TATIANA ALVES RIGAMONTE FERNANDES

INTERNALIZAÇÃO DA PERMEASE DE LACTOSE DE *Kluyveromyces lactis* EM RESPOSTA A FONTES DE CARBONO

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

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APROVADA: 12 de abril de 2010

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(Co-orientador)

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(Orientadora)

“Vede que grande amor nos tem concedido o Pai: que fôssemos chamados filhos de Deus; e nós o somos.”

I João 3:1

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BIOGRAFIA

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RESUMO

FERNANDES, Tatiana Alves Rigamonte, M. Sc., Universidade Federal de Viçosa, abril de 2010. **Internalização da permease de lactose de *Kluyveromyces lactis* em resposta a fontes de carbono.** Orientadora: Flávia Maria Lopes Passos. Co-orientadores: Luciano Gomes Fietto e Everaldo Gonçalves de Barros.

A permease de lactose de *Kluyveromyces lactis*, Lac12, media o transporte de lactose e o de galactose de baixa afinidade. Aqui é apresentado o estudo do efeito de fontes de carbono na internalização de Lac12 através do uso de linhagens contendo o gene quimérico *LAC12-GFP*. Quando células de *K. lactis* pré-cultivadas em galactose ou lactose foram transferidas para um novo meio, Lac12-GFP foi removida da membrana plasmática e localizada intracelularmente. Surpreendentemente, mesmo a presença de galactose ou lactose no novo meio de transferência causou essa internalização, e a resposta celular foi diferente para esse dois açúcares. Os resultados obtidos revelam que o processo de internalização é dependente do tipo de açúcar presente e de sua concentração. A internalização de Lac12-GFP causou redução nas taxas de captação de lactose[C^{14}] e também foi observada em uma linhagem mutante Kl*snf1*; portanto, esse evento independe da atividade de KlSnf1. Evidências indicam que glicose-6-fosfato é o sinal intracelular, uma vez que a internalização foi induzida por 2-deoxiglicose, e a inibição da atividade da enzima fosfoglimutase por lítio impediu a internalização por galactose, mas não por lactose ou glicose. A internalização não ocorreu em 6-deoxiglicose, e, em ausência de síntese protéica, o evento foi irreversível.

ABSTRACT

FERNANDES, Tatiana Alves Rigamonte, M. Sc., Universidade Federal de Viçosa, April 2010. **Internalization of *Kluyveromyces lactis* lactose permease in response to carbon sources.** Adviser: Flávia Maria Lopes Passos. Co-advisers: Luciano Gomes Fietto and Everaldo Gonçalves de Barros.

Kluyveromyces lactis Lac12 permease mediates lactose and low-affinity galactose transports. In this study we have investigated the effects of carbon sources on internalization of Lac12 by using a *LAC12-GFP* fusion construct. When galactose- or lactose-grown cells are shifted to a fresh sugar medium, Lac12-GFP is removed from the plasma membrane and localized intracellularly. Surprisingly, even galactose or lactose in the new media caused the internalization, and cells responded differently to theses two sugars. Our results reveal that this process is dependent of sugar species and also sugar concentration. Lac12-GFP internalization causes reduction on [C^{14}]lactose uptake rates and also occurs in a Kl*snf1* mutant strain; thereby, it is independent of KlSnf1 activity. We suggest that glucose-6-phosphate is the intracellular signal, since internalization was induced by 2-deoxyglucose and inhibition of phosphoglucomutase by lithium prevented galactose- but not lactose- or glucose-induced internalization. Lac12-GFP internalization was not triggered by 6-deoxyglucose, and was irreversible in absence of protein synthesis.

1. INTRODUÇÃO

A levedura *Kluyveromyces lactis* é uma das poucas leveduras capazes de assimilar lactose, o açúcar do leite. Entre suas aplicações biotecnológicas está a fermentação da lactose presente no soro de queijo, um abundante resíduo da indústria de laticínios. Ela também tem recebido atenção como um potencial hospedeiro para síntese heteróloga de proteínas e como um modelo para leveduras não-convencionais (Rubio-Texeira, 2005). Comparada a *Saccharomyces cerevisiae*, *K. lactis* utiliza um espectro mais amplo de açúcares, como pentoses, e dissacarídeos como celobiose, além da lactose (Freer, 1991; Breunig *et al.*, 2000).

A assimilação de lactose por *K. lactis* é mediada pelas proteínas Lac12 e Lac4, as quais, respectivamente, realizam o transporte e a hidrólise intracelular de lactose em glicose e galactose. Tem sido sugerido na literatura científica que a fermentação de açúcares por *Kluyveromyces* é limitada pela capacidade de transporte transmembrana (Milkowski *et al.*, 2001). Portanto, se o interesse é aumentar a capacidade fermentativa de *K. lactis* para produção de etanol a partir de lactose ou de biomassa lignocelulósica, é importante investigar como a regulação do transporte de açúcares nessa levedura é modulada.

A permease Lac12 media tanto o transporte de lactose quanto o de baixa afinidade de galactose, por um mecanismo de simporte com prótons (Dickson & Barr, 1983; Wiedemuth & Breunig, 2005). *LAC12* é regulado em nível transcricional e corregulado com os genes *GAL*, envolvidos no metabolismo de galactose (Dong & Dickson, 1997). Além da transcrição, a atividade e a estabilidade de proteínas Lac12 são reguladas (Wiedemuth & Breunig, 2005). Em *S. cerevisiae*, fonts de carbono fermentáveis podem causar inativação irreversível de permeases. A inativação catabólica induzida por glicose ocorre quando *S. cerevisiae* é transferida de um meio contendo uma

fonte de carbono alternativa para outro meio contendo glicose, e o processo envolve internalização por endocitose e proteólise vacuolar. A adição de glicose a *S. cerevisiae* cultivada em galactose causa monoubiquitinação do principal transportador de galactose, Gal2, seguida de internalização e proteólise vacuolar (Horak & Wolf, 1997).

A principal via de regulação transcricional por glicose em *S. cerevisiae* envolve a serina/treonina cinase Snf1, mediadora central da ativação transcricional de genes sob repressão catabólica, cujos produtos são relacionados ao metabolismo de fontes de carbono alternativas (Hedbacker *et al.*, 2003; Mayordomo *et al.*, 2003). A deleção de *SNF1* em *K. lactis* causa deficiência no direcionamento de Lac12 para a membrana plasmática; essa deficiência não é primeiramente devida a redução da transcrição de *LAC12*, mas a alteração na distribuição subcelular da permease (Wiedemuth & Breunig, 2005). Tais dados indicam um papel de KlSnf1 na regulação pós-transcricional em *K. lactis*.

O transporte transmembrana, mediado por permeases, é o primeiro alvo da regulação do metabolismo de carbono. Dependendo do meio em que as células se encontram, a atividade e/ou estabilidade dessas proteínas é afetada. Em *S. cerevisiae*, fontes de carbono fermentáveis, especialmente glicose, podem causar inativação irreversível dos transportadores de fontes alternativas. A inativação envolve internalização por endocitose e proteólise vacuolar (Horak & Wolf, 1997).

O controle pós-traducional da permease de lactose de *K. lactis* em resposta a diferentes fontes de carbono ainda não foi estudado. Nesse trabalho demonstramos que Lac12 é pós-transcricionalmente regulada por alteração em sua localização celular em resposta não somente à glicose, mas também a lactose e galactose.

2. REVISÃO DE LITERATURA

K. lactis é uma das poucas leveduras conhecidas capazes de assimilar o açúcar do leite lactose, e tal capacidade fisiológica determina muitas das aplicações biotecnológicas desta levedura. Entre elas, está a fermentação da lactose presente no soro de queijo, um abundante resíduo da indústria de laticínios. Ela também tem recebido atenção como um potencial hospedeiro para síntese heteróloga de proteínas e como um modelo para leveduras não-convencionais (Rubio-Texeira, 2006). Comparada a *Saccharomyces cerevisiae*, *K. lactis* utiliza um espectro mais amplo de açúcares, como pentoses, e dissacarídeos como celobiose, além da lactose (Freer, 1991; Breunig *et al.*, 2000).

