I. Tubulation activity analysis using total cell extracts

A. Preparation of *Escherichia coli* extracts.

Construction of the expression vector
The DNA sequence encoding the first 97 amino acids of *Penicillium chrysogenum* Pex11 (Pex11N) was amplified using the LMOPPcPex11 plasmid as a template and primers LMOp066 and LMOp067 containing *Nco*I and *Bgl*II sites, respectively. The obtained PCR product and expression vector pQE60 (Qiagen) were digested with *Nco*I and *Bgl*II and ligated, resulting in plasmid pQE60-Pex11N, encoding Pex11N fused to a His-Tag at the C-terminus. Correctness of the plasmid was confirmed by sequencing.

Protein expression and cell lysis
E. coli M15 cells (Qiagen) containing either the expression plasmid pQE60-Pex11N or the empty M15 host were grown in LB medium at 37°C, 200 rpm to an OD of 1 and then induced with IPTG (1mM) and incubated for 4 hours at 30°C, 200 rpm. Cells were harvested by centrifugation and the cell pellets were suspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris, 8M urea, pH 8.0) and disrupted by 2 cycles of stirring with a magnetic stirrer and French press. The soluble fractions were obtained upon centrifugation (13 000 rpm, 1 h, 12°C).
The presence of Pex11N in extracts of *E. coli* M15 cells expressing Pex11N was confirmed by Western blotting using α-His antibodies (indicated as a band of approximately 12 kD in Fig. 1B). This 12 kD band was absent in extracts prepared from cells of the empty *E. coli* M15 host (Fig. 1B).

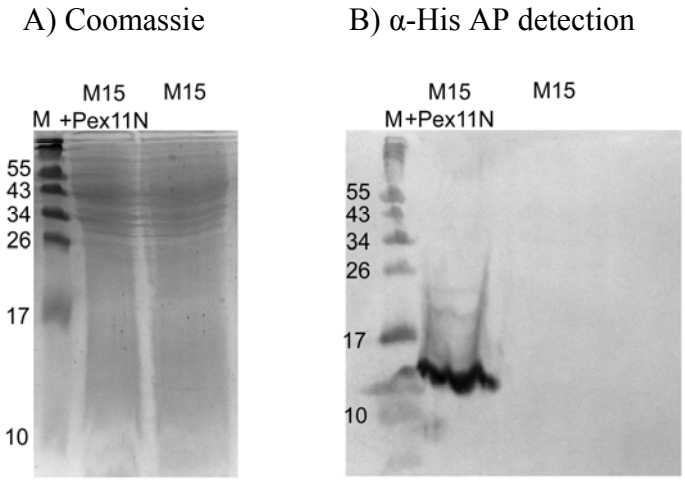

Fig.1. Analysis of the presence of Pex11N in crude extracts. A) Coomassie stained gel showing the soluble fraction of cell lysates of M15 cells expressing Pex11N (M15 + Pex11N) or empty M15 cells (M15); B) detection of Pex11N by Western blotting using α-His antibodies. Pex11N is present as indicated by a band of approximately 12 kD.

B. Liposome tubulation assay using total cell extracts.

Cell extracts were filtered through a 0.2 µm filter and used for *in vitro* tubulation assays using 100 nm diameter small unilamellar vesicles (SUVs) with a phospholipid content resembling that of the peroxisome membrane (0.2 mg/ml). The protein concentration used was 0.02 mg/ml; the final urea concentration was 32 mM. Reaction mixtures were incubated at room temperature, negatively stained and analyzed by electron microscopy. Extensive tubulation of liposomes was observed after addition of extracts containing Pex11N (Fig. 2, images at the left). In contrast tubulation of vesicles was not observed after addition of extracts from empty M15 cells (Fig. 2, images at the right).

