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Subunit interactions in ABC transporters: a conserved sequence in hydrophobic membrane proteins of periplasmic permeases defines an important site of interaction with the ATPase subunits

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The cytoplasmic membrane proteins of bacterial binding protein-dependent transporters belong to the superfamily of ABC transporters. The hydrophobic proteins display a conserved, at least 20 amino acid EAA---G---------I-LP region exposed in the cytosol, the EAA region. We mutagenized the EAA regions of MalF and MalG proteins of the *Escherichia coli* maltose transport system. Substitutions at the same positions in MalF and MalG have different phenotypes, indicating that EAA regions do not act symmetrically. Mutations in *malG* or *malF* that slightly affect or do not affect transport, determine a completely defective phenotype when present together. This suggests that EAA regions of MalF and MalG may interact during transport. Maltose-negative mutants fall into two categories with respect to the cellular localization of the MalK ATPase: in the first, MalK is membrane-bound, as in wild-type strains, while in the second, it is cytosolic, as in strains deleted in the *malF* and *malG* genes. From maltose-negative mutants of the two categories, we isolated suppressor mutations within *malK* that restore transport. They map mainly in the putative helical domain of MalK, suggesting that EAA regions may constitute a recognition site for the ABC ATPase helical domain.

Keywords: ABC transporters/binding protein-dependent transport/conserved protein sequences/cytoplasmic membrane proteins/interactions

Introduction

In bacteria, high affinity uptake of nutrients is achieved by complex substrate binding protein-dependent transport systems. These multi-component permeases consist of one periplasmic substrate-binding protein, two hydrophobic cytoplasmic membrane proteins and two subunits of a peripheral cytoplasmic membrane protein that displays ATP-binding motifs. The cytoplasmic membrane proteins form a complex with a stoichiometry of two ATP-binding subunits and two integral membrane subunits that mediates the ATP-dependent translocation of the substrates into the cytoplasm (Bishop *et al.*, 1989; Davidson and Nikaido, 1991). Such a characteristic organization is an intrinsic property of the superfamily of ABC (ATP Binding Cassette) transporters (Higgins, 1992) or Traffic ATPases (Ames and Lecar, 1992) widely distributed among living organisms.

The maltose transport system of *Escherichia coli* is one of the most studied bacterial ABC transporters and its functional mechanism is known to some extent (see Boos *et al.*, 1996 for a review). Genetic, and more recently biochemical approaches, have led to a functional model that is summarized below. Maltose and maltodextrins enter the periplasm by facilitated diffusion through a specific outer membrane porin coded for by the *lamB* gene, which is specifically required for maltose transport at submicromolar concentrations and for maltodextrins at all concentrations (Szmelcman and Hofnung, 1975). In the periplasm, maltose-binding protein (MBP) binds substrates at high affinity (K_D = 1 μM). Upon binding of substrates, MBP undergoes a conformational change (Szmelcman *et al.*, 1976) and interacts with a cytoplasmic membrane complex made of MalF, MalG and two subunits of MalK (MalFGK2), and very likely with hydrophobic membrane proteins MalF and MalG (Hor and Shuman, 1993) that carry also a substrate binding site(s) (Treptow and Shuman, 1988). Recent results obtained with a proteoliposome-reconstituted transport system suggest that MBP transmits through MalF and MalG a signal to MalK allowing it to hydrolyze ATP (Davidson *et al.*, 1992).

An important step in understanding maltose transport mechanism is the analysis of protein–protein interactions. The existence of mutations allowing maltose transport in the absence of MBP led to the identification of sites on MalF and MalG thought to be important for the recognition of MBP (Covitz *et al.*, 1994). We are interested in the identification of regions of hydrophobic membrane proteins involved in the interaction with the MalK ATPase. By comparing the sequences of hydrophobic membrane proteins from several binding protein-dependent transporters, we identified a conserved sequence (EAA---G---------I-LP) located at a distance of ~100 residues of their C-terminal ends (Dassa and Hofnung, 1985b). This 20 amino acid sequence (called thereafter EAA region) is hydrophilic and lies in a loop facing the cytosol in all proteins of known topology (Saurin *et al.*, 1994). It has been proposed that it might constitute a recognition site for a ligand or a partner common to binding protein-dependent permeases (Dassa and Hofnung, 1985a), which could be the most conserved component in such permeases, namely the ATP-binding proteins (Kerppola and Ames, 1992). This EAA region is probably of functional importance since several mutations have been characterized within that reduce or abolish transport in the maltose (Dassa, 1990, 1993), the iron (III) hydroxamate (Koster and Bohm, 1992) and the phosphate (Webb *et al.*, 1992) uptake systems.

To investigate the physiological relevance of the conserved EAA region, we generated substitution mutations in the corresponding regions of MalF and MalG. The

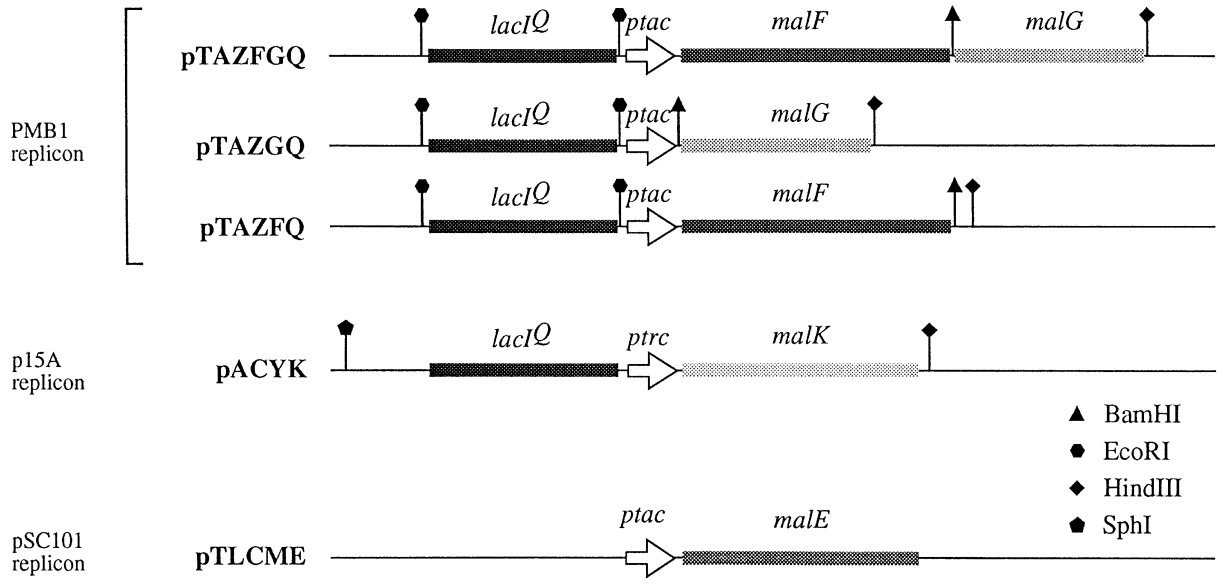

Fig. 1. Plasmids used in this work. Three compatible replicons have been used to produce simultaneously MalE, MalF and MalG, and MalK. The genes were placed under the control of the *ptac* or the *ptrc* promoters. Symbols represent the restriction enzymes used to generate these plasmids (for details see Materials and methods).

most conserved residues were modified and we analyzed the consequences of these changes on maltose and maltodextrins uptake, and on the expression of the proteins. The membrane association of MalK was altered in some uptake-defective mutants. We isolated suppressor mutations in *malK* that restore maltose uptake and MalK membrane association in these mutants. We discussed a model suggesting that EAA regions constitute a recognition site for the MalK subunits.

