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ompT Encodes the *Escherichia coli* Outer Membrane Protease That Cleaves T7 RNA Polymerase during Purification

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Bacteriophage T7 RNA polymerase is stable in *Escherichia coli* but very susceptible to cleavage by at least one endoprotease after cell lysis. The major source of this endoprotease activity was found to be localized to the outer membrane of the cell. A rapid whole-cell assay was developed to screen different strains for the presence of this proteolytic activity. Using this assay, we identified some common laboratory strains that totally lack the protease. Genetic and Southern analyses of these null strains allowed us to conclude that the protease that cleaves T7 RNA polymerase is OmpT (formerly termed protein *a*), a known outer membrane endoprotease, and that the null phenotype results from deletion of the OmpT structural gene. A recombinant plasmid carrying the *ompT* gene enables these deletion strains to synthesize OmpT and converts them to a protease-positive phenotype. The plasmid led to overproduction of OmpT protein and protease activity in the *E. coli* K-12 and B strains we used, but only weak expression in the *E. coli* C strain, C1757. This strain-dependent difference in *ompT* expression was investigated with respect to the known influence of *envZ* on OmpT synthesis. A small deletion in the *ompT* region of the plasmid greatly diminishes the amount of OmpT protein and plasmid-encoded protease present in outer membranes. Use of *ompT* deletion strains for production of T7 RNA polymerase from the cloned gene has made purification of intact T7 RNA polymerase routine. Such strains may be useful for purification of other proteins expressed in *E. coli*.

The DNA-dependent RNA polymerase of bacteriophage T7 is a single-chain enzyme with a molecular weight close to 100,000 that has stringent specificity for its own promoters (7, 33, 45). The gene specifying T7 RNA polymerase, T7 gene *1*, has been cloned, and the protein has been successfully expressed at high levels in *Escherichia coli* (8, 49). As one of the simplest RNA polymerases known, it is an attractive candidate for attempts at crystallization, both alone and in association with its promoter sequence, and for specific biochemical and mutational analysis to map functional regions.

Expression of cloned gene *1* in *E. coli* is very efficient, and high levels of intact enzyme rapidly accumulate in the cells. However, during purification the protein becomes accessible to an endoprotease that introduces one or more nicks into the polypeptide chain (8, 49). The most prevalent cuts occur between amino acid 172 (lysine) and amino acid 173 (arginine) (49) or between two consecutive lysines occurring at amino acid positions 179 and 180 (J. J. Dunn, unpublished results). Although the nicked enzyme can be partially resolved from the intact species by ion-exchange chromatography, T7 RNA polymerase preparations invariably contain a significant amount of nicked protein since the resulting amino and carboxyl fragments remain tightly associated under nondenaturing conditions (20). Such preparations, while very active and specific in transcribing DNA that has a T7 promoter (8, 21), are unsuitable for some studies, especially crystallization trials, because it is known that protein microheterogeneity can adversely affect crystal quality.

From a practical standpoint, inclusion of a variety of standard protease inhibitors during purification failed to prevent cleavage of T7 RNA polymerase. Clearly, a mutant devoid of protease activity would be one way to circumvent this problem. As a first step towards isolating mutants defective in the endoprotease, we decided to characterize the enzyme's cellular location. From these studies we were able to devise an assay that allowed for rapid screening of the activity. When various laboratory strains of *E. coli* were tested for their ability to cleave T7 RNA polymerase, some strains were identified that appeared to lack protease activity.

In this paper, we report the results of these studies and show that the protease that nicks T7 RNA polymerase is the product of the *ompT* gene, which encodes a previously known outer membrane protease of *E. coli*.

MATERIALS AND METHODS

Strains and media. The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1. The Hfr::Tn*10* strain kit was the kind gift of B. Bachmann (*E. coli* Genetic Stock Center, Yale University).

Tryptone broth (43) alone and supplemented with 0.4% glucose and 0.5% yeast extract was used to grow most cultures. Defined medium was M9 (43). When appropriate, the media were supplemented with amino acids (10 μg/ml) and antibiotics at the following concentrations: ampicillin, 40; kanamycin, 25; and tetracycline, 10 μg/ml.

Genetic manipulations. Hfr matings and P1 transductions were performed as described in Miller (31). BL21.1, a Kan^r *leu* derivative of BL21, was the recipient strain in most mating experiments. It was constructed by P1 transduction of BL21 to kanamycin resistance, using a lysate grown on D0250. Exconjugants were selected on M9-glucose plates supplemented with leucine, kanamycin, and tetracycline. In two matings where the Hfr strains would be expected to transfer a *leu*$^+$ gene, BL21 was used as the recipient and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Source or reference
E. coli K-12		
MC4100	*ompB*$^+$ operon	5
MH1461	MC4100 *envZ11*	17
MH1471	MC4100 *envZ473*	17
SG477	MC4100 *envZ22*	12
SG480Δ76	MC4100 Δ(*malT-ompB*)	13
RT3	MC4100 *malA*::Tn*5* *envZ3*	12
D0250	MC4100 *leu*::Tn*5*	D. Oliver
SY781	*ompB*$^+$ operon linked to Tn*10*	D. Oliver
BW490.12	*perA*$^+$	51
BW490.9	BW490.12 *perA*	51
SK596	r_K^+ m_K^+ with Tn*10* insertion between *hsd*$^+$ and *serB*	D. Oliver
W3110	*thy*	27
HMS174	*hsdR recA*	4
ED8739	*hsdS*	46
LE392	*hsdR*	27
DH1	*hsdR recA*	27
K803	*hsdS*	53
SG4038	*lon*$^+$	D. Oliver
SG4044	SG4038 *lon*	D. Oliver
KK2186	F$^+$, non-phage lysogen of JM103	54
UT2300	*ompT*$^+$	10
UT4400	Δ(*ompT*)	10
UT5600	Δ(*ompT*)	10
E. coli B		
B707	r_B^+ m_B^+ *metB*	53
B834	*hsdS metB*	53
BL21	B834 transduced to *met*$^+$	46; F. W. Studier
BL21.1	BL21 *leu*::Tn*5*	This study
B837	*hsdR metB*	53
E. coli C		
C1757	*hsdR hsdM*	47
Plasmid		
pAR1219	*lacI lac*UV5 promoter and T7 gene *1* in pBR322	8
pAT428	*ompB*$^+$ operon in pBR322	32
pML19	*ompT*$^+$ in pUC19	15; M. Lundrigan
pML19:*sma*	Derived from pML19	This study

