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The fission yeast Pvg1p has galactose-specific pyruvyltransferase activity

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ABSTRACT

***N*-Glycan from the fission yeast *Schizosaccharomyces pombe* contains outer-chain pyruvic acid 4,6-ketal-linked galactose (PvGal). Here, we characterized a putative *S. pombe* pyruvyltransferase, Pvg1p, reported to be essential for biosynthesis of PvGal. When *p*-nitrophenyl-β-Gal (*p*NP-β-Gal) was used as a substrate, the structure of the recombinant Pvg1p product was determined to be *p*NP-PvGal by one- and two-dimensional NMR spectroscopy. The recombinant Pvg1p transferred pyruvyl residues from phosphoenolpyruvate specifically to β-linked galactose.**

1. Introduction

N-Linked oligosaccharides of glycoprotein in *Schizosaccharomyces pombe* are elongated in the secretory pathway by addition of 50–100 sugar residues. The glycosylation strengthens the cell wall and plays an important role in cell–cell recognition, through sexual/non-sexual agglutination. *S. pombe N*-linked oligosaccharides comprise a $Man_9GlcNAc_2$ inner core, to which several α1,2-linked galactose (Gal) residues [3] and α1,3-linked Gal residues are added [23], and an outer chain, composed of an α1,6-linked mannose (Man) backbone decorated with α1,2-linked Gal [4] and pyruvylated galactose (PvGal) residues [1,9,10]. Few reports have documented the presence of pyruvylated sugar residues in eukaryotic polysaccharides and glycoprotein oligosaccharides [2,15,21]. Mutants of *S. pombe* lacking the pyruvyl group in PvGal have been isolated leading to the identification of five genes (*pvg1–5*$^+$) involved in PvGal biosynthesis [1]. While standard protein–protein BLAST searches established that the amino acid sequence of Pvg1p shares weak similarity with predicted bacterial pyruvyltransferases [1], to our knowledge, no reports on the enzymatic characterization of eukaryotic ketal pyruvyltransferases have been published.

In the present study, the enzymatic properties of recombinant Pvg1p were analyzed using phosphoenolpyruvate (PEP) as donor substrate and *p*-nitrophenyl-β-Gal (*p*NP-β-Gal) as acceptor substrate, with reaction products determined by NMR. This is the first report on the substrate specificity and enzymology of a eukaryotic pyruvyltransferase.

2. Materials and methods

2.1. Strains and media

The *S. pombe* strains used in this study were ARC039 (h- *leu1*-32 *ura4*-C190T) and derived *pvg1, pvg2, pvg3, pvg5* deletion mutants [17]. Cells were grown aerobically at 30 °C in YES medium [3% glucose, 0.5% yeast extract with minimal medium (MM) supplements] and in synthetic MM as described [18]. *Escherichia coli* BL21 (DE3) Δ*lacZ* was used to express recombinant protein and was grown aerobically at 37 °C in Luria–Bertani (LB) medium.

2.2. Plasmid construction

The pET32b plasmid (Novagen, USA) was used to construct a recombinant Pvg1p expression vector. *pvg1*$^+$ was amplified by PCR using the following primers including the indicated restriction sites (underlined): 5′-GTTTTCCATGGACTTGCAAACTTTGAAGAAC-3′ (*Nco*I) and 5′-GTTTTGCGGCCGCTTAAAAGTAGCCGGCCTC-3′ (*Not*I). The PCR fragment was digested with *Nco*I and *Not*I, and cloned into the corresponding sites of pET32b. The recognition region of enterokinase was replaced with that of HRV3C (Novagen, USA) in order to remove tags (thioredoxin, S, and 6-His tag) from recombinant Pvg1p (Fig. 1A). A PCR was performed using pET32b as template, and the following primers: 5′-TTCCAGGGGCCCGCCATGGACTTGCAAACTTTGAAG-3′ and 5′-CAGAACTTCCAGGGTACC

