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THE ENERGETICS OF D-FUCOSE TRANSPORT IN *SACCHAROMYCES FRAGILIS*

THE INFLUENCE OF THE PROTONMOTIVE FORCE ON SUGAR ACCUMULATION

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The protonmotive force in *Saccharomyces fragilis* has been estimated under various experimental conditions. The transmembrane potential has been monitored with tetraphenylphosphonium and 3,3′-dipropylthiadicarbocyanine. The distribution ratio of these cations between intracellular and extracellular water appeared to be governed by the electrical potential difference across the membrane of this yeast strain. The transmembrane pH difference was deduced from dimethyloxazolidinedione uptake experiments and from direct measurements of intracellular pH after freezing and boiling of the cells. Both methods yielded similar results. D-Fucose is transported by *S. fragilis* via H^+ symport, with a H^+/fucose stoichiometry of approximately 1. Accumulation of this sugar appeared to be closely correlated with the protonmotive force.

Introduction

Osmotic coupling between solute uptake and ion gradients, as proposed by Mitchell [1], seems to be a well established mechanism for uphill transport. For instance, in many microbial cells sugars are cotransported with protons (see, for example, Ref. 2).

A crucial point in Mitchell's theory is the thermodynamics of the reaction. The hypothesis predicts that net transport should cease when the gradient of the solute is balanced by the electrochemical gradient of the co-substrate. Thus accumulation of sugars, via H^+ symport systems, should proceed until equilibrium is achieved with the protonmotive force.

Such a direct thermodynamic coupling between sugar accumulation and protonmotive force could indeed be shown in bacteria [3,4]. In yeast, however, a similar correlation has not yet been shown unequivocally. This is mainly caused by the fact that the measurement of the transmembrane potential, being a component of the protonmotive force, is difficult in this organism. Because of the small size of most yeast cells, direct methods, using microelectrodes, have failed. The use of fluorescent dyes to monitor $\Delta\psi$ in yeast has led to some contradictory results. Using diS-C_3-(5), changes in membrane potential could be shown during sugar transport in *S. fragilis* [5]. However, with *Saccharomyces cerevisiae* apparently conflicting data were obtained [6–8]. Also the use of lipophilic cations for the measurement of $\Delta\psi$ in yeast sometimes seems somewhat problematic. For instance DDA and TPMP are transported across

Abbreviations: $\Delta\psi$, transmembrane potential; ΔpH, transmembrane pH difference; TPP, tetraphenylphosphonium; TPMP, triphenylmethylphosphonium; DDA, dibenzyldimethylammonium; diS-C_3-(5), 3,3′-dipropylthiadicarbocyanine CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide.

the plasma membrane of *S. cerevisiae* via the thiamine carrier [9]. At the moment TPP, which is probably not taken up via this thiamine carrier [10,11], appears to be the best probe for more-or-less quantitative measurements of the transmembrane potential in yeast cells [10–14].

In this paper it will be shown that the transmembrane potential of *S. fragilis* can be measured qualitatively, using diS-C_3-(5), or semi-quantitatively, using TPP. From data obtained with these probes and from measurements of the transmembrane pH difference, the total proton motive force has been estimated under various experimental conditions. Finally a rather close correlation between D-fucose accumulation and the protonmotive force was found.

Materials and Methods

S. fragilis was cultured, with glucose or lactose as carbon source, harvested and washed as described before [15].

D-Fucose transport, TPP distribution and intracellular pH were measured using 10% (w/v) yeast suspensions buffered with 0.2 M Tris-maleate, at 25°C.

In all experiments using radioactively labeled substances, intracellular concentrations were determined by filtration of 0.3 ml suspension on a cellulose nitrate filter (Schleicher&Schüll, 0.45 μm pore). The cells were washed three times with ice-cold water. After transferring the yeast into a counting vial with 1 ml 0.5% Triton X-100, the radioactivity was determined using Picofluor-30 Scintillation liquid (Packard).

Extracellular radioactivity was determined by counting an aliquot of the supernatants after centrifugation in an Eppendorf centrifuge 3200.

When TPP uptake was measured, the cells were washed with ice-cold $MgCl_2$ as described by Barts et al. [9] to prevent binding of radioactivity to cell walls.

