Multi enzyme systems involved in astin biosynthesis and their use in heterologous astin production

Project acronym: MESIAB
Project no: EIB.10.004
Project members

Dresden, D
Prof. Karl-Heinz van Pée
Dipl.-Biol. Liane Flor
Prof. Jutta Ludwig-Müller
Dipl.-Biol. Linda Jahn

Wageningen, NL
Prof. Willem van Berkel
M. Sc. Mieke Huijbers

Helsinki, Fl
Prof. Kaarina Sivonen
Dr. David Fewer

Tübingen, D
Prof. Wolfgang Wohlleben
Dr. Tilmann Weber
Dipl.-Biol. Thomas Schafhauser

Lille, F
Prof. Philippe Jacques
M. Sc. Thibault Caradec

Saint Beauzire, F
Dr. Jean-Yves Berthon

Fig. 1: http://www.digitale-europakarte.de/europakarte.png
Project aim

Enhancing the production of astins using molecular genetic tools and screening for new biological activities and novel applications of astins

General project approach

Detection, cloning and heterologous expression of the individual genes involved in astin biosynthesis

Combining the genes into a cluster, expression of the cluster in Streptomyces, yeast and hairy root cultures

Screening for new biological activities and novel applications for astins using cell cultures and transcriptomic technologies

Chemical structure of astin C
Detection of astins in *Aster tataricus*

LC-MS detection methods were established for astins

- We have detected astins from dried root samples of *Aster tataricus*
- These are mainly dichlorinated, but some do not have the same mass as published astins
Cyclochlorotarine from *Penicillium islandicum*

**Astin C**

- β-Amino phenylalanine
- Amino-butyric acid
- Dichloroproline

**Cyclochlorotarine**

- β-Amino phenylalanine
- Dichloroproline
Detection of astin/cyclochlorotrine biosynthetic genes

- Both biosynthetic pathways can be assumed to require highly similar enzymes.

- Working with *P. islandicum* should be faster and easier.

- Thus, it was decided to sequence the *P. islandicum* genome and use the detected genes for searching for the corresponding genes in *A. tataricus*. 
Candidate genes are present, but not clustered
Genome analysis

Macrocyclisation of NRPS

- Bacteria
  - Te
  - Literature data show that a terminal condensation (CT) domain is required for macrocyclisation of cyclic nonribosomal peptides in fungi
  - Du and Lou, 2009, Nat Prod Rep

- Fungi
  - CT
  - Gao et al., 2012, Nature ChemBiol

Specific signature

Present, ending the NRPS?

Cyclochlorotine

This information means that the NRPS involved in cyclochlorotine production will most likely contain a CT domain
Genetic engineering of *P. islandicum*

1. **Protoplasting successful**
   - mycelium
   - protoplasts
   - cellwall degrading enzymes

2. **Protoplast-transformation successful**
   - benomyl containing agar
   - test PCR
   - protos+H₂O protos+ben⁹-plasmid

3. **NHEJ deficient strain established**
   - non-homologous end joining (NHEJ)
   - ku70 mRNA → silencing
   - ku70 rev
   - Ku70 for
   - pST
   - dsRNA
   - Ku70 gene activity (qRT-PCR)
   - WT
   - clone 1
   - clone 2
   - clone 3
   - clone 4
   - -90%
Prolyl dehydrogenase (PRODH)

L-Pro + ATP → PPi + AMP

FAD + proline adenyltransferase → acyl-CoA dehydrogenase

FADH₂-dependent halogenase → H₂O + FAD
Prolyl dehydrogenase (PRODH)

Thermophilic proline dehydrogenase

- PRODH from *Thermus thermophilus*
- High overproduction of MBP-tagged flavoenzyme
- Active as fusion and after removal of MBP tag
- Enzyme fully stable at 80 °C
Prolyl dehydrogenase (PRODH)

Fungal proline dehydrogenase

- 3 Putative PRODH genes in *Penicillium islandicum*
- One PRODH produced in soluble form
- One-step purification of MBP-tagged flavoenzyme
- Active as fusion and after removal of MBP tag

