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Export of Maltose-Binding Protein Species with Altered Charge Distribution Surrounding the Signal Peptide Hydrophobic Core in *Escherichia coli* Cells Harboring *prl* Suppressor Mutations

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It is believed that one or more basic residues at the extreme amino terminus of precursor proteins and the lack of a net positive charge immediately following the signal peptide act as topological determinants that promote the insertion of the signal peptide hydrophobic core into the cytoplasmic membrane of *Escherichia coli* cells with the correct orientation required to initiate the protein export process. The export efficiency of precursor maltose-binding protein (pre-MBP) was found to decrease progressively as the net charge in the early mature region was increased systematically from 0 to +4. This inhibitory effect could be further exacerbated by reducing the net charge in the signal peptide to below 0. One such MBP species, designated MBP−3/+3 and having a net charge of −3 in the signal peptide and +3 in the early mature region, was totally export defective. Revertants in which MBP−3/+3 export was restored were found to harbor mutations in the *prlA* (*secY*) gene, encoding a key component of the *E. coli* protein export machinery. One such mutation, *prlA666*, was extensively characterized and shown to be a particularly strong suppressor of a variety of MBP export defects. Export of MBP−3/+3 and other MBP species with charge alterations in the early mature region also was substantially improved in *E. coli* cells harboring certain other *prlA* mutations originally selected as extragenic suppressors of signal sequence mutations altering the hydrophobic core of the LamB or MBP signal peptide. In addition, the enzymatic activity of alkaline phosphatase (PhoA) fused to a predicted cytoplasmic domain of an integral membrane protein (UhpT) increased significantly in cells harboring *prlA666*. These results suggest a role for PrlA/SecY in determining the orientation of signal peptides and possibly other membrane-spanning protein domains in the cytoplasmic membrane.

Proteins exported across the cytoplasmic membrane of *Escherichia coli* cells are usually synthesized with a cleavable, amino-terminal signal peptide, which is thought to be chiefly responsible for initiating the export process. This structure includes a central hydrophobic core followed by a somewhat less apolar signal peptidase processing site and preceded by a hydrophilic segment that exhibits a net positive charge due to the presence of one to three basic residues (39). The early mature region of such proteins generally lacks a net positive charge (40). For integral cytoplasmic membrane proteins, a region of net positive charge is usually found immediately adjacent to one end of each membrane-spanning stretch of hydrophobic amino acids (41). In both cases, the transmembrane orientation of the hydrophobic domains is thought to be primarily determined by the distribution of basic residues at either end, with the most positively charged end residing in the cytoplasm. This "positive-inside rule" was initially proposed by von Heijne (41).

In support of the positive-inside rule, several studies have shown that systematically reducing the net charge of the signal peptide hydrophilic segment to below +1 results in a progressive reduction in the efficiency of protein export (19, 30, 38). Likewise, the addition of basic residues, particularly arginine, to the early mature region of precursor proteins has been shown to have a similar adverse effect on export efficiency (21, 36, 45). In the case of integral membrane proteins, it has been found that the normal membrane topology can be altered by changing the charge distribution flanking hydrophobic domains (7, 27). These studies have been reviewed recently (8).

In addition to requiring the proper charge balance to orient the insertion of the signal peptide into the membrane, translocation of a precursor protein across the cytoplasmic membrane requires a specific interaction between the signal peptide and components of the cellular export machinery. This was initially suggested by the isolation of *prl* extragenic suppressor mutations, selected for their ability to restore export of proteins synthesized with a defective signal peptide (13, 32) and, more recently, by biochemical studies (1, 17, 22). The majority of suppressor mutations map to the *prlA* (*secY*) gene, encoding an essential, 49-kDa integral cytoplasmic membrane protein (5). Recent evidence suggests that PrlA/SecY is involved in mediating the actual translocation event (6). Suppressor mutations were also obtained in a locus designated *prlD* (32). These were subsequently demonstrated to be alleles of the *secA* gene (15), encoding an essential 101-kDa protein (28) peripherally associated with the cytoplasmic membrane by its affinity for acidic phospholipids, PrlA/SecY, and a second integral membrane protein, PrlG/SecE (17, 22). PrlD/SecA is the translocation ATPase that requires precursor proteins for maximum enzyme activity.

All of the *prlA* and *prlD* alleles characterized to date were selected by their ability to restore export of mutant LamB or maltose-binding protein (MBP) species with alterations in the hydrophobic core regions of their respective signal

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peptides (13, 32). In this study, site-specific mutagenesis has been used to construct various MBP species rendered export defective by an altered distribution of charged amino acid residues flanking the intact hydrophobic core and signal peptidase processing site regions. The export of these mutant proteins in *E. coli* cells harboring previously characterized *prlA* and *prlD* suppressor alleles has been investigated. In addition, Mal^+ revertants harboring extragenic suppressor mutations that restore MBP export have been isolated. The results clearly demonstrate that the PrlA/SecY protein can be altered to accommodate export of such mutant MBP species, suggesting that the positive-inside rule is at least partly determined by the specific interaction of exported proteins with the cell's secretion machinery in the cytoplasmic membrane.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* BAR1091, a derivative of MC4100, has been described previously (31). It harbors the *malEΔ312* mutation, an in-frame, nonpolar deletion that removes DNA sequences encoding residues 15 of the MBP signal peptide through 159 of the mature moiety. Strain DHB4 (7) is a Δ*phoA* derivative of MC4100 provided by Dana Boyd of Harvard Medical School. Strain ROB1 (Δ*rbs-7*) is also an MC4100 derivative that has been described previously (11). Isogenic derivatives of BAR1091, DHB4, and ROB1 harboring various *prl* alleles were constructed by P1 transduction. The *lacUV5 malE* plasmid pJF2 and derivatives pTL3 and pTL8 have been described elsewhere (14, 30). Plasmids encoding various ribose-binding protein (RBP)-MBP and MBP-RBP hybrid proteins have been described previously (11). Derivatives of plasmid pRJK10, encoding UhpT-PhoA hybrid proteins 57, 141, and 302 (23), were provided by Robert Kadner of the University of Virginia.