The ΔpH was measured via determination of the extracellular pH and the intracellular pH. The extracellular pH was measured with a conventional pH meter. The intracellular pH was deduced from the dimethyloxazolidinedione distribution between cells and medium, utilizing an initial dimethyloxazolidinedione concentration of 1.8 μM and pK 6.32 [16,17]. Alternatively the intracellular pH was measured by the freezing-boiling method as described by Borst-Pauwels and Dobbelman [18]. Prior to freezing the cells medium was removed by filtration, followed by washing three times with cold distilled water.

H^+ fluxes were measured as described before [19]. The presence of sugar phosphates in cellular extracts was determined using the conventional Ba-Zn method [20], or by performing descending paper chromatography of cellular extracts as described earlier [21].

Measurements of the intracellular water space were performed as described before [22] and revealed a value of 0.45 ml/g yeast (wet weight).

Fluorescence spectroscopy was measured in an Aminco Bowman Spectrofluorometer equipped with a scale expànder and an Aminco *X-Y* recorder (for spectra) or a Vitratron stripchart recorder (for kinetic measurements). Reaction conditions: 0.1% (w/v) yeast in 10 mM Tris-maleate/0.25 μM diS-C_3-(5).

Pyrithiamine was measured fluorimetrically as described by Fujita [23]. [^{14}C]DDA was prepared by Dr. H.T.A. Jaspers according to the method of Lombardi et al. [24].

[^{14}C]Dimethyloxazolidinedione, [^{14}C]TPP, [^{14}C]inulin and D-[^{3}H]fucose were supplied by Amersham International. DiS-C_3-(5) iodide was a gift of Dr. A.S. Waggoner, Amherst College, Amherst.

Results

Measurement of the transmembrane potential

In order to decide whether the uptake of lipophilic cations can be used as a measure of the transmembrane potential in *S. fragilis*, several conditions will have to be fulfilled. The probe should preferentially not be transported via a carrier system, as discussed before [9]. Further its distribution should respond in a predictable and reversible way to changes of the membrane potential.

In *S. fragilis*, DDA appeared to be poorly transportable, whereas TPP was readily taken up, reaching a steady state, at pH 5.5, in about 40 min. It has been shown that DDA and TPMP pass the plasma membrane of *S. cerevisiae* via the thiamine

translocator [9]. This does not apply to DDA and TPP uptake in *S. fragilis*. Growth in the absence of external thiamine [25] induced biosynthesis of the thiamine carrier in *S. fragilis*, as could be deduced from pyrithiamine uptake studies, but did not stimulate DDA and TPP uptake (results not shown). Because of this slow uptake DDA has not been used in further experiments (see also Ref. 26).

In further experiments the influence of $\Delta\psi$ on TPP accumulation was studied. The presence of either 5 μg/ml antimycin, 1 mM DCCD, 5 mM iodoacetate or 0.1 mg/ml diethylstilbestrol, inhibited TPP accumulation strongly. A similar inhibition was observed under anaerobic conditions.

Depolarization of the cells by uncouplers leads to efflux of accumulated TPP (Fig. 1). Increasing the concentration TPP also results in a lowered steady-state accumulation ratio. Both phenomena show that depolarization of the membrane reduces TPP uptake.

K^+, which lowers transmembrane potentials in various cells [12,27], causes reduced uptake of TPP (Fig. 2). When added 1 min before TPP addition uptake is strongly inhibited. However, when added after accumulation of TPP, only a slow efflux is observed, indicating that TPP transport is not readily reversible under these circumstances.

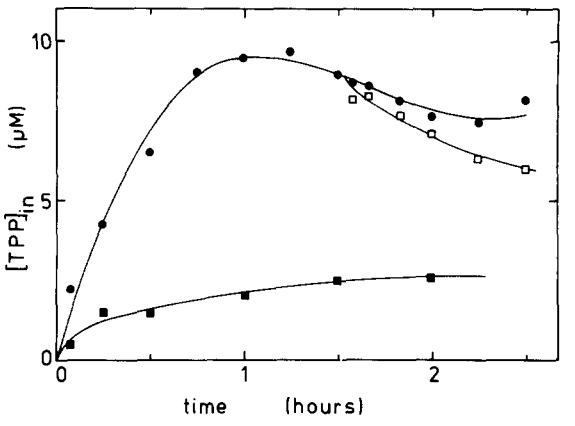

Fig. 2. Influence of KCl on TPP accumulation. Transport was measured, using lactose cultured yeast, aerobically at pH 5.0. 0.2 M KCl was added 1 min before TPP incubation (■———■) or 90 min after incubating cells with TPP (□———□). ●———● represents the control without KCl. Initial TPP concentration: 1 μM.