O transporte transmembrana, mediado por permeases, é o primeiro alvo da regulação do metabolismo de carbono. A permease de *K. lactis* Lac12 medeia tanto o transporte de lactose quanto o transporte de baixa afinidade de galactose, sendo ambos por um mecanismo de transporte ativo secundário de simporte com prótons (Dickson & Barr, 1983; Baruffini *et al.*, 2006). Tem sido bem documentado que *LAC12* é regulado em nível transcricional e co-regulado com os genes *GAL*, do metabolismo de galactose (Dong & Dickson, 1997).

Em *S. cerevisiae*, a principal via de regulação do metabolismo de carbono em nível transcricional envolve a atuação do complexo protéico Snf1, cujos estados fosforilado e não-fosforilado determinam, respectivamente, a ativação ou a repressão catabólica de genes relacionados à utilização de fontes alternativas de carbono (Hedbacker *et al.*, 2003). Para o estudo dessa via de regulação em *K. lactis*, Dong e Dickson (1997) construíram a linhagem mutante para o gene *KlSNF1* denominada JSD1. A partir de JSD1, Wiedemuth e Breunig (2005) construíram a linhagem JSD1R-LAC12GFP, que contém o gene *LAC12* fusionado ao da *GFP* (*Green Fluorescent Protein*). Seus estudos

demonstraram que esse mutante é deficiente em destinar Lac12 para a membrana plasmática, e que essa deficiência não se deve à redução da transcrição de *LAC12*, mas à alteração da distribuição subcelular da permease. No mutante, uma grande proporção da permease é localizada em vesículas intracelulares, o que evidencia a atuação de KlSnf1 em nível pós-traducional, no direcionamento subcelular das proteínas sintetizadas. Porém, ainda não se sabe se KlSnf1 atua na estabilização da permease na membrana, impedindo sua internalização, ou no direcionamento da proteína recém-sintetizada para a membrana.

Em nível pós-traducional, a regulação do metabolismo de carbono é alcançada pela modulação da atividade e estabilidade das enzimas. Uma das estratégias é a inativação irreversível das permeases, que é estimulada por fontes fermentáveis de carbono, mas não por fontes não fermentáveis como glicerol e etanol (Riballo *et al*., 1995). Quando *S. cerevisiae* é transferida de uma fonte de carbono alternativa (não-preferencial) para um meio contendo glicose, ocorre a internalização dos transportadores da fonte alternativa por endocitose e proteólise vacuolar – processo conhecido como “inativação catabólica induzida por glicose”. A adição de glicose a células de *S. cerevisiae* cultivada em galactose causa monoubiquitinação do principal transportador de galactose, Gal2, seguida de internalização e degradação vacuolar (Horak & Wolf, 1997). Por meio do estudo da permease de maltose Mal61, de *S. cerevisiae*, duas vias da inativação induzida por glicose foram elucidadas. Uma via é mediada pelo sensor de glicose extracelular Rgt2, e causa proteólise de Mal61 independentemente do transporte de glicose; a outra via depende do transporte de glicose e estimula a proteólise de Mal61, além de causar uma inibição do transporte de maltose. Essa inibição do transporte é uma resposta mais rápida que a proteólise (Jiang *et al*., 1997; Medintz *et al*., 1996). A via dependente do transporte de glicose é reconhecidamente estimulada pelas etapas iniciais da fermentação de açúcares, incluindo transporte e fosforilação. A sinalização do processo não é glicose-específica, e pode ser produzida pelo rápido transporte e fermentação de qualquer açúcar. Por exemplo, maltose é capaz de gerar inativação de sua própria permease (Jiang *et al*., 2000).

Estudos prévios sobre inativação em *Kluyveromyces* têm abordado principalmente a inativação catabólica induzida por glicose das enzimas

intracelulares isocitrato-liase (López *et al.*, 2004) e frutose-1,6-bisfosfatase (Zaror *et al.*, 1993). Até o momento, o controle pós-traducional da permease de lactose de *K. lactis* em resposta à glicose ou a outros açúcares não foi suficientemente estudado. Em estudos prévios, observamos que a adição de glicose a células previamente cultivadas em lactose ou galactose causa internalização da permease Lac12-GFP. Contudo, de modo semelhante a *S. cerevisiae*, em que o sinal para inativação não é glicose-específico, a adição de lactose e galactose também promoveu internalização. Esse processo se revelou dependente da fonte de carbono e de sua concentração, uma vez que células transferidas para meio fresco com baixa concentração de galactose (0,1 % p/v) não apresentaram internalização, mas para os meios com 0,1 % de lactose ou 0,5 % de galactose a internalização foi nítida.

O metabolismo de lactose é iniciado pela hidrólise intracelular, que gera glicose e galactose. A galactose é metabolizada pela via de Leloir, composta por uma série de reações catalisadas pelas enzimas galactocinase, galactose-1-fosfato uridiltransferase e uridina difosfato (UDP)-galactose 4-epimerase que converte galactose em glicose-1-fosfato (Csutora 2005). A confluência da via de Leloir com a via glicolítica ocorre pela conversão da glicose-1-fosfato em glicose-6-fosfato, que prossegue, então, na glicólise. A enzima fosfoglicomutase (PGM), responsável por essa conversão, é essencial no metabolismo de galactose. A perda da atividade da PGM em *S. cerevisiae*, por mutação ou por inibição por lítio, gera aumento significativo da razão entre glicose-1-fosfato e glicose-6-fosfato em células cultivadas em galactose e redução no nível de UDP-glicose em células cultivadas em glicose, o que impede o prosseguimento das vias biossintéticas que utilizam esse metabólito (Bro *et. al.,* 2003; Csutora *et. al.,* 2005). As vias de assimilação de galactose e glicose em *S. cerevisiae* estão esquematizadas na figura 1.

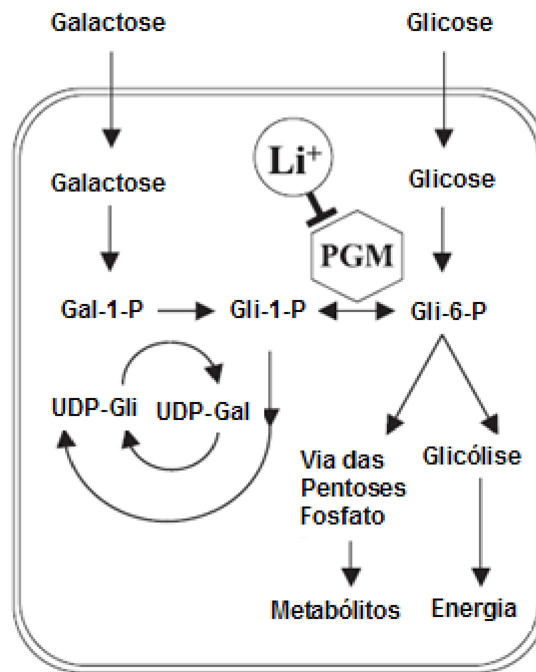

Figura 1 – Vias de assimilação de galactose e glicose em *Saccharomyces cerevisiae.* Glc: glicose; Glc-1-P: glicose-1-fosfato; Glc-6-P: glicose-6-fosfato; Gal-1-P: galactose-1-fosfato. Adaptado de Csutora *et. al.,* 2005.

Em resultados anteriores, demonstramos que a inibição da enzima PGM por cloreto de lítio impede a internalização da permease em galactose, mas não em lactose ou glicose. A inibição da PGM pelo lítio no meio contendo galactose causou um acúmulo de glicose-1-fosfato intracelular, que não pôde ser convertida a glicose-6-fosfato. Assim, os resultados indicam que a formação de glicose-6-fosfato nessas células é essencial ao processo de internalização. Trata-se de um dado relevante, uma vez que glicose-6-fosfato é reconhecidamente responsável pela repressão catabólica em *S. cerevisiae* (Ralsera *et al.*, 2008).