Reagents. Minimal medium M63 supplemented with a carbon source (0.2%) and thiamine (2 μg/ml) and TYE agar were prepared as described previously (25). When required, ampicillin was added to minimal and complex media at concentrations of 25 and 50 μg/ml, respectively. 5-Bromo-4-chloro-3-indolyl-phosphate (XP) (Sigma Chemical Co., St. Louis, Mo.) was used in agar plates at a concentration of 40 μg/ml. To induce *malE* genes under *lacUV5* promoter-operator control (31), isopropyl-β-D-thiogalactoside (IPTG) was used on agar plates and in liquid media at concentrations of 1 and 5 mM, respectively. [^{35}S]methionine (Expre^{35}s^{35}s) was obtained from New England Nuclear, Wilmington, Del. Rabbit anti-MBP and anti-RBP sera were described previously (11). Electrophoresis reagents were purchased from Life Technologies, Inc., Gaithersburg, Md. DNA-modifying enzymes were purchased from Life Technologies, Boehringer Mannheim Biochemicals, Indianapolis, Ind., or Promega, Madison, Wis. XAR film was purchased from Eastman Kodak Co., Rochester, N.Y.

Oligonucleotide-directed mutagenesis. To construct derivatives of plasmid pJF2 (encoding wild-type MBP), pTL3 (encoding MBP with a net charge of 0 in the hydrophilic segment of the signal peptide), and pTL8 (encoding MBP with a net charge of −3 in the hydrophilic segment), oligonucleotide-directed mutagenesis was performed as described before (46), except that a single mutagenic primer was used and the primer was extended in vitro at 0°C for 5 min, then at room temperature for 5 min, and finally at 37°C for 2 h. Mutagenic primers were prepared with an Applied Biosystems 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis (PAGE) as described by Hutchison et al. (18). Plasmids packaged as M13 particles were prepared as described previously (37). Mutations were confirmed by DNA sequencing as described by Bankier et al. (4).

Selection for Mal^+ revertants. Twelve independent cultures of BAR1091 cells synthesizing plasmid-encoded MBP−3/+3 were grown to late log phase in glycerol minimal medium supplemented with ampicillin. Cells were pelleted, washed once with M63 medium lacking carbon source, and resuspended in 0.5 volume of the same medium. Aliquots were then spread on maltose minimal agar plates containing ampicillin and IPTG (isopropylthiogalactopyranoside). Mal^+ revertants were obtained as colonies following 24 to 36 h of incubation at 37°C.

Amplification and cloning of *prlA* alleles. DNA sequences harboring the entire *prlA666* or *prlA*$^+$ gene were amplified from *E. coli* genomic DNA by the polymerase chain reaction (PCR) (26). Crude extracts containing genomic DNA were prepared as follows. Several colonies of the appropriate bacterial strain were suspended in 1.5 ml of L broth in an Eppendorf tube and centrifuged for 5 min. The supernatant was discarded, and the pelleted cells were resuspended in 0.5 ml of lysis buffer, containing 10 mM Tris (pH 8.0), 10 mM EDTA, 10 mM dithiothreitol, and 0.5% sodium dodecyl sulfate (SDS). Proteinase K (Boehringer Mannheim) was added to 150 μg/ml, and the lysate was incubated at 37°C for 1 h. The lysate was extracted twice with an equal volume of phenol-chloroform (1:1), and the genomic DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol. The DNA precipitate was pelleted by centrifugation for 30 min, washed once with 70% ethanol, and then resuspended in 50 μl of 10 mM Tris (pH 8.0)–0.1 mM EDTA overnight at 4°C. Then 1 μl of this DNA solution was used for amplification of *prlA* genes by PCR as described by Saiki (33).

Oligonucleotide primers 28 bases in length were synthesized as described above. The primers were designed so that the 5′ ends contained 4 bases of noncomplementary sequence preceding a 6-base *Bam*HI recognition sequence, followed by 18 bases of sequence complementary to *prlA*. Amplifications were carried out for 35 cycles (1 s at 94°C, 1 min at 50°C, and 2 min at 72°C) in a Perkin Elmer Cetus thermal cycler (Perkin Elmer Cetus, Norwalk, Conn.). Following amplification, a sample of the reaction mix was treated with *Bam*HI and resolved by electrophoresis in a 1% low-gelling-temperature agarose gel (FMC, Rockland, Maine). Gel slices containing DNA fragments harboring the *prlA* genes were excised, and the DNA was purified by adsorption to Geneclean silica beads (Bio 101, La Jolla, Calif.). The purified fragments were ligated into *Bam*HI-digested pBS+ (Stratagene, La Jolla, Calif.), and the plasmid DNA was transformed into competent cells of JM109. Single-stranded plasmid DNA was prepared from Lac^- colonies and subjected to DNA sequence analysis with *prlA*-specific oligonucleotide primers.

Radiolabeling, immunoprecipitation, SDS-PAGE, and autoradiography. Cultures were grown to mid-log phase in glycerol minimal medium supplemented with ampicillin and induced for synthesis of MBP, RBP, or hybrid proteins by the addition of IPTG to the culture medium. After 45 min, cells were labeled with [^{35}S]methionine for 15 s. Chase periods were initiated and terminated as described previously (32). For other experiments, cultures were radiolabeled for 10 min with [^{35}S]methionine. Radiolabeled proteins were immunoprecipitated with the appropriate antisera from solubilized cell extracts by procedures described previously

