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Proper Interaction Between at Least Two Components Is Required for Efficient Export of Proteins to the *Escherichia coli* Cell Envelope

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An *Escherichia coli* mutant carrying *ΔmalE12-18*, a 21-base pair deletion confined to the coding DNA of the maltose-binding protein signal peptide, is unable to export maltose-binding protein to the periplasm efficiently. Consequently, such a strain is defective for the utilization of maltose as a sole carbon source. We obtained 16 mutants harboring extragenic *ΔmalE12-18* suppressor mutations that exhibit partial restoration of export to the mutant maltose-binding protein. A genetic analysis of these extragenic suppressor mutations demonstrated that 15 map at *prlA*, at 72 min on the standard *E. coli* linkage map, and that 1 maps at a new locus, *prlD*, at 2.5 min on the linkage map. Our evidence indicates that the *prlA* and *prlD* gene products play an important role in the normal pathway for export of proteins to the cell envelope. Efficient execution of the secretory process requires that these *prl* gene products interact properly with each other so that a productive interaction of these gene products with the signal peptide also can occur. Our data suggest that proper assembly of a complex is required for efficient export of *E. coli* envelope proteins to their various extracytoplasmic compartments.

The export of proteins from their site of synthesis in the cytoplasm to the various extracytoplasmic compartments where they ultimately reside is a basic biological activity that is executed with remarkable fidelity. Rarely is an exported protein found to establish a permanent residence in more than one discrete subcellular compartment. Studies of secretion in eucaryotic systems have led to the formulation of the signal hypothesis to describe the initial steps in protein export (5–7). The salient features of this proposal include a direct role for the signal peptide in mediating the interaction of an exported protein with a cellular secretion machinery that facilitates cotranslational translocation of the protein across a membrane. The signal peptide is subsequently removed, an event termed processing or maturation, either before or shortly after completion of translation.

The localization of periplasmic and outer membrane proteins of *Escherichia coli* seems analagous to protein export in eucaryotic cells. A large body of experimental evidence has been gathered which indicates that the initial steps in the export of procaryotic proteins exhibit many features that are compatible with the signal hypothesis. These include the characterization of amino-terminal signal peptides that are structurally similar to their eucaryotic counterparts (22), the demonstration of cotranslational export and, in some cases, cotranslational processing (18, 19, 32, 33), and the observation that many procaryotic exported proteins are synthesized on membrane-bound polysomes (8, 29, 33). Studies of protein export in heterologous systems emphasize the high degree of conservation that is exhibited throughout the biological kingdom in the process of protein localization (14, 30, 34, 35).

Genetic studies in *E. coli* are beginning to identify the cellular components that are involved in protein export. Oliver et al. (25, 27) have isolated mutants which exhibit pleiotropic defects in protein export that identify two genetic loci, *secA* and *secB*. The biochemical properties of *secA* and *secB* (20) mutants strongly suggest a role for their gene products in facilitating the normal export of *E. coli* proteins. Other mutants have been isolated that are presumed to be altered for components of the normal cellular secretion machinery. These identify three loci, *prlA*, *prlB*, and *prlC* (11) and suggest that *E. coli* possesses a complex protein export machinery.

We describe here the isolation of additional mutants that are altered for components of a cellular secretion apparatus. The corresponding mutations include novel *prlA* alleles and an alteration in what appears to be a new locus (*prlD*) that encodes a component of *E. coli* secretion machinery. We present strong evidence for a direct interaction between the *prlA* and *prlD* gene products, a role for both of these products in the normal pathway of *E. coli* protein export, and a requirement for interaction between the *prlA* and *prlD* gene products for proper export of wild-type envelope proteins.

MATERIALS AND METHODS

Bacterial and phage strains, media, chemicals, and genetic techniques. The pertinent bacterial and phage strains used in this study are listed in Table 1. The complex media used included TYE and maltose-triphenyltetrazolium chloride (MalTTC) agar (24). The minimal base medium used was M63 (24) supplemented with the appropriate carbon sources and amino acids to final concentrations of 0.2% and 50 μg/ml, respectively. When required, kanamycin sulfate, streptomycin sulfate, tetracycline, and spectinomycin hydrochloride were added to concentrations of 30, 150, 20, and 200 μg/ml, respectively. All carbohydrates and antibiotics except spectinomycin hydrochloride were obtained from Sigma Chemical Co.; spectinomycin hydrochloride was purchased from The Upjohn Co. Amino acids were purchased from Fisher Scientific Co.; a uniformly ^{14}C-labeled amino acid mixture was purchased from ICN; and formalinized *Staphylococcus aureus* (IgGsorb) was obtained from The Enzyme Center, Inc. [^{35}S]methionine (translation grade; 1,110 Ci/mmol) was obtained from Amersham Corp. Electrophoresis chemicals were purchased from Bethesda Research Lab-

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oratories, Inc. Generalized transduction with phage P1*vir* and other standard genetic techniques have been described previously (24). The isolation of mutants suppressed for the Δ*malE12-18* export defect has also been described previously (2).

Construction of *malB prl* double-mutant strains. To introduce a *malE* or *lamB* signal sequence mutation into a *prl* strain, an extensive deletion of the *malB* region was introduced into the *prl* strain by cotransduction with a kanamycin resistance transposon, Tn*5*, which exhibits approximately 50% linkage to *malB*. This deletion, Δ*malB224*, encompasses the promotor-proximal portion of *malE*, including the entire coding region for the maltose-binding protein (MBP) signal peptide, and extends completely through the *malK-lamB* operon (31). Strains carrying Δ*malB224* are not able to transport maltose into the cell and are Mal$^-$. Transduction of such strains to Mal$^+$ with phage P1 lysates propagated on the appropriate *malE* or *lamB* signal sequence mutants required incorporation of the desired *malB* export-defective mutation into the genome of the recipient strain. Only Mal$^+$ transductants of the Δ*malB224 prl* recipient that had lost the linked Tn*5* by homologous recombination, thereby becoming Kan^s, were saved.

Construction of *prlA prlD* double-mutant strains. To introduce a *prlA* allele into a *prlD1* strain, the *prlD1* mutant was transduced to spectinomycin hydrochloride resistance (*rpsE*) with a phage P1 lysate propagated on strain S10. Spc^r transductants that scored Str^s were saved. These were transduced to Str^r (*rpsL*) with phage P1 lysates propagated on strains harboring the desired *prlA* alleles. The *prlA* locus is approximately 70% cotransducible with *rpsL* and more than 98% cotransducible with *rpsE* (11). As the gene order is *rpsL-rpsE-prlA*, Str^r transductants that acquired *rpsE*$^+$ (i.e., were Spc^s) almost certainly also acquired the *prlA* allele of interest. The acquisition of the desired *prlA* allele could be confirmed phenotypically and genetically, by transducing the suppressor allele, via cotransduction with *rpsL*, to a strain bearing an export-defective mutation whose phenotypic interaction with the suppressor is known.

