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secD, a New Gene Involved in Protein Export in *Escherichia coli*†

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New mutants of *Escherichia coli* altered in protein export were identified in *phoA-lacZ* and *lamB-lacZ* gene fusion strains by searching for mutants that showed an altered lactose phenotype. Several mutations mapped in a new gene, *secD*. These mutants were, in general, cold sensitive for growth, and the mutations led to an accumulation of precursor of exported proteins. The *secD* gene is closely linked to *tsx* on the *E. coli* chromosome, but separable from another gene proposed to be involved in export, *ssaD*, which maps nearby. A plasmid carrying *secD*$^+$ was identified and used to show that the mutations are recessive. The *secD* gene may code for a component of the cellular export machinery.

In vitro and genetic studies suggest that there exists in *Escherichia coli* a secretion machinery required for the export of proteins to the cell envelope. Studies by Muller and Blobel on an in vitro system have revealed the existence of a 12S particle required for the transfer of secretory proteins into membrane vesicles (14). Genetic analysis has led to the identification of at least three genes whose products appear to be required for protein export in vivo. Mutations in the *secA* (15), *secB* (10), and *prlA* (or *secY*) (6, 9) genes have pleiotropic effects on protein export, causing the accumulation in the cytoplasm of precursors of a variety of membrane and periplasmic proteins. Although it is not established that the protein products of these genes play a direct role in export, certain lines of evidence make this seem likely. One of these is the finding that mutations in the *prlA* gene can either enhance or inhibit protein export (6, 9). Furthermore, the demonstration that the SecA protein is regulated by the secretion needs of the cell suggests an important role for that protein in the export process (16). A variety of procedures which lead to a block in protein export cause a derepression of the synthesis of the SecA protein. It is as though the cell can sense when its secretion machinery is not sufficient and compensates by increasing the concentration of at least this component.

One approach to isolating mutants defective in the secretion machinery involves the use of strains carrying fusions between an exported protein and β-galactosidase (3). In many cases, such hybrid proteins are inbedded in the membrane. In this location they have very low β-galactosidase activity, and the strains exhibit a Lac$^-$ phenotype. Selection for Lac$^+$ derivatives of such strains is a selection for internalization of the hybrid protein and therefore a selection for mutants with secretion defects. In this paper, we describe the use of the Lac$^+$ selection with fusions of β-galactosidase to the periplasmic enzyme, alkaline phosphatase, and to the outer membrane protein LamB. Both selections have yielded a class of mutants in a gene which we have termed *secD*. *secD* mutations have pleiotropic effects on secretion similar to those with previously described *sec* mutations.

MATERIALS AND METHODS

Media. TYE, M63, and tetrazolium media were described by Miller (13). Labeling medium was M63 supplemented with a mixture of 18 of the 20 common amino acids at 50 μg/ml each. Other additions included thiamine (1 μg/ml) and either 0.4% glucose, maltose, or ribose. Ampicillin was used at a final concentration of 200 μg/ml. Tetracycline was added to 20 μg/ml. Streptomycin was used as described by Miller (13). Restriction enzymes were from New England BioLabs. 5-Bromo-4-chloro-3-indolylphosphate was obtained from Bachem, Inc.

Genetics. All bacterial strains are listed in Table 1.

To test the possibility that *secD1* was a mutation within the *phoA* gene, a P1 lysate grown on MPh44 was used to transduce MPh101 to Kan^r on TYE–5-bromo-4-chloro-3-indolylphosphate plates. All Kan^r transductants were cross-streaked against T6 phage to be sure the Tn*5* had not hopped. Of the 62 Kan^r T6^r transductants tested, 39 were white on the 5-bromo-4-chloro-3-indolylphosphate plates, demonstrating the recovery of the *phoA-proC* deletion. Of the 39 Δ(*phoA-proC*) derivatives, 1 was cold sensitive and was shown to accumulate the precursor to maltose-binding protein. Thus, the mutation did not map within the *phoA* gene, but was near the *tsx* gene, since 58 of 62 (93.5%) Kan^r transductants were cold resistant.

Tn*10* insertions near *secD* were obtained as described by Kumamoto and Beckwith (10).

