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Characterization of a maltose transport system in *Clostridium acetobutylicum* ATCC 824

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The utilization of maltose by *Clostridium acetobutylicum* ATCC 824 was investigated. Glucose was used preferentially to maltose, when both substrates were present in the medium. Maltose phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) activity was detected in extracts prepared from cultures grown on maltose, but not glucose or sucrose, as the sole carbon source. Extract fractionation and PTS reconstitution experiments revealed that the specificity for maltose is contained entirely within the membrane in this organism. A putative gene system for the maltose PTS was identified (from the *C. acetobutylicum* ATCC 824 genome sequence), encoding an enzyme II^{Mal} and a maltose 6-phosphate hydrolase. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 298–306.

Keywords: phosphotransferase system; maltose 6-phosphate hydrolase; *malPH*

Introduction

The possibility of the large-scale reintroduction of the traditional acetone–butanol–ethanol (ABE) fermentation industry has appeared increasingly more feasible in recent times [4,5]. As with other biotechnology industries (such as industrial enzyme production), a key aspect to the success of future ABE production plants will be the reliable and maximal conversion of a cheap substrate into product. In this respect we have considered the significance of substrate utilization in solventogenic clostridia. It has long been recognized that bacteria possess the capacity to preferentially utilize one substrate over another and have evolved numerous complex and often interactive mechanisms by which to achieve this goal. Solventogenic clostridia appear no different in this regard, and, as with other organisms, glucose appears to be a preferred substrate [9–11]. This has significant implications for the efficiency of bioconversion of growth substrates into product.

One source of cheap substrate is biological or agricultural waste, which can be high in starch content. Fermentation on starch-based media will generate maltose, which may either be further hydrolyzed to glucose, or, transported into the cell intact. Although little is currently known about maltose transport in solventogenic clostridia, numerous energy dependent mechanisms for maltose transport have been identified in other Gram-positive bacteria. For example, maltose is transported by a proton symport mechanism in *Bacillus licheniformis* [22,26], whereas in *Streptococcus bovis* maltose is transported *via* a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) [8]. The latter mechanism is of particular importance for anaerobic bacteria as it allows the concomitant transport and phosphorylation of a substrate, without the additional expenditure of ATP in a post-translocation phosphorylation reaction [7,13]. Furthermore, the PTS plays a significant role in gene regulation in other Gram-positive bacteria, such as *Bacillus subtilis* [3,6,18].

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The PTS is composed of two general cytosolic proteins, called enzyme I (EI) and HPr, as well as a substrate-specific enzyme complex called enzyme II (EII). The EII complex consists of three distinct functional domains, namely IIA, IIB and IIC. The IIA and IIB domains are involved in sequential phosphate transfer, whereas IIC is responsible for substrate translocation across the membrane. In a PTS reaction, phosphate is transferred from PEP to the substrate *via* EI, HPr and the EII domains. The product of the transport will be a phosphorylated substrate that must be metabolized further, and consequently PTS transport systems are generally associated with a product-specific enzyme or enzymes. PTS activity has been observed in a number of solventogenic clostridia [10]. We previously established the existence of a sucrose PTS in *Clostridium acetobutylicum* ATCC 824, where the EII^{Scr} is associated with a sucrose 6-phosphate hydrolase and a fructokinase. We further demonstrated both substrate induction and glucose-mediated catabolite repression of the *scr* operon, confirming that regulation of transport systems occurs in this organism [23]. In consideration of the potential significance of maltose utilization by clostridia growing on starch-based substrates, we also initiated a study of maltose transport in *C. acetobutylicum* ATCC 824, the findings of which are presented here.

Materials and methods

Organism and growth conditions

C. acetobutylicum ATCC 824 was maintained as a spore suspension at 4°C. Spores were resuscitated in reinforced clostridial medium (Oxoid) and working cultures were grown in clostridial basal medium, supplemented with the appropriate carbon source as described previously [23].

Assay of sugar concentration in culture supernatants

Culture samples (1 ml) were removed and centrifuged at stated intervals. Glucose concentration in supernatants was determined using a Sigma assay kit No. 510-A. Maltose was determined after hydrolyzing it to glucose as described previously [22].

Enzyme assays in cell-free extracts

The preparation and fractionation of cell-free extracts was by the method of Mitchell and Booth [12]. Sugar phosphorylation assays in cell-free extracts were carried out as described by Tangney *et al* [21], with radiolabelled substrate (α-D-maltose [U-^{14}C], ICN Biomedical Research Products, Basingstoke, UK) at a concentration of 0.2 mM. PTS activity was determined as the PEP-dependent phosphorylation of substrate. Protein concentration in cell extracts was determined by the microbiuret assay, as described by Zamenhof [34].

DNA computational analysis methods

Data bank searches were performed using the BLAST service at the National Center for Biotechnology Information [1]. The *C. acetobutylicum* ATCC 824 *malPH* sequence was identified from the genome sequence deposited by the Genome Therapeutics, Waltham, MA, USA. Alignments were produced using the program GeneJockey.

