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Cloned DNA Fragment Specifying Major Outer Membrane Protein *a* in *Escherichia coli* K-12

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Plasmid pMC44 is a recombinant plasmid that contains a 2-megadalton *Eco*RI fragment of *Escherichia coli* K-12 DNA joined to the cloning vehicle, pSC101. The polypeptides specified by plasmid pMC44 were identified and compared with those specified by pSC101 to determine those that are unique to pMC44. Three polypeptides specified by plasmid pMC44 were localized in the cell envelope fraction of minicells: a Sarkosyl-insoluble outer membrane polypeptide (designated M2), specified by the cloned 2-megadalton DNA fragment, and two Sarkosyl-soluble membrane polypeptides specified by the cloning plasmid pSC101. Bacteria containing plasmid pMC44 synthesized quantities of M2 approximately equal to the most abundant *E. coli* K-12 outer membrane protein. Evidence is presented that outer membrane polypeptide M2, specified by the recombinant plasmid pMC44, is the normal *E. coli* outer membrane protein designated protein *a* by Lugtenberg and 3b by Schnaitman.

The prominent phenotypes of the *capR* (*lon*) mutants are overproduction of capsular polysaccharide, characterized by mucoid colonies on minimal medium, and increased sensitivity to UV radiation (for review, see 27). In an attempt to clone the *capR* (*lon*) gene, an *Eco*RI restriction fragment of *Escherichia coli* K-12 DNA was cloned using plasmid pSC101. Three independent recombinant plasmids, designated pMC44, pMC52, and pMC303, were isolated that, when transformed into *capR* (*lon*) mutant bacteria, inhibited the overproduction of capsular polysaccharide but did not affect the UV sensitivity of the *capR* (*lon*) mutant (4). Analysis of the *Eco*RI-digested pMC44, pMC52, and pMC303 DNA by agarose gel electrophoresis revealed that the three plasmids each contained a 2-megadalton (Mdal) DNA fragment in addition to a 6.1-Mdal DNA fragment (pSC101; 4). The 2-Mdal fragment does not contain the *capR*$^+$ gene (36; B. Zehnbauer and A. Markovitz, manuscript in preparation).

The proteins coded by a plasmid can be selectively labeled using plasmid-containing minicells; the latter exclude the bacterial chromosome (10). To identify the proteins specified by plasmid pMC44, minicells that contained pMC44 or pSC101 were purified and labeled with [^{35}S]methionine. The ^{35}S-labeled polypeptides were then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). We expected that one or more plasmid pMC44-specified proteins could be membrane associated, since pMC44 decreased the polysaccharide synthesis in *capR* (*lon*) mutant bacteria. Polysaccharide synthesis occurs in the bacterial envelope (17) similar to the synthesis of lipopolysaccharide (18, 29). Also, polysaccharide polymerase activity in *capR* mutants has been detected in membrane preparations (17; M. M. Lieberman, Ph.D. thesis, University of Chicago, Chicago, Ill., 1969). Therefore, [^{35}S]methionine-labeled, pMC44-containing minicell membrane preparations were isolated and fractionated based on solubility in 1% sodium lauryl sarcosinate (Sarkosyl, a detergent that solubilizes the inner membrane proteins but not the "major" outer membrane proteins of *E. coli* [8]). Three radioactive polypeptides specified by plasmid pMC44 were localized in the cell envelope of minicells containing plasmid pMC44, a Sarkosyl-insoluble outer membrane protein of approximately 40,000 daltons (40 Kdal), designated M2, plus two Sarkosyl-soluble polypeptides of 34 and 26 Kdal. Only outer membrane protein M2 was specified by the 2-Mdal DNA fragment. The 34- and 26-Kdal polypeptides were also synthesized in minicells containing the cloning plasmid pSC101. Evidence is presented that the outer membrane protein (M2) specified by the recombinant plasmid pMC44 is identical to the normal *E. coli* outer membrane protein designated protein *a* by Lugtenberg et al. (23, 24) and 3b according to Schnaitman's nomenclature (3).

MATERIALS AND METHODS

Bacterial strains. All strains are derivatives of *E.*

coli K-12. *E. coli* K-12 minicell-producing strain χ984, obtained from R. Curtiss III (10), was used. Strains MC100, RGC103, and RGC121 have been described (4, 11, 12). All pMC44 and pSC101 plasmid-harboring strains were constructed by DNA transformation (4). Strain P400 was supplied by U. Henning (31). Bacteria supplied by J. Foulds were used exclusively to define the major outer membrane polypeptides. These include JF568 (6), a wild-type strain; AB1621, Lugtenberg's strain diminished in outer membrane polypeptides b and c (24), as defined by Lugtenberg's nomenclature (23); and JF643 (spontaneous mutant of AB1621 diminished in outer membrane polypeptides b, c, and d).

Media and chemicals. Complex medium was YET (5 g of yeast extract, 10 g of tryptone, 10 g of NaCl, and water to 1 liter) or SLBH (super-rich complex medium, which consists of 22.5 g of yeast extract, 11 g of tryptone, 4 ml of glycerol, and water to 1 liter, pH adjusted to 7.3). Minimal medium was M9 without $CaCl_2$ (1) supplemented with 0.6% glucose, thiamine, amino acids, and adenine as required. For growth of the minicell-producing strain χ984, M9 was supplemented with 0.5% (wt/vol) Casamino Acids (Difco Laboratories), adenine (50 μg/ml), and pyridoxine-HCl (10 μg/ml), hereafter designated M9-0.5% CAA. Proteins synthesized by purified minicells were labeled with [^{35}S]methionine, using quarter-strength methionine assay medium (Difco Laboratories) added to M9. [^{35}S]methionine (specific activity, approximately 400 Ci/mmol) was purchased from New England Nuclear. Tetracycline-HCl and tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride were purchased from Sigma. Acrylamide and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were obtained from Eastman. SDS, *N,N'*-methylenebisacrylamide, and ammonium persulfate were obtained from Bio-Rad Laboratories. *N,N'*-diallytartardiamide was purchased from Aldrich. Penicillin G sodium NF was purchased from Upjohn. Sodium lauryl sarcosinate (Sarkosyl), from Geigy Chemicals, was supplied by M. Stodolsky.

Isolation of plasmid-containing minicells. The procedure followed for the isolation of minicells was essentially that described by Frazer and Curtiss (9), with the addition of a penicillin G selection as described by Levy (20). The penicillin treatment of the partially purified minicells was in complex medium, YET or SLBH. The contaminating viable cells in the minicells were determined by plating 10 μl of the resuspended purified minicells (optical density at 620 nm = 1) on YET agar with or without tetracycline (Tc; 12.5 μg/ml). This purification procedure yielded minicell preparations that were essentially free of normal cells (one cell per 10^6 minicells).

