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Original Article

Glucose-PTS Involvement in Maltose Metabolism by *Streptococcus mutans*

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Abstract

Streptococcus mutans grows with starch-derived maltose in the presence of saliva. Maltose transported into the cells is mediated by the MalQ protein (4-alpha-glucanotransferase) to produce glucose and maltooligosaccharides. Glucose can be phosphorylated to glucose 6-phosphate, which can enter the glycolysis pathway. The MalQ enzyme is essential in the catabolism of maltose when it is the sole carbon source, suggesting the presence of a downstream glucokinase of the MalQ enzyme reaction. However, a glucokinase gene-inactivated mutant (*glk* mutant) grew with maltose as the sole carbon source, with no residual glucokinase activity. This left a phosphoenolpyruvate-dependent phosphotransferase system (PTS) as the only candidate pathway for the phosphorylation of glucose in its transport as a substrate. Our hypothesis was that intracellular glucose derived from maltose mediated by the MalQ protein was released into the extracellular environment, and that such glucose was transported back into the cells by a PTS. The mannose PTS encoded by the *manL*, *manM*, and *manN* genes transports glucose into cells as a high affinity system with concomitant phosphorylation. The purpose of this study was to investigate extracellular glucose by using an enzyme-linked photometrical method, monitoring absorbance changes at 340 nm in supernatant of *S. mutans* cells. A significant amount of glucose was detected in the extracellular fluid of a *glk*, *manLM* double mutant. These results suggest that the *glk* and *manLMN* genes participate in maltose catabolism in this organism. The significance of multiple metabolic pathways for important energy sources, including maltose, in the oral environment is discussed.

Key words: *Streptococcus mutans* — Glucokinase — Maltose metabolism — Glucose PTS — 4-alpha-glucanotransferase

Introduction

Streptococcus mutans is a major etiologic agent of human dental caries[4)], and its natural habitat is dental plaque, an oral biofilm, where it is continually subjected to alternating periods of abundance and depletion with respect to carbohydrate energy sources (the so-called

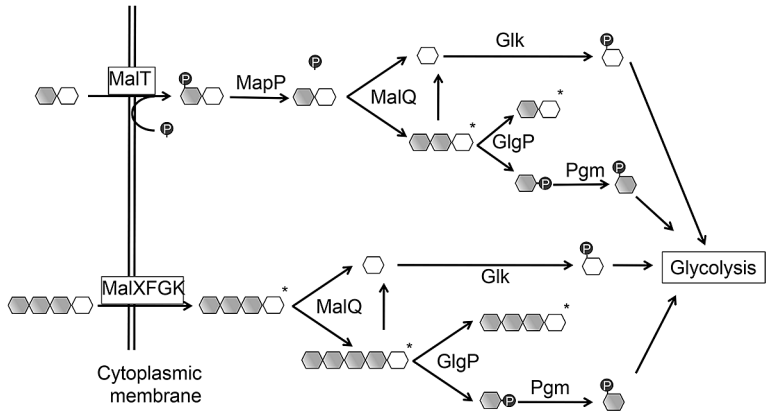

Fig. 1 Theoretical metabolic pathway for maltose and maltooligosaccharides from membrane transport to glycolysis

Asterisked maltooligosaccharides enter intracellular pool to repeatedly form substrates for 4-alpha-glucanotransferase (MalQ) and glycogen phosphorylase (GlgP) enzymes, releasing glucose and G1P from their reducing and non-reducing ends, respectively.
Symbols: open hexagon, glucose residues at reducing ends; shaded hexagon, non-reducing glucose residues.
Abbreviations: circled P, phosphate residues; MalT, maltose-PTS; MalXFGK, binding protein-dependent ABC transporter for maltooligosaccharides; MapP, putative maltose-6-phosphate phosphatase; MalQ, 4-alpha-glucanotransferase; Glk, glucokinase; GlgP, glycogen phosphorylase; Pgm, phosphoglucomutase.