Results

The experimental system

We constructed three plasmids to generate easily site-directed mutations within EAA regions. Plasmids pTAZFQ and pTAZGQ carry the *malF* and *malG* genes respectively under the control of the *tac* promoter. Plasmid pTAZFGQ carrying these two genes, was constructed by inserting the *Bam*HI–*Hin*dIII fragment from pTAZGQ into pTAZFQ. Each plasmid contains a copy of the *lacI*^{Q} gene in order to control the expression of cloned genes (Figure 1). pTAZGQ, pTAZFQ and pTAZFGQ were able to complement mutations in PMED34 (MalG^{-}), DHB4 (MalF^{-}) and ED170 (MalF^{-}G^{-}) respectively. To evaluate the expression of cloned genes, particulate fractions were prepared from strain MM140 (ED170 transformed with pTAZFGQ), analyzed by SDS–PAGE, and proteins were revealed by immunoblotting. We compared this amount of protein with the level of expression of MalF and MalG in a total extract of the fully induced maltose transport positive strain MC4100. We found that a concentration of 10 μM isopropyl β-D-thiogalactopyranoside (IPTG) was enough to ensure the production of 110 and 78% of the induced chromosomal levels for the MalF and MalG proteins respectively, as judged from the scanning of Western blots. We analyzed the cellular localization of MalF and MalG (Figure 2). The proteins partitioned in a particulate fraction, from which they could be partially

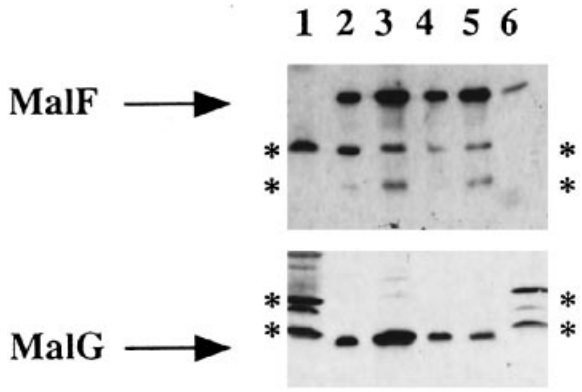

Fig. 2. Cellular localization of MalF and MalG proteins produced by plasmid pTAZFGQ. Strains MM140 and ED170 were grown in LB medium. Plasmid genes were induced using 10 μM IPTG. Cell fractionation was performed as described in Materials and methods and particulate fractions were extracted using 1% Triton X-100. Ten micrograms total protein of the different fractions were separated by SDS–PAGE and transferred onto nitrocellulose membranes. MalF (top) and MalG (bottom) proteins were revealed using polyclonal antibodies, horseradish peroxidase conjugate and ECL detection reagents. Only the relevant part of the membrane is shown. Lane 1, total cellular extract of ED170; lanes 2–6, fractions of MM140; lane 2, cell debris; lane 3, particulate fraction; lane 4, Triton insoluble fraction; lane 5, Triton soluble fraction; lane 6, cytoplasmic fraction. Arrows indicate the positions of MalF and MalG proteins. Stars indicate cross-reacting proteins.

extracted by a buffer containing 1% Triton X-100. As shown previously, a substantial amount of MalF and MalG is still not extracted by the detergent (Dassa, 1990; Panagiotidis *et al.*, 1993). Triton X-100 is a non-ionic detergent known to specifically solubilize cytoplasmic membrane proteins (Diedrich *et al.*, 1977), and has been used to show the membrane localization of the MalFGK2 complex. Thus, we interpret this result to indicate that, under our expression conditions, the bulk of the proteins is correctly assembled in the membrane.

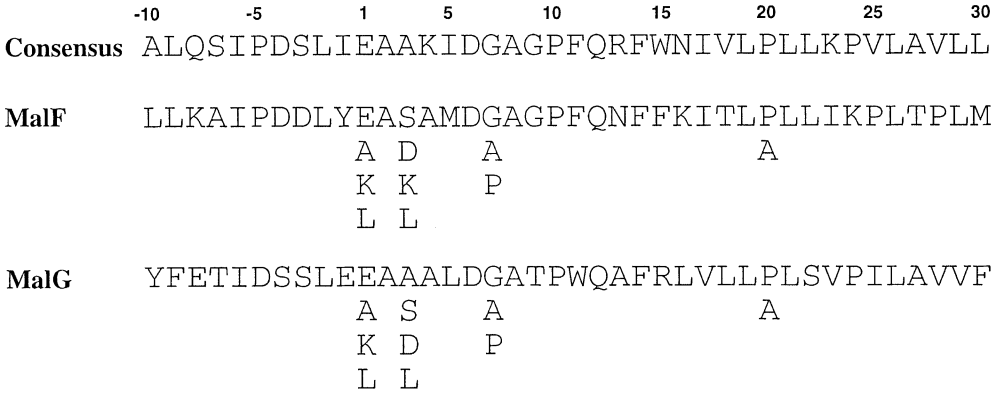

Fig. 3. Amino acid substitutions introduced in *malF* and *malG* genes. The sequence for the consensus of the EAA region of disaccharide uptake hydrophobic membrane proteins is shown on top with the relative positions of residues used to identify EAA mutations. The origin of the numeration is set on the E of the consensus sequence. The corresponding sequences of *malF* and *malG* are displayed below. Substituted amino acids in mutants are shown under the wild-type sequence.

Site-directed mutagenesis of conserved residues in the EAA region

Starting from the assumption that residues are conserved because they play an important functional role, we mutagenized the most conserved residues in the EAA regions of MalF and MalG. We modified residues located at positions 1 (E), 3 (A or S), 7 (G) and 20 (P) of the consensus of EAA motifs (Saurin *et al.*, 1994). In the absence of structural information on MalF and MalG, we made several changes at each position, modifying the size, the charge and the side chain mobility of the amino acids (Figure 3). The mutations in *malF* and *malG* were generated on plasmids pTAZFQ and pTAZGQ respectively, sequenced and eventually recombined on plasmid pTAZFGQ as described above. Each mutant was identified by a set of two symbols separated by a slash. The first symbol describes the changes effected in *malF* and the second those in *malG*. A non-mutated gene was represented by a hyphen. Numbers between letters point to the relative position of the residue in the consensus sequence. For example, E1A/A3L means that the E residue in the EAA region of MalF was changed to A and that the A residue in position 3 of MalG was changed to L.

Twenty three plasmids were constructed, each carrying a substitution in MalG (nine mutants), in MalF (nine mutants) or in both proteins (five mutants). The growth rate of mutants induced by 10 μM IPTG in glycerol-supplemented minimal medium and in ML medium (data not shown) was similar to that of strains MM141 ($MalF^-G^-$) and MM140 ($MalF^+G^+$). Hence, the expression of mutated proteins does not determine major growth defects in bacteria. Particulate fractions of mutants were separated on SDS–polyacrylamide gels and analyzed by immunoblotting (Figure 4 and Table I). Mutated proteins have electrophoretic mobilities similar to that of the wild-type. There are variations in protein amounts with a 2- to 3-fold difference between extreme values, with the exception of the MalG level in E1K/A3L, E1L/E1L and S3K/A3D mutants, where there is a 4-fold increase as compared to MM140. This higher level of expression was not investigated further. Each mutant protein was correctly inserted in the cytoplasmic membrane, as suggested by the fact that it was extracted from the particulate fraction by Triton X-100 as efficiently as wild-type proteins (data not shown).

Phenotypes of mutants

Table I shows the characteristics of mutants. Their phenotypes were scored on MacConkey plates supplemented with maltose or maltodextrins. No Mal^+ Dex^- or Dex^+ Mal^- mutants were found. Phenotypes were characterized by measuring growth rates on maltose minimal medium and by determining maltose uptake rates at a substrate concentration of 4 μM. Mutants were classified according to decreasing maltose uptake rates. Class I mutants have initial velocities ranging from 240 to 150, doubling times ranging from 220 to 240 min and they form deep red colonies on MacConkey plates. Class II mutants have initial velocities ranging from 60 to 145, doubling times comprised between 230 and 300 min and they form red to pink colonies. Class III mutants form white colonies, do not grow on minimum maltose medium and do not transport maltose. These three parameters are in general well correlated, with few exceptions that could be explained by the difference of maltose concentration used in phenotypic determinations, in growth rate measurements and in transport assays. Since the mutated proteins are not found in significantly lesser amounts than the wild-type proteins, the defects in maltose-uptake-deficient mutants are most probably not due to a reduced protein expression, nor to a defective localization.