Strain constructions. Strains ECB2170, -2172, -2176, -2177, and -2179 were constructed by transducing pop3299 to Lac$^+$ with P1 *vir* lysates prepared on the *malB*$^+$ cold-sensitive (Cs) transductants described below. Strain ECB2731 was made by first infecting MC4100 with P1 grown on SG46 (*proC*::Tn*10*), selecting tetracycline resistance (Tet^r), and then infecting this derivative with P1 grown on MPh44(*tsx*::Tn*5*) and selecting for kanamycin resistance (Kan^r). A Tet^r Pro$^-$ Kan^r T6^r transductant was called ECB2731. Strain ECB2735 was made by transducing ECB2731 to Pro$^+$ by using a P1 *vir* lysate prepared on

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† We dedicate this paper to Hiroshi Inouye, who died on 24 July 1986. Hiroshi's pioneering contributions on alkaline phosphatase and the *phoA* gene in this laboratory were essential to the work described here. His enthusiasm was an inspiration for all who knew him.

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TABLE 1. *E. coli* strains

Strain	Genotype	Origin
ECB2170	pop3299 *secD2170*	This study
ECB2172	pop3299 *secD2172*	This study
ECB2176	pop3299 *secD2176*	This study
ECB2177	pop3299 *secD2177*	This study
ECB2179	pop3299 *secD2179*	This study
ECB2731	MC4100 *proC*::Tn*10* *tsx*::Tn*5*	This study
ECB2735	MC4100 *secD2177*	This study
ECO	F (Ts) *lac*/Δ(*lac-pro*)XIII *supE*	Beckwith collection
EC150	Hfr [*tsx*::F(Ts) *lac*] Δ(*lac-pro*)XIII *supE*	This study
L7005	*thr* Δ(*ara-leu*)*1119 trp9605*(Am) *arg his-29*(Am) *tsx*	Beckwith collection
MC1000	F⁻ *araD139* (*ara-leu*)*7697* Δ*lac74 galE galK rpsL*	Beckwith collection
MC4100	F⁻ *araD*Δ*lacU169 relA rpsL*	Beckwith collection
MPh42	MC1000 *phoR*	Beckwith collection
MPh44	MC1000 *phoR* Δ(*phoA-proC*) *tsx*::Tn*5*	Beckwith collection
MPh101	MC1000 *phoR secD1*	This study
MPh102	MC1000 *zaj*::Tn*10*	This study
MPh103	MC1000 *zaj*::Tn*10 secD1*	This study
MPh1061	*araD169* Δ(*ara-leu*)*7697* Δ*lac74 galE galK phoA61 rpsL*	Beckwith collection
NF1706	*thr-1 leuB6 proA2 argE3 phr-1 recA1 uvrA6 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44 ilv*	Niels Fiil
pop3299	MC4100 λpφ*lamB-lacZ42-12*	7
SG46	MC4100 *proC*::Tn*10*	S. Garrett
X53	F⁻ *lac purE thi rpsL supE*	Beckwith collection

ECB2177. All Pro⁺ Tet^s Kan^s transductants exhibited the cold-sensitive phenotype.

An Hfr strain with F integrated in *tsx* was made by the method of Beckwith et al. (2). Briefly, a strain deleted for the lactose operon on its chromosome, harboring an F′ Ts *lac* episome, can be used to isolate an Hfr strain by simply screening for Lac⁺ at 42°C. If a culture of the strain is plated on a lawn of T6 bacteriophage at 42°C, then the Lac⁺ colonies appearing are candidates for those cells in which the F′ upon integration has interrupted the gene coding for the T6 phage receptor. Onto a tetrazolium lactose plate spread with 10^{11} T6 phage particles, 10^8 cells of strain ECO (grown in M63 lactose supplemented with the 18-amino-acid mix) were plated and incubated at 42°C. Large white colonies appeared after incubation and were purified and shown to be resistant to T6 by cross-streaking. These Hrf strains were shown to transfer the *leu* (2′) marker but not the *gal* (17′) and *purE* (12′) markers early in mating experiments with MC1000 and X53 (1).

