Genomic Wake-Up Call
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CHAPTER 6

Gene Deletion Analysis of Transporters of Siderophores in *Penicillium chrysogenum*

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Abstract

Siderophore transporters mediate iron acquisition and play an important role in iron homeostasis. In the filamentous fungus Penicillium chrysogenum, the genes encoding PsmA and PsmB-members of the ATP-binding cassette proteins (ABC), and PstA and PstB-a siderophore-iron transporters (sit) subfamily of the major facilitator superfamily (MFS) are present in siderophore biosynthetic genes clusters, and the respective genes are highly expressed when cells are grown under iron depletion conditions. Based on functional analysis in other filamentous fungi, PstA and PstB are likely responsible for siderophore uptake. Our data indicate that the ABC transporter PsmB participates in the excretion of fusarinine A and novel putative siderophores fusarinine M and fusarinine E, while PsmA only affects fusarinine E production. Interestingly, cultures of ΔpsmA mutant were consistently blue suggesting the presence of a Cu(II) complex in the medium likely because of a deficiency in uptake.
6.1 Introduction

Siderophores play a key role in the acquisition of metal ions, in particular iron. Siderophores transport was first studied in Gram-negative bacteria (Schalk [2008]). A TonB–ExbB–ExbD complex of inner and outer membrane protein bridges the periplasm, is responsible for the uptake of the ferri form siderophore into the periplasm, through a process driven by the proton motive force. Next the Fe-siderophore complex is actively transported into the cytoplasm by the ABC-binding cassette protein (Braun and Hantke [2011]). ABC transporters play a quintessential role in transport of the molecule across membranes in a thermodynamically unfavorable direction by using the energy from ATP hydrolysis (Rees et al. [2009]). Excretion of siderophores involves efflux pumps, which most likely belong to one of the following groups: a) the major facilitator superfamily (MFS), b) the resistance, nodulelation, and cell division (RND) superfamily, or c) the ATP-binding cassette (ABC) superfamily (Miethke and Marahiel [2007]). Most fungal species employ two general ways of iron acquisition: (i) extracellular reduction of ferric iron to ferrous iron or (ii) the direct uptake of Fe$^{3+}$-siderophores complexes (Kosman [2003]). In recent years, iron transport and homeostasis has become an area of interest in many Aspergilli species after it was extensively studied in Saccharomyces cerevisiae, scavenger for xenosiderophores, i.e., siderophores produced by other genera. Those findings presented a range of transporters, which are members of Major Facilitator Superfamily that are involved in the uptake of the ferri form of siderophores (Nelissen et al. [1997]). Here we have analyzed the role of a set of putative transporters of siderophores in iron acquisition by *P. chrysogenum*.

6.2 Materials

*Penicillium chrysogenum* strains were prepared according to Kovalchuk et al. (Kovalchuk et al. [2012]) using DS4465 strain as a host (Table 6.1). Primers used for plasmids construction are listed in Table 6.2. The correct genomic integration was verified by PCR using oligonucleotides listed in Table 6.3.

Table 6.1: Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS54465</td>
<td>Δku70</td>
<td>(Snoek et al. [2009])</td>
</tr>
<tr>
<td>ΔpsmA</td>
<td>Δku70, ΔPc16g03870::amdS</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpstA</td>
<td>Δku70, ΔPc16g03910::amdS</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpstB</td>
<td>Δku70, ΔPc22g20360::amdS</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpsmB</td>
<td>Δku70, ΔPc22g20390::Phleo</td>
<td>This study</td>
</tr>
</tbody>
</table>
**Table 6.2: Oligos used in plasmids constructs.**

<table>
<thead>
<tr>
<th>gene number</th>
<th>Oligos</th>
<th>Forward (5' → 3')</th>
<th>Reverse (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pc16g03870</td>
<td>5'-pDONR psmA</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
</tr>
<tr>
<td></td>
<td>3'-pDONR psmA</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGAAGCC CAGAACCCTCAG</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGAAGCC CAGAACCCTCAG</td>
</tr>
<tr>
<td>Pc16g03910</td>
<td>5'-pDONR 15 (5'FP-3'RP)</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
</tr>
<tr>
<td></td>
<td>3'-pDONR 16 (3'FP-3'RP)</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGAAGCC CAGAACCCTCAG</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGAAGCC CAGAACCCTCAG</td>
</tr>
<tr>
<td>Pc22g20360</td>
<td>5'-pDONR 17 (5'FP-3'RP)</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
</tr>
<tr>
<td></td>
<td>3'-pDONR 3' (3'FP-3'RP)</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGAAGCC CAGAACCCTCAG</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGAAGCC CAGAACCCTCAG</td>
</tr>
<tr>
<td>Pc22g20390</td>
<td>5'-pDONR psmB</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
</tr>
<tr>
<td></td>
<td>3'-pDONR psmB</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
<td>GGGGACAACCTTTGATAGA AAAGTTCGCTACAAGACAGC CAGAACCCTCAG</td>
</tr>
</tbody>
</table>

**Table 6.3: Oligos used in PCR verification of correct marker integration.**

<table>
<thead>
<tr>
<th>gene number</th>
<th>Forward (5' → 3')</th>
<th>Reverse (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>amdS</td>
<td>GGAAGACTCAGTGAAGAGAGGA</td>
<td>CTCTACCTACTCGAGAGAG</td>
</tr>
<tr>
<td>phleomycin</td>
<td>TCGTCCGTCCAGGCCTCTTC</td>
<td>CGGGCGTAGTTGGCCACTGCT</td>
</tr>
<tr>
<td>Pc16g03870</td>
<td>GTTTGACGTCTCCTCTGAGTAC</td>
<td>CTTAGCACTCATTCTGAGT</td>
</tr>
<tr>
<td>Pc16g03910</td>
<td>GAGCCTTTCTCCTGCTGCTGACG</td>
<td>CGATCAGTCAGCTACGCTATTGAC</td>
</tr>
<tr>
<td>Pc22g20360</td>
<td>GGCAACTTCTCTGGCTGAGTAC</td>
<td>CTTAGCACTCATTCTGAGT</td>
</tr>
<tr>
<td>Pc22g20390</td>
<td>GGAACGCACTTCTTGCTGAGTAC</td>
<td>CTTAGCACTCATTCTGAGT</td>
</tr>
</tbody>
</table>