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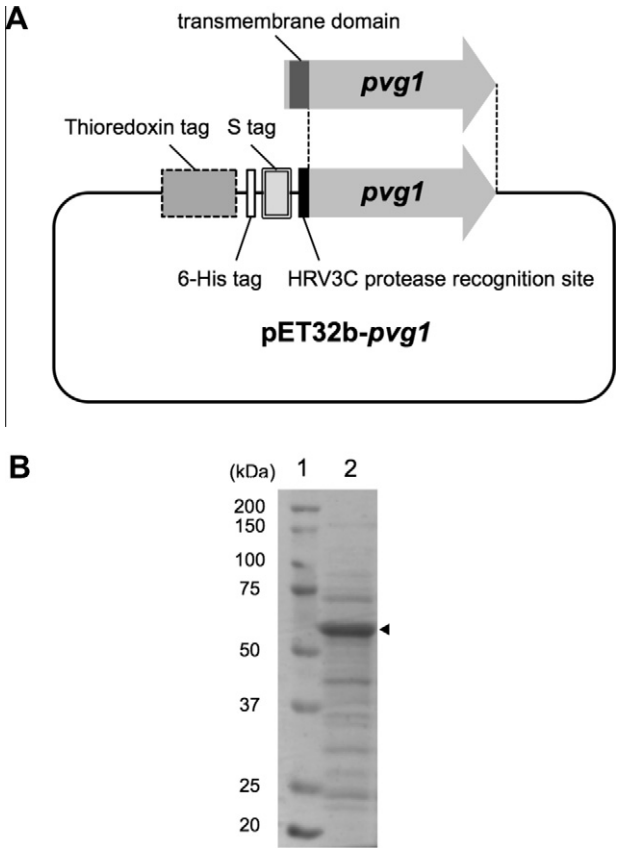

Fig. 1. Expression vector construct and analysis of purified recombinant Pvg1p by SDS–PAGE. (A) Structure of pET32b-*pvg1*. Pvg1p lacking the *N*-terminal region containing the transmembrane domain (amino acids12–29) was introduced into pET32b. (B) SDS–PAGE analysis of purified recombinant Pvg1p. Lanes: 1, protein standards; 2, fraction purified by Ni affinity column. Recombinant Pvg1p is indicated by the arrowhead.

CAGATCTGGGCTGTC-3′ to generate fragment A. Next, we amplified fragment B by PCR using fragment A as a template, and the following primers: 5′-GAAGTTCTGTTCCAGGGGCCCGCCATGG-3′ and 5′-CTGGAACAGAACTTCCAGGGTACCCAGATC-3′. Fragment B was ligated using In Fusion PCR (TaKaRa, Inc., Japan) to introduce the HRV3C recognition site at the 5′-terminus of *pvg1*$^+$ (pET32b-*pvg1*).

2.3. Expression and purification of recombinant Pvg1p

Cells from a single colony of *E. coli* BL21 Δ*lacZ* transformed with pET32b-*pvg1* were inoculated into 5 ml of LB medium containing 100 μg/ml ampicillin and incubated at 37 °C to stationary phase. The 5 ml culture was then inoculated into 1000 ml of LB medium containing 100 μg/ml ampicillin. The culture was allowed to grow at 37 °C to OD_{600} = 0.3 at which time isopropyl-β-D-thiogalactopyranoside (IPTG, Wako, Japan) was added to a final concentration of 0.1 mM. The culture was incubated for 48 h at 15 °C. Cells were harvested, separated from the broth by centrifugation, and resuspended in the cell-breakage buffer [100 mM MOPS-NaOH, pH 7.4, 0.01% TritonX-100, protease inhibitor cocktail (Roche, USA)]. Cells were then lysed by ultrasonication on ice. The cell lysate was centrifuged at 15 000 rpm for 10 min at 4 °C after which the supernatant was collected and loaded onto a Ni-affinity column (GE Healthcare, USA). The affinity-purified Pvg1p was washed in 100 mM MOPS-NaOH (pH 7.4), 500 mM NaCl, 30 mM imidazole and eluted with 100 mM MOPS-NaOH (pH 7.4), 500 mM NaCl, 500 mM imidazole. The purity and concentration of the recombinant Pvg1p were determined by SDS–PAGE and a BCA protein assay kit (Thermo Scientific, USA), respectively.