exconjugants were selected on the basis of being able to grow on M9-glucose plates (to select against the auxotrophic Hfr strains) supplemented with tetracycline (to kill BL21).

The restriction capability of various strains was determined by using lambda *cI* phage lysates (1) that had been grown on B834, an r_B^- m_B^- host (53). The ability of *hsd*$^+$-transduced strains to modify lambda was determined by measuring how well lysates prepared on these strains plaqued on an r_K^+ indicator strain, SK596, relative to B834 or the C strain, C1757.

DNA techniques. Restriction enzymes (New England BioLabs, Inc.) and T4 DNA ligase (Pharmacia) were used according to the manufacturers' specifications. Plasmid pML19:*sma* was constructed by digesting pML19 with *Sma*I as described previously (16). Prior to ligation, the appropriate fragment, pML19 minus the 300-base-pair internal *Sma*I fragment, was purified by electrophoresis on a 1% low-melting agarose gel. The plasmid was introduced into $CaCl_2$-treated HMS174 (4), characterized by restriction analysis, and then used to transform other strains.

Standard techniques (27) were used to purify chromosomal DNAs from stationary-phase cells. After restriction endonuclease digestion, the DNA fragments were separated electrophoretically on 0.5% agarose gels in Tris-acetate buffer (8) and transferred to nitrocellulose filters essentially as described (27). Prior to transfer, the gels were stained with ethidium bromide and photographed to record the position of phage lambda size standards.

The probe used in Southern analysis was prepared by nick translating (11) the 2-kilobase (kb) *Eco*RI-*Pst*I fragment of pML19. Blots were hybridized by using standard conditions (27).

Purification of T7 RNA polymerase. Cultures of BL21(pAR1219) were used as the source of T7 RNA polymerase (8). Typically, two 500-ml cultures were grown in shaking 1-liter flasks at 37°C in tryptone broth supplemented with M9 salts, 0.4% glucose, and ampicillin. When the cultures reached an $A_{600\ nm}^{1\ cm}$ of 0.5, isopropyl β-D-thiogalactopyranoside was added (final concentration, 0.5 mM) to derepress the *lac* UV5 promoter and allow T7 RNA polymerase to accumulate in the cells. After 4 h of induction, the cells were collected by centrifugation (Sorvall GS-3 rotor; 10 min at 10,000 × *g*), washed twice with 100 ml of 20 mM Tris hydrochloride (pH 8.1)–20 mM NaCl–2 mM trisodium EDTA, and stored as a cell pellet at −70°C. Usually about 8 g of packed cells was obtained per liter of culture.

The cells were suspended in 24 ml of cold buffer LB (50 mM Tris hydrochloride [pH 8.1], 20 mM NaCl, 2 mM trisodium EDTA, 1 mM dithiothreitol), and lysis was initiated by adding 6 ml of a fresh solution of egg white lysozyme (1.5 mg/ml) in buffer LB. (Note: *E. coli* BL21 lacks the major protease that nicks T7 RNA polymerase [see text]. However, since T7 RNA polymerase seems to be very sensitive to proteolysis, the protease inhibitors phenylmethylsulfonyl fluoride [50 μl of a 20-mg/ml solution in isopropanol] and leupeptin [20 μl of a 5-mg/ml aqueous solution] were added routinely to the lysate. In addition, phenylmethylsulfonyl fluoride [20 μg/ml] was generally included in the first dialysis and chromatography buffers, and all steps were carried out at 0 to 4°C.) After 20 min, 2.5 ml of 0.8% sodium deoxycholate was added and the mixture was allowed to stand for 20 min to complete lysis. The viscosity of the lysate was reduced by four 15-s sonications (10 relative output) from a Heat Systems W185D sonicator. Ammonium sulfate, 5 ml of a 2 M solution, was added to the lysate, which was then brought to 50 ml with buffer LB.

Polymin P (5 ml of a 10% solution that had been adjusted to pH 8 with concentrated HCl [3]) was added slowly with stirring on ice. After 20 min the precipitate that formed was removed by centrifugation for 15 min at 39,000 × *g* in a Sorvall SS-34 rotor. The supernatant was mixed slowly on ice with 0.82 volume of a saturated solution of enzyme grade ammonium sulfate (a saturated solution at room temperature was adjusted to pH 7 by addition of Tris base, passed through a 0.45-μm filter, and chilled to 4°C prior to use). After 15 min of stirring on ice, the precipitate was collected by centrifugation (12,000 × *g* for 10 min) and dissolved in 15 ml of buffer C (20 mM sodium phosphate [pH 7.7], 1 mM trisodium EDTA, 1 mM dithiothreitol, 5% glycerol) containing 100 mM NaCl. Buffer C was used during all subsequent steps in the purification procedure; only the NaCl concentration was varied.

