Metabolism of sucrose and its five isomers by *Fusobacterium mortiferum*

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***Fusobacterium mortiferum* utilizes sucrose [glucose-fructose in $\alpha(1\rightarrow2)$ linkage] and its five isomeric α -D-glucosyl-D-fructoses as energy sources for growth. Sucrose-grown cells are induced for both sucrose-6-phosphate hydrolase (S6PH) and fructokinase (FK), but the two enzymes are not expressed above constitutive levels during growth on the isomeric compounds. Extracts of cells grown previously on the sucrose isomers trehalulose $\alpha(1\rightarrow1)$, turanose $\alpha(1\rightarrow3)$, maltulose $\alpha(1\rightarrow4)$, leucrose $\alpha(1\rightarrow5)$ and palatinose $\alpha(1\rightarrow6)$ contained high levels of an NAD⁺ plus metal-dependent phospho- α -glucosidase (MalH). The latter enzyme was not induced during growth on sucrose. MalH catalysed the hydrolysis of the 6'-phosphorylated derivatives of the five isomers to yield glucose 6-phosphate and fructose, but sucrose 6-phosphate itself was not a substrate. Unexpectedly, MalH hydrolysed both α - and β -linked stereomers of the chromogenic analogue *p*-nitrophenyl glucoside 6-phosphate. The gene *malH* is adjacent to *malB* and *malR*, which encode an EII(CB) component of the phosphoenolpyruvate-dependent sugar:phosphotransferase system and a putative regulatory protein, respectively. The authors suggest that for *F. mortiferum*, the products of *malB* and *malH* catalyse the phosphorylative translocation and intracellular hydrolysis of the five isomers of sucrose and of related α -linked glucosides. Genes homologous to *malB* and *malH* are present in both *Klebsiella pneumoniae* and the enterohaemorrhagic strain *Escherichia coli* O157:H7. Both these organisms grew well on sucrose, but only *K. pneumoniae* exhibited growth on the isomeric compounds.**

Keywords: phospho- α -glucosidase, sucrose isomers, sucrose-6-phosphate hydrolase, *Klebsiella pneumoniae*, *Escherichia coli* O157:H7

INTRODUCTION

Many bacterial species, including *Klebsiella pneumoniae* (Sprenger & Lengeler, 1988; Titgemeyer *et al.*, 1996), *Bacillus subtilis* (Fouet *et al.*, 1987), *Lactococcus lactis* (Thompson & Chassy, 1981; Thompson *et al.*, 1991; Rauch & deVos, 1992), *Fusobacterium mortiferum* (Thompson *et al.*, 1992), *Escherichia coli* (Schmid *et al.*, 1988) and *Clostridium beijerinckii* (Tangney *et al.*, 1998; Reid *et al.*, 1999) translocate sucrose simultaneously

with phosphorylation at C-6 of the glucosyl moiety via the phosphoenolpyruvate-dependent sucrose:phosphotransferase system (PEP:PTS) (Meadow *et al.*, 1990; Postma *et al.*, 1993). Sucrose 6-phosphate is hydrolysed intracellularly by sucrose-6-phosphate hydrolase (S6PH) to yield glucose 6-phosphate and fructose, which are further metabolized via the glycolytic pathway. The multi-component sucrose-PEP:PTS and S6PH are also expressed by oral streptococci, including *Streptococcus mutans* (St Martin & Wittenberger, 1979; Slee & Tanzer, 1979) and *Streptococcus sobrinus* (Chen & LeBlanc, 1992) and dietary sucrose is fermented primarily to lactic acid. By its demineralizing action upon tooth enamel, this organic acid initiates or contributes to the aetiology of dental caries (Loesche, 1986; Van Houte, 1994).

The linkage between the two component sugars of sucrose, i.e. D-glucose and D-fructose, can be modified to

Abbreviations: FK, fructokinase; G6P, glucose 6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; HK, hexokinase; 4-MU- α -G6P, 4-methylumbelliferyl α -D-glucopyranoside-6-phosphate; PEP:PTS, phosphoenolpyruvate-dependent sucrose:phosphotransferase system; pNP- α -G6P, *p*-nitrophenyl α -D-glucopyranoside 6-phosphate; pNP- β -G6P, *p*-nitrophenyl β -D-glucopyranoside 6-phosphate; S6PH, sucrose-6-phosphate hydrolase.

Fig. 1. Molecular formulae of sucrose and its five isomeric α -D-glucosyl-D-fructoses, in both free and phosphorylated forms.

