JOURNAL OF BACTERIOLOGY, Jan. 1985, p. 285–291
0021-9193/85/010285-07$02.00/0
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Vol. 161, No. 1

Identification of Five New Essential Genes Involved in the Synthesis of a Secreted Protein in *Escherichia coli*

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Received 30 July 1984/Accepted 9 October 1984

To define additional components of the export machinery of *Escherichia coli*, I have isolated extragenic suppressors of a mutant [*secA*(Ts)] that is temperature sensitive for growth and secretion at 37°C. Suppressors that restored growth at 37°C, but that rendered the cell cold sensitive for growth at 28°C, were obtained. The suppressor mutations fall into at least seven loci, two of which (*prlA* and *secC*) have been previously implicated in protein secretion. The five remaining loci (*ssaD*, *ssaE*, *ssaF*, *ssaG*, and *ssaH*) have been mapped by P1 transduction and appear to define new genes in *E. coli*. All of the suppressor mutations allow both enhanced growth and protein secretion of the *secA*(Ts) mutant at 37°C, but not 42°C, indicating a continued requirement for SecA protein. Strains carrying solely the cold-sensitive mutations show reduced levels of certain periplasmic proteins when grown at low temperatures. In at least one case, that of maltose-binding protein, this defect is at the level of synthesis of the protein. Since mutants in any of seven genes as well as *secA* amber mutants halt or reduce the synthesis of an exported protein, it appears that *E. coli* may possess a general and complex mechanism for coupling protein synthesis and secretion.

The mechanisms by which secreted proteins pass through membranes to achieve their ultimate location have been the subject of much recent study. Such studies have indicated that protein export in procaryotic and eucaryotic systems have many features in common. First, many secreted proteins in eucaryotic cells and all bacterial envelope proteins examined to date are initially synthesized with an amino-terminal, signal sequence that is removed during translocation through the membrane (20, 29). Second, signal sequences from diverse sources have a number of structural similarities that apparently allow them to function across distant phylogenetic barriers (9, 27, 30).

By using a mammalian in vitro secretion system, Blobel and co-workers have been able to dissect the early steps in protein export into the endoplasmic reticulum (31–33). A soluble complex of proteins and RNA termed the signal recognition particle (SRP) causes a site-specific arrest of translation of a secreted protein, presumably by interaction with the emerging signal peptide (31–33). Synthesis of the protein is restored when the SRP-polysome complex binds to the SRP receptor or docking protein found in the endoplasmic reticular membrane (10, 19). This sequence of events couples protein synthesis to export and presumably prevents accumulation of presecretory proteins in the cytoplasm. It is interesting to note that the information present in signal sequences must be highly conserved, since a procaryotic exported protein shows the same SRP dependence and arrest features noted above when synthesized in the mammalian in vitro secretion system (22).

Genetic analysis of protein secretion in the gram-negative bacterium *Escherichia coli* and in the yeast *Saccaromyces cerevisiae* has been used to define the various steps in the protein secretion pathways of these organisms. In *S. cerevisiae*, 23 gene products have been genetically defined which are involved in passage of the exported protein from the endoplasmic reticulum to the cell surface (23), whereas only two gene products have thus far been implicated in translocation of the exported protein through the endoplasmic reticular membrane (6, 8). Strains with mutations in these latter two gene products accumulate inactive molecules of exported proteins stuck in the endoplasmic reticular membrane and are therefore blocked at a later step in the translocation process than that mediated by SRP and docking protein.

In contrast, genetic studies with *E. coli* have identified at least four gene products that appear to be involved in the earliest steps in protein secretion. The *prlA* and *prlC* mutants were isolated by their ability to suppress signal sequence mutations (5). The allele-specific suppression effects observed with these mutations would be compatible with a physical interaction of these gene products with the signal peptide and with a role as part of an SRP-like particle (4, 29). The use of gene fusions has allowed selections for export-defective mutants in two additional genes, *secA* (24, 25) and *secB* (16). Mutations in these genes result in pleiotropic defects in secretion. The *secA* gene product also appears to promote coupling between the synthesis and export of at least one secreted protein (25), and this coupling can be disrupted by signal sequence mutations (17). This strong parallel between the procaryotic and eucaryotic secretion systems regarding a coupling mechanism suggests that *secA* and *secB* gene products may function along with *prlA* and *prlC* gene products in an SRP and docking protein type of mechanism.

To define additional genes whose products form components of a common *E. coli* secretion machinery, I have used an approach originally suggested by Jarvik and Botstein (15) and Botstein and Maurer (1). This approach is based on the finding that mutations that render one component of a protein complex inactive can be suppressed by compensatory mutations that alter a second component of that complex. Such suppressor mutations can in practice help to define protein-protein interactions that occur within the cell (Maurer et al., submitted for publication).

