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Topical Review

The Use of Gene Fusions to Study Bacterial Transport Proteins

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Summary. The transport of solutes by bacteria has been studied for about thirty years. Early experiments on amino acid entry and galactoside accumulation provided concrete evidence that bacteria possessed specific transport systems and that these were subject to regulation. Since then a large number of transport systems have been discovered and studied extensively. Many of these use entirely different strategies for capturing or accumulating substrates. This diversity reflects variation in the availability of nutrients and ions in the different environments tolerated and inhabited by microorganisms. Examination of a few bacterial transport systems provides an opportunity to gain insight into a wide range of topics in the area of membrane transport. These include: the identification of carrier proteins and their arrangement in the membrane, the regulation of transport protein synthesis by environmental factors, and the localization of transport proteins to their extracytoplasmic destinations.

It has been possible to construct a number of bacterial strains in which the gene (*lacZ*) which codes for the cytoplasmic enzyme β-galactosidase is fused to genes which code for transport proteins. The following article is intended to illustrate how these gene fusions have been used to study the regulation and structure of transport proteins in *Escherichia coli.*

Key words: Bacterial transport, gene fusions, maltose transport, osmolarity regulation, porins, potassium transport.

The identification of bacterial transport proteins has been problematic. Efforts to purify and characterize these proteins have been hampered by the lack of a sensitive assay for transport activity and the inability to solubilize active carrier molecules. The greatest success to date has been the identification of the lactose carrier protein (M protein) from *E. coli.* This protein was originally identified by Fox and Kennedy who used a specific labeling procedure that relies on the susceptibility of the carrier protein to a sulfhydryl reagent (N-ethylmaleimide) in the absence but not in the presence of substrate [22]. Unfortunately, this approach has not been universally successful. A more general technique for labeling and identifying proteins is provided by gene fusions constructed with the *lacZ* gene, which codes for the soluble enzyme, β-galactosidase.

Labeling of two cytoplasmic membrane components of the maltose transport system with a fragment of β-galactosidase was accomplished by constructing fusions of the *lacZ* gene to the structural genes of the two proteins, *malF* and *malK.* Subsequently, antibody reagents were prepared that recognize the two previously unidentified transport proteins. These antibodies have been useful in studying the arrangement of these proteins in the membrane.

The regulation of transport protein synthesis in response to the external environment has been studied in two systems with the aid of gene fusions. In both cases changes in the osmolarity of the external media profoundly influence the synthesis of the transport system components. The first of these is the high affinity ATP-driven K^+ transport system coded for by genes of the *kdp* operon. Epstein and coworkers have isolated *kdp-lac* fusions to study the manner in which the expression of the *kdp* genes is regulated in response to changes in the osmotic pressure of the medium. The other transport proteins whose synthesis is regulated by the external environment are the porin proteins in the *E. coli* outer membrane. The osmolarity of the medium has been known to be important in determining the relative amounts of two of the porin proteins. Hall and Silhavy have constructed fusions of the *lacZ* to the genes which code for these proteins and have demonstrated that a pro-

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Table 1. A brief glossary of genetic nomenclature

Gene	Protein	Relevant properties	References
lacZ	β-galactosidase	Soluble cytoplasmic enzyme necessary for growth on lactose, polypeptide $M_r = 116{,}000$	[21, 32]
malE	Periplasmic maltose binding protein, MBP	Necessary for maltose transport $K_d = 10^{-6}$M for maltose	[28]
malF	Integral cytoplasmic membrane protein	Necessary for maltose transport $M_r = 40{,}000$	[49]
malK	Peripheral cytoplasmic membrane protein	Necessary for maltose transport $M_r = 40{,}000$	[48]
malG	Cytoplasmic membrane protein (?)	Not directly identified; appears to anchor MalK protein to membrane	[48]
lamB	Outer membrane protein	Forms maltose specific aqueous channel; also functions as a receptor for bacteriophage lambda	[40, 53]
ompF, ompC	Outer membrane protein porins Ia, Ib	Form nonspecific aqueous channels. Also function as receptors for phages	[36, 37]
ompB	?	Complex genetic locus necessary for regulation of *ompF* and *ompC* expression	[24, 44]
kdp A, B, C	High affinity potassium transport system, K^+ ATPase	Synthesis controlled by changes in medium osmolarity	[29, 42]
kdpD	Regulatory protein for K^+ transport	Membrane protein sensitive to changes in osmolarity	[30]