Diploid analysis. The KLF4 (Ara$^+$ SecA$^+$ PrlD$^+$) episome was introduced into *recA56* strains RL567 (Δ*malE12-18 prlD1*), RL619 (*malE10-1 prlD1*), and RL620 (*malE16-1 prlD1*) by selecting for growth on Ara minimal agar after mating with donor strain AB2463/KLF4. Several exconjugants were purified and tested for sensitivity to the male-specific coliphage R17. The R17-sensitive exconjugants were streaked onto MalTTC agar beside isogenic *prlD*$^+$ strains also carrying KLF4, and the preparations were incubated for 24 h at 30°C. The phenotypic dominance or recessiveness of *prlD1* to *prlD*$^+$ was then scored by reaction on MalTTC agar.

Isolation of λ*secA*$^+$ transducing phage from a *prlD1* strain. A *prlD1* strain deleted for the primary λ attachment site was constructed and designated I7023.2 (Table 1). This strain was lysogenized with λ16-25 (21), an event that integrates the λ*envA*$^+$ transducing phage into the *fts-envA* region of the host chromosome via homologous recombination (26). The site of integration was confirmed by mapping the prophage to this region with P1*vir* transduction crosses. The prophage was subsequently induced by UV irradiation. Five independently obtained lysates, which were derived from five independently obtained lysogens of λ16-25, were used to transduce a *secA*^{ts} strain (strain MM52) (Table 1) to Ts$^+$ at 42°C on TYE agar (multiplicity of infection, 0.2). One such Ts$^+$ lysogen was purified from each transduction. Genetic analysis by P1*vir* transduction demonstrated that the prophage in each of the five Ts$^+$ lysogens had integrated into the primary λ attachment site, a region of the genome that is not linked to the *envA-secA* region. Therefore, alleviation of the conditional *secA*^{ts} defect occurred by *trans* complementation, and the λ prophage each carried an intact *secA*$^+$ gene. The λ*secA*$^+$ phage were induced by UV irradiation, plaque purified, and retested for *secA*$^+$ transducing ability.

Radiolabeling and immune precipitation. In experiments where *prl*-mediated suppression of various MBP export-defective mutations was determined, 1-ml cultures of the appropriate strains were grown to logarithmic phase in MalM63 liquid medium at 30°C with shaking. These cultures were radiolabled with 5 μCi of a uniformly ^{14}C-labeled amino acid mixture for 10 min. Incorporation of the label was terminated by rapidly chilling the cultures in an ice water bath. The cells were subsequently pelleted at 4°C, washed once with 1 ml of ice-cold 10 mM Tris (pH 8.0), suspended in 50 μl of 10 mM Tris-hydrochloride (pH 8.0)–1% sodium dodecyl sulfate (SDS)–1 mM EDTA, and solubilized by heating for 3 min in a boiling water bath. The MBP was precipitated from the clarified supernatants with MBP antiserum and IgGsorb as previously described (10). This labeling and precipitation protocol was also used in experiments in which export of *malE* proteins harboring signal peptide alterations was investigated in *prlA prlD* double-mutant

TABLE 1. Bacterial and phage strains

Strain or phage	Genotype or bacterial gene(s) carried	Source and/or reference
Bacteria		
MC4100	F$^-$ Δ*lacU169 araD139 rpsL150 thi flbB5301 deoC7 ptsF25 relA1*	9
RL361	MC4100 Δ*malE12-18 lamBS60*	This study
RL414	MC4100 Δ*malE12-18 lamBS60 prlD1*	This study
RL545	MC4100 Δ*malE12-18 prlD1*	This study
RL567	MC4100 Δ*malE12-18 lamBS60 prlD1 srl-300*::Tn*10 recA56*	This study
RL619	MC4100 *malE10-1 prlD1 srl-300*::Tn*10 recA56*	This study
RL620	MC4100 *malE16-1 prlD1 srl-300*::Tn*10 recA56*	This study
RL13	MC4100 Δ*malB224 zjb*::Tn*5*	This study
MM52	MC4100 *secA51*(Ts)	D. Oliver (25)
AB2463/KLF4	*thi-1 thr-1 leu-6 argE3 his-4 proA2 recA13 mtl-1 xyl-5 ara-14 galK2 lacY1 str-31 tsx-33 supE44/F'104*	D. Oliver
S10	AP2246 Amp^r *lacUV5 relA*(Su) *rpsE lysA29 Δcya-854 argE*::Tn*10 ilv*::Tn*5*/ F'*lacZ*(Am)	D. Oliver
I7023.2	F$^-$ Δ*lacU169 rpsL* Δ(*gal-bio*) Δ*malE12-18 zjb*::Tn*5 leu*::Tn*10 prlD1*	This study
Phage		
λ*c*I		Laboratory stock
λ16-25	*fts*$^+$ *ftsZ*$^+$ *envA*$^+$ *secA'*	D. Oliver (21)
λD02	*ftsA*$^+$ *ftsZ*$^+$ *envA*$^+$ *secA*$^+$	D. Oliver (26)
ΔD020	*secA*$^+$	D. Oliver (26)
P1*vir*		Laboratory stock
R17		S. Short

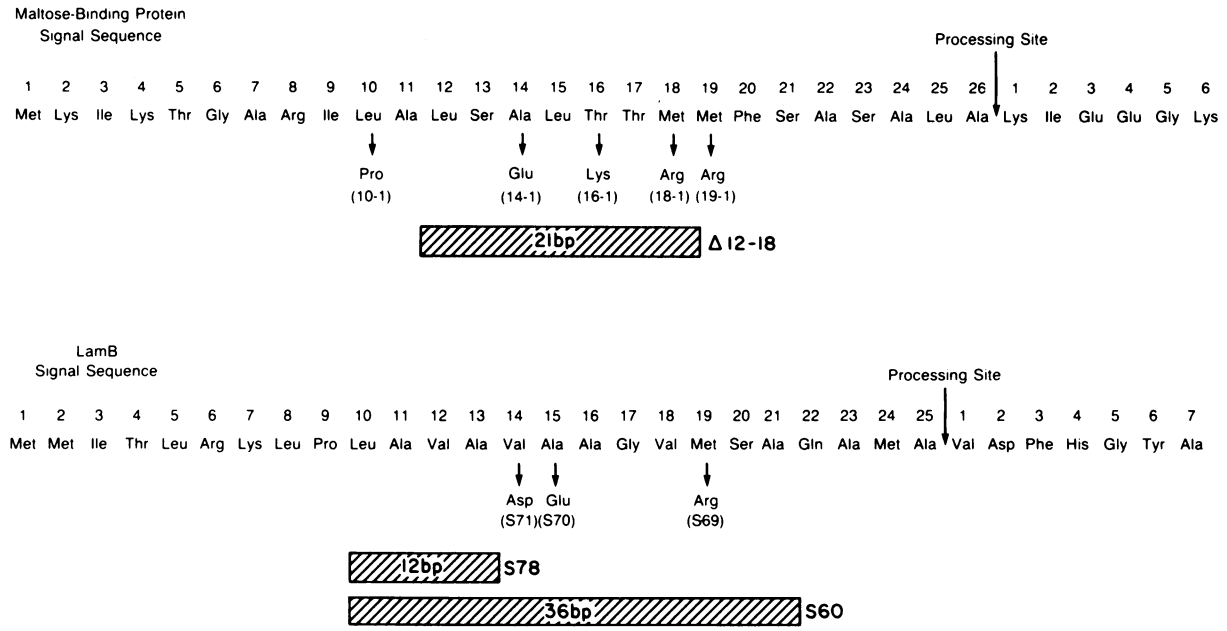

FIG. 1. Primary sequence of wild-type and mutant MBP and LamB signal peptides. The amino-terminal 32 residues of the MBP and LamB precursor proteins are shown, including the entire signal peptides and both processing sites. Single amino acid alterations that result in major export defects for either protein are indicated by arrows. Residues removed from the signal peptide by deletion mutations are indicated by the cross-hatched bars. The corresponding allelic designations are given. Additional details and references are provided in the text. bp, Base pairs.

strains, except that [^{35}S]methionine was used for radiolabeling.

