TARTU ÜLIKOOL

BIOLOOGIA-GEOGRAAFIATEADUSKOND
MOLEKULAAR- JA RAKUBIOLOOGIA INSTITUUT
GENEETIKA ÕPPETOOL

Katrin Viigand

Hansenula polymorpha MAL-lookus

Magistritöö

Juhendaja:
dots Tiina Alamäe, biol. kand.

TARTU
2006

Sisukord

KASUTATUD LÜHENDID

ap – aluspaari

ATP – adenosiintrifosfaat

CCCP – karbonüültsüaniid-*m*-klorofenüülhüdrasoon

EDTA – etüleendiamiintetraatsetaat

HPMAL1 – H. polymorpha maltaasi geen

HPMAL2 – H. polymorpha α-glükosiidi permeaasi geen

IPTG – isopropüül-β-tiogalaktosiid

kap – kiloaluspaari

LB – Luria-Bertani sööde

MAL61 – S. cerevisiae MAL6 lookuse maltoosi transporteri geen

MAL62 – S. cerevisiae MAL6 lookuse maltaasi geen

MAL63 – S. cerevisiae MAL6 lookuse *MAL*- aktivaatori geen

MAL-lookus – maltoosi kasutamise eest vastutav geneetiline lookus

OD – optiline tihedus

PNPG – *p*-nitrofenüül-α-D-glükopüranosiid

TAE – Tris-atsetaat-EDTA

Tris – trishüdroksümetüülaminometaan

X-Gal – 5-bromo-4-kloro-3-indolüül-β-D-galaktopüranosiid

YNB – minimaalsööde pärmidele

SISSEJUHATUS

Metülotroofsed pärmid on pärmide hulgas erandlikud oma võime poolest kasvada ühesüsinikulisel alkoholil - metanoolil. Seni on nad uurijatele huvi pakkunud peamiselt seetõttu, et nende väga tugevaid metanooliga indutseeritavaid promootoreid (näiteks alkoholi oksüdaasi promootorit) saab kasutada biotehnoloogias heteroloogiliste valkude ekspresseerimiseks. Üks liik metülotroofsetest pärmidest - *Hansenula polymorpha* - suudab kasvada ka disahhariididel, maltoosil ja sahharoosil. Meie grupis uuritakse maltoosi ja sahharoosi metabolismi *Hansenula polymorpha*'l alates 1997. aastast. Oleme näidanud, et mõlema disahhariidi kasutamist reguleeritakse glükoosi repressiooniga nagu mudelpärmil *Saccharomyces cerevisiae*. Maltoosi ja sahharoosi kasutamine on neil kahel pärmil siiski erinev. Pärmil *H. polymorpha* toimub mõlema substraadi hüdrolüüs rakusisese maltaasiga, mille geeni ja valku oleme eelnevalt iseloomustanud (Liiv *et al*., 2001). Pärmil *S. cerevisiae* toimub sahharoosi hüdrolüüs väljaspool rakku kestaseoselise invertaasiga, maltoos aga transporditakse maltoosi permeaasiga rakku ja hüdrolüüsitakse raku sees maltaasiga. Kuna maltoosi kasutamiseks vajalikke geene, valke ja nende ekspressiooni regulatsiooni on pärmil *S. cerevisiae* põhjalikult uuritud, siis pakub analoogilise süsteemi uurimine *H. polymorpha*'l põnevat võrdlusmaterjali hindamaks vastavate geenide, valkude ja regulatsiooni-mehhanismide evolutsioneerumist pärmidel. Pärmil *S. cerevisiae* on näidatud, et maltoosi kasutamiseks vajalikud geenid paiknevad genoomis kolmegeeniliste klastritena ning neid klastreid võib genoomis olla mitu. Sellised funktsionaalselt seotud geenide klastrid esinevad pärmidel harva ja meid huvitas, kas selline omapärane joon esineb näiteks ka *H. polymorpha*'l.

Antud töös 1) näidati, et *H. polymorpha*'l on maltoosi ja sahharoosi kasutamiseks vajalikud geenid (*MAL*-geenid) genoomis kõrvuti, moodustades *MAL*-lookuse; 2) uuriti *MAL*-lookuse geenide promootorite regulatsiooni ning tõestati lookuses paikneva α-glükosiidi permeaasi geeni *HPMAL2* katkestamisega, et ta vastutab maltoosi, sahharoosi ja trehaloosi transpordi eest; 3) iseloomustati HPMAL2 permeaasi omadusi; 4) uuriti *S. cerevisiae* maltaasi geeni ekspressiooni pärmis *H. polymorpha* ning otsiti *H. polymorpha* geenijärjestusi, mis võiksid kodeerida *S. cerevisiae MAL* geenide transkriptsiooniliste regulaatorvalkude homolooge.

Sooviksin tänada oma juhendajat Tiina Alamäed, kes aitas mind katsete planeerimisel ning antud töö koostamisel. Samuti tänan laborikaaslasi, kes aitasid töövõtete omandamisel.

1 Kirjanduse ülevaade

1.1 Pärmide *MAL*-geenid

Pärmid suudavad kasvada paljudel erinevatel suhkrutel. Eelistatuimad on glükoos ja fruktoos, kuid paljud pärmid kasvavad ka galaktoosil, maltoosil ja sahharoosil, mõned ka laktoosil ja tärklisel (Boekhout ja Kurtzman, 1996). Kuna maltoosi fermentatsioonil põhinevad nii pagari- kui pruulimistööstus, siis on maltoosi kasutamist pärmidel uuritud juba alates 1940ndatest aastatest. Eriti põhjalikult on sellest aspektist uuritud pagaripärmi *Saccharomyces cerevisiae*.
Maltoosi kasutamises osalevaid geene on hakatud pagaripärmil nimetama *MAL*-geenideks. *Saccharomyces*'e erinevatel tüvedel on leitud viis genoomset lookust: *MAL1, MAL2, MAL3, MAL4* ja *MAL6*, mis sisaldavad *MAL*-geene. Need järjestuselt sarnased lookused (*MAL* lookused) asuvad klastrina viie erineva kromosoomi telomeeri läheduses ja sisaldavad vähemalt kolme erinevat geeni, mis kodeerivad vastavalt maltoosi permeaasi, maltaasi ja MAL-aktivaatorvalku. Selleks, et pärm suudaks kasvada maltoosil on vaja vähemalt ühte *MAL*-lookust (Needleman, 1991). Lisaks maltoosi kasutamiseks vajalikele lookustele on pagaripärmil ja talle lähedastel pärmidel telomeersed ka lookused, mis on vajalikud sahharoosi ja melibioosi kasutamiseks (*SUC*- ja *MEL*- lookused) (Korshunova *et al*. 2005).
Kõige rohkem on uuritud *S. cerevisiae* kaheksandas kromosoomis paikneva *MAL6*-lookuse geene.
MAL61 kodeerib maltoosi permeaasi (Dubin *et al*., 1985). Maltoosi permeaas on kõrge afiinsusega transporter, mis transpordib maltoosi rakku sümpordis prootonitega (Chang *et al*, 1988; Lagunas, 1993; vt ka pt 1.3). *MAL62* kodeerib maltaasi, mis hüdrolüüsib maltoosi kaheks glükoosi molekuliks (Dubin *et al*., 1985). Maltaas on laia substraadispetsiifilisusega ensüüm, mis suudab lisaks maltoosile hüdrolüüsida ka teisi α-glükosiide (Needleman *et al*., 1978). *MAL63* kodeerib MAL-aktivaatorit (Zn-sõrmvalk), mis on *MAL*-geenide promootoritele järjestusspetsiifiliselt seonduv transkriptsiooni aktivaator. MAL-aktivaator vahendab *MAL*-geenide induktsiooni maltoosiga ning tema transkriptsioon on glükoosiga repressеeritav (Chang *et al*., 1988; Kim ja Michels, 1988; Needleman, 1991).
Lisaks *MAL6*-lookusele on uuritud ka *S. cerevisiae MAL1*-lookust. *MAL1*-lookus on sarnane *MAL6*-lookusele ning sisaldab samuti kolme geeni: *MAL11* (kodeerib maltoosi permeaasi), *MAL12* (kodeerib maltaasi) ja *MAL13* (kodeerib MAL-aktivaatorit). *MAL1* ja *MAL6* lookuste geenid on järjestustelt sarnased ning võimelised üksteist funktsionaalselt asendama (Charron

et al., 1986). Maltoosil on võimelised kasvama ka mitmed teised pärmid: *Klyuveromyces lactis, Candida albicans, Schwanniomyces occidentalis* ja *Hansenula polymorpha.*

Pärmil *Candida albicans* hüdrolüüsib nii maltoosi kui ka sahharoosi rakusisene ensüüm maltaas. Sellel pärmil ei ole leitud invertaasi, mille abil toimub sahharoosi kasutamine pärmidel *S. cerevisiae, Klyuveromyces lactis, Schizosaccharomyces pombe* ja *Schwanniomyces occidentalis* (Trumbly, 1986; Goffrini *et al*., 1995; Moreno *et al*., 1990; Rose, 1995). Pärmil *S. cerevisiae* ei toimu *MAL*-geenide induktsiooni sahharoosil kasvavates rakkudes, kuigi maltaas suudab edukalt sahharoosi rakusiseselt hüdrolüüsida (Wang ja Needleman, 1996).

Ka pärmil *K. lactis* on kirjeldatud maltaasi geen (*MAL22*) ning maltoosi permeaasi geen (*MAL21*). Mõlemad geenid on järjestuselt sarnased *S. cerevisiae* vastavate geenidega (Goffrini *et al*., 2002) ning paiknevad genoomis üksteise kõrval (vt järjestus geenipangas tunnusnumbri AF261762 all). Alves-Araujo *et al*. (2004) näitasid, et ka pärmil *Torulaspora delbrueckii* on genoomis maltaasi ja maltoosi permeaasi geenid kõrvuti. Seega tundub, et *MAL*-geenide klasterdumine on pärmidele tüüpiline.

Metülotroofsetest pärmidest suudab maltoosil kasvada vaid *Hansenula polymorpha*, kellel toimub nii maltoosi kui ka sahharoosi kasutamine rakusisese maltaasi abil ning invertaasi ei ole sel pärmil leitud (Alamäe ja Liiv, 1998). *H. polymorpha* maltaasi geen *HPMAL1* on kloneeritud ja sekveneeritud ning sellelt tuletatud valk on järjestuselt vastavalt 58% ja 47% identne maltaasidega pärmidest *C. albicans* ning *S. cerevisiae* (Liiv *et al*., 2001).

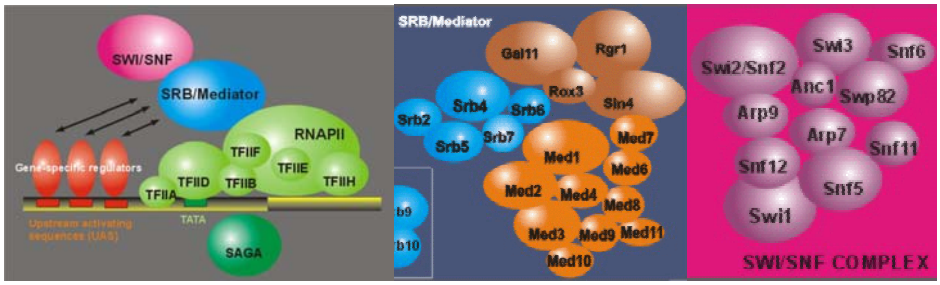
1.2 *MAL*-geenide regulatsioon pärmidel

Maltoosil kasvanud *S. cerevisiae* rakkudes on maltoosi permeaasi ja maltaasi sünteesi induktsioon kuni mitmesajakordne võrreldes glükoosil kasvanud rakkudes (Vanoni *et al*., 1989; Wang ja Michels, 2004). Maltoosi permeaasi ja maltaasi geenide vahel on ühine divergentne promootorala (Needleman, 1991). Reportergeenide abil on näidatud, et sellelt kahesuunaliselt promootorilt toimub koordineeritud (süsinikuallikaga reguleeritud) transkriptsioon mõlemas suunas (Levine *et al*., 1992; Bell *et al*., 1995).

Pärmil *S. cerevisiae* vahendab *MAL*-geenide induktsiooni maltoosiga Zn-sõrmvalk, mida nimetatakse MAL-aktivaatoriks (Needleman, 1991). See valk seostub aktivaatorjärjestustele (UAS_{MAL}) maltaasi ja maltoosi permeaasi geenide ühises divergentses promootoralas ning aktiveerib transkriptsiooni nendelt (Needleman, 1991; Wang *et al*., 1997). Kui *MAL*-aktivaatori geenid deleteerida, siis ei suuda sellised mutandid maltoosil kasvada (Charron *et*

al., 1986). Oletatakse, et maltoos seostub MAL-aktivaatorvalguga ja muudab tema konformatsiooni, mille tagajärjel suudab aktivaatorvalk efektiivselt seostuda *MAL*-geenide promootoritele (Danzi *et al*., 2000).

MAL-geenide induktsiooniks maltoosiga on vajalik ka mediaatorkompleks. Mediaatorkompleks vahendab geenispetsiifiliste regulaatorite seostumist RNA polümeraasiga ja basaalsete transkriptsioonifaktoritega (Myers ja Kornberg, 2000; vt ka joonis 1). Wang ja Michels on näidanud, et *MAL*-geenide induktsiooni maltoosiga vahendavad mediaatorkompleksi valgud GAL11 ja MED2. Protsessiks on vajalikud ka kromatiini ümberkorraldumises osalevad SWI/SNF kompleksi valgud. Mediaatorkompleksi valgud SIN4 ja RGR1 suruvad alla *MAL*-geenide basaalset ekspressiooni, kuid ei mõjuta nende indutseeritavust maltoosiga (Wang ja Michels, 2004).



Joonis 1. Transkriptsioonikompleksi valgud pärmidel (Holstege *et al*., 1998; http://biochemie.web.med.uni-muenchen.de/YTFD/RNApolii_complet.htm). Jooniselt võib leida *MAL*-geenide aktivatsioonis osalevad mediaatorkompleksi valgud GAL11 ja MED2. Näidatud on ka kromatiini modelleerimises osaleva SWI/SNF kompleksi koostis.

Bali jt. (2003) näitasid, et MAL-aktivaatorvalgu stabiilsuseks on vajalik Hsp90 valk (tšaperonvalk) ja mutandid, mis ei sünteesi seda valku, kasvavad väga vaevaliselt maltoosil, eriti siis, kui selle kontsentratsioon on söötmes madal.

MAL61-62 promootori uurimine on näidanud, et maltoosiga aktiveeritavaid järjestusi on seal kolm ning piirkonda promootoris, milles need järjestused sisalduvad nimetatakse UAS_{MAL} piirkonnaks. MAL-aktivaatori seostumiseks promootoritele on olulised kaks suhteliselt sarnast järjestust $CGGN_9CGG$ (piirkond 1) ning $CGCN_9CGC$ (piirkond 2) (Ni ja Needleman, 1990; Gancedo, 1998). Wang jt. (1997) leidsid ka kolmanda piirkonna *MAL61-MAL62* promootorist, kuhu seostub MAL-aktivaator, selle piirkonna järjestus on $CGCN_9GGG$. MAL-aktivaatori äratundmisjärjestus *MAL63* promootoris on $CGGN_9CGC$ ning konsensuseks MAL-aktivaatori seondumiseks erinevates *MAL*-lookustes loetakse järjestust $c/aGCN_9c/aGC/g$, kusjuures N_9 ala on AT-rikas (Sirenko ja Needleman, 1995).

MAL6 lookuse *MAL61-62* geenide vahealas esinevale UAS_{MAL} järjestusele väga sarnane järjestus on olemas ka *S. cerevisiae* α-glükosiidide transporteri geeni *AGT1* promootoralas (Han *et al*., 1995).

Kõrge glükoosi kontsentratsioon keskkonnas pärsib maltoosi kasutamise (Gancedo, 1998). Glükoos mõjutab maltoosi kasutamist pärmidel kahel moel: 1) inaktiveerib maltoosi permeaasi valgu, takistades sellega *MAL*-geenide induktori jõudmise rakku ja 2) represseerib maltoosi transporteri, maltaasi ja nende geenide aktivaatorvalku kodeerivate geenide transkriptsiooni (Needleman, 1991; Gancedo, 1998).

Maltoosi ning ka teiste glükoosile alternatiivsete suhkrute (näiteks sahharoosi ja galaktoosi) kasutamise eest vastutavate geenide promootoralast on pärmil *S. cerevisiae* leitud valgu MIG1 seostumiskohad ning on näidatud nende geenide transkriptsiooni repressioon selle valguga (Klein *et al*., 1998). MIG1 on Zn-sõrmstruktuuriga repressorvalk, mida kodeeriv geen kirjeldati Ronne poolt (Ronne, 1995) ning mille homoloogid on leitud ka pärmidest *Kluyveromyces lactis* (Cassart *et al*., 1995)*, Candida albicans* (Zaragoza *et al*., 2000)*, Candida utilis* (Delfin *et al*., 2001), *Scwanniomyces occidentalis* (Carmona *et al*., 2002) ja hallitusseenest *Aspergillus* (Gancedo, 1998; Klein *et al*., 1998). MIG1 valk paikneb kas tuumas või tsütoplasmas, olenevalt sellest, kas ta on fosforüleeritud või mitte. MIG1 valk juhib mittespetsiifilised transkriptsioonilised repressorid CYC8 (SSN6) ja TUP1(SSNG) glükoosiga represseeritavatele promootoritele (Keleher *et al*., 1992). Glükoosi lõppemisel söötmest toimub MIG1 valgu fosforüleerimine proteiinkinaasi Snf1 kompleksi vahendusel ning sellega kaasneb tema liikumine tuumast tsütoplasmasse (Gancedo, 1998).

MIG1 valguga represseeritavates promootorites on leitud konsensusjärjestus (G/C)(C/T)GG(G/A)G, millele eelneb 5´suunas AT-rikas ala (Lundin *et al*., 1994; Klein *et al*., 1998). MIG1 valgu seondumiskohad esinevad nii maltoosi permeaasi, maltaasi kui ka *MAL*-aktivaatori geenide promootorites (Hu *et al*., 1995). MIG1 seostumiskohtade kõrvaldamine *MAL63* promootorist muudab maltoosi permeaasi ja maltaasi sünteesi glükoosiga mitterepresseritavaks (Wang ja Needleman, 1996).

Pärmil *Candida albicans* on maltaasi süntees indutseeritud sahharoosil ja maltoosil kasvanud rakkudes ning represseeritud glükoosil kasvanud rakkudes (Kelly ja Kwon-Chung, 1992). *C. albicans*'il on lisaks maltaasi geenile *CAMAL2* (Geber *et al*., 1992) kirjeldatud ka *MAL*-aktivaatori geen *CASUC1* (Kelly ja Kwon-Chung, 1992). *CASUC1* heteroloogiline ekspressioon *S. cerevisiae MAL*-aktivaator-negatiivses mutandis näitas, et ta suutis ilmselt seostuda *MAL*-geenide promootoritele ja neid aktiveerida, kuna vastavatel transformantidel taastus võime kasvada maltoosil (Kelly ja Kwon-Chung, 1992).

Pärmist *C. albicans* on kloneeritud ka *S. cerevisiae MIG1* geeni homoloog *CaMIG1*(Zaragoza *et al*., 2000). *CaMIG1* suutis komplementeerida *MIG1* puudumist pärmis *S. cerevisiae*, kuid tema katkestamine *C. albicans*'i genoomis ei mõjutanud maltaasi geeni repressiooni glükoosiga. Ka ei ole *C. albicans*'i maltaasi geeni promootorist leitud järjestusi, mis oleksid sarnased *S. cerevisiae* MIG1 valgu seostumisjärjestustele (Zaragoza *et al*., 2000).

Sarnaselt *C. albicans*'iga on ka pärmil *H. polymorpha* maltaasi süntees indutseeritud nii maltoosil kui ka sahharoosil kasvanud rakkudes (Alamäe ja Liiv, 1998). Maltaasi süntees on glükoosiga represseeritav, glütseroolil ja etanoolil kasvanud rakkudes toimub maltaasi sünteesi osaline derepressioon (Alamäe ja Liiv, 1998).

HPMAL1 geen ekpresseerus enda promootorilt ka pagaripärmi *S. cerevisiae* maltaasnegatiivses mutandis 100-1B ning teda reguleeriti seal samamoodi nagu pagaripärmi enda maltaasi geeni – indutseeriti maltoosiga ja represseeriti glükoosiga (Alamäe *et al*., 2003). See näitab, et regulaatorvalgud, mis vahendavad *MAL*-geenide regulatsiooni pärmis *S. cerevisiae*, võiksid seostuda ka *H. polymorpha* maltaasi geeni promootorile. *HPMAL1* geeni promootori analüüs näitas, et seal on *S. cerevisiae* MIG1 valgu ja MAL-aktivaatori potentsiaalsed seostumisjärjestused (Alamäe *et al*., 2003).

Pärmi *Torulaspora delbrueckii* maltoosi permeaasi promootor on samuti hästi äratuntav pagaripärmis: maltoosi permeaasi geeni viimine *S. cerevisiae* maltoosi permeaasi katkestusmutanti taastas viimase kasvu maltoosil ning võime transportida maltoosi. Promootorist leiti ka *S. cerevisiae* MIG1 valgu ja MAL-aktivaatori potentsiaalsed seostumisjärjestused (Alves-Araujo *et al*., 2004).

Siiani ei ole täpselt teada, kuidas jõuab glükoosi repressiooni signaal MIG1 valguni ja mis vallandab selle signaali. Pärmil *S. cerevisiae* on näidatud, et glükoosi repressiooniks on vajalik heksokinaasi ühe isovormi (PII) olemasolu rakus (Zimmermann ja Scheel, 1977) ning heksokinaas PI ja glükokinaas ei suuda glükoosi repressiooni signaali edastada (Entian, 1997). On arvatud, et glükoosi ja ATP seondumisel heksokinaasi PII valguga ning järgneval fosforüüljäägi ülekandel glükoosile toimub valgu konformatsiooniline muutus, mis võimaldab tal kontakteeruda teiste glükoosi repressiooni signaaliahelas osalevate valkudega (Kraakman *et al*., 1999). Hiljuti ilmus töö, milles on näidatud, et heksokinaas PII võib seostuda MIG1 valguga, et koos liikuda tuuma ning osaleda repressorkompleksi moodustamises glükoosiga represseeritavatel promootoritel (Ahuatzi *et al*., 2004).

Pärmil *H. polymorpha* aga suudavad glükoosi repressiooni vahendada nii heksokinaas kui ka glükokinaas, kuid fruktoosi repressiooniks on vaja heksokinaasi, mis on ainus fruktoosi fosforüüliv ensüüm sellel pärmil (Kramarenko *et al*., 2000; Laht *et al*., 2002). Nii näiteks ei

ole maltaasi süntees fruktoosiga represseeritav *H. polymorpha* heksokinaasnegatiivsetes mutantides (Kramarenko *et al*., 2000).

1.3 Maltoosi transport pärmidel ning vastavad permeaasid

Glükoos on eelistatuim süsiniku- ja energiaallikas enamusele rakkudele, ka pärmidele. Esimeseks etapiks glükoosi metabolismis on glükoosi transport rakku. Pagaripärmil on 20 geeni, mis kodeerivad potentsiaalseid heksooside transportereid. Nende geenide ekspressioon on reguleeritud glükoosi hulgaga keskkonnas. Glükoosi transport rakku toimub vahendatud difusiooniga, ei vaja lisaenergiat ning enamus glükoosi permeaasidest transpordib rakku ka fruktoosi (Özcan ja Johnston, 1995). Suhteliselt hiljuti leiti, et pagaripärmil on olemas ka aktiivtransport (sümport prootonitega) fruktoosile. Vastavat permeaasi kodeerib *FSY1* geen (Goncalves *et al*., 2000). Prootonsümport glükoosile on kirjeldatud ka pärmidel *Schizosaccharomyces pombe* (Lichtenberg-Frate *et al*., 1997) ja *Hansenula polymorpha* (Karp ja Alamäe, 1998).

Maltoosi transport pagaripärmi rakku oli esimene näide suhkru aktiivtranspordist pärmidel (Loureiro-Diaz ja Peinado, 1984). Maltoos transporditakse rakku koos prootonitega vahekorras 1:1 ning transporteri K_m maltoosile on vahemikus 2-5 mM (Leeuwen *et al*., 1992). Busturia ja Lagunas (1985) avastasid lisaks sellele kõrge afiinsusega maltoosi transporterile ka oluliselt madalama afiinsusega transportsüsteemi. Hilisemad uuringud on näidanud, et sellise tulemuse võis anda tüves esinev laia susbstraadispektriga α-glükosiidi permeaas, mille afiinsus maltoosile on tõesti madal (Cheng ja Michels, 1991).

S. cerevisiae maltoosi permeaasi geen *MAL61* kloneeriti aastal 1989. Geen kodeerib valku, mille pikkus on 614 aminohapet ja tal ennustatakse 12 transmembraanset domääni. Valgu N- ja C-terminaalsed otsad on vastavalt 100 ja 67 ah pikad ning asuvad tsütoplasma poolel (Cheng ja Michels, 1989).

