Regulation of the *Hansenula polymorpha* maltase gene promoter in *H. polymorpha* and *Saccharomyces cerevisiae*¹

Tiina Alamäe *, Pille Pärn, Katrin Viigand, Helen Karp

Institute of Molecular and Cell Biology, University of Tartu, Riia 23, 51010 Tartu, Estonia

Received 20 December 2002; received in revised form 11 April 2003; accepted 14 April 2003

First published online 17 May 2003

Abstract

*Hansenula polymorpha* is an exception among methylotrophic yeasts because it can grow on the disaccharides maltose and sucrose. We disrupted the maltase gene (*HPMAL1*) in *H. polymorpha* 201 using homologous recombination. Resulting disruptants HP201HPMAL1v failed to grow on maltose and sucrose, showing that maltase is essential for the growth of *H. polymorpha* on both disaccharides. Expression of *HPMAL1* in HP201HPMAL1Δ from the truncated variants of the promoter enabled us to define the 5'-upstream region as sufficient for the induction of maltase by disaccharides and its repression by glucose. Expression of the *Saccharomyces cerevisiae* maltase gene *MAL62* was induced by maltose and sucrose, and repressed by glucose if expressed in HP201HPMAL1Δ from its own promoter. Similarly, the *HPMAL1* promoter was recognized and correctly regulated by the carbon source in a *S. cerevisiae* maltase-negative mutant 100-1B. Therefore we suggest that the transcriptional regulators of *S. cerevisiae* MAL genes (*MAL* activator and Mig1 repressor) can affect the expression of the *H. polymorpha* maltase gene, and that homologues of these proteins may exist in *H. polymorpha*. Using the *HPMAL1* gene as a reporter in a *H. polymorpha* maltase disruption mutant it was shown that the strength of the *HPMAL1* promoter if induced by sucrose is quite comparable to the strength of the *H. polymorpha* alcohol oxidase promoter under conditions of methanol induction, revealing the biotechnological potential of the *HPMAL1* promoter.

© 2003 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: *Hansenula polymorpha*; *HPMAL1*; Maltase; Gene disruption; Alcohol oxidase promoter

1. Introduction

*Hansenula polymorpha* is a popular host for the production of foreign proteins. Mostly, the promoter of the alcohol oxidase gene (*MOX*) has been used to drive the expression of various genes in a carbon source-regulated manner. This promoter is glucose-repressed, moderately derepressed during growth on glycerol, and strongly induced by methanol, thus enabling tightly regulated production of the protein of interest [1]. The nitrogen-regulated promoters of nitrate and nitrite utilization genes of *H. polymorpha* have also been characterized [2] and can potentially be used in biotechnological expression systems. In contrast to other methylotrophic yeasts, *H. polymorpha* grows on the disaccharides maltose and sucrose. High maltase activity is induced in maltose- and sucrose-grown *H. polymorpha*, and the induction is repressed by glucose [3]. In our previous work we have cloned and characterized the maltase gene *HPMAL1* from *H. polymorpha* [4] with the aim of studying its regulation from the aspect of glucose repression. Genetics and regulation of maltose utilization have been thoroughly studied in *Saccharomyces cerevisiae* [5] as maltose is the main sugar in baking and brewing. Growth of *S. cerevisiae* on maltose requires three genes encoding a maltose permease (*MALx1*), a maltase (*MALx2*) and an activator of these genes (*MALx3*), 'x' designating the number of the *MAL* locus [5]. The above-mentioned *MAL* genes are clustered to five unlinked *MAL* loci: *MAL1*, *MAL2*, *MAL3*, *MAL4* and *MAL6*, situated near telomeres of five different chromosomes [5–7]. Glucose repression of maltose utilization in
S. cerevisiae is executed by binding of a zinc finger repressor protein Mig1 to the promoters of all three MAL genes [8,9]. In addition, in S. cerevisiae hexokinase PI1 protein is crucial for the primary signaling of glucose repression [10] and mutants deficient in this protein show reduced glucose repression of maltase [11], whereas glucokinase cannot substitute hexokinase PI1 in glucose repression [12]. Our previous data have shown that H. polymorpha differs from S. cerevisiae in primary sensing of glucose because both hexose kinases of H. polymorpha, hexokinase and glucokinase, are able to transmit the glucose repression signal in this yeast [13,14]. The current paper studies the carbon source-dependent expression of the H. polymorpha maltase gene HPMA1 in H. polymorpha and in S. cerevisiae in order to compare its regulation in these two yeasts. The strength of the promoter is evaluated and possibilities of using the HPMA1 promoter in biotechnology are discussed.

