J. Mol. Microbiol. Biotechnol. (2002) 4(3): 301–307.

JMMB Symposium

Network Regulation of the *Escherichia coli* Maltose System

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Abstract

The genes of the *Escherichia coli* maltose regulon are controlled by MalT, the specific transcriptional activator which, together with the inducer maltotriose and ATP, is essential for *mal* gene transcription. Network regulation in this system affects the function of MalT and occurs on two levels. The first concerns the expression of *malT*. It has long been known that *malT* is under catabolite repression and thus under the control of the cAMP/CAP complex. We found that, in addition, the global regulator Mlc is a repressor for *malT* transcription. The repressor activity of Mlc is controlled by the transport status of the glucose-specific enzyme EIICB of the PTS that causes sequestration (and inactivation as a repressor) of Mlc when glucose is transported. The second level of MalT regulation affects its activity. MalT is activated by maltotriose which is not only formed when the cells are growing on any maltodextrin but also, in low amounts, endogenously when the cells grow on non-maltodextrin carbon sources. Thus, cellular metabolism, for instance degradation of galactose or trehalose, can cause *mal* gene induction. It was found that unphosphorylated internal glucose takes part in endogenous maltodextrin biosynthesis and is therefore a key element in endogenous *mal* gene expression. In addition to the maltotriose-dependent activation, MalT can interact with three different enzymes that lead to its inactivation as a transcriptional activator. The first is MalK, the energy transducing ABC subunit of the maltodextrin transport system. Transport controls the interaction of MalK and MalT thus affecting gene expression. The second enzyme is MalY, a pyridoxal phosphate containing enzyme exhibiting cystathionase activity. The crystal structure of MalY was established and mutations in MalY that reduce *mal* gene repression map in a hydrophobic MalT interaction patch on the surface of the enzyme. The last enzyme is a soluble esterase of as yet unknown function. When overproduced, this enzyme specifically reduces *mal* gene expression and affects the activity of MalT in an *in vitro* transcription assay.

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Introduction

The *E. coli* maltose regulon (Boos *et al.*, 1998) consists of 10 genes in five operons whose products are four enzymes, five transport proteins and one periplasmic protein, MalM (Gilson *et al.*, 1986), the function of which remains obscure. The enzymes (MalP, MalQ, MalS and MalZ) catalyze the degradation of maltose and maltodextrins to glucose and glucose-1-phosphate. The transport proteins consist of the λ receptor (maltoporin) in the outer membrane (Schirmer *et al.*, 1995) as well as a binding protein-dependent ABC transporter composed of the periplasmic maltose binding protein, MalE, the translocation complex, MalF/G, in the membrane, and the associated ATP-hydrolyzing subunit, MalK. All *mal* genes are controlled by MalT, the specific *mal* gene activator of the system which, together with the inducer maltotriose and ATP, is needed for their expression (Richet *et al.*, 1989). MalT is in an equilibrium of inactive monomers and active multimers, the latter being stabilized by maltotriose (Schreiber *et al.*, 1999). The system is induced by the presence of maltose or any maltodextrin in the medium, but only maltotriose which is always an intermediate of maltodextrin metabolism can activate MalT (Raibaud *et al.*, 1987). Mutants lacking MalT cannot grow on maltose and mutations in *malT* exist that exhibit constitutive *mal* gene expression (*malT*^{c} mutants). Figure 1 is an overview of the genetic organization of the maltose regulon. For reviews on the maltose system, see (Schwartz, 1987) (Boos *et al.*, 1998).