yield five isomeric compounds (Fig. 1) that trivially are designated trehalulose, turanose, maltulose, leucrose and palatinose (Lichtenthaler & Rönninger, 1990; Lichtenthaler *et al.*, 1991; Immel & Lichtenthaler, 1995). Until recently (Thompson *et al.*, 2001a), there were no reports of the bacterial utilization of the five sucrose isomers. Indeed, the inability of mutans streptococci to metabolize these comparatively sweet disaccharides suggests their use as non-cariogenic substitutes for dietary sucrose (Ooshima *et al.*, 1983, 1991; Ziesenitz *et al.*, 1989; Minami *et al.*, 1990; Peltroche-Llacsahuanga *et al.*, 2001). In light of these reports, we were surprised to discover that *K. pneumoniae* readily utilized all five α -D-glucosyl-D-fructoses as energy sources for growth (Thompson *et al.*, 2001a, b), and that the enzymes encoded by the sucrose (*scr*) operon (Titgemeyer *et al.*, 1996) did not participate in dissimilation of these compounds. Remarkably, the sucrose isomers and structurally related α -glucosides (including maltose, isomaltose, maltitol and methyl α -D-glucoside) are first translocated by an α -glucoside-specific EII(CB) transport protein, and the accumulated 6-phospho- α -D-glucosides are hydrolysed by a metal-requiring, NAD⁺-dependent phosphoglucosyl hydrolase belonging to family 4 of the glycosylhydrolase superfamily (Henrissat, 1991). In *K. pneumoniae*, the genes for the EII(CB) transport protein (*aglA*) and the phospho- α -glucosidase (*aglB*) lie adjacent, and are chromosomally encoded (Thompson *et al.*, 2001b).

Fusobacteria are Gram-negative anaerobic rods that are usually described as weakly or asaccharolytic, and most species, including *Fusobacterium nucleatum*, use amino acids as fermentable energy sources (Robrish *et al.*, 1987; Robrish & Thompson, 1990). The products of metabolism (acetic, butyric and propionic acids) may penetrate periodontal tissue, thereby contributing to the aetiology of gingivitis and periodontal disease. In contrast to other species, *F. mortiferum* ferments an extraordinarily wide variety of carbohydrates (Robrish *et al.*, 1991). Previously, in studies of maltose metabolism in *F. mortiferum*, we cloned and expressed a gene (*malH*) whose deduced sequence exhibits ~75% residue identity with the phospho- α -glucosidase of *K. pneumoniae* (Thompson *et al.*, 1995; Bouma *et al.*, 1997). In the present report, we show that the gene adjacent to *malH* (designated *malB*) also encodes a putative EII(CB) protein that is ~60% identical with AglA of *K. pneumoniae*. Coincident with our studies, publication of the complete genome sequence of enterohaemorrhagic *E. coli* O157:H7 (Perna *et al.*, 2001) also revealed two adjacent genes with extensive homology to those found in *K. pneumoniae*. It was of interest, therefore, to determine whether possession of these genetic elements would also permit growth of *F. mortiferum* and *E. coli* O157:H7 on the five isomers of sucrose. Our findings are summarized in this communication. Additionally, we describe the purification, and some unexpected properties, of the phospho- α -

glucosidase (MalH) that catalyses the hydrolysis of phosphorylated sucrose isomers in *F. mortiferum*.

METHODS

Materials and reagents. Sucrose isomers were obtained from the following sources: trehalulose, Südzucker, Mannheim/Ochsenfurt; turanose, Pfanstiehl Laboratories; maltulose, TCI America; leucrose, Fluka; palatinose, Wako Chemicals. Other sugars and glucosides were purchased from Sigma and Pfanstiehl Laboratories. Phosphorylated derivatives were biosynthesized via the PEP:PTS of permeabilized (palatinose-grown) cells of *K. pneumoniae* and were purified by Ba²⁺/ethanol precipitation, ion-exchange and paper chromatography (Thompson *et al.*, 2001a). The chromogenic and fluorogenic substrates *p*-nitrophenyl α -D-glucopyranoside 6-phosphate (pNP- α -G6P), *p*-nitrophenyl β -D-glucopyranoside 6-phosphate (pNP- β -G6P) and 4-methylumbelliferyl α -D-glucopyranoside-6-phosphate (4-MU- α -G6P) were prepared by selective phosphorylation (at C6-OH) of the parent glucosides with phosphorus oxychloride in trimethyl phosphate containing small proportions of water (Thompson *et al.*, 1995). Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and hexokinase (HK, EC 2.7.1.1) were purchased from Boehringer Mannheim, and Ultrogel AcA-44 and Tris-Acryl M-DEAE from Sigma.

Bacterial strains and culture media. *K. pneumoniae* ATCC 23357, *E. coli* O157:H7 (EDL 933) and *F. mortiferum* ATCC 25557 were obtained from the American Type Culture Collection. *K. pneumoniae* and *E. coli* O157:H7 were grown in a medium of the following composition (per litre): Na₂HPO₄, 7.1 g; KH₂PO₄, 1.5 g; (NH₄)₂SO₄, 3 g; MgSO₄·7H₂O, 0.1 g; FeSO₄·7H₂O, 5 mg. Filter-sterilized sugars were added to autoclaved media (pH 7.4) to a final concentration of 4 g per litre. Cells of *K. pneumoniae* were grown in standing cultures, but *E. coli* O157:H7 was grown with vigorous aeration on a rotary shaker (~250 r.p.m.). *E. coli* PEP43(pCB4.11) was grown with aeration in Luria-Bertani (LB) medium supplemented with 50 µg kanamycin ml⁻¹. *F. mortiferum* was grown anaerobically (GasPak, BBL) in a medium comprising (per litre): Tryptone (Difco), 17 g; Protease Peptone (Difco), 3 g; Na₂HPO₄, 2.5 g; NaCl, 5 g; final pH 7.3.