"feast and famine" cycle)[6]. A major carbon source during a "feast" period is dietary starch, although this does not act as a direct carbon source for its growth; rather, *S. mutans* grows well with starch-derived maltose or maltooligosaccharides in the presence of saliva. These starch derivatives are imported through two sugar transport systems, the phosphoenolpyruvate-dependent maltose-phosphotransferase system (PTS) and the binding protein-dependent ABC transporter system for maltooligosaccharides, which are encoded by the *malT* (symbolized as *ptsG* in the genome data)[15] and *malXFGK* genes[8,16], respectively, in the genome of this organism. Maltose transported *via* the maltose-PTS (MalT) is phosphorylated to maltose 6-phosphate, which was recently demonstrated to be dephosphorylated by a novel maltose 6-phosphate phosphatase (MapP) in *Enterococcus faecalis*[9]. The *mapP* gene is located downstream from the enterococcal *malT* gene encoding the maltose-specific PTS. The chromosomal *malT* and putative *mapP* gene (SMU_2046c) arrangement in *S. mutans* is the same as that in *E. faecalis*. This suggests that extracellular maltose is also transported and subsequently metabolized as intracellular maltose in *S. mutans*.

Our group recently characterized the *malQ* and *glgP* genes, which encode 4-alpha-glucanotransferase and glycogen phosphorylase, respectively[10]. The MalQ protein catalyzes maltose and maltooligosaccharides, resulting in their conversion to glucose and different-sized malto-oligomers, while the GlgP protein degrades these oligomers from their non-reducing ends to produce glucose 1-phosphate (Fig. 1). This indicates that maltose- and ABC transporter system (MalXFGK)-derived maltooligosaccharides repeatedly enter an intracellular maltooligosaccharide pool to become substrates for the MalQ and GlgP proteins. Meanwhile, glucose and glucose 1-phosphate (G1P) act as substrates for enzymes in the glycolysis pathway, thus serving as energy sources (Fig. 1). It was demonstrated that, unlike GlgP, the MalQ protein was essential when maltose or maltooligosaccharide was

the only available carbon source. This suggests that, for glycolysis to take place, a kinase is essential to mediate reactions downstream of the MalQ enzyme. The *glk* gene in *S. mutans* was identified as a potential candidate for this. However, in characterizing the *glk* gene, which encodes glucokinase, it was found that this was not the case. Therefore, it was necessary to raise another hypothesis with regard to the phosphorylation mechanism for glucose, and the candidates were the PTSs underlying transportation of glucose as a substrate. One glucose-transporting PTS, the mannose-PTS, encoded by the *manL*, *manM*, and *manN* genes, has been reported as a high affinity system to glucose[2]. Growth of the *glk* and *manLM* double mutant was partially inhibited in the presence of maltose as the sole carbon source, and glucose was detected in the extracellular fluid of this mutant.

The possibility that a glucose-PTS substitutes for the glucokinase reaction in the maltose catabolism of *S. mutans* not only in the *glk*-negative condition, but also under physiological conditions, is discussed.

Materials and Methods

1. Bacterial strains

The *S. mutans* strains used were UA159[3] and its mutants cvU8 (*glk*), cmU1 (*manLM*), cmvU1 (*glk*, *manLM*), and blcmvU1 (*pgm*, *glk*, *manLM*). Streptococci were maintained on Todd-Hewitt (TH) broth/agar plates with or without appropriate antibiotics. *Escherichia coli* strain TOP10 was used as a host with the vector pBAD/HisA for the expression of the cloned gene as a N-terminal histidine-tagged protein.