Several curious observations concerning *mal* gene expression have been noticed in the past but have not obtained sufficient attention at the time. They were the start point for the studies on network regulation of the maltose system outlined in this review:

1. Sugars other than maltodextrins are able to induce the maltose system. For instance galactose or lactose are inducers in a mutant lacking phosphoglucomutase (Pgm) (Adhya *et al.*, 1971). Trehalose metabolism induces the system even though trehalose is neither a substrate of the transport system nor an effector of MalT (Klein *et al.*, 1993). This indicated that the inducer maltotriose can be formed endogenously in the absence of maltodextrins in the medium. In fact, it turned out that there are at least two ways by which maltotriose can be synthesized endogenously; one was by degradation of glycogen and the other seemed to involve unphosphorylated internal glucose as well as

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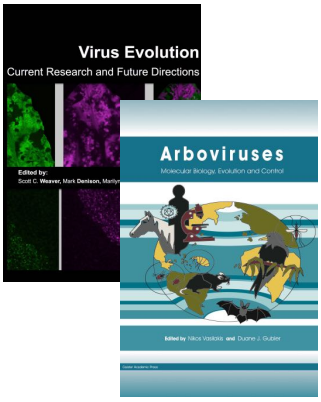

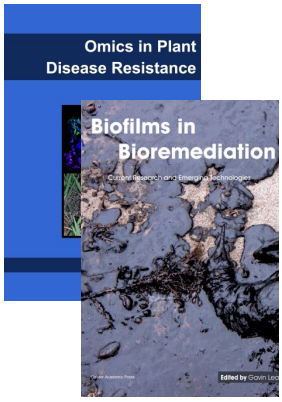

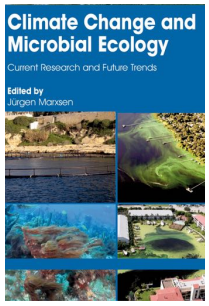

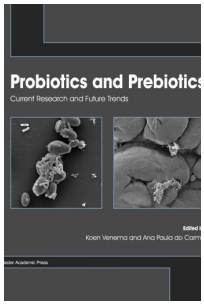

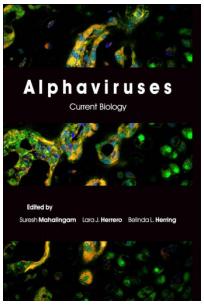

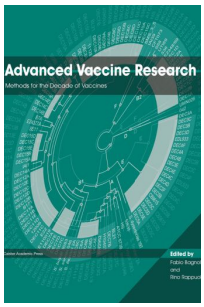

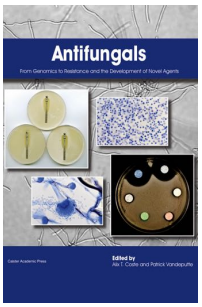

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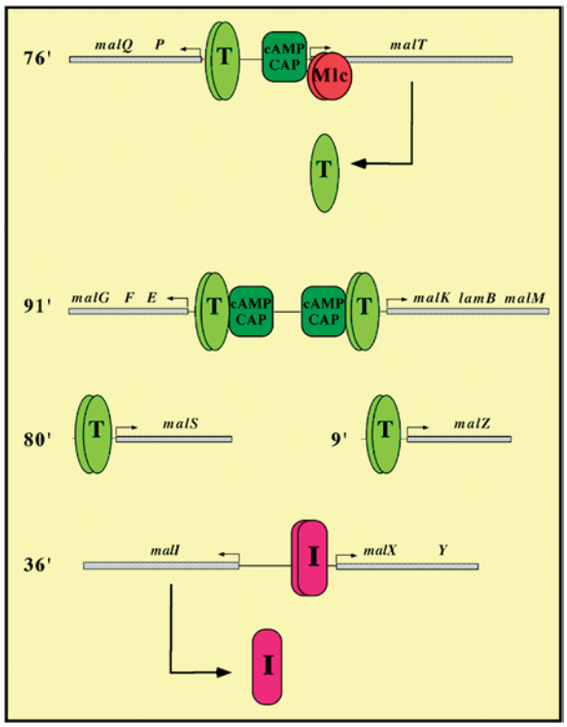

Figure 1. Genetic organization of the *mal* genes and the *malI/malXY* gene cluster. Boxes depict genes or operons; their location in min on the *E. coli* map is given on the left. Transcriptional start points and the direction of transcription are indicated by small arrows. Transcription enhancing proteins are in green, repressors in red. One-letter abbreviations stand for the corresponding *mal* genes or proteins.