To define additional components that interact with SecA protein to mediate early steps in secretion, I have isolated and characterized a large number of extragenic suppressors of a *secA*, temperature-sensitive mutation [*secA*(Ts)]. Suppressors that restored growth at 37°C, but that rendered the cell cold sensitive for growth at 28°C, were obtained. It has

TABLE 1. Bacterial strains

Strain	Genotype	Source
MC4100	F⁻ Δ*lacU169 araD136 relA rpsL thi*	Ito et al. (14)
MC4100Spc^r	MC4100 *rpsE*	E. Brinkman
MM52	MC4100 *secA51*(Ts)	Oliver and Beckwith (24)
DO301	MM52 *ssaD1*(Cs)	This study
DO302	MM52 *prlA22*(Cs)	This study
DO303	MM52 *ssaE1*(Cs)	This study
DO304	MM52 *secC4*(Cs)	This study
DO305	MM52 *ssaF1*(Cs)	This study
DO306	MM52 *ssaG1*(Cs)	This study
DO307	MM52 *ssaH1*(Cs)	This study
DO308	MM52 *secC21*(Cs)	This study
DO309	MC4100 *ssaD1*(Cs)	This study
DO310	MC4100 *prlA22*(Cs)	This study
DO311	MC4100 *ssaE1*(Cs)	This study
DO312	MC4100 *secC4*(Cs)	This study
DO313	MC4100 *ssaF1*(Cs)	This study
DO314	MC4100 *ssaG1*(Cs)	This study
DO315	MC4100 *ssaH1*(Cs)	This study
DO316	MC4100 *secC21*(Cs)	This study
AC6081	*dsdA thr leu thi*	E. McFall
AE1122	Hfr *zeb-1*::Tn*10 metB1 argG6 rpsL104 thyA*	P. Silverman
AW1003	MC4100 *tsx*::Tn*5*	A. Wright
CK1640	MC4100 *zia*::Tn*10 purE mtl*	C. Kumamoto
D21 (λ)	F⁻ *ampA1 proA trp his rpsL*(λ)	J. Walker
DB821	MC4100 *zgj*::Tn*10 nusA1*	M. Syvanen
DC101	*bglR*$^+$ *glmS rbs ara lac thi gyrA*	A. Wright
DF2000	F⁻ *zwfA2 pgiZ2*	McEwen and Silverman (18)
DO284	MC4100 *ilv*::Tn*10*	S. Brown
EM1003	*aroC purF his trp*	E. McFall
MS461.1	F⁻ *tnaA*::Tn*10 couA2* Δ(*lac-pro*) *supE*/F′ *lacZ*::Tn*5 pro*$^+$	A. Wright
N35	F⁻ *lir-35* (*acrA*) *met*	J. Walker
NK6073	*purF*::Tn*10 pro endL hsdR*⁻	N. Kleckner
RS3338	*fadL71*::Tn*10 fadR*	R. Simon
SG4044	F⁻ Δ*lon*-100 Δ(*gal-bio*) *rpsL lac*	S. Gottesman
SM103	MC4100 *proC*::Tn*5*	S. Michaelis
SY798	F⁻ *zje*::Tn*10 hfl-1* Δ*lacM445 lac-1 his argE rpsL mtl xyl* Δ(*recA-srl*)/pColE1 *recA*$^+$ *srl*	M. Syvanen

already been shown that such suppressors are located in at least two loci, *prlA* and *secC* (2, 7). In the present study I have identified five additional genes that can be mutated to allow suppression of a *secA*(Ts) mutant. Furthermore, strains containing solely the cold-sensitive mutations show reduced levels of certain exported proteins at the nonpermissive temperature. In at least one case, that of the periplasmic maltose-binding protein (MBP), this defect is at the level of synthesis of the protein. These results point to a general and complex mechanism in *E. coli* in which synthesis and secretion are tightly coupled for at least certain exported proteins.

MATERIALS AND METHODS

Media and reagents. The media used have been described previously (11, 21). Coumermycin and salicin were obtained from Sigma Chemical Co. L-[^{35}S]methionine (specific activity, 1,050 Ci/mmol) was purchased from Amersham Corp. Igsorb was obtained from the New England Enzyme Center. Antiserum to MBP or SecA protein was obtained from W. Boos or was available in the laboratory, respectively.

Bacteria. The bacterial strains used are listed in Table 1. The procedures for performing Hfr crosses and P1 transductions were essentially as described by Miller (21).

