CLONING AND PURIFICATION OF A REPRESSOR PROTEIN FROM *ARTHROBACTER NICOTINOVORANS* PAO1

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

Keywords: *Arthrobacter,* repressor, carbohydrates
Abstract: The pAO1 megaplasmid of *A. nicotinovorans* consists of 165 ORF's related mainly to nicotine degradation, uptake and utilization of carbohydrates, amino acids and sarcosine. The putative sugar catabolic pathway consists of 11 ORF's organized as a single operon and coding for an ABC-type sugar-transport system and several putative oxidoreductases and dehydrogenases. The current work is focused on orf32, a putative PdhR related protein, most probably involved in the control of the whole operon. The approx. 700 kb *orf32* gene was cloned in the pH6EX3 plasmid vector and the gene product purified to homogeneity as a 29 kDa His-tagged recombinant protein. As indicated by GPC, it consists of a monomeric protein with a native molecular weight of 32 kDa. The specific UV/Vis spectra showed only a single peak at 280 nm common for all proteins and did not indicated the presence of any colored cofactors. This is in good agreement with the fact that PdhR-family proteins contain a winged helix-turn-helix (wHTH) domain responsible for DNA binding, and not a Zn-finger or any other metal containing domains.

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 a 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. All of these genes are clearly organized as a single operon, with *orf32,* a putative DNA binding -protein having an opposite orientation and thereby being the perfect candidate for the repressor involved in the control of the whole operon (figure 1).

Figure 1. General organization of the putative carbohydrate utilization gene cluster from pAO1

Our current study is focused further characterization the ORF32 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, to easily purify it to homogeneity and, using antibodies developed in rats, to indicate a possible inducer by Western-blot.

MATERIAL AND METHODS

Strains and growth conditions. For all recombinant DNA-techniques and protein purifications, *E. coli* XL1 Blue grown on Luria-Bertani (LB) medium was used. Arthrobacter nicotinovorans pAO1+ and pAO1- were a kind gift from prof. Dr. Brandsch R, and were grown an citrate medium supplemented with carbohydrates at 10 mM final concentration.
Isloation and cloning of *orf32*. The *orf32* 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 *orf42* in the pH_6EX_3 vector was achieved by using *BamHI* 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 *orf32*

	Sequence
Orf32forw	5′-GGCCGAGGATCCATGGACG-3′
Orf32rev	5′-CGCTACCACTCGAGGCTGACC-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 et. Al., 2002) on Fast-Flow Ni-chelating Sepharose (Amersham Biosciences, Sweden). **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). **Antibodies** against the purified protein were developed in rats and used as primary antibodies in Western-Blots. **Carbohydrate metabolism** assay was performed with the API 50CHL (Biomerieux, France) per producer's indications.

RESULTS AND DISCUSSIONS

***ORF32* encodes a monomeric protein.** The recombinant protein obtained by cloning orf39 in pH6EX3 has the N-terminal sequence as follows: HHHHHLVPRGSEA**L**, where the leucine in bold is the native start codon. This allowed for a 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. 2)

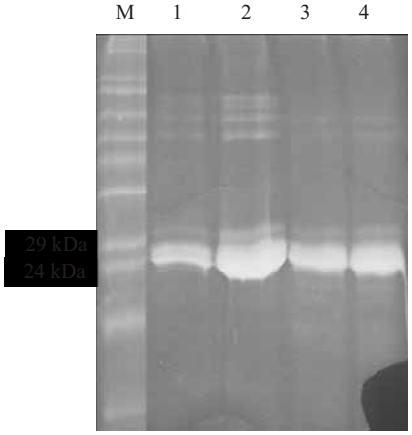

Figure 2. Orf32 encoded protein was purified to homogenity.
M –Molecular Weight Marker Sigma Wide Range
1,2- Purified protein, 200 mM imidasol elution
3,4 – Purified protein, 500 mM imidasol elution

Repressor proteins are usually tetramers or dimers in solution. In order to establish native state of this protein a

gel permeation chromatography was performed. Approximately 1.6 mg purified ORF40 were injected on a HiLoad 16/60 Superdex 200 column. The chromatogram is presented in figure 3. The protein eluted as a single peak, corresponding to a molecular weight of 32 kDa. This indicates that surprisingly, ORF32 is monomer in solution.

