Molecular analysis of the mannitol operon of 
Clostridium acetobutylicum encoding a 
phosphotransferase system and a putative 
PTS-modulated regulator

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Clostridium acetobutylicum DSM 792 accumulates and phosphorylates mannitol via a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). PEP-dependent mannitol phosphorylation by extracts of cells grown on mannitol required both soluble and membrane fractions. Neither the soluble nor the membrane fraction could be complemented by the opposite fraction prepared from glucose-grown cells, indicating that the mannitol-specific PTS consists of both a soluble (IIA) and a membrane-bound (IICB) component. The mannitol (mtl) operon of C. acetobutylicum DSM 792 comprises four genes in the order mtlARFD. Sequence analysis of deduced protein products indicated that the mtlA and mtlF genes respectively encode the IICB and IIA components of the mannitol PTS, which is a member of the fructose-mannitol (Fru) family. The mtlD gene product is a mannitol-1-phosphate dehydrogenase, while mtlR encodes a putative transcriptional regulator. MtlR contains two PTS regulatory domains (PRDs), which have been found in a number of DNA-binding transcriptional regulators and in transcriptional antiterminators of the Escherichia coli BglG family. Also, near the C-terminus is a well-conserved signature motif characteristic of members of the IIAR/IIAATIIANtr PTS protein family. These regions are probably the sites of PTS-dependent phosphorylation to regulate the activity of the protein. A helix–turn–helix DNA-binding motif was not found in MtlR. Transcriptional analysis of the mtl genes by Northern blotting indicated that the genes were transcribed as a polycistrionic operon, expression of which was induced by mannitol and repressed by glucose. Primer extension experiments identified a transcriptional start point 42 bp upstream of the mtlA start codon. Two catabolite-responsive elements (CREs), one of which overlapped the putative −35 region of the promoter, were located within the 100 bp upstream of the start codon. These sequences may be involved in regulation of expression of the operon.

Keywords: mtl genes, catabolite-responsive elements, PTS regulatory domain (PRD), carbohydrate accumulation

INTRODUCTION

During the first half of the twentieth century, the Clostridium acetobutylicum acetone-butanol (AB) fermentation was operated successfully on an industrial scale to produce commodity chemicals (Jones & Woods, 0002-4258 © 2001 SGM
1986). Although the fermentation subsequently declined for economic reasons, it remains as an example of the utility of biological production methods. The prospect of a revival of the process will be enhanced by a greater understanding of the genetics and physiology of the organism, leading to the ability to manipulate both the organism and fermentation conditions to advantage.

One of the attractions of the clostridia as industrial bacteria lies in their ability to utilize a wide range of carbohydrates as a source of carbon and energy (Dürre & Bahl, 1996). The industrial fermentations used maize mash (starch) or molasses as feedstock (Jones & Keis, & Bahl, 1996). The industrial fermentations used maize carbohydrates as a source of carbon and energy (Du

A major mechanism for carbohydrate accumulation in the clostridia is the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), which catalyses both uptake and phosphorylation of the substrate (Mitchell, 1998). It is clear from both functional studies and gene sequencing that the clostridial PTS is similar to the PTS of other bacteria (Mitchell, 1993; Mitchell, 1998; Tangney et al., 1998; Reid et al., 1999; Tangney & Mitchell, 2000). Thus, the PTS is composed of two general cytoplasmic proteins or domains, enzyme I (EI) and histidine-containing protein (HPr), which participate in the phosphorylation of all PTS carbohydrates, and a substrate-specific enzyme II typically comprising three domains (A, B and C), which are found as one or more polypeptide chains. The similarities shown by the clostridial system raise the possibility that, in addition to its role in carbohydrate accumulation, the PTS will also be involved in regulation of metabolism as has been clearly documented for other bacteria (Postma et al., 1993; Saier & Reizer, 1994; Saier et al., 1996). It is well established that catabolic operons in clostridia are subject to induction and repression (Mitchell, 1996, 1998; Tangney et al., 1998; Reid et al., 1999; Tangney & Mitchell, 2000) but the mechanisms responsible have not yet been described. Both catabolite-responsive elements (CRE) and catabolite control protein (CcpA), which contribute to a global mechanism of catabolite repression in low-GC Gram-positive bacteria (Saier et al., 1996), have been identified in clostridia (Davison et al., 1995; Mitchell, 1998; Reid et al., 1999; Tangney & Mitchell, 2000). However, their relevance in regulation of gene expression has not been verified experimentally, and in no case has the interplay between induction and repression mechanisms been investigated. A greater understanding of this aspect of clostridial physiology will require the molecular analysis of a range of genes and operons.