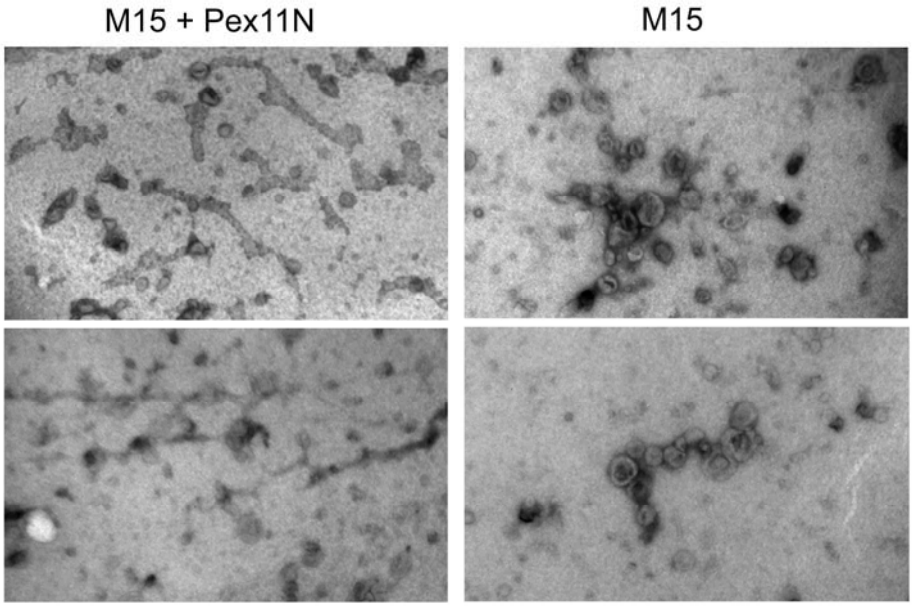

Fig. 2. Liposome tubulation assay using bacterial extracts. Extract from M15 cells expressing Pex11N (M15 + Pex11N) and from the empty M15 host (M15) were used in a liposome tubulation assay with SUVs (100 nm diameter) with a phospholipid content resembling that of the peroxisomal membrane.

II. Liposome tubulation assay using fractions highly enriched in Pex11N

Cell lysates were prepared as described above. Pex11N was subsequently purified using Ni-NTA affinity chromatography, followed by ion exchange chromatography using a MonoQ column. Essentially the same procedure was performed using a cell extract prepared from cells of the empty M15 host.

a. Induction and preparation of cell lysates

Induction and preparation of cell lysates was performed as described above. Fractions were separated by SDS-PAGE, blotted onto nitrocellulose. A total protein stain was performed using Ponceau-S. Subsequently the same blot was decorated with anti-His antibodies.

Upon induction the expected protein band at approx. 12 kD was observed on the Western blot (Fig. 3B). A portion of the protein appeared in the pellet fraction, but the bulk was present in the soluble fraction. The appearance of an extra protein band upon induction was not evident on the Ponceau-S stained blot (Fig. 3A), indicating that expression levels were relatively low.

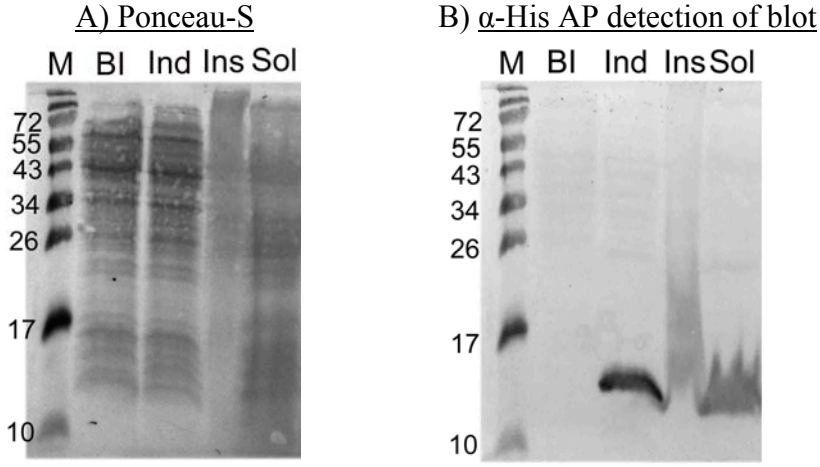

Fig. 3. Expression of Pex11N. A) Ponceau-S stained blot; B) detection of blot using α-His antibodies. **BI** – total cell lysate before induction, **Ind** – total cell lysate after induction with IPTG, **Ins** – pellet fraction after cell disruption, **Sol** – soluble fraction after cell disruption.