Procedure for labeling proteins in minicells. Purified minicells were resuspended to an optical density at 620 nm of 1 (approximately 4×10^9 minicells per ml) in quarter-strength methionine assay medium in M9. Fifty to 100 μCi of [^{35}S]methionine plus 0.2 μg of cold methionine per ml (final specific activity, 200 to 400 μCi/μg of methionine) were added to the 1 ml of minicell suspension. Labeling was accomplished by incubating the purified minicells with the labeled amino acid for 2 h at 37°C in a rotary shaking water bath. Once labeled, the minicells were centrifuged, washed twice in 0.01 M PO_4 buffer, and then stored at −20°C. Ten-microliter samples were taken in duplicate during the labeling, precipitated with 5% chilled trichloroacetic acid, and filtered on GF/C fiber filters. The filters were washed twice with 5% trichloroacetic acid and once with 70% ethanol-3% potassium acetate, dried, and counted in a liquid scintillation counter (12).

PAGE. Two different SDS-PAGE recipes were followed. (i) A 15% SDS-PAGE recipe was used for the experiments reported in Fig. 3 and 5, in which the main gel was 8.5 cm in length, as described by Heine et al. (13). The main gel contained 15% acrylamide, 0.4% *N,N'*-diallytartardiamide, 0.1% SDS, 0.375 M Tris-hydrochloride (pH 8.8), 0.5% TEMED (vol/vol), and 0.034% (wt/vol) ammonium persulfate. The stacker gel (approximately 1 cm in length) contained 4% acrylamide, 0.4% *N,N'*-diallytartardiamide, 0.1% SDS, 0.125 M Tris-hydrochloride (pH 7.0), 0.5% TEMED (vol/vol), and 0.07% (wt/vol) ammonium persulfate. Electrode buffer consisted of 0.025 M Tris-hydrochloride (pH 8.5)–0.1% SDS–0.192 M glycine. The protein samples were solubilized by heating at 100°C for 5 min in the presence of 2% SDS, 5% β-mercaptoethanol, and 50 mM Tris-hydrochloride (pH 7.0) (Dis buffer I). Solubilized samples were applied to the gel in 50- to 100-μl volumes so that the same amount of isotopically labeled polypeptide was applied, usually between 5,000 and 20,000 cpm. Gels were subjected to electrophoresis at a constant current of 8 mA for 8 h. Polypeptides in the gel were fixed and stained with 0.4% Coomassie brilliant blue in 10% acetic acid and 25% isopropanol. Destaining was in 10% acetic acid–10% isopropanol. Analysis of radioactive polypeptides in the acrylamide gel was done by a fluorographic technique (5). The gels were dried and placed in contact with Dupont-Cronex 2DC medical X-ray film in a mechanical press. (ii) Electrophoresis was also performed utilizing the discontinuous system as described by Laemmli (19), modified as follows and used in Fig. 2, 6, and 7. The main gel was polymerized in a solution containing 0.37 M Tris-hydrochloride (pH 7.8)–0.1% SDS–0.03% (vol/vol) TEMED–0.33% (wt/vol) ammonium persulfate, with concentrations of acrylamide ranging from 15 to 30% maintaining a *N,N'*-methylenebisacrylamide–acrylamide ratio of 1:173. The stacker consisted of 0.14 M Tris-hydrochloride (pH 6.8)–5.7% acrylamide–0.15% *N,N'*-methylenebisacrylamide–0.1% SDS–0.05% (vol/vol) TEMED–0.05% (wt/vol) ammonium persulfate. The electrode buffer at pH 8.5 contained 0.05 M Tris, 0.384 M glycine, and 0.1% SDS. The gels were run at 20 mA at constant current until the dye front reached the bottom of the base gel. Samples were solubilized by heating at 100°C for 5 min in the presence of 4% SDS, 0.1 M Tris-hydrochloride (pH 6.8), 10% β-mercaptoethanol, 5% glycerol, and 0.025% bromophenol blue (Dis buffer II). Gels were stained in a solution containing final concentrations of 0.1% Coomassie brilliant blue, 50% methanol, and 10% acetic acid and destained in a solution of 5% methanol and 10% acetic acid. The separation of unlabeled outer membrane proteins was performed in a gel apparatus in which the main gel was 20 cm in length and the stacker gel was approximately 2 cm in length. The separation of cyanogen

bromide (CNBr)-produced fragments (see below) was performed in a 15 to 25% linear gradient acrylamide gel in which the main gel was 8.5 cm in length.

Isolation of membrane preparations. Purified minicells, from strains grown in either minimal (M9-0.5% CAA) or complex (SLBH) medium and labeled with [^{35}S]methionine as described above, were lysed using the conditions described by Levy et al. (22). Since freezing the minicells made them more difficult to lyse, the minicells were lysed immediately after labeling (10; personal observation). The minicells were ruptured by passing them through a French pressure cell twice at 16,000 lb/in^2. The envelope fractions (Fig. 3) were prepared from minicells isolated from M9-grown bacteria that were lysed and then fractionated as follows. Large membrane fragments and unlysed minicells were pelleted by centrifugation at 27,000 × *g* for 15 min (27,000 × *g* pellet fraction). The small membrane fragments which remained in the supernatant were pelleted by high-speed centrifugation at 4°C for 2 h at 200,000 × *g* (high-speed supernatant fraction). Envelope membrane from the 27,000 × *g* pellet fraction was separated from unlysed minicells by suspending it in 1 ml of 15% (wt/wt) sucrose in 5 mM ethylenediaminetetraacetate (EDTA), pH 7.5, layering it onto 9 ml of 55% sucrose in 5 mM EDTA, pH 7.5, in an SW41 centrifuge tube, and centrifuging at 124,000 × *g* for 2 h. The interphase band of purified membrane was removed by puncturing the side of the tube immediately below the band and withdrawing the visible material into a syringe. The unbroken minicells pelleted to the bottom of the tube.

Minicells obtained from bacteria grown in complex media (Fig. 4 and 5) were more easily lysed and yielded greater quantities of membrane fraction. Either passage through the French pressure cell or sonic disruption to approximately 10% of the original absorbance yielded almost complete disruption. Thus, Sarkosyl solubilization could be performed (8) without preliminary sucrose gradient fractionation.

Isopycnic centrifugation of the minicell envelopes and minicell outer membrane (Sarkosyl-insoluble material) was performed as described by Osborn et al. (30) as modified by Levy (21). The gradients were collected by puncturing the bottom of the centrifuge tube with a long 18-gauge needle and collecting 0.2-ml fractions (10 drops). Trichloroacetic acid-precipitable radioactivity was determined from a 10-μl sample of each fraction as described above. The density of the sucrose was determined with a Zeiss refractometer. For SDS-PAGE analysis, the fractions were pooled, centrifuged, and resuspended in 7 mM EDTA.