In this paper we present an analysis of the mannitol (mtl) operon of C. acetobutylicum DSM 792, which was isolated fortuitously during the screening of a genomic library containing partially Sau3A-digested DNA fragments in pUC9 (Gerischer & Dürre, 1990). The operon, which is induced by mannitol and repressed by glucose, encodes a mannitol PTS, the enzyme mannitol-1-phosphate dehydrogenase, and a putative regulator protein. The domain structure of the regulator, MtlR, suggests that it may be involved in PTS-dependent induction and repression mechanisms.

METHODS

Bacterial strains, plasmids and growth conditions. Clostridium acetobutylicum DSM 792 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was used throughout this study. Cultures were maintained under strict anaerobic conditions on Clostridium basal medium (O’Brien & Morris, 1971) at 37 °C when used as a source of genomic DNA. For the purpose of RNA preparation and transport studies, cells were grown on minimal medium containing, per litre: K$_2$HPO$_4$, 3H$_2$O, 0.5 g; KH$_2$PO$_4$, 0.5 g; MgSO$_4$·7H$_2$O, 0.4 g; NaCl, MnSO$_4$·H$_2$O, FeSO$_4$·7H$_2$O and p-amino benzoic acid, 10 mg each; biotin, 1 mg; ammonium acetate, 22 g; carbon source, 20 g. Carbon sources were sterilized separately and added to the medium to a final concentration of 2% (w/v).

Escherichia coli JM109 (Yanisch-Perron et al., 1985) was used as a cloning host, and the plasmids pUC9 ( Vieira & Messing, 1982) and pUC18 (Yanisch-Perron et al., 1985) served as vectors for cloning experiments. E. coli was propagated and manipulated using standard methods (Sambrook et al., 1989).

Preparation of cell extracts. Cultures were harvested at the mid-exponential phase of growth. Cells were centrifuged (10000 g, 10 min, 4 °C), washed and resuspended in 50 mM potassium phosphate buffer, pH 7.0 (4 ml per g wet weight); MgCl$_2$ and dithiothreitol (DTT) were then added to 5 mM and 1 mM respectively. Breakage of cells in a French press and preparation and fractionation of extracts into soluble extract and membranes by ultracentrifugation were as described by Mitchell & Booth (1984), with the modification that the membranes were concentrated twofold with respect to the original extract. Protein concentrations in extracts were estimated by a microburet method, with bovine serum albumin as the standard (Zamenhof, 1957).

Assay of mannitol phosphorylation. Sugar phosphorylation was assayed by precipitation of radiolabelled sugar phosphate in ethanolic barium bromide as described by Mitchell & Booth (1984). Standard assay mixtures contained, in a volume of 1.0 ml, 50 mM potassium phosphate (pH 7.0), 2 mM DTT, 5 mM MgCl$_2$, 12 mMKF, 0.1–0.3 mM cell extract, 0.1 mM D-[1-3H]mannitol [1 Ci (37×10$^{6}$) Bq] mol$^{-1}$], and 1 mM PEP or ATP. Reactions were started by addition of the substrate after 5 min preincubation at 37 °C. For glucose-inhibition experiments, 1 mM glucose was added simultaneously with the substrate. At timed intervals, samples (150 µl) were removed, added to 2 ml 1% (w/v) barium bromide in 80% (v/v) ethanol, filtered through glass-fibre discs (Whatman GF/F), and washed with 5 ml 80% (v/v) ethanol. Discs were dried under a heat lamp, and their radioactivity was measured in scintillation cocktail O (BDH Scintran). All experiments were done in duplicate.
DNA preparation and manipulation. Chromosomal DNA of *C. acetobutylicum* DSM 792 was isolated by the method of Marmur (1961) modified by Bertram & Dürr (1989). Plasmid DNA from *E. coli* was prepared by the method of Birnboim & Doly (1979) or with the Qiagen Midi Kit (Diagen). DNA was manipulated by standard methods (Sambrook *et al.*, 1989). Restriction enzymes were obtained from Gibco-BRL or Pharmacia LKB, and calf intestinal alkaline phosphatase was from Boehringer. All enzymes were used according to the manufacturer’s instructions.

Polymerase chain reactions (PCR) for the verification of internal restriction sites of the analysed DNA region and the generation of specific DNA probes were performed under the following conditions: predenaturation at 95 °C, 5 min and 85 °C, 5 min, followed by 30 cycles of 95 °C, 30 s (denaturation), 42–55 °C, 30–40 s (annealing), 72 °C, 30–90 s (amplification) and finally 72 °C for 5 min. Vent Polymerase (Biolabs) was added during the 85 °C step of predenaturation (hot start). Optimal primer annealing temperatures (*Tm*) were calculated as described by Chester & Marshak (1993).

