HETEROLOGUS OVER-EXPRESSION AND *IN-SILICO* CHARACTERISATION OF A PUTATIVE 2-KETO-GLUCONATE DEHYDROGENASE FROM *ARTHROBACTER NICOTINOVORANS* pAO1

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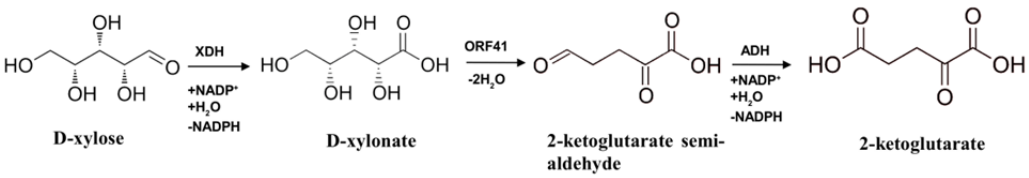
Abstract: *Arthrobacter nicotinovorans* is a gram positive soil actinobacteria which is able to grow on nicotine contaminated soils due to the presence of a large plasmid - pAO1. It has been shown that pAO1 encodes not only the pathway for nicotine mineralization, but also newly described oxidative xylose-catabolic pathway. Part of the genes cluster encoding this last pathway is also *gdh*, a putative 2-keto-gluconate dehydrogenase with no experimentally shown function. Based on sequence homology, a 3D model of the GDH protein was generated. The protein over-expression was tested on two growth mediums, but was found to be satisfactory only on LB medium when using 0.1 mM IPTG. Using immobilized metal affinity chromatography, GDH was purified to homogeneity, but the yield was extremely low and did not allowed for further characterization of the protein.

INTRODUCTION

As lignocellulose is the most abundantly available raw material on the Earth, using it for the production of bio-ethanol is promising alternative to replace petroleum-based chemicals and fuels. Since a rather abundant (20%, (Aristidou and Penttilä, 2000)) fraction of the lignocellulosic biomass consists of the mono aldopentose D-xylose, it is essential that this fraction be utilized efficiently in biotechnological processes (Meijnen et al., 2009). So far, D-xylose has been found to have very few bio-technological uses (Ko et al., 2006, Meijnen et al., 2009) due to the lack of suitable enzymes that can be applied into biotechnological and industrial processes.

The newly identified oxidative pathway for the degradation of xylose found on the pAO1 megaplasmid of *Arthrobacter nicotinovoran*s might offer new alternatives for utilization of this valuable resource by providing new and interesting enzymes. The pathway is encoded by a *xyl*-operon and is similar to the Weimberg pentose pathway, consisting of several oxidore-reductive steps (Figure 1). It was postulated that the D-xylose is taken up by bacterial cells by the ABC-type transporter system and then oxidized by xylose-dehydrogenase (XDH) to D-xylonate, which may be further dehydrated by ORF41 (GI:25169063). The 2-ketoglutarate semi- aldehyde formed is further oxidized by the ORF39 protein (GI:25169061, aldehyde dehydrogenase) to 2- ketoglutarate. The latter compound is then reintegrated into the general metabolism of the cell (Mihasan et al., 2013a; Mihasan et al., 2013b).

A.



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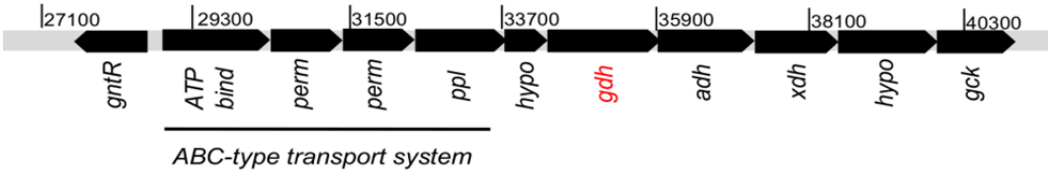

Figure 1. The postulated xylose oxidative pathway (A) encoded by the the *xyl*-operon (B) from pAO1 of *Arthrobacter nicotinovorans*. *ATP-bind* - a putative ATP binding protein, *perm* - pore-forming membrane proteins, *ppl* - periplasmic sugar binding protein, *hypo* – hypothetical protein, unknown function, *gdh* – putative keto-gluconate dehydrogenase, *adh* – aldehyde dehydrogenase, *xdh* – xylose-dehydrogenase, *gck* – putative glicerate-kinase.