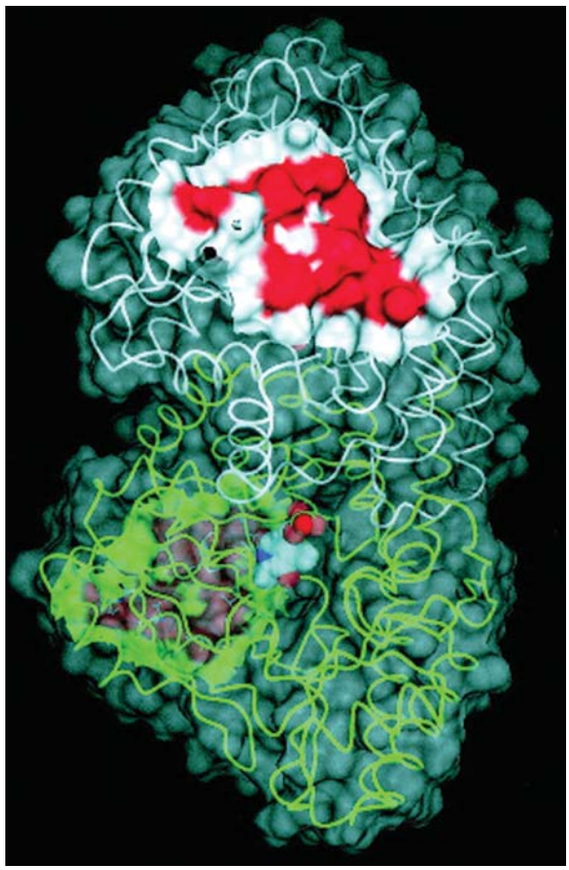

Figure 2. Representation of the crystal structure of the MalY dimer. The carbon backbone traces of the monomers, shaded green and white, are overlaid with a transparent surface. Highlighted as a white solid surface is the MalT-interaction patch that has been defined by repression negative mutations (red). The pyridoxal-5′-phosphate cofactor is depicted in the van der Waals representation. Note that the MalT binding face and the cystathionase active site are on opposite sides of the individual monomers.

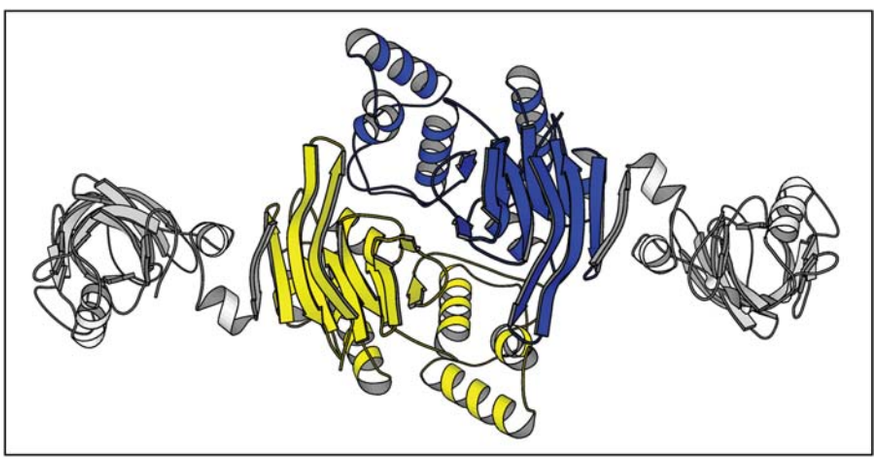

Figure 4. The crystal structure of the MalK dimer, the ABC subunit of the trehalose/maltose transporter of *Thermococcus litoralis* as a ribbon representation. The ABC domains of the individual monomers are shown in blue and yellow. The distinct regulatory barrel-like domains are shown in grey. We propose that *E. coli* MalK harbours a similar regulatory domain carrying the MalT interaction site. For details on the crystal structure see (Diederichs *et al.*, 2000).