2.4. Pvg1p activity assay

The activity of recombinant Pvg1p was determined in a total volume of 50 μl containing 100 mM MOPS-NaOH (pH 6.0), 1.0 mM PEP monopotassium salt (Wako, Japan), 1.0 mM *p*NP-β-Gal (Sigma–Aldrich, USA) and purified recombinant Pvg1p at 35 °C. Reactions were terminated by boiling after which samples were centrifuged at 15 000 rpm for 10 min at 4 °C. Supernatants were analyzed by HPLC.

Separation of *p*NP-saccharides was carried out by HPLC using a GL-7400 HPLC system equipped with a UV spectrophotometer (GL-Sciences, Japan). Size-fractionation HPLC was performed using a reverse-phase column COSMOSIL 5C_{18}-P (4.6 × 150 mm) (Nacalai tesque, Japan) at a rate of 1.0 ml/min at 30 °C. The column was equilibrated with developing solvent (0.3% ammonium acetate, pH 7.4, 13% acetonitrile) after which the purified sample solution (10 μl) was loaded onto the column. *p*NP-saccharides were detected at 265 nm.

2.5. NMR spectroscopy

The HPLC-purified Pvg1p product (~28 mg) was exchanged five times with D_2O by repeated lyophilization and dissolved in 150 μl of 99.97% D_2O. ^1H and ^{13}C, one-dimensional and two-dimensional spectra were recorded using a Varian Inova-600 NMR spectrometer (Agilent Technologies, Inc., Wilmington, DE, USA).

2.6. Transfer of pyruvate to the cell wall and alcian blue staining

In the present study, 1 OD_{600} of log-phase *pvg* mutant cells grown at 30 °C was harvested and washed three times with water. Washed cells were suspended in 500 μl of reaction solution (100 mM MOPS-NaOH, 20 mM PEP monopotassium salt, 200 μg of recombinant Pvg1p) and incubated for 1 h at 30 °C. Following the incubation, cells were stained with alcian blue to determine the presence of negatively-charged glycan. Standard methods for alcian blue staining of yeast cells were used [7,17,19].

3. Results

3.1. Expression and purification of recombinant Pvg1p

The expression vector pET32b-*pvg1* was constructed as shown in Fig. 1A. To prevent degradation of *p*NP-β-Gal by endogenous LacZ (β-D-galactosidase), *E. coli* BL21 Δ*lacZ* was used as the expression host. Because Pvg1p is a type II transmembrane protein that has one transmembrane domain (amino acids 12–29), a 90 bp *N*-terminal region of *pvg1*$^+$ was deleted. The recombinant Pvg1p was expressed in *E. coli* transformed with pET32b-*pvg1* and was purified by nickel affinity chromatography (GE Healthcare, USA). By SDS–PAGE (Fig. 1B), the purified recombinant Pvg1p appeared as a 56 kDa band consistent with its theoretical value.

3.2. Recombinant Pvg1p has pyruvyltransferase activity

Although information about bacterial pyruvyltransferases is limited [16], substrates and the reaction mechanism for Pvg1p have not been described. Because PEP has a high-energy phosphate bond, we chose PEP as the donor substrate. *p*NP-β-Gal was chosen as the acceptor substrate because the pyruvyl residue of PvGal is linked to β-Gal [9–11]. Purified recombinant Pvg1p was mixed with the substrates and incubated at 35 °C for 30 min. The reaction

