NOTE

Contribution of Peroxisomes to Penicillin Biosynthesis in *Aspergillus nidulans*^{\dagger}\textsuperscript{†}

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Peroxisomal localization of the third enzyme of the penicillin biosynthesis pathway of *Aspergillus nidulans*, acyl-coenzyme A:IPN acyltransferase (IAT), is mediated by its atypical peroxisomal targeting signal 1 (PTS1). However, mislocalization of IAT by deletion of either its PTS1 or of genes encoding proteins involved in peroxisome formation or transport does not completely abolish penicillin biosynthesis. This is in contrast to the effects of IAT mislocalization in *Penicillium chrysogenum*.

The production of penicillin has been reported only for *Aspergillus nidulans* and *Penicillium chrysogenum* since both filamentous fungi possess acyl-coenzyme A (CoA):IPN acyltransferase (IAT; encoded by the *aatA* [penDE] gene) as one prerequisite for converting isopenicillin N to penicillin by exchange of the hydrophilic l-α-aminoacidsic acid side chain for a hydrophobic acyl group (2, 4). Based on transcriptome analyses, in *P. chrysogenum* a substantial contribution of peroxisomes—single-membrane organelles containing specialized enzymes involved in a wide range of metabolic activities—to penicillin production was recently suggested (21). Whereas for this fungus the importance of IAT localization within functional peroxisomes has been shown (14), the contribution of these organelles to penicillin biosynthesis in *A. nidulans* has been studied only in connection with the cytoplasmic protein AatB, which is also involved in penicillin biosynthesis in *A. nidulans* (16). This study already hinted that peroxisomes are not absolutely essential for penicillin biosynthesis in *A. nidulans*, a theory the present study is focused on. Differences between these species are also important from an applied point of view since most basic research is done with the model organism *A. nidulans* whereas *P. chrysogenum* is used for industrial production.

Peroxisins are proteins required for peroxisome biogenesis and division and for import of proteins into the peroxisomal matrix (15). Attempts to isolate *P. chrysogenum* peroxin deletion mutants have so far not been successful (8, 9). However, high peroxisome abundance caused by overproduction of peroxin Pc-Pex11p led to a twofold increase in penicillin production (10), emphasizing the importance of these organelles for *P. chrysogenum*. Recently, different *A. nidulans* peroxin mutant strains have been characterized (7). Of particular interest for the present study were the mutant unable to form peroxisomal structures caused by a disruption of the *Saccharomyces cerevisiae* *pex3* ortholog (*pexC:*bar strain) and mutants impaired in import of matrix proteins carrying peroxisomal targeting signal 1 (PTS1) or PTS2 (*pexEΔ* and *pexG14* strains, respectively) due to deletion/mutation of the respective receptors *PexE* and *PexG* (13, 17).

Usually, PTS1 sequences comprise three C-terminal amino acids of the form (S/A)(R/K)(L/M) although the context of the C-terminal sequence can greatly affect targeting (5). As depicted in Fig. 1A, the *P. chrysogenum* but not the *A. nidulans* IAT possesses such a motif. However, some peroxisomal proteins have cryptic PTS1 sequences (see, e.g., reference 11). By transformation of different *A. nidulans* strains (1) with a *gfp-aatA* gene fusion carried on plasmid pALX-aatA (Fig. 1B1) (18) and subsequent fluorescence microscopy, we showed that localization of the *A. nidulans* IAT is PTS1 dependent (Fig. 1B2 to 5) (strains are listed in Table S1 in the supplemental material). Compared to its mainly peroxisomal localization in the wild-type strain, indicated by punctate dots (Fig. 1B2), mislocalization of the green fluorescent protein (GFP)-IAT fusion protein was observed in strains without peroxisomal structures (Fig. 1B4) and without PTS1 import (*pexEΔ* strain) (Fig. 1B5). The effects of these peroxin mutations clearly defined the punctate dots of the wild-type strain as peroxisomes. Localization was unaffected in the *pexG14* strain, which lacked PTS2 import (Fig. 1B3). Thus, PTS1 and not PTS2 targeting is responsible for localizing IAT to peroxisomes. Additionally, the atypical putative PTS1 sequence (ANI) is essential for this import, because a deletion of this sequence (GFP-IAT\textsuperscript{ANI}) led to mislocalization of the protein in the wild-type strain (Fig. 1B6). We therefore conclude that the *A. nidulans* IAT is transported to the peroxisomes via its atypical PTS1 by the *PexE*-dependent import machinery.

