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The ABC transporter MalFGK$_2$ sequesters the MalT transcription factor at the membrane in the absence of cognate substrate

Evelyne Richet[1,2,*], **Amy L. Davidson**[3], and **Nicolas Joly**[1,2,4,5]

[1]Institut Pasteur, Unité de Génétique Moléculaire, Département de Microbiologie, 25 rue du Dr. Roux, F-75015 Paris, France

[2]CNRS, ERL3526, 25 rue du Dr. Roux, F-75015 Paris, France

[3]Purdue University, 560 Oval Dr. West Lafayette, IN, USA

[4]Univ. Paris Diderot, Sorbonne Cité, Cellule Pasteur, rue du Dr. Roux, F-75015 Paris, France

SUMMARY

MalK, the cytoplasmic component of the maltose ABC transporter from *Escherichia coli* is known to control negatively the activity of MalT, the activator of the maltose regulon, through complex formation. Here we further investigate this regulatory process by monitoring MalT activity and performing fluorescence microscopy analyses under various conditions. We establish that, under physiological conditions, the molecular entity that interacts with MalT is not free MalK, but the maltose transporter, MalFGK$_2$, which sequesters MalT to the membrane. Furthermore, we provide compelling evidence that the transporter's ability to bind MalT is not constitutive, but strongly diminished when MalFGK$_2$ is engaged in sugar transport. Notably, the outward-facing transporter, i. e. the catalytic intermediate, is ineffective in inhibiting MalT compared to the inward-facing state, i. e., the resting form. Analyses of available genetic and structural data suggest how the interaction between one inactive MalT molecule and MalFGK$_2$ would be sensitive to the transporter state, thereby allowing MalT release upon maltose entrance. A related mechanism may underpin signalling by other ABC transporters.

INTRODUCTION

Over the last decade, dissection of regulatory circuitries in bacteria have unveiled the roles played by transporters in signal transduction. Notably, bacterial transporters often couple the expression of a set of genes to substrate internalization via the control of a transcriptional regulator. As shown for phosphotransferase system transporters (PTS) (Deutscher et al., 2006) and the FecA-type outer-membrane transporters (Brooks and Buchanan, 2008), coupling between substrate translocation and signalling involves regulator sequestration. Sequestration occurs when the transporter is in a specific conformation. The anti-terminator BglG is specifically sequestered by the inner-membrane β-glucoside PTS EIIB^{Bgl} transporter in its resting state (Amster-Choder, 2005; Raveh et al., 2009). In contrast, the

*Corresponding author: erichet@pasteur.fr.
[5]Present address: Institut Jacques Monod, CNRS UMR 7592, Université Paris 7- Denis Diderot, Batiment Buffon, 15 rue Helene Brion, 75205 Paris Cedex 13, France.

Mlc repressor, a glucose signalling protein, is sequestered by the PTS $EIIB^{Glc}$ enzyme only when transport is occurring, with the non-phosphorylated form of $EIIB^{Glc}$, a signature of active transport, specifically interacting with Mlc (Lee et al., 2000; Nam et al., 2001; Tanaka et al., 2000). Signal transduction by the outer-membrane Fe-citrate FecA transporter involves a more complex chain of events. Substrate binding to the transporter induces a TonB/ExbD/ExbB-dependent conformational change that is sensed by FecR, an inner membrane protein and leads to the activation of FecI, a σ factor bound to FecR (Brooks and Buchanan, 2008).

ATP-binding cassette (ABC) transporters might also mediate signalling, as suggested by extensive studies of the *Escherichia coli* maltose transporter (Bordignon et al., 2010) as well as recent findings on peptide-7 ABC transporters from Firmicutes (Dintner et al., 2011; Hiron et al., 2011). The maltose transporter, which depends on the periplasmic maltose-binding protein (MalE), comprises two inner membrane proteins (MalF and MalG) and two copies of MalK, a cytoplasmic protein. MalK is made of an N-terminal nucleotide-binding domain (NBD) and a C-terminal regulatory domain (RD) that also contributes to the dimerization of MalK within the transporter complex. In addition to hydrolyzing ATP to provide energy for transport, MalK negatively controls the activity of MalT, a transcriptional activator dedicated to the maltose regulon (Hofnung et al., 1974; Joly et al., 2004; Reyes and Shuman, 1988). MalT cycles between an ADP-bound, monomeric resting form and an ATP-bound, multimeric form which cooperatively binds the MalT boxes present in the target promoters (Fig. 1) (Marquenet and Richet, 2007). Maltotriose, the inducer, binds to MalT, stabilizing the active form, while MalK binds to and stabilizes the resting form of MalT, thereby antagonizing maltotriose (Böhm et al., 2002; Joly et al., 2004; Marquenet and Richet, 2007; Schlegel et al., 2002). In this way, MalK can prevent activation of MalT by endogenous maltotriose, a glycogen degradation product (Dippel et al., 2005), so that induction of the maltose regulon can be coupled to cognate substrate internalization (Boos and Böhm, 2000).

The coordinate conformational changes that drive the translocation of a maltose molecule by $MalFGK_2$, and how they are coupled to ATP binding and hydrolysis by MalK, are understood at the atomic level (Khare et al., 2009; Oldham and Chen, 2011; Oldham et al., 2007). The transporter rests in an inward-facing conformation where a gated substrate-binding site born by MalF and MalG is exposed to the cytoplasm and the NBDs in the MalK dimer are open. The binding of closed, maltose-bound MalE to MalF/MalG on the periplasmic side drives ATP-dependent closure of the MalK NBDs, with the bound ATPs buried at the dimer interface, which converts the transporter to an outward-facing state where the substrate-binding site is exposed to the opposite side of the membrane and pries open maltose-bound MalE. Maltose is transferred to the gated binding site in this conformational state. Upon ATP hydrolysis, the transporter returns to the resting state and maltose is released in the cytoplasm. In contrast, the molecular details of the mechanism whereby MalK controls MalT activity remains elusive. It is unclear whether MalT control is mediated by free MalK or by the transporter and how MalT inhibition is relieved upon maltose transport. Based on in vivo data, Panagiotidis et al. (1998) suggested that the

regulatory function of MalK and transport are coupled and that only the resting form of the transporter interacts with MalT.

Using in vivo approaches, we demonstrate here that MalT inhibition actually involves sequestration of the activator by the maltose transporter, with MalK mediating the interaction with MalT. We also analyse under well controlled in vivo conditions the effects of mutations that change the proportion of the transporter population that is in the resting state and provide compelling evidence that MalT preferentially interacts with the resting transporter, thereby confirming the notion of a coupling between transport and signalling by this ABC transporter. A model is proposed which accounts for the specific interaction between the resting transporter and the transcriptional activator.

RESULTS

Efficient MalT inhibition by MalK involves the maltose transporter

To determine whether MalT inhibition by MalK involves the maltose transporter or a cytoplasmic form of MalK, we analysed the effect of a deletion of the *malG* gene, which encodes one of the inner membrane components of the transporter, on MalT control by MalK. For this purpose, we used a strain (pop7234) harbouring the *lac* operon under the control of *malEp*Δ*92*, a MalT-dependent promoter (Richet, 1996), which allows monitoring MalT activity via measurement of the β-galactosidase level. In this strain, hereafter referred to as the MalT reporter strain, the MalT-dependent promoters that drive *malEFG* and *malKlamBmalM* expression have been replaced by constitutive promoters to avoid the possibility that the release of MalT inhibition by MalK be partially masked by the concomitant increase in the concentrations of the maltose transporter components. Whereas *malEp* was replaced by P_{tac}, a strong promoter, *malKp* was substituted by a weak promoter, $P_{KAB\text{-}TTGG}$ (Burr et al., 2000) in order to obtain a low level of *malK* expression, similar to that prevailing in a wild-type strain when MalT is inhibited by MalK. This ensured a physiological concentration of the maltose transporter and avoided the presence of free MalK. When bacteria were grown in M9 minimal medium supplemented with glycerol, i. e., under conditions in which MalT was inhibited by MalK, MalT activity was 31-fold lower in the reporter strain than observed in a Δ*malK* derivative (Fig. 2). In contrast, the level of β-galactosidase made in a Δ*malG* reporter strain was only 2-fold lower than that measured in the Δ*malK* reporter strain, suggesting that the ability of MalK to inhibit MalT was dramatically reduced in the absence of MalG (Fig. 2). A two-fold decrease in MalT activity was also observed when both MalF and MalG were absent, which excluded the possibility that residual inhibition was due to a MalK/MalF sub-complex (Kennedy et al., 2004). Controls showed that the levels of β-galactosidase measured in the Δ*malK* and the Δ*malFG* Δ*malK* strains were identical (Fig. S1). Furthermore, based on immunoblots analyses, the amounts of MalK present in the cells were the same irrespective of MalF/MalG presence (see hereinafter and Fig. S2). From all of these data, we therefore infer that the residual inhibition observed in the absence of MalF and MalG results from the weak ability of free MalK to interact with MalT, as observed in vitro (Joly et al., 2004) and that free MalK is much less effective (13-fold less) than the MalF/MalG-associated MalK in inhibiting MalT. Under physiological conditions, MalT inhibition is hence primarily ensured by transporter-

associated MalK, most likely via the formation of a MalT/transporter complex. The levels of β-galactosidase activity were the same in the wild-type and the Δ*malE* reporter strains (Fig. 2), which indicates that the periplasmic maltose-binding protein is not involved in MalT inhibition by the transporter (The Δ*malE444* allele does not alter the expression levels of the downstream genes *malF* and *malG* (Shuman, 1982) (data not shown)). We also verified that *malK-G346S* and *malT-N637T*, two mutations that specifically prevent MalK from interacting with MalT (Kühnau et al., 1991; Richet et al., 2005), alleviated MalT inhibition to the same extent as the *malK* deletion (Fig. 2).