DNA sequence analysis. Automated DNA sequencing incorporating Big Dye terminators was used to sequence *malB*, and an adjacent upstream gene (*malR*), directly from genomic DNA of *F. mortiferum*. From data previously reported by Bouma *et al.* (1997), a reverse primer 1R1 (5'-AACTCT-CTCTAACTTGTGGTACTGAAAGTC-3') was designed to obtain initial sequence information. Subsequent data were obtained by the primer synthesis and the chromosomal 'walking' technique. PCR primers were designed for sequencing of the second strand, and for amplification (from genomic DNA) of the fragment encoding the two genes by use of *Taq* DNA polymerase. The amplicon was cloned into the TOPO TA cloning vector (Invitrogen). All sequencing was performed by BioServe Biotechnologies (Laurel, MD, USA), and the MacVector 7.0 sequence analysis package (Genetics Computer Group, Madison, WI, USA) was used to assemble and analyse the data.

Metabolism of sugars by washed cells of *F. mortiferum*. To maintain anaerobic conditions, centrifuge tubes were flushed with a gas mixture (5% CO₂, 5% H₂, 90% N₂, by vol.) prior to harvesting of the sucrose-grown cells (5000 g for 10 min at 5 °C). The supernatant fluid was discarded, and the cell pellet

was resuspended as quickly as possible in 30 ml anaerobically prepared wash solution [50 mM potassium phosphate buffer (pH 7) containing 1 mM MgCl₂]. After centrifugation, the washed cells were resuspended in 5 ml wash buffer, and the mixture was maintained at 0 °C under anaerobic conditions until required. For studies of disaccharide utilization, the washed cells (equivalent to 30 mg total cell protein) were added to 10 ml of wash buffer containing the desired sugar (sucrose or isomer) at a final concentration of 10 mM. The cell suspensions were incubated at 37 °C in 100 ml serum bottles filled with anaerobic gas and, at intervals, 1 ml samples were withdrawn by insertion of a gas-flushed tuberculin syringe through the butyl rubber cap. The cells were removed by filtration through Millex-GS filter units (0.22 µm pore size; Millipore) and filtrates were collected. Samples were heated in 1 M HCl for 1 h at 100 °C, cooled, and neutralized with 1 M KOH. Glucose (equivalent to disaccharide remaining) was determined by the ATP-G6PDH/HK-NADP⁺ coupled enzyme assay.

Preparation and analysis of *F. mortiferum* extracts. Cells of *F. mortiferum* grown on the different sugars were harvested from 400 ml anaerobic culture. The cell pellets (~1–2 g wet weight) were resuspended with 3 vols 25 mM Tris/HCl buffer (pH 7.5) containing 0.1 mM NAD⁺ and 1 mM MnCl₂ (designated TNM buffer). The cells were disrupted by sonication at 0 °C (6 × 15 s bursts in a Branson instrument, model 185), and centrifuged at 14000 r.p.m. for 20 min at 5 °C in an Eppendorf bench-top instrument. The clarified supernatants were assayed for S6PH, FK and phospho- α -glucosidase activities.

Enzyme assays. The activities of S6PH, FK and phospho- α -glucosidase (with disaccharide phosphate substrates) were determined from the formation of glucose, fructose 6-phosphate and G6P, respectively, in the appropriate reaction mixture. Production of the three metabolites was coupled to the enzymic reduction of NADP⁺ (measured as A₃₄₀), and rates were determined in a Beckman DU 640 recording spectrophotometer. In all calculations, a molar absorption coefficient (ϵ) of 6220 M⁻¹ cm⁻¹ was assumed for NADPH.

S6PH. This enzyme catalyses the hydrolysis of both sucrose 6-phosphate (to G6P and fructose) and sucrose (to glucose and fructose), albeit with significantly different *K_m* for the two compounds (0.1 mM and ~100 mM, respectively; see Thompson *et al.*, 1992). Because of the limited supply of sucrose 6-phosphate, sucrose was used as substrate for the spectrophotometric assay of S6PH in cell extracts. The standard 1 ml assay contained: 0.1 M potassium phosphate buffer (pH 7.2); 50 mM sucrose; 5 mM ATP; 10 mM MgCl₂; 1 mM NADP⁺, ~3 U each of G6PDH/HK, and cell extract.

FK. Activity was determined in a similar mixture to that used for S6PH, containing 10 mM fructose as substrate, 3 U G6PDH and 5 U phosphoglucose isomerase.

Phospho- α -glucosidase (MalH). Activity was determined by two methods in which either chromogenic analogues or phosphorylated disaccharides served as substrates. Cofactors NAD⁺ and Mn²⁺ were included in both reaction mixtures. Throughout the purification of MalH, enzyme activity was determined in a discontinuous assay with pNP- α -G6P and pNP- β -G6P as substrates. The 2 ml reaction mixture (at 37 °C) contained: 50 mM Tris/HCl buffer (pH 7.5); 0.5 mM NAD⁺; 1 mM MnCl₂; and 0.5 mM of the chromogenic substrate. After enzyme addition, samples (0.25 ml) were removed at intervals throughout a 3 min period of incubation, and were immediately added to 0.75 ml 0.5 M Na₂CO₃ solution containing 0.1 M EDTA to stop the reaction. The A₄₀₀ was

Fig. 2. Structural organization of the putative α -glucoside operons of: *F. mortiferum* (GenBank accession number U81185); *E. coli* O157:H7 (*yidP*, GenBank AAG58886; *glvA*, GenBank AAG58885; *glvG*, GenBank AAG58884) and *K. pneumoniae* (GenBank AF337811). The numbers below the arrows are predicted amino acid residues, with calculated molecular masses (Da) of the encoded polypeptides in parentheses. Dotted lines indicate incomplete sequence for *aglR* of *K. pneumoniae*.