The dissolved ammonium sulfate fraction was dialyzed overnight against 1-liter changes of buffer C plus 100 mM

NaCl, clarified by centrifugation at 12,000 × *g*, and diluted with sufficient buffer C without added NaCl to produce a conductivity equal to that of buffer C plus 50 mM NaCl. The diluted sample was then passed through a 25-ml bed of Trisacryl-SP (LKB Instruments, Inc.) which had been equilibrated with buffer C containing 50 mM NaCl. The column was washed with 4 volumes of starting buffer to remove proteins that failed to bind. The bound T7 RNA polymerase was eluted with buffer C containing 200 mM NaCl. The peak fractions, about 35 ml, were pooled and dialyzed against 500-ml volumes of buffer C plus 25 mM NaCl. After about 3 h of dialysis, the sample was diluted with buffer C so as to have a conductivity equal to that of buffer C plus 25 to 30 mM NaCl and then applied to a 15-ml column of TSK-CM 650 (EM Science) equilibrated with buffer C plus 25 mM NaCl. T7 RNA polymerase was recovered in the flow-through, and essentially all applied enzyme was recovered by washing the carboxymethyl column with starting buffer. T7 RNA polymerase was further purified by chromatography of the carboxymethyl flowthrough on a 10-ml column of TSK-DEAE 650 (EM Science) that had been equilibrated with buffer C plus 25 mM NaCl. The pooled carboxymethyl fraction was loaded onto the DEAE column, and the bound protein was eluted by application of a 200-ml gradient of NaCl from 25 to 250 mM in buffer C. The peak fractions, eluting near 100 mM NaCl, were pooled, concentrated by dialysis versus buffer C containing 100 mM NaCl and 50% (vol/vol) glycerol, and then stored at −20°C.

The above purification procedure routinely yields approximately 50 mg of essentially pure, highly active T7 RNA polymerase. The purified enzyme has a molar extinction coefficient, ξ_{280}, of $1.4 \pm 0.1 \times 10^5$ M^{-1} (22).

Protease assay. Cells were grown overnight in tryptone broth supplemented with 0.4% glucose and 0.5% yeast extract at 37°C. Samples, 1.5 ml, were collected by centrifugation, washed in an equal volume of wash buffer (10 mM Tris hydrochloride [pH 8.0], 20 mM NH_4Cl), and recentrifuged. The cell pellets were suspended in 75 μl of wash buffer, and the $A^{1\ cm}_{600\ nm}$ was determined.

The standard reaction mixture contained 0.25 absorbance unit of cells and 5 μg of intact T7 RNA polymerase, purified from induced cultures of BL21(pAR1219), in 60 μl of reaction buffer (10 mM Tris hydrochloride [pH 8.0], 20 mM NH_4Cl, 10 mM $MgCl_2$). After 1 h at 37°C, the reaction was chilled on ice and then centrifuged in an Eppendorf centrifuge for 2 min to pellet the cells. A 20-μl portion of the supernatant was removed and transferred to 10 μl of 3× loading buffer (1× is 50 mM Tris hydrochloride [pH 6.8], 1% sodium dodecyl sulfate, 2 mM trisodium EDTA, 1% 2-mercaptoethanol, 10% glycerol, 0.1% bromphenol blue). The samples were heated for 2 min at 100°C and then analyzed by electrophoresis in a discontinuous buffer system containing sodium dodecyl sulfate on 10 to 20% polyacrylamide gradient gels (44), followed by staining with Coomassie blue.

RESULTS

Localization of the endoprotease. Electrophoresis of T7 RNA polymerase on polyacrylamide gels in the presence of sodium dodecyl sulfate is a sensitive assay for determining whether the enzyme has been split into the M_r ~20,000 and M_r ~80,000 cleavage products. Our initial studies (8), and those of Tabor and Richardson (49), indicate that T7 RNA polymerase is nicked only after cell lysis, suggesting that the protease activity responsible for nicking may be external to the cytoplasm. As a first step in characterizing the location of the protease, we prepared total extracts of *E. coli* HMS174 by lysozyme freeze-thaw lysis or by passage of the cell suspension through a French press. We then incubated a small portion of each extract with a sample of T7 RNA polymerase containing mostly intact protein (8). As expected, these extracts had very high levels of the endoprotease that splits T7 RNA polymerase into a small amino-terminal and a large carboxyl-terminal fragment. Further studies indicated that the bulk of the endoprotease activity could be recovered in the membrane fraction of each extract by centrifugation at 20,000 × *g*.

Very little if any endoprotease activity could be detected in cytoplasmic or periplasmic cell fractions (35). Clearly, the endoprotease is not protease I, which is known to be localized in the periplasmic space (24), nor does it seem to be one of the proteases known to be localized in the soluble fraction of the cell (48). It also appears that the endoprotease detected by this assay is expressed constitutively in *E. coli*, as opposed to being induced by the presence of T7 RNA polymerase, since these protease-positive extracts were prepared from cells lacking the gene *1* plasmid.

When *E. coli* total membranes were fractionated into inner and outer membranes by centrifugation through discontinuous sucrose gradients (37), the endoprotease was found to be associated almost entirely with the outer membrane fraction (data not shown). An alternative procedure for obtaining outer membrane vesicles, by shaking whole cells with glass beads at 45°C (29), also gave membrane preparations which were highly active when assayed for the presence of the endoprotease. From these studies we conclude that the endoprotease is localized primarily in the outer membrane of *E. coli*.