A GFP-MalT chimera is localized to the membrane in the absence of maltose in the growth medium

To obtain direct evidence that MalT was sequestered by the maltose transporter in the absence of maltose in the growth medium, we fused the GFP protein to the N-terminus of MalT in order to localize the activator in the bacterium by fluorescence microscopy. A Δ*malT* strain (pop7212) that harboured pOM185, a one-copy plasmid containing a *gfp-malT* fusion gene under the control of a CRP-independent *malTp* promoter derivative, was grown in M63 minimal medium supplemented with glycerol. As revealed by fluorescence microscopy analyses, the GFP-MalT chimera was localized mainly to the membrane (Fig. 3A). Immunoblot analyses of total cell extracts with anti-MalT and anti-GFP antibodies confirmed the presence of the GFP-MalT fusion protein together with two major degradation products of 115 and 70 kDa, respectively. Little if any GFP and MalT polypeptides were however observed (Fig. 3B). A comparison with the profiles obtained with a GFP-MalT variant lacking the DNA-binding domain, which is located at the C-terminus of the protein, indicated that the main degradation products were truncated at their N-termini (see Fig. 5) and hence corresponded to a non-functional 70 kDa ‘MalT fragment and an ~ 115 kDa ‘GFP-MalT polypeptide. Based on quantitative immunoblot analyses (data not shown), the level of the latter fragment represented less than 1/8 of the full-length chimera level, which excludes alteration of the localization pattern of the full-length fusion protein.

As shown by immuno-blot analyses, GFP-MalT was slightly overproduced compared to MalT in a wild-type strain grown in the same conditions (Fig. 3B). The steady-state level of GFP-MalT was ~ 6-fold higher than the chromosomal MalT level observed in a *malT*$^+$ strain harbouring an empty vector (pop7212 (pJM241)), when grown in the presence of glycerol, while the level of the 115 kDa fragment was similar to that of MalT in a wild-type strain (Fig. 3B). Further analyses revealed that the maltose regulon was almost fully induced in pop7212 (pOM185) despite the absence of maltose in the growth medium (data not shown), as typically observed when MalT is overproduced (Schwartz, 1987). MalT overproduction in cells grown in the absence of maltose actually increases the concentrations of both resting and active MalTs, with the balance between both forms maintained due to the MalT/MalK regulatory loop. As a result, the concentration of active MalT is high enough for promoter binding and transcription activation although a fraction of MalT is still bound to and maintained in the resting form by MalK. GFP-MalT presence in the cytoplasm is consistent with the weak contrast between membrane fluorescence and cytoplasm fluorescence (Fig.

3A), compared to fully membrane-associated GFP-fused proteins, such as GFP-MalK in the presence of MalF/MalG (data not shown).

GFP-MalT is released from the membrane upon maltose addition

To obtain evidence that the observed GFP-MalT localization to the membrane was physiologically significant and reflected MalT sequestration by the $MalFGK_2$ transporter, we tested whether it depended on the MalT moiety and whether the chimera was released upon maltose addition. To address these questions, we used a Δ*malT* strain (pop7216), hereafter referred to as the GFP reporter strain, in which the MalT-dependent promoters controlling the maltose-transporter encoding genes had been replaced by strong, constitutive promoters, with P_{tac} and P_{con} replacing *malEp* and *malKp*, respectively (Deuschle et al., 1986). These constitutive promoters ensured the high level of transporter expression that was found to be required to see membrane localization of the GFP-MalT chimera by fluorescence microscopy and uncoupled the synthesis of transporter components from the level of MalT activity. When the GFP reporter strain harbouring pOM185 was grown at 32°C in M9 minimal medium in the absence of maltose, the GFP-MalT chimera was mainly localized to the membrane (Fig. 4A).

In contrast, when the reporter strain contained a pOM185 derivative expressing GFP only, the cells were uniformly fluorescent (Fig. 4C), which reflected a purely cytoplasmic localization of GFP and therefore indicated that the MalT moiety was responsible for the chimera localization at the membrane. When maltose was added to the growth medium, no membrane localization was observed; the chimera was fully clustered in discrete foci that were centrally located in the cells with one or two fluorescent foci per bacterium (Fig. 4B). Such a pattern also depended on the MalT moiety because GFP was evenly distributed in the cytoplasm in the presence of maltose when the MalT encoding region was deleted (Compare Fig. 4B and D).

To test the hypothesis that the maltose-induced clusters corresponded to activated GFP-MalT bound to chromosomal DNA, we asked whether they were still observed when the MalT DNA-binding domain (DBD), which is located at the C-terminus of the protein, was missing. In the absence of maltose in the growth medium, the GFP-$MalT^{\Delta DBD}$ variant was sequestered to the membrane like the wild-type chimera (Fig. 4E). However, when maltose was present in the growth medium, GFP-$MalT^{\Delta DBD}$ was not clustered in central foci as observed with GFP-MalT, but clustered in thin rods instead that were located in the cytoplasm close to the membrane (Fig. 4F, G). Based on immunoblot analyses, neither maltose addition nor deletion of the MalT DNA-binding domain changed the steady-state levels of GFP-MalT, MalF and MalK, thereby excluding the possibility that the observed changes in chimera localization were caused by a dramatic alteration in the chimera or transporter levels (Fig. 5).

Taken together, these data demonstrated that, as predicted, GFP-MalT was not localized to the membrane in the presence of maltose and that the central clusters formed by GFP-MalT under these conditions reflected a DNA-bound state. The foci observed in the presence of maltose most likely represent large-size GFP-MalT homopolymers, whose inducer-triggered assembly was mediated by the NOD module of MalT (Fig. 1) and whose binding to

chromosomal DNA was nucleated by the MalT-box arrays present in the MalT-dependent promoters. When activated MalT is present in excess, MalT box arrays are known to nucleate cooperative, non-specific binding of the activator to adjacent DNA sequences via homopolymer extension and to interfere with promoter activation by MalT (Larquet et al., 2004; Raibaud et al., 1989; Richet and Raibaud, 1991; Schwartz, 1987). This event, which is probably not physiologically relevant, would have occurred here because of the high concentration of active MalT moieties resulting from the high GFP-MalT expression level and maltose presence. Consistently, maltose addition to pop7212 (pOM185) triggered GFP-MalT clustering in central foci and inhibited the expression of *malEFG* and *malKlamBmalM* (data not shown). Likewise, the rods formed by the ΔDBD chimera in the presence of maltose most likely represent long GFP-MalT$^{\Delta DBD}$ homopolymers that resulted from self-association of the activated MalT$^{\Delta DBD}$ moieties (Larquet et al., 2004).

GFP-MalT is sequestered to the membrane by the maltose transporter via a MalK/MalT interaction

To obtain direct evidence that GFP-MalT sequestration to the membrane was mediated by the maltose transporter, we looked at the effects of mutations known to interfere with MalT inhibition by MalK. In a Δ*malFG* GFP-reporter strain grown in the absence of maltose, the GFP-MalT chimera was not localized to the membrane, but evenly distributed in the cytoplasm (Compare Fig. 6A and E). In a Δ*malK* GFP-reporter strain grown under the same conditions, the GFP-MalT chimera was cytoplasmic, with a noticeable fraction of the cell population additionally exhibiting one or two central foci (Fig. 6B). Substitutions MalK-G346S and MalT-N637T, which disrupt MalT/MalK interaction, caused the same subcellular localization pattern as *malK* deletion (Fig. 6C, D). Based on western blot analyses, the GFP-MalT levels were not altered by the mutations introduced, nor were the MalK and MalF levels altered by any of the mutations except for the deletion of the cognate gene (Fig. S2). These results hence demonstrated that chimera sequestration to the membrane depended on the integrity of the maltose transporter and that MalK mediated the interaction with MalT.

The discrete central foci exhibited by a fraction of the cells grown in the absence of MalK or when the MalK/MalT interaction was disrupted by substitutions (Figs. 6B–D) reflected clusters of DNA-bound MalT-GFP that formed upon activation of the MalT moieties by endogenous maltotriose. Note that only a fraction of the Δ*malK* cells exhibited foci in the absence of maltose in the growth medium while all of the wild-type reporter bacteria contained foci in the presence of maltose, consistent with the observation that Δ*malK* cells grown in the absence of maltose displayed a lower MalT activity than the wild-type strain grown in the presence of maltose (cf Fig. 2). This is most likely due to the fact that the cytoplasmic concentration of maltotriose is lower in the absence of maltose. As a result, the MalT equilibrium would be less shifted toward activated species than observed when maltose is added to the growth medium. Nucleation of MalT polymerization along the DNA from the MalT box arrays would then occur only in the cells in which the concentration of active MalT moieties is above a given threshold.

The cytoplasmic localization of GFP-MalT in Δ*malFG* cells and the quasi absence of cells exhibiting fluorescent clusters compared to the Δ*malK* bacteria is best explained by the residual ability of free MalK to bind MalT and compete with inducer binding (Joly et al., 2004). Albeit weak, this competition would be sufficient to bring the concentration of active MalT moieties below the threshold above which clusters are formed, consistent with the weaker MalT activity measured in a Δ*malFG* strain compared to a Δ*malK* strain (cf Fig. 2).