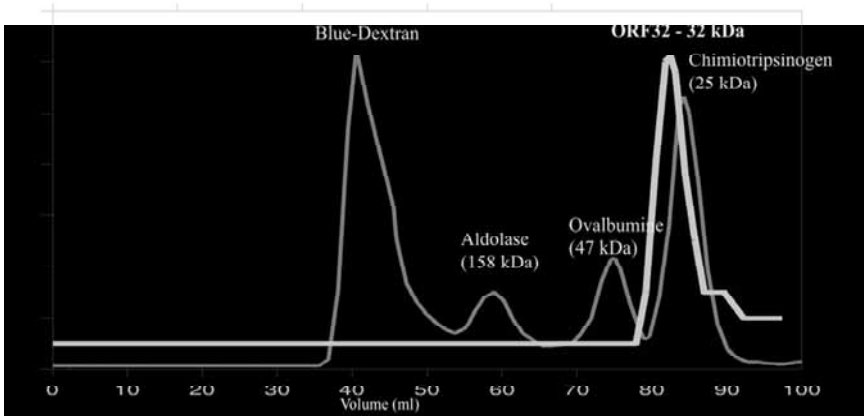

Figure 3. Native molecular mass determination of ORF32 protein. 1.6 mg purified ORF32 protein was injected on a HiLoad 16/60 Superdex 200 previosly calibrated using Blue-Dextran, Aldolase (158 kDA, Ovalbumine (43 kDa), Chimiotripsinogen (25 kDa).

ORF32 contains a helix-turn-helix (HTH) domain. A BLAST search performed with the amino acid sequence of ORF32 indicates that the protein belongs to the GntR family of transcriptional regulators. This family consists of several subfamilies (Rigali et al. 2002), but most of the members were described as repressors. Using SwissModeller, a computer generated ORF32 model was obtained. The putative 3D structure follows the same general organization with two domains as the LdlR transcriptional factor from *Corynebacterium glutamicum* (Gao et al. 2008). The N-terminal LdlR region contains a winged helix-turn-helix (wHTH) domain characteristic for GntR family proteins which is in a way conserved in the ORF32 model. The two helices and the connecting turn are present and are forming an HTH domain, but the beta sheets forming the wing are missing (figure 4). This HTH domain is usually responsible for the DNA binding activity which one might expect from a repressor protein. This is in good accordance with the experimentally observed fact that specific UV/Vis spectra of ORF32 do not indicate the presence of a Zn finger.

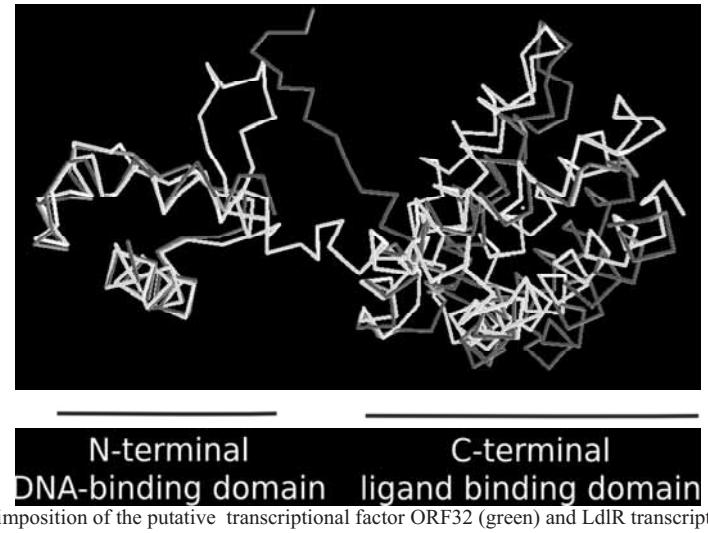

Figure 4. Superimposition of the putative transcriptional factor ORF32 (green) and LdlR transcriptional factor from *Corynebacterium glutamicum* (black, 2di3)