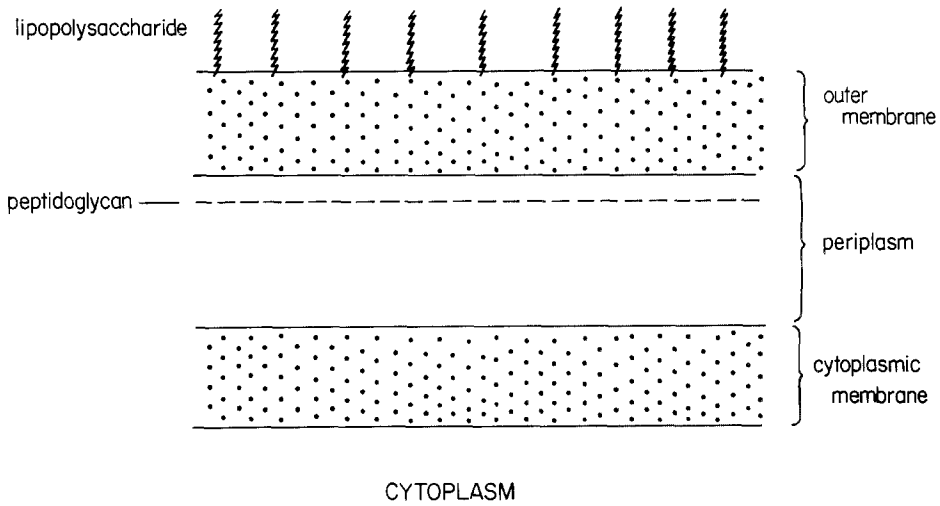

Fig. 1. The cell envelope of a gram-negative cell such as *Escherichia coli*

tein coded for by a third locus regulates the expression of the porin genes [23, 24].

Finally, gene fusions have proved to be valuable in determining how some transport proteins are correctly localized to their extracytoplasmic locations. Studies with fusions of the *lacZ* gene to the gene which codes for the periplasmic maltose binding protein, *malE* and to the gene that codes for the outer membrane component of the matose transport system, *lamB* are providing a detailed genetic analysis of protein localization in bacteria. Since this topic has recently been the subject of a number of excellent reviews, it will not be considered here [15, 50].

The following review begins with a brief description of the gram-negative cell envelope and an outline of the construction and properties of gene fusions. Table 1 is a list of the genetic symbols which are used and a description of protein products of each gene.

The Gram-Negative Cell Envelope

Before departing on any discussion of transport proteins in *E. coli*, it is necessary to consider the overall arrangement of the cell's extracytoplasmic compartments.

The cell envelope of *E. coli* and other gram-negative bacteria is a complex trilaminar structure consisting of: (1) an innermost cytoplasmic membrane, (2) a peptidoglycan network, (3) an outer membrane,

and (4) the space bounded by the two membranes, the periplasm (*see* Fig. 1).

The cytoplasmic membrane which constitutes the main permeability barrier of the cell is a typical plasma membrane containing approximately equivalent amounts of phospholipid and protein. This membrane is the site of specific transport systems, catabolic dehydrogenases, phospholipid biosynthetic enzymes, and is also the location of electron transport and oxidative phosphorylation. Consistent with this multiplicity of functions, many different protein species are found in this membrane.

The peptidoglycan is a network of aminoglycosides that is crosslinked by oligopeptides. It is attached to the outer membrane (*see* below) via a lipoprotein, which is both located in the outer membrane and covalently bound to the peptidoglycan [9]. This structure provides the cell with rigidity, shape, and resistance to osmotic stress.

The outer membrane is atypical with respect to most biological membranes due to the presence of large amounts of lipopolysaccharide in its outer leaflet. The permeability properties of this membrane suggest that it functions as a molecular sieve. Compounds in excess of 900–1000 mol wt are excluded by this membrane, 600–900 mol wt compounds are partially retarded, and compounds smaller than 600 mol wt pass through [36]. The proteins of this membrane have been shown to consist of only a few major and minor species. Some of the major protein species are able to form aqueous channels in artificial membranes which then have the same molecular sieving properties as whole cells [37]. These proteins are commonly referred to as porins. Some of the outer membrane proteins are components of specific transport systems and provide "pores" for specific substrates. Many of these proteins serve as receptors for a variety of bacteriophage and colicins (for a review, *see* [3] and [39]).

The periplasmic space contains a number of water soluble degradative enzymes and substrate binding proteins [33, 38]. Many of the binding proteins participate in active transport of their substrate. (For a review, *see* [8].) This aqueous compartment has been likened to the more sophisticated lysosome that is present in eukaryotic cells. Substrates that enter the periplasm via the aqueous channels of the outer membrane can either be "trapped" by binding proteins and/or partially degraded before being transported across the cytoplasmic membrane. This arrangement is well suited for scavenging and concentrating growth substrates. Other organisms in the immediate vicinity do not have access to nutrient molecules that have been partially degraded or "trapped" by the extracytoplasmic protein components of the periplasm.

Fusions of the *lac* Operon

The lactose operon is one of the best characterized systems in *E. coli*. The regulation of *lac* gene expression and the protein products of the *lac* genes have been studied in detail (for an extensive review, *see* [32].) The enzyme β-galactosidase is the product of the *lacZ* gene. There are a variety of substrates for this enzyme which are useful for detecting enzymatic activity *in vivo* and *in vitro*. Other galactosides can used to screen for and select mutant strains in which the amount of β-galactosidase is either increased or decreased.

Since some gene products such as transport proteins and regulatory proteins are impractical to assay, many other systems cannot be studied as easily as the *lac* operon. In order to facilitate the study of these systems it has been possible to fuse the genes of the *lac* operon to the genes which code for the system of interest. The *lac* genes are then expressed in a manner determined by the gene to which they are fused. For example, if the regulation of gene X is to be studied but there is no assay for X protein, fusions of the *lac* genes to gene X can be constructed. In a strain which contains such a fusion the *lac* genes are expressed in the manner determined by the promoter of gene X. In order to study expression of gene X it is then sufficient to assay β-galactosidase activity. Mutants in which gene X expression is altered can be isolated and analyzed using standard *lac* operon technology.

