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Novel *secA* Alleles Improve Export of Maltose-Binding Protein Synthesized with a Defective Signal Peptide

JOHN D. FIKES† AND PHILIP J. BASSFORD, JR.*

Department of Microbiology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

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Mutations previously designated *prlD* were described that suppressed *malE* signal sequence mutations and were located in the vicinity of the *secA* gene on the *Escherichia coli* chromosome. In this study, we demonstrated that four such independently isolated *prlD* mutations represented three unique single-base substitutions in *secA*, resulting in alterations at residues 111, 373, and 488 of the 901-residue SecA protein. Heretofore, the only mutations that had been described for *secA* were located early in the gene and resulted in a general protein export defect. Insertion mutations in the cloned gene X-*secA* operon that reduced or eliminated suppression by a *prlD* mutation also have been obtained. The properties of these suppressor and insertion mutations provide some insight into the role of SecA in the protein export process.

The localization of proteins to sites beyond the cytoplasm of *Escherichia coli* cells is an efficient and highly selective process. Proteins destined for export to extracytoplasmic locations are generally synthesized with an amino-terminal extension that has been referred to as a signal peptide or leader sequence (5, 24). This structure is thought to be chiefly responsible for initiating the export process, either cotranslationally or posttranslationally, and is proteolytically removed during or immediately following translocation of the protein across the cytoplasmic membrane. The structural features important for signal peptide function have been studied in detail (for a recent review, see reference 35).

Translocation of a protein across the cytoplasmic membrane may require a specific interaction between the signal peptide and components of the cellular export machinery. Mutants exhibiting a pleiotropic protein export defect have been isolated, and the genetic loci (designated *sec*) identified by these mutations are thought to encode components involved in the export process (for recent reviews, see references 3, 5, and 24). For three of these genes, the product has been identified and the subcellular location has been determined. The *secA* product is a large, 101-kilodalton hydrophilic protein that resides in the cytoplasm or is peripherally associated with the cytoplasmic membrane (20, 29). In response to a block in protein export, the intracellular level of SecA protein increases 10- to 20-fold (20, 26, 27). The *secB* product (16) is a 17-kilodalton cytoplasmic protein that was recently shown to promote export of the maltose-binding protein (MBP) by retarding the premature folding of the MBP into an export-incompetent conformation in the cytoplasm (7). The *secY* locus encodes a 49-kilodalton integral cytoplasmic membrane protein (13) that may be responsible for mediating the actual protein translocation event. The products of two recently identified loci, *secD* and *secE*, have not yet been characterized (11, 26).

Another class of mutants consists of those harboring extragenic suppressor mutations that can restore the export of proteins synthesized with a defective signal peptide. Although these *prl* loci represent a class of mutations very different from the mutations that result in a pleiotropic export defect, it was believed that the isolation of such suppressor mutations would also help to identify components of the export pathway, particularly those that interact in some way with the signal peptide. A majority of the suppressor mutations identified in genetic selections of this nature map to the *prlA* gene, which was first described by Emr et al. (10). In fact, *secY* mutations that were later isolated proved to represent new alleles of the *prlA* locus (31). Extragenic suppressor mutations designated *prlB*, *prlC*, and *prlD* have also been mapped to at least three other locations on the *E. coli* chromosome (2, 10). One of these, *prlD*, was closely linked to the *secA* gene (2). However, several lines of evidence indicated that *prlD1*, the first *prlD* allele studied in detail, was not a *secA* mutation. A second suppressor mutation in this region, designated *prlD2* (28), and additional extragenic suppressors subsequently obtained (*prlD3*, *-D4*, and *-D5*; J. P. Ryan, Ph.D. dissertation, University of North Carolina, Chapel Hill, 1985) were shown to cotransduce with markers in the *secA* region of the chromosome but were not studied further with respect to their precise genetic identity.

In this study, the extragenic suppressor phenotypes conferred by *prlD2*, *-D3*, *-D4*, and *-D5* are shown to actually be the result of three unique mutations within the *secA* gene. Such suppressor alleles represent a phenotype that has not previously been described for mutations in *secA* and should prove useful in elucidating the exact role of SecA in the protein export process.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, reagents, and genetic techniques. The pertinent bacterial strains, phages, and plasmids used in this study are shown in Table 1. All bacterial strains except RY2422, Q359, and GE1033 are derivatives of strain MC4100 (6). The minimal medium used was M63 (18), supplemented with maltose at a final concentration of 0.2%. The complex media used included TYE, maltose-triphenyltetrazolium agar (MalTTC), and maltose-MacConkey agar, prepared as previously described (18). When required, kanamycin sulfate, tetracycline, ampicillin, and chloramphenicol were added at final concentrations of 30, 20, 50, and 12.5 μg/ml, respectively. Gigapack Plus in