ORF32 is a continuously expressed in *Arthrobacter nicotinovorans pAO1*+ cells. As a DNA/binding assay could not be performed in order to directly address the function of this protein, an indirect approach was chosen. In a series of experiments, the conditions in which this protein is expressed *in-vivo* were studied. Cell free extracts of *A. nicotinovorans* pAO1+ cells grown on citrate medium supplemented with various sugars were separated by SDS-PAGE and the levels of ORF32 proteins were detected by Western-Blot using in the house raised antibodies. In all tested conditions, in the presence or in the absence of the sugars, the ORF32 protein was expressed in equal amounts. This is understandable, as a repressor protein should always be expressed in order to efficiently inhibit the transcription of operon. When the inducer appears, the DNA binding capacity of the repressor is abolished and the transcription can take place.

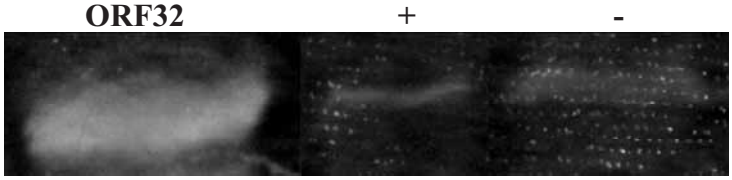

Figure 5. Detection of ORF32 by Western-Blot in the cell free extracts of *Arthrobacter nicotinovorans* pAO1 growth on citrate media (-) and on citrate media supplemented with D-xylose (+)

CONCLUSIONS

The ORF32 from pAO1 was cloned, expressed and purified to homogeneity. It consists of a HTH repressor protein of 29 kDa, which surprisingly is a monomer in solution.

Acknowledgements. This work was supported by CNCSIS-UEFISCSU, project number PN II- RU 337/2010.

REFERENCES

Ausubel M Frederick, Brent Roger, Kingston E Robert , Moore D David,Seidman J G,Smith A John, Struhl Kevin (2002): *Short protocols in molecular biology*, , a-5-a ed., vol. , John Wiley & Sons.

Berthold H,, Scanarini M,, Abney CC,, Frorath B,, Northemann W, (1992): *Purification of recombinant antigenic epitopes of the human 68-kDa (U1) ribonucleoprotein antigen using the expression system pH6EX3 followed by metal chelating affinity chromatography*. Protein Expr Purif, 3(), 50-56,