b. **Ni-NTA affinity chromatography**.
The soluble fraction was loaded onto a Ni-NTA column. The resin was washed with lysis buffer and proteins were eluted with elution buffer (100 mM NaH_2PO_4, 10 mM Tris, 8M urea, pH 4.5). Figure 4 shows the elution profiles of both lysates (*E. coli* M15 expressing Pex11N and the empty M15 cells as a control). 2 ml fractions were eluted until the A_{280} had decreased to 0.

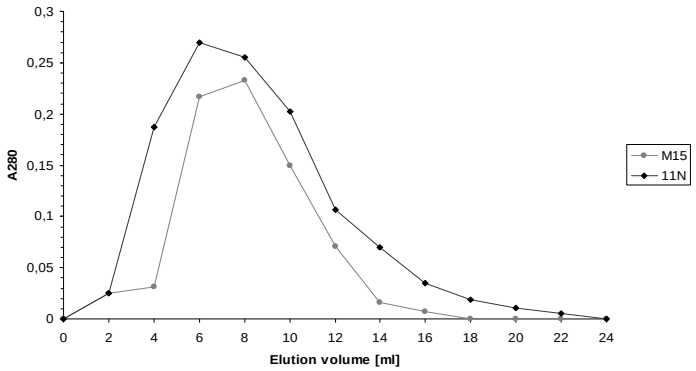

Fig. 4. Elution profiles from the Ni-NTA column for both empty M15 and M15 + Pex11N.

All fractions were analyzed by Western blotting. Blots were first stained with Ponceau-S (Fig. 5A) and subsequently decorated with anti-His antibodies (Fig. 5B).

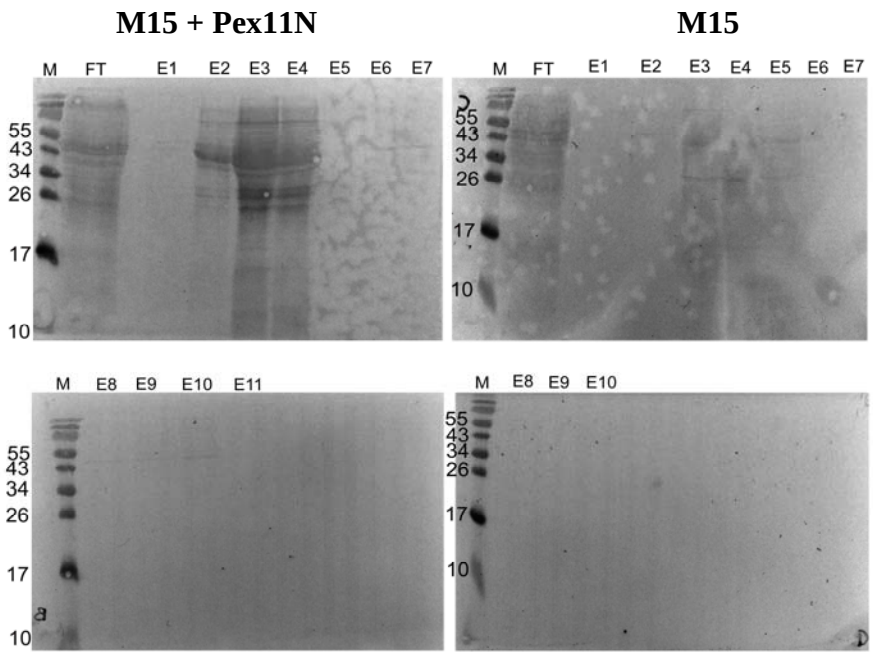

Fig. 5. A. Ponceau S staining of fractions obtained upon Ni-NTA affinity chromatography. FT – flow through. The numbers (E1-E11) correspond to the fractions shown in figure 4. The blots at the left are from M15+Pex11N, the blots at the right are from the empty M15 host.

