

Transport of organic anions by the lysosomal sialic acid transporter: a functional approach towards the gene for sialic acid storage disease

Adrie C. Havelaar, Cecile E.M.T. Beerens, Grazia M.S. Mancini, Frans W. Verheijen*

Department of Clinical Genetics, Erasmus University, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

Received 11 January 1999; received in revised form 1 February 1999

Abstract Transport of sialic acid through the lysosomal membrane is defective in the human sialic acid storage disease. The mammalian sialic acid carrier has a wide substrate specificity for acidic monosaccharides. Recently, we showed that also non-sugar monocarboxylates like L-lactate are substrates for the carrier. Here we report that other organic anions, which are substrates for carriers belonging to several anion transporter families, are recognized by the sialic acid transporter. Hence, the mammalian system reveals once more novel aspects of solute transport, including sugars and a wide array of non-sugar compounds, apparently unique to this system. These data suggest that the search for the sialic acid storage disease gene can be initiated by a functional selection of genes from a limited number of anion transporter families. Among these, candidates will be identified by mapping to the known sialic acid storage disease locus.

© 1999 Federation of European Biochemical Societies.

Key words: Lysosomal transporter; Sialic acid; Organic anion; Major facilitator superfamily; Salla disease

1. Introduction

Recent work has led to the characterization of specific transport systems for monosaccharides in the mammalian lysosomal membrane [1,2]. One of these, the sialic acid transporter has an essential metabolic function in the disposal of acid sugars from the lysosomal compartment after degradation of glycoproteins, glycosaminoglycans and glycolipids. In the human genetic disorders Salla disease and infantile sialic acid storage disease (SASD) (OMIM 269920), the function of this transporter is impaired and a progressive accumulation of acid sugars occurs in the lysosomal compartment [3,4]. The responsible SASD gene(s) is (are) not known but linkage was demonstrated for both phenotypes to chromosome 6q14-q15 [5]. The lysosomal transporter from human fibroblasts and rat liver recognizes monocarboxylic anionic sugars (e.g. sialic acid, glucuronic acid and iduronic acid) and other aliphatic monocarboxylated anions (e.g. L-lactate), showing functional similarities with the previously characterized family of monocarboxylate transporters (MCTs) [1,4,6].

Membrane transporters have been classified into distinct families on the basis of sequence similarities [7]. Within each

family, most proteins have a similar substrate specificity, indicating that substrate specificity frequently correlates with phylogeny. In this way several different anion transporter families have been characterized [8]. The MCT family seems structurally and phylogenetically distinct from other families of organic anion transporters. So far, the molecular structure of the lysosomal sialic acid transporter is not known, but initial functional characterization showed some similarities with members of the different anion transporter families [6]. Therefore, in this paper we have compared in more detail the functional properties of the lysosomal sialic acid transporter with those of carriers from different families of anion transporters. Our final aim is to identify and clone the sialic acid transporter gene causing SASD, which can be initiated by this functional approach.

2. Materials and methods

2.1. Materials

Rat liver lysosomes were isolated by differential centrifugation, and highly purified membrane vesicles were prepared as described [1]. The lysosomal membrane vesicles were suspended at a protein concentration of 8–10 mg/ml in 20 mM NaHEPES, 0.1 mM EDTA at pH 7.4 and were stored at -70°C . [^3H]GlcA (specific activity 6.6 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Most chemicals were purchased from Sigma or as indicated.

2.2. Transport assays

For a 'zero-trans' uptake assay, lysosomal membrane vesicles were rapidly thawed at 37°C and pre-equilibrated for 10 min in 20 mM NaHEPES, 10 mM KCl and 10 μM valinomycin at 20°C . All uptake studies were performed for 30 s at 20°C and in the presence of an inward-directed proton gradient ($\text{pH}_{\text{out}} = 5.5 < \text{pH}_{\text{in}} = 7.4$) as described earlier [4]. For *cis*-inhibition studies 10 μl pre-equilibrated vesicles were incubated with 10 μl of substrate solution containing 0.5 μCi of radiolabelled GlcA (final concentration 2.5 μM) in 20 mM NaHEPES, 80 mM Mes (free acid), resulting in an extravesicular pH of 5.5 and 10 μl of 21 mM of several organic anions (final concentration 7 mM), titrated with NaOH to pH 5.5. The following organic anions were used: α -ketoglutarate, *p*-aminohippurate, urate, salicylate, valproate, methotrexate, folate, glutamate, tetraethylammonium, phosphate, sulfate, D-glucosamine-2-sulfate, D-galactose-6-sulfate, D-glucose-6-phosphate, *N*-acetylglucosamine-1-phosphate. The blank value was determined by incubation of vesicles with 7 mM unlabelled GlcA and subtracted from all determinations. Incubations were stopped by the addition of 70 μl of ice-cold stop-buffer (13 mM NaHEPES, 27 mM Mes (free acid) and 10 mM KCl, pH 5.5) and 100 μl was immediately applied to a Sephadex G50 fine (Pharmacia Biotech) column (Pasteur pipettes, 0.5×5 cm), equilibrated in cold stop-buffer at 4°C . Vesicles were eluted with 1 ml ice-cold stop-buffer. Vesicle-associated radioactivity was determined by liquid scintillation counting in 10 ml Instagel (Packard).