Bradford, M (1976): *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the*

principle of protein-dye binding. Anal. Biochem, 72(), 248-254,
Brandsch Roderich. (2006): *Microbiology and biochemistry of nicotine degradation*. Appl. Microbiol. Biotechnol, 69(), 493-498,
Bruns, C M;Hubatsch, I;Ridderström, M;Mannervik, B;Tainer, J A (1999): *Human glutathione transferase a4-4 crystal structures and mutagenesis reveal the basis of high catalytic efficiency with toxic lipid peroxidation products*. J Mol Biol, 288(3), 427-439,
Caballero E., Baldoma L., Ros J., Boronat A., Aguilar J. (1983): *Identification of lactaldehyde dehydrogenase and glycoaldehide dehydrogenase as functions of the same protein in escherichia coli*. Journal of Biological Chemistry, 258(), 7783-7792,
Cuff, J. A., Barton, G. J. (2000): *Application of enhanced multiple sequence alignment profiles to improve protein secondary structure prediction*. Proteins:Structure, Function and Genetics, 40(), 502-511,
Eaton A. (2001): *Plasmid-Encoded Phthalate Catabolic Pathway in Arthrobacter keyseri 12B*. Journal of Bacteriology, 183(), 3689-3703,
Farrbs J., Wang X., Takahashis K., Cunningham S. J., Wangn T. , Weiner H., (1994): *Effects of changing glutamate487 to lysine in rat and human liver mitochondrial aldehyde dehydrogenase*. The Journal of Biological Chemistry, 269(), 13854-13860,
Ganas Petra,, Sachelaru Paula,, Mihasan Marius,, Igloi Gabor L,, Brandsch Roderich, (2008): *Two closely related pathways of nicotine catabolism in Arthrobacter nicotinovorans and Nocardioides sp. strain JS614*. Arch Microbiol, 189(5), 511-517,
Gao Y., Suzuki H., Itou H., Zhou Y., Tanaka Y., Wachi M., Watanabe N., Tanaka I. & Yao M. 2008. *Structural and functional characterization of the LldR from Corynebacterium glutamicum: a transcriptional repressor involved in L-lactate and sugar utilization*. Nucleic Acids Res. 36: 7110-23.
Gouet, P., Courcelle, E., Stuart, D.I. and Metoz, F (1999): *Espript: multiple sequence alignments in postscript*. Bioinformatics, 15(), 305-308,
Hahn G., Kaup B., Bringer-Meyer S., Sahm H. (2003): *A zinc-containing mannitol-2-dehydrogenase from leuconostoc pseudomesenteroides atcc 12291: purification of the enzyme and cloning of the gene*. Arch. Microbiol., 179(), 101-107,
Igloi GL,, Brandsch R, (2003): *Sequence of the 165-kilobase catabolic plasmid pAO1 from Arthrobacter nicotinovorans and identification of a pAO1-dependent nicotine uptake system*. J Bacteriol, 185(6), 1976-1986,
Kimura E., (1993): *Roles of zn(ii) ion in zinc enzymes*. Pure & Appl. Chem. , 65(), 356-359,
Marchal, S; Branlant, G; (1999): *Evidence for the chemical activation of essential cys-302 upon cofactor binding to nonphosphorylating glyceraldehyde 3-phosphate dehydrogenase from streptococcus mutans*. Biochemistry, 38(), 12950-12958,
Mihasan M., Ungureanu E., Artenie V. (2007): *Optimum parameters for overexpression of recombinat proteins from tac promotors on autoinducible medium*. Roumanian Biotechnological letters, 12(6), 3473-3482,
Mihasan Marius, Artenie Vlad, (2008): *Computer-based modeling for sugar preferences of an oxidoreducatse from Arthrobacter nicotinovorans Pao1 plasmid*. Analele Stiintifice ale Universitatii Alexandru Ioan Cuza, Sectiunea Genetica si Biologie Moleculara, IX(3), 129-133,
Mihasan Marius,, Artenie Vlad,, Brandsch Roderich, (2009): *Purification of a novel aldehide-dehidrogenase with wide substrate specificity*. Analele Stiintifice ale Universitatii Alexandru Ioan Cuza, Sectiunea Genetica si Biologie Moleculara, X(2), 9-13,
Rigali S., Nothaft H., Noens E. E. E., Schlicht M., Colson S., Müller M., Joris B., Koerten H. K., Hopwood D. A., Titgemeyer F. & van Wezel G.P. 2006. *The sugar phosphotransferase system of Streptomyces coelicolor is regulated by the GntR-family regulator DasR and links N-acetylglucosamine metabolism to the control of development*. Mol. Microbiol. 61:1237-51.
Rosselló-Mora R A,, Lalucat J,, García-Valdés E, (1994): *Comparative biochemical and genetic analysis of naphthalene degradation among Pseudomonas stutzeri strains*. Appl Environ Microbiol, 60(3), 966-972,
Sambrook J, Fritsch EF, Maniatis T (1989): *Molecular cloning - a laboratory manual*, , ed., vol. , Cold Spring Harbour Laboratory Press.
Sullivan R., Zhao H. (2007): *Cloning, characterization and mutational analysis of a highly active and stable L-arabinitol 4-dehydrogenase from Neurospora crassa*. Appl. Microbiol. Biotechnol., 77(), 845-852,

1. University Al I Cuza Iasi, Romania
2. University Albert Ludwigs Freiburg im Breisgau, Germany
* marius.mihasan@uaic.ro