Neste trabalho, apresentamos evidências de que a internalização da permease de lactose é sinalizada pela concentração intracelular de glicose-6-fosfato, independe de Snf1p, e determina a redução do transporte transmembrana de lactose.

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4. ARTIGO CIENTÍFICO

Os resultados obtidos neste trabalho estão apresentados sob a forma de artigo científico, elaborado de acordo com as normas da revista FEMS Yeast Research.

Lactose permease in *Kluyveromyces lactis* is internalized not only in response to glucose, but also to lactose and galactose

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Short running title: Internalization of *Kluyveromyces lactis* lactose permease

Kluyveromyces lactis Lac12 permease mediates lactose and low-affinity galactose transports. Here we report our study of the effects of carbon sources on internalization of Lac12 by using a *LAC12-GFP* fusion construct. When galactose- or lactose-grown cells are shifted to a fresh sugar medium, Lac12-GFP is removed from the plasma membrane and localized intracellularly. Surprisingly, even galactose or lactose in the new media caused the internalization, and cells responded differently to theses two sugars. Our results reveal that this process is dependent of sugar species and also sugar concentration. Lac12-GFP internalization causes reduction on [C^{14}]lactose uptake rates and occurs in a Kl*snf1* mutant strain; thereby, it is independent of KlSnf1 activity. We suggest that glucose-6-phosphate is the intracellular signal, since internalization was induced by 2-deoxyglucose and inhibition of phosphoglucomutase by lithium prevented galactose- but not lactose- or glucose-induced internalization. Lac12-GFP internalization was not triggered by 6-deoxyglucose, and was irreversible in absence of protein synthesis.

Keywords: *Kluyveromyces lactis*, lactose permease, Lac12, posttranslational control, catabolite inactivation, sugar transport.

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31 **Introduction**

32

33 The yeast *Kluyveromyces lactis* is one of the few yeasts capable to assimilate
34 the milk sugar lactose. One of its biotechnological applications is the fermentation of
35 lactose present in cheese whey, an important residue from the dairy industry. It is
36 also used as a host for heterologous protein synthesis, and has emerged as a model
37 for non-conventional yeasts (Rubio-Texeira, 2006). Compared to *Saccharomyces*
38 *cerevisiae*, *K. lactis* is able to use a more diverse range of sugars, such as pentoses
39 and the disaccharide cellobiose (Freer, 1991; Breunig *et al.*, 2000).
40 Lactose metabolism in *K. lactis* is mediated by the proteins Lac12 and Lac4,
41 which mediate lactose transport and hydrolysis, respectively. It has been suggested
42 that fermentative utilization of sugars in *Kluyveromyces* is limited by transport
43 capacity (Milkowski *et al.*, 2001). Consequently, improvement of *K. lactis* fermentative
44 potential might be reached by modulating sugar uptake.
45 Lac12 permease mediates both lactose and low affinity galactose transport by
46 a symport mechanism (Dickson & Barr, 1983; Wiedemuth & Breunig, 2005). *LAC12* is
47 regulated at the transcriptional level and co-regulated with genes *GAL*, mediating
48 galactose metabolism (Dong & Dickson, 1997). In addition, the activity and stability of
49 Lac12 may be regulated (Wiedemuth & Breunig, 2005). In *S. cerevisiae*, fermentable
50 carbon sources can cause irreversible inactivation of transporters. Glucose-induced
51 catabolite inactivation occurs when *S. cerevisiae* is shifted from alternative carbon
52 sources to glucose, and involves internalization by endocytosis and vacuolar
53 proteolysis. Addition of glucose to galactose-growing *S. cerevisiae* triggers
54 monoubiquitination of the major galactose transporter, Gal2, followed by
55 internalization and vacuolar degradation (Horak & Wolf, 1997).
56 There are two signaling pathways that stimulate the glucose-induced
57 inactivation, elucidated by study of *S. cerevisiae* maltose permease Mal61: one is
58 mediated by the extracellular glucose sensor Rgt2 and causes Mal61 proteolysis
59 independently on glucose transport; the other pathway is dependent on transport and
60 stimulates Mal61 proteolysis and also an inhibition of maltose transport that is faster
61 than can be explained by proteolysis alone (Jiang *et al*., 1997; Medintz *et al*., 1996).

62 The transport-dependent pathway is stimulated by the initial steps of sugar
63 metabolism including transport and phosphorylation. However, as it has been
64 recognized, the signaling of the process is not glucose-specific, and can be produced
65 by fast transport and phosphorylation of many other sugars. For example, maltose
66 can trigger inactivation of its own permease (Jiang *et al*., 2000).

67 The main transcriptional regulation by glucose in *S. cerevisiae* depends on the
68 trimeric serine/treonine Snf1 kinase, the central mediator of transcription release of
69 genes under catabolic repression, whose products are related to alternative carbon
70 sources metabolism (Hedbacker *et al.*, 2003; Mayordomo *et al.*, 2003). Mutant *snf1 K.*
71 *lactis* cells presented lower growth rates in glucose, sucrose, lactose and galactose,
72 and no growth was obtained in raffinose, sorbitol, maltose or glycerol (Dong &
73 Dickson, 1997). Furthermore, deletion of Kl*SNF1* causes deficiency in accumulate
74 Lac12 at cellular membrane; it has also been shown that this deficiency is not
75 primarily due to reduction on *LAC12* transcription, but to alteration on subcellular
76 distribution of the permease (Wiedemuth & Breunig, 2005). These data indicate the
77 role of KlSnf1 in posttranslational regulation in *K. lactis*.

78 The posttranslational control of *K. lactis* lactose permease in response to
79 glucose or other sugars has not been studied in detail. In this study we demonstrate
80 that Lac12 is posttranslationally regulated by change in its subcellular localization in
81 response to the carbon source, as previously reported for *S. cerevisiae* galactose and
82 maltose permeases.

83

84 **Material and methods**

85

86 **Yeast strains and growth conditions**

87 The *K. lactis* strains used in this work were JA6-LAC12GFP (*MATα ade1-600 adeT-*
88 *600 ura3-12 trp1-11 LAC9-2 LAC12-EGFP*) and JSD1R-LAC12GFP (*MATα ade1-600*
89 *adeT-600 ura3-12 trp1-11 LAC9-2* Kl*snf1*::*ura3 LAC12-EGFP*) (Wiedemuth &
90 Breunig, 2005). In both strains, Lac12-GFP fusion protein is expressed under control
91 of its own *LAC12* promoter. Cells were grown in synthetic complete medium (SC)
92 (0.67% w/v yeast nitrogen base supplemented with amino acids and nucleobases)
93 containing 2 % (w/v) galactose or lactose as carbon and energy sources. Cultures
94 were maintained in rotatory shaker at 200 rpm, and 30 ^{o}C.

95

96 **Effects of carbon source concentration and presence of 2-DG and 6-DG on**
97 **Lac12-GFP localization**

98 *K. lactis* JA6-LAC12GFP was pre-cultured in SC medium containing 2 % galactose or
99 lactose to late log phase (optical density at 600 nm [$O.D._{600nm}$] 3 to 4). Then, cells
100 were harvested by centrifugation, washed with sterile water and resuspended in SC
101 media with 0.1, 0.5 or 2 % galactose, glucose or lactose. Resuspension of pre-
102 cultured cells corresponded to time zero. The nonfermentable carbon source glycerol
103 3 % (v/v) was used as a negative control of the inactivation process. In addition, cells
104 were resuspended in SC medium with 2 % of the non-metabolizable glucose
105 analogues 2-deoxy-D-glucose (2-DG) or 6-deoxy-D-glucose (6-DG) in order to
106 determine the minimal number of metabolic steps required for inactivation of Lac12
107 permease. 2- and 6-DG were obtained from Sigma Chemical Co.

108

109 **Fluorescence microscopy**

110 At selected time intervals, the localization of the permease was observed by the
111 presence of fluorescence in cellular compartments, as both strains contain the
112 chimerical gene *LAC12-GFP*. The microscope was Olympus BX50 with oil immersion
113 objective (100X).