Methods of protein analysis. Bacteria were grown in M63 medium containing 0.4% glycerol and 0.4% maltose at the appropriate temperature to a cell density of 2×10^8 to 4×10^8 cells per ml. Samples of 1 ml were labeled with 20 μCi of L-[^{35}S]methionine for 2 min, when the samples were placed on ice. The immunoprecipitation procedure for analysis of MBP and SecA protein was as described previously (25). To isolate periplasmic proteins the cells from a 1-ml culture were pelleted in an Eppendorf centrifuge at 4°C for 1 min and suspended in 100 μl of cold 20% sucrose buffered with 30 mM Tris (pH 8). A 10-μl sample of lysozyme (1 mg/ml in 100 mM EDTA, pH 8) was added, followed by mixing, and the sample was incubated on ice for 30 min. Cells were then pelleted in an Eppendorf centrifuge at 4°C for 5 min, and the supernatant was used as the periplasmic fraction. All proteins were analyzed on gels of 10% acrylamide and 0.13% bisacrylamide. For autoradiography, the gels were fixed in 10% glacial acetic acid for 0.5 h, rinsed in water for 0.5 h, and soaked in 1 M salicylate (pH 6.0) for 1 h. The gel was dried and allowed to expose X-ray film (Kodak XAR-5) at −70°C for 12 to 48 h.

Isolation of suppressors. Each of 60 colonies of MM52 [*secA*(Ts)] were inoculated into 5 ml of LB medium and grown overnight at 30°C. A 0.1-ml sample of a 10^{-4} dilution of each culture was spread onto a MacConkey lactose plate and incubated for 24 h at 37°C. Approximately 12,000 revertants arising on these 60 plates were screened by replica plating at 28 and 37°C for cold-sensitive mutants. The replicas were scored after 7 to 9 h of incubation. A total of 28 cold-sensitive mutants were obtained; 13 that had a stronger cold-sensitive phenotype have been analyzed in detail.

RESULTS

Isolation of extragenic suppressors of a *secA*(Ts) mutant. Extragenic suppressor mutations of an export-defective, *secA*(Ts) mutant were sought by selecting for temperature-resistant derivatives that could now grow at 37°C. The *secA*(Ts) mutant will grow normally at low temperatures (below 32°C), but not at higher temperatures (37°C and above). We have previously found that temperature-resistant derivatives selected at 42°C appear to be revertants in the *secA* gene (2), whereas most derivatives selected at 37°C still harbor the *secA*(Ts) mutation and cannot grow at 42°C (7; present study).

I have sought extragenic suppressors that themselves have an independent phenotype, namely, they confer on the cell carrying the suppressor mutation a cold sensitivity for growth. This limits the study of such suppressors to genes coding for essential cellular proteins, but allows a genetic and physiological analysis of the suppressor mutant in an otherwise wild-type strain background.

Sixty independent cultures of the *secA*(Ts) mutant were plated at 37°C and gave rise to 12,000 colonies, which were screened for cold-sensitive mutants by replica plating at 28 and 37°C. A total of 28 mutants that could grow at 37°C, but not 28°C, were found, and 13 of the strongest mutants have been analyzed in detail. All 13 cold-sensitive mutants grew at 37°C, but not at 28 or 42°C, suggesting that such strains still contain the original *secA*(Ts) mutation. Apparently the suppressor mutation is able to compensate for a partial defect found in the SecA(Ts) protein at 37°C, but not a more complete defect found at 42°C. The presence of the *secA*(Ts)

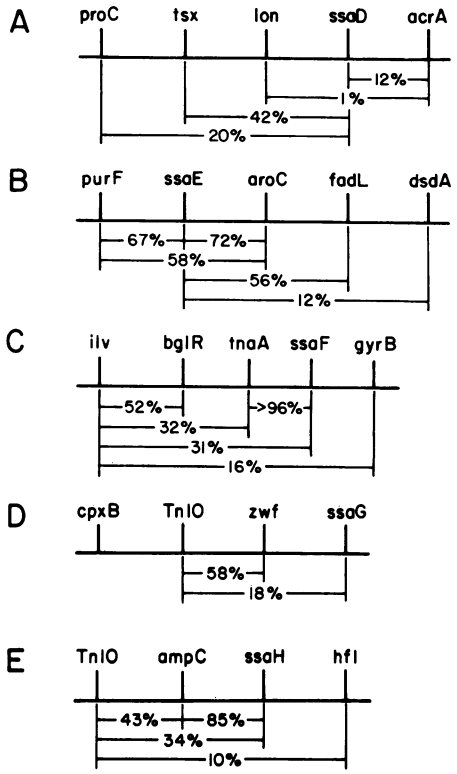

FIG. 1. Genetic map positions of *ssa* mutants. The linkage between various genes is expressed as the percent cotransduction by phage P1. The numbers given are based on genetic crosses where a minimum of 48 and a maximum of 121 transductants were examined for each cross.

mutation in all of these strains was confirmed by a genetic back-cross by P1 transduction (data not shown).

