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ORIGINAL ARTICLE

Glucose dehydrogenase of a rhizobacterial strain of *Enterobacter asburiae* involved in mineral phosphate solubilization shares properties and sequence homology with other members of enterobacteriaceae

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Abstract Glucose dehydrogenase (GDH) of Gram-negative bacteria is a membrane bound enzyme catalyzing the oxidation of glucose to gluconic acid and is involved in the solubilization of insoluble mineral phosphate complexes. A 2.4 kb glucose dehydrogenase gene (*gcd*) of *Enterobacter asburiae* sharing extensive homology to the *gcd* of other enterobacteriaceae members was cloned in a PCR-based directional genome walking approach and the expression confirmed in *Escherichia coli* YU423 on both MacConkey glucose agar and hydroxyapatite (HAP) containing media. Mineral phosphate solubilization by the cloned *E. asburiae gcd* was confirmed by the release of significant amount of phosphate in HAP containing liquid medium. *gcd* was over expressed in *E. coli* AT15 (*gcd*::*cm*) and the purified recombinant protein had a high affinity to glucose, and oxidized galactose and maltose with lower affinities.

The enzyme was highly sensitive to heat and EDTA, and belonged to Type I, similar to GDH of *E. coli.*

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Introduction

Numerous soil bacteria such as *Achromobacter, Agrobacterium, Bacillus, Enterobacter, Erwinia, Escherichia, Flavobacterium, Mycobacterium* and *Pseudomonas,* solubilize phosphates and are highly efficient at dissolving insoluble mineral phosphates in the soil[1] making phosphorous available to the plants. In Gram-negative bacteria this mineral phosphate solubilization (MPS) phenotype is attributed predominantly to the secretion of low molecular weight organic acids such as gluconic and 2-ketogluconic acids in the periplasmic space[9].

Glucose dehydrogenase (GDH) of Gram-negative bacteria, is a member of quinoproteins, catalyzing the oxidation of glucose to gluconic acid, requiring pyrrolo quinoline quinone (PQQ) and also metal ions such as Ca^{+2} (or Mg^{+2} *in vitro*) for its activity[4]. Membrane GDHs (m-GDHs) from various bacteria are about 88 kDa monomeric proteins that are similar in primary structure to each other, though they differ slightly in properties such as substrate specificities[19]. The m-GDH has an N-terminal hydrophobic domain (residues 1–150) consisting of five transmembrane segments that ensures a strong anchorage of the protein to the membrane, and a large conserved PQQ-binding C-terminal domain which has the catalytic function[18].

In addition to providing carbon for intracellular metabolism, GDH plays a key regulatory and bioenergetic role in these bacteria. The protons generated in the oxidation

contribute directly to the trans-membrane proton motive force (PMF), which results in the uptake of exogenous amino acids and other compounds[5]. Hence, this oxidative glucose pathway might be important for the survival of enteric bacteria in aerobic, low-phosphate, aquatic environments[8].

The MPS$^-$ mutants of *Enterobacter asburiae*, deficient in the GDH activity, failed to release phosphate from alkaline soils indicating that GDH activity is required to solubilize phosphate[11]. A five-fold increase in the GDH activity upon phosphate starvation, though the activity was not completely repressed by the presence of available phosphate in the medium, indicated the regulation of MPS trait[11]. GDH of *E. asburiae* PSI3 oxidizes a broad substrate-range of both mono-and disaccharides and releases the corresponding organic acids that solubilize the rock phosphate[15].

In this paper we report the isolation of glucose dehydrogenase (*gcd*) gene of *E. asburiae* and over expression in *E. coli* to study the enzyme properties and role in MPS.

Materials and Methods

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in the present study are listed in Table 1. Luria Bertani (LB) medium was used for culturing the bacteria at either 37°C or 28°C. Medium of Nautiyal[12] having Hydroxyapatite (HAP) in place of tri calcium phosphate (TCP), was used to evaluate the MPS ability of the clones at 28°C. MacConkey glucose agar (2% peptone, 5% sodium tauroglycocholate, 1% glucose, 2% neutral red), was used for the GDH complementation studies. 2XYT medium (1.6% tryptone, 1% yeast extract and 5% sodium chloride) was used for over expression and purification of glucose dehydrogenase.