114

115 **Measurement of [C^{14}]lactose uptake rates**

116 The initial uptake rates of [D-glucose-1-^{14}C] lactose were measured as previously
117 described elsewhere (Loureiro-Dias & Peinado, 1984). Cell suspension (20 μL; about
118 0.6 mg dry wt), 20 μL Tris/citrate buffer (pH 5.0, 100 mM) and 10 μL of a solution of
119 [D-glucose-1-^{14}C] lactose (Amersham) were incubated at 28 ^{o}C for 10 s (the range of
120 final concentrations of lactose was 0.05 to 5 mM and the specific activity was about
121 50 GBq.mol^{-1}). Incorporation was stopped by addition of 5 mL ice-cold water. Cells
122 were immediately filtered and washed with ice-cold water on GF/C Whatman glass-
123 fiber filters. Radioactivity was counted in a liquid scintillation system (Beckman LS
124 6000SC). Controls were prepared by addition of 5 mL cold water prior to addition of
125 labeled lactose. In order to check whether the measurements were good estimates of
126 initial uptake rates, linearity of incorporation with time for periods up to 40 s was
127 confirmed. All assays were done in duplicate. When transport rates at different points
128 of the time course of growth were evaluated, the final lactose concentration utilized
129 was 0.15 mM. The procedure was as described above.
130

131 **Effect of LiCl on *K. lactis* growth and on Lac12-GFP internalization**

132 *K. lactis* was pre-cultured in SC medium containing 2 % galactose to late log phase.
133 Cells were harvested and diluted for plating in YP 2 % galactose or glucose agar
134 media containing 0, 30 or 100 mM LiCl to investigate the effect of
135 phosphoglucomutase inhibition on *K. lactis* growth in galactose. Colonies were
136 examined after 48 hours of incubation at 30 ^{o}C. To test the effect of lithium on Lac12-
137 GFP internalization, galactose-grown cells were shifted to SC medium containing 2 %
138 galactose, lactose or glucose, and 30 mM LiCl. Samples were taken and cellular
139 fluorescence was observed.
140

141 **Results**

142

143 **Lac12-GFP localization is altered during *K. lactis* growth**

144

145 The subcellular localization of Lac12-GFP was analyzed by fluorescence
146 microscopy during 12 hours of growth with samples being taken each hour. Figure 1
147 shows yeast cells sampled after 2 and 4h at log growth phase, and after 10 and 12h,
148 at reduced growth rates. The images reveal permease internalization within the first
149 hours in fresh medium. At later time points the intracellular fluorescence was reduced,
150 whereas a strong fluorescence signal in the plasma membrane reappeared.
151 Besides glucose, the sugars galactose and lactose also caused internalization
152 of Lac12-GFP permease, even though galactose and lactose are substrates for the
153 permease. In lactose, the cells emitted fluorescence from intracellular compartments
154 in all samples taken from the cultures during 12 hours of growth (Fig. 1A). The
155 punctuated staining may reflect fragmented vacuoles or intracellular vesicles. When
156 cells were grown in galactose, practically no internal fluorescence was observed after
157 10 hours and the signal at the plasma membrane was very intense (Fig. 1B).
158 To analyze the influence of sugar concentration on Lac12-GFP internalization,
159 galactose-grown cells were shifted to fresh media containing galactose or lactose at
160 concentrations of 0.1, 0.5 or 2 % (Fig. 2). In 0.1 and 0.5 % galactose, most of the
161 fluorescence signal remained at the membrane after shifting, and only a slight
162 intracellular signal appeared; 2 % galactose caused a strong internalization. Lactose
163 caused rapid internalization of the permease even at concentration of 0.1 %. Glycerol,
164 which is a non-fermentable carbon source, did not cause internalization of the
165 permease.

166

167

168 **Permease internalization is followed by reduction on lactose uptake rates**

169

170 Galactose-grown cells were harvested at log phase and inoculated to an initial
171 O.D.$_{600nm}$ of 0.2 into a fresh 2 % galactose medium. Subcellular Lac12-GFP

172 distribution was then followed by fluorescence microscopy. Samples were taken after
173 2, 5 and 10 hours of inoculation for analysis of [C^{14}]lactose uptake rates. The rates
174 (Fig. 3) were coherent with the phenomenon of internalization of the permease as
175 presented in Fig. 1: samples of yeast cells at time zero (pre-culture) showed higher
176 transport activity, while after two hours in fresh medium the transport rate decreased.
177 Transport rates tend to increase, paralleling the return of the permease to the
178 membrane.

179 Lactose uptake rates from galactose-grown cells were higher than those from
180 cells lactose-grown (Table 1, "pre-culture" line). This is expected since fluorescence
181 signal at membrane is more intense in galactose than in lactose (Fig. 1). Cells
182 transferred to fresh 2 % lactose or galactose media showed reduction on lactose
183 uptake rates, which can be explained by internalization. The uptake rates in cells
184 transferred to 0.1 % galactose medium was reduced in the first hour, but increased
185 later (Table 1), in accordance with the fluorescence images (Fig. 2).
186
187

188 **Lac12-GFP internalization depends on the formation of glucose-6-phosphate**
189 **but is independent of the glycolytic flow**
190

191 To address the question whether galactose-induced internalization requires
192 galactose metabolism, we tested the influence of lithium on yeast growth and on
193 Lac12 internalization. Lithium is known to inhibit phosphoglucomutase (PGM)
194 (Masuda *et al.*, 2001), which is essential for galactose metabolism, as it catalyzes the
195 interconversion of glucose-1-phosphate into glucose-6-phosphate. In *S. cerevisiae*
196 grown in glucose, the lithium inhibitory concentration (IC_{50}) is 100 mM, while in
197 galactose the IC_{50} is 6 mM (Bro *et al.*, 2003). Growth assays in the presence of LiCl
198 confirmed that lithium inhibits the growth of *K. lactis* in galactose but not in glucose
199 medium, for the two concentrations used: 30 and 100 mM (Fig. 4A). The effect of LiCl
200 on Lac12-GFP internalization was determined in galactose-grown cells shifted to
201 fresh SC media containing 30 mM LiCl and 2 % galactose, lactose or glucose. As
202 shown in Fig. 4B, lithium prevented galactose- but not lactose- or glucose-induced

203 Lac12 internalization. In lactose, the enzymatic hydrolysis of the disaccharide
204 releases intracellular glucose, which generates the signal for Lac12-GFP
205 internalization even when galactose channeling into glycolysis is blocked.
206
207
208 **2- deoxyglucose, but not 6-deoxyglucose, induces Lac12-GFP internalization**
209
210 *K. lactis* cells pre-grown on galactose were shifted to fresh media containing 2
211 % 2-deoxyglucose (2-DG), 6-deoxyglucose (6-DG) or galactose. 2-DG is a glucose
212 analogue that cannot be metabolized via the glycolytic pathway beyond 2-DG-6-
213 phosphate, known to cause catabolite repression in *S. cerevisiae* and growth
214 inhibition in galactose, maltose, ethanol and lactate in *K. lactis* strains (Betina *et al.*,
215 2001; Brondijk *et al.*, 2001). 6-DG is a glucose analogue that is transported into the
216 cell but cannot be phosphorylated (Bisson & Fraenkel, 1983). After 2h in fresh media
217 the reduction of the fluorescence signal at the plasma membrane in the presence of
218 galactose (positive control) or 2-DG indicates that 2-DG can cause internalization of
219 Lac12-GFP. No internalization was observed in cells shifted to 6-DG.
220
221
222 **Lac12 internalization is independent of KlSnf1 activity**
223
224 In *K. lactis*, the protein Snf1 is needed for stabilization of Lac12 in the plasma
225 membrane, revealing its role in posttranslational regulation of the permease
226 (Wiedemuth & Breunig, 2005). To test if Lac12 internalization is dependent on KlSnf1
227 activity, the *K. lactis snf1* mutant strain JSD1R-LAC12GFP was grown in SC 2 %
228 galactose to late log phase and shifted to fresh SC 2 % galactose or 3 % glycerol
229 media. After 1 and 2 hours (Fig. 6), Lac12-GFP internalization occurred in 2 %
230 galactose medium, but no internalization was observed in glycerol, similarly to results
231 obtained with strain JA6-LAC12GFP.
232