Pagaripärmis on maltoosi võimeline transportima ka α-glükosiidi transporter AGT1. AGT1 on laia substraadispetsiifikaga prooton-sümporter, mis transpordib mitmeid α-glükosiide: maltoosi, trehaloosi, maltotrioosi, α-metüülglükosiidi, meletsitoosi ja sahharoosi (Stambuk *et al*., 1999). AGT1 transporteril on kõrge afiinsus trehaloosile (K_m ~7 mM) ning madalam afiinsus maltoosile (K_m ~18 mM), maltotrioosile (K_m ~18 mM) ning α-metüülglükosiidile (K_m ~35 mM) (Stambuk ja Araujo, 2001). Seevastu *MAL2* lookusega kodeeritava maltoosi permeaasi MAL21 K_m trehaloosile on 90 mM ning maltoosile ~5 mM (Stambuk ja Araujo, 2001; vt ka tabel 1). Seega on pagaripärmil kahte tüüpi permeaase: 1) maltoosi permeaasid,

mis transpordivad peamiselt maltoosi ning mille afiinsus maltoosile on kõrge ning 2) α-glükosiidi permeaasid, mille afiinsus maltoosile on madal ning mille põhifunktsioon on ilmselt teiste α-glükosiidide rakku transportimine. Hiljuti leiti pagaripärmil veel kaks oletatavat maltoosi permeaasi geeni: *MPH2* ja *MPH3* (Day *et al*., 2002). Need geenid kodeerivad kahte sama järjestusega valku, mille identsus *S. cerevisiae* maltoosi permeaasidega MAL31 ja MAL61 on 75% ja AGT1 permeaasiga 55%. Katsed näitasid, et MHPx (x = 2 või 3) permeaas on võimeline transportima maltoosi (K_m ~4.4 mM), maltotrioosi (K_m ~7 mM), turanoosi ja α-metüülglükosiidi (Day *et al*., 2002).

Maltoosi kasutamist on uuritud ka pärmil *Torulaspora delbrueckii.* Alves-Araujo oma uurimisgrupiga (2004) kirjeldas *T. delbrueckii* geeni *TdMAL11*, millelt tuletatud valk oli sarnane maltoosi transporteritega pärmidest *S. cerevisiae* (identsus 71%) ja *K. lactis* (identsus 57%). *TdMAL11* ekspressioon on süsinikuallikaga reguleeritud nii nagu pagaripärmis: indutseeritud maltoosiga ja represseeritud glükoosiga. *TdMAL11* geeni katkestamine *T. delbrueckii* genoomis näitas, et nimetatud pärmil on vähemalt kaks maltoosi permeaasi geeni.

Reinders ja Ward (2001) kirjeldasid α-glükosiidi transporteri SUT1 pärmist *Schizosaccharomyces pombe*. See transporter on kõige sarnasem taimede transporteritele. SUT1 permeaasi omadusi uuriti selle heteroloogilisel ekspresseerimisel pärmis *S. cerevisiae* ning leiti, et tema afiinsus maltoosile on kõrgem kui sahharoosile, erinedes sellepoolest taimede SUT valkudest. Sarnaselt teistele α-glükosiidi transporteritele on *SUT1* ekspressioon glükoosiga represseeritav (Reinders ja Ward, 2001).

Maltoosi permeaas on pagaripärmil üks valkudest, mille hulka rakus reguleeritakse ka posttranslatsiooniliselt. Nimelt, glükoosi või fruktoosi lisamine maltoosil kasvavatele rakkudele inaktiveerib maltoosi permeaasi täielikult juba 90 minutiga (Busturia ja Lagunas, 1985). Brondijk jt. (2001) näitasid, et permeaasi inaktivatsiooniks on vajalik inaktivatsiooni indutseeriva glükoosi metaboliseerimine vähemalt glükoos-6-fosfaadini.

Maltoosi permeaasi inaktiveerimisel toimub esmalt valgu kiire ubikvitineerimine ja seejärel tema edasine lagundamine vakuoolis (Medintz *et al*., 1996; Medintz *et al*., 1998). Glükoosi lisamisel keskkonda maltaasi aga ei inaktiveerita ja tema ekspressiooni reguleeritakse vaid transkriptsiooni tasemel.

Tabel 1. Maltoosi ja α-glükosiidi permeaasid pärmidel.

Pärm	Permeaas	Transporditavad substraadid	K_m (mM)			Viide
			PNPG	Maltoos	Sahharoos	
S. cerevisiae	MAL21	Maltoos, sahharoos, turanoos	Ei transpordi	5	120	Stambuk ja Araujo, 2001; Stambuk *et al*., 2000; Hollatz ja Stambuk, 2001
S. cerevisiae	MAL61	Maltoos, turanoos	Ei transpordi	2-4	Ei transpordi	Han *et al*., 1995
S. cerevisiae	MPHx	Maltoos, maltotrioos, turanoos, α-metüülglükosiid	-	4.4	Ei transpordi	Day *et al*., 2002
S. cerevisiae	AGT1	Trehaloos, sahharoos, maltoos, maltotrioos, α-metüülglükosiid, PNPG, turanoos, isomaltoos, palatinoos, meletsitoos	3	18	7	Stambuk ja Araujo, 2001; Hollatz ja Stambuk, 2001; Han *et al*., 1995
S. pombe	SUT1	Maltoos, sahharoos	-	6.5	36	Reinders ja Ward, 2001
S. carlsbergensis	MTY1	Maltoos, maltotrioos	-	61-88	-	Salema-Oom *et al*., 2005
T. delbrueckii	TdMAL11	Maltoos	-	2-3	-	Alves-Araujo *et al.*, 2004

- andmed puudusid

2 Eksperimentaalosa

2.1 Töö eesmärgid

1. uurida *S. cerevisiae* maltaasi geeni *MAL62* ekspressiooni pärmis *H. polymorpha;*
2. teha kindlaks *H. polymorpha MAL*-lookuse struktuur;
3. kloneerida *H. polymorpha MAL*-lookusest α-glükosiidi permeaasi geen ja testida tema funktsionaalsust;
4. uurida *MAL*-lookuse promootorite regulatsiooni reportergeenide abil;
5. iseloomustada *H. polymorpha* α-glükosiidi permeaasi.

2.2 Materjal ja metoodika

2.2.1 Kasutatud tüved ja plasmiidid

Töös kasutatud tüved on esitatud tabelis 2.1 ja plasmiidid tabelis 2.2.

Tabel 2.1. Töös kasutatud pärmi- ja bakteritüved

Tüvi	**Genotüüp**	**Viide**
Hansenula polymorpha		
A16	*Hansenula polymorpha* tüvi ATCC 34438 *leu2-2 HPMAL1*	Veale *et al*., 1992
HP201	*leu2-2 ura3-1 met4-220 HPMAL1*	Saadud K. Lahtchevilt
HP201HPMAL1Δ	*leu2-2 ura3-1 met4-220 HPMAL1::HPURA3*	Pärn, 2003
HP201HPMAL2Δ	*leu2-2 ura3-1 met4-220 HPMAL2::HPURA3*	Käesolev töö
Saccharomyces cerevisiae		
CMY 1001	*MATα MAL61/HA MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200*	Saadud C. Michelsilt (Wang *et al*., 2002)
CMY 1050	*MATα MAL12 MAL13 GAL leu2 ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-200 mal61Δ::HIS3*	Saadud C. Michelsilt (Wang *et al*., 2002)
Escherichia coli		
DH5α	*supE44 ΔlacU169(ø80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi relA1*	Miller, 1972

Tabel 2.2. Töös kasutatud plasmiidid

Plasmiid	Kirjeldus ja kommentaarid	Viide
pYT3	*E. coli/H. polymorpha* süstikvektor	Tan *et al*., 1995
p51	Isoleeritud *H. polymorpha* CBS4732 genoomsest raamatukogust. Sisaldab plasmiidis pYT3 ~ 5.6 kap suurust inserti *HPMAL1* geeniga.	Liiv *et al*., 2001
pY6	Sisaldab *S. cerevisiae* maltaasi geeni *MAL62*, *MAL61-MAL62* promootorala ja osa maltoosi permeaasi *MAL61* geenist.	Charron *et al*., 1989
pRS425	*E. coli/S. cerevisiae/H. polymorpha* süstikvektor	Christianson *et al*., 1992
pRS425-p51SpeI-SmaI	Plasmiidist p51 kloneeriti *HPMAL1* geeni sisaldav *Spe*I-*Sma*I fragment plasmiidi pRS425 polülinkeri *Spe*I ja *Sma*I lõikekohtadesse.	Käesolev töö
pRS425-MAL62	Plasmiidist pY6 kloneeriti *S. cerevisiae MAL62* geeni sisaldav *Spe*I-*Eco*105I fragment plasmiidi pRS425 polülinkeri *Spe*I ja *Sma*I lõikekohtadesse.	Käesolev töö
pRS425 HPMAL1	Plasmiidilt pRS425-p51SpeI-SmaI amplifitseeriti *HPMAL1* geen praimeritega T7 ja MalpromSpeFw. Produkti lõigati ensüümidega *Spe*I ja *Sma*I ja kloneeriti plasmiidi pRS425 *Spe*I ja *Sma*I lõikekohtadesse.	Käesolev töö
pRS425 malpromFw HPMAL1	Plasmiidilt pRS425-p51SpeI-SmaI amplifitseeriti *HPMAL1* geeni promootor praimeritega T3 ja MalpromSpeRev. Produkti lõigati ensüümiga *Spe*I ja kloneeriti plasmiidi pRS425 HPMAL1 *Spe*I lõikekohta. Sisaldab *HPMAL1* geeni pärisuunas promootoriga.	Käesolev töö
pRS425 malpromRev HPMAL1	Plasmiidilt pRS425-p51SpeI-SmaI amplifitseeriti *HPMAL1* geeni promootor praimeritega T3 ja MalpromSpeRev. Produkti lõigati ensüümiga *Spe*I ja kloneeriti plasmiidi pRS425 HPMAL1 *Spe*I lõikekohta. Sisaldab *HPMAL1* geeni ümberpööratud promootoriga.	Käesolev töö
pRS425 MalaktpromFw HPMAL1	Kloonilt BB0AA011B12 praimeritega Malakt7 ja Malakt8 amplifitseeritud *MAL*-aktivaatori promootorit lõigati *Spe*I-ga ning kloneeriti vektori pRS425 HPMAL1 *Spe*I lõikesaiti. Sisaldab *MAL*-aktivaatori geeni promootorit *HPMAL1* geeni ees samas suunas kui *MAL*-aktivaatori geeni ees.	Käesolev töö
pRS425 MalaktpromRev HPMAL1	Kloonilt BB0AA011B12 praimeritega Malakt7 ja Malakt8 amplifitseeritud *MAL*-aktivaatori promootorit lõigati *Spe*I-ga ning kloneeriti vektori pRS425 HPMAL1 *Spe*I lõikesaiti. Sisaldab *MAL*-aktivaatori geeni promootorit *HPMAL1* geeni ees vastassuunas.	Käesolev töö
pHPURA3	*H. polymorpha HPURA3* geen plasmiidis pBluescript SK	Saadud J. Siverio´lt
pBluescript SK	Kloneerimisvektor	Stratagene
pBB12::HpURA3	Praimeritega HPURA3FwNheI ja HPURA3RevNheI plasmiidilt pHPURA3 amplifitseeritud *HPURA3* geeni lõigati *Nhe*I-ga ja kloneeriti plasmiidi BB0AA011B12 *Spe*I ja *Nhe*I saitide vahele.	Käesolev töö
pRS425 Mal61promHPMAL2	Plasmiidilt pRS425 MAL62 praimeritega MAL62algusRevSpe ja Mal61promSpe amplifitseeritud *MAL61-MAL62* promootorit lõigati ensüümiga *Spe*I ja kloneeriti plasmiidi pRS425 BamHI-BglII *Spe*I saitide vahele.	Käesolev töö
BB0AA021D05	*H. polymorpha* raamatukogu kloon, sisaldab inserdis *H. polymorpha* α-glükosiidi permeaasi geeni lõppu ja oletatava *MAL*-aktivaatori geeni algust, vt joonis 5.	Saadud S. Casaregolalt (Blandin *et al*., 2000)
BB0AA011B12	*H. polymorpha* raamatukogu kloon, sisaldab inserdis *H. polymorpha* α-glükosiidi permeaasi geeni, vt joonis 5.	Saadud S. Casaregolalt (Blandin *et al*., 2000)
pRS425 BamHI-BglII	Plasmiidist BB0AA011B12 kloneeritud *H. polymoprha* α-glükosiidi permeaasi geeni sisaldav *Bam*HI-*Bgl*II fragment plasmiidi pRS 425 *Bam*HI lõikekohas.	Käesolev töö

2.2.2 DNA eraldamine ja transformatsioon bakterisse ja pärmi

Plasmiidse DNA eraldamiseks *E. coli* rakkudest kasutati Eppendorfi minikolonne (*Perfectprep Plasmid Mini*) ja valmistajapoolset standardprotokolli.

Transformeerimiseks kasvatati bakterirakke LB söötmes tiheduseni 0.2-0.5 (λ=580 nm). Rakud muudeti transformatsioonikompetentseks Hanahani (1983) meetodil. Kompetentsetele rakkudele lisati ligikaudu 1 μg DNAd, seejärel hoiti rakke 30 min jääl ning pärast kuumašokki (5 min 37 °C) veel 10-15 min jääl. Seejärel külvati rakud 1.5 ml LB söötmesse, inkubeeriti loksutil üks tund, tsentrifuugiti söötmest välja ja külvati LB tardsöötmele. *E. coli* transformantide selekteerimiseks lisati söötmesse vastavalt vajadusele ampitsilliini (0.1 mg/ml).

Vajadusel kasutati transformantide selektsiooniks IPTG-d (lõppkontsentratsioon söötmes 75 μg/ml) ja X-Gal-i (lõppkontsentratsioon söötmes 60 μg/ml).

S. cerevisiae transformatsiooniks kasutati CMY1001 ja CMY1050 eksponentsiaalse kasvufaasi rakke ning transformatsioon viidi läbi Gietz *et al*. (1995) protokolli järgi.

H. polymorpha transformatsiooniks kasutati HP201, HP201HPMAL1Δ ja HP201HPMAL2Δ eksponentsiaalse faasi rakke. Rakud kasvatati üles YPD (1% pärmiekstrakti, 2% peptooni, 2% glükoosi) vedelsöötmes 37 ºC juures. Transformatsioonil lähtuti *S. cerevisiae* transformeerimise protokollist (Gietz *et al*., 1995), mida kohandati *H. polymorpha'*le. Pärmirakud muudeti transformatsioonikompetentseteks LiTE-ga (10 mM Tris-HCl pH 7.4; 100 mM Li-atsetaat; 1 mM EDTA). Ühe transformatsiooni jaoks tsentrifuugiti eksponentsiaalsest kasvavast kultuurist ($OD_{600\ nm}$ ~0.8-1.0) toatemperatuuril välja 2 ml rakke. Rakud suspendeeriti 1 ml-s LiTE ning inkubeeriti 5 min 30 ^{o}C juures. Rakud sadestati toatemperatuuril ning rakusademele lisati järgnevalt 240 μl PEG 4000 (50%), 36 μl 1 M Li-atsetaati, 20 μl (5 mg/ml) üheahelalist DNA-d ning 1-5 μg plasmiidset DNA-d ning segati Vortex-mikseril. Seejärel inkubeeriti segu 30 ºC juures 30-60 min. Kuumašokk tehti rakkudele 45 ºC juures 15-20 min. Järgnevalt külmutati transformatsioonisegu vedelas lämmastikus ja sulatati toatemperatuuril. Rakke pesti 1 ml destilleeritud veega ning plaaditi YNB tardsöötmele (vt pt 2.2.3), mis sisaldas 2% glükoosi ja vajadusel uratsiili ning aminohappeid. Rakke kasvatati 30 ºC juures kuni kolooniate moodustumiseni (3-6 päeva).

2.2.3 Söötmed ja rakkude kasvatamine

E. coli rakke kasvatati 37 ºC juures kas tahkel või vedelas Luria-Bertani (LB) söötmes. Vedelsöötmes kasvatamisel aereeriti kultuuri loksutil. Transformantide kasvatamisel lisati söötmesse vastavalt vajadusele ampitsilliini (0.1 mg/ml).

Maltaasi ja α-glükosiidi permeaasi aktiivsuste määramiseks kasvatati *H. polymorpha* rakke 37 ºC juures 0.67% YNB söötmes (Yeast Nitrogen Base without amino acids), millele lisati erinevaid süsinikuallikaid, ning vajadusel uratsiili ja aminohappeid (20-30 mg/l). Kultuure aereeriti loksutil. Ensüümiaktiivsuste mõõtmiseks koguti rakud eksponentsiaalsest kasvufaasist (OD_{600} ~2.0).

2.2.4 Rakuekstraktide ja –suspensioonide valmistamine

Pärmirakud eraldati söötmest tsentrifuugimisega Sorvall RC-5B tsentrifuugiga 4 ^{o}C juures 6000 p/min 10 minuti jooksul. Rakke pesti kaks korda destilleeritud veega ning suspendeeriti seejärel 1 ml 100 mM K-fosfaatpuhvris (pH 6.5), mis sisaldas 0.1 mM EDTA-d. Rakud purustati klaaskuulidega (diameeter 450 μm) Vortex-mikseril 4 korda á 1 minut. Iga minutilise töötluse järel hoiti segu vähemalt 1 minuti jooksul jääl. Klaaskuulid, purunemata rakud ning rakukestad sadestati tsetrifuugimisega Eppendorfi tsentrifuugis (4 ^{o}C, 12000 p/min) 30 minuti jooksul. Supernatanti kasutati ensüümpreparaadina.

α-glükosiidi permeaasi aktiivsuse määramiseks tehti rakususpensioon 200 mM K-fosfaatpuhvrisse (pH 4.5) tihedusega ~10 mg/ml. Transpordi pH sõltuvuse uurimiseks tehti rakususpensioon vette. Katse vältel hoiti rakususpensiooni jäävannis magnetsegajal. Rakususpensiooni kuivainesisaldus arvutati välja lahjendatud rakususpensiooni optilise neeldumise kaudu 600 nm juures.

2.2.5 Maltaasi aktiivsuse määramine

Maltaasi aktiivsust mõõdeti *p*-nitrofenooli tekke järgi *p*-nitrofenüül-α-D-glükopüranosiidist (PNPG) (Zimmermann *et al*., 1977) 37 ^{o}C juures. Inkubatsioonisegu sisaldas järgmisi komponente: 100 mM K-fosfaatpuhver (pH 6.5), 0.1 mM EDTA, 1 mM PNPG ning rakuekstrakt. Reaktsioon algatati PNPG lisamisega ning peatati õlgkollase värvuse tekkimisel 1 M naatriumkarbonaadi lisamisega. Aeg fikseeriti ning moodustunud kollase produkti (*p*-

nitrofenooli) hulk mõõdeti spektrofotomeetril lainepikkuse 400 nm juures. Ensüümi eriaktiivsus väljendati ühikutes nmooli/mg/min (mU/mg).

Metaboolsete mürkide mõju uurimiseks maltaasile lisati rakuekstraktile 3 min enne PNPG lisamist kas karbonüültsüaniid-*m*-klorofenüülhüdrasoon (CCCP, lõppkontsentratsioonis 0.05 mM) või Na-asiid (lõppkontsentratsioonis 0.02 mM ja 2 mM).

Valgu kontsentratsioon rakuekstraktis määrati Lowry jt. (1951) järgi.

2.2.6 α-glükosiidide transpordi kiiruse mõõtmine

α-glükosiidide transpordi kiiruse mõõtmisel rakkudesse kasutati transporditava substraadina *p*-nitrofenüül-α-D-glükopüranosiidi (PNPG), sest pärmide α-glükosiidi transporterid transpordivad seda kromogeenset ainet (Sims *et al*., 1984; Hollatz ja Stambuk, 2000). Permeaas transpordib PNPG rakku, kus ta hüdrolüüsitakse maltaasiga glükoosiks ja *p*-nitrofenooliks. Glükoos kataboolitakse ja *p*-nitrofenool difundeerub rakust välja. Rakust väljadifundeerunud nitrofenooli hulga kaudu hinnatakse rakku transporditud PNPG hulka. Kuna rakusisese PNPG hüdrolüüsi kiirus maltaasiga on kuni 1000 korda kiirem kui PNPG transpordi kiirus pärmidel, siis limiteerib PNPG kasutamise kiirust rakkudel transport ja seega saab rakust eritatud *p*-nitrofenooli hulga järgi hinnata PNPG transpordi kiirust (Sims *et al*., 1984; Hollatz ja Stambuk, 2000). Meie lähtusime enda katsetes Sims jt. (1984) ja Stambuki (2000) töödes esitatud metoodikatest, kohandades neid järgnevalt. Reaktsioon viidi läbi Eppendrofi tuubides loksutustermostaadis 37 ^{o}C juures, et tagada ühtlane temperatuur ja rakkude ühtlane kontakt substraadiga. Inkubatsioonisegu (kokku 200 µl) sisaldas järgmisi komponente: 200 mM K-fosfaatpuhver (pH 4.5), 2 mM PNPG ning rakususpensioon (ca 1 mg rakke (kuivaines)/ml). Puhvris suspendeeritud rakke eelsoojendati loksutustermostaadis 1000 p/min 2 minutit, reaktsioon algatati PNPG lisamisega ning peatati kindlatel ajamomentidel 1 ml 1 M naatriumkarbonaadi lisamisega. Rakud tsentrifuugiti kohe põhja ning *p*-nitrofenooli hulk supernatandis mõõdeti spektrofotomeetril 400 nm juures. PNPG transpordi kiirus väljendati rakku transporditud PNPG hulgana ühikutes nmooli/mg/min (mU/mg).

α-glükosiidi permeaasi afiinsuse hindamiseks PNPG-le määrati PNPG transpordi kiirust selle erinevatel kontsentratsioonidel (0.2 mM-10 mM) ning K_m väärtus leiti Eadie-Hofstee graafikult.

α-glükosiidi permeaasi substraadispetsiifilisuse uurimiseks kasutati konkurentsimeetodit (Karp ja Alamäe, 1998; Hollatz ja Stambuk, 2000). Potentsiaalsed PNPG transporteriga konkureerivad ained lisati termostateeritud suspensioonile üks minut enne PNPG

(lõppkontsentatsioon 1 mM) lisamist. Konkurentidena kasutati maltoosi, sahharoosi, glükoosi, trehaloosi, α-metüülglükosiidi ja fruktoosi lõppkontsentratsioonides 1 mM ja 10 mM.

Metaboolsete mürkide mõju uurimiseks PNPG transpordile lisati rakususpensioonile 3 min enne PNPG lisamist kas CCCP-d (lõppkontsentratsioonis 0.05 mM) või Na-asiidi (lõppkontsentratsioonis 0.02 mM ja 2 mM).

Et uurida PNPG transpordi sõltuvust keskkonna pH-st, mõõdeti 2 mM PNPG transpordi kiirust kahes erineva pH-ga (4.5 ja 7.0) 200 mM K-fosfaatpuhvris.

2.2.7 Muud meetodid

Plasmiidse DNA restriktsiooni ja kloneerimise, sekveneerimise ning polümeraasi ahelreaktsiooni (PCR) metoodika on kirjeldatud lisas 2, pt 2.3 ja 2.4. Töös kasutatud arvutiprogrammid ja andmebaasid on esitatud lisas 2, pt 2.5.

PCR metoodikat kasutati *H. polymorpha MAL*-lookuse uurimiseks, *HPMAL1-HPMAL2* promootori, *MAL*-aktivaatori promootori ja *S. cerevisiae MAL61-MAL62* promootori, *HPMAL1* geeni ja *HPURA3* geeni kloneerimiseks ning α-glükosiidi permeaasi geeni katkestamiseks *H. polymorpha* genoomis. Kasutatud praimerid ja nende järjestused on esitatud tabelites 2.2 ja 3.

Tabel 3. Töös kasutatud praimerid ja nende järjestused. Allajoonitult on näidatud *Spe*I ja *Nhe*I lõikekohad praimerites.