Part of the xyl-operon is the *gdh* gene encoding a putative 2-keto-gluconate dehydrogenase (Igloi and Brandsch, 2003). The gene is located between the ABC-type system and the postulated last enzyme of the pathway, the aldehyde dehydrogenase. Precisely the same gene arrangements have been identified in another *Arthrobacter* strain, namely *A. phenanthrenivorans* Sphe3. It is The function of this gene in this genetic context is still unknown.

Our previous efforts to purify this protein were unsuccessful, as the protein was found to be insoluble when over-expressed in *Escherichia coli (*Mihasan et al., 2007*)*. Thereby, this study is a second-generation effort to express and characterize the *gdh* protein and its expression in an attempt to further shed some light on the xylose metabolism in *A. nicotinovorans*.

MATERIAL AND METHODS

Chemicals. All chemicals were purchased from well-known suppliers and were of greatest purity available.

Strains and growth conditions. For all recombinant DNA-techniques and protein purifications, *E. coli* XL1 Blue (Stratagene) was grown on Luria-Bertani (LB) or autoinducible medium (ZYP5052) supplemented with appropriate antibiotics. *Arthrobacter nicotinovorans* (strain ATCC 4991) harboring the pAO1 megaplasmid was grown on citrate medium supplemented with 0,05% nicotine and 0,005% minerals solution (Eberwein et al., 1961) on a rotary shaker at 28^0C/190 rpm. When required, the growth of the culture was followed at 660 nm using a Beckman Coulter DU700 Life Science spectrophotometer.

Cloning of gdh. The *gdh* was isolated by PCR using the primers in table 1 and a suspension of *Arthrobacter nicotinovorans* cells as template. Directional cloning (Sambrook, 2001) of the fragment containing the *gdh* in the pH_6EX_3 vector was achieved by using *BamHI* şi *SalI* (NEB, U.K) enzymes and Rapid DNA ligation Kit, Roche). Transformed *E. coli XL1* Blue competent cells were selected on plates containing ampiciline (50 µg/ml) and the recombinant plasmid was checked for the presence of insert by restriction enzyme digestion.

Table 1. Oligo-nucleotides used for isolation of *gdh*

Primer	Sequence
gdhforw	5´-CGTGACCGAGCATCCAAGGACAGC-3´
gdhrev	5´-GCTGGTAGGTCGACGGAACGGAATG-3´

Protein over-expression was achieved using either ZYP5052 auto-inducible medium as described elsewhere (Mihasan et al., 2007) or the standard IPTG-induction method described in the Maniatis manual (Sambrook et al., 1989).

Protein solubility assay. The levels of protein expression and protein solubility were monitored using standard SDS-PAGE. Samples were taken at precise time-intervals and cells were harvested by centrifugation. Cells were further solubilised in SDS-loading buffer and boiled for 10 min. at 95C (total proteins) or lysed using a combination of osmotic-shock/detergents, centrifuged and the supernatant mixed with SDS-loading buffer and boiled (soluble proteins).

Protein purification was achieved using standard IMAC techniques (Ausubel et al., 2002). Briefly, cells were harvested by centrifugation, lysed by osmotic-shock/detergents treatment and the cell free extract was loaded on a Fast-Flow Ni-chelating Sepharose (Amersham Biosciences, Sweden) column. Protein was eluted using a step gradient of imidazole.

Protein concentration was assayed using the dye-binding method of Bradford.
Protein modeling. The 3D homology model of GDH was build using Swiss-model Server (Arnold et al., 2006) and visualized using PyMol (Schrödinger, LLC, 2010).

RESULTS AND DISCUSSIONS

Putative 3D structure of GDH. The amino acid sequence of GDH (GenBank: CAD47896.1) contains 522 aminoacids and, based on it primary structure, the theoretical pI of 6.20 and molecular weight of 56526.29 Da was calculated using the Expasy server. The amino acid sequence was also submitted on the Swiss-model Server in order to identify suitable candidates for homology modeling. Several structures have been identified in PDB that have enough levels of homology with GDH and could serve as templates for model building (Table 2). For the actual modeling, we have selected 3 representative templates from each type of protein that, beside a known structure also have a known, experimentally shown structure: 1coy - cholesterol-oxidase, 2jbv – choline-oxidase and 4miq – pyranose-1-oxidase.

Table 1. GDH sequence homologs identified in PDB. With bold are highlighted the chosen templates.