A detailed description of the construction of gene fusions can be found in a paper by Casadaban [11]. A brief description of this technique is provided in Fig. 2. Two types of gene fusion can be constructed. The first type is referred to as an "operon fusion." In strains containing an operon fusion, the expression of a normal copy of the *lacZ* gene is controlled by the gene X promoter (site of transcription initiation); since the *lacZ* gene has its own translation initiation site, normal β-galactosidase is produced. The second type of gene fusion is called a "protein fusion." In strains that contain a protein fusion, the deletion which generated the fusion has extended into the *lacZ* gene (*see* Fig. 2). Since the translation site of the *lacZ* gene has been deleted, a hybrid protein is produced. The amino-terminal portion of the hybrid protein is coded for by gene X and the carboxyl-terminal portion is coded for by *lacZ*. Due to the technique used to construct protein fusions the hybrid proteins always retain β-galactosidase activity [11, 35]. The difference between the two types of fusion is illustrated in Fig. 3.

Operon fusions have been particularly useful in studying the regulation of gene expression because

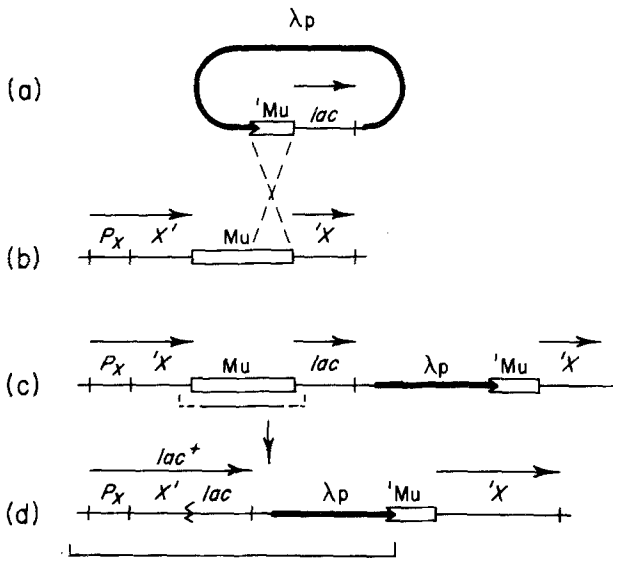

Fig. 2. Steps leading to fusion of the *lac* operon to other bacterial genes. (*a*), (*b*), and (*c*) depict the way in which the *lac* genes are transposed to a site adjacent to the gene (X) of interest. First, a copy of the bacteriophage Mu genome is inserted in gene X in a strain which is deleted for the *lac* genes (*b*). This strain is then lysogenized with a special λ phage which carries a copy of the *lac* genes and a piece of bacteriophage Mu (*a*). This λ phage also lacks the normal attachment site at which it usually integrates into the host chromosome. Therefore, integration into the bacterial chromosome frequently occurs *via* homologous recombination between the sequences. The structure of the gene X-*lac* region of the chromosome in the resulting lysogen is shown in (c). The *lac* genes are now separated from gene X and its promoter by the Mu genome. The Mu genome prevents expression of the *lac* genes from the gene X promoter because it contains at least one transcription barrier. Cells which have gained the ability to express the *lac* genes at the same time that they lose the Mu genome are usually the result of a deletion event which fuses the *lac* genes to gene X and its promoter (*c*) and (*d*). P_X indicates the promoter for gene X. The normal *lac* promoter has been eliminated by mutation. Horizontal arrows indicate the direction of transcription. (Reprinted from [2], with permission)

the amount of β-galactosidase activity is a direct measure of the transcription originating at the promoter to which the *lacZ* gene is fused [2]. The hybrid proteins, which are produced in strains containing protein fusions, have been extremely valuable in characterizing the products of the genes to which the *lacZ* gene has been fused. In addition, protein fusions have been an essential tool in studying post-transcriptional events in the synthesis of noncytoplasmic proteins.

Construction of a protein fusion results in an amino-terminal region of a given protein becoming labeled with an active fragment of β-galactosidase. Several properties of β-galactosidase make it ideally suited for this purpose. First, the amino terminal part of the enzyme is unnecessary for enzymatic activity. This was recognized initially when it was observed that inactive enzyme from some *lacZ* mutant strains could be activated by anti-β-galactosidase antibodies [1]. The only mutant enzymes that could be activated were produced in strains in which an amino-terminal part of the protein was removed by a small deletion entirely within the *lacZ* gene. Subsequently it was shown that the amino terminal region of galactosidase could be replaced by a large fragment of the *lac* repressor protein and the resulting hybrid protein retained enzymatic activity [35]. The isolation of a variety of β-galactosidase hybrid proteins suggests that even though the amino terminal region is not necessary for enzymatic activity it must be replaced by some other protein sequence to maintain enzymatic activity. By mapping the fusion site in a large number of independently isolated *lacZ* gene fusions, it has been estimated that the nonessential region of β-galactosidase extends to residue 20–30 [10].