Departure from the resting state relieves MalT inhibition

Panagiotidis et al. (1998) found that mutations causing futile ATP hydrolysis cycles in a Δ*malE* strain and hence decreasing the fraction of the transporter in the resting state strongly enhanced MalT activity level under physiological conditions, thereby suggesting that only the resting transporter is able to interact with MalT. When assessing the effect of *malF500*, one of these mutations (which corresponds to the double substitution G338R and N505I), in a Δ*malE* derivative of our MalT reporter strain grown in the absence of maltose (Fig. 2), we observed the same increase in MalT activity. We further verified that this enhancement did not result from a decreased stability of the mutated-transporter components. The cellular level of MalK was not altered by *malF500* while MalF level was decreased by 20 % (Fig. S3). However this small reduction in the amount of MalF should not change the total amount of transporter assembled given that MalF and MalG were widely in excess with respect to MalK in the Δ*malE* MalT-reporter strain.

To obtain additional evidence that MalT specifically interacts with the transporter resting form, we examined whether MalT inhibition was relieved when the transporter was frozen in a conformation distinct from the resting state. By substituting a glutamine for the catalytic glutamate in MalK (E159), $MalFGK_2$ was frozen at a pre-hydrolytic state, i. e., in an outward-facing state with the MalK dimer closed (Oldham et al., 2007). The effect of the substitution on MalT activity was tested by growing the MalT reporter strain and its *malK-E159Q* derivative in M9 medium supplemented with glycerol. The substitution led to a 10-fold enhancement of MalT activity (Fig. 2), which points at a reduced ability of the transporter to sequester MalT when trapped in an outward-facing state. Mutation *malK-E159Q* altered neither the levels of MalK nor that of MalF (Fig. S3), which ruled out the possibility that *malK-E159Q* decreased the stability of the mutated-transporter components.

However, the possibility remained that the increases in MalT activity caused by mutation *malK-E159Q* in a wild-type context and by mutation *malF500* in a Δ*malE* context reflected PTS $EIIA^{Glc}$ enzyme-mediated effects instead of a reduced transporter ability to bind MalT. The non-phosphorylated form of EIIA, which is increased when cells are grown in the presence of glucose, is known to block maltose transport by interacting with MalK (Dean et al., 1990). Although the transporter form recognized by $EIIA^{Glc}$ has not been identified, we presumed that any mutation changing the transporter equilibrium between its different states might modify its affinity for non-phosphorylated $EIIA^{Glc}$. This might indirectly affect MalT interaction with the transporter if both proteins cannot bind MalK simultaneously. This might also alter the concentration of phosphorylated $EIIA^{Glc}$, and ultimately those of active Mlc repressor and CRP activator via an effect on $EIIB^{Glc}$ phosphorylation and cAMP synthesis, respectively (Deutscher et al., 2006), which could deeply affect the level of active

MalT. Both regulators indeed control the *malT* promoter (Decker et al., 1998; Raibaud et al., 1991) while *malEp*Δ*92*, the reporting promoter, is synergistically controlled by MalT and CRP (Vidal-Ingigliardi et al., 1991). Moreover, endogenous maltotriose production might possibly be modulated by any one of these regulators.

To rule out any EIIA-mediated effect, the effects of mutations *malK-E159Q* and *malF500* on the MalT activity level were analysed in pop7403, a derivative of pop7234 (the MalT reporter strain used above) bearing a deletion of *crr*, the EIIA-encoding gene. Cells were grown in the presence of cAMP to circumvent the reduced adenylate cyclase activity caused by EIIA absence (van der Vlag et al., 1994); this ensured a proper cellular level of CRP/cAMP, which plays a critical role in the activation of both the *malT* promoter and the MalT-reporting promoter (*malEp*Δ*92*), as mentioned above. Comparison of the levels of β-galactosidase in pop7403 and the isogenic *malK-E159Q* strain confirmed the increase in the level of MalT activity caused by the *malK* mutation trapping the transporter in the outward-facing state. The substitution increased MalT activity 5-fold in this genetic context, the level reached corresponding to 1/3 of that measured for pop7403 Δ*malK* (Fig. 7A). Likewise, mutation *malF500* strongly increased the transcriptional activity of MalT in pop7403 Δ*malE* (Fig. 7A). The β-galactosidase level measured in the *malF500* Δ*malE* strain was slightly higher than that observed in the Δ*malK* Δ*malE* strain. These results therefore establish that mutations decreasing the fraction of the transporter population that is in the resting state partially relieve MalT inhibition and that these effects cannot be accounted for by transporter instability nor are they mediated by EIIA. This fully supports the notion that the entity responsible for MalT sequestration is the transporter resting form.

A mutation that blocks the transporter in the resting state does not confer a “super-repressor” phenotype

MalK substitution G137A, which prevents MalK closure due to the alteration of the ABC signature (Chen et al., 2003), was described as conferring a “super-repressor” phenotype (Panagiotidis et al., 1998; Schmees et al., 1999) in a context where MalT inhibition was exerted by overproduced free MalK. Having established that the molecular species responsible for MalT sequestration is the transporter, we investigated the effect of substitution G137A on its ability to inhibit MalT. Since the resting transporter is characterized by an open MalK dimer (Khare et al., 2009), we predicted that the substitution should not confer a “super-repressor” phenotype when assessed under physiological conditions, i. e., under which the transporter is the active species regarding MalT inhibition. For this purpose, we used strain pop7342 (pOM252), a derivative of the MalT reporter strain (pop7234), which constitutively expressed the maltose transporter at a level close to that prevailing in a wild-type strain when MalT is inhibited, while ensuring the absence of free MalK. Strain pop7342 contains a *malPp-lac* fusion instead of a *malEp*Δ*92-lac* fusion and a deletion of the chromosomal *malT* gene. MalT was provided in *trans* from a one-copy number plasmid (pOM252) bearing the *malT* gene under the control of a constitutive promoter. In the absence of maltose in the growth medium, pop7342 (pOM252) exhibits a higher residual level of MalT activity than pop7234, thereby allowing a better assessment of enhanced MalT inhibition. When bacteria were grown in M9 minimal medium supplemented with glycerol, the residual levels of MalT activity represented 5 % and 6 % of

that measured in the Δ*malK* strain for the *malK-G137A* and the wild-type reporter strains, respectively (Fig. 7B). We verified that substitution G137A did not alter the cellular level of MalK (data not shown). Alteration of the signature motif therefore barely increases the ability of the transporter to inhibit MalT, consistent with the notion that MalT is specifically bound by the resting form of the transporter. The minor effect of substitution G137A on the ability of the transporter to inhibit MalT contrasts with the dramatic increase in the inhibitory effect of the substitution observed with overproduced cytoplasmic MalK (50 and ≥ 2 fold, respectively ((Panagiotidis et al., 1998; Schmees et al., 1999)).

Residual MalT inhibition by the maltose transporter when engaged in transport

To evaluate the extent to which MalT inhibition is alleviated when the transporter undergoes transport cycles, we determined whether mutations that specifically interfere with MalT/MalK interaction without altering other protein properties further increase MalT activity when the MalT reporter strain was grown in the presence of maltose. As shown in Fig. 2, both *malK-G346S* and *malT-N637T* enhanced the transcriptional activity of MalT to 150 % of the wild-type level, thereby indicating that inhibition of the activator is not fully relieved by transport. The residual inhibition observed in the wild type might be caused by the small fraction of transporter that is in the resting state at any time when maltose is transported inside the cells. Alternatively, the transporter may exhibit a residual affinity for MalT when engaged in an enzymatic cycle.

DISCUSSION

The maltose transporter, the entity responsible for MalT inhibition

In this paper, we established that, under physiological conditions, free MalK had little ability to modulate MalT activity in the absence of maltose in the growth medium and that inhibition of the activator involved its sequestration to the membrane by the maltose transporter $MalFGK_2$, with MalK mediating the interaction with MalT. First, we showed that MalT inhibition depended on the integrity of the transporter when MalK was present at a concentration close to that prevailing under conditions where MalT was inhibited by MalK. At this concentration, MalT inhibition by MalK dropped by a factor of 13 in the absence of MalF/MalG. Second, as revealed by fluorescence microscopy experiments, a GFP-MalT chimera was localized to the membrane in the absence but not in the presence of maltose. This localization also depended on the integrity of the transporter, and was not observed when MalT or MalK were altered by substitutions specifically interfering with negative control MalT. The MalT form that is sequestered is the ADP-bound, resting form (Joly et al., 2004; Marquenet and Richet, 2007, 2010) with its sequestration by the transporter shifting the equilibrium toward MalT inactive forms and preventing MalT activation by endogenous maltotriose (Fig. 1).

It is interesting to note that, although the very first analyses pointing at an inhibitory function of MalK indicated that mutations in *malEFG* (then referred to as *malJ*) partially induced the maltose regulon (Hofnung et al., 1974), the role of MalF and MalG in the control of MalT activity has been overlooked since then and in vivo studies of MalT control

by MalK generally involved free MalK forms, with MalK overproduction compensating for the weak ability of the isolated protein to bind MalT.

Departure from the resting state leads to the release of MalT inhibition

Most importantly, we provided compelling evidence that, as previously suggested by Panagiotidis et al. (1998), the ability of $MalFGK_2$ to sequester MalT was not constitutive but specific to the resting form. In other words, when the transporter is busy translocating a sugar molecule, its affinity for MalT is reduced. Panagiotidis et al. (1998) observed that the futile ATP hydrolysis cycles caused by *malF500* in a Δ*malE* strain induce the maltose regulon. Here, we found that MalT inhibition was partially released when the transporter was trapped in the pre-hydrolytic state by mutating the catalytic glutamate, i. e., in a conformation where the transporter faced outward with the MalK dimer closed. Furthermore, we excluded the possibilities that the increases in the levels of MalT activity caused by *malF500* or *malK-malE159Q* resulted from a decreased stability of the mutated proteins or was mediated by the EIIA protein. It is also unlikely that the effects observed reflects a defect in the assembly of the mutated transporters; $MalF^{500}GK_2$ is indeed as stable as wild-type $MalFGK_2$ (Sharma et al., 2005) and purified $MalFGK_2^{E159Q}$ recovery is as high as that of the wild-type transporter (Oldham et al., 2007).