Sarkosyl-insoluble membranes from whole cells were isolated from bacteria grown overnight in M9-0.05% CAA at 37°C. Cells from 10 ml were pelleted by centrifugation at 12,000 × *g* for 10 min, suspended in 5 ml of 0.01 M potassium phosphate buffer, pH 7.4, centrifuged again, and finally resuspended in 2 ml of the same buffer. The cells were lysed by sonic disruption. The unlysed cells were removed by centrifugation at 500 × *g* for 10 min, and the cell envelopes were pelleted by centrifugation at 20,000 × *g* for 15 min. The envelope-containing pellet was then suspended in 1 ml of 7 mM EDTA, pH 7.5, and Sarkosyl was added to 1%. After incubation at 37°C for 20 min, the membranes were centrifuged again at 20,000 × *g* for 15 min, resuspended in 2 ml of 7 mM EDTA, pH 7.5, and treated again for 20 min at 37°C with 1% Sarkosyl. The Sarkosyl-insoluble membranes were then pelleted, washed in 5 ml of 7 mM EDTA, pH 7.5, and stored at 4°C. Isolation of outer membranes using Triton X-100 was as described by Schnaitman (32).

Procedure for labeling outer membrane proteins of whole cells. Cells were grown in M9-0.5% CAA containing [^{14}C]leucine for 3 h (approximately three generations). The cells were then centrifuged and sonically disrupted, and Sarkosyl-insoluble membranes were extracted as described. The specific activities of the Sarkosyl-insoluble membrane preparations were 261 cpm/μg of protein for strain MC100 and 130 cpm/μg of protein for strain MC100/pMC44.

CNBr digestion of eluted outer membrane proteins *a* and M2. Purified authentic *a* and M2 proteins were isolated from Sarkosyl-insoluble outer membrane preparations of labeled strains MC100 and MC100/pMC44, using the elution procedure described by Cleveland et al. (7). The outer membrane polypeptides were separated on a 20-cm preparatory gel (PAGE procedure II), and the polypeptides were visualized in the gel by staining with Coomassie brilliant blue for 30 min and destaining for 1 h. The polypeptide bands corresponding to *a* and M2 were cut out of the gel and eluted into dialysis bags by electrophoresis. Approximately 20 μg of each polypeptide was isolated into a final volume of 1 ml of electrophoresis buffer. Ovalbumin (final concentration, 100 μg/ml) was added to avoid losses of the ^{14}C-labeled outer membrane polypeptides. The contents were cooled to 4°C, and the protein was precipitated by the addition of trichloroacetic acid to a final concentration of 20%. The precipitate was pelleted, washed sequentially with 1 ml of 95% ethanol and then with 1 ml of ethyl ether, and air dried in a desiccator.

CNBr digestion of polypeptides *a* and M2 was performed in 70% formic acid and 5 mg of CNBr per ml for 16 h at 25°C. The reaction was halted by diluting 10-fold with water. The samples were lyophilized, and the CNBr peptide fragments were suspended in Dis buffer II and separated by SDS-PAGE, method II, on a 10 to 25% gradient gel as described above.

RESULTS

Identification of the proteins unique to plasmid pMC44. pMC44- and pSC101-containing minicells were isolated and incubated in the presence of radioactive isotopes. The incorporation of [^{3}H]uridine and [^{35}S]methionine into trichloroacetic acid-insoluble material in pMC44- and pSC101-containing minicells is illustrated in Fig. 1. pMC44-containing minicells incorporated both isotopes five to seven times more efficiently than pSC101-containing minicells. The increased transcription and translation could be caused by a new efficient promoter for RNA synthesis or more copies of plasmid pMC44 per minicell than of pSC101. Since the increased transcription caused by plasmid

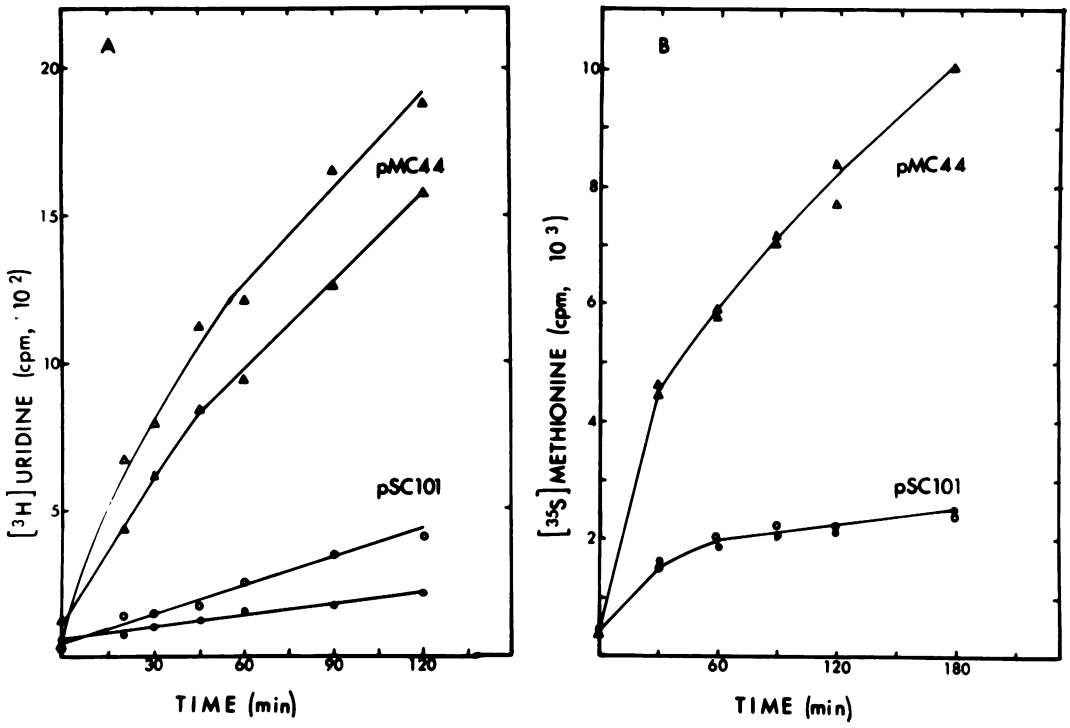

FIG. 1. *Isotope incorporation by pSC101- and pMC44-containing minicells. Minicells of χ984/pMC44 and χ984/pSC101 were purified and suspended in minimal medium supplemented with quarter-strength methionine assay medium (Difco). The minicell suspensions were incubated at 37°C in the presence (△, ○) or absence (▲, ●) of Tc (final concentration, 12.5 μg/ml). Duplicate samples were taken at intervals, and the radioactivity was determined. (A) Incorporation of [^{3}H]uridine (50 μCi/ml; specific activity, 244 μCi/mmol) in minicells suspended at an absorbancy of 0.5 at 620 nm. (B) Incorporation of [^{35}S]methionine (80 μCi/ml; specific activity, 20 Ci/mmol) in minicells suspended at an absorbancy of 1 at 620 nm.*

pMC44 is paralleled by increased translation, it appears likely that the plasmid DNA is coding for the mRNA specifying the polypeptides synthesized. Such mRNA may or may not be stable (vide infra). The presence of Tc stimulated [^{3}H]uridine incorporation by both pMC44- and pSC101-containing minicells but had no effect on [^{35}S]methionine incorporation in the experiment presented (Fig. 1). Occasionally Tc caused some inhibition of [^{35}S]methionine incorporation.

