MOLECULAR GENE CLONING OF NICOTINE-DEHYDROGENASE FROM THE pAO1 MEGAPLASMID OF *ARTHROBACTER NICOTINOVORANS*

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Abstract: 6-hydroxi-L-nicotine (6HNic) has an important potential as a drug for neuro-degenerative disorders and a suitable simple and reliable method for obtaining contaminant-free 6HNic preparations is required. Here, we envision the *in-vitro* production of 6HNic by using purified nicotine-dehydrogenase (NDH) followed by HPLC or capillary electrophoresis techniques and we focus on the isolation and cloning of the three genes coding the NDH enzyme. A PCR protocol was established for easy amplification and the DNA fragment containing the *ndhLSM* genes was directionally cloned into the pART2 vector.

INTRODUCTION

It is estimated that Alzheimer's disease (AD) alone contributes 11,2 % of years lived with disability in people aged 60 years and older; more than other age-related diseases (stroke, musculoskeletal disorders, cardio-vascular disease and all forms of cancer) (Ferri et al., 2005). The recent implication of nicotinic acetylcholine receptors (nAChR) subtypes α7 and α4β2 in AD pathogenesis (Parri, Hernandez, & Dineley, 2011) has led to the proposal of a new approach in AD therapies: by using nAchR modulators to increase the availability of receptors for acetylcholine, the loss of cholinergic neurons can be overcome, and thereby the cognitive and non-cognitive functions can be improved. Nicotine, the well-known agonist of nAchR, has proven negative effects on various other organs such as lungs and did not impose itself as a feasible therapeutic agent for AD. Nevertheless, the beneficial effects of nicotine has led to an unprecedented search for nicotine-based derivative with biotechnological and medical applications.

As shown by recent studies performed in our lab (Mihasan, Luminita, Neagu, Stefan, & Hritcu, 2013) (Mihasan, Stefan, Brandsch, & Hritcu, 2012), the nicotine derivate 6-hydroxi-L-nicotine (6HNic) has the ability to sustain spatial memory formation by decreasing brain oxidative stress in rats (Hritcu, Stefan, Brandsch, & Mihasan, 2011). This fact is having an important potential as a drug for neuro-degenerative disorders. Although the tested 6HNic was a product of chemical synthesis, this compound is a natural product occurring is in the first steps of the nicotine-catabolic pathway from *Arthrobacter nicotinovorans (Roderich Brandsch, 2006)*. 6HNic is formed in a hydroxylation reaction performed by nicotine-dehydrogenase (NDH), a trimeric enzyme containing molybdopterin dinucleotide, FAD and Fe-S clusters as cofactors (Grether-Beck et al., 1994) (Freudenberg, Konig, & Andreesen, 1988). NDH is encoded by three genes (*ndhS - 0,5 kb, ndhM – 0,85 kb, ndhM – 2,4 kb*), part of the nicotine-gene cluster placed on the pAO1 megaplasmid (R Brandsch & Decker, 1984). The arrangement and localization of the three NDH genes is depicted in figure 1.

Figure 1. Nicotine-gene cluster and the localization of the NDH genes.

Considering the above mentioned potential of 6HNic as a protective agent in neuro-degenerative disorders such as Alzheimer's disease, a simple suitable and reliable method for obtaining contaminant-free 6HNic preparations is required. As the sequences of the NDH genes are readily available (Igloi & Brandsch, 2003), we envision the *in-vitro* production of 6HNic by using purified NDH followed by HPLC or capillary electrophoresis techniques. In this direction, the current work focuses on the isolation and molecular cloning of the NDH genes in a suitable vector which would allow simple downstream purification of this enzyme in an active form.

MATERIAL AND METHODS

Chemicals. All chemicals were of highest purity available. Kanamycin and tetracycline were 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, Stefan, & Zenovia, 2012) with appropriate antibiotics (tetracycline 12,5 μg/ml for the *wt* strain and kanamycin 35 μg/ml for the pART2 harboring cells). *Arthrobacter nicotinovorans* pAO1+ was a kind gift from professor *Dr. Brandsch R* and was grown on citrate minimal medium (Brühmüller, Möhler, & Decker, 1972) supplemented with 0,05% nicotine and 70 μg/ml kanamycin.