2. PCR amplification of fragments to express or inactivate specific genes in *S. mutans*

The PCR primers used in this study are listed in Table 1. All amplification reactions were carried out with high fidelity DNA polymerase, KOD-Plus (Toyobo, Osaka, Japan), without terminal deoxynucleotidyl transferase activity. Regions corresponding to the *glk* gene in strain UA159 were amplified with the primer set expglk5/expglk3. Amplified fragments were purified with the PureLink Quick PCR Purification Kit (Invitrogen, Lohne, Germany), digested with *Xho*I, and subcloned into *Xho*I/*Pvu*II-double digested pBAD/HisA. Splicing was performed by the overlapping extension method[7] to construct the linear fragments used for transformation of *S. mutans*, in which a markerless mutagenesis method reported by Xie *et al.*[17] was employed to construct some mutants. Briefly, these markerless mutants were constructed by a two-step transformation procedure using the IFDC2 cassette containing the negative- and positive-selection markers (p-Cl-Phe^s and Em^r) for the first step screening and the two homologous upstream and downstream fragments directly ligated together without the intervening IFDC2 cassette for the second step. The resulting markerless mutants were p-Cl-Phe^r and Em^s. Gene arrangements at the target sites in these mutants were confirmed by PCR amplification.

3. Monitoring growth of *S. mutans* and preparation of culture supernatant

Growth of *S. mutans* strains and mutants in BTR-sugar broth[11] was measured at an optical density (OD) of 660 nm with the Ultrospec 500 pro spectrophotometer (GE Healthcare Life Sciences, Uppsala, Sweden). Values of OD660 nm were recorded at 1-hr intervals following inoculation of cultures into screw-capped glass tubes containing BTR-sugar broth. Sugars included 2.75 mM maltose or 5.5 mM glucose. Aliquots of BTR-maltose broth cultures at appropriate growth points were collected and centrifuged. The supernatant was filtrated through a disposable membrane filter unit Dismic-03CP045AN (Advantec, Tokyo, Japan) to obtain samples.

4. Preparation of permeabilized cells of *S. mutans*

Cells from the BTR-maltose or glucose broth culture at the mid-exponential phase (OD660 nm, 0.4–0.5) were harvested following centrifugation, washed twice, and sus-

Table 1 Primers used in this study

Primer designations	Sequences (5′>3′)	Purpose or target region	Reference
expglk5	ATCTCGAGGCTAAGAAACTTTTAGGGATTGATC	*E. coli glk* clone	This study
expglk3	GAAATGATAGAGATAATTGACATAATTTTCCT	*E. coli glk* clone	This study
ldhF	CCGAGCAACAATAACACTC	IFDC2 amplification	17)
ermR	GAAGCTGTCAGTAGTATACC	IFDC2 amplification	17)
dpr51	GGCACATGGGATAAATCAATAACT	*S. mutans glk* IFDC2 and markerless mutants	This study
3Tglk5R	GCTAAATGACGTGCGTAAAATCAATTCAGCA	*S. mutans glk* IFDC2 mutant	This study
5Tglk3F	ATTTTACGCACGTCATTTAGCAGAAGA	*S. mutans glk* IFDC2 mutant	This study
glk31	ACACGAAAATAATTCCAAACAAA	*S. mutans glk* IFDC2 and markerless mutants	This study
3Tglk5R	GCTAAATGACGTGCGTAAAATCAATTCAGCA	*S. mutans glk* markerless mutant	This study
5Tglk3F	ATTTTACGCACGTCATTTAGCAGAAGA	*S. mutans glk* markerless mutant	This study
manL50	ATTAAACGGAAAAACACAACACAATAA	*S. mutans manLM* kan^r mutant	This study
kanTmanL3	GGGTTTATCCGGGATCCTGGCGATAACGATTCCGA	*S. mutans manLM* kan^r mutant	This study
kanF	GGATCCCGGATAAACCCAG	*S. mutans manLM* kan^r mutant	This study
kanR	GCGGATCCCGAGCTTTT	*S. mutans manLM* kan^r mutant	This study
kanTmanM5	AGCTCGGGATCCGCTCACTCAACTGGTAAAACCAT	*S. mutans manLM* kan^r mutant	This study
manM30	GCAATGGTAATACCTTTTTGTGAAAA	*S. mutans manLM* kan^r mutant	This study
pgm51	GCTTATGCTAAACTTCCCGA	*S. mutans pgm* Em^r mutant	This study
EmTpgm5R	GATACTGCACTATCAACACACTCTTCACCATAAACTTTGTAACCA	*S. mutans pgm* Em^r mutant	This study
Em50	AAGAGTGTGTTGATAGTGCAGTATC	*S. mutans pgm* Em^r mutant	This study
Em30	GGCGCTAGGGACCTCT	*S. mutans pgm* Em^r mutant	This study
EmTpgm3F	AGAGGTCCCTAGCGCCGAAAATCAAATTCTACAT	*S. mutans pgm* Em^r mutant	This study
pgm31	GATACACGAACAGAAATCTTGGT	*S. mutans pgm* Em^r mutant	This study