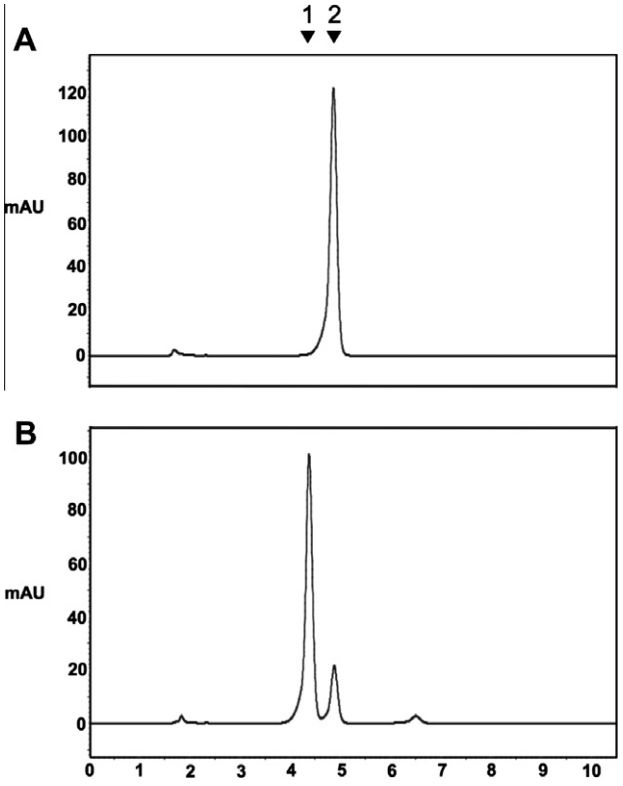

Fig. 2. HPLC profile of Pvg1p reaction. (A) 0 min; (B) 30 min. 1: product of the reaction, 2: *p*NP-β-Gal. The reaction was performed in a volume of 50 μl containing100 mM MOPS-NaOH (pH 6.0), 1.0 mM PEP monopotassium salt, 1.0 mM *p*NP-saccharide and 80 μg purified enzyme at 35 °C.

solution was then analyzed by HPLC. A peak for *p*NP-β-Gal was detected at 4.8 min (Fig. 2A). After incubation with Pvg1p, a new peak was detected at 4.3 min while the *p*NP-β-Gal peak decreased (Fig. 2B). The new peak increased with time, and was not detected when PEP or recombinant Pvg1p was omitted from the reaction. The new peak was also not detected when pyruvate was used as the donor substrate. These results suggested that the new peak was a product of the pyruvyltransferase reaction.

3.3. NMR spectroscopy

Structural analysis of the Pvg1p product (pyruvylated Gal-β-*p*NP) was performed by one- and two-dimensional NMR spectroscopy. Spectral assignments for the Pvg1p product were conducted by cross-checking high resolution one-dimensional, two-dimensional COSY, HSQC and HMBC spectra and are summarized in Table 1. Pyruvyl transfer at the β-Gal residue is suggested by the presence of signals attributed to the CH_3 at 1.33 ppm for ^1H NMR and 24.7 ppm for ^{13}C NMR, and were confirmed by the signal correlation in the HSQC spectrum (Fig. 3A). All assigned chemical shift values are almost identical to those described for a 4,6-*O*-(carboxyethylidene)-β-galactopyranoside [12] except for the signal from the benzyl group. Furthermore, pyruvate attachment at least at the Gal C-6 position was confirmed by the pyruvyl O–C–O cross peak at 100.4 ppm and the Gal H-6 residue at 3.82 ppm in the HMBC spectrum (Fig. 3B). For the 4,6-pyruvyl acetals on the Gal residue, the methyl carbon signal at 24.7 ppm is characteristic of the *R* configuration for acetal carbon**.** The acetal carbon in the pyruvate residue in the *S* configuration has a chemical shift at 15.0–18.0 ppm [8]. Taken together, these results indicate the presence of a Gal residue containing a 4,6-*O*-carboxyethylidene cyclic acetal (Fig. 3C).

Table 1
^1H and ^{13}C chemical shifts for Pvg1p product.

