Arthrobacter nicotinovorans pAO1

a tool to produce neuroactive compounds

ALEXANDRU IOAN CUZA UNIVERSITY of IAŞI www.uaic.ro

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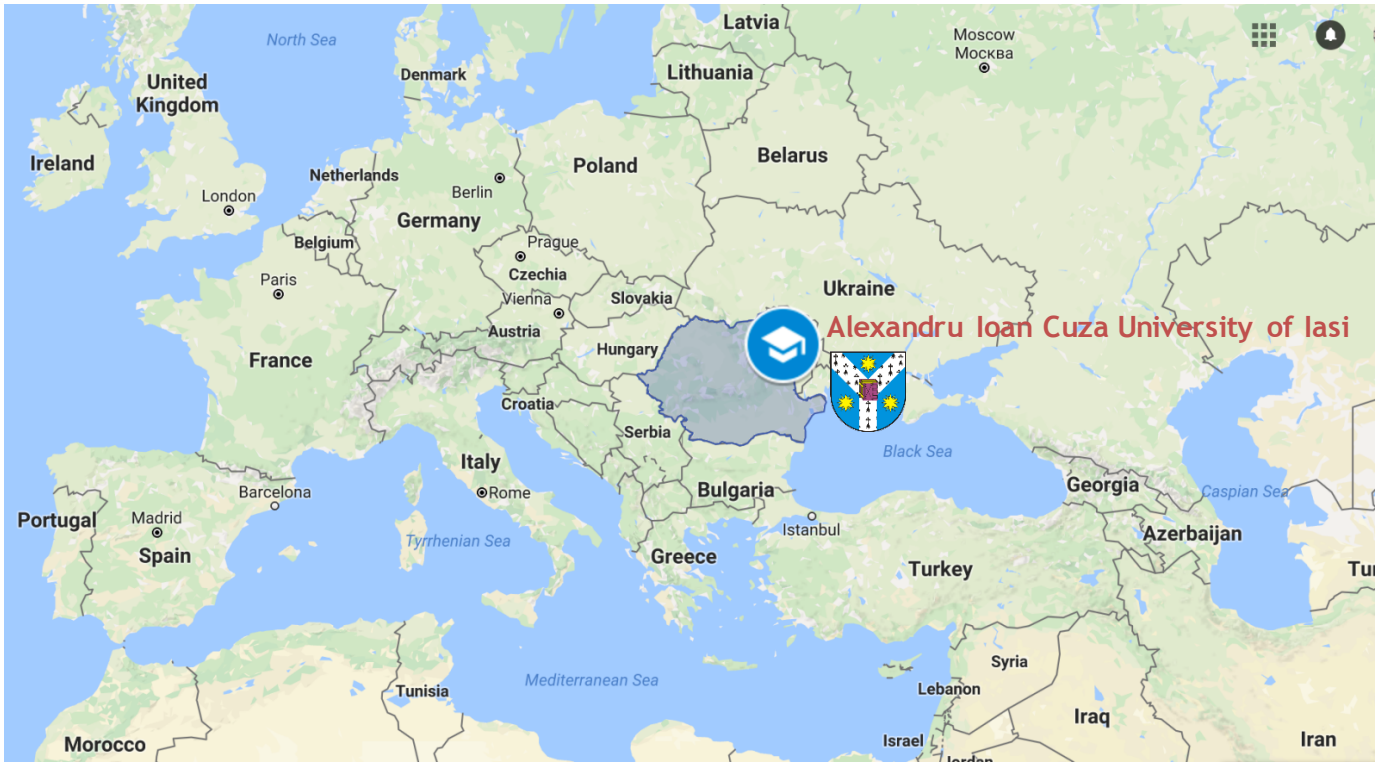
A bit about my home country



Romania

Area: 92,043 sq mi
Population: 20,121,641

Transylvania

Chemistry

Lazăr Edeleanu
the first to synthesize amphetamine

Vintilă Ciocâlteu
co-developed the Folin-Ciocalteu reagent

Nicolae Teclu
the Teclu burner

Biology

Microbiology: **Victor Babeș**
more than 50 types of bacteria

Bio speleology: **Emil Racoviță**
the first to study the arctic life

Cell Biology: **Emil Palade**
the most influential cell biologist ever

Facts and figures

- ✓ The **oldest** Romanian university
- ✓ **Diplomas recognized** all over Europe
  - ✓ **15** departments
  - ✓ **93** Bachelor programs
  - ✓ **176** Master programs
  - ✓ **26** PhD programs

Sciences

- ✓ Biology
- ✓ Chemistry
- ✓ Computer Science
- ✓ Geography and Geology
- ✓ Mathematics
- ✓ Physics

Social Sciences & Humanities

- ✓ Economics and Business Administration
- ✓ History
- ✓ Law
- ✓ Letters
- ✓ Orthodox Theology
- ✓ Philosophy and Social – Political Sciences
- ✓ Physical Education and Sports
- ✓ Psychology and Education Sciences
- ✓ Roman – Catholic Theology
- ✓ Center for European Studies

Department of Biology

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Founded in **1948**

- ✓ **758** students:
  - **20** PhD students
- ✓**46** full-time faculty members
- ✓ **16** technicians and administrative staff

Programs of Study

BACHELOR

B — 6 (8) semesters — ECTS 180 - 240 — Years of study 3

- Biology
- Biochemistry
- Environmental science

MASTER

M — 4 semesters — ECTS 120 — Years of study 2

- Biology — Molecular genetics; Cellular and microbial biotech.; Biodiversity Conservation
- Environment Counceling

PhD

D — 6 semesters — ECTS 180 — Years of study 3

- PCS (Scientific research program) — Semester 6 5
- PCS (Scientific research program) — Semester 4 3
- PPUA (Advanced University Training Program) — Semester 2 1

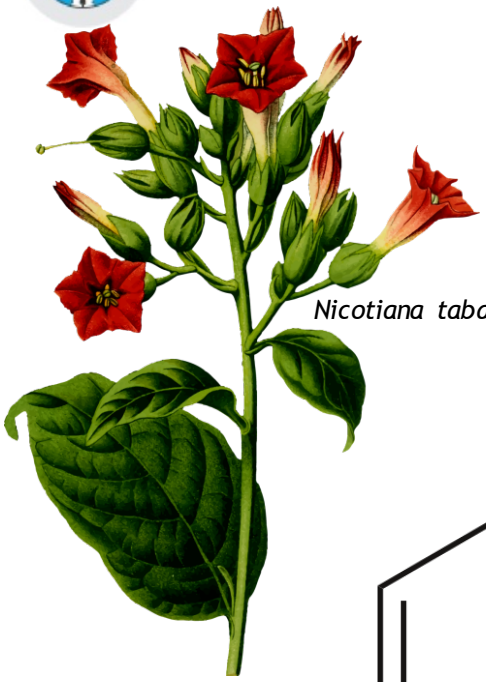
Why I am in Potsdam?

Sponsor: U.S. Department of State
Romanian-U.S. Fulbright Commission
Exchange Visitor Program Number G-1-00005

- **Observe and learn the dynamics of a research group here in US**
- **Attend lectures and understand the US academic system**
- **Identify all the nicotine-induced proteins in *Arthrobacter nicotinovorans* by means of mass-spectrometry**

6 months in Darie's Biochemistry & Proteomics Group
Department of Chemistry & Biomolecular Science, Clarkson University

