The N-terminus of amine oxidase of *Hansenula polymorpha* contains a peroxisomal targeting signal

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Abstract Here we describe the identification of the targeting sequence of peroxisomal amine oxidase (AMO) of *H. polymorpha*. Deletion analysis revealed that essential targeting information is located within the extreme N-terminal 16 amino acids. Moreover, this sequence can direct a reporter protein to the peroxisomal matrix of *H. polymorpha*. The N-terminal 16 amino acids of AMO contain a sequence with strong homology to the conserved PTS2 sequence. Therefore, AMO is considered to be a PTS2 protein.

Key words: Peroxisome; Topogenic signal; Amine oxidase; PTS2; β-Lactamase; Watermelon malate dehydrogenase

1. Introduction

The yeast *Hansenula polymorpha* can utilize primary amines (e.g. methyl- and ethylamine) as a sole nitrogen source for growth. The key enzyme of the metabolism of these compounds is the peroxisomal matrix enzyme amine oxidase (AMO) which converts primary amines into their corresponding aldehydes, ammonium and hydrogen peroxide. The synthesis of AMO is fully repressed by ammonium ions but is induced during growth of cells on primary amines as the sole N source [1].

Two classes of peroxisomal targeting signals (PTS) have been identified so far. The first one (PTS1) was found at the extreme C-terminus of firefly luciferase which comprises the three amino acids SKL-COOH. In apparent contradiction to its small size, a high level of degeneracy of the PTS1 was shown by site-directed mutagenesis experiments, and variants of this sequence are present at the C-terminus of many peroxisomal proteins [2,3]. Also in *H. polymorpha*, import of the majority of the peroxisomal matrix proteins is mediated by a PTS1 signal, for example ARF in alcohol oxidase, NKL in dihydroxyacetone synthase and SKI in catalase [4,5].

The second type of peroxisomal targeting signals (PTS2) was found in the presequence of peroxisomal thiolase from rat [6]. Comparable sequences have been found in the N-termini of other peroxisomal proteins, e.g. human and yeasts thiolases, watermelon malate dehydrogenase and *Trypanosoma brucei* aldolase. These sequences, which also contain peroxisomal targeting information, conform to the general consensus RL-X_3-H/QL [2,7,8].

Based on the similarity between the N-terminus of AMO and the PTS2 consensus sequence [2], we have studied targeting of AMO in detail by determining the intracellular location of a truncated form of AMO and several hybrid proteins consisting of C- or N-terminal parts of AMO fused to heterologous reporter proteins. The results indicate that the targeting information of AMO resides within the first 16 amino acids of the N-terminus of the protein.

2. Materials and methods

2.1. Microorganisms and growth conditions

All strains used in this study are listed in Table 1. Transformants carrying truncated or hybrid genes under the control of the *H. polymorpha* alcohol oxidase promoter (P_{AOX}) are derivatives of *H. polymorpha* A16 [9]. Strain A17 is an *ura*$^-$ derivative of A16 obtained by selection on 5-fluoric acid (FOA) (J. Cregg, unpublished). To obtain expression of truncated or hybrid genes, the cells were grown in batch cultures in mineral medium [10] on 0.1% (v/v) glycerol, 0.5% (v/v) methanol mixtures supplemented with 0.20% (w/v) methylamine as sole nitrogen source.

2.2. Molecular genetics and yeast transformation

All standard genetic procedures were according to [11]. Mutated DNA fragments and fusion points of the hybrid genes were sequenced using the T7 sequencing kit from Pharmacia (Uppsala, Sweden). Yeast cells were transformed by electroporation [12].

2.3. Plasmid constructions

All truncated and hybrid genes (see Fig. 1) were inserted in vector pHIPX2 [13], containing the *Saccharomyces cerevisiae LEU2* gene for selection and autonomous replication in *H. polymorpha* [14] and the P_{AOX} for driving the expression of heterologous genes. Bacterial β-lactamase and the mature part of watermelon (glyoxysomal) malate dehydrogenase (gMDH) [15] were used as reporter proteins. The gene coding for bacterial β-lactamase (βlac) was adapted for in-frame fusions by site- directed mutagenesis, hereby introducing a *Sma*I–*Xma*I site preceding the N-terminal coding region, or a *Xba*I site following the C-terminal coding region. Restriction sites used for constructing the truncated and hybrid proteins, and insertion of these genes in pHIPX2, are given in Fig. 1.