A similar poor reversibility was observed after adding antimycin to cells, which had accumulated TPP aerobically and after changing from aerobic to anaerobic conditions. A suchlike observation has been described before by Hauer and Höfer for *Rhodotorula gracilis* [12].

Finally the pH dependence of TPP uptake was studied. At high pH uptake is much faster than at

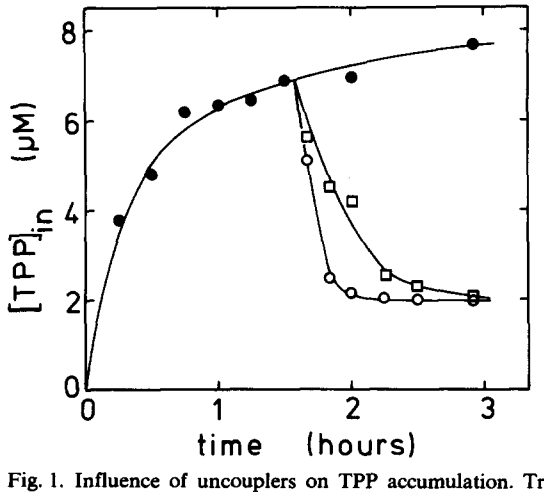

Fig. 1. Influence of uncouplers on TPP accumulation. Transport was measured, using lactose cultured yeast, aerobically at pH 5. After incubating the cells for 95 min with TPP, 100 μM CCCP (○———○) or 100 μM 2,4-dinitrophenol (□———□) were added. To the control (●———●) no addition was made. Initial TPP concentration: 1 μM.

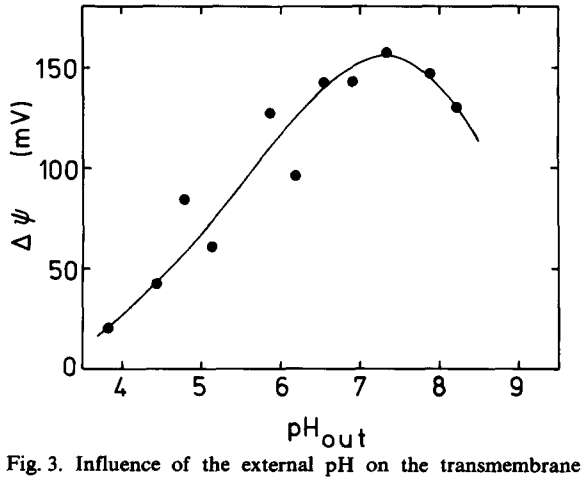

Fig. 3. Influence of the external pH on the transmembrane potential. Lactose-cultured yeast was incubated aerobically with 1 μM TPP at several pH values. The TPP accumulation ratio was determined by measuring the concentration TPP both intra- and extracellularly. $\Delta\psi$ (in mV) was calculated following the equation: $\Delta\psi = 59.1 \log ([TPP]_{in}/[TPP]_{out})$.

low pH. Also steady-state accumulation increases strongly with increasing pH, as shown in Fig. 3.

From these experiments it may be concluded that TPP behaves as a probe of the transmembrane potential in *S. fragilis*. However, the fact that under some conditions a poor reversibility is observed, places some limitations on its use as a quantitative probe.

Therefore a second type of $\Delta\psi$ probe was investigated. DiS-C_3-(5), belonging to the group of carbocyanine dyes, is transported across the plasma membrane of many cells in accordance with the $\Delta\psi$ [28–30]. Intracellularly the fluorescence of this cationic dye is quenched, e.g., by binding to cell constituents and by aggregation. Uptake of this probe can thus be monitored continuously by measuring fluorescence at fixed excitation and emission wavelengths.

For *S. fragilis* this is shown in Fig. 4. Addition of yeast to a solution of diS-C_3-(5) reduced fluorescence to about 10–20% of the original level. Subsequent addition of 50 μM DNP or 5 mM TPP yielded fluorescence enhancement, indicating efflux of accumulated dye. Maximal fluorescence change after depolarization is obtained with 0.1% yeast and at a concentration diS-C_3-(5) of 0.25 μM. Higher concentrations of this probe led to a percentually reduced effect of depolarizing agents, indicating de-energization of the membrane by the probe itself. Since fluorescence enhancements greater than 35% (100% = fluorescence of probe without yeast) were never observed, this apparently represents maximal effect.