Underlined sequences are those of added nucleotides for restriction endonuclease reactions or those necessary for splicing by overlapping extension method.

pended in 1/100 volume of 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM 2-mercaptoethanol. Cell density was adjusted to approximately 30 (OD660 nm) and permeabilized with toluene according to the method reported by Vadeboncoeur and Trahan[14].

5. Preparation of supernatant samples to determine released glucose from intact and permeabilized cells suspended in buffer containing maltose

The permeabilized cells, as well as intact cells, were suspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 13.75 mM maltose at an OD660 of 3 and incubated at 37°C for 30 min. Cells were centrifuged immediately after the incubation period and the supernatant filtrated as described above as an extracellular fluid sample for the glucose assay.

6. Enzyme assays for glucokinase activity and released glucose from cells

NADP-linked glucose-6-phosphate dehydrogenase (G6PDH) enzyme reactions were conducted throughout these assays[10]. Enzyme assays for glucokinase activity in an *E. coli glk* clone, *S. mutans* UA159, and its *glk* mutant were performed in a 1-ml cuvette containing 80 mM TrisCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM NADP, 2 IU G6PDH (Oriental Yeast Co., Ltd., Tokyo, Japan), 5.5 mM glucose, and a crude extract. The cuvette was pre-warmed in a cuvette holder (JASCO HMC-358, JASCO Corporation, Tokyo, Japan) incorporated in

Table 2 Glucokinase activities of *E. coli glk* clone and *S. mutans glk* mutant

E. coli		*S. mutans*	
pBAD/HisA (vector)	ZFG7 (*glk* clone)	cvU8 (*glk*)	UA159 (wild type)
0.111 ± 0.019*	22.5 ± 5.9*	0.00753 ± 0.00269**	0.573 ± 0.123**

(IU)

Crude extract added in cuvette: ZFG7 (*E. coli glk* clone), 1.8–4.7 μg; pBAD/HisA (*E. coli* vector clone), 5.1–10.6 μg; cvU8 (*S. mutans glk* mutant), 34–49 μg; UA159 (wild type), 37–74 μg. Mean ± SD. Data represent results from three or four independent experiments.
*$p<0.05$, **$p<0.01$

a spectrophotometer (JASCO U-660) and reactions commenced with addition of 0.5 mM ATP or other potential phosphate donors. Changes in absorbance (ΔA) at 340 nm due to generation of NADPH in the cuvette were recorded. An initial rate of ΔA/min was determined by using the enzyme activity measurement program supplied with the spectrophotometer.

Glucose in aliquots of culture supernatant or cell-suspension was also spectrophotometrically determined by the end-point method of ΔA at 340 nm due to generation of NADPH in the assay mixture, as recommended by the supplier of the enzymes (Oriental Yeast Co., Ltd.). The standard enzyme reaction mixture for glucose-assays comprised 80 mM TrisCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM NADP, 0.5 mM ATP, 20- or 100-μl aliquots of supernatant, and 2 IU G6PDH. Glucose assays were started with the addition of 2 IU hexokinase. Aliquots of culture supernatant containing maltose were treated with 0.1 IU/100 μl yeast alpha-glucosidase (Oriental Yeast Co., Ltd.) at 37°C for 10 min; glucose derived from maltose and pre-existing glucose from before glucosidase treatment was determined as total glucose. The amount of maltose was calculated following subtraction of pre-existing glucose from total glucose.

7. Statistical analysis

A paired analysis with the student *t*-test was performed using the Microsoft Excel program.