We also observed that substitution G137A which alters the signature motif in MalK and is expected to prevent MalK dimer closure, thereby restricting the transporter to the resting state conformation, barely enhances MalT inhibition under physiological conditions, i. e., when the molecular entity that down-modulate MalT activity is the maltose transporter. Given that $MalFGK_2$ is known to rest with the MalK dimer open in the absence of substrate, the very mild effect of substitution G137A is consistent with a specific interaction between the resting state and MalT.

MalT sequestration by the resting transporter would also nicely account for the weak ability of free MalK to interact with MalT and for the “super-repressor” phenotype specifically conferred to free MalK by the signature mutation. Based on ATP concentration in the cell and the *Km* of free MalK for ATP (150 μM) (Walter et al., 1992), soluble MalK dimers are expected to undergo futile ATP hydrolysis cycles, and the dimer may be only transiently in the open state during nucleotide exchange. In contrast, in the context of the transporter, the MalK dimer is kept in the open conformation by MalF and MalG - as long as maltose-bound MalE is not around – even though the nucleotide binding site may be occupied. As a result, at any time, only a small fraction of soluble MalK dimer would be in the resting state, accounting for its low affinity for MalT, and mutation of the signature motif could have a dramatic effect on the affinity for MalT and its inhibition, by interfering with dimer closure and stabilizing free MalK in the resting state.

In conclusion, the notion of a specific interaction between MalT and the resting transporter provides a cogent explanation to all of these observations, albeit quite different in nature, which strongly argues for the proposed model. As a result, when maltose is internalized, the maltose transporter population able to interact with MalT decreases and the activator is partially released from the membrane. We however predict that full induction also critically depends on an increase in inducer concentration that would result from maltose or

maltodextrin internalization and catabolism. As judged from the MalT activity enhancement caused by substitutions MalT-N637T and MalK-G346S in the presence of maltose, MalT is still substantially inhibited by $MalFGK_2$ when the latter is engaged in transport. Given that induction leads to ~ 30-fold increase in transporter concentration in a wild-type strain, it follows that net induction requires a quasi-complete neutralization of its ability to sequester MalT, which would be achieved by both an increase in inducer concentration and a decrease in the transporter ability to sequester MalT.

Molecular basis of the specific interaction between MalT and the resting transporter

MalT sequestration is known to be mediated by the MalK regulatory domain. Most of the substitutions that decrease MalT inhibition without impairing transport alter residues that are located on the regulatory domains (Böhm et al., 2002; Kühnau et al., 1991; E. R., unpublished). (The sole exception is substitution P72L which alters MalK NBD (Böhm et al., 2002), but we have not reproduced the stimulatory effect described (data not shown)). We note that the altered residues whose side-chains are exposed actually delineate two distinct areas on the MalK regulatory domains, defined by residues A248, A250, I251, Q253, Q255, W267 and residues L291, D297, E350, respectively (Fig. 8A). In the case of W267G and D297G, the substituted amino-acids eliminate the side-chain, which points at missing contacts and indicates that these areas actually represent two interaction patches. We speculate that they might be the counterparts of the two MalT surface determinants that are known to be involved in MalK binding, which are located on the NBD-HD and the sensor domain, respectively.

Whether the two MalK patches contacted by MalT are borne by the same regulatory domain or whether one MalT molecule contacts both regulatory domains, with each of them contributing one patch type, is unknown. But, the latter possibility would account for the specificity of the interaction between the activator and the resting transporter. The conformational changes that occur during one transport cycle indeed involve distortions of the dimeric structure formed by the MalK regulatory domains while leaving their intrinsic conformation unaltered. As illustrated in Fig. 8, these distortions change the relative positions of the patches borne by the two MalK protomers and are large enough to break the complex formed by MalT and the resting transporter if each protomer provides one interaction patch. It is worth pointing out that inhibition of the maltose transporter by $EIIA^{Glc}$ is predicted to follow a similar scenario wherein $EIIA^{Glc}$ would freeze $MalFGK_2$ in a specific state by complexing one face of the transporter via a contact with the NBD of one MalK subunit and a contact with the regulatory domain of the other subunit (Bordignon et al., 2010; Samanta et al., 2003). We presume that a signalling mechanism that relies on the sensing of the rigid-body rotations that characterize substrate translocation by ABC transporters might apply as well to other ABC transporters endowed of regulatory functions, such as the peptide-7 transporters (Dintner et al., 2011; Hiron et al., 2011).

EXPERIMENTAL PROCEDURES

Bacterial strains

The *E. coli* K12 strains used in this study (Table 1) are derivatives of MC4100 (Casadaban, 1976). Most of the mutations altering *malT* or genes encoding maltose transporter components were introduced in the bacterial chromosome by using M13 derivatives as shuttles (Danot and Raibaud, 1994). When the shuttle used was a derivative of M13mp7, -mp10 or -mp11, lyzogens were screened for as ColE1-resistant and M13mp18-resistant clones, as described (Danot and Raibaud, 1994). When the shuttle used was a derivative of M13Zeo2, a zeocin-resistant M13 variant, lysogens were selected on LB plates + 25 μg.ml^{-1} zeocin, except for the lysogens of pop7216 derivatives that were selected on LB plates + 6 μg.ml^{-1} zeocin. Cells that had undergone phage DNA excision and allelic exchange were screened by growing the lysogenic cells in the presence of 0.2 % deoxycholate and plating them on MacConkey plates + maltose (or lactose).

Replacement of the divergent, chromosomal *malEp* and *malKp* promoters by constitutive promoters

malEpKpΔ99::P_{tac}-P_{con} corresponds to a 261 bp deletion of the *malEp-malKp* intergenic region, that extends from position -6 with respect to the *malEp* transcription start site to position - 6 with respect to the *malKp* transcription start site, with the divergent *malEp* and *malKp* promoters replaced by P_{tac} and P_{con} (Deuschle et al., 1986), respectively. The substitution of *malEp-malKp* for *malEpKpΔ99*::P_{tac}-P_{con} was obtained by a two step recombination event involving pOM172. Strain pop4137, which is Mal^{-} because of the deletion of the CRP sites 1 and 2 in the *malEp-malKp* intergenic region (Vidal-Ingigliardi and Raibaud, 1991), was transformed with pOM172 and bacteria that had integrated pOM172 into the chromosome by homologous recombination in the *malB* region were selected on M63 minimal medium plates containing 0.4 % maltose and 100 μM IPTG. Excision and allele exchange were obtained by transducing pop4137 to Mal^{+} with a P1 stock from pop4137:: pOM172, and by selecting for Mal^{+} clones on M63 minimal medium plates containing 0.05 % maltose + 100 μM IPTG. This yielded strain pop4139, in which *malEFG* and *malK lamB malM* expression is driven by the constitutive P_{tac} and P_{con} promoters. See Fig. S4 for the sequence of the *malEpKpΔ99*::P_{tac}-P_{con} region.

*malEpKp*Δ100::P_{tac}-$P_{KAB\text{-}TTGG}$ corresponds to a 260-bp deletion of the *malEp-malKp* intergenic region, that extends from position -6 with respect to *malEp* to position - 7 with respect to *malKp*, with the divergent *malEp* and *malKp* promoters replaced by P_{tac} and $P_{KAB\text{-}TTGG}$ (Burr et al., 2000), respectively. See Fig. S5 for the sequence of the *malEpKp*Δ100::P_{tac}-$P_{KAB\text{-}TTGG}$ region.

Media

Minimal media M63 and M9 are described (Miller, 1992). The pBR322 and the pJM241 derivatives were maintained in the presence of 100 and 30 μg.ml^{-1} ticarcillin, respectively. Zeocin was used at 25 μg.ml^{-1} unless specified.

Plasmids and M13 derivatives

The plasmids and M13 derivatives used in this work are described in Table 2 and Supporting Information. The sequences of the primers used for cloning are given in Table S1.

Growth and processing of cells for fluorescence microscopy

Strain pop7216 (pOM185) or derivatives thereof were precultured at 32°C in L broth + 30 μg.ml^{-1} ticarcillin to A_{600} ≈ 1–2. M9 medium supplemented with glycerol + 30 μg.ml^{-1} ticarcillin was inoculated at A_{600} ≈ 0.002 with L broth cultures and bacteria were grown overnight at 30°C. Overnight cultures were diluted to A_{600} = 0.07 in the same medium and grown at 32°C to A_{600} ≈ 0.5. When indicated, 0.4 % maltose was added one hour before cell collect. Cells were centrifuged for 3 min × 16 000 g and resuspended in the growth medium so as to get A_{600} ≈ 10. Bacteria were immobilized on dried-agarose coated glass slides and observed with a Zeiss Axioplan2 fluorescence microscope mounted with a Hamamatsu CCD camera. For strain pop7212 (pOM185), M63 medium instead of M9 medium was used, and the cultures were incubated at 30°C.

β-galactosidase assays

Cells were grown in M9 medium supplemented with 0.4 % glycerol ± 0.4 % maltose, 0.01 % tryptophan, 1 mg/ml thiamine and 0.5 % L broth. The cultures were inoculated to a low cell density (A_{600} = 8.10^{-5} for cultures without maltose and 2.10^{-5} for cultures with maltose) and grown overnight at 32°C up to A_{600} 0.7–0.8. β-galactosidase was assayed at 28°C as described by Miller (1972), by using chloroform and 0.002 % SDS to permeabilize the cells. Specific enzyme activity is given in Miller units. All of the values represent the average of assays performed in duplicate on at least two independent cultures and are corrected for background values as measured with an isogenic Δ*malT* strain.