233 **The return of Lac12-GFP to the plasma membrane is dependent of protein**
234 **synthesis**
235
236 *K. lactis* cells pre-grown in galactose and transferred to fresh galactose
237 medium show internalization of Lac12-GFP in the first hours and increase of the
238 fluorescence signal at the plasma membrane after some hours. To test if this increase
239 was due to protein synthesis, galactose-grown cells were shifted to fresh SC 2%
240 galactose, or SC 2 % galactose + hygromycin (final concentration 200 μg mL^{-1}).
241 Hygromycin is an inhibitor of protein synthesis that affects mRNA translation. After 1 h
242 of transference, Lac12-GFP internalization was observed in both media; after 4 h,
243 only in the medium without hygromycin the fluorescence signal returned to the
244 plasma membrane (Fig. 7). This pattern also occurred in lactose. As a control,
245 galactose-grown cells were also shifted to SC 0.1 % galactose or SC 0.1 % galactose
246 + hygromycin, and no internalization occurred in these media, indicating that
247 hygromycin did not cause the internalization in 2 % sugar. So, it is concluded that the
248 return of the fluorescence to plasma membrane is dependent of protein synthesis –
249 likely *de novo* synthesis of Lac12-GFP.
250

251 **Discussion**

252

253 *K. lactis*, unlike *S. cerevisiae*, is one of the few yeasts capable to assimilate the
254 milk sugar lactose, and this physiological ability determines many of the
255 biotechnological applications of this yeast. The aim of this research was to investigate
256 the internalization of *K. lactis* lactose/galactose permease Lac12, as little is known
257 about the posttranslational control of this permease.

258 Our data showed that *K. lactis* lactose permease has a differential response to
259 lactose and galactose, although both sugars are substrates for Lac12. Lac12-GFP
260 internalization was observed in concentration as low as 0.1 % (w/v) lactose. In 2 %
261 galactose the internalization occurred, but not in 0.1 or 0.5 %. Also, fluorescence in
262 intracellular compartments in cells cultured in lactose was detected during all phases
263 of growth, while in galactose the intracellular fluorescence disappears at the end of
264 the log phase.

265 The absence of internalization in 0.1 and 0.5 % galactose indicates that *K.*
266 *lactis* cells respond not only to the sugar species, but specifically to its concentration.
267 Thus, the gradual return of the fluorescence signal to the membrane, observed while
268 culture approached the desacceleration growth phase in either galactose or lactose,
269 may be a consequence of reduction on sugar concentrations.

270 [C^{14}]lactose uptake rates reveal that sugar transport is reduced in the first
271 hours of cell shifting to 2 % sugar medium, paralleling the internalization of Lac12-
272 GFP. The rates tend to increase with time, which may be caused by the return of the
273 permease to the membrane. Further, [C^{14}]lactose uptake also confirm that lactose
274 causes more intense internalization than galactose, and that 0.1 % galactose is not
275 sufficient to trigger the process.

276 Galactose is metabolized via the Leloir pathway, whose confluence with the
277 glycolytic pathway occurs at the conversion of glucose-1-phosphate to glucose-6-
278 phosphate, catalyzed by phosphoglucomutase (PGM). Glucose-6-phosphate (G6P) is
279 already known to be a signal for catabolite repression in *S. cerevisiae* (Meijer *et al.*,
280 1998), and our results suggest that G6P is also the signaling molecule to
281 internalization. The first evidence is that 2-deoxyglucose (2-DG) but not 6-

282 deoxyglucose caused Lac12-GFP internalization. 2-DG is a non-metabolizable
283 glucose analogue that is transported and phosphorylated in the cell, but does not
284 proceed along the glycolytic pathway and, so, accumulates as 2-DG-6-phosphate
285 (Lascaris *et al.*, 2005). 2-DG is highly toxic to *S. cerevisiae*, and causes growth
286 inhibition in galactose, maltose, ethanol and lactate in *K. lactis* strains (Betina *et al.*,
287 2001; Brondijk *et al.*, 2001). In *S. cerevisiae*, inactivation of maltose permease Mal61
288 is also caused by addition of 2-DG (Harma *et al.*, 2001), and requires not only
289 maltose transport, but also the activity of maltase (Mal12) and, consequently, the
290 production of intracellular glucose (Jiang *et al.*, 2000). The second evidence for
291 signaling by G6P is that lithium, known to inhibit PGM activity, blocked internalization
292 by galactose, but not by lactose or glucose. In lactose and glucose media,
293 intracellular glucose-6-phosphate could be formed by phosphorylation of the glucose
294 transported or released by lactose hydrolysis. The occurrence of internalization in
295 these two sugars also confirms that none of the possible indirect effects of lithium
296 affected the cellular capability to promote internalization of the permease, and, so,
297 galactose-induced internalization did not occur because galactose metabolism was
298 impaired. The loss of PGM activity in *S. cerevisiae*, by mutation or lithium inhibition,
299 generates a considerable increase in Glu-1P/Glu-6P ratio in cells growing on
300 galactose (Csutora *et. al.,* 2005; Bro *et. al.,* 2003). However, we cannot conclude that
301 G6P is the signal, since addition of 2-DG also promotes a slight formation of two other
302 metabolites, likely dideoxytrehalose-6P and dideoxytrehalose.
303 The protein kinase Snf1 is one of the central regulators of carbon metabolism
304 in *S. cerevisiae*, triggering activation of genes associated to alternative carbon
305 sources metabolism. Mutant *snf1 K. lactis* cells presented lower growth rates in
306 glucose, sucrose, lactose and galactose, and no growth was obtained in raffinose,
307 sorbitol, maltose or glycerol (Dong & Dickson, 1997). In both *K. lactis* strains JA6-
308 LAC12GFP and JSD1R-LAC12GFP (mutant *snf1*) the chimerical *LAC12-GFP* is
309 under control of its own promoter. Induction of Lac/Gal regulon is only weakly
310 reduced in the Kl*snf1* mutant, and these cells present much less fluorescence at the
311 plasma membrane and more fluorescence in internal vesicles (Wiedemuth & Breunig,
312 2005), showing that a KlSnf1-independent pathway induces the regulon, and that the

313 kinase has an important posttranslational role on location of the permease in the
314 cells. Considering the role of KlSnf1 in carbon metabolism and specifically in Lac12
315 posttranslational control, we analyzed whether Lac12 internalization is dependent of
316 KlSnf1 activity. Mutant Kl*snf1* cells JSD1R-LAC12GFP pre-grown on galactose were
317 shifted to fresh 2 % galactose medium, and Lac12-GFP was internalized. Further, no
318 internalization was noted in 0.1 % galactose or in glycerol. Thereby, the data show
319 that the pathway leading Lac12 internalization is not under KlSnf1 control.
320 The observed return of the fluorescence to the plasma membrane after the
321 permease internalization is dependent on protein synthesis, as it did not occur in cells
322 transferred to 2 % galactose or lactose when hygromycin, an inhibitor of protein
323 synthesis, was present. Cells transferred to 0.1 % galactose with or without
324 hygromycin showed no internalization, allowing the conclusion that it was not the
325 inhibitor the cause of Lac12-GFP internalization. So, during the cultivation, the sugar
326 concentration decreases with time, causing diminution on rates of sugar transport and
327 glucose-6-phosphate formation. Then, the fluorescence signal at the membrane is
328 gradually increased, probably due to induction of Lac regulon and Lac12-GFP *de*
329 *novo* synthesis. The need for *de novo* protein synthesis also suggests that Lac12-
330 GFP is degraded after internalization.
331
332
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334
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338

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407

408 **Legends**

409

410 Figure 1 – Subcellular localization of the permease Lac12-GFP during growth of *K.*
411 *lactis* in lactose and galactose. Yeast cells were pre-grown in SC medium plus 2 %
412 lactose (A) or 2 % galactose (B) and shifted to fresh lactose (A) or galactose (B)
413 media, for initial O.D.$_{600nm}$ of 0.2. Lac12-GFP fluorescence was analyzed at indicated
414 times with fluorescence microscope.