E at position 1 is conserved in 50% of EAA sequences and is substituted by D in 12% sequences. Position 3 (A) is conserved in 75% of EAA sequences or is substituted by S, T or C in 20% sequences (Saurin *et al.*, 1994). Our data show that any change affecting the E residue in MalF or in MalG leads to a reduction by 2- to 3-fold in transport rates. Changes made to the third residue of the region (A or S) promote a reduction in transport rates that is related to the nature of the side chain of the replacing residue. A change to a charged residue (K or D) is more detrimental for transport than a change to a short side-chain polar (S) residue. The G residue, invariant in EAA motifs, could be changed to A without incidence on transport. This is in agreement with earlier observations made on the FhuB protein (Koster and Bohm, 1992) and establishes that conserved EAA motifs play similar roles in binding protein-dependent transport systems. The highly conserved P residue at position 20 is not essential for transport. This residue is predicted to be in a transmembrane segment in

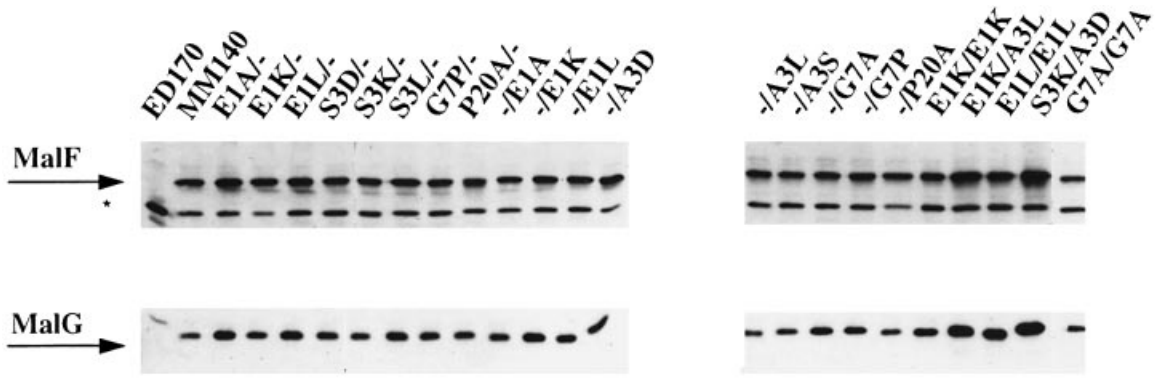

Fig. 4. Immunodetection of mutated MalF and MalG proteins. Strain ED170 transformed with wild-type or mutated pTAZFGQ was grown in LB medium. Plasmid genes were induced using 10 μM IPTG and particulate fractions were recovered as described in Materials and methods. Approximately 5 μg of particulate fraction of each construction were separated on SDS–PAGE and transferred onto nitrocellulose membranes. MalF and MalG proteins were revealed as described in Figure 2. Mutants are identified using the conventions described in the text. Only the relevant part of the membrane is shown. Arrows indicate the positions of MalF and MalG proteins. The star indicates a cross-reacting protein.

Table I. Characteristics of EAA mutants

Plasmids[a]	Phenotype[b]	Doubling time[c] (min)	Initial rate of maltose uptake[d]	Relative amount of protein[e]	
				MalF	MalG
pTAZFGQ	+++	238	210 ± 20	1.00	1.00
pTZ18R	–	>700	<1	0	0
Class I					
P20A/–	+++	233	240±20	1.15	1.23
–/A3S	+++	227	239±18	0.88	1.20
–/G7A	+++	221	214±13	1.11	2.25
G7A/–	+++	ND	ND	ND	ND
S3L/–	+++	231	208±10	1.10	2.34
G7A/G7A	+++	221	184±1	0.73	1.36
G7P/–	+++	234	177±7	0.98	1.92
–/P20A	+++	224	157±2	0.99	1.62
Class II					
–/A3L	+	269	145±1	1.06	0.86
S3K/–	+	295	104±4	0.96	1.50
–/E1K	++	238	100±2	1.08	2.83
–/E1A	+++	230	94±5	0.95	1.66
–/E1L	++	262	85±5	1.18	2.19
E1A/–	+++	264	79±4	1.24	2.87
S3D/–	+++	282	75±4	0.86	1.87
E1K/–	++	288	61±2	1.15	1.76
E1L/–	++	285	60±1	1.28	2.70
Class III					
–/A3D	–	>700	2±0.3	1.44	2.17
–/G7P	–	>700	4±0.6	1.21	2.05
E1K/E1K	–	305	2±0.2	1.30	2.34
E1K/A3L	–	>700	<1	1.87	4.31
E1L/E1L	–	>700	1±0.1	1.62	4.09
S3K/A3D	–	>700	ND	1.83	4.53

All experiments are performed in strain ED170.
[a]Mutant plasmids are designated with the conventions described in the text.
[b]Maltose and maltodextrins utilization is assayed on MacConkey plates containing 2% maltose or 1% maltodextrins. No difference was found in the utilization of these sugars. +++, deep red colonies; ++, red colonies; +, pink colonies; –, white colonies.
[c]Doubling times, in min, are measured in synthetic medium 63 supplemented with 0.4% maltose. The turbidity at 600 nm is measured every 30 min or every hour depending of the growth rate and the resulting plots are fitted on an exponential equation.
[d]Maltose uptake is measured at 4 μM final concentration of maltose as described in Materials and methods on strains transformed by the indicated plasmid. Values expressed in pmol maltose/min/10^8 bacteria are from single experiments. Errors represent the error estimated by fitting to a linear equation the amount of radioactivity incorporated during the experiment. In some cases three independent experiments were made and the standard error of the mean was <15%.
[e]Films of the immunoblots in Figure 4 were scanned using a MasterScan Interpretive densitometer (Scanalytics), quantities of MalF and MalG were normalized to the quantity present in MM140.
ND, not determined.

Table II. Relative amounts of MalG and MalF proteins in presence of pACYK

Plasmids	WT	E1L/–	–/E1L	E1K/–	–/A3L	E1L/E1L	E1K/A3L	–/A3D	–/G7P
MalF	1.00	0.97	0.66	1.16	0.66	1.15	1.23	0.56	1.28
MalG	1.00	1.20	0.82	1.27	1.02	1.54	1.67	0.51	1.10

Particulate fractions of strain ED170 bearing pACYK and wild-type or mutated pTAZFGQ plasmids were analyzed as in Figure 4. Films of the immunoblots were scanned using a MasterScan Interpretive densitometer (Scanalytics), quantities of MalF and MalG were normalized to the quantity in MM142. WT, wild-type genes.

MalF and in MalG (Boyd and Beckwith, 1989; Dassa and Muir, 1993). It may be concluded that transport is not dependent on a peculiar property of this residue such as a *cis–trans* isomerization.

Substitutions made at the same positions in *malG* or in *malF* affect MalG more severely than MalF. At position 3, a substitution to D has a more pronounced effect in MalG (transport defective) than in MalF (reduced transport rate). At position 7, a change to P leads to a completely defective MalG protein while MalF is unaffected. All double mutants having a mutation in *malF* and in *malG*, except G7A/G7A, are unable to carry out transport (Class III). However, the single mutants from which the double mutants are made have a transport activity of ~50–30% of the wild-type (Class II).