First fungal prolyl dehydrogenase
Flavin-dependent halogenases

Pyr29_Streptomyces_vitaminophilus

ChlB4_Streptomyces_antibioticus

HrmQ_Streptomyces_griseoflavus_W-384

PcpE_Actinoplanes_sp._ATCC_33002

SyrB2_Pseudomonas_syringae

PyrM_Pseudomonas_fluorescens_Pf-5

PyrM_Pseudomonas_aeruginosa_M18

Putative halogenases from fungi

Pyr16_Streptomyces_vitaminophilus

HalA_PcpI_Actinoplanes_sp._ATCC_33002

HalB_PcpK_Actinoplanes_sp._ATCC_33002

KtzR_Kutzneria_sp._744

PyrH_Streptomyces_rugosporus_LL-42D005

Sth_Streptomyces_toxytricini_strain_NRRL_15443

ThiH_Streptomyces_albogriseolus_MJ286-76/F7

RebH_Lechevalieria_aerocolonigenes

KtzQ_Kutzneria_sp._744

PmA_Pseudomonas_fluorescens

XP_002537682_Ricinus_communis

Tryptophan halogenases

RadH_Aspergillus_niger_CBS_513.88

Halogenase_Penicillium_islandicum_WF-38-12

Gsfl_Penicillium_aethiopicum

RadH_Aspergillus_oryzaeRIB40

XP_002151004_Penicillium_marneffei_ATCC_18224
Halogenase from *P. islandicum*

Purification of the halogenase

50 µM IPTG, over night at 20 °C
GST fusion protein: 83.6 kDa

Halogenase can be purified using a maltose-binding protein tag.
Demonstration of halogenase activity

Activity assay using a chemically synthesised substrate mimic has not been successful, yet.

Complementation of pyoluteorin biosynthesis in *Pseudomonas fluorescens*.

The pyrrole halogenase gene *pltA* is inactivated and will be substituted by the halogenase gene *cycH* from the cyclochlorotrine producer *P. islandicum* to show halogenating activity *in vivo* by complementing pyoluteorin biosynthesis.

![Chemical structures](image-url)
Localisation of astins in *A. tataricus*

Presence of astins depends on plant origin

Astin composition and concentration

- astin A/B
- astin C
- astin F
- astin H/E
- astin G
Astins, plant or fungal metabolites?

Cyclochlorotines were identified from strains of *P. islandicum* isolated from soil or spoiled food; none of them produces astins.

Cyclochlorotines were identified from strains of *P. islandicum* isolated from soil or spoiled food; none of them produces astins.
Astins, plant or fungal metabolites?

**A. tataricus** (Austria) roots

- Wash
- Surface sterilize

Fungal endophytes

Ribosomal DNA

HPLC-MS analysis of extracts

Astins could not be detected in any of the fungi isolated from *A. tataricus* roots.
Is there astin production in sterile plants?

Cultivation of sterile seeds on MS medium without any hormones

Cultivation of sterilised inflorescence tissue on MS medium (naphthyl acetic acid or benzyl amino purine) to induce regeneration of small, sterile plants

These plants turned out to be not really sterile!
Generation of sterile \textit{in vitro} cultures of \textit{Aster tataricus} (Austrian) via hormone treatment (auxin and cytokinin for inducing shoot and roots)

After a few months, growth of a pink fungus could be observed. This fungus could not be removed by treatment with antimycotica like amphotericin.

\textbf{Isolation of a novel fungus}
Astin C is the main compound. Astins produced by the new fungus are identical to those isolated from dried A. tataricus roots.
An endophyte which produces astins

Astins were identified in a fungal endophyte isolated from A. tataricus which is not closely related to P. islandicum.
# Genome sequencing of endophyte

**Astin producing endophyte**

<table>
<thead>
<tr>
<th>Genome Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>28 MB</td>
</tr>
<tr>
<td>GC Content</td>
<td>53 %</td>
</tr>
<tr>
<td>Coverage</td>
<td>57 fold</td>
</tr>
<tr>
<td>Scaffolds</td>
<td>395</td>
</tr>
</tbody>
</table>

**Penicillium islandicum**

<table>
<thead>
<tr>
<th>Genome Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>34 MB</td>
</tr>
<tr>
<td>GC Content</td>
<td>45.22 %</td>
</tr>
<tr>
<td>Coverage</td>
<td>41 fold</td>
</tr>
<tr>
<td>Scaffolds</td>
<td>330</td>
</tr>
</tbody>
</table>

## Predicted Secondary Metabolite Gene Clusters (by antiSMASH)

<table>
<thead>
<tr>
<th>Type</th>
<th>Astin producing endophyte</th>
<th>Penicillium islandicum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKS</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td>NRPS</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>(1/5 is 5-modular)</td>
<td></td>
<td>(2/17 are 5-modular)</td>
</tr>
<tr>
<td>NRPS-PKS</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Terpene</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Other</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td><strong>Sum</strong></td>
<td><strong>33</strong></td>
<td><strong>60</strong></td>
</tr>
</tbody>
</table>
Partial astin gene cluster
Comparison of gene clusters

There is a gene cluster present in the new fungus with high similarity to two gene clusters in the cyclochlorototine producer *P. islandicum*. 

**Genes with high similarity**
Summary

Modified proposal
- Cyclochlorotines
  - *P. islandicum*
- Genome sequencing
- Identification of NRPS gene cluster
- Cloning of individual genes
- Enzymatic activity and gene inactivation

Proposal
- Astins
  - *A. tataricus*
- Identification of individual biosynthetic genes
- Expression and enzymatic activity
- Construction of gene cluster
- Heterologous expression
- Production of astins
- Bioactivities

What we achieved
- Astins
- Isolation of a novel fungus producing astins
- Draft genome sequence
- Astins can now be produced by fermentation

What we achieved
- Astins