Additional experiments revealed that the membrane-associated protease activity could be solubilized following treatment of isolated outer membranes with 1% Triton X-100 or 10 to 30 mM *n*-octylglucoside. We also found that a substantial amount of protease activity could be solubilized by extracting whole cells with 30 mM *n*-octylglucoside, a result which seemed to indicate that the protease might be present on the surface of the cell. To test this idea, we incubated washed unpermeabilized cells with purified T7 RNA polymerase and then pelleted the cells by brief centrifugation. When the supernatant was analyzed by gel electrophoresis, it was clear that the gene *1* protein had been cleaved into the 20- and 80-kilodalton (kDa) fragments. This observation suggested that a rapid simple assay for screening *E. coli* strains for this endoprotease might be to incubate whole cells with purified T7 RNA polymerase and then determine by gel electrophoresis whether the added protein was cleaved. The patterns shown in Fig. 1 are representative of those obtained when this procedure was used to assay some common laboratory strains for protease activity. The supernatants usually contain a variable, but low, amount of cellular proteins which presumably are derived from cells that lyse during incubation (Fig. 1). However, their presence does not interfere with the ability to detect even minor amounts of the 80- and 20-kDa T7 RNA polymerase cleavage products (see lane 5). Control experiments demonstrated that these supernatants have very low levels of the protease that nicks T7 RNA polymerase (data not shown), indicating that most of the cleavages detected by the assay are mediated by whole cells.

Every K strain we initially tested cleaved T7 RNA polymerase, although one K strain, DH1 (lane 5), consistently

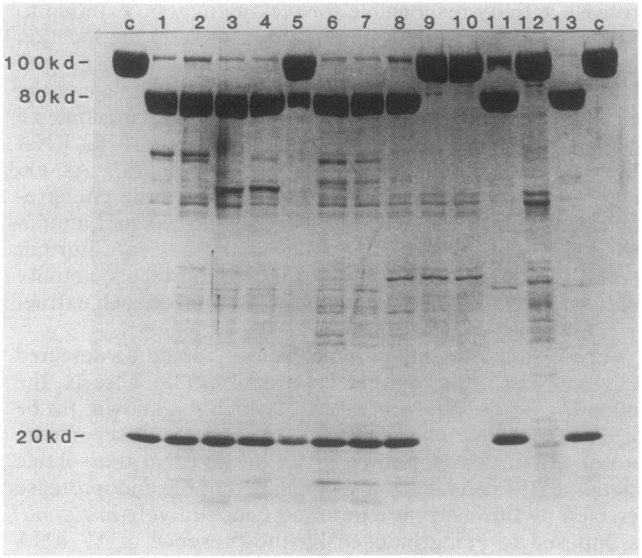

FIG. 1. Screening of various *E. coli* strains for their ability to cleave purified T7 RNA polymerase. The samples applied to lanes c of the sodium dodecyl sulfate-polyacrylamide (10 to 20%) gradient gel are controls of T7 RNA polymerase incubated in buffer alone. The samples applied to lanes 1 to 13 were obtained by incubating T7 RNA polymerase with washed cells from cultures of the following: (1) W3110; (2) HMS174; (3) ED8739; (4) LE392; (5) DH1; (6) SG4038; (7) SG4044; (8) B707; (9) B834; (10) BL21; (11) HB101; (12) C1757; (13) KK2186. Equal $A_{600\ nm}^{1\ cm}$ units were used in each assay as described in Materials and Methods. Numbers to the right indicate molecular size in kilodaltons (kd).

showed greatly reduced levels of endoprotease. Cleavage of T7 RNA polymerase was not attenuated if the strain used in the assay carried a deletion that removes the *lon* gene (lane 7) (2). However, as can be seen from the gel pattern, two B strains, B834 and BL21 (lanes 9 and 10), both apparently lack active endoprotease on their cell surface, as does the one C strain we tested, C1757 (lane 12). Additional experiments showed that the failure to detect active protease on these cells was correlated with a significantly reduced ability of the corresponding total cell extracts to cleave added T7 RNA polymerase. Thus, it is very likely that the protease detected by this assay represents the major endoproteolytic activity of *E. coli* that is able to cleave T7 RNA polymerase.

The structural gene for the outer membrane protease is *ompT*. The most striking feature of the above survey is that strain B707 can cleave T7 RNA polymerase whereas two derivatives of this strain, B834 and BL21, lack the protease. The only previously known difference between these strains was the presence (in B707) or absence (in B834 and BL21) of *E. coli* B restriction and modification capabilities (53). Interestingly, the only other strain we found in this initial screening that completely lacks the protease was C1757, which, like B834 and BL21, is unable to restrict or modify lambda phage DNA. However, there does not seem to be a direct correlation between the presence of the protease and an *hsd*$^+$ locus since all of the K strains used in Fig. 1 have the protease even though many of them have mutations in the *hsd* structural genes (Table 1).

To see if the gene encoding the protease might be closely linked to the *hsd* locus, we introduced into both B834 and C1757 an *hsd*$^+$ genotype, via P1 transduction, from a protease-positive strain, SK596 (Table 1), which has the tetracycline-resistant transposon Tn*10* tightly linked to *hsd*. Of the 40 tetracycline-resistant transductants examined for each strain, 80% became *hsdR*$^+$ when tested for their ability to restrict unmodified lambda phage; however, none of these transductants had acquired the protease that cleaves T7 RNA polymerase. From these results it seems clear that the protease gene is not closely linked to *hsd* and that, in the case of strain B834, the loss of protease activity must have occurred by a genetic event independent of the loss of restriction and modification capabilities.

Since we had in our collection the protease-positive parent of BL21, we decided to use BL21 for further experiments to map the protease gene. As a first step, we constructed by P1 transduction a kanamycin-resistant derivative of BL21, BL21.1, to serve as the recipient in a series of Hfr matings. Because acquisition of the protease activity could not be selected directly, the donors we used were Hfr strains each carrying a Tn*10* insertion near their respective origin of transfer. Exconjugates were selected by their ability to grow in the presence of kanamycin and tetracycline and then tested for protease activity by the whole-cell assay. The results we obtained were consistent with the protease gene mapping between 10 and 20 min.