Praimeri nimetus	Praimeri järjestus 5' - 3' suunas	Lõikekoht praimeris
TRANS 1	TCAGAAGAACAAGGATTGCCG	-
TRANS 3	ATCAGAATGCACGTGTTTATGC	-
TRANS 4	TCGACGAGTGCGTCTTGGGC	-
TRANS 5	ATTGCCTCGGCTAGCGGAGC	-
TRANS 6	GCAATAATAAAGACATCTACGG	-
T 3	ATTAACCCTCACTAAAG	-
T 7	AATACGACTCACTATAG	-
SQMAL 18	AGTGGTCCCAAGCTGTTTTGG	-
SQMAL 19	TTTCTGGAAAGCAAACATAGGC	-
MALAKT 4	GCCACTTCCTTCTAGCAG	-
MALAKT 7	ATATTATACTAGTTGTTGATGG	*Spe*I
MALAKT 8	ATCACTAGTAAAAATGTCTCG	*Spe*I
HPURA3FwNheI	GGGTAAAGCTAGCAGGACGGG	*Nhe*I
HPURA3RevNheI	ACACTGCTAGCAATCCAATATCC	*Nhe*I
MalpromSpeFw	TAAACTAGTATGACTATCGAGTCTCAAGAACC	*Spe*I
MalpromSpeRev	AACACTAGTGGAATTAATATTGTCAAGAGGG	*Spe*I
Mal61promSpe	ATAGTTAATTACTAGTCTTGGATG	*Spe*I
Mal62algusRevSpe	GATCAGAACTAGTCATTTATG	*Spe*I
HPURA2	GGGTGGGAAGCTTACAAGCG	-

-, lõikesait praimeris puudub

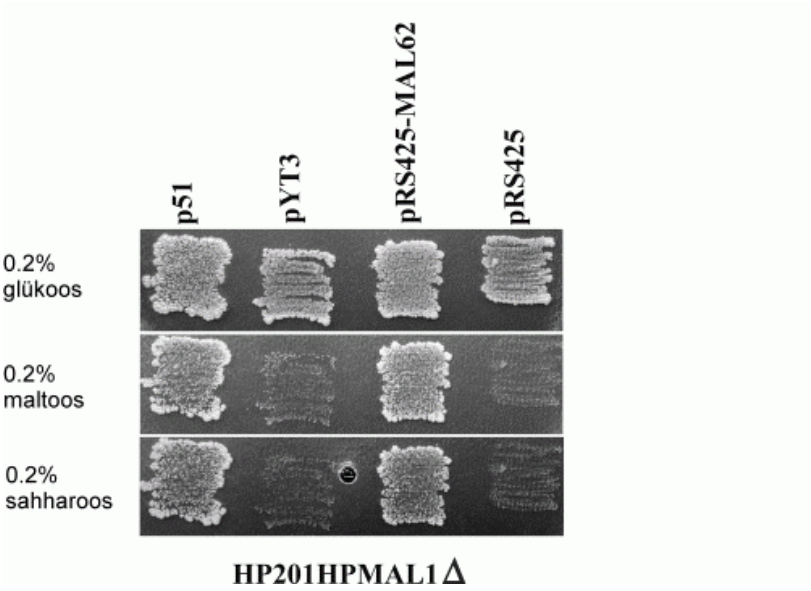
2.3 Tulemused ja arutelu

2.3.1 *S. cerevisiae* maltaasi geeni ekspressiooni uurimine pärmis *H. polymorpha*

Meie grupi varasematel andmetel (Pärn, 2003; Alamäe *et al*., 2003; lisa 1) on *H. polymorpha* maltaasi geeni *HPMAL1* promootor äratuntav ning korrektselt reguleeritav pärmis *S. cerevisiae.* Seega on tõenäoline, et *S. cerevisiae* regulaatorvalgud, mis vahendavad *MAL*-geenide ekspressiooni, võivad reguleerida ekspressiooni ka *H. polymorpha* maltaasi geeni promootorilt (vt peatükk 1.2) ning et pärmis *H. polymorpha* võivad esineda nende regulaatorvalkude homoloogid.

Et saada kaudseid tõendeid selle hüpoteesi tõestuseks, otsustasime testida, kas *S. cerevisiae* maltaasi geeni promootor tuntakse ära ja kuidas teda reguleeritakse pärmis *H. polymorpha*.

Selleks tehti konstrukt pRS425-MAL62, mis sisaldab *S. cerevisiae* maltaasi geeni *MAL62* ja selle täispikka promootorit ning transformeeriti *H. polymorpha* maltaaskatkestusmutanti HP201HPMAL1Δ. Kuna maltaasi abil hüdrolüüsitakse pärmis *H. polymorpha* nii maltoosi kui ka sahharoosi (Liiv *et al*., 2001), siis ei kasva HP201HPMAL1Δ neil suhkrutel. Kontrollina transformeeriti maltaaskatkestusmutanti ka *H. polymorpha* maltaasi geeni *HPMAL1* sisaldava plasmiidiga p51 ning vastavate tühjade vektoritega.



Joonis 2. *H. polymorpha* maltaasi geeni *HPMAL1* (p51) ja *S. cerevisiae* maltaasi geeni *MAL62* (pRS425-MAL62) sisaldava *H. polymorpha* maltaaskatkestusmutandi HP201HPMAL1Δ kasv glükoosil, maltoosil ja sahharoosil. Kontrollidena on kasutatud transformante, mis sisaldasid vastavaid tühje vektoreid (pYT3 ja pRS425).

Jooniselt 2 on näha, et *S. cerevisiae* maltaasi geen *MAL62* taastas sarnaselt *HPMAL1* geeniga *H. polymorpha* maltaaskatkestusmutandil võime kasvada nii maltoosil kui ka sahharoosil. *S. cerevisiae* ise maltaasi sahharoosi omastamiseks ei kasuta, sest sahharoos hüdrolüüsitakse tal väljaspool rakku rakukestaga seotud invertaasiga ning moodustuvad produktid (glükoos ja fruktoos) transporditakse rakku (Tkacz ja Lampen, 1973). *S. cerevisiae* rakusisene maltaas aga suudab sahharoosi hüdrolüüsida (Krakenaite ja Klemzha, 1983).

Kuna *S. cerevisiae* maltaasi geen *MAL62* taastas tüvel HP201HPMAL1Δ võime kasvada maltoosil ja sahharoosil, siis uurisime järgmisena *MAL62* regulatsiooni pärmis *H. polymorpha*. *S. cerevisiae* maltaasi geeni sisaldavaid transformante HP201HPMAL1Δ (pRS425-MAL62) ning tühja vektorit sisaldavaid transformante HP201HPMAL1Δ (pRS425) kasvatati erinevatel süsinikuallikatel ning eksponentsiaalse faasi rakkudes mõõdeti maltaasi aktiivsus. Tulemused on esitatud tabelis 4.

Tabel 4. *S. cerevisiae* maltaasi geeni *MAL62* regulatsioon süsinikuallikaga *H. polymorpha* maltaaskatkestusmutandis HP201HPMAL1Δ. Esitatud on kahe erineva tranformandi analüüsil saadud keskmised tulemused. Standardhälve oli alla 20%.

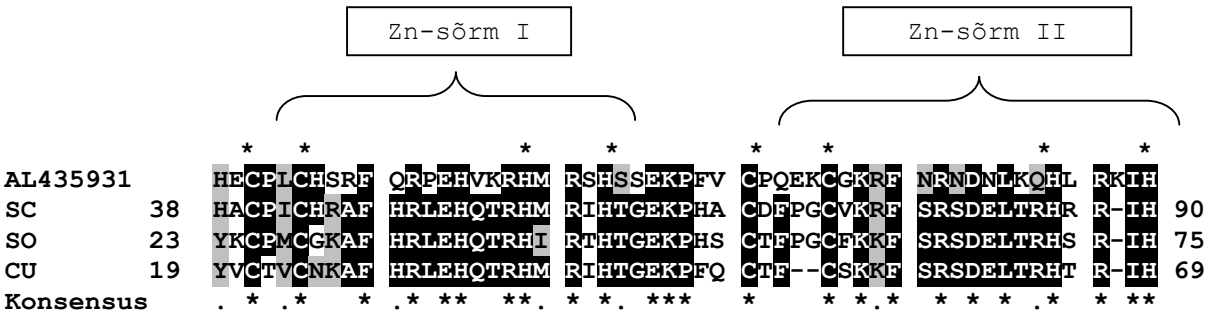
Plasmiid	**Plasmiidi iseloomustus**	**Maltaasi eriaktiivsus (mU/mg)**				
		G	M	G+M	S	G+S
pRS425-MAL62	Sisaldab *S. cerevisiae* maltaasi geeni *MAL62*	2	928	2	992	13
pRS425	Tühi vektor (kontroll)	AM	Ei kasva	AM	Ei kasva	AM

AM, allpool määramispiiri; G, 2% glükoos; M, 2% maltoos; G+M, 2% glükoos + 2% maltoos; S, 2% sahharoos; G+S, 2% glükoos +2% sahharoos.

Tabelist 4 on näha et *S. cerevisiae* maltaasi geeni *MAL62* reguleeriti pärmis *H. polymorpha* samamoodi nagu *H. polymorpha* enda maltaasi geeni: indutseeriti sahharoosil ja maltoosil kasvavates rakkudes ning represseeriti glükoosiga. Seega võib oletada, et *H. polymorpha*'l on olemas *S. cerevisiae* MAL-aktivaatori ja MIG1 repressori homoloogid. See on võimalik, sest *S. cerevisiae* MIG1 valgu homoloogid on leitud näiteks pärmidest *Kluyveromyces lactis* (Cassart *et al*., 1995), *Candida albicans* (Carmona *et al*., 2002), *Schwanniomyces occidentalis* (Zaragoza *et al*., 2000) ja *Candida utilis* (Delfin *et al*., 2001) ja kõigi nende pärmide *MIG1* geenid olid võimelised komplementeerima *MIG1* defekti pärmil *S. cerevisiae*. Pärmist *C. albicans* on leitud ka MAL-aktivaatori homoloog CASUC1 (Kelly ja Kwon-Chung, 1992).

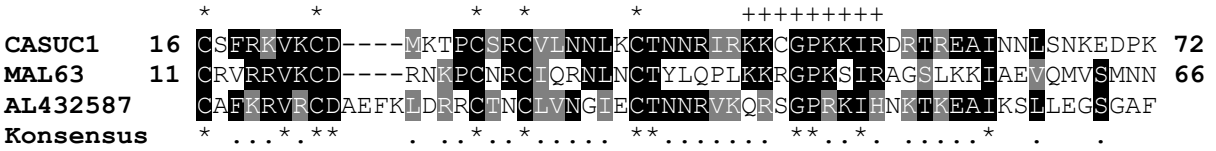
Kuna pärmi *H. polymorpha* genoom on ca 50% ulatuses sekveneeritud (Blandin *et al*., 2000) ja vastavate geenifragmentide järjestused on olemas nii geenipangas (Gen Bank) kui ka mittetraditsiooniliste pärmide andmebaasis (http://natchaug.labri.u-bordeaux.fr/Genolevures), siis otsisime nendest andmebaasidest *H. polymorpha* järjestusi, mis võiksid kodeerida *S. cerevisiae* MIG1 ja MAL-aktivaatori valkude homolooge. Selgus, et *H. polymorpha*

genoomselt järjestuselt AL435931 (kokku 979 ap) tuletatud valk oli sarnane pärmide *S. cerevisiae, Candida albicans* ja *Schwanniomyces occidentalis* MIG1 valkude N-terminaalse osaga. Glükoosi repressioonis võtmepositsioonil asuvad MIG1 valgud (vt pt 1.2) on eriti konserveerunud oma N-terminustes, kus asuvad DNA-ga seonduvad Zn-sõrmed. Valkude võrdlemisel saadud joondused on esitatud joonisel 3.



Joonis 3. Pärmide *S. cerevisiae* (SC), *S. occidentalis* (SO) ja *C. utilis* (CU) MIG1 valkude N-terminaalsete osade võrdlemine *H. polymorpha* AL435931 (tunnusnumber geenipangas) järjestuselt tuletatud oletatava MIG1 homoloogiga. Valkude võrdlemiseks kasutati programmi Clustal W (Thompson *et al*., 1994). Identsed ja sarnased aminohapped on joonise all ära märgitud vastavalt tärnide ja punktidega. Joondatud valkude identsus ja sarnasus *H. polymorpha* oletatava MIG1 valguga on ära toodud vastavalt mustal ja hallil taustal. Zn-sõrmedes konserveerunud tsüsteiini ja histidiini jäägid on tärnidega ära märgitud joonise kohal.

H. polymorpha genoomselt järjestuselt AL432587 tuletatud valk on sarnane pärmide *C. albicans* ja *S. cerevisiae* MAL-aktivaatorite N-terminaalse osaga. Pärmide transkriptsiooni aktivaatorid sisaldavad N-terminaalses osas kuute tsüsteiini jääki, mis osalevad DNA-ga seonduva Zn-sõrme moodustumises (Kim ja Michels, 1988; Kelly ja Kwon-Chung, 1992). *H. polymorpha* AL432587 järjestuselt tuletatud valgust leidsime me tsüsteiini jäägid, mis olid konserveerunud nii *C. albicans*'i kui ka *S. cerevisiae* vastavates aktivaatorvalkudes (joonis 4).



Joonis 4. Pärmide *S. cerevisiae* (SCMAL63) ja *C. albicans* (CASUC1) MAL-aktivaatorite võrdlemine *H. polymorpha* genoomselt järjestuselt AL432587 (tunnusnumber geenipangas) tuletatud MAL-aktivaatori homoloogiga. Valkude võrdlemiseks kasutati programmi Clustal W (Thompson *et al*., 1994). Identsed ja sarnased aminohapped on joonise all ära märgitud vastavalt tärnide ja punktidega. Joondatud valkude identsus ja sarnasus *H. polymorpha* oletatava MAL-aktivaatoriga on näidatud vastavalt musta ja halli taustaga. Joonise ülaosas on tärnide ja ristikestega tähistatud vastavalt konserveerunud tsüsteiinid ja oletatav tuumaminekusignaal (Kim ja Michels, 1988; Kelly ja Kwon-Chung, 1992).

Tuumasignaaljärjestused sisaldavad positiivselt laetud aminohappeid lüsiini (K) ja arginiini (R), mis piiravad kolmest aminohappest (üks nendest kindlasti proliin) koosnevat lõiku (Herrero *et al*., 1998 ja seal tsiteeritud viited). Oletatav tuumasignaal on leitud pärmi *S. cerevisiae* MAL-aktivaatorist MAL63 (KKRGPKSIR) (Kim ja Michels, 1988) ja *C. albicans*'i CASUC1 valgust (RKKCGPKKI) (Kelly ja Kwon-Chung, 1992), ning sarnane järjestus on olemas ka oletataval *H. polymorpha* MAL-aktivaatoril (vt joonis 4).

2.3.2 *H. polymorpha MAL*-lookuse struktuuri uurimine

Kõige enam on uuritud maltoosi kasutamist pärmil *S. cerevisiae*. Maltoosi kasutamises osalevad maltoosi permeaas, maltaas ja MAL-aktivaator ning nimetatud geenid paiknevad klastrina *MAL*-lookuses, kusjuures maltaasi ja maltoosi permeaasi geenidel on ühine divergentne promootorala (vt pt 1.1). Maltaasi ja maltoosi permeaasi geenide paigutus on samasugune ka pärmides *Kluyveromyces lactis* (vt järjestus Geenipangas tunnusnumbri AF261762 all), *Candida albicans* (http://genolist.pasteur.fr/CandidaDB/) ja *Torulaspora delbrueckii* (Alves-Araujo *et al*., 2004).

H. polymorpha maltaasi geen *HPMAL1* (Geenipangas numbri all AF261762) on meie töögrupil kloneeritud ja sekveneeritud (Liiv *et al*., 2001). *Southern blot* analüüsiga ning geenikatkestusega näidati, et pärmil *H. polymorpha* on ainult üks maltaasi geen (Liiv *et al*., 2001).

HPMAL1 geeni promootorala edasine sekveneerimine plasmiidi p51 inserdis (vt joonis 5) näitas, et ka pärmil *H. polymorpha* võivad maltaasi ja α-glükosiidi permeaasi geenid asuda genoomis kõrvuti ning neid võidakse transkribeerida ühiselt promootoralalt (Alamäe *et al*., 2003; lisa 1, joonis 1). Et teha kindlaks, kas nende kahe geeni naabruses võiks ka *H. polymorpha*'l paikneda *MAL*-aktivaatori geen, tegime otsinguid geenijärjestuste andmebaasides.

"Genolevures" projekti raames sekveneeriti osaliselt (ca 50% ulatuses) 13 mittetraditsioonilist pärmi, nende hulgas ka *H. polymorpha*. α-glükosiidi permeaasi järjestusega plasmiidis p51 (vt skeemi joonisel 5) kattuva järjestuse leidsime *H. polymorpha* genoomsest kloonist BB0AA021D05: see sisaldas α-glükosiidi permeaasi geeni lõppu (geenipangas numbri AL434103 all) ning järjestust AL434102, mis võiks kodeerida MAL-aktivaatorit (vt joonis 5). Edasisel järjestuste võrdlemisel programmiga Blastn leidsime *H. polymorpha* raamatukogu klooni BB0AA011B12 (vt joonis 5), mille genoomse inserdi ühes otsas on *H. polymorpha* maltaasi geeni algusega identne järjestus ning teises otsas on järjestus, mis võib kodeerida

MAL-aktivaatorit (Geenipangas on need järjestused tähistatud vastavalt AL432586 ning AL432587). Analüüsides *MAL*-geenide paigutust erinevates genoomsetes kloonides oletasime, et kloonis BB0AA011B12 (joonis 5) võiks maltaasi ja *MAL*-aktivaatori geenide vahel paikneda α-glükosiidi permeaasi geen.

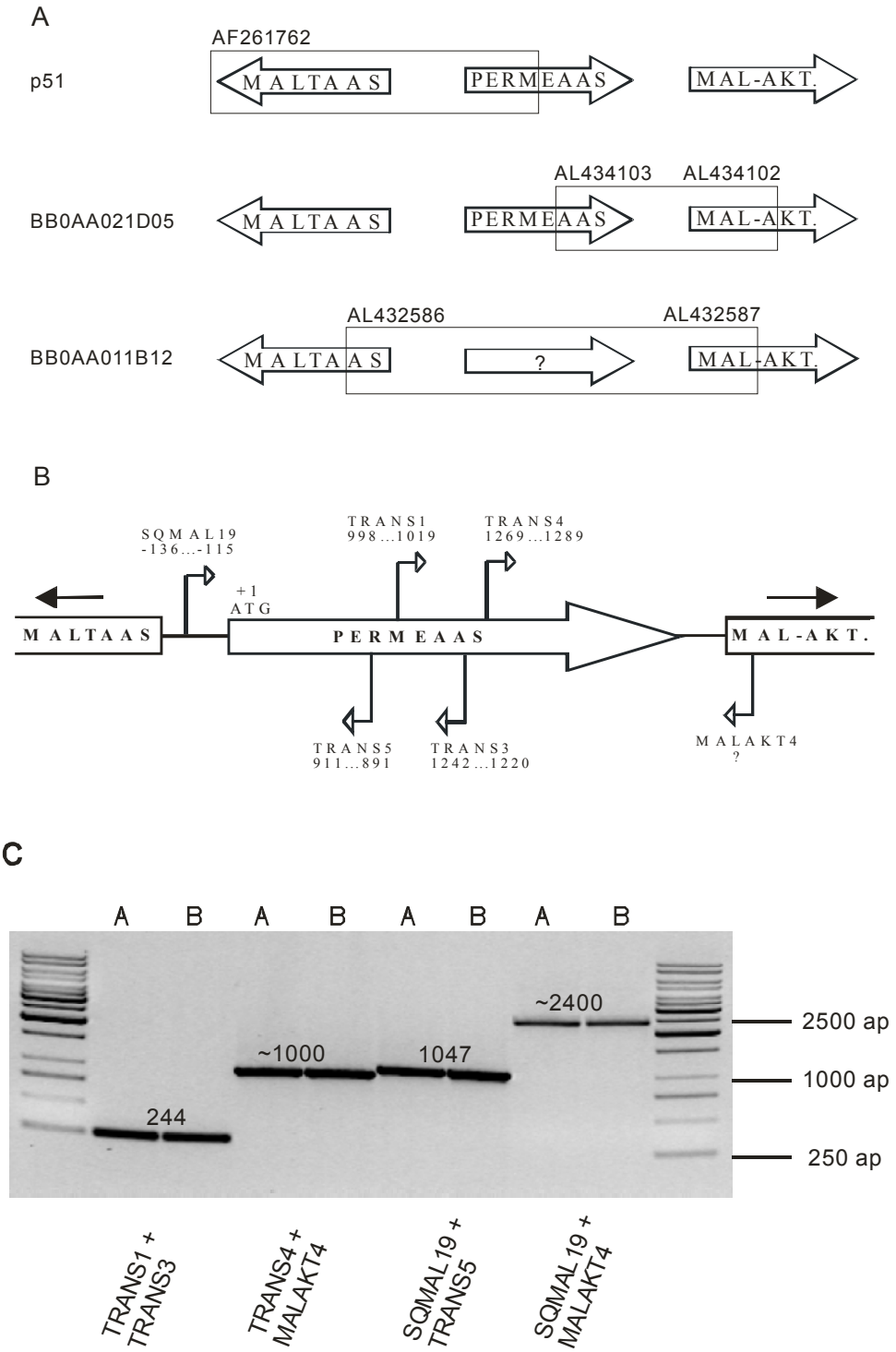
Et seda tõestada, kasutasime polümeraasi ahelreaktsiooni *H. polymorpha MAL*-geenide spetsiifiliste praimeritega. Esmalt amplifitseerisime *H. polymorpha* tüvest A16 eraldatud genoomselt DNA-lt (Rose *et al*., 1990) ~2400 ap pikkuse lõigu praimeritega SQMAL19 (seostub maltaasi geeni promootorile) ja MALAKT4 (seostub oletatavale *MAL*-aktivaatori geenile) ning puhastasime saadud produkti. Järgnevalt kontrollisime, kas saadud produktilt on võimalik amplifitseerida produkte praimeritega, mis seostuvad α-glükosiidi permeaasi geenile. Kasutatud praimerite paarid ja arvestuslikud produktide pikkused (vt ka tabel 3 ja joonis 5) olid järgnevad:

SQMAL19 + MALAKT4 = ~2400 ap

SQMAL19 + TRANS5 = 1047 ap

TRANS1 + TRANS3 = 244 ap

TRANS4 + MALAKT4 = ~1000 ap



Joonis 5.

A: *H. polymorpha MAL*-geenide paiknemine plasmiidis p51 ning genoomsetes kloonides BB0AA021D05 ja BB0AA011B12. Raami sees on näidatud nimetatud kloonide genoomses inserdis sisalduv osa *MAL*-lookusest. Järjestuste juures on esitatud ka vastavad tunnusnumbrid Geenipangas.

B: *H. polymorpha* maltoosi permeaasi geeni detekteerimiseks kasutatud praimerid ja nende seondumiskohad näidatuna α-glükosiidi permeaasi geeni ATG koodoni suhtes. Praimeri MALAKT4 täpne seondumiskaugus permeaasi geeni kodeeriva järjestuse algusest ei ole teada.

C: *H. polymorpha* tüve A16 genoomselt DNA-lt (A) ja genoomselt kloonilt BB0AA011B12 (B) α-glükosiidi permeaasile seonduvate praimeritega amplifitseeritud produktid, lahutatuna 1% agaroosgeelis. DNA fragmentide suuruse hindamiseks kasutati 1 kap suurusmarkerit firmalt Fermentas.

Jooniselt 5C on näha, et amplifitseerunud produktid on oodatud pikkusega ning et sama pikkusega produktid on saadud nii *H. polymorpha* genoomselt DNA-lt kui ka kloonilt BB0AA011B12. Järelikult on *H. polymorpha MAL*-lookuse struktuur selline nagu oletasime. Kuna kloonis BB0AA011B12 võiks sisalduda terve α-glükosiidi permeaasi geen, siis otsustasime lookuse skeemi kontrollida sekveneerimisega. Oletatav α-glükosiidi permeaasi geen (*HPMAL2*) sekveneeriti genoomses kloonis BB0AA011B12. Geeni järjestus deponeeriti Geenipangas tunnusnumbri AY917133 all. *HPMAL2* ja oletatava *MAL*-aktivaatori geenide vaheala on kõigest 238 ap pikkune. *H. polymorpha´*l paiknevad klastrina veel nitraadi kasutamises osalevad geenid ning nende geenide vahealad on isegi lühemad (Avila *et al*., 1998). Nimetatud geeniklastrist 95.8% moodustab kodeeriv ala.

HPMAL2 geenilt tuletatud valk (582 ah) oli 39-57% identne teiste pärmide maltoosi permeaasidega. Kui valkude võrdlusest varieeruvad N- ja C-terminaalsed osad välja jätta, siis oli valkude identsus oluliselt suurem (vt lisa 2, tabel 1 ja joonis 2). Kõige suurem identsus (57%) leiti pärmi *D. hansenii* ühe maltoosi permeaasiga. 51% identsus leiti *C. albicans´*i maltoosi permeaasiga. Ka *H. polymorpha* maltaas on üpris sarnane maltaasidega samadest pärmidest: 62% ulatuses indentne maltaasiga pärmist *D. hansenii* ja 58% ulatuses identne *C. albicans´*i maltaasiga. *H. polymorpha* HPMAL2 permeaasi joondus maltoosi permeaasidega pärmidest *D. hansenii, S. cerevisiae* ja *C. albicans* on näidatud lisas 2 joonisel 2a. HMMTOP programmiga (http://www.enzim.hu/hmmtop/) võib ennustada, et α-glükosiidi permeaasil on 11 transmembraanset domeeni ning et N-terminaalne ots paikneb väljaspool rakumembraani.