Trans-stimulation of [^3H]GlcA uptake was studied at $\text{pH}_{\text{in}} = \text{pH}_{\text{out}} = 5.5$ in the presence of the ionophore monensin. 15 μl of vesicles were pre-equilibrated 60 min at 20°C with 40 mM Mes (free acid), 12 mM NaHEPES, 4 mM KHEPES, 1 mM NaGlcA or other organic anions, 10 μM valinomycin and 10 μM monensin (final concentrations). The assay was started by adding 75 μl of an equivalent

*Corresponding author. Fax: (31) (10) 4089489.
E-mail: verheijen@ikg.fgg.eur.nl

Abbreviations: GlcA, glucuronic acid; Neu5Ac, *N*-acetylneuraminic acid, sialic acid; PAH, *p*-aminohippurate; TEA, tetraethylammonium; GlcNH₂-2-S, D-glucosamine-2-sulfate; Gal-6-S, D-galactose-6-sulfate; Glu-6-P, D-glucose-6-phosphate; GlcNAc-1-P, *N*-acetylglucosamine-1-phosphate

buffer containing 2 μCi of radiolabelled GlcA (final concentration 3 μM). Control experiments were performed by pre-equilibration of the membranes with the same buffer without Na-GlcA or other organic anions. To give the same extravesicular substrate concentration in both experiments, 0.25 mM unlabelled organic anion was added together with radiolabelled substrate at the start of the assay.

For competitive inhibition studies, initial proton dependent transport rates (30 s, 20°C) of 10 μM [^3H]GlcA were measured at increasing unlabelled GlcA concentrations (0.05, 0.1, 0.2, 0.3, 0.5 and 1 mM final concentrations) in the presence or absence of different inhibitors. Details about the used concentrations of inhibitors are described in the legend. All experiments were performed in duplicate or triplicate.

3. Results

In earlier substrate specificity studies, we have shown that the lysosomal sialic acid transporter recognizes structurally different types of organic anions, like monocarboxylic (aldonic, hexuronic and *N*-substituted anionic) sugars and aliphatic monocarboxylates [1,6]. Recent genome sequencing data and a wealth of biochemical and molecular genetic investigations have revealed the occurrence of many families of primary and secondary transporters [7,8]. Since in the lysosomal system a proton gradient provides the driving force for secondary active transport [1], transporter families in which organic anions are known to be transported by a proton symport mechanism are of special interest. In this paper, we investigated which other organic anions are recognized by the lysosomal sialic acid transporter. We tested a variety of typical substrates of previously identified members of the different families which transport organic anions. As representatives of the novel multispecific organic anion transporters (OATs) we tested *p*-aminohippurate (PAH), α -ketoglutarate, urate, salicylate, methotrexate, folate and valproate [9,10]. The dicarboxylate α -ketoglutarate is also a substrate of the α -ketoglutarate: H^+ symport permease of *Escherichia coli*, which belongs to a different family [8]. Additionally, we tested substrates of the mammalian inorganic anion transporters of the sulfate permease (SulP) and phosphate: H^+ symporter (PHS) family (i.e. sulfate and phosphate) [11–13], of the acidic amino acid transporter of the proton-dependent oligopeptide transporter (POT) family (i.e. glutamate) [8,11], of the organic cation transporters (OCTs) (i.e. tetraethylammonium) [14] and of the bacterial hexose phosphate transporters of the organophosphate: P_i antiporter (OPA) family (i.e. several sugar-phosphates) [15]. As shown in Fig. 1, the lysosomal sialic acid transporter is strongly *cis*-inhibited by the dicarboxylate α -ketoglutarate, the prototype substrate of organic anion transporters PAH, the purine metabolism endproduct urate and

