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Overproduction of MalK Protein Prevents Expression of the *Escherichia coli mal* Regulon

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The *mal* regulon of *Escherichia coli* comprises a large family of genes whose function is the metabolism of linear maltooligosaccharides. Five gene products are required for the active accumulation of maltodextrins as large as maltoheptaose. Two cytoplasmic gene products are necessary and sufficient for the intracellular catabolism of these sugars. Two newly discovered enzymes have the capacity to metabolize these sugars but are not essential for their catabolism in wild-type cells. A single regulatory protein, MalT, positively regulates the expression of all of these genes in response to intracellular inducers, one of which has been identified as maltotriose. In the course of studying the mechanism of the transport system, we have placed the structural gene for one of the transport proteins, MalK, under the control of the P*trc* promoter to produce large amounts of this protein. We found that although high-level expression of MalK was not detrimental to *E. coli*, the increased amount of MalK decreased the basal-level expression of the *mal* regulon and prevented induction of the *mal* system even in the presence of external maltooligosaccharides. Constitutive mutants in which MalT does not depend on the presence of the internal inducer(s) were unaffected by the increased levels of the MalK protein. These results are consistent with the idea that MalK protein somehow interferes with the activity of the MalT protein. Different models for the regulatory function of MalK are discussed.

Maltooligosaccharides derived from startch are probably an important carbon source for *Escherichia coli* and other members of the family *Enterobacteriaceae*. The accumulation of these sugars and their conversion to glucose and glucose 1-phosphate are mediated by the products of the *mal* regulon (23). The transport system for maltodextrins catalyzes the efficient accumulation of maltose and longer maltodextrins as large as maltoheptaose. This system is composed of five proteins including an outer membrane component (LamB, lambda receptor) (5, 20, 23), a periplasmic component, maltose-binding protein (8, 14), and three cytoplasmic membrane proteins, MalF, MalG, and MalK (1, 6, 11, 24, 25). The intracellular catabolism of the accumulated sugars is carried out by an amylomaltase (MalQ) and a maltodextrin phosphorylase (MalP). Amylomaltase transfers a donor maltodextrin to the nonreducing end of an acceptor maltodextrin and releases a molecule of glucose from the reducing end of the donor. Maltodextrin phosphorylase uses inorganic phosphate to remove the nonreducing glucose residue from maltodextrins as glucose 1-phosphate. The glucose and glucose 1-phosphate are then metabolized via the glycolytic pathway (23).

The synthesis of the *mal* gene products is controlled by the MalT protein, which acts as positive activator of transcription (7, 19). MalT protein stimulates transcription of *mal* genes only in the presence of inducers. One inducer compound has been identified as maltotriose; it is able to stimulate MalT in an S-30-coupled transcription-translation system (19). Interestingly, maltose is unable to stimulate MalT. A newly discovered gene, *malI*, has been shown to be necessary for the induction of the *mal* regulon in vivo (9). The *malI* gene product is an enzyme which produces sugars in the cytoplasm that may act as inducers. Some of these sugars have been identified by thin-layer chromatography of crude lysates, and their concentrations in the cytoplasm correlate with the level of *mal* gene expression (9).

A relationship between the regulation of the *mal* regulon and the maltose transport system has been recognized since the earliest studies of the *mal* system. It was initially found that *malK* null mutants exhibited an increased level of *mal* gene expression in the absence of any exogenous inducers (M. Schwartz, Thesis, Doctorat es Sciences, Université de Paris, Paris, France). This partial constitutive expression can be as much as 30 to 40% of the fully induced levels of *mal* gene expression in wild-type cells. Mutations in genes that code for other components of the transport system do not result in constitutive gene expression. Speculations about the ability of MalK either to pump an "internal inducer" out of the cell or to degrade it were advanced to explain the increased levels of gene expression in *malK* mutants (23). More recently, Boos and co-workers found that the basal, uninduced levels of *mal* gene expression could be influenced by a variety of factors that change the levels of intracellular glucosides. These include the ability of the cell to synthesize glycogen, the presence or absence of the MalQ enzyme, the presence of the MalI enzyme, and the osmolarity of the growth medium (3, 9).