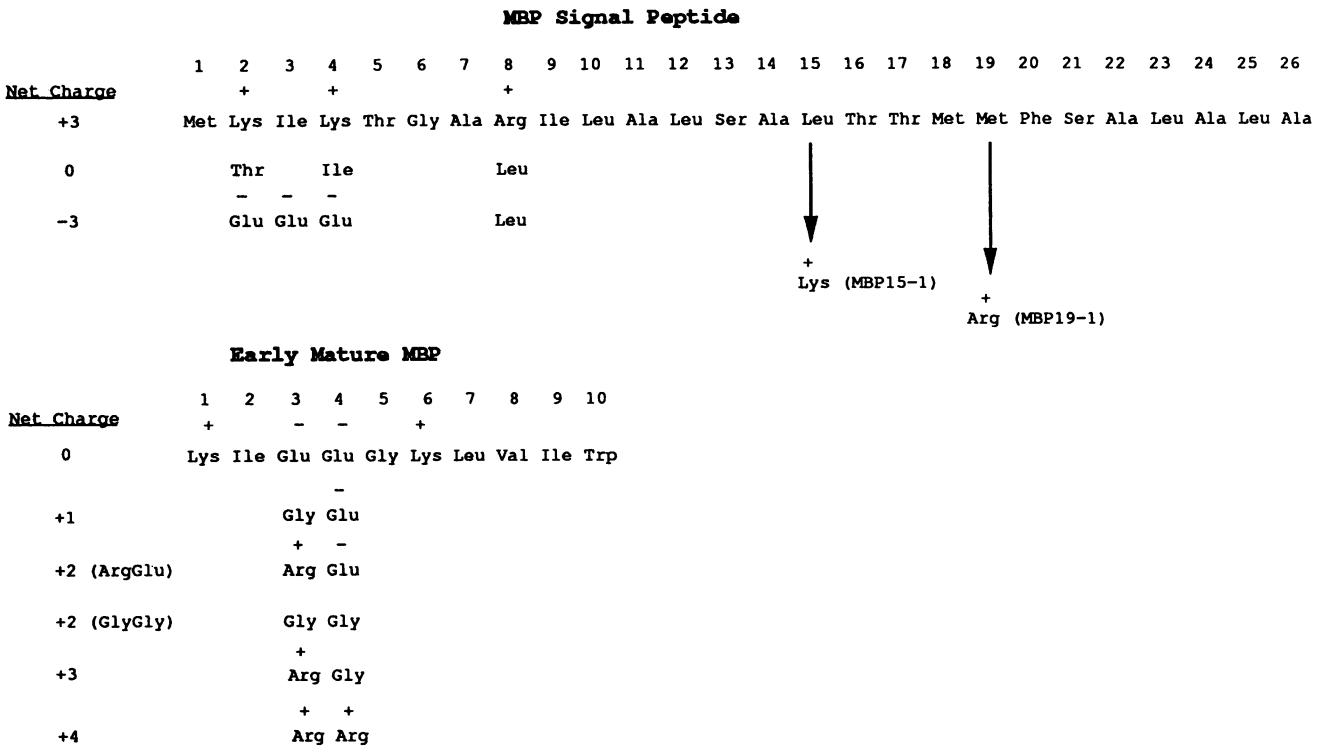

FIG. 1. Primary amino acid sequence of the MBP signal peptide and early mature region. Basic (+) and acidic (−) residues are indicated. Derivatives of wild-type MBP with alterations introduced by oligonucleotide-directed mutagenesis (see Materials and Methods) are indicated below the wild-type sequence shown on the top line. For both the hydrophilic segment of the signal peptide and the early mature region, the net charge of each wild-type or mutant sequence is indicated at left. The MBP species used in this study were designated on the basis of the net charge exhibited by these two regions, with the net charge of the signal peptide preceding that of the early mature region. For example, by this scheme, wild-type MBP is designated MBP+3/0. Note that a plasmid encoding MBP with a wild-type signal peptide and a net charge of +3 in the early mature region was not constructed.

(32). Immunoprecipitates were resolved by SDS-PAGE and autoradiography, also as described previously (32). For quantitation of protein export, an Ambis radioanalytic imaging system was used (AMBIS Systems, San Diego, Calif.). Counts were adjusted for the loss of methionine residues when precursor proteins were processed to their mature forms.

Enzyme assay. Alkaline phosphatase (PhoA) enzyme activity was determined by a modification of the method described by Brickman and Beckwith (9). Substrate was added to a final concentration of 15 mM, and the increase in A_{420} was measured over time with a Guilford recording spectrophotometer. PhoA activity was determined by the following formula: 1,000 × (change in OD_{420} per min/OD_{600}) × dilution factor.

RESULTS

Mutational alterations generating a net negative charge in the early mature region of pre-MBP. The early mature region (first 10 residues) of pre-MBP exhibits an overall neutral charge owing to the presence of two basic Lys residues at positions 1 and 6 and two acidic Glu residues at positions 3 and 4 (Fig. 1). If the latter Glu residues were changed to neutral or basic residues, then this region would exhibit a net positive charge. To this end, in vitro oligonucleotide-directed mutagenesis was used to substitute Arg or Gly codons for the appropriate Glu codons in the wild-type *malE* gene on plasmid pJF2 (14) (see Materials and Methods). Plasmids encoding four different mutant MBP species were obtained in this manner; the alterations in the early mature moiety of pre-MBP are shown in Fig. 1.

In previous studies, the processing of pre-MBP to mature MBP has correlated well with MBP export (e.g., see references 10, 11, 14, 30, 31, and 32). The rate of processing of wild-type MBP and mutant species with alterations at residues 3 and/or 4 of the mature moiety was determined by pulse-chase analysis. Cells were pulse-labeled for 15 s with [^{35}S]methionine and then incubated in a chase solution containing unlabeled methionine. The chase was terminated after 1 or 10 min, and the labeled MBP species present at each chase point were analyzed by immunoprecipitation, SDS-PAGE, and autoradiography (see Materials and Methods) (Fig. 2). As expected, wild-type MBP was exported very rapidly, with very little pre-MBP detectable at the 1-min chase point and no pre-MBP discernible after 10 min of chase. It was observed that, as the net positive charge of the early mature MBP moiety increased, MBP export efficiency progressively decreased. Indeed, when Arg replaced both of the Glu residues at positions 3 and 4, the resultant MBP species, designated MBP+3/+4 (see legend to Fig. 1), was found to be strongly export defective, so that mature MBP was just barely detectable at the 10-min chase point. Cells expressing MBP+3/+4 displayed a strongly Mal$^-$ phenotype on maltose tetrazolium agar but could grow slowly on maltose minimal medium, indicative of export of a very small amount of MBP to the periplasm.

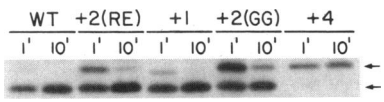

FIG. 2. Export of wild-type MBP and mutant species with alterations in the early mature region. Cells of strain BAR1091 harboring various plasmids were pulse-radiolabeled for 15 s with [^{35}S]methionine and chased with excess unlabeled methionine. After either 1 or 10 min of chase, equal portions were removed, the chase was terminated, and the MBP was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Only the relevant portion of the autoradiograph is shown. Positions of pre-MBP and mature MBP are indicated by the upper and lower arrows, respectively. For each MBP species, the net charge of the early mature region is indicated at the top (see Fig. 1). WT indicates wild-type MBP having a net charge of 0 in this region.