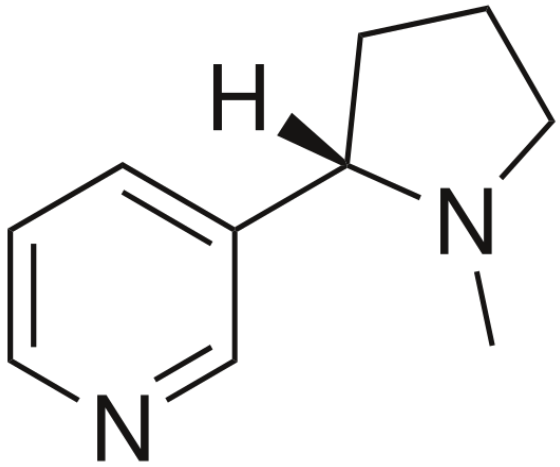
Nicotine and nicotine-containing waste

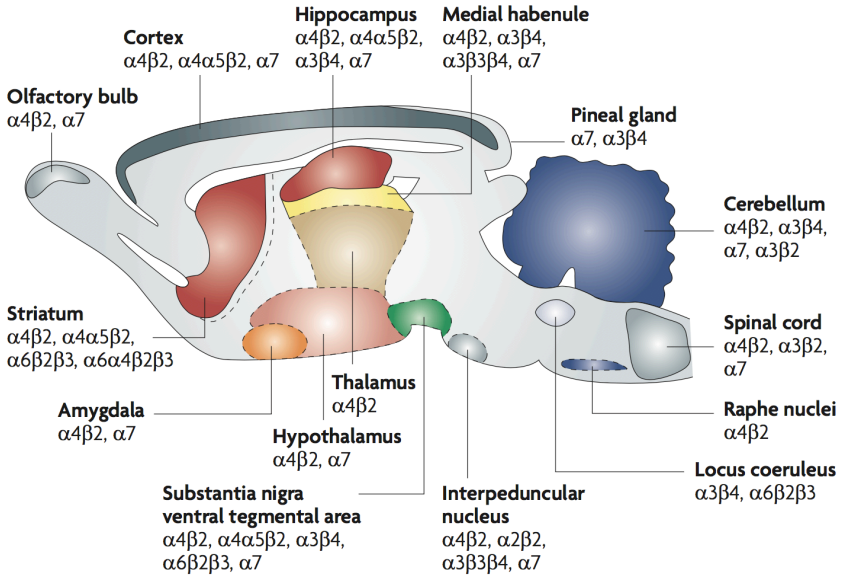


Nicotiana tabacum

Nicotine

- Produced by the **tobacco plant** to prevent consumption by herbivores
- **Responsible for smoking addiction** - by releasing dopamine
- **Potentially lethal to humans** (LD_{50} 0.5 – 1.0 mg/kg)
- **Improves cognition, alertness, memory**





nAChRs - Nicotine acetylcholine receptors

ligand-gated ion channel superfamily of neurotransmitter receptors

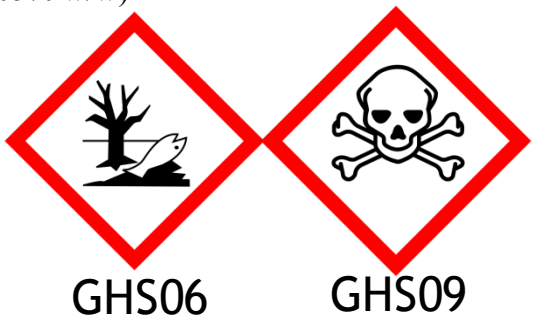
Nicotine is a lead compound used for developing drugs for cognitive dysfunctions

Levin ED and Rezvani AH. Nicotinic treatment for cognitive dysfunction. Curr DrugTargets CNS Neurol Disord. 2002;1(4):423-31.
Taly et. all. Nicotinic receptors: allosteric transitions and therapeutic targets in the nervous system Nat. Rev. Drug Discov.2009, 8, 733-750.

Nicotine and nicotine-containing waste

Tobacco industry produces 300 000 tons of non-recyclable nicotine-containing waste, 18 g nicotine/per kg

- According to EPA the waste is **Toxic Release Inventory (TRI)** chemicals
- According to the European Union Regulations (EUR) - **"toxic and hazardous"**(when nicotine content is over 0.05% w/w)



GHS06 GHS09

This waste is simply discarded in the environment

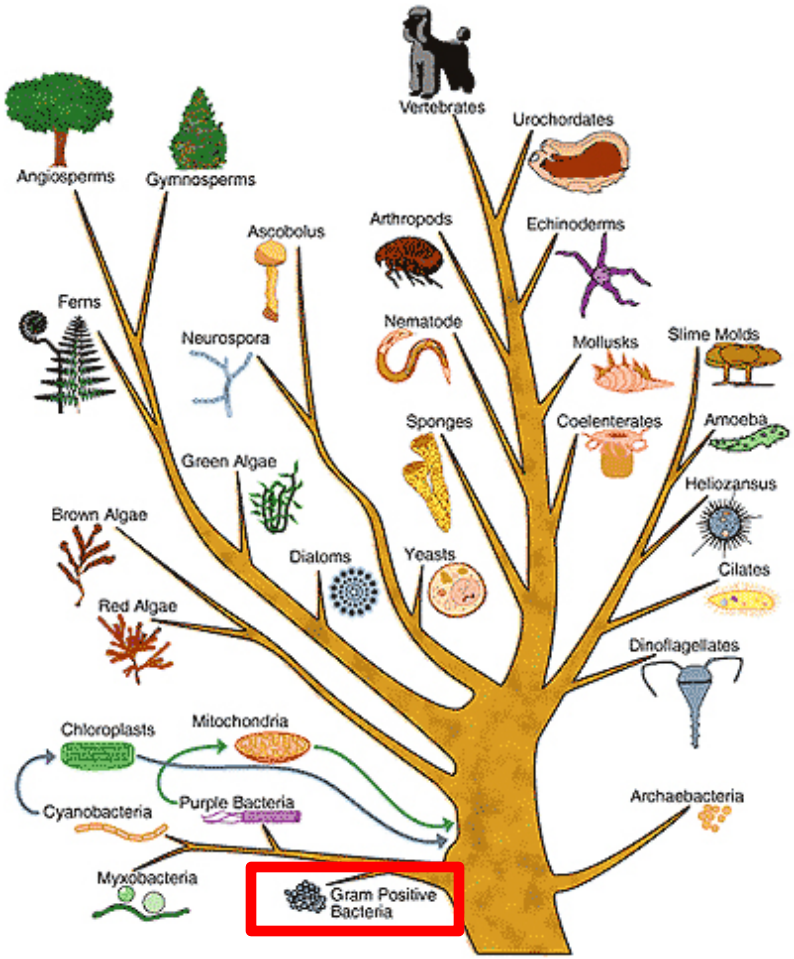
Country or region	Production in thousands of tons
World	7176
China	2995
Brazil	862
India	720
United States	397
European Union	262

According to FAOSTAT data, 2014

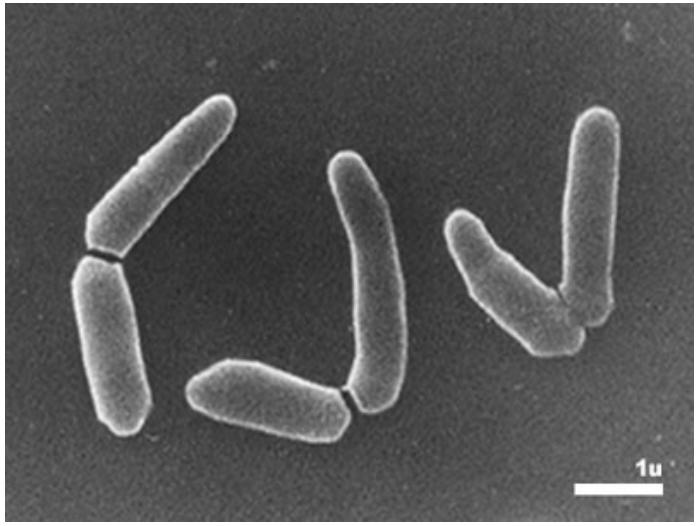
Why not using this waste to produce nicotine-based chemicals and drugs?

Novotny TE et al. The environmental and health impacts of tobacco agriculture, cigarette manufacture and consumption. Bull World Health Organ.. 2015;93(12):877-880.

What is *Arthrobacter nicotinovorans*?