Plasmids and primers. The NDH genes were isolated by PCR using the primers in table 1 and a suspension of *Arthrobacter nicotinovorans* pAO1+ cells as template. Directional cloning (Sambrook, Fritsch, & Maniatis, 1989) of the *ndhLSM* fragment was achieved using the pART2 shuttle vector (*Sandu, Chiribau, Sachelaru, & Brandsch, 2005*) . 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 *ndhLSM*

Oligo's name	Sequence*
ForNDHmbcl	5'AGTGAAGGAT*TGATCA*ACCTGCTATC'3
RevNDHlxba	5'CTCCCTGTC*TCTAGA*GCCGCGATC'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 Ca^{2+}-method as described by *Sambrook et al., 1989*. Following digestion, the vector and the 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 kanamycin 35 μg/ml and the recombinant plasmid was checked for the presence of insert by restriction enzyme digestion.

RESULTS AND DISCUSSIONS

NDH and pART2. Previous work on the nicotine dehydrogenase from *Arthrobacter nicotinovorans* has revealed that the enzyme is formed from three subunits, corresponding to a molecular weight of about 82 000, 30 000 and 15 000 Da (Freudenberg et al., 1988). Interestingly, the structure of the enzyme has been solved and it has been shown that the smallest subunit (NDHS) carries two iron-sulphur cluster, the middle-sized subunit (NDHM) binds noncovalently a flavin adenine dinucleotide (FAD) molecule and the largest NDH subunit (NDHL) contains the molybdopterin cytosine dinucleotide cofactor (MCD) giving a $(\alpha\beta\gamma)_2$ complex. These structural details have an important implication in our attempt to clone and purify the enzyme as a recombinant protein. MCD is a rather rare cofactor and it seems like NDH is extensively linked with pAO1. The megaplasmid contains all the genes required for the synthesis of MCD and its insertion in the MCD-containing enzyme (Sachelaru, Schiltz, & Brandsch, 2006). Although molybdopterin cofactor synthesis pathways have been described in *E. coli,* this strain produces a slightly different form, namely molybdopterin guanosine dinucleotide cofactor (MGD) (Leimkühler, Wuebbens, & Rajagopalan, 2011). Thereby, *E. coli* is not a suitable host for over-expression and purification of active NDH.

The identification of some nicotine-gene cluster regulators (Sandu, Chiribau, & Brandsch, 2003) has led to the tailoring of novel pART2 shuttle vectors (Malphettes et al., 2005) which allows the usage of species belonging to the *Arthrobacter* genus as expression hosts. The fact that the molybdopterin cytosine dinucleotide cofactor synthesis genes are located on the pAO1 implies that only *Athrobacter nicotinovorans* could be used as host for NDH over-expression.

PCR isolation and amplification of the *ndhLSM* fragment. As depicted in table 1, the NDH genes are placed on the complementary strand. The first gene is *ndhM* which starts at position

996683 and ends at position 95832. It is then followed by *ndhS* wich starts at posititon 95835 and ends at position 95338 (Igloi & Brandsch, 2003). Interestingly, *ndhL*, the last gene, starts at 95345 and ends at 92895, which indicates a frame-shift in translation. Using the primers indicated in table 1 and a PCR cycle consisting of – denaturation 95^0C, 30 s; annealing variable temperature, 30 s; synthesis 72^0C, 4 minutes repeated 30 times, the whole approximately 3,8 kb DNA fragment was successfully amplified as shown in figure 2. Although it seems that the most specific amplification was performed at $51,4^0$C, the amplification efficiency is rather low and we have preferred to use 49^0C as annealing temperature and to further isolate the 3,8 kb fragment from the agarose gel.

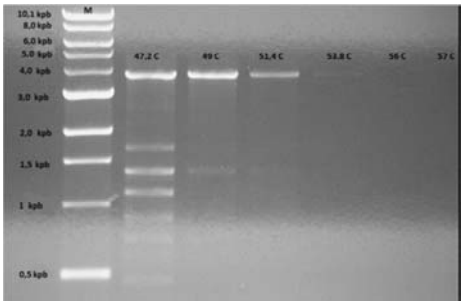

Figure 2. PCR amplification yield and the annealing temperature. M – 1kb DNA ladder, further lanes- PCR product at the indicated temperatures.