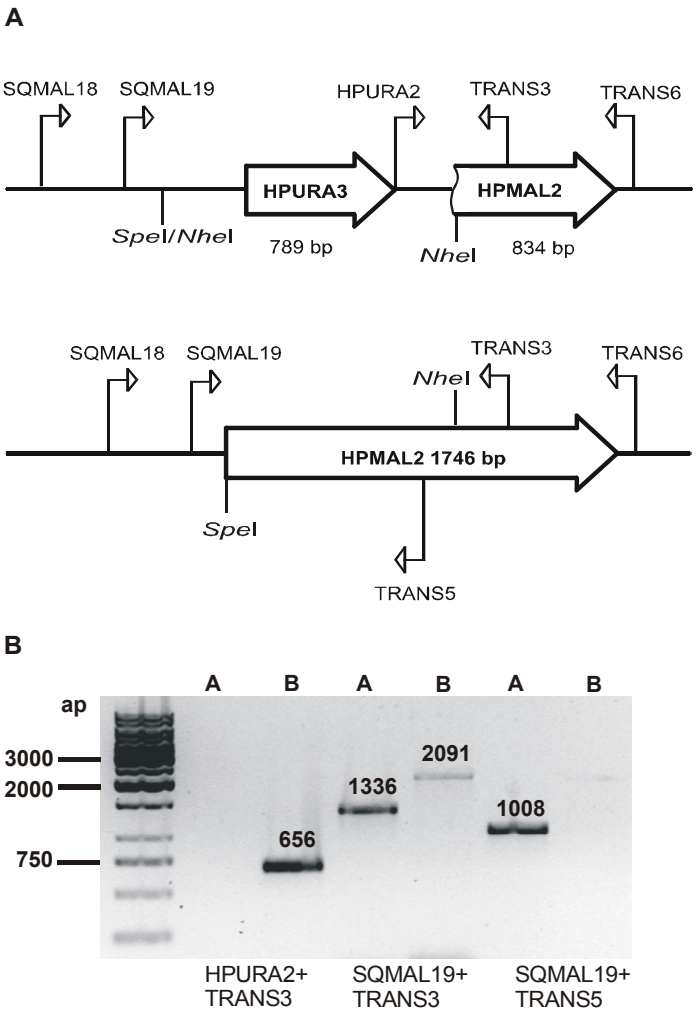
2.3.3 *H. polymorpha* α-glükosiidi permeaasi geeni kloneerimine *MAL*-lookusest ja selle funktsionaalsuse kontrollimine

Meie varasemad katsed (Alamäe ja Liiv, 1998) on näidanud, et pärmis *H. polymorpha* toimub maltoosi ja sahharoosi hüdrolüüs maltaasi abil ning rakukestaga seotud invertaas puudub. Oletasime, et pärmis *H. polymorha* võiks ka maltoosi ja sahharoosi transport toimuda sama kandja vahendusel ja seda permeaasi võiks kodeerida *HPMAL2* geen. Et saada tõendeid oletuse kontrollimiseks, konstrueeriti *HPMAL2* geeni katkestusmutant ning uuriti saadud mutandi võimet kasvada maltoosil ja sahharoosil.

Geeni katkestamiseks asendati osa permeaasi geeni kodeerivast alast (912 ap) plasmiidis BB0AA011B12 *H. polymorpha HPURA3* geeniga (inserdi suurus 1667 ap) (vt joonis 6). Saadud konstruktilt pBB12::HPURA3 amplifitseeriti praimeritega TRANS6 ja SQMAL18 (tabel 3) fragment, mis transformeeriti *H. polymorpha* uratsiil/leutsiin/metioniin kolmikauksotroofsesse tüvesse HP201. Transformandid plaaditi uratsiili mittesisaldavale

minimaalsöötmele glükoosiga. Eeldati, et *HPURA3* geeniga katkestatud *HPMAL2* integreerub homoloogilise rekombinatsiooni teel genoomi, vahetades välja funktsionaalse *HPMAL2* geeni. Uratsiilprototroofsete kolooniate kasvu kontrolliti glükoosil, maltoosil ja sahharoosil. Kõik uratsiilprototroofsed kolooniad, mis kasvasid glükoosil, kuid mitte maltoosil, ei kasvanud ka sahharoosil. Oletatavad *H. polymorpha* α-glükosiidi permeaasi katkestusmutandid tähistati HP201HPMAL2Δ.

Järgnevalt eraldati HP201 ja HP201HPMAL2Δ rakkudest kromosomaalne DNA ning amplifitseeriti sellelt lõigud, mille pikkuse järgi saaks spetsiifiliselt kinnitada *HPMAL2* geeni katkestumist genoomis. Kasutatud praimerid ning geelelektroforeesi pilt on esitatud joonisel 6.



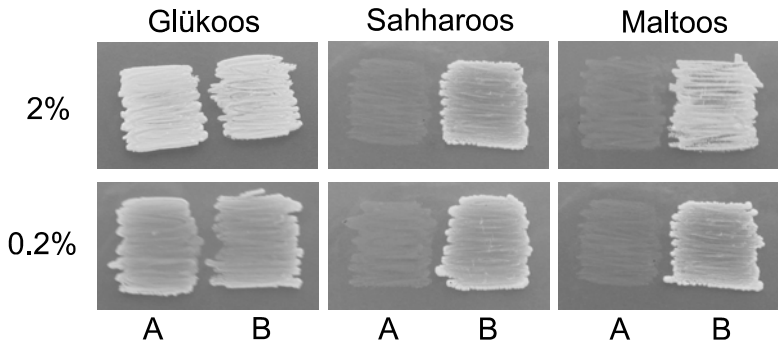
Joonis 6.

A: *H. polymorpha HPMAL2* geeni katkestamise skeem. Joonisel on näidatud kloneerimisel kasutatud *Spe*I ja *Nhe*I lõikekohad ning geeni katkestamiseks ja katkestuse kontrollimiseks kasutatud praimerite seondumiskohad.

B: α-glükosiidi permeaasi geeni katkestuse kontroll genoomse DNA PCR analüüsiga. *H. polymorpha* tüve HP201 (A) ja α-glükosiidi permeaasi katkestusmutandi HP201HPMAL2Δ (B) genoomselt DNA-lt amplifitseeritud fragmendid lahutatuna 1% agaroosgeelis. DNA fragmentide suuruse hindamiseks kasutati 1 kap suurusmarkerit firmalt Fermentas. Arvestuslikud fragmentide suurused (ap) on näidatud joonisel nende kohal.

Jooniselt 6B on näha, et α-glükosiidi permeaasi geeni katkestamine genoomis õnnestus. Praimeripaariga HPURA2 ja TRANS3 oli võimalik produkti saada vaid HP201HPMAL2Δ mutandi DNA-lt, kuna kasutati spetsiifilist *HPURA3* geenile seonduvat praimerit. Praimeripaariga SQMAL19 ja TRANS5 saadi produkt vaid metsiktüvelt HP201, kuna TRANS5 seondumiskoht lõigati katkestuskonstruktist kloneerimisel välja. SQMAL19 ja TRANS3 praimeripaariga saadi HP201 ja HP201HPMAL2Δ genoomselt DNA-lt erineva pikkusega fragmendid, kuna maltoosi permeaasi geeni kodeerivast alast välja lõigatud 912 ap asendati 1667 ap pikkuse *HPURA3* geeni sisaldava fragmendiga.

Et kontrollida α-glükosiidi permeaasi geeni katkestuse komplementatsiooni *H. polymorpha* α-glükosiidi permeaasi geeniga, transformeeriti *H. polymorpha* α-glükosiidi permeaasi katkestumutanti *HPMAL2* geeni sisaldava plasmiidiga pRS425 BamHI-BglII ning vastava tühja vektoriga pRS425. Saadud transformandid külvati glükoosi, maltoosi ja sahharoosi sisaldavale tardsöötmele. Jooniselt 7 on näha, et tühja vektorit sisaldavad HP201HPMAL2Δ transformandid kasvasid ainult glükoosil. Plasmiidil *HPMAL2* geeni kandvad HP201HPMAL2Δ transformandid kasvasid ka maltoosil ja sahharoosil. Järelikult vastutab maltoosi permeaas nii maltoosi kui sahharoosi transpordi eest *H. polymorpha´s.*



Joonis 7. *H. polymorpha* α-glükosiidi permeaasi katkestusmutandi HP201HPMAL2Δ transformantide kasv erinevatel süsinikuallikatel. Mutanti transformeeriti *H. polymorpha* α-glükosiidi permeaasi geeni sisaldava pRS425 BamHI-BglII plasmiidiga (B) ja vastava tühja vektoriga pRS425 (A).

Meie andmed näitavad, et *H. polymorpha´*l on ainult üks *MAL*-lookus, milles paiknevad kõrvuti maltaasi geen *HPMAL1* ja α-glükosiidi permeaasi geen *HPMAL2*. Lookuses permeaasi geeni kõrval paikneb suure tõenäosusega *MAL*-aktivaatori geen, mille funktsionaalsust tuleb veel tõestada.

Kuna on teada, et *S. cerevisiae* maltaasi geen *MAL62* ja tema promootor on äratuntav ja korrektselt reguleeritud pärmis *H. polymorpha* (vt pt 2.3.1) ning ka vastupidi: *H. polymorpha* maltaasi geen *HPMAL1* ja tema promootor on äratuntav pagaripärmis (Alamäe *et al.,* 2003),

siis otsustasime testida, kas *H. polymorpha* α-glükosiidi permeaasi geen funktsioneerib pärmis *S. cerevisiae*. Selleks transformeeriti *S. cerevisiae* α-glükosiidi permeaasi suhtes negatiivset tüve CMY1050 *HPMAL2* geeni sisaldava plasmiidiga pRS425 BamHI-BglII ning uuriti transformantide kasvu maltoosil. Kontrolliks kasutasime tühja vektoriga transformeeritud CMY1050 ja metsiktüve CMY1001. Kõik transformandid kasvasid glükoosisöötmel, kuid ainult metsiktüvi kasvas maltoosisöötmel. Samuti ei näinud me maltaasi induktsiooni galaktoosi ja maltoosi segul kasvavates rakkudes. Seda induktsiooni testi on eelnevalt kasutatud maltoosi permeaasi funktsioneerimise kontrollimiseks pagaripärmis. Nimelt, maltoosi transport rakku on vajalik maltaasi sünteesi indutseerumiseks (Wang *et al*., 2002). Oletasime, et *HPMAL2* promootor ei töötanud pagaripärmis ning otsustasime asendada selle *S. cerevisiae* maltoosi permeaasi geeni promootoriga. Selleks konstrueeritud plasmiidiga pRS425 Mal61promHPMAL2 transformeeriti uuesti *S. cerevisiae* tüve CMY1050. Seekord kasvasid transformandid maltoosil ning neil esines ka maltaasi induktsioon maltoosiga (vt lisa 2, tabel 2). Järelikult on *HPMAL2* geeniga kodeeritav valk funktsionaalne pagaripärmis, kuid *H. polymorpha HPMAL1-HPMAL2* geenide ühine promootorala on pagaripärmis äratuntav vaid maltaasi geeni suunas.

2.3.4 *H. polymorpha MAL*-lookuse promootorite regulatsiooni uurimine reportergeenide abil

On teada, et *S. cerevisiae MAL61-MAL62* vaheline promootorala on mõlemas suunas süsinikuallikaga sarnaselt reguleeritud (Levine *et al*., 1992, Bell *et al*., 1995). Seetõttu otsustasime uurida *H. polymorpha* maltaasi ja α-glükosiidi permeaasi geenide ühise promootorala regulatsiooni. Selleks tegime konstruktid pRS425 malpromFwHPMAL1 ja pRS425 malpromRevHPMAL1, milles maltaasi geeni (*HPMAL1*) ees on *H. polymorpha HPMAL1-HPMAL2* promootorala vastavalt maltaasi ja α-glükosiidi permeaasi geeni suunas. Kontrollina kasutasime konstrukti pRS425 HPMAL1, mis sisaldab promootorita maltaasi geeni. Nimetatud plasmiididega transformeerisime *H. polymorpha* maltaasnegatiivset mutanti HP201HPMAL1Δ. Promootorita maltaasi geeni sisaldavad HP201HPMAL1Δ transformandid kasvasid glükoosil ja glütseroolil, kuid ei kasvanud ei maltoosil ega sahharoosil. Glütseroolil kasvanud HP201HPMAL1Δ (pRS425 HPMAL1) transformantides puudus ka maltaasi aktiivsus (Viigand *et al*., 2005).

Transformandid, mis sisaldasid plasmiidil maltaasi geeni ees *HPMAL1-HPMAL2* promootorala, suutsid kasvada mõlemal disahhariidil – maltoosil ja sahharoosil, sõltumata promootori orientatsioonist (vt lisa 2, joonis 3).

Järgnevalt kasvatasime nimetatud transformante erinevatel süsinikuallikatel: 2% maltoosil (indutseerivates tingimustes), 2% glükoosil (represseerivates tingimustes) ja 2% glütseroolil (derepressiooni tingimustes) ning määrasime rakkudes maltaasi aktiivsuse.

Katse näitas, et *HPMAL1-HPMAL2* promootorala on mõlemas suunas maltoosiga indutseeritav, glükoosiga represseeritav ning et ekspressioon on maltaasi geeni suunas pisut suurem (vt lisa 2, joonis 4).

H. polymorpha MAL-lookuses paikneb peale maltaasi ja α-glükosiidi permeaasi geenide veel kolmas geen – oletatav *MAL*-aktivaatori geen, mille promootorala on kõigest 238 ap pikk. Eeldasime, et *MAL*-aktivaatori geeni promootor võiks olla süsinikuallikaga reguleeritav samamoodi, kui *HPMAL1-HPMAL2* promootor. Selle kontrollimiseks tegime kaks konstrukti, milles *MAL*-aktivaatori geeni promootor paiknes kahes erinevas orientatsioonis ning reporterina kasutasime jällegi *H. polymorpha* maltaasi geeni *HPMAL1*. Saime konstruktid pRS425 MalaktpromFwHPMAL1 ja pRS425 MalaktpromRevHPMAL1, milles *MAL*-aktivaatori geeni promootor on maltaasi geeni ees vastavalt samas suunas kui *MAL*-aktivaatori geeni ees ning vastupidises suunas. Nende plasmiididega transformeerisime *H. polymorpha* maltaasnegatiivset mutanti HP201HPMAL1Δ ning kasvatasime transformante erinevatel süsinikuallikatel. Eeldasime, et uuritav promootor võiks olla glükoosiga represseeritav ning maltoosi ja sahharoosiga indutseeritav. Transformandid kasvatati eksponentsiaalsesse kasvufaasi ning rakkudes määrati maltaasi aktiivsus. Tulemused on esitatud tabelis 5.

Tabel 5. *H. polymorpha MAL*-aktivaatori promootori regulatsioon süsinikuallikaga *H. polymorpha* maltaaskatkestusmutandis HP201HPMAL1Δ. Esitatud on kahe erineva transformandi analüüsil saadud keskmised tulemused. Standardhälve oli alla 20%.

Plasmiid	Maltaasi eriaktiivsus (mU/mg)			
	2% glükoos	2% glütserool	2% maltoos	2% sahharoos
pRS425 MalaktpromFwHPMAL1	11	63	479	380
pRS425 MalaktpromRevHPMAL1	4	40	1494	1747
pRS425 HPMAL1	AM	2	Ei kasva	Ei kasva

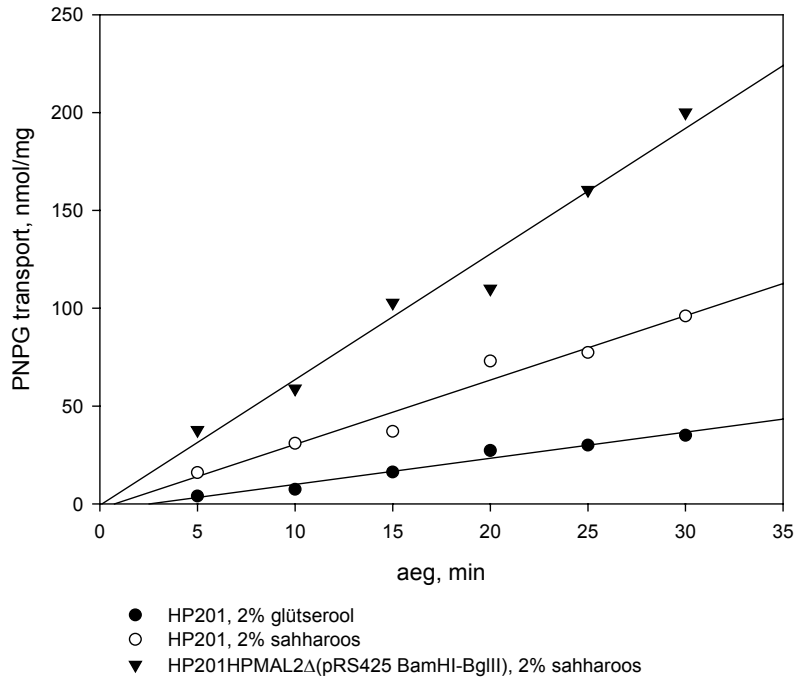
AM, allpool määramispiiri

Tabelist 5 on näha, et *MAL*-aktivaatori promootor on mõlemas suunas reguleeritud sarnaselt *HPMAL1-HPMAL2* promootorile: represseeritud glükoosil, indutseeritud disahhariididel ja nõrgalt derepresseeritud glütseroolil kasvavates rakkudes. Miks on *MAL*-aktivaatori promootor funktsionaalne ning reguleeritav mõlemas suunas? Seda võiks ehk põhjendada sellega, et Zn-sõrmvalkudel, mis võiksid vahendada selle promootori regulatsiooni, on

näidatud promootori orientatsioonist sõltumatut aktiveerivat toimet (Bell *et al*., 1995; Schjerling ja Holmberg, 1996). Näiteks pagaripärmi galaktoosi kasutamises osalevate geenide *GAL1-GAL10* kahesuunaline promootor on mõlemas suunas galaktoosiga indutseeritav ning induktsiooni vahendab Zn-sõrmvalk GAL4, mis seondub promootori ühises divergentses UAS alas palindroomsetele seondumisjärjestustele (**CGG**N_{11}**CCG**) dimeerina (Carey *et al*., 1989; Gancedo, 1998).

2.3.5 *H. polymorpha* α-glükosiidi permeaasi omaduste uurimine

H. polymorpha α-glükosiidi permeaasi omaduste uurimiseks kasutasime tüve HP201 ja selle permeaaskatkestusmutanti HP201HPMAL2Δ. Esmalt kasvatasime α-glükosiidi permeaasnegatiivset mutanti HP201HPMAL2Δ, vastavat metsiktüve HP201 ja α-glükosiidi permeaasi geeniga komplementeeritud katkestusmutante HP201HPMAL2Δ (pRS425 BamHI-BglII) sahharoosil ja glütseroolil ning määrasime rakkudes PNPG transpordi kiiruse. Tulemused on esitatud joonisel 8.



Joonis 8. PNPG transport HP201 ja HP201HPMAL2Δ (pRS425 BamHI-BglII) glütseroolil ja sahharoosil kasvanud rakkudes.

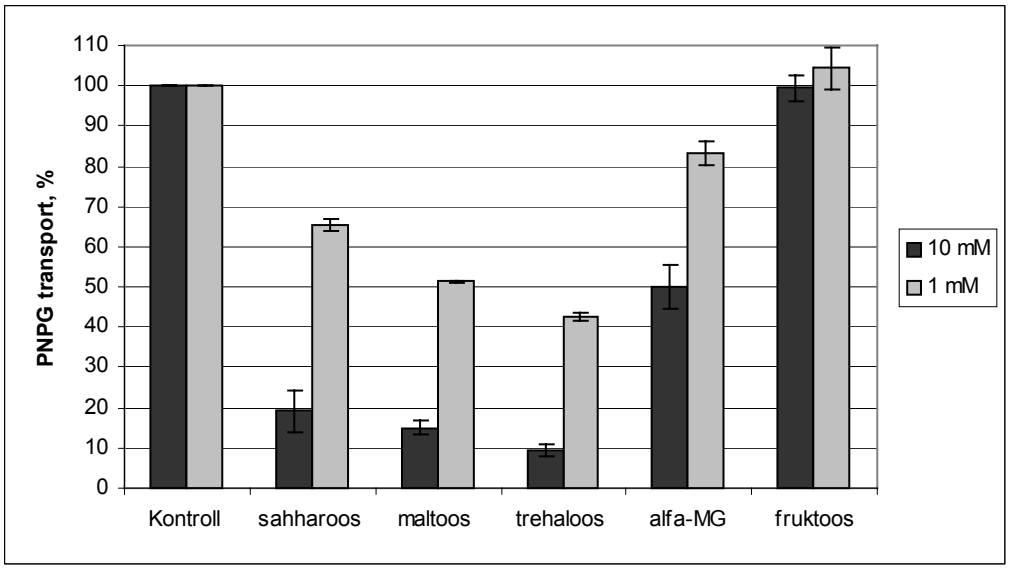
Jooniselt 8 on näha, et PNPG transport on sahharoosil kasvanud HP201 rakkudes ~3 korda kiirem kui glütseroolil kasvanud rakkudes. See on vastavuses meie varasemate andmetega, mis näitavad, et *MAL*-geenide ekspressioon on glütseroolil kasvanud rakkudes palju madalam, kui disahhariididel (maltoosil ja sahharoosil) kasvanud rakkudes (lisa 2, joonis 4;

tabel 5 osas 2.3.4). Permeaasnegatiivne mutant ei kasva sahharoosil. Seetõttu kontrollisime tema võimet PNPG-d transportida glütseroolil kasvanud rakkudega ja nägime, et mutant PNPG-d rakku ei transpordi (andmeid ei esitata). Plasmiidil *HPMAL2* geeni kandvatel katkestusmutantidel HP201HPMAL2Δ (pRS425 BamHI-BglII) taastus võime kasvada sahharoosil ning PNPG transpordi kiirus sahharoosil kasvanud rakkudes oli neil kuni kaks korda suurem, kui metsiktüvel HP201. See on ilmselt seletatav sellega, et transformantidel on maltoosi permeaasi geeni rakus rohkem kui üks koopia.

α-glükosiidi permeaasi afiinsuse määramiseks PNPG suhtes mõõtsime PNPG transpordi kiirust metsiktüves HP201 erinevatel PNPG kontsentratsioonidel (0.2 mM – 5 mM). Permeaasi K_m PNPG-le tuletatuna Eadie-Hofstee graafikult on 0.6±0.1 mM. Pärmi *S. cerevisiae* α-glükosiidi permeaasi AGT1 afiinsus PNPG-le on mõnevõrra madalam (K_m 3 mM). Sama permeaasi afiinsus tema looduslikele substraatidele, maltoosile ja sahharoosile on madalam kui PNPG-le - K_m väärtused vastavalt 18 ja 7 mM (tabel 1; Han *et al*., 1995; Stambuk ja Araujo, 2001; Hollatz ja Stambuk, 2001).

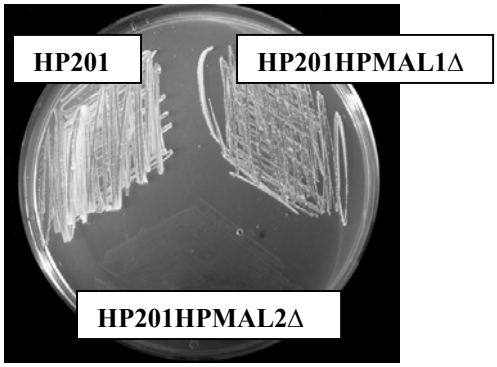
PNPG on substraadiks ka maltaasidele. Ka maltaaside puhul kehtib see, et nende afiinsus PNPG suhtes on kõrgem, kui nende loomulike substraatide maltoosi ja sahharoosi suhtes. Näiteks *H. polymorpha* maltaasi K_m PNPG-le on 0.51 mM, samas kui K_m maltoosile ja sahharoosile on vastavalt 95 mM ja 42 mM (Liiv *et al*., 2001).

H. polymorpha α-glükosiidi permeaasi substraadispetsiifilisuse uurimiseks kasutasime erinevaid substraate, mida ta võiks potentsiaalselt transportida ning testisime nende võimet konkureerida PNPG-ga. Konkureerivate substraatidena kasutasime maltoosi, sahharoosi, trehaloosi, α-metüülglükosiidi, glükoosi ja fruktoosi lõppkontsentratsioonides 1 mM ja 10 mM, vahekorras vastavalt 1:1 ja 1:10 PNPG kontsentratsiooni suhtes. Glükoosi ja fruktoosi kasutasime negatiivsete kontrollidena, kuna eeldasime, et maltoosi permeaas neid monosahhariide transportida ei tohiks.



Joonis 9. *H. polymorpha* α-glükosiidi permeaasi substraadispetsiifilisus. PNPG (1 mM) transpordiga konkureerivate suhkrutena kasutati maltoosi, sahharoosi, trehaloosi, α-metüülglükosiidi ja fruktoosi lõppkontsentratsioonides 1 mM ja 10 mM. Kontrollina (võrdsustatud 100%-ga) on kasutatud PNPG transpordi kiirust variandis, kus konkurente ei lisatud. PNPG transpordi kiirus kontrollvariandis oli 3.6 nmol/mg/min. Näitatud on kahe erineva katse tulemused ja standardhälve.

Jooniselt 9 selgub, et kõige tugevamini konkureerisid PNPG transpordiga trehaloos, maltoos ja sahharoos. Maltoos ja sahharoos on substraadiks ka *H. polymorpha* maltaasile, trehaloos aga mitte (Liiv *et al*., 2001). Testisime ka *H. polymorpha* metsiktüve ning maltaas- ja permeaaskatkestusmutandi kasvu trehaloosil ning nägime, et ainult permeaaskatkestumutant ei suutnud trehaloosil kasvada (vt joonis 10). Sellest võib järeldada, et α-glükosiidi permeaas võiks olla ainus trehaloosi transportija *H. polymorpha*'l. Katse tõestas ka veelkord, et et maltaas pole vajalik trehaloosi kasutamiseks. Arvame, et trehaloosi hüdrolüüsitakse raku sees spetsiifilise ensüümi – trehalaasiga nii nagu pagaripärmilgi (Stambuk *et al*., 1996).