2.4. Disruption of the AMO gene

A 1624 bp *Hin*dIII–*Hpa*I (−32 to +1592) fragment containing the translation initiation site and the coding region of AMO up to codon Val^{531} [15] was replaced by a 2.2-kb *Eco*RI–*Bam*HI fragment containing the *Candida albicans LEU2* gene. A 3.3-kb *Pvu*II–*Sac*I fragment containing the *LEU2* and *AMO* flanking regions was used to transform strain A17, forcing homologous recombination at the genomic *AMO* locus (Fig. 5A).

2.5. Biochemical methods

Crude extracts were prepared as described [17]. Protein concentrations were determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. Activities of alcohol oxidase [18], amine oxidase [19], cytochrome *c* oxidase [20] and β-lactamase [21] were assayed as described. Peroxisomes were purified by differential and sucrose density centrifugation of homogenized protoplasts [20]. Crude extracts and subcellular fractions were analyzed by SDS-PAGE [22]

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Fig. 2. Subcellular location of hybrid and truncated proteins, determined by cytochemical procedures. Polyclonal antibodies against β-lactamase (A, B and D), gMDH (C) or AMO (F) were used in combination with gold-conjugated goat anti-rabbit antibodies. AMO activity was localized by Ce^{3+} activity staining (E). (A) β-Lac-C_{39}AMO; (B) N_{63}-AMO-β-lac; (C) N_{63}AMO-gMDH; (D) N_{16}-AMO-β-lac; (E and F) ▲N_{16}AMO. Only cells in which the hybrid proteins containing N-terminal parts of AMO were expressed showed clear peroxisomal labelling, whereas the truncated AMO was excluded from the peroxisomes. The inset in (F) shows the labelling pattern of WT AMO. N, nucleus; P, peroxisome; V, vacuole. Bars = 0.5 μm. →

followed by Western blotting, using the protoblot immunoblotting system (Promega Biotec) and polyclonal antibodies against *H. polymorpha* AMO, β-lactamase and watermelon gMDH.

2.6. Electron microscopy

The methods for the preparation of cells for electron microscopy have been described earlier [10]. Immunocytochemical experiments were performed on ultrathin sections of unicryl-embedded cells, using specific antibodies against AMO, β-lactamase and gMDH, and gold conjugated goat anti-rabbit antibodies.

3. Results

Efficient import of PTS2 proteins in *H. polymorpha* is dependent on the induction of a specific import machinery by amine substrates [13]. Therefore, all transformed strains were grown on media containing methylamine as the sole N source and glycerol/methanol mixtures as C sources.

3.1. The C-terminus of AMO does not contain a PTS

Earlier we reported that deleting the C-terminal 12 amino acids, which contain an internal PTS1 sequence (SRL), does not interfere with normal targeting of the mutant protein [23]. To confirm that targeting information is indeed absent from the C-terminus of AMO, a hybrid protein was constructed consisting of the 39 C-terminal amino acids of AMO and β-lactamase (βlac-C_{39}AMO). Cells synthesizing the hybrid protein were subjected to cell fractionation studies. These showed that βlac-C_{39}AMO was predominantly present in the soluble fraction, and co-fractionated with the cytosolic protein formaldehyde dehydrogenase (data not shown). The cytosolic location of the hybrid protein was confirmed immunocytochemically using specific antibodies against β-lactamase (Fig. 2A). In addition, a significant amount of label was found in the nucleus, an observation which is not uncommon if cytosolic proteins are expressed to high levels in *H. polymorpha* [8].