2. Materials and methods

2.1. Strains and plasmids

H. polymorpha 201 (leu2-2 ura3-1 met4-220) provided by K. Luhitech (Sofia, Bulgaria) was used as a strain in which the maltase gene HPMA1 was disrupted. The maltase-negative strain S. cerevisiae 100-1B (Mata ura3-52 leu2 MAL13 MAL11 MAL12::LEU2) [6] was used for the heterologous expression of HPMA1. Escherichia coli strains TG1 and DH5α were used in DNA amplification and cloning procedures. p51 is a library clone with a genomic insert of approximately 5.6 kb containing HPMA1 in pYT3 [4]. HPMA1 was subcloned from p51 to S. cerevisiae E. coli high-copy vectors pRS425 and pRS426 [15] to study the regulation of HPMA1 in maltase disruption mutants of H. polymorpha and S. cerevisiae. pRS425 can replicate in H. polymorpha because it contains the S. cerevisiae LEU2 gene that has ARS activity in H. polymorpha [16]. The SpeI-SmaI fragment of the genomic insert in p51 (see Fig. 11) was cloned to pRS425 and pRS426 opened with the same enzymes resulting in pRS425-p51SmaI and pRS426-p51SmaI, respectively. In these vectors the HPMA1 gene is behind the 1242 bp of the 5’-upstream region (Fig. 1), further named the full-length promoter. Constructs harboring HPMA1 behind the medium-length promoter (containing the region up to -315 bp from the maltase gene ATG) were designed starting from pSK51MunI [4]. This plasmid contains a 2064-bp MunI fragment (see Fig. 1) of p51 in the SmaI site of pBluescript SK polynlinker.

The above-mentioned fragment was excised from pSK51MunI using NotI-EcoRI digestion and inserted between the same sites in pRS426 polynlinker resulting in pRS426-p51MunI. The NotI-SalI fragment of pRS426-p51MunI was moved to pRS425 cut with the same enzymes, yielding pRS425-p51MunI. The maltase gene promoter region in pRS425-p51MunI was further shortened to the NruI site at position -127 bp from the maltase gene ATG, by restriction of pRS425-p51MunI with NruI and SalI and religating the ends, resulting in pRS425-p51NruI-MunI (HPMA1 behind the short promoter; Fig. 1). The S. cerevisiae maltase gene MAL62 was cloned as the SpeI-Eco105I fragment (besides MAL62 includes the entire MAL61-MAL62 intergenic region and part of the MAL61 gene) from pY6 [7] into pRS425 opened with SmaI and SpeI resulting in pRS425-MAL62. To study the expression of HPMA1 from the H. polymorpha alcohol oxidase promoter, the HindIII-MunI fragment (Fig. 1) including the open reading frame of HPMA1 and 68 bp of the 5’-upstream region was excised from pSK51MunI and inserted into pX4-HNBEX, a derivative of pHIPX4 (see http://www.biol.rug.nl/em) opened with HindIII and EcoRI yielding pX4-HPMA1. To create a plasmid that has a similar plasmid backbone as pX4-HPMA1 but harbors the H. polymorpha maltase gene promoter instead of the alcohol oxidase promoter, the NotI-SalI fragment of pRS425-p51SmaI containing HPMA1 with its full-length promoter was inserted into NotI-SalI-cut pHIPX8 (see http://www.biol.rug.nl/em/) creating pHIPX8-p51SmaI. The plasmid pSK51MunI-HPURA3 used for the genomic disruption of HPMA1 was designed from