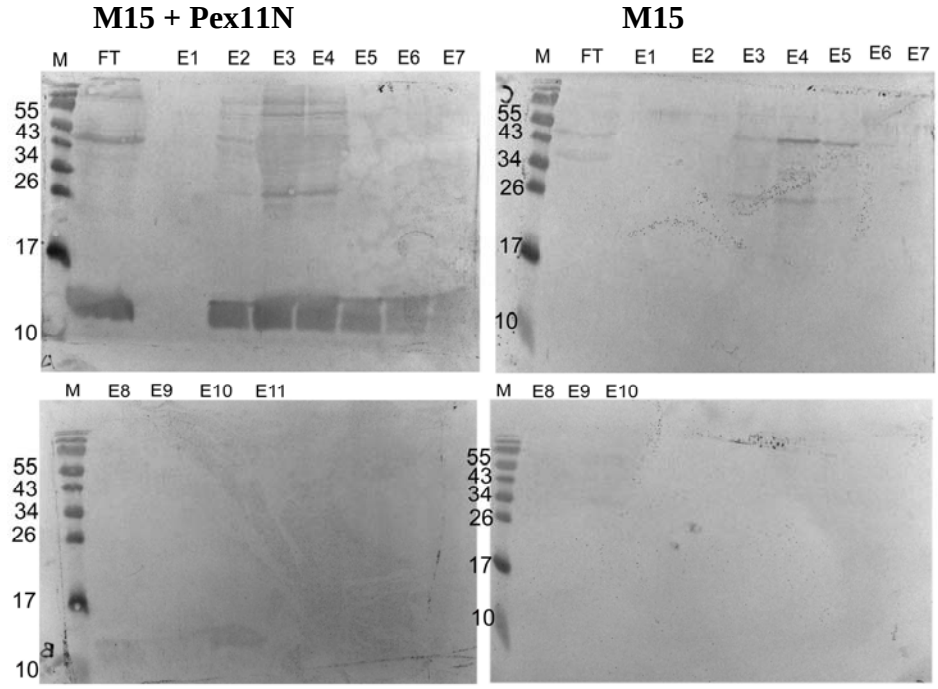

Fig. 5. **B** Anti-His detection of blots shown in Figure 5A. A protein band of approx. 12 kDa is present in fractions E2-E7 of the elution of M15+Pex11N. This band is absent in the eluate of the control.

For M15+Pex11N, the fractions E2 and E5 -10 were pooled and concentrated on a Amicon Ultra-4 YM-3 filter. Fractions E3 and E4 were excluded because of relatively high contamination. For the control (M15) the fractions E2-E7 were pooled and concentrated. The concentrated fractions were used for ion exchange chromatography.

