CLONING AND PURIFICATION OF A TETRAMERIC OXIDOREDUCTASE FROM *ARTHROBACTER NICOTINOVORANS* PAO1

MARIUS MIHĂŞAN[1,*], VLAD ARTENIE[1,] RODERICH BRANDSCH[2]

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Abstract: The pAO1 megaplasmid of *Arthrobacter nicotinovorans* encodes two different pathways: one for nicotine metabolism and an putative sugar catabolic pathway. One open reading frame, orf40, from the latter pathway was cloned, purified to homogenity and partially characterized. It consist of an tetrameric oxidoreductase containing atoms of zinc per molecule of monomer. A possible role in the metabolism of *Arthrobacter nicotinovorans* is postulated.

INTRODUCTION

Plasmids are simple genetic elements, independent from the bacterial chromosome, involved both in vertical and horizontal-gene transfer. Most of the time, the plasmids encode different properties (resistance to antibiotics, to highly toxic compounds) which give the host cell an evolutionary advantage. The ability to use less common compounds as carbon and nitrogen sources is such an advantage, allowing the bacteria to be present in many environments as natural autochthonous microflora with a high potential for bioremediation of pollutants. Several plasmid-encoded pathways were described (ex: for metabolism of phthalate (Eaton A., 2001) or naphthalene (Rosselló-Mora, Lalucat & García-Valdés, 1994)) but only few are completely elucidated.

The presence of the 165- kb pAO1 megaplasmid inside the cells of the gram positive soil bacteria *Arthrobacter nicotinovorans* allows this microorganism to use nicotine as sole carbon and nitrogen sources. The complete sequence of this plasmid was determined and two putative pathways could be described (Igloi & Brandsch,2003): on one hand the nicotine-degrading pathway, fully characterized by Brandsch (Brandsch Roderich.,2006) and on the other hand an yet unknown putative sugar-catabolic pathway. The overall GC content of the pAO1 plasmid indicates that nicotine-catabolism gene clusters are a new acquisition, being attached during the evolution to an older plasmid, containing the sugar-catabolic pathway. Recently shown analogies of the pAO1 encoded pathway for nicotine metabolism and the chromosome encoded one from *Nocardioides* sp. strain js614 (Ganas *et al.*, 2008) would suggest a horizontal gene transfer.

The sugar-catabolic pathway is comprised of several genes, among which an putative cellulase, an ABC-transporter system gene cluster and a cluster of several dehydrogenases and oxidoreductases. This last cluster probably encodes the last steps of the pathway, connecting it to the general metabolism of the cell. A part of this cluster are ORF40, an putative oxidoreductase. By means of protein structure modelling and docking we showed that ORF40 is an sugar dehidrogenase able to bind various ketohexoses (Mihasan & Artenie, 2008).

Our current study is focused further characterization the ORF40 protein and elucidating its possible role in the cell. By cloning the gene in the expression vector pH_6EX_3 (Berthold *et al.*, 1992), we were able to express it as a recombinant His-tagged protein and to easily purify it to homogeneity.

MATERIAL AND METHODS

Isloation and cloning of orf39. The *orf40* was isolated by PCR using the primers in table 1 and a suspension of *Arthrobacter nicotinovorans* cells as template. Directional cloning (Sambrook J, Fritsch EF, Maniatis T,1989) of the fragment containing the *orf40* in the pH_6EX_3 vector was achieved by using *HindIII* şi *XhaI* (NEB, U.K) enzymes and Rapid DNA ligation Kit, Roche). Transformed *E. coli XL1* Blue competent cells were selected on plates containing ampiciline (50 microg/ml) and the recombinant plasmid was checked for the presence of insert by restriction enzyme digestion.

Table 1. Oligo-nucleotides used for isolation of *orf40*

	Sequence
Orf40forw	5´-C TCT GAG G*AA* GCT TTG ACT AAA ACA GC-3´
Orf40rev	5´-G GAA GGA TG*C TCG AG*G TCA TTA GAG C-3´

Protein expresion was done using auto-inducible medium as described elsewhere. (Mihasan, Ungureanu & Artenie,2007)

Protein purification was achieved using standard IMAC techniques (Ausubel M Frederick, Brent Roger, Kingston E Robert , Moore D David,Seidman J G,Smith A John, Struhl Kevin,2002) on Fast-Flow Ni-chelating Sepharose (Amersham Biosciences, Sweden). All buffers used in the purification precess had 10 mM β-mercaptoethanol final concentration. **Native molecular weight determination** was done using gel permeation chromatography on an HiLoad 16/60 Superdex 200 column connected to an AKTA Basic FPLC system. **Protein concentration** was assayed using the dye-binding method of Bradford (Bradford,1976). **SDS-PAGE** was performed using the discontinuous system of Laemlli fallowing the procedure described by Sambrook, 1989(Sambrook J, Fritsch EF, Maniatis T,1989).
Enzyme assay was developed by fallowing the guidelines described by de Caballero et. Al 1983, Marchal and Branlant,1999, Farrbs et al., 1994. The assay mixture was phosphate buffer 100 mM pH 8,4, 10 mM β-mercaptoethanol, 1 mM NAD(P)+ and 20 µg purified enzyme The reaction was started by adding the substrate at 33 mM final concentration. The formation of NAD(P)H was monitored at 340 nm for 2 min. Enzyme activity was expressed as nanomoles NADH formed per minute per microgram enzyme (molar extinction coeficient for NAD 6220 M-1*cm-1).