Genetic characterization of the cold-sensitive mutations. To genetically characterize the cold-sensitive mutations, an *secA*$^+$ allele was introduced into each strain carrying the cold-sensitive mutation by P1 transduction. Out of 13 such strains, 10 now grew at 37 and 42°C, but still not at 28°C, indicating that the *secA*(Ts) mutation was, in fact, responsible for the temperature-sensitive phenotype of these strains, and that the cold-sensitive mutation still maintained its phenotype in the presence of the *secA*$^+$ allele. Interestingly, three strains remained both temperature and cold sensitive despite the presence of the *secA*$^+$ allele, whose presence was confirmed by a genetic back-cross with P1 transduction (data not shown). It has been shown by transductional analysis that this double phenotype is due to a single suppressor mutation mapping in or near the recently identified *secC* gene (7).

The 13 strains containing the cold-sensitive mutations alone were screened by P1 transductional analysis to determine whether these mutations mapped near genes previously implicated in protein secretion. Two mutations showed greater than 95% linkage to *rpsE* and are therefore probably alleles of *prlA* (2, 28). Six mutations were approximately 50% linked to a Tn*10* insertion at 69 min on the chromosome and are likely to be alleles of the recently described *secC* gene (7). For the purposes of this paper, it will be assumed that these mutations are alleles of *prlA* and *secC*, respectively. The five remaining mutations neither mapped to these two locations nor were linked to a Tn*10* insertion that is 90% cotransducible with the *secB* gene (16) and therefore represent mutations in new loci.

The five new mutations were mapped by Hfr crosses with a set of 11 different Hfr strains that have different origins and directions of transfer on the *E. coli* chromosome. Spot matings were carried out first, followed by more precise interrupted matings to determine the location of the *cs*$^+$ marker within 5 min. The results showed that the five cold-sensitive mutations were completely unlinked (data not shown). The cold-sensitive mutations were therefore designated *ssaD*, *ssaE*, *ssaF*, *ssaG*, and *ssaH*, where *ssa* designates suppressor of *secA*.

The new *ssa* mutations were mapped by P1 transductions (Fig. 1, Table 2). *ssaD* maps around min 10 on the *E. coli* chromosome and is located between *proC* (9 min) and *acrA* (10.5 min) (Fig. 1A, Table 2). Since the gene coding for the major cytoplasmic protease, *lon* (12), also maps in this region, it is possible that the *ssaD* mutation could be an allele of *lon* which would serve to stabilize the SecA(Ts) protein at 37°C. This seems unlikely since a *lon* marker shows significantly less linkage to *acrA* than does *ssaD* (1

TABLE 2. Transductional mapping of *ssa* mutants[a]

Donor/recipient	Selected marker	Unselected markers	No. obtained	Marker order
proC::Tn*5*	Tn*5*	*ssaD*$^-$ *acrA*$^+$	1	*proC*,
ssaD$^-$ *acrA*$^+$/		*ssaD*$^+$ *acrA*$^-$	120	*ssaD*,
proC$^+$ *ssaD*$^+$		*ssaD*$^-$ *acrA*$^-$	29	*acrA*
acrA$^-$		*ssaD*$^+$ *acrA*$^+$	1	
proC::Tn*5*	Tn*5*	*ssaD*$^-$ *acrA*$^+$	103	*proC*,
ssaD$^+$ *acrA*$^-$/		*ssaD*$^+$ *acrA*$^-$	0	*ssaD*,
proC$^+$ *ssaD*$^-$		*ssaD*$^-$ *acrA*$^-$	0	*acrA*
acrA$^+$		*ssaD*$^+$ *acrA*$^+$	27	
purF::Tn*10*	Tn*10*	*ssaE*$^-$ *aroC*$^+$	21	*purF*,
ssaE$^-$ *aroC*$^+$/		*ssaE*$^+$ *aroC*$^-$	18	*ssaE*,
purF$^+$ *ssaE*$^+$		*ssaE*$^-$ *aroC*$^-$	6	*aroC*
aroC$^-$		*ssaE*$^+$ *aroC*$^+$	3	
purF::Tn*10*	Tn*10*	*ssaE*$^-$ *aroC*$^+$	25	*purF*,
ssaE$^+$ *aroC*$^-$/		*ssaE*$^+$ *aroC*$^-$	60	*ssaE*,
purF$^+$ *ssaE*$^-$		*ssaE*$^-$ *aroC*$^-$	0	*aroC*
aroC$^+$		*ssaE*$^+$ *aroC*$^+$	11	
ilv$^+$ *tnaA*$^-$	*ilv*$^+$	*tnaA*$^-$ *ssaF*$^+$ *gyrB*$^-$	15	*ilv*, *tnaA*, *ssaF*,
ssaF$^+$ *gyrB*$^-$/				
ilv$^-$ *tnaA*$^+$ *ssaF*$^-$ *gyrB*$^+$		*tnaA*$^+$ *ssaF*$^-$ *gyrB*$^+$	64	*gyrB*
		tnaA$^-$ *ssaF*$^+$ *gyrB*$^+$	14	
		tnaA$^-$ *ssaF*$^-$ *gyrB*$^+$	2	
		tnaA$^+$ *ssaF*$^+$ *gyrB*$^+$	1	
ilv::Tn*10* *bglR*$^+$	Tn*10*	*bglR*$^+$ *ssaF*$^+$	33	*ilv*, *bglR*,
ssaF$^+$ *ilv*$^+$		*bglR*^{0} *ssaF*$^-$	42	*ssaF*
bglR^0 *ssaF*$^-$		*bglR*$^+$ *ssaF*$^-$	19	
		bglR^0 *ssaF*$^+$	2	
zeb::Tn*10* *zwf*$^+$	Tn*10*	*zwf*$^+$ *ssaG*$^-$	12	*zeb*::Tn*10*,
ssaG$^-$ *tet*^{s}		*zwf*$^-$ *ssaG*$^+$	28	*zwf*,
zwf$^-$ *ssaG*$^+$		*zwf*$^-$ *ssaG*$^-$	0	*ssaG*
		zwf$^+$ *ssaG*$^+$	26	
zje::Tn*10*	Tn*10*	*ampC*$^+$ *ssaH*$^+$	30	*zje*::Tn*10*,
ampC$^+$ *ssaH*$^+$		*ampC*$^-$ *ssa*$^-$	52	*ampC*,
tet^s *ampC*$^-$		*ampC*$^+$ *ssaH*$^-$	11	*ssaH*
ssaH$^-$		*ampC*$^-$ *ssaH*$^+$	3	