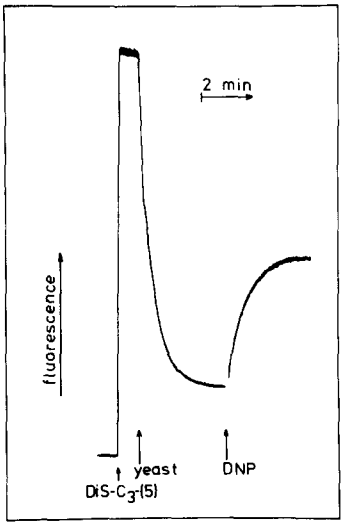

Fig. 4. Fluorescence of diS-C_3-(5) in the presence of yeast. Lactose grown *S. fragilis* (0.125% w/v) is added to a 0.25 μM diS-C_3-(5) solution in 10 mM Tris-maleate pH 4.5. 50 μM 2,4-dinitrophenol (DNP) is added after obtaining a constant fluorescence level. Excitation: 622 nm, emission: 670 nm.

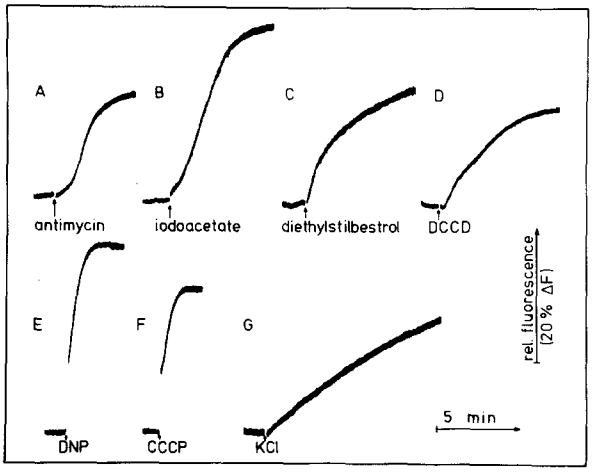

Fig. 5. Influence of inhibitors, uncouplers and KCl and diS-C_3-(5) fluorescence in the presence of yeast. Lactose grown yeast is incubated at pH 4.5 with diS-C_3-(5). After reaching a constant fluorescence level additions are: (A) 0.5 μg/ml antimycin, (B) 3.5 mM iodoacetate, (C) 6 μg/ml diethylstilbestrol, (D) 0.5 mM DCCD, (E) 50 μM 2,4-dinitrophenol, (F) 5 μM CCCP and 200 mM KCl. The fluorescence is expressed in percentage of fluorescence of diS-C_3-(5) in the absence of yeast. Excitation: 622 nm, emission: 670 nm.

The quenching of fluorescence is, at least partially, reversed by de-energization of the membrane, as depicted in Fig. 5. Also an increase of the extracellular pH from 4.5 to 7.5 caused a decrease in the percentage fluorescence (relative to the original level in the absence of yeast) from 8.86 to 0.96, indicating that $\Delta\psi$ (negative inside) strongly increases with increasing extracellular pH. This is in accordance with the results shown in Fig. 3. These results indicate that diS-C_3-(5) is a useful probe for monitoring qualitatively changes of $\Delta\psi$ in *S. fragilis*.

Influence of protonmotive force on D-fucose accumulation

Transport of D-fucose by *S. fragilis* is concentrative. With an initial sugar concentration in

