

GENE CLONING OF A PUTATIVE PERIPLASMIC SUGAR-BINDING PROTEIN FROM THE pAO1 MEGAPLASMID OF *ARTHROBACTER NICOTINOVORANS*

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Abstract: D-xylose is a very important fraction of lignocellulose and it provides a promising renewable resource for production of bio-ethanol or other various chemicals. Recently, the pAO1 megaplasmid of *Arthrobacter nicotinovorans* has been linked with the ability of this microorganism to metabolize D-Xylose through a less common oxidative pathway. The operon encoding the *A. nicotinovorans* oxidative xylose-catabolic pathway has been identified and some enzymes have been isolated and characterized. So far, the mechanisms underlying operon activation and xylose transport have been neglected. The *xyl* operon contains all the components of a ABC-type transport system that must be involved in cross-membrane transport of D-Xylose. In this work, a PCR protocol for the isolation of the putative periplasmic binding protein (ppl) component of the ABC-type system has been established and the DNA fragment containing the *ppl* gene has been cloned into the pH6EX3 expression vector.

INTRODUCTION

Xylose, as the main component of the hemicellulose xylan, is a rather abundant pentose, comprising up to 20% of some plants biomass (Aristidou and Penttilä, 2005). Although a very important resource in terms of availability, D-xylose has found very few bio-technological uses: production of bioethanol, xylitol (Ko et al., 2006) or production of tricarboxylic-acid (TCA)-cycle-derived chemicals (e.g. C4 building blocks (Meijnen et al., 2009)). This situation is due to the lack of suitable enzymes that can be applied into biotechnological and industrial process.

Recently, we have shown that the soil bacteria *Arthrobacter nicotinovorans* is able to degrade D-Xylose due to the presence of a *xyl* operon on the 165 kb megaplasmid pAO1 (Mihasan et al., 2013). It seems that the pathway is not unique to this species, as its existence has also been inferred from the DNA sequence of *A. phenanthrenivorans* (Mihasan and Brandsch, 2013). The degradation pathway consists of an oxidative stepped-mechanism similar to the Weimberg pathway, in which D-Xylose is oxidized by a xylose-dehydrogenase (XDH) to D-xylonate, which is dehydrated to 2-ketoglutarate semi-aldehyde and further oxidized to 2-ketoglutarate by an aldehyde-dehydrogenase. Part of the *xyl*-operon is also a ABC-type transport system, involved most probably in the ATP-dependent transport of D-xylose across the membrane into the bacterial cell. The ABC-type transport system genes cluster contains all the components of a typical class 3 ABC system (Davidson AL et al. 2008) (Figure 1): a putative ATP binding protein gene (*ATP-bind*), two genes for pore-forming membrane proteins (*perm*) and a periplasmic sugar binding protein gene (*ppl*).

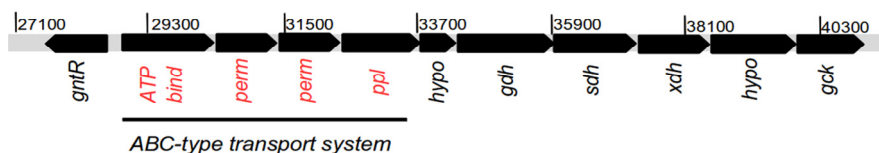


Figure 1. The *xyl* operon in pAO1 of *Arthrobacter nicotinovorans*. *ATP-bind* - a putative ATP binding protein, *perm* - pore-forming membrane proteins, *ppl* - periplasmic sugar binding protein, *hypo* - hypothetical protein, unknown function, *gdh* - putative keto-gluconate dehydrogenase, *sdh* - semi-aldehyde dehydrogenase, *xdh* - xylose-dehydrogenase, *gck* - putative glicerate-kinase.

A BLAST search using the amino acid sequence of the periplasmic sugar binding protein (PSBP) indicates that the protein is part of the type I periplasmic binding protein superfamily and that it contains the cd06300 conserved domain (Marchler-Bauer et al. 2013). Members of this group are predicted to be involved in the transport of sugar-containing molecules across cellular and organellar membranes; however their substrate specificity is not yet known in detail (Davidson et al., 2008). A putative model for the 3D structure of the PSBP protein has been previously generated using homology modeling. The overall structure of the model follows well the characteristics of the bacterial PBP structural superfamily: a bilobate structure known as Venus flytrap (Acher and Bertrand, 2005) formed by two pseudo-symmetric domains linked by a hinge. *In-silico* docking experiments have shown that the protein is able to bind not only

xylose and its derivatives, but also other less common sugars and derivatives such as: tagatose, xylulose or trehalose (Mihasan, 2010).