3.2. The N-terminus of amine oxidase mediates protein translocation into peroxisomes

Hybrid genes were constructed encoding the 63 N-terminal amino acids from AMO fused to either β-lactamase (N_{63}AMO-βlac) or the mature part of watermelon glyoxysomal malate dehydrogenase (N_{63}AMO-gMDH), proteins which in earlier studies were found in the cytosol when expressed in *H. polymorpha* [4,8]. Cell fractionation studies performed on strains expressing these proteins showed that a significant amount of the hybrid proteins was observed in the particulate fraction (shown for N_{63}AMO-gMDH, Fig. 3). Immunocytochemically, specific peroxisomal labelling was found using antibodies against the reporter proteins (Fig. 2B and C). However, cytosolic labelling was also evident, indicating that the hybrid proteins were only partially imported. To delimitate the topogenic information, we subsequently fused a smaller N-terminal fragment of AMO to β-lactamase. Cells, synthesizing this hybrid protein, N_{16}AMO-βlac, were analyzed biochemically and immunocytochemically. Again, a significant amount of β-lactamase activity was found in the organellar fraction (P_4). After sucrose density centrifugation of the P_4 fraction, the β-lactamase activity showed a similar distribution pattern as endogenous AMO (Fig. 4), indicating that at least part of the hybrid protein is peroxisome-bound. Immunocytochemical experiments confirmed these results (Fig. 2D). The peroxisomal labelling was often observed in specific parts of the peroxisomal matrix, indicating that the hybrid protein may have aggregated, a phenomenon observed earlier when β-lactamase was fused to a PTS1 signal and expressed in *H. polymorpha* [21].

3.3. A short deletion of the N-terminus of AMO abolishes peroxisomal targeting

In order to seek further evidence that the topogenic information of AMO indeed resides within its N-terminal 16 amino acids, a truncated AMO gene, lacking the DNA fragment encoding the N-terminal amino acids, was constructed

Table 1
H. polymorpha strains

Strain	Genotype	Ref.
A16	*leu2*	[10]
A17	*leu2 ura3*	Gregg, unpublished
GF54	A16 + pGF154 (N_{16}AMO-β-lactamase)	This study
GF55	A16 + pGF155 (N_{63}AMO-β-lactamase)	This study
GF57	A16 + pGF188 (β-lactamase-C_{39}AMO)	This study
GF76	*ura3 amo::Leu2*$_{C.\ albicans}$	This study
GF96	GF76 + pGF237 (▲N_{16}AMO)	This study
GF99	A16 + pGF242 (N_{63}AMO-gMDH)	This study

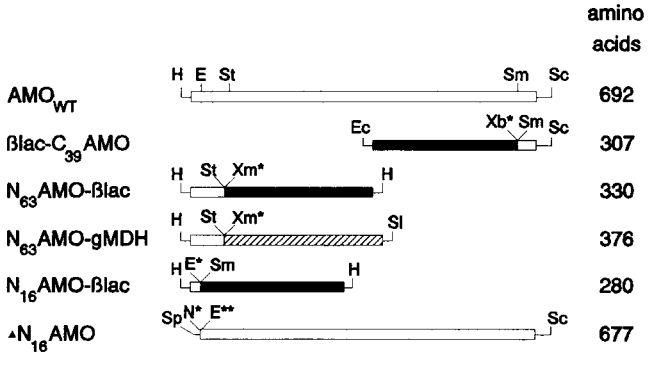
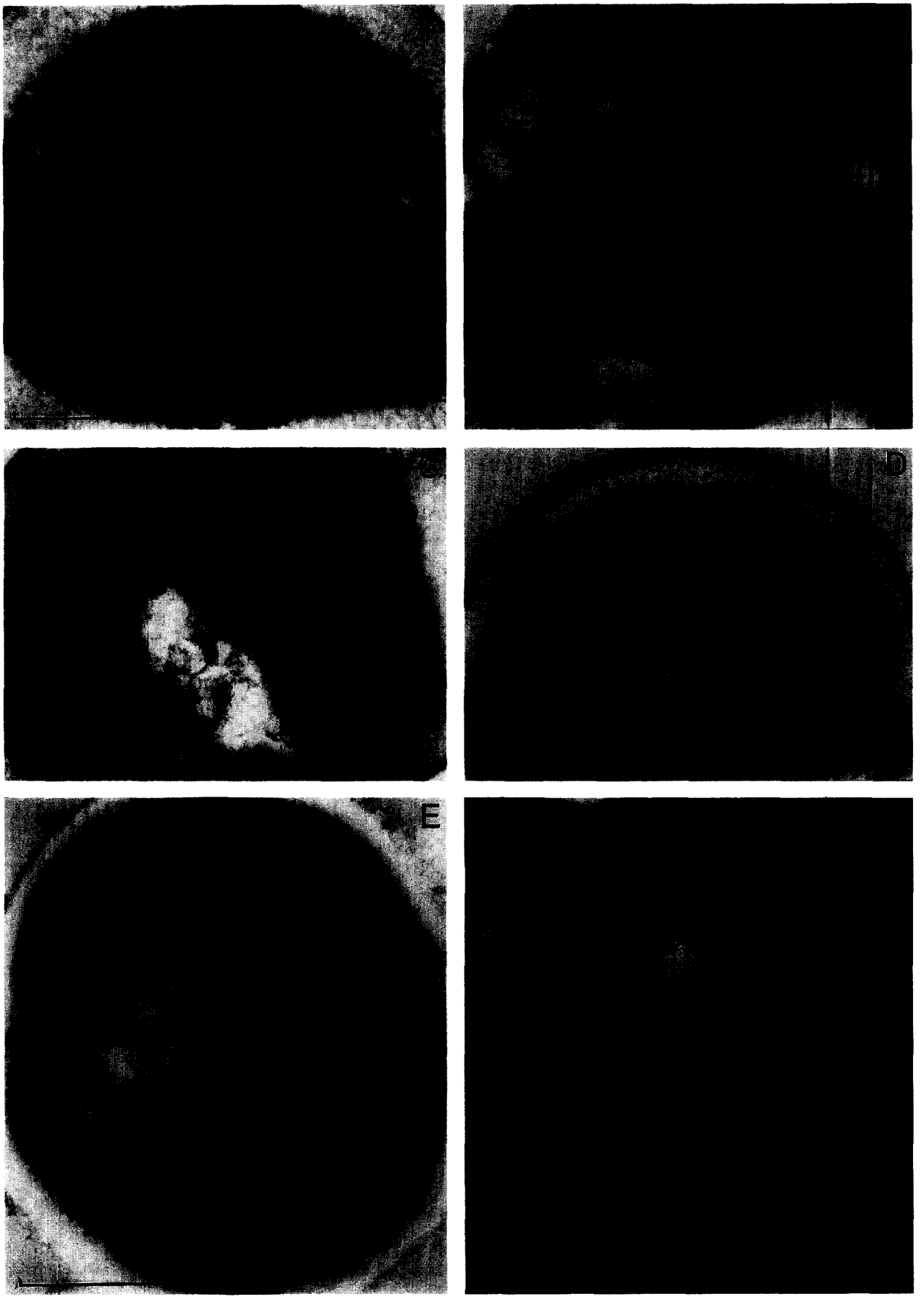


