We have followed the synthesis and secretion of a number of periplasmic and outer membrane proteins in three strains of *Escherichia coli*, a secA amber mutant, a secA temperature-sensitive mutant, and a strain that blocks protein secretion due to a high level of expression of an export-defective hybrid protein between maltose-binding protein and β-galactosidase (MalE-LacZ). Our results show that after several hours under nonpermissive conditions the specificity and extent of the export blocks in the secA temperature-sensitive mutant and the strain producing the MalE-LacZ hybrid protein are identical, affecting at least four major outer membrane proteins and most but not all periplasmic proteins. The secA gene product, therefore, appears to be an essential component of the major export pathway in *E. coli* which is used by many envelope proteins independent of whether they are cotranslationally or post-translationally secreted. In contrast, the synthesis of only a subset of these envelope proteins is reduced in the secA amber mutant after shift to the nonpermissive condition. These results indicate that the SecA protein serves roles both in the synthesis and the secretion of certain cell envelope proteins.

Recent studies in the field of protein secretion indicate that the initial stages of protein export in procaryotic and eucaryotic organisms bear certain similarities. First, many secreted proteins are made as larger precursors containing an amino-terminal signal sequence which is structurally and functionally conserved across distant phylogenetic barriers (Talmadge *et al.*, 1980). Second, additional export information is contained within the mature portion of secreted proteins either to stop their transfer through membranes (Davis *et al.*, 1985; Yost *et al.*, 1983) or to target their translocation to other subcellular locations (Benson *et al.*, 1984). Finally, protein secretion in both systems requires a cellular export machinery. Export into the endoplasmic reticulum of higher eucaryotic cells requires at least two components, the signal recognition particle and the docking protein, which serve to couple the export of secreted proteins to their synthesis (Walter *et al.*, 1984). Export in procaryotes also requires at least one soluble and one membrane bound component (Chen *et al.*, 1985; Müller and Blobel, 1984), although their similarity to the eucaryotic counterparts awaits their characterization.

Genetic selections have been developed in *Escherichia coli* in order to identify components of the protein export machinery. Temperature-sensitive mutations in two genes, *prlA* (secY) and secA, have been isolated which result in the accumulation of unsecreted cytoplasmic precursors for a number of exported proteins (Oliver and Beckwith, 1981; Shibata *et al.*, 1984). Although the extent of these export defects have not been investigated in detail, mutants in these two genes effect export to all three envelope layers (Garwin and Beckwith, 1982; Hayashi and Wu, 1985; Wolfe *et al.*, 1985). In contrast, secB mutants block the export of only a limited number of envelope proteins (Kumamoto and Beckwith, 1985), indicating that not all components required for export are shared in common, and different secreted proteins appear to have different export requirements.

In order to determine the breadth and specificity of the cellular secretion system that utilizes the SecA gene product, we have investigated the synthesis and secretion of a large number of periplasmic and outer membrane proteins in secA amber (secA<sup>am</sup>) and secA temperature-sensitive (secA<sup>t</sup>) mutants. This study was particularly warranted since secA<sup>am</sup> and secA<sup>t</sup> mutant strains appear to have different phenotypes, since the former strain is defective in the synthesis of certain envelope proteins while the latter strain shows a defect in the secretion of envelope protein precursors from the cytoplasm (Oliver and Beckwith, 1981, 1982). We have compared these genetic export defects to a different sort of export block, namely one found in a strain that produces a large amount of MalE-LacZ hybrid protein that is abortively secreted into the inner membrane where it interferes with normal cellular protein secretion (Ito *et al.*, 1981). Our results show that the specificity and extent of the export blocks found in the secA<sup>am</sup> mutant and in the strain containing the malE-lacZ fusion are identical, affecting at least four major outer membrane proteins and most, but not all, periplasmic proteins. Therefore, SecA appears to be an essential component of the major export pathway in *E. coli*, although minor pathways appear to exist. Furthermore, the synthesis of only a subset of these envelope proteins is reduced in the secA<sup>am</sup> mutant, indicating that envelope proteins show different requirements for SecA to promote their synthesis and secretion.

