Evidence for rapid uptake of D-galacturonic acid in the yeast Saccharomyces cerevisiae by a channel-type transport system

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D-Galacturonic acid is a major component of pectins but cannot be metabolized by Saccharomyces cerevisiae. It is assumed not to be taken up. We show that yeast displays surprisingly rapid low-affinity uptake of D-galacturonic acid, strongly increasing with decreasing extracellular pH and without saturation up to 1.5 M. There was no intracellular concentration above the extracellular level and transport was reversible. Among more than 160 single and multiple deletion mutants in channels and transporters, no strain was affected in D-galacturonic acid uptake. The uptake was not inhibited by any compound tested as candidate competitive inhibitor, including D-glucuronic acid, which was also transported. The characteristics of D-galacturonic acid uptake are consistent with involvement of a channel-type system, probably encoded by multiple genes.

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1. Introduction

D-Galacturonic acid is the major constituent of pectin in plant cell walls. While pectin is normally present at levels lower than 5%, some plants and industrial waste streams, like sugar beet pulp, citrus peel, apple pulp, cacti . . . contain up to 24% pectin [1,2]. Several bacteria, yeasts and filamentous fungi degrade pectin and metabolize pectin degradation products. In general, bacterial exo-polygalacturonase enzymes produce mostly digalacturonic acid residues, while fungal enzymes produce monogalacturonates [3]. Several metabolic pathways for D-galacturonic acid and other pectinolytic degradation products are known in bacteria like Escherichia coli, Erwinia spp. and Agrobacterium tumefaciens [2]. Only one D-galacturonic acid degradation pathway is known in filamentous fungi [2].

The uptake of pectin degradation products is well characterized in bacteria. Oligomers are transported by the ABC-transporter TogMNA or by the cation symporter TogT [4]. D-Galacturonic acid monomers are transported by the ExuT-transporter. The D-galacturonic acid transport system in Erwinia chrysanthemi and Erwinia carotovora is able to concentrate the substrate in the cell 1000-fold and was saturable with a $K_m$ of 25–50 µM and a $V_{max}$ of 38 nmol min$^{-1}$ (mg protein)$^{-1}$. Inhibition by energy-depleting compounds like cyanide, carbonyl cyanide-m-chlorophenyl (CCCP) and 2,4-dinitrophenol (DNP) suggests that the uptake requires energy. It was found that D-galacturonate, polygalacturonate or pectin are required for induction of the ExuT transporter. Furthermore, ExuT also transports D-glucuronate and is unable to transport di- galacturonic acid [5]. Unlike in bacteria, very few studies have been conducted on D-galacturonic acid uptake in fungi. Sclerotinia fructigena, a mould causing brown rot of apple and pear, was found to take up D-galacturonic acid, though in an unspecific (K$m > 50$ mM) and energy independent way [6]. Another study revealed three transporters that were induced in Aspergillus niger grown on D-galacturonic acid or pectin. Two of them showed similarity to hexose transporters and the third to monocarboxylate transporters [7].

Uptake of D-galacturonic acid is an important requirement for genetically modified yeast strains able to ferment pectin-rich substrates. However, there are no reports of Saccharomyces cerevisiae strains able to grow on D-galacturonic acid. Because of the latter reason and because of the absence of genes with clear homology to genes of the fungal galacturonic pathway, it was postulated in the literature that S. cerevisiae is unable to take up D-galacturonic acid [8]. While this paper was in submission, a report appeared in which D-galacturonic acid was suggested to enter via the galactose transporter Gal2 since it inhibited galactose, xylose and arabinose fermentation at an extracellular pH of 3.5 [9].

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Abbreviations: CCCP, cyanide, carbonyl cyanide-m-chlorophenyl; DNP, 2,4-dinitrophenol; pH$_i$, intracellular pH; GFP, green fluorescent protein; d.w., dry weight.
We now report that D-galacturonic acid is taken up by S. cerevisiae at a surprisingly high rate. The transport displays unexpected characteristics for a sugar-like molecule, is not affected by inactivation of hexose transport, nor by deletion of any other transporter or combination of transporters tested. Based on the uptake characteristics, we suggest a channel-type transport mechanism, likely encoded by multiple genes.