* Corresponding author.
† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

TABLE 1. Bacterial strains, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Relevant genotype	Source or reference
Bacterial strains		
MC4100	F⁻ *lacU169 araD139 rpsL150 thi flbB5301 deoC7 ptsF25 relA1*	6
CC161	*malE14-1*	4
CC299	*malE10-1*	4
CC212	*malE14-1 recA srl-300*::Tn*10*	This study
CC214	*malE14-1 leu*::Tn*10*	2
CC276	Δ*malB224 prlD2*	This study
CC284	*malE14-1 prlD1*	2
CC165	*malE14-1 prlD2*	28
CC261	*malE14-1 prlD3*	J. P. Ryan
CC262	*malE14-1 prlD4*	J. P. Ryan
CC263	*malE14-1 prlD5*	J. P. Ryan
CC275	*malE14-1* λ⁺	This study
MM52	*secA51*(Ts)	D. Oliver (19)
CC287	*secA51*(Ts) λ⁺	This study
RY2422	*rpsL*⁺ *recA* F'101	R. Young
CC285	*malE14-1 ara*⁺ *leuB* λ2G1 F'101	This study
CC286	*malE14-1 ara*⁺ *leuB* λ1H1 F'101	This study
Q359	P2⁺	R. Maurer (8)
Bacteriophages		
P1 *vir*		Laboratory stock
λSE6		R. Maurer (8)
λDO20	*secA*⁺	D. Oliver (21)
λ⁺		Laboratory stock
Plasmids		
pNK972	Tn*10* transposase	G. Weinstock
pZ152		37
pTA108		N. Trun (34)
pLG552	*secA*⁺	D. Oliver (15)
F'101	*secA*⁺ *leu*⁺	18
pJF33	pZ152 *prlD2*	This study
pJF35	pZ152 *secA*⁺	This study
pJF39	pTA108 *secA*⁺	This study
pJF42	pTA108 *prlD2*	This study

vitro packaging extracts were purchased from Stratagene. [^{35}S]methionine (Translabel) was obtained from ICN Pharmaceuticals Inc. Rabbit anti-MBP serum has been described previously (9). Electrophoresis reagents, restriction enzymes, T4 DNA ligase, and large fragment of DNA polymerase I were purchased from Bethesda Research Laboratories, Inc. XAR film was obtained from Eastman Kodak Co. Standard genetic techniques were performed as previously described by Silhavy et al. (32).

Molecular cloning of *prlD2*. An *E. coli* genomic library in λSE6 (8) was prepared by using strain CC276 (*prlD2* Δ*malB224*) as the source of chromosomal DNA. High-molecular-weight chromosomal DNA was partially digested with *Sau*3A1 and size fractionated by agarose gel electrophoresis. Restriction fragments ranging between 12 and 18 kilobases (kb) were isolated and ligated into *Bam*HI-cleaved λSE6 DNA. Following in vitro packaging, the library was amplified once on strain Q359. Growth on this strain, a phage P2 lysogen, selected for Spi⁻ recombinant phages. In cells expressing the λ *cI* repressor gene, the recombinant phage replicated as a low-copy-number plasmid that could be maintained by selection for kanamycin resistance (Km^r) (8). Therefore, unless otherwise stated, cells carrying a λSE6-derived recombinant phage were also lysogens of wild-type phage λ, providing a source of *cI* repressor.

The library was screened for recombinant phage able to suppress the signal sequence mutation, *malE14-1*, by infecting cells of strain CC275 and selecting for Km^r lysogens on maltose-MacConkey indicator plates. Numerous colonies exhibiting a Mal⁺ phenotype characteristic of *prlD2* suppression were purified, and the phenotype was confirmed by testing for improved growth on minimal agar containing maltose. To test linkage between the *prlD2* phenotype and the recombinant phages, lysates were prepared from 33 putative *prlD2* clones by UV induction (32) and used to relysogenize strain CC275. The *prlD2* phenotype was linked to the phage in each case, and one recombinant phage, designated λ2G1, was chosen for further analysis. A recombinant phage that did not suppress the *malE14-1* mutation was randomly selected to serve as a negative control and designated λ1H1.

To determine if the DNA harbored on λ2G1 had been cloned from the 2.5-min region of the chromosome, an attempt was made to recombine the suppressor mutation from this phage onto F'101 (18), an F plasmid carrying DNA from that region. Strains CC285 (F'101 and λ2G1) and CC286 (F'101 and λ1H1) were mated with strain CC214, as described by Miller (18), selecting for *leu*⁺ (F'101) and Tc^r (to counterselect donor). To screen for suppression of the *malE14-1* mutation, colonies were replica plated onto maltose-tetrazolium indicator agar. In addition, the λ2G1 and λ1H1 phages were tested for the ability to complement the recessive *secA51*(Ts) mutation present in strain CC287. Lysogens were streaked onto TYE plates containing kanamycin and incubated for 36 h at 42°C.

Transposon mutagenesis of λ2G1. The insertional mutagenesis of λ2G1 recombinant phage using the defective transposon Tn*10d*-Cam (8a) was conducted as described by Elledge and Walker (8). Phage harboring Tn*10d*-Cam insertions in λ2G1 that adversely affected *prlD2*-mediated suppression of *malE14-1* were identified by lysogenizing strain CC275 and selecting for growth on MalTTC indicator agar supplemented with chloramphenicol.