Western blotting

Total extracts from pop7216 (pOM185) and pop7234 derivatives grown as described for GFP localization experiments and β-galactosidase assays, respectively, were analysed on 9 % SDS polyacrylamide gels (acrylamide:bisacrylamide, 37.5:1). Western blotting were performed with rabbit polyclonal anti-MalT, anti-MalK, anti-MalF or anti-GFP (BD Living Colors) and horseradish peroxidase-conjugated secondary antibodies to rabbit immunoglobulin G, followed by ECL Plus (GE Healthcare) detection on a PhosphoImager. The immunoblot analyses were performed under linear dose-response conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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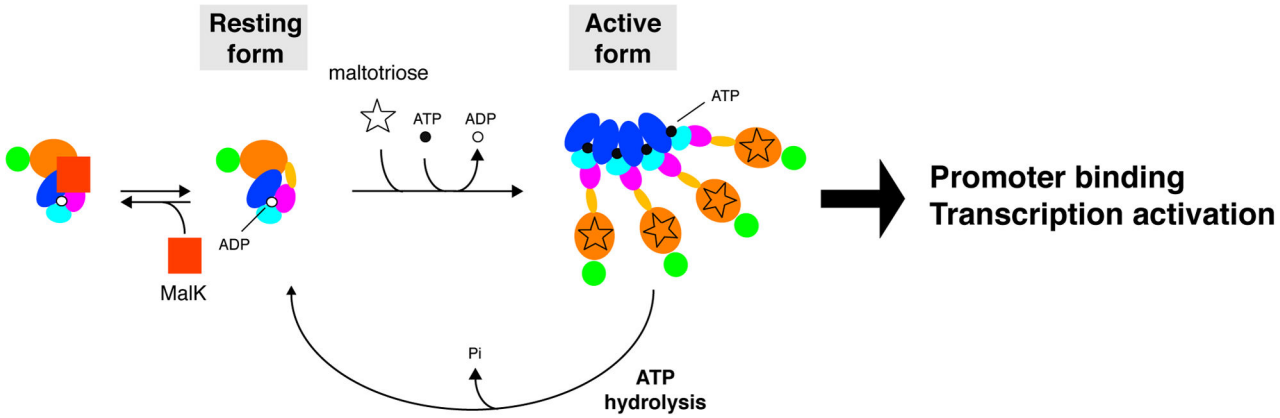

Fig. 1.

MalT cycle. MalT is made of a NOD module comprising the nucleotide-binding domain (NBD, blue), the helical domain (HD, cyan) and the winged-helix domain (WHD, magenta), an arm (yellow), a sensor domain (orange) and a LuxR-type DNA binding-domain (DBD, green). The resting form is characterized by a closed NOD conformation which buries the bound ADP and which is thought to be stabilized by the unliganded sensor domain. The resting form is further stabilized by the binding of negative effectors: MalK, MalY and Aes. The active form presents an open NOD that self-associates via head-to-tail interactions into polydisperse homopolymers with the bound ATP at the protomer interface. By allowing cooperative binding of the MalT boxes present in the targeted promoters, multimerization ensures promoter occupancy.

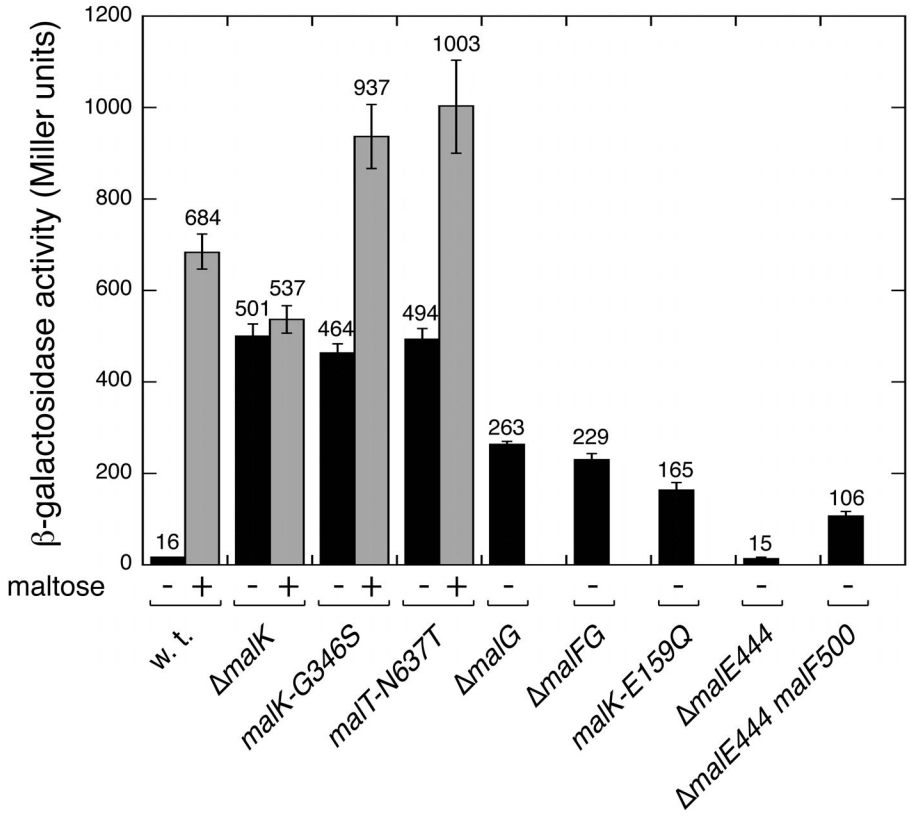

Fig. 2.

Effect of mutations in the genes encoding maltose-transporter components on MalT activity in vivo. The levels of β-galactosidase in various derivatives of the MalT reporter strain (pop7234) grown at 32°C in M9 medium supplemented with glycerol ± maltose were measured as described in Experimental Procedures. The values are the means ± SD of results from at least two independent experiments and are corrected for the background values measured with a Δ*malT* derivative (pop7237) (9 and 8 Miller units in the absence and in the presence of maltose, respectively).

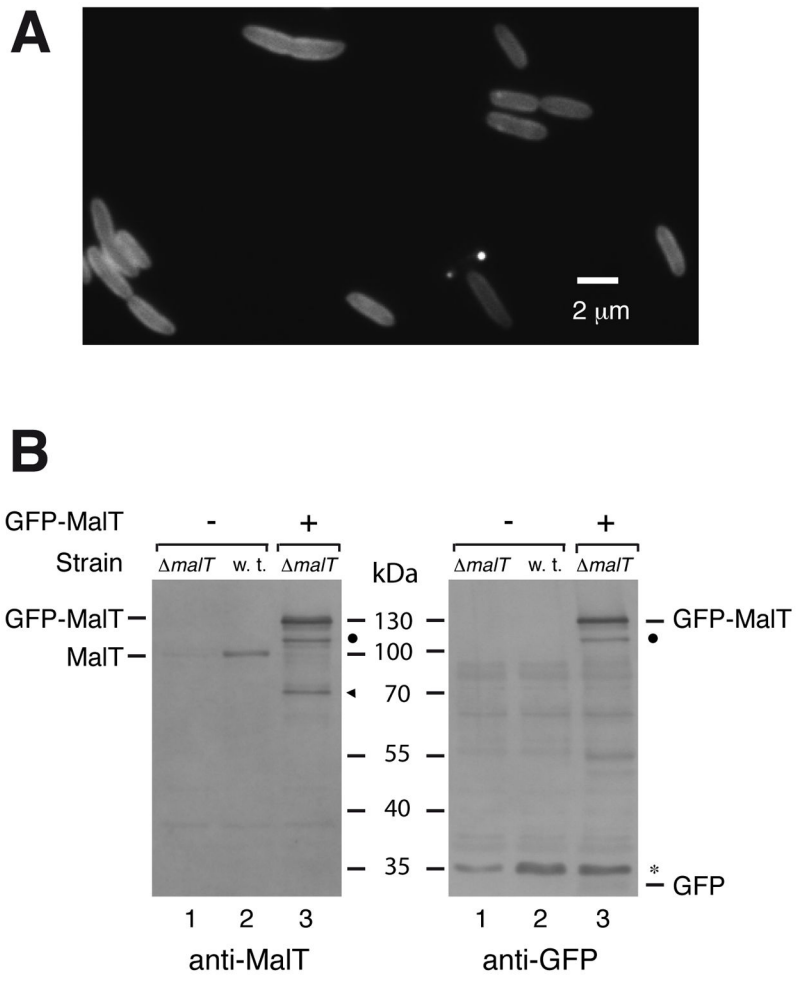

Fig. 3. Localization of a GFP-MalT fusion protein in a Δ*malT* strain in the absence of maltose in the growth medium

A. A Δ*malT* strain (pop7212) bearing a plasmid expressing a GFP-MalT chimera (pOM185) was grown at 30°C in M63 medium supplemented with glycerol and analysed by fluorescent microscopy as described in Experimental Procedures.

B. Immunoblot analyses of the total cell extracts with anti-MalT and anti-GFP antibodies. pop7212 (pJM241) (lanes 1), pop1951 (pJM241) (lanes 2) and pop7212 (pOM185) (lanes 3) were grown as in (A) and total cell extracts were analysed by SDS-PAGE and developped with anti-MalT and anti-GFP antibodies. The marked bands are the 115 kDa ‘GFP-MalT (closed circle) and 70 kDa ‘MalT (closed triangle) degradation products and an anti-GFP crossreacting band (*).