Nucleotide sequence accession number

The DNA sequence data described in this paper have been deposited in GenBank with the accession number AF290982.

Results

Sugar utilisation by C. acetobutylicum ATCC 824

Initial growth experiments established that *C. acetobutylicum* is capable of growth in minimal medium supplemented with maltose as the sole carbon source (data not shown). Experiments with pairs of substrates revealed that glucose is preferred over maltose as a substrate. In the experiment shown in Figure 1, cells, which were initially grown on glucose, were diluted into a medium supplemented with both maltose and glucose and the utilization of each substrate was followed as described in Materials and methods. Glucose was specifically depleted from the medium, concomitant with cell growth, whereas there was no perceptible utilization of maltose (Figure 1). Cultures that were pregrown on maltose also demonstrated preferential utilization of glucose when presented with both substrates (data not shown). We therefore conclude that glucose can regulate maltose metabolism in *C. acetobutylicum*.

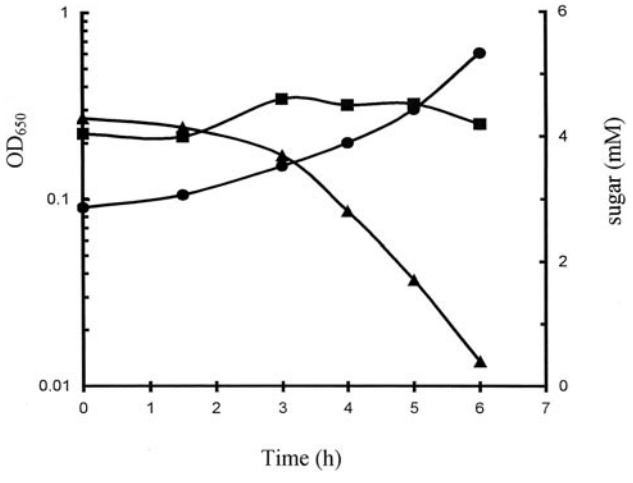

Figure 1 Culture growth and carbohydrate utilisation by *C. acetobutylicum* ATCC 824. Cells grown on glucose were inoculated into clostridial basal medium containing glucose (▲) and maltose (■). The OD_{650} (●) was monitored throughout growth, and the concentration of glucose and maltose in the culture supernatant was determined at intervals.

Sugar phosphorylation in cell-free extracts

To determine the mechanism by which maltose is transported in *C. acetobutylicum* we employed cell-free extracts in sugar phosphorylation assays. Extracts prepared from cultures grown on maltose as the sole carbon source were assayed for PEP-dependent phosphorylation of [^{14}C]maltose. PEP, but not ATP, stimulated phosphorylation demonstrating the presence of maltose PTS activity in this organism (Figure 2A). Maltose PTS activity was observed only in extracts prepared from cultures grown on maltose, but not glucose or sucrose (Figure 2B).

We have previously demonstrated that sucrose is transported *via* a PTS mechanism in *C. acetobutylicum* ATCC 824. Using combinations of purified membrane and cytosol fractions of appropriate cell-free extracts, we determined that the specificity for the substrate was contained within the membrane of the cell [23]. The absence of maltose PTS activity in glucose- and in sucrose-grown cells further allowed us to investigate the architecture of the maltose PTS by similar methodology. Extracts were prepared from cultures grown on maltose as the sole carbon source and the membrane and cytosol fractions of the cell-free extracts were then separated. Each fraction when assayed separately was devoid of maltose PTS activity; however, activity was reconstituted by combining the two cell fractions (Figure 3A). The purified membranes were then added to extracts prepared from either glucose- or sucrose-grown cells and the combinations were again assayed for maltose PTS activity. As shown earlier, neither extract alone possessed maltose PTS activity; however, the specific addition of membranes from maltose-grown cells was both sufficient and necessary to bestow maltose PTS activity upon extracts prepared from either sucrose- or glucose-grown cultures (Figure 3B). Thus the specificity for maltose PTS activity resides within the membrane in *C. acetobutylicum* ATCC 824.

Sequence analysis of the C. acetobutylicum ATCC 824 genome

The product of the maltose PTS will almost certainly be maltose 6-phosphate, which must be further metabolised by the cell. Maltose 6-phosphate could be hydrolysed by a 6-phospho-α-D-glucosidase. Typically, gene systems for disaccharide PTS substrates have a phosphoglucosidase (hydrolase) gene adjacent to the gene for the enzyme II. We have previously identified such a gene system for the sucrose PTS in *C. acetobutylicum*, where the *ScrB* gene (encoding the sucrose 6-phosphate hydrolase) lies downstream of the *scrA* gene (which encodes the enzyme II^{Scr}) in the *scrAKB* operon [23]. An analysis of the *C. acetobutylicum* genome sequence reveals the putative gene system depicted in Figure 4. The tandem ORFs, which we tentatively term *malP* and *malH*, encode putative enzyme II^{Mal} and 6-phospho-α-D-glucosidase proteins, respectively.