Plasmid constructions. Standard recombinant DNA techniques were performed as described by Silhavy et al. (32). Plasmid pJF33 was constructed by subcloning the 0.8- and 2.5-kb *Eco*RI fragments known to carry the *secA* gene (21, 29) from λ2G1 DNA into the *Eco*RI site of the high-copy-number vector pZ152 (37). A plasmid carrying the *secA*⁺ gene (obtained from λDO20 [21]) was constructed in an identical manner to that of pJF33 and designated pJF35. Plasmid pLG552 (15) carries a 14-kb *Bam*HI fragment from *E. coli* that harbors *secA*⁺ and approximately 10.5 kb of flanking DNA. This *Bam*HI fragment was subcloned into pTA108 (34) to generate plasmid pJF39.

By the transductional allele exchange method of Trun and Silhavy (34), the *prlD2*, *-D3*, *-D4* and *-D5* mutations each were genetically recombined from the chromosome onto plasmid pJF39. A phage P1 lysate was prepared on cells harboring pJF39 and one of the chromosomal *prlD* alleles. This lysate was then used to transduce strain CC161 (*malE14-1*), selecting for Amp^r on MalTTC agar containing ampicillin. Transfer of pJF39 to a recipient strain by P1 transduction is dependent on integration of the plasmid into the chromosome via homologous recombination between *E. coli* DNA on each replicon (a relatively rare occurrence).

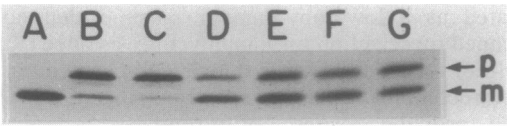

FIG. 1. Immunoprecipitation of radiolabeled MBP14-1 from cells harboring *prlD* suppressor alleles. Experimental details are provided in Materials and Methods. Lanes: A, *malE*$^+$; B, *malE14-1*; C, *malE14-1 prlD1*; D, *malE14-1 prlD2*; E, *malE14-1 prlD3*; F, *malE14-1 prlD4*; G, *malE14-1 prlD5*. The positions of pMBP (p) and mMBP (m) are shown.

Excision of the plasmid DNA from the chromosome by a different homologous crossover event can result in transfer of the *prlD* mutation from the chromosome to the plasmid. Plasmids harboring *prlD* mutations were identified by screening Amp^r transductants of strain CC161 for the appropriate Mal$^+$ phenotype. To ensure homogeneity, plasmids were isolated and reintroduced into strain CC161. Recombination of *prlD1* onto pJF39 was attempted in an identical manner to that described above, except that strain CC299 (*malE10-1*) was employed as the recipient strain (see Results).

DNA sequencing. The cloning of DNA restriction fragments into M13 vectors and DNA sequence analyses were performed as described by Hutchison et al. (12).

Radiolabeling, immunoprecipitation, SDS-PAGE, and autoradiography. Cells were grown in minimal medium containing glycerol at 30°C to mid-log phase, and synthesis of MBP was induced by the addition of 0.2% maltose to the culture medium. After 45 min, cells were labeled with [^{35}S]methionine for 10 min, and MBP was immunoprecipitated from solubilized cell extracts by procedures previously described (28). Immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography as previously described (28). Quantitation of the ratio of precursor MBP (pMBP) to mature MBP (mMBP) was performed as described by Collier et al. (7). The percentages reported here are based on the average of data from three or more experiments. Methods for kinetic analysis of MBP export by pulse-chase experiments have been described previously (28).

RESULTS

Molecular cloning of *prlD2*. The effect of *prlD* suppressor mutations on the export of an MBP species with a defective signal peptide (MBP14-1) is shown in Fig. 1. Mid-log-phase cells induced with maltose were radiolabeled for 10 min with [^{35}S]methionine, and then MBP was immunoprecipitated from solubilized cell extracts and analyzed by SDS-PAGE and autoradiography. The export of MBP from the cytoplasm to the periplasmic space was monitored by following the processing of pMBP to mMBP. A decrease in the pMBP/mMBP ratio in the presence of a particular suppressor allele provides an accurate indicator of the strength of suppression (2, 9, 28). Consistent with an earlier study (2), *malE14-1 prlD1* cells did not exhibit a biochemically detectable change in the pMBP-to-mMBP ratio compared with *malE14-1 prl*$^+$ cells. However, significantly more mMBP was precipitated from cells of *malE14-1* strains harboring either *prlD2*, *-D3*, *-D4* or *-D5*, clearly demonstrating the increased efficiency of MBP export in these strains. No effect of *prlD* mutations on export of wild-type MBP could be discerned (data not presented).

The decision was made to initially clone *prlD2*, since this was the best characterized of the various *prlD* alleles exhibiting strong suppression of *malE14-1* (28). Chromosomal DNA was prepared from a Δ*malB224* deletion strain (no *malE* gene) harboring the *prlD2* gene, and 11- to 18-kb restriction fragments from a *Sau*3A1 partial digest were cloned into the phage vector λSE6 (8). Following in vitro packaging and a single amplification, this library was screened for cloned DNA sequences able to suppress *malE14-1*. Approximately 1 in 350 Km^r colonies exhibited the phenotype characterisitc of *prlD2* suppression, and this phenotype was linked to the vector in each of 33 clones tested (data not shown). One of these clones, designated λ2G1, was chosen for further study.