Table 1 Details of the bacterial cultures and plasmids used.

Strain or Plasmid [description]	Source
Strains	
E. asburiae [Wild type]	Dr. Naresh Kumar, G
E. coli JM109	Lab Stock
E. coli PP2419 [*E. coli* JM101 (*gcd*::*cm*)]	Dr. Goosen, N
E. coli M15 (pREP4)	Lab Stock
E. coli AT15 (pREP4) [*E. coli* M15 (*gcd*::*cm*)]	This work
E. coli YU423 [FB8 Δ(*ptsH ptsI crr*) *gal P*::Tn*10 gcd*::*cm*]	Dr. Yamada, M
Plasmids	
pUC18 [amp^r]	Lab Stock
pDrive [amp^r kan^r]	Qiagen
pQE30 [amp^r]	Lab Stock
pATNE [amp^r *E. asburiae gcd*]	This work

Table 2 Purification table of *E. asburiae* GDH from the membranes of *E. coli* AT15 harboring pATNE.

	Total protein (mg)	Total Activity* (U)	Specific Activity (U)	Recovery %
Membrane fraction	114	1334	11.7	100
Soluble fraction	24	576	24	43.1
Ni-NTA column	3.65	400.5	110	30

*Activity is defined as one unit of the enzyme required to reduce 1 µmol of DCIP, or oxidize 1 µmol of D-glucose per minute at 25 °C. Sodium azide (1 mM) was included in the reaction when the enzyme activity of the membrane fraction was determined.

Amplification and cloning of *gcd*

(a) Cloning of a partial gcd sequence: A 1.2 kb partial *gcd* sequence of *E. asburiae* was amplified using *Pfu* DNA polymerase at an annealing temperature of 55°C using the primers P1 Fp (5′-CCCGAATTCGGCGTGATCCGTGGTT-3′) and P2 Rp (5′-ATGCGTCGACTAGTCGCCCATCTT-3′) and cloned in pUC18. The sequence homology was compared with the other reported *gcd* sequences.

(b) Directional genome walking: The full-length *gcd* of *E. asburiae* was isolated by directional genome walking approach[14]. Ten micrograms of the genomic DNA of *E. asburiae* was digested to completion with *Bam* HI and the 5′overhangs were partially end-filled with dGTP and dATP using Klenow enzyme. One microgram of the partially double stranded genome walker adapters made by annealing walking adapter primers WAP-1 (5′-CTTAATACCACTCACATAGGGCGGCCGCCCGGGC-3′) and WAP-2 (5′-TCGCCCGGGCG-3′), were ligated to the partially end-filled restriction fragments. The adapter ligated DNA was used as template in a PCR at an annealing temperature of 55°C and polymerization for 4 min using the primers WP-1 (5′-CTAATACGACTCACTATAGGG-3′) and a 5′ biotinylated specific primer BSP-1 (5′-CAGCTTCGCGTCATACGCTGC-3′) designed in the above known 1.2 kb sequence. The amplified biotinylated PCR products were immobilized on to 50 µL of streptavidin-linked paramagnetic beads (Roche Molecular Biochemicals), which were washed thrice in 1X PCR buffer, and the non-biotinylated DNA was washed off. The immobilized DNA was denatured with 0.15 M NaOH

for 10 min at room temperature and after magnetic separation of the biotinylated strand, the unbound complimentary strand was collected and stored in 20 µL of TE buffer. One µL of this primary PCR product was used as a template in a nested PCR using walker primer 2 (WP 2) (5′-GGGCGGCCGCCCGGGCGATC-3′) and a locus specific inner primer 1 (IP-1) (5′-CGATGGGATCGCGTTAGGATCT-3′) to walk upstream of the 1.2 kb partial sequence.