Results

1. Glucokinase activities of *E. coli glk* clone and *S. mutans glk* mutant

The *S. mutans* Glk protein was initially purified as a His-tagged protein. The purified protein was clearly visualized with Coomasie-stained SDSPAGE. However, no glucokinase activity was observed. The Glk protein was inactivated during the purification procedure. Therefore, crude extracts from both *E. coli* and *S. mutans* were used. Glucokinase activity assays were carried out immediately after sample preparation for *S. mutans* and within 24 hr of preparation for the *E. coli* extracts. An *E. coli glk* clone (ZFG7) exhibited a more than 100-fold glucokinase activity value compared with a mock clone harbouring vector plasmid pBAD/HisA ($p<0.05$, Table 2). The activity of the *S. mutans glk* mutant (cvU8) grown with maltose was significantly lower ($p<0.01$) than that of wild type strain UA159 (Table 2). These results suggest that the *S. mutans glk* gene encodes the glucokinase enzyme, and that there is no other gene expressing glucokinase activity in this organism.

Four-alpha-glucanotransferase encoded by the *malQ* gene is essential for maltose metabolism in *S. mutans*[10]. This enzyme degrades maltose, resulting in glucose, which requires glucokinase for metabolism *via* the Embden-Meyerhof pathway. Therefore, glucokinase also appears to be essential for maltose catabolism. However, one *S. mutans glk* mutant, cvU8, was able to grow in the presence of

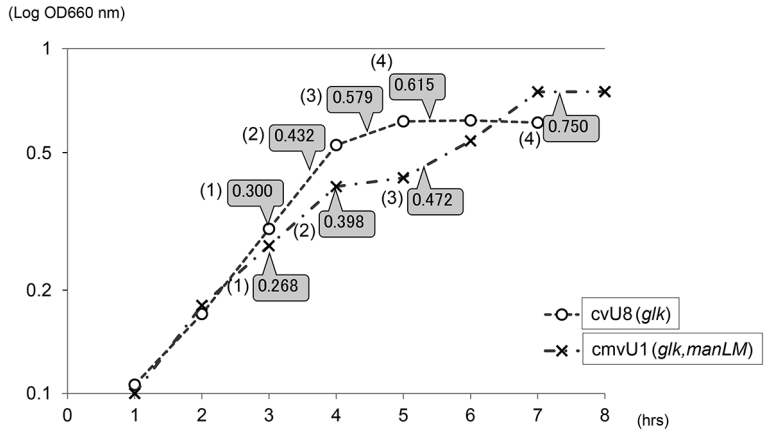

Fig. 2 Growth curves of *glk* mutant (cvU8) and *glk*, *manLM* double mutant (cmvU1)
Representative growth curves from 4 independent experiments. Cloud icons indicate time points and values of OD660 nm at which samples were obtained for glucose assays. Determined glucose concentrations of samples obtained at (1) to (4) cloud positions correspond to Table 3.

maltose as the sole carbon source, although its growth rate and yield were slightly lower than those of wild type UA159. Adenosine triphosphate was the sole phosphate donor for *S. mutans* glucokinase, and this activity in wild type strain UA159 was not detectable with any other high-energy phosphate compound, including guanosine triphosphate, polyphosphate, or phosphoenolpyruvate. This suggests another phosphorylation pathway for glucose.

2. Possible phosphorylation pathway for intracellular glucose

One candidate for the phosphorylation pathway for glucose was a glucose-PTS following intracellular glucose release by some unknown mechanism. Therefore, a mutation was introduced into the *manLM* gene, which encodes a major high-affinity glucose-PTS[2] in the *glk* mutant cvU8. The resultant mutant was designated cmvU1. When mutants cvU8 and cmvU1 were grown in BTR-maltose media, cmvU1 exhibited diauxic growth (Fig. 2), although the diauxie-like mechanism involved remains to be determined. Culture supernatant fluid was isolated at appropriate growth points and glucose concentration determined as described in Materials and Methods. The data in Table 3 corresponding to those in Fig. 2 indicate that very low concentrations of glucose were detected from the supernatants at any growth stage in cvU8. In contrast, glucose was detected in cmvU1 at the initiation of exponential growth and at subsequent plateau phases. This suggests that the *glk-manLM* mutant cmvU1 released glucose into the extracellular milieu and was not able to metabolize it further, and that mutant cvU8 transported the released glucose back into the cell with concomitant phosphorylation by the glucose-PTS.