We found that cells which overproduce the MalK protein under the control of the P*trc* promoter are phenotypically Mal$^-$ and Dex$^-$. Different models are discussed to explain how excess MalK may prevent expression of the *mal* regulon. Our results are consistent with the idea that the excess MalK protein prevents induction of the *mal* regulon by removing the inducer compounds normally present in the cytoplasm of the cell.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are listed in Table 1. The *lacI*^{q1} gene contains a promoter mutation that results in a 50- to 100-fold increase in the amount of Lac repressor.

Media and genetic techniques. Rich medium (LB), minimal medium M63, and indicator medium have been described previously (17). Antibiotics were added at final concentra-

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TABLE 1. Bacterial strains

Strain	Genotype	Origin or reference
HS3134	F⁻ Δ*lacU169 araD139 rpsL flbB5301 rel malP-lacZ*⁺	7
pop3301	HS3134 *malT1*(Con)	7
HS3205	F⁻ *lacI*^{q1} Δ*malK16 recA1*	This study
HS3349	F⁻ Δ*lacU169 araD139 rpsL flbB5301 rel* (F′ *lacI*^{q1})	This study
HS3322	HS3349 *malT1*(Con)	
HS3331	HS3349 *malTp1p7*	This study

tions of 100 μg/ml for carbenicillin and 25 μg/ml for kanamycin and chloramphenicol. Standard genetic techniques were carried out as described in Miller (17). Plasmid transformation was performed by $CaCl_2$ treatment as described before (16).

Construction of pMR10 and pMR11. The construction described below was carried out with *mal* DNA which originated from a λ transducing phage, λap*malB*13, which was kindly provided by C. Marchal and M. Hofnung of the Institut Pasteur. Restriction endonuclease digestions and DNA ligations were carried out in the buffer conditions suggested by the supplier. Solutions and buffers were prepared with distilled water and sterilized.

The plasmid vector pKK233-2 contains a hybrid promoter, P*trc*, comprising the −35 region of the *trp* promoter and the −10 region and operator of the *lacUV5* promoter. The precise structure of this vector has been described in detail elsewhere (2). In the presence of the Lac repressor, transcription from P*trc* is repressed and is inducible by isopropyl-β-D-thiogalactoside (IPTG).

The construction of a plasmid which expresses the *malK* gene under the control of the P*trc* promoter was complicated by the fact that the *malK* gene is adjacent to the *malB* promoter region. Since the nucleotide sequence of this region is known, it was possible to devise a way to separate the coding region of the *malK* gene from the *malB* promoter and fuse *malK* to an artificial promoter and ribosome-binding site. First, a *Hin*fI fragment which contains the *malB* promoter and the *malK* gene was inserted into the *Hin*cII site of plasmid pACYC177 (Fig. 1). This plasmid was called pHS4. Next, a *Pvu*II-*Hin*dIII fragment was purified from pHS4 which contained all of the *malK* gene except for the first 12 nucleotides at the 5′ end of the gene. In order to connect this fragment to the pKK233-2 vector, we used a synthetic oligonucleotide linker. The structure of this linker is 5′-CATGGCGAGCGTACAG-3′, 3′-CGCTCGCATGTC-5′. The staggered end of this fragment is one half of an *Nco*I site and the blunt end is half of a *Pvu*II site. The fragment also contains the first ATG and subsequent four codons of the *malK* sequence. This oligonucleotide was then ligated with pKK233-2 DNA that had been restricted with *Nco*I and *Hin*dIII and the *Pvu*II-*Hin*dIII fragment of pHS4. This is illustrated in Fig. 1. The ligation mixture was then transformed into the *lacI*^{q1} Δ*malK* strain HS3205, and carbenicillin-resistant transformants were selected. Mal⁺ transformants were identified by replica plating onto MacConkey-maltose medium containing carbenicillin. Plasmid DNA from a Mal⁺ transformant was isolated, and the presence of the *Nco*I, *Pvu*II, and *Hin*dIII restriction sites was confirmed by restriction digestion. This plasmid was called pMR10. In addition, the orientation of the *malK* gene was established by digestion of the plasmid DNA with *Sal*I. In order to construct pMR11, the *Bam*HI fragment of pMR10, which contains the