Microorganism isolated from tobacco cultivated soil



Arthrobacter Photo by: T. Tamura, T. Nishii & K. Hatano

Arthrobacter ***nicotinovorans*** can grow on media containing up to **6 g/L** nicotine

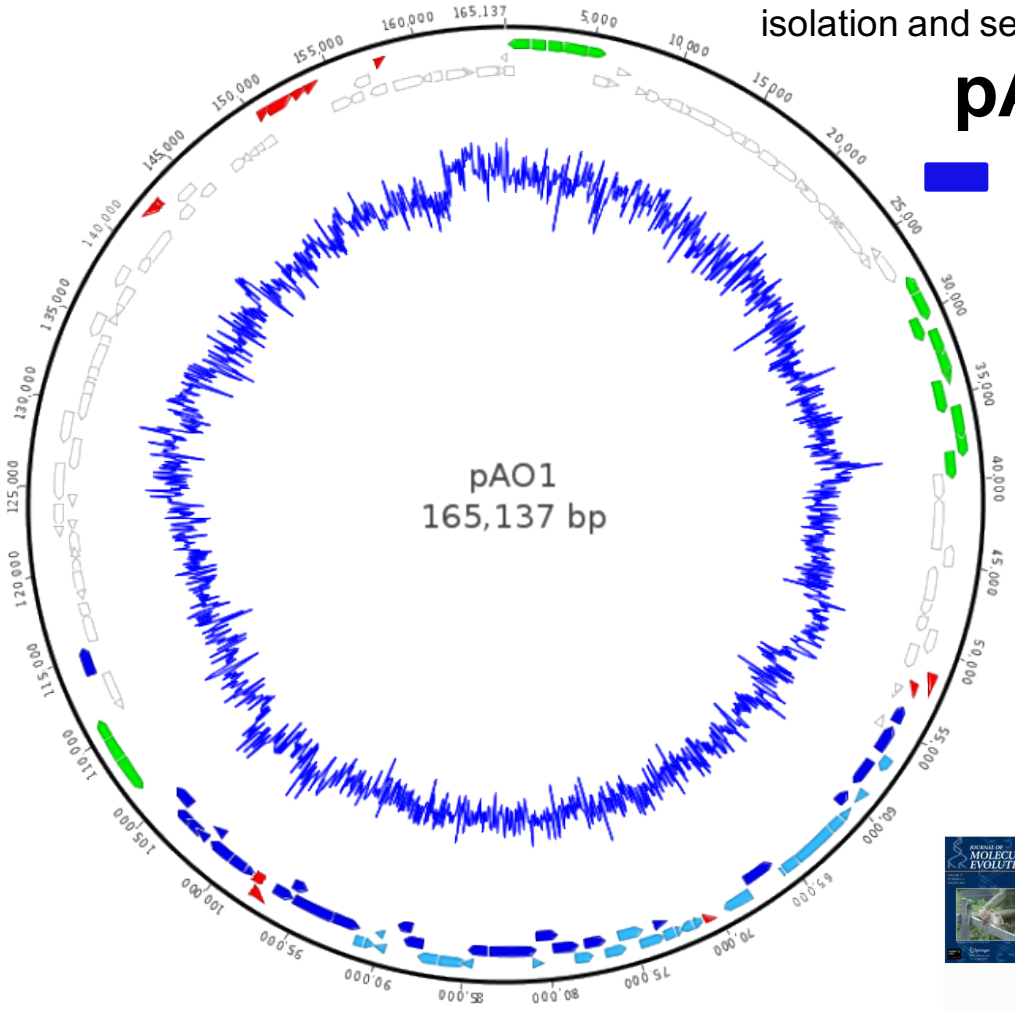
Dana Krempels, Introduction to Evolution, http://www.bio.miami.edu/dana/107/107F10_5print.html

Arthrobacter nicotinovorans and nicotine degradation

Brandsch group, Albert Ludwigs University of Freiburg, Germany

isolation and sequencing of the **p**lasmid **A**rthrobacter **O**xydans 1 –

pAO1



- **Nicotine metabolism**
- **Carbohydrate catabolism**
- Transposons and insertion elements
- Plasmid replication, partition and conjugation;

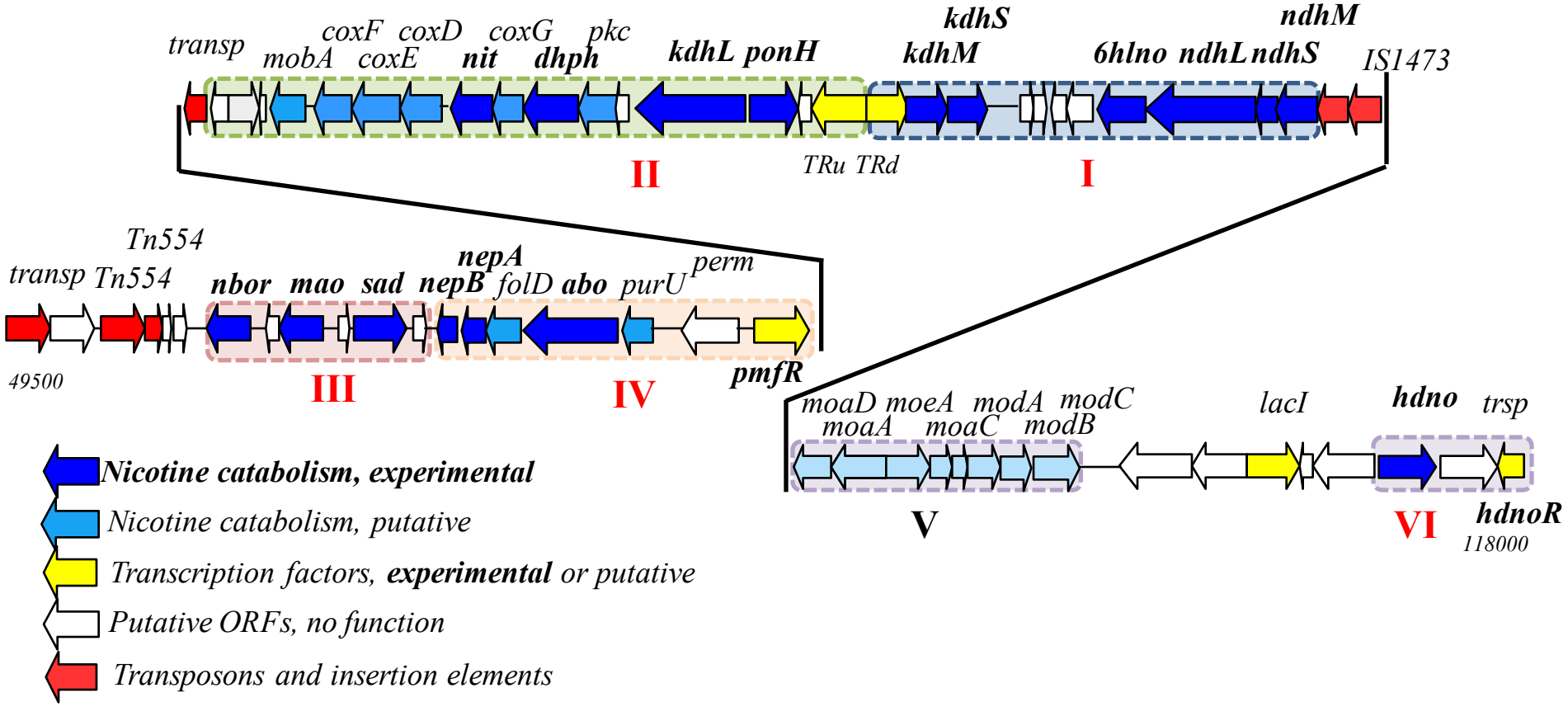
Journal of Molecular Evolution
August 2013, Volume 77, Issue 1, pp 22–30

pAO1 of *Arthrobacter nicotinovorans* and the Spread of Catabolic Traits by Horizontal Gene Transfer in Gram-Positive Soil Bacteria