Another property of β–galactosidase which makes it ideally suited for labeling protein fragments is the large molecular weight of the polypeptide. The molecular weight of the intact monomer is 116,349 [21]. The hybrid protein products of *lacZ* gene fusions are usually at least this large, and one has a molecular weight of 170,000 [16]. In most instances the hybrid proteins are among the very largest polypeptides in the cell and can easily be visualized by electrophoresis of whole cell extacts on polyacrylamide gels containing SDS. The large molecular weight of these proteins has also facilitated their purification [49]. Some of the purified hybrid proteins have been useful in determining sequences of the amino terminal extensions present only in the precursor forms of secreted proteins [6, 34, 45].

Two Cytoplasmic Membrane Components of the Maltose Transport System

The efficient utilization of maltose (glucose α(1→4) glucoside) and longer α(1→4) glucose polymers (dextrins, maltodextrins) by *E. coli* requires the products of eight genes. These include: a positive regulatory protein, the product of the *malT* gene [13], two catabolic enzymes, maltodextrin phosphorylase and amylomaltase, the products of genes *malP* and *malQ* [25], respectively, and finally five proteins devoted to substrate active transport, the products of genes *malE*, *F*, *G*, *K*, and *lamB*. The *malE* gene codes for a periplasmic maltose binding protein (MBP) [28], and the *lamB* gene codes for an outer membrane protein that is both the receptor for bacteriophage lambda and a component of the transport system [40, 53].

The complexity of this transport system is both interesting and enigmatic. Although two of the proteins (MBP and λ receptor) have been purified and studied in detail, the remaining three components

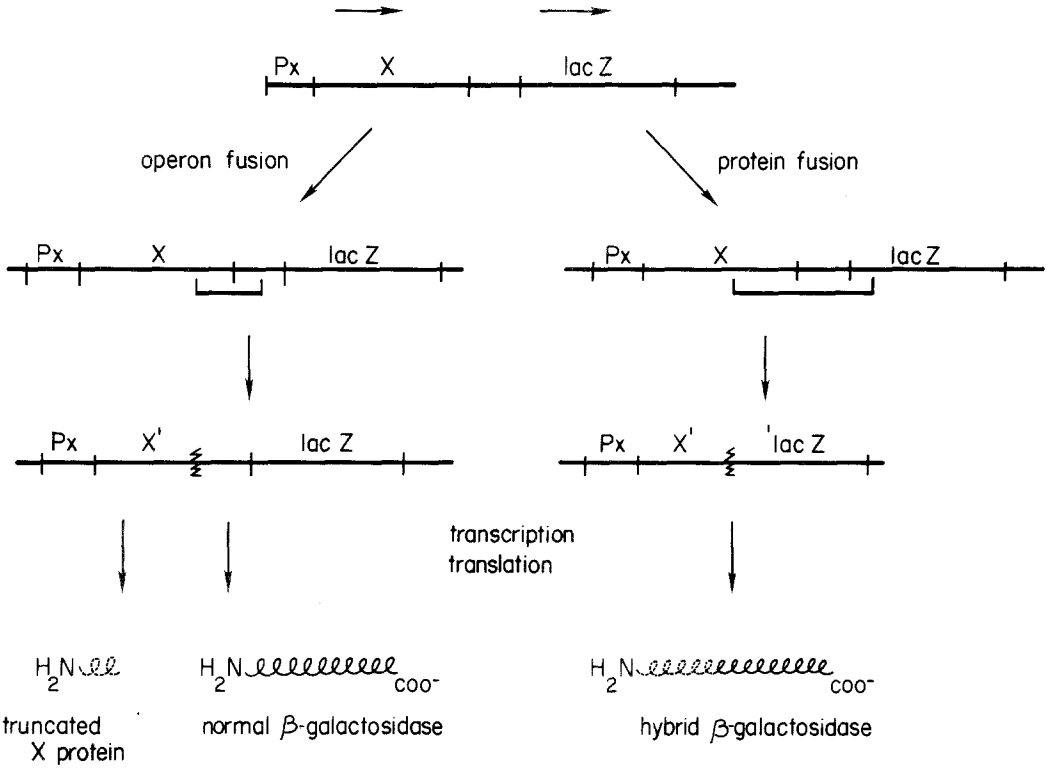

Fig. 3. Differences between operon and protein fusions. A deletion which fuses the *lac* genes to gene X can result in the production of either wild-type β-galactosidase (operon fusions) or hybrid β-galactosidase (protein fusions). The scheme for producing fusions has been simplified in this figure for clarity

proved elusive. In addition, the source of energy for substrate accumulation is not an electrochemical gradient of cations but some undefined form of high energy phosphate [7,26]. Finally, the role of the periplasmic binding proteins is obscure. The binding protein has an affinity for substrate of approximately 10^{-6}M [28]. It is also essential for substrate translocation across the membrane but its exact role is unclear [28, H.A. Shuman, *unpublished*]. In order to study this complex system in more detail, it became necessary to first identify the remaining components of the transport system and determine their cellular location.