In order to confirm the release of intracellular glucose, resting cells were prepared as well as permeabilized cells, as described in Materials and Methods. When permeabilized cells of cvU8 and cmvU1 were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 13.75 mM (corresponding to 27.5 mM glucose moiety concentration) maltose at 37°C for 30 min, approximately 4 mM glucose was detected in the extracellular fluids of both mutants (Table 4). No glucose was detected in the extracellular fluid of *malQ* mutant aeU1 permeabilized cells, indicating that these glucose molecules were generated from maltose by 4-alpha-glucanotransferase

Table 3 Extracellular glucose detected at (1)–(4) growth points in Fig. 3 (*S. mutans glk* mutants grown in BTR-maltose broth)

	(1)	(2)	(3)	(4)
cvU8 (Glucose mM)	0.109	0.128	0.132	0.125
cmvU1 (Glucose mM)	1.087	1.623	1.721	0.087
cmvU1 (Maltose mM*)	0.645	0.309	ND	ND

*Data are converted into glucose moiety mM concentrations. ND, not detected.

Table 4 Presence of extracellular glucose (mM) in *S. mutans glk* mutants

intact cells			permeabilized cells		
cvU8 (*glk*)	cmvU1 (*glk*, *manLM*)	cmU1 (*manLM*)	cvU8 (*glk*)	cmvU1 (*glk*, *manLM*)	aeU1 (*malQ*)
0.06±0.02*	2.49±0.30	0.17±0.07*	3.97±0.42	3.98±0.55	ND*

*Five-fold volumes of samples (100 μl) were used in assays. $p<0.05$. ND, not detected.

enzyme activity encoded by the *malQ* gene. Using intact resting cells under the same experimental conditions, the extracellular glucose concentration was 2.49 mM with mutant cmvU1 (*glk*, *manLM*). In contrast, the glucose concentrations of cvU8 (*glk*) and cmU1 (*manLM*) were 1/40 (0.06 mM) and 1/15 (0.17 mM) lower than that of cmvU1, respectively. These results reinforced our hypothesis concerning glucose release from the cells and the involvement of a glucose-PTS for phosphorylation of glucose.

3. Involvement of glycogen phosphorylase (GlgP)/phosphoglucomutase (Pgm) pathways in maltose metabolism

A maltose PTS encoded by the *malT* gene was induced and actively mediated maltose transport in mutant cmvU1. Table 3 shows that cmvU1 cells consumed almost all of the extracellular maltose up to its plateau phase. Therefore, the first growth phases of both mutants were supported by the catabolism of the imported maltose. To confirm this, we constructed a *pgm* mutant from the double mutant cmvU1 (*glk*/*manLM*). The resultant triple mutant, blcmvU1, did not grow in the presence of maltose, but grew with glucose, as shown in Fig. 3.

Discussion

Transport of maltose and maltooligosaccharide as starch-degradation products in *S. mutans* is mediated by salivary amylase in conjunction with the maltose-PTS (*malT*)[15] and binding protein-dependent ABC transport system (*malXFGK*)[8,16]. Maltose is predominantly taken up through the former system with concomitant phosphorylation, resulting in maltose 6-phosphate, which is presumed to be dephosphorylated by a putative maltose-6-phosphatase encoded by the SMU.2046c gene[3,9]. Intracellular maltose is then subsequently metabolized by 4-alpha-glucanotransferase encoded by the *malQ* gene, giving rise to glucose and maltooligosaccharides[10]. In order to catabolize intracellular glucose through the Embdem-Meyerhof pathway, it must be phosphorylated to glucose 6-phosphate by glucokinase (Fig. 1). In our preliminary study, we demonstrated that ATP was the sole phosphate donor in glucokinase activity encoded by the *glk* gene (SMU.542) in *S. mutans*, which differs to such activity encoded by *glk* in *Actinomyces naeslundii*[13]. Therefore, the *glk* gene may have been proposed to be essential, since no other pathway to intracellular phosphorylate glucose was