To measure the export efficiency for certain wild-type envelope proteins in *prlA prlD* double-mutant strains, 1-ml cultures of the strains were grown to logarithmic phase in MalM63 liquid medium at 30°C with shaking. These cultures were labeled for 1 min with 5 μCi of the ^{14}C-labeled amino acid mixture. Incorporation of the label was terminated by injecting 900 μl of the culture into 450 μl of ice-cold 15% trichloroacetic acid. The trichloroacetic acid precipitates were pelleted, washed twice with 1 ml of ice-cold acetone, dried in vacuo, and suspended in 100 μl of the solubilization buffer (see above). The pellets were solubilized as described above. Immediately before labeling, a small sample of each culture was streaked for isolation onto TYE agar and incubated for 16 h at 37°C. The percentage of colonies that were significantly larger than the miniscule colonies characteristic of the *prlA prlD1* double mutant (see below) indicated the degree of reversion to "healthiness." Cultures exhibiting a degree of reversion in excess of 10% were not used in the subsequent immune precipitations. MBP, ribose-binding protein (RBP), and LamB were immune precipitated from the clarified supernatants with the corresponding antisera and IgGsorb, as previously described (10). Preparation of rabbit anti-MBP serum has been described previously (10). Rabbit anti-RBP serum was provided by Carol Kumamoto and Jonathan Beckwith, Harvard Medical School, Boston, Mass. Goat anti-LamB serum was a gift from Thomas Silhavy, Frederick Cancer Research Center, Frederick, Md. OmpA was precipitated with rabbit anti-OmpA serum provided by Paul Ray, Wellcome Research Laboratories, Research Triangle Park, N.C. Kinetic studies of MBP export in *prlA prlD* double-mutant strains were performed by using the pulse-chase experimental procedure described previously (2). The method used for precipitating MBP is described above.

Polyacrylamide gel electrophoresis. Immune precipitates were resolved by SDS-polyacrylamide gel electrophoresis and autoradiography (16). The gel dimensions used have been described previously (2). We used 12.5% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 30:0.8) with NaCl incorporated into the separation gel at a final concentration of 70 mM. The gels were stacked and run at 15 and 30 mA (regulated current) per gel, respectively.

RESULTS

Isolation and genetic characterization of mutants suppressed for the *ΔmalE12-18* export defect. The isolation of mutants blocked for the export of a particular envelope protein to its proper extracytoplasmic location has made it possible to devise selections for mutants that are suppressed for such an export defect. Intragenic suppressor mutations have proven to be valuable in dissecting the nature of export signals within a secreted protein (2, 13). Extragenic suppressor mutations, however, are presumed to identify loci that encode components of a cellular machinery that is involved in facilitating protein export (11).

Strain RL361 carries *ΔmalE12-18*, a deletion of seven residues from within the hydrophobic core of the MBP signal peptide (Fig. 1). This deletion represents the strongest export-defective MBP mutation known. It does not, however, completely block MBP export, as a very small fraction (less than 1%) of the total MBP made in a *ΔmalE12-18* strain is probably localized and processed. This slight leakiness enables strain RL361 to grow very slowly in maltose-containing minimal medium. Nevertheless, the essentially Mal$^-$ character of a *ΔmalE12-18* strain permits the isolation of phenotypically Mal$^+$ mutants (2). Strain RL361 also harbors *lamBS60*, a deletion of 12 residues from within the hydrophobic core of the LamB signal peptide (12) (Fig. 1). The rationale for using strain RL361 for obtaining Mal$^+$ phenotypic revertants was threefold. First, *ΔmalE12-18* is incapable of true reversion, and mutants suppressed for this

deletion represent the desired pseudorevertants. Second, Δ*malE12-18 lamBS60* double mutants grow more slowly in maltose-containing minimal medium than isogenic *lamB*$^+$ strains. This property facilitates the isolation of Mal$^+$ pseudorevertants. Third, *lamBS60* provides a rapid means for identifying certain classes of extragenic suppressor mutations. *lamBS60* strains are totally resistant to bacteriophage λ as a result of their inability to export LamB to the outer membrane (12). Mal$^+$ derivatives of strain RL361 that are also λ sensitive (λ^s) exhibit pleiotropic suppression of *malE12-18* and *lamBS60*. Such pleiotropic suppressors must act in *trans*.

A total of 28 spontaneous Mal$^+$ pseudorevertants of strain RL361 were obtained and categorized into a variety of phenotypic classes, based upon color reaction on MalTTC indicator agar. Upon cross-streaking against phage λ*c* on maltose-containing minimal agar, an environment that maximizes *lamB* expression, 12 of the 28 pseudorevertants proved to be λ^s. Thus, these 12 λ^s Mal$^+$ pseudorevertants were considered to harbor extragenic suppressor mutations. Each of the remaining 16 λ^r Mal$^+$ derivatives was used as a donor in phage P1*vir*-mediated transduction of Δ*malB224* strain RL13 to Mal$^+$. If a particular donor Δ*malE12-18* suppressor mutation actions were intragenic, every Mal$^+$ transductant in this cross should have acquired the suppressor phenotype. For 12 of the 16 λ^r Mal$^+$ pseudorevertants, this proved to be the case. These 12 intragenic Δ*malE12-18* suppressor mutations have been characterized in detail previously (2) and are not discussed further here. The remaining four donor strains yielded recipient transductants that were only weakly Mal$^+$ on minimal medium and exhibited the parental Mal$^-$ phenotype on MalTTC agar. These data indicated that an additional 4 of the 16 λ^r Mal$^+$ pseudorevertants also carry extragenic Δ*malE12-18* suppressor mutations.

Three genetic loci have been described in *E. coli* on the basis that suppressor mutations restore export to proteins with nonfunctional signal peptides; these loci have been designated *prlA*, *prlB*, and *prlC* (11). The overwhelming majority and the most efficient of these extragenic suppressor mutations map at *prlA*, a gene located on the promotor-distal portion of the Pspc operon at 72 min on the *E. coli* linkage map. Mutations in *prlA* are more than 98% P1 contransducible with *rpsE*. The 16 Mal$^+$ pseudorevertants harboring extragenic suppressor mutations were used as recipients in phage P1*vir*-mediated transduction to Spc^r. The donor strain was strain S10 (*rpsE prlA*$^+$). Several Spc^r transductants were purified from each cross and tested for the suppressor phenotype on MalTTC agar. If a suppressor mutation mapped at *prlA*, it should have been lost from the great majority of the Spc^r transductants. This was found to be the case for all 12 of the λ^s Mal$^+$ pseudorevertants and for 3 of the 4 λ^r Mal$^+$ pseudorevertants, indicating that 15 of the 16 extragenic Δ*malE12-18* suppressor mutations mapped at *prlA*.