Joonis 10. *H. polymorpha* metsiktüve, maltaaskatkestusmutandi HP201HPMAL1Δ ja permeaaskatkestusmutandi HP201HPMAL2Δ kasv 0.5% trehaloosil.

PNPG transporti inhibeeris ka α-metüülglükosiid, kuigi tunduvalt vähem, kui maltoos, trehaloos ja sahharoos. Huvitav on see, et *H. polymorpha* siiski α-metüülglükosiidil ei kasva, kuigi ka maltaas on võimeline α-metüülglükosiidi hüdrolüüsima. Võimalik, et α-metüülglükosiid ei indutseeri maltaasi ja maltoosi permeaasi sünteesi ning seetõttu ei ole kasv sellel substraadil võimalik.

Et kaudselt hinnata *H. polymorpha* α-glükosiidi permeaasi afiinsust maltoosi suhtes, võrdlesime *H. polymorpha* tüve HP201 ja *S. cerevisiae* tüve CMY1001 (sisaldab maltoosi permeaasi geeni *MAL61*) kasvu 1 mM, 5 mM ja 10 mM maltoosil. Kasvu visuaalsel hindamisel nägime, et HP201 kasvas 1mM ja 5mM maltoosil oluliselt paremini kui CMY1001. Teades, et *S. cerevisiae* MAL61 permeaasi K_m maltoosile on 2-4 mM (vt tabel 1; Han *et al*., 1995) võib järeldada, et HPMAL2 afiinsus maltoosile on suurem. Oletame, et *H. polymorpha* α-glükosiidi permeaasi K_m maltoosile võiks kindlasti olla alla 2 mM. Lähtudes sellest, et 1 mM maltoosi lisamine vähendas 1 mM PNPG transporti umbes kaks korda (joonis 9), võiks α-glükosiidi permeaasi K_m maltoosile olla ~1 mM.

Nii nagu ennustasime, ei konkureerinud fruktoos PNPG-ga transpordi pärast, kuid glükoosi lisamine rakkususpensioonile alandas PNPG transporti 40%-ni kontrolltasemest. Arvame, et α-glükosiidi permeaas ei transpordi glükoosi rakku, kuid viimane suudab ilmselt permeaasiga seostuda ja seetõttu PNPG transporti inhibeerida. Selle oletuse kasuks räägib see, et *H. polymorpha* HPMAL2 permeaaskatkestusmutant kasvab glükoosil sama hästi, kui metsiktüvi. Oleme varem näidanud, et ilmselt sama mehhanismiga konkureerib *H. polymorpha*'l glükoosi transpordiga maltoos (Karp ja Alamäe, 1998).

Konkurentsikatses kasutatud ained võivad potentsiaalselt konkureerida ka maltaasiga, takistades PNPG rakusisest hüdrolüüsi. PNPG hüdrolüüsil maltaasiga moodustub *p*-nitrofenool, mille rakust väljadifundeerumise järgi PNPG transpordi kiirust mõõdeti. Seetõttu hindasime ka samade ainete konkureerimist PNPG-ga selle rakusisesel hüdrolüüsil maltaasireaktsioonis. Tulemused näitasid, et kui maltaasi kiiruse mõõtmise reaktsioonisegule lisati PNPG suhtes kümnekordses ülehulgas transpordikatses kasutatud konkurente, siis mingit PNPG hüdrolüüsi inhibeerumist ei ilmnenud. See on kooskõlas meie grupi varasemate andmetega, mis näitavad, et *H. polymorpha* maltaasi afiinsus PNPG-le on kõrge, kuid sahharoosile, maltoosile ja α-metüülglükosiidile väga palju madalam (Liiv *et al*., 2001). Seega arvame, et PNPG transpordi uurimises konkurentsikatse tulemused iseloomustavad α-glükosiidi permeaasi substraadispetsiifilisust.

Kõik senikirjeldatud maltoosi permeaasid ja α-glükosiidi permeaasid pärmidel ja taimedel on prootonsümpordid (Leeuwen *et al*., 1992; Stambuk *et al*., 2000; Hollatz ja Stambuk, 2001; Reinders ja Ward, 2001). Nende funktsioneerimist mõjutavad ained, mis kas takistavad

membraanse prootongradiendi tekkeks vajaliku ATP sünteesi või lõhuvad membraanil juba moodustunud prootongradiendi (Brondijk *et al*., 2001; Hollatz ja Stambuk, 2001). Uurimaks, kas ka *H. polymorpha* α-glükosiidi permeaas on prootonsümporter, testisime prooton-sümportereid mõjutavate metaboolsete mürkide (CCCP ja Na-asiidi) mõju PNPG transpordile. Tulemused on esitatud tabelis 6.

Tabel 6. Metaboolsete mürkide mõju PNPG transpordile ja rakusisesele hüdrolüüsile tüves HP201.

Mürk	**PNPG**	
	Transport (%)	**Hüdrolüüs (%)**
Kontroll (mürke ei lisatud)	100 [a]	100 [b]
CCCP (0.05 mM)	14.9±2.4	101.8±2.5
Na-asiid (0.2 mM)	1.5±0.4	97.7±2.8
Na-asiid (2 mM)	0.3±0.1	98.6±1.5

Mürgid lisati rakususpensioonile 3 min enne PNPG lisamist (vt pt 2.2.5 ja 2.2.6).
[a], 100% = 5.3 nmooli/mg x min; [b], 100%= 635.9 nmooli/mg/min.

Tabelist 6 on näha, et PNPG rakusisene hüdrolüüs on üle saja korra kiirem kui PNPG transport rakku. Seega on PNPG kasutamisel limiteerivaks etapiks PNPG transport. Mõlemad kasutatud metaboolsed mürgid mõjutasid tugevasti PNPG transporti, kuid nende toime maltaasile (PNPG rakusisesele hüdrolüüsile) oli minimaalne. Seda, et sedatüüpi mürgid ei inhibeeri PNPG rakusisest hüdrolüüsi, on varem näidatud ka pagaripärmil (Stambuk, 2000). Seega võib antud katsetest järeldada, et ka *H. polymorpha* α-glükosiidi permeaas on kindlasti prooton-sümporter. Seda tõestab ka katse, milles mõõtsime PNPG transpordi kiirust HP201 sahharoosil kasvatatud rakkudes, kasutades rakkude suspendeerimiseks kahte erineva pH väärtusega (4.5 ja 7.0) fosfaatpuhvrit. Happelises puhvris oli PNPG transpordi kiirus üle 5 korra suurem kui neutraalses puhvris.

KOKKUVÕTE JA JÄRELDUSED

Käesoleva töö eesmärkideks oli:

1. uurida *S. cerevisiae* maltaasi geeni *MAL62* ekspressiooni pärmis *H. polymorpha;*
2. teha kindlaks *H. polymorpha MAL*-lookuse struktuur;
3. uurida *MAL*-lookuse promootorite regulatsiooni reportergeenide abil;
4. kloneerida *H. polymorpha MAL*-lookusest α-glükosiidi permeaasi geen ja testida tema funktsionaalsust;
5. iseloomustada *H. polymorpha MAL*-lookusega kodeeritavat permeaasi.

Läbiviidud eksperimentide põhjal võib teha järgmised järeldused:

1. *S. cerevisiae* maltaasi geen ja tema promootor olid *H. polymorpha'*s funktsionaalsed, kuna taastasid plasmiidil ekspresseerituna *H. polymorpha* maltaaskatkestusmutandi kasvu maltoosil ja sahharoosil;
2. *S. cerevisiae* maltaasi geeni promootorit reguleeriti *H. polymorpha*'s süsinikuallikaga samuti nagu pagaripärmis. See lubab oletada, et pärmis *H. polymorpha* võivad olla *S. cerevisiae* MIG1 ja MAL-aktivaatorvalgu homoloogid;
3. *H. polymorpha* genoomis on olemas järjestused, mis võiksid kodeerida tema MIG1 valku ja MAL-aktivaatorit;
4. *H. polymorpha MAL*-geenid paiknevad klastrina *MAL*-lookuses;
5. *H. polymorpha MAL*-lookuses paiknevate geenide promootorid on sarnaselt reguleeritud: indutseeritud maltoosi ja sahharoosiga, represeeritud glükoosiga ja mõõdukalt derepresseeritud glütseroolil kasvanud rakkudes. *HPMAL1-HPMAL2* geenide kahesuunaline promootorala on mõlemas suunas koordineeritult reguleeritav;
6. Kuna *H. polymorpha HPMAL2* geeni katkestusmutant ei kasva maltoosil, sahharoosil ja trehaloosil, siis temaga kodeeritav permeaas transpordib kindlasti kõiki neid α-glükosiide rakku. Seega peaks *HPMAL2* geeniga kodeeritavat permeaasi nimetama α-glükosiidi permeaasiks;
7. HPMAL2 on ainus α-glükosiidi permeaas *H. polymorpha*`l;
8. *H. polymorpha* α-glükosiidi permeaas on prooton-sümporter ja tema afiinsus *p*-nitrofenüül-α-D-glükosiidile on 0.6±0.1 mM.

SUMMARY

Methylotrophic yeasts are suitable organisms for the production of heterologous proteins because of their extremely strong and regulated promoters of methanol-specific genes. *Hansenula polymorpha* is an exception among methylotrophic yeasts because it grows on disaccharides - maltose and sucrose, and uses intracellular maltase for their hydrolysis. We have shown that induced strength of the *H. polymorpha* maltase gene promoter is quite close to the induced strength of the *H. polymorpha* alcohol oxidase gene promoter that is considered the strongest yeast promoter available at the moment.

Thus, regulation of the maltase gene promoter should certainly be studied because of its biotechnological potential. Besides, as genetics and biochemistry of maltose and sucrose utilization has been thoroughly studied in the model yeast *Saccharomyces cerevisiae*, addressing similar problem in a phylogenetically distant yeast, *H. polymorpha*, may yield interesting data on different aspects of evolution of genome and metabolic regulation.

As our previous work showed that expression of the *H. polymorpha* maltase gene is correctly regulated in *S. cerevisiae*, we decided to investigate expression of the *S. cerevisiae* maltase gene *MAL62* in a *H. polymorpha* maltase disruption mutant. Since it was correctly regulated in *H. polymorpha* we presume that *H. polymorpha* possesses homologues of *S. cerevisiae* regulatory proteins. Inspection of *H. polymorpha* genomic data availalbe in the GenBank resulted in sequences that may encode homologues of *S. cerevisiae* MAL activator and MIG1 repressor proteins. Further analysis of sequences in different genomic clones and sequencing of the *HPMAL2* gene showed that three *MAL*-genes in *H. polymorpha* (the maltase gene *HPMAL1*, the permease gene *HPMAL2* and the gene for a putative MAL activator) are genomically clustered to a locus (*MAL* locus) similarly to some other yeasts. The promoters of all three *MAL* genes are regulated similarly: induced by maltose and repressed by glucose.

The *HPMAL2* gene was disrupted in the *H. polymorpha* genome resulting in strains unable to grow on maltose, sucrose and trehalose, and the deficiency was complemented by transformation of the mutant with the cloned *HPMAL2* gene. Our data show that *H. polymorpha* has only one *MAL* locus that contains at least two functional *MAL* genes encoding for maltase and a permease that is needed for the transport of maltose, sucrose and trehalose into the cell. Properties of the permease were studied using *p*-nitro-phenyl-α-glucoside as a substrate. Based on sensitivity of the transport to protonophore CCCP, the permease encoded by *HPMAL2* is a proton-symporter. The affinity of the permease to PNPG is 0.6±0.1 mM. Substrate specificity assay of the permease suggests that it is an α-glucoside permease that transports some other α-glucosides besides maltose.

KASUTATUD KIRJANDUS

Ahuatzi D., Herrero P., de la Cera T., Moreno F.: The glucose-regulated nuclear localization of hexokinase 2 in *Saccharomyces cerevisiae* is Mig1-dependent. J Biol Chem 279 (2004) 14440-14446.

Alamäe T., Liiv L.: Glucose repression of maltase and methanol-oxidizing enzymes in the methylotrophic yeast *Hansenula polymorpha*: isolation and study of regulatory mutants. Folia Microbiol 43 (1998) 443-452.

Alamäe T., Pärn P., Viigand K., Karp H.: Regulation of the *Hansenula polymorpha* maltase gene promoter in *H. polymorpha* and *Saccharomyces cerevisiae*. FEMS Yeast Res 4 (2003) 165-173.

Alves-Araujo C., Hernandez-Lopez M. J., Sousa M. J., Prieto J. A., Randez-Gil F.: Cloning and characterization of the *MAL11* gene encoding a high-affinity maltose transporter from *Torulaspora delbrueckii*. FEMS Yeast Res 4 (2004) 467-476.

Avila J., Gonzalez C., Brito N., Siverio J. M.: Clustering of the YNA1 gene encoding a Zn(II)2Cys6 transcriptional factor in the yeast Hansenula polymorpha with the nitrate assimilation genes YNT1, YNI1 and YNR1, and its involvement in their transcriptional activation. Biochem J 335 (1998) 647-652.

Bali M., Zhang B., Morano K., Michels C. A.: The Hsp90 molecular chaperone complex regulates maltose induction and stability of the *Saccharomyces MAL* gene transcription activator Mal63p. J Biol Chem 278 (2003) 47441-47448.

Bell P. J., Bissinger P. H., Evans R. J., Dawes I. W.: A two-reporter system for the analysis of bi-directional transcription from the divergent MAL6T-MAL6S promoter in *Saccharomyces cerevisiae.* Curr Genet 28 (1995) 441-446.

Blandin G., Llorente B., Malpertuy A., Wincker P., Artiguenave F., Dujon B.: Genomic exploration of the hemiascomycetous yeasts: 13. *Pichia angusta*. FEBS Lett. 487 (2000) 76-81.

Boekhout T., Kurtzman C.P.: Principles and methods used in yeast classification, and an overview of currently accepted yeast genera. Nonconventional yeasts in biotechnology. (1996) 1-67.

Brondijk T. H. C., Konings W. N., Poolman B.: Regulation on maltose transport in *Saccharomyces cerevisiae.* Arch Microbiol 176 (2001) 96-105.

Busturia A., Lagunas R.: Identification of two forms of the maltose transport system in *Saccharomyces cerevisiae* and their regulation by catabolite inactivation. Biochim Biophys Acta 820 (1985) 324-326.

Carey M., Kakidani H., Leatherwood J., Mostashari F., Ptashne M.: An amino-terminal fragment of GAL4 binds as a dimer. J Mol Biol 209 (1989) 423-432.

Carmona T. A., Barrado P., Jimenez A., Lobato M. F.: Molecular and functional analysis of MIG1 homologue from the yeast *Schwanniomyces occidentalis*. Yeast 19 (2002) 459-465.

Cassart J. P., Georis I., Ostling J., Ronne H., Vandenhaute J.: The MIG1 repressor from Kluyveromyces lactis: cloning, sequencing and functional analysis in *Saccharomyces cerevisiae*. FEBS Lett 371 (1995) 191-194.

Cassart J.-P., Georis I., Östling J., Ronne H., Vandehaute J.: The MIG1 repressor from *Kluyveromyces lactis*: cloning, sequencing and functional analysis in *Saccharomyces cerevisiae.* FEBS Lett. 371 (1995) 191-194.

Chang Y. S., Dubin R. A., Perkins E., Forrest D., Michels C. A., Needleman R.B.: *MAL63* codes for a positive regulator of maltose fermentation in *Saccharomyces cerevisiae*. Curr Genet 14 (1988) 201-209.

Charron M. J., Dubin R. A., Michels C. A.: Structural and functional analysis of the *MAL1* locus of *Saccharomyces cerevisiae*. Mol Cell Biol 11 (1986) 3891-3899.

Charron M. J., Read E., Haut S. R., Michels, C.: Molecular evolution of telomere-associated *MAL* loci of *Saccharomyces*. Genetics 122 (1989) 307-316.

Cheng Q., Michels C. A.: *MAL11* and *MAL61* encode the inducible high-affinity maltose transporter of *Saccharomyces cerevisiae.* J Bacteriol 173 (1991) 1817-1820.

Cheng Q., Michels C. A.: The maltose permease encoded by the *MAL61* gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters. Genetics 123 (1989) 477-484.

Christianson T. W., Sikorski R. S., Dante M., Shero J. H., Hieter, P.: Multifunctional yeast high-copy-number shuttle vectors. Gene 110 (1992) 119-122.

Concalves P., Rodrigues de Sousa H., Spencer-Martins I.: *FSY1*, a novel gene encoding a specific fructose/H^+symporter in the type strain *Saccharomyces carlsbergensis*. J Bacteriol 182 (2000) 5628-5630.

Danzi S. E., Zhang B., Michels C. A.: Alterations in the *Saccharomyces* MAL-activator cause constitutivity but can be suppressed by intragenic mutations. Curr Genet 38 (2000) 233-240.

Day R. E., Higgins V. J., Rogers P. J, Dawes I. W.: Characterization of the putative maltose transporters encoded by YDL247w and YJR160c. Yeast 19 (2002) 1015-1027.

Delfin J., Perdomo W., Garcia B., Menendez J.: Isolation and sequence of the MIG1 homologue from the yeast *Candida utilis*. Yeast 18 (2001) 597-603.

Dubin R.A., Needleman R.B., Gossett D., Michels C.A.: Identification of the structural gene encoding maltase within the *MAL6* locus of *Saccharomyces carlsbergensis*. J Bacteriol 164 (1985) 605-610.

Entian K. D.: Sugar phosphorylation in yeast. in: Zimmerman, F. K.., Entian, K. D. (Eds.). Yeast sugar metabolism. technomic publishing, Lancaster/Basel (1997) 67-79.

Gancedo J.M.: Yeast carbon catabolite repression. Microbiol Mol Biol Rev 62 (1998) 334-361.

Geber A., Williamson P.R., Rex J.H., Sweeney E.C., Bennett J.E.: Cloning and characterization of a *Candida albicans* maltase gene involved in sucrose utilization. J Bacteriol 174 (1992) 6992-6996.

Gietz R.D, Schiestl R.H., Willems A.R., Woods R.A.: Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11 (1995) 355-360.

Goffrini P., Ferrero I., Donnini C.: Respiration-dependent utilization of sugars in yeasts: a determinant role for sugar transporters. J Bacteriol 184 (2002) 427-432.

Goffrini P., Ficarelli A., Ferrero I.: Hexokinase activity is affected in mutants of *Kluyveromyces lactis* resistant to glucose repression. Microbiol 141 (1995) 441-447.

Han E. K., Cotty F., Sottas C., Jiang H., Michels C.A.: Characterization of *AGT1* encoding a general alpha-glucoside transporter from *Saccharomyces*. Mol Microbiol 17 (1995) 1093-1107.

Hanahan D.: Studies on the transformation of *E. coli* with plasmids. J Mol Biol 166 (1983) 577-580.

Herrero P., Martinez-Campa C., Moreno F.: The hexokinase 2 protein participates in regulatory DNA-protein complexes necessary for glucose repression of the *SUC2* gene in *Saccharomyces cerevisiae*. FEBS Lett 434 (1998) 71-76.

Hollatz C., Stambuk B. U.: Colorimetric determination of active α-glucoside transport in *Saccharomyces cerevisiae.* J Microbiol Methods 46 (2001) 253-259.

Holstege F. C., Jennings E. G., Wyrick J. J., Lee T. I., Hengartner C. J., Green M. R., Golub T. R., Lander E. S., Young R. A.: Dissecting the regulatory circuitry of a eukaryotic genome. Cell 95 (1998) 717-728.

Hu Z., Nehlin J.O., Ronne H., Michels C.A.: MIG1-dependent and MIG1-independent glucose regulation of *MAL* gene expression in *Saccharomyces cerevisiae*. Curr Genet 28 (1995) 258-266.

Karp H., Alamäe T.: Glucose transport in a methylotrophic yeast *Hansenula polymorpha*. FEMS Microbiol Lett 166 (1998) 267-273.

Keleher C.A., Redd M. J., Schultz J., Carlson M., Johanson A. D.: Ssn6- Tup1 is a general repressor of transcription in yeast. Cell 68 (1992) 709-719.

Kelly R., Kwon-Chung K.J.: A zinc finger protein from *Candida albicans* is involved in sucrose utilization. J Bacteriol 174 (1992) 222-232.

Kim J., Michels C.A.: The MAL63 gene of *Saccharomyces* encodes a cysteine-zinc finger protein. Curr Genet 14 (1988) 319-323.

Klein C.J., Olsson L., Nielsen J.: Glucose control in *Saccharomyces cerevisiae*: the role of Mig1 in metabolic functions. Microbiology 144 (1998) 13-24.

Korshunova I. V., Naumova E. S., Naumov G. I.: Comparative molecular genetic analysis of β-fructosidases of yeasts *Saccharomyces*. Mol Biol 39 (2005) 366-371.

Kraakman L.S., Winderickx J., Thevelein J.M., De Winde J.H.: Structure-function analysis of yeast hexokinase: structural requirements for triggering cAMP signalling and catabolite repression. Biochem J 343 (1999) 159-168.

Krakenaite R.P., Glemzha A.A.: Some properties of two forms of alpha-glucosidase from *Saccharomyces cerevisiae*-II. Biokhimiia 48 (1983) 62-68.

Kramarenko, T., Karp, H., Järviste, A., Alamäe, T.: Sugar repression in the methylotrophic yeast *Hansenula polymorpha* studied by using hexokinase-negative, glucokinase-negative and double kinase-negative mutants. Folia Microbiol. 45 (2000) 521-529.

Lagunas R.: Sugar transport in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 10 (1993) 229-242.

Laht S., Karp H., Kotka P., Järviste A., Alamäe T.: Cloning and characterization of glucokinase from a methylotrophic yeast *Hansenula polymorpha*: different effects on glucose repression in *H. polymorpha* and *Saccharomyces cerevisiae*. Gene 296 (2002) 195-203.

Levine J., Tanouye L., Michels C. A.: The UAS(MAL) is a bidirectional promotor element required for the expression of both the *MAL61* and *MAL62* genes of the *Saccharomyces MAL6* locus. Curr Genet 22 (1992) 181-189.

Lichtenberg-Frate H., Naschen T., Heiland S., Hofer M.: Properties and heterologous expression of the glucose transporter GHT1 from *Schizosaccharomyces pombe*. Yeast 13 (1997) 215-224.

Liiv L., Pärn P., Alamäe T.: Cloning of maltase gene from a methylotrophic yeast, *Hansenula polymorpha*. Gene 265 (2001) 77-85.

Loureiro-Diaz M., Peinado J. M.: Transport of maltose in *Saccharomyces cerevisiae.* Biochem J 222 (1984) 293-298.

Lowry O.N., Rosenbrough A., Farr K., Randall K.: Protein measurement with the Folin phenol reagent. J Biol Chem 193 (1951) 265-275.

Lundin M., Nehlin J. O., Ronne H.: Importance of a flanking AT-rich region in target site recognition by the GC box-binding zinc finger protein MIG1. Mol Cell Biol 14 (1994) 1979-85.

Medintz I., Jiang H., Han E-K., Cui W., Michels C. A.: Characterization of the glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae.* J Bacteriol 178 (1996) 2245-2254.

Medintz I., Jiang H., Michels C. A.: The role of ubiquitin conjugation in glucose-induced proteolysis of *Saccharomyces* maltose permease. J Biol Chem 273 (1998) 34454-34462.

Miller J. H.: Eksperiments in Molecular Genetics. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY, 1972.

Moreno S., Sanchez Y., Rodriguez L.: Purification and characterization of the invertase from *Schizosaccharomyces pombe*. Biochem J 267 (1990) 697-702.

Myers L. C., Kornberg R. D.: Mediator of transcriptional regulation. Annu Rev Biochem 69 (2000) 729-749.

Needleman R. B., Federoff H. J., Eccleshall T. R., Buchferer B., Marmur J.: Purification and characterization of an alpha-glucosidase from *Saccharomyces carlsbergensis*. Biochemistry 17 (1978) 4657-4661.

Needleman R. B.: Control of maltase synthesis in yeast. Mol Microbiol 5 (1991) 2079-2084.

Ni B. F., Needleman R. B.: Identification of the upstream activating sequence of MAL and the binding sites for the MAL63 activator of *Saccharomyces cerevisiae*. Mol Cell Biol 10 (1990) 3797-3800.

Pärn P.: Pärmi *Hansenula polymorpha* maltaas, Tartu Ülikool, 2003, Magistritöö.

Reinders A., Ward J. M.: Functional characterization of the α-glucoside transporter Sut1p from *Schizosaccharomyces pombe*, the first fungal homologue of plant sucrose transporters. Mol Microbiol 39 (2001) 445-454.

Ronne H.: Glucose repression in fungi. Trends Genet 11 (1995) 12-17.

Rose M. D., Winsto, F., Hieter P.: Methods in yeast genetics a laboratory course manual. Cold Spring Harbor Laboratory Press (1990) 131-132.

Rose M.: Molecular and biochemical characterization of the hexokinase from the starch-utilizing yeast *Schwanniomyces occidentalis*. Curr Genet 27 (1995) 330-338.

Salema-Oom M., Pinto V. V., Goncalves P., Spencer-Martins I.: Maltotriose utilization by industrial *Saccharomyces* strains: characterization of a new member of the α-glucoside transporter family. Appl Environ Microbiol 71 (2005) 5044-5049.