Cytoplasmic localization of MalK in maltose-uptake-defective mutants

The EAA region is located in a cytoplasmic hydrophilic loop in all hydrophobic membrane proteins with a known topology (Saurin *et al.*, 1994). This location makes it a candidate for an interaction site with the ATPase subunit MalK. We reasoned that the phenotypes of our mutants might be explained by a defective or a non-productive interaction with MalK. To test this hypothesis, we analyzed the cellular localization of MalK in Class II and III mutants. As in MM140 and its mutant derivatives the *malK* gene is chromosomal and under the control of the *pmal* promoter, maltose-uptake-negative mutants would have reduced to undetectable levels of protein MalK. To overcome this problem, we constructed plasmid pACYK that carries the *malK* gene under the control of the *trc* promoter. We found that in MM142 (ED170 transformed with pTAZFGQ and pACYK), a 10 μM IPTG concentration determines an amount of MalK 77% of that in maltose-induced MC4100. In these conditions the amount of MalF is 90% of that in 10 μM IPTG-induced MM140. The presence of pACYK does not alter the phenotype of MM142, since maltose uptake rates at 4 μM maltose and growth rates on maltose minimal medium are identical to those of MM140 (data not shown).

We transformed with pACYK a selected set of Class II and Class III mutants and we used strains MM142 and MM143 as controls. We found that particulate fractions of mutants and controls have relative amounts of MalF and MalG as described in Table II. These relative amounts are somewhat different to what was observed without pACYK but remain in the same range of a 2- to 3-fold difference between extreme values.

The cellular localization of MalK was determined as described in Materials and methods (Figure 5). In MM142 ($MalF^+G^+$), 80% of MalK is found in the particulate fraction and it is efficiently extracted by Triton X-100. In

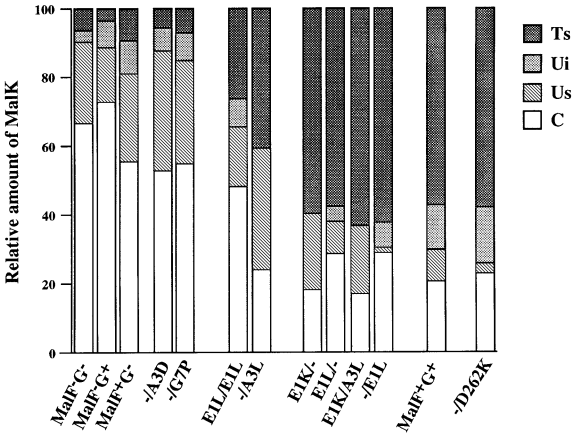

Fig. 5. Cellular localization of MalK. Strain ED170 transformed with pACYK and wild-type or mutated pTAZFGQ were grown in LB medium, plasmid genes were induced using 10 μM IPTG. Cell fractionation, Triton X-100 and urea solubilizations were performed as described in Materials and methods. Equal volumes of the different fractions, corresponding to ~20 μg of total protein, were separated on SDS–PAGE and transferred onto nitrocellulose membranes. MalK protein was revealed using a polyclonal antibody and the ECL detection kit. The amount of protein in each fraction was evaluated using a MasterScan Interpretive densitometer (Scanalytics), and the percentage of protein located in each fraction was deduced. The graph shows these deduced percentages in Class II and Class III mutants and control strains. Only the fractions recovered at the end of the fractionation procedure are shown. C, cytoplasmic fraction; P, particulate fraction; Ts, Triton X-100 soluble fraction of P; Ti, Triton X-100 insoluble fraction of P; Us, urea soluble fraction of Ti; Ui, urea insoluble fraction of Ti. $MalF^+G^+$, MM142; $MalF^-G^-$, MM143; $MalF^+G^-$, MM144; $MalF^-G^+$, MM145.

MM143 ($MalF^-G^-$), ~60% of MalK is found in a soluble fraction. The residual MalK protein present in the particulate fraction is hardly extracted by Triton X-100, but the Triton-insoluble pellet could be solubilized by using 6 M urea. By comparison, the Triton-insoluble fraction of MalG is insoluble in urea, consistent with its membrane association (data not shown). This suggests that the Triton-insoluble MalK fraction might constitute aggregates that sediment artefactually with the membranes as has been previously observed by other groups (Reyes and Shuman, 1988; Walter *et al.*, 1992a). Thus we interpret our results as follows: the amount of MalK found in the Triton X-100 soluble fraction (Figure 5, Ts) represents the fraction correctly associated in the membrane, whereas the amount of protein found in the soluble fraction (Figure 5, C) and in the Triton insoluble–urea soluble fraction (Figure 5, Us) represents the fraction of MalK which is not associated with the membrane and is consequently located in the cytoplasm. The Triton insoluble–urea insoluble fraction (Figure 5, Ui) contains membrane proteins not extractable by Triton X-100 and most notably outer membrane proteins (data not shown).

We analyzed the cellular localization of MalK in strains MM144 ($MalF^{-}G^{+}$) and MM145 ($MalF^{+}G^{-}$). In both cases, the bulk of MalK was present in the soluble and in the Triton insoluble–urea soluble membrane fraction. The roles of MalF and MalG in the membrane association of MalK are controversial. Earlier observations showed that MalK was found in a cytoplasmic fraction in a mutant defective in the *malG* gene (Shuman *et al.*, 1980). More recently, the same group showed that MalF alone was able to direct MalK to the cytoplasmic membrane (Panagiotidis *et al.*, 1993). Our results support the idea that both MalF and MalG are needed for the correct membrane association of MalK.

The cellular localization of MalK in mutants was also evaluated. Two of the five Class III mutants tested, –/A3D and –/G7P, behaved as $MalF^{-}G^{-}$ deficient strains although they expressed these proteins. One Class III mutant, E1K/A3L, and most of the Class II mutants, E1K/–, E1L/– and –/E1L behaved as the wild-type strain. In mutants E1L/E1L, –/A3L of Class III and Class II respectively, MalK had an intermediate distribution. The fact that one of the Class III mutants behaved as the wild-type strain suggests that there are at least two different types of defects introduced by mutations in the EAA region, one that results in the cytoplasmic localization of MalK and one that maintains the correct membrane association of MalK.

It is noticeable that the two mutations that completely dislocate MalK are substitutions in the EAA region of MalG. This is consistent with the observation that MalG is important for the constitution of the membrane complex. We asked whether substitutions located outside the EAA region might have the same effects. We analyzed the cellular localization of MalK in a strain carrying a derivative of pTAZFGQ, having a substitution D262K affecting a residue located in the last transmembrane segment of MalG (P.Lambert and E.Dassa, unpublished). Despite the fact that this strain is completely defective for maltose uptake, the cellular localization of MalK is not affected.

Mutations in the malK gene restore transport in maltose-negative mutants

We then asked whether mutations of the *malK* gene would restore maltose uptake. Plasmid pACYK was mutagenized *in vitro* by hydroxylamine and was used to transform ED170 harboring maltose-negative mutants –/G7P, E1K/A3L and E1L/E1L. These strains carry a chromosomal wild-type copy of *malK*. We decided to isolate mutants in such a background for the following reasons. First, mutant –/G7P has a wild-type allele of *malF* that might not function in the presence of a mutated allele of *malK* able to correct the defect present in *malG*. Second, as observed above, mutations in *malF* produce proteins able to support maltose transport to some extent by contrast with *malG* mutations that lead to completely defective proteins. We therefore suspected that in double mutants E1K/A3L and E1L/E1L, mutated MalF proteins would not accommodate *malK* mutations able to correct defects of *malG* mutations.