![Fig. 1. Map of the 5’-upstream region of the H. polymorpha maltase gene HPMA1. The location of the putative maltose permease gene is indicated. The intergenic region is drawn to scale. The restriction sites used for the subcloning and disruption of HPMA1 as well as the annealing positions of primers MAL5 and MAL9 used to verify maltase gene disruption are also shown.](image-url)
pSK51MunI by replacing the 456-bp Bsp911-BglII fragment (Fig. 1) of the HPMAL1 coding region with the H. polymorpha URA3 gene on a 1795-bp BamHI-BglII fragment. The linear MunI fragment with disrupted HPMAL1 (Fig. 1) was transformed to H. polymorpha 201 to disrupt the genomic HPMAL1.

2.2. Cultivation of cells

Yeasts were grown on 0.67% Difco yeast nitrogen base medium without amino acids with adequate auxotrophic supplements or in 1% yeast extract-2% peptone medium. Agar was added at 2% for solid media. Carbon sources were supplemented at concentrations shown in the text. Disaccharides were autoclaved separately in distilled water and added before the inoculation. E. coli was grown in Luria–Bertani medium by adding ampicillin (0.1 mg ml\(^{-1}\)) or kanamycin (0.05 mg ml\(^{-1}\)) when required. The cultivation temperature of H. polymorpha and E. coli was 37°C. S. cerevisiae was grown at 30°C. Cells for the enzymatic activity measurements were harvested from the exponential growth phase (OD\(_{600}\) nm \(\sim\) 2.0).

For comparison of the strength of the promoters of the HPMAL1 and MOX genes, the transformants of HP201HPMAL1\(\Delta\) carrying HPMAL1 on a plasmid behind its native promoter or the MOX promoter were grown as follows. Cells were cultured on 2% glycerol medium until the exponential growth phase and a culture sample was withdrawn to register the initial level of maltose activity (zero time points in Fig. 5). Then methanol (final concentration 0.5%) was added to the culture of HP201HPMAL1\(\Delta\) (pX4-HPMAL1) to induce the alcohol oxidase promoter and suspension samples were collected after further cultivation for 2.5, 5.0 and 7.5 h to measure the maltase activity. The maltase gene promoter in the transformants HP201HPMAL1\(\Delta\) (pRS425-p51SpeI3maI) was induced with either 0.5% or 2.0% of a disaccharide (sucrose or maltose) and the cells were harvested at the same time points as indicated above.

2.3. Isolation of DNA, restriction digestions, DNA sequencing and transformation

DNA manipulations were carried out using standard methods [17]. Bacterial plasmid DNA was purified using Perfectprep plasmid minikit (Eppendorf). Yeast genomic DNA was isolated as shown by us earlier [4]. Restriction endonuclease digestions and DNA ligation were performed according to the manufacturers’ recommendations. DNA was sequenced from both strands using an ABI Prism® 377 DNA sequencer (Perkin Elmer) and DYEnamic® ET terminator cycle sequencing kit (Amersham). E. coli was transformed with plasmid DNA according to Hanahan [18]. H. polymorpha and S. cerevisiae were transformed using Li-acetate [19], while the heat shock for H. polymorpha was performed at 45°C.

2.4. Assay of maltase activity

Cells for the preparation of extracts were harvested by centrifugation at 4°C, washed twice in 100 mM K-phosphate buffer (pH 6.5) containing 0.1 mM EDTA, suspended in 200–400 μl of the same buffer and disrupted using glass beads. Supernatant obtained after the centrifugation (30 min at 12 000 \(\times\) g at 4°C) was used as cell extract. Maltase activity in the extract was measured using p-nitrophenyl-\(\alpha\)-D-glucopyranoside as a substrate [4] and expressed as nmol of substrate transformed per minute per mg of protein (mU mg\(^{-1}\)). Protein concentration in the cell extract was determined according to the Lowry method. Maltase values presented are the average values of triplicate measurements with cell extracts from two or three distinct transformants. Standard deviations were less than 20%.