The two genes are in the same orientation, but in different reading frames, and are separated from each other by 80 bases. The genes are contained between two potential transcription terminators as shown in Figure 4. Upstream of the first gene, *malP*, is located a putative promoter region that is in good agreement with the

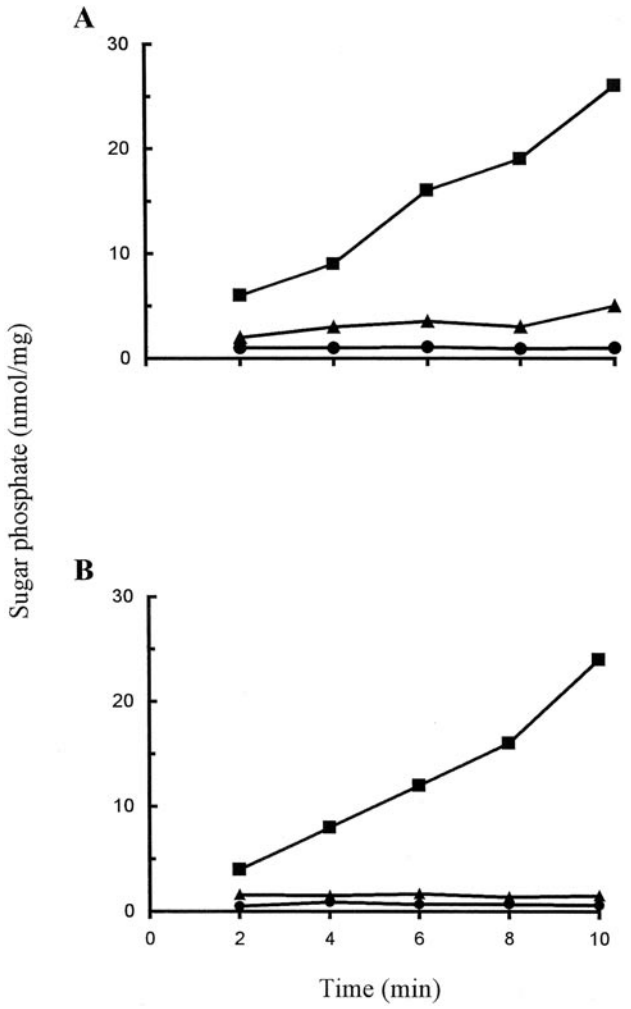

Figure 2 Maltose phosphorylation in cell-free extracts of *C. acetobutylicum* ATCC 824. (A) Extracts, prepared from cultures grown on maltose, were assayed for maltose phosphorylation with the following additions: 1 mM PEP (■); 1 mM ATP (▲); no addition (●). (B) Extracts prepared from cultures grown on maltose (■), glucose (▲), or sucrose (●) were assayed for maltose PTS activity in the presence of 1 mM PEP.

consensus clostridial promoter sequence [33]; with a −35 sequence "TTGAAA" and a −10 sequence "TAATAT" separated by 17 bases. Overlapping the putative −35 region is an imperfect palindrome that closely resembles a catabolite responsive element (CRE). In organisms such as *B. subtilis*, the CRE has been shown to be the target region for catabolite repression *via* the catabolite control protein, CcpA [3,6]. The potential CRE sequence at the putative −35 region has two mismatches relative to the proposed consensus sequence [31]. This genetic organization suggests that the *malP* and *malH* genes may be coordinately regulated and expressed as part of the same transcription unit.

Analysis of the predicted protein sequences, MalP and MalH

The *malH* gene was identified previously and the predicted protein has been assigned by Thompson *et al* [30] to Family 4 of the glycosylhydrolase superfamily on the basis of sequence homology. The gene is preceded by a putative ribosome binding site (rbs), AGGAGG, and encodes a product of 441 amino acids with a predicted molecular mass of 49 977 Da. The predicted protein is remarkably closely related to MalH of *Fusobacterium mortiferum* (with 81% identity and 91% conservation) and GlvA of *B. subtilis*