[a] The donor and recipient strains were constructed by P1 transductions starting with the strains given in Table 1. Kanamycin (20 μg/ml), tetracycline (20 μg/ml), or glucose minimal agar was used to select for Tn*5*, Tn*10*, or *ilv*$^+$, respectively. After purification of the transductants, the unselected markers were scored as follows: *ssaD*, *ssaE*, *ssaF*, *ssaG*, and *ssaH*, (28°C); *acrA* (1% sodium dodecyl sulfate); *aroC* (glucose minimal agar); *tnaA*::Tn*10* (20 μg of tetracycline per ml); *gyrB* (50 μg of coumermycin per ml); *bglR* (MacConkey salicin agar); *zwf* (glucose minimal agar); *ampC* (15 μg of ampicillin per ml).

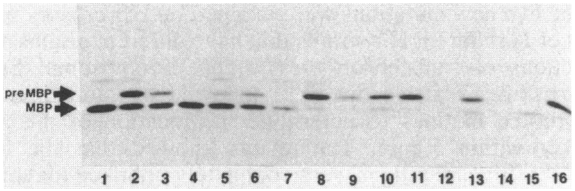

FIG. 2. Suppressors correct the *secA*(Ts) secretion defect. The suppressor strains carrying the *secA*(Ts) mutation were grown in M63 medium supplemented with 0.4% glycerol and 0.4% maltose at 37°C until the midlogarithmic phage, when 0.5 ml of each culture was labeled with 10 μCi of [^{35}S]methionine for 2 min. For comparison, a strain carrying solely the *secA*(Ts) mutation was grown in the same medium at 30°C and shifted to 37°C for 3 h before labeling. In the second part of the experiment these same strains were shifted from 37°C (for the suppressor strains) or 30°C [for the *secA*(Ts) mutant] to 42°C for 3 h before labeling. MBP was prepared from each sample by immunoprecipitation as described in the text. Samples of 20 μl were run on a 10% sodium dodecyl sulfate–polyacrylamide gel, which was fluorographed and used for autoradiography. Lanes: 1 through 7, *secA*(Ts) strains containing suppressors in *ssaD*, *prlA*, *ssaE*, *secC*, *ssaF*, *ssaG*, and *ssaH*, respectively at 37°C; 8, *secA*(Ts) strain at 37°C; 9 through 16, same as lanes 1 through 8, except at 42°C. The positions of MBP precursor (preMBP) and the mature form (MBP) are given.

versus 12%, Fig. 1A), although transductional analysis with *lon* mutants may show some irregularities. Furthermore, the SecA(Ts) protein appears to be stable in vivo at 37°C (data not shown), suggesting a mechanism of restoration of function other than lowered proteolysis. Finally, to rule out the possibility that a *lon* mutation could stabilize the *secA*(Ts) protein at 37°C, a *lon*$^-$ *secA*(Ts) double mutant was constructed by using a *lon* deletion and shown to be temperature sensitive at 37°C (data not shown).

ssaE maps in min 50 on the *E. coli* chromosome between *purF* (50 min) and *aroC* (50.5 min) (Fig. 1B, Table 2). *ssaF* maps in min 83 on the *E. coli* chromosome in a region densely packed with known genetic markers. A four-factor P1 cross reveals that *ssaF* appears to map between *tnaA* and *gyrB* (Fig. 1C, Table 2), although this order is somewhat uncertain due to the strong linkage between *tnaA* and *ssaF*. A three-factor P1 cross shows that *ssaF* definitely maps to the counterclockwise side of *bglR* (26) (Table 2) and is therefore at least between *bglR* and *gyrB*.

ssaG maps in min 41 on the *E. coli* chromosome. By using a Tn*10* insertion near the *cpxB* gene (18), *ssaG* has been mapped to the clockwise side of *zwf* (Fig. 1D, Table 2) in a region sparse with known genetic markers.

ssaH maps in min 94 on the *E. coli* chromosome. Using a Tn*10* insertion in this region (Fig. 1E), *ssaH* was mapped to the clockwise side of *ampC* (Table 2), thus placing it between *ampC* and *hfl*.