measured, and the amount of pNP formed (substrate hydrolysed) was calculated from the molar absorption coefficient of the yellow *p*-nitrophenolate anion, $\epsilon = 18300 \text{ M}^{-1} \text{ cm}^{-1}$. A continuous NADP⁺-coupled assay was used to measure G6P formed by MalH-catalysed hydrolysis of phosphorylated disaccharides. The assay mixture contained in 1 ml: 0.1 M HEPES buffer (pH 7.5); 1 mM MgCl₂; 1 mM MnCl₂; 1 mM NAD⁺; 1 mM NADP⁺; 2 mM disaccharide phosphate, 3 U G6PDH and purified MalH (35 μg protein).

Purification of MalH from *E. coli* PEP43(pCB4.11). A 2.2 kb *Sau*3AI chromosomal DNA fragment of *F. mortiferum* that includes the *malH* gene and its promoter has previously been cloned and the enzyme has been expressed from plasmid pCB4.11 (Bouma *et al.*, 1997). This plasmid was transferred by electroporation to *E. coli* PEP43 $\Delta\text{cel } \Delta(\text{bgl-pho}) \text{ leu metA}$ or *B. his rpsL lacZ* $\Delta 4680 \text{ lacY}^+ \text{ arbT}^+ \text{ Tn10::bglA dTn10}_{\text{cam}}::\text{ebgA5100 ebgR}^+ \text{ L532}$ (B. G. Hall, Biology Department, University of Rochester, NY, USA, laboratory collection). *E. coli* PEP43 expresses no phospho- β -glucosidases because the *cel* and *bglGFB* operons have been deleted, *bglA* is disrupted by Tn10 and the *asc* operon is cryptic.

Cells of *E. coli* PEP43(pCB4.11) (~25 g wet weight) were resuspended with 40 ml TNM buffer, and the organisms were disrupted by 2 $\times$ 1.5 min sonication with a Branson model 350 instrument. The preparation was clarified by ultracentrifugation (180000 *g* for 2 h at 5 $^{\circ}\text{C}$), and the supernatant fluid was dialysed against 4 litres of TNM buffer. The dialysed material was transferred (~0.6 ml min⁻¹) to a column of TrisAcryl M-DEAE (2.6 $\times$ 10 cm) that had been equilibrated with TNM buffer. The column was washed to elute non-adsorbed material, and then MalH activity was eluted with 500 ml of a linear, increasing concentration gradient of NaCl (0–150 mM) in TNM buffer. Fractions of 5 ml were collected, and those containing highest MalH activity (54–65 inclusive) were pooled and concentrated in an Amicon pressure cell to ~3 ml. The concentrated sample was transferred (0.15 ml min⁻¹) to an Ultrogel AcA-44 gel filtration column (1.6 $\times$ 94 cm; linear fractionation range, 10–130 kDa) previously equilibrated with TNM buffer containing 0.1 M NaCl. Fractions of 2 ml were collected, and tetrameric MalH (~200 kDa) was eluted at the void volume of the column. Fractions that contained a single protein by SDS-PAGE (47–50, inclusive) were pooled, and concentrated to yield ~5 mg

purified MalH [specific activity 2.9 $\mu\text{mol pNP-}\alpha\text{-G6P hydrolysed min}^{-1} (\text{mg protein})^{-1}$].

Analytical methods. Protein concentrations were determined by the BCA protein assay (Pierce). The Novex X-Cell system was used for both native (nonreducing) and SDS-PAGE. For SDS-PAGE experiments, precast NuPage (4–12%) Bistris gels and MES-SDS running buffer (pH 7.3) were used with Novex Mark 12 protein size standards. Proteins were stained with Coomassie brilliant blue R-250. Electrophoresis of cell extracts under nonreducing conditions was carried out at 10 $^{\circ}\text{C}$ in Tris/glycine (4–20%) precast gels from Novex, with Tris/glycine (pH 8.3) supplemented with 1 mM MnCl₂ and 0.1 mM NAD⁺ as the running buffer. For detection of phospho- α -glucosidase activity, the gel was immersed in 30 ml of a solution that contained 25 mM Tris/HCl buffer (pH 7.5); 1 mM MnCl₂; 0.1 mM NAD⁺ and 0.1 mM 4MU- α -G6P. After ~5 min incubation, the gel was photographed under long-wave UV light with Ektapan Kodak film (2 min exposure with a green filter). For Western blot analysis, proteins in the cell extracts together with pre-stained markers (SeeBlue from Novex), were first separated by SDS-PAGE and then transferred to a nitrocellulose membrane. Immunodetection of phospho- α -glucosidase was performed with polyclonal antibody to MalH from *F. mortiferum* as described previously (Thompson *et al.*, 1995). Molecular dynamics simulations and procedures for the determination of solvent-accessible surfaces have been described previously (Immel & Lichtenthaler, 1995; Thompson *et al.*, 2001b).