Chemical shifts, δ								
β-Galactose							Pyruvate	
C-1	C-2	C-3	C-4	C-5	C-6		CH_3	O–C–O
H-1	H-2	H-3	H-4	H-5	H-6a	H-6b		
99.1	69.5	70.8	70.3	66.1	64.2		24.7	100.4
5.13	3.84	4.13	4.13	3.73	3.82	3.94	1.33	

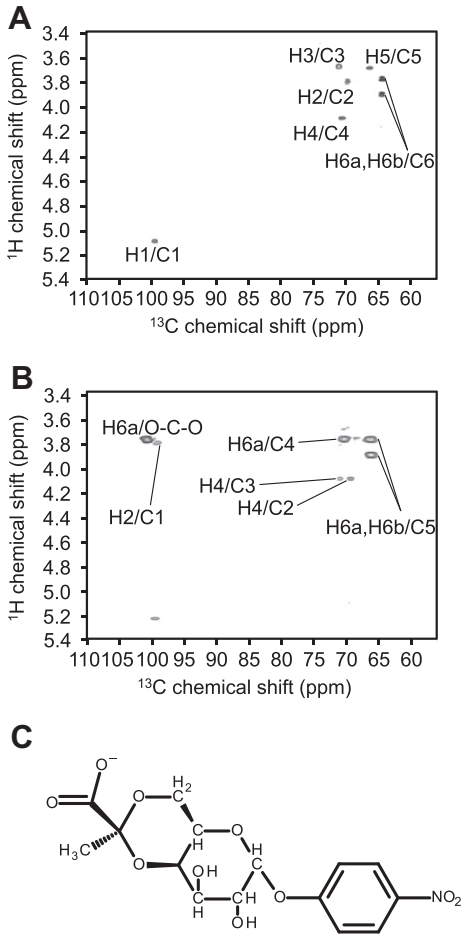

Fig. 3. The HSQC and HMBC spectra of Pvg1p product. (A) $^1H/^{13}C$ HSQC spectrum of the Pvg1p product. The cross peaks are indicated with the name of the residues. (B) $^1H/^{13}C$ HMBC spectrum of the Pvg1p product. (C) Structure of *p*-nitrophenyl-4,6-*O*-(carboxyethylidene)-β-galactopyranoside.

3.4. Enzymatic properties of recombinant Pvg1p

pH and temperature optima were determined for Pvg1p. The recombinant Pvg1p in MOPS-NaOH buffer exhibited >80% of maximum activity between pH 5.5 and pH 6.5, with a defined peak at pH 6.0 (data not shown). The optimum temperature was around 35 °C.

The effect of several divalent cations on Pvg1p activity was also determined. Because activity did not change significantly upon addition of EDTA (10 mM), we presume that metal ions are not required. However, some divalent cations (10 mM) inhibited activity (e.g., Co^{2+}; 44.6%, Ni^{2+}; 9.5%, Cd^{2+}; 6.8%, Cu^{2+} and Zn^{2+}; 0%).

The acceptor substrate specificity of recombinant Pvg1p was analyzed. As shown in Table 2, *p*NP-β-Gal and *p*NP-β-Lac served as good acceptors for recombinant Pvg1p. On the other hand, activity was not observed in the case of *p*NP-α-Gal, *p*NP-α,β-Glc, *p*NP-

Table 2
Substrate specificity of recombinant Pvg1p.

Acceptor	Relative activity (%)
*p*NP-α-Gal	0.0
*p*NP-β-Gal	100.0
*p*NP-α-Glc	0.0
*p*NP-β-Glc	0.0
*p*NP-α-Man	0.0
*p*NP-β-Man	0.0
*p*NP-β-GalNAc	0.0
*p*NP-β-Lac	217.8 ± 5.4

The reaction solution consisted of 100 mM MOPS-NaOH (pH 6.0), 1.0 mM PEP monopotassium salt, 1.0 mM *p*NP-sugar, and purified enzyme in a total volume of 50 μl.