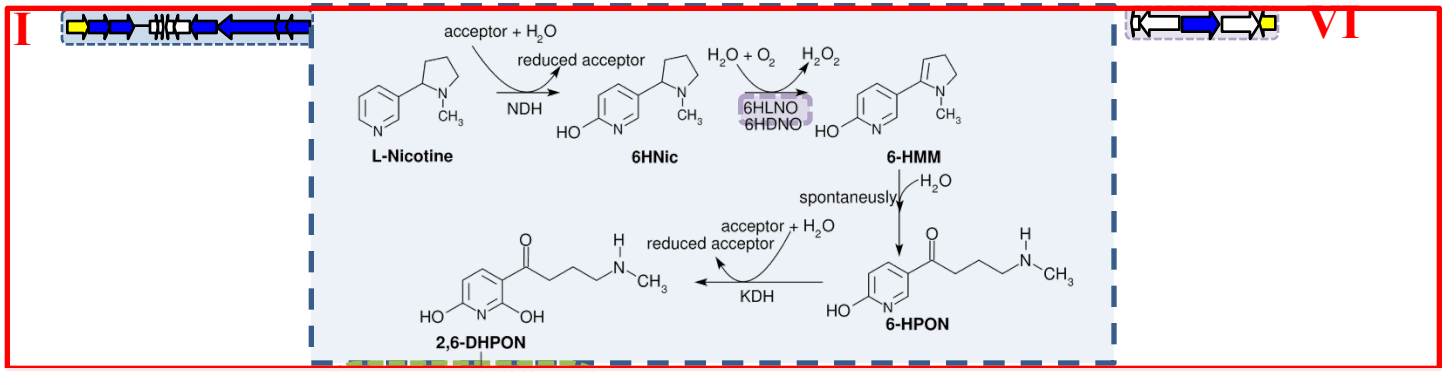
Authors Authors and affiliations

Marius Mihasan, Roderich Brandsch

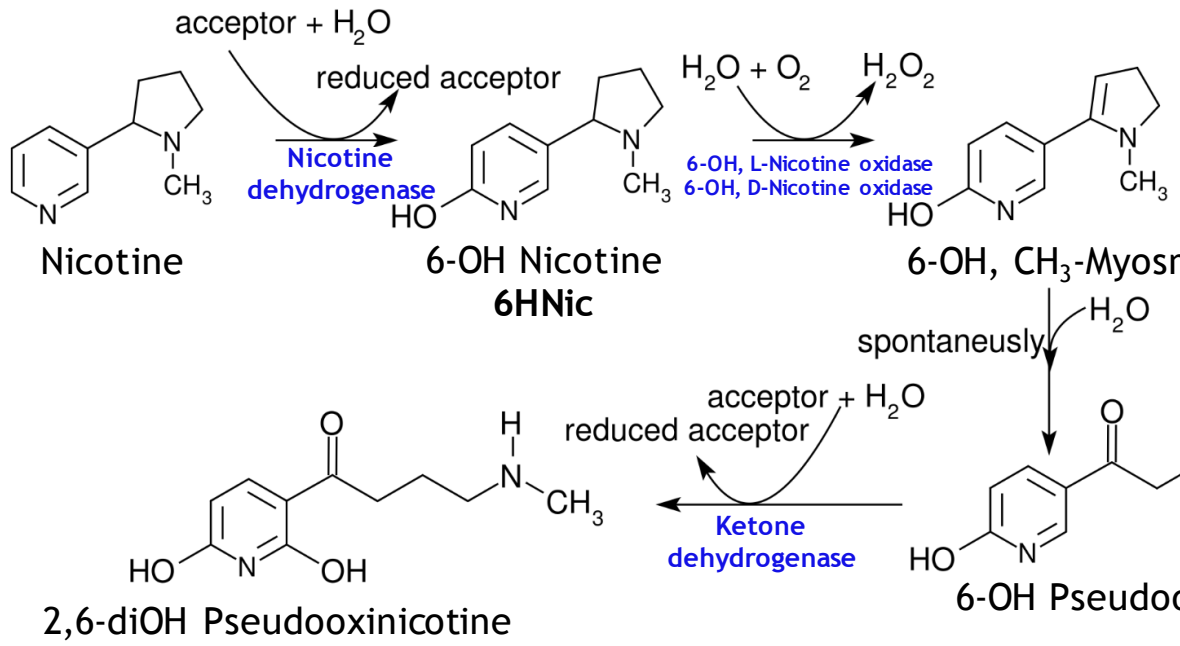
The *nic* gene cluster on pAO1

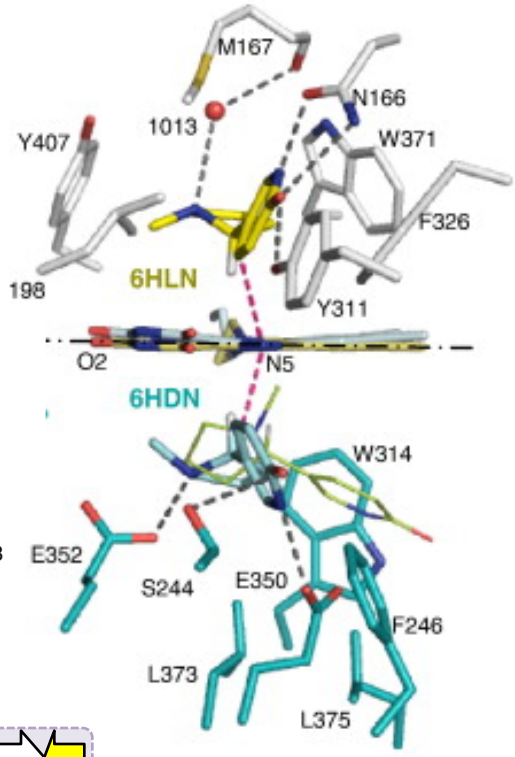


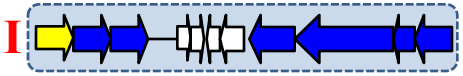
The nicotine break-down pathway in *A. nicotinovorans*



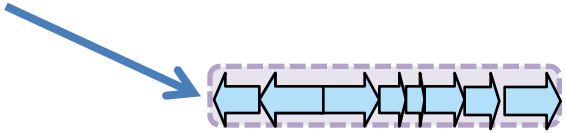
Upper nicotine pathway in *A. nicotinovorans*

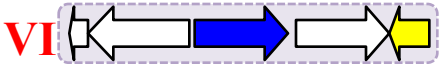






NDH - nicotine dehydrogenase
KDH - ketone dehydrogenase
heterotrimeric $(\alpha\beta\gamma)_2$ enzymes
FAD, MCD and FeS clusters

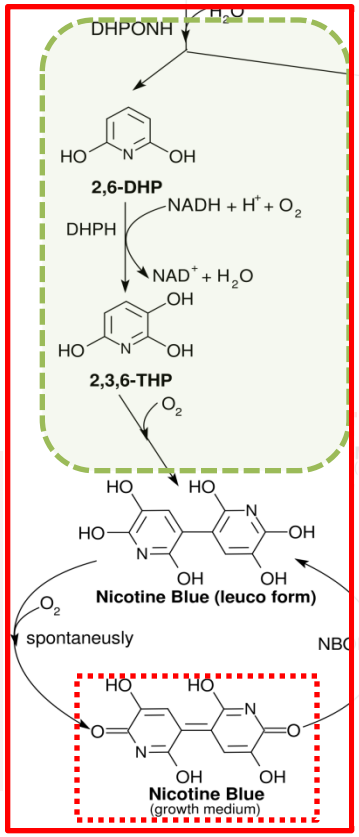




6-OH **L**-Nicotine oxidase
and
6-OH **D**-Nicotine oxidase
absolute stereospecificity,
genetically and structurally
unrelated flavoenzymes