415

416 Figure 2 – Subcellular localization of the permease Lac12-GFP in SC media
417 containing 0.1%, 0.5 % or 2 % galactose, lactose or glucose, or 3 % glycerol. Pre-
418 grown cells in SC 2 % galactose medium were shifted to specified carbon
419 concentration. Lac12-GFP fluorescence was analyzed as in Fig. 1.

420

421 Figure 3 – [C^{14}]lactose transport by *K. lactis* during growth. Pre-grown cells in SC 2 %
422 galactose medium were shifted to a fresh medium. At indicated times, samples were
423 collected for [C^{14}]lactose transport analysis. Time zero corresponds to lactose
424 transport rate from pre-culture. Final lactose concentration utilized in this experiment
425 was 0.145 mM.

426

427 Table 1. Lactose uptake rates (μmol.h^{-1}.g^{-1}) in *K. lactis* cells in lactose and different
428 galactose concentrations
429 *K. lactis* JA6-Lac12GFP was pre-cultivated in SC 2 % lactose or galactose media.
430 Lactose uptake rates were measured from pre-cultures, and after 1 and 3 hours of
431 transference to the indicated carbon media. The data are the slant of the linear
432 relation between intracellular [C^{14}]lactose and time. Final lactose concentration
433 utilized in this experiment was 0.145 mM.

434

435 Figure 4 – Effect of LiCl on *K. lactis* growth and on Lac12 internalization. (A) Pre-
436 grown cells at log phase in SC 2 % galactose medium were harvested and diluted for
437 plating in YP 2 % galactose or glucose agar media, containing 0, 30 or 100 mM LiCl.
438 (B) Pre-grown cells at log phase in SC 2 % galactose medium were shifted to fresh

439 SC media with 2 % of the indicated sugars. Lac12-GFP fluorescence was analyzed
440 as in Fig. 1.
441
442 Figure 5 – The effect of the glucose analogues 2-DG and 6-DG on Lac12-GFP
443 localization. Pre-grown cells in SC 2 % galactose medium at log phase were shifted
444 to SC medium with 2 % 2-DG, 6-DG or galactose. Lac12-GFP fluorescence was
445 analyzed as in Fig. 1. Images were obtained after 2h in fresh media.
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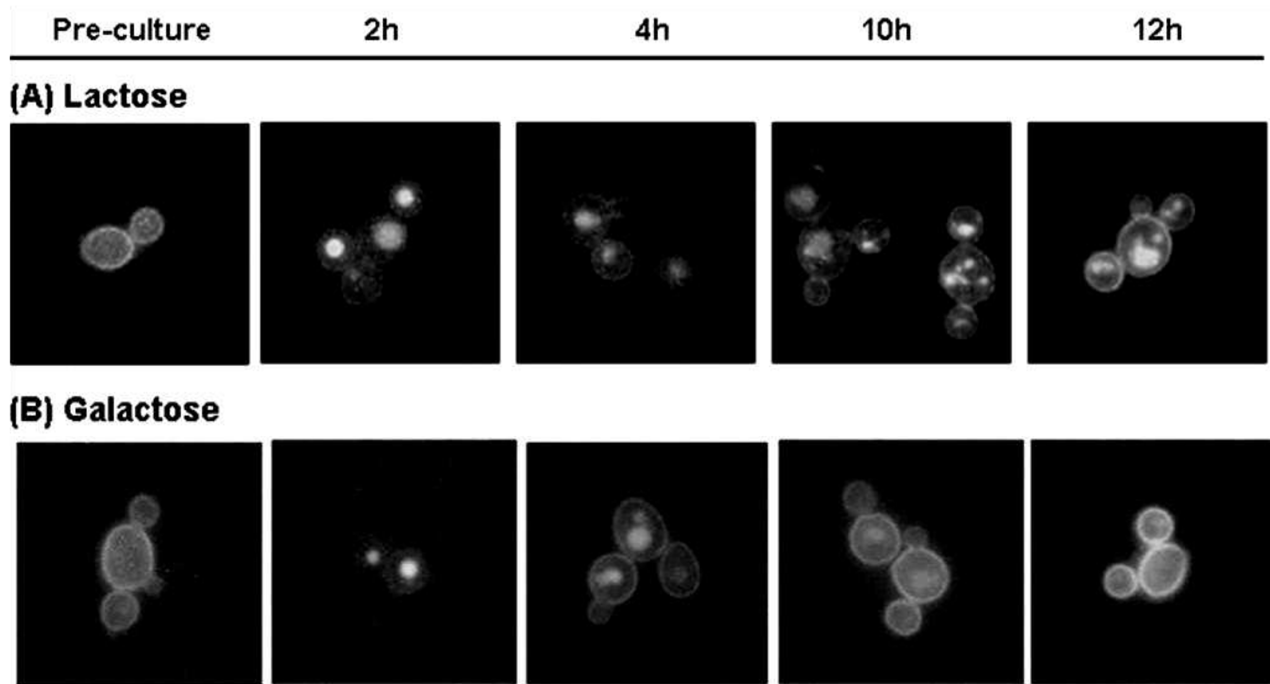
447 Figure 6 – Lac12 internalization in Kl*snf1* mutant strain. Cells were pre-grown in SC 2
448 % galactose to late log phase and shifted to fresh SC 2 % galactose or SC 3 %
449 glycerol media. At indicated times, samples were collected and analyzed as in Fig. 1.
450
451 Fig. 7 – Effect of protein synthesis impairment on Lac12-GFP internalization.
452 Galactose-grown *K. lactis* cells were shifted to fresh SC galactose or SC galactose +
453 hygromycin. Lactose-grown cells were shifted to fresh SC lactose or SC lactose +
454 hygromycin. Sugar concentration was 2 % w/v and hygromycin final concentration
455 was 200 µg mL^{-1}. At indicated times, samples were collected and analyzed as in Fig.
456 1.
457

458 **Figures**

459



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467 Figure 1 – Subcellular localization of the permease Lac12-GFP during growth of *K.*
468 *lactis* in lactose and galactose. Yeast cells were pre-grown in SC medium plus 2 %
469 lactose (A) or 2 % galactose (B) and shifted to fresh lactose (A) or galactose (B)
470 media, for initial O.D.$_{600nm}$ of 0.2. Lac12-GFP fluorescence was analyzed at indicated
471 times with fluorescence microscope.

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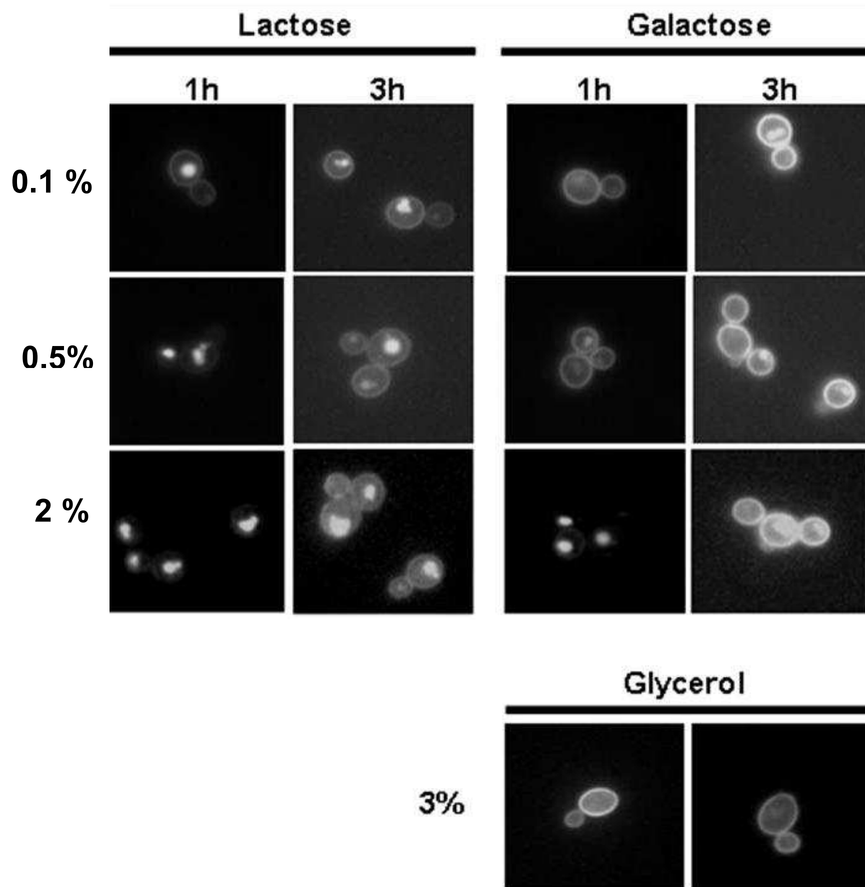