The DNA fragment present on phage λ2G1 was shown to be derived from the *prlD* region by the demonstration that the suppressor activity could be genetically recombined from the phage onto F′101 (18), which carries this region of the *E. coli* chromosome (data not shown). This event requires homologous recombination between the *E. coli* DNA sequences carried on each replicon. A randomly selected phage from this library not harboring the *prlD2* mutation, designated λ1H1, also was tested to ensure that the suppressed phenotype of Mal$^+$ exconjugates was dependent on λ2G1. To further characterize λ2G1, the ability of this clone to complement the *secA51*(Ts) mutation (19) in strain CC287 was tested. The *prlD1* mutation was previously shown to be closely linked to *secA* by P1 transduction (2). When the λ2G1 phage was introduced into strain CC287, the temperature-sensitive phenotype associated with the recessive *secA51*(Ts) mutation was relieved. The λ1H1 phage did not rescue strain CC287 at the nonpermissive temperature. Analysis of λ2G1 DNA revealed a 14-kb insert with restriction sites that correspond to the region of the chromosome that begins within the *ftsZ* gene (36) and extends clockwise to approximately 7 kb beyond the *secA* gene (including the two *Eco*RI fragments encompassing the *secA* gene shown in Fig. 2).

Insertional mutagenesis of λ2G1 using Tn*10d*-Cam was utilized to identify the region of this DNA fragment that was responsible for the PrlD2 phenotype. The λ2G1 phage was grown lytically on a strain harboring the Tn*10d*-Cam and a plasmid encoding the enzymatic activity necessary for transposition of the defective transposon (8a). Clones harboring Tn*10d*-Cam insertions that affected suppression were isolated by lysogenizing a *malE14-1* strain and selecting for chloramphenicol resistance on MalTTC indicator agar. Three classes of insertion events were identified: (i) Tn*10d*-Cam insertions that had no effect on suppression of the *malE14-1* mutation by λ2G1, (ii) transposon insertions that partially reduced suppression of the signal sequence mutation, and (iii) insertions that totally eliminated the PrlD2 phenotype. Two Cm^r clones from the two latter classes were subjected to restriction analysis, and each Tn*10d*-Cam insertion mapped within or immediately upstream of the *secA* gene (see below). These findings strongly suggested that the PrlD2 phenotype was the result of a mutation within either *secA* itself or a gene just downstream from *secA* but in the same operon.

***prlD2* is an allele of *secA*.** To determine whether *prlD2* was an allele of *secA*, the *secA* gene from λ2G1 was subcloned and tested for the ability to suppress *malE14-1*. An *Eco*RI partial digest of λ2G1 DNA was used to introduce the *secA* gene into the high-copy-number plamid pZ152 (37). The resultant plasmid, designated pJF33, carried the 0.8- and 2.5-kb *Eco*RI restriction fragments previously shown to harbor the entire *secA* gene (21, 29). When pJF33 was introduced into a *malE14-1* strain, strong suppression of the

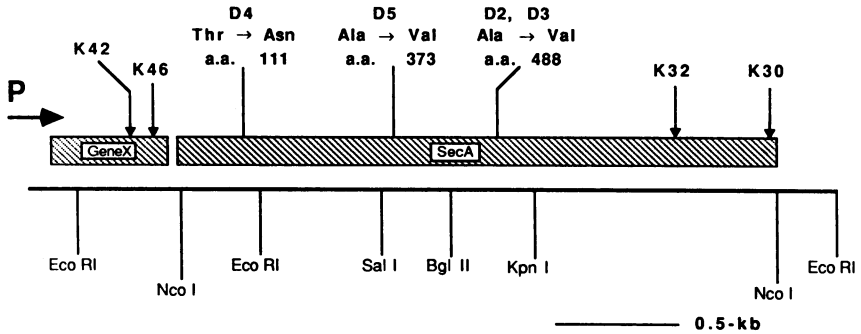

FIG. 2. Location of point mutations and Tn*10d*-Cam insertions. The gene X-*secA* operon with restriction sites pertinent to this study is shown. The positions of amino acid substitutions in the SecA protein resulting from the *prlD2*, *-D3*, *-D4*, and *-D5* mutations are indicated at the top of the figure. Downward-pointing arrows represent transposon insertions, and the large rightward-pointing arrow labeled P denotes the direction of transcription. a.a., Amino acid.

signal sequence mutation was observed on MalTTC agar. When the wild-type *secA* gene was subcloned in a similar manner from the *secA*$^+$-transducing phage λDO20 (21) to form pJF35, some suppression of the *malE14-1* mutation also could be discerned, although the reaction was significantly less than that resulting from the presence of the *prlD2* allele in a single copy or multiple copies. In addition to the *secA* gene, plasmid pJF33 also harbored the 3′ end of gene X and the 5′ end of a downstream open reading frame (29). However, because suppression of *malE14-1* by pJF33 occurred in a *recA*-deficient background that prevented recombination with the chromosome, it seemed unlikely that these truncated open reading frames were responsible for *prlD2*-mediated suppression (data not shown).

