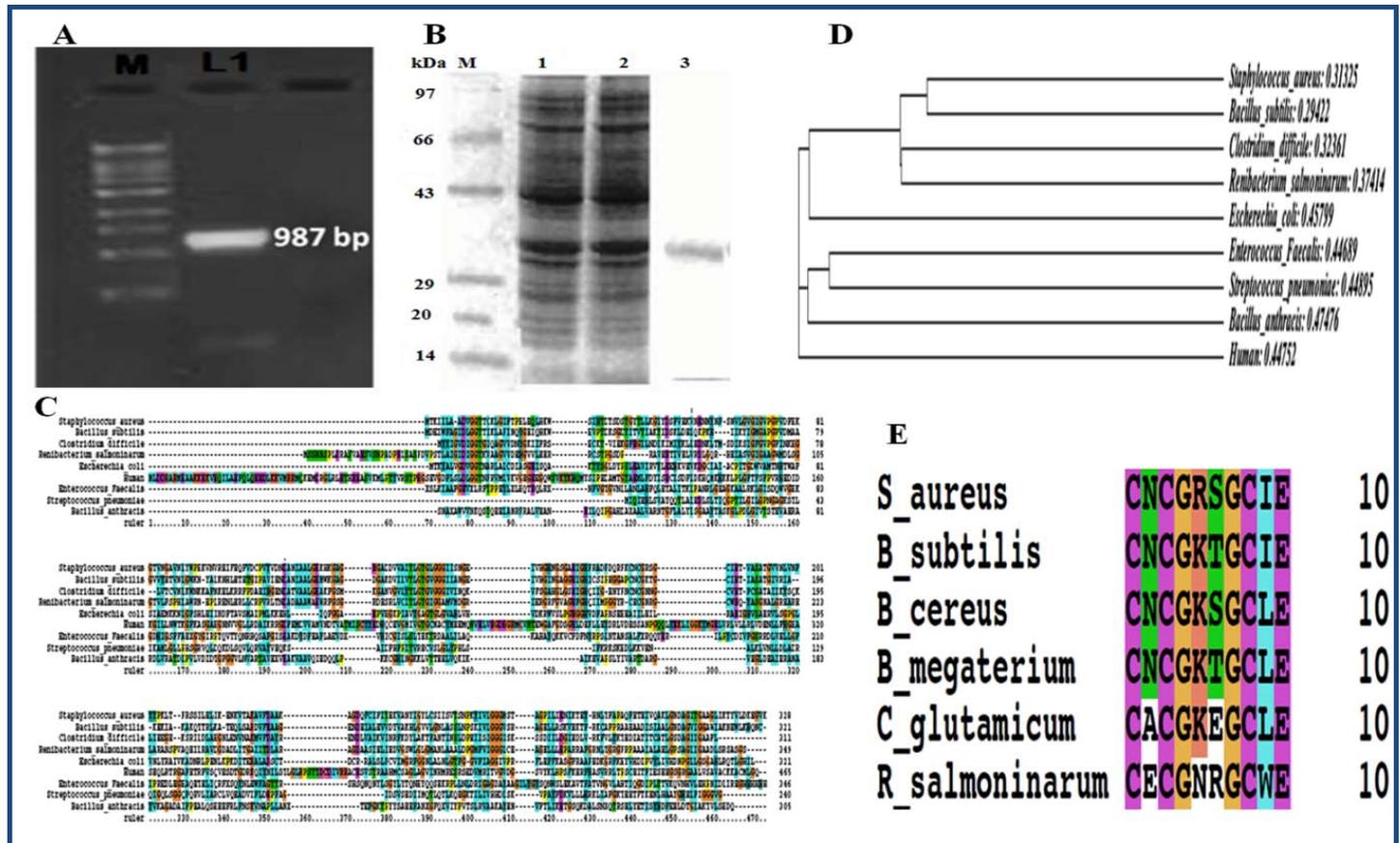


Figure 1: A) PCR amplification of glk A gene (0.987kb) from clone glk A1; B) SDS-PAGE analysis of analysis glk A protein from recombinant clone glk A 1; C) Sequence alignment of bacterial glk A sequences and human glk A sequence; D) Phylogenetic analysis of *S. aureus* with other Gram positive & Gram negative bacterial glk A; E) Multiple sequence alignment of *S. aureus* with other Gram positive and Gram negative bacterial glk A which is having ROK CXCGX(2)GCXE motif.

Methodology:

Bacterial strains and culture conditions

Staphylococcus aureus ATCC12600 and *Escherichia coli* DH5 α were obtained from Bangalore Genei Pvt Ltd. *S. aureus* was grown on modified Baird Parkar media at 37°C. After overnight incubation, a single black shiny colony with distinct zone was picked, inoculated in Brain heart infusion (BHI) broth and incubated at 37°C for overnight. Thus, grown *S. aureus* ATCC12600 culture was used to characterize Glucokinase and for the isolation of chromosomal DNA for glk A gene amplification. *E. coli* DH5 α was used in the expression of *S. aureus* glucokinase cloned in pQE 30 vector [8, 9].