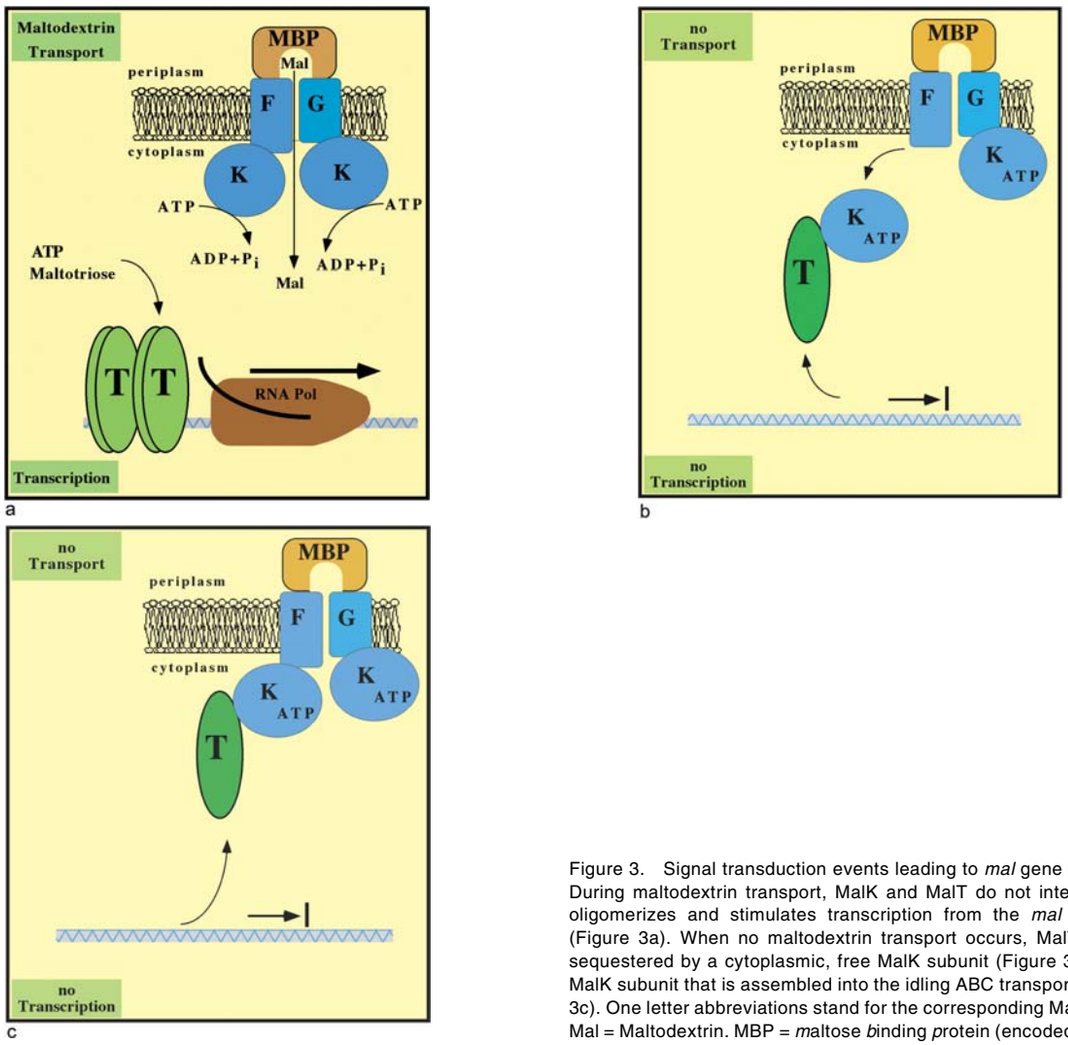

Figure 3. Signal transduction events leading to *mal* gene regulation. During maltodextrin transport, MalK and MalT do not interact; MalT oligomerizes and stimulates transcription from the *mal* promoters (Figure 3a). When no maltodextrin transport occurs, MalT is either sequestered by a cytoplasmic, free MalK subunit (Figure 3b) or by a MalK subunit that is assembled into the idling ABC transporter (Figure 3c). One letter abbreviations stand for the corresponding Mal proteins. Mal = Maltodextrin. MBP = *m*altose *b*inding *p*rotein (encoded by *malE*).