Kachalova et al., 2010, J Molec Biol., 396(3):785-799

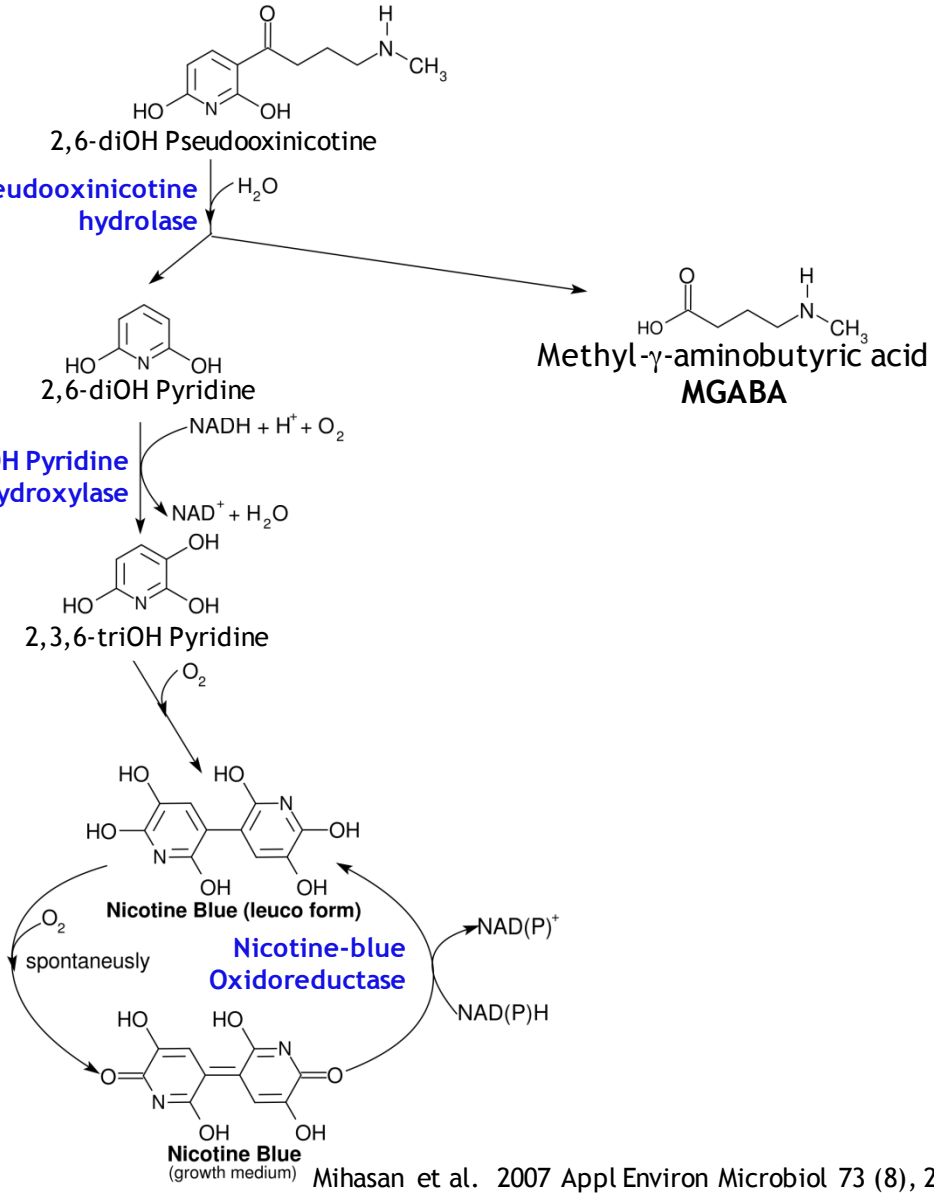
The nicotine break-down pathway in *A. nicotinovorans*



Pyridine ring metabolism in *A. nicotinovorans*

II 

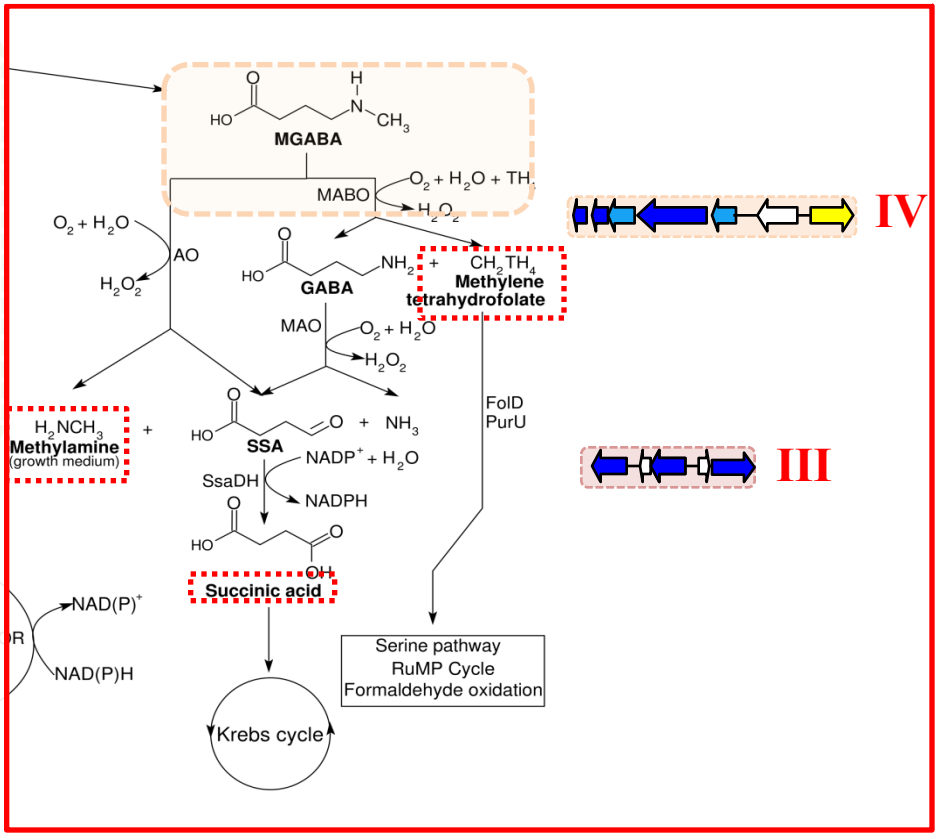
An α/ß fold C-C bond hydrolase
no sequence similarity to known bacterial *meta*-cleavage c-c bond hydrolases



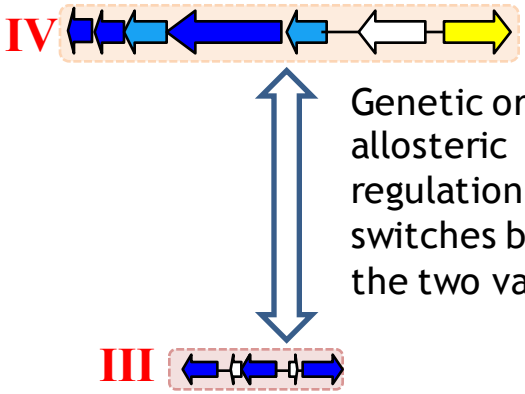
Schleberger et al., 2006, J Molec Biol., 367(2):409-418

Mihasan et al. 2007 Appl Environ Microbiol 73 (8), 2479-2485.

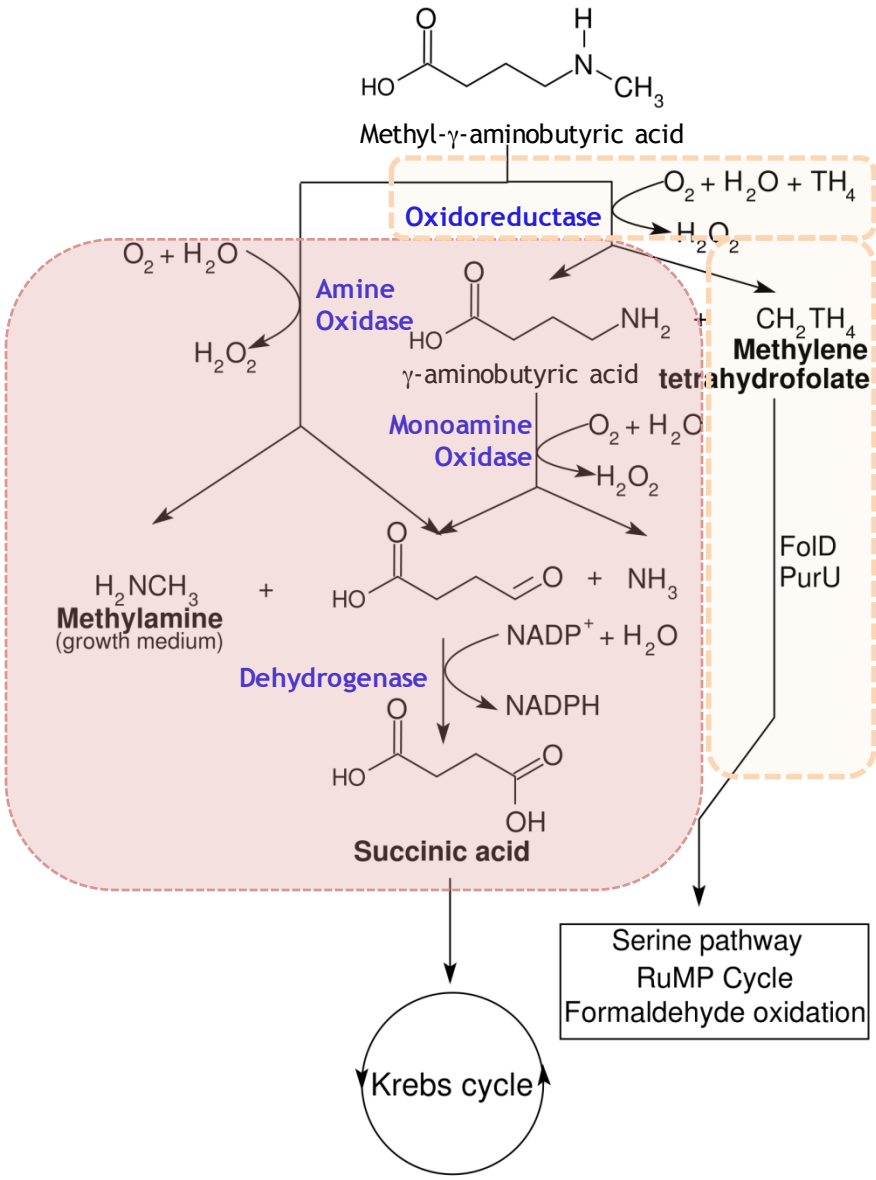
The nicotine break-down pathway in *A. nicotinovorans*