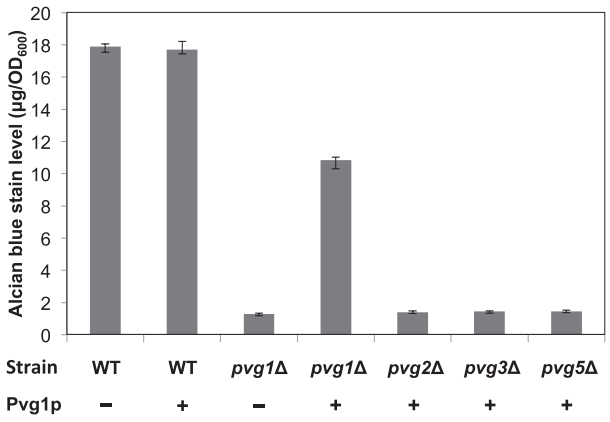

Fig. 4. Alcian blue staining of WT, *pvg1*Δ, *pvg2*Δ, *pvg3*Δ and *pvg5*Δ cells following a 1 h incubation with Pvg1p and PEP at 30 °C. (+): Cultivated cells added to mixture of Pvg1p and PEP. (−): Pvg1p not added.

α,β-Man or *p*NP-β-GalNAc. These results suggest that β-Gal is essential for Pvg1p activity.

3.5. Pvg1p is able to transfer pyruvic acid to the surface of live yeast cells

It was previously reported that the pyruvyl group of PvGal disappeared from *N*-linked glycan from the *pvg1*$^-$ mutant and that PvGal disappeared from *N*-linked glycan from *pvg2*$^-$, *pvg3*$^-$ and *pvg5*$^-$ mutants [1]. Alcian blue specifically binds negatively-charged glycans, such as PvGal [17] and mannosylphosphate of *Saccharomyces cerevisiae* [7,19]. Because no phosphate or sulfate has previously been detected in *S. pombe N*-linked glycan [9], pyruvate appears to be the only negatively-charged functional group on the cell surface. Wild-type cells bound alcian blue (about 18 μg/OD_{600} cells), whereas *pvg1*Δ cells to which recombinant Pvg1p had not been added did not. When recombinant Pvg1p was added to the reaction with *pvg1*Δ cells, about 11 μg of alcian blue/OD_{600} cells were bound. Addition of recombinant Pvg1p to the reactions involving *pvg2*Δ, *pvg3*Δ *and pvg5*Δ cells did not result in binding of alcian blue (Fig. 4). These results indicate that recombinant Pvg1p transferred pyruvate from PEP to outer chains of *N*-linked glycan of live *pvg1*Δ cells.

4. Discussion

Pyruvylated hexoses have rarely been found in eukaryotic polysaccharides and glycoprotein oligosaccharides, but have been found in a limited number of prokaryotes (e.g. *Rhizobium leguminosarum* bv. *viciae*: 4,6-PvGal and 4,6-PvGlc [13,22], *Bacillus anthracis*: 4,6-PvManNAc [6], *Xanthomonas campestris*: 4,6-PvMan [14]). Whereas many yeast species have phosphate groups linked to extracellular glycan [5,11,20], the *N*-linked glycan of *S. pombe* has 4,6-PvGal on a portion of the outer-chain Galα1,2-residues [9]. Andreishcheva et al., isolated *pvg* (Pyruvylated Gal-β-1,3-epitope synthesis) mutants and identified five genes (*pvg1*$^+$ to *pvg5*$^+$) involved in PvGal biosynthesis in *S. pombe* [1]. Pvg1p shares a ~300- to 350-amino acid stretch of about 30% identity and about 45% similarity with nearly 20 predicted bacterial pyruvyltransferases.

In the present study, we confirmed that Pvg1p transferred a pyruvyl residue from PEP to *p*NP-β-Gal resulting in the synthesis of 4,6-ketal-linked PvGal indicating that Pvg1p has pyruvyltransferase activity. Pvg1p was severely inhibited by addition of Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+}. While Pvg1p recognized *p*NP-β-Gal and *p*NP-β-Lac as acceptor substrates, it did not recognize *p*NP-α-Gal, *p*NP-α,β-Glc, *p*NP-α,β-Man or *p*NP-β-GalNAc (Table 1). These results indicate that β-linked Gal is an essential structure for recognition by Pvg1p.

