Mutations affecting the expression of the \textit{MOX} gene encoding peroxisomal methanol oxidase in \textit{Hansenula polymorpha}

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\textbf{Abstract} In this study, aimed at identifying genetic factors acting positively upon the \textit{MOX} gene, we report the isolation and characterisation of several methanol utilisation-defective (Mut\textsuperscript{−}) mutants of \textit{Hansenula polymorpha}. These fall into 12 complementation groups, eight of which show significant reductions in alcohol (methanol) oxidase activity in methanol. Three of these groups, identifying the \textit{MUT3}, \textit{MUT5} and \textit{MUT10} loci, exhibit extremely low levels of \textit{MOX} promoter activity, not only in methanol medium, but also during growth in glycerol or methyamine. We suggest that these loci play a significant role in the derepression of the \textit{MOX} gene expression. One of these genes (\textit{MUT10}) also seems to be involved in the utilisation of carbon sources other than methanol, and it is apparent that the same gene plays some role in the biogenesis or in the enlargement of the peroxisome. Three other genes (\textit{MUT7}, \textit{MUT8} and \textit{MUT9}) appear to be involved in peroxisome biogenesis, whereas most other mutants harbour lesions that leave the peroxisome biogenesis and proliferation unaffected.

\textbf{Key words} \textit{MOX} expression \cdot Mut\textsuperscript{−} mutants \cdot Peroxisome \cdot \textit{Hansenula polymorpha}

\textbf{Introduction}

Peroxisomes are single-membrane bound, multi-functional organelles found throughout the eukaryotes. Their importance in cellular metabolism has long been neglected, until their significance in plants, fungi and mammals was recognised (Van den Bosch et al. 1992). The recent discovery of human peroxisomal disorders raised several questions, primarily regarding microbody biogenesis and the control of their proliferation (Moser et al. 1991 and references therein; Masters and Crane 1992 and references therein).

Methylotrophic yeasts, such as \textit{Hansenula polymorpha} (syn. \textit{Pichia angusta}) are excellent models for the study of peroxisomes, due to their highly controllable peroxisome proliferation (Berardi 1997 and references therein). This is related to their metabolism for methanol utilisation (\textit{C\textsubscript{1}} metabolism), which employs methanol as the growth substrate (Veenhuis et al. 1983; Veenhuis and Harder 1991). In the peroxisome, methanol is oxidised to formaldehyde by means of an \textit{H\textsubscript{2}O\textsubscript{2}}-producing, flavin adenine dinucleotide-dependent alcohol (methanol) oxidase (AO), encoded by the \textit{MOX} gene (Ledeboer et al. 1985). This enzyme occurs in the peroxisomal matrix as octameric molecules with identical subunits arranged in two alternating layers, forming a highly regular structure, often referred to as crystalloid AO (reviewed in van der Klei et al. 1991).

AO can only take part in \textit{C\textsubscript{1}} metabolism if confined in the peroxisome, as shown by some peroxisome-deficient mutants which are unable to utilise methanol, even if their AO is active and correctly assembled (Cregg et al. 1990; Tan et al. 1995). The amount of AO parallels the extent of peroxisome proliferation and varies considerably, according to growth conditions (Veenhuis et al. 1983; Veenhuis and Harder 1991). Therefore, common regulatory mechanisms synchronising peroxisome proliferation and the synthesis of peroxisomal enzymes can be assumed. Maximum AO levels and peroxisome proliferation are observed during growth on methanol or on other derepressing substrates (e.g. glycerol) and in chemostat cultures at low dilution rates, when cells may contain over 20 tightly packed cuboid organelles, which occupy a large proportion of the cytoplasmic volume. The presence of other carbon sources, such as glucose or ethanol, represses both \textit{C\textsubscript{1}} metabolism (including the synthesis of AO) and the proliferation of \textit{C\textsubscript{1}}
peroxisomes, even in the presence of methanol (Veenhuis et al. 1983; Veenhuis and Harder 1991). In order to understand the mechanisms controlling peroxisome proliferation, the regulation of the expression of one or more genes encoding peroxisomal enzymes can be tackled. Steady-state mRNA studies indicate that the MOX gene is transcriptionally controlled and remarkably strong (Pereira 1994; Parpinello et al. 1998). Its regulation seems to involve mainly repression–derepression, rather than induction mechanisms (see Parpinello et al. 1998 and references therein). Functional analysis of the MOX promoter led to the identification of a complex promoter structure with some cooperating cis elements, and at least three specific DNA-binding proteins (Godecke et al. 1994).

Identifying and isolating the genetic factors involved in these processes can increase knowledge of these regulatory mechanisms. To this end, methanol utilisation-defective (Mut−) mutants of *H. polymorpha*, in which methanol utilisation is impaired, may serve not only to identify peroxisome-deficient mutants (Cregg et al. 1990; Tan et al. 1995), but also to unveil the mechanism activating peroxisome biogenesis and proliferation.