Correlation of the cold-sensitive mutation with the suppressor mutation. In isolating the *ssa* mutations described here, it was assumed that the cold-sensitive phenotype was due to the suppressor mutation. Having accurately mapped the various cold-sensitive mutations, I have tested this assumption by measuring the linkage between the cold-sensitive and suppressor mutations. This linkage was measured by transducing the double-mutant strains to *cs*$^+$ and determining whether the strain still could grow at 37°C. Tn*5* or Tn*10* insertions near the *cs*$^+$ alleles of the donor strain were used to transduce the recipient to kanamycin or tetracycline resistance at 30°C. All such transductants tested were no longer able to form single colonies at 37°C (24 transductants tested for each), indicating greater than 95% linkage of the cold-sensitive and suppressor mutations. Interestingly, transductants arising when the *secA*(Ts) *ssaD*(Cs) recipient strain was transduced to *ssaD*$^+$ did grow somewhat better at 37°C than did the *secA*(Ts) control strain, indicating the presence of a second, weaker suppressor mutation in this one strain that is unlinked to the *ssaD*(Cs) suppressor mutation. However, by transducing solely the *ssaD*(Cs) mutation into the *secA*(Ts) strain, it was possible to show that this suppressor alone is strong enough to restore growth at 37°C.

Physiology of strains containing the suppressor mutations. Suppression of the temperature-sensitive defect in the *secA*(Ts) mutant could be achieved by a variety of mechanisms, some of which may not involve direct protein-protein interaction. To test whether the suppressors not only correct the temperature-sensitive defect of the *secA*(Ts) mutant, but also correct the secretion defect, the secretion of MBP was checked in these strains. It has been shown previously that the accumulation of MBP precursor in the *secA*(Ts) mutant is due to a secretion block (24), and therefore the amount of MBP processing serves as a measure of the secretion proficiency of these strains. MBP secretion and processing is at least partially restored at 37°C in *secA*(Ts) strains containing the suppressor mutations compared with the *secA*(Ts) parent (Fig. 2, compare lanes 1 through 7 with lane 8). Certain mutations are stronger suppressors of the secretion defect than others as judged by the amount of MBP precursor remaining in strains containing these mutations. For example, *secA*(Ts) strains containing suppressor mutations in *ssaD* and *secC* show little or no MBP precursor accumulation (Fig. 2, lanes 1 and 4), whereas *secA*(Ts) strains containing *ssaE*, *ssaF*, and *ssaG* suppressors still contain low levels of MBP precursor (Fig. 2, lanes 3, 5, and 6). In two cases, MBP secretion continues to show major defects. In the *secA*(Ts) strain containing the *prlA* suppressor MBP secretion remains blocked by as much as 50% (Fig. 2, lane 2), whereas the *secA*(Ts) strain containing the *ssaH* suppressor makes only lower levels of mature MBP (Fig. 2, lane 7).

Since the suppressor mutations restored growth of the *secA*(Ts) mutant at 37°C, but not at 42°C, the secretion proficiency of these strains has also been checked at 42°C. At this higher temperature, all of the double-mutant strains show defects in MBP secretion, synthesis, or a combination of both. For example, *secA*(Ts) strains containing suppressors in *ssaD*, *prlA*, *ssaE*, and *ssaF* when grown at 42°C now synthesize mainly unsecreted MBP precursor, but often in reduced amounts when compared with the *secA*(Ts) parent (Fig. 2, compare lanes 9, 10, 11, and 13 with lane 16). Other strains containing suppressors in *secC* and *ssaG* show little or no MBP present (Fig. 2, lanes 12 and 14), whereas the strain containing the *ssaH* suppressor does make a low level of mature MBP (Fig. 2, lane 15). This reduction in synthesis is specific for MBP and certain other proteins, since incorporation of [^{35}S]methionine into most cellular proteins is normal in these strains (data not shown). Ferro-Novick et al. (7) have recently demonstrated that *secC*(Cs) single mutants are defective in the synthesis of several periplasmic and outer membrane proteins, including MBP, when grown at low temperatures. This effect is presumably similar to what is seen here in the double mutant background, where the suppressor mutation may be having an epistatic effect on the *secA*(Ts) mutant. I conclude that the temperature-sensitive phenotype and the secretion proficiency of the doubly mutant strains are directly correlated: growth and secretion are enhanced at 37°C, but not at 42°C.