Schjerling P., Holmberg S.: Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. Nucleic Acids Res 24 (1996) 4599-4607.

Sims A P., Kopetzki E., Schulz B., Barnett A. A.: The use of phenolic glycosides for studying the aerobic or anaerobic transport of disaccharides into yeast. J Gen Microbiol 130 (1984) 1933-1940.

Sirenko O. I., Ni B., Needleman R. B.: Purification and binding properties of the Mal63p activator *of Saccharomyces cerevisiae*. Curr Genet 27 (1995) 509-516.

Stambuk B U.: A simple laboratory exercise illustrating active transport in yeast cells. Biochem Mol Biol Educ 28 (2000) 313-317.

Stambuk B. U., Araujo P. S.: Kinetics of active α-glucoside transport in *Saccharomyces cerevisiae*. FEMS Yeast Res 1 (2001) 73-78.

Stambuk B. U., Batista A. S., de Araujo P. S.: Kinetics of active sucrose transport in *Saccharomyces cerevisiae.* J Biosci Bioeng 89 (2000) 212-214.

Stambuk B. U., da Silva M A., Panek A D., de Araujo P. S.: Active α-glucoside transport in *Saccharomyces cerevisiae.* FEMS Microbiol Lett 170 (1999) 105-110.

Stambuk B. U., de Araujo P. S., Panek A. D., Serrano R.: Kinetics and energetics of trehalose transport in *Saccharomyces cerevisiae.* Eur J Biochem 237 (1996) 876-881.

Zaragoza O., Rodriguez C., Gancedo C.: Isolation of the *MIG1* gene from *Candida albicans* and effects of its disruption on catabolite repression. J Bacteriol 182 (2000) 320-326.

Zimmerman F. K., Kaufmann I., Rasenberger H., Haussmann H.: Genetics of carbon catabolite repression in *Saccharomyces cerevisiae* genes involved in the derepression process. Mol Gen Genet 151 (1977) 95-103.

Zimmermann F. K., Scheel I.: Mutants of *Saccharomyces cerevisiae* resistant to carbon catabolite repression. Mol Gen Genet 154 (1977) 75-82.

Tan X., Waterham H. R., Veenhuis M., Cregg J. M.: The *Hansenula polymorpha PER8* gene encodes a novel peroxisomal integral membrane protein involved in proliferation. J Cell Biol 128 (1995) 307-319.

Thompson J. D., Higgins D. G., Gibson T. J.: Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22 (1994) 4673-4680.

Tkacz J. S., Lampen J.O.: Surface distribution of invertase on growing *Saccharomyces* cells. J Bacteriol 113 (1973)1073-1075.

Trumbly R. J.: Isolation of *Saccharomyces cerevisiae* mutants constitutive for invertase synthesis. J Bacteriol 166 (1986) 1123-1127.

Van Leeuwen C. C. M., Weusthuis R. A., Postma E., van den Broek P. J. A., van Dijken J. P.: Maltose/proton co-transport in *Saccharomyces cerevisiae.* Biochem J 284 (1992) 441-445.

Wang J., Needleman R.: Removal of Mig1p binding site converts a MAL63 constitutive mutant derived by interchromosomal gene conversion to glucose insensitivity. Genetics 142 (1996) 51-63.

Wang J., Sirenko O., Needleman R.: Genomic footprinting of Mig1p in the *MAL62* promoter. Binding is dependent upon carbon source and competitive with the Mal63p activator. J Biol Chem 272 (1997) 4613-4622.

Wang X., Bali M., Medintz I., Michels C. A.: Intracellular maltose is sufficient to induce *MAL* gene expression in *Saccharomyces cerevisiae.* Eukaryot Cell 1 (2002) 696-703.

Wang X., Michels C. A.: Mutations in *SIN4* and *RGR1* cause constitutive expression of *MAL* structural genes in *Saccharomyces cerevisiae.* Genetics 168 (2004) 747-757.

Vanoni M., Sollitti P., Goldenthal M., Marmur J.: Structure and regulation of the multigene family controlling maltose fermentation in budding yeast. Prog Nucleic Acid Res Mol Biol 37 (1989) 281-322.

Veale R. A., Giuseppin M. L., van Eijk H. M., Sudbery P. E., Verrips C. T.: Development of a strain of *Hansenula polymorpha* for the efficient expression of guar alpha-galactosidase. Yeast 8 (1992) 361-72.

Viigand K., Tammus K., Alamäe T.: 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. FEMS Yeast Res 5 (2005) 1019-1028.

Özcan S., Johnston M.: Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. Mol Cell Biol 15 (1995) 1564-1572.

KASUTATUD INTERNETIAADRESSID

http://biochemie.web.med.uni-muenchen.de/YTFD/RNApolii_complet.htm
http://genolist.pasteur.fr/CandidaDB/
http://natchaug.labri.u-bordeaux.fr/Genolevures
http://www.enzim.hu/hmmtop/

ELSEVIER

FEMS Yeast Research 4 (2003) 165–173

FEMS Yeast Research

www.fems-microbiology.org

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 HP201HPMAL1Δ 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.

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

* Corresponding author. Tel.: +372 (7) 375013; Fax: +372 (7) 420286.
E-mail address: talamae@ebc.ee (T. Alamäe).

[1] The sequence of the *HPMAL1* gene and the 5′-upstream sequence is available in GenBank under accession number AF261762.

Abbreviations: *HPMAL1*, the maltase structural gene of *Hansenula polymorpha*; HP201HPMAL1Δ, the maltase gene disruption strain of *H. polymorpha* 201; ORF, open reading frame; PCR, polymerase chain reaction

1567-1356/03/$22.00
doi:10.1016/S1567-1356(03)00142-9

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 PII 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 PII 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 *HPMAL1* 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 *HPMAL1* 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. Lahtchev (Sofia, Bulgaria) was used as a strain in which the maltase gene *HPMAL1* was disrupted. The maltase-negative strain *S. cerevisiae* 100-1B (*Matα ura3-52 leu2 MAL13 MAL11 MAL12::LEU2*) [6] was used for the heterologous expression of *HPMAL1*. *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 *HPMAL1* in pYT3 [4]. *HPMAL1* was subcloned from p51 to *S. cerevisiae*/*E. coli* high-copy vectors pRS425 and pRS426 [15] to study the the regulation of *HPMAL1* 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 *Spe*I-*Sma*I fragment of the genomic insert in p51 (see Fig. 11) was cloned to pRS425 and pRS426 opened with the same enzymes resulting in pRS425-p51SpeISmaI and pRS426-p51SpeISmaI, respectively. In these vectors the *HPMAL1* gene is behind the 1242 bp of the 5′-upstream region (Fig. 1), further named the full-length promoter. Constructs harboring *HPMAL1* 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 *Mun*I fragment (see Fig. 1) of p51 in the *Sma*I site of pBluescript SK polylinker. The above-mentioned fragment was excised from pSK51MunI using *Not*I-*Eco*RI digestion and inserted between the same sites in pRS426 polylinker resulting in pRS426-p51MunI. The *Not*I-*Sal*I 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 *Nru*I site at position −127 bp from the maltase gene ATG, by restriction of pRS425-p51MunI with *Nru*I and *Sal*I and religating the ends, resulting in pRS425-p51NruI-MunI (*HPMAL1* behind the short promoter; Fig. 1). The *S. cerevisiae* maltase gene *MAL62* was cloned as the *Spe*I-*Eco*105I fragment (besides *MAL62* includes the entire *MAL61-MAL62* intergenic region and part of the *MAL61* gene) from pY6 [7] into pRS425 opened with *Sma*I and *Spe*I resulting in pRS425-MAL62. To study the expression of *HPMAL1* from the *H. polymorpha* alcohol oxidase promoter, the *Hin*dIII-*Mun*I fragment (Fig. 1) including the open reading frame of *HPMAL1* and 68 bp of the 5′-upstream region was excised from pSK51MunI and inserted into pX4-HNBESX, a derivative of pHIPX4 (see http://www.biol.rug.nl/em/) opened with *Hin*dIII and *Eco*RI yielding pX4-HPMAL1. To create a plasmid that has a similar plasmid backbone as pX4-HPMAL1 but harbors the *H. polymorpha* maltase gene promoter instead of the alcohol oxidase promoter, the *Not*I-*Sal*I fragment of pRS425-p51SpeISmaI containing *HPMAL1* with its full-length promoter was inserted into *Not*I-*Sal*I-cut pHIPX8 (see http://www.biol.rug.nl/em/) creating pHIPX8-p51-SpeISmaI. The plasmid pSK51MunI-HPURA3 used for the genomic disruption of *HPMAL1* was designed from

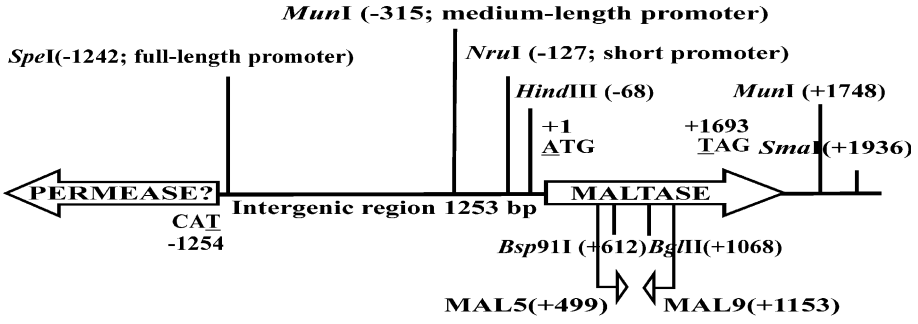


Fig. 1. Map of the 5′-upstream region of the *H. polymorpha* maltase gene *HPMAL1*. The location of the putative maltose permease gene is indicated. The intergenic region is drawn to scale. The restriction sites used for the subcloning and disruption of *HPMAL1* as well as the annealing positions of primers MAL5 and MAL9 used to verify maltase gene disruption are also shown.

pSK51MunI by replacing the 456-bp *Bsp*91I-*Bgl*II fragment (Fig. 1) of the *HPMAL1* coding region with the *H. polymorpha URA3* gene on a 1795-bp *Bam*HI-*Bgl*II fragment. The linear *Mun*I 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 ($OD_{600\,nm}$ ~2.0).

For comparison of the strength of the promoters of the *HPMAL1* and *MOX* genes, the transformants of HP201HPMAL1Δ carrying *HPMAL1* on a plasmid behind its native promoter or the *MOX* promoter were grown as follows. Cells were cultivated on 2% glycerol medium until the exponential growth phase and a culture sample was withdrawn to register the initial level of maltase activity (zero time points in Fig. 5). Then methanol (final concentration 0.5%) was added to the culture of HP201HPMAL1Δ (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 HP201HPMAL1Δ (pRS425-p51SpeISmaI) 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 minikits (Eppendorf). Yeast genomic DNA was isolated as shown by us earlier [4]. Restriction endonuclease digestions and DNA ligations 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 × *g* at 4°C) was used as cell extract. Maltase activity in the extract was measured using *p*-nitrophenyl-α-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 *Mun*I 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 HP201HPMAL1Δ) 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

grows on glucose, but not on maltose or sucrose. The *HPMAL1* on a plasmid corrected the growth defect of the disruptant on both maltose and sucrose. Therefore, the maltase protein is responsible in *H. polymorpha* for the utilization of both disaccharides, maltose and sucrose, thus verifying our earlier assumption [3]. Fig. 3 also shows that the growth deficiency of HP201HPMAL1Δ on disaccharides was corrected by the *S. cerevisiae* maltase gene *MAL62* on a plasmid and that transformation of the *S. cerevisiae* maltase disruption mutant 100-1B with plasmid pRS426-p51MunI (harbors *HPMAL1*) restored the growth of the *S. cerevisiae* mutant on maltose. Thus, the promoters of the maltase genes of these two yeast species were crosswise recognized in a heterologous partner and functional maltase proteins were produced resulting in the phenotypic complementation of the respective mutations. Notably, the *S. cerevisiae* maltase gene *MAL62* complements the growth deficiency of HP201HPMAL1Δ on sucrose despite the fact that in *S. cerevisiae* maltase is not responsible for sucrose utilization – in this yeast sucrose is hydrolyzed extracellularly by a cell wall-bound invertase [20]. However, yeast maltases are able to hydrolyze sucrose, and their affinity for sucrose is typically even higher than that for maltose [4,21,22]. Thus, if a certain yeast does not possess invertase but can transport sucrose into the cell, it may use intracellular maltase for the hydrolysis of sucrose as has been shown for the human pathogenic yeast *Candida albicans* [22].

3.2. *Sequence analysis of the 5′-neighborhood of the* HPMAL1 *gene in p51 reveals beginning of a putative divergently transcribed maltose permease gene*

In different *MAL* loci of *S. cerevisiae* the structural

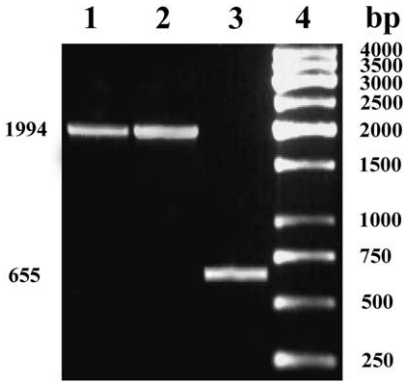


Fig. 2. Verification of the genomic disruption of the *H. polymorpha* maltase gene *HPMAL1*. Genomic DNA was isolated from the wild-type *H. polymorpha* 201 (lane 3) and from two distinct putative *HPMAL1* disruptants (lanes 1 and 2) and used as a template for the PCR amplification with the primers MAL5 and MAL9. The resulting PCR products were separated in a 2% agarose gel containing ethidium bromide and photographed under UV illumination. The Fermentas 1-kb DNA Ladder (lane 4) was used as a size marker. The calculated sizes of the PCR products from either the intact (655 bp) or the disrupted (1994 bp) maltase gene are shown to the left.

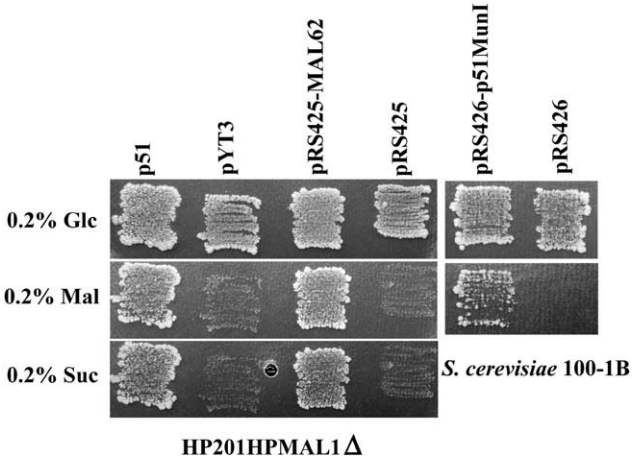


Fig. 3. Growth properties of *H. polymorpha* HP201HPMAL1Δ and *S. cerevisiae* 100-1B transformed with plasmids containing either the *H. polymorpha* maltase gene *HPMAL1* (plasmids p51 and pRS426-p51MunI) or the *S. cerevisiae* maltase gene *MAL62* (plasmid pRS425-MAL62). The transformants harboring the respective empty vectors (pYT3, pRS425 and pRS426) served as controls. The transformants were grown on 0.2% glucose medium and replicated to media containing 0.2% maltose or 0.2% sucrose. The plates were photographed after 48 h of growth.

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 *HPMAL1* 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* HPMAL1 *promoter in HP201HPMAL1Δ using promoter truncations*

In order to define the minimum-length promoter region

Table 1
Regulation of the *H. polymorpha* maltase gene *HPMAL1* and the *S. cerevisiae* maltase gene *MAL62* in the *H. polymorpha* maltase disruption mutant HP201HPMAL1Δ

Plasmid	Description of the construct	Maltase activity (mU mg^{-1})				
		G	M	G+M	S	G+S
pRS425-p51MunI	*HPMAL1* behind the medium-length promoter	5	2330	3	1970	9
pRS425-p51SpeISmaI	*HPMAL1* behind the full-length promoter	5	2350	4	993	7
pRS425-MAL62	*S. cerevisiae* maltase gene *MAL62*	2	928	2	992	13
pRS425	Empty vector (control)	BD	No growth	BD	No growth	BD

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.

sufficient for the growth of HP201HPMAL1Δ transformants on maltose and sucrose, 5′ truncations in front of the *HPMAL1* coding region were made. Restriction sites used for the truncations are shown in Fig. 1. Transformation of HP201HPMAL1Δ with the *HPMAL1* behind the short promoter (truncated to the *Nru*I site at −127 bp from the maltase gene ATG) did not result in maltose- and sucrose-assimilating transformants. Thus, the short promoter was considered nonfunctional. However, the *HPMAL1* behind the medium-length promoter (truncated to the *Mun*I site at −315 bp from the maltase gene ATG) supported the growth of HP201HPMAL1Δ on maltose as well as on sucrose, and the growth of *S. cerevisiae* 100-1B on maltose, while used for the transformation of these strains. As a next experiment, we compared the regulation of the medium-length and full-length promoters (the latter includes the 5′-upstream region of *HPMAL1* to the *Spe*I site; Fig. 1) of *HPMAL1* in HP201HPMAL1Δ. The respective transformants were grown on (1) 2% maltose; (2) 2% sucrose; (3) 2% glucose; (4) 2% glucose+2% maltose; and (5) 2% glucose+2% sucrose. Data on maltase activity of the transformants are presented in Table 1. The table shows that HP201HPMAL1Δ carrying the empty vector pRS425 displays no maltase activity if grown either on glucose or on the mixture of glucose and a disaccharide. Regulation of the plasmid-originating maltase gene in HP201HPMAL1Δ from the medium- and full-length promoters proved quite similar. There was a strong induction of maltase expression during growth of the transformants on maltose or sucrose. In glucose-grown transformants the maltase activity was very low, and the presence of glucose in the medium suppressed the induction of maltase by disaccharides. According to these data, the 5′-upstream region of *HPMAL1* present in the medium-length promoter should harbor *cis*-elements needed for the induction and repression of maltase gene transcription in *H. polymorpha*.

3.4. *Regulation of the* S. cerevisiae *maltase gene* MAL62 *in HP201HPMAL1Δ*

As the *S. cerevisiae* maltase gene *MAL62* restored growth of HP201HPMAL1Δ on maltose and sucrose when expressed from its own promoter (Fig. 3), we were interested in the regulation of *MAL62* in HP201MAL1Δ. The transformants of HP201HPMAL1Δ harboring the *MAL62* on pRS426 were grown as described in Section 3.3 and maltase activity was measured in the cells. Table 1 shows that the *S. cerevisiae MAL62* promoter was perfectly recognized and correctly regulated in *H. polymorpha* – it was properly induced by disaccharides and strongly repressed by glucose. Therefore we presume that *H. polymorpha* possesses homologues of the proteins that are able to regulate the transcription of *MAL62*. According to the literature, transcriptional regulators of maltase genes from other yeasts can function in *S. cerevisiae*. For example, the Mig1 repressor homologues from *Candida utilis* [27], *K. lactis* [28] and *Schwanniomyces occidentalis* [29] complement the Mig1 deficiency in *S. cerevisiae*, and the *MAL*

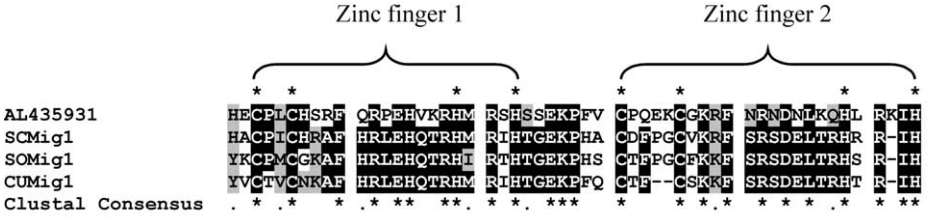


Fig. 4. Alignment of the N-terminal zinc finger regions of Mig1 proteins from *S. cerevisiae* (SCMig1), *S. occidentalis* (SOMig1) and *C. utilis* (CUMig1) with the protein sequence deduced from a genomic sequence of *H. polymorpha* AL435931 (GenBank accession number). The alignment was performed using 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 *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.

Table 2
Regulation of the *H. polymorpha* maltase gene *HPMAL1* in a *S. cerevisiae* maltase disruption mutant 100-1B

Plasmid	Description of the construct	Maltase activity (mU mg^{-1})		
		G	M	G+M
pRS426-p51MunI	*HPMAL1* behind the medium-length promoter	108	1260	145
pRS426-p51SpeISmaI	*HPMAL1* behind the full-length promoter	5	862	5
pRS426	Empty vector (control)	BD	No growth	BD

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* HPMAL1 *promoter in* S. cerevisiae *100-1B*

Fig. 3 shows that *HPMAL1* 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 *HPMAL1* in *S. cerevisiae*, mutant 100-1B was transformed with *HPMAL1* 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 *HPMAL1* 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 *HPMAL1* promoter and thus activate the transcription. The recognition sequence for the *S. cerevisiae MAL* activator protein has been reported to be c/aGCN$_9$c/aGC/g [33]. A search for putative binding sites for the *S. cerevisiae MAL* activator in the *HPMAL1* promoter region did not result in a perfect match with this consensus. However, the motif CGGGCAGTTTTCCGG 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 (GTATTAAACCCGGGGTA; 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 *HPMAL1* are presented in Table 3.

Table 3
Cis-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*

Element and distance from the maltase gene ATG	Putative function
−310 **TTGACAGCAATTTCAGCGAGAATTAAATT** −282	−35 and −10 hexamers (underlined) for the binding of *E. coli* σ^{70} protein
−213 **GGTACAGATAACAATCAAGTAACTATTAT** −185	−35 and −10 hexamers (underlined) for the binding of *E. coli* σ^{70} protein
−265 **CTGAAATAATTAGGC** −251	*S. cerevisiae MAL* activator binding
−486 CGGAGTTTTGTAGCG −472	*S. cerevisiae MAL* activator binding
−759 CGGGCAGTTTTCCGG −745	*S. cerevisiae MAL* activator binding
−824 CGCGAAATAAATGCG −838	*S. cerevisiae MAL* activator binding
−674 CCCCGC −669	*S. cerevisiae* Mig1 repressor binding
−748 CCGGGG −743	*S. cerevisiae* Mig1 repressor binding
−775 GCGGGG −770	*S. cerevisiae* Mig1 repressor binding
−1185 GTGGGG −1180	*S. cerevisiae* Mig1 repressor binding

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* σ^{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 HP201-HPMAL1Δ 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.

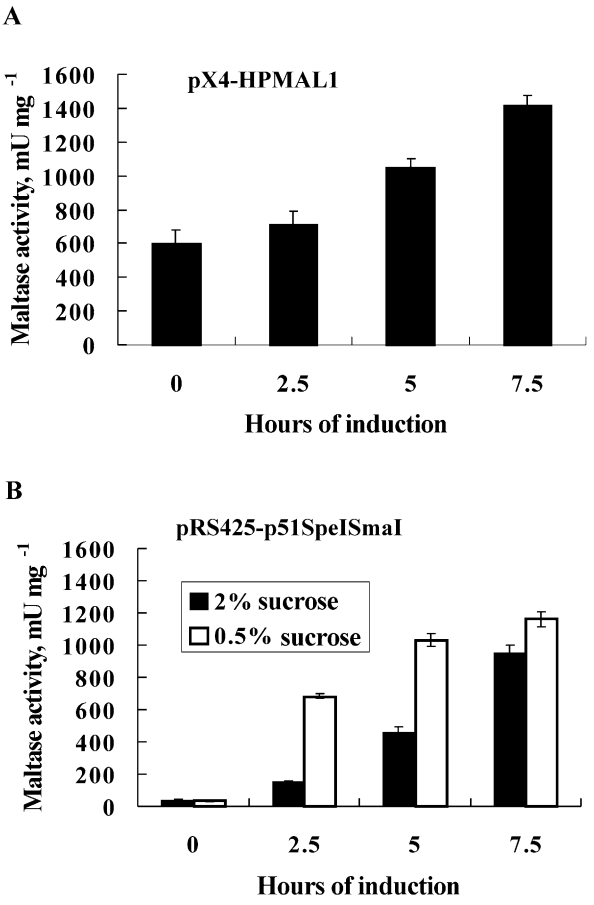


Fig. 5. Comparison of the induced strengths of the promoters of *H. polymorpha* maltase and alcohol oxidase genes. Glycerol-growing HP201-HPMAL1Δ transformants were supplemented with an inducer (0.5% methanol, 2% sucrose or 0.5% sucrose) and further cultivated for 7.5 h. At the indicated time points, 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.

