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SHORT CONTRIBUTION

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Isolation of *Penicillium chrysogenum PEX1* and *PEX6* encoding AAA proteins involved in peroxisome biogenesis

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Abstract In *Penicillium chrysogenum*, key enzymes involved in the production of penicillin reside in peroxisomes. As a first step to understand the role of these organelles in penicillin biosynthesis, we set out to isolate the genes involved in peroxisome biogenesis. Here we report the cloning and characterization of *P. chrysogenum PEX1* and *PEX6*, which encode proteins of the AAA family of ATPases. The second AAA module, which is essential for the function of Pex1p and Pex6p in peroxisome biogenesis, is highly conserved in both PcPex1p and PcPex6p. *PcPEX1* and *PcPEX6* contain three and two introns, respectively.

Introduction

Penicillins are members of the β-lactam class of peptide antibiotics synthesized by many filamentous fungi. The biosynthesis of these antibiotics has been well characterized at the biochemical and genetic level (reviewed by Martín and Gutiérrez 1995). The enzymes involved in the first steps of this process, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthase and isopenicillin *N* synthase are thought to be cytosolic enzymes (Müller et al. 1991; Van de Kamp et al. 1999). However, the enzymes isopenicillin *N*:acyl CoA acyltransferase (IAT) and phenylacetyl-CoA ligase were shown to contain putative peroxisomal targeting sequences (Barredo et al. 1989; Gledhill et al. 1998) and indeed for IAT it has been shown that this signal is essential to target the enzyme to peroxisomes (Müller et al. 1992).

Microbodies (peroxisomes, glyoxysomes, and glycosomes) are vital organelles, present in virtually all eukaryotic cells. The organelles are involved in various metabolic pathways including cholesterol biosynthesis, glycolysis, and the primary metabolism of certain carbon or organic nitrogen sources (Veenhuis and Harder 1991; Van den Bosch et al. 1992; Reddy et al. 1996). The biogenesis of these organelles has been under study for the past 15 years using mainly yeasts as model systems (reviewed by Subramani 1998). In contrast, only a few studies have analyzed the role of peroxisomes in filamentous fungi (see e.g. Berteaux-Lecellier et al. 1995). Nevertheless, the importance of these organelles in penicillin biosynthesis has now been recognized. Indeed, a correlation was observed between the number of peroxisomes per cell and the amount of penicillin produced by certain *Penicillium chrysogenum* and *Aspergillus nidulans* strains (Müller et al. 1991; Valenciano et al. 1998).

In order to analyze the role of peroxisomes in penicillin biosynthesis in filamentous fungi in more detail, we set out to isolate the genes involved in peroxisome biogenesis in *P. chrysogenum*. Using a polymerase chain reaction (PCR) approach, we isolated the *P. chrysogenum PEX1* and *PEX6* genes, both encoding members of the "ATPases associated with various cellular activities" (AAA) protein family. The present paper describes a genetic analysis of these genes.

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Materials and methods

Strains, gene libraries, and plasmids

P. chrysogenum GB8 was cultured as described (Gouka et al. 1991). *Escherichia coli* DH5α (Sambrook et al. 1989) and XL1-Blue (Stratagene, La Jolla, Calif.), grown according to Sambrook et al. (1989), were used for plasmid constructions and bacteriophage

propagations, respectively. A gene library in λZAPII (Stratagene) was constructed according to Gouka et al. (1991) and contained partially *Sau*3A-digested *P. chrysogenum* genomic DNA (insert size 7–12 kb; 1.6×10^5 primary clones, representing approximately 60 times the *P. chrysogenum* genome). Custom-made cDNA libraries were constructed by Life Technologies (Rockville, Md.) in pCMVSPORT4, using mRNA from a high penicillin-yielding *P. chrysogenum* strain cultured on penicillin production medium (at least 10^7 primary clones).