Since previous studies had shown that the structural gene for OmpT, a known outer membrane protease of *E. coli*, is located at 12.5 min (10, 15, 42), the question arose as to whether OmpT might be the endoprotease that cleaves T7 RNA polymerase. Therefore, we obtained from C. Earhart (University of Texas, Austin) a set of K strains having either the wild-type *ompT* structural gene or deletions that eliminate the *ompT* locus (10). The strain having *ompT* (UT2300) cleaves T7 RNA polymerase, but neither deletion strain (UT4400 or UT5600) cuts the added protein (Fig. 2). Because the deletions in these strains are large, the possibility still existed that a second protease gene, or a regulatory gene for some other protease, had also been deleted. To address this

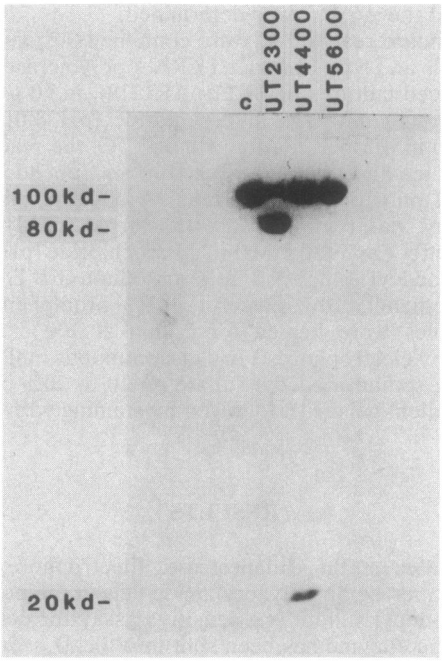

FIG. 2. *ompT* deletion strains do not cleave T7 RNA polymerase. Lanes: (c) T7 RNA polymerase control; (UT2300) *ompT*$^+$; (UT4400) *ompT* deletion; (UT5600) *ompT* deletion.

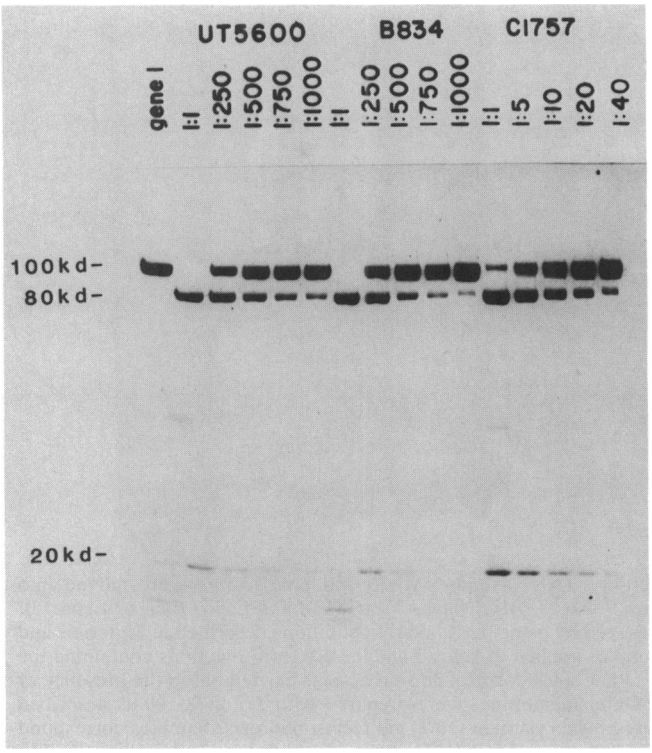

FIG. 3. Restoration and high-level expression of protease activity by transformation with a plasmid encoding OmpT. Protease-minus strains UT5600, B834, and C1757 harboring the *ompT* plasmid pML19 were assayed as described in Materials and Methods (1:1) or after dilution, as indicated, with wash buffer. Gene *1* is the intact T7 RNA polymerase control.

question, we took advantage of the availability of a pUC-based plasmid, pML19, constructed by M. Lundrigan (University of Virginia, Charlottesville) that contains a 2-kb *Eco*RI-*Pst*I fragment of *E. coli* DNA carrying sequences that code for all of OmpT (15, 16). When plasmid pML19 was used to transform UT5600 and B834, the resulting ampicillin-resistant colonies had not only the ability to cleave T7 RNA polymerase, but also dramatically increased levels of protease activity relative to nontransformed protease-positive strains such as B707 or HMS174. Only small amounts of UT5600(pML19) or B834(pML19) cells need be present in the assay to cleave an appreciable fraction of the added T7 RNA polymerase (Fig. 3). It also is apparent (Fig. 3) that some further hydrolysis of the added T7 RNA polymerase can occur when cells with elevated expression of the OmpT protease are used in the assay. The smaller amino-terminal fragment appears to be particularly sensitive to further cleavage. However, specific cleavage is observed when these cells are diluted prior to the assay.

Plasmid pML19 also transforms the null strain C1757 to a protease-positive phenotype. However, in this strain pML19 does not appear to cause marked overproduction of OmpT. In fact, the level of protease detected on C1757(pML19) cells (Fig. 3) is very similar to that observed for most nontransformed protease-positive strains.

Because pUC-based plasmids are usually present in high copy number, it was not unexpected that clones containing pML19 would synthesize higher than normal levels of OmpT. In fact, the OmpT protein encoded by pML19 in UT5600 and B834 can be easily detected as a major cellular protein in both whole-cell lysates and isolated membranes (see Fig. 4b), whereas the OmpT protein was undetectable in these strains prior to transformation. Other experiments showed that C1757(pML19) produced much less OmpT protein. Although we do not know why OmpT is not overproduced in C1757, control experiments showed that the copy number of pML19 in all three strains was similar.