The location of the remaining suppressor mutation was determined by a combination of conjugational mapping and phage P1*vir*-mediated transduction crosses. Genetic crosses in which we used various Hfr donor strains positioned this Δ*malE12-18* suppressor locus between 98 and 6 min on the *E. coli* linkage map (data not shown). This suppressor locus was designated *prlD* as it was clearly distinct from both of the remaining *prl* loci that have been described previously (i.e., *prlB* at 84 min and *prlC* between 69 and 71 min) (11). Subsequent analysis by phage P1*vir*-mediated transduction established that *prlD1* was 33% cotransducible with *ara* and 94% cotransducible with *envA*, at 2.5 min on the *E. coli* linkage map (1). Data obtained from three-factor crosses placed *prlD1* clockwise from *envA*, indicating a gene order of *leu-ftsA-envA-prlD* (data not shown). Thus, *prlD* lies in the immediate vicinity of *secA*, a gene previously described as encoding a component of the cellular secretion machinery by a different set of criteria (25, 26). Evidence indicating that *prlD1* is not an allele of *secA* is presented below.

Characterization of novel *prlA* alleles. Of the 16 extragenic Δ*malE12-18* suppressor mutations isolated, we presumed that 15 represented *prlA* mutations by virtue of their tight linkage to *rpsE*. The majority of these suppressor *prlA* mutations were not studied further since their isolation was expected. However, six alleles did exhibit some noteworthy properties. Three alleles (*prlA403*, *prlA406*, and *prlA421*) were unique in that they did not detectably suppress *lamBS60*. All other *prlA* suppressor alleles that were isolated exhibited some suppression of all known *lamB* and *malE* signal sequence mutations. Three other *prlA* alleles were of greater interest and were recognized by comparing their phenotypic suppression of Δ*malE12-18* and *lamBS60* with the suppression exhibited by *prlA4*, the strongest of the *prlA* suppressor mutations previously characterized (10, 11). Phenotypic suppression of Δ*malE12-18* was initially determined on MalTTC agar, whereas suppression of *lamBS60* was determined by testing for λ^s on TYE agar. The latter conditions are not conducive for maximal induction of *lamB* expression and are useful for distinguishing stronger phenotypic suppressors of *lamBS60* (10). The three *prlA* alleles of interest distinguished themselves by phenotypically suppressing Δ*malE12-18* at least as efficiently as *prlA4* did. Also, *lamBS60* strains bearing these alleles were detectably λ^s on TYE agar. These observations suggested that *prlA401*, *prlA402*, and *prlA409* were more efficient suppressors than *prlA4*.

To investigate the efficiency of these suppressors, known MBP signal sequence mutations (Fig. 1) were introduced into isogenic *prlA* strains. MBP was immune precipitated from ^{14}C-labeled cells and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Whereas precipitation of radiolabeled MBP from a *malE*$^+$ strain (regardless of *prl* genotype) yielded only the mature form of the protein (mMBP), precipitates obtained from *prl*$^+$ strains synthesizing export-defective MBP yielded characteristic amounts of the precursor MBP (pMBP), in addition to the mMBP (3, 4) (Fig. 2). For example, Δ*malE12-18*, *malE18-1*, and *malE19-1* exert very strong MBP export defects. The labeled MBP precipitated from *prl*$^+$ strains carrying these mutations migrated on SDS-polyacrylamide gel electrophoresis gels almost exclusively as pMBP. The *malE10-1*, *malE14-1*, and *malE16-1* alterations caused somewhat less severe export defects, as demonstrated by the larger amounts of mMBP precipitated from strains harboring these mutations. The ratio of pMBP to mMBP provides a reliable operational indicator of the severity of a particular MBP export defect. A shift in this ratio toward the mMBP, in the presence of a particular suppressor allele, is an accurate indicator of the strength of suppression (10, 11).

As shown in Fig. 2, *prlA4* suppressed Δ*malE12-18* significantly but inefficiently; 18% of the radiolabeled MBP precipitated from a Δ*malE12-18 prlA4* strain was exported and processed. The *prlA4* allele also suppressed the five *malE* point mutations to varying extents. Note that *malE10-1* and *malE16-1* were particularly well suppressed (more than 98% MBP precipitated in the mature form). In contrast, significant amounts of pMBP were still detected in the other

mutants, although clearly MBP export efficiency was greatly improved in each instance. The suppression patterns observed for *prlA401*, *prlA402*, and *prlA409* strains indicated that these alleles exhibited more efficient suppression of *malE* signal sequence mutations than *prlA4* did (Fig. 2). In particular, the *prlA402* allele mediated more than 90% suppression of all of the *malE* point mutations.

Characterization of *prlD1*-mediated suppression. In terms of reaction on MalTTC agar and growth rate in maltose-containing minimal medium, *prlD1* represents the weakest phenotypic suppressor of Δ*malE12-18*. Nevertheless, the effect of *prlD1* on the ability of a Δ*malE12-18* strain to catabolize maltose was significant. A Δ*malE12-18 lamBS60 prlD1* strain, strain RL414, exhibited a doubling time of 128 min in maltose-containing minimal liquid medium at 37°C, compared with 220 min for an isogenic *prlD*$^+$ strain (strain RL361) cultured under identical conditions. To investigate further the suppressor capabilities of *prlD1*, isogenic *prlD*$^+$ and *prlD1* strains carrying each of the export-defective *malE* mutations shown in Fig. 1 were constructed and analyzed both phenotypically and biochemically. On MalTTC agar, *prlD1* elicited a weak phenotypic suppression of Δ*malE12-18*, *malE10-1*, and *malE16-1*. Phenotypic suppression was not observed for *malE18-1*, *malE19-1*, or *malE14-1*. These findings were supported by analyzing the MBP species precipitated from labeled *prlD*$^+$ and *prlD1* strains harboring each of these six mutations (Fig. 3). The MBP profiles observed for *malE18-1*, *malE19-1*, and *malE14-1* strains were not affected by *prlD1*. In contrast, a detectable increase in the radiolabeled mMBP fraction was observed for Δ*malE12-18*, *malE10-1*, and *malE16-1* strains harboring *prlD1*. Quantitation of the mMBP fractions in *prlD1* and *prlD*$^+$ strains revealed a proportion of 3 to 1% for Δ*malE12-18*, 55 to 25% for *malE10-1*, and 63 to 41% for *malE16-1*. To the limit of resolution of the SDS-polyacrylamide gel electrophoresis system used, processing of both wild-type and mutant pMBPs appeared authentic in *prlD1* strains.