Side chain metabolism in *A. nicotinovorans*



Genetic or allosteric regulation probably switches between the two variants



The FEBS Journal

Final steps in the catabolism of nicotine

Deamination versus demethylation of γ-*N*-methylaminobutyrate

Calin-Bogdan Chiribau, Marius Mihasan, Petra Ganas, Gabor L. Igloi, Vlad Artenie, Roderich Brandsch

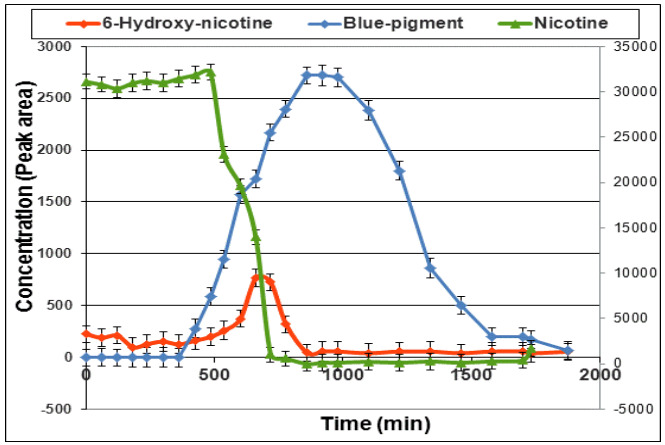
First published: 16 March 2006 Full publication history
DOI: 10.1111/j.1742-4658.2006.05173.x View/save citation
Cited by: 10 articles Citation tools

View issue TOC
Volume 273, Issue 7
April 2006
Pages 1528–1536

Blank spots in nicotine metabolic pathway *in A. nicotinovorans*

1. Is Nicotine-Blue (NB) the final product of the pathway?

A. Nicotine-blue is consumed in old cultures



A pyridine ring cleaving enzyme must exist

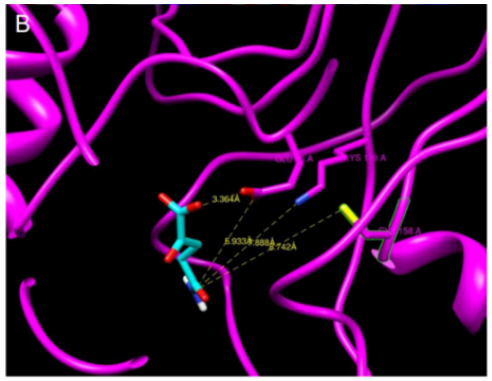
Unknown enzyme?

Chromosomal gene?

Razavn B., B.Sc Doina G. B.Sc

Andreea A., M.Sc

B. A ω-amidase for α-ketoglutaramate is part of the *nic*-gene cluster



Flexible targeted docking

Cobzaru, C., et al. (2011) *Research in Microbiology*, *162*(3), 285-91.

Petra G.

Blank spots in nicotine metabolic pathway of *A. nicotinovorans*

2. How is nicotine transported across the cell membrane?

L-nicotine uptake in *A. nicotinovorans*:
- **Dependent** on nicotine degradation but **not on pAO1**
- **Energy-independent**
- **Saturable** with a K(m) of 6.2 microM and a V(max) of 0.70 micromol/ min/mg protein)

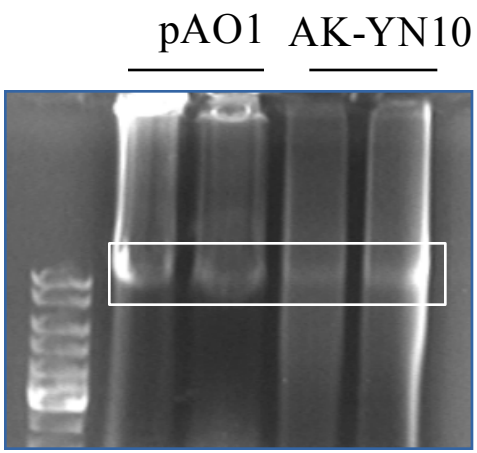
Ganas & Brandsch, 2009, *Microbiology*, *155*: 1866-77

=>

facilitated diffusion
driven by the nicotine gradient due to its intracellular degradation
by a unknown
chromosomal permease

3. Several other bacteria have the *nic* gene cluster, but not a functional nicotine catabolism

Victor B., M.Sc. France
AK-YN10 contains a plasmid



Razvan B, M.Sc

99% sequence identity between key *nic*-genes in *A. nicotinovorans* and AK-YN10.
Same nic-genes but no nicotine metabolism

Some chromosomal genes must be directly/indirectly involved in nic catabolism

Potential applications of the nic-pathway

1. Decontamination of nicotine-containing waste, soil and water from tobacco industry

2. Engineering the *nic*-pathway for the production of value-added chemicals

Sci Rep. 2015; 5: 16411.
Published online 2015 Nov 17. doi: 10.1038/srep16411
PMCID: PMC4647180

Sustainable production of valuable compound 3-succinoyl-pyridine by genetically engineering *Pseudomonas putida* using the tobacco waste

Weiwei Wang,[1] Ping Xu,[1] and Hongzhi Tang[a,1,*]

6-hydroxy-L-nicotine – a neuroactive agent?

Nicotine improves memory by binding to **nAChRs subtypes α4β2**

Murray and Abeles, *Aging & Mental Health*. 2002, 6, 129–138.