The 2-kb restriction fragment of *E. coli* DNA cloned in pML19 carries the structural gene for OmpT and another protein, designated M5, whose function is unknown (15, 16). To demonstrate that functional OmpT is necessary for cleavage of T7 RNA polymerase, we constructed a deletion mutant of pML19 by removing an internal 300-base-pair *Sma*I fragment known to lie within the *ompT* coding sequence (16). When strains UT4400, UT5600, and BL21 carry the deletion plasmid, only a trace of protease activity against T7 RNA polymerase could be detected on their cell surfaces (Fig. 4A), in contrast to the high levels present when these same strains carry pML19 having an intact *ompT* gene (Fig. 3). Electrophoretic analysis of the proteins present in the membrane fraction of cells harboring the *Sma*I deletion plasmid showed no evidence of full-length OmpT protein (Fig. 4B). However, it is worth noting that the *Sma*I deletion does not completely eliminate plasmid-encoded protease activity.

Hybridization analysis of protease-minus strains. Southern hybridization analysis (27) was performed to determine whether the protease-minus strains B834, BL21, and C1757 have an *ompT* structural gene. The 2-kb *Eco*RI-*Pst*I fragment of pML19 was used to probe *Pst*I-digested DNAs from these strains and similar digests from a strain known to have the *ompT* gene, UT2300, or a deletion strain, UT5600, lacking this locus. As expected, the probe hybridized to a single 3.3-kb *Pst*I fragment of UT2300 DNA, but not to any fragments of UT5600 DNA (Fig. 5). The same probe failed to hybridize to any fragments present in digests of B834, BL21, or C1757 DNAs. However, it did hybridize to a 3.3-kb *Pst*I fragment of B707 DNA, the protease-positive parent of B834 and BL21. It also hybridized to a 6-kb *Pst*I fragment of DH1 DNA, a weakly protease-positive strain. From these results we concluded that the B834, BL21, and C1757 assay as protease minus because they lack the structural gene for OmpT. These data also show that the *ompT* gene is not entirely missing in DH1.

Regulation of *ompT* expression. Previous studies have shown that *perA* mutants have diminished quantities of a protein in the cell's outer membrane which is thought to be OmpT (26). The *perA* mutation is an allele of *envZ* which, together with *ompR*, comprises the *ompB* operon, located at 75 min on the *E. coli* K-12 chromosome (17, 32). The genetics of this locus are complex, and a number of different alleles have been described that depress the expression of various noncytoplasmic proteins (6, 50, 51). We have tested a number of *envZ* strains, including a *perA* strain, for their ability to cleave T7 RNA polymerase (Fig. 6). Two mutants, MH1461 and MH1471, were found to have much lower levels of protease activity than did the *envZ*$^+$ parent strain MC4100. However, three other *envZ* strains, RT3, SG477, and SG480Δ76, showed somewhat increased levels of activity relative to MC4100. Surprisingly, the *perA* strain, BW490.9, did not appear to have a significant reduction in protease activity relative to the isogenic *perA*$^+$ strain BW490.12 or to MC4100 (see Discussion).

To further characterize *ompT* expression in these strains, we transformed them with pML19, the recombinant plasmid

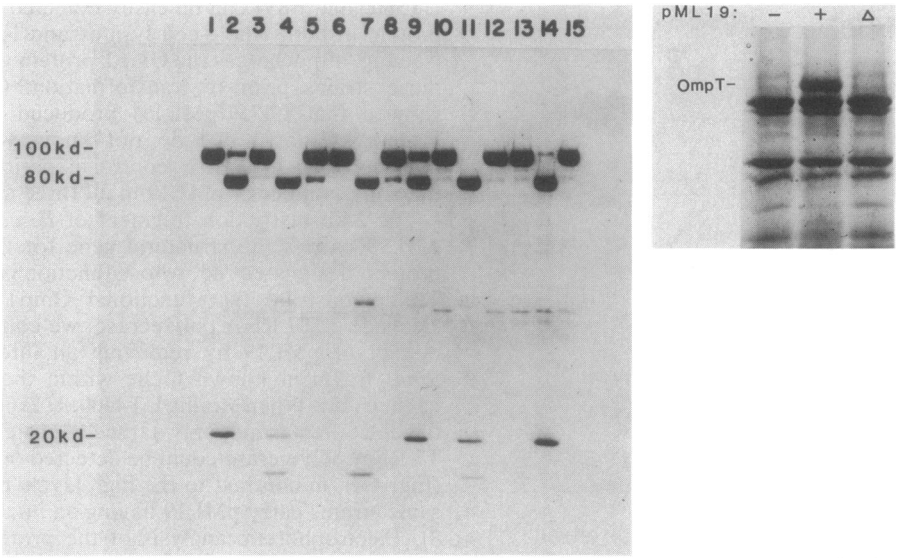

FIG. 4. Evidence that pML19:*sma* does not restore full protease activity. (A) Plasmids pML19 and pML19:*sma* were moved into protease-minus strains UT4400 (lanes 4 and 5, respectively), UT5600 (lanes 7 and 8), BL21 (lanes 11 and 12), and C1757 (lanes 14 and 15). The resulting transformants and their nontransformed parents were then assayed by using the standard conditions described in Materials and Methods. Lane 1 is a control showing intact T7 RNA polymerase. The samples applied to lanes 2 and 9 were from reactions containing the nontransformed protease-positive strains UT2300 and B707, respectively. Other lanes correspond to reactions carried out in the presence of nontransformed UT4400 (3), UT5600 (6), B121 (10), and C1757 (12). (B) Outer membranes were prepared with Triton X-100 as described previously (36), and the proteins were separated by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis. Samples correspond to membranes obtained from equal amounts of (−) nontransformed UT5600, (+) UT5600(pML19), and (Δ) UT5600(pML19:*sma*). The position of OmpT is indicated.

carrying the *ompT* structural gene. The resulting strains all had increased levels of protease activity; however, the increases were not uniform. *envZ* mutant strains that initially had a strong *ompT*$^+$ phenotype showed greater increases in activity relative to those strains that initially had reduced *ompT* expression.