Digestion, ligation and transformation. After the successful amplification of the NDH genes, the fragment was sequentially digested using the enzyme pair XbaI/BclI. The pART2 vector was also digested with the enzyme pair BamHI/XbaI which leads to compatible ends and assures a precise orientation of the fragment (Mihasan, Stefan, Artenie, & Brandsch, 2010). Following ligation and transformation, a number of 12 colonies were obtained on kanamycine containing plates. The plasmid DNA from these colonies was isolated and analyzed by agarose gel electrophoresis (figure 3). From all 12 colonies, only colony 7 showed a different running pattern and was considered for further clone verification.

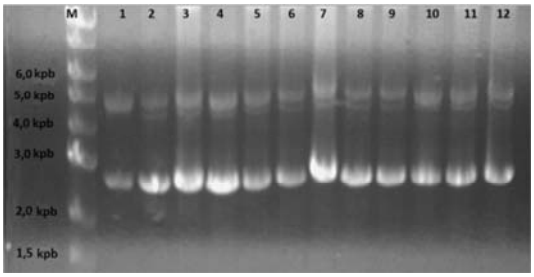

Figure 3. Screening of the putative colonies harboring the recombinant pART2*ndhLMS* plasmid. M – 1kb DNA ladder, further lanes - plasmid DNA from the indicated colonies. Colony 7 clearly runs different than the rest.

Cloning verification. Although the best way of checking a positive clone is by sequencing, a controlled enzymatic digestion which would take advantage of an cutting site existing only on the cloned fragment is as well as relevant (Mihasan, 2011)(Chiribau, Sandu, Fraaije, Schiltz, & Brandsch, 2004). In order to check the isolated colonies, a double digest was performed using XbaI/ApaL1 enzyme pair. As shown in figure 4, all 8 tested colonies migrated similar with the pART2 plasmid, and only in the colony 7 a large 3,6 kb fragment could be identified. This indicated that from all the tested colonies, onlz number 7 is harboring the recombinat pART2*ndhLMS* and the rest contain just the re-circularized pART2 plasmid.

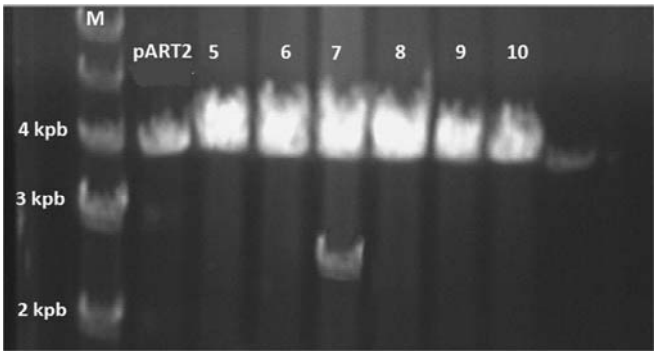

Figure 4. Controled enyzmatic digestion of the isolated plasmid DNA using XbaI/ApaL1. M - 1kb DNA ladder, pART2 – wt plasmid, further lanes (, 5, 6, 7, 8, 9, 10) – corresponding colonies.

CONCLUSION

The *ndhLSM* genes from pAO1 was isolated and cloned in pART2. The recombinant vector will be further used in downstream applications to purify the NDH enzyme and obtain 6HNic.

REFERENCES

Brandsch, R, & Decker, K. (1984). *Isolation and partial characterization of plasmid DNA from Arthrobacter oxidans.* Arch Microbiol, 138(1), 15–17.

Brandsch, Roderich. (2006). *Microbiology and biochemistry of nicotine degradation. Appl Microbiol Biotechnol*, 69(5), 493–498.

Brühmüller, M., Möhler, H., & Decker, K. (1972). *Covalently bound flavin in D-6-hydroxynicotine oxidase from Arthrobacter oxidans. Purification and properties of D-6-hydroxynicotine oxidase.* European Journal Of Biochemistry / *FEBS*, 29(1), 143–51.

Chiribau, C. B., Sandu, C., Fraaije, M., Schiltz, E., & Brandsch, R. (2004). *A novel gamma-N-methylaminobutyrate demethylating oxidase involved in catabolism of the tobacco alkaloid nicotine by Arthrobacter nicotinovorans pAO1*. Eur J Biochem, 271(23-24), 4677–4684.