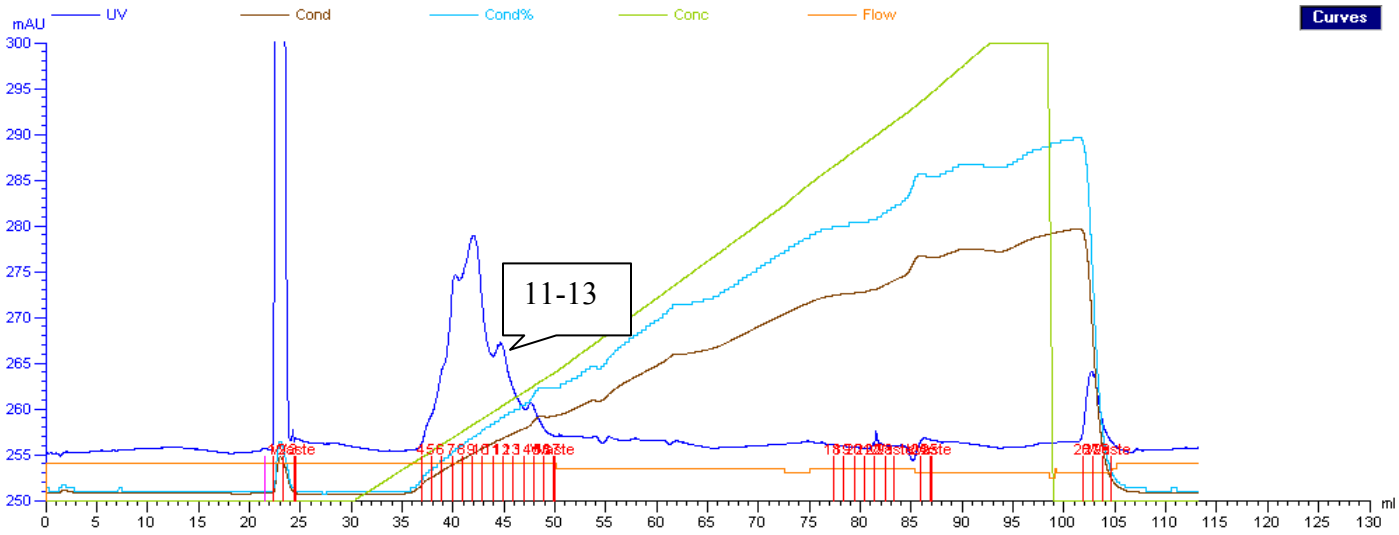

Fig. 6A. Elution profiles from Mono-Q column (FPLC) using the pooled fractions obtained after Ni-NTA chromatography of the M15 + Pex11N lysate. The fractions marked with a box contain Pex11N (see blots Figure 7). The dark blue line represents the absorbance at 280 nm. The green line represents the salt gradient (0 – 2M NaCl).

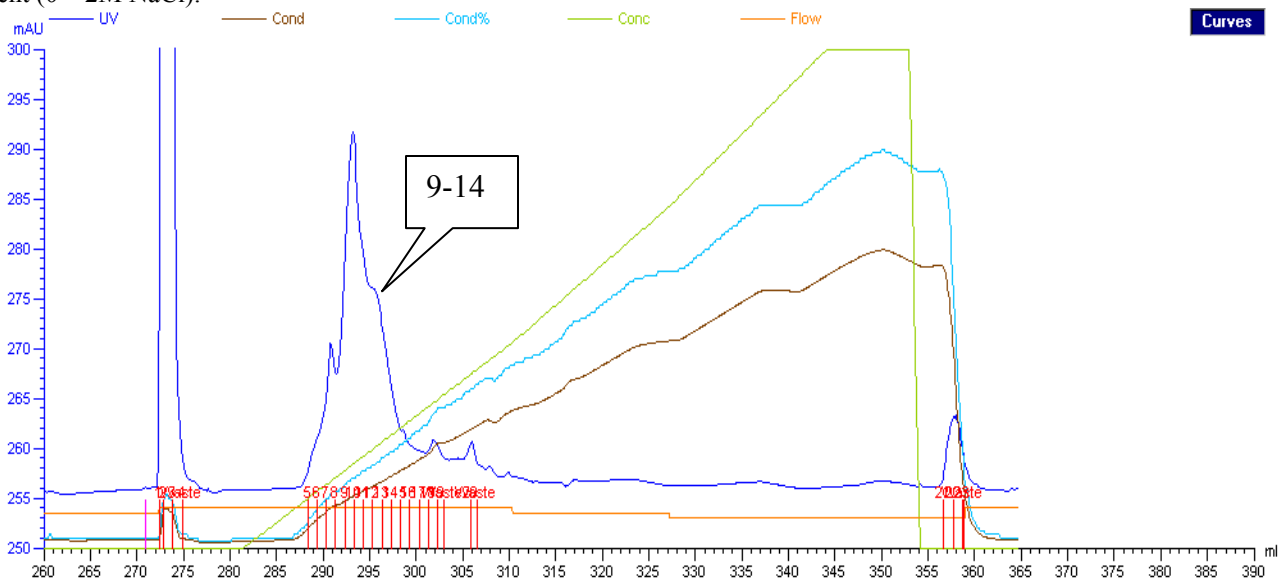

Fig. 6B. Elution profile from the Mono-Q column of the pooled fractions of the M15 empty host. The fractions marked with a box were pooled, concentrated and used as a control.