Kinetic study of Glucokinase

The enzyme assay was performed on a Cyber lab spectrophotometer (USA). Glucokinase is exclusively present in cytoplasm of bacteria therefore cytoplasmic fraction was isolated from the bacteria [10]. 2ml of reaction mixture contains the following components 60Mm Tris-HCl buffer pH-7.5, 0.5

mM MgCl₂, 0.2M ATP, 0.9mM NAD, 1unit of Glucose-6-phosphate dehydrogenase, 12mM Glucose (substrate) and 10 μ l of crude (isolated from *S. aureus* ATCC 12600) or pure enzyme and incubated for 30 minutes at 37°C. The absorbance was measured at 340 nm against blank. Enzyme activity was expressed as the micro moles of NADH formed per ml. per minute which is equivalent to the amount of G-6-P formed [11].

Calculation of Hills coefficient

The Hills coefficient was calculated by plotting the graph with log [V_i / V_{max} - V_i] on Y-Axis and log [S] on X- axis where V_i is the velocity at different substrate concentrations, V_{max} is the maximum velocity of the enzyme at which the enzyme is fully saturated with the substrate concentration [11].

glk A gene Amplification and sequencing from Staphylococcus aureus ATCC12600

glk A gene was amplified from *S. aureus* chromosomal DNA using the primers glk A -F: 5'-CGCT GAT GTA GGC GGG

ACG 3¹ and glk A -R: 5¹-GGT CTT GAT TAA TCC 3¹ which were designed from the Glucokinase gene sequence of *S. aureus* Mu 50 strain [12]. The reaction mixture contained in a final volume of 50µl which consisted of 100pmoles of each primers, 100µmol of dNTPS mix, 10mM Tris- HCl (pH 8.8), 1.5mM MgCl₂, 1U of hot start Taq DNA Polymerase (Bangalore Genei pvt ltd) and 0.25µg of chromosomal DNA. Amplification parameters included an initial denaturation step for 10 min at 94°C; 35 cycles of 94°C for 60 seconds of denaturation, 60 seconds of annealing at 46°C and 100 seconds of amplification at 72°C which was followed by a final extension step at 72°C for 5 min in a Mastercycler gradient Thermocycler (Eppendorf). Amplified products were purified with NP-PCR Purification kit, Taurus Scientific, USA and were sequenced by dye terminating method at MWG Biotech India Ltd. Thus, obtained glk A gene sequence was deposited at Gen Bank (<http://www.ncbi.nlm.nih.gov/nuccore/JN645812>).

Cloning, expression and purification of Glucokinase

glk A gene was cloned in the Sma I site of pQE 30 and transformed into *E. coli* DH5α, the resultant clone was named as glk A1. The insert in the clone was sequenced and after ascertaining the sequence of glk A was over expressed with 1mM IPTG. The rgk A was purified from the cytosolic fraction of glk A1 clone by passing through nickel metal chelate agarose column (by following QIA express expression system protocol) and protein was eluted using 300mM imidazole hydrochloride. The product was analysed on 10% SDS-PAGE [8]. The enzyme kinetics of purified rgk A was performed as described in earlier section.

Sequence Analysis of glk A

The glk A sequences were retrieved for *Staphylococcus aureus* (AEY84930.1), *Bacillus subtilis* (YP_004877992.1), *Bacillus cereus* (AAU16254.1), *Bacillus megaterium* (ADF41316.1), *Corynebacterium glutamicum* (AAF80161.1), *Renibacterium salmoninarum* (YP_001625658.1) from Genbank. All these structures were scanned against PROSITE data base to predict the structures of ROK motif. The predicted ROK motifs of all these glk A sequences were aligned using clustalX tool [13] to find out the ROK signature and dissimilarities among them.

Results:

Cloning, expression and characterization of glucokinase gene

In the present study glk A gene was cloned, expressed and characterized from *S. aureus* ATCC 12600. glk A gene was amplified from the chromosomal DNA of *S. aureus* ATCC 12600 and the PCR product of (0.987kb) (Figure 1A) was cloned in the Sma I site of pQE 30 vector and transformed in *E. coli* DH5α and the clone was named as glk A1. The insert in the clone was confirmed by PCR using the same primers and the PCR product was sequenced (Accession number: JN645812) and the BLAST results resembled with all the glk A gene sequence reported for other strains of *S. aureus*. The PCR product obtained was cloned in “-1” frame and successfully induced with 1mM IPTG for glk A expression in clone glk A1. The 10% SDS PAGE results clearly indicated the expression of rgk A with a molecular weight 33 KDa corresponding to the insert cloned (Figure 1B). The enzyme kinetics of pure recombinant glk A exhibited in the cytosol was 0.1053±0.01mM of NADPH/ml/min and K_m 5.22±0.17mM with Hill coefficient of 1.71±0.025mM which is close to