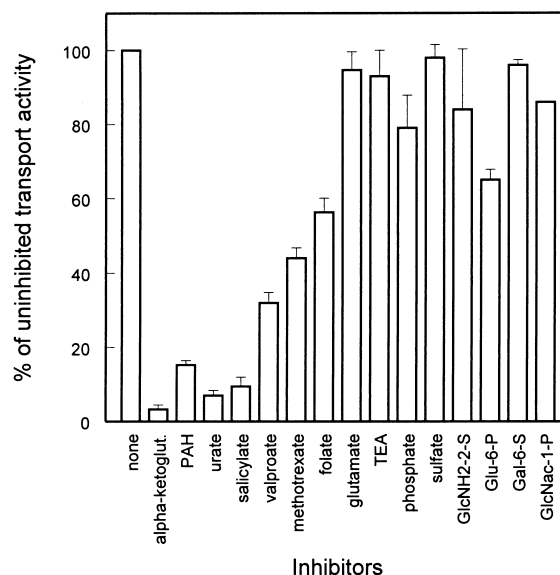


Fig. 1. *Cis*-inhibition of [^3H]GlcA uptake into lysosomal membrane vesicles by different anions. Lysosomal membrane vesicles were pre-equilibrated for 10 min, 20°C in 20 mM NaHEPES, 10 mM KCl and 10 μM valinomycin. A sample of 10 μl pre-equilibrated vesicles were incubated for 30 s at 20°C with 2.5 μM [^3H]GlcA in the presence of an inward-directed proton gradient and 7 mM of the indicated compounds. The blank value was subtracted from all determinations. Data are presented as percentage of the uninhibited transport activity. Values ($n = 3$) are mean \pm S.D.

the acidic monocarboxylated drug salicylate. Moderate inhibition (25–60% of control) was shown by the anti-epileptic drug valproate and the anionic drugs methotrexate and folate. No significant inhibition was found for the acidic amino acid glutamate, the organic cation tetraethylammonium (TEA), the inorganic anions phosphate and sulfate and phosphorylated sugars. This demonstrates that the lysosomal sialic acid transporter recognizes different organic anions, but not acidic amino acids, organic cations, inorganic anions or sugar-phosphates.

In order to test which of the *cis*-inhibitors can be transported by the lysosomal sialic acid transporter, we investigated their *trans*-stimulation effect on the uptake of [^3H]GlcA. For these experiments, vesicles were pre-loaded with unlabelled compound and the uptake of [^3H]GlcA was measured in the absence of a proton gradient. In our experience, significant *trans*-stimulation represents at least a two-fold increase above the basal uptake rate under the current condi-

Table 1
Trans-stimulation of GlcA uptake by different anions

anionic compound	Transport Activity		<i>Trans</i> -stimulation factor
	+Preloading	–Preloading	
	(pmol/mg/30 s)		
GlcA	6.53 \pm 0.28	0.99 \pm 0.04	6.6
Neu5Ac	5.90 \pm 0.07	0.84 \pm 0.07	7.0
α -Ketoglutarate	4.35 \pm 0.03	0.88 \pm 0.07	4.9
PAH	2.53 \pm 0.17	1.56 \pm 0.03	1.6
Salicylate	1.87 \pm 0.06	1.05 \pm 0.16	1.8
Valproate	1.84 \pm 0.09	1.16 \pm 0.03	1.6
Methotrexate	1.94 \pm 0.17	1.62 \pm 0.05	1.2
Urate	2.09 \pm 0.14	1.57 \pm 0.07	1.3
Glutamate	1.74 \pm 0.05	1.32 \pm 0.17	1.3