Alterations in the hydrophilic segment of the signal peptide can exacerbate the effect of alterations in the early mature region of the MBP. A previous study had shown that, as the net charge in the hydrophilic segment at the amino terminus of the MBP signal peptide progressively decreased below +1, MBP export efficiency decreased in a similarly progressive manner (30). The effects that such substitutions would have on MBP export when placed in *cis* to an altered early mature MBP moiety exhibiting a net positive charge were investigated in this study. A series of plasmids encoding MBP species with three different signal peptides (+3 [wild type], 0, or −3 net charge in the hydrophilic segment) and two different early mature moieties (0 [wild type] or +2 [GG] net charge) were constructed (see Materials and Methods), and MBP export efficiency was analyzed as above. As shown in Fig. 3, both MBP+3/+2 (wild-type signal peptide and a net charge of +2 in the early mature region) and MBP0/0 (mutant signal peptide without any charged residues in the hydrophilic segment and a wild-type mature moiety) both exhibit an obvious export defect; however, the great majority of both species was found to be processed after 10 min of chase. In contrast, MBP0/+2, with the same charge alterations in both the signal peptide and early mature region, was found to be exported at a greatly decreased efficiency; after 10 min of chase, greater than 60% of the MBP remained in its unprocessed form. MBP−3/0 was somewhat less export competent than MBP0/0 but was still more than 60% processed by 10 min of chase. However, MBP−3/+2 exhibited a very severe export defect, so that mature MBP was undetectable at the 10-min chase point. These results revealed that changes in one region of pre-MBP could exacerbate the export defect resulting from changes in the other region, well beyond that expected if the effects were simply additive.

Extragenic suppressor mutations that restore export of MBP−3/+3. It was of interest to determine whether Mal$^+$ revertants of cells synthesizing MBP species rendered export defective by major charge alterations in the signal peptide hydrophilic segment and the early mature region would yield new classes of *prl* extragenic suppressor mutations. Cells synthesizing MBP−3/+2 were still capable of growing on maltose minimal medium, albeit slowly, indicating that some small amount of MBP was exported to the periplasm. However, an MBP species with a net charge of −3 in the hydrophilic segment and +3 in the early mature region, designated MBP−3/+3 (Fig. 1), was found to be sufficiently export defective that cells of strain BAR1091 synthesizing MBP−3/+3 were unable to utilize maltose as a sole carbon source (data not presented). This facilitated direct selection for Mal$^+$ revertants (see Materials and Methods). Twelve independently obtained Mal$^+$ isolates were characterized initially. When growth on maltose clearly resulted from restoration of MBP export due to an extragenic (i.e., non-*malE*-linked) suppressor mutation, bacteriophage P1-mediated cotransduction with *rpsE* strongly indicated that the suppressor mutations mapped to the *prlA* locus (data not presented).

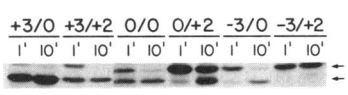

FIG. 3. Export of MBP species with charge alterations in both the signal peptide and early mature region. Cells of strain BAR1091 harboring various plasmids were pulse-radiolabeled, and the MBP was immunoprecipitated and analyzed as described in the legend to Fig. 2. The net charge of the hydrophilic segment and early mature region for each MBP species is indicated at top (see Fig. 1). Positions of pre-MBP and mature MBP are indicated by the upper and lower arrows, respectively.

Amplification of *prlA* genes by PCR and DNA sequence analysis. To identify the *prlA* mutations responsible for the suppressor phenotype, the DNA sequences encoding the entire *prlA* gene from the parental strain and the two strongest Mal$^+$ revertants (as determined by growth on maltose minimal medium and restoration of pre-MBP processing) were amplified by PCR (see Materials and Methods). Multiple PCR amplifications were performed with duplicate samples to minimize the chances of identifying mutations introduced by *Taq* polymerase error. PCR primers were designed to incorporate *Bam*HI restriction sites at both ends of the amplified fragment. Following their isolation by agarose gel electrophoresis, amplified DNA fragments were digested with *Bam*HI and cloned into the multipurpose plasmid vector pBS+ (Stratagene). Plasmid DNA was transformed into competent cells of strain JM109, and several clones of the wild-type *prlA* allele and each mutant allele were chosen for DNA sequencing. Analysis of the entire *prlA* reading frame of each clone revealed that both suppressor alleles harbored the identical base change. This mutation changed codon 67 from TTC (Phe) to TCC (Ser) and was designated *prlA666*. Note that a previously identified mutation, designated *prlA3*, changes codon 67 to TGC (Cys) (34).

Plasmid pBS+ harboring either the amplified *prlA*$^+$ or *prlA666* allele was introduced into *E. coli* cells expressing MBP15-1, a strongly export-defective MBP species with an alteration in the hydrophobic core (16) (Fig. 1). A Mal$^+$ phenotype was observed for cells harboring the *prlA666* plasmid but not the *prlA*$^+$ plasmid (data not presented). Since the two cloned *prlA* genes differed only by the single nucleotide change in codon 67, this result clearly established that this point mutation was responsible for the suppressor phenotype exhibited by *prlA666* cells.

***prlA666* mediates suppression of a broad range of MBP export defects.** The suppressor activity of *prlA666* was compared with that of three other well-characterized *prlA* alleles, *prlA3*, *prlA4*, and *prlA402* (13, 32, 34), using cells synthesizing a variety of export-defective MBP species. The results with four different MBP species are shown in Fig. 4. In the case of MBP19-1, having an altered hydrophobic core (Fig. 1), the efficiency of MBP export to the periplasm, as indicated by the ratio of pre-MBP to mature MBP precipitated, was noticeably improved in cells harboring any one of the four *prlA* alleles tested. However, MBP19-1 export clearly was most efficient in *prlA666* cells. The most efficient export

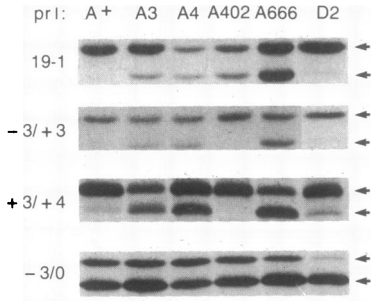

FIG. 4. Export of four different mutant MBP species in cells harboring different *prl* suppressor alleles. Cells were radiolabeled for 10 min with [^{35}S]methionine (5 min for the MBP-3/0 species), and the MBP was immunoprecipitated and analyzed as described in the legend to Fig. 2. The MBP species analyzed in each panel is indicated at left. The relevant *prl* allele is indicated above each lane. Positions of pre-MBP and mMBP are indicated by the upper and lower arrows for each autoradiograph, respectively. Because of differences in the amounts of each mutant protein synthesized (30), exposure times for the various autoradiographs shown were not equivalent. See text for additional experimental details.