RESULTS

Gene organization in *F. mortiferum*, *K. pneumoniae* and *E. coli* O157:H7

The genes that constitute the putative α -glucoside operons, and their organization in the three bacterial species, are shown in Fig. 2. In *K. pneumoniae*, adjacent chromosomal genes *aglA* and *aglB* encode, respectively, an EII(CB) transport protein and phospho- α -glucosidase. These proteins promote the phosphorylative translocation and hydrolysis of sucrose isomers by this organism (Thompson *et al.*, 2001a, b). The partial

Table 1. Enzyme activities in extracts prepared from cells of *F. mortiferum* grown previously on various carbohydrates

Growth sugar	Enzyme specific activity†		
	Phospho- α -glucosidase‡	FK§	S6PH
Trehalulose*	11.6	2.6	2.2
Sucrose	ND	26.3	63.4
Turanose*	3.5	0.7	1.9
Maltulose*	3.4	1.6	1.6
Leucrose*	12.7	2.4	6.5
Palatinose*	3.2	1.2	4.0
Glucose	ND	2.1	ND
Fructose	ND	1.0	3.6
Maltose	8.5	3.1	2.8
Methyl α -D-glucoside	23.9	ND	2.8

* Sucrose isomers.

† The same cell extracts were used for the assay of the three enzyme activities; values are means of two separate assays. ND, No detectable activity.

‡ nmol pNP- α -G6P hydrolysed min⁻¹ (mg protein)⁻¹.§ nmol fructose phosphorylated min⁻¹ (mg protein)⁻¹.|| nmol sucrose hydrolysed min⁻¹ (mg protein)⁻¹. **Fig. 3.** Studies of the metabolism of sucrose and two of its isomers by sucrose-grown cells of *F. mortiferum*. Washed cells were resuspended, under anaerobic conditions, in buffered solution containing the disaccharides at an initial concentration of 10 mM. Samples were removed at intervals and residual disaccharide was determined by the enzymic assay of glucose produced by acid hydrolysis. ●, Sucrose; ○, turanose; ■, palatinose.

sequence of the regulatory gene, *aglR*, was compiled from our own work, together with data obtained from the Washington University (St Louis) sequencing project of the *K. pneumoniae* genome (<http://genome.wustl.edu>). The recently published genome sequence of enterohaemorrhagic *E. coli* O157:H7 (Perna *et al.*, 2001) revealed the same organization of the three genes (designated *yidP*, *glvA* and *glvG*) as described for *K. pneumoniae*. The amino acid sequence deduced from

Table 2. Expression of S6PH and FK during growth of *K. pneumoniae* on different sugars

Growth sugar	Enzyme specific activity	
	FK†	S6PH‡
Trehalulose*	69	289
Sucrose	103	247
Turanose*	64	342
Maltulose*	54	170
Leucrose*	51	184
Palatinose*	87	289
Maltose	15	29
Trehalose	3	ND
Melibiose	9	18
Cellobiose	2	6
Maltitol	1	3
Glucose	ND	2
Methyl α -D-glucoside	1	ND
Galactose	8	6

ND, No detectable activity.

* Sucrose isomers.

† nmol fructose phosphorylated min⁻¹ (mg protein)⁻¹.‡ nmol sucrose hydrolysed min⁻¹ (mg protein)⁻¹.

yidP of *E. coli* O157:H7 predicts a polypeptide of 238 residues that exhibits 91% overall identity with the 233 residues deduced by translation of the incomplete gene *aglR* of *K. pneumoniae*. At their N-termini, the products of *aglR* and *yidP* contain a helix–turn–helix (HTH)

Fig. 4. Demonstration by PAGE of the expression, *in situ* activity, and purification of phospho- α -glucosidase (MalH) from *F. mortiferum*. (A) Immunodetection of MalH expression during growth of *F. mortiferum* on different sugars, by Western blotting using polyclonal antibody to MalH. Note the absence of immunoreactive polypeptide (~ 50 kDa, arrow) in the extract from sucrose-grown cells. (B) Zymogram demonstration of MalH activity in cell extracts by hydrolysis (arrow) of the fluorogenic substrate 4MU α G6P. Again, note the absence of fluorescence in the lane containing the extract from sucrose-grown cells. (C) Purification and determination of the molecular mass of MalH. Samples from each of the three stages of purification were denatured, and polypeptides were resolved by SDS-PAGE: lane 1, dialysed high-speed supernatant; lane 2, TrisAcryl M-DEAE; lane 3, purified MalH (molecular mass ~ 50 kDa) obtained by AcA-44 gel filtration chromatography. The asterisks in panel A indicate sucrose isomers.

motif that assigns the two proteins to the GntR family of transcriptional regulators. The complete sequence of the phospho- α -glucosidase gene (*malH*) of *F. mortiferum*, together with a partial sequence for the gene (*malB*), were described in an earlier report (Bouma *et al.*, 1997). The entire sequences for *malB*, and the upstream gene (*malR*), have now been obtained by chromosome 'walking' (GenBank accession no. U81185). Translation of *malR* predicts a 106-residue polypeptide that shows extensive homology with the 13 members of the UPF0087 family of regulatory proteins.