1 The abbreviations used are: secA<sup>am</sup>, secA<sup>t</sup>, and prlA<sup>t</sup>, amber and temperature-sensitive mutations in the indicated gene; SRP, signal recognition particle; MalE-LacZ, hybrid protein between maltose-binding protein and β-galactosidase; OmpA, OmpF/C, PrlA, SecA, protein products of the indicated gene; CysBP, GalBP, LivK, MalE, RbsB, XBP, periplasmic binding proteins for cystine, galactose, leucine, maltose, ribose, and xylose, respectively; Bla, TEM β-lactamase; LamB, lambda receptor protein; LepB, leader peptidase; Lpp, Braun lipoprotein; proLpp, preLamB, preMalE, preOmpA, and preOmpF/C, precursors for the respective proteins; TEMED, N,N,N',N'-tetramethylmethylenediamine.
**EXPERIMENTAL PROCEDURES**

**Media and Reagents**—The M63 medium has been described previously (Miller, 1972). [³⁵S]Methionine (approximately 1050 Ci/mol) was purchased from Amersham Corp. IgG Sorb used for immunoprecipitations was obtained from the New England Enzyme Center. Antiserum to MalE and Lpp were gifts from W. Boos, Department of Biology, University of Konstanz, Konstanz, W. Germany, and M. Inouye, Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, NY, respectively. Antiserum to PhoA, LamB, OmpA, OmpF/C, and SecA protein were prepared in the laboratory.

**Bacterial Strains**—The bacterial strains used in this study are all isogenic derivatives of MC4100 (F-, ΔlacU169, araD139, relA, rpsL, thi) MM15 (MC4100 Δp72-g77) carrying the malE-lacZ fusion has been described previously (Ito et al., 1981). MM52 (MC4100, secA⁴⁺) and MM113 (MC4100, secA⁴⁺, supF, rpsL, thi, livK) have been described (Kumamoto et al., 1984; Oliver and Beckwith, 1981). Strains carrying pBR322 and pOXT, a plasmid containing the lacZ gene (Oxender et al., 1980), were constructed by transformation.

**Periplasmic Proteins**—Periplasmic proteins were isolated from lys-ozyme/EDTA-treated spheroplasts as described by Oliver and Beckwith (1981).

**Immunoprecipitations**—Antibody precipitations were performed as described by Oliver and Beckwith (1982). One to five microliters of the different antisera were used in each sample.

**Gel Electrophoresis**—Preparation, electrophoresis, and processing of polyacrylamide gels were performed as described by Oliver and Beckwith (1982). Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (1975). The gels used for lipoprotein analysis were prepared as follows: 9.4 g of urea dissolved in a mixture of 12.4 ml of 40% acrylamide/0.14% bisacrylamide and 3.3 ml of 3 M Tris, 0.708 M NaCl (pH 8.8), and the volume was adjusted to 25 ml with deionized water. This solution was degassed under vacuum and 0.24 ml of 10% sodium dodecyl sulfate, 60 μl of 10% ammonium persulfate, and 0.63 ml of 1 M Tris-HCl (pH 6.8), and the volume was adjusted to 10 ml with deionized water. The solution was degassed under vacuum and 0.1 ml of 10% sodium dodecyl sulfate, 30 μl of 10% ammonium persulfate, and 10 μl of TEMED were added before pouring the stacking gel.

**RESULTS**

**Periplasmic Proteins**—In order to characterize the breadth and specificity of the cellular secretion system that utilizes the secA gene product, we have determined which periplasmic and outer membrane proteins showed synthetic or secretion defects in secA⁴⁺ and secA⁴⁺ mutants, respectively. The secA⁴⁺ strain, MM113, contains a temperature-sensitive, amber suppressor so that SecA levels decline after temperature shift to 41 °C, resulting in failure to synthesize at least one secreted protein (MalE) (Oliver and Beckwith, 1982). In contrast, the secA⁴⁺ strain, MM52, is defective in the secretion of a number of envelope proteins at 41 °C and accumulates their corresponding precursors within the cytoplasm of the cell (Oliver and Beckwith, 1981). We have also compared these export-specific defects to a different kind of defect, namely the secretion defect present in a strain containing a malE-lacZ fusion, MM18. MM18 makes large amounts of a MalE-LacZ hybrid protein during maltose induction that is abortively secreted into the inner membrane where it interferes with the export of a number of envelope proteins (Ito et al., 1981).

MM18, MM52, and MM113 are all isogenic derivatives of the wild type strain, MC4100. In this paper we will assume that reduction in the level of individual proteins to the periplasmic space and outer membrane is due to a synthetic block in MM13 and export blocks in MM18 and MM52, although this has not been rigorously proven for several envelope proteins in these mutants.