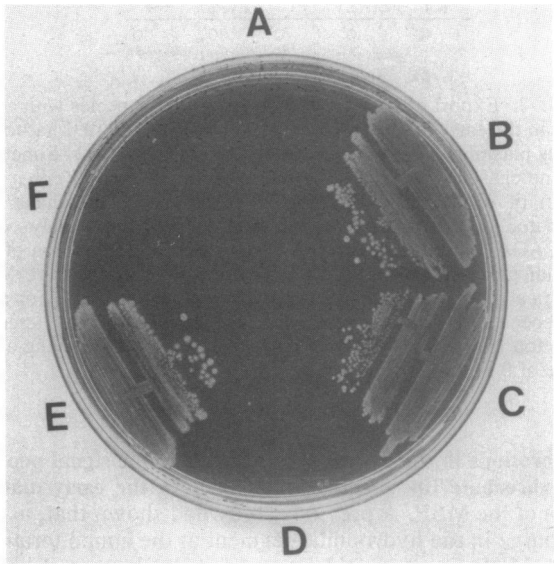

FIG. 5. Growth on maltose minimal agar of isogenic derivatives of strain BAR1091 harboring various *prlA* alleles and plasmid pUZ226 encoding MBPΔ2-26. The plate was incubated for 48 h at 37°C. Sector: A, *prlA*$^+$; B, *prlA3*; C, *prlA4*; D, *prlA402*; E, *prlA666*; F, *prlA666* with no plasmid.

of MBP-3/+3 export also was achieved in *prlA666* cells. MBP-3/+3 export clearly was less efficient in both *prlA3* and *prlA4* cells and was undetectable in cells harboring the *prlA402* allele. For MBP+3/+4, similar results were obtained; export was most efficient in *prlA666* cells and undetectable in *prlA402* cells. In marked contrast, MBP-3/0 export was not improved in cells harboring any of the *prlA* alleles tested. In fact, as reported previously (30), MBP-3/0 export was even less efficient in *prlA4* cells than in *prlA*$^+$ cells. The results with the various *prlA* suppressor alleles and seven different mutant MBP species are summarized in Table 1.

It was shown previously that, in marked contrast to *prlA* alleles, *prlD* alleles exhibit the ability to efficiently suppress mutations that result in a net negative charge in the hydrophilic segment of the MBP signal peptide but are generally weak suppressors of *malE* mutations altering the hydrophobic core (15, 30, 32). As shown in Fig. 4 and Table 1, MBP-3/0 export was substantially improved in cells harboring *prlD2*, the most efficient of the *prlD* suppressor alleles (15), whereas MBP19-1 export was only very slightly improved. Export of MBP-3/+3 and MBP+3/+4 in *prlD2* cells was found to resemble that of MBP19-1 in that only marginal improvement in export efficiency could be discerned compared with that found in *prl*$^+$ cells.

TABLE 1. Restoration of MBP export in cells harboring *prl* suppressor alleles

MBP species	Suppression[a]				
	prlA3	*prlA4*	*prlA402*	*prlA666*	*prlD2*
MBP15-1	++	++	+++	++++	−
MBP19-1	++	+++	+++	++++	+/−
MBP-2/0	−	−[b]	−	−	+++
MBP-3/0	−	−[b]	−	−	+++
MBP+3/+4	++	++	−	++++	+
MBP-3/+3	++	++	−	+++	+/−
MBPΔ2-26	+	+	−	++	−

[a] Suppression was scored by observing changes in MBP export efficiency by pulse-chase analysis, changes in ability to promote growth on maltose minimal agar, or both. Scores are relative to export observed for same MBP species in isogenic *prl*$^+$ cells. Symbols: −, no suppression; +/−, very weak suppression detected; ++++, the strongest suppression detected.

[b] Export of MBP-2/0 and MBP-3/0 was actually less efficient in *prlA4* cells than in *prl*$^+$ cells, as reported previously (30).

MBPΔ2-26 is an MBP species deleted for its entire signal peptide except for the initiating methionine residue (43). It recently was found that MBPΔ2-26 and an analogous signal peptide-less PhoA species can be exported to the periplasm, albeit somewhat inefficiently, in cells harboring *prlA4* (12). Thus, the ability of *prlA666* and other *prl* alleles to promote the export of MBPΔ2-26 was investigated, using growth on maltose minimal medium as a qualitative assay for MBP export. Cells synthesizing MBPΔ2-26 and harboring *prlA3*, *prlA4*, or *prlA666* but not *prlA*$^+$ or *prlA402* alleles clearly were capable of using maltose as a carbon source (Fig. 5). Colony formation by *prlA666* cells was most rapid. Cells harboring any *prlA* suppressor allele but not synthesizing an MBP species were totally Mal$^-$, demonstrating that growth on maltose minimal medium is dependent on export of functional MBP. Note that *prlD2* cells synthesizing MBPΔ2-26 also were unable to grow on maltose minimal medium (Table 1).

PrlA666 can alter the enzymatic activity of PhoA fused to an integral cytoplasmic membrane protein. It was of interest to determine whether PrlA666 could influence the transmembrane orientation of the hydrophobic domains of an integral cytoplasmic membrane protein, since such orientation is thought to be largely determined by the distribution of basic amino acids flanking the membrane-spanning stretches. A commonly employed method to determine the topology of integral bacterial membrane proteins has been the use of PhoA fusions (recently reviewed in reference 24). When PhoA is fused to periplasmic domains, it exhibits higher enzymatic activities than when it is fused to cytoplasmic domains of the same membrane protein. Lloyd and Kadner (23) recently used PhoA fusions to help determine the topology of the UhpT protein in the *E. coli* cytoplasmic membrane. In this study, the enzymatic activity of three

