Kinetic characterization of the glucose-6-phosphate dehydrogenases from *Pseudomonas putida* KT2440

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Abstract: *Pseudomonas putida* KT2440 is a bacteria with potential biotechnological applications. However, its mechanisms to accomplish the cofactor balance are poorly known. The gene *zwf*-1, encoding for the glucose-6-phosphate dehydrogenase (G6PDH), increases its expression level during growth on glucose and upon oxidative challenges (Kim *et al.*, 2008). Besides *zwf*-1, two other alleles had been annotated. Although it is believed that the enzymes encoded by these three alleles are NADP-specific, there are no kinetic data confirming this assumption so far. Using tools from molecular biology and enzyme kinetics we cloned, expressed and purified the G6PDH encoded by *zwf*-1 gene. The kinetic parameters for the oxidation of glucose-6-phosphate, using NAD or NADP as the cofactor, were determined using the purified enzyme. The results indicated that, in physiological conditions, G6PDH-1 could be a source of both NADH and NADPH instead of an exclusive NADPH producer enzyme. Because there are three G6PDH alleles with apparently different properties, the assessment of how much NADH and NADPH is produced in the reaction catalyzed by these enzymes seems to be a complex task.

Glucose uptake pathways in *P. putida* KT2440

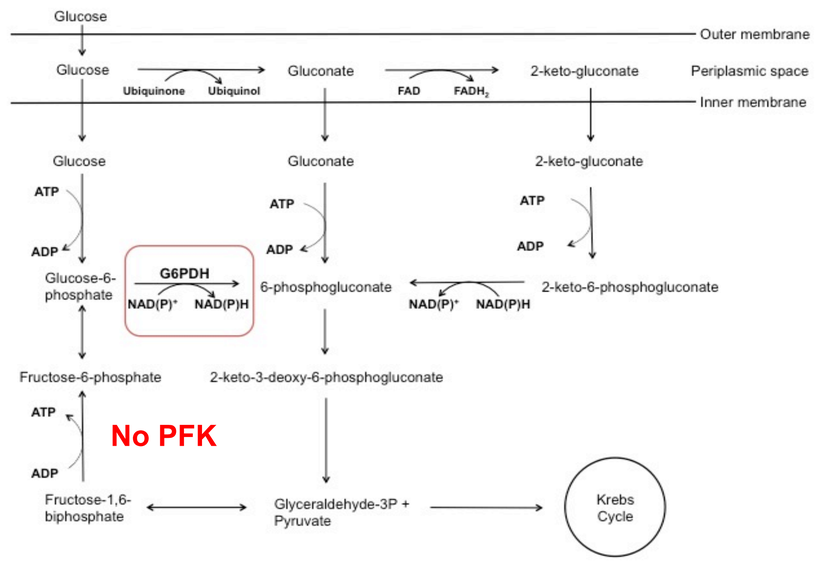


Table 1. Association between the cofactor preference* of the glucose-6-phosphate dehydrogenases and the activity of phosphofructokinase (PFK) in 16 bacterial species.

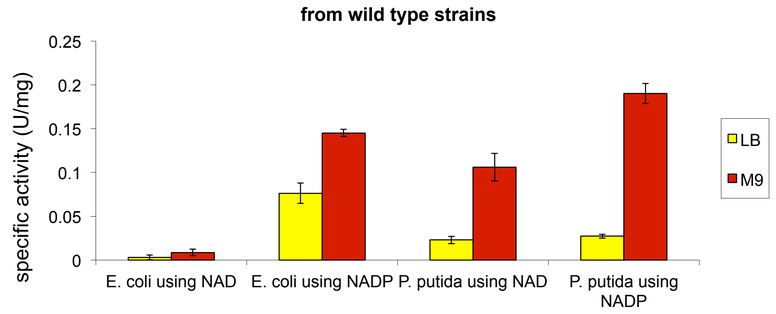
Species	$\frac{(k_{cat}/K_M)^{NADP}}{(k_{cat}/K_M)^{NAD}}$	Observations**
Gluconacetobacter hansenii	0.2	No PFKs
Pseudomonas fluorecens	0.34***	2.7.1.56 a.n.c. ****
Burkholderia multivorans	1	2.7.1.11 a.n.c.
Pseudomonas vinelandii	2	2.7.1.11 a.n.c.
Pseudomonas aeruginosa	2	No PFKs
Kitasatospora aureofaciens	2	No PFKs
Leuconostoc mesenteroides	9	No PFKs
Burkholderia cepacia	13	No PFKs
Methylomonas sp.	13	No PFKs
Neisseria gonorrhoeae	16	No PFKs
Aquifex aeolicus 70°C	6	2.7.1.11 a.n.c.
Aquifex aeolicus 40°C	21	
Acetobacter suboxydans	48	No PFKs
Thiobacillus ferrooxydans	60	2.7.1.11 has very low activity
Geobacillus stearothermophilus	102	2.7.1.11 and 2.7.1.56
Escherichia coli	410	2.7.1.11 and 2.7.1.56
Thermotoga maritima	1268	2.7.1.11 and 2.7.1.56

