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Clustering of *MAL* genes in *Hansenula polymorpha*: Cloning of the maltose permease gene and expression from the divergent intergenic region between the maltose permease and maltase genes

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Abstract

Hansenula polymorpha uses maltase to grow on maltose and sucrose. Inspection of genomic clones of *H. polymorpha* showed that the maltase gene *HPMAL1* is clustered with genes corresponding to *Saccharomyces cerevisiae* maltose permeases and *MAL* activator genes orthologues. We sequenced the *H. polymorpha* maltose permease gene *HPMAL2* of the cluster. The protein (582 amino acids) deduced from the *HPMAL2* gene is predicted to have eleven transmembrane domains and shows 39–57% identity with yeast maltose permeases. The identity of the protein is highest with maltose permeases of *Debaryomyces hansenii* and *Candida albicans*. Expression of the *HPMAL2* in a *S. cerevisiae* maltose permease-negative mutant CMY1050 proved functionality of the permease protein encoded by the gene. *HPMAL1* and *HPMAL2* genes are divergently positioned similarly to maltase and maltose permease genes in many yeasts. A two-reporter assay of the expression from the *HPMAL1–HPMAL2* intergenic region showed that expression of both genes is coordinately regulated, repressed by glucose, induced by maltose, and that basal expression is higher in the direction of the permease gene.

Keywords: Maltose permease; Maltase; *MAL* activator; Bidirectional promoter; Gus reporter

1. Introduction

Methylotrophic yeasts, including *Hansenula polymorpha*, are popular hosts for the production of foreign proteins under the control of extremely powerful regulated promoters [1,2]. They are also perfect objects to study evolution of glucose repression mechanisms among yeasts due to the presence of numerous glucose-repressed functions [2]. Phylogenetic analysis has revealed a large evolutionary distance between *H. polymorpha* and the most thoroughly studied yeast *Saccharomyces cerevisiae* (http://natchaug.labri.u-bordeaux.fr/Genolevures/), suggesting that complex phenotypic traits in these two species may also differ. Our research data on hexose kinases and glucose repression in *H. polymorpha* [3,4] has confirmed that at least primary signaling of glucose repression in *H. polymorpha* should be different from that described for *S. cerevisiae* [5]. We initiated the study of maltose utilization in *H. polymorpha* [6,7] in order to perform comparative analysis of a glucose-repressed enzymatic system present in many yeasts, including *S. cerevisiae*. Growth of yeasts on maltose is based on intracellular hydrolysis of this disaccharide by maltase [7 and references therein]. It has turned out that in *H. polymorpha*, as in the human pathogen *Candida albicans*, maltase protein is also responsible for the utilization sucrose, and no invertase

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is present [8–10]. We showed that the promoter of the *H. polymorpha* maltase gene *HPMAL1* is up-regulated by maltose and sucrose, and its induced strength is up to 70% of that of the *MOX* promoter [8]. Thus, it certainly has a biotechnological potential. Sequencing of the 5′-noncoding region of the maltase gene in the library plasmid p51 suggested that the maltose permease gene may locate aside [8]. In *S. cerevisiae* the maltose-specific (*MAL*) genes are genomically clustered to the *MAL* loci *MAL1*, *MAL2*, *MAL3*, *MAL4* and *MAL6*, to be found in different chromosomes [11,12]. Each locus contains a cluster of three different genes encoding a maltose permease (*MALx1*), a maltase (*MALx2*) and an activator of these genes (*MALx3*), "*x*" designating the number of the *MAL* locus [11]. However, only one fully functional locus, particularly *MAL1*, is present in standard laboratory strains [13]. The *MAL61* and *MAL62* genes are coordinately transcribed from a bidirectional promoter region [14,15]. Similarly, expression from the *GAL1–GAL10* genes of *S. cerevisiae* [16] and nitrate-specific specific genes niaD–niiA of *Aspergillus nidulans* [17] is directed from a bidirectional promoter. The nitrate utilization genes *YNT1*, *YNI1*, *YNA1* and *YNR1* are also clustered in *H. polymorpha*. But differently from the situation in *Aspergillus*, the direction of the transcription of all four genes is the same [18]. As cited in Levine et al. [14], eukaryotic genes that are coordinately regulated commonly map to unlinked positions in the genome. However, recent inspection of the human genome has revealed an abundance of bidirectional promoters, and many of them contain shared elements that regulate both genes [19]. In this paper we present the sequence and functionality analysis of the maltose permease gene *HPMAL2* detected between the maltase gene *HPMAL1* and the putative *MAL* activator gene in a Génolevures clone BB0AA011B12. We also evaluate carbon source-dependent expression from the *HPMAL1–HPMAL2* divergent promoter region in two directions, using a single- and a two-reporter test system. As genomic sequences are available for *Debaryomyces hansenii* (http://natchaug.labri.u-bordeaux.fr/Genolevures/) and *C. albicans* (http://genolist.pasteur.fr/CandidaDB/) that are phylogenetically close to *H. polymorpha*, we also analyze the spectrum and genomic neighbourhood of maltose-specific genes in these yeasts.