If the suppressor mutations are restoring the secretion

proficiency of the *secA*(Ts) strain by altering a component of the secretion machinery which directly interacts with the SecA protein, then strains containing solely these mutations (*secA*$^+$ background) may show defects in the synthesis or secretion of exported proteins at low temperatures. This prediction has been verified. The wild-type and cold-sensitive mutant strains were grown at 40°C and shifted to 23°C for 1 h, when they were pulse-labeled with [^{35}S]methionine; protein export to the periplasmic space was analyzed (Fig. 3, lanes 1 and 3 through 10). The periplasmic protein profile for the cold-sensitive mutants is complex: certain proteins are reduced in amount (62K and MBP), others are increased in amount, whereas others show little quantitative change when compared with the wild type. That the level of certain periplasmic proteins is unaffected by the cold-sensitive mutations indicates that these mutants do not simply possess a leaky outer membrane or are less susceptible to the spheroplasting procedure used. Although all of the mutants show a reduced amount of the 62K protein and MBP, this reduction varies between two extremes, with the *ssaH* mutant showing the greatest reduction and the *ssaG* mutant showing the least reduction in these two proteins (Fig. 3, lanes 9 and 8, respectively). For comparison, the periplasmic proteins present in a *secA*(Ts) mutant grown at the nonpermissive temperature are included in this experiment (Fig. 3, lane 2). The secretion block seen in the *secA*(Ts) mutant is in general stronger and affects a greater number of periplasmic proteins than the blocks found in most of the cold-sensitive mutants, with the exception of the *ssaH* mutant (Fig. 3, compare lane 2 with lane 9).

I have determined whether the reduced amount of periplasmic MBP present in the cold-sensitive mutants is due to a defect at the level of synthesis or secretion. Total MBP was prepared by immunoprecipitation (Fig. 3). It is apparent that the reduced level of periplasmic MBP found in the cold-sensitive strains is due to a reduction in the synthesis of this protein, since all of these strains make less MBP than the wild type (Fig. 3, compare lanes 13 through 20 with lane 11), with the possible exception of the *ssaG* mutant (lane 18). As a control, it was found that [^{35}S]methionine incorporation into most cellular proteins in these mutants remained normal (data not shown). An *secA* amber mutant strain also shows marked reduction in MBP synthesis (25). Therefore, it is possible that the suppressor mutations are reducing MBP synthesis by lowering SecA levels in the strains. To test this possibility, SecA synthesis was measured in the cold-sensitive mutants and found to be as great as that in the wild type (Fig. 3, compare lanes 23 through 30 with lane 21). In contrast, the *secA*(Ts) mutant continues to make normal levels of MBP, which accumulates as a precursor despite a dramatic increase in SecA synthesis (Fig. 3, lanes 12 and 22). I conclude that the cold-sensitive mutants show defects in MBP synthesis at the nonpermissive temperature even though SecA levels appear to be at least as great as those of the wild type.

DISCUSSION

Genetic selections with *E. coli* have identified four genes, *prlA* and *prlC* (5, 29) and *secA* and *secB* (16, 24), that are involved in protein secretion. A genetic approach involving extragenic suppressor mutations has been used here in an attempt to identify additional genes involved in protein export. Suppressors that correct the temperature-sensitive defect found in an *secA*(Ts) mutant fall into at least seven genes, two of which (*prlA* [2] and *secC* [7]) have been previously implicated in protein secretion. The five remaining loci (*ssaD*, *ssaE*, *ssaF*, *ssaG*, and *ssaH*) have been mapped by P1 transduction and appear to define new genes in *E. coli*. Since only a single mutant in each of the latter five genes was isolated, additional genes may well be identified with this approach.

Although the cold-sensitive suppressor mutants show reduced levels of certain periplasmic proteins at the nonpermissive temperature, the magnitude of this effect varies for the different mutants and is in general not as dramatic as the