These results are for the most part consistent with the idea that the *envZ* gene product can influence *ompT* expression. If this is the case, one possible reason why introduction of the pML19 plasmid into C1757 results in such a low level of protease activity is that C1757 is an *envZ* mutant. This possibility was tested by introducing an *envZ*$^+$ locus into C1757(pML19) by P1 transduction. The lysate was grown on

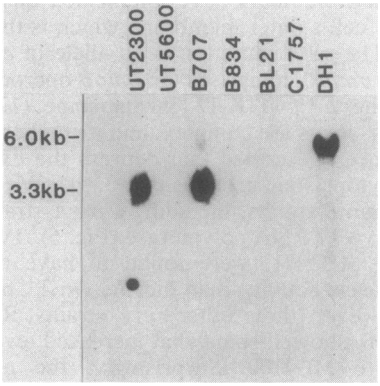

FIG. 5. Autoradiogram from a Southern blot of *Pst*I-digested chromosomal DNA from various protease-positive and protease-minus *E. coli* strains. The filter was probed with ^{32}P-labeled *ompT* DNA from the *Pst*I-*Eco*RI fragment of pML19. Fragment sizes are given at the left.

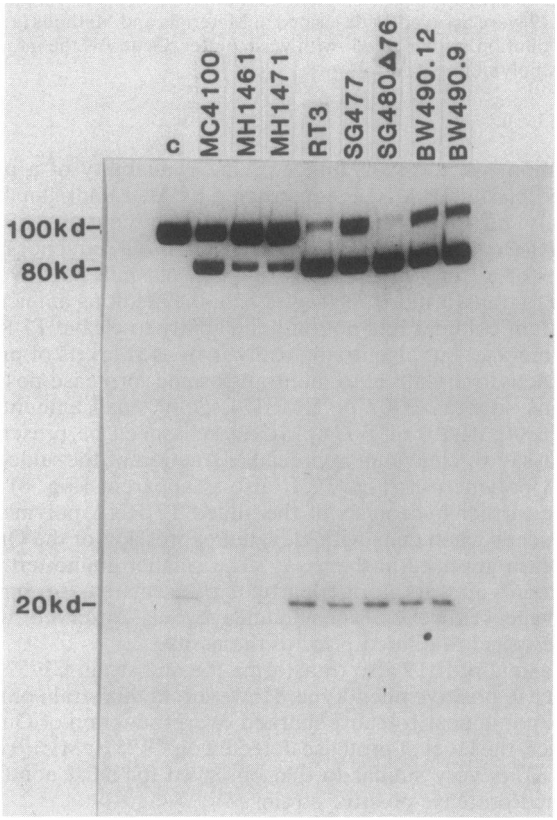

FIG. 6. Protease assay of *envZ*, *envZ*$^+$, *perA*, and *perA*$^+$ strains. Lane c, Intact T7 RNA polymerase. Strains MC4100 and BW490.12 have a wild-type *envZ* gene, while the others all carry a mutation in this locus (Table 1).

SY781, which has a Tn*10* insertion 90% cotransducible with the *envZ*$^+$ structural gene (D. Oliver, personal communication). None of the 14 tetracycline-resistant transformants displayed a further increase in the level of *ompT* expression, but 14 of 16 similar transductants of a known *envZ* mutant, SG480Δ76, regained the wild-type *envZ* phenotype. From these results, it appears that introduction of the *envZ*$^+$ gene into C1757(pML19) is insufficient by itself to confer high-level *ompT* expression.

We also introduced a wild-type *envZ* allele into DH1 to see if its presence increased the basal level of protease activity. In this case, a plasmid, pAT428, carrying the entire *ompB*$^+$ operon was used (32). However, the resulting DH1(pAT428) transformants showed no increase in protease activity, although the same plasmid was able to increase *ompT* expression when moved into the *envZ* mutant strain MH1461. While the mutation in DH1 could not be complemented by introduction of the wild-type *envZ* allele, it does not prevent high-level synthesis of OmpT when transformed with plasmid pML19 (data not shown). These results, together with those of Southern blotting, raise the possibility that DH1 may actually carry a mutation in or near the *ompT* coding sequence.

DISCUSSION

Previous biochemical and genetic studies have shown that OmpT, formerly called protein *a*, is a trypsinlike protease with narrow specificity (18). OmpT is synthesized as a 42-kDa proprotein that is processed in the membrane to the mature 40-kDa form (14). Since its synthesis is reduced significantly in cells grown at or below 32°C, the structural gene is designated *ompT* in reference to the temperature-dependent appearance of its product (28). However, when we tried to purify T7 RNA polymerase from an *ompT*$^+$ strain grown at 30°C, we still had problems with proteolysis, presumably because some OmpT protein still was present. We only have been successful in purifying intact T7 RNA polymerase from an *ompT* deletion strain, such as BL21.