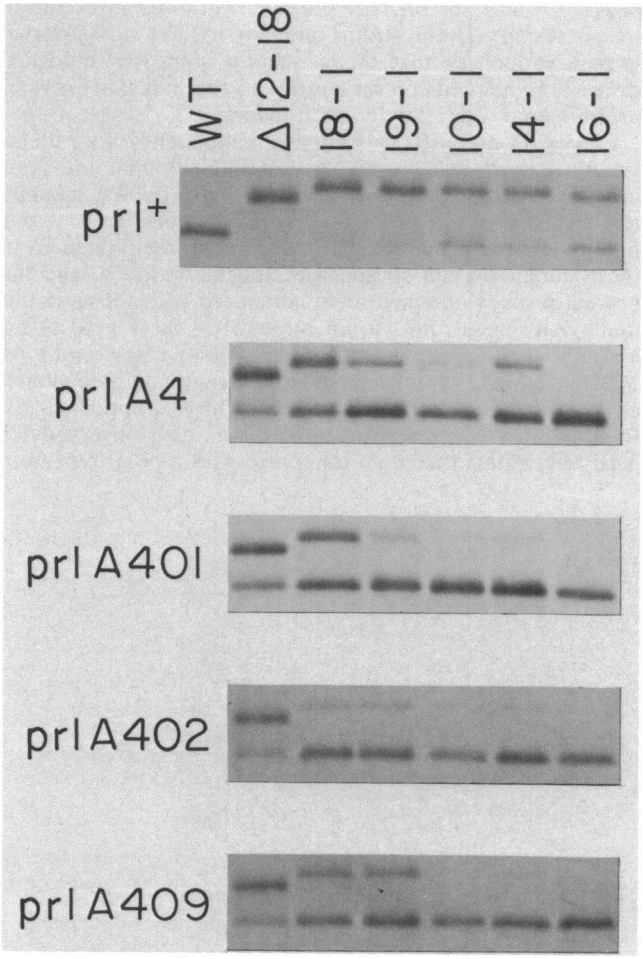

FIG. 2. Immune precipitation of radiolabeled MBP from various *prlA* strains. Experimental details are provided in the text. The *malE* genotypes are given at the top, and the *prlA* genotypes are indicated on the left. WT, Wild type.

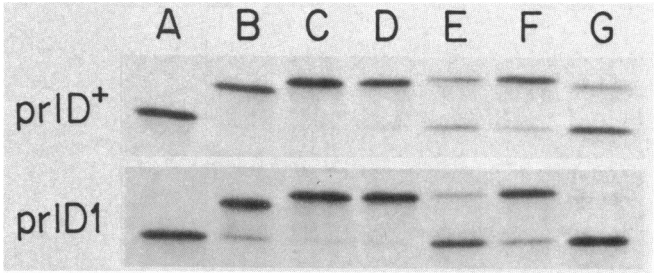

FIG. 3. Immune precipitation of radiolabeled MBP from various *prlD* strains. See text for details. The *prlD* genotypes are given on the left. Lane A, *malE*$^+$; lane B, Δ*malE12-18*; lane C, *malE18-1*; lane D, *malE19-1*; lane E, *malE10-1*; lane F, *malE14-1*; lane G, *malE16-1*.

The *prlD1* mutation also affected the LamB phenotype of strains bearing one of several strong *lamB* signal sequence mutations that have been previously described (12) (Fig. 1). One of these, *lamBS71*, was weakly suppressed. As noted about, *lamBS60* was not phenotypically suppressed by *prlD1*. However, the export defects exerted by *lamBS69*, *lamBS70*, and *lamBS78* were actually exacerbated by *prlD1*. These phenotypic effects on mutant LamB export were observed by comparing the sensitivities of isogenic *prlD1 prlD*$^+$ strain pairs with phage λ*c* on maltose-containing minimal agar. Such a phenotypic analysis is an exquisitely sensitive means for qualitative determination of suppression of LamB export defects (10).

***prlD1* is not an allele of *secA*.** We obtained two independent lines of evidence which indicated that *prlD* and *secA* are not allelic. The first line of evidence stems from dominance studies with *prlD1 prlD*$^+$ merodiploid strains. Merodiploid analysis with the KLF4 episome indicated that *prlD1* was phenotypically dominant to *prlD*$^+$ for suppression of *malE10-1* and *malE16-1*. However, a similar analysis indicated that *prlD1* was phenotypically recessive to *prlD*$^+$ for suppression of Δ*malE12-18*. An explanation for this apparent paradox is offered below. Nevertheless, the recessive phenotype permitted a direct test of the possible allelic relationship between *prlD* and *secA*. If *prlD* and *secA* are distinct genes, introduction of *secA*$^+$ into a Δ*malE12-18 prlD1* strain should not regenerate the parental Δ*malE12-18 prlD*$^+$ phenotype. Strain RL545 was lysogenized with λD020, a *secA*$^+$ transducing phage. λD020 was constructed in vitro and carries a 3.6-kilobase pair insertion of bacterial DNA composed almost entirely of *secA*$^+$ structural and regulatory DNA sequences (26). Strain RL545 lysogens of λD020 retained the suppressed phenotype, as judged on MalTTC agar, indicating that the λ*secA*$^+$ phage does not carry *prlD*$^+$. The same result was obtained when strain RL545 was lysogenized with λD02, an *envA*$^+$ *secA*$^+$ transducing phage carrying an additional 10-kilobase pair of bacterial DNA that lies clockwise

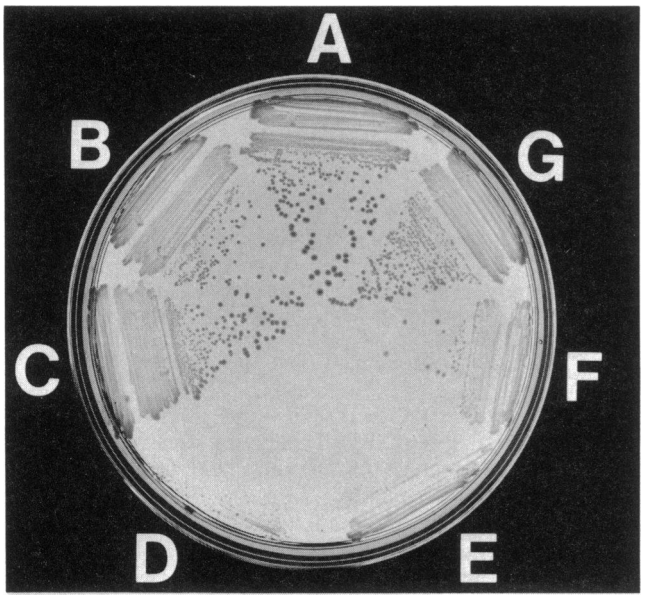

FIG. 4. Growth properties of various *prlA prlD* double-mutant strains. The isogenic double mutants were constructed as described in the text, streaked for isolation onto TYE agar, and incubated for 24 h at 37°C. Position A, *prlA*$^+$ *prlD1* strain; position B, *prlA2 prlD1*; position C, *prlA3 prlD1*; position D, *prlA4 prlD1*; position E, *prlA401 prlD1*; position F, *prlA402 prlD1*; position G, *prlA409 prlD1*. The sparsely distributed larger colonies readily apparent among the almost invisible microcolonies characteristic of the *prlA4 prlD1* and *prlA401 prlD1* strains represent healthy pseudorevertants (see text for additional details).

from and adjoins *secA* (26). This observation indicated a gene order of *envA-secA-prlD* proceeding in a clockwise direction on the *E. coli* genetic map.