3. Results and discussion

3.1. Disruption of the HPMAL1 in H. polymorpha 201

Previously we have shown that the synthesis of maltase in H. polymorpha is induced during growth of the cells on both maltose and sucrose [3], indicating that growth of the yeast on these disaccharides may depend on maltase. To verify the former hypothesis, the maltase gene HPMAL1 [4] was disrupted in H. polymorpha 201 using the disruption plasmid pSK51MunI-HPURA3. The linear MunI fragment of pSK51MunI-HPURA3 (see Section 2.1 and Fig. 1) was used to transform H. polymorpha 201 to uracil prototrophy. About 30% of glucose-grown uracil-prototrophic colonies did not grow on maltose or sucrose, indicating the possible disruption of the maltase gene. Then genomic DNA was isolated from several independent uracil-prototrophic/maltose-negative clones and polymerase chain reaction (PCR) analysis was carried out using the HPMAL1-specific primers MAL5 and MAL9 (annealing positions of the primers shown in Fig. 1). In the case of the intact maltase gene, the specific PCR product amplified from the genomic DNA by using these primers should be 655 bp long, whereas in the case of expected genomic disruption it should be much longer (1994 bp) due to the insertion of the H. polymorpha URA3 gene into the coding region of HPMAL1. Agarose gel electrophoresis of specific PCR fragments shown in Fig. 2 indicated the predicted genomic disruption of HPMAL1. As a next step, the HPMAL1 disruptant of H. polymorpha 201 (further designated HP201HPMAL1\(\Delta\)) was transformed with p51 containing HPMAL1 and with the respective empty vector pYT3 to study complementation of the gene disruption with HPMAL1 on a plasmid. The cells were streaked to glucose-containing medium and thereafter replicated to maltose- and sucrose-containing media. Fig. 3 shows that the disruptant harboring the empty vector pYT3
3.2. Sequence analysis of the 5′-neighborhood of the MAL62 gene in p51 reveals beginning of a putative divergently transcribed maltose permease gene

In different MAL loci of S. cerevisiae the structural genes for maltase and maltose permease reside adjacent and are divergently transcribed from a shared promoter region [5]. This situation is similar in Kluyveromyces lactis (see sequence data under GenBank accession number AJ007636). To find out if the structure of the MAL locus in H. polymorpha might be alike, we further sequenced the upstream region of the HMPAL1 gene in the genomic insert of plasmid p51 and revealed the beginning of the open reading frame (ORF) transcribed from the opposite strand. The protein sequence deduced from the ORF revealed a high similarity to maltose-transporting proteins from S. cerevisiae, a maltose permease Mal31p and an α-glucoside transporter [23]. Unfortunately, p51 contained approximately only half of the putative maltose permease gene. Therefore, further work has to be done to obtain the full sequence and to verify the functionality of the gene. Our data indicated that the length of the intergenic region between the H. polymorpha maltase gene and a putative maltose permease gene was 1253 bp (Fig. 1). The respective region between the Saccharomyces carlsbergensis MAL61-MAL62 genes is 884 bp long [24], but in industrial strains of S. cerevisiae it is longer due to the tandem insertion of several 147-bp repeats [25]. The length of the intergenic region of the K. lactis maltose permease and maltase genes is 1107 bp (GenBank accession number AF261762). It is interesting to note that in H. polymorpha the genes needed for nitrate assimilation are also clustered [26].

3.3. Study of the regulation of the HMPAL1 promoter in HP201HMPAL1Δ using promoter truncations

In order to define the minimum-length promoter region
Table 1
Regulation of the \textit{H. polymorpha} maltase gene \textit{HPMAL1} and the \textit{S. cerevisiae} maltase gene \textit{MAL62} in the \textit{H. polymorpha} maltase disruption mutant \textit{HP201HPMAL1\Delta}