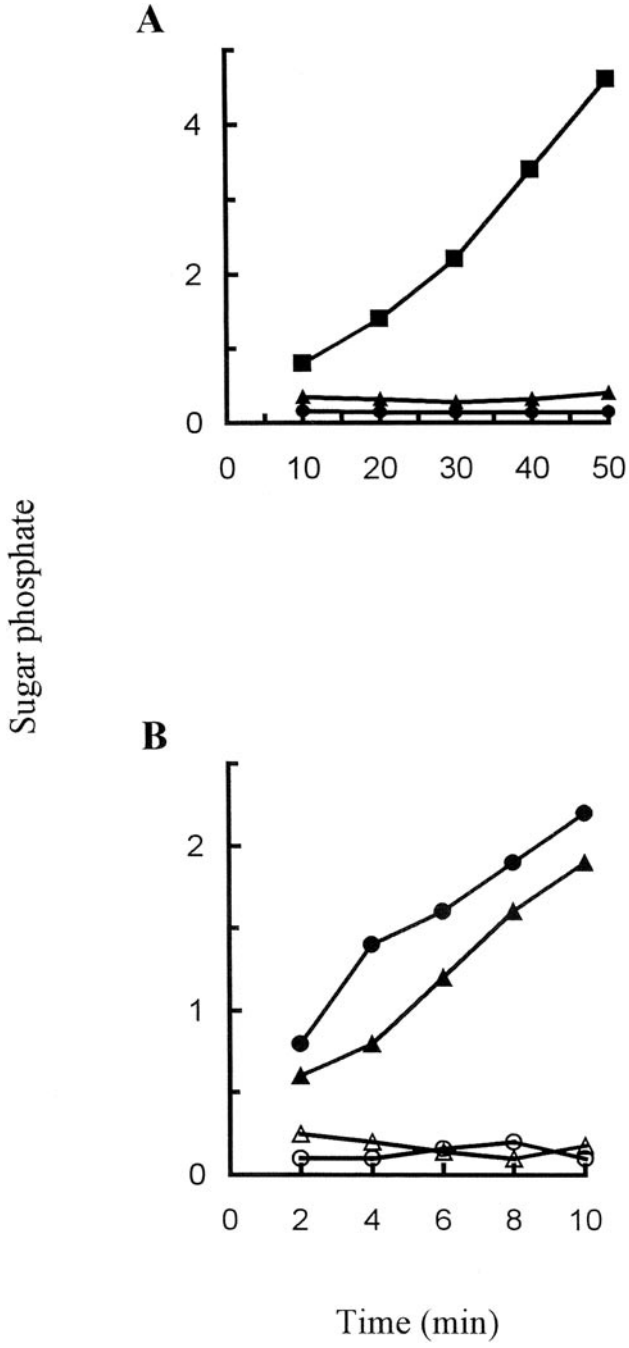

Figure 3 Maltose phosphorylation in reconstituted cell-free extracts of *C. acetobutylicum* ATCC 824. (A) Extracts prepared from cultures grown on maltose were fractionated into membrane and cytosol components. Purified membranes (●), cytosol (▲), or a combination of membranes and cytosol (■) were assayed for maltose PTS activity. Activity is expressed as nanomoles maltose phosphorylated. (B) Extracts prepared from cultures grown on glucose (▲,△) or sucrose (●,○) were assayed for maltose PTS activity in the presence (closed symbols) or absence (open symbols) of purified maltose membranes. Activity is expressed as nanomoles maltose phosphorylated per milligram cell extract protein.