In order to identify the products of the *malF* and *malK* genes, *malF-lacZ* and *malK-lacZ* protein fusions were constructed. Originally two classes of *malF-lacZ* gene fusions were found. The first class contained only a small portion of the *malF* gene. The hybrid β-galactosidase produced by this class is found exclusively in the cytoplasm of the cell. The second class of *malF-lacZ* fusions contained almost all of the *malF* gene. The hybrid β-galactosidase produced by this class is located in the cytoplasmic membrane of the cell. This suggested that if the MalF protein itself is located in the cytoplasmic membrane, then the portion of the *malF* gene present in the fusion is sufficient to localize this protein to the cytoplasmic membrane [51].

The *malK-lacZ* gene fusion was constructed in a manner that resulted in a complex fusion which contains almost the entire *malK* gene, a small portion of the *lamB* gene, and the *lacZ* gene. The hybrid protein produced by this fusion is also found in the cytoplasmic membrane. The role of the small portion of *lamB* sequence in determining the location of the hybrid protein is not known [16].

The hybrid protein products from the gene fusion strains could be identified after electrophoresis of membrane extracts on polyacrylamide gels containing SDS. In addition, it was shown that these proteins could be precipitated by anti-β-galactosidase antibodies.

The molecular weight of the MalF-hybrid protein is approximately 150,000 and that of the MalK hybrid is approximately 170,000 [49, 16]. This indicates that both of the hybrids contain substantial portions of the MalF and MalK proteins, respectively.

The MalF and MalK proteins were identified using antibodies that recognize these proteins. The antibodies were prepared by immunizing rabbits with purified MalF or MalK hybrid protein. Some of the antibodies produced by the rabbits would recognize

lacZ coded β-galactosidase sequences, but others would recognize sequences coded for by the *malF* or *malK* gene. The MalF or MalK-specific antibodies could then be used to detect the MalF and MalK proteins in wild-type cells.

The hybrid proteins have been purified by a simple procedure based on the fact that each is the largest polypeptide species in the membranes of the respective fusion strain. A crude membrane fraction was prepared from broken cells of each strain. The proteins from each membrane fraction were then solubilized and denatured in SDS at 100 °C. The polypeptides in each extract were then separated according to size by gel filtration on a column of agarose beads. Milligram quantities of purified hybrid proteins were obtained using this procedure [49].

Rabbits were then immunized with each hybrid protein. The immune response was followed by monitoring the ability of serum samples to precipitate the appropriate hybrid protein. After it was determined that antibodies were being produced, serum samples were used to detect the MalF and MalK proteins in immune precipitation experiments.

This was accomplished by labeling cells with radioactive amino acids, preparing whole cell extracts, and adding antibody to the extracts. After incubating this mixture, the immune complexes were collected and subjected to electrophoresis on SDS-polyacrylamide gels. Radioactive antigens which had combined with the antibody were then detected in the gels by fluorography. The MalF and MalK proteins were detected as maltose inducible proteins that were precipitated by the appropriate antibody preparation. In addition, the MalF protein was absent in *malF* mutants and the MalK protein was absent in *malK* mutant stains. The various *malB* mutant strains were grown in maltose to induce expression of any *malB* region genes which were not inactivated. This was made possible by providing the *malB* mutant stains with a secondary maltose transport system [47]. Finally it was shown that the MalF and MalK proteins crossreacted immunologically with their respective hybrid proteins.

The molecular weights of these two proteins have been estimated by their electrophoretic mobility on SDS-polyacrylamide gels. Both the MalF and MalK proteins have a molecular weight of approximately 41,000–43,000 [48, 49].

The location of the MalF and MalK proteins was determined by fractionating radioactively labeled wild-type cells and testing each fraction for the presence of the proteins by immune precipitation.

The MalF protein was shown to be a cytoplasmic membrane protein because it sedimented with the total membrane fraction but could be solubilized by Triton X-100 in the absence of EDTA. (This is one of the standard techniques for distinguishing cytoplasmic membrane proteins from outer membrane proteins [46]).

The MalK protein was shown to be located in the envelope fraction in wild-type cells. When the location of this protein was examined in different *malB* mutant strains, it was found that in *malG* mutants the MalK protein was not located in the membrane fraction but could be recovered from the cytoplasm [48]. In addition, it has been shown that this protein can be solubilized from wild-type membranes by Triton X-100 [4] und can be removed from membranes by sonication (H.A. Shuman, *unpublished*). These results are interpreted to indicate that the MalK protein is peripherally associated with the inside surface of the cytoplasmic membrane via an interaction with the MalG protein. This location of the MalK protein implies that it is unlikely to participate in substrate recognition or binding protein: substrate recognition. A more reasonable role for the MalK protein would be coupling metabolic energy to substrate transport.

The proposed interaction of the MalG and MalK proteins can be tested either with biochemical or genetic experiments. Biochemical tests of this model would include experiments with bifunctional reagents to see if the MalK and MalG proteins can be crosslinked. Genetic evidence for the interaction of these two proteins would be provided if a defect in one of the proteins which had been altered by mutation could be "phenotypically suppressed" by a secondary mutation in the gene coding for the other protein.