RESULTS AND DISCUSSIONS

***ORF40* encodes a tetrameric protein.** The recombinant protein obtained by cloning orf39 in pH6EX3 has the N-terminal sequence as fallows: HHHHHLVPRGSEA**L**, where the leucine 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. The purified enzyme had a relative molecular weight of 47 kDa, in good accordance with the theoretical mass. The purity of our preparations was very high (over 95% on SDS-PAGE, fig. 1)

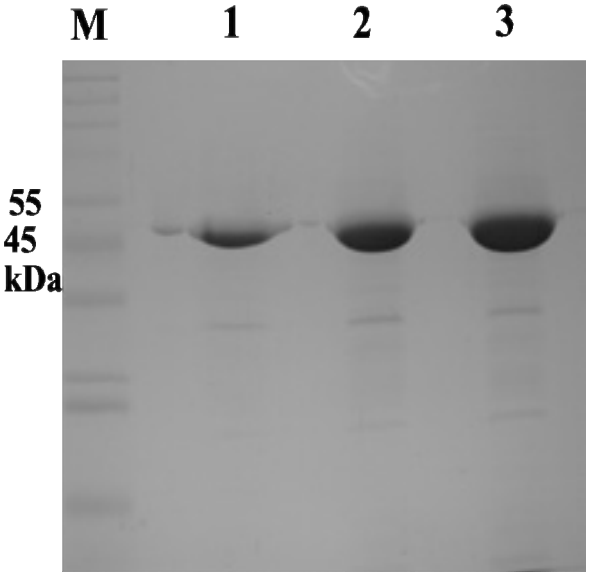

Figure 1. Orf40 encoded protein was purified to homogenity.
M –Molecular Weight Marker Sigma Wide Range
1,2,3 – Purified protein 5, 10, 15 microg respectivelly)

A BLAST search performed at the NCBI servers has shown that the ORF40 is similar at the sequence level with several oxidoreductases. Enzymes from this class are in various states of oligomerisation: tetramers, dimers or monomers. In order to establish native state of this enzyme in solution, a gel permeation chromatography was performed. Approximately 1.6 mg purified ORF40 were injected on a HiLoad 16/60 Superdex 200 column. The cromatogram is presented in figure 2. The protein eluted as two peaks, a small one corresponding to the void volume and second one, corresponding to a molecular weight of 163 kDa. This indicate that the enzyme is an tetramer in solution.

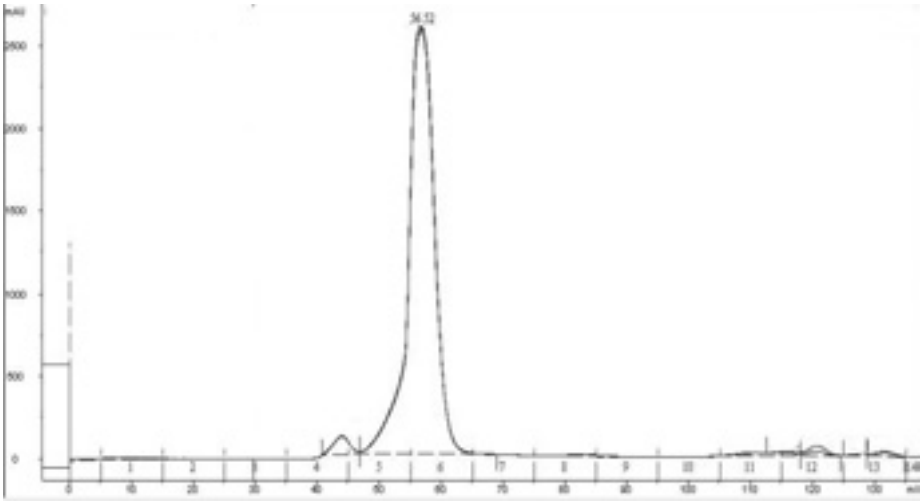

Figure 2. The determination of native molecular mass of ORF40 protein. 1.6 mg purified ORF40 protein was injected on a HiLoad 16/60 Superdex 200 previosly calibrated using Blue-Dextran, Feritine (440 kDa), Catalase (232 Kda), Aldolase (158 kDA), Albumine (67 kDa), Ovalbumine (43 kDa), Ribonuclease (17.4 kDa).