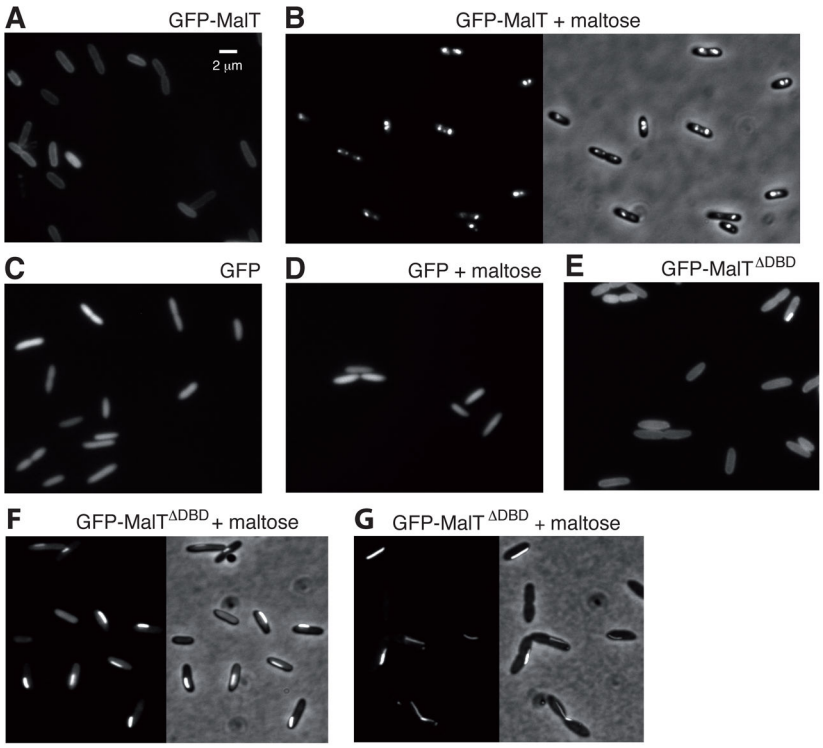

Fig. 4.

Effect of maltose on GFP-MalT localization. The GFP reporter strain (pop7216) containing pOM185 or derivatives thereof were grown at 32°C in M9 minimal medium supplemented with glycerol ± maltose and analysed by fluorescence microscopy as described in Experimental Procedures, except that maltose was added to the culture one hour before cells were collected. In (B) and (G), the exposure duration was 3 sec instead of 4 sec. The rightward images of panels B, F and G correspond to merged fluorescent and phase-contrast images.

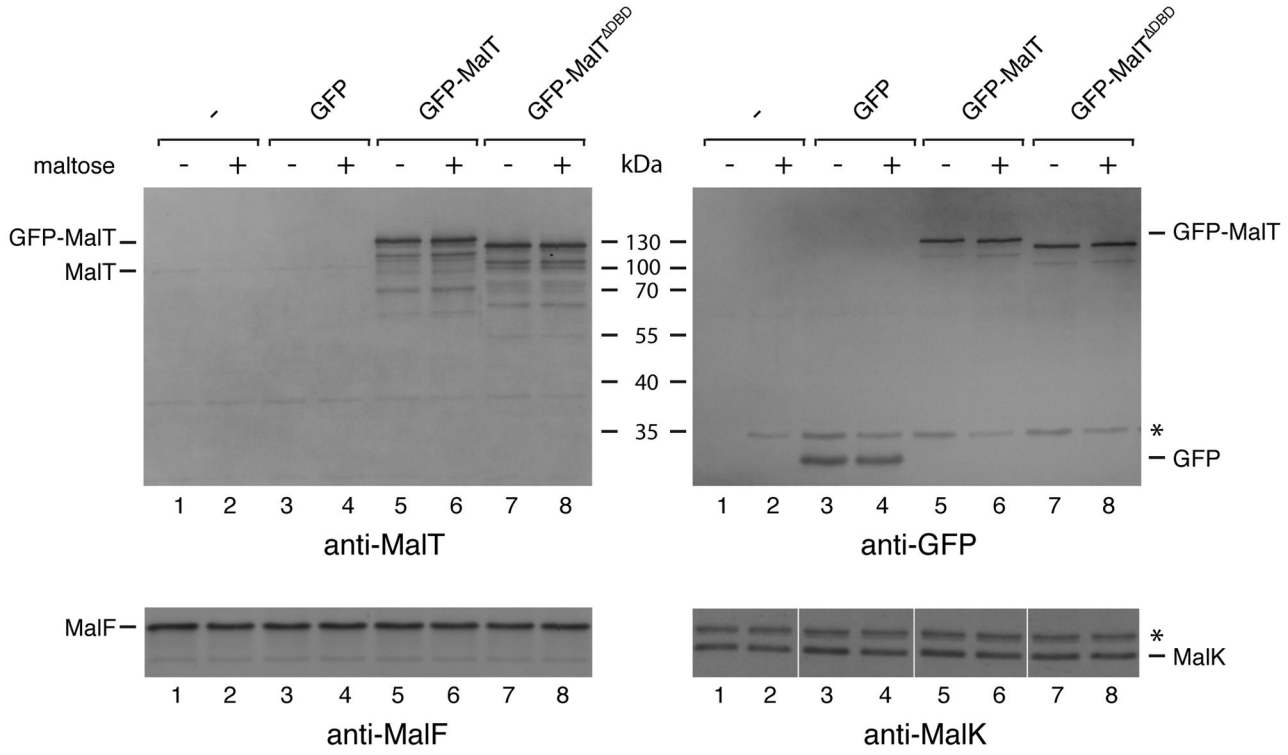

Fig. 5.

No effect of maltose on the levels of GFP-MalT, MalK and MalF. The GFP reporter strain (pop7216) containing pOM185 or derivatives thereof was grown at 32°C in M9 minimal medium supplemented with glycerol ± maltose and total cell extracts were analysed by SDS-PAGE and developed with anti-MalT, anti-GFP, anti-MalF and anti-MalK antibodies. pJM241, vector; pOM185-Δ*malT*, pGFP; pOM185, pGFP-MalT; pOM185-ΔDBD, pGFP-MalT$^{\Delta DBD}$. The lanes shown in the anti-MalK panel were cut out from the same immunoblot. The marked bands are crossreacting bands (*).

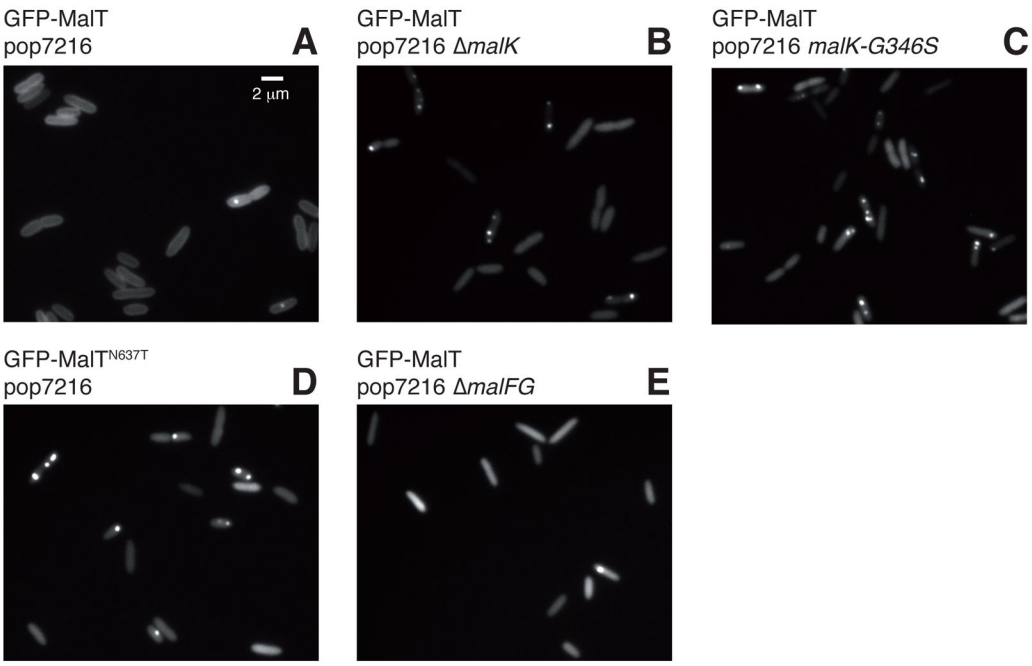

Fig. 6.

Effect of various mutations on GFP-MalT localization to the membrane. The GFP reporter strain (pop7216) or Δ*malK*, *malK-G346S*, and Δ*malFG* derivatives thereof (pop7293, pop7303, and pop7307, respectively) containing pOM185 or pOM185-N637T were grown at 32°C in M9 minimal medium supplemented with glycerol and analysed by fluorescence microscopy.