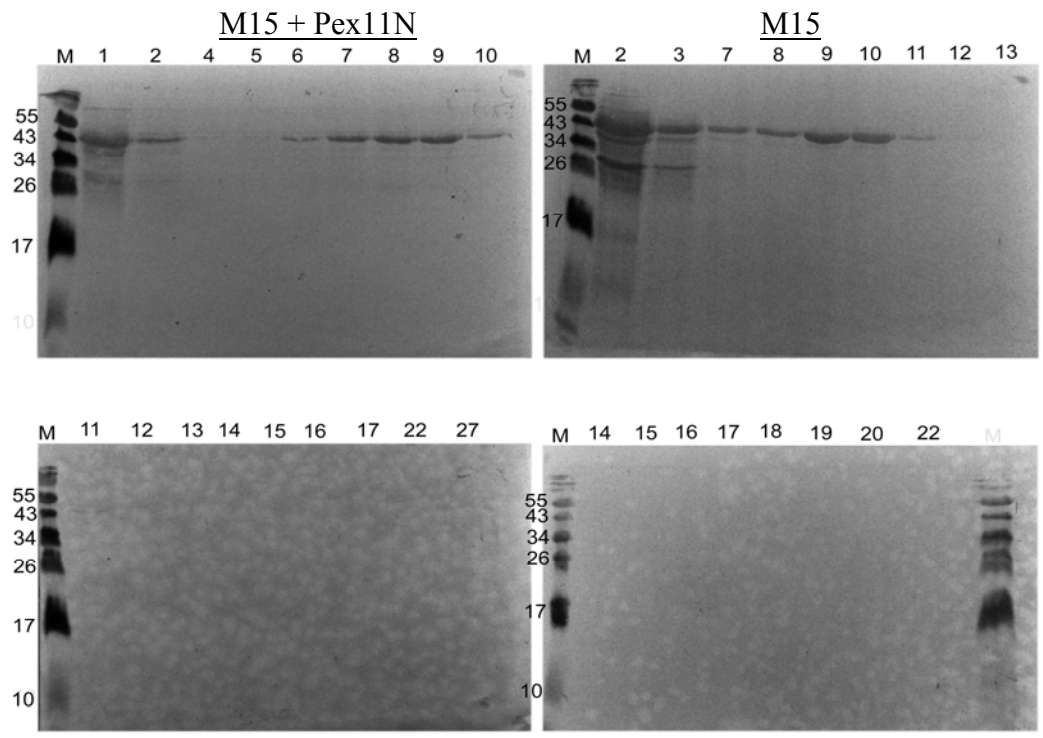

Fig. 7A. Ponceau S staining of Western blots of the fractions obtained upon MonoQ ion exchange chromatography.