Sequence alignment of phospho- α -glucosidase and EII(CB) proteins

Alignment of the amino acid sequences predicted for the phospho- α -glucosidase(s) and EIIs reveals a high degree of similarity among these proteins (data not shown). MalH from *F. mortiferum* exhibits $\sim 75\%$ residue identity with the phosphoglucosyl hydrolase(s) from *E. coli* O157:H7 and *K. pneumoniae*. The EII(CB) transport protein of *F. mortiferum* (MalB) shows $\sim 60\%$ amino acid identity throughout its length with GlvA and AglA in *E. coli* O157:H7 and *K. pneumoniae*, respectively. For the two enteric species, phospho- α -gluc-

osidase and EII(CB) proteins exhibit overall identities of 89% and 81%, respectively. By sequence-based alignment (Henrissat, 1991) and signature pattern (P-X-[SA]-X-[LIVMFY](2)-[QN]-X(2)-N-P-X(4)-[TA]-X(9,10)-[KRD]-X-[LIV]-[GN]-X-C), the three phospho- α -glucosidases can be assigned to family 4 of glycosyl-hydrolases (see <http://www.expasy.ch/cgi-bin/lists?glycosid.txt> and <http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html>). By their composition and modular structure, proteins MalB, GlvA and AglA, can be assigned to the EII^{Glc/Ser} family of PTS transporters (Lengeler *et al.*, 1994; Lanz & Erni, 1998).

Growth studies with *F. mortiferum*, *K. pneumoniae* and *E. coli* O157:H7

Growth studies were performed to determine if possession of the putative operons would permit growth of the three organisms on sucrose isomers and other α -glucosides. Both *K. pneumoniae* and *F. mortiferum* showed excellent growth on all sugars tested, including glucose, fructose, methyl α -glucoside, maltose, maltitol, sucrose and all five α -D-glucosyl-D-fructoses. *E. coli* O157:H7 grew well on glucose, fructose, maltose and

sucrose, but the pathogen was unable to grow on methyl α -glucoside, maltitol or any of the sucrose isomers.

Enzyme expression during growth of *F. mortiferum* on sucrose and its isomers

S6PH and ATP-dependent fructokinase (FK) are induced by growth of *F. mortiferum* on sucrose (Robrish *et al.*, 1991; Thompson *et al.*, 1992). Because sucrose and its isomers comprise the same hexose moieties, it was of interest to determine whether sucrose-grown cells of *F. mortiferum* would also metabolize the isomeric compounds. As expected, sucrose-grown cells readily fermented sucrose, but there was no detectable metabolism of the five isomers, including palatinose and turanose (Fig. 3). Furthermore, whereas sucrose-grown cells of *F. mortiferum* contained high levels of S6PH and FK activity (Table 1), after growth on the isomers, the activities of the two enzymes were not significantly greater than the constitutive levels found in glucose- or fructose-grown cells. These findings contrast markedly with the high levels of S6PH and FK that are expressed during growth of *K. pneumoniae* on the isomeric compounds (Table 2).

Phospho- α -glucosidase (MalH) is expressed during growth of *F. mortiferum* on sucrose isomers

From the results of fermentation studies and enzymic analyses (Fig. 3 and Table 1, respectively), it was evident that that dissimilation of the sucrose isomers by *F. mortiferum* was via a route that was separate from that encoded by the *scr* regulon. Because previous studies with *K. pneumoniae* (Thompson *et al.*, 2001a) showed that growth on the sucrose isomers induced high-level expression of phospho- α -glucosidase (AglB), the various cell extracts of *F. mortiferum* were accordingly assayed for phospho- α -glucosidase (MalH) activity (Table 1). Extracts prepared from organisms grown previously on the sucrose isomers and other α -glucosides (e.g. maltose and methyl α -glucoside) readily hydrolysed pNP- α -G6P (Table 1). However, there was no detectable hydrolysis of the chromogenic analogue by similarly prepared extracts from organisms grown on glucose, fructose or sucrose. The results of a Western blot (Fig. 4A), performed with polyclonal antibody to MalH, confirmed expression of the phospho- α -glucosidase (molecular mass $\sim$ 50 kDa) during growth on sucrose isomers and other α -glucosides. Significantly, the immunoreactive protein (MalH) was not detectable in either sucrose- or glucose-grown cell extracts. The data presented in Fig. 4(B) established the co-identity of the immunoreactive polypeptide and the enzymically active protein. In this experiment, samples of the various cell extracts of *F. mortiferum* were electrophoresed under non-denaturing conditions, prior to *in situ* staining for phospho- α -glucosidase activity using the fluorogenic substrate 4-MU- α -G6P. The zymogram (Fig. 4B) yielded three significant results: (i) the intensely fluorescent aglycone (4-methylumbelliferone) was generated only

Table 3. Cofactor requirements for the hydrolysis of chromogenic substrates pNP- α -G6P and pNP- β -G6P by purified MalH from *F. mortiferum*

Assay composition and procedures are described in Methods. NAD⁺, metal ions and chromogenic substrates were present at 1 mM final concentration. Hydrolysis rates, expressed as μ mol pNP formed min⁻¹ (mg protein)⁻¹, are the means of two determinations. ND, No detectable activity.

Additions to assay	Activity with chromogenic analogue:	
	pNP- α -G6P	pNP- β -G6P
None	ND	ND
NAD ⁺	0.11	0.08
Mn ²⁺	1.61	1.19
NAD ⁺ + Mn ²⁺	2.91	1.56
NAD ⁺ + Mg ²⁺	0.39	0.16
NAD ⁺ + Ni ²⁺	0.51	0.54
NAD ⁺ + Co ²⁺	1.47	2.28

by those extracts that contained the immunoreactive protein (MalH); (ii) formation of a single zone of fluorescence (at the same migration distance in the gel) provided evidence for only one species of phospho- α -glucosidase in the extracts, and (iii) absence of fluorescence in lane 2 was consistent with the inability of *F. mortiferum* to express MalH during growth on sucrose.