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description of the construct</th>
<th>Maltase activity (mU mg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS425-p51MunI</td>
<td>\textit{HPMAL1} behind the medium-length promoter</td>
<td>G  2330 M 3 G+M 1970 S 9 G+S 7</td>
</tr>
<tr>
<td>pRS425-p51SpeI</td>
<td>\textit{HPMAL1} behind the full-length promoter</td>
<td>BD 2350 M 4 S 993 G 13</td>
</tr>
<tr>
<td>pRS425-MAL62</td>
<td>\textit{S. cerevisiae} maltase gene \textit{MAL62}</td>
<td>BD 928 BD 2 BD No growth BD 13</td>
</tr>
<tr>
<td>pRS425</td>
<td>Empty vector (control)</td>
<td>BD No growth BD No growth BD 13</td>
</tr>
</tbody>
</table>

Transformants carrying the respective maltase genes on plasmid pRS425 were grown on different carbon sources until the mid-exponential growth phase and maltase activity was measured in cell extracts. Medium data of two to three distinct transformants are shown. Standard deviation was less than 20%.

BD, activity was below detection; G, 2\% glucose; M, 2\% maltose; G+M, 2\% glucose+2\% maltose; S, 2\% sucrose; G+S, 2\% glucose+2\% sucrose.

Fig. 4. Alignment of the N-terminal zinc finger regions of Mig1 proteins from \textit{S. cerevisiae} (SCMig1), \textit{S. occidentalis} (SOMig1) and \textit{C. utilis} (CUMig1) with the protein sequence deduced from a genomic sequence of \textit{H. polymorpha} AL435931 (GenBank accession number). The alignment was performed using the Clustal W program [36]. Identical residues and similar residues in all sequences are denoted below the alignment by asterisks and dots, respectively. Identity and similarity of aligned proteins with the putative Mig1 protein of \textit{H. polymorpha} is shown by black and gray shading, respectively. Crucial cysteine and histidine residues of zinc fingers 1 and 2 are designated by asterisks above the alignment.

3.4. Regulation of the \textit{S. cerevisiae} maltase gene \textit{MAL62} in \textit{HP201HPMAL1\Delta}

As the \textit{S. cerevisiae} maltase gene \textit{MAL62} restored growth of \textit{HP201HPMAL1}\Delta on maltose and sucrose when expressed from its own promoter (Fig. 3), we were interested in the regulation of \textit{MAL62} in \textit{HP201HPMAL1\Delta}. The transformants of \textit{HP201HPMAL1\Delta} harboring the \textit{MAL62} on pRS426 were grown as described in Section 3.3 and maltase activity was measured in the cells. Table 1 shows that the \textit{S. cerevisiae} \textit{MAL62} promoter was perfectly recognized and correctly regulated in \textit{H. polymorpha} – it was properly induced by disaccharides and strongly repressed by glucose. Therefore we presume that \textit{H. polymorpha} possesses homologues of the proteins that are able to regulate the transcription of \textit{MAL62}. According to the literature, transcriptional regulators of maltase genes from other yeasts can function in \textit{S. cerevisiae}. For example, the Mig1 repressor homologues from \textit{Candida utilis} [27], \textit{K. lactis} [28] and \textit{Schwanniomyces occidentalis} [29] complement the Mig1 deficiency in \textit{S. cerevisiae}, and the \textit{MAL}
Table 2

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description of the construct</th>
<th>Maltsate activity (mU mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS426-p51MunII</td>
<td>HPMAL1 behind the medium-length promoter</td>
<td>108 1260 145</td>
</tr>
<tr>
<td>pRS426-p51SpeSmaI</td>
<td>HPMAL1 behind the full-length promoter</td>
<td>5 862 5</td>
</tr>
<tr>
<td>pRS426</td>
<td>Empty vector (control)</td>
<td>BD No growth BD</td>
</tr>
</tbody>
</table>

Transformants carrying HPMAL1 on the plasmid pRS426 were grown on different carbon sources until the mid-exponential growth phase and maltase activity was measured in cell extracts. Medium data of two to three distinct transformants are shown. Standard deviation was less than 20%.