478 Figure 2 – Subcellular localization of the permease Lac12-GFP in SC media
479 containing 0.1%, 0.5 % or 2 % galactose, lactose or glucose, or 3 % glycerol. Pre-
480 grown cells in SC 2 % galactose medium were shifted to specified carbon
481 concentration. Lac12-GFP fluorescence was analyzed as in Fig. 1.
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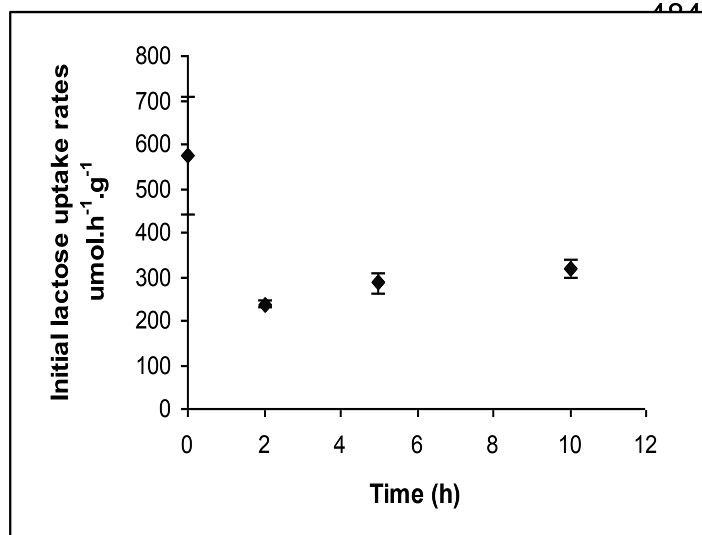

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486 Figure 3 – [C^{14}]lactose transport by *K. lactis* during growth. Pre-grown cells in SC 2 %
487 galactose medium were shifted to a fresh medium. At indicated times, samples were
488 collected for [C^{14}]lactose transport analysis. Time zero corresponds to lactose
489 transport rate from pre-culture. Final lactose concentration utilized in this experiment
490 was 0.145 mM.

491

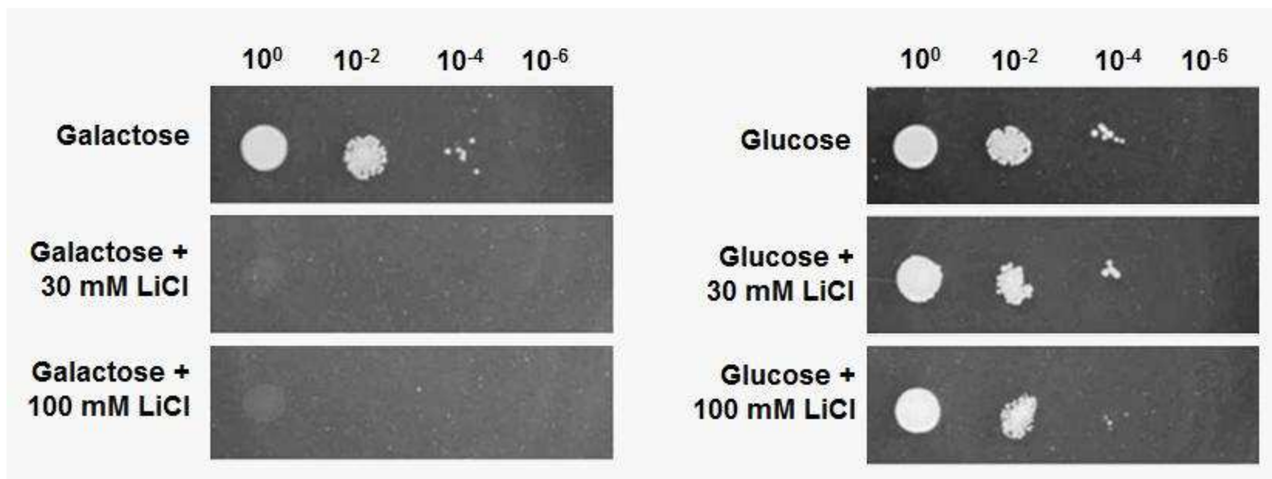
492 Table 1. Lactose uptake rates (μmol.h^{-1}.g^{-1}) in *K. lactis* cells in lactose and different
493 galactose concentrations

	Lactose	Galactose	
0h (pre-culture)	477.57	787.48	
		Galactose 2 %	Galactose 0.1 %
1h	317.68	735.51	677.33
3h	202.77	487.00	835.37

494

495 *K. lactis* JA6-Lac12GFP was pre-cultivated in SC 2 % lactose or galactose media.
496 Lactose uptake rates were measured from pre-cultures, and after 1 and 3 hours of
497 transference to the indicated carbon media. The data are the slant of the linear
498 relation between intracellular [C^{14}]lactose and time. Final lactose concentration
499 utilized in this experiment was 0.145 mM.
500

501 **(A)**



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503

504 Figure 4 – Effect of LiCl on *K. lactis* growth and on Lac12 internalization. (A) Pre-
505 grown cells at log phase in SC 2 % galactose medium were harvested and diluted for
506 plating in YP 2 % galactose or glucose agar media, containing 0, 30 or 100 mM LiCl.
507 (B) Pre-grown cells at log phase in SC 2 % galactose medium were shifted to fresh
508 SC media with 2 % of the indicated sugars. Lac12-GFP fluorescence was analyzed
509 as in Fig. 1.