Immunoprecipitations. Cultures (5 ml) were grown at 37°C, shifted to 23°C for 1 or 2 h, and pulse-labeled with [^{35}S]methionine for 30 s. Immunoprecipitations were performed, and samples were analyzed on 10% polyacrylamide gels as previously described (8). Antiserum to maltose-binding protein was kindly provided by Winfried Boos; antisera to OmpA, OmpF, and LamB were provided by Tom Silhavy; antiserum to ribose-binding proteins was provided by Jeff Garwin; and to antiserum to SecA was provided by Don Oliver. Anti-alkaline phosphatase antibody was raised in rabbits with help from Steve Lory.

Cloning of *secD*. Strain MPh101 was transformed with a plasmid library of partially *Sau*3A digested *E. coli* chromosomal fragments inserted into the *Bam*HI site of pBR322, kindly provided by Yu-Mei Chen. Clones were selected by looking for fast-growing transformants at the nonpermissive temperature (23°C) on TYE-ampicillin plates. The largest plasmid, pCG169, also complemented the *ssaD* mutant. Two of the plasmids were found to have the *tsx* gene as determined by transformation of NF1706 and L7005.

Selection of Lac⁺ mutants of a *lamB-lacZ* gene fusion. Strain pop3299 carries the *lamB-lacZ42-12* fusion, which encodes a hybrid protein containing 241 residues of LamB fused to a functional β-galactosidase moiety (5). Strains carrying this fusion are sensitive to the inducer maltose (Mal^s) and are unable to grow on the sugar lactose (Lac⁻) (7). We selected for a Lac⁺ phenotype on lactose-tetrazolium medium and screened for retention of the Mal^s phenotype as described previously (4). Maltose sensitivity was assayed as described previously, except the plates were incubated at 30°C (4). Genetic analysis of the five cold sensitive mutants revealed that the Lac⁺ phenotype was due to two mutations, one linked to the gene fusion and one unlinked. The mutation conferring the cold-sensitive phenotype mapped to the unlinked mutation in all cases.

RESULTS

Selections with a *phoA-lacZ* fusion strain. Strain MPh100 (12) has a *phoA-lacZ* gene fusion carried by a lambda transducing phage inserted by homology adjacent to the *phoA* gene. This strain is Lac⁻ due to the membrane location of the hybrid protein. We isolated 100 independent Lac⁺ mutants from this strain. Of these, 74 carried mutations which were genetically linked to the gene fusion; most or all were signal sequence mutations. The remaining 26 were genetically unlinked to the fusion.

The unlinked mutations were candidates for mutations in genes involved in the secretion process. We examined these mutants to determine whether there was any defect in protein export. First, the strains were cured of the lambda phage carrying the gene fusion so that only an intact *phoA* gene was present. We then precipitated alkaline phosphatase with antibody to see whether there were any defect in the export of this protein. One of these mutants, MPh101, accumulated alkaline phosphatase precursor (Fig. 1) and was studied further. We examined MPh101 to see whether the mutation had pleiotropic effects on protein export. Antibody to maltose-binding protein, another periplasmic protein, was used to precipitate pulse-labeled proteins; again, small amounts of maltose-binding protein precursor were observed (Fig. 2).

Strain MPh101 exhibits a cold-sensitive phenotype; it grows at 37°C, albeit more slowly than the wild type, but barely at all at 23°C. When cells of this strain were shifted from 37 to 23°C for 2 h and pulse-labeled with [^{35}S]methionine, a much stronger defect in maltose-binding protein export was observed (Fig. 2). Under the same conditions, precursors to proteins LamB, OmpF, ribose-binding protein, and OmpA were seen (Fig. 3; data shown only for ribose-binding protein and OmpF). We have named the mutation in MPh101 *secD1*.

The properties of this mutant are quite similar to those of the *secA*(Ts) mutant and of the *secY*(Ts) mutant allele of the *prlA* gene, both of which inhibit the export of a number of cell envelope proteins. In addition, the *secA* and *secY* mutations cause a substantial increase in the synthesis of