Coulc

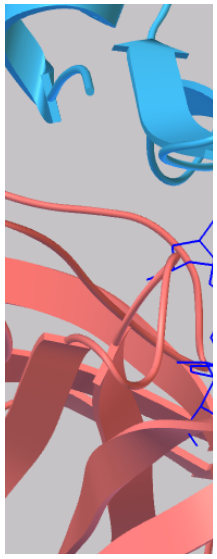
Arthr

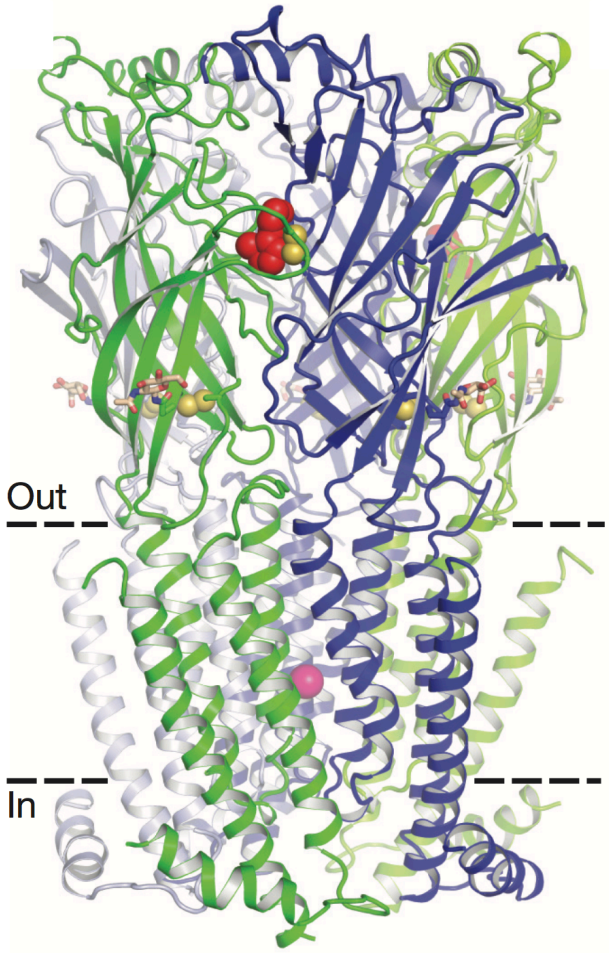
***In silico* - T**

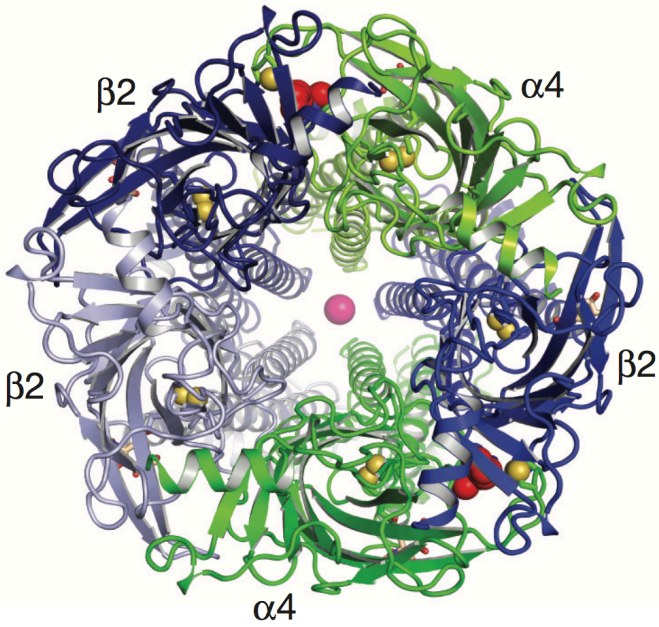
– *rigid receptor* su

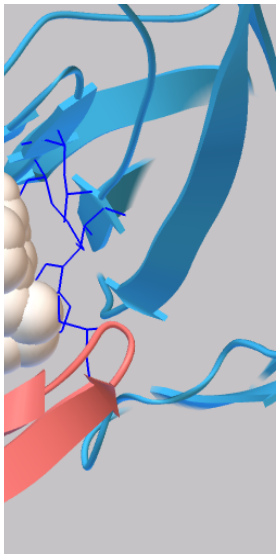
- *targeted region* -

- *flexibile ligands*









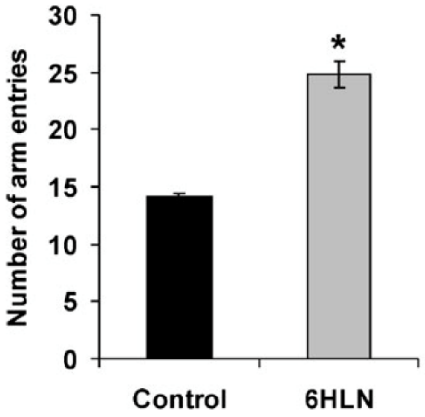
Morales-Perez C et al., *Nature*, 2016, 538 (7625): 411–415

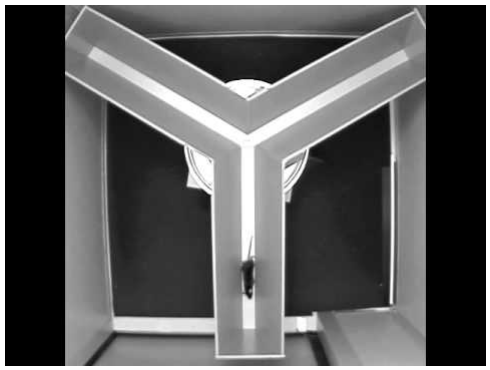
L-N [illegible] -Nicotine

6-hydroxy-L-nicotine – a neuroactive agent?

Animals tests

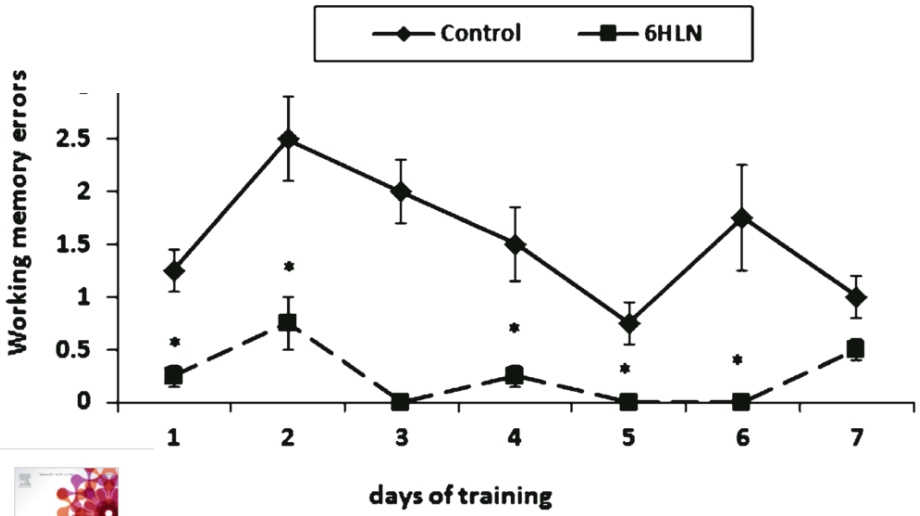
- male Wistar rats (3-4 months old)
- 6-hidroxy-L-nicotine was injected intraperitonealy, 0.3 mg/kg b.w, daily, for 7 consecutive days.





Y-maze task Values are mean ± S.E.M. (n=10 animals per group), *p<0.0001 vs. control group

Radial arm-maze task Values are mean ± S.E.M. (n=10 animals per group), *p<0.0001 vs. control group

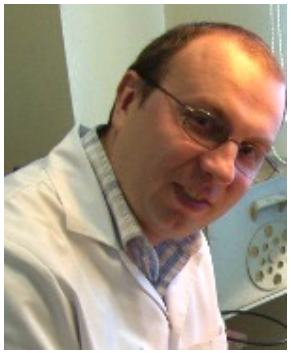


ELSEVIER
Biomedicine & Pharmacotherapy
Volume 86, February 2017, Pages 102–108

Original article

Nicotine versus 6-hydroxy-L-nicotine against chlorisondamine induced memory impairment and oxidative stress in the rat hippocampus

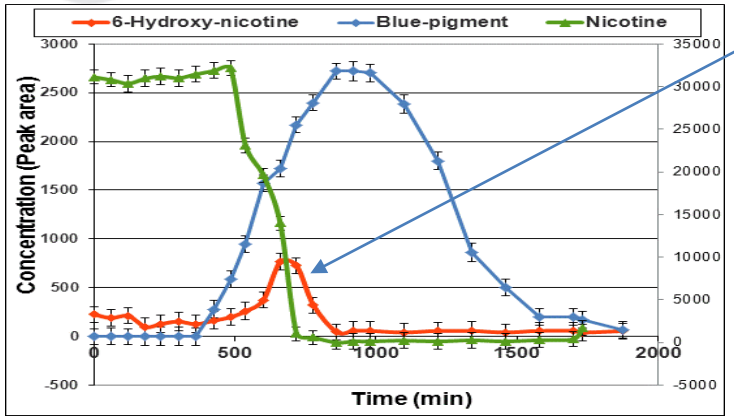
Lucian Hritcu, Radu Ionita, Diana Elena Motei, Cornelia Babii, Marius Stefan, Marius Mihasan