2. Materials and methods

2.1. Strains and plasmids

A maltase disruption mutant HP201HPMAL1Δ [8] derived from *H. polymorpha* 201 (*leu2-2 ura3-1 met4-220*) provided by K. Lahtchev (Sofia) was used throughout the experiments. Functionality of the *H. polymorpha* maltose permease gene was verified using complementation of the *S. cerevisiae* maltose permease-negative mutant CMY1050 (*mal61*Δ::HIS3). Isogenic wild-type strain CMY1001 (*MAT*a *MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1*-Δ63 *his3-200*) was used as a control (strains kindly provided by C. Michels; see also [20]). *Escherichia coli* DH5α served as a host strain in DNA amplification and cloning procedures. p51 (insert ~5.6 kpb) is a clone isolated from a *H. polymorpha* genomic library [21]. It contains part of the *H. polymorpha MAL* locus in pYT3 ([7]; see also Fig. 1(a)). The *H. polymorpha* genomic clones BB0AA011B12, BB0AA02D05 (see Fig. 1(a)) and BB0AA002C11 of Génolevures [22] were kindly sent by S. Casaregola.

The maltose permease gene was subcloned from the library clone BB0AA011B12 on a *Bam*HI–*Bgl*II fragment inserted between the same sites of pRS425 yielding pRS425-B12 BamHIBglII. The promoter of the *H. polymorpha* maltose permease gene in pRS425-B12 BamHIBglII was changed with that of the *S. cerevisiae MAL61* as follows. Using the primers 5′gatcagaactagtcatttatg 3′ and 5′ atagttaattactagtcttggatg 3′ (*Spe*I sites underlined) the *MAL61–MAL62* promoter was amplified from the plasmid pRS425-MAL62 [8], cut with *Spe*I and cloned into pRS425-B12BamHIBglII to replace the *Spe*I fragment with the *HPMAL2* promoter, resulting in pRS425-MAL61promHPMAL2. Orientation of the *HPMAL1–HPMAL2* intergenic region in front of the *H. polymorpha* maltase gene *HPMAL1* was switched in the polylinker of the pRS425 plasmid. First, the maltase gene ORF was amplified from pRS425-p51SpeISmaI [8] with Pfu DNA polymerase (Fermentas, Vilnius, Lithuania) using the primers MalpromSpeFw 5′ taaactagtatgactatcgagtctcaagaacc 3′ (the *Spe*I site is underlined) and T7 5′ taatacgactcactataggg 3′, the product was cut with *Spe*I and *Sma*I and inserted between the same sites of the pRS425 polylinker, yielding pRS425-MAL1 – the promoterless maltase gene in pRS425. Next, the *HPMAL1–HPMAL2* promoter region was amplified from pRS425-p51SpeISmaI by PCR using primers T7 (5′ taatacgactcactataggg 3′) and MalpromSpeRev (5′ aacactagtggaattaatattgtcaagaggg 3′), cut with *Spe*I (the site is underlined in the primer) and cloned into the *Spe*I site in front of the maltase gene in both orientations. The cloning resulted in two different constructs: (1) pRS425-MalpromFwMAL1 containing the *HPMAL1–HPMAL2* intergenic region in front of the maltase reporter in the orientation of the maltase gene, (2) pRS425-MalpromRevMAL1 containing the same intergenic region in reverse orientation, i.e., in the direction of the maltose permease gene. To make a two-reporter plasmid pRS425-GUSMalpromFwMAL1 the promoterless *gusA* gene of *E. coli*, originating from pGUS102 [23],