Purification, cofactor requirements, and substrate specificity of MalH

The data presented thus far (although suggestive), did not establish a functional role for MalH in dissimilation of the α -D-glucosyl-D-fructoses by *F. mortiferum*. Clearly, it was necessary to purify MalH, and demonstrate hydrolysis of either free or phosphorylated derivatives of the isomers by the enzyme. To this end a plasmid (pCB4.11) containing the cloned *malH* gene under its own promoter (Bouma *et al.*, 1997) was transformed into *E. coli* strain PEP43. Importantly, the latter strain is deficient in all phospho- β -glucosidase activities, and an extract of these cells is unable to hydrolyse the chromogenic substrate pNP- β -G6P. Unexpectedly, after expression of MalH in *E. coli* PEP43(pCB4.11), the resultant cell extract caused the hydrolysis of both pNP- α - and pNP- β -G6P. Hydrolysis of both compounds was observed throughout purification of MalH (Fig. 4C), and the same cofactors (divalent metal ion and NAD⁺) were required for cleavage of both chromogenic substrates by electrophoretically pure enzyme (Table 3). We conclude that a single protein (MalH) is responsible for the hydrolysis of the two stereomers, pNP- α -G6P and pNP- β -G6P. Studies of substrate specificity revealed that neither purified MalH, nor extracts prepared from cells of *F. mortiferum* grown previously on the isomers, were

Table 4. Hydrolysis of phosphorylated sucrose isomers by MalH from *F. mortiferum*

Assay procedures are described in Methods. Phosphorylated compounds were present at a concentration of 2 mM. Enzyme activity is expressed as $\mu\text{mol G6P formed min}^{-1} (\text{mg protein})^{-1}$; values are means of two determinations. ND, No detectable hydrolysis.

Disaccharide phosphate in assay	Specific activity
Trehalulose-6'P*	0.15
Sucrose-6P	ND
Turanose-6'P*	0.68
Maltulose-6'P*	0.40
Leucrose-6'P*	0.08
Palatinose-6'P*	0.11
Maltose-6'P	0.15
Cellobiose-6'P†	ND
Gentiobiose-6'P‡	ND

* Sucrose isomers.

† Cellobiose: 4-O- β -D-glucopyranosyl-D-glucopyranose.

‡ Gentiobiose: 6-O- β -D-glucopyranosyl-D-glucopyranose.

able to hydrolyse the free (non-phosphorylated) forms of the isomeric compounds (data not shown). However, the 6'-O-phosphorylated derivatives of the five α -D-glucosyl-fructoses were hydrolysed by MalH (Table 4), albeit at a rate considerably slower than that determined for the α -linked chromogenic substrate, pNP- α -G6P. Significantly, sucrose 6-phosphate itself was not a substrate for MalH, and the enzyme also failed to hydrolyse β -O-linked phosphorylated disaccharides such as cellobiose 6'-phosphate and gentiobiose 6'-phosphate (Table 4).

DISCUSSION

Here we report the metabolism of sucrose isomers by *F. mortiferum*, and provide insight into the enzymic and genetic basis for growth on these isomers. Presently, *F. mortiferum* and *K. pneumoniae* are the only organisms known to ferment the five α -D-glucosyl-D-fructoses. Earlier we showed that MalH from *F. mortiferum* hydrolysed maltose 6'-phosphate (Thompson *et al.*, 1995), and now we provide evidence for the cleavage of the phosphorylated isomers of sucrose by this enzyme. The phospho- α -glucosidase gene (*malH*) is adjacent to the gene *malB*, whose now completed sequence predicts a polypeptide that in size, domain structure, and conserved motifs (GITE and CATRLR) is characteristic of an EII(CB) transporter of the PEP:PTS. Genes *malB* and *malH* are homologous to *aglA* and *aglB*, respectively, of *K. pneumoniae*. We suggest that the polypeptides encoded by these genetic elements are required for growth of *F. mortiferum* and *K. pneumoniae* on the isomers of sucrose and related α -glucosides, including maltose. A common feature of these genetic units is the absence of a gene encoding a third (and usually sugar-specific) protein (EIIA) that is required for operation of

all PTS systems. Interestingly, both sucrose:PTS and trehalose:PTS operons in *Bacillus subtilis* also lack the expected EIIA genes and, for these systems, it is believed that EIIA^{Glc} can serve as substitute (Sutrina *et al.*, 1990; Dahl, 1997). A similar cross-complementation may also occur between the EIIA^{Glc} and EII(CB) proteins of *F. mortiferum* and *K. pneumoniae* to yield a functional α -glucoside:PTS in these species.