Any model of the maltose transport system should take into account the stoichiometry of the components. The number of copies of maltose binding protein and functional units of LamB protein per cell have been estimated to be approximately 3×10^4 [14].

Only very crude estimates of the number of MalF and MalK protein molecules per cell have been made. Based on the amount of radioactivity present in the MalF and MalK bands after immunoprecipitation from radioactively labeled extracts, there appear to be approximately 500–1000 copies of each of these proteins per cell [48; H.A. Shuman, *unpublished*)]. This is at least 10–50 times less than the amount of maltose binding protein and LamB protein per cell.

A model for the maltose transport system based on currently available information and some speculation is presented in Fig. 4. This model will have to be amended as more information about the disposition of the MalF, MalK and MalG proteins in the cytoplasmic membrane becomes available.

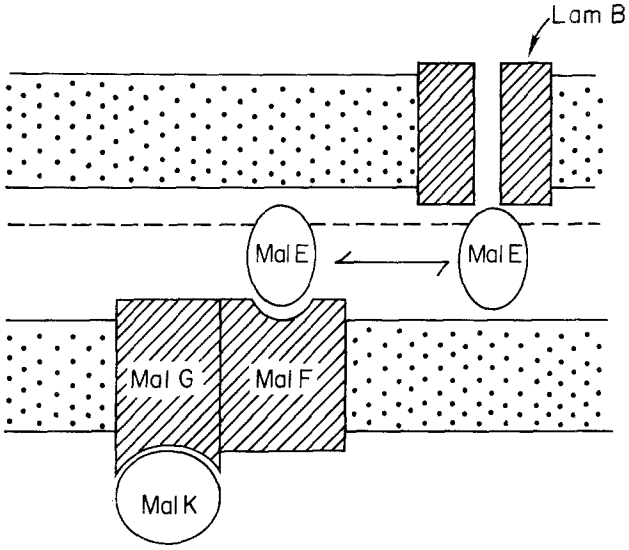

Fig. 4. Arrangement of the maltose transport system in the *E. coli* cell envelope. There is extensive evidence for the outer membrane location of the LamB protein and the periplasmic location of the maltose binding protein. The MalF and MalK proteins have both been shown to be located in the cytoplasmic membrane. It is not known whether the MalF protein spans the bilayer. The location of the MalG protein is inferred from its role in anchoring the MalK protein to the membrane. Evidence for the location of the components is discussed in the text

Turgor Pressure Regulates K^+ Transport

E. coli cells maintain an internal osmolarity which is much higher than the external environment, usually on the order of three atmospheres [19]. This internal-positive osmotic pressure has been called turgor pressure. The rigidity of the peptidoglycan prevents the cell from exploding. Epstein and coworkers have demonstrated that constant turgor pressure is maintained primarily by adjusting the amount of K^+ inside the cell [19]. There are two K^+ transport systems in *E. coli*. The first of these is the Trk system and the other is the Kdp system [43]. The Trk system has a relatively low affinity for K^+ ($K_m = 1.5$ mM) but a rather high maximal rate of transport. The Kdp system, however, has a high affinity for K^+ ($K_m = 2$ μM) and is therefore ideal for scavenging K^+ [43]. The Trk system is responsible for K^+ transport in cells growing in the presence of high external concentrations of K^+. This system is complex, requiring six components [43]. Although the energy for K^+ accumulation via the Trk system is provided by the electrochemical gradient of protons, ATP seems to be an essential regulator of transport activity [41].

The Kdp system is normally repressed in cells which are growing in the presence of added K^+. This system has been studied in greater detail than the Trk system. The Kdp system can be studied in strains in which the Trk system has been eliminated by mutation. The Kdp system consists of three cytoplasmic membrane proteins coded for by the genes of the *Kdp* operon: *kdpA*, *kdpB* and *kdpC* [29]. A fourth protein, the product of the *kdpD* gene seems to be [42] a positive regulator protein for *Kdp* gene expression. The source of energy for K^+ transport via the Kdp system is ATP. A K^+-stimulated ATPase activity has been demonstrated to be associated with this transport system [20]. The 90,000 mol wt *kdpB* gene product has been shown to be phosphorylated by ATP in a reversible manner [17]. Mutations which affect the affinity of the transport system for K^+ map in the *kdpA* gene. This suggests that the 47,000 mol wt KdpA protein provides at least part of the K^+ binding site [20]. Proteolysis studies have shown that the KdpB protein is exposed predominantly on the cytoplasmic side of the inner membrane while the KdpA protein appears to span the bilayer [18].

To facilitate studies on the regulation of *kdp* operon expression, a *kdpA-lacZ* operon fusion was constructed [30]. The amount of β-galactosidase in strains containing this fusion is a direct measure of the *kdp* operon transcription. The effect of varying the external K^+ concentration on *kdp* gene expression was evaluated by assaying β-galactosidase in strains grown in media containing different concentrations of K^+. In strains which have neither an active Kdp nor Trk transport system (the *kdpA-lacZ* fusion inactivates the Kdp system), the expression of the *kdpA* gene was observed to increase at approximately the same concentration of K^+ that became limiting for growth, 40 mM. In a strain containing the *kdpA-lacZ* fusion and a functional TrkA system *kdp* gene expression was stimulated at 10 mM K^+, the same concentration at which K^+ is limiting for growth in the presence of the TrkA system. These results show that the expression of *kdp* operon is stimulated when the ability of the cell to accumulate K^+ is rate limiting for growth rather than by the absolute concentration of K^+ in the medium.