2. Materials and methods

2.1. Strains, plasmids and growth media

The S. cerevisiae strains used in this work were BY4741, BY4742, CEN.PK2-1C, the multiple deletion strains EBY.VW1000 and ABY.VW4000 [10], and single deletion strains derived from BY4742 (Euroscarf deletion collection, http://web.uni-frankfurt.de/fbi15/mikro/euroscarf). The arn1-4A-strain was made by crossing and subsequent segregation of BY4741 enb1::KanMX4, BY4742 sit1::KanMX4 and BY4742 arn2::KanMX4. ARN1 was deleted by PCR-mediated gene disruption with a loxp-KIURA3-loxp cassette amplified from pUG72. All cultures were grown to exponential phase (OD_600 = 1.5–3) in YPD-medium or YP-maltose (for glucose uptake deficient strains) at 30 °C. For determination of the pH, BY4741 was transformed with the plasmid pVT100U [11] and a transformant was grown in SC-Ura with 2% glucose.

2.2. Transport assays

0.5-Galacturonic acid uptake in intact cells was assayed with [3H(D)]-D-galacturonic acid, custom made by Vitrax, Placentia, USA. Product identity for this lot was confirmed by HPLC co-elution with authentic standard, detection was done at 210 nm. The product identity was also verified by Mass Spec analysis, which confirmed the presence of a predominant mono tritiated species. The nature of the reaction work-up and purification method would not allow for any exchangeable protons to remain on the molecule. This includes all the secondary hydroxyl protons, as well as the carboxylic acid proton. For all transport assays, D-galacturonic acid (Sigma–Aldrich) solutions were adjusted to the desired pH with KOH. The protocol was adapted from [12]. The cell suspension (90 mg wet weight per ml) and the radioactively labelled substrate were both buffered with 100 mM potassium phosphate buffer at the desired pH value. Cell suspension (66 μl) was pre-incubated at 30 °C in a water bath for 5 min. For determination of D-galacturonic acid uptake in the presence of inhibitors 33 μl of buffered cell suspension (180 mg wet weight per ml) was pre-incubated with 33 μl of the inhibitor solution for 10 min. DNP and CCCP were dissolved in 1% dimethyl sulfoxide. Dimethyl sulfoxide (1%) without inhibitor was used as a negative control. The uptake was initiated by addition of 33 μl of radioactively labelled D-galacturonic acid. The initial uptake rate was determined for 1 min at 30 °C. For time course experiments 100 μl samples were taken in the reaction volume incubated in a shaking water bath. The reaction was stopped by addition of 5 ml ice-cold stop buffer consisting of non-labelled D-galacturonic acid. The cells were collected on a glass-fibre filter (Whatman, GF/F) pre-wet with stop buffer. The filter was washed three times with 5 ml milliQ-water and radioactivity retained on the filter was measured by liquid scintillation counting (Beckman Coulter LS6500). The initial D-glucose uptake rate was determined for 5 s as previously described [12]. In all uptake assays, blanks were determined as previously described [12]. Data represent mean values ± standard deviation (SD). For estimation of the intracellular D-galacturonic acid concentration, the cell dry weight was converted to cell volume by use of the conversion factor of 2.2 μl/mg dry weight [13].

2.3. Determination of intracellular pH

The measurement of intracellular pH was done according to previously published procedures [11] in a SynergyHT microplate reader (BioTek). Cell suspension (100 μl) was added to 100 μl of substrate solution buffered with potassium phosphate buffer at the desired pH value. The negative control consisted of potassium phosphate buffer without substrate. All assays were done in quintuplicate. The substrate solution before addition of cells was used as blank. Data represent mean values ± SD.

2.4. Reproducibility of the results

The collection of deletion strains and the collection of candidate inhibitors were completely screened once for D-galacturonic acid transport. All deletion strains or compounds causing a decrease of more than 20% relative to the wild-type or the control condition, respectively, were retested. All other experiments were performed at least twice.

3. Results

3.1. D-Galacturonic acid uptake in S. cerevisiae is pH-dependent and increases linearly with the substrate concentration

We have measured the initial uptake rate of radioactively labeled D-galacturonic acid at pH values of 3–5.5 in laboratory strain BY4741 (Fig. 1A). D-Galacturonic acid has a dissociation constant (pK_a) of about 3.5 [6]. We used a relatively high concentration of 100 mM because of the presumed absence of high uptake activity in yeast [8]. The uptake strongly increases at lower pH, largely following the dissociation curve of D-galacturonic acid as a function of pH, especially in the lower pH range, suggesting that it is the acid form that is taken up and not the anion. At pH values higher than 4.5, the uptake rate is clearly higher than would be expected if only the acid form would be taken up. Hence, at higher pH transport of the anionic form is likely.