For abbreviations see footnote to Table 1.

activator of C. albicans (CASUC1) can substitute the function of MAL63 activator protein in S. cerevisiae [30]. We inspected the GenBank data resulting from the partial genomic sequencing project of H. polymorpha [31] in order to find nucleotide sequences that might encode homologues of S. cerevisiae MAL activator and Mig1 repressor proteins. We found that the protein sequence deduced from a H. polymorpha genomic sequence AL431330 (1013 bp in total) shows similarity to the MAL activators CASUC1 from C. albicans and Mal13 from S. cerevisiae. In addition, the protein fragment deduced from a H. polymorpha genomic sequence AL435931 (979 bp in total) exhibited good homology to N-terminal zinc fingers of Mig1 proteins from S. cerevisiae, S. occidentalis and C. utilis (Fig. 4). The above-mentioned DNA binding zinc finger domain is strongly conserved among the Mig1 homologues of different yeasts [29,32]. Thus, we hypothesize that homologues of the MAL activator and Mig1 proteins may exist in H. polymorpha.

3.5. Regulation of the HP Mal1 promoter in S. cerevisiae 100-1B

Fig. 3 shows that HP MAL1 corrects the maltose growth deficiency in the S. cerevisiae maltase disruption mutant 100-1B if expressed from its own promoter. To analyze the regulation of HP MAL1 in S. cerevisiae, mutant 100-1B was transformed with HP MAL1 behind the full- and medium-length promoters. Table 2 shows that both promoter variants were maltose-inducible in a heterologous host S. cerevisiae 100-1B with the medium-length promoter showing slightly higher induction compared to the full-length one. If the cells were grown on either glucose or glucose+maltose, expression of HP MAL1 from the full-length promoter was strongly glucose-repressed while the medium-length promoter showed clearly reduced glucose repression. We suggest that the MAL activator of S. cerevisiae can bind the HP MAL1 promoter and thus activate the transcription. The recognition sequence for the S. cerevisiae MAL activator protein has been reported to be c/aGCN9c/aGC/g [33]. A search for putative binding sites for the S. cerevisiae MAL activator in the HP MAL1 promoter region did not result in a perfect match with this consensus. However, the motif CGGCGAGTTTTCCGG at position −759 to −745 shares some similarity within the GC-rich borders with the Mal63 binding site 1 in the MAL61-MAL62 promoter [9]. Interestingly, this motif partially overlaps with the CCGGGG box at position −748 to −743 that might bind the Mig1 repressor protein. A similar GC box that is present in the promoter of the S. cerevisiae MAL63 gene (GTATTAAACCAGGGTGTA; GC box underlined) binds Mig1 protein strongly [8]. It should be noted that overlap of the binding sites for the MAL activator and Mig1 repressor is described in the MAL61-MAL62 divergent promoter, and it results in competition of these two proteins for the binding [9]. The above-mentioned as well as other putative binding sites for the S. cerevisiae Mig1 repressor and MAL activator proteins found in the 5′-upstream region of HP MAL1 are presented in Table 3.

Table 3

<p>| Cx-elements in the 5′-upstream region of the H. polymorpha maltase gene that might be important for the expression of the gene in E. coli [4] and in S. cerevisiae |</p>
<table>
<thead>
<tr>
<th>Element and distance from the maltase gene ATG</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>−310 TGGACGCAATTTCGCGCCGAGATTATT</td>
<td>−282 TGGACGCAATTTCGCGCCGAGATTATT</td>
</tr>
<tr>
<td>−213 GTGACGATTAATTACCAATAGATTTAATTAT</td>
<td>−185 GTGACGATTAATTACCAATAGATTTAATTAT</td>
</tr>
<tr>
<td>−265 CTGAAATTATAGCC</td>
<td>−251 CTGAAATTATAGCC</td>
</tr>
<tr>
<td>−486 CGCAGTTTTGTTAGCC</td>
<td>−472 CGCAGTTTTGTTAGCC</td>
</tr>
<tr>
<td>−759 CGCAGTTTTTTCGG</td>
<td>−745 CGCAGTTTTTTCGG</td>
</tr>
<tr>
<td>−824 CGCAGATTAAATTCGG</td>
<td>−838 CGCAGATTAAATTCGG</td>
</tr>
<tr>
<td>−674 CCCCGC</td>
<td>−669 CCCCGC</td>
</tr>
<tr>
<td>−748 CGCAGG</td>
<td>−743 CGCAGG</td>
</tr>
<tr>
<td>−775 CGCAGG</td>
<td>−770 CGCAGG</td>
</tr>
<tr>
<td>−1185 GTGCGG</td>
<td>−1180 GTGCGG</td>
</tr>
</tbody>
</table>