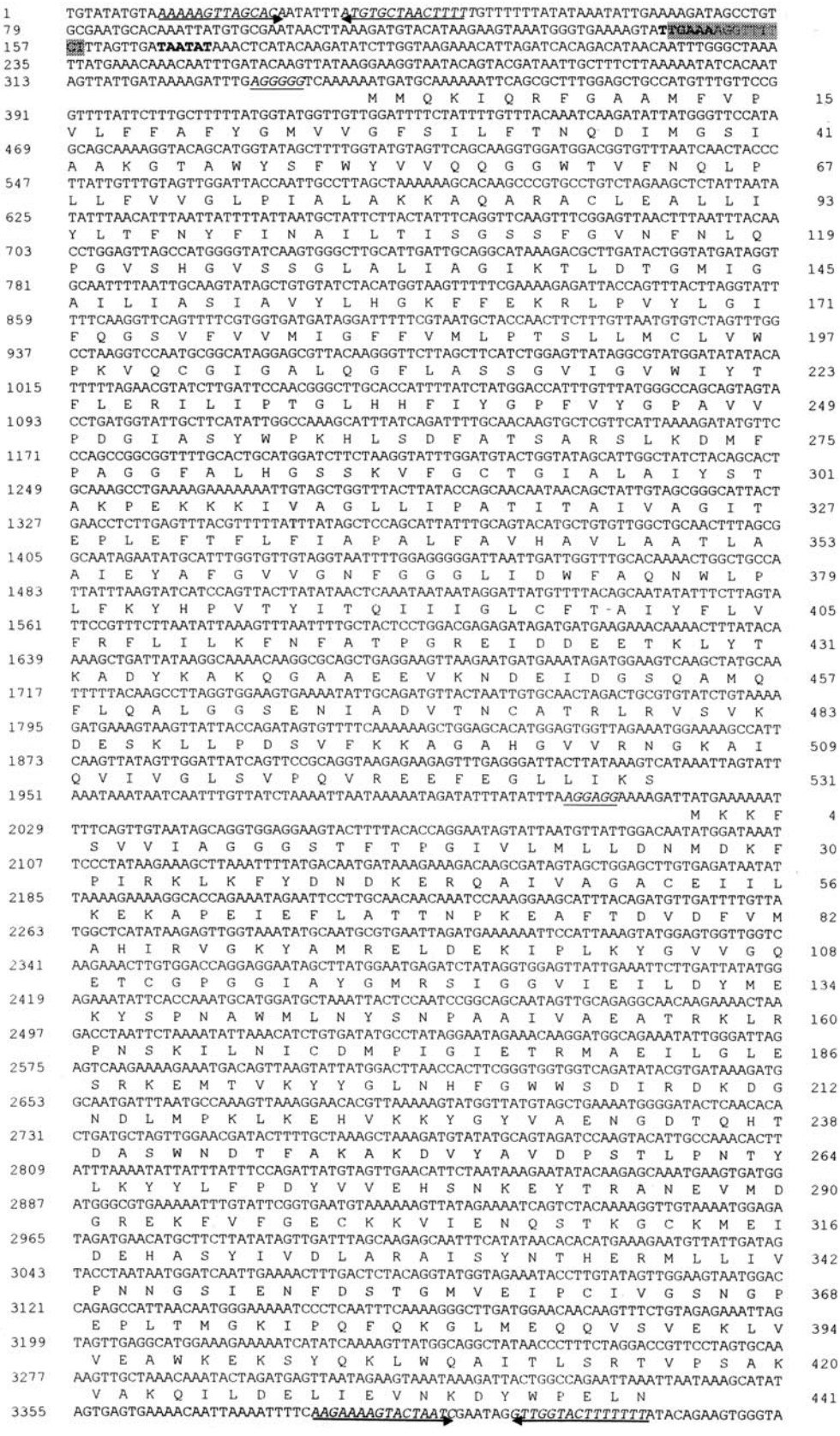

Figure 4 Nucleotide and deduced amino acid sequence of the *C. acetobutylicum* ATCC 824 *malPH* genes. The deduced amino acid sequence of the two ORFs is presented in single-letter code below the coding sequence. Putative ribosome binding sites are underlined and in italics. Putative −35 and −10 promoter sequences are in bold text; potential terminator sequences are in italics and indicated with arrows; a putative CRE sequence is shaded.

(with 75% identity and 88% conservation), both of which have been purified and shown to be 6-phospho-α-D-glucosidase enzymes that hydrolyse maltose 6-phosphate [2,29,30]. An alignment of the *C. acetobutylicum* MalH aa sequence with these well-characterized proteins shows that all three sequences are strikingly related to each other throughout their entire length (Figure 5), and provides compelling evidence that the *malH* gene from *C. acetobutylicum* also encodes a 6-phospho-α-D-glucosidase.

The *malP* gene is preceded by a putative ribosome binding site (rbs), AGGGGG (Figure 4). It encodes a protein of 531 amino acids with a predicted molecular mass of 57 684 Da. The deduced amino acid sequence has most significant homology to the enzyme II^{Glc} family of proteins (which includes the maltose transport proteins) having 65% and 59% identity to the GlvC proteins from *B. subtilis* and *Escherichia coli*, respectively [16,32]. By homology, and from computer analysis of the hydrophobicity/hydrophilicity profile (data not shown), the predicted *C. acetobutylicum* MalP domain structure is in the order IICB. The deduced MalP IIB domain also shares a striking 67% identity in an 80 amino acid overlap with the incomplete IIB^{Mal} sequence (MalB), which is encoded immediately upstream