Ferri, C. P., Prince, M., Brayne, C., Brodaty, H., Fratiglioni, L., Ganguli, M., … Scazufca, M. (2005). *Global prevalence of dementia: a Delphi consensus study.* Lancet, 366(9503), 2112–7.

Freudenberg, W., Konig, K., & Andreesen, J. R. (1988). *Nicotine dehydrogenase from Arthrobacter oxidans: A molybdenum-containing hydroxylase.* FEMS Microbiology Letters, 52(1-2), 13–17

Grether-Beck, S., Igloi, G. L., Pust, S., Schilz, E., Decker, K., & Brandsch, R. (1994). *Structural analysis and molybdenum-dependent expression of the pAO1-encoded nicotine dehydrogenase genes of Arthrobacter*

nicotinovorans. Molecular Microbiology, 13(5),

Hritcu, L., Stefan, M., Brandsch, R., & Mihasan, M. (2011). 6-hydroxy-L-nicotine from Arthrobacter nicotinovorans sustain spatial memory formation by decreasing brain oxidative stress in rats. Journal of Physiology and Biochemistry Vol. 69, pp. 25–34).

Igloi, G. L., & 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.

Leimkühler, S., Wuebbens, M. M., & Rajagopalan, K. V. (2011). *The History of the Discovery of the Molybdenum Cofactor and Novel Aspects of its Biosynthesis in Bacteria.* Coordination Chemistry Reviews, 255(9-10), 1129–1144.

Malphettes, L., Weber, C. C., El-Baba, M. D., Schoenmakers, R. G., Aubel, D., Weber, W., & Fussenegger, M. (2005). *A novel mammalian expression system derived from components coordinating nicotine degradation in Arthrobacter nicotinovorans pAO1.* Nucleic Acids Research, 33(12),

Mihasan, M. (2011). *Megaplasmidul pAO1 - Structura si Functie.* (M. Mihasan, Ed.) (p. 125). Editura Universitatii Alexandru Ioan Cuza din Iasi.

Mihasan, M., Luminita, C., Neagu, E., Stefan, M., & Hritcu, L. (2013). *In-silico identification of 6-hydroxy-L-nicotine as a novel neuroprotective drug*. Romanian biotechnological letters, 18(3), 8333–8340.

Mihasan, M., Stefan, M., Artenie, V., & Brandsch, R. (2010). *Cloning and purification of a repressor protein from arthrobacter nicotinovorans pao1*. Analele Stiintifice ale Universitatii Alexandru Ioan Cuza; din Iasi Sec. II a. Genetica si Biologie Moleculara; Vol 12, No 3,

Mihasan, M., Stefan, M., Brandsch, R., & Hritcu, L. (2012). *Identification of 6-hydroxy-L-nicotine as a novel neuroprotective drug*. In Buletinul Societatii Romane de Biologie Celulara, Nr. 40, iulie 2012, p. 34.
Mihasan, M., Stefan, M., & Zenovia, O. (2012). Biologie moleculara - metode experimentale (p. 350). Iaşi: Editura Univ. Al. I. Cuza Iasi.

Parri, H. R., Hernandez, C. M., & Dineley, K. T. (2011). *Research update: Alpha7 nicotinic acetylcholine receptor mechanisms in Alzheimer's disease*. Biochemical Pharmacology, 82(8), 931–42.

Sachelaru, P., Schiltz, E., & Brandsch, R. (2006). *A functional mobA gene for molybdopterin cytosine dinucleotide cofactor biosynthesis is required for activity and holoenzyme assembly of the heterotrimeric nicotine dehydrogenases of Arthrobacter nicotinovorans*. Applied And Environmental Microbiology, 72(7),

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (3 Volume Set). Cold Spring Harbor Laboratory Pr.

Sandu, C., Chiribau, C. B., & Brandsch, R. (2003). *Characterization of HdnoR, the transcriptional repressor of the 6-hydroxy-D-nicotine oxidase gene of Arthrobacter nicotinovorans pAO1, and its DNA-binding activity in response to L- and D-nicotine Derivatives*. J Biol Chem, 278(51), 51307–51315.

Sandu, C., Chiribau, C.-B., Sachelaru, P., & Brandsch, R. (2005). *Plasmids for nicotine-dependent and -independent gene expression in Arthrobacter nicotinovorans and other Arthrobacter species.* Applied And Environmental Microbiology, 71(12), 8920–4.

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