We have determined the decline in the appearance of newly synthesized proteins in the periplasmic space in MM18, MM52, and MM113 after initiation of the export-specific blocks. These strains were first grown under permissive conditions and then shifted to nonpermissive conditions, where at 15-min intervals aliquots were withdrawn and pulse-labeled with [³⁵S]methionine for 2 min in order to assess the extent of the export-specific blocks at a given time. Periplasmic fractions were prepared from these samples and are shown in Fig. 1. As previously reported, MM52 showed a delay before protein export to the periplasmic space became completely blocked (Oliver and Beckwith, 1981). However, by 60 min after temperature shift, there was a reduction in the export of most periplasmic proteins, and by 120 min, export was reduced severely. The decline in the export of most proteins to the periplasmic space followed kinetics similar to MalE, although the export of individual periplasmic proteins was affected to somewhat different extents (see below). A similar result was seen for MM18, where a reduction in the export of most periplasmic proteins occurred 45–60 min after maltose induction and reached a very low level by 120 min. In contrast, within 15 min after shifting MM113 to 41 °C, the synthesis of MalE declined severely and remained low. However, the synthesis and export of most other periplasmic proteins remained relatively unaffected, with some reduction occurring by 105 min after shift up, at which time SecA levels should have fallen to approximately 15% of the wild type level.

In order to more carefully determine the effect of the secA⁴⁺ mutation on the secretion of individual periplasmic proteins, we have compared periplasmic fractions from MC4100 and MM52 on two-dimensional gels. We have looked at newly synthesized periplasmic proteins in these strains grown at 30 °C or shifted to 41 °C for 90 min prior to labeling. Individual periplasmic proteins were identified on these gels by physiological or genetic criteria or by using the radioactive ligand-binding technique described by Copeland et al. (1982). In this way we could measure the effect of the secA⁴⁺ mutation on the secretion of binding proteins for cystine, galactose, leucine, maltose, and ribose. The results are shown in Fig. 2. It can be seen that the pulse-labeled, periplasmic protein profiles for MC4100 and MM52 grown at 30 °C were nearly identical (panels A and C), indicating that periplasmic protein export in MM52 is essentially normal at the permissive temperature. One curious finding is that the isogenic derivative for RbsB is shifted in the acidic direction in MM52. That protein is in fact RbsA is confirmed by immunoprecipitation with an appropriate antiserum followed by two-dimensional gel electrophoresis (data not shown). In contrast, the export of most periplasmic proteins was markedly reduced in MM52 after shift to 41 °C for 90 min (compare panels B and D). However, the extent of this reduction varied for different periplasmic proteins. Certain abundant proteins were essentially undetectable (see X and Y in Fig. 2), while others showed moderate reduction (LivK, MalE, and Z in Fig. 2). Since many of the periplasmic proteins that were absent in MM52 after temperature shift are normally less abundant, it is difficult to determine whether their export is strongly or only moderately affected. Finally, certain proteins were present in nearly equal amounts in both strains despite the export block (RbsB, CysBP, and arrows in Fig. 2). The secretion of RbsA does become blocked in MM52 by 120 min after temperature shift (Garwin and Beckwith, 1982). The existence of periplasmic proteins that are less affected by this export block supports the notion that the absence or reduction in the level of most periplasmic proteins in MM52 grown at 41 °C is indeed due to an export-specific block and not to artifacts arising from poor labeling or release of periplasmic proteins.
SecA Requirement for Bacterial Protein Secretion

FIG. 1. Synthesis and export of proteins to the periplasmic space after induction of export-specific blocks. MM18 was grown in M63 medium supplemented with 0.4% glycerol at 30 °C until early logarithmic phase, when maltose (final concentration of 0.4%) was added to 1-ml aliquots of the culture in 15-min intervals for up to 120 min. MC4100, MM52, and MM113 were grown in M63 medium supplemented with 0.4% glycerol and 0.4% maltose at 30 °C until early logarithmic phase, when 1-ml aliquots of the culture were shifted to 41 °C in 15-min intervals for up to 120 min. Each aliquot was labeled with 20 μCi of [35S]methionine for 2 min and placed on ice, and the periplasmic fraction was prepared as described under "Experimental Procedures." 10 μl of each periplasmic fraction was analyzed on the polyacrylamide gel shown. In a similar experiment shown in panel A, MalE protein was analyzed by immunoprecipitation as described under "Experimental Procedures." 20 μl of each immunoprecipitate was analyzed on the polyacrylamide gel shown.

From this mutant. Of interest, there were a couple of proteins in the periplasm of the temperature-shifted mutant that were absent from the wild type strain, and these were also detected in reduced amounts in MM52 grown at 30 °C (see N in Fig. 2). Since the number of these proteins is small, this result is probably not due to contamination of this fraction with other cellular proteins, but rather to the induction of new gene products or to different fractionation properties of this mutant strain.