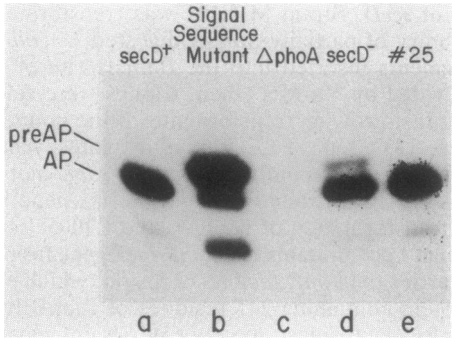

FIG. 1. Effect of *secD1* on alkaline phosphatase. Lanes: a, MPh42 (*secD*$^+$); b, MPh1061 (signal sequence mutant); c, MPh44 [Δ(*phoA-proC*)]; d, MPh101 (Lac$^+$ derivative of MPh100, cured of λ456); f, no. 25, an independent Lac$^+$ mutant of strain MPh100. This strain is another Lac$^+$ derivative of MPh100 in which no precursor to alkaline phosphatase is observed. Cells were grown to the early log phase at 37°C and pulse-labeled for 30 s with [^{35}S]methionine.

SecA protein at the nonpermissive temperature (16; Liss and Oliver, personal communication). Here we show that a dramatic increase in the amount of SecA protein is also seen when a strain with the *secD* mutation is shifted to the nonpermissive temperature (Fig. 4).

Mapping of *secD*. We next tested whether the mutation in MPh101 was linked to other loci known to be involved in protein secretion, *secA*, *secB*, and *prlA* (*secY*). Using P1 lysates made on strains carrying transposon insertions linked to each of these genes, we showed that the new mutation was not near any of these loci.

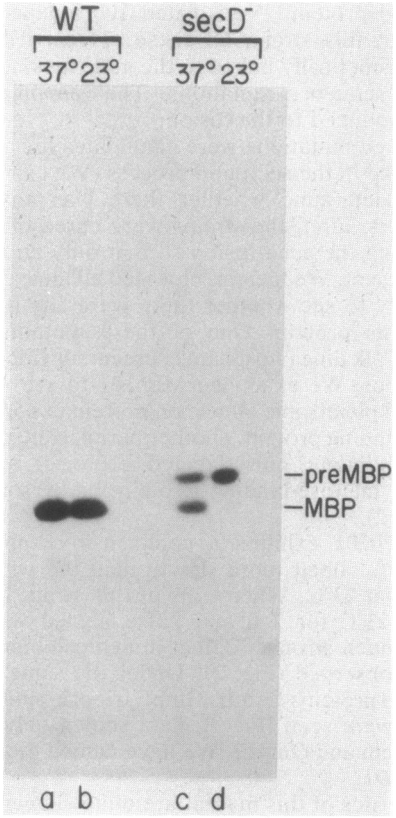

FIG. 2. Effect of *secD1* on maltose-binding protein (MBP), including temperature shift. Lanes: a, MPh42 at 37°C; b, MPh42 shifted to 23°C for 2 h before labeling; c, MPh101 at 37°C; d, MPh101 shifted to 23°C for 2 h before labeling.

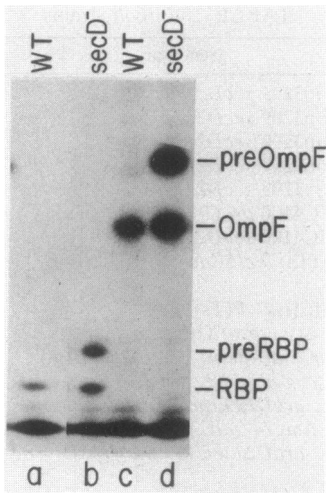

FIG. 3. Effect of *secD1* on ribose-binding protein (RBP; lanes a and b) and OmpF (lanes c and d) in strains MPh42 (lanes a and c) and MPh101 (lanes b and d). Both strains were shifted to 23°C for 2 h before labeling.

In other crosses involving the *proC* region, we fortuitously discovered that the cold sensitivity was linked to *proC* but clearly separable from *phoA*. Further, in all transductions, the cold sensitivity and secretion defects segregated together, indicating that a single mutation was responsible for both phenotypes.