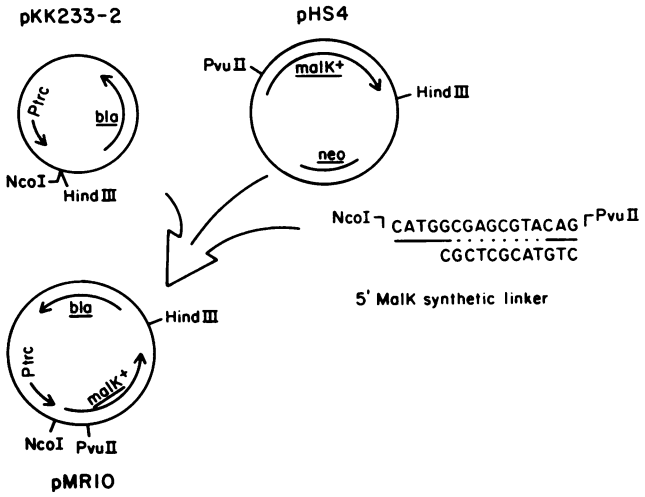

FIG. 1. Construction of plasmid pMR10. See Materials and Methods for details.

P*trc-malK* construction, was cloned into the *Bam*HI site of pACYC184.

Cell fractionation. In order to separate the soluble and particulate fractions of the cells, the following procedure was employed. Cells (200 ml of culture at an A_{600} of 0.8) were converted to spheroplasts by the procedure of Witholt et al. (26). The volume of the spheroplast suspension was adjusted to a final volume of 10 ml, and the spheroplasts were lysed by sonication at 30% output from a Heat Systems sonicator. Cell debris and unbroken cell were removed by centrifugation at 5,000 × *g* for 10 min. The particulate fraction of the cell was then collected by centrifugation at 200,000 × *g* for 2 h. The supernatant from this centrifugation contained the contents of the periplasm and the cytoplasm. The pellet material contained the inner membrane, the outer membrane, and other insoluble material.

Separation of inner and outer membranes by density gradient centrifugation was performed as described before (18).

Gel electrophoresis. Gel electrophoresis was carried out by the method of Laemmli (15). Samples were diluted in sample buffer containing 5% (vol/vol) 2-mercaptoethanol and heated in a boiling water bath for 5 min. Electrophoresis was carried out in 10% polyacrylamide gels. Proteins were detected by staining the gels with 0.2% Coomassie brilliant blue R-250 in methanol-acetic acid-water (5:1:5) and destaining in 7.5% acetic acid–5% methanol.

RESULTS

Overproduction of MalK in cells containing plasmid pMR10. In order to evaluate the degree to which the P*trc* construction caused increased production of the MalK protein, cells were induced with 1 mM IPTG for various lengths of time and extracts were prepared and examined by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS). We found that we could detect increased production of a polypeptide with an apparent molecular weight of 40,000 as soon as 30 min after the addition of IPTG (data not shown). Initial fractionation studies showed that the 40,000-molecular-weight material sedimented very rapidly at 30,000 × *g*. A more careful examination of the degree of overproduction was undertaken

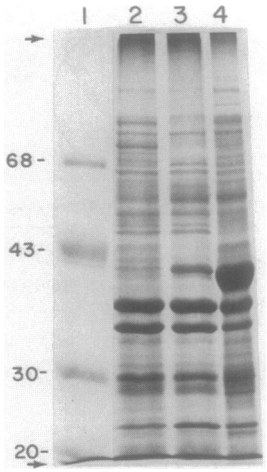

FIG. 2. Induction of MalK overproduction by IPTG. HS3328 was grown in M63 containing 0.5% Casamino Acids, 0.2% maltose, and carbenicillin (100 μg/ml). At time zero a 200-ml sample was removed from the culture and IPTG was added to a final concentration of 1 mM to the remainder. At 4 and 16 h, 200-ml samples of the culture were also removed. A particulate fraction was prepared from all three samples as described in the Materials and Methods section. A portion of each particulate fraction was subjected to electrophoresis on polyacrylamide gels containing SDS. Lane 1 contains molecular weight marker proteins (M_r in thousands); bovine serum albumin, 68,000 M_r; ovalbumin, 43,000 M_r; carbonic anhydrase, 31,000 M_r; soybean trypsin inhibitor, 20,100 M_r. Lane 2, Sample from time zero. Lane 3, Sample from 4 h. Lane 4, Sample from 16 h. Small arrows indicate the top of the separating gel and the bromphenol blue dye front.