*It is variable according to concentration of G6P.
**We considered the following E.C.: 2.7.1.11, 2.7.1.56, 2.7.1.105 and 2.7.1.146.
***Data referred to the G6PDH committed to the Entner-Doudoroff pathway. There is also a NADP-preferring G6PDH in the same organism.
****a.n.c: annotated but not characterized

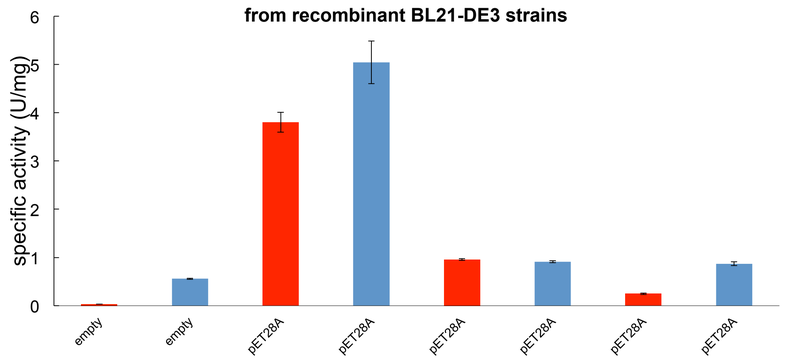
For the 16 analyzed bacteria, the presence of a dual-preferring G6PDH was associated with the absence, non-confirmed functionality or very weak phosphofructokinase activity. Phosphofructokinase activity has been reported in the three organisms where the G6PDHs were 100 times more specific for NADP than for NAD (Olavarria *et al*, 2012). ***What happens in P. putida KT2440?***

Specific activities in crude extracts

[G6P]=1 mM [NAD]=500 uM [NADP]=500 uM



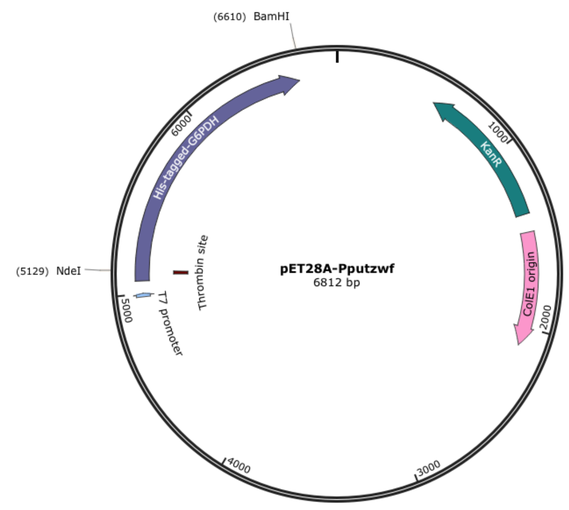
The results suggest that in *P. putida*, the use of the cofactors NAD and NADP changes according with the carbon source.



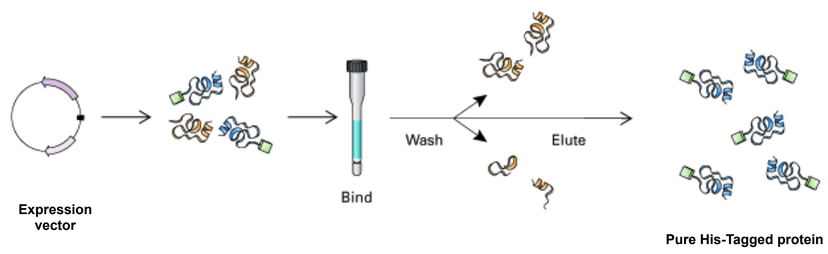
Different alleles, different specificities.

Molecular Biology

- After the analysis of the annotated genome of *P. putida* KT2440, primers were designed to amplify the sequences of *zwf*-1, *zwf*-2 and *zwf*-3, taking advantage of the PCR reactions to add the targets for the restriction enzymes *Nde*I and *Bam*HI at the ends of the PCR-formed molecules.
- The corresponding PCR products were ligated into the plasmid pET28A (Invitrogen). The amino acid encoding sequences were cloned in frame with a sequence encoding for a N-terminal 6xHis-thrombine site.
- The resultant plasmids were transformed into *E. coli* BL21-DE3 competent cells.