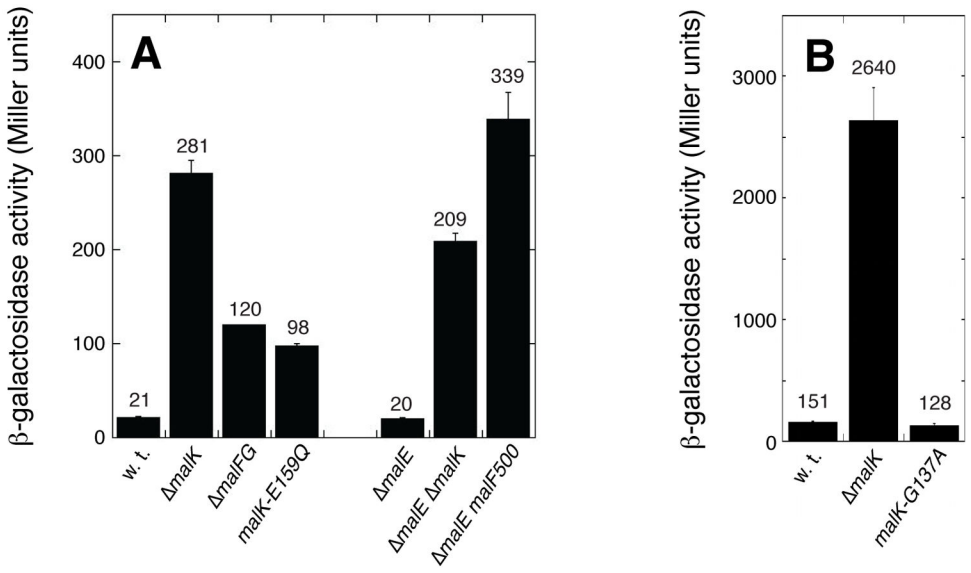

Fig. 7. Effect of mutations altering the fraction of the transporter in the resting state on its ability to inhibit MalT

A. Derivatives of strain pop7403 (a Δ*crr glgA*$^{-}$ variant of pop7234, the MalT reporter strain) containing the indicated mutations were grown at 32°C in M9 medium supplemented with glycerol + 5 mM cAMP and β-galactosidase activity levels were measured as described. The values obtained are corrected for the background value (6 Miller units), as measured with a Δ*malT* isogenic strain (pop7405).

B. *malK*$^{+}$, Δ*malK* and *malK-G137A* derivatives of a Δ*malT* strain containing a *malPp-lac* fusion (pop7342, pop7352 and pop7355, respectively) and bearing pOM252 (a plasmid expressing MalT from a constitutive promoter) were grown in M9 medium + glycerol + 30 μg.ml^{-1} ticarcillin at 32°C and their β-galactosidase levels measured as described. For each strain, the value obtained was corrected for the background value, as determined with the pJM241 vector (102–110 Miller units).

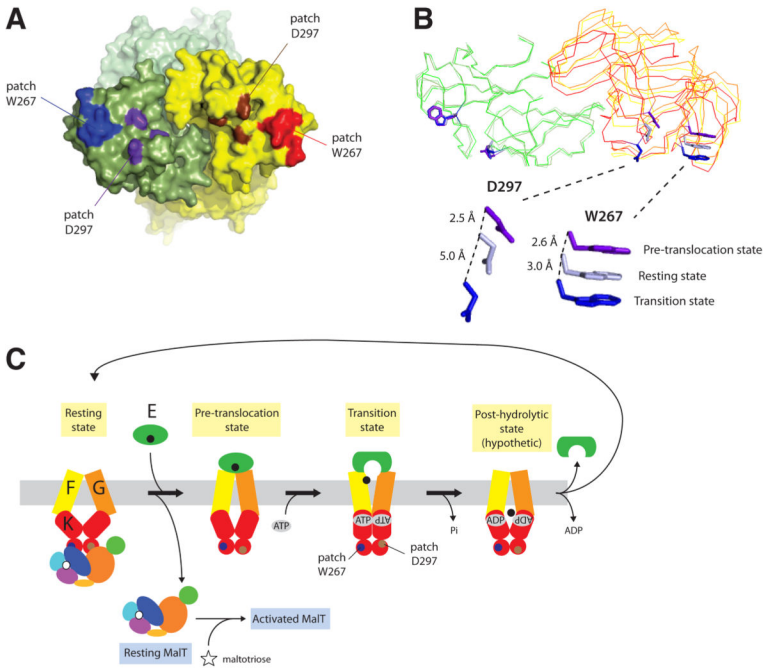

Fig. 8. Model for the coupling between transport and MalT release

A. Locations of the W267 and the D297 patches on the regulatory domains of open MalK (in resting MalFGK2). Patches W267 and D297 are defined by residues A248, A250, I251, Q253, Q255, W267 and residues L291, D297, E350, respectively, which have been identified by mutagenesis and whose side-chains are surface-exposed (Böhm et al., 2002; Kühnau et al., 1991) E. R., unpublished).

B. View of the MalK regulatory domain dimers of MalFGK2 in the resting, pre-translocation and catalytic states superpositioned via one regulatory domain protomer (in green). The changes in the relative orientation of the two regulatory domains is highlighted by the displacement of the second-protomer residues W267 and D297.

C. Cartoon of the maltose transport cycle based on available crystal structures, with the transporter interacting with MalT specifically when it is in the resting state. Although each RD displays both patch W267 and patch D297, only one pair is shown, that thought to be bound by MalT.

Table 1

Strains used in this study

Strains	Genotype	Source
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	Casadaban (1976)
KM512	MC4100 *glgA::Tn*10 ϕ(malK::lacZ)hyb113 *malI malK malY*	Decker et al. (1993)
TST6	MC4100 *malF::Tn*10	T. Silhavy (Princeton University)
pop1951	MC4100 F^+	Lab collection
pop1966	MC4100 F' [$lacI^q$ Δ*lacM15* pro^+ *Tn*10]	Lab collection
pop2348	MC4100 *glpD aroB* F^+	Lab collection
pop2349	MC4100 *glpD aroB malEpKp*Δ*CRP1+2* F^+	M13mp7::*malEpKp*Δ*CRP1+2* X pop2348 -> screen Mal^-
pop2974	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} *trp*::[Kan^r-*malEp*Δ*92-lac*]$_{op}$ F^+	P1 (MC4100) X pop7146 -> $AroB^+$ Gly^+ (O. Danot, Institut Pasteur)
pop3218	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} *trp*::[Kan^r-*malEp*Δ*92-lac*]$_{op}$ *malK-G346S* F^+	M13mp11del::'*malK-G346S* X pop2974 -> screen Lac^+
pop4137	MC4100 *malEpKp*Δ*CRP1+2* F' [$lacI^q$ Δ*lacM15* pro^+ *Tn*10]	M13mp7::*malEpKp*Δ*CRP1+2* X pop1966 -> screen Mal^-
pop4139	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} F' [$lacI^q$ Δ*lacM15* pro^+ *Tn*10]	see Experimental Procedures
pop4140	MC4100 *gly aroB malEpKp*Δ99::P_{tac}-P_{con} F^+	P1 (pop4139) X pop2349 -> Mal^+
pop7146	MC4100 *gly aroB malEpKp*Δ99::P_{tac}-P_{con} *trp*::[Kan^r-*malEp*Δ*92-lac*]$_{op}$ F^+	P1 (pop7165) X pop4140 -> $Kana^r$
pop7165	MC4100 Δ*malB107 trp*::[Kan^r-*malEp*Δ*92-lac*]$_{op}$ F^+	Richet et al. (2005)
pop7190	MC4100 Δ*malB107 trp*::[Kan^r-*malEp*Δ*92-lac*]$_{op}$ Δ*malT220*	Richet et al. (2005)
pop7199	MC4100 Δ*malB107 trp*::[Kan^r-*malEp*Δ*92-lac*]$_{op}$ Δ*malT220 glgA::Tn*10	P1 (KM512) X pop7190 -> Tet^r
pop7202	MC4100 Δ*malB107 trp*::[Kan^r-*malPp-lac*]$_{op}$	Marquenet and Richet (2007)
pop7212	MC4100 Δ*malT224*::Zeo^r F^+	M13mp11::*malT*Δ*malT224*::Zeo^r X pop1951 -> screen Mal^-
pop7213	MC4100 *malF::Tn*10 F^+	P1 (TST6) X pop1951 -> Tet^r
pop7215	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} F^+	P1 (pop7140) X pop7213 -> Mal^+
pop7216	MC4100 Δ*malT224*::Zeo^r *malEpKp*Δ99::P_{tac}-P_{con} F^+	P1 (pop7212) X pop7215 -> Zeo^r [(a)]
pop7233	MC4100 *malEpKp*Δ99::P_{tac}-P_{CON}-'*malK trp*::[Kan^r-*malEp*Δ*92-lac*]$_{op}$ F^+	M13mp11del::P_{con}-'*malK*' X pop2974 -> screen Mal^-
pop7234	MC4100 *malEpKp*Δ99::P_{tac}-$P_{KAB\text{-}TTGG}$ *trp*::[Kan^r-*malEp*Δ*92-lac*]$_{op}$ F^+	M13zeo2::$P_{KAB\text{-}TTGG}$-*malK*' X pop7233 -> screen Lac^-
pop7237	pop7234 Δ*malT224::Zeo*^{r}	P1 (pop7212) X pop7234 -> Zeo^r
pop7257	pop7234 *malK-E159Q*	M13mp11del::'*malK-E159Q* X pop7234 -> screen Mal^-
pop7258	pop7234 *malK-G346S*	M13mp11del::'*malK-G346S* X pop7234 -> screen Lac^+