**Construction and screening of genomic libraries.** DNA of *C. acetobutylicum* DSM 792 was digested to completion with the appropriate restriction enzymes. For partial gene banks the resulting DNA fragments were separated by sucrose density centrifugation (10–40%, w/v). Fractions containing DNA of the desired size range were dialysed against TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA) and ethanol-precipitated. DNA fragments were ligated to suitably digested and dephosphorylated vectors. Competent *E. coli* cells were transformed with the microdialysed ligation mixtures by electroporation in a Gene Pulser (Bio-Rad). White colonies on IPTG/X-Gal (48 µg ml⁻¹ and 40 µg ml⁻¹ respectively) plates, harbouring plasmids with an insert, were screened with the appropriate x-[^32P]dATP-labelled probe by colony hybridization. Inserts of positive clones were sized by restriction endonuclease digestion and were sequenced.

**DNA sequencing and sequence analysis.** DNA was sequenced by the dideoxy-chain termination method of Sanger *et al.* (1977) using [α-^32P]dATPα (Hartmann Analytic) and a T7 Sequencing kit from Pharmacia LKB or a Sequenase version 2.1 T7 DNA Polymerase Kit from USB. Plasmids were sequenced on both strands. Sequencing was started with commercially available M13/pUC universal sequencing forward primer and reverse primer. Oligonucleotides (17-mer) complementary to the ends of already sequenced templates were synthesized via a Gene Assembler Plus (Pharmacia LKB) according to the manufacturer’s instructions and used as primers for continued sequencing. The separation of the dideoxy-terminated fragments was attained on 55 cm wedge-shaped thickness gradient gels (0.2–0.4 mm, 6%, w/v, polyacrylamide) with a Macrophor sequencing unit (Pharmacia LKB) as recommended by the manufacturer.

The nucleotide sequence and the deduced proteins were analysed using DNA Strider Software on a Macintosh LCII computer. Sequence comparisons were done using the BLAST suite of programs (Altschul *et al.*, 1997) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). Multiple alignment of proteins was conducted using CLUSTAL W (Thompson *et al.*, 1994) and results of phylogenetic analysis obtained using the TreeView 1.5 software package (Page, 1996).

**RNA preparation and Northern blot analysis.** Total RNA was isolated from *C. acetobutylicum* DSM 792 by the hot phenol/chloroform procedure described by Oelmüller *et al.* (1990). To obtain higher yields of RNA, cells were not washed in acetate/EDTA buffer, and the extraction in 60 °C hot phenol/chloroform was extended to 10 min and repeated once.

For Northern blot analysis, RNA (10–20 µg) was electrophoresed in 1% (w/v) formaldehyde/MOPS agarose gels and transferred to nylon membranes (GeneScreen Plus; Dupont, NEN Research Products). An RNA ladder (0-24, 1-4, 2-4, 4-4, 7-5 and 9-5 kb; Gibco-BRL) was included as a standard for size determination. The DNA probes were radiolabelled with x-[^32P]dATP (Hartmann Analytic) by using a nick-translation kit (Gibco-BRL) and fragments were purified by column chromatography on Sephadex G-25. Prehybridization and hybridization were performed at 42 °C in the presence of formamide in a hybridization solution containing 25% (w/v) dextran sulfate, 50% (v/v) formamide, and 1% (w/v) SDS. Prehybridization for 2-6 h was followed by hybridization in the presence of the radiolabelled denatured probe (0.2–0.5 Ci ml⁻¹) for 15–20 h. Finally the membranes were washed twice in 2 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min at room temperature and subjected to autoradiography. If necessary the blot was washed twice in 2 x SSC, 1% (w/v) SDS at 55 °C for 30 min and twice in 0.1 x SSC at room temperature for 30 min.

**Primer extension analysis.** Transcriptional start sites were determined by primer extension analysis using oligonucleotides (17-mer) complementary to the 5' ends of the respective primary transcript. Radiolabelling of 2 pmol oligonucleotide (17-mer) was performed in a total volume of 10 µl in the presence of 10 µCi [γ-^32P]ATP (Hartmann Analytic), 10 units T4 polynucleotide kinase (Gibco-BRL) and PNK buffer according to the manufacturer for 1 h at 37 °C. The reaction was stopped by heating for 2 min at 95 °C.