Subsequently, we measured accumulation of D-galacturonic acid in wild type yeast cells as a function of time using either radioactively labeled D-galacturonic acid or by HPLC measurement of intracellular metabolites (data not shown). The cells were incubated with 10 or 100 mM D-galacturonic acid at an extracellular pH of 3 or 6.5 (Fig. 1B). At pH 3 the estimated intracellular D-galacturonic acid concentration stabilized at about half of the extracellular concentration. The same pattern of uptake was observed for D-glucuronic acid as measured by HPLC analysis of intracellular metabolites (data not shown).

We subsequently showed that transport is reversible by measuring D-galacturonic acid efflux from the cells. The cells were loaded with radioactively labeled D-galacturonic acid at an extracellular concentration of 100 mM and pH 3. After washing, resuspension of the cells in buffer resulted in rapid loss of D-galacturonic acid from the cells, indicating that the transport is bidirectional (Fig. 1C).

Determination of the initial D-galacturonic acid uptake rate as a function of the extracellular concentration revealed a linear relationship without any indication for saturation up to a concentration of 1.5 M at both pH 3, 4 and 6.5 (Fig. 1D).

3.2. Non-competitive inhibition of D-galacturonic acid uptake by small organic acids and modest inhibition by CCCP and DNP

We subsequently tested a wide variety of compounds for possible competitive inhibition of D-galacturonic acid uptake. A number of sugars (D-glucose, D-galactose, D-glucuronate, D-mannose, L-galactonate, L-arabinose, L-rhamnose, D-xylene, D-fructose, D-malt-
ose, D-trehalose, glycerol, and the di- and trisaccharides α-D-GalA-(1→4)-α-D-GalA and α-D-GalA-(1→4)-α-D-GalA-(1→4)-α-D-GalA and organic acids (formate, acetate, propionate, succinate, DL-malate, D,L-lactate, L-glutamate, L-aspartate, phtalate) were tested in a concentration of 50 mM for inhibition of the uptake of 5 mM D-galacturonic acid both at pH 3 and 6.5 (see supplementary data Table S1). None of these compounds had any significant effect, except for the small organic acids, formate, acetate and propionate, which inhibited D-galacturonic acid uptake by 67%, 26% and 19%, respectively. The inhibitory effect was only present at pH 3 and subsequent analysis showed that it was not competitive (data not shown).

Uncouplers of the proton gradient, DNP and CCCP, caused a partial inhibition of 100 mM D-galacturonic acid uptake at pH 3 (Fig. 2A). At 1 mM DNP or 0.1 mM CCCP a decrease in uptake of about 50% was observed. A higher DNP-concentration of 2 mM did not result in more inhibition.

3.3. The buffering capacity of the cytosol supports intracellular trapping of the anion

If the undissociated acid is indeed the preferred substrate for the transport system (Fig. 1A), the imported D-galacturonic acid could become trapped inside the cell, because of its dissociation into the anion due to the higher intracellular pH (pHi) [14–16]. The removal of the undissociated acid inside the cell would delay reaching an equilibrium in the D-galacturonic acid concentration across the plasma membrane and could thus help to drive the uptake of D-galacturonic acid. A requirement for significant trapping of D-galacturonate anions inside the cell is sufficient buffering capacity of the cytosol. To evaluate the latter, we have measured pHi for 30 min after addition of D-galacturonic acid using the fluorescent probe pHluorin, a pH-sensitive form of GFP, which is expressed from a plasmid [11]. Addition of 1, 10 or 100 mM D-galacturonic acid at pH 3 or 6.5 did not cause any drop in the pHi compared to addition of buffer at the same pH (Fig. 2B and C). In contrast, acetic acid, which was added as a control, rapidly lowered pHi. This indicates that the buffering capacity of the cytosol is high enough to neutralize and trap the imported D-galacturonic acid in the anionic form. This was supported by the fact that D-galacturonic acid did not inhibit growth on glucose on plates (up to 470 mM, pH 4 and 6), nor in liquid medium (50 mM, pH 5.5) (data not shown).