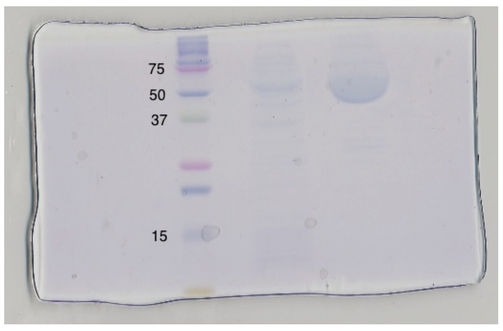


Protein purification



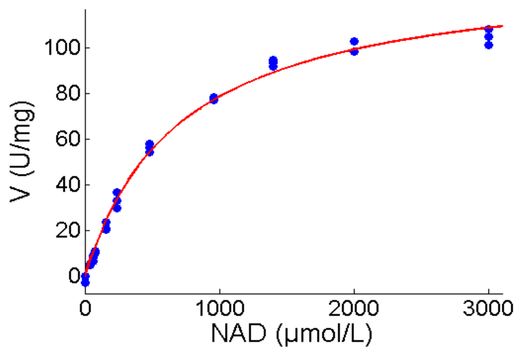
The recombinant BL21-DE3 cells were inoculated in LB-Kan medium and incubated at 37ºC with agitation. IPTG 1mM was added to the culture when the OD reached 0,5. After 4h of induction, the biomass was collected by centrifugation and re-suspended in Tris 50 mM, $MgCl_2$ 10 mM, NaCl 5 mM, glycerol 10% v/w, 2-mercaptoethanol 10 mM, pH 8. The cells were disrupted by sonication and the crude extract were obtained by centrifugation.

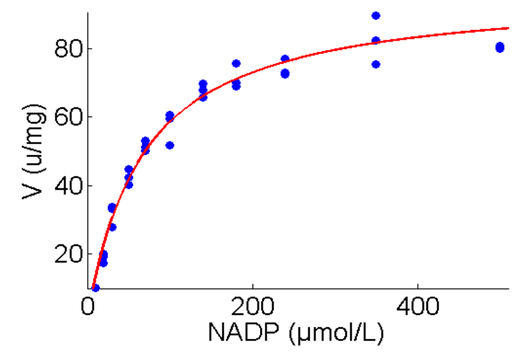
The crude extract was loaded into a Ni^+ charged HisTrap column (GE Healthcare). The elution was performed using an imidazole gradient (0.025-1M).
The fractions with higher NAD-dependent G6PDH activity were pooled and concentrated. Purity was checked by electrophoresis in SDS-PAGE.



SDS-PAGE electrophoresis of samples representing different stages of the purification procedure.
A: Precision Plus Protein Kaleidoscope standards (Biorad); **B:** cellular extract after induction with IPTG; **C:** after purification with HisTrap column.

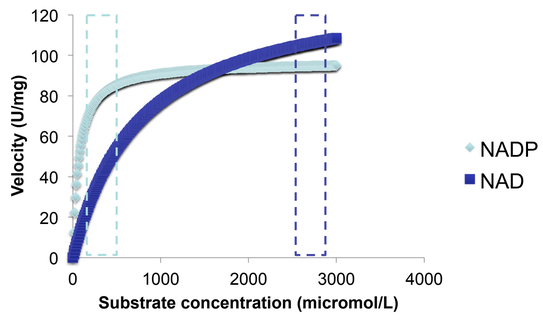
Enzyme kinetics





	NAD	NADP
$*K_M$ (µM)	712 ± 83	66 ± 9
$*k_{cat}$ (s^{-1})	135 ± 6	97 ± 4
$\frac{k_{cat}/K_M^{NADP}}{k_{cat}/K_M^{NAD}}$	≈ 8	

*Apparent values for [G6P]=1mM



The dashed lines represent the physiological concentrations of the respective cofactors. It is possible to note that under physiological conditions the generation of NADH could be higher than the generation of NADPH. However, further kinetic details (product inhibition, allosteric effects etc) are required to complete this analysis

Conclusions:

1) The three alleles of G6PDH from *P. putida* KT2440 seem to have different kinetic properties
2) G6PDH-1 is a dual preferring enzyme.
3) The kinetic data obtained so far suggest that, in the physiological conditions, the oxidation of G6P by G6PDH-1 could be coupled with the co-generation of NADH and NADPH.

Perspectives:

1) Study the kinetic behavior at different G6P concentrations.
2) Study the product (NADH, NADPH) inhibition.
3) Expand these studies to the products of the other two *zwf* alleles.

References:

- **Kim, J., Jeon, C. O. & Park, W. (2008).** Dual regulation of zwf-1 by both 2-keto-3-deoxy-6-phosphogluconate and oxidative stress in Pseudomonas putida. *Microbiology* **154**, 3905-3916.
- **Olavarria, K., Valdes, D. & Cabrera, R. (2012).** The cofactor preference of glucose-6-phosphate dehydrogenase from Escherichia coli- modeling the physiological production of reduced cofactors. *Febs Journal* **279**, 2296-2309.
- **Olavarria, K., De Ingeniis, J., Zielinski, D. C., Fuentealba, M., Muñoz, R., McCloskey, D., Feist, A. M. & Cabrera, R. (2014)**. The Metabolic Impact of a NADH-producing Glucose-6-phosphate Dehydrogenase in Escherichia coli. *Microbiology*.

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