ORF40 contains un-covalently bound Zn^{2+}. After the concentration of the purified protein up to 34 µg /µL using a Vivaspin MWCO 3000 centrifugal device , a brown color could be observed. The UV-Vis spectrum of the concentrated solution (figure 3) indicates an large peak at 275 nm which is characteristic for proteins, as well as an shoulder at 405 nm. This would indicate the presence of an metal co-factor. The presence of Mn, Zn si Fe was investigated by mass spectrometry. Although negligible amounts of Mn and Fe could be detected, this is due to contamination in the buffers. Although the the purification buffer contains traces amounts of Zn, the protein and the flow-through contains high amounts of this metal (table 2).

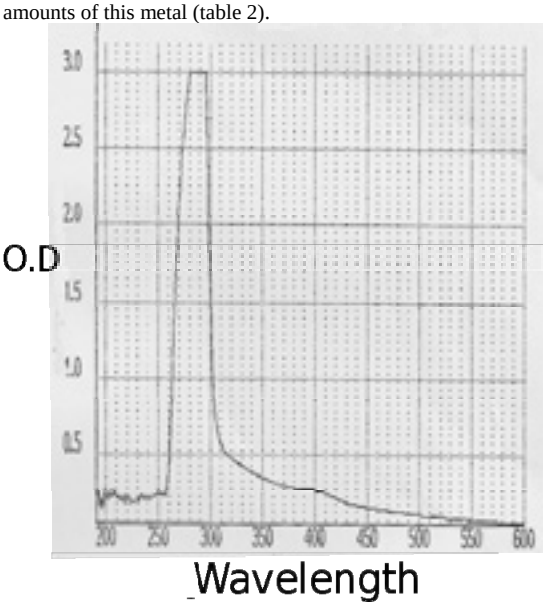

Figure 3. UV-Vis spectrum of the ORF40 protein

Table 2. Zn content in the analyzed samples

Sample	Zn concentration (mg/L)
Purification buffer Hepes 40 mM, NaCl 500 mM	1,7
Flow-through	27,2
ORF40 protein (15 mg/ml)	43,2

The concentrated protein solution kept at 4^{o}C for more than one week forms on small brown precipitate. If the protein is precipitated by boiling or by ATC 5% treatment, the precipitated is white. This is in god accordance with the fact that Zn was detected in the flow-through and indicated the metal is un-covalently bound to the protein.

The amount of Zn detected corresponds to a ration of about 2 Zn atoms per monomer molecule. Such ratio was previously reported also for L-arabinitol 4-dehidrogenase from *Neurospora crassa* (Sullivan R.,2007) or manitol-dehydrogenase from *Leuconostoc pseudomesenteroides* . Usually one Zn atom is implicated in the enzymatic reaction and an other has a structural role (Hahn G., Kaup B., Bringer-Meyer S., Sahm H. ,2003).

ORF40 is part of the GFO/IDH/MocA family of enzymes, which have one substrate binding domain and a second metal binding domain. Such a region with homology to this metal binding domain was nevertheless not detected on the ORF40.

Zn containing enzymes can be devided in 5 categories: DNA and RNA polimerases, alkaline phosphatases, peptidases, carbonic anhydrases and alcohol-dehydrogenases (Kimura E.,1993). This is in good accordance with our previously molecular modeling reports (MIHASAN & ARTENIE,2008) that the protein codified by ORF40 to be an alchool-dehydrogenase that act on a sugar substrate forming an aldehyde and shortening the C chain. The formed aldehyde is then further dehydrogenated by ORF39 (Mihasan, Artenie & Brandsch,2009), the resulting acid being further integrated in the tricarboxilic acid cycle. This way the two enzymes ORF39 and ORF40 are the final steps of this sugar catabolic pathway, integrating this plasmid encoded patway into the general chromosome encoded metabolic network of the cell.

CONCLUSIONS

The ORF40 was cloned, expressed and purified to homogeneity. It consists of an novel sugar-oxidoreductase of 47 kDa, which is an tetramer as assayed by gel-permeation chromatography. The brown color of the purified enzyme preparations indicated that it contains an co-factor. Mass spectroscopy indicated that the enzyme contains 2 atoms of Zn per molecule of monomer, classifying the enzyme as a alcohol dehydrogenase. In order to fully establish the biotechnological potential of this enzyme, serious efforts are undertaken to develop a suitable method for assaying the enzyme activity and to further characterise the enzyme (heat stability, pH stability, Km, Kcat,).

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1. University Al I Cuza Iasi, Romania
2. University Albert Ludwigs Freiburg im Breisgau, Germany

* marius.mihasan@uaic.ro