Proteins encoded by the *scr* operons of *K. pneumoniae* and *F. mortiferum* are expressed during growth of both organisms on sucrose (Sprenger & Lengeler, 1988; Thompson *et al.*, 1992). Hydrolysis of sucrose 6-phosphate by S6PH yields G6P and fructose, and for *K. pneumoniae*, fructose is believed to be the inducer of the *scr* operon (Jahreis & Lengeler, 1993). Hydrolysis of the phosphorylated isomers by AglB of *K. pneumoniae* also yields G6P and fructose, and formation of the latter ketohexose is consistent with the high levels of S6PH and FK present in cells grown on the isomers (Table 2). Surprisingly, similar studies with *F. mortiferum* showed that, for this organism, growth on the isomeric compounds did not induce significant expression of either S6PH or FK (Table 1). These findings explain why these cells were unable to metabolize sucrose, and additionally, the data point to sucrose 6-phosphate (rather than fructose) as the likely inducer of the *scr* operon in *F. mortiferum*.

Substrate specificity and hydrolysis of chromogenic substrates by MalH

MalH is an oligomeric protein comprising four identical subunits (molecular mass ~ 50 kDa) that, by sequence-based alignment, is assigned to family 4 of the glycosylhydrolase superfamily. As reported for other members of this unusual family, MalH is inherently unstable and Mn^{2+} and NAD^{+} are prerequisite cofactors for activity (Nagao *et al.*, 1988; Thompson *et al.*, 1998, 1999; Raasch *et al.*, 2000). Whether the nucleotide and metal ion fulfil catalytic or structural functions has not been ascertained for any member of family 4. Phosphorylation at O-6 of the glucosyl moiety of the isomers is necessary for substrate cleavage, and MalH is unable to hydrolyse the corresponding non-phosphorylated compounds. MalH is also exacting with respect to the α -O linkage of its PTS-derived substrates (see below) and, because there is no detectable hydrolysis of β -O-linked stereoisomers such as cellobiose 6'-phosphate and gentiobiose 6'-phosphate (Table 4), the enzyme may reasonably be classified as a phospho- α -glucosidase. In this context, it is not clear why MalH should hydrolyse both pNP- α -G6P and pNP- β -G6P with comparable efficiency (Table 3). Co-purification of MalH with a phospho- β -glucosidase resident in the host (*E. coli* PEP43) can be discounted because of gene inactivation or crypticity, and analysis of the final preparation by SDS-PAGE provided evidence for only a single polypeptide. That the same cofactors should also be required for catalysis is further evidence that the same enzyme hydrolyses both α - and β -forms of the chromogenic compound(s).

Unlike the phosphorylated sucrose isomers (where G6P is linked to a fructose moiety), the essential G6P moiety of the chromogenic substrates is attached to *p*-nitrophenol. Perhaps the aromatic aglycone exerts an effect (electron-withdrawing?) upon the O-linkage such that α/β conformation is no longer a determinant of substrate specificity. It is of comparative interest to note that cellobiose-6-phosphate hydrolase (CelF) from *E. coli* is also a member of glycosylhydrolase family 4 (Thompson *et al.*, 1999). In contrast to MalH, this NAD⁺- and metal-dependent phospho- β -glucosidase hydrolyses only pNP- β -G6P.

Molecular basis for substrate recognition by MalH

Sucrose 6-phosphate and its phosphorylated isomers are not commercially available, but all of these derivatives were recently prepared in our laboratory (Thompson *et al.*, 2001a). Studies of substrate specificity showed that whereas MalH hydrolysed all of the phosphorylated isomers, the enzyme failed to hydrolyse sucrose 6-phosphate itself. Insight into the molecular basis for this remarkable discrimination among potential substrates was gained by molecular dynamics simulations, which revealed the probable solution-state geometries of the various disaccharide phosphates (Thompson *et al.*, 2001b). Molecular dynamics simulations and determination of solvent-accessible surfaces indicate pronounced conformational differences between sucrose 6-phosphate and its five isomeric 6'-phosphates. By virtue of an interresidue water bridge between Glc-2-O $\cdots$ H₂O $\cdots$ O-1-Fru, both sucrose and sucrose 6-phosphate assume a compact, globular shape in solution (Immel & Lichtenthaler, 1995; Thompson *et al.*, 2001b). This water bridge is not present in the 6'-phosphoglucosyl-fructoses and, in consequence, the phosphorylated isomers adopt a more linear (extended) molecular geometry. The specificity of MalH for the isomeric phosphates presumably reflects recognition by the enzyme's binding domain of both the shape and the molecular lipophilicity potential of the contact surfaces of these particular molecules (Thompson *et al.*, 2001b).

Conclusions

Both *F. mortiferum* and *K. pneumoniae* readily metabolize the five isomers of sucrose. In contrast, *E. coli* O157:H7 (which grows well on sucrose) failed to grow on any of the isomeric compounds. These results were surprising, because this enterohaemorrhagic strain has three genes (*yidP*, *glvA* and *glvG*) whose organization and deduced amino acid sequences are virtually identical to those of *aglR*, *aglA* and *aglB*, respectively, in *K. pneumoniae*. Although contrary to expectation, the results obtained for *E. coli* O157:H7 were nevertheless important. First, the data established for *E. coli* O157:H7 (as for the other species), that the sucrose-PTS/S6PH pathway is neither induced by, nor does it provide a route for dissimilation of, sucrose isomers. Secondly, the data indicate that possession of genes encoding α -glucoside-specific EII(CB) and phospho- α -

glucosidase (while necessary), may not be entirely sufficient for dissimilation of α -D-glucosyl-D-fructoses by micro-organisms.

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