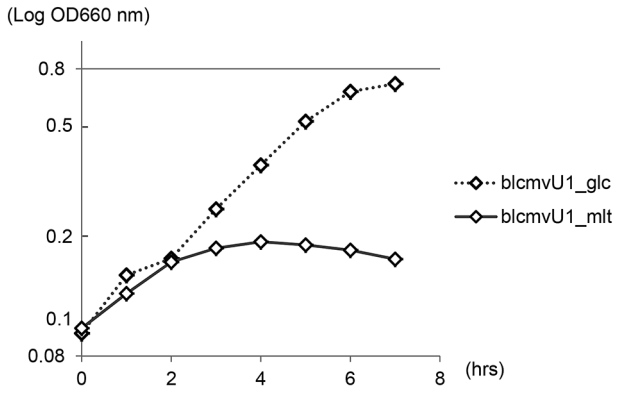

Fig. 3 Growth curves of *pgm, glk, manLM* mutant blcmvU1

Representative growth curves from 3 independent experiments. Mutant was grown in BTR-glucose (dotted line) or maltose (solid line) broth.

apparent. However, the present observation that the *glk* mutant grew in the presence of maltose as the sole carbon source questions this supposition.

In order to explain this inconsistency, it was hypothesized that a glucose-PTS pathway substituted for glucokinase activity, and extracellular glucose was therefore investigated. However, this necessitated the assumption of a mediator to facilitate glucose export from the cells or the compromise of membrane integrity in small populations of this organism. Examination of the *glk* and *manLM* double mutants revealed glucose in the supernatants of the BTR-maltose cultures and resting cell-suspensions containing maltose (Fig. 2 and Tables 3, 4). These results suggest that the detected extracellular glucose was released from the cells. It is possible that the mutant cvU8 (*glk*) took up glucose through the high affinity glucose-PTS encoded by the *manLMN* genes[1,2] immediately after release of glucose from the cells, which would explain why glucose was detected only at trace levels. In contrast, the double mutant cmvU1 (*glk*, *manLM*) was not able to transport glucose at low concentrations. Therefore, the released glucose accumulated until other glucose transport systems were activated to transport the sugar. This suggests that the detected glucose, therefore, was not the result of extracellular enzymatic activity, but rather of intracellular maltose, as no glucose was detected from the permeabilized *malQ* mutant, aeU1.

It was demonstrated that the glucose-PTS was able to substitute for the glucokinase reaction in maltose metabolism. Starch degradation products must constitute an important energy source for oral streptococci, including *S. mutans*, where carbohydrate energy sources are subjected to continual cycles of abundance and depletion. Therefore, these organisms may have developed multiple mutually complementary metabolic pathways involving these important carbohydrates, including maltose.

Small amounts of glucose were detected in the extracellular fluids of mutants cvU8 (*glk*) and cmU1 (*manLM*) (Table 4). Glucose assays on volumes (100 μl) initially obtained from these two mutants showed values close to the detection limit in contrast to a sample from the *glk/manLM* double mutant cmvU1. Therefore 500-μl samples of these two mutants were used for the assays. These values are small, but reproducible and consistent, suggesting that even *S. mutans* wild-type strains, as well as the *glk* mutant, release glucose and take it up through the ManLMN glucose-PTS when utilizing maltose under

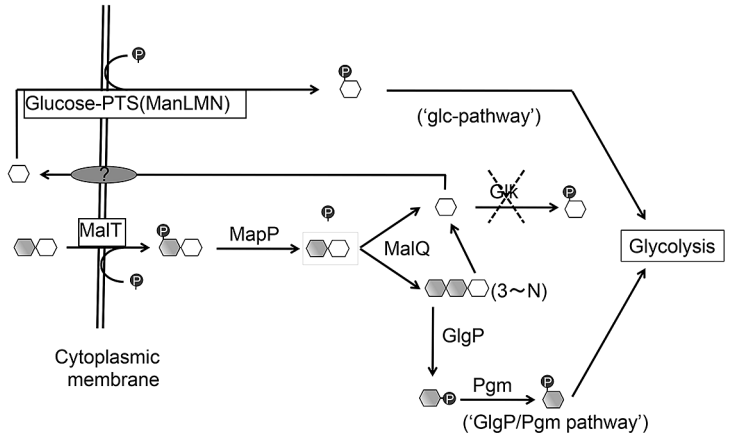

Fig. 4 Suggested pathways for phosphorylation of intracellular glucose in *glk* mutant from membrane transport to glycolysis