If maltose was used as the inducer instead of sucrose, induction of maltase activity in HP201HPMAL1Δ carrying pRS425-p51SpeISmaI was much less rapid and reached only 160 mU mg^{-1} by 7.5 h of induction (not shown). However, the same transformants exhibited a high maltase activity during prolonged growth on maltose (see Table 1). Thus, the repressing effect of maltose, if added to glycerol-grown culture, seems to be higher than that of sucrose. However, as during prolonged cultivation of *H. polymorpha* on disaccharides the maltase activity is high, the cells should be able to balance their metabolism,

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 HP201HPMAL1Δ. 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

[1] Gellissen, G. (2000) Heterologous protein production in methylotrophic yeasts. Appl. Microbiol. Biotechnol. 54, 741–750.
[2] Brito, N., Pérez, M.D., Perdomo, G., González, C., García-Lugu, P. and Siverio, J. (1999) A set of *Hansenula polymorpha* integrative vectors to construct *lacZ* fusions. Appl. Microbiol. Biotechnol. 53, 23–29.
[3] Alamäe, T. and Liiv, L. (1998) Glucose repression of maltase and methanol-oxidizing enzymes in the methylotrophic yeast *Hansenula polymorpha*: isolation and study of regulatory mutants. Folia Microbiol. 43, 443–452.
[4] Liiv, L., Pärn, P. and Alamäe, T. (2001) Cloning of maltase gene from a methylotrophic yeast *Hansenula polymorpha*. Gene 26, 77–85.
[5] Needleman, R. (1991) Control of maltase synthesis in yeast. Mol. Microbiol. 5, 2079–2084.
[6] Charron, M.J., Dubin, R.A. and Michels, C. (1986) Structural and functional analysis of the *MAL1* locus of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 6, 3891–3899.
[7] Charron, M.J., Read, E., Haut, S.R. and Michels, C. (1989) Molecular evolution of telomere-associated *MAL* loci of *Saccharomyces*. Genetics 122, 307–316.
[8] Hu, Z., Nehlin, J.O., Ronne, H. and Michels, C.A. (1995) *MIG1*-dependent and *MIG1*-independent glucose regulation of *MAL* gene expression in *Saccharomyces cerevisiae*. Curr. Genet. 28, 258–266.
[9] Wang, J., Sirenko, O. and Needleman, R. (1997) Genomic footprinting of Mig1p in the *MAL62* promoter. J. Biol. Chem. 272, 4613–4622.
[10] Rolland, F., Winderickx, J. and Thevelein, J.M. (2002) Glucose-sensing and -signaling mechanisms in yeast. FEMS Yeast Res. 2, 183–201.
[11] Entian, K.-D. (1997) Sugar phosphorylation in yeast. In: Yeast Sugar Metabolism (Zimmermann, F.K. and Entian, K.-D., Eds.), pp. 67–79. Technomic, Lancaster.
[12] Rose, M., Albig, W. and Entian, K.D. (1991) Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinases PI and PII. Eur. J. Biochem. 199, 511–518.
[13] Kramarenko, T., Karp, H., Järviste, A. and Alamäe, T. (2000) Sugar repression in the methylotrophic yeast *Hansenula polymorpha* studied by using hexokinase-negative, glucokinase-negative and double kinase-negative mutants. Folia Microbiol. 45, 521–529.
[14] Laht, S., Karp, H., Kotka, P., Järviste, A. and Alamäe, T. (2002) Cloning and characterization of glucokinase from a methylotrophic yeast *Hansenula polymorpha*: different effects on glucose repression in *H. polymorpha* and *Saccharomyces cerevisiae*. Gene 296, 195–203.
[15] Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H. and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene 110, 119–122.

[16] Berardi, E. and Thomas, D.Y. (1990) An effective transformation method for *Hansenula polymorpha*. Curr. Genet. 18, 169–170.
[17] Sambrook, J., Fritsch, E.T. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Hanahan, D. (1983) Studies on the transformation of *E. coli* with plasmids. J. Mol. Biol. 166, 577–580.
[19] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153, 163–168.
[20] Tkacz, J.S. and Lampen, J.O. (1973) Surface distribution of invertase on growing *Saccharomyces* cells. J. Bacteriol. 113, 1073–1075.
[21] Krakenaite, R.P. and Glemzha, A.A. (1983) Some properties of two forms of α-glucosidase from *Saccharomyces cerevisiae*-II (in Russian). Biokhimija 48, 62–68.
[22] Geber, A., Williamson, P.R., Rex, J.H., Sweeney, E.C. and Bennett, J.E. (1992) Cloning and characterization of a *Candida albicans* maltase gene involved in sucrose utilization. J. Bacteriol. 11, 1992–6996.
[23] Han, E.-K., Cotty, F., Sottas, C., Jiang, H. and Michels, A. (1995) Characterization of *AGT1* encoding a general α-glucoside transporter from *Saccharomyces*. Mol. Microbiol. 17, 1093–1107.
[24] Hong, S.H. and Marmur, J. (1986) Primary structure of the maltase gene of the *MAL6* locus of *Saccharomyces carlsbergensis*. Gene 41, 75–84.
[25] Bell, P.J., Higgins, V.J., Dawes, I.W. and Bissinger, P.H. (1997) Tandemly repeated 147 bp elements cause structural and functional variation in divergent *MAL* promoters of *Saccharomyces cerevisiae*. Yeast 13, 1135–1144.
[26] Avila, J., González, C., Brito, N. and Siverio, J.M. (1998) Clustering of the *YNA1* gene encoding a $Zn(II)_2Cys_6$ transcriptional factor in the yeast *Hansenula polymorpha* with the nitrate assimilation genes *YNT1*, *YNI1* and *YNR1*, and its involvement in their transcriptional activation. Biochem. J. 335, 647–652.
[27] Delfin, J., Perdomo, W., Garcia, B. and Menendez, J. (2001) Isolation and sequence of the *MIG1* homologue from the yeast *Candida utilis*. Yeast 18, 597–603.
[28] Cassart, J.-P., Georis, I., Östling, J., Ronne, H. and Vandehaute, J. (1995) The MIG1 repressor from *Kluyveromyces lactis*: cloning, sequencing and functional analysis in *Saccharomyces cerevisiae*. FEBS Lett. 371, 191–194.
[29] Carmona, T.A., Barrado, P., Jimenez, A. and Lobato, M.F. (2002) Molecular and functional analysis of MIG1 homologue from the yeast *Schwanniomyces occidentalis*. Yeast 19, 459–465.
[30] Kelly, R. and Kwon-Chung, K.J. (1992) A zinc finger protein from *Candida albicans* is involved in sucrose utilization. J. Bacteriol. 174, 222–232.
[31] Blandin, G., Llorente, B., Malpertuy, A., Wincker, P., Artiguenave, F. and Dujon, B. (2000) Genomic exploration of the hemiascomycetous yeasts: 13. *Pichia angusta*. FEBS Lett. 487, 76–81.
[32] Nehlin, J.O. and Ronne, H. (1990) Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. EMBO J. 9, 2891–2898.
[33] Sirenko, O.I., Ni, B. and Needleman, R.B. (1995) Purification and binding properties of the Mal63p activator of *Saccharomyces cerevisiae*. Curr. Genet. 27, 509–516.
[34] van Dijk, R., Faber, K.N., Kiel, J.A.K.W., Veenhuis, M. and van der Klei, I. (2000) The methylotrophic yeast *Hansenula polymorpha*: a versatile cell factory. Enzyme Microb. Technol. 26, 793–800.
[35] Cox, H., Mead, D., Sudbery, P., Eland, R.M., Mannazzu, I. and Evans, L. (2000) Constitutive expression of recombinant proteins in the methylotrophic yeast *Hansenula polymorpha* using the *PMA1* promoter. Yeast 16, 1191–1203.
[36] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

ELSEVIER

FEMS Yeast Research 5 (2005) 1019–1028

FEMS Yeast Research

www.fems-microbiology.org

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

Katrin Viigand, Kersti Tammus, Tiina Alamäe *

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

Received 10 February 2005; received in revised form 19 May 2005; accepted 17 June 2005

First published online 3 August 2005

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

* Corresponding author. Tel.: +372 7 375013; fax: +372 7 420286.
E-mail address: talamae@ebc.ee (T. Alamäe).

1567-1356/$22.00
doi:10.1016/j.femsyr.2005.06.003

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],

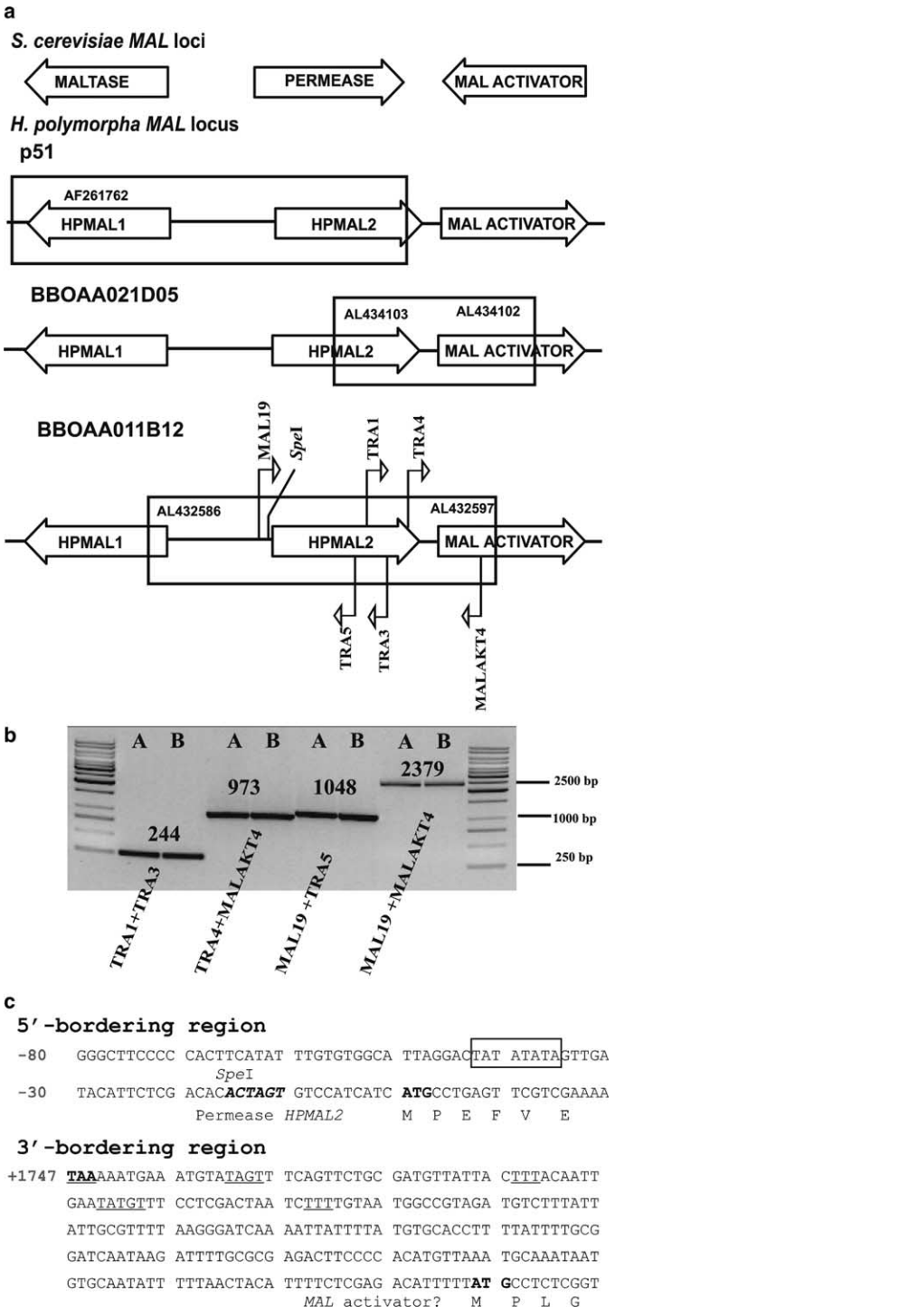


Fig. 1. Clustering of *MAL* genes in *H. polymorpha* (a) Localization of the genes for maltase (*HPMAL1*), maltose permease (*HPMAL2*) and a putative *MAL* activator in *H. polymorpha* genomic clones p51, BB0AA021D05 and BB0AA011B12. The GenBank accession numbers of sequences are presented and the fragment of the *MAL* locus in the particular clone is shown inside the frame. In the plot of BB0AA011B12 the approximate annealing positions of gene-specific primers are designated. For comparison, the structure of *MAL* loci in baker's yeast [11] is shown in the upper part of the figure. (b) PCR analysis of the *H. polymorpha MAL* locus. DNA fragments were amplified from the genomic DNA of *H. polymorpha* (A) or from the Génolevures clone BB0AA011B12 (B) using gene-specific primers (see Section 2.4). The fragments were separated in 1% agarose gel containing ethidium bromide and photographed under UV illumination. The 1 kbp DNA marker (Fermentas) was used as a fragment size reference. Calculated sizes of DNA fragments are shown above the bands. (c) 5′- and 3′-bordering regions of the *HPMAL2* gene. Start and stop codons are in bold. The A nucleotide of the *HPMAL2* ATG codon is numbered as +1. The T nucleotide of the *HPMAL2* stop codon TAA is numbered as +1747. The putative TATA region (starting from the position −43) of the *HPMAL2* is boxed. Beginning of the *HPMAL2* ORF and the ORF of a putative *MAL* activator is shown. Sequences in the 3′-bordering region that may function in transcription termination according to Zaret and Sherman [30] are underlined. The *Spe*I site in front of the *HPMAL2* promoter used in cloning (see Section 2.1) is designated.

was cloned on a *Not*I–*Sac*I fragment to the polylinker of pRS425, resulting in pRS425-GUS – the promoterless β-glucuronidase gene in pRS425. Next, the *H. polymorpha* maltase gene and the divergent promoter region, excised from pRS425-MalpromFwMAL1 using *Not*I and *Nar*I digestion, was inserted between the same sites in pRS425-GUS, yielding pRS425-GUSMalpromFwMAL1. In this construct, expression from the bidirectional promoter region in the maltase direction can be evaluated by maltase activity, and in the permease direction by β-glucuronidase activity.

2.2. Growth of bacteria and yeasts

Yeasts were grown in 0.67% Yeast Nitrogen Base medium without amino acids (Difco, Detroit, MI) with adequate auxotrophic supplements or in 1% yeast extract – 2% peptone medium. 2% agar was added for solid media. Carbon sources were supplemented at concentrations shown in the text. Disaccharides were autoclaved separately in distilled water and then added to media. *E. coli* was grown in Luria–Bertani (LB) medium containing ampicillin (0.1 mg ml^{-1}) when required. Cultivation temperature of *H. polymorpha* and *E. coli* was 37 °C. *S. cerevisiae* was grown at 30 °C.

Routinely, exponentially growing cultures of yeasts ($OD_{600} \sim 1.5$) were used in carbon source-dependent enzyme expression studies. To study maltase induction in *S. cerevisiae*, the strains CMY1001 and CMY1050, carrying the empty vector pRS425 or the plasmid pRS425-MAL61promHPMAL2, were grown in YNB medium with 2% galactose up to mid-exponential growth phase, and then 2% of maltose was added as the inducer. Cells were harvested for maltase assay before the addition of maltose and after 6 h of induction.

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

DNA manipulations were carried out using standard methods [24]. Bacterial plasmid DNA was purified using Perfectprep plasmid minikits (Eppendorf, Hamburg, Germany). Yeast genomic DNA was isolated as shown by us earlier [7]. Restriction endonuclease digestions and DNA ligations were performed according to the manufacturers (Fermentas) recommendations. DNA was sequenced from both strands using an ABI Prism™ 377 DNA sequencer (Perkin–Elmer, Foster City, CA) and DYEnamic™ ET terminator cycle sequencing kit (Amersham, Sunnyvale, CA). *E. coli* was transformed with plasmid DNA according to Hanahan [25]. *H. polymorpha* and *S. cerevisiae* were transformed using Li-acetate [26]. The *HPMAL2* sequence was deposited in the GenBank under the Accession No. AY917133.

2.4. Oligonucleotides used for the gene clustering assay

MALAKT4, 5′ gccacttccttctagcag 3′; TRA1, 5′ tcagaagaacaaggattgccg 3′; TRA3, 5′ atcagaatgcacgtgtttatgc 3′; TRA4, 5′ tcgacgagtgcgtcttgggc 3′; TRA5, 5′ attgcctcggctagcggagc 3′ and MAL19, 5′ tttctggaaagcaaacataggc 3′ were used as oligonucleotide primers in PCR analysis of the *MAL* locus in different clones. Approximate annealing positions of the primers are shown in Fig. 1(a). PCR (25 cycles) was carried out as follows: denaturation at 96 °C for 1 min, annealing of the primers at 52 °C for 1 min, amplification at 72 °C for 1–2 min. The chemicals and instructions for PCR were from Fermentas.

2.5. Computer databasis and programs used

Génolevures (http://natchaug.labri.u-bordeaux.fr/Genolevures/), CandidaDB (http://genolist.pasteur.fr/CandidaDB/), GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and Swiss Prot (http://www.expasy.org/sprot/) databases were used for searching and retrieving DNA and protein sequences. BLAST and ORF Finder programs at the National Center for Biotechnology Information (NCBI) were used for nucleotide sequence analysis, deduction of amino acid sequences and database search. Protein sequences were aligned employing the CLUSTAL W program. The topology of the *HPMAL2* protein was predicted using the HMMTOP program (http://www.enzim.hu/hmmtop/) elaborated by Tusnády and Simon [27].

2.6. Assay of enzyme activity

Cells for the preparation of extracts were harvested by centrifugation at 4 °C, washed twice in distilled water and suspended in 200–400 μl of 100-mM K-phosphate buffer (pH 6.5) containing 0.1 mM EDTA (maltase buffer). Yeast cells were disrupted using glass beads and ultrasonication was used for *E. coli*. Supernatant obtained after the centrifugation (30 min at 12,000*g* at 4 °C) was diluted with appropriate buffer and used as crude cell extract. Maltase activity in the cell extract was measured in maltase buffer using *p*-nitrophenyl-α-D-glucopyranoside as a substrate [7]. Activity of β-glucuronidase was measured in 100-mM K-phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1 mM *p*-nitrophenyl-β-D-glucuronide essentially as in the case of maltase assay [7]. Specific activity was expressed as nanomoles of substrate transformed per min per mg of protein (mU mg^{-1}). Protein concentration in cell extract was determined according to the Lowry method. Specific enzyme activities presented are average values of measurements obtained with cell extracts from two to five distinct transformants. Standard deviations were less than 20%.

3. Results and discussion

3.1. Detection of the maltose permease gene in a Génolevures clone BB0AA011B12

In several yeasts such as *S. cerevisiae* [11–15], *Torulaspora delbrueckii* [28], and *Kluyveromyces lactis* (see GenBank data under Accession No. AJ007636), the genes for maltase and maltose permease constitute a bidirectional gene pair. In *S. cerevisiae*, a gene encoding a transcriptional activator of maltose-specific genes is located nearby [11,12,29]. Our previous data [8] suggested that in *H. polymorpha* the maltose permease gene may be genomically clustered with the maltase gene. To find out whether *H. polymorpha* possesses a *MAL* locus similar to that in *S. cerevisiae*, we took advantage of the Génolevures database. In the Génolevures project, *H. polymorpha* (*Pichia angusta*) was partially sequenced along with other twelve nonconventional yeasts [22]. Analysis of *H. polymorpha* genomic clones (Fig. 1(a)) showed that they contained different fragments of a maltose-specific cluster. The Génolevures clone BB0AA011B12 (Fig. 1(a)) was the most interesting, because we presumed that its genomic insert might contain the entire maltose permease gene. Sequence AL432586 of the clone aligned perfectly with the *H. polymorpha* maltase gene sequence [7,8] within the promoter region and the beginning of the coding region, and the opposite end sequence of the clone was suggested to encode a *MAL* activator (see Section 3.5, Figs. 1(a) and 2(b)). To localize the maltose permease gene in BB0AA011B12 we performed PCR analysis with gene-specific primers (Fig. 1(a)), using genomic DNA as a reference. Fig. 1(b) shows that permease-specific oligonucleotides annealed to the BB0AA011B12 sequence and amplified produced products of expected size. According to our analysis, the *MAL* genes in the genomic insert (~4.3 kbp) of BB0AA011B12 are organized as shown in Fig. 1(a).

3.2. Sequence analysis of the maltose permease

The putative maltose permease gene designated by us as *HPMAL2* was sequenced in the genomic insert of BB0AA011B12 from both strands using the primer walking method. The permease fragment in the library clone p51 (Fig. 1(a)) revealed full sequence identity with the respective region in BB0AA011B12. In BB0AA011B12, the 1746-bp ORF of *HPMAL2* is positioned divergently from the truncated ORF of the maltase gene *HPMAL1* (Fig. 1(a)). A TATATATA stretch starting at position −43 from the permease gene ATG codon may serve as a TATA-element. Close to the termination codon TAA, a potential Zaret and Sherman termination motif TA(T)GT...TTT [30] is present twice (Fig. 1(c)). The intergenic region between the *HPMAL2* and putative *MAL* activator gene is AT-rich and rather short (238 bp) (Fig. 1(c)). However, the intergenic distances between the convergently transcribed nitrate-specific genes in the nitrate cluster of *H. polymorpha* are even smaller: in this cluster 95.8% of the genomic sequence is coding [18]. Comparison of the amino-acid sequence of the *H. polymorpha* maltose permease protein (582 aa in total) deduced from *HPMAL2* revealed 39–57% identity with yeast maltose permeases (Table 1). The moderate overall identity between the permeases is mostly due to variable N- and C-termini (Fig. 2(a)). High variability in N-termini among yeast maltose permeases has been reported earlier by other authors [28,31]. If variable termini were excluded from the comparison (designated in Fig. 2(a)), the identity values between the permeases increased significantly (Table 1). The closest homologue of the *H. polymorpha* *HPMAL2* protein is one of the maltose permeases of *D. hansenii* (identity 57%; see also Table 2). 51% Identity was detected with *C. albicans* maltose permease. Notably, maltase proteins of these yeasts are also similar: maltase of *H. polymorpha* (Q9P8G8) is 62% identical to maltase from *D. hansenii* (Q6BXY6) and 58% identical to maltase from *C. albicans* (QO2751). Phylogenetic trees based on comparison of ribosomal RNA genes also confirm close relatedness between these three yeast species (http://natchaug.labri.u-bordeaux.fr/Genolevures/, see also [32]). Sequence alignment of the *HPMAL2* protein with maltose permeases from *D. hansenii*, *S. cerevisiae* and *C. albicans* is presented in Fig. 2(a). The topology analysis of the *H. polymorpha* maltose permease protein with the HMMTOP programme [27] suggested eleven transmembrane helices that are designated in Fig. 2(a). The N-terminus of the protein was predicted to be outside the cell membrane. The *HPMAL2* and the recently cloned *HpGCR* [33] are the first sugar transporter family genes sequenced from *H. polymorpha*.