The putative binding sites retained in the medium-length promoter are shown in bold.
Table 3 also shows the putative binding sites for the E. coli $\sigma^{70}$ protein in the 5′-upstream region of HPMAL1. We have shown earlier that the promoter of the HPMAL1 gene is perfectly recognized in E. coli resulting in a high expression of the H. polymorpha maltase protein in this prokaryotic host [4].

We consider that the absence of putative binding sites for the S. cerevisiae Mig1 protein in the medium-length promoter of HPMAL1 (Table 3) may explain its reduced glucose repression in S. cerevisiae 100-1B (Table 2). Glucose will presumably still down-regulate the expression of the MAL activator and maltose permease in this strain, which might account for the remaining amount of glucose repression of HPMAL1 in these transformants. However, the medium-length promoter of HPMAL1 is perfectly glucose-repressed in H. polymorpha (Table 1). Further analysis is needed to explain this fact.

3.6. Comparison of the strengths of the promoters of alcohol oxidase and maltase genes in H. polymorpha

The powerful promoter of the H. polymorpha alcohol oxidase gene (MOX) has often been used for the regulated expression of heterologous proteins in H. polymorpha [1]. Thus we compared the induced strengths of the maltase and alcohol oxidase promoters using the H. polymorpha maltase gene HPMAL1 as a reporter and HP201HPMAL1Δ as a host. HP201HPMAL1Δ transformed with either pX4-HPMAL1 (contains HPMAL1 behind the alcohol oxidase promoter) or pRS425-p51SpeISmaI (contains HPMAL1 behind its own full-length promoter) was pregrown on glycerol and the initial maltase activity was registered in the cells. Thereafter the specific inducers of the respective promoters (methanol or a disaccharide) were added to the cultures, the cells were further cultivated for 7.5 h and sampled for the measurement of maltase activity. The HP201HPMAL1Δ transformed with pX4-HPMAL1 exhibited derepressed maltase activity if grown on glycerol (see zero time points in Fig. 5A), in good agreement with the regulation of the alcohol oxidase promoter in H. polymorpha [34]. Addition of methanol to the glycerol-grown culture resulted in a further increase of maltase activity in the cells reaching about 1500 mU mg$^{-1}$ by 7.5 h of induction (Fig. 5A). Moreover, even higher maltase activity (up to 3000 mU mg$^{-1}$) was observed during prolonged cultivation of these transformants on methanol.

Glycerol-grown HP201HPMAL1Δ transformants carrying pRS425-p51SpeISmaI had a moderately derepressed maltase activity (about 40 mU mg$^{-1}$) (Fig. 5B). Addition of 2% sucrose to these cells (Fig. 5B) resulted in a continuous increase of maltase activity reaching over 950 mU mg$^{-1}$ by 7.5 h of induction. Induction by 0.5% sucrose was even more rapid and yielded a high maltase level already by 2.5 h of induction. We hypothesize that if sucrose is added to a glycerol-grown culture of H. polymorpha at a high concentration, its intracellular hydrolysis may cause transient repression of maltase by monosaccharides. This can explain a delayed and lower level of maltase induction at higher sucrose concentrations.

If maltose was used as the inducer instead of sucrose, induction of maltase activity in HP201HPMAL1Δ transformants were supplemented with an inducer (0.5% methanol, 2% sucrose or 0.5% sucrose) and further cultivated for 7.5 h. At the indicated time points, cells were harvested to measure the maltase activity. Zero time point designates the maltase activity in glycerol-grown cells before the addition of the inducer. A: HP201HPMAL1Δ was transformed with pX4-HPMAL1 harboring the H. polymorpha maltase gene behind the MOX promoter. B: HP201HPMAL1Δ was transformed with pRS425-p51SpeISmaI carrying the H. polymorpha maltase gene behind its own full-length promoter. Medium data and standard deviation for two to three distinct transformants are presented.