In this study, aimed at identifying genetic factors acting positively upon the MOX gene, we report the isolation and characterisation of several Mut− mutants of *H. polymorpha*. These fall into 12 complementation groups, eight of which show significant reductions of AO activity in methanol. In particular, we set out to find mutations causing an extremely defective phenotype, i.e. low MOX promoter activities not only in methanol, but also in other derepressing conditions (glycerol growth, methylyamine growth). Three complementation groups, identifying the MUT3, MUT5 and MUT10 loci, fulfil these requirements, exhibiting extremely low levels of MOX promoter activity not only in methanol medium, but also during growth in glycerol or methylyamine.

### Materials and methods

#### Strains

All strains used in this work are derivatives of *H. polymorpha* homothallic haploidNCYC-495. L1 (leu1-1) is a Mut− strain used to isolate all the Mut− mutants and as a control in all experiments (Gleeson et al. 1986). M6 (met6-1 and A11 (ade1-1), obtained from P. Sudbery (University of Sheffield, Sheffield, UK), are also Mut− strains used to obtain Mut−/Mut+ diploids. NLAM (ade1-1, met6-1, Amox) and NLAC (ura3-1, Acat), obtained from M. Veenhuis (University of Groningen, Groningen, the Netherlands), are Mut− strains with deletions in the MOX or cat genes, used as “testers” to identify which of the Mut− mutants were mox or cat. The *Escherichia coli* strain MC1061 [hisD rcr mcrB araD139 araABC-lexA7679 lacZ74 galU galK rpsL thi] was used in plasmid construction.

#### Media

YPD medium contained 2% glucose, 1% yeast extract and 2% peptone. ME medium contained 2% malt extract. The minimal media (DM1, DM2, E1, G1, M1, M2 and X1) contained 0.5% ammonium sulfate, 0.2% yeast nitrogen base without amino acids or ammonium sulfate (Difco); when necessary 0.006% leucine, 0.002% methionine, 0.004% adenine or 0.002% uracil were added. In addition DM1 contained 2% glucose, E1 contained 1% ethanol, G1 contained 1% glycerol, M1 contained 0.5% methanol, M2 contained 1% methanol and X1 contained 2% xylose. DM2 had the same composition as DM1, but 0.25% methylamine replaced the ammonium sulfate. When necessary, 1.8% agar was added.

#### Cultures

All cultures were grown in 250-ml flasks with 100 ml medium, using an orbital incubator at 37 °C and 220 rpm.

#### Induction experiments

After 12 h pre-cultivation in YPD, the cells were inoculated in an induction medium (DM2, G1 or M1). Cell number (direct count) and enzyme activities were determined when necessary.

#### Genetic methods

Mut− mutants were obtained by negative selection. 2 × 10^4 L1 cells were plated on DM1, UV-irradiated (mortality: 95%) and incubated at 37 °C for 3 days. Colonies were purified and tested for methanol growth by replica plating on M2. All colonies that failed to grow on methanol plates were streak-purified on YPD plates and then re-tested to confirm the Mut− phenotype. Crosses and sporulations were on ME plates, as described by Gleeson and Sudbery (1988).

#### Biochemical methods

Plate colony assays, used for the preliminary visualisation of AO activity in the mutants, were done on methanol plates as described by Titorenko et al. (1995). o-Dianisidine (0.5 mg/ml), instead of 2,2′-azinobis-3-ethylbenzthiazoline-sulfonic acid, was used as a chromogenic agent.

Crude extracts were obtained by glass bead vortexing (eight times, 30 s each time) in 50 mM potassium phosphate buffer, pH 7.0 (1 vol. Sigma No. G-9268 beads:1 vol. cells). Enzyme activities of methanol oxidase (AO; EC 1.1.3.13), amine oxidase (EC 1.4.3.4), glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6) were assayed by established procedures (Lück 1963; Large and Haywood 1990; van der Klei et al. 1990; Hodgkins et al. 1993).

Protein concentrations were determined using a Bio-Rad kit (Cat. No. 500-0006; Bio-Rad, USA) with bovine serum albumin as the standard.

#### Transformants and molecular methods

DNA manipulations were carried out according to standard methods (Sambrook et al. 1989). *H. polymorpha* was transformed by the LiAc method of Berardi and Thomas (1990). The transformating plasmid, pHHL-31, harbours the reporter cassette (MOXp::xMF::GOD) described by Hodgkins et al. (1993). It was obtained by joining the 5-kbp BamHI fragment of plasmid pWG31 (Hodgkins et al. 1993) to the replicative vector pHHL-01.

#### MOX mRNA levels

RNA was extracted using the method of Rose et al. (1990) and was analysed by Northern blotting, using formaldehyde as a denaturing agent (Sambrook et al. 1989). RNA was transferred to a Hybond-N+ membrane (Amersham, UK) and hybridised using standard conditions with probes made by the random primer method (Sambrook et al. 1989), using DNA fragments derived from the