Walking was done in a similar fashion even down stream of the 1.2 kb sequence using the adapter specific primers WP-1, WP-2 and appropriate gene primers BSP-2 (5′-AACCGCAGATAACTACCTGCC-3′) and IP-2 (5′-CAACGGTGAGAAACTGTGGCA-3′). The sequence of the amplicons generated by the walking both upstream and downstream of the 1.2 kb sequence was used to generate primers to amplify the full-length gene. The gene specific primers E Fp (5′-CGCGGATCCATGGCAATTAACAATACAGG-3′) and E Rp (5′-CCCAAGCTTTTACTTCACATCATCCGGCA-3′) thus designed, were used to amplify the full-length *gcd* from the genomic DNA of *E. asburiae* using *Pfu* polymerase at an annealing temperature of 58°C and extension at 72°C for 5 min and cloned into Qiagen pDrive cloning vector (QIAGEN, Germany) according to the manufacturers' instructions. The clone was sequenced using automated DNA sequencer at BioServe Biotechnologies India Pvt. Ltd., Hyderabad, India. The sequence was deposited in GenBank (GenBank Acession no. **AY353710**). The gene was later cloned in the expression vector pQE30 and the resulting plasmid was designated as pATNE.

Expression and purification of glucose dehydrogenase

E. coli YU423 was transformed with pATNE and spotted on MacConkey glucose agar and HAP containing agar medium supplemented with 100 µg mL^{-} ampicillin, 0.1 mM IPTG, and 200 nM PQQ and incubated for 24 h at 37°C, and for 1 week at 28°C, respectively. To quantify the phosphate released in the medium, the pATNE clone was cultured in HAP containing liquid medium. Cell-free supernatant of the samples, withdrawn after every 24 h for 1 week, were used for inorganic phosphate estimations[2]. *E. coli* YU423 (pQE30) served as negative control.

A *gcd* insertion mutant of *E. coli* M15 was generated by P1 transduction using *E. coli* PP2419 as the donor and the resulting strain, designated as *E. coli* AT15 (*gcd*::*cm*), was used as the host for expression and recombinant protein purification. *E. coli* AT15 (pATNE) was cultured in 1 litre of 2XYT medium with ampicillin (100 µg mL^{-}), kanamycin (25 µg mL^{-}), 200 nM PQQ, 5 mM $MgCl_2$ and incubated at 37°C with constant shaking. The cells were induced with IPTG at a final concentration of 1 mM. The cells were harvested after 3½ h by centrifugation at 6,000 g for 10 min and washed with 0.85% saline, and 50 mM potassium phosphate buffer pH-6.0. The cell pellet was stored at –20°C overnight. The pellet was resuspended in 10 mM potassium phosphate buffer pH-7.0 containing 5 mM $MgCl_2$ (10 ml buffer/g cell pellet). PMSF and DNase were added at a final concentration of 1 mM and 1 µg mL^{-} respectively and the cells were disrupted by sonication. The protein was released from the membranes as described by Yamada *et al.,*[18]. The supernatant was applied onto Ni-NTA column equilibrated with the binding buffer (10 mM potassium phosphate buffer pH 7.0, 500 mM KCl and 0.2% Triton-X 100). The column was washed with 2 bed volumes of the wash buffer (10 mM potassium phosphate buffer pH 6.0, 500 mM KCl and 0.2% Triton-X 100) and the enzyme was eluted with the elution buffer (10 mM potassium phosphate buffer pH 6.0, 500 mM KCl and 0.2% Triton-X 100) having 50 mM imidazole. The eluted enzyme was dialyzed against 10 mM potassium phosphate buffer pH 7.0, containing 0.2% Triton X-100 and the activity determined. The protein was quantified[6] and also detected by western blot using anti-His antibodies (Amersham Biosciences) as per the manufacturer's instructions.