The [^{35}S]methionine-labeled polypeptides synthesized by pMC44- and pSC101-containing minicells were analyzed by SDS-PAGE. Examination of the gel band patterns in Fig. 2 indicates that at least 10 polypeptides were synthesized in pSC101-containing minicells and at least 5 additional polypeptides were synthesized in pMC44-containing minicells. Bands that are associated with the presence of plasmid pSC101 are labeled P1 through P10 from the largest to the smallest. P7 initially appeared as a single band in a 15% acrylamide gel (see Fig. 3) and probably separated into three bands (labeled 7a, b, and c; Fig. 2) when analyzed in a 15 to 30% gradient gel. Similarly, light minor bands that appeared (labeled P2 and P4) on analysis in 15% SDS-PAGE gels (Fig. 3a and b) did not appear upon analysis with 15 to 30% gradient gels. The respective molecular weights of the polypeptides of Fig. 2 are approximately: P1, 34,000; P3, 26,000; P5, 20,000; P6, 18,000; P7(a, b, c), 16,000; P8, 14,000; P9 and P10, <10,000. Polypeptides that appear to be new and specified by the 2-Mdal fragment of plasmid pMC44 are labeled M1 through M5. The major polypeptide bands reproducibly observed were M1, M2, and M5. Two minor polypeptide bands, labeled M3 and M4, were resolved when labeled polypeptides were analyzed on a 15% linear PAGE gel (Fig. 3); however, only polypeptide M4 has remained distinct upon analysis on a 15 to 30% gradient gel. M3 appears to resolve into three or four minor bands due to the better resolution of the 15 to 30% gradient SDS-PAGE system. The minor bands composing M3, as well as other minor bands observed in fluorograms of polypeptides separated by 15 to 30% gradient SDS-

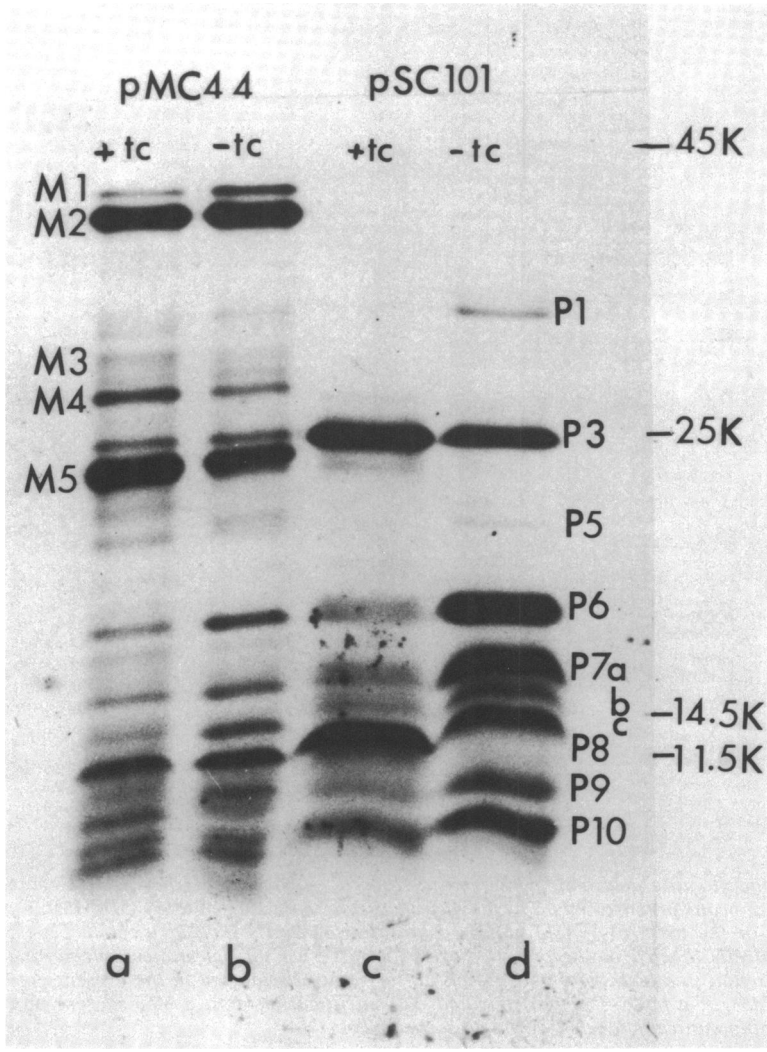

FIG. 2. *SDS-acrylamide gels of [^{35}S]methionine-labeled polypeptides from pSC101- and pMC44-containing minicells and the effect of Tc. The minicells were isolated from strain χ984 grown in the absence of Tc. The polypeptides were separated on a 15 to 30% SDS-PAGE gel (procedure II, see text). The polypeptides synthesized by both pMC44- and pSC101-containing minicells are labeled with the prefix P. The new polypeptides specified by plasmid pMC44 are labeled with the prefix M. Equal amounts of radioactivity (20,000 cpm) were applied to each slot. The amount of total protein applied to slots a, b, c, and d was approximately 25, 25, 140, and 310 μg, respectively, calculated from the minicell absorbancy at 620 nm. Standards were ovalbumin (45 Kdal), chymotrypsin (25 Kdal), lysozyme (14.5 Kdal), cytochrome c (11.5 Kdal).*

PAGE, have not been labeled since they were somewhat variable. We speculate that some of these minor polypeptides may be processing and/or degradation products of the major polypeptides. The molecular weights of the pMC44-specific polypeptides are approximately: M1, 42,000; M2, 40,000; M4, 28,000; M5, 25,000.

Equal amounts of radioactivity were applied to each slot in Fig. 2, in order to better observe the radioactive polypeptides of pSC101. Thus, six to ten times more total protein was applied to the pSC101 slots as compared to pMC44 slots. Therefore, a simple visual comparison in Fig. 2 underemphasizes the increased synthesis of pMC44-specified polypeptides compared to pSC101-specified polypeptides.