No.	PDB ID	Protein	Homology level (%)	Species	Experimental function
1	**1coy**	**Cholesterol Oxidase**	**21.59**	***Brevibacterium sterolicum***	**Yes**
2	1mxt	Cholesterol Oxidase	20.58	*Streptomyces sp*	No
3	1cc2	Cholesterol Oxidase	20.4	*Streptomyces sp*	No
4	3cnj	Cholesterol Oxidase	20.27	*Streptomyces sp. SA-COO*	Yes
5	**2jbv**	**Choline Oxidase**	**20.17**	***Arthrobacter globiformis***	**Yes**
6	3gyj	Cholesterol Oxidase	20.13	*Streptomyces sp. SA-COO*	Yes
7	1cbo	Cholesterol Oxidase	20.13	*Streptomyces sp.*	No
8	3ljp	Choline Oxidase	20.13	*Arthrobacter globiformis*	Yes
9	3gyi	Cholesterol Oxidase	20.09	*Streptomyces sp. SA-COO*	Yes
10	3b6d	Cholesterol Oxidase	19.91	*Streptomyces sp. SA-COO*	Yes
11	1b8s	Cholesterol Oxidase	19.91	*Streptomyces sp*	No
12	3nne	Choline Oxidase	19.83	*Arthrobacter globiformis*	Yes
13	3b3r	Cholesterol Oxidase	19.69	*Streptomyces sp. SA-COO*	Yes
14	3q9t	formate oxidase	19.45	*Aspergillus oryzae RIB40*	Yes
15	**4mig**	**Pyranose 2-oxidase**	**19.39**	***Phanerochaete chrysosporium***	**Yes**
16	1gal	Pyranose 2-	19.28	*Aspergillus niger*	Yes

No.	PDB ID	Protein	Homology level (%)	Species	Experimental function
		oxidase			
17	1tzl	Pyranose 2-oxidase	19.07	*Peniophora sp. SG*	Yes
18	3bg7	Pyranose 2-oxidase	19.03	*Trametes ochracea*	Yes
19	2f6c	Pyranose 2-oxidase	18.95	*Peniophora sp. SG*	Yes
20	2f5v	Pyranose 2-oxidase	18.88	*Peniophora sp. SG*	Yes

All the structural homologs identified are FAD-containing proteins, indicating that it might be the case of GDH also. The selected templates were used as models and the putative catalytic amino-acids were identified (Figure 2). No matter what template was used for modeling, the putative models are very similar, with a RMSD between models between 4.411 -5.396 for about 2500 atoms.

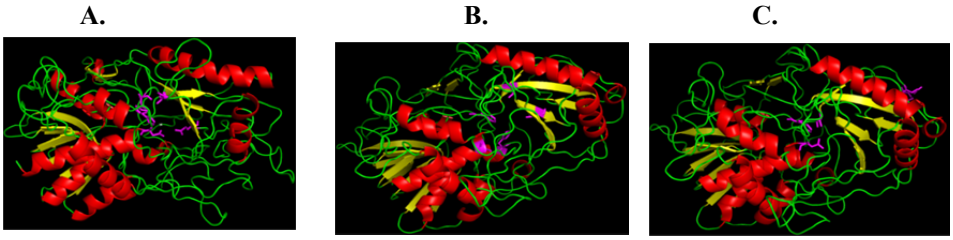

Figure 2. Generated models of the GDH tridimensional structure. With magenta are marked the putative catalytic amino-acids. A. Template: 1coy, catalytic amino-acids: His454; Asp497; Glu347; His303; Thr305, B. Template: 2jbv, catalytic amino-acids: His454; Thr499; Ile349; Thr305; Phe9, C. Template: 4mig, catalytic amino-acids: His454; Asp497; Gln368; Lys372; Ser452

Testing for protein over-expression levels and solubility. The *E. coli* XL1 Blue strain harboring the recombinant plasmid pH_6EX_3*gdh* was grown on two different medium in order to test for differences expression levels and protein solubility. At first, the YZP5052 auto-inducible medium was used. This medium was chosen as it contains a mixture of lactose that glucose that allow a mild expression of genes under the control of *lac* or *tac* promoters and it is known to promote protein solubility (Studier, 2005) At 37°C, the *lac* promoter from pH_6EX_3*gdh* failed to activate even after 9 hours of cultivation and no clear signs of over-expressed protein could be identified. On the other hand, when the strain was cultivated on LB medium and the synthetic lactose analog IPTG was used as inducer, a clear sign of over-expressed protein could be observed on SDS-PAGE gels. Even after 24 hours of cultivation, the expression levels were found to be high and the protein was soluble (Figure 3).