In most cases, glycan moieties associated with these glycoproteins provide cell surfaces with a net negative charge. The negative surface charge has been predicted to play a significant role in cell–cell interactions, including cell aggregation and adhesion of pathogens. For example, PvGal is required for cellular recognition and adhesion in the marine sponge *Microciona prolifera* [21]. The nodule-forming bacterium *Rhizobium leguminosarum* bv. *viciae* requires PvGal in exopolysaccharides for establishment of a nitrogen-fixing symbiosis with the pea [13]. In *N*-glycan from *S. pombe*, PvGal appears to be the only negatively-charged functional group on the cell surface. The negative charge was lost by disruption of *pvg1*$^+$. However, growth of *pvg1*Δ cells was not significantly different than that of wild-type cells. In a recent study, we found that pyruvylation of *N*-linked glycan in *S. pombe* negatively regulates non-sexual flocculation [18]. These results suggest that pyruvylation of *N*-linked glycan plays an important role in cell–cell recognition in *S. pombe*.

Pvg1p was able to transfer pyruvic acid to the surface of live *S. pombe pvg1* cells in vitro. However, the rate of transfer of pyruvate to the cell wall was approximately 60% under these conditions (Fig. 4). In *S. pombe* cells, Pvg1p localizes to the Golgi apparatus and addition of puruvate to Gal residues occurs in the lumen of Golgi. Therefore, we speculate that Pvg1p efficiently recognizes and attaches pyruvate to β-linked Gal residues of sugar chains in the Golgi apparatus by an as-yet undefined mechanism.

Very recently, we succeeded in determining the three-dimensional crystal structure of recombinant Pvg1p (our unpublished results). Our ongoing studies are focused on the reaction mechanism of Pvg1p/puruvyltransferase and the physiological function of PvGal in *S. pombe*.

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References

[1] Andreishcheva, E.N., Kunkel, J.P., Gemmill, T.R. and Trimble, R.B. (2004) Five genes involved in biosynthesis of the pyruvylated Galβ1,3-epitope in *Schizosaccharomyces pombe N*-linked glycans. J. Biol. Chem. 279, 35644–35655.
[2] Araki, S., Abe, S., Satake, M., Hayashi, A., Kon, K. and Ando, S. (1991) Novel phosphonoglycosphingolipids containing pyruvylated galactose from the nervous system of Aplysia kurodai. Eur. J. Biochem. 15, 689–695.
[3] Ballou, B.C., Ballou, L. and Ball, G. (1994) *Schizosaccharomyces pombe* glycosylation mutant with altered cell surface properties. Proc. Natl. Acad. Sci. USA 91, 9327–9331.