Are there stable mRNA's in minicells containing pMC44? EDTA-permeabilized minicells

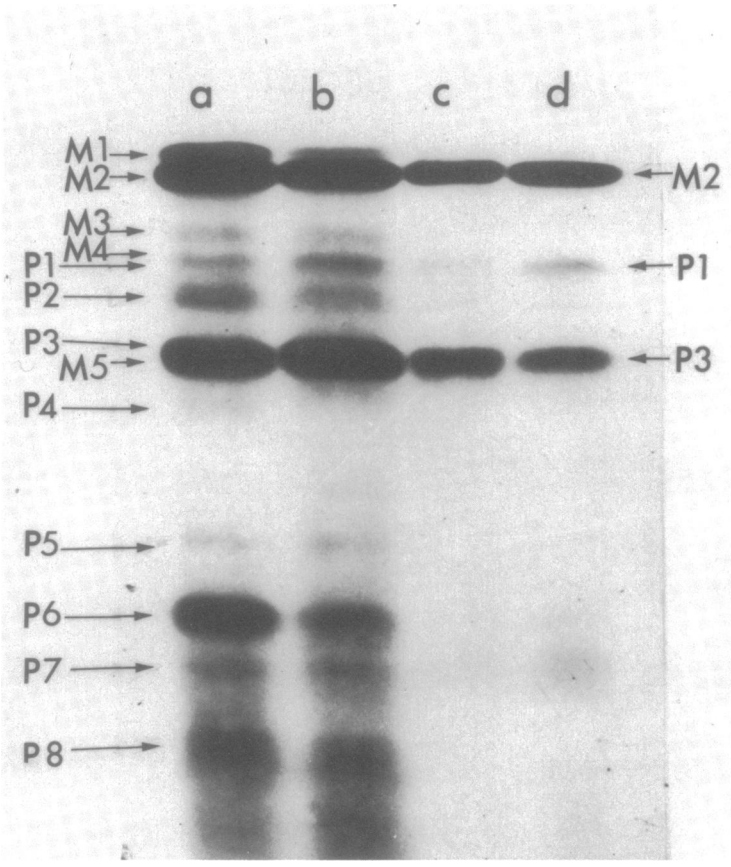

FIG. 3. *SDS-acrylamide gels of [^{35}S]methionine-labeled polypeptides from pMC44-containing minicells and minicell membrane preparations. The polypeptides were separated on a 15% SDS-PAGE gel by procedure I as described in the text. (a) Whole pMC44-containing minicells labeled without Tc; (b) whole pMC44-containing minicells labeled in the presence of 12 μg of Tc per ml; (c) minicell membranes of high-speed supernatant fraction (see text) from pMC44-containing minicells labeled in the presence of Tc; (d) minicell membranes (27,000 × g pellet fraction) purified by centrifugation onto a 55% sucrose pad (see text) from pMC44-containing minicells labeled in the presence of Tc.*

were prepared (34), and [^{35}S]methionine incorporation was measured with and without a 15- and 30-min pretreatment with rifampin (200 μg/ml). Incorporation was reduced by more than 90% in rifampin-treated minicells containing pMC44. The residual incorporation was largely into polypeptides M2 and M5, suggesting that M2 and M5 mRNA is relatively stable compared to mRNA specifying pSC101-coded polypeptides (in pMC44-containing minicells). Further consideration of these results will be presented in the Discussion.

Identification of the membrane proteins specified by pMC44- and pSC101-containing minicells. Minicells from strains χ984/pMC44 and χ984/pSC101 were isolated and labeled for 2 h in [^{35}S]methionine. The minicells were then immediately lysed, and minicell envelopes (containing both the inner and outer membrane) were isolated as described in Materials and Methods. Figure 3 presents a fluorogram of a 15% SDS-PAGE analysis of the labeled membrane polypeptides from pMC44-labeled minicells. Three radioactive polypeptides synthesized in pMC44-containing minicells remained associated with the membrane fraction: M2, P1, and P3. Although the polypeptide labeled M5 is not distinctly resolved from P3 on the 15% gel, analysis of a similar membrane preparation on a 15 to 30% linear gradient gel, as in Fig. 2, demonstrated that M5 was not membrane associated. Furthermore, when pSC101-containing minicell membranes were similarly isolated, P1 and P3 were again associ-

ated with the membrane fraction (data not shown).

Several attempts to separate pMC44-containing membranes into outer and inner membranes by the Osborn procedure (30), using isopycnic sucrose gradient centrifugation, were unsuccessful. The procedure resulted in only a broad band of radioactivity with a density below 1.23 (Fig. 4a). Thus, a differentiation of pMC44 membrane polypeptides was performed by Sarkosyl detergent solubilization. If any of the labeled membrane polypeptides of pMC44-harboring minicells were in the outer membrane, they should not have been efficiently solubilized by Sarkosyl detergent. Treatment of ^{35}S-labeled minicell membranes with 1% Sarkosyl resulted in the solubilization of 75 to 85% of the ^{35}S radioactivity, with 15 to 25% of the radioactivity remaining in the Sarkosyl-insoluble pellet. SDS-PAGE analysis of the Sarkosyl-insoluble fraction revealed that most of the inner membrane proteins had been solubilized. When the pMC44-containing minicell membrane, which was insoluble in 1% Sarkosyl, was purified by isopycnic sucrose gradient centrifugation, a radioactive peak was found at the density (ρ = 1.22) of purified outer membrane (Fig. 4b). A 15% SDS-PAGE analysis of this radioactive peak from the sucrose gradient demonstrated that the radioactive peak contained only the labeled polypeptide M2 (Fig. 5a and b). In addition, the results of Fig. 5 show that radioactive membrane not treated with Sarkosyl and with a density greater than 1.22 contains whole minicell membrane, since both P3 and M2 were present.