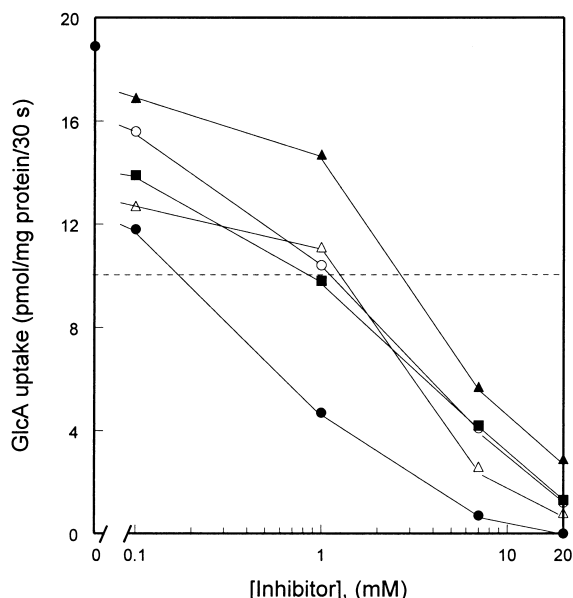


Fig. 2. Concentration dependent inhibition of [^3H]GlcA uptake. Transport of 10 μM [^3H]GlcA was measured in the presence of α -ketoglutarate (●), valproate (○), PAH (■), folate (▲) and urate (△) at the following concentrations: 100 μM , 1, 7 and 20 mM (plotted on a logarithmic x-axis). Transport assays ($n=3$) were performed as described in the legend of Fig. 1. The blank value was subtracted from all determinations. The dotted line corresponds with 50% of the uninhibited rate. The uninhibited rate is indicated on the y-axis (●).

tions. As shown in Table 1, α -ketoglutarate clearly *trans*-stimulated GlcA uptake, like GlcA itself and Neu5Ac. PAH, salicylate, valproate, methotrexate, urate and glutamate did not show a significant *trans*-stimulation at the tested concentrations. This indicates that the lysosomal sialic acid transporter can exchange GlcA for α -ketoglutarate.

Further kinetic studies were performed to determine the mode of inhibition. As shown in Fig. 2, all compounds inhibited in a concentration dependent manner. In subsequent kinetic inhibition studies the following concentrations, close to IC_{50} , were used: 0.5 mM α -ketoglutarate, 1 mM PAH, 1 mM valproate, 5 mM urate and 5 mM folate. The initial uptake of [^3H]GlcA was measured at increasing GlcA concentrations in voltage clamped membranes with K^+ /valinomycin in the absence and presence of unlabelled compounds. As illustrated in Fig. 3, all compounds showed a competitive mode of inhibition of glucuronic acid transport. The calculated K_i for α -ketoglutarate, valproate and PAH were respectively 0.46 mM, 0.64 mM and 0.73 mM ($K_i = K_t[I]/(-1/x) - K_t$ }, in which K_t is the K_m for GlcA, $[I]$ is the inhibitor concentration, x is the intercept on the abscissa). For urate and folate, we found a K_i of respectively 1.77 mM and 4.6 mM, indicating that these last compounds have a much lower affinity for the transporter.

4. Discussion

The lysosomal sialic acid transporter shows functional similarities with the MCT family [6]. Both, MCTs and our carrier, are energized by proton symport, have an overlap in substrate specificity in compounds like L-lactate, and are sensitive to specific inhibitors like cyano-cinnamates. The MCT

family is classified as a subfamily of the major facilitator superfamily (MFS) [7,8]. This superfamily contains many different prokaryotic and eukaryotic anion transporters. Many homologues of bacterial genes have also been found in higher animals (mammals). Members of the MFS are transporting small solutes, including sugars, often in response to ion gradients and are recently classified into 18 distinct subfamilies [8]. There can be significant overlap in substrate specificity between members of the different subfamilies.

In the experiments, described in this paper, several substrates of the OATs [9,10,16–18], like PAH, α -ketoglutarate, folate and valproate showed competitive *cis*-inhibition of GlcA uptake by the lysosomal sialic acid transporter. *Trans*-stimulation studies demonstrated that α -ketoglutarate is not only recognized but also actually translocated across the membrane by the mammalian sialic acid transporter. The OATs have homology with members of the sugar porter subfamily of the MFS [16,18]. Better phylogenetic analysis could reveal whether these transporters indeed belong to the sugar porter family. This shows that our observation of a mammalian carrier for both sugars and anions is not completely unexpected. We do not know whether compounds like α -ketoglutarate, PAH, urate, salicylate or valproate are physiological substrates in lysosomes or not. It would be interesting to study in more detail the role of the sialic acid transporter in the translocation of these drugs or their metabolites across the