For the annealing reaction, total RNA (10 µg) was incubated with 0.2 pmol labelled oligonucleotide and 12.5 units RNAseIn (Gibco-BRL) in 500 mM KCl/20 mM Tris/HCl (pH 7.9) buffer for 3 h at 30 °C in a final volume of 10 µl. The primer extension reaction was initiated by adding 10 µl reverse transcriptase buffer (Gibco-BRL), 0.5 µl 0.1 mM dithiothreitol (DTT), 10 µl of a 10 mM dNTP mix, 2.5 µl actinomycycin (5 mg ml⁻¹), and 200 units Superscript® RNaseH⁻ reverse transcriptase (Gibco-BRL) to the annealing mixture and incubated for 1 h at 37 °C. The resulting RNA–DNA hybrids were extracted with phenol/chloroform, ethanol-precipitated, and analysed on 6% (w/v) urea sequencing gels. The length of each primer-extension product was calculated by running a sequencing reaction with the identical primer on the same gel.

**RESULTS**

Mannitol transport and phosphorylation in *C. acetobutylicum* extracts

Crude extract of *C. acetobutylicum* DSM 792 prepared from cells grown on mannitol was assayed for mannitol phosphorylation in the presence of either ATP or PEP. The results (Fig. 1a) clearly indicate that phosphorylation of mannitol is dependent on the availability of PEP, whereas mannitol was not phosphorylated in the presence of ATP. Extracts prepared from cells grown on glucose were completely inactive for mannitol phosphorylation. To further confirm that PEP-dependent phosphorylation was due to a PTS system specific for mannitol, soluble and membrane fractions were...
prepared and examined in phosphorylation assays. While each fraction alone was inactive (not shown), restoration of PEP-dependent mannitol phosphorylation was achieved following reconstitution of the complete extract (Fig. 1b). On the other hand, as expected no phosphorylation activity was observed when the combination of soluble extract and membranes from cells grown on mannitol or glucose were assayed for PEP-dependent phosphorylation of mannitol. Mannitol membranes plus mannitol soluble extract; △, glucose membranes plus mannitol soluble extract; ○, mannitol membranes plus glucose soluble extract.

**Fig. 1.** Mannitol phosphorylation in cell extracts of *C. acetobutylicum* DSM 792. (a) PEP-dependent phosphorylation of mannitol. Crude extracts from cells of *C. acetobutylicum* grown on mannitol as sole carbon source were assayed for phosphorylation of mannitol with addition of 1 mM PEP (△) or 1 mM ATP (○). Extracts of glucose-grown cells were assayed in the presence of 1 mM PEP (●). (b) Membranes and soluble extracts from cells of *C. acetobutylicum* grown on mannitol or glucose were assayed for PEP-dependent phosphorylation of mannitol. Membranes plus soluble extract; ○, mannitol membranes plus mannitol soluble extract; △, mannitol membranes plus glucose soluble extract.

Taken together, the results show that *C. acetobutylicum* DSM 792 contains a mannitol PTS involved in uptake and phosphorylation of this substrate. The fact that neither the soluble nor the membrane fraction of glucose-grown extracts is capable of supporting mannitol phosphorylation in the presence of the opposite fraction from mannitol-grown extracts indicates that each lacks a component of the mannitol-specific enzyme $\text{II}^\text{M1}$.

**Cloning and sequencing of mannitol PTS genes of *C. acetobutylicum***

Sequence analysis of a 2.6 kbp *Sau*3A fragment in the plasmid pSB30, belonging to a genomic library of *C. acetobutylicum* DSM 792, revealed the presence of an ORF which encoded a protein with a deduced amino acid sequence showing significant similarity to the IIA$^\text{M1}$ protein of mannitol-specific PTSs of several Gram-positive and Gram-negative bacteria. The availability of this clone provided the opportunity for a genetic analysis of the mannitol PTS of *C. acetobutylicum*. Downstream of the identified ORF, which was tentatively designated *mtlF*, was the 5′ end of another ORF (*mtlD*) predicted to encode a mannitol-1-phosphate dehydrogenase. Cloning of the complete *mtlD* gene was achieved by chromosome walking, resulting in the isolation of pSB31 from a subgenomic library of *C. acetobutylicum* containing HindIII-digested DNA fragments in pUC18. The DNA region upstream of *mtlF* was present on pSB32. The arrangement of all fragments resulting from digestion at internal *Sau*3A sites (Fig. 2) was confirmed to be reflected on the bacterial chromosome by Southern hybridization of digested chromosomal DNA and by PCR (data not shown). The complete DNA sequence of the *mtl* gene region of *C. acetobutylicum* DSM 792, which is shown schematically in Fig. 2, has been submitted to GenBank (accession number U53868).