Evidence that M2 is identical with a nor-

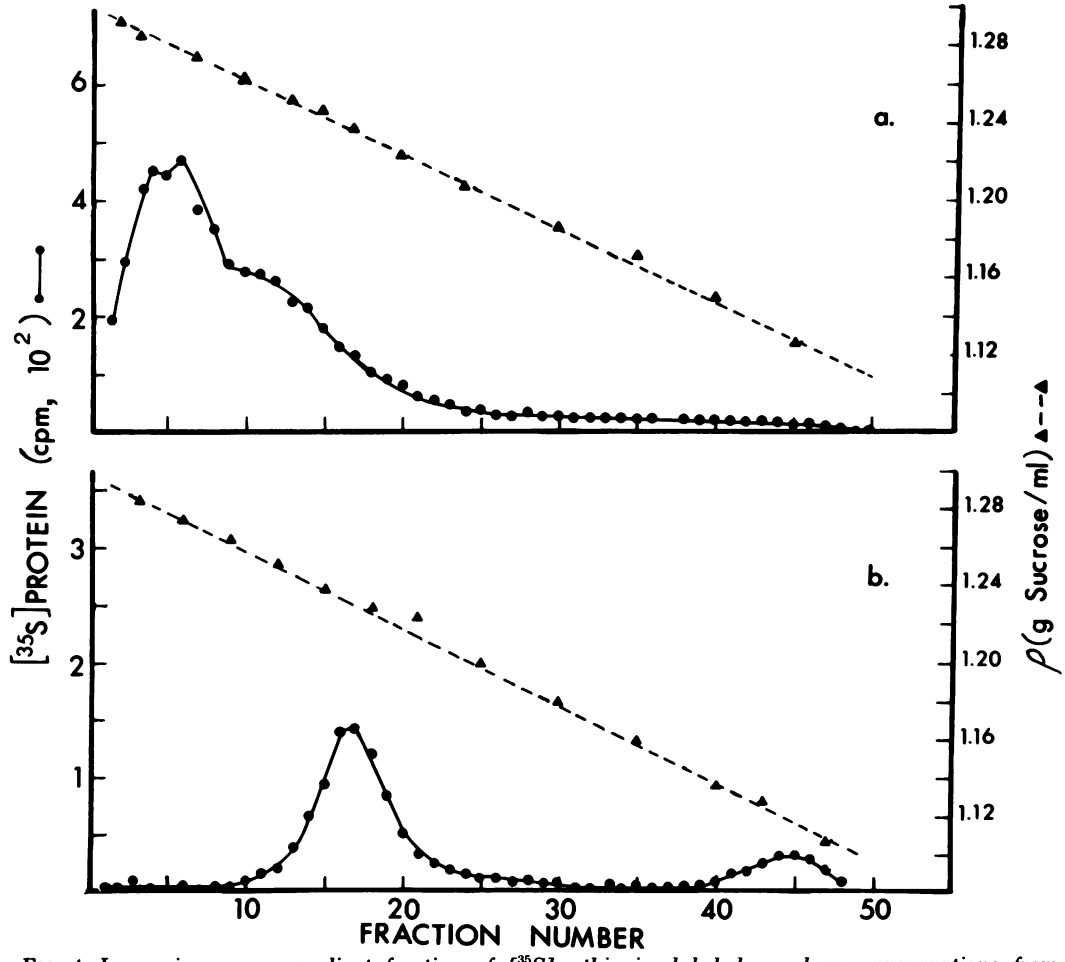

FIG. 4. *Isopycnic sucrose gradient fraction of [^{35}S]methionine-labeled membrane preparations from pMC44-containing minicells. Bacteria were grown in complex medium. (a) Labeled minicell membrane fraction from lysed pMC44-containing minicells; (b) same membrane preparation as in (a) but extracted with 1% Sarkosyl before centrifugation.*

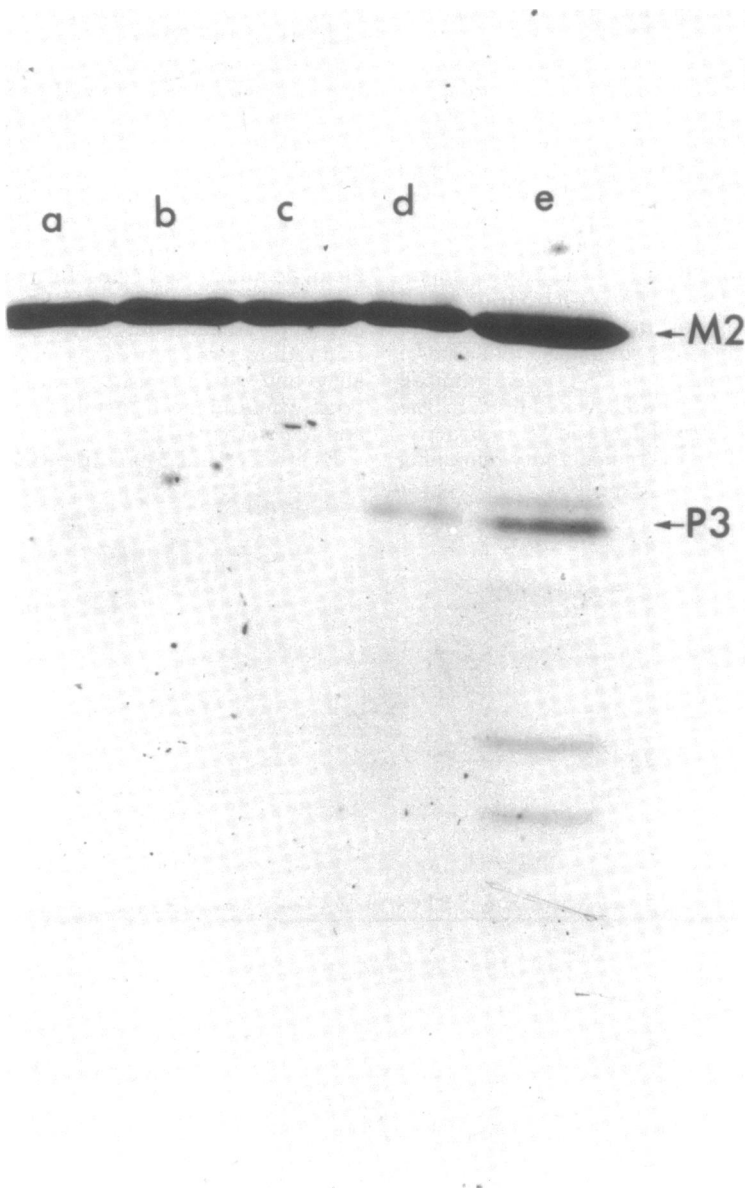

FIG. 5. *SDS-acrylamide gel of [^{35}S]methionine-labeled polypeptides from pMC44-containing minicell membrane preparations separated by isopycnic sucrose gradient centrifugation. The polypeptides were analyzed by procedure I. (a and b) Sarkosyl-insoluble membrane fractions with a density of 1.22 (Fig. 4b); (c and d) membrane fractions of minicell membrane not treated with Sarkosyl that had densities of 1.24 and 1.26, respectively (Fig. 4a); (e) polypeptides of unfractionated, labeled pMC44-containing minicells from the same experiment. (M2 is the major polypeptide synthesized in complex medium; compare with Fig. 2 and 3, where the pMC44-containing minicell strains were grown in M9-0.5% CAA.)*

mal *E. coli* K-12 outer membrane protein. The M2 polypeptide was synthesized in large amounts in minicells and could be distinguished after Coomassie brilliant blue protein staining of SDS-PAGE of pMC44-containing minicells. The amplification of M2 polypeptide in the total cell protein extracts was also observed in whole-cell extracts from strains containing plasmid pMC44. The M2 (= *a*) polypeptide is amplified in the outer membrane compared to other outer mem-