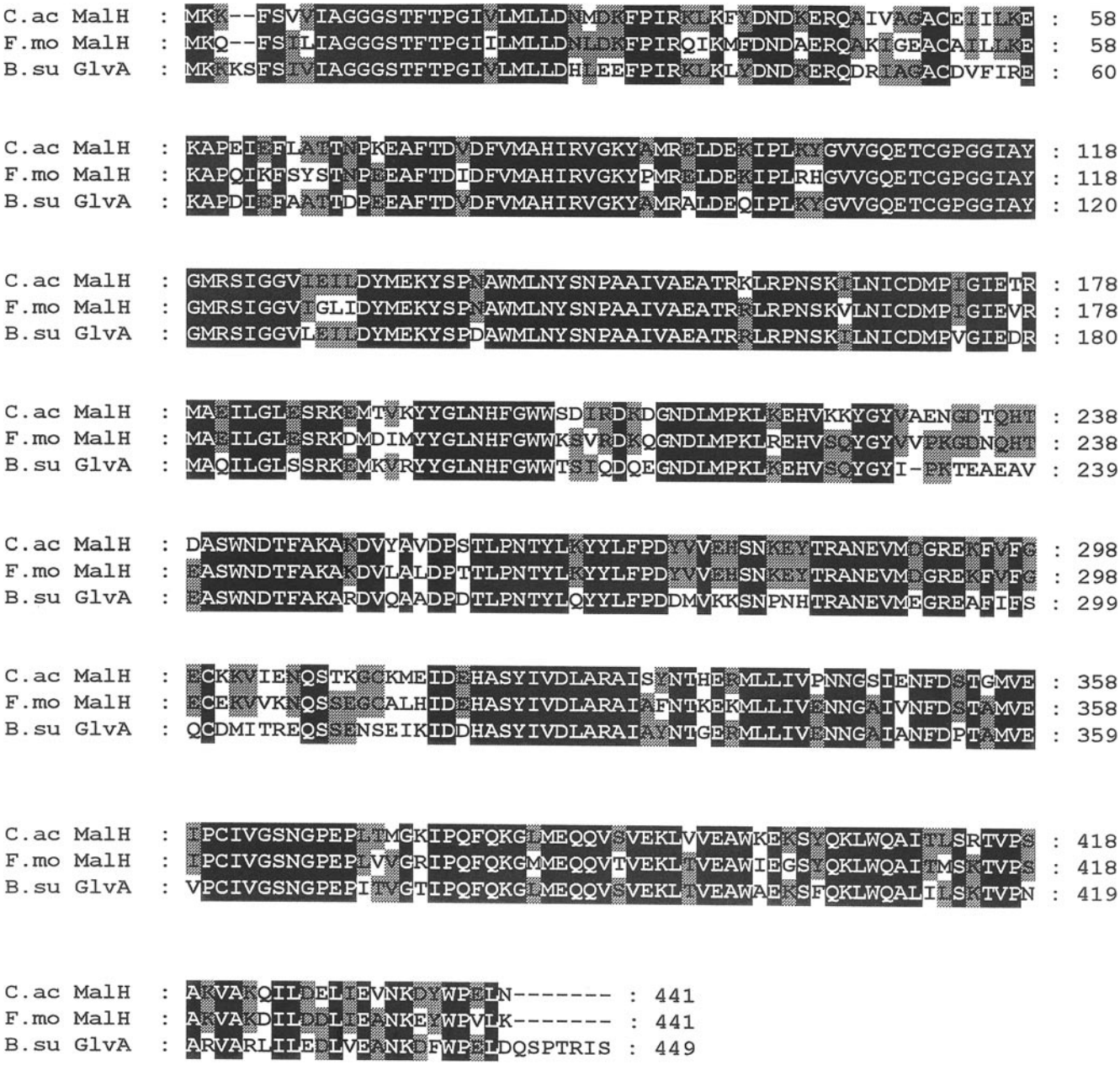

Figure 5 Multiple alignment of the *C. acetobutylicum* ATCC 824 MalH sequence with known maltose 6-phosphate hydrolases. The deduced amino acid sequence of the *C. acetobutylicum malH* is aligned with the MalH protein of *F. mortiferum* and the GlvA protein of *B. subtilis*. Dashes within the sequences indicate gaps giving optimal alignment. Identical residues are within black boxes and conserved residues (present in two of the three sequences) are shaded.