the native glk A of *S. aureus* ATCC12600 (0.20817±0.04mM of NADPH/ml/min and K_m 5.1±0.06mM with Hill coefficient of 1.66±0.032mM). The close K_m and Hill coefficient values of native glk A and rgk A corroborated the sequence results indicating presence of only one kind of glk A in the *S. aureus* Table 1 (see supplementary material). These results also explain that both native glk A and rgk A showed positive cooperativity towards glucose. The above results also reflected on the functional properties of the glk A, with human GCK showing very high K_m compared with *S. aureus* K_m suggesting lower affinity of substrate for the enzyme Table 2 (see supplementary material). From the results it can be concluded that *S. aureus* glk A and human GCK are dissimilar enzymes.

Sequence analysis of *S. aureus* glk A

The glk A protein sequence of *S. aureus* ATCC12600 showed highly conserved regions with both Gram negative (*E. coli*) and positive (*Bacillus subtilis*) bacteria, while very low identity (21%) was observed with human GCK gene (Figure 1C). A clear distinction between *S. aureus* glk A and other glucokinases of both Gram positive and Gram negative pathogens was observed in multiple sequence alignments and phylogenetic analysis (Figure 1D) which showed that phylogenetically not very close relationship occurs among bacteria and humans. Further, the glk A sequence of *S. aureus* showed typical ATP binding site and ROK motif which is indicated by three conserved C residues in the motif CNCGRSGCIE (Figure 1E) such ATP dependent glk A having ROK motif is the property of glk A of Archaea. Therefore, it was exciting to identify the role of the amino acid sequences that are uniquely conserved in glk A of *S. aureus* which is having very low homology with other glk A of Gram positive and Gram negative bacteria indicating variable rate of G-6-P formation which also reflects on variability in the pathogenesis.

Discussion:

In *Staphylococcus aureus* the glk A functions optimally at higher glucose concentration which probably leads to increased reductive conditions in the pathogen probably resulting in upregulation of butanediol pathway, cell wall biosynthesis, Polysaccharide Intracellular Adhesion (PIA) molecules, exopolysaccharide synthesis and dTMP levels, these are the characteristic features Small Colony Variants (SCV) which are usually observed in relapsed episodes of *S. aureus* infections in humans [14, 15].

The kinetic results of the present study were closed to native glk A and exhibited higher affinity for glucose compared to other pathogenic bacteria (Table 2) these results correlated with the multiple sequence alignment and phylogenetic analysis of glk A (Figure 1D) indicating the rate of G-6-P formation is different in all these organisms. The pure glucokinase was obtained from glk A1 [8, 9] which exhibited molecular weight of 33 kDa similar to native protein [4]. The sequence analysis of *S. aureus* glk A showed typical ATP binding site and ROK motifs similar to the glk A present in Archaea [6] which is different in both Gram positive and negative pathogenic bacteria indicating the glk A in *S. aureus* is a regulated enzyme.

The human GCK counter part of *S. aureus* glk A showed absence of ROK motif and exhibited higher K_m (Table 2) these

differences between glk A of *S. aureus* and human GCK probably suggests that in *S. aureus* glucose is rapidly phosphorylated and used more in the anabolic pathways such as increased nucleotide biosynthesis, cell wall biosynthesis resulting in rapid proliferation and formation of SCV which are one of the key pathogenic factors of this pathogen [16-21].

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Supplementary material:

Table1: Enzyme kinetics of glk A

S.NO	Source of glk A	Enzyme Activity ($\mu\text{M}/\mu\text{g}/\text{ml}$)	V_{max} ($\mu\text{M}/\text{ml}/\text{min}$)	K_m (μM)	HillCoefficient $S_{0.5}$ (mM)
1	Cytosolic fraction of <i>S. aureus</i> ATCC12600	0.1053 \pm 0.01	2.24 \pm 0.06	5.22 \pm 0.17	1.71 \pm 0.025
2	rglk A	0.20817 \pm 0.04	2.19 \pm 0.05	5.1 \pm 0.06	1.66 \pm 0.032

Values are the mean \pm SD from three determinations

Table 2: Enzyme kinetics comparison of *S. aureus* glk A with other bacterial and human glk A

S.NO	Source of glk A	K_m	References
1	<i>E. coli</i> K-12	0.78mM	[16]
2	<i>Ranibacterium salmoninarum</i>	0.266mM	[17]
3	<i>Bacillus subtilis</i>	0.24mM	[18]
4	<i>Bacillus sphaericus</i>	0.31mM	[19]
5	Human	7.234mM	[20]
6	<i>S. aureus</i>	5.22 mM	In the present study