The dependence of *kdp* gene expression on the internal concentration of K^+ was also examined. The amount of β-galactosidase was measured at three different internal concentrations of K^+. Since the internal concentration of K^+ is known to vary with the osmolarity of the medium, different internal concentrations of K^+ could be produced by varying the osmolarity of the external medium. It was found that *kdp* gene expression varied with the internal K^+ concentration in a complex fashion which also depended on the external K^+ concentration.

Since the maintenance of constant turgor pressure appears to be the major role of internal K^+ in *E. coli*, the expression of the *kdp* operon was examined following a sudden increase in the osmolarity of the medium (this will transiently decrease the turgor pressure). It was found that following a short lag (due to temporary disruption of protein synthesis) *kdp* expression was stimulated by the transient decrease in turgor pressure. The extent of stimulation was shown to correlate with the magnitude of the decrease in turgor pressure; larger increases in external osmolarity produced a greater stimulation of β-galactosidase activity. Addition of 230 mM glucose caused an increase in β-galactosidase activity of approximately 40-fold over a period of 30 min. If the somewhat decreased rate of total protein synthesis is taken into account, the differential rate of β-galactosidase synthesis (and therefore *kdp* transcription) following the decrease in turgor pressure is quite large.

Laimins et al. have proposed a model to explain how changes in osmotic pressure stimulate expression of the *kdp* genes [30]. The model requires a protein in the cell which can sense changes in osmotic pressure and transduce them into a signal to stimulate *kdp* gene transcription. The properties of the *kdpD* gene product suggest that this protein could fill this role. As mentioned above there is considerable genetic evidence that the *kdpD* gene product is a positive regulator of the *kdp* operon expression. In addition the KdpD protein probably exists as an oligomer since complementation can be observed between different *kdpD* mutant alleles [42]. Recently, the *kdpD* gene product has been identified as a 90,000 mol wt cytoplasmic membrane protein (L. Laimins and W. Epstein, *unpublished results*). The model proposed by Laimins et al. states that the conformation of the *kdpD* gene product changes in response to alterations in turgor pressure. In the conformational state produced by lowered turgor pressure, the KdpD protein stimulates the transcription of the *kdp* operon genes *A*, *B*, *C*. Any regulatory events that take place post-transcriptionally would not have been revealed by studies with *kdp* operon fusions. Examination of the effect of *kdpD* mutant alleles on the amount of β-galactosidase in strains containing *kdp-lacZ* fusions would clarify the role of the kdpD protein. If mutations in *kdpD* decrease the amount of β-galactosidase, it would strengthen the argument that KdpD protein is a positive transcription factor.

In addition, the *kdp-lac* fusions should be useful in selecting mutant strains with altered responses to changes in turgor pressure. Hopefully these would help in elucidating the mechanism of how mechanical information is transmitted into a genetic regulatory signal.

Osmolarity Regulates Porin Synthesis

As mentioned above, the outer membrane of gram-negative bacteria is composed of a limited variety of proteins in addition to phospholipids and LPS. Most of the proteins play a role in accessability of small molecules to the periplasm and cytoplasmic membrane. The porins form aqueous channels which extend from the exterior of the cell to the periplasm. Although two major species of porins are present in the membrane, their relative amount fluctuates in a complex manner. The fluctuations seem to occur in response to the composition of the medium in which the bacteria are grown. Although the exact determinants of the fluctuation are not understood, some generalizations have been made: (i) cells tend to keep the total amount of protein in the outer membrane constant, probably by feedback control of porin synthesis [12, 31]; (ii) the presence of a fermentable carbon source favors the synthesis of the OmpC protein; (iii) the OmpC protein is preferentially expressed in media of high osmolarity and the OmpF protein is preferentially expressed in media of low osmotic strength [27, 54].

In an effort to understand the regulation of porin synthesis, Hall and Silhavy have constructed a number of strains which contain operon fusions of the *ompC* and *ompF* genes to the *lacZ* gene [23, 24].

In strains containing either an *ompC-lacZ* or an *ompF-lacZ* fusion, β-galactosidase activity fluctuates in a manner which closely resembles that of the original porin. The ratio of β-galactosidase activity in high osmolarity medium (TSB) to that in low osmolarity medium (NB) is ~4 in strains with *ompC-lacZ* fusions and is approximately 0.25 in strains with *ompF-lacZ* fusions. Increasing the osmolarity of a low osmotic strength medium by the addition of a nonpenetrating substance such as sucrose increases the β-galactosidase activity in a *ompC-lacZ* fusion strain and decreases the activity in a *ompF-lacZ* fusion strain. Since the effect of changes in osmolarity can be observed with operon fusions, regulation by osmolarity must be exerted at the level of transcription [29].