A complementary line of evidence demonstrating that *prlD* and *secA* are not allelic was derived from our ability to obtain specialized λ*secA*$^+$ transducing phages from a *prlD1* strain. Five such independently obtained phages were used to lysogenize *prlD*$^+$ strains carrying either *malE10-1* or *malE16-1*, two mutations whose suppression by *prlD1* is a dominant trait. Such lysogens were expected to harbor the λ*secA*$^+$ prophage at the primary λ attachment site which is not linked to the *envA* region of the genome. P1*vir*-mediated transduction experiments confirmed this for all of the lysogens generated, indicating that *prlD1* function was being tested in *trans*. A phenotypic analysis of the resulting lysogens revealed that none of the five independently obtained λ*secA*$^+$ phages carried an intact and functional *prlD1* allele. Thus, our ability to transduce an intact *secA*$^+$ allele from a *prlD1* strain to a *prlD*$^+$ strain without transducing the suppressor indicated that *prlD* and *secA* are not allelic. Since λ16-25 does carry a portion of *secA*$^+$ (27), we cannot totally discount the possibility that *prlD* and *secA* are allelic, but the suppressor mutation was not picked up in the aberrant excision event that generated the λ*secA*$^+$ phage. Such an occurrence would be a very rare event. The observation that all five independently obtained λ*secA*$^+$ phages failed to transduce *prlD1* makes this possibility extremely remote.

Properties of *prlA prlD1* double-mutant strains. To investigate the relationship between *prlA* and *prlD*, we constructed a number of strains that carried both *prlD1* and one of several suppressor *prlA* alleles. During our attempts to construct various *prlA prlD* double-mutant strains, it became obvious that *E. coli* had difficulty in accommodating certain *prlA* alleles in a *prlD1* genetic background. Such difficulties were manifested by the altered growth properties of certain *prl* double mutants on complex or minimal media. Figure 4 illustrates this point. The *prlD1* strains harboring *prlA2*, *prlA3*, *prlA402*, or *prlA409* grew at wild-type rates on all media tested. These strains did not exhibit any unusual growth properties that might suggest some physiological distress. In marked contrast, *prlD1* strains carrying *prlA4* or *prlA401* grew very slowly on all media.

Since *prlA* and *prlD* may encode components of a cellular protein export machinery, we felt it likely that the poor growth of *prlA4 prlD1 and prlA401 prlD1* double mutants reflected a problem in normal protein export. To test this prediction, four envelope proteins were precipitated from these double-mutant strains with specific antisera, and the protein profiles obtained were compared with those exhibited by an isogenic *prl*$^+$ strain, strain MC4100 (Fig. 5). After a 1-min labeling period with [^{35}S]methionine, the wild-type MBP precipitated from cells of a *prl*$^+$ strain migrated almost exclusively as the mature species. Only a minor pMBP fraction was detected. This profile was also observed for MBP precipitated from an otherwise wild-type *prlD1* strain

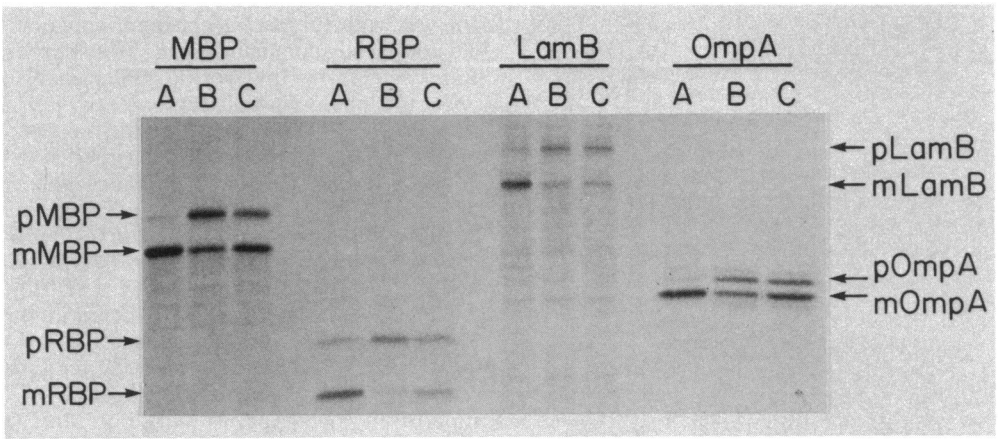

FIG. 5. Processing of *E. coli* envelope proteins in slow-growing *prlA prlD* double-mutant strains. See text for details. The envelope proteins are indicated at the top, and the locations of the precursor and mature protein forms are indicated at the sides. The *prl* genotypes are indicated below the envelope proteins as follows: A, *prl*$^+$; B, *prlA4 prlD1*; C, *prlA401 prlD1*. Note that RBP synthesis in strain MC4100 derivatives is constitutive (15).

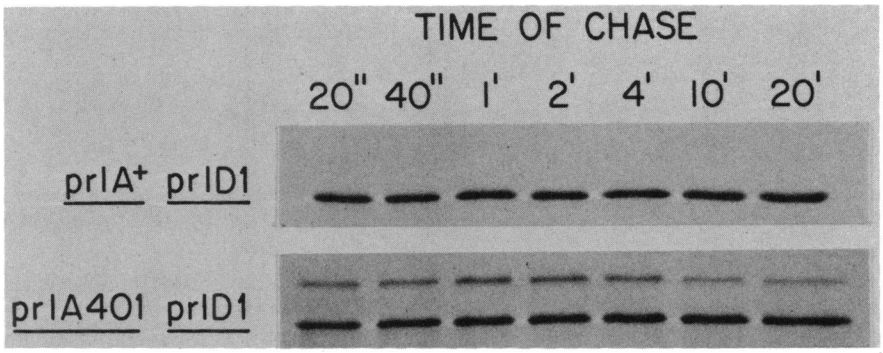

FIG. 6. Kinetics of wild-type MBP export in *prlA401 prlD1* strains. The strains were pulse-labeled for 15 s with [^{35}S]methionine, and the chase was initiated by incorporating excess unlabeled methionine and terminated with trichloroacetic acid at the times indicated at the top, as previously described (2). The MBP was subsequently immune precipitated from the solubilized protein (see text). The relevant strain genotypes are indicated at the left.

and strains carrying any of the efficient suppressor *prlA* alleles utilized in this study (data not shown). However, a significant amount of radiolabeled pMBP was precipitated from each of the *prlA prlD1* double mutants exhibiting a severe growth defect. For the *prlA4 prlD1* strain, fully 60% of the labeled MBP precipitated was precursor. The *prlA401 prlD1* strain exhibited 40% of the total labeled MBP in the precursor fraction. The same general pattern was observed for the periplasmic RBP and outer membrane proteins OmpA and LamB. In each case, the *prlA prlD1* strains exhibited markedly increased fractions of these envelope proteins migrating in their precursor forms compared with the isogenic *prl*$^+$ strain. The *prlA prlD1* double mutants that grew normally did not exhibit such a generalized accumulation of precursors.

An undesirable feature of both *prlA4 prlD1* and *prlA401 prlD1* strains was their instability. Each double mutant rapidly accumulated healthy revertants that exhibited normal growth properties. This caused several technical difficulties. First, it required reconstruction of the appropriate double mutant immediately before use. Second, it did not permit experimental analysis of a pure culture. Although the degree of reversion in a culture could be determined easily, contamination was always detectable and could be substantial. Quantitatively reproducible results required that the revertant population be kept below 10% of the total population.