We tested for P1 transduction linkage of the *secD* gene to other markers in the *phoA* region. When a P1 lysate made on a strain carrying a Tn*5* insertion in *tsx* (closely linked to *proC*) was used to transduce a *secD*(Cs) strain, 93.5% of the Kan^r transductants were cold resistant. By using an Hfr

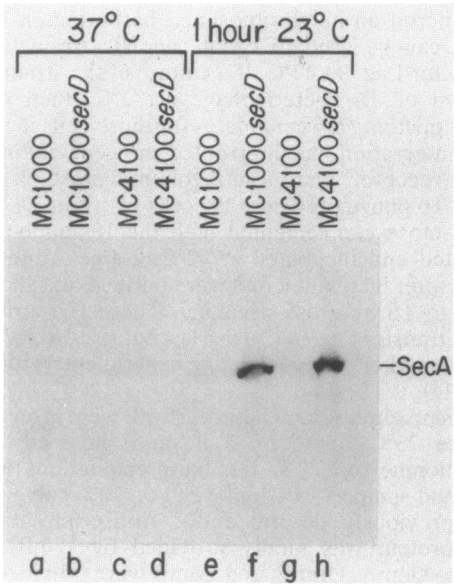

FIG. 4. Regulation of SecA protein in the *secD1* mutants. Lanes, a through d, 37°C; e through f, shifted for 1 h to 23°C before labeling; a and e, MPh42; b and f, MPh101; c and g, MC4100 *zaj*::Tn*10* *secD*$^+$; d and g, MC4100 *zaj*::Tn*10* *secD1*.

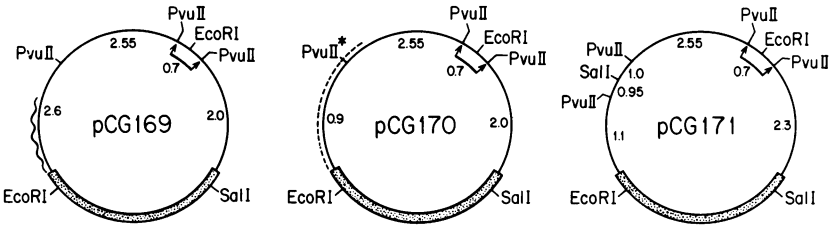

FIG. 5. Plasmids carrying the *secD* gene. The thick dotted line shows the pBR322 backbone. Numbers represent kilobases. Plasmids pCG169 and pCG170 also carry the *tsx* gene. The largest plasmid pCG169 complements the cold-sensitive defect of the *ssaD* mutant. *secD* appears to lie between the *Pvu*II* and *Sal*I sites. *tsx* lies in the region with the dotted line. The most likely location of *ssaD* is within the *Eco*RI-proximal end of the 2.6-kilobase *Eco*RI-*Pvu*II fragment of pCG169.

strain, EC150, which donates counterclockwise and with a point of origin in *tsx* (Materials and Methods), we showed that *secD* maps on the *proC* side of *tsx*. Another gene in this region which was suggested to be involved in secretion, *ssaD*, appears to lie on the other side of the origin of EC150 from *secD*.

Selections with a *lamB-lacZ* fusion strain. Strain pop3299 carries the *lamB-lacZ* fusion 42-12, which encodes a hybrid protein containing 241 amino acid residues of the LamB protein fused to the amino terminus of β-galactosidase. The protein has little or no β-galactosidase enzymatic activity because of its membrane location (5). In a selection somewhat more complex than that described for the *phoA-lacZ* fusion strain, we took advantage of the Lac$^-$ phenotype to select mutant derivatives with secretion defects (see Materials and Methods). Of about 1,500 Lac$^+$ mutant derivatives of strain pop3299, 5 showed a growth defect at 25 to 28°C. In each case the cold-sensitive phenotype was unlinked to the *lamB-lacZ* fusion. Hfr crosses suggested that the cold sensitivity mutations were linked to *proC*; this was confirmed by P1 transductions. Additional crosses showed that these mutations were closely linked to *tsx*, like the *secD* mutation described above. One mutation was crossed into MC4100 and shown to synthesize maltose-binding protein precursor at the nonpermissive temperature (data not shown).

Cloning of the *secD* gene. Using an *E. coli* clone bank prepared with plasmid pBR322 (Materials and Methods), we have found three plasmids which complement the *secD* mutation in strain MPh101. The restriction maps of these plasmids are shown in Fig. 5. Two of the plasmids also complement a *tsx* mutation, whereas one does not. The plasmids which complement the *secD* mutation share two common adjacent *Pvu*II fragments. Subclones carrying portions of this fragment also complement the *secD* mutation (data not shown).

Since the same plasmids restored a cold-resistant phenotype to the *secD* mutant affecting *lamB-lacZ* expression, we conclude that the mutations identified here map in the same gene or in adjacent genes. However, only one of the three plasmids complemented *ssaD*, which therefore appears to define a different gene.