To localize the *prlD2* mutation within the *secA* gene, restriction fragments were exchanged between pJF33 (*prlD2*) and pJF35 (*prlD*$^+$). The PrlD2 suppressor phenotype was localized to the *Sal*I-*Kpn*I restriction fragment which lies entirely within the borders of the *secA* gene (Fig. 2). DNA sequence analysis of this 702-base-pair region from pJF33 revealed a single mutation resulting in a C-to-T transition at base 1463 of the *secA* gene. This mutation would result in the substitution of a valine for an alanine at residue 488 of the SecA protein. The DNA sequence of the corresponding region of the *secA* gene carried on pJF35 (*secA*$^+$) was identical to that reported by Schmidt et al. (29).

The extragenic suppressors *prlD3*, *-D4*, and *-D5* are also alleles of *secA*. The possibility that other *prlD* mutations also were located within the *secA* gene was investigated. By the method of Trun and Silhavy (34), it was found that the *prlD3*, *-D4*, and *-D5* mutations could be genetically recombined from the chromosome onto a low-copy-number plasmid, pJF39, carrying the *secA* region. From each of these plasmids, an *Nco*I fragment that brackets almost the entire *secA* gene (Fig. 2) was isolated and used to replace the corresponding fragment in the *secA*$^+$ plasmid pJF35. Cells of a *malE14-1* strain were transformed with these recombinant plasmids and plated on MalTTC agar containing ampicillin. In each case, the transformants exhibited the appropriate PrlD suppressor phenotype, indicating that the responsible mutation moved with the *Nco*I fragment and, thus, represented an alteration within the *secA* gene. DNA sequence analysis revealed that the *prlD3* mutation was identical to *prlD2*. These two alleles also exhibited an identical level of suppression when colonies were judged by color reaction on MalTTC agar. However, the other two mutations were unique. The *prlD4* mutation was a C-to-A transversion at base 332 that substituted Asn for Thr at residue 111 of the SecA protein, and *prlD5* was a C-to-T transition at base 1118 that replaced Ala with Val at residue 373.

Attempts also were made to recombine *prlD1* from the chromosome onto plasmid pJF39. However, recombinant plasmids conferring the PrlD1 phenotype were not obtained.

Additional observations. As reported above, Tn*10d*-Cam insertions in λ2G1 were isolated that altered suppression of *malE14-1* by *prlD2*. Two of the clones analyzed, λK30 and λK32, exhibited a total loss of suppression, and two others, λK42 and λK46, retained partial suppression as scored on MalTTC agar. To further investigate the effect of each insertion on suppression by λ2G1, MBP was immunoprecipitated from radiolabeled cells and analyzed by SDS-PAGE and autoradiography (Fig. 3). Quantitation of pMBP processing from cells harboring the control phage λ1H1 demonstrated that, under these labeling conditions, approximately 16 to 18% of MBP14-1 was exported in the absence of a suppressor mutation. The presence of λ2G1 in a *malE14-1* strain improved the efficiency of export to 39%. In agreement with the phenotypes scored on MalTTC indicator plates, the Tn*10d*-Cam insertions within λ2G1 designated K30 and K32 were found to totally eliminate suppression. The sensitivity of MalTTC indicator agar provided the best means to distinguish between this class of λ2G1 derivatives and the class represented by λK42 and λK46. The latter suppressed *malE14-1* to a level that was easily distinguishable by color reaction on MalTTC agar but was only barely detectable by direct determination of pMBP/mMBP ratios.

In addition to being analyzed for suppression of *malE14-1*, the four λ2G1 Tn*10d*-Cam derivatives described above were

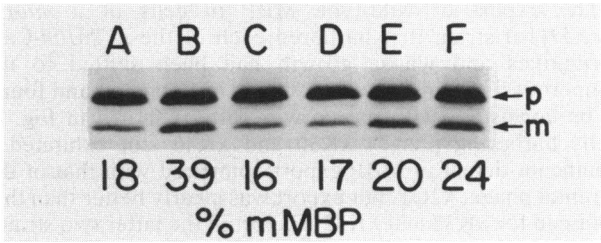

FIG. 3. Immunoprecipitation of radiolabeled MBP14-1 from cells harboring various λ2G1 derivatives. See Materials and Methods for details. All strains carried *malE14-1* on the chromosome and were lysogens of one of the following phages. Lanes: A, λ1H1 (negative control); B, λ2G1; C, λK30; D, λK32; E, λK42; F, λK46. The percentage of total radiolabeled MBP precipitated as mMBP is indicated below each lane. The positions of pMBP (p) and mMBP (m) are shown.