Nineteen maltose-positive colonies, scored on MacConkey maltose medium, appeared after 2 days incubation on strains –/G7P, E1K/A3L and E1L/E1L. pACYK DNA was purified from these clones and transformed again into strain ED170 carrying the respective pTAZFGQ mutated plasmids. Eleven transformants became maltose positive in these conditions, indicating that the phenotype was indeed linked to the mutagenized pACYK plasmid. We replaced the wild-type gene of pACYK by *malK* genes from the mutated plasmids and transformed the corresponding pTAZFGQ mutants with these constructs. Eight subclones were maltose positive, thereby demonstrating that a mutation(s) in *malK* was responsible for the phenotypic change. The three remaining subclones *malK301*, *malK306* and *malK308*, all isolated in a E1L/E1L background, formed white colonies on MacConkey maltose plates. This is probably due to the presence of a mutation outside of *malK* in the original plasmid. To assess if such a mutation could be sufficient to restore maltose transport, we replaced the *malK* allele of these plasmids by a wild-type copy from pACYK. The resulting phenotype was white. These experiments strongly suggest that a mutation in *malK* and an additional mutation(s) elsewhere on the plasmid are needed to determine a maltose-positive phenotype in these clones. Such a mutation would probably raise the cellular level of the mutated MalK proteins, either by increasing the transcriptional level of the gene or the plasmid copy number. Indeed, we found that the expression of MalK in these three alleles was at least twice the normal level (Table III). DNA sequencing of the mutants (see below) revealed that no mutation was found in the *ptrc* promoter of *malK*.

We analyzed the cellular localization of MalK from revertants *malK104*, *malK106*, *malK301*, *malK303*, *malK306* and *malK308*, selected with mutants –/G7P and E1L/E1L, and for which an aberrant localization of MalK was observed. In the presence of mutated MalF and MalG, the mutated MalK proteins were correctly associated to the membrane (data not shown).

Each *malK* allele was co-transformed with pTAZFGQ plasmids bearing different uptake-defective *malF* and/or *malG* mutants in strain ED170. Phenotypes were recorded on MacConkey maltose plates (Figure 6). All *malF* and *malG* mutants tested cannot ferment maltose in the presence of wild-type *malK*. By contrast, all *malK* suppressors are able to function with wild-type copies of *malF* and *malG*. There is no strict allele specificity but *malK* suppressor isolated in a double *malF* and *malG* mutant background is not able to suppress single mutant –/G7P and vice versa. To evaluate the influence of the chromosomal copy of *malK* in the ED170 background, we constructed strain ED169 that harbors a large *malBΔ107* deletion. In this context, the *malE* gene was provided by plasmid pTLCME. This strain was co-transformed with the same plasmid combinations as ED170. Phenotypes were recorded on MacConkey maltose plates. The phenotypes observed on ED169 are similar to those seen on ED170 (data not shown). This demonstrates that the chromosomal *malK* copy of ED170 is dispensable for the restoration of maltose uptake by *malK* suppressors and is consistent with the observation that all suppressors could function with wild-type MalF and MalG. No suppressor can restore a maltose-positive phenotype in the absence of pTLCME (data not shown). Consequently, these suppressors do not display an MBP-independent phenotype suggesting that

Table III. Identification of the *malK* mutations that restore maltose uptake in EAA mutants

malK allele		Phenotype after subcloning[a]	Transport rate[b]	MalK protein[c]	Sequence change[d]
Selected on	Allele number				
–/G7P	*malK104*	++	47.85	0.74	V149M
	malK106	++	61.05	0.81	V149I
E1K/A3L	*malK201*	+++	174.57	ND	V117M, L268L, A361V
	malK202	++	55.11	0.66	A85M
	malK203	+++	160.05	ND	V117M
	malK204	+++	152.79	ND	V117M
	malK205	+++	ND	ND	V117M
E1L/E1L	*malK303*	+++	231.99	0.46	V117M
	malK301	–	ND	1.96	V154I
	malK306	–	ND	2.65	G210S, S293D, D294D, A341T
	malK308	–	ND	2.50	M187I

The *malK* alleles and the EAA mutations from which they were selected are given in the two first columns.
[a]Color on MacConkey maltose plates of strain ED170 cotransformed with pTAZFGQ carrying the given EAA mutations and plasmid pACYK bearing the subcloned *malK* gene from suppressors. Conventions are the same as in Table I.
[b]Transport rates were measured on the same strain at 4 μM maltose final concentration and were expressed in pmol maltose/min/10^8 bacteria.
[c]Relative amount of MalK protein produced by the different mutated pACYK after *in vivo* pulse labelling of strain ED169 bearing mutated or wild-type pACYK. MalK bands of the autoradiography were scanned using an interpretive densitometer and amounts of protein were normalized to the quantity produced by a wild-type pACYK.
[d]Mutations identified by sequencing *malK* suppressors. In *malK201* the mutation responsible for the restoration of maltose uptake was identified by subcloning the different mutations separately (underlined). *malK306* was not subcloned because of the lack of appropriate restriction enzyme sites. ND, not determined.

they do not constitutively hydrolyze ATP (Davidson *et al.*, 1992).

Most MalK mutants able to relieve defects in EAA fall into the so-called helical domain of ABC transporters

The 11 mutated *malK* genes were completely sequenced to determine if specific regions of MalK were important for suppressing the defects in MalF or MalG. The results of these experiments were reported in Table III. The –/G7P suppressors *malK104* and *malK106* carry a V149M and V149I substitution respectively. The E1K/A3L suppressors *malK203*, *malK204* and *malK205* have the same V117M substitution. *malK201* carries three substitutions. We subcloned the mutations and we found that the phenotype was exclusively dependent on the N-proximal mutation V117M. E1L/E1L suppressor *malK303* has also a V117M substitution. Suppressors *malK301*, *malK306* and *malK308* were also sequenced, even if these changes in *malK* needed higher levels of expression to determine the phenotype. We identified two new suppressors V154I and M187I from *malK301* and *malK308* respectively. *malK306* carries four substitutions and we were not able to subclone the different mutations because of the lack of appropriate restriction enzymes sites. All substitutions correspond to CG→TA transitions known to be specifically induced by hydroxylamine (Lawley, 1967). Mutations are located between codons 85 and 210 of MalK, and the same V117M mutation was found in five suppressors isolated from the E1K/A3L and the E1L/E1L backgrounds. This distribution argues against a random localization of reversion mutations, indicating that alteration of specific regions of MalK suppresses the defects in $MalF^-G^-$ mutants.

Most mutations are located between codons 85 and 154 of *malK*. According to current secondary structure models of ABC ATPases, this area corresponds to Loop 2–3 (Hyde *et al.*, 1990) or to the so-called helical domain (Mimura *et al.*, 1991) of ABC ATPases. This region constitutes the main difference between the predicted secondary structure models of ABC domains and the known structure of adenylate kinase. It has been proposed to be involved in the coupling of ATP hydrolysis to transport (Hyde *et al.*, 1990) or to constitute a site of interaction between ABC ATPases and hydrophobic membrane proteins (Ames *et al.*, 1990).

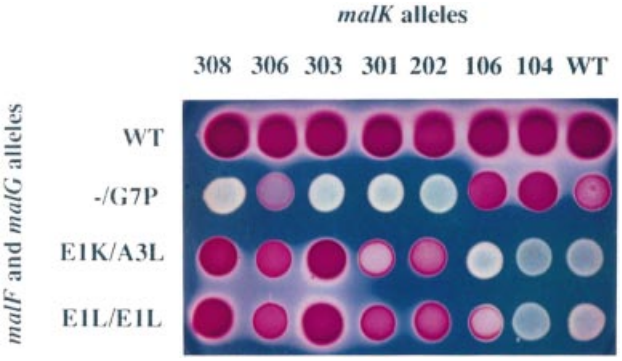

Fig. 6. Allele specificity of *malK* suppressors for EAA mutations. Phenotypes of strain ED170 bearing the different combinations of plasmids were evaluated on MacConkey maltose plates. Lines correspond to mutated or wild-type (WT) *malF* and *malG* genes and columns to wild-type or mutated *malK* genes. *malK* allele numbers beginning with 1, 2 and 3 were selected on mutants –/G7P, E1K/A3L and E1L/E1L respectively. Alleles *malK303*, *malK201*, *malK203*, *malK204* and *malK205* bear the same V117M substitution and behaved identically in this test. Only *malK303* is represented. A red color indicates a full complementation and a white color no complementation.