Fig. 1. Schematic drawing of the truncated and hybrid proteins. The restriction sites used for the construction of the hybrid/truncated genes, and for the insertion into the expression vector pHIPX2, are indicated. Open box, AMO sequences; solid box, β-lactamase sequences; hatched box, gMDH sequences. E, *Eag*I; Ec, *Eco*RI; H, *Hin*dIII; N, *Nco*I; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; St, *Stu*I; Xb, *Xba*I; Xm, *Xma*I; *, sticky ends filled in using Klenow enzyme; **, sticky ends removed using Mung bean.


D
E

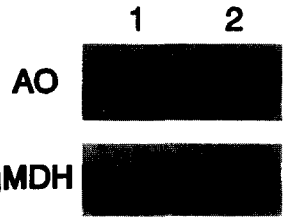


Fig. 3. Western blot analysis of subcellular fractions of strain GF99 expressing N_{63}AMO-gMDH. Homogenized protoplasts were fractionated by differential centrifugation into a cytosolic fraction (30,000 × *g* supernatant, lane 1) and an organellar fraction (30,000 × *g* pellet, lane 2). 20 μg of protein was loaded in each lane. Antibodies against alcohol oxidase (AO; upper panel) and gMDH (lower panel) were used.

($\blacktriangle N_{16}$AMO). To avoid misinterpretation of its subcellular sorting due to the synthesis of endogenous AMO, the truncated AMO protein was synthesized in an *AMO* disruption strain. This *AMO* disruption strain was obtained by transforming strain A17 with a linear DNA fragment containing the *LEU2* gene from *C. albicans* flanked by fragments homologous to the genomic *AMO* locus of *H. polymorpha*, forcing transplacement integration at that site. One strain was selected (designated GF76), which fully lacked AMO, and Southern blot analysis showed that site specific integration had occurred (Fig. 5B). A plasmid carrying the $\blacktriangle N_{16}$AMO gene behind the P_{AOX} was used to transform strain GF76, yielding GF96. Upon growth of these cells in glycerol/methanol/methylamine-containing medium, synthesis of the $\blacktriangle N_{16}$AMO was demonstrated by Western blot analysis; furthermore, the truncated AMO still displayed enzyme activity (data not shown). The subcellular location of $\blacktriangle N_{16}$AMO was determined by biochemical and ultrastructural methods. After differential centrifugation of homogenized protoplasts from strain A16 and GF96, cytosolic (S_4) and organellar fractions (P_4) were obtained. Western blot analysis revealed that, in contrast to WT AMO, $\blacktriangle N_{16}$AMO was predominantly present in the cytosolic fraction (S_4) (Fig. 5C). This result was confirmed cytochemically and immunocytochemically (Fig. 2E and F). Whereas WT AMO was strictly peroxisomal (Fig. 2F, inset), the truncated AMO was only detected in the cytosol, indicating that the N-terminal 16 amino acids are essential for targeting of AMO in *H. polymorpha* but do not abolish protein activity.