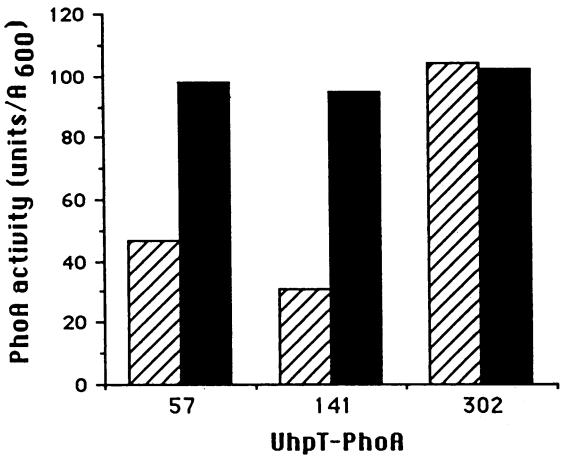

FIG. 6. PhoA enzymatic activities for isogenic *prl*$^+$ (hatched bars) and *prlA666* (solid bars) cells synthesizing different UhpT-PhoA hybrid proteins. PhoA activity was determined for cells grown in TYE medium at 37°C to mid-log phase. See Materials and Methods for details.

different UhpT-PhoA hybrid proteins in isogenic *prl*$^+$ and *prlA666* cells was determined (see Materials and Methods). As shown in Fig. 6, the hybrid protein designated 302, exhibiting the highest activity in *prl*$^+$ cells (i.e., PhoA domain in periplasm), did not significantly change in activity when expressed in *prlA666* cells. In marked contrast, the two hybrid proteins exhibiting the lowest activity in *prl*$^+$ cells (i.e., PhoA domain in cytoplasm), designated 57 and 141, were found to substantially increase in activity when expressed in *prlA666* cells, so that their activity was nearly equivalent to that of hybrid protein 302. The PhoA activities measured for these fusion strains grown in liquid medium correlated very well with the intensity of blue color when grown on TYE-XP agar plates (data not shown). Thus, PrlA666 was substantially better than wild-type PrlA in facilitating export of the PhoA domain of hybrid proteins 57 and 141 across the cytoplasmic membrane.

Suppression of RBP signal sequence mutations by *prlA666*. Export-defective RBP species with three different mutational alterations in the signal peptide hydrophobic core region were recently characterized (11). It was found that, in contrast to analogous export-defective MBP species, export was not substantially improved in cells harboring *prlA402*. This was true whether or not the RBP signal peptide was attached to mature RBP or mature MBP (designated RBP-MBP). Since *prlA666* was found to exhibit very strong suppression of a variety of *malE* signal sequence mutations, the export of the same RBP and RBP-MBP species in cells harboring *prlA666* was investigated. The results are presented graphically in Fig. 7. Analogous to previous findings (11), the export of proteins with an export-defective RBP signal peptide was significantly less efficient in *prlA666* cells than that of proteins with a similarly altered MBP signal peptide. For example, both the RBP15-1 and MBP15-1 signal peptides have a charged amino acid substituted for a leucine at residue 15 and both result in a total export defect in *prlA*$^+$ cells (Fig. 7E). Note that MBP15-1 and MBP15-1-RBP were exported with much greater efficiency in *prlA666* cells than either RBP15-1 or RBP15-1MBP (Fig. 7F). Of proteins with a mutant RBP signal peptide, RBP12-1 and RBP12-1-MBP were exported with the greatest efficiency in *prlA666* cells (Fig. 7D). However, the relative increase in export efficiency in *prlA666* cells compared with *prl*$^+$ cells was decidedly less than that observed for each of the proteins with mutant MBP signal peptides.

It was anticipated that a direct selection for extragenic suppressors of mutations affecting the RBP signal peptide might yield a new class of *prl* mutations. Cells synthesizing RBP15-1-MBP are unable to utilize maltose as a sole carbon source. A number of Mal$^+$ revertants were obtained that harbored extragenic suppressor mutations, all of which were closely linked to the *prlA* locus. The mutant allele exhibiting the strongest suppressor activity was amplified by PCR as described above, and the DNA sequence was determined. Interestingly, analysis of the entire *prlA* reading frame revealed that this allele harbored the identical base change previously identified for *prlA666*.

DISCUSSION

Introduction of a net positive charge into the early mature moiety of pre-MBP was found to have an adverse effect on the efficiency of MBP export to the periplasm. An MBP species with an overall net charge of +1 in this region exhibited a minor export defect. Export efficiency progressively decreased as the net charge was systematically increased to +4. Export of the MBP with a net charge of +4 was extremely inefficient and was detectable only by the ability of the mutant protein to promote slow growth of cells on maltose minimal medium. These results are entirely consistent with the positive-inside rule (41) and previous studies demonstrating the adverse effects of basic residues in the early mature region on the export of various *E. coli* envelope proteins (21, 36, 45). A net positive charge just following the MBP signal peptide presumably interferes with the ability of this structure to insert properly into the membrane, with the amino terminus in the cytoplasm and the cleavage site exposed on the periplasmic surface. This orientation originally was proposed in the loop model (20), is well supported by a variety of experimental evidence, and is considered crucial to the initiation of the protein translocation process (29). It is not exactly clear why basic residues immediately following the signal peptide have this adverse effect; several explanations have been offered (recently reviewed in reference 8).

Reducing the net charge at the amino terminus of the signal peptide to below +1 was shown previously to adversely affect MBP export, although not as severely as charge changes in the early mature region. For example, when the charge in the signal peptide was reduced from +3 to −3, more than half of the MBP was still exported (30). In this study, the export of MBP species with charge alterations in both the signal peptide and early mature region was investigated. The results indicated that the net effect of charge changes in both regions was significantly greater than the sum of the individual effects. For example, MBP−3/+2 exhibited a nearly total export defect, whereas greater than 50% of MBP−3/0 and 90% of MBP+3/+2 was exported to the periplasm (Fig. 3). For MBP−3/+2 and MBP−3/+3, the reversal of the charge polarity flanking the hydrophobic core may have caused the signal peptide to insert into the membrane opposite to its normal orientation. The end result could be that pre-MBP was converted from a protein targeted to the periplasm to a type I bitopic integral membrane protein, with its amino terminus in the periplasm and the signal peptide serving as an anchor sequence (29). This would be analogous to the recent finding that reversal of the normal distribution of basic residues in the *E. coli* Lep