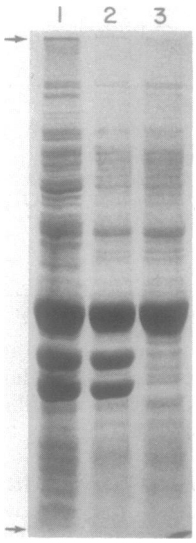

FIG. 3. Triton insolubility of the overproduced MalK protein. Approximately 200 μg of the particulate fraction derived from the 16-h time point described in the legend to Fig. 2 was incubated with 10 ml of the following solutions: lane 1, 50 mM Tris hydrochloride, pH 8; lane 2, 50 mM Tris hydrochloride (pH 8) containing 1% Triton X-100; lane 3, 50 mM Tris hydrochloride (pH 8) containing 1% Triton X-100 and 10 mM disodium EDTA. After incubation on ice for 1 h, the samples were centrifuged at 40,000 × *g* for 1 h and the supernatants were discarded. The pellets were heated in gel sample buffer and subjected to electrophoresis on polyacrylamide gels containing SDS. Small arrows indicate the top of the separating gel and the bromphenol blue dye front.

by inducing for various times and isolating the particulate fraction of the cells. These results are shown in Fig. 2. It should be noted that there was no detectable decrease in the growth rate of the bacteria after addition of IPTG, nor did the cells appear to form filaments. Because the bulk of the overproduced protein appeared to cosediment with the particulate fraction of the cell and the MalK protein has been reported to be located in the cytoplasmic membrane of wild-type cells, we attempted to define the location of the overproduced protein. To do this we performed two types of experiments that have been used to study the distribution of material between the cytoplasmic and outer membranes of gram-negative bacteria: differential detergent solubilization and equilibrium density centrifugation. First, the material shown in lane 3 of Fig. 2 was treated with Triton X-100 in either the absence or presence of EDTA. Triton X-100 is known to solubilize cytoplasmic membrane proteins in the absence of EDTA but is unable to solubilize outer membrane proteins unless divalent cations are chelated by EDTA (21, 22). Figure 3 shows that the 40,000-molecular-weight protein was not solubilized by Triton X-100 even in the presence of EDTA, although the other components of the particulate fraction were efficiently solubilized. To determine whether the bulk of the 40,000-molecular-weight protein was associated with the membrane, we examined the distribution of the 40,000-molecular-weight protein in sucrose gradients. The MalK protein was exclusively found at an apparent density of 1.23 g/ml together with 3-deoxy-D-*manno*-octolosonic acid and the major outer membrane proteins. These results indicate that the excess MalK is in an aggregated form which may associate with the outer membrane nonspecifically during cell breakage.

Homogeneous preparations of the 40,000-molecular-weight protein could be recovered by cutting out the band from SDS-polyacrylamide gels of the Triton X-100-insoluble material. This material was subjected to automated Edman degradation. The first 11 amino acid residues of the protein corresponded to the predicted amino acid sequence from the *malK* gene (11). In addition to its ability to participate in maltose transport in vivo, the similarity in molecular weight and the identity of the amino acids strongly suggested that the protein was identical to wild-type MalK protein.

Cells which overproduce MalK are Mal$^-$. We wanted to find out whether cells which overproduce MalK were able to transport maltose. Plasmid pMR11 was introduced into HS3348, and its Mal phenotype was examined on MacConkey-maltose plates in the presence and absence of 1 mM IPTG. Surprisingly, colonies of this strain were completely Mal$^-$ (white) in the presence of IPTG, while in the absence of IPTG the bacteria had a completely normal Mal$^+$ phenotype. This result was confirmed by tests on minimal maltose medium. Overproduction of MalK had no effect on the ability of bacteria to grow on other sugars, such as glycerol, galactose, and sorbitol. When maltose transport assays were performed on cells grown on L broth and maltose with or without IPTG, the bacteria grown in the presence of IPTG had undetectable levels of maltose transport, significantly below the levels present in uninduced wild-type cells (Fig. 4). When the transport activity of cells grown on L broth and maltose was measured at various times after induction with IPTG, it was found that as soon as 30 min after the addition of IPTG there was a significant decrease in transport activity (data not shown). These results could be explained if the overproduced MalK interfered with the activity of the transport proteins synthesized after the onset of MalK overproduction or if the excess MalK affected *mal* gene expression and no new transport proteins could be synthesized.