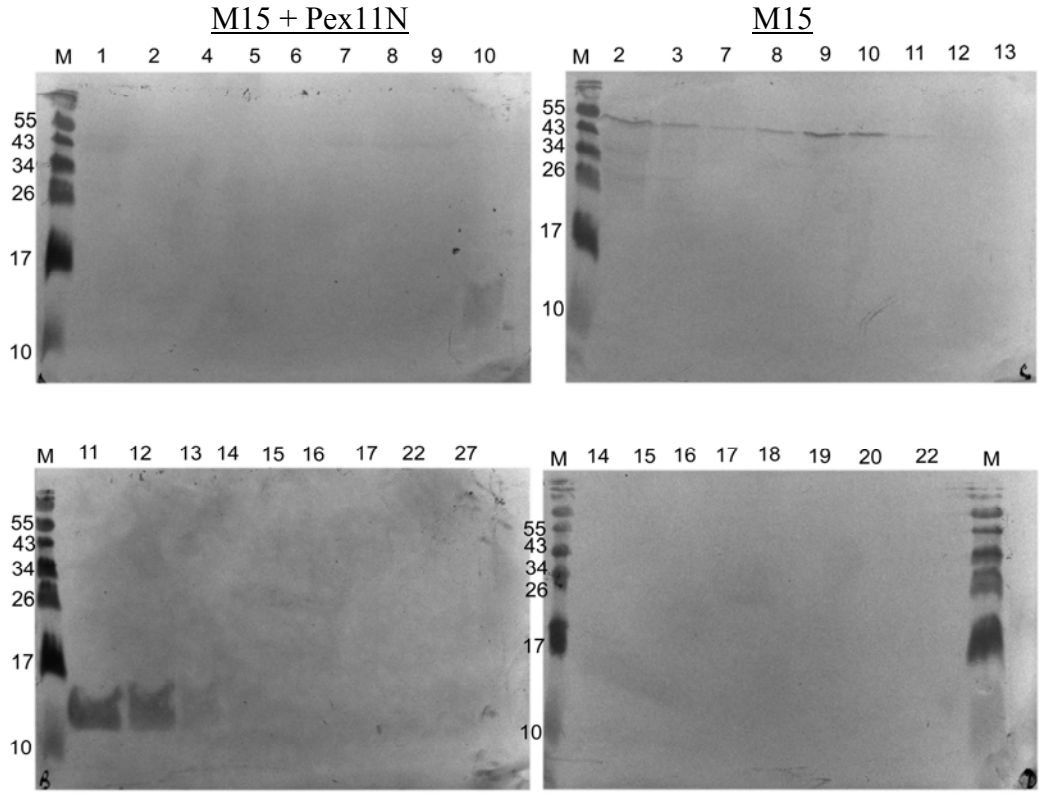

Fig. 7B. Detection of the blots shown in Fig. 7A using α-His antibodies. Left panels – purification from M15 + Pex11N, right panels- purification from empty M15 cells.

All fractions were analyzed by Western blotting. As shown in Figure 7 B, Pex11N was present in fractions 11, 12 and 13. These fractions were pooled. For the control (M15) the corresponding fractions were pooled (fractions 9-14). The pooled fractions were concentrated on an Amicon Ultra-4 YM-3 filters to protein concentrations of 80 µg/ml for M15 + Pex11N (300 µl in total) and 1.1 mg/ml for M15 (600 µl in total). Equal amounts of proteins (1.6 µg) were loaded on 15% acrylamide gels and used for Coomassie and Silver staining and for Western blots (Fig. 8).