4. Discussion

Here we describe that the N-terminus of peroxisomal AMO of *H. polymorpha* contains topogenic information for subcellular sorting. The N-terminal 16 amino acids of AMO are able to direct the cytosolic reporter protein, bacterial β-lactamase, to peroxisomes of *H. polymorpha*. Moreover, a truncated form of AMO, lacking the first 16 N-terminal amino acids, remains in the cytosol. The AMO N-terminus contains a sequence which shows homology to the proposed PTS2 consensus sequence RL-X_5-H/QL [2]. In AMO, the second leucine residue is replaced by an alanine; RL-X_5-QA. Hybrid proteins, consisting of N-terminal parts of AMO and a reporter protein (either β-lactamase or the mature part of malate dehydrogenase (gMDH) were, however, only partially imported into peroxisomes of *H. polymorpha*. This might indicate that AMO contains additional targeting information elsewhere in the protein. However, the truncated form of AMO ($\blacktriangle N_{16}$AMO) remained in the cytosol, implying that no additional targeting information is present in AMO which individually can act as a PTS. Therefore, we conclude that the essential targeting information in AMO resides within the N-terminal sequence.

The inhibitory effect of heterologous protein fragments on efficient targeting is, however, not uncommon and was also observed when fragments of *S. cerevisiae* catalase [24], human

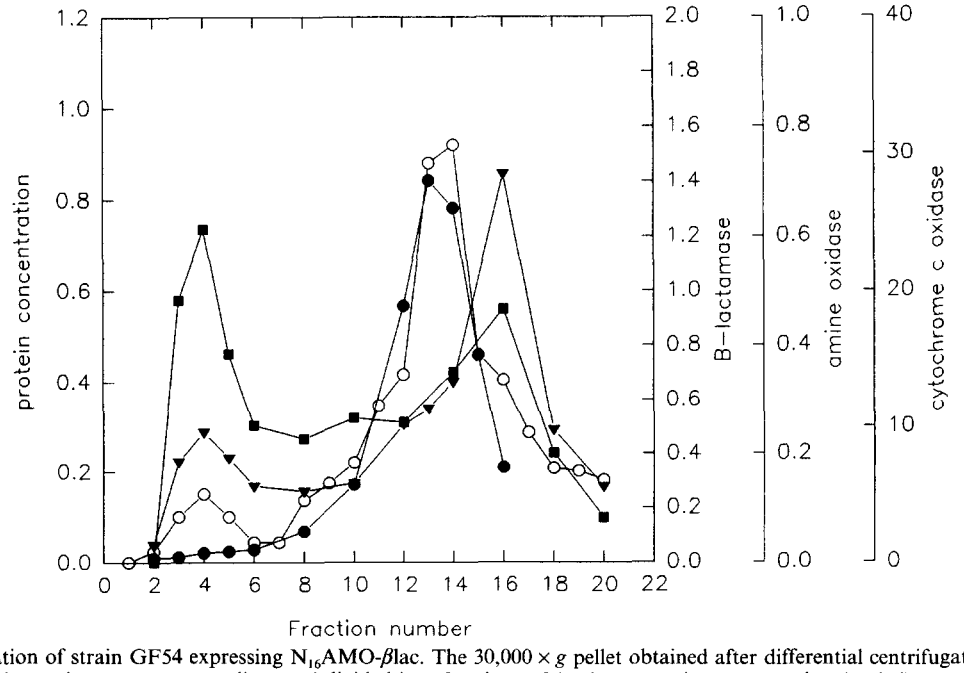


Fig. 4. Cell fractionation of strain GF54 expressing N_{16}AMO-βlac. The 30,000 × *g* pellet obtained after differential centrifugation of homogenized protoplasts was fractionated on a sucrose gradient and divided into fractions of 1 ml. ○, protein concentration (mg/ml); ●, cytochrome *c* oxidase activity ($\Delta E_{550} \cdot min^{-1} \cdot ml^{-1}$); ■, amine oxidase activity ($\Delta E_{420} \cdot min^{-1} \cdot ml^{-1}$); ▼, β-lactamase activity ($\Delta E_{486} \cdot min^{-1} \cdot ml^{-1}$).