Cells which express the *mal* regulon constitutively are

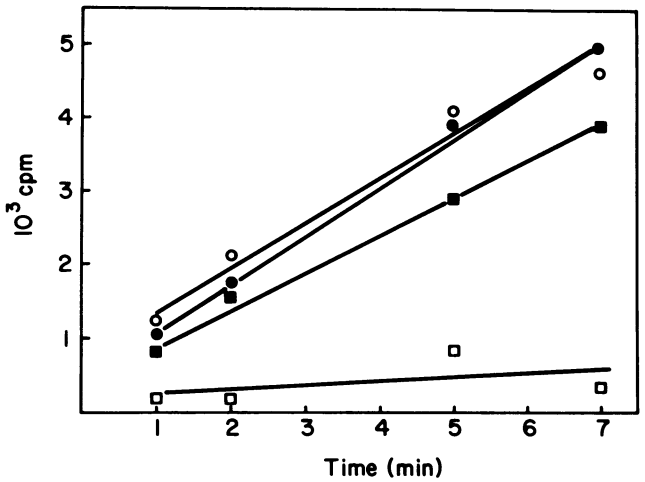

FIG. 4. Overproduction of MalK prevents maltose uptake. HS3349 was transformed with either pACYC184 or pMR11. Cultures were grown in LB medium containing 0.2% maltose and chloramphenicol (50 μg/ml). IPTG was present when indicated at a concentration of 1 mM. After overnight growth the cells were centrifuged, washed three times with M63 salts, and suspended in M63 salts containing spectinomycin (50 μg/ml). [^{14}C]Maltose (365 mCi/mmol) uptake was measured by filtration of 10^7 cells at an external concentration of 1 μM. Symbols: ●, pACYC184 in HS3349; ○, pACYC184 in HS3349 plus IPTG; ■, pMR11 in HS3349; □, pMR11 in HS3349 plus IPTG.

insensitive to excess MalK. If excess MalK interferes with the induction of the *mal* system by removing internal inducer, cells which express the *mal* regulon constitutively should be insensitive to excess MalK. In contrast, if excess MalK directly blocks the activity of the transport system and prevents sugar entry, cells which express the *mal* regulon constitutively would still be sensitive to excess MalK and remain Mal$^-$. As mentioned above, the MalT protein is known to stimulate transcription of *mal* genes in the presence of inducers (19). Two genetic alterations of the *malT* gene result in constitutive expression of the *mal* regulon. Missense mutations in *malT* have been identified which result in mutant MalT proteins that stimulate high levels of *mal* gene expression even in the absence of exogenous inducers (7). Promoter mutations which raise the concentration of MalT also result in high levels of *mal* gene expression in the absence of exogenous inducers (4). We examined the effects of excess MalK in both kinds of constitutive strains. Strain HS3438 contains the *malT1*(Con) allele (7) and HS3440 contains the *malTp1p7* promoter mutation, which results in approximately 50-fold-higher levels of MalT than in wild-type bacteria (4). When grown in the absence of maltose, both strains exhibited levels of *mal* gene expression that were comparable to those of fully induced wild-type strains. Plasmid pMR11 was transformed into both strains. In both cases the resulting bacteria were completely Mal$^+$ even in the presence of 1 or 5 mM IPTG. Under these conditions, the levels of MalK overproduction were indistinguishable from those in the *malT*$^+$ strains described above. These results indicate that excess MalK does not interfere with maltose catabolism by directly blocking maltose transport but probably acts by preventing the action of wild-type MalT.