Glucose dehydrogenase assay

The holoenzyme of purified recombinant protein was reconstituted by incubating the enzyme solution in 10 mM potassium phosphate buffer pH 7.0, containing 5 µM PQQ, 1 mM $MgCl_2$ and 0.2% Triton X-100 at 25°C for 20 min. The holoenzyme was then incubated in 10 mM potassium phosphate buffer (pH 7.0), 0.6 mM phenazine methosulfate (PMS), 0.06 mM 2,6-dichloroindophenol (DCIP), and 0.2% Triton X-100 as described by Sode *et al.,*[17]. The reaction was initiated with the injection of 20 µL of 1 M glucose and monitored by the decrease in the absorption of DCIP at OD_{600} in a Shimadzu UV-visible spectrophotometer.

Analysis of substrate specificity, heat and EDTA tolerance of GDH

The affinity of glucose dehydrogenase to glucose (1–20 mM), galactose (20–200 mM) and maltose (20–200 mM) was tested and the K_m was derived from the Lineweaver-Burk plot.

The heat tolerance of the glucose dehydrogenase was determined by incubating the reconstituted holoenzyme at different temperatures ranging from 25–50°C for 10 min. The sample was chilled on ice for 1 min and the residual activity was calculated as a percentage of the activity at 25°C.

The EDTA tolerance was determined by incubating the reconstituted holoenzyme, with different concentrations of

EDTA (2–10 mM). After 3 min incubation, at specified concentration, the residual activity was calculated as a percentage of the activity at 25°C.

Results and Discussion

Isolation of *gcd* of *E. asburiae*

Microorganisms play an important role in mineralization of phosphate by secreting organic acids into the external medium, which in turn releases phosphate from the insoluble complex forms thereby making phosphate available to the plants. *E. asburiae* is highly efficient in solubilizing phosphate by the release of gluconic acid, which is the direct oxidation product of glucose by the membrane bound glucose dehydrogenase[11]. The reported *gcd* sequence of Enterobacteriaceae members such as *E. coli*, *Serratia marcescens*, *Salmonella* sp. and *Shigella* sp. is highly conserved. In this study a 1.2 kb partial sequence of *E. asburiae gcd* was initially amplified with primers designed for the conserved enterobacteriaceae *gcd* internal sequences. Subsequently, walking upstream and downstream of this 1.2 kb sequence by the directional genome walking approach resulted in 1.3 kb and 1.1 kb amplicons, respectively. Using gene specific primers designed from the sequence of these amplicons, ~2.4 kb sequence was amplified. The nucleotide sequence of the 2.4 kb amplicon shared 92%, 84% and 91% homology to *gcd* of *E. coli*, *Salmonella* sp. and *Shigella* sp., respectively, confirming the isolation of *gcd* of *E. asburiae*. The deduced protein sequence shared 97% homology with the reported GDH sequences of *E. coli*, *S. typhimurium* and *S. flexneri*. The conservation of the *gcd* gene across the enterobacteriaceae members indicates the significance of the enzyme in sugar metabolism. In addition, such bacteria would also use the alternative oxidative pathway in conditions where growth becomes difficult and energy needs to be released from the substrate in a short time.

GDH complementation studies

In *E. coli* PTS⁻ (phosphate transferase system) mutants where the utilization of several carbohydrates including glucose was impaired by the deterioration of the PTS system[7,13], glucose was directly oxidized to gluconic acid, which is in turn, presumably catabolized *via* the Entner-Doudoroff pathway, thus providing an alternative route to glucose utilization[10]. Since glucose can be utilized either by the PTS pathway or by the GDH pathway, it would be ideal to study the *in vivo* activity of GDH in both PTS and GDH deficient mutant. GDH complementation studies were,

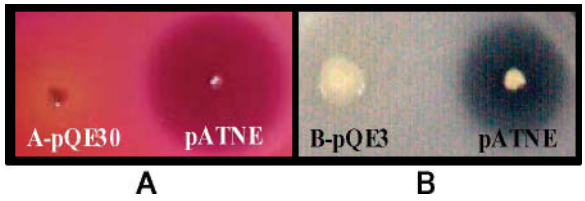

Fig. 1 Expression of *E. asburiae* GDH in *E. coli* YU423 (PTS⁻ GDH⁻). YU423 harboring either pATNE or pQE30 were spotted on either on **(A)** MacConkey glucose agar and incubated for 24 h at 37°C or **(B)** NBRIP agar and incubated for 1 week at 28°C.