A third locus, *ompB* has been known to play a role in the expression of the porin proteins [44, 55]. Mutations at the *ompB* locus can impair synthesis of either the OmpF or OmpC protein. Mutations mapping at the *ompB* locus have been isolated with widely different phenotypes [56, 57]. In order to gain some understanding of the role that the product(s) of the *ompB* locus play in porin regulation, the level of β-galactosidase activity was determined in *ompF-lacZ and ompC-lacZ* fusion strains containing different *ompB* alleles. In strains containing an *ompB* allele which prevents synthesis of both the OmpF and

OmpC proteins, β-galactosidase activity is dramatically reduced in both *ompF* and *ompC* fusions when grown in all media. This result demonstrates that a product of the *ompB* locus is necessary for the transcription of both the *ompF* and *ompC* genes. The amount of β-galactosidase activity was also examined in fusion strains containing *ompB* alleles which affect the synthesis of only OmpF or OmpC. An *ompB* allele which has a $OmpF^-OmpC^+$ phenotype decreases the β-galactosidase activity in the *ompF-lacZ* fusion, but this basal activity can still be induced in certain culture media. The same effect is seen with $OmpF^+$ $OmpC^-$ *ompB* alleles with *ompC-lacZ* fusions.

An *ompB* allele which is $OmpF^+$ $OmpC^-$ has an interesting effect on the level of β-galactosidase in an *ompF-lacZ* fusion strain. In this case the β-galactosidase activity is always synthesized at the fully induced level even in media of high osmolarity where *ompF* expression would be very low in the wild-type. Similar results were obtained in *ompC-lacZ* fusions with $OmpF^-$ $OmpC^+$ *ompB* alleles. These results indicate that the fluctuation of *ompF* and *ompC* gene expression occurs at the level of transcription and is mediated by a product of the *ompB* locus.

The number of genes at the *ompB* locus is unknown but is likely to be more than one. Strains have been isolated which contain *ompB* alleles that result in a pleiotropic phenotype. These strains in addition to being $OmpF^-$ $OmpC^+$ are impaired in the localization of a number of periplasmic proteins [57], or have decreased expression of the genes coding for the maltose transport system [56]. This may be because an *ompB*-coded protein performs an essential membrane related function. In support of this notion, it has not been possible to isolate *ompB* insertion mutations with a $OmpF^-OmpC^+$ phenotype despite extensive efforts (M. Hall and T. Silhavy, *personal communication*) even though insertion mutations at *ompB* with the other phenotypes have been isolated.

Clearly, some component coded by the *ompB* locus must sense changes in medium osmolarity and effect changes in the expression of the *ompF* and *ompC* genes. A number of observations and hypotheses concerning the regulation and biosynthesis of the OmpF and OmpC proteins could be accommodated by the speculation that the *ompB* locus codes for a protein(s) which is part of, or located at, the zones of adhesion which are known to exist between the inner and outer membrane [5]. First, it has been shown by Smit and Nikaido that the OmpC and OmpF proteins appear in the outer membrane at discrete sites, dispersed over the entire cell surface, which appear to coincide with the sites of adhesion [52]. Second, changes in medium osmolarity could easily affect the overall conformation of these adhesion sites thus providing a control system analogous to that hypothesized in the *kdp* system. Third, it is likely that these sites are indispensible for viability since they are the site of LPS translocation to the outer membrane and form a part of the cell envelope structure. Fourth, if the adhesion sites are involved in both regulating the expression of the *ompF* and *ompC* genes and are the sites of cotranslational export of their protein products to the outer membrane, it is easy to formulate a feedback inhibition mechanism. Only those adhesion sites which are not occupied by porin proteins in transit to the outer membrane would be available to stimulate transcription of the *ompF* or *ompC* genes.

Experimental evidence bearing on this hypothesis will most likely result from studies with hybrid proteins coded for by *ompF-lacZ* and *ompC-lacZ* protein fusion and further genetic analysis of the *ompB* locus. Mutations at the *ompB* locus with a conditionally lethal, temperature-sensitive phenotype would be particularly informative. If such mutants are obtained it could be ascertained whether the structure of the adhesion sites is altered at the restrictive temperature.

Conclusion

Many bacterial transport proteins cannot be detected or characterized easily because they function only in a closed membrane system or become inactivated during solubilization. In order to facilitate the study of these proteins, gene fusions have been constructed between the *lacZ* gene (which codes for β-galactosidase) and the genes which code for a variety of transport proteins.

The hybrid proteins produced by protein fusions have been used to prepare antibody reagents for two of the maltose transport system components. These antibodies have provided a way to detect these proteins and study their arrangement in the membrane. Protein fusions constructed between *lacZ* and the genes for exported proteins have been an essential tool in elucidating the pathway(s) of protein localization in prokaryotes.

The influence of the external environment on the regulation of gene expression has been studied by measuring the β-galactosidase activity in operon fusion strains grown in various media. These experiments have demonstrated that changes in the medium osmolarity can influence the transcription of the genes which code for the high affinity K^+ transport system and the outer membrane porin proteins. The manner in which this regulation takes place is not currently understood.

Since fusions of the *lacZ* gene can be constructed to most *E. coli* genes, it should be possible to study other transport systems with the aid of β-galactosidase.

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