DNA procedures

Standard recombinant DNA manipulations according to Sambrook et al. (1989) were used throughout. *P. chrysogenum* genomic DNA was isolated essentially as described by Kolar et al. (1988). Southern blot analysis was performed using the ECL direct nucleic acid labelling and detection system according to the supplier (Amersham, Arlington Heights, Ill.). Plaque hybridization was performed according to Sambrook et al. (1989). PCR-mediated DNA amplification using degenerate primers (obtained from Eurogentec, Seraing, Belgium) was performed on a GeneAmp 2400 DNA amplifier (Perkin Elmer, Norwalk, Conn.) with Gold Star RED Taq polymerase (Eurogentec), using an annealing temperature of 55 °C. cDNA fragments were isolated from the gene libraries using the Expand high fidelity PCR system (Boehringer, Mannheim, Germany) and gene-specific oligonucleotides (20-mers; Gibco Life Technologies, Breda, The Netherlands). DNA sequencing of genomic *P. chrysogenum* DNA fragments was carried out on a ABI 373A DNA sequencer using the BigDye Terminator system (Perkin Elmer/Applied Biosystems). PCR-derived cDNA fragments were cloned in pBluescript II SK+ (Stratagene) and sequenced using standard primers (Baseclear, Leiden, The Netherlands). For analysis of the DNA sequences and deduced amino acid sequences, the PC-GENE program (release 6.80, Intelligenetics, Mountain View, Calif.) was used. Amino acid sequences were aligned using the ClustalX program (Thompson et al. 1997). Hydropathy profile alignments were performed using the MemGen program (Lolkema and Slotboom 1998).

GenBank accession numbers

The nucleotide sequences of *P. chrysogenum PEX1* and *PEX6* have been deposited at GenBank and were assigned the accession numbers AF233276 (*PcPEX1*) and AF233277 (*PcPEX6*).

Results and discussion

AAA proteins contain so-called AAA modules characterized by a putative ATP-binding site and a second region of homology with an unknown function (Confalonieri and Duguet 1995). The two AAA proteins essential for peroxisome biogenesis, the peroxins Pex1p and Pex6p, each contain two of these AAA modules. To isolate *P. chrysogenum PEX1* and *PEX6*, we used the primary sequences of the second, highly conserved AAA modules in yeast Pex1p and Pex6p (for references see Fig. 1) in the design of degenerate PCR primers. To obtain a *P. chrysogenum PEX1* fragment, the sense primer PcPEX1F (5′ TAC GGY TAC CCY GGY TGY GGN AAR AC 3′) – based on the amino acid sequence of the Walker A motif in the second AAA module, YGYPGCGKT – was used together with the anti-sense primer PcPEX1R (5′ TGG TTG ACG ACR CGG TCN GTN ACN CCN GT 3′) – based on the amino acid sequence TGVTDRVVNQ – using *P. chrysogenum* genomic DNA as the target. This resulted in a specific DNA fragment of approximately 250 bp that was highly similar to yeast *PEX1* genes (data not shown). In a similar way, a 450-bp *PcPEX6*-specific PCR fragment was isolated using the sense primer PEX6F (5′ AAC ATG TAC ATT GGC GAG TCT GAR GCN AAY GT 3′) – based on the amino acid sequence NMYIGESEANV – and the antisense primer PEX6R (5′ AGC ATG GCA TCA GAG CAC AGN GCR TAR AAR TC 3′) – based on the amino acid sequence DFYALCSDAML (data not shown).

To demonstrate that the PCR fragments truly specified parts of the *P. chrysogenum* genome, the fragments were used as probes in Southern blot analyses of *P. chrysogenum* genomic DNA. In all cases single hybridization signals were observed, indicating that both genes are present as single copies in the *P. chrysogenum* genome (data not shown). Subsequently, the genomic regions comprising the complete *P. chrysogenum PEX1* and *PEX6* genes were isolated from a *P. chrysogenum* gene library in λZAPII by plaque hybridization, using the PCR fragments as probes. Fragments containing the hybridizing regions were sequenced.