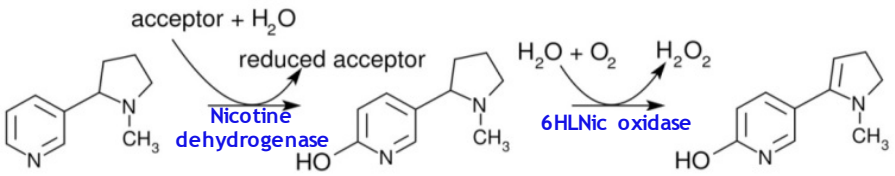
Prof. Hritcu L., PhD

Radu I., PhD student

An *A. nicotinovorans* based biotechnology for the production of 6HLNic



6-HNic accumulates in the medium for a short period of time



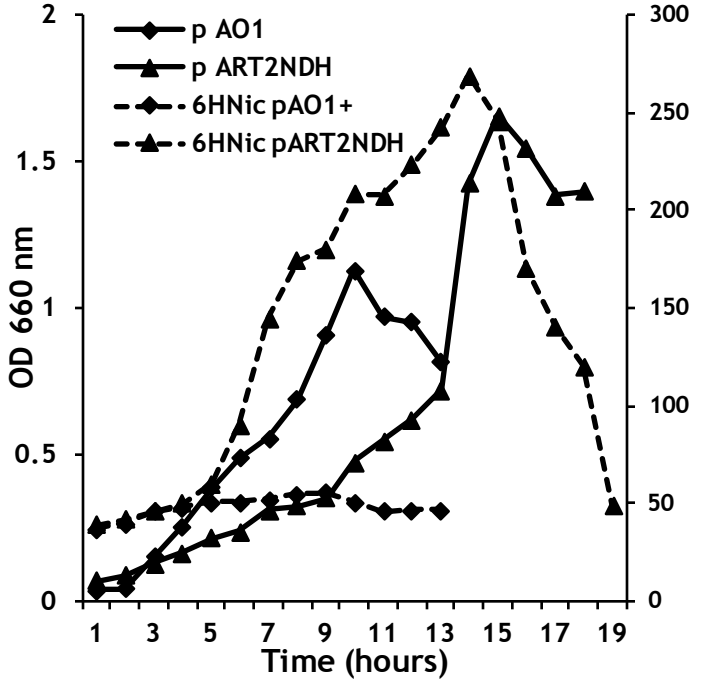
L-Nicotine — 6-OH L-Nicotine **6HLNic** — 6-OH, CH_3-Myosmine

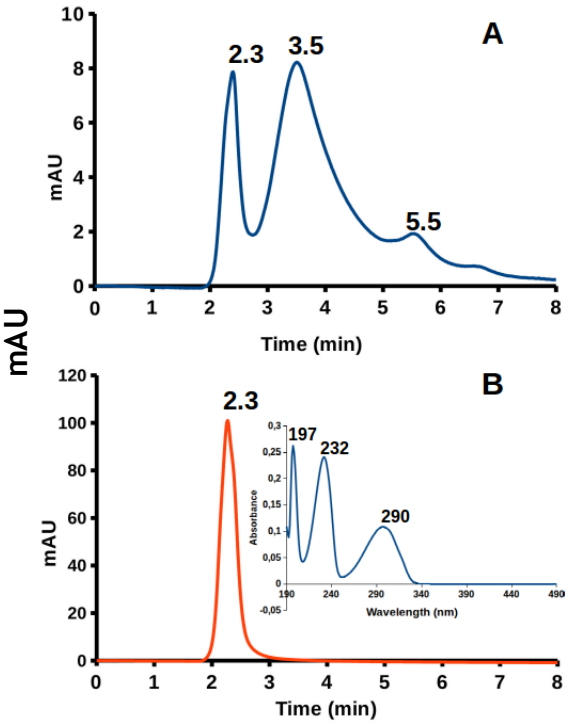
Julie R., M.Sc, France

Prof. Stefan M., PhD

Cornelia B., PhD student

Diana M., B.Sc.

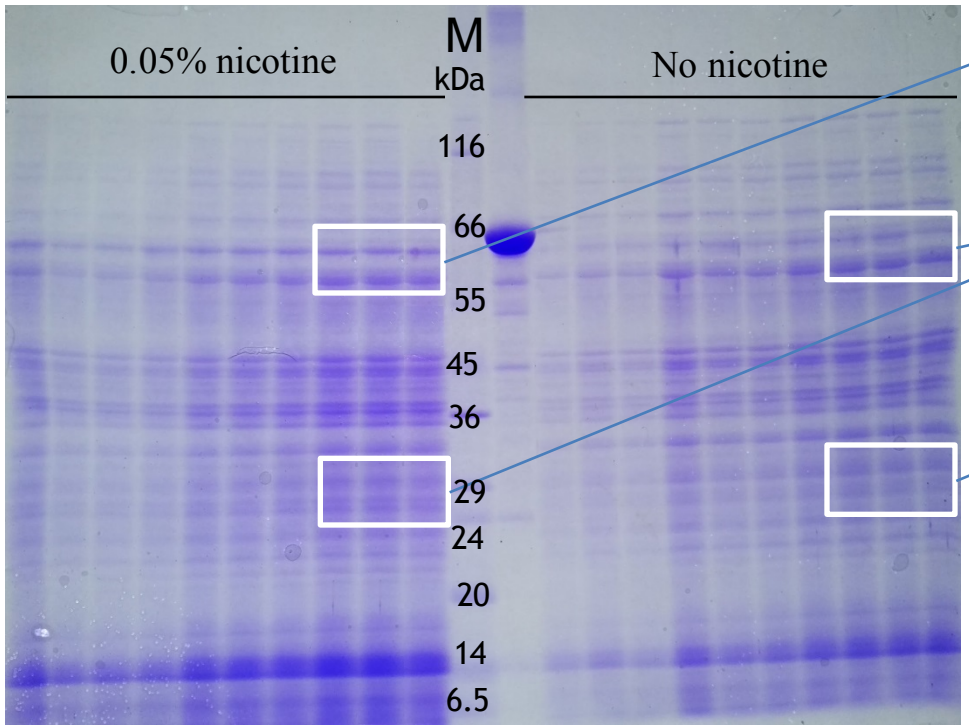


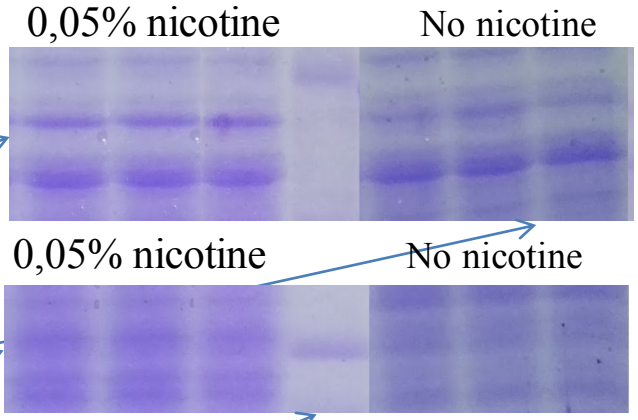


Mihalache et al. (2016) Steps towards an *A. nicotinovorans* based biotechnology for production of 6-hidroxy-nicotine." FEBS Journal 283: 174.

Proteome – the entire set of proteins expressed by Arthrobacter nicotinovorans

Arthrobacter nicotinovorans cell lysates





Several gels containing lysates at **4** different time points and on **2** growth media

Identify the entire set of proteins expressed by *Arthrobacter nicotinovorans* = **the proteome**

1. Identify all **nicotine-induced proteins** encoded by pAO1 genes

2. Focus on **transcription factors and transporters** related to nicotine catabolism (plasmidial or chromosomal)

3. Identify some candidates for the **pyridine ring cleaving enzyme**

Summary

Tobacco industry produces large amounts of toxic and hazardous nicotine-containing waste

Arthrobacter nicotinovorans **can break-down nicotine from the waste due to enzymes encoded by the pAO1 megaplasmid**

The major steps in the pAO1 nicotine metabolic pathway are known, but some key players such as pyridine ring cleaving enzyme, major regulators or nicotine transporters are still missing

The pAO1 pathway could be used to transform nicotine-containing waste in useful chemicals such as 6-hidroxy-L-nicotine

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-6HNic testing on lab rats
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Questions?