We have also determined the effect of the secA-ts mutation on the export of xylose binding protein (XBP) and the TEM β-lactamase (Bla) to the periplasmic space. These results are shown in Fig. 3. In MM52 the secretion of XBP was more strongly blocked than MalE, since little XBP was seen in the periplasm of the mutant when MalE was still readily detectable (Fig. 3A, lanes 4 versus 8). The export of Bla was at least as strongly blocked as MalE or LivK in this mutant (Fig. 3B, lanes 10 and 12). Bla is somewhat unusual in that, like coat protein of bacteriophage M13, its secretion through the cytoplasmic membrane occurs only after its synthesis is complete (Ito et al., 1979; Koshland and Botstein, 1982). Therefore, there is a requirement for SecA to promote the export of post-translationally as well as cotranslationally secreted proteins.

Outer Membrane Proteins—We have determined whether the synthesis or export of the major outer membrane proteins LamB, Lpp, OmpA, and OmpF/C is blocked in strains MM18, MM52, and MM113. The three strains were grown under permissive conditions and shifted to nonpermissive conditions where aliquots were withdrawn at 0, 1, 2, and 3 h and pulse-labeled with [35S]methionine for 2 min in order to assess the extent of the export-specific block at a given time. The synthesis and export of individual outer membrane proteins was followed by immunoprecipitation of total protein with specific antisera. In these experiments we have used precursor processing as an indicator of secretion since precursor accumulation in MM18 and MM52 has been shown previously to be due to secretion defects (Ito et al., 1981; Oliver and Beckwith, 1981). The results of these studies are shown in Fig. 4. Similar to the previous studies with periplasmic proteins, MM52 and MM18 showed similar kinetics in the appearance of the secretion block for individual outer membrane proteins. At least some precursor to the four major outer membrane proteins was detected in these two strains by 2 h after imposing the secretion block. However, the secretion of individual outer membrane proteins was inhibited at different rates. For example, the export of Lpp was almost completely blocked in these two strains by 2 h, whereas to achieve a similar result with OmpF/C took approximately 3 h (Fig. 4, panels A and D). It has been shown that the Lpp precursor accumulating in MM18 and MM52 is unmodified with glycerol, implying a more proximal block in export (Hayashi and Wu, 1985). By comparison with Lpp and OmpF/C, the rate of inhibition of export of OmpA and LamB was slower and less complete, since no more than approximately 50% precursor was seen in these two strains by 3 h after commencement of the export block (Fig. 4, panels B and C). Longer incubation times under nonpermissive conditions resulted in a more complete export block for these latter two proteins (data not shown). In doing these experiments we noticed that it was important to prepare...
the different proteins from a single culture, since there was some variability in the rate of appearance of the export blocks in different cultures. This probably accounts for the difference between our results and those of Wolfe et al. (1985), who found that OmpA secretion was completely blocked in MM52 after 3 h at 42 °C.

The four outer membrane proteins examined showed very different responses to the synthetic block that occurs in MM113 at 41 °C. Lpp and OmpA showed little or no reduction in their synthesis and export during the time course of this experiment (Fig. 4, panels A and B). Presumably these proteins require only low levels of SecA for their synthesis and secretion, since SecA levels are approximately 8% of wild type after growth for 3 h at 41 °C. However, similar to the results reported for MalE, synthesis of LamB declined within 1 h after shift to 41 °C and remained low (Fig. 4, panel C). One interpretation of this result is that MalE and LamB require higher levels of SecA for their synthesis than for their secretion, since in MM113 the reduced amounts of these proteins are found in the processed form. Synthesis of OmpF/C may also have decreased somewhat within 1 h at 41 °C, but clearly not as much as the MalE control (Fig. 4, panel D). Since total protein synthesis declined somewhat in MM113 after 3 h at 41 °C, it was difficult to determine whether the synthesis of OmpF/C was preferentially affected at later times or not. We conclude that envelope proteins fall into at least two classes depending on whether they are affected or not by the synthetic block arising in MM113.