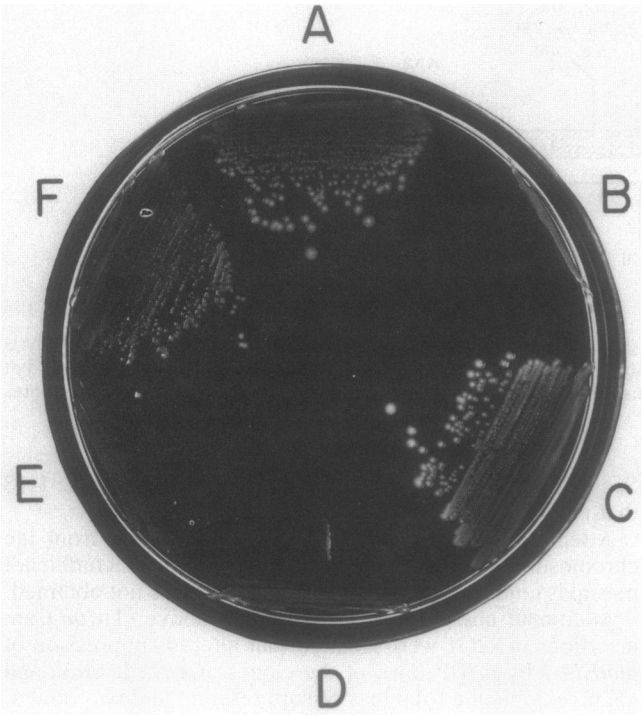

FIG. 4. Growth properties of *secA51*(Ts) derivatives harboring various derivatives of λ2G1. Cells of each strain were streaked for isolation on TYE agar supplemented with chloramphenicol and incubated for 36 h at 42°C. The prophage carried by each strain was as follows: λ2G1 (A), λ1H1 (B), λK30 (C), λK32 (D), λK42 (E), and λK46 (F).

tested for their ability to complement *secA51*(Ts). Each phage was introduced into strain CC287, and Cm^r lysogens were scored for growth at the nonpermissive temperature (42°C). Phage λ2G1, but not control phage λ1H1, complemented the *secA51*(Ts) mutation (Fig. 4). One of the two Tn*10d*-Cam derivatives that did not suppress the *malE14-1* signal sequence mutation, λK30, also allowed normal growth of CC287 at 42°C. Only a few pinpoint colonies were detectable for λK32 lysogens. Similarly, CC287 cells harboring λK42, a phage that retained partial suppression of *malE14-1*, exhibited virtually no growth. In contrast to λK42, λK46 restored near-normal growth of strain CC287 at the nonpermissive temperature. Identical results were obtained in a *recA* derivative of strain CC287 (data not shown).

The export of wild-type MBP in cells of a $malE^+$ *secA51*(Ts) strain that harbored each of these Tn*10d*-Cam derivatives and whose growth had been shifted to the nonpermissive temperature also was investigated and found to be consistent with the growth pattern shown in Fig. 4. Cells harboring phages λK30 and λK46 still exhibited a significant defect in MBP export compared with that of the parental phage, λ2G1, but export was clearly better than that obtained for λK32 and λK42. Export in the latter two strains was essentially identical to that obtained for cells of the *secA51*(Ts) strain harboring no phage or the control phage, λ1H1 (data not shown).

DNA sequence analysis was used to determined the exact site of insertion in each of the four λ2G1 derivatives. The transposons present in λK30 and λK32 had inserted following bases 2680 (disrupting residue 894) and 2308 (disrupting residue 769) at the end of the *secA* gene, respectively (Fig. 2). The Tn*10d*-Cam insertions carried on λK42 and λK46 were not found within the *secA* gene. Instead, these insertions were located within the nonessential open reading frame upstream of *secA* that has been referred to as gene X (29, 36).

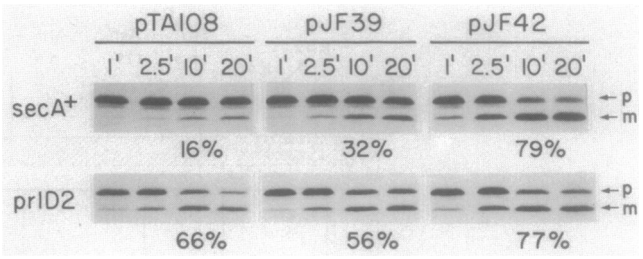

FIG. 5. Kinetics of MBP14-1 export in strains harboring various combinations of *secA* and *prlD2*. All strains carried *malE14-1* and either $secA^+$ or *prlD2* on the chromosome (as indicated to the left of the gel). In addition, cells contained either pTA108 (vector control), pJF39 ($secA^+$), or pJF42 (*prlD2*). The percentage of the total radiolabeled MBP precipitated as mMBP at the 10-min chase point is indicated below lane 10' for each chase series. As found previously (28), the pMBP/mMBP ratios did not change appreciably with longer chase times. See Materials and Methods for experimental details.