Triple hexagon as product of 4-alpha-glucanotransferase (MalQ) represents 3 to N multimers of maltooligosaccharides. Question mark indicates hypothetical transporter for glucose release from cells.

Hexagons and abbreviations are same as those in Fig. 1.

physiological conditions. This may explain why the detected glucose concentration in the extracellular fluid of cmU1 (*manLM*) was higher than that of cvU8 (*glk*) (Table 4, $p<0.05$). Release of glucose would be disadvantageous for this organism when the cells were in the planktonic state, which would be similar to the experimental conditions employed in this study. However, when cells are present in biofilm, released glucose may not immediately diffuse, but rather remain concentrated near those cells.

The growth curve of the mutant cmvU1 (*glk*, *manLM*) exhibited a diauxie-like pattern (Fig. 2). The second growth phase observed in cmvU1 may be regarded as the contribution of another glucose-PTS, the cellulose-PTS reported by Zeng and Burne[18], or an as yet unidentified glucose-PTS. However, further experiments will be necessary to elucidate the underlying mechanism of diauxic growth in cmvU1. It may also be necessary to determine what type of glucose transport system (PTS or non-PTS) is involved in the second growth phase of this mutant.

Concerning the generation of glucose and maltooligosaccharides by the MalQ protein[10], we tentatively designate the "glc-pathway" for the former and the "GlgP/Pgm pathway" for the latter (Fig. 4). Even though the glc-pathway was inhibited, as in the *glk* mutants, the GlgP/Pgm pathway would still remain active, supporting the first phase growth of cmvU1 with the generation of glucose 6-phosphate by glycogen phosphorylase and phosphoglucomutase. The mutant blcmvU1 (*pgm*, *manLM*, *glk*) did not grow in the presence of maltose, but grew well with glucose (Fig. 3). This suggests that the GlgP/Pgm pathway is involved in first-phase growth of the *manLM*, *glk* mutant (cmvU1) in the presence of maltose as the sole carbon source. Taken together with the diauxic growth of cmvU1 in the presence of maltose, these results suggest that the first phase growth of cmvU1 was supported by extracellular maltose and intracellular maltooligosaccharides, primarily mediated by the MalQ enzyme and GlgP/Pgm pathway, and that the second growth phase was supported by extracellular glucose derived through the glc-pathway. In addition, maltooligosaccharide generated by the MalQ enzyme may function as a short-term energy reservoir in contrast to glycogen-like intracellular polysaccharides,

which may serve as a long-term energy reservoir[5]. There are two genes, *glgP* and *phsG*, annotated as glycogen phosphorylase on the distinct genome loci of *S. mutans*. GlgP and PhsG glycogen phosphorylase may be physiologically involved in the degradation of maltooligosaccharide and glycogen-like polysaccharides, respectively, as the *phsG* gene is located in a cluster including other genes which participate in the synthesis of glycogen-like polysaccharides[3,5,12].

In this study, it was proposed that, once released from the cell, glucose was taken up by a glucose-PTS not only in mutants with a *glk*-negative background, but also in *glk*-positive organisms, although molecular evidence for the involvement of a glucose-PTS in the uptake of glucose remains to be obtained. This suggests that a glucose-PTS contributes to the metabolism of maltose, even under physiological conditions. A mediator for the transport of glucose from the cell to the extracellular environment also remains to be identified. Further study, therefore, will be needed to investigate the role of membrane integrity in full cell populations in this respect. Further work is needed to clarify these remaining questions.

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