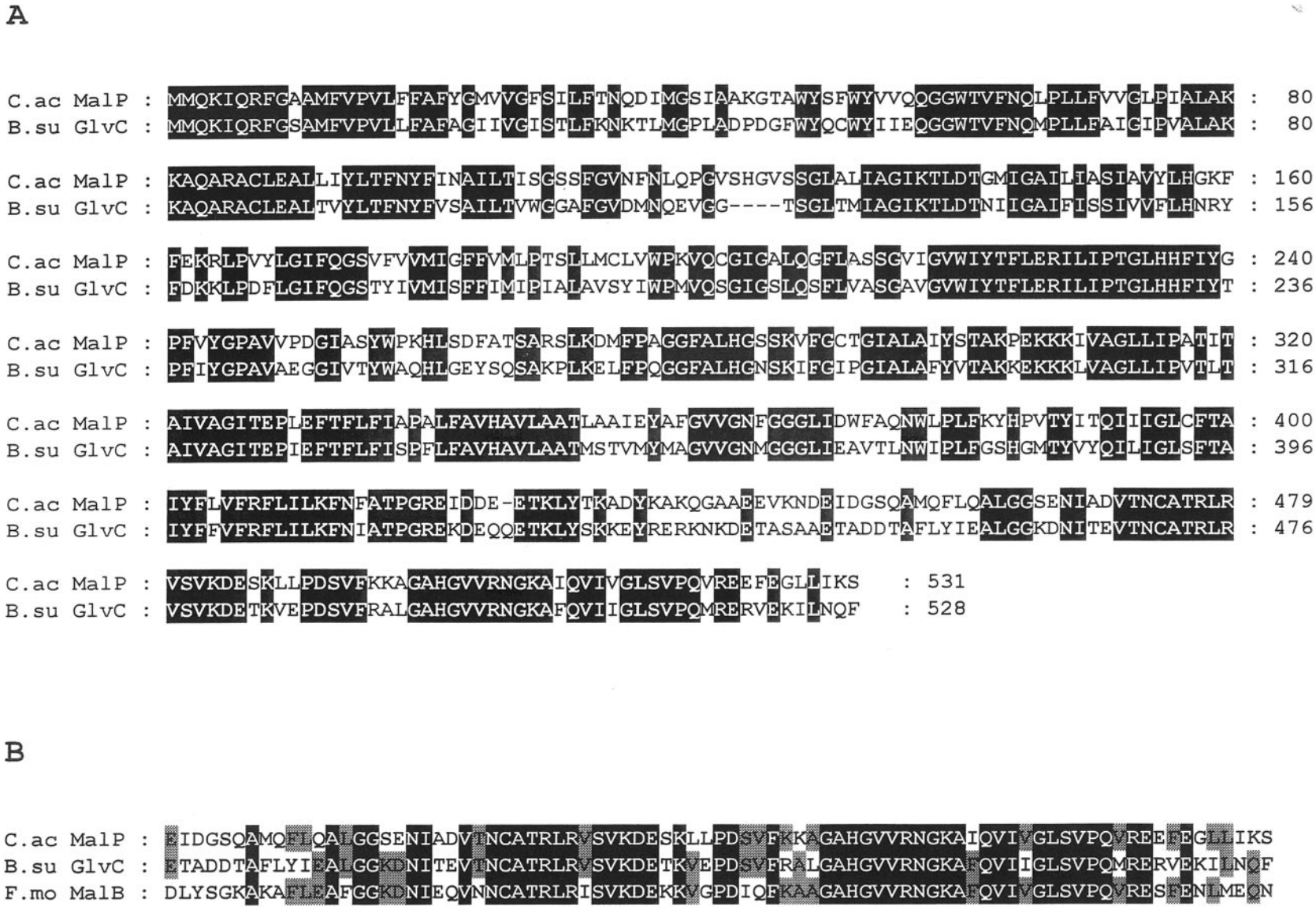

Figure 6 Sequence alignment of the *C. acetobutylicum* ATCC 824 MalP protein. (A) The deduced amino acid sequence of the *C. acetobutylicum* MalP is aligned with the full GlvC protein of *B. subtilis*. (B) Alignment of the partial MalB sequence of *F. mortiferum* with the equivalent regions of the two sequences aligned in (A). Dashes within the sequences indicate gaps giving optimal alignment. Identical residues are within black boxes. Conserved residues, present in two of the three sequences aligned in (B), are shaded.

of the *malH* gene in *F. moritiferum* [2]. An alignment of the GlvC from *B. subtilis* with the *C. acetobutylicum* protein is shown in Figure 6A, and the partial MalB sequence is included in an alignment with the equivalent regions (the IIB domains) of these proteins in Figure 6B. The results imply that the *malP* gene encodes an EII^{Mal}, which in association with *malH* is part of a two-gene maltose utilization system in *C. acetobutylicum*.

Discussion

Starch-based substrates have traditionally been used in the ABE fermentation and are once again attracting interest as the drive to reestablishing the industry gains momentum. In this context the metabolism of maltose is a significant consideration. Here we present for the first time a detailed analysis of maltose transport in a solventogenic *Clostridium*. We find that *C. acetobutylicum* ATCC 824 is capable of growth on maltose as a sole carbon source and has a PTS mechanism for maltose transport. This is only the second demonstration of a PTS mechanism in this strain, the first being a sucrose PTS [23]. Similar to other PTS operons, it is likely that the maltose PTS is inducible by its substrate, as we have only observed maltose PTS activity in maltose-grown cells. It is clear that the maltose PTS is subject to regulation by glucose, as was observed for the sucrose PTS in this organism [23]. This is consistent with the observation that glucose is a preferred substrate in other solventogenic clostridia, such as *Clostridium beijerinckii* [9–11].

Different regulatory mechanisms have been identified for substrate-mediated induction (or de-repression) of PTS operons in clostridia. For example, the *C. acetobutylicum scr* operon is induced *via* a BglG-type antitermination system [23], whereas the equivalent operon in *C. beijerinckii* NCIMB 8052 is negatively regulated by a repressor protein [14]. Both gene systems are subject to an overriding repression by glucose, but the mechanism(s) by which glucose-mediated catabolite repression is achieved in clostridia is not known. In *B. subtilis* a general system for catabolite repression has been proposed. Here the organism responds to the presence of a rapidly metabolized substrate, such as glucose, by phosphorylating the PTS component HPr at a regulatory serine residue *via* a metabolite-activated ATP-dependent kinase. In this state the HPr can interact with the catabolite control protein, CcpA, which effects repression by binding to specific imperfect palindromic DNA sequences, known as CRE sequences [3,6,18]. Interestingly, CRE-like sequences were identified in both of the clostridial *scr* gene systems [14,23]. We have identified, from the *C. acetobutylicum* genome sequence, all of the genes required for such a catabolite repression mechanism in this organism and have experimental evidence for ATP-dependent phosphorylation of HPr (Tangney *et al*, unpublished results). It is therefore significant that a putative CRE sequence was also identified in the proposed *mal* gene system presented in this work. That the CRE sequence overlaps the proposed −35 sequence of a putative promoter is also significant, as it is easy to envisage how the binding of a CcpA-like protein to this region could inhibit transcription of the downstream genes.

The *malPH* gene system was identified (on the basis of homology) from the *C. acetobutylicum* genome sequence. The linked EII and hydrolase gene system is typical of PTS operons. In *F. mortiferum* maltose is also transported *via* a PTS mechanism, where MalH catalyses the hydrolysis of the product of the PTS transport, maltose 6-phosphate [17,28]. The deduced amino acid sequence of the proposed *C. acetobutylicum malH* gene product is strikingly homologous to the *F. mortiferum* MalH protein, as well as the *B. subtilis* GlvA, both of which have been purified and demonstrated to hydrolyze maltose 6-phosphate [28,29]. It is therefore almost certain that the *C. acetobutylicum malH* gene encodes a similar function. In accordance with its role in maltose metabolism we deemed it appropriate to adopt the terminology used in *F. mortiferum*, and therefore designated this gene *malH*.