Finally, in the course of this study, it was observed that the copy numbers of $secA^+$ and *prlD2* in *malE14-1* cells affected the efficiency of MBP14-1 export. The kinetics of MBP14-1 export in strains harboring various combinations of chromosomal and plasmid-borne copies of these two alleles was studied in detail. For each strain tested, the chromosomal copy of the essential *secA* gene was either $secA^+$ or *prlD2*. The plasmid carried was either the vector, pTA108, pJF39 ($secA^+$), or pJF42 (*prlD2*). The copy number of pTA108 has been estimated at four to five copies per cell (1, 34). Log-phase cells were pulse-labeled with [^{35}S]methionine for 15 s, and MBP was immunoprecipitated from cells solubilized at various times following the addition of unlabeled methionine. The precipitates were subsequently analyzed by SDS-PAGE and autoradiography (Fig. 5). When the chromosomal $secA^+$ gene was the only copy present, approximately 16% of MBP14-1 had been exported (as indicated by conversion to the mMBP form [28]) by the 10-min time point. As reported previously for MBP14-1, export was accomplished in a slow, posttranslational manner (28). Increasing the copy number of $secA^+$ by introducing pJF39 resulted in a reproducible twofold increase in the amount of MBP14-1 exported, and there was a fivefold increase for cells harboring plasmid pJF42 (*prlD2*). However, in these latter two instances, the posttranslational nature of MBP14-1 export did not appear to be significantly altered. The pTA108 vector did not influence suppression by the chromosomal copy of *prlD2* (compare Fig. 1 and Fig. 5). However, the presence of pJF39 ($secA^+$) in the same strain slightly reduced suppression by *prlD2*. Cells carrying *prlD2* both on the chromosome and on pJF42 exported MBP14-1 slightly more efficiently than cells harboring only a chromosomal *prlD2* copy. In the presence of pJF42 (*prlD2*), MBP14-1 export was virtually identical in strains harboring either $secA^+$ or *prlD2* on the chromosome. It should be noted that in each of these cases, particularly the increased suppression of *malE14-1* obtained by simply increasing the copy number of $secA^+$, the observed changes in the efficiency of MBP export strongly correlated with changes in the color reaction of colonies of these strains cultured on MalTTC agar.

DISCUSSION

This study demonstrates that four mutations previously characterized as extragenic signal sequence suppressor mutations and designated as alleles of the *prlD* gene are actually located within the *E. coli secA* gene. This represents the second instance when such suppressor mutations have been selected in a gene and when conditional lethal alleles also have been obtained that result in cells exhibiting an obvious defect in protein export under nonpermissive growth conditions. Mutations conferring both phenotypes have been recognized previously in the *prlA/secY* locus (10, 30, 31). Oliver and co-workers (17, 19, 20, 21, 27, 29) have isolated and extensively characterized a number of *E. coli secA*(Ts) mutants. Under nonpermissive conditions, the export of most periplasmic and outer membrane proteins, including all of the major outer membrane proteins, is strongly blocked (17, 19). DNA sequence analysis of nine *secA*(Ts) alleles revealed that they were all point mutations substituting what were predicted to be structurally disruptive amino acids at one of three tightly clustered sites in the first 170 residues of the SecA protein (29). In contrast, the three unique suppressor mutations whose sequence was determined in this study were more broadly dispersed throughout the *secA* gene, generating alterations at residues 111, 373, and 488 of this 901-residue protein.

The suppressor mutations *prlD2*, *-D3*, *-D4*, and *-D5* are similar to *prlA* suppressor mutations in several respects. (i) Each one suppresses, to some degree, a number of different *malE* mutations that alter the hydrophobic core of the MBP signal peptide (Ryan, Ph.D. dissertation). (ii) Suppressor-mediated MBP export exhibits posttranslational kinetics (28; also this study). (iii) Their presence has no obvious effect on the export kinetics of wild-type MBP. However, none of these *prlD* mutations exhibit a suppression efficiency that approaches the efficiency achieved by the stronger *prlA* alleles. For example, several *prlA* alleles have been obtained that will facilitate the export of MBP14-1 with nearly 100% efficiency (2). In contrast, for *prlD2*, the strongest of the *prlD* suppressors, only about two-thirds of the MBP14-1 synthesized is eventually exported. It is impossible to speculate as to the functional significance of the actual amino acid substitutions in SecA resulting from the *prlD* mutations. They are different from the mutational alteration induced by *prlA4* (Ile to Asn), the only *prlA* suppressor mutation for which the sequence has been reported (33).

The finding that SecA can be altered in such a way as to increase the export efficiency of MBP with a defective signal peptide may indicate that SecA directly interacts with this structure in facilitating protein export. A similar argument has been made for the PrlA protein (5, 9, 10). However, in the absence of direct biochemical evidence for such an interaction, the genetic evidence in these cases can only be considered suggestive. Genetic evidence indicating a direct interaction between two different proteins is usually based on the demonstration of strong allele specificity in the restoration of function when both structural genes harbor mutations that, when individually expressed, induce a negative phenotype (14, 23). However, as reviewed extensively by Randall et al. (24), there is little evidence supporting the existence of true allele-specific interactions between *prlA* suppressor alleles and *malE* and *lamB* signal sequence mutations. The same interpretation currently applies to *prlD* suppressor mutations. The latter do not impair export of wild-type MBP, and the proportion of MBP exported when any given *prlD* allele is combined with various *malE* signal sequence mutations largely depends on the severity of the signal sequence defect (Ryan, Ph.D. dissertation). One also must keep in mind, however, that there is little primary sequence homology between signal peptides of different exported proteins (35). Therefore, proteins that recognize or otherwise interact with signal peptides probably will not do so by the kind of lock-and-key fit that would be expected to give rise to a high degree of allele specificity.