To localize the intron positions in both genes and thus be able to deduce the primary sequences of both peroxins, we designed sets of gene-specific PCR primers to isolate specific fragments of approximately 500–1000 bp from *P. chrysogenum* cDNA libraries (data not shown), which were then sequenced. Analysis of the sequences revealed that *PcPEX1* contained three introns in the 5′ end of the gene, while *PcPEX6* contained a single intron in its 5′ end as well as a second intron in its 3′ end. Both the size of the introns in *PcPEX1* and *PcPEX6* (53–78 nt) and the intron sequences (splice donor and acceptor sites and the lariat) are similar to those observed in 51 introns found in 22 *P. chrysogenum* genes present in GenBank (size 50–139 nt; consensus sequence: GT(A/G)AGT-$(N)_{26–118}$-CT(A/G)A(C/T)-$(N)_{5–18}$-(C/T)AG).

PcPEX1 and *PcPEX6* encode proteins of 1,213 and 1,459 amino acids, respectively, that are quite similar to, but larger than orthologs isolated from several yeasts and man (28–36% and 32–40% overall identity, respectively). As depicted in Fig. 1A, B, the proteins are highly similar to their orthologs in the region containing the second AAA domain. In contrast, the N-terminal region of approximately 500 amino acid residues shows only little conservation. Figure 1C shows a comparison of the Walker A and B motifs of the ATP-binding sites present in the AAA modules of Pex1p and Pex6p. Clearly, the ATP-binding site in the second AAA domain in both Pex1p and Pex6p is highly conserved. This ATP-binding site has been shown to be essential for the function of both Pex1p and Pex6p (Krause et al. 1994; Tsukamoto et al. 1995; Yahraus et al. 1996).

The peroxins Pex1p and Pex6p have been demonstrated to functionally and physically interact in vivo (Faber et al. 1998; Geisbrecht et al. 1998; Tamura et al.

A

Pc-Pex1p	N	AAA	AAA	C
Yl-Pex1p	29.6	30.1	73.8	27.2
Pp-Pex1p	27.5	30.1	73.2	28.7
Hp-Pex1p	24.9	27.3	75.0	26.0
Sc-Pex1p	21.7	24.6	66.9	26.4
Hs-Pex1p	20.4	25.8	61.7	17.2

B

Pc-Pex6p	N	AAA	AAA	C
Yl-Pex6p	20.8	28.3	77.8	17.8
Pp-Pex6p	22.2	25.4	80.0	26.5
Hp-Pex6p	20.3	27.2	78.8	20.0
Sc-Pex6p	18.6	19.8	75.2	16.2
Hs-Pex6p	17.6	25.2	62.8	10.3
Rn-Pex6p	18.4	25.7	62.8	9.7

C

	AAA-module 1		AAA-module 2	
	Walker A	**Walker B**	**Walker A**	**Walker B**
Pc-Pex1p	560 GGLGAGKT	629 EDLD	879 GFPGCGKT	938 DEFD
Yl-Pex1p	499 GSRGSGKS	561 DDID	768 GYPGCGKT	826 DEFD
Pp-Pex1p	523 GTSGSGKS	585 EDLD	840 GYPGCGKT	899 DEFD
Hp-Pex1p	504 GASGSGKT	567 ENLD	771 GYPGCGKT	830 DEFD
Sc-Pex1p	461 GKQGIGKT	525 DNVE	738 GYPGCGKT	797 DEFD
Hs-Pex1p	599 GGKGSGKS	662 DDLD	881 GPPGTGKT	940 DEFE
	* * **•	• ••	* ** ***	***•
Pc-Pex6p	795 QQRHIGKA	861 RHIE	1070 GPPGTGKT	1129 DELD
Yl-Pex6p	477 AKRGVGKS	536 QHLE	760 GPPGTGKT	819 DELD
Pp-Pex6p	570 LSRAIGKS	631 KHIE	859 GPPGTGKT	918 DELD
Hp-Pex6p	571 MARCVGKA	632 RHIE	853 GPPGTGKT	912 DELD
Sc-Pex6p	483 TTNNVGKA	548 AHLD	772 GPPGTGKT	831 DEID
Hs-Pex6p	470 GPPGCGKT	529 TAVD	744 GPPGTGKT	803 DELD
Rn-Pex6p	470 GPPGSGKT	529 TALD	742 GPPGTGKT	801 DELD
	**•	••	********	**•*
Consensus	GxxGxGKT A S	DexD E		