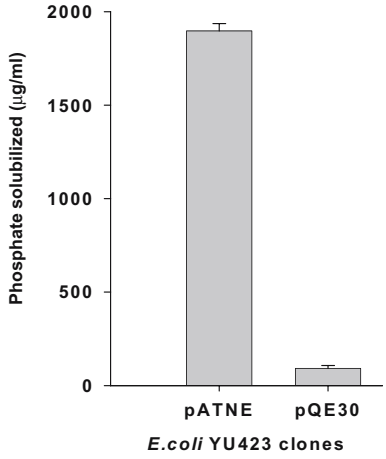

Fig. 2 Phosphate release in NBRIP liquid medium by, *E. coli* YU423 (pATNE) and *E. coli* YU423 (pQE30) after 1-week incubation at 28°C. Values are averages of three independent experiments. The vertical bar indicates standard deviation.

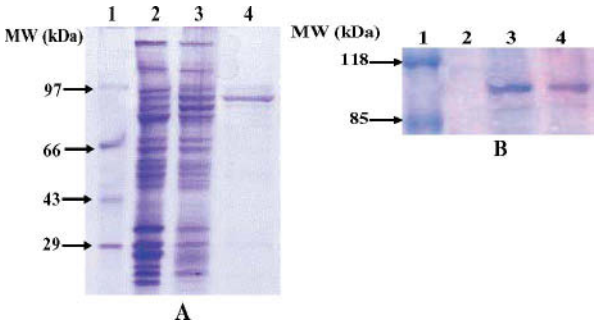

Fig. 3 SDS-PAGE and western blot of *E. asburiae* GDH expressed in *E. coli* AT15. **(A)** 10 % SDS polyacrylamide gel stained with coomassie blue (Lane 1: molecular weight marker; lane 2: membrane fraction; lane 3: Solubilized fraction; lane 4: Ni-NTA column fraction) **(B)** Western blot of the membrane fractions of *E. coli* AT15 harboring the various plasmids using anti-His antibodies. Lane 1: Prestained protein molecular weight marker; lane 2: pQE30 (– ve control); lane 3: *E. coli gcd* (+ ve control) and lane 4: *E. asburiae gcd* (pATNE).

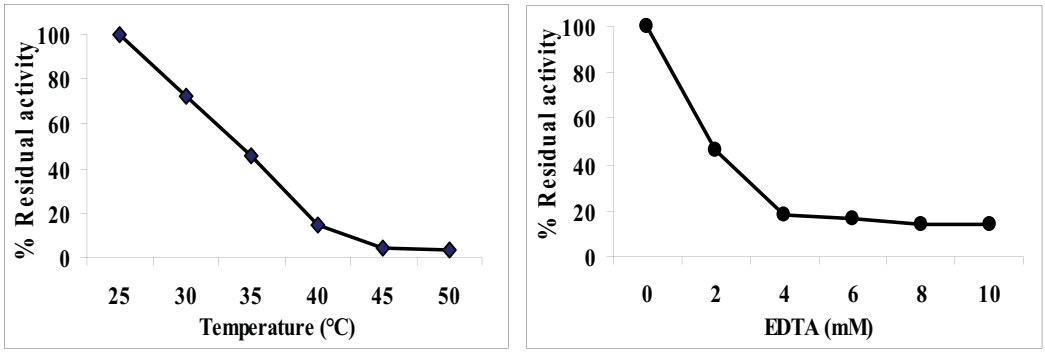

Fig. 4 Heat and EDTA tolerance of *E. asburiae* GDH. The reconstituted holoenzyme of *E. asburiae* was subjected to **(A)** different temperatures (25–50°C) for 10 min and **(B)** different EDTA concentrations (2–10 mM) for 3 min and the % residual activity expressed at 25°C.

therefore, carried out in *E. coli* YU423 (PTS^- GDH^-). Colonies of *E. coli* YU423 (pATNE) on MacConkey glucose agar were pink with a purple halo, after 24 h incubation at 37°C, whereas, the negative control remained colourless indicating the expression of cloned *gcd* of *E. asburiae* (Fig. 1a). GDH oxidizes glucose to gluconic acid and brings about a change in the pH of the medium imparting a pink colour to the colony.