DISCUSSION

Using comparable selections for the internalization of hybrid proteins composed of β-galactosidase and either alkaline phosphatase or LamB protein, we have obtained a set of mutants with cold sensitivity mutations which have pleiotropic effects on protein export. Genetic mapping and cloning studies indicate that these mutations are very tightly linked and may be in the same gene. Since previous mutants obtained by this selection were given the *sec* nomenclature, we will call this new gene *secD*.

The *secD* gene is tightly linked to the *tsx* gene. Both three- and four-factor crosses and mapping with an Hfr, the origin of which is in the *tsx* gene, indicate that *secD* maps on the *proC* side of *tsx*. A second locus connected to secretion, *ssaD*, has been identified in this region (17). An *ssaD* mutation suppresses the temperature sensitivity and secretion defects of a *secA*(Ts) mutation. However, crosses with the Hfr strain described above and complementation studies with *secD* clones indicate that *ssaD* is a distinct gene mapping on the other side of *tsx* from *secD*. Recent studies show that most of the suppressors of the *secA*(Ts) mutation alter components of the protein-synthesizing machinery and exert their suppression indirectly by reducing the rate of protein synthesis (11, 18). Thus, it is possible that the *ssaD* mutation is in a gene for such a component.

Mutations which cause pleiotropic defects in protein secretion have been described now in four genes, *secA*, *secB*, *secD*, and *prlA* (the *secY* allele). These genes map at four separate sites on the *E. coli* chromosome (Fig. 6). An exhaustive use of the Lac$^+$ selection with a strain carrying a *malE-lacZ* gene fusion yielded only *secA* and *secB* mutations (10). Selections reported here with *phoA-lacZ* and *lamB-lacZ* fusion strains revealed only *secD* mutations. The *secY* alleles of the *prlA* gene were found only after localized mutagenesis of the *prlA* region of the chromosome and a hunt for temperature-sensitive mutants (9). Yet, the *secA*, *secD*, and *secY* mutations all reduce the secretion of maltose-binding protein, LamB protein, and alkaline phosphatase. (*secB* mutations only affect a subset of cell enve-

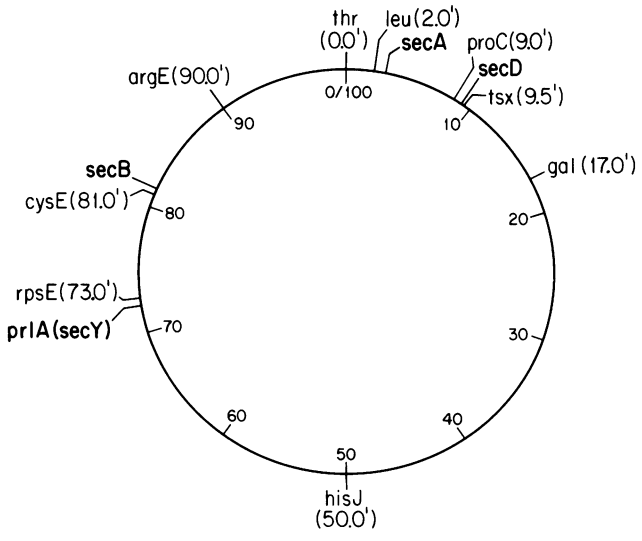

FIG. 6. Location of *sec* genes on the *E. coli* chromosome (1).

lope proteins.) It is surprising, then, that all three classes of mutations were not detected in each selection. At any rate, the results show the utility of using several systems for such selections.

Only 1 of 26 unlinked mutations which gave a Lac^+ phenotype in combination with the *phoA-lacZ* fusion had an observable effect on protein secretion. The other 24 may either have very weak effects or may restore a Lac^+ phenotype by some other mechanism. For instance, the alkaline phosphatase–β-galactosidase hybrid protein is somewhat unstable. Some of the unlinked mutations may reduce the breakdown of this protein.

Although various lines of evidence suggest that the *sec* genes detected so far are required for protein secretion, there is no direct confirmation of this supposition. Final proof of their role will come from the correlation of the effects of such mutants with the functioning of the *sec* gene products in an in vitro system.

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