The DNA locus reported here comprises four complete ORFs (*mtlARFD*) closely spaced or overlapping (*mtlRF*) one another. A further ORF, designated *orfP* and truncated at its 5′ end, was identified 260 bp upstream of these genes. Putative ribosome-binding sites with reasonable homology to others found in clostridia (Young et al., 1989) could be identified upstream of all complete ORFs, although the site upstream of *mtlR* (5′-GGTGAG) was somewhat unusual. A search for secondary structures at the mRNA level resulted in the detection of several hairpin-loops with free energy values ranging between $-26$ and $-96$ kJ mol$^{-1}$ [calculated with the computer program FoldRNA (Zuker, 1989) according to the determination of Freier et al. (1986)]. The strongest stem–loop structure ($-95.8$ kJ mol$^{-1}$) is located 12 bp downstream of *orfP* and is followed by a sequence of several Ts, thus probably forming a prokaryotic rho-independent transcription terminator (Platt, 1986). Further hairpin structures, with free energy
values of $-48 \text{ kJ mol}^{-1}$ or less, were identified within the mtlA, mtlR and mtlF genes, and two were found downstream of mtlD ($-26 \text{ kJ mol}^{-1}$ and $-33 \text{ kJ mol}^{-1}$). These structures do not resemble prokaryotic rho-independent terminators. Interestingly, two copies of a 10 bp sequence (5'-'TAGTTAGGAGC') with the same orientation and spaced by 20 bp were found to follow the first stem–loop in mtlA, at a distance also of 20 bp. Two nucleotide sequences with high homology to the catabolite-responsive element (CRE) of Gram-positive bacteria were identified 96 bp (CRE1, 5'-'TGAAAAACGTATACC) and 63 bp (CRE2, 5'-'TGAAAAACGTACGAA) upstream of the putative ATG start codon of the mtlA gene. The CRE2 sequence overlaps the proposed -35 region of the promoter (see Fig. 6).

Analysis of mtl genes in C. acetobutylicum

The deduced amino acid sequences of all ORFs in the mtl gene region of C. acetobutylicum were aligned to sequences available in protein databases. The products from the first and third complete ORF revealed high similarity (56% identity, 78% similarity and 45% identity, 66% similarity, respectively) to the mannitol-specific PTS components IICB$^{M}$ and IIA$^{M}$ of Bacillus stearothermophilus. Consistent with the genetic nomenclature of other phosphotransferases, these genes were named mtlA and mtlF. In addition, homology of MtlA (481 aa, predicted molecular mass 50.8 kDa) and MtlF (139 aa, predicted molecular mass 15.7 kDa) was demonstrated with the corresponding domains of other mannitol phosphotransferases including the MtlA proteins of E. coli, Bacillus subtilis and Klebsiella pneumoniae, which are single polypeptides with the domain structure IICBA. The histidine and cysteine residues at which IIA and IIB domains of the PTS are derivatized during PTS-mediated phosphoryl transfer are both conserved in MtlF (His64) and MtlA (Cys398) of C. acetobutylicum. Furthermore MtlA and MtlF are also homologous to fructose-specific PTS components, and MtlF showed a more distant relationship to IIA$^{N}$ proteins of Gram-negative bacteria. The IIA, IIB and IIC domains of mannitol- and fructose-specific phosphotransferases have been shown to belong to a single family of PTS permeases (Saier & Tseng, 1999), and MtlA and MtlF of C. acetobutylicum are clearly members of this family.

It has been proposed that all phosphotransferases have arisen from a common ancestral PTS, and that throughout evolution the various hydrophilic and hydrophobic domains have been repeatedly fused in different orders and combinations, spliced apart and duplicated to give rise to the range of systems observed today (Saier & Reizer, 1992). The phylogenetic position of the individual domains of the MtlA (IICB$^{M}$) protein was investigated by performing an analysis of members of the fructose-mannitol PTS family. As shown in Fig. 3(a), the IIB$^{M}$ domain of C. acetobutylicum is part of a cluster comprising IIB domains which are found in mannitol-specific permeases with the domain order IICB or IICBA. The domains in this cluster are more distantly related to IIB domains found in fructose-specific permeases, or putative fructose-specific permeases, which have variable domain structure. The same was observed following analysis of the corresponding IIC domains (Fig. 3b), although it is noticeable that the C. acetobutylicum MtlA domain occupies a different position within the mannitol cluster. These findings indicate that the separation of substrate specificity most likely occurred as an early event in the evolution of the fructose-mannitol PTS family, and suggest that the domains of the C. acetobutylicum mannitol-specific PTS may have evolved independently of each other. A
Fig. 3. Phylogenetic analysis of (a) IIB domains and (b) IIC domains of members of the fructose-mannitol family of PTS permeases. Unrooted phylogenetic trees are presented. Abbreviations are as follows, with domain structure and accession number indicated in parentheses. Mannitol permeases: C. acetobutylicum MtlA, Clostridium acetobutylicum (IICB); B. stearothermophilus MtlA, Bacillus stearothermophilus (IICB, U18943); B. subtilis MtlA, Bacillus subtilis (IICBA, P42956); E. coli MtlA, Escherichia coli (IICBA, P00550); K. pneumoniae MtlA, Klebsiella pneumoniae (IICBA, AAD45385); E. coli PtyC, Escherichia coli cryptic (IICB, P32059); S. carnosus MtlA, Staphylococcus carnosus (IICB, P71580); M. pneumoniae MtlA, Mycoplasma pneumoniae (IIBC, S73517); M. capricolum MtlEII, Mycoplasma capricolum (IIC, BAA05968); S. mutans MtlA, Streptococcus mutans (IIB', AF210133). The B' domain is a duplicated B domain. Fructose and fructose-like permeases: M. pneumoniae PtflA, Mycoplasma pneumoniae (IIBC, P71580); B. burgdorferi FruA-1, Borrelia burgdorferi (IIBC, AAC6687); B. burgdorferi FruA-2, Borrelia burgdorferi (IIBC, AAC6687); E. coli PtyC, Escherichia coli (IIC,
broadly similar phylogenetic relationship was observed between 35 IIA domains of the IIA$_{Prf}$/IIA$_{Mlt}$/IIA$_{Sti}$ family (data not shown; Powell et al., 1995).