Excess MalK decreases the basal, uninduced level of *malP* gene expression. In order to measure the effects of excess MalK on *mal* gene expression, we took advantage of a strain which contains a *malP-lacZ*$^+$ transcriptional fusion (7). In this strain, production of β-galactosidase is dependent on the *malPQ* promoter. We transformed the pMR11 plasmid into strains that contained this gene fusion and either the *malT*$^+$ or the *malT*(Con) allele described above. In these strains the *malK* gene was expressed at a maximum rate in the absence of IPTG because there was no *lacI* gene to control the activity of the P*trc* promoter. We measured the levels of β-galactosidase (Miller units [17]) in these strains after growth in LB in either the absence or presence of maltose. These results are shown in Table 2. The presence of excess MalK decreased the uninduced level of *malP* expression approximately 10-fold. This effect cannot be due to a decrease in maltose transport activity, because there was no maltose in the medium. The excess MalK must be decreasing *malP* expression by affecting the ability of MalT to stimulate transcription. When the *malT*$^+$ cells were grown in the presence of maltose, there was no increase in β-galactosidase activity. This is consistent with the Mal$^-$ phenotype of HS3348 in the presence of excess MalK. The expression of the *malP* gene in strains that contained the *malT1*(Con) allele was unaffected by excess MalK. This is also consistent with the Mal$^+$ phenotype of strain HS3338 in the presence of IPTG. Different ways in which excess MalK might interfere with the activity of MalT are discussed below.

TABLE 2. Effect of excess MalK on β-galactosidase activity in a *malP-lacZ*$^+$ gene fusion strain

Strain	Plasmid	0.2% Maltose in medium	β-Galactosidase activity (U)
HS3134	pKK233-2	−	101
		+	995
	pMR10	−	9
		+	11
pop3301	pKK233-2	−	789
		+	1,210
	pMR10	−	669
		+	1,115

DISCUSSION

We have constructed a plasmid in which expression of the *malK* gene is under the control of the P*trc* promoter. When transcription of *malK* from P*trc* is not maximal (i.e., in the absence of IPTG), sufficient MalK was produced to complement the Δ*malK16* mutation. In the presence of 1 mM IPTG, this plasmid resulted in significant overproduction of the MalK protein. The overproduced protein had the same molecular weight as MalK produced in wild-type bacteria, and the first 11 amino acids of the overproduced MalK corresponded to those predicted by the nucleotide sequence of the *malK* gene. When the maximum rate of MalK synthesis was induced, the protein formed an aggregate which sedimented rapidly and was insoluble in Triton X-100. The aggregated material appeared to associate nonspecifically with the outer membrane.

Maximum expression of MalK renders bacteria which are genetically *mal*$^+$ phenotypically Mal$^-$. We showed that bacteria which either (i) overproduce wild-type MalT 50-fold or (ii) produce a mutant form of MalT which does not require inducer to stimulate transcription remain Mal$^+$ in the presence of excess MalK. In addition, excess MalK decreased the uninduced, basal level of a *malP-lacZ*$^+$ transcriptional fusion. These results rule out the idea that excess MalK prevents maltose catabolism in otherwise wild-type bacteria by interfering with the activity of the maltose transport system. The results are consistent with the idea that MalK

has a second regulatory function in addition to its role in active maltose transport. MalK may regulate *mal* gene expression in a variety of ways. It may act directly as a repressor and interact with sites on *mal* genes. Alternatively, it may interact with the MalT protein and modulate its ability to stimulate transcription. Finally, MalK may bind, enzymatically degrade, or pump out of the cell the inducer substance(s) which is necessary for the activity of MalT. Preliminary data suggest that excess MalK does not pump inducers out of the cell. The effect of excess MalK on a *malF-lacZ* gene fusion strain was examined. This strain does not contain either active MalF or MalG, the two integral membrane components of the maltose transport system. The ability of excess MalK to lower the β-galactosidase activity of the *malF-lacZ* fusion strain was the same whether or not wild-type MalF and MalG were supplied in *trans*. Therefore, if indeed excess MalK were able to pump the inducer(s) out of the cell, it must be able to do so without the participation of MalF and MalG.

The MalK protein contains a potential nucleotide-binding fold found in a variety of proteins which are known to bind ATP or GTP (11–13). It is tempting to think that the ability to bind and hydrolyze a high-energy phosphate-containing compound could contribute the energy for active transport. A second potential function for such a site might be to participate in the phosphorylation of internal substrate molecules. MalK is somewhat larger than the homologous proteins of other transport systems that operate with a periplasmic binding protein. The extra sequence at the carboxy-terminal part of MalK not found in the other proteins might be the location of a site with the ability to mediate the regulatory function of MalK. Further genetic and biochemical investigation of the structure and functional domains of the MalK protein will be necessary to understand its dual role in active transport and regulation of *mal* gene expression.

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