Fig. 5. Comparison of the induced strengths of the promoters of H. polymorpha maltase and alcohol oxidase genes. Glycerol-grown HP201HPMAL1Δ transformants were supplemented with an inducer (0.5% methanol, 2% sucrose or 0.5% sucrose) and further cultivated for 7.5 h. At the indicated time points, cells were harvested to measure the maltase activity. Zero time point designates the maltase activity in glycerol-grown cells before the addition of the inducer. A: HP201HPMAL1Δ was transformed with pX4-HPMAL1 harboring the H. polymorpha maltase gene behind the MOX promoter. B: HP201HPMAL1Δ was transformed with pRS425-p51SpeISmaI carrying the H. polymorpha maltase gene behind its own full-length promoter. Medium data and standard deviation for two to three distinct transformants are presented.
thereby reducing or eliminating the maltase repression by its reaction products.

As we used multicopy plasmids for the evaluation of the strengths of the alcohol oxidase and maltase gene promoters, we next compared the copy numbers of the plasmids pRS425-p51SpeISmaI and pX4-HPMAL1 in HP201HPMAL1. These plasmids do not possess any ARS element originating from Hansenula, but contain the S. cerevisiae LEU2 gene as a selection marker. It has been shown that the coding region of this gene or its close vicinity is responsible for the ARS activity in H. polymorpha, enabling autonomous replication of S. cerevisiae LEU2-containing plasmids in H. polymorpha [16]. To evaluate the copy number of the plasmid pX4-HPMAL1 in HP201HPMAL1Δ, we designed a construct pHIPX8-p51SpeISmaI (see Section 2.1), which has the same plasmid backbone as pX4-HPMAL1, but the maltase gene on it is controlled by its own full-length promoter instead of the MOX promoter. The plasmid pHIPX8-p51SpeISmaI was introduced into HP201HPMAL1Δ and several distinct transformants were cultivated on 2% sucrose to measure the activity of maltase in exponentially growing cells. Medium maltase activity in these transformants was 1160 mU mg\(^{-1}\), that is even higher than the activity in sucrose-grown HP201HPMAL1Δ transformed with pRS425-p51SpeISmaI (see Table 1). Therefore we presume that the copy number of the plasmid pX4-HPMAL1 in HP201HPMAL1Δ is not lower than that of pRS425-p51SpeISmaI. According to our data, the strength of the HPMAL1 promoter under sucrose induction is quite comparable to the strength of the MOX promoter under methanol induction. It has earlier been shown that the strength of the promoter of the plasma membrane H\(^+\)-ATPase gene (PMA1) is also comparable to that of the MOX promoter in H. polymorpha [35]. However, the PMA1 promoter is constitutive and its expression cannot be controlled by a specific carbon source.

Summing up, we assume that the MAL genes in H. polymorpha are clustered similarly to those in S. cerevisiae and K. lactis. Data on the regulation of the H. polymorpha maltase gene encourage us to isolate the genes of H. polymorpha that encode homologues of the S. cerevisiae MAL activator and Mig1 repressor proteins to go on with the study of glucose repression in H. polymorpha. Results of the present work also show that the HPMAL1 promoter might find application as a component in H. polymorpha expression cassettes. Therefore it could be used like the MOX promoter for the production of proteins that might be toxic to the cell: the production of the protein of interest can be switched on by the addition of sucrose after a high cell density is achieved at the expense of a carbon source repressing the expression from the maltase promoter (glucose) or allowing its low or moderate expression (glycerol). The attractive feature of the promoter is its functionality in E. coli [4] and S. cerevisiae. Thus, we should also recommend the HPMAL1 promoter for the design of a wide-host-range expression plasmid that can be used to choose an appropriate host to produce the protein of interest.

Acknowledgements

The authors would like to thank K. Lahtchev, R. Needleman, M. Veenhuis, J. Siverio and C. Michels for sending us the yeast strains and plasmids and S. Kuuse for technical help. This work was supported by Grant 3923 from the Estonian Science Foundation.

References