The last ORF in the mtl region spans 1155 bp to encode a protein of 384 aa with a molecular mass of 43.9 kDa. Analysis of the deduced amino acid sequence revealed homology to mannitol-1-phosphate dehydrogenase enzymes from several Gram-positive bacteria, and _E. coli_. Within the particularly well-conserved N-terminal region, two possible overlapping NAD$^+$-binding motifs could be identified beginning at residues 7 and 12, a feature of mannitol-1-phosphate dehydrogenases which has been described before (data not shown; Fischer et al., 1991). The first potential NAD$^+$-binding site exhibits higher correlation to the postulated consensus sequence (Wiepenga et al., 1986).

The fourth gene of the mtl region, located between _mtlA_ and _mtlF_, encodes a protein of 684 aa with a predicted molecular mass of 79.4 kDa. This protein is most closely related to transcriptional regulators and antitermini-nators, and has accordingly been designated MtlR. Overall sequence similarity with these regulators is low, although their domain structures exhibit common features (Fig. 4a). MtlR of _C. acetobutylicum_ exhibits greatest identity (25.4%) to the MtlR protein of _B. stearothermophilus_ (Henstra et al., 1999). Similar transcriptional regulators are encoded within the lic (lichenan) operon of _B. subtilis_ (LicR; Tobisch et al., 1997), by the ydaA gene of _B. subtilis_, for which the name _mtlR_ has recently been proposed (Stülke et al., 1998), and by the _mtlR_ and _srlR_ genes of the _Streptococcus mutans_ mannitol and sorbitol operons respectively (Honeyman & Curtiss, 2000; Boyd et al., 2000). The central part of MtlR comprises two PTS regulatory domains (PRDs), which are found in each of these proteins, as well as in the _B. subtilis_ transcriptional regulator LevR and in transcriptional antiterminator proteins of the BglG family (Tortosa et al., 1997; Stülke et al., 1998). The second domain (PRD-II; residues 334–410) in _C. acetobutylicum_ MtlR is the more closely related to PRDs in other proteins. PRDs are regarded as being the targets of PTS-mediated phosphorylation which serves to moderate transcriptional regulation activity. Three of the conserved histidine residues within the PRDs, which have been implicated in phosphorylation, are conserved in MtlR (Fig. 4a). It therefore seems likely that MtlR activity will also be determined by the phosphorylation state of the protein.

The C-terminal part of MtlR shows a low, but significant, similarity to proteins of the IIA$_{Prf}$/IIA$_{Mlt}$/IIA$_{Sti}$ family. This has also been noted previously for the LicR protein of _B. subtilis_ and the SrlR protein of _S. mutans_, but has not been reported for _MtgR_ of _B. stearothermophilus_, _MtR_ of _S. mutans_, or YdaA of _B. subtilis_. An alignment of these proteins indicated that all contained a relatively well-conserved signature sequence for this protein family (Powell et al., 1995; Fig. 4b). The conserved histidyl residue within this sequence in _A. niger_ proteins has been shown to be derivatized during transfer of phosphate along the PTS chain (Reiche et al., 1988; Pas & Robillard, 1988); it is therefore possible that the transcriptional regulators can be phosphorylated at this site, as has in fact been suggested by mutagenic analysis of _B. subtilis_ LicR (Tobisch et al., 1999).

Curiously, the MtR protein of _C. acetobutylicum_ does not contain a recognizable helix–turn–helix motif associated with DNA binding, as predicted by the method of Dodg & Egan (1990).