3.4. D-Galacturonic acid is not transported by the hexose carriers

We have subsequently tried to identify the genes encoding the D-galacturonic acid uptake system. A previous report suggested that in the filamentous fungus A. niger, D-galacturonic acid is taken up by hexose carriers and/or a monocarboxylate transporter [7]. Hence, we first tested the multiple hexose carrier deletion strains EBY.VW1000 (hxt1-17Δ galA2Δ) and EBY.VW4000 (hxt1-17Δ galA2Δ stl1Δ agt1Δ mph2A mph3A) [10], which lack hexose uptake (Fig. 3). The uptake of D-galacturonic acid was not affected at all in the hxt.A0null strains, both at pH 3 and 6.5. Hence, uptake of D-galacturonic acid clearly does not require the hexose carriers. Single deletion mutants for genes with similarity to the A. niger monocarboxylate transporter and to the bacterial D-galacturonic acid transporter ExuT were also not affected in D-galacturonic acid transport.
Because of the diffusion-like characteristics of the transport system, we have measured the initial \( \alpha \)-galacturonic acid uptake rate in single deletion strains with the BY-background of the 11 channel proteins that are documented as being localized in the yeast plasma membrane: \textit{Aqy1}, \textit{Aqy2}, \textit{Fps1}, \textit{Yl054c}, \textit{Mid1}, \textit{Cch1}, \textit{Tok1}, \textit{Ctr1}, \textit{Mep1}, \textit{Ahr2} and \textit{Mni2} [17]. In addition, two multiple deletion strains, \textit{fps1A yf054cA aqy1A} and \textit{fps1A ycf1A acr3A} [18], in the W303-background were tested. None of these strains displayed significantly reduced \( \alpha \)-galacturonic acid uptake.

We subsequently tested all 165 viable single deletion strains (see supplementary data Fig. S1) of the remaining known and predicted plasma membrane transporters and transporters with unknown localization [17] for deficiency in \( \alpha \)-galacturonic acid uptake at pH 3 and 6.5. However, none of the tested deletion strains showed a significant reduction in \( \alpha \)-galacturonic acid uptake. A strain deficient in the pleiotropic drug resistance transporters, \textit{Pdr1}, \textit{Pdr3}, \textit{Pdr5}, \textit{Pdr10}, \textit{Pdr11}, \textit{Yor1}, \textit{Snq2} and \textit{Ycf1}, [19], was also not affected. Finally, we tested two multiple deletion strains deficient in transporters related to siderophore uptake (\textit{Arn1}, \textit{Arn2}, \textit{Sit1} and \textit{Enb1}) and heme uptake (\textit{Flc1}, \textit{Flc2} and \textit{Flc3}) [20], since a role for \( \alpha \)-galacturonic acid in Fe(III)-uptake via a siderophore-like chelation was described in the literature [21]. None of these genes were found to be involved in \( \alpha \)-galacturonic acid uptake.

4. Discussion

While the uptake systems for \( \alpha \)-galacturonic acid are well documented in bacteria [5,22], there are only two reports in fungi, suggesting hexose carriers as possible \( \alpha \)-galacturonic acid transporters in \textit{A. niger} [7] and recently the \textit{Gal2} transporter in \textit{S. cerevisiae} [9]. We have shown that \textit{S. cerevisiae} transports \( \alpha \)-galacturonic acid at an unexpectedly high rate given its inability to metabolize this compound as a substrate. In contrast to what has been suggested in the literature, the hexose carriers, including Gal2, are apparently not involved in the uptake. The high rate of uptake contradicts involvement of simple diffusion. Moreover, \( \alpha \)-galacturonic acid is more hydrophilic and is as large as e.g. \( \beta \)-glucose or \( \beta \)-rhamnose, for which it is well known that they cannot diffuse through membranes [23,24].

The uptake of \( \alpha \)-galacturonic acid was strongly enhanced at low pH, which might have been an indication for symport. However, there was only modest inhibition by the protonophores DNP and CCCP, which in the literature have been shown to inhibit symport systems completely at even lower concentrations [14,15]. In addition, we did not observe intracellular concentration of \( \alpha \)-galacturonic acid above the extracellular substrate levels, nor a low \( K_m \)-value as has been reported previously for classical symport systems [25,26]. For these reasons, we think that \( \alpha \)-galacturonic acid is not transported by symport. Moreover, it is doubtful that \textit{S. cerevisiae} would spend energy to transport a molecule that it cannot metabolize.

Low-pH induction of the transport protein can be ruled out as an explanation for the strong pH effect since the handling time during the transport assay is only a few minutes. Preference of the transport protein for the undissociated \( \alpha \)-galacturonic acid, on the other hand, fits with the close correlation between uptake rate at low pH and the ratio of undissociated/dissociated \( \alpha \)-galacturonic acid. A similar explanation was given for the enhanced uptake of acetic acid by \textit{Fps1} at low pH [16]. The pH measurements showed that the buffering capacity of the cytosol is high enough to fully counteract the dissociation of \( \alpha \)-galacturonic acid inside the cell, even at high extracellular concentration and low pH. Hence, \( \alpha \)-galacturonic acid could become trapped in its dissociated form inside the cells. This may give an explanation for the partial inhibition of the uptake in the presence of compounds that lower the pH, like the protonophores and weak acids. In this case, lowering of the pH may have resulted in the partial reduction of \( \alpha \)-galacturonic acid uptake. The strong inhibition by formate may also be due to additional mechanisms of toxicity. The stability of the pH of the \( \alpha \)-galacturonic acid addition also indicates that the recently described inhibitory effect of \( \alpha \)-galacturonic acid on galactose, xylose and arabinose fermentation is likely not due to intracellular acidification [9]. Efflux of \( \alpha \)-galacturonic acid from the cells was rapid, especially at pH 6.5, in spite