brane proteins from non-plasmid-containing strains (Fig. 6, column 1). This fact permitted us to compare the outer membrane polypeptide M2 with the previously identified major outer membrane polypeptides. The outer cell membrane of *E. coli* K-12 is unique in that it contains only a few species of protein in rather high concentrations. According to the nomenclature of Hindennach and Henning (14), the major proteins are I, II*, III, and IV, with apparent molecular weights of 38,000, 33,000, 17,000, and 7,000. Using a modified Laemmli discontinuous gel system, the outer membrane proteins between 40 and 30 Kdal have been resolved into four polypeptides by Lugtenberg et al. (23). They were designated: *a*, 40 Kdal; *b*, 38.5 Kdal; *c*, 38 Kdal; and *d*, 33 Kdal. These proteins have recently been reconciled with the nomenclature of Schnaitman (33) and Schmitges and Henning (31) (see reference 3). Briefly, polypeptide bands *b* and *c* (23) correspond with two protein bands of I (Ia, Ib; 31) of Henning and 1a and 1b of Schnaitman (33). Polypeptide band *d* corresponds with II* of Henning and 3a of Schnaitman. Polypeptide band *a* corresponds with Schnaitman's band 3b.

The initial SDS-PAGE analysis on 8.5-cm-length gels suggested that M2 was distinct from the major outer membrane proteins *b*, *c*, and *d*. However, the gel procedure did not resolve protein *a*. To increase the resolution, we changed to a modified Laemmli procedure and increased the gel length to 20 cm. Figure 6 illustrates the resolution of the major outer membrane proteins of several strains. Using several bacterial mutants either missing or expressing poorly the major outer membrane proteins *b*, *c*, and *d*, these proteins were identified in the outer membrane preparation. Lugtenberg's protein *a*, the least abundant major outer membrane protein, is also visible in Fig. 6 and can be resolved in all of the strains tested, including strain P400, a strain which has been studied by Schmitges and Henning (31) and Manning and Reeves (25). Importantly, the increased outer membrane pro-

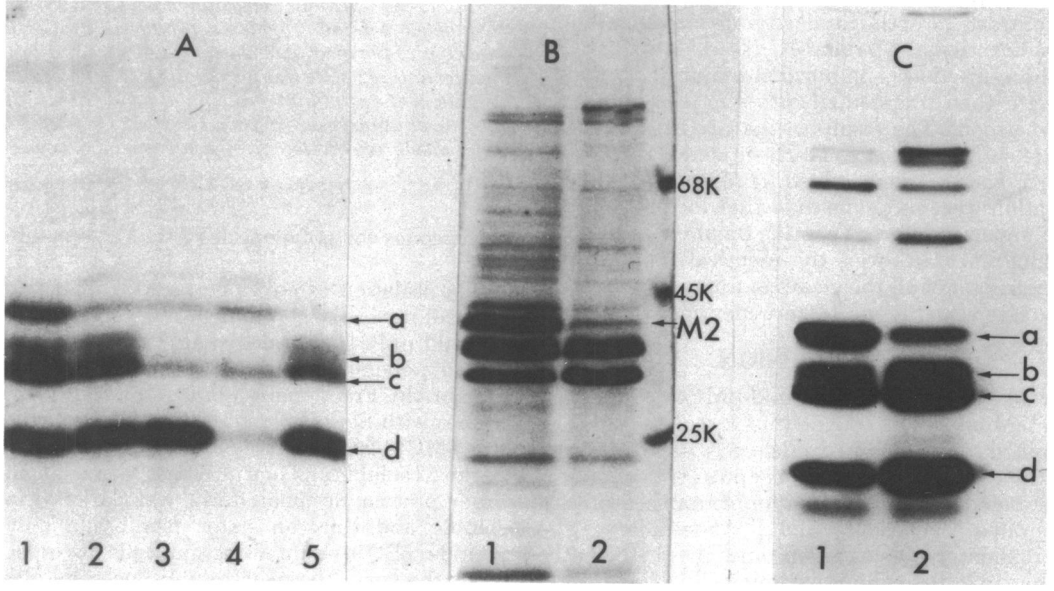

FIG. 6. *SDS-acrylamide gels of outer membrane polypeptides. The polypeptides were separated on 15% acrylamide by procedure II. (A) Sarkosyl-insoluble outer membrane polypeptides from various E. coli K-12 strains, using a 20-cm slab gel. Bands a, b, c, and d are labeled according to Lugtenberg et al. (23). All samples contained 100 μg of protein. Polypeptides were visualized using Coomassie brilliant blue. (1) MC100/pMC44, plasmid-containing strain in which protein a is amplified; (2) P400, wild type; (3) AB1621, mutant strain diminished in proteins b and c; (4) JF643, mutant strain diminished in proteins b, c, and d; (5) JF568; wild type. (B) Comparison of the Triton X-100-insoluble outer membrane preparations of strain MC100/pMC44 grown at 37°C (1) and 32°C (2). The polypeptides were separated on an 8.5-cm slab gel. Both samples contained 50 μg of protein. The molecular weights of the standard proteins are indicated: bovine serum albumin (68,000), ovalbumin (45,000), chymotrypsin (25,000). Polypeptides were visualized using Coomassie brilliant blue. (C) Autoradiogram of the Sarkosyl-insoluble ^{14}C-labeled membrane preparations from which a and M2 were isolated for CNBr digestion. The polypeptides were separated on a 20-cm slab gel. (1) MC100/pMC44, 205 μg of protein with approximately 30,000 cpm; (2) MC100, 475 μg of protein with approximately 107,000 cpm.*

tein observed in strain MC100/pMC44 (Fig. 6, column 1) comigrated with the band designated *a*.

A distinctive property of protein *a* (3b) is that its synthesis is temperature dependent. That is, protein *a* was not visible on SDS-PAGE gels of *E. coli* outer membrane isolated from bacteria grown at 32°C but was observed in outer membrane fractions isolated from bacteria grown at 37°C (24, 26). Such a temperature-dependent effect on synthesis was also found for M2 with pMC44-harboring bacteria (Fig. 6B). Much less M2 was observed in outer membrane from pMC44-harboring bacteria grown at 32°C than in those grown at 37°C. The data suggested that M2 and *a* were identical.