**mRNA analysis of the mtl gene region**

Total RNA from _C. acetobutylicum_ DSM 792 cells grown on mannitol or glucose, or in the presence of both carbon sources, was isolated, electrophoresed, blotted and hybridized to a _mtlR_-specific DNA probe (758 bp HindIII–ClaI fragment isolated from pSB30). This Northern blot experiment revealed that the _mtlARFD_ genes constitute a polycistronic operon and that expression clearly is induced by the substrate mannitol and is subject to catabolite repression by glucose (Fig. 5). Only in the presence of mannitol and in the absence of glucose could a major transcript of about 6 kb be detected, which is in good agreement with the size of the _mtlARFD_ genes (see Fig. 2). Essentially the same results were obtained with a _mtID_-specific probe (data not shown), but in this case the background was much stronger, possibly indicating the presence of degradation products of the long transcript.

In accordance with the data obtained by Northern blot experiments, the 5′ end of the _mtlARFD_ transcript (the putative transcription start site of the operon) was located 42 bp upstream of the ATG start codon of _mtlA_ by primer extension analysis (Fig. 6). The corresponding promoter sequence (5′-TTGAAA-17 bp-TATCAT) showed reasonable homology to the consensus suggested for Gram-positive bacteria and clostridia (Graves & Rabinowitz, 1986; Young et al., 1989) and partially overlapped the CRE2 DNA sequence found in this position (Fig. 6). As observed by Northern blot analysis, induction of _mtl_ gene expression by mannitol and strong repression by glucose were confirmed by the primer extension experiments (Fig. 6). No transcription start sites could be identified upstream of either _mtlF_ or _mtlD_ (data not shown).
**DISCUSSION**

In this report the molecular analysis of the mannitol operon of *C. acetobutylicum* DSM 792 has been presented. With respect to the architecture, the order of genes (Fig. 2), and the apparent mechanisms involved in the regulation of *mtl* gene expression, the mannitol PTS of *C. acetobutylicum* clearly differs from that in *E. coli* (Davis *et al.*, 1988) but rather reflects the structural organization which has been proposed for Gram-
positive micro-organisms (Fischer et al., 1991; Henstra et al., 1996). Our results indicate that the four genes mtlARFD of C. acetobutylicum constitute a poly-cistronic operon, and that transcription is initiated upstream of mtlA (Fig. 6). The appearance of several transcripts observed by Northern blot analysis, however, remains to be clarified. Their generation might be due to RNA processing, possibly controlling the rate of expression of the mtl genes.

In contrast to the E. coli EIICBA\textsubscript{Mtl} permease, the mannitol-specific PTS of C. acetobutylicum and other Gram-positive bacteria consists of two separate proteins. By demonstrating the existence of a mannitol-specific PTS component in both soluble and membrane fractions of cell extracts, our data obtained in phosphorylation studies confirm a separation of mannitol enzyme II into membrane-bound EIICB\textsubscript{Mtl} and cytoplasmic IIA\textsubscript{Mtl} components. The corresponding genes mtlA and mtlF are separated by the mtlR gene encoding the putative multi-domain regulator of the mannitol loci in several Gram-positive bacteria exhibit similar characteristics and gene arrangements (Henstra et al., 1999; Honeyman & Curtiss, 2000). On the other hand, OrfX of Enterococcus faecalis, encoded by a gene found in the same position as mtlR within the mannitol gene region of this bacterium, does not show similarity to the other regulators. Whereas the C-termini of MtlR of C. acetobutylicum, B. stearothermophilus and S. mutans exhibit similarity to the EIIA\textsuperscript{Ntr} signature sequence of PtsN proteins (Powell et al., 1995; Fig. 4b), some similarity of OrfX from Ent. faecalis to EIICB\textsubscript{Mtl} domains was observed (Fischer et al., 1991).

Sequencing of the genome of C. acetobutylicum ATCC 824 has recently been completed. Comparison of the gene products encoded by the mtl operons of strains DSM 792 and ATCC 824 shows 99.4%, 99.85%, 82.2% and 100% identity between the MtlA, MtlR, MtlF and MtlD proteins respectively. The MtlF proteins, which showed the lowest overall identity, were identical for the first 117 aa. The near total identity between the Mtl
proteins clearly reflects the close taxonomic relationship of the two *C. acetobutylicum* strains (Keis et al., 1995; Johnson et al., 1997).