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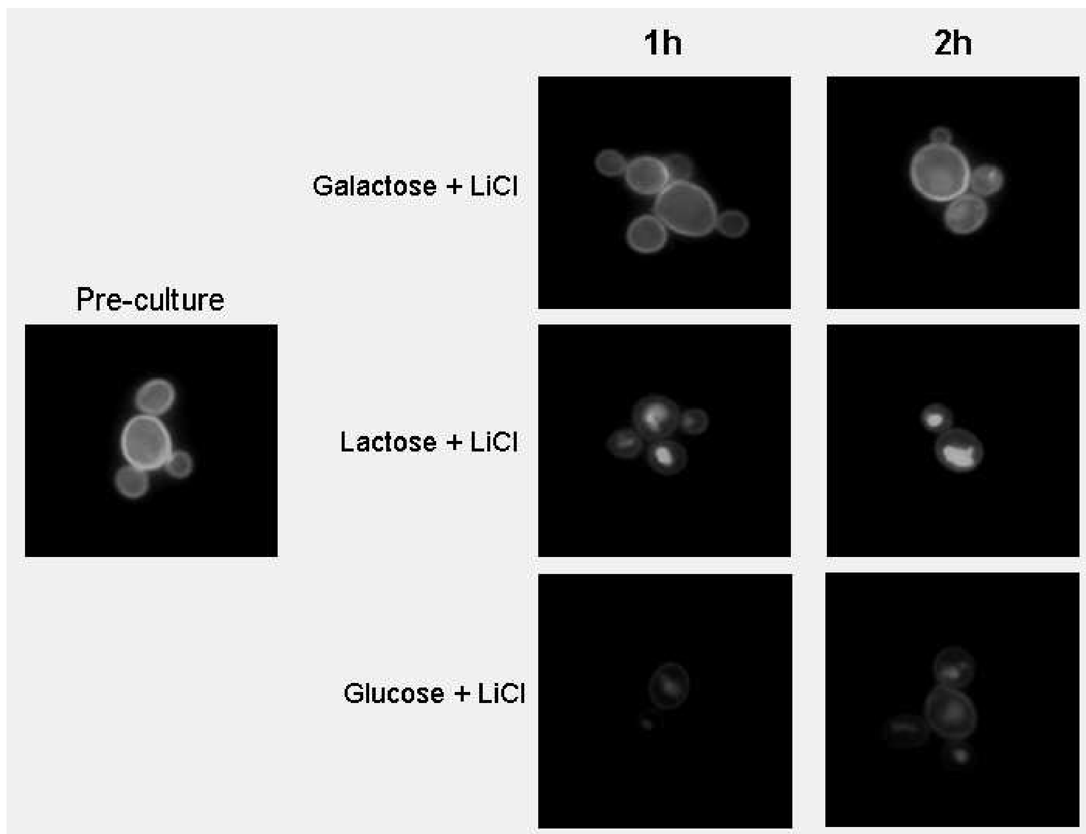
511 **(B)**



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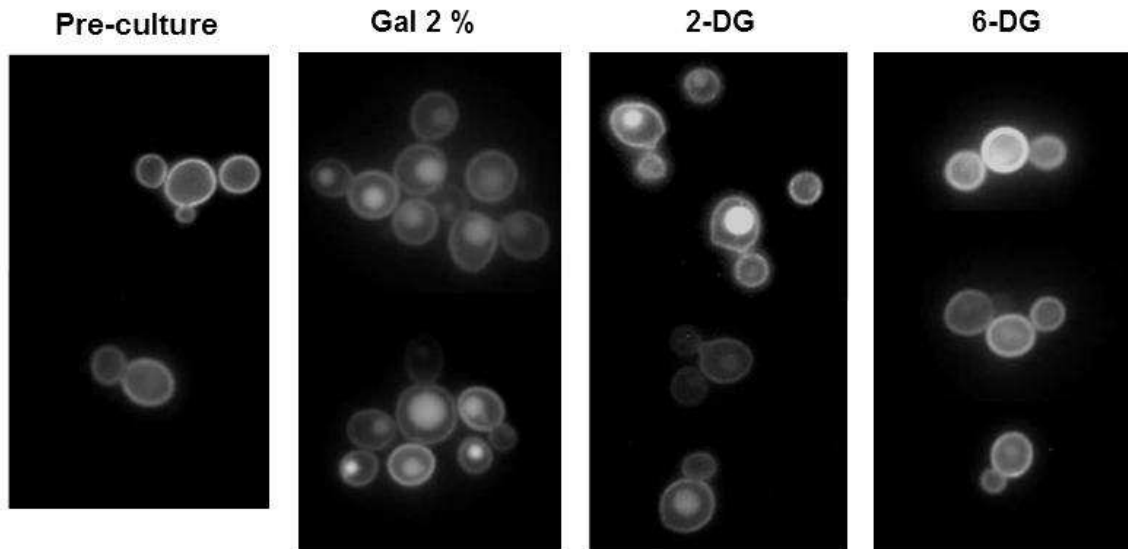

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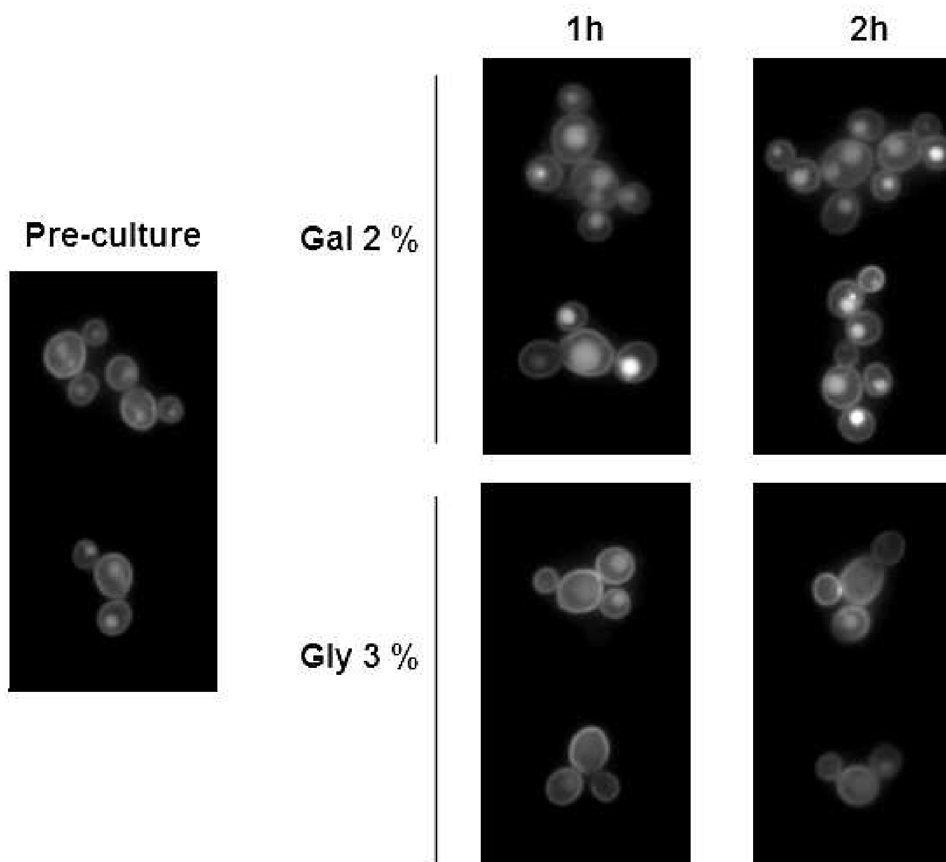

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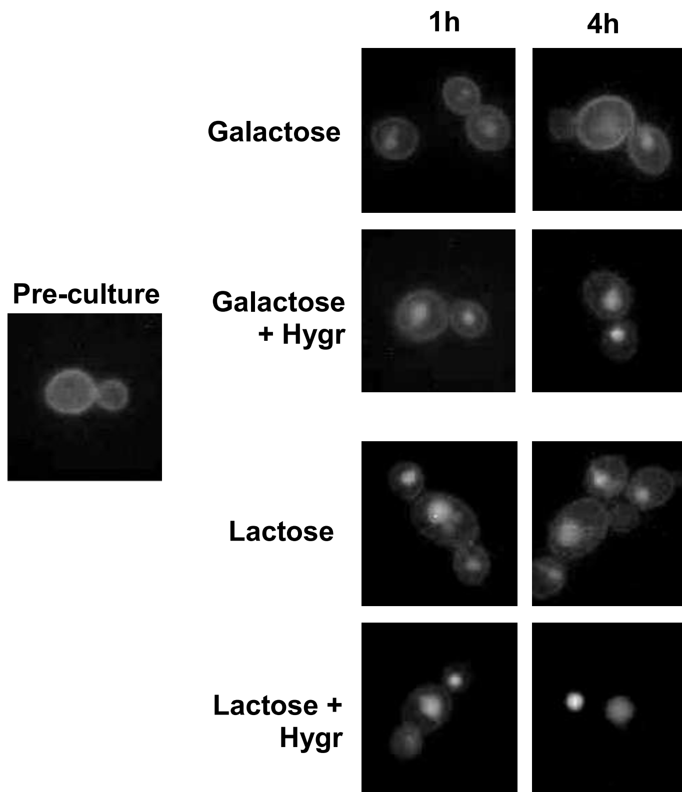

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