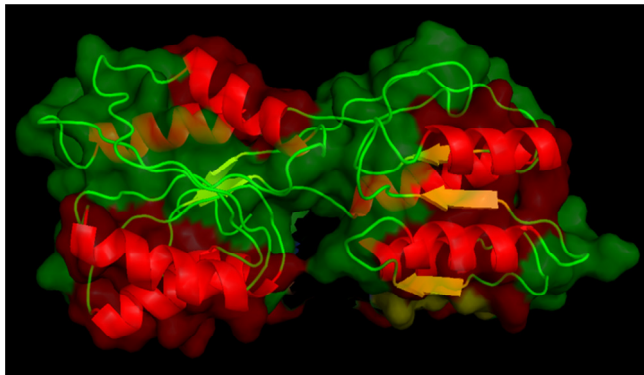


Figure 2. The 3D model of the PSPB showing characteristics of bacterial PBP structural superfamily protein: a bilobate structure formed by two pseudo-symmetric domains linked by a molecular hinge. Image generated with PyMol molecular modeling and visualization software (Schrödinger, 2010).

The PSPB is thereby a rather interesting protein from several points of view. Despite the fact the *in-silico* experiments have shown that the best ligand is tagatose, its localization in the *xyl*-operon would indicate that the physiological substrate is xylose. No signature sequence for protein export has been detected on PSPB, but for sure the protein is exported outside the cell in order to fulfill its role in sugar transport. The lack of fully characterized protein family members is also intriguing. Thereby, the current work focuses on the isolation and molecular cloning of the *ppl* gene in a suitable vector for simple downstream over-expression and purification of this protein.

MATERIAL AND METHODS

Chemicals. All chemicals used were of highest purity available. Ampicillin and kanamycin was from Sigma-Aldrich, Germany. HEPES, yeast extract, peptone from caseine, EDTA and DTT were from Carl Roth, Germany. All restriction enzymes were from NEB, U.K.

Strains and growth conditions. For all recombinant DNA-techniques and plasmids harvesting, *E. coli* XL1 Blue (Stratagene) was grown on LB-nutrient broth (Mihasan et al., 2012) with appropriate antibiotics (ampicline 50 µg/ml). *Arthrobacter nicotinovorans* pAO1+ was a kind gift from prof. Dr. Brandsch R and was grown on citrate minimal medium (Brühmüller et al., 1972) supplemented with 0,05% nicotine and 70 µg/ml kanamycin.

Plasmids and primers. The *ppl* gene was isolated by PCR using the primers in table 1 and a suspension of *Arthrobacter nicotinovorans* pAO1+ cells as template. Directional cloning (Sambrook et al., 1989) of the *ppl* fragment was achieved using the pH6EX3 expression vector (Berthold et. al., 1992). PCR clean-up, plasmid mini-preps and DNA-gel extraction were performed with the kits from Zymo Research, Germany. All DNA separations were performed using standard horizontal agarose-gel electrophoresis (Sambrook et al., 1989). The DNA was visualized using etidium-bromide and a Biorad Gel-Doc system.

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

Oligo's name	Sequence*
ForPplBam	5' <u>GCGGTACTAGGATCCGCCCATG</u> 3
RevNDH1xba	5' <u>CCCTGTGCGCTCGAGAATGACC</u> 3

*nucleotides written in italics indicate mutated nucleotides, underlined nucleotides denote the engineered restriction sites,

Ligation, transformation and clone selection. Competent *E. coli* XL1 blue cells were prepared using the standard Ca²⁺-method as described by (Sambrook et al., 1989). Following digestion, the vector and amplified fragment were ligated using the Rapid DNA ligation Kit, Roche, Germany and directly used for the transformation reaction. The putative clones were selected on plates containing ampicillin 50 µg/ml and the recombinant plasmid was checked for the presence of insert by restriction enzyme digestion.

RESULTS AND DISCUSSIONS

Sequence-inferred properties of PSPB. As shown previously (Igloi and Brandsch, 2003), the *ppl* gene is placed on the direct strand, with the START codon at the position 32506 and the STOP codon at the position 33684. The gene has 1,2 kb and it encodes 292 aminoacids. According to ProtParam on the ExPASy server (Gasteiger et al., 2005) the computed molecular weight of PSPB is 42.2 kDa and the theoretical pI is 4.63. The instability index (II) was calculated to be 36.42 which classifies the protein as stable and makes PSBP a suitable candidate for overexpression and purification.