The role of *E. asburiae* GDH in phosphate solubilization was also verified and confirmed on both HAP containing agar and liquid medium. Clear zones of phosphate solubilization by *E. coli* YU423 (pATNE) compared to *E. coli* YU423 (pQE30), after 1 week of incubation, was due to the expression of *gcd* and the oxidation of glucose to gluconic acid (Fig. 1b). *E. coli* YU423 (pQE30) which served as the negative control released only 92 µg mL^- of phosphate in HAP containing liquid medium, whereas *E. coli* YU423 (pATNE) in HAP containing liquid medium released 1897 µg mL^- of soluble phosphate after 1 week incubation at 28°C, signifying the role of GDH in MPS (Fig. 2).

Purification and properties of glucose dehydrogenase

E. asburiae gcd cloned in pQE30 was expressed in the *E. coli* AT15. PQQ was included in the medium since the supplementation of the cofactor PQQ greatly improved the production of *E. coli* PQQGDH[16]. The protein was expressed with N-terminal His-tag fusion facilitating the purification by the Ni-NTA column (Table 3). SDS-PAGE analysis showed GDH of *E. asburiae* as a distinct ~90 kDa, protein which was also detectable with anti-His antibodies (Fig. 3B).

Sharma *et al.,*[15] using whole cells of *E. asburiae* as the source of glucose dehydrogenase, demonstrated broad substrate range, including mono- and disaccharides. Here we used the purified enzyme to test its affinity to different substrates (Table 3). The purified glucose dehydrogenase showed highest affinity to glucose and low affinity to maltose similar to GDH of *E. coli*. *E. asburiae* GDH showed a higher affinity to galactose than GDH of *E. coli*. The heat and EDTA tolerance of *E. asburiae* GDH was lower than that of *E. coli* GDH (Tripura and Podile, unpublished). GDH of *E. asburiae* was very sensitive to the incubation at elevated temperatures, retaining only 15% of the original activity, at 40°C for 10 min (Fig. 4A). The enzyme was totally inactive at temperatures beyond 40°C. GDH of *E. asburiae* was highly sensitive to EDTA, and retained only 14% activity at 10 mM EDTA for 3 min (Fig. 4B). The enzyme lost more than 55% activity when incubated with 2 mM EDTA. Based on the sensitivity to EDTA and as described by Dokter *et al.,*[3] glucose dehydrogenase of *E. asburiae* may be grouped under Type I

A full-length glucose dehydrogenase of *E. asburiae* was cloned and expressed as a His-tag fusion facilitating the purification using the Ni-NTA column. Since the enzyme had a wide substrate range *in vitro*, genetic manipulation of *gcd* to further improve the temperature or EDTA tolerance without compromising on the enzyme activity holds great promise for a variety of applications.

Table 3 Substrate affinity of *E. asburiae* GDH and *E.coli* GDH.

Substrate / Enzyme	Glucose K_m (mM)	Galactose K_m (mM)	Maltose K_m (mM)
E. coli PQQGDH	1.13 ± 0.25	26 ± 1.0	30.8 ± 2.25
E. asburiae PQQGDH	1.36 ± 0.25	19.3 ± 2.9	28.8 ± 3.0

E. coli and *E. asburiae* GDH were tested for their affinity to glucose, galactose and maltose. The K_m was determined from the Lineweaver–Burk reciprocal plots. The values are average of three independent estimations of GDH activity (for details refer to text).

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