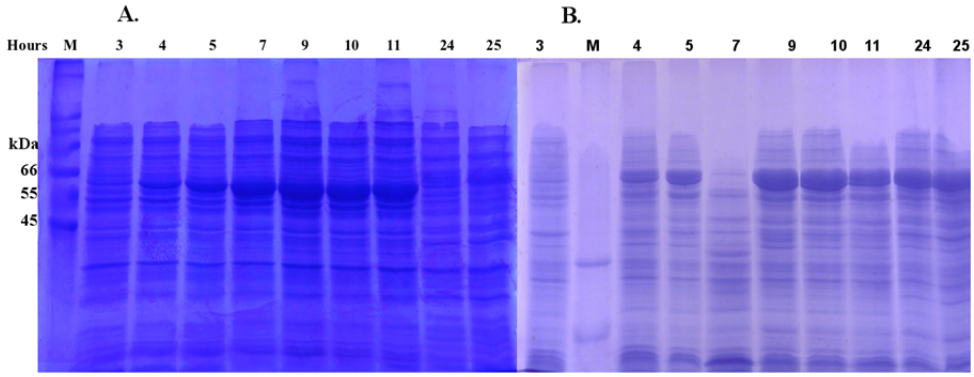

Figure 3. Protein expression profile of *E.coli* XL1 harboring the pH_6EX_3*gdh* plasmid on LB medium. A - total proteins. B - soluble proteins.

Purification of GDH. The recombinant protein obtained by cloning *gdh* in pH_6EX_3 has the N-terminal sequence as follows: HHHHHLVPRGSKDSR**M**KKYDLTDD, where the methionine in bold is the native START codon. This allowed for an one step purification process of the protein from the *E.coli* cell lysate using mobilized metal affinity chromatography (Figure 4). The purified enzyme had a relative molecular weight of 58 kDa, in good accordance with the theoretical mass calculated based on the amino-acid sequence. Despite the fact that high levels of protein expression have been reached, the purification lead to really low recovery. Also, the protein runs as two bands on SDS-PAGE indicating that it might contain a on di-sulfur bridge between two Cys residues (Schägger, 2006).

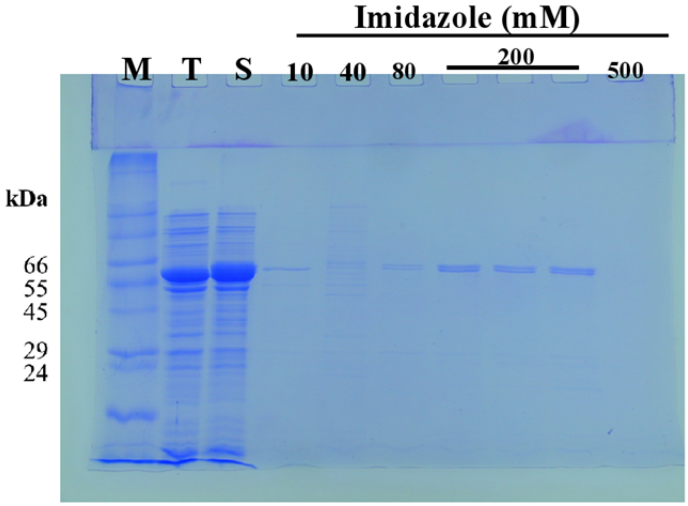

Figure 4. Purification of GDH. M – Sigma Weight Marker, T – total proteins, S – Soluble proteins.

The low yield of the purification method used does not allow for further characterization of the isolated protein. Thereby, we are currently focusing on overcoming and improving the

recovery of the method. One issue is that the cell lysis is performed using osmotic shock and detergents which require 1mM EDTA. The IMAC resin should be compatible with this EDTA concentration, but some Ni stripping of the column could be observed during purification. Thereby, the first step will be identifying a lysis method that does not require EDTA and keeps the protein soluble.

CONCLUSIONS

A 3D homology model of GDH of *Arthrobacter nicotinovorans* was successfully generated, indicating a possible function as a oxido-reductase of this protein. The conditions for heterologus expression of GDH were also established as well as a initial purification protocol. GDH protein was purified to homogenity, albeit with a low yield.

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