The expression of enzymes necessary for the uptake and metabolism of alternative carbon sources in most bacteria is regulated by transcriptional control mechanisms which allow for substrate induction and catabolite repression (Postma et al., 1993). Results obtained in Northern experiments and phosphorylation studies demonstrate that both mechanisms are involved in the coordinate expression of the *mtlARFD* genes of *C. acetobutylicum*. The MtlR protein is a member of a class of transcriptional activators and antiterminiators, found in a variety of bacteria, which contain PTS regulatory domains (PRDs). These regulators control the expression of PTs, and a general mechanism of regulation of their activity has been proposed based on multiple phosphorylation of the PRDs by components of the PTS (Stülke et al., 1998). Thus, in the absence of an inducer, the regulator is phosphorylated by its corresponding EII and is inactive. When inducer is present, it is transported and phosphorylated by the PTS and the regulator becomes dephosphorylated and thereby activated. As a result, induction is achieved in response to the presence of the PTS substrate. In addition, some PRD-containing regulators must also be phosphorylated by the general PTS phosphocarrier protein HPr in order to be active. This activation is prevented if phosphate is diverted from HPr to support transport and phosphorylation of a readily metabolized sugar such as glucose. The resulting inactivation of the regulator thus underlies a form of catabolite repression. In the case of the MtlR protein of *B. stea*tothermophilus, DNA binding has been shown to be stimulated by HPr-dependent phosphorylation and decreased on phosphorylation by EIIICB<sub>III</sub>, thus demonstrating a direct link between the phosphorylation state of the regulator and transcriptional regulation activity (Henstra et al., 1999).

Direct labelling and mutagenesis studies have indicated that PRD-containing regulators are phosphorylated on conserved histidine residues. However, despite the overall homology shown by PRDs and the conservation of potential phosphorylation sites, the position(s) of phosphorylation and the effects on regulator activity appear to be highly variable. For example, the antitermini LicT and SacY of *B. subtilis* have each been shown to be phosphorylated on three sites by HPr (only two of which are equivalent); however, the role of phosphorylation at these sites is not yet fully understood, and while LicT is activated SacY is, unusually, negatively regulated by HPr-mediated phosphorylation (Tortosa et al., 1997; Lindner et al., 1999). With respect to the transcriptional regulators shown in Fig. 4(a), the LevR protein of *B. subtilis* is inactivated via LevE (IIB<sup>Rev</sup>)-mediated phosphorylation on his869 in PRD-II, and activated by HPr-mediated phosphorylation of His585 within domain B, which contains PRD-I (Martin-Verstraete et al., 1998). Mutagenesis studies have implicated all four conserved histidine residues in the two PRDs of the LicR protein of *B. subtilis* in phosphorylation by HPr, while IIB<sup>Rev</sup>-dependent phosphorylation appears to occur within the C-terminal IIA domain (Tobisch et al., 1999). A similar mutagenic approach has suggested that MtlR from *B. stea*tothermophilus is phosphorylated on the two histidine residues in PRD-I by IICB<sub>III</sub> and on those in PRD-II by HPr (Henstra et al., 1999). Thus, although MtlR of *C. acetobutylicum* contains corresponding histidine residues and its activity is therefore likely to be controlled by phosphorylation, the mechanistic details cannot be predicted with certainty and their description must await further experimental analysis.

The apparent absence of a helix–turn–helix DNA-binding motif from *C. acetobutylicum* MtlR is intriguing, and raises the possibility that MtlR exerts its effects indirectly, perhaps by influencing the DNA-binding affinity of another protein. The identification of two well-conserved CREs partially overlapping the promoter region upstream of *mtlA* (Fig. 6) suggests that catabolite repression of the *mtl* operon in *C. acetobutylicum* may follow the general model proposed for low-GC Gram-positive bacteria (Hueck & Hillen, 1995; Saier et al., 1996). Proteins (RegA and RegB) homologous to the catabolite control protein (CcpA) of *B. subtilis* have recently been detected in *C. acetobutylicum* P262 and *C. beijerinckii* NCIMB 8052 (Davison et al., 1995; Reid et al., 1999). However, whether the putative regulatory sites CRE1 and CRE2 indeed serve as targets for the binding of a CcpA-like protein involved in glucose regulation of the *mtl* operon remains to be confirmed by experimental data. MtlR might influence such binding in a manner which depends on its phosphorylation state, thus providing a coordinated induction and repression mechanism to control *mtl* gene expression.

In addition to MtlR of *C. acetobutylicum* DSM 792 described in this paper, PRD-containing antiterminiators have recently been identified in the β-glucoside operon of the rumen bacterium *Clostridium lansiosisporum* and the sucrose operon of *C. acetobutylicum* ATCC 824 (Brown & Thomson, 1998; Tangney & Mitchell, 2000). The emergence of this class of regulator among the clostridia is a further indication of the importance of the PTS as a mechanism of carbohydrate accumulation in these bacteria.

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