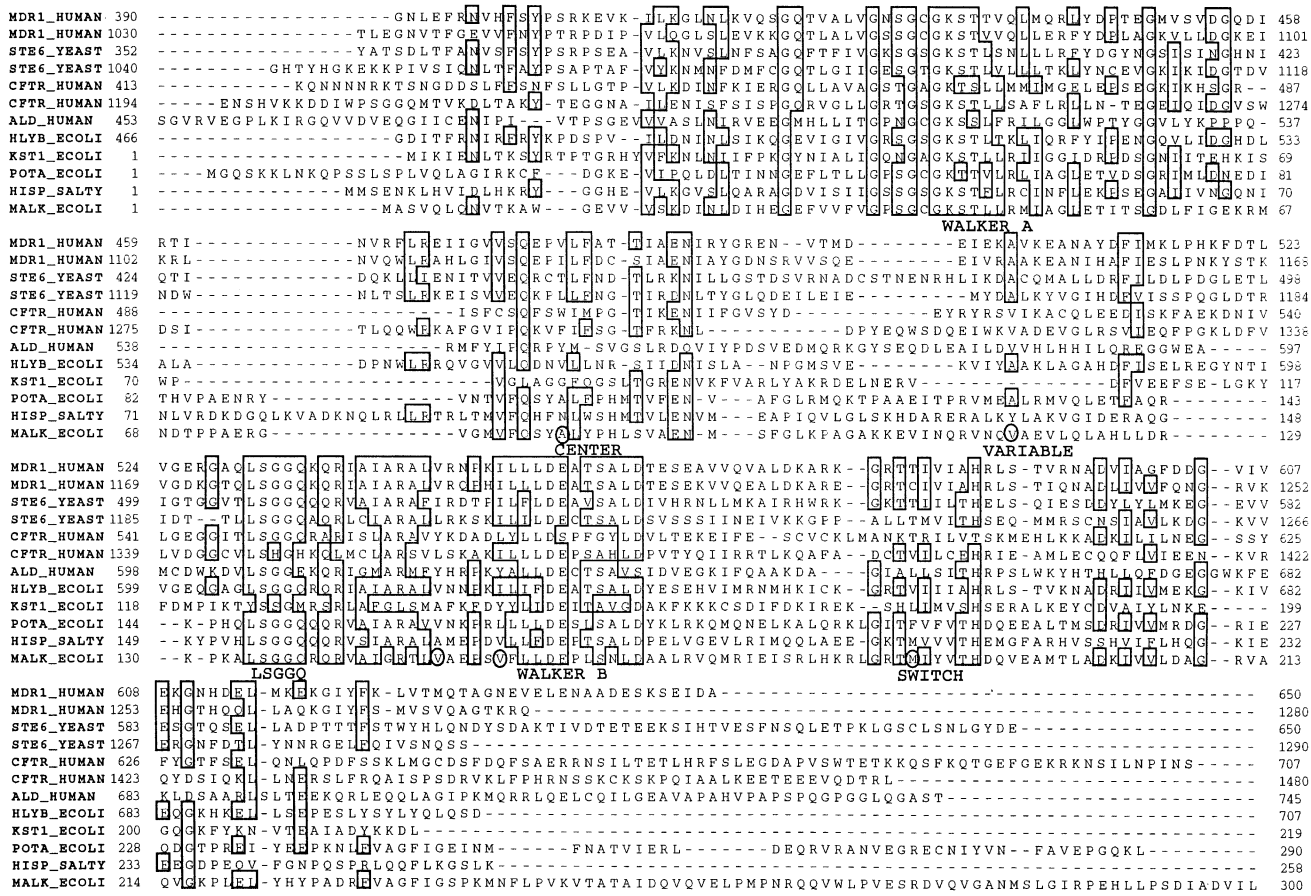

Fig. 7. Sequence alignment of nucleotide binding domain of ABC transporters. Sequences were retrieved from the SWISSPROT database and aligned using software PileUp (Genetic Computer Group, Inc.). Boxes represent >50% amino acid identity. The different regions discussed in the text are indicated in bold characters under the corresponding sequences. Locations of *malK* mutations that restore maltose uptake in transport-defective EAA mutants are indicated with circles. The ABC transporters considered are: MDR1_HUMAN, multidrug resistance P-glycoprotein (human); STE6_YEAST, mating **a** factor secretion protein (*Saccharomyces cerevisiae*); CFTR_HUMAN, cystic fibrosis transmembrane regulator (human); ALD_HUMAN, adrenoleukodystrophy linked peroxisomal protein (human); HLYB_ECOLI, hemolysin A secretion protein (*E.coli*); KST1_ECOLI, capsular polysialic acid secretion protein (*E.coli*); POTA_ECOLI, polyamine uptake ATPase (*E.coli*); HISP_SALTY, histidine uptake ATPase (*Salmonella typhimurium*).

Discussion

We analyzed by a genetic approach the functional role of EAA motifs, present in all hydrophobic membrane proteins of known binding protein-dependent permeases. Twenty-three mutants were constructed by site-directed mutagenesis in MalF and in MalG. No change obviously affects the stability and the membrane location of mutated proteins. Five mutations severely impairing transport were characterized. Hence, this region appears to play an important physiological role.

Two single substitutions (A3D and G7P) in MalG completely abolish transport while identical changes made in MalF have no effect. This suggests that EAA regions, and perhaps the MalG and MalF proteins, do not function symmetrically whereas it is generally admitted that the two proteins play similar roles in the constitution of a substrate binding site (Shuman and Panagiotidis, 1993). We characterized mutants carrying mutations in both MalF and MalG that are completely defective in transport. The reduction of initial uptake rates in such double mutants is greater than that determined by the corresponding single mutations. This suggests that the EAA regions of MalF and MalG might cooperate or interact together, directly or indirectly, in the constitution of a functionally important site. A cooperative effect was observed in double EAA mutants in FhuB, a hydrophobic iron-hydroxamate uptake protein having two EAA motifs (Koster and Bohm, 1992). Recently, cooperativity in ATP hydrolysis was demonstrated in the MalFGK2 complex (Davidson *et al.*, 1996) and was interpreted as the consequence of interactions between MalK subunits. This effect was not detected on purified MalK (Walter *et al.*, 1992a). We speculate that it might be mediated by MalF and MalG.

As we obtained two types of Class III mutants with regard to the membrane association of MalK, we tentatively propose that EAA regions participate in at least two functions. First, they might play a central role in the interactions that allow the membrane association of MalK and the constitution of a functional transporter since some EAA mutations result in a cytoplasmic localization of MalK. This result is strengthened by the fact that revertants were selected in *malK*, restoring maltose transport and membrane association. Recently, Bohm *et al.* (1996) concluded from a genetic study that the EAA region of protein FhuB could be involved in an interaction with another partner of the iron-hydroxamate transport system.

Second, EAA regions might participate to the constitution of a substrate binding site or a site that transduces a signal to MalK. The membrane association of MalK is normal in maltose-uptake-negative mutant E1K/A3L. As *malK* revertants can be selected from this mutant, we suspect that it is affected in a functional interaction with MalK.

These functions might be directly determined by EAA regions, but we cannot exclude indirect effects, since there is not a strict allele specificity in the restoration of transport which would be expected if mutated MalK was interacting separately with either MalF or MalG. Mutations in EAA loops might induce a conformational change in another region of the proteins more directly involved in the interaction with MalK. Alternatively, EAA mutations might affect the formation of the MalF–MalG heterodimer, resulting in the release of MalK in the cytoplasm. This would be consistent with the aggravated phenotypes of double mutants and in this hypothesis, *malK* suppressor mutations might induce a conformational change in mutated MalF and MalG proteins allowing them to interact properly. Eventually, EAA mutations might alter a functional site involving the three proteins MalF, MalG and MalK. Extensive genetic and biochemical investigations will be required to choose between these hypotheses. Nevertheless, we report here for the first time that in an ABC transporter, mutations in a cytoplasmic loop of hydrophobic membrane proteins are corrected by mutations in the ATPase.