### 6.3 Experimental Procedures

The procedure of media preparation (Section 5.2.1), qPCR analysis (Section 5.2.4) and LC-MS(/MS) detection (Section 5.2.5) are described in chapter 5.
6.4 Results - Transporters of Extracellular Siderophores

A set of transporters was identified whose genes localize siderophores biosynthetic gene clusters in *P. chrysogenum* and these were targeted for gene inactivation (Table 6.1). This concerned the ABC transporter PsmA (Pc16g03870) and putative siderophore transporters PstA (Pc16g03910) that are clustered together with the pssA gene responsible for coprogen formation (Fig. 5.1A). Likewise, the genes encoding the ABC transporter PsmB (Pc22g20390) and the MFS permease PstB (Pc22g20360) localize in the *pssB* gene cluster (Fig. 5.1B) which is responsible for fusarinine A production (see chapter 5). The *psmA* and *psmB* genes were high up-regulated during iron deprivation growth conditions (Fig. 6.1). In a culture supernatant of the *psmB* deletion strain, fusarinine A and fusarinine M were no longer detected (Fig. 6.2). The putative novel siderophore fusarinine E was no longer detected in the culture broths of ∆*psmA* and ∆*psmB* (Fig. 6.2), while the ∆*pstA* and ∆*pstB* strains showed no particular loss of the potential siderophores despite being highly expressed under iron deficient condition (data not shown Fig. S5.3). Cultures of the ∆*psmA* strain (Table 6.1, Fig. 6.3) turned blue during cultivation under low iron conditions, which might be related to copper acquisition.

6.5 Discussion

In *P. chrysogenum* biosynthetic genes are organized in clusters that are often regulated by iron accessibility (Haas [2003]; Winterberg et al. [2010]). Apart from genes encoding for enzymes needed for biosynthesis, the clusters also includes genes encoding for transporters, i.e. ABC multidrug transporters (MDR) and transporters of the Major Facilitator Superfamily (MFS) (Fig. 5.1). These transporters could be involved in the uptake of the siderophore-metal ion complexes or needed for the secretion of the siderophores. Deletion of the transporter genes and subsequent metabolomics analysis of the culture broth analysis shed light on the function of these transporters in *P. chrysogenum*.

The siderophore-iron transporters (sit) subfamily of MFS have been extensively studied in *Neurospora crassa*, *Aspergillus nidulans* (Haas et al. [2003]), *Aspergillus fumigatus* (Raymond-Bouchard et al. [2012]) and *Cryptococcus neoformans* (Tangen et al. [2007]), and now in *P. chrysogenum* they are represented by PstA and PstB (this study). These two proteins show more than 95% similarity to the MirB transporter of coprogen, ferricrocin, and TAFC in *A. fumigatus* (Raymond-Bouchard et al. [2012]).

One proposed model of iron-acquisition acts via ferreted siderophores shuttling entire complexes followed by intracellular endosome trafficking (Kosman [2003]; Raymond-Bouchard et al. [2012]). However, coprogen recognition by these trans-
Porters has been reported to be highly stereospecific, rather than size dependent (Wong et al. [1983]; Huschka et al. [1985]; Huschka et al. [1986]; Matzanke et al. [1988]). All siderophores secreted by the parental strains were still detected in the culture of the deletion strains ∆pstA and ∆pstB. It is therefore likely that these transporters participate in internalization of a Fe³⁺-siderophore complexes, although this needs to be further investigated. Future studies, using radioisotopes need to be conducted to further understand the mechanism of the PstA and PstB action.

Thus far little is known about the role of fungal ABC-binding cassette proteins in transport of iron-chelating molecules. Here we have shown that psmA and psmB are commonly up-regulation when cells are grown under iron depleted condition (Fig. 5.1, Fig. 6.1) along with the structural genes responsible for siderophore production. Furthermore, based on the metabolites profile in culture broth of deletion strains, PsmB is required for export of fusaridine A and novel putative siderophores fusaridine M and fusaridine E, while PsmA is required exclusively for fusaridine E (Fig. 6.2). These observations might also relate to fusaridine C and fusaridine B, which due to their fragility and processing procedures, could not be detected in this study. Although we cannot yet determine whether PsmA and PsmB are involved directly or indirectly in the excretion processes, it appears that fusaridine E utilized either of these transporters for product secretion. In addition, the ∆psmA mutant consistently grew as a blue culture (Fig. 6.3). Such a phenotype indicates that the iron and copper metabolic pathways are interconnected (Kosman [2003]), and are indicative for a deficiency in copper ion aquisition leaving the blue copper complex in the medium.
In conclusion, we have shown the presence of two groups of transporter Pst and Psm. While the Pst are rather involved in siderophores-iron uptake as members of the sit subfamilies in other fungi, our data present a newly described function of the Psm in the excretion of fusarinine A and novel putative siderophores fusarinine M and fusarinine E.
Figure 6.3: Effect of Pc16g03870 deletion (ΔpsmA) on the blue color of culture broth. The ΔpsmA strain and the wild type DS54465 were grown in triplicates for 7 days in iron deficient media in a rotary incubator.


References