Kinetics of MBP maturation in *prl* double-mutant strains. To determine whether the abnormal accumulation of envelope protein precursors observed in certain *prlA prlD* double-mutant strains represented a transient or permanent block in maturation, the kinetics of wild-type MBP and OmpA processing were studied in a *prlA401 prlD1* strain. As shown in Fig. 6, approximately 30% of the pulse-labeled MBP migrated as precursor at 20 s after initiation of chase. A major fraction of the MBP matured very rapidly. The pMBP fraction, however, remained essentially constant through 4 min of chase. Only a minor reduction in the pulse-labeled pMBP fraction was observed at 10 and 20 min postchase. A reciprocal increase in the labeled mMBP fraction did not occur. Thus, it seemed that the accumulated pulse-labeled pMBP did not chase to the mature form. Note that an identical analysis of MBP maturation in the isogenic *prlA*$^+$ *prlD1* strain demonstrated that all of the pulse-labeled MBP was found as the mature species after 20 s of chase. An additional point of interest was the stability of the nonexported wild-type pMBP. Very little degradation of this pulse-labeled species was observed, even after 20 min of chase.

Biochemical analysis of MBP export defects in *prlA prlD* double-mutant strains. To investigate further the relationship between *prlA* and *prlD* and how the respective gene products interact with the MBP signal peptide, we analyzed the export of three export-defective MBPs in *prlA401 prlD1* strains. These MBP export defects, resulting from Δ*malE12-18*, *malE10-1*, and *malE16-1*, were suppressed by either *prlA401* or *prlD1* in an otherwise *prl*$^+$ genetic background. We considered the possibility that introduction of both of the suppressor *prl* alleles into strains carrying one of these *malE* signal sequence mutations might at least result in enhanced suppression of the MBP export defects, even if export of other envelope proteins was partially impaired (Fig. 7).

As noted above, Δ*malE12-18* is a very strong signal sequence mutation. Essentially all of the radiolabeled MBP precipitated from a Δ*malE12-18 prl*$^+$ strain migrated as the truncated pMBP. Introduction of *prlD1* elicited a slight but detectable increase in the labeled mMBP fraction. Similarly, Δ*malE12-18 prlA401* strains exhibited even greater amounts of properly exported mMBP. However, precipitation of radiolabeled MBP from a Δ*malE12-18 prlA401 prlD1* strain yielded exclusively the truncated pMBP. The same general pattern was observed in the case of *malE10-1* and *malE16-1*. Although *prlD1* and *prlA401* individually suppressed both of these mutations quite efficiently, the *prlA401 prlD1* double

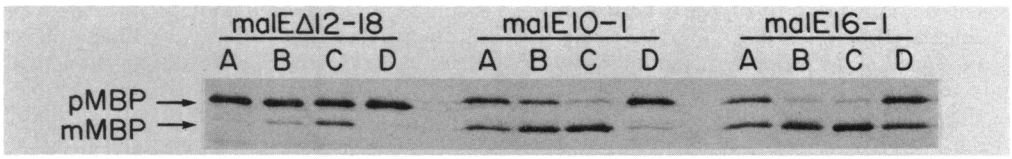

FIG. 7. Export of mutant MBP in *prlA401 prlD1* strains. The experimental conditions are described in the text. The *malE* alleles are indicated at the top. The *prl* genotypes are indicated above the lanes as follows: A, *prl*$^+$; B, *prlD1*; C, *prlA401*; D, *prlA401 prlD1*. The locations of the precursor and mature species are indicated at the left.

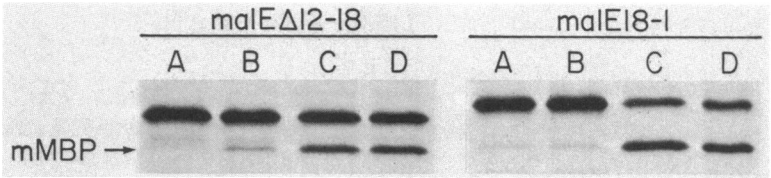

FIG. 8. Export of mutant MBP in *prlA409 prlD1* strains. See text for experimental details. The *malE* alleles are indicated at the top, and the *prl* genotypes are indicated above the lanes as follows: A, *prl*$^+$; B, *prlD1*; C, *prlA409*; D, *prlA409 prlD1*. The location of the mature protein form is also indicated.

mutants exhibited more pronounced pMBP fractions that the corresponding *prl*$^+$ strains.

We also studied the suppression of MBP export defects in *prlD1* strains carrying a compatible suppressor *prlA* allele. In marked contrast to the results described above, suppression of an MBP export defect was preserved in strains carrying a compatible *prlA prlD1* combination. This was the case whether the MBP export defect was suppressed by both the *prlD1* and compatible suppressor *prlA* alleles or by the *prlA* allele alone (Fig. 8). For instance, the Δ*malE12-18* export defect was suppressed weakly by *prlD1* and quite efficiently by *prlA409*. The Δ*malE12-18 prlA409 prlD1* strain exhibited a radiolabeled MBP profile that was indistinguishable from that exhibited by the Δ*malE12-18 prlA409* strain. Densitometric scanning indicated that in each case 34% of the labeled MBP was found as the mature species. On the other hand, the *malE18-1* export defect was not suppressed by *prlD1* but was efficiently suppressed by *prlA409*. The labeled MBP profile observed for the *malE18-1 prlA409 prlD1* strain was indistinguishable from that observed for the *malE18-1 prlA409* strain. In both cases, 67% of the radiolabeled MBP was found in the mature form.

DISCUSSION

One genetic approach for identifying cellular components that may serve to facilitate protein export involves obtaining mutants capable of exporting a protein that carries a defective signal peptide. Previous studies have indicated that an intact signal peptide is required for efficient translocation of a polypeptide across the cytoplasmic membrane (4, 12, 23). Restoration of proper localization to a protein with a nonfunctional signal peptide can be envisioned to occur via mutational alteration of an element of the cellular secretion machinery that normally interacts with a functional signal peptide. This strategy was initially applied to mutants synthesizing an export-defective LamB and identified three loci (*prlA*, *prlB*, and *prlC*) thought to encode components of a cellular secretion machinery (11). Since the MBP and LamB are localized to different extracellular compartments (the periplasm and outer membrane, respectively), we considered the possibility that selection for mutants exhibiting more efficient localization of an export-defective MBP may identify new loci that were not recognized in the original LamB selection. We expected to obtain in this selection mutants harboring suppressor *prlA* or *prlC* alleles, as these have been previously demonstrated to suppress both LamB and MBP export defects (10).

The 16 extragenic Δ*malE12-18* suppressor mutations analyzed in this study fell into two categories on the basis of genetic mapping; 15 mapped at *prlA*, and the remaining suppressor mutation, designated *prlD1*, mapped at 2.5 min on the *E. coli* genetic map and identified a new locus whose product seems to participate in facilitating protein export. In all cases, suppression of Δ*malE12-18* was manifested biochemically by increased fractions of periplasmic mMBP that could be precipitated from the suppressor strains. Particularly noteworthy in their suppression ability were three new *prlA* suppressor alleles (*prlA40*, *prlA402*, and *prlA409*). On the other hand, *prlD1* was a fairly weak suppressor; its ability to suppress several MBP export defects and at least one LamB export defect indicates a general suppressor capability for *prlD*. However, none of the new *prlA* or *prlD* suppressor alleles which we obtained had any detectable effect on wild-type protein export. The failure to demonstrate an effect by suppressor *prlA* alleles or *prlD1* on export of wild-type envelope proteins can be construed as evidence that these mutations do not alter the normal pathway of protein export.