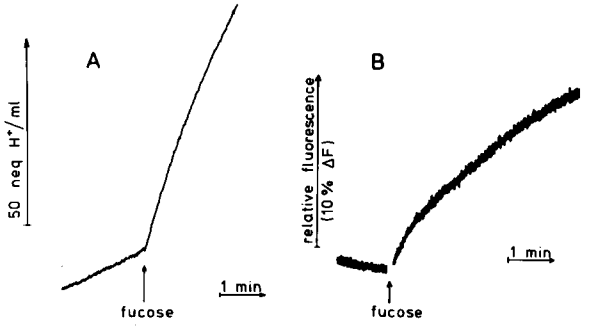

Fig. 6. The influence of fucose uptake on the H^+ flux and the transmembrane potential. A. 10% (w/v) yeast in incubated anaerobically in 1 mM Tris-maleate. Initial pH is 4.4. After recording a baseline, 1 mM fucose is added. The curve is calibrated by addition of known amounts of HCl. Upward deflection = H^+ influx. B. 0.1% (w/v) yeast is incubated with diS-C_3-(5) in 10 mM Tris-maleate, pH 4.5. After reaching a constant fluorescence level 10 mM fucose is added. Fluorescence is expressed in percent relative to fluorescence of diS-C_3-(5) in the absence of yeast. Excitation: 622 nm, emission: 670 nm.

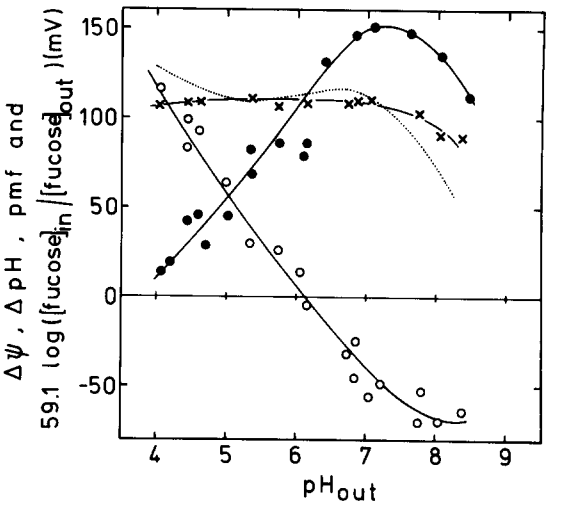

Fig. 7. Influence of the external pH on the protonmotive force (pmf) and on fucose accumulation. Yeast is incubated aerobically with 0.1 mM fucose, 1 μM TPP and 1.8 μM dimethyloxazolidinedione. $\Delta\psi$ is determined from TPP distribution, ΔpH (multiplied with 59.1 to obtain mV) from dimethyloxazolidinedione distribution. ●——●, $\Delta\psi$; ○——○, ΔpH; ×——×, 59.1 log ($[fucose]_{in}/[fucose]_{out}$) and ···, protonmotive force (calculated by summation of ΔpH and $\Delta\psi$).

the medium of 0.1 mM, the cells accumulated fucose up to 2.0–2.1 mM intracellularly. This accumulation is completely prevented by 1 mM CCCP. With the Ba-Zn method and with descending paper chromatography it could be shown that cellular extracts contained no fucose phosphates and that all the accumulated sugar was present in the cells as free fucose.

Because of the strong influence of uncouplers it was tested whether the energy for uphill transport could be obtained from an electrochemical H^+ gradient. Fig. 6 shows that addition of fucose to a weakly buffered yeast suspension results in a stimulation of H^+ influx. Under anaerobic conditions the H^+:fucose stoichiometry appeared to be 1.17 ± 0.30. As shown in Fig. 6, addition of fucose to a yeast suspension caused an increase of diS-C_3-(5) fluorescence, indicating depolarization during sugar influx. Therefore it is obvious that fucose uptake is coupled to electrogenic H^+ influx, indicating H^+-fucose symport. Kinetic analysis of initial influx, with the method described previously [31] revealed that transport proceeds mainly via a single transport system. At pH 5.2 a $K_{app} = 2.2$ mM and $V_{app} = 2.13$ μmol/g per min was measured. A second transport system with low affinity accounted at low sugar concentrations (0.1 mM) for 5–25% of total uptake (depending on pH). Since the high affinity system represents the H^+ symport, it can be stated that the accumulation ratio should be almost completely determined by the protonmotive force. To test this experimentally, the protonmotive force and the fucose accumulation ratio were determined simultaneously. The protonmotive force was calculated from the $\Delta\psi$ as measured with TPP and ΔpH, determined from the dimethyloxazolidinedione distribution. Analogous experiments using the freezing-boiling method to determine pH_{in} [18] yielded values identical to that obtained from dimethyloxazolidinedione distribution.

Fig. 7 summarizes the experimental results. Apparently the protonmotive force remains relatively constant over a wide pH range. The decrease of ΔpH with increasing pH_{out} is for the greater part compensated by an increase of the transmembrane potential. Also, fucose accumulation is relatively pH-independent, in accordance with the expected close correlation between fucose uptake and the magnitude of the protonmotive force.