In *F. mortiferum* the *malH* gene is immediately preceded by an ORF that is thought to encode the associated EII^{Mal} protein of the maltose PTS in this organism [2]. Only a partial sequence (encoding 83 amino acids), is available but, as can be seen in Figure 6, it has significant homology to the proposed IIB domain of the *C. acetobutylicum* MalP. This suggests that the gene order is also conserved between the two organisms. In *B. subtilis* there is an associated gene believed to encode an EII^{Mal} (GlvC), which is related to the *C. acetobutylicum* MalP throughout its sequence and to the *F. mortiferum* MalB through its IIB^{Mal} domain (Figure 6). A *glvC* mutant of *B. subtilis* grew markedly slower on maltose (but not on glucose, glucosamine or *N*-acetylglucosamine), than does the wild type [15]. It was proposed that *glvC* should be renamed *malP* to signify that it is in fact part of a maltose PTS in *B. subtilis*, and accordingly we designated the *C. acetobutylicum* gene *malP*. It is notable that the *glvC* mutant is capable of growth on maltose as the sole carbon source, albeit at a reduced rate, demonstrating that an alternative pathway must exist for growth on this substrate. In a previous study we presented evidence for the likely existence of a protonmotive force (PMF)-dependent maltose transport system in *B. subtilis*; although under our experimental conditions we were unable to demonstrate maltose PTS activity, we did observe that a strain with a temperature sensitive mutation in the *ptsI* gene encoding enzyme I was unable to accumulate maltose at the nonpermissive temperature, confirming the involvement of the PTS in maltose transport in this organism [20]. Such multiplicity of transport systems for a single substrate is not unique in *Bacillus*, for example both a PMF-dependent glucose transport system and a glucose PTS transport mechanism are found in log phase cells of *B. licheniformis* [24], although only the PTS mechanism appears to be active in stationary phase cells [27].

In *B. subtilis, glvC* is separated from *glvA* by another gene, *yfiA* [32]. The function of YfiA is not known, but there is a homologous ORF upstream of *malP* in *C. acetobutylicum*. The predicted amino acid sequence of this ORF exhibits 36% identity to the *B. subtilis* YfiA protein. Both predicted proteins contain potential helix–turn–helix DNA-binding motifs and have low homology to putative transcriptional regulator proteins. It is conceivable that these proteins may play a role in the regulation of either or both of these gene systems. However, between the *yfiA* homolog and the *malPH* genes, we have identified a transcription terminator that is followed by a promoter (reading into the *malPH* gene system) as shown in Figure 4. The involvement therefore, if any, of the YfiA homolog in maltose transport in *C. acetobutylicum* remains to be established.

It should also be noted that a second gene system, which could potentially encode a maltose PTS, is also present in the *C. acetobutylicum* genome. A second putative hydrolase was also assigned by Thompson *et al* [30] to Family 4 of the glycosylhydrolase superfamily on the basis of sequence homology. However, in this case the predicted amino acid sequence has a relatively low

50% identity (65% conservation) to the *F. mortiferum* MalH — compared with the 81% identity (91% conservation) exhibited by the proposed *C. acetobutylicum* MalH; and 48% identity and 65% conservation to GlvA — compared to 75% identity (88% conservation) for the proposed MalH. Not surprisingly the gene is also associated with a gene encoding a putative EII, but this has only weak homology to the EII^{Glc} family. Furthermore this particular gene system encodes a unique IIA protein, which is inconsistent with our data concerning the architecture of the *C. acetobutylicum* maltose PTS.

We find no evidence for a separate IIA^{Mal} component in *C. acetobutylicum*. However, neither is there a IIA domain encoded by the *malP* gene. This gene organization is also found in *B. subtilis*, and the indication is that *F. mortiferum* will also have the same domain arrangement. The source of the IIA activity is therefore unknown. Interestingly, it has been shown that the IIA component of the glucose PTS can substitute for the IIA^{Scr} activity in sucrose grown cells in *B. subtilis* [19], and this has also been suggested to occur for the sucrose PTS in *C. beijerinckii* [23,25]. We have confirmed the existence of a glucose PTS in *C. acetobutylicum* (unpublished results) and it is therefore possible that the IIA^{Glc} provides this activity for the maltose PTS in this organism.

In conclusion, we present evidence for a maltose PTS in *C. acetobutylicum* ATCC 824 and identify a putative gene system encoding this transport system. The observation that maltose utilization is subject to catabolite repression by glucose has implications for substrate utilization when using starch as a growth substrate in the ABE fermentation. The identification of a strong candidate gene system for the maltose PTS highlights the usefulness of the availability of the *C. acetobutylicum* ATCC 824 genome sequence, and how it can potentially provide significant information when contemplating the genetic manipulation of strains to improve productivity.

Acknowledgements

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