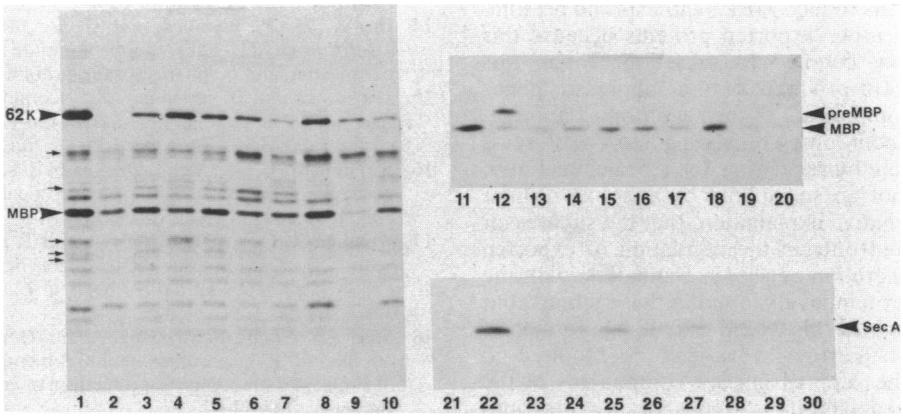

FIG. 3. Cold-sensitive mutants show reduced levels of certain periplasmic proteins. Wild-type (MC4100) or cold-sensitive mutant strains were grown in M63 medium supplemented with 0.4% glycerol and 0.4% maltose at 40°C until the early logarithmic phase, when they were shifted to 23°C for 1 h before labeling. For comparison, the *secA*(Ts) mutant was grown in the same medium at 30°C and shifted to 40°C for 100 min before labeling. A 1-ml sample of each culture was labeled with 20 μCi of [^{35}S]methionine for 2 min and placed on ice. Periplasmic proteins, MBP, and SecA were prepared from each sample as described in the text. Samples of 20 μl were run on 10% sodium dodecyl sulfate–polyacrylamide gels, which were fluorographed and used for autoradiography. Lanes: 1 through 10, periplasmic proteins; 1, MC4100; 2, *secA*(Ts) mutant at 40°C; 3 through 10, cold-sensitive mutations in *prlA*, *secC*(Cs4), *secC*(Cs21), *ssaD*, *ssaF*, *ssaG*, *ssaH*, and *ssaE*, respectively; 11 through 20, MBP immunoprecipitates from the total cultures given in lanes 1 through 10, respectively; 21 through 30, SecA immunoprecipitates from the total cultures given in lanes 1 through 10, respectively. The positions of a 62-kilodalton protein (62K), MBP precursor (preMBP) and the mature form (MBP), and SecA protein (SecA) are given. The small arrows indicate protein species that are increased in amount in certain mutant strains.

secretion block seen for the *secA*(Ts) mutant. However, the genetic selection used to obtain the suppressor mutations had two requirements: correction of the *secA*(Ts) defect and the acquisition of an additional cold-sensitive defect. This double requirement may have severely limited the types of mutations that could be obtained in the relevant genes. Now that the various *ssa* genes have been identified, it is possible to use more direct approaches to obtain more general types of mutations (3, 13) and to study their effect on protein synthesis and secretion.

Strains containing the cold-sensitive suppressor mutations halt or reduce MBP synthesis when shifted down to the nonpermissive temperature. This defect is apparently at the level of synthesis, since the procedure used here (pulse-labeling and sodium dodecyl sulfate denaturation at 100°C) should have minimized any differences in MBP levels due to degradation. Analogous results have been obtained with *secA*(Am) and *secC*(Cs) mutants (7, 25). In the case of a *secC*(Cs) mutant this block was shown to affect the synthesis of two periplasmic proteins, MBP and RBP, and two outer membrane proteins, OmpF and LamB. These findings, taken together, indicate that *E. coli* may possess a general and complex mechanism for coupling protein synthesis and secretion.

Although suppressor mutations can be used to infer direct interactions between two different proteins, suppression can occur by other, less direct mechanisms. The limited physiology presented here does cast some light on the possible mechanisms of suppression. First, since the suppressors enhance both the growth and secretion of the *secA*(Ts) mutant, they apparently are allowing the cell to regain some SecA function or some other export-related function. Since they do not suppress these defects at 42°C, they evidently have not bypassed a cellular requirement for some level of SecA function. Second, since the suppressor mutations alone reduce the levels of certain periplasmic proteins and these same protein species are subject to secretion blocks in the *secA*(Ts) and *secB* mutants (Fig. 3) (16), all of these genes may code for or regulate a common export pathway. If this explanation is correct, then it must be assumed that the cold-sensitive mutations reduce MBP synthesis and presumably the synthesis of other exported proteins because this synthesis is somehow coupled to secretion. Given this assumption, the protein products of the suppressor genes could be structural components of the secretion machinery, or they could affect secretion by regulating SecA activity. If the suppressors operate by regulating SecA protein activity, they apparently do not do so directly by affecting cellular SecA levels. An alternative explanation, that the suppressor mutations are involved only in the regulation of exported proteins and not in secretion per se, is compatible with the altered periplasmic protein levels found in these strains, but provides no clue as to how this would lead to suppression of the *secA*(Ts) mutant. Further work will be required to determine whether the suppressors are components of the export machinery, regulate its activity, or are tied into protein synthesis and export in an as yet undefined way.

ACKNOWLEDGMENTS

I thank Susan Ferro-Novick, Mary Honma, and Jon Beckwith as well as Russell Maurer, Barbara Osmond, and David Botstein for sending their manuscripts before publication. I also thank Laura Liss for help in mapping *ssaD*, Judy Wong for technical assistance, and Phyllis Leder and Sandra Burns for preparation of this manuscript.

This work was supported by Public Health Service grant GM32958 from the National Institute of General Medical Sciences.

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