We mapped *malK* suppressors on an alignment of some ABC ATPases for which functional information is available (Figure 7). Interestingly, reversion mutations located between codons 85 and 154 affect the so-called helical domain of ABC ATPases, located between ATP-binding motifs Walker A and Walker B (Mimura *et al.*, 1991). Studies using a chimeric MalK–HisP protein showed that the MalK helical domain was critical for restoration of maltose uptake in strains lacking the *malK* gene (Schneider and Walter, 1991). Mutation A85M affects a region named 'center' containing a highly conserved Q residue and a predicted catalytic carboxylate residue (Yoshida and Amano, 1995). Mutations in this area in HisP and in MalK affect primarily transport while ATP-binding is essentially unaffected (Shyamala *et al.*, 1991; Walter *et al.*, 1992b). This behaviour is consistent with a defective interaction between the ATPase and hydrophobic subunits. Mutation V117M falls in a variable region where an HlyB suppressor able to export a secretion-defective HlyA mutant is located. Since most suppressors of such mutated HlyA protein have mutations in transmembrane segments of the HlyB exporter, it was concluded that mutations with similar phenotypes in the ATPase fall in regions interacting with transmembrane domains (Sheps *et al.*, 1995). Mutations V149M, V149I and V154I are near the LSGGQ region, also called linker peptide. In this area, mutations altering the drug specificity of the Mdr transporter have been described and were interpreted as defective in an interaction between nucleotide-binding domains and transmembrane domains (Beaudet and Gros, 1995). As most *malK* suppressors in our study map in the helical domain of MalK, our results give more direct evidence that this region is involved in interactions with the membrane embedded subunits.

Mutations of suppressors *malK301* (M187I) and *malK306* are more difficult to interpret since they need an increased level of protein expression to restore transport. This might indicate that the mutated regions, located outside the helical domain, are not directly involved in an interaction with the membrane components but are none the less undergoing conformation changes during these interactions. It has been proposed that in the RecA ATPase, the corresponding region is a 'switch region' involved in the propagation of a conformational change triggered by ATP hydrolysis (Yoshida and Amano, 1995). It is tempting to speculate that such a region in ABC transporters might also undergo conformational changes during the exchange of signals with hydrophobic membrane subunits.

In conclusion, our study provides evidence that cytoplasmic loops of the hydrophobic domains of an ABC transporter interact functionally with nucleotide binding domains, and particularly the so-called helical domain. This finding might shed new light on the study of ABC transporters since such interactions have often been postulated. The fact that we obtained two types of negative mutants regarding the strength of association of MalK with mutated MalF–MalG suggests that these interactions may be important for the proper assembly and also for the correct function of the transporter.

Materials and methods

Bacterial strains

Strains were derivatives of *E.coli* K-12 and are described in Table IV. Miscellaneous genetics techniques were performed as described (Miller, 1972). Strain PMED34 was constructed by introducing F′ *lacI*^{Q} *lacZ*::Tn*5* into strain PMED35. Strain ED169 was constructed by co-transduction of Δ*malB107* with the *metA*$^{+}$ gene of pop5218 into pop6952. Strain ED170 was constructed by transducing *srl*::Tn*10recA* of PMED35 into pop6484.

Media, chemicals and culture conditions

Strains carrying plasmids were grown at 30°C in order to minimize possible undesirable effects due to the elevated expression of mutant membrane proteins. Media were described in Miller (1972). The synthetic medium 63 was used for growth rate determinations, transport assays and *in vivo* pulse labeling. It was supplemented with 0.4% glycerol or maltose (Merck), 0.5 μg/ml thiamine and 0.4% Bacto-Casamino Acids (Difco) or with a 0.004% mix of amino acids and nucleotides (Sigma) without methionine and cysteine for labeling experiments. Ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (30 μg/ml) and tetracycline (10 μg/ml) were added when required. Rich medium LB was used for plasmid preparation and for mutant protein identification. Maltose and maltodextrins utilization was determined on MacConkey medium containing Maltose (2%) or 1% of the 'malto-oligosaccharide mixture' from Pfanstiehl Laboratories Inc. (USA) respectively.

Recombinant DNA methods

Restriction enzymes were used according to the instructions of the suppliers. Site-directed mutagenesis was performed as described (Kunkel, 1985). Mutagenic oligonucleotides are described in Table V. Single strand templates from different plasmids were prepared after infection of CJ236 with the M13MKO7 helper phage. Mutagenized plasmids were introduced into strain PMPM5 as described (Chung *et al.*, 1989). DNA from randomly selected clones was sequenced and plasmids bearing the desired mutation were transformed into strain ED170. Nucleotide sequencing was performed by the Sequenase method (USB). α-^{33}P- and ^{35}S-nucleotides and [^{14}C]maltose were obtained from Amersham (UK).

Plasmid constructions

Plasmids are described in Figure 1 and were constructed as described below.

Plasmid pTAZGQ. An *Eco*RI–*Ssp*I fragment carrying the *malG* gene downstream of the *ptac* promoter and an *Eco*RI fragment bearing the

Table IV. Strains used in this work

Strain	Genotype	Origin
MC4100	F⁻ Δ*lacU169 araD139 rpsL relA thi flbB*	Lab. collection
DHB4	F⁻ Δ*(ara-leu)7697 araD139 lacX14 phoA* Δ[*Pvu*II] *phoR galR galK rpsL thi malF3* (F′ *lacI*^{Q} *pro*)	Boyd *et al.* (1987)
CJ236	F⁻ *dut1 ung1 thi1 relA1* (pCJ105)	Bio-Rad kit
PMPM5	F⁻ *mutL*::Tn*10 malT ara* Δ(*lac-pro*) *thi* (F′ *lacI*^{Q} *lacZ*::Tn5)	Lab. collection
PMED34	F′ *thr1 leuB6 tonA21 supE44 rpoB malG1 srl*::Tn*10 recA* (F′ *lacI*^{Q} *lacZ*::Tn5)	This work
pop5218	HfrC Δ(*gal-bio*) Δ*malB107 thi rpoB*	Lab. collection
pop6952	F⁻ Δ*lacU169 araD139 rpsL relA thi flbB metA*	Lab. collection
ED169	F⁻ Δ*lacU169 araD139 rpsL relA thi flbB* Δ*malB107*	This work
PMED35	F⁻ *thr1 leuB6 tonA21 supE44 rpoB malG1 srl*::Tn*10 recA*	Dassa and Muir (1993)
pop6484	F⁻ Δ*lacU169 araD139 rpsL relA thi flbB rpoB malFv49*$_{och}$ (polar on *malG*)	Lab. collection
ED170	F⁻ Δ*lacU169 araD139 rpsL relA thi flbB rpoB malFv49*$_{och}$ *srl*::Tn*10recA*	This work
MM140	ED170 transformed with pTAZFGQ	
MM141	ED170 transformed with pTZ18-R	
MM142	MM140 transformed with pACYK	
MM143	MM141 transformed with pACYK	
MM144	ED170 transformed with pTAZFQ and pACYK	
MM145	ED170 transformed with pTAZGQ and pACYK	

Table V. Sequences of mutagenic oligonucleotides

Mutagenic oligonucleotide sequence (5′→3′)	Sequence change in	
	MalF	MalG
CGATTTGTAT**GCA**GCCTCAGCAATGG	E1→A	
CGATTTGTAT**AAA**GCCTCAGCAATGG	E1→K	
CGATTTGTAT**CTA**GCCTCAGCAATGG	E1→L	
GTATGAAGCC**GAT**GCAATGGATGGC	S3→D	
GTATGAAGCC**AAA**GCAATGGATGGC	S3→K	
GTATGAAGCC**TTA**GCAATGGATGGC	S3→L	
CCTCAGCAATGGAT**GCC**GCAGGTCCG	G7→A	
CCTCAGCAATGGA<u>C</u>**CCC**GCAGGTCCG	G7→P	
GATTACGCTG**GCG**CTGCTGATTAAACCG	P10→A	
CGCTGGAA**GCA**GCTGCTGCGC		E1→A
CGCTGGAA**AAA**GCTGCTGCGC		E1→K
GTTCGCTGGAA**TTA**GCTGCTGCGC		E1→L
GAAGAGCT**GAT**GCGCTGGATGG		A3→D
GGAAGAAGC<u>C</u>**TCT**GCGCTGGATGG		A3→S
GGAAGAAGCT**CTT**GCGCTGGATGG		A3→L
GCGCTGGAT**GCT**GCGACACCG		G7→A
GCGCTGGA<u>C</u>**CCT**GCGACACCG		G7→P
GCCTTGTCCTGTTG**GCG**CTGTCAGTACCG		P10→A

Bold characters represent mutated codons. Underlined nucleotides were silent changes made to avoid introduction of undesirable *Bam*HI or *Hin*dIII restriction sites.

lacI^{Q} gene, both isolated from plasmid pDG (Dassa and Muir, 1993), were inserted successively into phagemid pTZ18-R (Pharmacia Biotech).