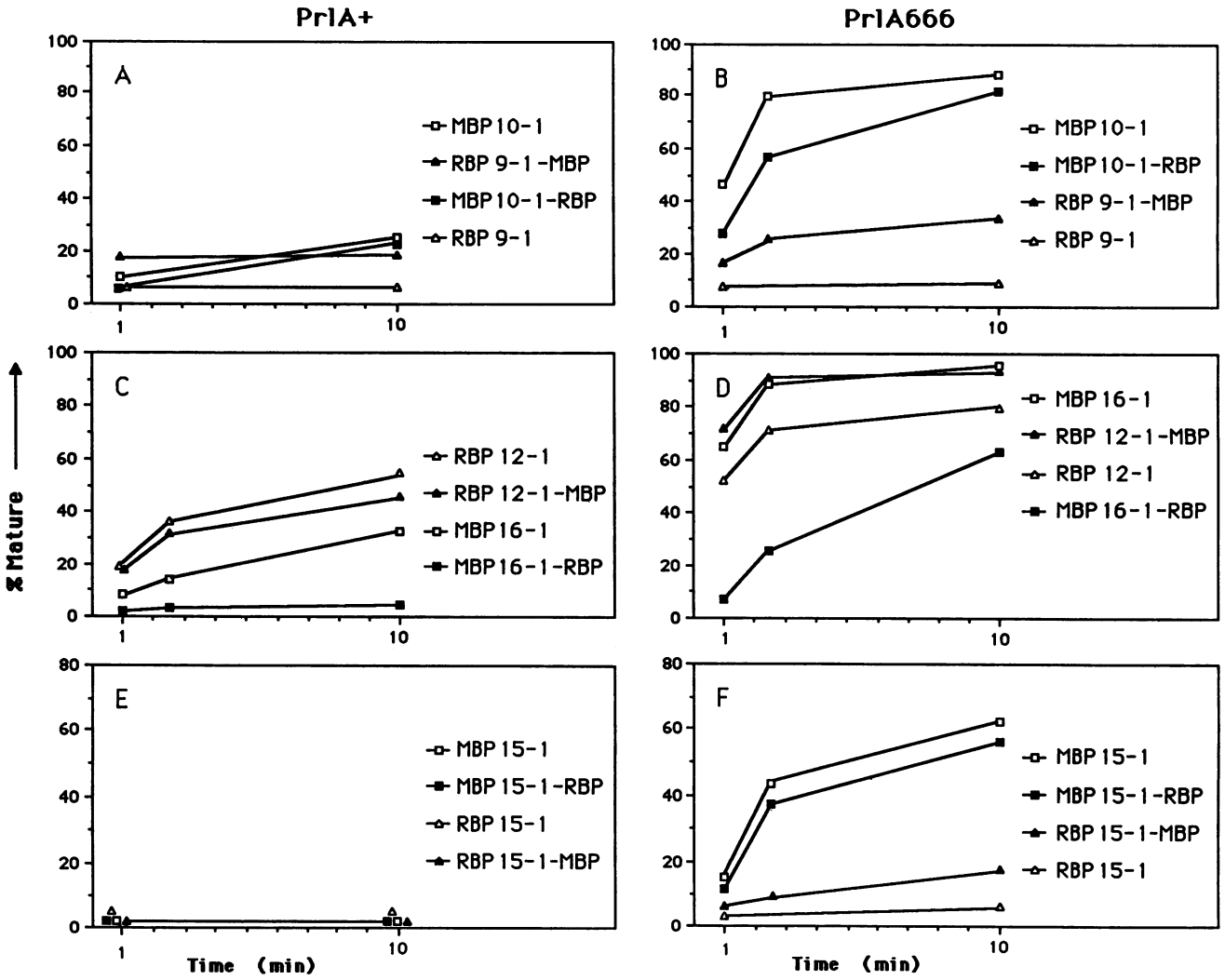

FIG. 7. Export kinetics of various RBP, MBP, and hybrid protein species in *prlA*$^+$ and *prlA666* cells. Experimental conditions were as described in the legend to Fig. 2 except that cells of strain ROB1 or ROB1 *prlA666* were used when appropriate. The results of these experiments are presented graphically. Each chase point represents the percentage of total radiolabeled protein precipitated in its mature form (see Materials and Methods). The mutant RBP and MBP signal peptides are described in reference 11.

protein results in a protein with an inverted membrane topology (27, 42).

Once we had generated MBP species with charge alterations flanking the signal peptide hydrophobic core, the major goals of this study were to analyze their export in cells harboring previously characterized *prlA* and *prlD* suppressor mutations and to determine whether direct selection for mutations that improve the export of these mutant proteins would identify new *prl* genes or unique alleles of known *prl* loci. In this way, cellular components that specifically interact with the early mature region of pre-MBP might be identified. This work was prompted in part by an earlier study which revealed that export of MBP species with a net charge of −2 or −3 in the hydrophilic segment exhibited no or only marginal improvement in cells harboring *prlA* alleles but was markedly improved in cells harboring *prlD2*. It was suggested that the integral membrane protein PrlA/SecY interacts more strongly with the signal peptide hydrophobic core and the peripheral membrane protein PrlD/SecA interacts more strongly with the positively charged hydrophilic segment (30). Additional support for the latter was provided more recently by biochemical studies on the interaction of purified PrlD/SecA protein with OmpF-Lpp (1).

In this study, export of MBP species with a net positive charge in the early mature moiety showed little improvement in *prlD2* cells but was improved significantly in cells harboring *prlA3*, *prlA4*, or *prlA666*. Thus, the suppression activity conferred by these *prlA* mutations is not limited to precursor proteins with altered hydrophobic core structures. These results illustrate that the positive-inside rule is not absolute. There are other known exceptions to this rule (8) which likely depend, as is the case with mutant MBP species interacting with mutant PrlA proteins, on exactly how these proteins interact with the export machinery in the cytoplasmic membrane. With regard to suppressor-mediated export of these mutant MBP species, it is presumed that the mutant PrlA/SecY protein is promoting insertion of the signal peptide into the cytoplasmic membrane in the same orientation as the wild-type MBP signal peptide, since both MBP translocation and processing are restored.

MBP−3/+3 export was also substantially improved in cells harboring *prlA* suppressor mutations (except *prlA402*). In this case, it was probably the export defect specifically resulting from the net positive charge in the early mature MBP that was suppressed. (Note that the export efficiency of MBP−3/+3 in *prlA* cells is still less than that exhibited by MBP−3/0.) The finding that the same *prlA* mutations exhibit little ability to restore export of MBP species with a net negative charge in the hydrophilic segment further suggests that PrlA/SecY interacts primarily with the hydrophobic core and, in addition, with residues in the early mature moiety of pre-MBP. This should not be considered surprising since, according to the loop model (20), the early mature region is inserted into the membrane along with the signal peptide hydrophobic core and cleavage site to initiate translocation (8, 29, 36).