Strains	Genotype	Source
pop7264	pop7234 Δ*malK*	M13Zeo2::$P_{KAB\text{-}TTGG}$-*lamB'* X pop7234 -> screen Mal^-
pop7268	pop7234 Δ*malE444*	M13mp11::Δ*malE444* X pop7234 (pMM13) -> screen Mal^- *(b)*
pop7269	pop7234 Δ*malG*	M13Zeo2::Δ*malG* X pop7234 -> screen Mal^-
pop7291	pop7234 *malT-N637T*	M13mp11del::'*malT-N637T* X pop7234 -> screen Lac^+
pop7292	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} Δ*malK* F^+	M13mp11del::P_{con}-*lamB'* X pop7215 -> screen Mal^-
pop7293	pop7216 Δ*malK*	P1 (pop7212) X pop7292 -> Zeo^r *(a)*
pop7302	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} *malK-G346S* F^+	P1 (pop3218) X pop7292 -> Mal^+
pop7303	pop7216 *malK-G346S*	P1 (pop7212) X pop7302 -> Zeo^r *(a)*
pop7304	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} *malK-E159Q* F^+	M13mp11del::'*malK-E159Q* X pop7215 -> screen Mal^-
pop7305	pop7216 *malK-E159Q*	P1 (pop7212) X pop7304 -> Zeo^r *(a)*
pop7306	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} Δ*malFG* F^+	M13Zeo2::Δ*malFG* X pop7215 -> screen Mal^-
pop7307	pop7216 Δ*malFG*	P1 (pop7212) X pop7306 -> Zeo^r *(a)*
pop7308	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} *malF500* F^+	M13Zeo2::'*malFG'-malF500* X pop7215 -> Mal^- *(c)*
pop7312	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} Δ*malE1444 malF500* F^+	M13Zeo2::Δ*malE1444* X pop7308 -> screen $Mal^\pm$
pop7313	pop7216 Δ*malE1444 malF500*	P1 (pop7212) x pop7312 -> Zeo^r *(a)*
pop7316	pop7234 Δ*malFG*	M13Zeo2::Δ*malFG* X pop7234 -> screen Mal^-
pop7319	pop7234 Δ*malE444 malF500*	M13Zeo2::'*malFG'-malF500* X pop7268 -> screen $Mal^\pm$ $Lac^\pm$ *(c)*
pop7322	MC4100 *malEpKp*Δ99::P_{tac}-P_{con} Δ*malE1444* F^+	M13Zeo2::Δ*malE1444* X pop7215 -> screen Mal^-
pop7323	pop7216 Δ*malE1444*	P1 (pop7212) X pop7322 -> Zeo^r *(a)*
pop7331	MC4100 *malEpKp*Δ99::P_{tac}-$P_{KAB\text{-}TTGG}$ F^+	P1 (MC4100) X pop7234 -> $Trypt^+$
pop7340	MC4100 *malEpKp*Δ99::P_{tac}-$P_{KAB\text{-}TTGG}$ *trp*::[Kan^r-*malPp-lac*]$_{op}$ F^+	P1 (pop7202) X pop7331 -> Kan^r
pop7342	MC4100 *malEpKp*Δ99::P_{tac}-$P_{KAB\text{-}TTGG}$ *trp*::[Kan^r-*malPp-lac*]$_{op}$ Δ*malT224::Zeo*^{r} F^+	P1 (pop7212) X pop7340 -> Zeo^r
pop7348	MC4100 *malEpKp*Δ99::P_{tac}-$P_{KAB\text{-}TTGG}$ *trp*::[Kan^r-*malPp-lac*]$_{op}$ Δ*malK* F^+	M13Zeo2::$P_{KAB\text{-}TTGG}$-*lamB'* X pop7340 -> screen Lac^+
pop7349	MC4100 *malEpKp*Δ99::P_{tac}-$P_{KAB\text{-}TTGG}$ *trp*::[Kan^r-*malPp-lac*]$_{op}$ *malK-G137A* F^+	M13mp11del::'*malK-G137A* X pop7340 -> screen Mal^-
pop7352	pop7342 Δ*malK*	P1 (pop7212) X pop7348 -> Zeo^r
pop7355	pop7342 *malK-G137A*	P1 (pop7212) X pop7349 -> Zeo^r
pop7389	pop7234 Δ*malK* Δ*malFG*	M13Zeo2::$P_{KAB\text{-}TTGG}$-*lamB'* X pop7316 -> screen Lac^+
pop7393	pop7234 *glgA::Tn10*	P1 (pop7199) X pop7234 -> Tet^r

Strains	Genotype	Source
pop7395	pop7234 *glgA::Tn10* Δ*malT220*	M13mp11::*malT*Δ*malT220* X pop7393 -> screen Mal$^-$
pop7396	pop7234 *glgA::Tn10* Δ*malK*	M13Zeo2::$P_{KAB\text{-}TTGG}$-*lamB*' X pop7393 -> screen Mal$^-$
pop7397	pop7234 *glgA::Tn10* Δ*malFG*	M13Zeo2::Δ*malFG* X pop7293 -> screen Mal$^-$
pop7398	pop7234 *glgA::Tn10* Δ*malK-E159Q*	M13mp11del::'*malK-E159Q* X pop7293 -> screen Mal$^-$
pop7399	pop7234 *glgA::Tn*10 Δ*malE444*	P1 (pop7199) X pop7268 -> Tet^r + screen Mal$^\pm$
pop7400	pop7234 *glgA::Tn*10 Δ*malE444* Δ*malK*	M13Zeo2::$P_{KAB\text{-}TTGG}$-*lamB*' X pop7399 -> screen Lac$^\pm$
pop7401	pop7234 *glgA::Tn*10 Δ*malE444 malF500*	M13Zeo2::'*malFG*'-*malF500* X pop7399 -> screen Mal$^\pm$ Lac$^\pm$ *(c)*
pop7403	pop7234 *glgA::Tn10* Δ*crr*::Zeo^r	M13mp11::Δ*crr*::Zeo^r X pop7393
pop7405	pop7403 Δ*malT220*	P1 (pop7403) X pop7395 -> Zeo^r
pop7406	pop7403 Δ*malK*	P1 (pop7403) X pop7396 -> Zeo^r
pop7407	pop7403 Δ*malFG*	P1 (pop7403) X pop7397 -> Zeo^r
pop7408	pop7403 Δ*malK-E159Q*	P1 (pop7403) X pop7398 -> Zeo^r
pop7409	pop7403 Δ*malE444*	P1 (pop7403) X pop7399 -> Zeo^r
pop7410	pop7403 Δ*malE444* Δ*malK*	P1 (pop7403) X pop7400 -> Zeo^r
pop7411	pop7403Δ*malE444 malF500*	P1 (pop7403) X pop7401 -> Zeo^r

(a) Zeo^r clones were selected on LB plates containing 6 μg.ml^{-1} zeocin.

(b) Followed by plasmid curing.

(c) The presence of the two *malF* substitutions comprising the *malF500* mutation (G338R and N505I) in the chromosome of the constructed strain was verified by PCR amplification and sequencing.

Table 2

Plasmids used

Plasmids	Type	Relevant features	Source
pDSW209	pBR322	P_{trc}-*gfp*, Amp^r, a vector for fusing proteins to the C-terminus of GFP	Weiss et al. (1999)
pJM241	single-copy R1 run-away plasmid	Amp^r	Norregaard-Madsen et al. (1995)
pJM241-NruI	single-copy R1 run-away plasmid	Cla^- Nru^+, Amp^r	This study *(a)*
pKN101	pBR322	BglII-ClaI '*malE-malKlamB*', Amp^r	K. Nikaido
pMM13	pACYC184	$lacI^q$, Tet^r	Ehrmann et al. (1997)
pOM2	pBR322	*malTp-malT*, Tet^r	Raibaud et al. (1985)
pOM95	pBR322	P_{trc}-*gfp-malT*, Amp^r GFP-MalT linker: YKEFLTMLI	This study *(a)*
pOM172	pBR322	P_{con}-*malK*'-T7 *Te* and P_{tac}-*malE*' divergently inserted, Amp^r	This study *(a)*
pOM174b-*G137A*	pBR322	A pET28b(+) derivative encoding $(His)_6$-$MalK^{G137A}$, $Kana^r$	This study *(a)*
pOM175	single-copy R1 run-away plasmid	$P_{KAB\text{-}TTGG}$-*malK*, Amp^r $P_{KAB\text{-}TTGG}$ being a constitutive promoter (Burr et al., 2000)	This study *(a)*
pOM181	single-copy R1 run-away plasmid	*malTp-malT*, Amp^r	Richet et al. (2005)
pOM183	single-copy R1 run-away plasmid	*malTp-malT* with a silent SacI site at +2381 in the *malT* coding sequence, Amp^r	This study
pOM184	single-copy R1 run-away plasmid	*truncated* $malTp^{Tp1\ Tp7}$-*gfp-malT* *(b)* Amp^r GFP-MalT linker: YKEFLTMLI	This study *(a)*
pOM184-ΔDBD	single-copy R1 run-away plasmid	*truncated* $malTp^{Tp1\ Tp7}$-*gfp-malT*$^{\Delta DBD}$ *(b)* Amp^r GFP-MalT linker: YKEFLTMLI	This study *(a)*
pOM185	single-copy R1 run-away plasmid	*truncated* $malTp^{Tp1\ Tp7}$-*gfp-malT* *(b)* Amp^r GFP-MalT linker: YKGLI	This study *(a)*
pOM185-ΔDBD	single-copy R1 run-away plasmid	*truncated* $malTp^{Tp1\ Tp7}$-*gfp-malT*$^{\Delta DBD}$ *(b)* Amp^r GFP-MalT linker: YKGLI	This study *(a)*
pOM185-Δ*malT*	single-copy R1 run-away plasmid	*truncated* $malTp^{Tp1\ Tp7}$-*gfp* *(b)*, Amp^r	This study *(a)*
pOM215	single-copy R1 run-away plasmid	$P_{KAB\text{-}TTCT}$-*malT*, Amp^r $P_{KAB\text{-}TTCT}$ being a constitutive promoter (Burr et al., 2000)	This study *(a)*
pOM252	single-copy R1 run-away plasmid	$P_{KAB\text{-}TTGG}$-*malT*, Amp^r $P_{KAB\text{-}TTGG}$ being a constitutive promoter (Burr et al., 2000)	This study *(a)*

(a) See Supporting Information for more details.

(b) *malTp1* and *malTp7* are promoter and Shine and Dalgarno up mutations, respectively (Chapon, 1982).