[^{14}C]leucine-labeled authentic *a* from cells not containing a plasmid (strain MC100) and similarly labeled M2 from MC100 containing pMC44 were prepared, and M2 and *a* were purified from a preparatory gel (see Materials and Methods; Fig. 6C). Twenty percent of the total outer membrane protein radioactivity was recovered with the M2 fraction in pMC44-containing MC100. In contrast, 2% of the total outer membrane protein radioactivity of strain MC100 was recovered as the *a* fraction. The purified *a* and M2 proteins were then fragmented into peptides by CNBr treatment. The results of the CNBr experiment are shown in Fig. 7. Thirteen identical radioactive bands were resolved. If M2 protein preparation were different than that for protein *a*, a number of different peptide fragments would be apparent. However, the identical number and migration of all the cleavage fragments strongly suggests that *a* and M2 are identical.

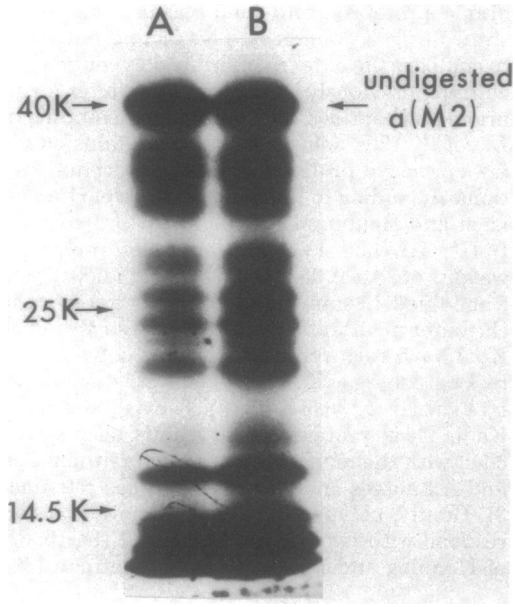

FIG. 7. *SDS-acrylamide gradient gel (10 to 25%) of ^{14}C-labeled peptide fragments produced by CNBr digestion of purified outer membrane protein a (authentic) and M2. (See text for details.) The molecular weights of the standard proteins are indicated: M2 (40,000), chymotrypsin (25,000), lysozyme (14,500). (A) Outer membrane protein a; (B) outer membrane protein M2.*

DISCUSSION

The recombinant plasmid pMC44 consists of a 2-Mdal *Eco*RI fragment of *E. coli* K-12 DNA joined to the cloning vehicle pSC101. Results of studies on gene expression of pMC44 in minicells revealed that pMC44-containing minicells incorporated [^{3}H]uridine and [^{35}S]methionine approximately five to seven times more efficiently than pSC101-containing minicells. Thus, plasmid pMC44 caused increased synthesis of RNA and protein compared to plasmid pSC101. SDS-PAGE analysis of the labeled polypeptides revealed that there were five new polypeptides in pMC44-containing minicells, in addition to pSC101-coded polypeptides. When the 2-Mdal DNA fragment was transferred to another cloning vehicle (mini-ColE1 [2]), the major polypeptides M1, M2, M4, and M5 were synthesized (R. C. Gayda, H. Avni, P. E. Berg, and A. Markovitz, manuscript in preparation). The simplest explanation of these results is that the 2-Mdal fragment codes for polypeptides M1, M2, M4, and M5.

The isolation of envelope preparations from labeled minicells containing the recombinant plasmid pMC44 revealed that three of the obtained polypeptides were associated with the membrane. Fractionation of the minicell membranes with Sarkosyl demonstrated that two of the pMC44-specified membrane proteins were Sarkosyl soluble and one pMC44-specified membrane protein, designated M2, was Sarkosyl insoluble and thus an outer membrane polypeptide. pSC101-containing minicells also specified the two Sarkosyl-soluble polypeptides. Therefore, the 2-Mdal DNA fragment of *E. coli* K-12 that was cloned appeared to specify an outer membrane polypeptide.

The outer membrane polypeptide expressed by plasmid pMC44 is a normal *E. coli* K-12 outer membrane polypeptide. A comparison of the protein profiles of the Sarkosyl-insoluble membrane proteins of wild-type bacteria and pMC44-containing strains indicated that M2 migrated on SDS-PAGE gels with the same mobility as the normal *E. coli* outer membrane protein designated *a* by Lugtenberg et al. (23,

24). The synthesis of M2 in pMC44-containing bacteria was temperature dependent (see Results), as is authentic *a* (24, 26). Furthermore, CNBr digestion of labeled M2 and the corresponding normal outer membrane protein *a* yielded identical fragments. Thus, plasmid-specified protein M2 and the outer membrane protein *a* are identical, and we conclude that a gene for a normal *E. coli* outer membrane protein has been cloned.

Since the mRNA for at least one outer membrane protein, the lipoprotein, is quite stable (15, 16), an alternative to M2 and M5 being coded by the 2-Mdal fragment of pMC44 is the following: plasmid pMC44 codes for an unidentified polypeptide that increases transcription of chromosomally specified mRNA's coding for polypeptides M1 through M5 (or some combination thereof), and these mRNA's are stable upon segregation into minicells. None of our results rigorously exclude the above explanation, but it is difficult to reconcile with the following. Our results demonstrated that increased transcription caused by plasmid pMC44 in isolated minicells was paralleled by increased translation (Fig. 1), suggesting that the mRNA being synthesized specifies the polypeptides being synthesized, including M1 through M5 (Fig. 2 and 3). If preexisting mRNA were a major source of polypeptides, this result would not be expected. More than 90% of [^{35}S]methionine incorporation into pMC44-containing minicells was prevented by rifampin, supporting the concept that continuing mRNA synthesis is required for efficient protein synthesis. Some residual polypeptide synthesis for mainly M2 and M5 was observed in rifampin-treated minicells containing pMC44, suggesting that the mRNA for these polypeptides is relatively stable compared to the mRNA for pSC101-coded polypeptides (in pMC44-containing minicells). The relative stability of M2 and M5 mRNA may be related to the fact that polypeptide M2 (*a*) is an outer membrane protein (15, 16). We conclude that, although mRNA for M2 and M5 are relatively stable, synthesis of polypeptides M2 and M5 is dependent on continued mRNA synthesis directed by plasmid pMC44; i.e., the 2-Mdal fragment specifies polypeptides M1 through M5.

Tc stimulates the synthesis of three polypeptides in purified pSC101-containing minicells (28, 34). These pSC101-coded polypeptides have molecular weights of 34,000 (P1), 26,000 (P3), and 14,000 (P8) and pellet with the envelope fraction (34). Either the 34-Kdal or the 18-Kdal polypeptide or both appear necessary for pSC101-conferred Tc resistance (28, 34). In this paper we report that P1 and P3 are associated with the minicell membrane fraction but are Sarkosyl soluble (i.e., probably inner membrane associated). The location of P1 and P3 may be relevant to understanding the mechanism by which pSC101 confers Tc resistance, since Tc-resistant strains transport Tc poorly into the cell (34, 35).

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