One other result obtained in this study also at least suggests an interaction at some level between SecA and the signal peptide. It was found that simply increasing the copy number of wild-type $secA^+$ in the cell led to a significant increase in the amount of MBP14-1 exported (Fig. 5). Although the normal haploid level of SecA in the cell does not appear to be limiting for export of wild-type proteins, it could be that a protein, such as MBP14-1, with a signal peptide defect cannot compete effectively for the available SecA unless the amount of this protein is increased. In other words, at least one reason that MBP14-1 is export defective is the inability of its signal peptide to efficiently interact with SecA.

Thus, despite the lack of truly convincing evidence, it is still tempting to propose a direct interaction between SecA and the signal peptide. One cannot rule out the possibility that SecA interacts with another region of the exported precursor. Various studies have suggested a role for the mature moiety in protein export (for a review, see reference 3), and there is good evidence that the SecB protein interacts with the pMBP at a site within the mature region (7). However, the signal peptide remains the only structurally defined feature characteristic of exported proteins and clearly has an essential role in this process. SecA is a soluble protein that can be found either in the cytoplasm or loosely associated with the cytoplasmic membrane, depending on the experimental conditions (20). It could be that SecA is involved in the recognition of the signal peptide of nascent or newly synthesized precursor proteins and in the formation of a translocation-competent complex with other components of the export machinery on the cytoplasmic membrane.

There is some evidence for an interaction between PrlA in the cytoplasmic membrane and SecA. Oliver and Liss (22) reported that *prlA*-mediated suppression of *malE* signal sequence mutations is affected by intracellular SecA levels, and Ryan and Bassford (28) demonstrated that *prlD2* and *prlA4* mutations present in the same strain act synergistically to suppress a *malE* signal sequence mutation. Such a role for SecA also is quite consistent with an earlier observation by Rasmussen and Bassford (25), who found that the synthesis of wild-type exported proteins was switched from occurring predominantly on membrane-bound polysomes to occurring almost exclusively on free polysomes following the shift of *secA*(Ts) cells to the nonpermissive growth temperature. It is expected that studies with SecA in an in vitro protein-translocating system, currently under way in several laboratories, will provide much more insight into its actual role in the export process.

In this study, several insertion mutations in the gene X-*secA* operon were obtained that reduced or eliminated suppression of *malE14-1* by *prlD2*. Two insertions that were found to reduce suppression, K42 and K46, were shown to reside in gene X and presumably reduced expression of *prlD2* (*secA*) by a polarity effect, as does an amber mutation in gene X (29). The earlier of the two insertions, which would be expected to be more strongly polar, was totally unable to restore growth to *secA51*(Ts) cells at the nonpermissive temperature, whereas the latter did provide partial comple-

mentation. The finding that these insertions still exhibited some *prlD2* suppressor activity but could not fully complement *secA51*(Ts) would rule out one possible explanation for the *prlD* mutations that was not mentioned above, that these mutations somehow markedly elevate the intracellular level of SecA, a protein that is known to be regulated by the secretion requirements of the cell (20, 26, 27).

Two insertions (K32 and K30) within the *secA* gene were found to totally eliminate *prlD2* suppression, which would be expected. However, the K30 insertion that would replace the last eight residues of SecA was still able to strongly complement *secA51*(Ts) for growth (Fig. 4) and to partially complement it for restoring wild-type pMBP export at the nonpermissive temperature. Schmidt et al. (29) reported that a protein fusion derivative of *secA* that replaced the last 3 residues of SecA with 22 residues of another protein exhibited a similar complementing activity. The significance of these observations is not known, but it may indicate that the carboxyl terminus of this protein, a region in which alterations caused by point mutations have not been found, may be involved in recognition of the exported polypeptide.

Finally, there remains the question of the identity of *prlD1*, the first suppressor mutation isolated mapping in the *secA* region (2). Earlier studies indicated that this was not an allele of *secA*, and attempts in this study to recombine *prlD1* into the $secA^+$ plasmid pJF39 were unsuccessful. This mutation differs from the other *prlD* mutations in several respects. First, it is a generally weaker suppressor mutation. Second, it exhibits no suppressor activity against several *malE* signal sequence mutations, including *malE14-1*, that are clearly suppressed by *prlD2*, *-D3*, *-D4*, and *-D5*. Third, when *prlD1* is introduced into strains harboring certain *prlA* suppressor alleles, the combination results in cells that manifest a severe defect in normal protein export and grow extremely slowly (2). This latter phenomenon was not encountered with any other *prlD* allele (data not presented). Thus, while these other *prlD* alleles must now be reclassified as alleles of the *secA* gene, it could well be that *prlD1* represents a mutation in another, as yet uncharacterized, gene. This possibility is currently being investigated.

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