**DISCUSSION**

We have followed the synthesis and secretion of a large number of periplasmic and outer membrane proteins in three strains that have defects in the synthesis or export of envelope proteins. It has been shown previously that MM113 is defective in the synthesis of an envelope protein (MalE), while MM18 and MM52 are defective in the export of a number of periplasmic and outer membrane proteins (Ito et al., 1981; Oliver and Beckwith, 1981, 1982). We have extended these findings by showing that induction of export blocks in MM18 and MM52 results in secretion defects for four major outer membrane proteins (LamB, Lpp, OmpA, and OmpF/C) and most but not all periplasmic proteins. MM52 is also defective in the export of at least one cytoplasmic membrane protein,
Logarithmic phase, when 1-ml aliquots of the culture were shifted to 30 °C until early 12 pCi of [35S]methionine for 2 min and placed on ice, and Lpp, OmpA, OmpF, and preLamB were added to 1-ml aliquots of the culture in 1-h intervals for up to 3 h. Visualization of OmpF/C and MalE. Only the relevant portions of total cell protein from 10-fold concentrated cultures was also analyzed on the polyacrylamide gel shown in panel D which allowed for the detection of protein bands.

LepB, after shift to the nonpermissive temperature (Wolfe et al., 1985). Since blocking export by either inactivation of SecA or by production of an export-defective, hybrid protein leads to such a broad secretion defect, we conclude that SecA appears to be an essential component of the major export pathway in E. coli for envelope protein secretion. Since prlA mutants are also defective in the secretion of a number of these envelope proteins (LepB, MalE, Lpp, OmpA, and OmpF) (Shiba et al., 1984; Wolfe et al., 1985), PrlA and SecA are probably both components of the same export pathway.

This contention is supported by the fact that the efficiency of prlA-mediated suppression of signal sequence mutations is affected by cellular SecA levels (Oliver and Liss, 1985). Also, the export defects of secA mutants can be suppressed by certain types of prlA mutations (Brinkman et al., 1984), implying a possible interaction between these two gene products.

In following the export of a large number of periplasmic and outer membrane proteins, we have found that individual envelope proteins show somewhat different patterns in the rate at which they respond to the secretion blocks in MM18 and MM52. One likely explanation for this result is that different envelope proteins can compete more or less effectively for export machinery components which become rate-limiting after induction of these export blocks, since envelope proteins normally show significant differences in their export kinetics (Jøsæs and Randall, 1981). Despite the wide ranging secretion defects found in MM18 and MM52 several hours after induction of the export blocks, there are certain periplasmic proteins whose export is relatively unimpaired. This result probably indicates that the secretion of these proteins occurs by a minor pathway not requiring SecA and other components inactivated by the export-defective hybrid protein. Alternatively, like RbsB, it may take longer for the secretion of certain proteins to be affected by the export blocks induced in MM18 and MM52. If the secretion of these proteins is truly unaffected in these two strains, then their secretion may require an alternate export machinery or may occur autocatalytically as proposed in the membrane trigger hypothesis (Wickner, 1983). The secretion of MC13 coat protein occurs normally in MM52 grown at 42 °C (Wolfe et al., 1985). However, we have found that the export of another post-translationaly secreted protein, Bla, is blocked in MM18 and MM52. Clearly, therefore, utilization of the export pathway defined by SecA is not determined by whether a protein is cotranslationally or post-translationally secreted.

We have found that the synthesis of certain envelope proteins in MM113 is substantially reduced (MalE and LamB), while the synthesis of others is relatively unaffected (Lpp and OmpA). However, in all cases the proteins that are synthesized in MM113 are exported as indicated by signal peptide processing. Therefore, it would appear that envelope proteins differ in the level of SecA required to promote their synthesis, and that lower SecA levels are sufficient to promote export. The fact that MM52 shows only export and not synthetic defects is probably a result of the constraint of the genetic background of the export-defective hybrid protein (Oliver and Beckwith, 1981). Whether the requirement for high SecA levels in envelope protein synthesis is at the transcriptional or translational level is unclear. The molecular nature of the SecA requirements for the synthesis and export of envelope proteins is currently being studied.

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Fig. 4. Synthesis and export defects for outer membrane proteins in MM113, MM18, and MM52. MM18 was grown in M63 medium supplemented with 0.4% glycerol at 30 °C until early logarithmic phase, when maltose (final concentration of 0.4%) was added to 1-ml aliquots of the culture in 1-h intervals for up to 3 h. MC100, MM52, and MM113 were grown in M63 medium supplemented with 0.4% glycerol and 0.4% maltose at 30 °C until early logarithmic phase, when 1-ml aliquots of the culture were shifted to 41 °C in 1-h intervals for up to 3 h. Each aliquot was labeled with 20 μCi of [35S]methionine for 2 min and placed on ice, and Lpp, OmpA, and LamB were analyzed by immunoprecipitation of total protein as described under “Experimental Procedures.” 20 μl of each immunoprecipitate was analyzed on the polyacrylamide gel shown in panel D which allowed for the visualization of OmpF/C and MalE. Only the relevant portions of each gel are shown.