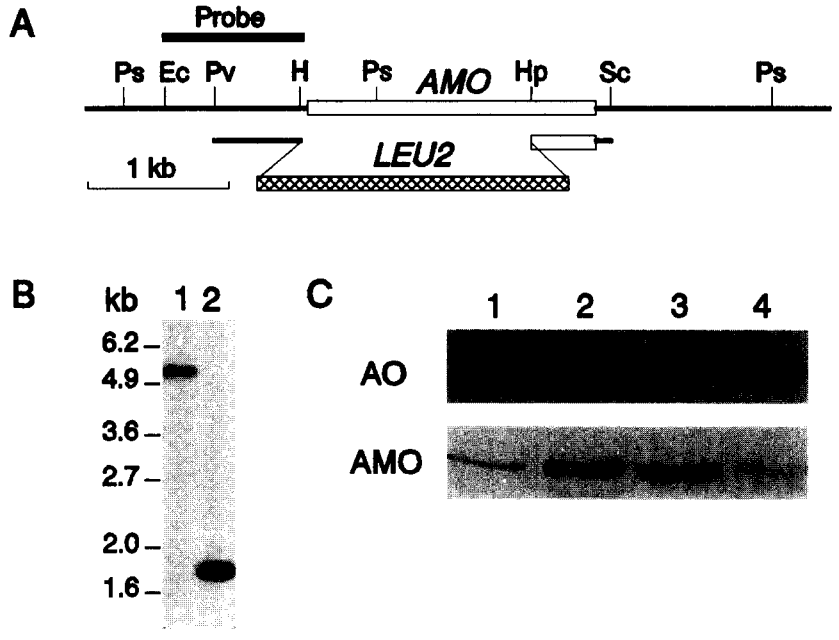


Fig. 5. Analysis of the *AMO* disruption strain (GF76) and strain GF96 expressing the truncated AMO protein, $\blacktriangle N_{16}$AMO. (A) Schematic representation of site-specific integration of the linear DNA fragment. (B) Southern blot analysis of *Pst*I-digested chromosomal DNA from strain GF76 (lane 1) and A16 (lane 2). A 1.0-kb *Eco*RI–*Hin*dIII fragment, identical to the 5′ region of the AMO structural gene, was used as a probe. (C) Western blot analysis of subcellular fractions of strain A16 (lanes 1 and 2) and GF96 (lanes 3 and 4). Cytosolic fractions (lanes 1 and 3) and organellar fractions (lanes 2 and 4) were obtained after differential centrifugation of homogenized protoplasts. 20 μg protein was loaded in each lane. Antibodies against alcohol oxidase (AO; upper panel) and AMO (lower panel) were used.

catalase [25] or *H. polymorpha* alcohol oxidase [26] were fused to reporter proteins. These results imply that proper recognition of a PTS (either PTS1 or PTS2) is dependent on the conformational context.

The PTS2 import machinery appears to be conserved in *H. polymorpha*. Not only have other, endogenous proteins containing a PTS2 been identified, e.g. Per1p [21] and Per6p (M. Tuijl, unpublished), but heterologous PTS2 proteins, like watermelon malate dehydrogenase [27] and *S. cerevisiae* thiolase [13], are normally sorted when they are expressed in this organism. In addition, the PTS2 import pathway in *H. polymorpha* appears to be inducible by amine substrates [13].

The specificity of the import components to recognize the PTS2 is probably strongly organism dependent. For instance, *H. polymorpha* AMO or *T. brucei* aldolase are not sorted to *S. cerevisiae* peroxisomes [2,28]. Both proteins contain a sequence which is slightly aberrant from the PTS2 consensus, RL-X_5-QA or RV-X_5-HL, which might cause the mislocation in *S. cerevisiae*. Actually, mutation analysis of the PTS2 sequence of *S. cerevisiae* thiolase confirmed that the RL-to-RV substitution in the PTS sequence results in a cytosolic location of the mutant protein [7].

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