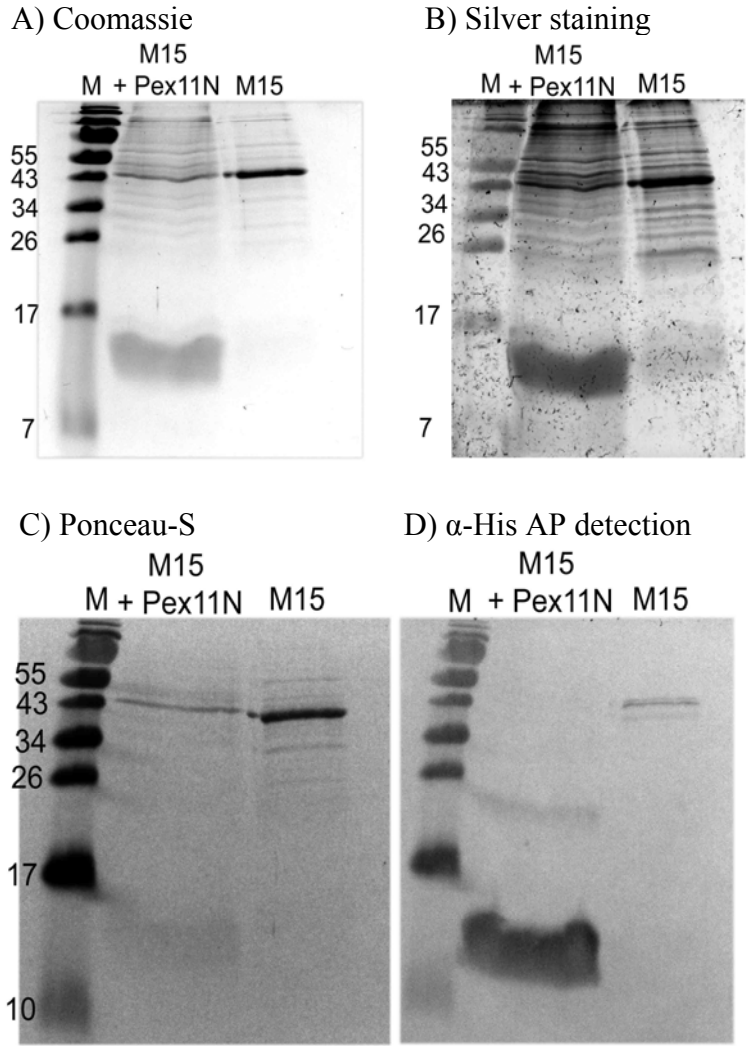

Fig. 8. Analysis of the final protein fraction obtained after purifications and concentration. A) Coomassie stained gel; B) Silver stained gel; C) Ponceau-S stained blot; D) detection of the blot with α-His antibodies; purification from empty M15 cells (M15) and M15 cells expressing Pex11N (M15 + Pex11N).

Protein fractions containing Pex11N and corresponding fractions obtained from cell extracts of the empty M15 host were rapidly diluted in liposome buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) in the presence of liposomes with a phospholipid content resembling that of peroxisome membranes (0.2 mg/ml), to a final protein concentration of 0.2 mg/ml and a urea concentration 0.8 M. Reaction mixtures were incubated for 15 minutes at room temperature and then placed on carbon coated grids and post stained with 0.5% uranyl acetate.

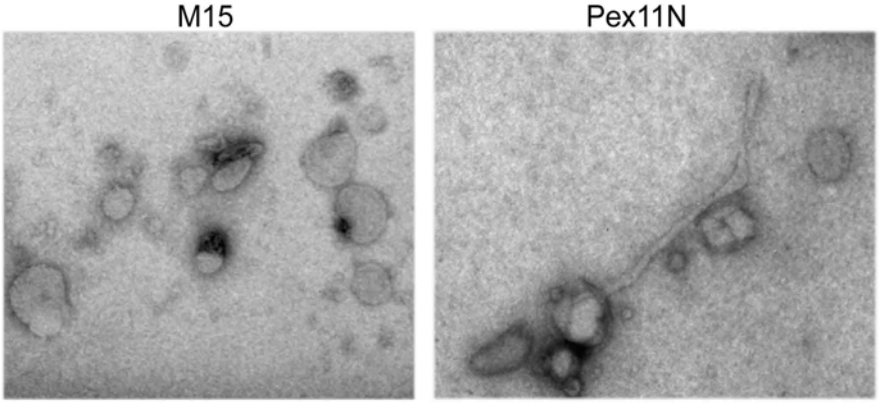

Fig. 9. Liposome tubulation assay purified Pex11N. Fractions containing purified Pex11N (Pex11N) and contaminating proteins present in Pex11N purified from empty M15 strain (M15) (both at 0.2 mg/ml, urea 0.8 M) were used in liposome tubulation assay with SUVs (100 nm diameter) with phospholipid content resembling peroxisomal membrane (0.2 mg/ml).

Tubulation of liposomes was only observed using the Pex11N fraction. Tubulation was never observed when the corresponding fraction of the M15 host was used (Fig. 9).