Suppression mediated by *prlD1* exhibited several interesting features. Merodiploid analysis indicated that *prlD1* was dominant to *prlD*$^+$ for phenotypic suppression of *malE10-1* and *malE16-1*, yet was recessive to *prlD*$^+$ for phenotypic suppression of Δ*malE12-18*. These seemingly paradoxical results can be reconciled if *prlD1* and *prlD*$^+$ are considered to act codominantly. Since *prlD1* suppressed *malE10-1* and *malE16-1* rather efficiently, introduction of *prlD*$^+$ into such strains may not sufficiently attenuate suppression so that a nonsuppressed *malE* phenotype is observed. However, suppression of Δ*malE12-18* was fairly weak. Any attenuation of this suppression may be sufficient to regenerate the Mal$^-$ phenotype of the Δ*malE12-18 prl*$^+$ parent.

A particularly intriguing property of *prlD1*-mediated suppression was its remarkable allele specificity. For example, *prlD1* suppressed only three of the six MBP signal peptide alterations tested. These represent the strongest (Δ*malE12-18*) and weakest (*malE16-1*) of the six and one (*malE10-1*) that is intermediate. The remaining MBP defects were not affected by *prlD1*. An even more impressive relationship was observed between *prlD1* and *lamB* signal sequence mutations. This suppressor allele did not detectably affect *lamBS60*, and it suppressed *lamBS71* weakly and rendered the *lamBS69*, *lamBS70*, and *lamBS78* export defects even more severe than the defect which is observed in isogenic *prl*$^+$ strains. Such a striking allele specificity argues persuasively for a direct interaction between the *prlD1* gene product and the defective MBP and LamB signal peptides (see reference 28 for a discussion of allele-specific interaction). Likewise, *prlA* mutations have been shown previously to suppress MBP and LamB mutations in an allele-specific manner, and similar conclusions were drawn (10, 11).

If both the *prlA* and *prlD* products do interact directly with signal peptides, then it could be that these products directly interact with each other as well. Indeed, this seems to be the case, as evidenced by the observation that *prlA* and *prlD* exhibit allele-specific interactions. Double-mutant strains carrying *prlD1* and either *prlA4* or *prlA401*, two very efficient suppressor *prlA* alleles, exhibited a severe growth defect, yet *prlD1* strains bearing either *prlA402* or *prlA409* appeared to grow normally, even though both of these *prlA* alleles are also very strong suppressors.

We believe that the very slow growth rate characteristic of certain *prlA prlD1* double mutants reflects cellular distress at a generalized protein export defect. These double mutants accumulated a significant fraction of precursors for a number of wild-type envelope proteins. These included proteins destined for localization to either the periplasm or the outer membrane. We believe that the pMBP which accumulated in these double-mutant strains was located in the cytoplasm. This presumption is based, in part, on a large body of evidence obtained from various sources which demonstrates that processing constitutes an absolute indicator of proper localization of the MBP to the periplasm (2, 10; data not shown). Kinetic studies indicated that, at least in the case of MBP, export of the unprocessed precursor was permanently blocked. Therefore, the secretion-stressed *prlA prlD1* double mutants synthesized two classes of pMBP, that which was exported and processed normally and that which was not exported at all. Revertants obtained from these double mutants that exhibit normal growth properties are alleviated for these export defects (V. A. Bankaitis, D. Collier, and P. Bassford, unpublished data). The clear demonstration of a major defect in general protein export in certain *prlA prlD* double mutants indicates a role for both of the products in the normal cellular pathway for protein export. Experimental evidence suggesting such a function for the *prlA* gene product has been reported previously (17).

We interpret the data described above to indicate that the *prlA* and *prlD* products are obligatorily involved in facilitating the localization of proteins to the cell envelope. Considering how the genetic loci were identified, one can speculate that the *prlA* and *prlD* products are involved in the initial recognition of the signal peptide as it emerges from the ribosome, with subsequent delivery of the nascent chain-ribosome complex to the cytoplasmic membrane. In order for this process to occur efficiently, the *prlA* and *prlD* products must execute two operations. The first entails the correct and efficient interaction of these components with each other. Another, perhaps coupled, operation is the productive interaction of these components with the signal peptide. In this instance, we define productive to indicate a functional priming of the signal peptide for the subsequent step in the secretory pathway (translocation?). Failure to correctly execute the former operation results in failure to perform the latter. Such difficulties are manifested as general export defects for proteins that utilize this pathway for localization to the periplasm (e.g., MBP and RBP) or the outer membrane (e.g., LamB and OmpA). This concept is intriguing from the standpoint that proper assembly of a complex for faithful execution of protein export is indicated.

In *prlA*$^+$ *prlD*$^+$, *prlA prlD*$^+$, *prlA*$^+$ *prlD1*, and healthy *prlA prlD1* strains, the *prl* products interact properly and consequently are able to interact efficiently with signal peptides in a productive fashion. Protein export in these strains is executed in the normal manner. For certain *prlA prlD1* double-mutant strains, however, a proper interaction between the *prlA* and *prlD1* products occurs at a fraction of the normal frequency. As a consequence, productive interactions of these components with signal peptides also occur at similarly reduced frequencies. In the case where a productive interaction with a signal peptide does occur, the polypeptide is exported normally. However, in the case where interaction with a signal peptide is not productive, the polypeptide remains in the cytoplasm as the unprocessed precursor species.

The markedly different behavior of export-defective MBP exhibited in stressed versus healthy *prlA prlD1* double mutants is also well accounted for within the framework of this hypothesis. We believe that *prlA*- or *prlD1*-mediated suppression of some MBP export defect reflects an increase in the frequency of productive interaction of the *prl* gene products with the defective MBP signal peptide. In a stressed *prlA prlD1* double mutant, the increased efficiency of productive interaction with the defective signal peptide by these components is totally negated by the inability of these components to interact properly with each other. In a healthy *prlA prlD1* double mutant, the *prl* gene products interact efficiently, and the increase in the frequency of productive interaction with the defective signal peptide is preserved. Also, the demonstratable interaction of export-defective proteins with the *prlA* and *prlD* products strongly suggests that these defective proteins utilize, albeit inefficiently, the normal route of export. Such evidence provides justification for using export-defective proteins to study the normal process of secretion in *E. coli*.

Finally, which other elements directly interact with the *prlA prlD* complex in facilitating protein export remains to be determined. The *prlC*, *secA*, and *secB* products are possible candidates. Other genetic approaches for addressing this question may be provided by reversion analyses of the *prlA prlD1* double mutants. It appears certain that *E. coli* possesses at least one type of cellular machinery that is required for the proper and efficient export of wild-type proteins to the cell envelope. In this regard, procaryotes and eucaryotes appear to share similar mechanistic features in effecting protein export. Walter and Blobel have characterized a eucaryotic nucleoprotein complex that interacts with nascent secretory polypeptides shortly after emergence of the signal peptide from the large ribosomal subunit (36, 37). Further genetic characterization of this procaryotic machinery should provide a basis for obtaining a greater understanding of bacterial protein export in biochemical terms.

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