Fig. 1A–C Sequence similarity between *Penicillium chrysogenum* Pex1p and Pex6p and their counterparts in other organisms. **A** *P. chrysogenum* Pex1p (*Pc*-Pex1p) was aligned with its orthologs from *Yarrowia lipolytica* (*Yl*; Titorenko et al. 2000), *Pichia pastoris* (*Pp*; Spong and Subramani 1993), *H. polymorpha* (*Hp*; Kiel et al. 1999), *Saccharomyces cerevisiae* (*Sc*; Erdmann et al. 1991) and man (*Hs*; Reuber et al. 1997). **B** Similarly, *P. chrysogenum* Pex6p was aligned with its orthologs from *Y. lipolytica* (Nuttley et al. 1994), *P. pastoris* (Heyman et al. 1994), *H. polymorpha* (Kiel et al. 1999), *S. cerevisiae* (Voorn-Brouwer et al. 1993), man (Yahraus et al. 1996) and rat (*Rn*; Tsukamoto et al. 1995). The sequence identities (%) between PcPex1p (panel **A**) and PcPex6p (panel **B**) and their respective orthologs in the different domains of the proteins are indicated. Note that the weak similarity in the C-terminal region of PcPex6p is mainly due to the long C-terminal extension of the *P. chrysogenum* protein. Panel **C** shows a sequence comparison between the ATP binding motifs observed in the AAA modules in Pex1p and Pex6p orthologs. The Walker A and B motifs of P-loop ATPases are indicated. Identical residues are indicated by an *asterisk*, similar residues by a *dot*. The Walker A and B consensus motifs are also shown

1998; Kiel et al. 1999). Localization studies in different organisms indicate that the Pex1p/Pex6p complex may be located either in the cytosol (Yahraus et al. 1996; Tamura et al. 1998) or at the cytosolic face of membranes (Tsukamoto et al. 1995; Faber et al. 1998; Kiel et al. 1999). We performed a hydropathy profile analysis of the entire Pex1p and Pex6p families using the MemGen program; and we confirm that these AAA proteins

do not contain membrane-spanning regions (data not shown). Although the exact function of the Pex1p/Pex6p complex is not yet known, the complex is essential for peroxisome assembly, since absence or inactivation of either of the peroxins leads to the mis-localization of the bulk of the peroxisomal matrix proteins into the cytosol, resulting in the appearance of peroxisomes which lack their contents (cf. Kiel et al. 1999).

Although the isolation of the *P. chrysogenum PEX1* and *PEX6* genes has provided the first tools for initiating molecular studies on the role of peroxisomes in penicillin biosynthesis in this industrially important fungus, attempts to disrupt *PcPEX1* or *PcPEX6* have so far been unsuccessful. Our results indicate that we have to take into account that a complete disruption of the process of peroxisome biogenesis in filamentous fungi may cause undesirable side effects and may even produce non-viable cells. In line with this, it was observed that a mutation in the *PEX2* (*CAR1*) gene affected caryogamy in the fungus *Podospora anserina* (Berteaux-Lecellier et al. 1995). So far, mutants completely devoid of peroxisomes have not been isolated in filamentous fungi [cf. De Lucas et al. (1997)]. To be able to better determine the role of peroxisomes in penicillin biosynthesis, we are currently isolating *P. chrysogenum PEX* genes, whose disruption will affect peroxisome biogenesis to a lesser extent – i.e. genes that function solely in the import of certain subclasses of peroxisomal matrix proteins or in peroxisome proliferation.

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