PCR isolation and amplification of the *ppl* fragment. Using the primers indicated in table 1 and a PCR cycle consisting of denaturation 95°C, 30 s; annealing variable temperature, 45 s; synthesis 72°C, 1.5 min repeated 30 times, the whole approx. 1.4 kb DNA fragment was successfully amplified (figure 3A). Despite all efforts, a very specific amplification was not possible and a supplementary DNA purification was required. The DNA fragment corresponding to the expected molecular weight was excised from the gel and extracted.

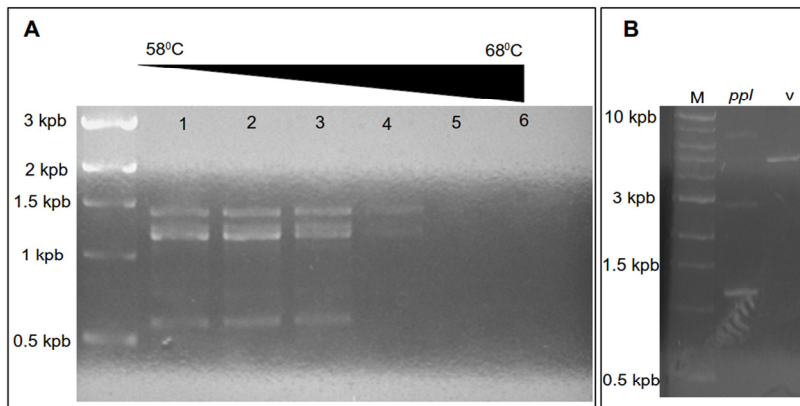


Figure 3. Isolation of the *ppl*- containing fragment. **A.** Unspecific amplification of the *ppl*-containing fragment. On top, the temperature gradient used in the annealing step of the PCR-program.

B. v – linear pH6EX3 vector and *ppl*- DNA fragment containing the *ppl* gene after digestion with the restriction enzymes specified in the main text and purification.

Digestion, ligation and transformation. After the successful amplification and isolation of the *ppl* gene, the fragment was digested using the enzyme pair BamHI/XhoI in Neb buffer 3 following the indications of the producer. The pH6EX3 vector was also digested with the enzyme pair BamHI/XhoI which leads to compatible ends and assures a precise orientation of the fragment (Mihasan et al., 2010). Following ligation and transformation, a number of only 3 colonies were obtained on ampicillin containing plates. The plasmid DNA from these colonies was isolated and analyzed by agarose gel electrophoresis (figure 4, A).

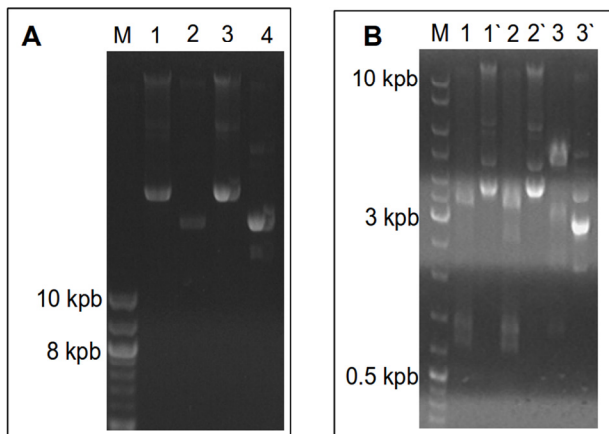


Figure 4. Screening of the putative colonies harboring the recombinant pH6EX3*ppl* plasmid. **A.** Circular plasmid DNA isolated from transformed colonies. M – 1kb DNA ladder, further lanes - plasmid DNA from the indicated colonies, 4 – circular pH6EX3 vector. Colony 1 and 3 run differently than the circular pH6EX3 indicating a recombinant plasmid molecule. **B.** Controlled enzymatic digestion of the isolated plasmid DNA using PstI/XhoI. M - 1kb DNA ladder, 1, 2 – digested pH6EX3*ppl* recombinant vector; 1', 2' - circular pH6EX3*ppl* recombinant vector; 3 - digested pH6EX3 vector; 3' - circular pH6EX3 vector.

Cloning verification. Although the best way of checking a positive clone is by sequencing, a controlled enzymatic digestion which would take advantage of a cutting site existing only on the cloned fragment is as well as relevant (Chiribau et al., 2004). In order to check the isolated colonies, a double digest was performed using PstI/XhoI enzyme pair. As shown in figure 4, B, the plasmid DNA from the two tested colonies an approx. 1.4 kb fragment could be identified. This indicated that both of the tested colonies are harboring the recombinant pH6EX3*ppl* plasmid.

CONCLUSION

The *ppl* gene from pAO1 was isolated and cloned in pH6EX3. The recombinant vector will be further used in downstream applications to over-express and purify the PSBP protein.

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