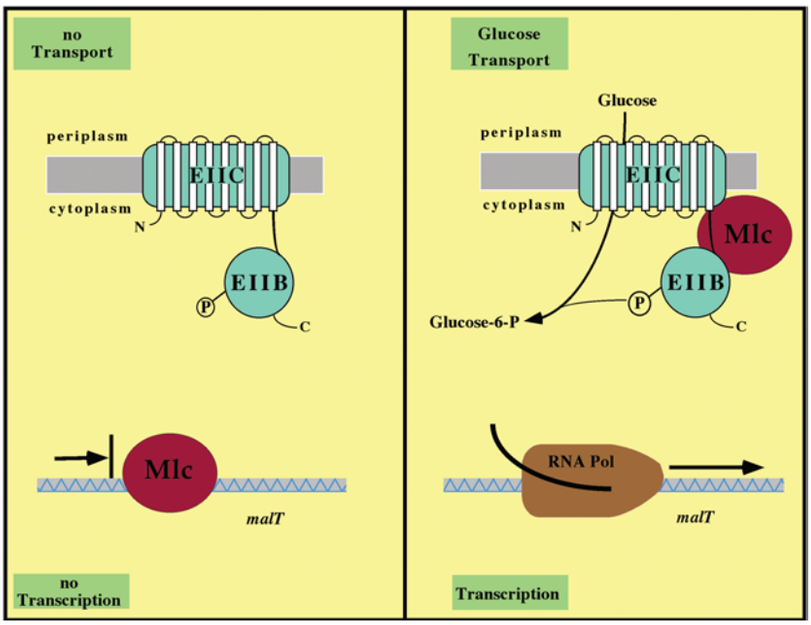

Figure 5. Glucose transport via the EIICB of the PTS leads to derepression of *malT*. When no glucose is transported by EIICB, Mlc binds to the *malT* promoter and represses transcription (left). During glucose transport, Mlc is sequestered from the *malT* promoter by unphosphorylated EIICB and transcription can occur (right). Phosphate groups are depicted as circled ''P''.

glucose-1-phosphate (Decker *et al.*, 1993). Thus, gluconeogenesis had to play a role in the endogenous induction of the maltose system.

2. It had been noticed early on that some mutants defective in maltose transport showed elevated expression of *malQ* and *malP* genes (Hofnung *et al.*, 1974). Later on, it became clear that it was MalK that was associated with this phenomenon. *malK* mutants cause constitutive expression (Bukau *et al.*, 1986) whereas the overproduction of MalK caused repression of the system. In fact, strains harboring *malK* expression plasmids can no longer grow on maltose (Reyes *et al.*, 1988). The C-terminus of MalK was found to be responsible for repression (Kühnau *et al.*, 1991). The mechanism by which MalK acted as a repressor was unclear.
3. In an attempt to isolate mutants in the still unknown enzyme(s) involved in the biosynthesis of the internal inducer we had isolated the *malI* (for *mal*tose *i*nductor) mutation that abolished the constitutivity of *malK* mutants (Ehrmann *et al.*, 1987). The sequencing of *malI* revealed that it did not encode an enzyme in maltotriose synthesis but an as yet unknown repressor (Reidl *et al.*, 1989). Subsequently, it became clear that MalI was the repressor of an adjacent operon consisting of *malX* and *malY*. *malX* encodes an enzyme IIBC of the phosphotransferase system (PTS), *malY* a pyridoxal phosphate-containing enzyme. It was the derepressed synthesis of MalY in the *malI* mutant that caused the repression of the *mal* genes (Reidl *et al.*, 1991). How this enzyme interfered with *mal* gene expression was unclear.