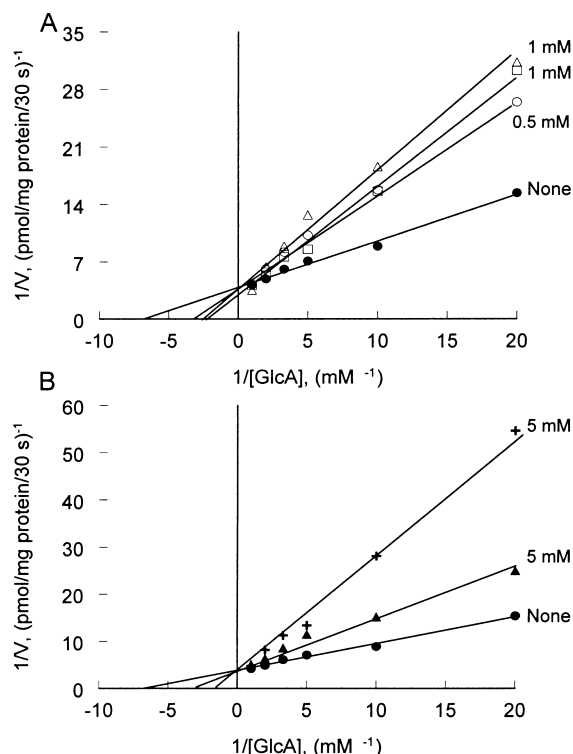


Fig. 3. Inhibition kinetics of [^3H]GlcA transport by different anions. Initial uptake rates of 10 μM [^3H]GlcA (30 s, 20°C) were measured at increasing GlcA concentrations in the presence of a proton gradient as described in the legend of Fig. 1. The uptake medium contained the following inhibitors: (A) 0.5 mM α -ketoglutarate (○), 1 mM PAH (□) or 1 mM valproate (△). (B) 5 mM of folate (▲) or urate (+). As a control, the uptake medium contained no inhibitor (●). The blank value was subtracted from all determinations. Data are plotted double reciprocally.

lysosomal membrane in future experiments, in relation to Salla disease.

α -Ketoglutarate is also a known substrate of a different MFS family, the metabolite: H^+ symporter (MHS) family [8]. Substrates of these carriers all possess at least one carboxyl group and the carriers function as proton symporters.

Sialic acid is an important substrate of our transporter. Two putative sialic acid permeases are known in the prokaryotes *E. coli* [19] and *Haemophilus influenza*. They form the sialate: H^+ symporter (SHS) family [8]. Interestingly, the yeast *Saccharomyces cerevisiae* homologue (JEN1) of the *E. coli* permease [20], although functionally characterized as a carboxylic acid transport protein (lactate transport, [21]), was classified on the basis of sequence similarities as a member of this SHS family.

The hexuronate glucuronic acid is another main substrate of the lysosomal sialic acid transporter. Few other transporters for hexuronates and glucarate (saccharate) are known. They belong to the anion:cation symporter (ACS) family of the MFS [8]. Saccharic acid 1,4 and 3,6 lactons are strong *cis*-inhibitors of GlcA transport by the lysosomal sialic acid transporter [22]. The lysosomal sialic acid transporter is the only mammalian carrier known to transport free hexuronates like glucuronate, galacturonate and iduronate [1,6].

The aldonic acid sugars gluconate and galactonate are also recognized by our transport protein [1]. They are substrates of the recently identified gluconate: H^+ symporter family (GntP), which is not a member of the MFS [23].

The tested compounds methotrexate and folate are known substrates of another phylogenetic different superfamily, distinct from the MFS, called the organic anion transporting polypeptides (oatps) [24,25]. As shown in Fig. 1, methotrexate and folate mildly inhibited GlcA transport, with a very high K_i for folate. Since the oatps do not recognize PAH and α -ketoglutarate and the lysosomal sialic acid transporter does not seem to transport or recognize with a high affinity substrates like methotrexate and folate, it is very unlikely that our transporter belongs to this family. Since sulfate, phosphate, glutamate, tetraethylammonium and several sugar-phosphates are not recognized by the lysosomal sialic acid transporter (*cis*-inhibition studies), the SulP family [11,12], the PHS family [11,13], the POT family [8,11], the OCT family [14] and the OPA family [15] are not of particular interest to us.

With the present studies we intended to extend the knowledge on the function of the lysosomal sialic acid carrier which can be helpful in the identification of the transporter gene involved in SASD. Our recent biochemical studies suggested homology between this transporter and the monocarboxylate transporters, belonging to the MCT family. Here we present data which show functional similarities with α -ketoglutarate transporters within the OAT and MHS family. On its functional basis, the transporter could also belong to the SHS, ACS or GntP family. This means that the search for the

human sialic acid transporter gene should not be limited to members of the MCT family.