Discussion

Transport of tetraphenylphosphonium ions by *S. fragilis* seems to be governed by the transmembrane potential. As has been shown for other yeasts [8–14], transport of this probe is energy-dependent, uncoupler-sensitive, reversed by K^+ and strongly concentration-sensitive, due to depolarization of the membrane by high concentrations of the probe itself. Direct measurements of $\Delta\psi$ in *S. fragilis*, utilizing microelectrodes, are as yet impossible, because of the small size of these yeast cells. Therefore $\Delta\psi$ measurements can be done only indirectly, utilizing a suitable probe. As TPP responds under many different experimental conditions in parallel to the theoretically expected $\Delta\psi$ shifts, it seems justified to use TPP as a probe of the transmembrane potential in this yeast. Using microelectrodes, Vacata et al. [32] measured the $\Delta\psi$ in a giant yeast. Comparison of this method with the TPP equilibrium technique showed that TPP distribution might overestimate this potential, presumably by intracellular binding. Such intracellular binding could also be shown to occur in some bacteria [33,34]. The incomplete reversibility of uptake under some experimental conditions, as described in this study and by Hauer and Höfer [12] may be related to this phenomenon. On the other hand, the fact that TPP uptake exhibits a strong pH sensitivity points to only a marginal binding of this probe. If extensive intracellular binding took place it should be expected to occur at all pH values. Therefore it seems probable that the TPP distribution actually reflects the transmembrane potential in *S. fragilis* in a semi-quantitative way.

As shown in this paper, the use of diS-C_3-(5) in *S. fragilis* also seems to yield reliable estimates of $\Delta\psi$ changes, confirming earlier results on *S. fragilis* [5] and *S. cerevisiae* [6]. Other, contradictory, results with *S. cerevisiae* [7,8] are probably due to incorrect reaction circumstances. For instance, the concentration diS-C_3-(5) used in Ref. 8 (5 μM) will lead to uncoupled cells, since the concentration of the probe should not exceed 0.4 μM [35].

As suggested by Kovác and Varečka [6], accumulation of diS-C_3-(5) is at least partially due to transport into the mitochondria. This indeed concerns another problem in experiments on yeast. Intracellular compartments will have separate and additive effects on the accumulation of membrane potential probes. The drastic change in probe accumulation at different pH values, however, indicates that uptake of diS-C_3-(5) (and also of TPP) represents predominantly the plasma membrane-induced distribution. The two probes can be used in a complementary fashion. DiS-C_3-(5) appears to be useful in monitoring fast changes of $\Delta\psi$ qualitatively, whereas TPP which is taken up less rapidly, can be used to estimate $\Delta\psi$ more or less quantitatively.

The transmembrane potential in *Saccharomyces* is probably formed by a plasma membrane H^+-ATPase. Inhibitors of this enzyme, such as DCCD and diethylstilbestrol [7,14] depolarize the membrane, indicating that an electrogenic ion flux coupled to the membrane ATPase brings about the transmembrane potential. Results, depicted in Fig. 5, showing depolarization after addition of DCCD and diethylstilbestrol are in accordance with this notion.

The measurement of the transmembrane pH difference by use of dimethyloxazolidinedione distribution [16,17] or the freezing-boiling method [18] yielded identical values. Although these methods can give small deviations from the actual values [36], it is likely that ΔpH measured in this way gives a good approximation.

The total protonmotive force, calculated from dimethyloxazolidinedione and TPP distributions, appears to be relatively constant over a wide range of pH values (see also Refs. 11, 13). At high medium pH values the increased $\Delta\psi$ compensates the decreased ΔpH.

Probably the physiological significance is that several cellular functions can proceed at an optimal velocity and extent over a large pH range. For instance, D-fucose, which is transported via H^+-symport, is highly accumulated over the whole pH range studied (Fig. 7).

A relatively close correlation between protonmotive force and fucose accumulation could be observed. The fact that at high pH values fucose accumulation exceeds the protonmotive force is presumably caused by an underestimation of ΔpH [36]. Nevertheless, the correlation between protonmotive force and fucose accumulation clearly indicates that the protonmotive force is indeed the driving force for fucose transport in *S. fragilis*.

Acknowledgement

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