Conclusions

The Role of Glucokinase

We had suspected that one pathway of endogenous maltotriose biosynthesis involved unphosphorylated internal glucose. Evidence came from a triple mutant that was unable to phosphorylate glucose due to mutations in *ptsG*, *ptsM* (encoding EII's of the PTS specific for glucose) and a mutation in *glk* (encoding glucokinase). Glucose can enter this strain only via the galactose permease (without phosphorylation) and the addition of glucose to the growth medium causes induction of the maltose system (Decker *et al.*, 1993). The glucose-dependent induction in this strain can be abolished by overexpression of plasmid encoded glucokinase. Overexpression of glucokinase also abolishes the constitutivity of *malK* mutants by removing internal free glucose via phosphorylation. Overexpression of glucokinase has no effect on the constitutivity of mutants in *malQ*, the gene coding for amylomaltase, which is due to the formation of maltotriose from glycogen where glucose is not an intermediate. Thus, these experiments strongly support the idea that *E. coli* does contain free internal glucose and that this internal glucose plays a decisive role in the glycogen-independent synthesis of endogenous maltotriose (Meyer *et al.*, 1997). At present it is not clear which reaction leads to glucose-dependent maltotriose synthesis. One possibility, supported by the phenotype of *pgm* mutants, was that the critical enzyme is a maltose/maltotriose phosphorylase that would produce maltose from glucose and glucose-1-phosphate and maltotriose from maltose and a second glucose-1-phosphate. Yet, no maltose phosphorylase with the expected specificity could be found to date in cellular extracts. Another possibility, suggested by the kinetics of maltotriose formation (a very early reaction product) from ^{14}C glucose is that an unknown maltodextrin precursor, possibly protein bound, would transfer maltosyl residues onto the non reducing end of ^{14}C glucose. The origin of free unphosphorylated glucose in the cell is still an enigma.

The MalY-MalT Intermezzo

The overproduction of MalY in *malI* mutants (or when expressed from plasmids) abolishes *mal* gene expression (Reidl, 1992). The identification of MalY as a βC-S lyase (cystathionase) did not give any clue about its function in *mal* gene regulation. The enzyme when induced can complement a *metC* mutation that is normally auxotrophic for methionine since *metC* encodes a cystathionase involved in the biosynthesis of methionine. Exchanging the lysine residue that is critical for pyridoxal phosphate binding yielded a mutant MalY protein that had lost cystathionase activity but was still functioning as a *mal* gene repressor. On the other hand, mutations in MalY were isolated that exhibit reduced *mal* gene repression but are still fully active in cystathionase activity. These regulation minus mutations cluster on three different locations along the polypeptide chain. Taken together, these studies showed that enzymatic activity and repressor function are separable entities. How MalY represses *mal* gene expression became clear when it was demonstrated that purified MalY binds to monomeric MalT and inactivates it in an *in vitro* transcription assay using purified proteins. The interaction of MalY and MalT as well as the strength of inhibition is counteracted by the addition of maltotriose (Schreiber *et al.*, 2000). Thus, the overproduction of MalY leads to sequestration and a reversible inactivation of MalT. MalY was crystallized and its three-dimensional structure determined (Figure 2). It is a homodimer; each monomer is composed of two clearly separable domains. The regulation minus mutations in MalY cluster in a hydrophobic patch on the surface of the protein that is surrounded by a circle of hydrophilic residues. This patch must represent the site where MalY interacts with MalT (Clausen *et al.*, 2000). Conversely, it is the N-terminal portion of MalT (AA 1-250) that is the minimal target for MalY (A. Schlegel, unpublished observations). At present, it is unknown what the natural substrate of MalY is. The clustering of *malY* with *malX* in an operon and the common regulation by MalI (whose inducer is also unknown) indicates that an unknown sugar, possibly a cysteinyl derivative, might be the substrate of these gene products. One could imagine that the carbon flux through this system exerts

a specific repression on the maltose system. A *malI/malX/malY* region with high sequence identity (67%) exists in *Vibrio furnissii* (Bouma *et al.*, 1996). In this organism, the MalX homologue encodes the only glucose transporter.