Plasmid pTAZFQ. A fragment containing the *malF* gene downstream of the *ptac* promoter was amplified from plasmid pTFG (E.Dassa, unpublished) using two primers. One of the primers was designed to introduce a *Bam*HI site 3′ to *malF*. The amplified fragment was digested with *Eco*RI and *Bam*HI and inserted in pTZ18-R. Then the *Eco*RI fragment bearing the *lacI*^{Q} gene was inserted in the *Eco*RI site.

Plasmid pTAZFGQ. A *Bam*HI–*Hin*dIII fragment from pTAZGQ carrying the *malG* gene was inserted into pTAZFQ.

Plasmid pACYK. This was constructed in two steps. First the *malK* gene from pKN101, a plasmid harbouring the *malK* and the *lamB* genes, was amplified using a set of primers that introduced a *Nco*I site at the ATG codon of *malK* and a *Hin*dIII site 3′ to *malK*. The fragment was digested by these two enzymes and inserted into plasmid pTrc99A (Pharmacia Biotech) giving rise to plasmid pTrK, where *malK* was under the control of the *trc* promoter. Second, an *Sph*I–*Sca*I fragment from pTrK containing the *lacI*^{Q} gene, the *trc* promoter and *malK* was inserted into plasmid pACYC184, generating plasmid pACYK compatible with the pTAZ series plasmids.

Plasmid pTLCME. This was obtained by cloning the *malE* gene under the control of the *ptac* promoter (Jean-Michel Betton, unpublished) in pWSK129, a plasmid carrying the pSC101 origin of replication (Wang and Kushner, 1991).

Hydroxylamine mutagenesis

Plasmid pACYK was treated with 0.8 M hydroxylamine (Sigma) at 70°C for 1 h using a procedure modified from Humphrey *et al.* (1976). Briefly, 20 μl of plasmid (1 μg/μl) was incubated with an equal volume of freshly prepared hydroxylamine stock solution (1.6 M hydroxylamine, 1 mM EDTA, pH 5.8) at 70°C. After 1 h incubation, the reaction was stopped by precipitating the DNA (70% ethanol, 0.1 M sodium acetate). Precipitated DNA was dissolved in 50 μl H_2O and stored at –20°C until use. We used 2 μl of mutagenized DNA to transform strain ED170. Mutants were screened as described in Results.

Transport and enzyme assays

Transport assays were performed essentially as described (Szmelcman and Hofnung, 1975) with the following changes. Strains were grown in medium 63 supplemented with 0.4% glycerol, 0.4% maltose, 100 μg/ml ampicillin, 25 μg/ml chloramphenicol and 0.2% Bacto-Casamino acids. At A_{600} = 0.2, 10 μM IPTG was added to induce expression of the *mal* genes on plasmids. Cells were harvested at A_{600} = 0.6 (~3×10^8 cells/ml), washed twice and resuspended in 0.2 volume of medium 63 containing 30 μg/ml kanamycin. A mixture of [^{14}C]maltose and non-

labelled maltose was added to 500 µl of suspension (final concentration 4 µM). Samples of 150 µl were withdrawn several times and quickly filtered through HAWP Millipore membrane filters of 0.45 µm pore size. Filters were thoroughly washed with medium 63, dried and counted.

Total extracts and cell fractionation

Strains were grown in 5 ml LB. At A_{600} = 0.2, 10 µM IPTG was added, and cells were grown to A_{600} = 1.0, chilled and recovered by centrifugation. Total cellular extracts were prepared as follows: a culture volume equivalent to 0.5 ml culture at A_{600} = 1.5 was centrifuged in a bench-top microcentrifuge and resuspended in 20 µl 50 mM Tris–HCl pH 8, 150 mM NaCl. Twenty µl of double strength SDS–PAGE loading buffer were added and the homogenized mixture was boiled for 5 min in a water bath. For cell fractionation, bacteria were resuspended in 10 mM Tris–HCl pH 7.5 containing 0.75 M sucrose and lysed by addition of egg-white lysozyme (1/8 volume of a 2 mg/ml solution in 0.1 M EDTA pH 7). After 30 min incubation at 0°C, spheroplasts were recovered by a 30 min centrifugation. Five hundred µl of 50 mM Tris–HCl pH 8, 150 mM NaCl, 2 mM EDTA were added to the spheroplast pellet and lysis was completed by three cycles of freezing and thawing. After addition of DNase I and a 15 min incubation at room temperature, cell debris was removed by low speed centrifugation. Particulate fractions were recovered from the extract by a 1 h centrifugation at 200 000 *g*. Triton X-100 extracts were performed essentially as described by Diedrich *et al.* (1977). Triton X-100 insoluble pellets were solubilized with freshly prepared 50 mM Tris–HCl pH 8, 150 mM NaCl, 6 M urea for 1 h at room temperature. Urea insoluble fractions were recovered by a 1 h centrifugation at 200 000 *g*. Each fraction was collected in equal volumes, and a subfraction was taken at each step of the fractionation/solubilization process for SDS–PAGE analysis.

In vivo pulse-labeling

Strains ED169 transformed with the mutated pACYK were grown in 5 ml M63 medium containing glycerol, nucleotides and amino acids without methionine and cysteine. At A_{600} = 0.2, 10 µM IPTG was added, and cells were grown for 1 h. Cultures were normalized to an A_{600} of 0.4 and 200 µl of culture was labeled with 8 µCi of TRAN^{35}S-LABEL (ICN) for 10 min at room temperature. Two hundred µl of double strength SDS–PAGE loading buffer were added and the homogenized mixture was boiled 5 min in a water bath. Samples were run on an SDS–polyacrylamide gel, the gel was fixed 30 min at room temperature in 10% acetic acid and 25% isopropanol, dried and subjected to autoradiography.

Polyacrylamide gel analysis and immune detection of proteins

We used the BCA protein kit (Pierce) to determine the protein concentration of samples. Membrane extracts were solubilized in a buffer containing 1% SDS. The calibration curve was made by using bovine serum albumin diluted in the same buffer.

Protein samples were separated on SDS–polyacrylamide slab gels prepared as described (Lugtenberg *et al.*, 1975). Immunodetection of MalF, MalG and MalK was performed by immunoblotting (Towbin *et al.*, 1979; Dassa, 1990). Antibodies were specific to MalG (Dassa, 1990), MalF (gift of Beth Traxler) and MalK (gift of Erwin Schneider). Electrophoretically separated acellular extracts were transferred onto nitrocellulose membrane filters and probed successively with the specific antibody and with a horseradish peroxidase anti-rabbit immunoglobulin conjugate (Bio-Rad). Immune complexes were revealed by ECL Western Blotting Detection Reagents (Amersham, UK).

For quantitative analyses, gels or autoradiography were scanned with a MasterScan Interpretive densitometer (Scanalytics).

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During the processing of the manuscript, Wilken *et al*. [*Mol. Microbiol*. (1996), **22**, 655–666] reported mutations allowing LacK, an ABC ATP-ase homologous to MalK, to interact with MalF and MalG. These mutations map into the helical domain of LacK.