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It has been shown previously that disruption of the *aatA* gene has a severe impact on penicillin biosynthesis in *A. nidulans*, with biosynthesis yielding only minimal amounts (below 20% in a bioassay) due to AatB activity (16). In *P. chrysogenum*, however, mutants possessing a mislocalized IAT failed to produce penicillin (14). Therefore, penicillin production by the *A. nidulans* peroxin mutants (7) and by a strain in which the *aatA* gene was replaced by an *aatA* allele lacking the PTS1-encoding sequence (AatA<sup>PTS1</sup>, generated in this study by homologous integration) (see Table S1 in the supplemental material) was assayed (3) (Fig. 1C). Mislocalization of IAT due to mutations of peroxins or the enzyme itself resulted in a decrease of the penicillin titer (compare to Fig. 1B). However, this decrease was much smaller than that in a strain completely lacking *aatA*-encoded IAT (16), indicating that the mislocalized IAT is at least partially functional. Furthermore, a strain without peroxisomes (*peK*::*bar* strain) was still able to produce penicillin. The penicillin titers of a strain lacking the PTS1 transporter (*peK*Δ strain) were reduced to 50% of wild-type levels, whereas the mutation of the PTS2 transporter in the *peK*Δ strain had no significant effect. A similar reduction in penicillin production was also observed in mutants impaired in both PTS1- and PTS2-mediated import (*pexA*9, *pexF*23, and *pexFM*15 strains) (7) (data not shown). This suggested that, in *A. nidulans*, transport of the peroxisomal proteins involved in penicillin biosynthesis, either directly or indirectly, is mainly PTS1 dependent. But the proper localization of these proteins and, moreover, the presence of functional peroxisomes are not absolutely required for penicillin production. The beneficial role of peroxisomes was further confirmed by the reduced penicillin production of a strain with only a small number of enlarged peroxisomes caused by deletion of *peK* (7), the ortholog of *Pe-pex11* (data not shown).

Besides IAT, one of the peroxisomal proteins involved in penicillin biosynthesis could be the PTS1 containing 17631.4, a proposed homolog of the *P. chrysogenum* phenylacetetyl-CoA ligase (Phl) which was shown to affect penicillin biosynthesis in *P. chrysogenum* by supplying IAT with phenylacetetyl-CoA (12). However, disruption of the associated gene in *A. nidulans* had no clear effect on penicillin biosynthesis (data not shown). It is likely that this is due to redundant enzymes, which are predicted to be both peroxisomal (e.g., enzymes encoded by genes with accession no. AN5990.4 and AN11034.4) and cytoplasmic (e.g., enzymes encoded by genes with accession no. AN2549.4 and AN3490.4). Phl-like cytoplasmic enzyme activity could also provide a mislocated IAT with acyl-CoA, thus enabling penicillin production in *A. nidulans*.

In contrast to what was found for *P. chrysogenum*, mislocalization of IAT in strain AatA<sup>PTS1</sup> did reduce but did not abolish penicillin biosynthesis (Fig. 1C), leading to the hypothesis that the *A. nidulans* IAT is also functional in the cytoplasm but may possess reduced activity. As mentioned above, we recently identified a cytoplasmic IAT-like protein (AatB) involved in penicillin production and showed that only inactivation of both the peroxisomal IAT and the cytoplasmic AatB reduced the penicillin titer to below the liquid chromatography-mass spectrometry detection level (16). To rule out the possibility that residual penicillin production by the AatA<sup>PTS1</sup> strain was due only to AatB activity, the strain was crossed (20) with the *aatB* disruption AatB-disr strain (16) and penicillin production of the parental strains and a strain carrying both mutant genes (A<sup>PTS1</sup>/B-disr strain) (see Table S1 in the supplemental material) was analyzed (Fig. 2). The double mutant was still able to produce penicillin, and production compared...
to that of the AatB-disr strain was significantly decreased; the extent of the reduction (approximately 20%) was the same as that for the AatA<sup>PTS1</sup> strain compared to the wild-type strain. Therefore, the impact of the mislocalized IAT was the same for both strains and independent of AatB activity. Taken together, these data indicate that, in <i>A. nidulans</i>, despite reduced activity, a mislocalized IAT is still functional. Obviously, differences in the cellular environment are better tolerated by the <i>A. nidulans</i> IAT than by the <i>P. chrysogenum</i> IAT; this is presumably due to sequence differences (19).

However, it is clear that the contribution of peroxisomes to penicillin biosynthesis in <i>A. nidulans</i> is more than the provision of a better environment for IAT function, since absence of peroxisome formation or of the essential PTS1 transport led to a more severe reduction of the penicillin titer (Fig. 1C). In these strains, mislocalization of additional peroxisomal proteins might provoke metabolic impairments that indirectly influence penicillin production. Therefore, compartmentalization of the final step seems to be advantageous with respect to the yield of the whole process. It is likely that keeping enzymes and substrates in close proximity or facilitating the catalyzed reactions, e.g., the exchange for a hydrophobic acyl side chain, by the more hydrophobic environment within the peroxisomes could increase efficiency of penicillin production. In addition, the slightly alkaline pH of the peroxisomal lumen (22, 23) might also contribute to this increase. Overall, this study has shown that the necessity for IAT being localized to functional peroxisomes in <i>A. nidulans</i> is different from that in <i>P. chrysogenum</i>. A detailed analysis of both the enzyme activity tolerance and the range of phenylacetyl-CoA ligases is necessary for elucidating this unexpected discrepancy.

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REFERENCES


FIG. 2. Penicillin titer of <i>A. nidulans</i> Aat<sup>PTS1</sup>/B-disr strain (see Table S1 in the supplemental material) compared to those of the wild type (set at 100%) and parental AatA<sup>PTS1</sup> and AatB-disr strains (16). Statistical significance is indicated by the *P value. Since the Aat<sup>PTS1</sup>/B-disr strain still produced penicillin, production by the AatA<sup>PTS1</sup> strain was not due to AatB alone.