[4] Ballou, L. and Ballou, C. (1995) *Schizosaccharomyces pombe* mutants that are defective in glycoprotein galactosylation. Proc. Natl. Acad. Sci. USA 92, 2790–2794.
[5] Cutler, J.E. (2001) *N*-glycosylation of yeast, with emphasis on *Candida albicans*. Med. Mycol. 39 (Suppl. 1), 75–86.
[6] Forsberg, L.S., Adshire, T.G., Friedlander, A., Quinn, C.P., Kannenberg, E.L. and Carlson, R.W. (2012) Localization and structural analysis of a conserved pyruvylated epitope in *Bacillus anthracis* secondary cell wall polysaccharides and characterization of the galactose-deficient wall polysaccharide from avirulent *B. anthracis* CDC 684. Glycobiology 22, 1103–1117.
[7] Friis, J. and Ottolenghi, P. (1970) The genetically determined binding of alcian blue by a minor fraction of yeast cell walls. C. R. Trav. Lab. Carlsberg 37, 327–341.
[8] Garegg, P.J. and Lindberg, B. (1979) Preparation and N.M.R. studies of pyruvic and related acetals of pyranosides: configuration at the acetal carbon atoms. Carbohydr. Res. 77, 71–78.
[9] Gemmill, T.R. and Trimble, R.B. (1996) *Schizosaccharomyces pombe* produces novel pyruvate-containing *N*-linked oligosaccharides. J. Biol. Chem. 271, 25945–25949.
[10] Gemmill, T.R. and Trimble, R.B. (1998) All pyruvylated galactose in *Schizosaccharomyces pombe* *N*-glycans is present in the terminal disaccharide, 4,6-*O*-[(*R*)-(1-carboxyethylidine)]-Galβ1,3Galα1. Glycobiology 8, 1087–1095.
[11] Gemmill, T.R. and Trimble, R.B. (1999) Overview of *N*- and *O*-linked oligosaccharide structures found in various yeast species. Biochim. Biophys. Acta 1426, 227–237.
[12] Gonçalves, A.G., Ducatti, D.R.B., Duarte, M.E.R. and Noseda, M.D. (2002) Sulfated and pyruvylated disaccharide alditols obtained from a red seaweed galactan: ESIMS and NMR approaches. Carbohydr. Res. 337, 2443–2453.
[13] Ivashina, T.V., Fedorova, E.E., Ashina, N.P., Kalinchuk, N.A., Druzhinina, T.N., Shashkoh, A.S., Shibaev, V.N. and Ksenzenko, V.N. (2010) Mutation in the *pssM* gene encoding ketal pyruvate transferase leads to disruption of *Rhizobium leguminosarum* bv. *viciae-Pisum sativum* symbiosis. J. Appl. Microbiol. 109, 731–742.
[14] Jansson, P.E., Kenne, L. and Lindberg, B. (1975) Structure of the exopolysaccharide from *Xanthomonas campestris*. Carbohydr. Res. 45, 275–282.
[15] Liao, M.L., Chiovitti, A., Munro, S.L.A., Craik, D.J., Kraft, G.T. and Bacic, A. (1996) Sulfated galactans from Australians specimens of the red alga *Phacelocarpus peperocarpos* (Gigartinales, Rhodophyta). Carbohydr. Res. 296, 237–247.
[16] Marzocca, M.P., Harding, N.E., Petroni, A., Cleary, J.M. and Ielpi, L. (1991) Location and cloning of the ketal pyruvate transferase gene of *Xanthomonas campestris*. J. Bacteriol. 173, 7519–7524.
[17] Matsuzawa, T., Yoritsune, K. and Takegawa, K. (2012) MADS box transcription factor Mbx2/Pvg4 regulates invasive growth and flocculation by inducing $gsf2^{+}$ expression in fission yeast. Enkaryot. Cell 11, 151–158.
[18] Matsuzawa, T., Morita, T., Tanaka, N., Tohda, H. and Takegawa, K. (2011) Identification of a galactose-specific flocculin essential for non-sexual flocculation and filamentous growth in *Schizosaccharomyces pombe*. Mol. Microbiol. 82, 1531–1544.
[19] Odani, T., Shimma, Y., Wang, X.H. and Jigami, Y. (1997) Mannosylphosphate transfer to cell wall mannan is regulated by the transcriptional level of the *MNN4* gene in *Saccharomyces cerevisiae*. FEBS Lett. 420, 186–190.
[20] Parolis, L.A., Duus, J.O., Parolis, H., Meldal, M. and Bock, K. (1996) The extracellular polysaccharide of *Pichia* (*Hansenula*) *holstii* NRRL Y-2448: the structure of the phosphomannan backbone. Carbohydr. Res. 293, 101–117.
[21] Spillmann, D., Hard, K., Thomas-Oates, J., Vliegenthart, J.F., Misevic, G., Burger, M.M. and Finne, J. (1993) Characterization of a novel pyruvylated carbohydrate unit implicated in the cell aggregation of the marine sponge *Microciona prolifera*. J. Biol. Chem. 268, 13378–13387.
[22] Sadykov, M.R., Ivashina, T.V., Kanapin, A.A., Shliapnikov, M.G. and Ksenzenko, V.N. (1998) Structure-functional organization of exopolysaccharide biosynthetic genes in *Rhizobium leguminosarum* bv. *viciae* VF39. Mol. Biol. (Moscow) 32, 797–804.
[23] Ziegler, F.D., Cavanagh, J., Lubowski, C. and Trimble, R.B. (1999) Novel *Schizosaccharomyces pombe* *N*-linked $GalMan_9GlcNAc$ isomers: role of the Golgi *GMA12* galactosyltransferase in core glycan galactosylation. Glycobiology 9, 497–505.