The results obtained with *prlA402* cells were somewhat intriguing. This allele was originally characterized as an unusually strong suppressor of mutations altering the hydrophobic core of the MBP signal peptide (32). It now appears that the suppression activity of *prlA402* is very specific for hydrophobic core alterations, since other types of export-defective MBP species do not show any improvement in export in *prlA402* cells. Thus, although its sequence has not yet been determined, *prlA402* may help to define a region of the PrlA/SecY protein that interacts very specifically with the hydrophobic core. This is being investigated further.

Analysis of the new *prlA* suppressor allele, designated *prlA666*, obtained in this study yielded several interesting results. First, it was found to be generally stronger than any other *prl* allele in restoring export of MBP species with alterations in the hydrophobic core or early mature region. Second, sequence analysis revealed that *prlA666* substitutes Ser for Phe at residue 67 of PrlA/SecY. A somewhat weaker suppressor allele, *prlA3*, previously had been shown to encode a different substitution, Cys, at the same position (34). According to the topological model for PrlA/SecY proposed by Akiyama and Ito (2), residue 67 is located in the first periplasmic domain of this cytoplasmic membrane protein, containing 10 hydrophobic transmembrane regions (2, 3). The studies of Bieker and Silhavy (6) and Hartl et al. (17) indicate that PrlA/SecY functions at a relatively late step in the export pathway. Assuming that the topology of PrlA/SecY has been correctly deciphered, the finding that an alteration in the periplasmic domain partially restores MBP−3/+3 export suggests that it must still partially navigate the export pathway in order to interact with mutant PrlA protein. Alternatively, suppressor mutations such as *prlA666* may modify the interaction between PrlA/SecY and other components of the export machinery, e.g., PrlD/SecA and PrlG/SecE, and somehow influence how those proteins recognize export-defective precursor proteins.

Understanding the mechanism of PrlA-mediated suppression is made even more complicated by the finding that MBPΔ2-26 can be exported by cells harboring *prlA666* and certain other *prlA* suppressor mutations. Recognition and entry of MBPΔ2-26 into the export machinery is probably facilitated by the cytoplasmic chaperone SecB and by SecA, both of which have affinity for the mature domains of precursor proteins as well as for each other (10, 17, 22, 43). Export of MBPΔ2-26 in *prlA* mutants is known to be strictly SecB dependent (12). These results suggest that *prl* suppressor mutations function by allowing those proteins that already interact with the cytoplasmic components of the export apparatus by virtue of the nature of their mature moieties to proceed all the way through the export pathway despite the lack of a functional signal peptide. Thus, although it is most tempting to believe that *prlA* suppressor mutations restore a specific interaction between the Prl/Sec machinery and export-defective precursor proteins, as suggested above, the lack of true allele specificity in the suppression pattern (5) and the finding that such mutations can bypass the requirement for any signal peptide whatsoever (12) are not easily explained. This clearly requires additional investigation.

It was interesting to find that the enzymatic activity of two UhpT-PhoA hybrid proteins was significantly elevated in cells harboring *prlA666*. For fusion 141, PhoA is thought to be fused at the cytoplasmic end of the fourth membrane-spanning domain of UhpT. For fusion 57, PhoA was fused downstream of the first membrane-spanning domain within a predicted periplasmic loop of UhpT. The unexpectedly low enzymatic activity of this fusion in *prl*$^+$ cells was attributed to the presence of three charged residues (two basic, one acidic) immediately preceding the PhoA fusion joint (23). The results indicate that interaction of these UhpT-PhoA hybrid proteins with PrlA666 can reorient their hydrophobic domains in the cytoplasmic membrane, so that the PhoA domain is translocated across the membrane into the periplasm. The activity of PhoA fused to a predicted periplasmic region later in UhpT was not further elevated in *prlA666* cells, demonstrating that PrlA666 is not just stimulating PhoA activity through an effect other than promoting transfer across the membrane. Thus, these results suggest that PrlA can interact specifically with domains of integral cytoplasmic membrane proteins such as UhpT. Previous studies have indicated a role for PrlA/SecY in the biogenesis of at least certain *E. coli* integral membrane proteins (3, 16, 44). However, these results may also simply reflect an interaction of the export machinery with the mature PhoA domain, since export of PhoA synthesized without a signal peptide can occur in cells harboring certain *prlA* suppressor mutations (12).

The ability of various *prlA* mutations to suppress RBP signal sequence mutations was also investigated. As found previously (11), restoration of RBP export was generally inefficient compared with that of MBP species with very similar alterations in the signal peptide hydrophobic core. This was true even for cells harboring *prlA666*, the strongest *prlA* suppressor allele isolated to date. These results were somewhat surprising, since *prlA* alleles have been shown previously to efficiently suppress signal sequence mutations in genes encoding a variety of *E. coli* envelope proteins (for review, see reference 5). The poor suppression of RBP signal sequence mutations is not related to properties of the mature RBP moiety, since the same suppression pattern was obtained with export-defective RBP-MBP and MBP-RBP hybrid proteins. The strongest suppressor of RBP signal sequence mutations obtained by direct selection was fairly inefficient and proved to be identical to *prlA666*. Although it exhibits the same three functionally conserved regions typical of most procaryotic signal peptides (39), these results strongly suggest that the interaction of the RBP signal peptide with the export machinery may be unique.

Finally, the initial screen of mutants selected for the restoration of MBP−3/+3 export revealed that every extragenic mutation identified was strongly linked to the *prlA* locus. This does not totally rule out the possibility that such suppressor mutations can be obtained in other loci, since the great majority of extragenic suppressors of mutations altering the signal peptide hydrophobic core also reside in *prlA* (6). A more extensive analysis of Mal$^+$ revertants is under

way in an attempt to identify *prlA* suppressor mutations that restore export only of MBP species with alterations in the early mature region (analogous to *prlA402* and hydrophobic core alterations) as well as to determine whether mutations restoring efficient MBP−3/+3 export can occur in other genes, e.g., *prlD*, *prlG* (35), or other known or unknown loci.

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