Fig. 2. Inhibition of \( \alpha \)-galacturonic acid uptake by protonophores and stability of the intracellular pH upon addition of \( \alpha \)-galacturonic acid. (A) Wild-type yeast cells (BY4741) were incubated for 10 min in the absence and presence of 0.1 mM CCCP or 1 mM DNP prior to incubation with 100 mM radiolabeled \( \alpha \)-galacturonic acid solution buffered at pH 3. (B, C) BY4741 cells were transformed with plasmid pVT100U expressing the pH-sensitive GFP-protein (pHluorin) and pre-grown on SC-Ura with 2% glucose. pHi was estimated by fluorescence measurement. The cells were incubated for 30 min with 1, 10 or 100 mM \( \alpha \)-galacturonic acid buffered at pH 3 or 6.5 or in 100 mM potassium phosphate buffer at the same pH. Acetic acid was added as a control and rapidly lowered pHi.

We can subsequently test all viable single deletion strains (see supplementary data Fig. S1) of the remaining known and predicted plasma membrane transporters and transporters with unknown localization [17] for deficiency in \( \alpha \)-galacturonic acid uptake at pH 3 and 6.5. However, none of the tested deletion strains showed a significant reduction in \( \alpha \)-galacturonic acid uptake. A strain deficient in the pleiotropic drug resistance transporters, Pdr1, Pdr3, Pdr5, Pdr10, Pdr11, Yor1, Snq2 and Ycf1, [19], was also not affected. Finally, we tested two multiple deletion strains deficient in transporters related to siderophore uptake (Arn1, Arn2, Sit1 and Enb1) and heme uptake (Flc1, Flc2 and Flc3) [20], since a role for \( \alpha \)-galacturonic acid in Fe(III)-uptake via a siderophore-like chelation was described in the literature [21]. None of these genes were found to be involved in \( \alpha \)-galacturonic acid uptake.
of the predicted preferential presence of the dissociated form inside the cells. This can be explained by the strong dilution when α-galacturonic acid leaves the cells and at the extracellular pH of 6.5 by a reverse trapping effect into the dissociated form.

Because of the diffusion-like characteristics of the uptake and the hydrophilic nature of α-galacturonic acid, a channel-type uptake system appears to be most likely. This would also explain why we were unable to find any competitive inhibitor of the uptake. Even α-glucuronic acid, which was also accumulated in high concentration by the yeast cells, has a very similar chemical structure and is taken up in bacteria by the same carrier [5,27], did not cause any inhibition of α-galacturonic acid uptake. In spite of many efforts, we were unable to identify the gene(s) that encode the transport system. Several explanations are possible. The channel protein may not be included in our selection based on plasma membrane localization or the transport system is encoded by multiple genes. The high rate of the uptake remains intriguing in view of the inability of S. cerevisiae to metabolize uronic acids. Either α-galacturonic and α-glucuronic acid are transported fortuitously by the channel-type protein or the molecules serve a function as such, like the use as a chelating compound for the uptake of metal ions [21].

Finally, although we have not been able to identify the genes underlying α-galacturonic acid transport, we unexpectedly discovered that the molecule is rapidly taken up at an extracellular pH of 4.5–5, which is typical for bioethanol fermentations. In comparison to other non-metabolized compounds in S. cerevisiae, α-galacturonic acid uptake at pH 6.5 is about 10 times faster than α-arabinose [28] and at least 250 times faster than the poorly transported α-rhamnose [24]. At pH 3, α-galacturonic acid uptake is in the same range as α-xylose uptake [29]. Moreover, the uptake will apparently not be inhibited by any other sugar present in the hydrolysates, which is an important advantage. Xylose uptake for instance is carried out by the hexose carriers and strongly outcompeted by any glucose present in the hydrolysate [30]. Uptake of the sugar substrate is considered a major limiting factor in bioethanol production with engineered strains [30,31]. Hence, our results provide an important contribution to the development of genetically engineered yeast strains able to convert pectin hydrolysates into bioethanol, since they show that S. cerevisiae has a high-capacity and independent α-galacturonic acid uptake system and therefore introduction of a heterologous α-galacturonic acid transporter does not seem to be required.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.06.012.
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