Acknowledgements: This work was supported by the Dutch Organization for Scientific Research (NWO).

References

- [1] Mancini, G.M.S., de Jonge, H.R., Galjaard, H. and Verheijen, F.W. (1989) *J. Biol. Chem.* 264, 15247–15254.
- [2] Mancini, G.M.S., Beerens, C.E.M.T. and Verheijen, F.W. (1990) *J. Biol. Chem.* 265, 12380–12387.
- [3] Gahl, W.A., Schneider, J.A. and Aula, P.P. (1995) in: *The Metabolic and Molecular Basis of Inherited Disease* (Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D., Eds.), 7th edn., pp. 3763–3797, McGraw-Hill, New York.
- [4] Mancini, G.M.S., Beerens, C.E.M.T., Aula, P.P. and Verheijen, F.W. (1991) *J. Clin. Invest.* 87, 1329–1335.
- [5] Schleutker, J., Leppänen, P., Månsson, J.E., Erikson, A., Weissenbach, J., Peltonen, L. and Aula, P. (1995) *Am. J. Hum. Genet.* 57, 893–901.
- [6] Havelaar, A.C., Mancini, G.M.S., Beerens, C.E.M.T., Souren, R.M.A. and Verheijen, F.W. (1998) *J. Biol. Chem.* 273, 34568–34574.
- [7] Paulsen, I.T., Sliwinski, M.K. and Saier Jr., M.H. (1998) *J. Mol. Biol.* 277, 573–592.
- [8] Pao, S.S., Paulsen, I.T. and Saier Jr., M.H. (1998) *Microbiol. Mol. Biol. Rev.* 62, 1–34.
- [9] Sekine, T., Watanabe, N., Hosoyamada, M., Kanai, Y. and Endou, H. (1997) *J. Biol. Chem.* 272, 18526–18529.
- [10] Uwai, Y., Okuda, M., Takami, K., Hashimoto, Y. and Inui, K. (1998) *FEBS Lett.* 438, 321–324.
- [11] Pisoni, R.L. and Thoene, J.G. (1991) *Biochim. Biophys. Acta* 1071, 351–373.
- [12] Bissig, M., Hoogenbuch, B., Stieger, B., Koller, T. and Meier, P.J. (1994) *J. Biol. Chem.* 269, 3017–3021.
- [13] Bun-Ya, M., Nishimura, M., Harashima, S. and Oshima, Y. (1991) *Mol. Cell. Biol.* 11, 3229–3238.
- [14] Pritchard, J.B. and Miller, D.S. (1993) *Physiol. Rev.* 73, 765–796.
- [15] Ambudkar, S.V. and Maloney, P.C. (1984) 259, 12576–12585.
- [16] Sweet, D.H., Wolff, N.A. and Pritchard, J.B. (1997) *J. Biol. Chem.* 272, 30088–30095.
- [17] Simonson, G.D., Vincent, A.C., Roberg, K.J., Huang, Y. and Iwanij, V. (1994) *J. Cell Sci.* 107, 1065–1072.
- [18] Sekine, T., Ho Cha, S., Tsuda, M., Apiwattanakul, N., Nakajima, N., Kanai, Y. and Endou, H. (1998) *FEBS Lett.* 429, 179–182.
- [19] Martinez, J., Steenbergen, S. and Vimr, E. (1995) *J. Bacteriol.* 177, 6005–6010.
- [20] Paulsen, I.T., Sliwinski, M.K., Nelissen, B., Goffeau, A. and Saier Jr., M.H. (1998) *FEBS Lett.* 430, 116–125.
- [21] Tzermia, M., Horaitis, O. and Alexandraki, D. (1994) *Yeast* 10, 663–679.
- [22] Mancini, G.M.S., Beerens, C.E.M.T., Galjaard, H. and Verheijen, F.W. (1992) *Proc. Natl. Acad. Sci. USA* 89, 6609–6613.
- [23] Peekhaus, N., Tong, S., Reizer, J., Saier Jr., M.H., Murray, E. and Conway, T. (1997) *FEMS Microbiol. Lett.* 147, 233–238.
- [24] Jacquemin, E., Hagenbuch, B., Stieger, B., Wolkoff, A.W. and Meier, P.J. (1994) *Proc. Natl. Acad. Sci. USA* 91, 133–137.
- [25] Noé, B., Hagenbuch, B., Stieger, B. and Meier, P.J. (1997) *Proc. Natl. Acad. Sci. USA* 94, 10346–10350.