The Esterase Connection

In search for the gene encoding a maltose phosphorylase which we expected to be involved in the synthesis of endogenous maltotriose, we screened a plasmid bank of *E. coli* genes (under their own promoter) to confer a Mal^+ phenotype to a *malQ* mutant. A *malQ* mutant is unable to grow on maltose (due to the loss of amylomaltase) and is sensitive to maltose. We found a clone that conferred a resistant phenotype on McConkey maltose indicator plates. However, the plasmid did not encode a maltose phosphorylase nor did it allow growth on maltose. Instead, it encodes a protein that represses the maltose system. This became clear when the plasmid was tested in a *malK-lacZ* strain. Analysis of the plasmid-encoded protein revealed that it is a cytoplasmic enzyme exhibiting homology to human lipases including a soluble conserved signature sequence. The purified protein (now called Aes for **A**cetyl **es**terase) is able to hydrolyze para-nitrophenyl acetate but is not a lipase. So far nothing has been learned about the control of monocistronic *aes*. Activity of *aes-lacZ* fusions does not vary significantly under different growth conditions (Peist *et al.*, 1997) (Kanaya *et al.*, 1998). Like MalY, Aes interferes with transcription initiation in an *in vitro* system using purified components and inhibits maltotriose binding by MalT *in vitro* (E. Richet, unpublished) which suggests that Aes too directly interacts with MalT.

The MalK (or the MalT) Cycle

Whereas the physiological relevance of the control exerted by MalY and Aes on MalT activity remains unclear, the role of MalK in controlling MalT activity seems to be logical. Obviously, it makes sense to repress a metabolic system when its substrate is not transported and to relieve repression when the transport machinery is active. Transport of any maltodextrin by the maltose/maltodextrin ABC transporter will free MalT from its inactive stage and may be regarded as a prelude for the final induction by increasing concentrations of internal maltotriose.

The decisive insight into the repressor[1] function of MalK came through the demonstration that MalT, biotinylated at its C-terminus and bound to Avidin coated beads, specifically retained MalK (Panagiotidis *et al.*, 1998). The interaction of MalK with MalT indicated that MalK, like MalY and Aes, is able to inactivate MalT as a transcriptional activator. Indeed, preliminary experiments with purified MalK in the MalT-dependent transcription assay support this notion. Obviously, soluble cytoplasmic MalK can exert repression since the plasmid-encoded protein abolishes any MalT-dependent transcription in a *malEFG* background (Decker, 1998). But also when in complex with the other transport components MalK participates in MalT regulation and its ATPase activity must play a decisive role in controlling its regulatory activity. This was concluded from the effects of mutations in *malF* (*malF* 500) causing a MalE-independent transport activity. This mutant transport complex is uncoupled for its ATPase activity and causes *mal* gene transcription to become partially constitutive, despite being wild type in MalK (Panagiotidis *et al.*, 1998). More evidence comes from the G137A mutation within the ATP binding motif of MalK that leads to loss of ATP hydrolysis but not ATP binding. This MalK mutant is an even stronger repressor (''super-repressor'') than the wild type protein (Panagiotidis *et al.*, 1998). Thus, in the absence of transport, i.e., in the absence of ATP hydrolysis, MalK has an increased affinity for MalT sequestrating it from its transcriptional activity and leading to *mal* gene repression.