3.3. Expression of the H. polymorpha maltose permease gene in a S. cerevisiae maltose permease deletion strain

As reported by Wang et al. [20], presence of intracellular maltose is crucial for the induction of maltase in *S. cerevisiae*: even plant sucrose permease, transporting maltose, restores maltase induction to a maltose permease-deficient *S. cerevisiae* mutant. Therefore, we used *S. cerevisiae* strains CMY1001 and CMY1050 explored in [20] to verify functionality of the *H. polymorpha* maltose permease encoded by *HPMAL2*. First, the maltose permease-negative mutant CMY1050 was transformed with the *H. polymorpha* maltose permease gene in pRS425-B12 BamHIBglII (see Section 2.1). For a reference, both *S. cerevisiae* strains were transformed with empty vector pRS425. All transformants showed perfect growth on minimal glucose medium lacking leucine (not shown), but only wild-type CMY1001 carrying the empty vector grew on maltose (Table 2). The maltase

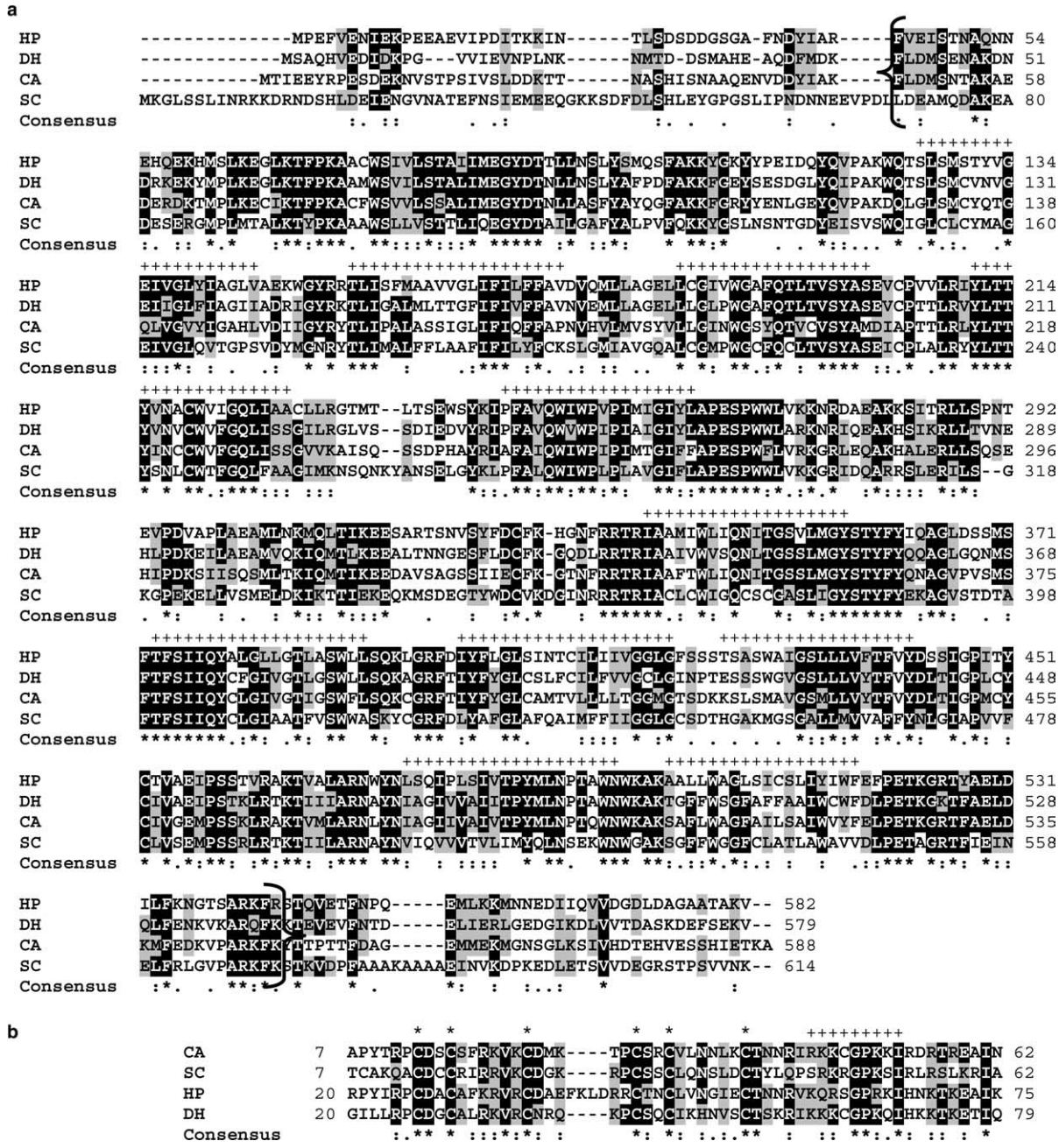


Fig. 2. Alignment of full-length (a) or N-terminal (b) protein sequences using the CLUSTAL W program. Identity and similarity of residues in proteins is designated by shading and consensus below the alignment. (a) HP, maltose permease of *H. polymorpha* deduced from *HPMAL2* (AY917133); DH, maltose permease of *D. hansenii* (Q6BYN5); CA, maltose permease of *C. albicans* (Q5AK39); SC, maltose permease (P15685) of *S. cerevisiae* encoded by *MAL61*. Crosses above the alignment designate the eleven transmembrane domains of the *H. polymorpha* maltose permease predicted by the HMMTOP program [27]. Conserved core regions of permeases used in identity calculations (see Table 1) are shown between the brackets. (b) HP, putative *MAL* activator of *H. polymorpha* (AL432587); SC, MAL13 from *S. cerevisiae* (P53338); CA, CASUC1 from *C. albicans* (P33181); DH, hypothetical *MAL* activator from *Debaryomyces hansenii* (Q6BYN4). Six cysteine residues of zinc fingers are shown by asterisks above the alignment. The crosses above the alignment designate the nuclear transport motif of the CASUC1 protein [22].

inducibility assay revealed that the mutant CMY1050 carrying pRS425-B12BamHIBglII did not transport maltose – addition of maltose to a galactose-grown culture did not result in maltase induction. In good accordance with the literature data [20], the wild-type strain CMY1001 exhibited perfect maltase induction (Table 2). Then, we decided to change the promoter of the *H. polymorpha* maltose permease gene in pRS425-B12 BamHIBglII with that of the *S. cerevisiae MAL61* (see Section 2.1). Table 2 shows that CMY1050 transformants harbouring pRS425-Mal61promHPMAL2 gained the ability to grow on maltose and exhibited perfect

Table 1
Identity of amino acid sequences between yeast maltose permeases

	HP	DH	CA	KL	SCAGT1	SCMAL61
HP (AY917133)	100	57 (63)	51 (57)	43 (52)	41 (46)	39 (45)
DH (Q6BYN5)		100	47 (52)	42 (50)	38 (45)	39 (45)
CA (Q5AK39)			100	43 (49)	38 (45)	40 (46)
KL (Q9Y845)				100	49 (58)	48 (56)
SCAGT1 (P53048)					100	54 (61)
SCMAL6 1 (P15685)						100

The sequences were retrieved from Swiss Prot, TrEMBL and GenBank (the accession numbers shown in the first column of the table) and compared using the Clustal W programme. The identity values shown in parentheses were obtained by excluding the highly variable N- and C-terminal regions of proteins from the alignment (see Fig. 2(a)). HP, *Hansenula polymorpha*; DH, *Debaryomyces hansenii*; CA, *Candida albicans*; KL, *Kluyveromyces lactis*; SCAGT1, *Saccharomyces cerevisiae* α-glucoside transporter; SCMAL61, maltose permease of *Saccharomyces cerevisiae MAL6* cluster.

Table 2
Expression of the *H. polymorpha* maltose permease gene *HPMAL2* in a *Saccharomyces cerevisiae* maltose permease-negative mutant

S. cerevisiae strain and genotype	Plasmid introduced	Growth on maltose[b]	Maltase activity[a] (mU mg^{-1})	
			2% galactose[c]	2% galactose + 2% maltose[d]
CMY1001 (*MAL61MAL12MAL12*)	pRS425	+	20	493
CMY1050 (*mal61ΔMAL12 MAL13*)	pRS425	−	14	13
CMY1050 (*mal61ΔMAL12 MAL13*)	pRS425-B12 BamHIBglII	−	12	19
CMY1050 (*mal61ΔMAL12 MAL13*)	pRS425-Mal61promHPMAL2	+	54	753

[a] Medium maltase activity of two transformants is presented. Standard deviation was less than 15%.
[b] Growth of transformants was checked on solid minimal medium containing 2% maltose after 48 h of incubation at 30 °C.
[c] Transformants were grown in liquid YNB medium with 2% galactose up to early exponentially phase and maltase activity was measured in the cells before the addition of maltose.
[d] 2% of maltose were added to galactose-grown transformants and they were further grown for 6 h. Then maltase activity was measured in the cells.

maltase induction. Thus, the protein encoded by *HPMAL2* is certainly a functional maltose permease.

3.4. Reporter gene assay of the expression from the HPMAL1–HPMAL2 intergenic region in H. polymorpha

Reporter gene assay has shown that expression from the *S. cerevisiae MAL61–MAL62* bidirectional intergenic region is coordinately regulated by carbon sources [14,15]. We decided to use the *H. polymorpha* maltase gene in a single-reporter assay of the expression from the *HPMAL1–HPMAL2* intergenic region. Orientation of the intergenic region in front of the promoterless maltase gene was switched in a plasmid to be expressed in a *H. polymorpha* maltase disruption mutant HP201HPMAL1Δ. The mutant HP201HPMAL1Δ transformed with the promoterless maltase gene did not grow on maltose or sucrose, while it showed perfect growth on glucose or glycerol (data on glucose growth are presented in Fig. 3). Expectedly, no maltase activity was detected in glycerol-grown HP201HPMAL1Δ transformants carrying the promoterless maltase gene (data not shown). If the *HPMAL1–HPMAL2* intergenic region was inserted in front of the maltase gene, the transformants gained the ability to grow on maltose and sucrose, irrespective of the orientation of the promoter region in the plasmid (see data on maltose growth in Fig. 3). The transformants carrying single-reporter

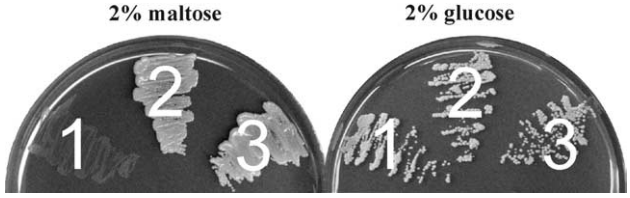


Fig. 3. Expression from the *HPMAL1-MPMAL2* intergenic region is activated by maltose in both orientations. The *H. polymorpha* maltase-disruption mutant HP201HPMAL1Δ carrying the promoterless maltase gene on a plasmid (1) grows on glucose, but not on maltose. The growth of the transformants on maltose is restored if the *HPMAL1-MPMAL2* bidirectional promoter region is inserted in front of the promoterless maltase gene in the orientation of either the maltase gene (2) or the maltose permease gene (3). All transformants grow on glucose, because the maltase protein is not needed for glucose growth.

plasmids were grown under induced (on 2% maltose), repressed (on 2% glucose) and uninduced (on 2% glycerol) conditions, and expression from the *HPMAL1–HPMAL2* intergenic region was evaluated by maltase activity. As shown in Fig. 4(a) and (b), expression from the *HPMAL1–HPMAL2* intergenic region is maltose-induced and glucose-repressed in both orientations, induction being slightly higher in the maltase direction. Inspired by a two-reporter system developed by Bell et al. [15,34], we designed a two-reporter test plasmid for *H. polymorpha* (see Section 2.1). The *H. polymorpha* maltase gene and the *E. coli* β-glucuronidase gene (*gusA*)

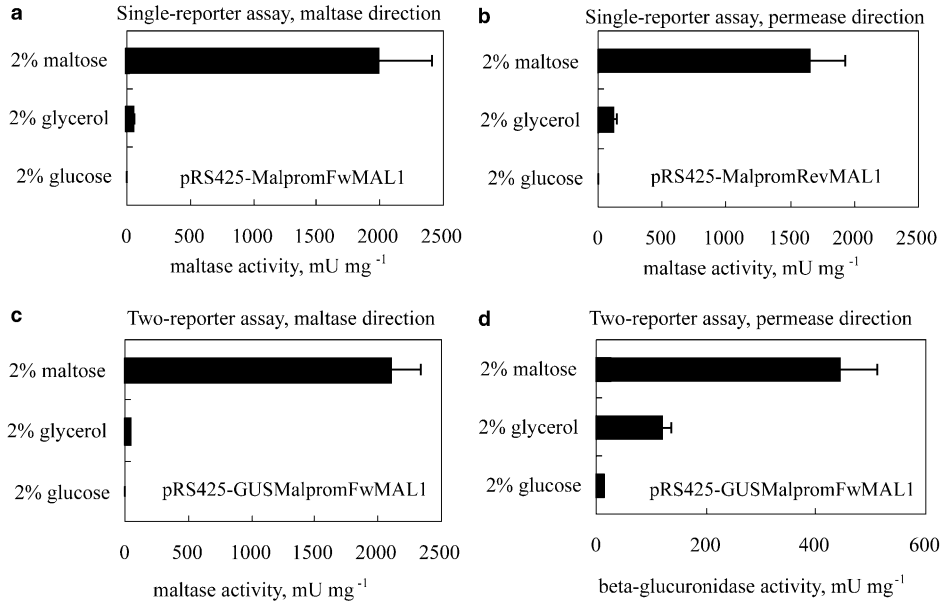


Fig. 4. Expression from the *HPMAL1–MPMAL2* intergenic region studied using a single- (a and b) and a two-reporter (c and d) assay. Transformants of HP201HPMAL1Δ carrying plasmids indicated in the figure were grown on different carbon sources till mid-exponential growth phase and specific activity of maltase was measured in cell extracts. Medium data and standard deviation for three to five distinct transformants are shown.

were chosen as reporters to follow expression from the *HPMAL1–HPMAL2* intergenic region in the direction of maltase and maltose permease, respectively. According to our knowledge, a bacterial β-glucuronidase reporter has been used in the methylotrophic yeast *Pichia pastoris* [35], but not in *H. polymorpha*. Our tests showed that *H. polymorpha* had no endogeneous β-glucuronidase activity and that the promoterless β-glucuronidase gene on a plasmid pRS425-GUS did not express in *H. polymorpha* (data not shown). A two-reporter plasmid pRS425-GUSMalpromFwMAL1 was introduced to HP201HPMAL1Δ, and the transformants were grown as in the case of the single-reporter experiment. Fig. 4(c) and (d) shows that expression from the *HPMAL1–HPMAL2* intergenic region is simultaneously induced by maltose and repressed by glucose in both directions. Both, single- and two-reporter studies showed considerably high basal expression in the direction of the permease gene (see data for glycerol-grown transformants in Fig. 4(b) and (d)). This result disagrees with respective data on *S. cerevisiae*: basal expression from the *S. cerevisiae MAL61–MAL62* bidirectional promoter is more pronounced in the maltase direction [15]. We consider that an elevated basal level of maltose permease protein may be beneficial for a yeast that has only one or few copies of maltose permease genes, because it will allow immediate transport of maltose into the cells if it suddenly becomes available. Only after maltose appears inside the cell, *MAL* genes will be induced [20] and growth on the disaccharide can be initiated.

3.5. Maltose permease genes and their maltose-related genomic neighbourhood in C. albicans, D. hansenii and H. polymorpha

In a Génolevures clone BB0AA011B12 the maltose permease gene *HPMAL2* is flanked by an N-terminal

Table 3
Genomic data[a] on five potential maltose permeases in *Debaryomyces hansenii*

Designation of the genetic locus	Protein accession number	Protein length (aa)	Similar to maltose permease from	Flanking maltose-specific gene and its predicted function
DEHA0A08316g	Q6BYN5	579	*Kluyveromyces lactis*	DEHA0A08338g (*MAL* activator, similar to *Candida albicans* P33181)
DEHA0B16269g	Q6BVW8	552	*Aspergillus oryzae*	No
DEHA0B00517g	Q6BXS9	548	*Aspergillus oryzae*	No
DEHA0E00649g	Q6BR19	582	*Candida albicans*	DEHA0E00627g (α-glucosidase, similar to *Aspergillus oryzae* Q8J2T6 and yeast maltases)
DEHA0E01232g	Q6BR02	539	*Candida albicans*	No

[a] The data were extracted from Génolevures database http://natchaug.labri.u-bordeaux.fr/Genolevures/.

fragment of a putative *MAL* activator gene (Fig. 1(a)). Comparative alignment of the protein sequence deduced from this genomic fragment with N-terminal sequences of yeast *MAL* activators is presented in Fig. 2(b). The *MAL* activators of *S. cerevisiae* and *C. albicans* contain conserved zinc fingers in N-termini [36,37] that are also present in the protein deduced from the *H. polymorpha* genomic sequence (Fig. 2(b)). The *MAL* activator of *C. albicans* can replace the function of the *MAL* activator of *S. cerevisiae* [37]. Hopefully, the functionality of the *H. polymorpha MAL* activator gene can also be tested by complementation of a respective *S. cerevisiae* mutant. As genome sequences of *C. albicans* and *D. hansenii* are available, we searched these genomes for the presence of maltose permease genes and inspected their genomic neighbourhood. The *C. albicans* genome (http://genolist.pasteur.fr/CandidaDB/) revealed presence of a divergently positioned gene pair for maltose permease and maltase with a long intergenic region, about 2900 bp, between them. The *CASUC1* gene encoding a *MAL* activator of *C. albicans* [37] is not a member of this cluster. The *D. hansenii* genomic sequence predicts presence of five potential maltose permease proteins of 539, 548, 552, 579 and 582 aa (Table 3). Clustal W alignment revealed mostly a low identity (23–40%) between these proteins. Exceptionally, permeases Q6BYN5 (579 aa) and Q6BR19 (582 aa) showed 57% identity. The maltose permease Q6BYN5 is the closest homologue of the *H. polymorpha* maltose permease *HPMAL2* (Table 1). Two of the *D. hansenii* maltose permease genes have flanking maltose-related genes (Table 3). One of the flanking genes encodes a sole putative *MAL* activator homologue of *D. hansenii*. In addition, the *D. hansenii* genome predicts presence of a maltase gene (genomic locus DEHA0A14300g) that has no maltose-specific genes aside. So, in the genome of *D. hansenii* we detected two potentially maltose-related gene pairs (Table 3), but did not find genomic clusters with all three *MAL* genes. Multiplicity of putative maltose permeases in *D. hansenii* was not surprising, because this yeast is especially rich in transporter genes, and its genome is highly redundant [38].

At the moment, the number of functional maltose-specific genes and loci in *H. polymorpha* is not clear. Our earlier study had shown that there is only one maltase gene, *HPMAL1*, in *H. polymorpha* [8]. It was verified by Southern analysis and efficient gene disruption. In the present study, we showed that *HPMAL1* is flanked by a functional maltose permease gene *HPMAL2* and that a functional *MAL* activator gene may locate adjacent to the permease gene. The number of *MAL* activator genes in yeasts varies. For example, in genomes of *C. albicans* and *D. hansenii*, we found only one *MAL* activator gene. At the same time, *MAL6* and *MAL3* loci of *S. cerevisiae* have duplicated *MAL* activator genes [11,29]. The data we have so far support the assumption that there is only one *MAL* locus in *H. polymorpha* and that it contains all three *MAL* genes.

Acknowledgements

The authors thank K. Lahtchev, J. Kiel, C. Michels and A. Mäe for providing plasmids and yeast strains. This work was supported by the Grants 3923 and 5676 from Estonian Science Foundation. The sequence of the *HPMAL2* gene is available in GenBank under Accession No. AY917133.

References

[1] Gellissen, G. (2000) Heterologous protein production in methylotrophic yeasts. Appl. Microbiol. Biotechnol. 54, 741–750.
[2] van Dijk, R., Faber, K.N., Kiel, J.A.K.W., Veenhuis, M. and van der Klei, I. (2000) The methylotrophic yeast *Hansenula polymorpha*: a versatile cell factory. Enzyme Microb. Technol. 26, 793–800.
[3] Laht, S., Karp, H., Kotka, P., Järviste, A. and Alamäe, T. (2002) Cloning and characterization of glucokinase from a methylotrophic yeast *Hansenula polymorpha*: different effects on glucose repression in *H. polymorpha* and *Saccharomyces cerevisiae*. Gene 296, 195–203.
[4] Karp, H., Järviste, A., Kriegel, T.M. and Alamäe, T. (2003) Cloning and biochemical characterization of hexokinase from the methylotrophic yeast *Hansenula polymorpha*. Curr. Genet. 44, 268–276.
[5] Ahuatzi, D., Herrero, P., de la Cera, T. and Moreno, F. (2004) The glucose regulated nuclear localization of hexokinase 2 in *Saccharomyces cerevisiae* is Mig1 dependent. J. Biol. Chem. 279, 14440–14446.
[6] Alamäe, T. and Liiv, L. (1998) Glucose repression of maltase and methanol-oxidizing enzymes in the methylotrophic yeast *Hansenula polymorpha*: isolation and study of regulatory mutants. Folia Microbiol. 43, 443–452.
[7] Liiv, L., Pärn, P. and Alamäe, T. (2001) Cloning of maltase gene from a methylotrophic yeast *Hansenula polymorpha*. Gene 26, 77–85.
[8] Alamäe, T., Pärn, P., Viigand, K. and Karp, H. (2003) Regulation of the *Hansenula polymorpha* maltase gene promoter in *H. polymorpha* and *Saccharomyces cerevisiae*. FEMS Yeast Res. 4, 165–173.
[9] Geber, A., Williamson, P.R., Rex, J.H., Sweeney, E.C. and Bennett, J.E. (1992) Cloning and characterization of a *Candida albicans* maltase gene involved in sucrose utilization. J. Bacteriol. 11, 1992–6996.
[10] Williamson, P.R., Huber, M.A. and Bennet, J.E. (1993) Role of maltase in the utilization of sucrose by *Candida albicans*. Biochem. J. 291, 765–771.
[11] Needleman, R. (1991) Control of maltase synthesis in yeast. Mol. Microbiol. 5, 2079–2084.
[12] Novak, S., Zechner-Krpan, V. and Marić, V. (2004) Regulation of maltose transport and metabolism in *Saccharomyces cerevisiae*. Food Technol. Biotechnol. 42, 213–218.
[13] Michels, C.A. and Needleman, R.B. (1984) The dispersed, repeated family of *MAL* loci in *Saccharomyces* spp. J. Bacteriol. 157, 949–952.
[14] Levine, J., Tanouye, L. and Michels, C.A. (1992) The UAS_{MAL} is a bidirectional promotor element required for the expression of both the *MAL61* and *MAL62* genes of the *Saccharomyces MAL6* locus. Curr. Genet. 22, 181–189.

[15] Bell, P.J.L., Bissinger, P.H., Evans, R.J. and Dawes, I.W. (1995) A two-reporter gene system for the analysis of bi-directional transcription from the divergent *MAL6T–MAL6S* promoter in *Saccharomyces cerevisiae*. Curr. Genet. 28, 441–446.

[16] West Jr., R.W., Yocum, R.R. and Ptashne, M. (1984) *Saccharomyces cerevisiae GAL1–GAL10* divergent promoter region: location and function of the upstream activating sequence UASG. Mol. Cell. Biol. 4, 2467–2478.

[17] Punt, P.J., Strauss, J., Smit, R., Kinghorn, J.R., van den Hondel, C.M.J.J. and Scazzocchio, C. (1995) The intergenic region between the divergently transcribed *niiA* and *niaD* genes of *Aspergillus nidulans* contains multiple NirA binding sites which act bidirectionally. Mol. Cell. Biol. 15, 5688–5699.

[18] Avila, J., González, C., Brito, N. and Siverio, J.M. (1998) Clustering of the *YNA1* gene encoding a $Zn(II)_2Cys_6$ transcriptional factor in the yeast *Hansenula polymorpha* with the nitrate assimilation genes *YNT1*, *YNI1* and *YNR1*, and its involvement in their transcriptional activation. Biochem. J. 335, 647–652.

[19] Trinklein, N.D., Aldred, S.F., Hartman, S.J., Schroeder, D.I., Otillar, R.P. and Myers, R.M. (2004) An abundance of bidirectional promoters in the human genome. Genome Res. 14, 62–66.

[20] Wang, X., Bali, M., Medintz, I. and Michels, C.A. (2002) Intracellular maltose is sufficient to induce *MAL* gene expression in *Saccharomyces cerevisiae*. Eukaryot. Cell 1, 696–703.

[21] Tan, X., Waterham, H.R., Veenhuis, M. and Cregg, J.M. (1995) The *Hansenula polymorpha* PER8 gene encodes a novel peroxisomal integral membrane protein involved in proliferation. J. Cell Biol. 128, 307–319.

[22] Blandin, G., Llorente, B., Malpertuy, A., Wincker, P., Artiguenave, F. and Dujon, B. (2000) Genomic exploration of the hemiascomycetous yeasts: 13. *Pichia angusta*. FEBS Lett. 487, 76–81.

[23] Marits, R., Tshuikina, M., Pirhonen, M., Laasik, E. and Mäe, A. (2002) Regulation of the expression of prtW::gusA fusions in *Erwinia carotovora* subsp. *carotovora*. Microbiology 148, 835–842.

[24] Sambrook, J., Fritsch, E.T. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[25] Hanahan, D. (1983) Studies on the transformation of *E. coli* with plasmids. J. Mol. Biol. 166, 577–580.

[26] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153, 163–168.

[27] Tusnády, G.E. and Simon, I. (1998) Principles governing amino acid composition of integral membrane proteins: application to topology prediction. J. Mol. Biol. 283, 489–506.

[28] Alves-Araujo, C., Hernandez-Lopez, M.J., Sousa, M.J., Prieto, J.A. and Randez-Gil, F. (2004) Cloning and characterization of the *MAL11* gene encoding a high-affinity maltose transporter from *Torulaspora delbrueckii*. FEMS Yeast Res. 4, 467–476.

[29] Charron, M.J., Read, E., Haut, S.R. and Michels, C.A. (1989) Molecular evolution of telomere-associated *MAL* loci of *Saccharomyces*. Genetics 122, 307–316.

[30] Zaret, K.S. and Sherman, F. (1982) DNA sequence required for efficient transcription termination in yeast. Cell 28, 563–573.

[31] Day, R.E., Higgins, V.J., Rogers, P.J. and Dawes, I.W. (2002) Characterization of the putative maltose transporters encoded by YDL247w and YJRI60c. Yeast 19, 1015–1027.

[32] Wong, S., Fares, M.A., Zimmermann, W., Butler, G. and Wolfe, K.H. (2003) Evidence from comparative genomics for a complete sexual cycle in the 'asexual' pathogenic yeast *Candida glabrata*. Genome Biol. 4, R10.

[33] Stasyk, O.V., Stasyk, O.G., Komduur, J., Veenhuis, M., Cregg, J.M. and Sibirny, A.A. (2004) A hexose transporter homologue controls glucose repression in the methylotrophic yeast *Hansenula polymorpha*. J. Biol. Chem. 279, 8116–8125.

[34] Bell, P.J.L., Higgins, V.J., Dawes, I.W. and Bissinger, P.H. (1997) Tandemly repeated 147 bp elements cause structural and functional variation in divergent *MAL* promoters of *Saccharomyces cerevisiae*. Yeast 13, 1135–1144.

[35] Sears, I.B., O'Connor, J., Rossanese, O.W. and Glick, B.S. (1998) A versatile set of vectors for constitutive and regulated gene expression in *Pichia pastoris*. Yeast 14, 783–790.

[36] Kim, J. and Michels, C. (1988) The *MAL63* gene of *Saccharomyces cerevisiae* encodes a cysteine-zinc finger protein. Curr. Genet. 14, 319–323.

[37] Kelly, R. and Kwon-Chung, K.J. (1992) A zinc finger protein from *Candida albicans* is involved in sucrose utilization. J. Bacteriol. 174, 222–232.

[38] Lépingle, A., Casaregola, S., Neuvéglise, C., Bon, E., Nguyen, H.-V., Artiguenave, F., Wincker, P. and Gaillardin, C. (2000) Genomic exploration of the hemiascomycetous yeasts: 14. *Debaryomyces hansenii* var. *hansenii*. FEBS Lett. 487, 82–86.