It is less clear how this regulation is realized on the molecular level. One could imagine that upon binding to MalT, MalK dissociates from the membrane and keeps MalT in an inactive (monomeric) state (Figure 3b). The presence of substrate-loaded binding protein on the periplasmic side of the membrane would increase the affinity of the complex for MalK, initiate transport and set MalT free for transcriptional activity (Figure 3a). The binding affinity of isolated MalK for MalT is in favor of such a scenario. Alternatively, the close connection of transport and regulation is in strong favor of a model where MalT is sequestrated by the idling ABC transporter (Figure 3c) (Boos and Böhm, 2000). The recent structure determination of *Thermococcus litoralis* MalK, a functional homologue of the *E. coli* protein, has revealed the C-terminus as an independent domain, forming a β-barrel (Diederichs *et al.*, 2000). Even though nothing is known about a possible homologue of MalT in *T. litoralis*, it is suggested from the sequence homology as well as from the structural prediction of *E. coli* MalK that the C-termini in both proteins have similar functions and might constitute a new type of regulation module (Figure 4). MalK is also known to interact with $EIIA^{Glc}$, a component of the phosphotransferase system (PTS) that is mediating catabolite repression and inducer exclusion in non PTS systems (van der Vlag *et al.*, 1994). From the analysis of mutations in MalK rendering maltose transport insensitive to inducer exclusion it is clear that the binding sites in MalK for $EIIA^{Glc}$ and MalT are close but not overlapping (Kühnau *et al.*, 1991; Böhm *et al.*, J. Biol. Chem. 2002, in press).

Sequestration of a Global Repressor as a Means of *mal* Gene Regulation

In the attempt to identify proteins needed for transmission of the repressor function of MalK we searched for mutants that would abolish the repressing effect of

[1]We used the term repressor for the function of MalK, MalY and Aes when inhibiting the transcriptional activity of MalT, even though these proteins are not repressors in the sense of DNA binding proteins. But since they reduce gene transcription by interaction with a gene activator the term repressor is kept in lack of a better term.

overproduced MalK on *mal* gene expression. Such a mutant was found. It had an insertion in *mlc*, a gene involved in the regulation of glucose utilization (Hosono *et al.*, 1995). But instead of mediating the MalK-MalT interaction Mlc was shown to be a protein with repressor function; it binds to the regulatory region of *malT* at a sequence with palindromic structure, the Mlc-box. Thus, Mlc did not interfere at the level of MalT activity but *malT* expression. Increasing the level of MalT was successful in over-riding the inhibiting activity of MalK (Decker *et al.*, 1998). In search of a potential inducer of Mlc, glucose-6-phosphate or a metabolic product were suspected, but their involvement could not be proven. This prompted us to probe for another possibility. Since it was known that transport of glucose via enzyme IICB for glucose (PtsG) of the PTS was needed for derepression of MalT it seemed possible that Mlc was bound by transporting PtsG and would thus be sequestrated from the DNA. Indeed, we found that Mlc was bound to PtsG-containing membranes and that phosphorylation of PtsG controlled Mlc binding. Elements necessary for Mlc binding were the B domain extending into the cytoplasm and the hinge region connecting the B domain and the membrane-bound C domain. In addition, the *in vitro* activity of PtsG in phosphorylation of α–methyl glucoside, a substrate analogue of glucose, was inhibited by increasing concentrations of Mlc (Lee *et al.*, 2000) (Figure 5). The effect of Mlc on the maltose system appears paradoxical. On one hand, in catabolite repression, transport of glucose reduces the expression of *malT* by lowering the cAMP concentration, on the other hand, transport of glucose *via* PtsG leads to sequestration of Mlc, a repressor for *malT*, thus dampening the effect of catabolite repression. The teleology behind this arrangement is at present unclear.

Acknowledgment

This work was only possible due to the generous collaboration of several laboratories: Howard Shuman and his crew at Columbia University in New York (MalK), Tim Clausen and collaborators at the Max Planck Institut in München (crystallization of MalY), Evelyne Richet and her coworkers at the Institut Pasteur in Paris (MalT) and Jackie Plumbridge at the Institut de Biologie Physico-chimique in Paris (Mlc).

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