The effect of low temperature on OmpT synthesis may be partially caused by altered posttranslational processing of the 42-kDa proprotein to a 20-kDa polypeptide (14). Gordon et al. (16) have proposed that the effect of temperature may also be at the level of initiation of OmpT proprotein synthesis and that EnvZ is a positive regulatory protein activating translation of *ompT* mRNA. We have examined the effect of a variety of different *envZ* mutations on expression of OmpT protease activity. Some *envZ* mutants did show reduced levels of OmpT activity, while others had elevated levels relative to the wild type. Moreover, when these mutants were transformed with pML19, they continued to show differential effects on *ompT* expression. Further work is needed to determine whether these differences in *ompT* expression all are caused by subtle changes in the ability of the *envZ* gene product to promote translation of *ompT* mRNA or whether any are mediated by EnvZ-specific changes in other outer membrane proteins.

Our failure to increase OmpT levels in C1757(pML19) by introducing a wild-type *envZ* gene suggests that C1757 may carry a mutation at some other locus which, in this strain, is limiting *ompT* expression. Why the *perA* strain we tested seems to have normal levels of protease activity is not certain. One explanation may be that this strain has undergone further mutation or that it has on its surface an increased concentration of another, as yet unidentified, protease that can cleave T7 RNA polymerase. Antibodies against purified OmpT are being raised to investigate the identity of this protease.

Southern analysis showed that the *ompT* structural gene is absent in at least two laboratory strains of *E. coli* B, B834 and BL21, as well as the C strain, C1757. In this regard, these strains are similar to the *ompT* deletion mutants of K-12 that Earhart has isolated (10, 30). These latter strains have spontaneous deletions beginning near 13 min that extend counterclockwise towards *purE*. The deletions not only remove the *ompT* structural gene, but also the gene for an 81-kDa outer membrane protein that serves as the receptor for colicins B and D (10, 30). Although the deletions can be large (0.3 to 0.7 min), they apparently do not remove any essential genes.

Presumably, a similar deletion event took place during Wood's original selection of strain B834 from its OmpT-positive parent B707 (53). We have tested two other *hsd* strains that he isolated for OmpT protease activity. Both K803 and B837 (Table 1) showed normal levels of activity. Thus, loss of the *ompT* locus from B834 must have been wholly independent of the selection for lesions in *hsd*.

Typically, the two proteolytic fragments of T7 RNA polymerase detected in our assay are present in stoichiometric amounts. However, when OmpT-overproducing strains are assayed, further degradation products become evident unless the number of cells used in the assay is reduced. This indicates that the 20- and 80-kDa fragments are not limit digestion products of OmpT cleavage. Recent experiments (J. J. Dunn, unpublished data) demonstrate that a number of other proteases with differing specificities all have primary cleavage sites at, or very near to, the same residues which serve as the primary sites for OmpT. Presumably, T7 RNA polymerase is folded so as to leave the OmpT protease-sensitive sites exposed and highly susceptible to cleavage. T3 RNA polymerase, which is highly homologous to T7 RNA polymerase, is also cleaved in vitro by purified OmpT into 20- and 80-kDa fragments (34). As in the case of T7 RNA polymerase, T3 RNA polymerase can be purified intact from an *ompT* deletion strain (34). SP6 RNA polymerase, which has only partial homology with T7 and T3 RNA polymerases, also seems to be sensitive to proteolysis during purification (23). It will interesting to determine whether the SP6 enzyme remains intact when it is purified from an *ompT* deletion strain.

The OmpT protease seems to be different from the previously reported membrane-associated proteases IV and V of *E. coli* (38, 39) and from the signal and leader peptidases (19, 52). However, its reported inhibitor profile (18; our unpublished studies), molecular weight, and elution profile from a benzamidine affinity matrix all are similar to those of a recently characterized activity termed protease VI (40). The availability of *ompT* deletion strains with and without an active plasmid-encoded *ompT* gene should help in determining whether protease VI and OmpT are related and whether either is identical to the enzyme present in *E. coli* membranes which can activate plasminogen to plasmin (25). Preliminary studies, carried out in collaboration with W. Mangel (Brookhaven National Laboratory), indicate that only *ompT*$^+$ strains can activate plasminogen.

BL21 is being used routinely for expression of T7 and T3 RNA polymerases and cloned genes under control of T7 genetic signals (46). As a B strain, it is naturally deficient in *lon* protease (2, 9, 41). Furthermore, it has no known growth requirements and it grows well in minimal medium. All of these properties suggest that BL21 might be useful for

expression of cloned gene products that are sensitive to hydrolysis during purification.

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ADDENDUM IN PROOF

The nucleotide sequence of *ompT* has been determined. It encodes a 317-amino-acid protein of M_r 35,567. The sequence found for the first 16 amino acids at the amino-terminal end of OmpT was exactly that predicted from the nucleotide sequence for a protein synthesized as a precursor with a 20-amino-acid signal sequence. (J. Grodberg, M. D. Lundrigan, D. L. Toledo, W. F. Mangel, and J. J. Dunn, Nucleic Acids Res., in press).

LITERATURE CITED

1. **Borck, K., J. D. Beggs, W. J. Brammar, A. S. Hopkins, and N. E. Murray.** 1976. The construction *in vitro* of transducing derivatives of phage lambda. Mol. Gen. Genet. **146**:199–207.
2. **Bukhari, A. I., and D. Zipser.** 1973. Mutants of *Escherichia coli* with a defect in the degradation of nonsense fragments. Nature (London) New Biol. **243**:238–241.
3. **Burgess, R. R., and J. J. Jendrisak.** 1975. A procedure for the rapid, large-scale purification of *Escherichia coli* DNA-dependent RNA polymerase involving polymin P precipitation and DNA-cellulose chromatography. Biochemistry **14**:4634–4638.
4. **Campbell, J. L., C. C. Richardson, and F. W. Studier.** 1978. Genetic recombination and complementation between bacteriophage T7 and cloned fragments of T7 DNA. Proc. Natl. Acad. Sci. USA **75**:2276–2280.
5. **Casadaban, M. J.** 1976. Transposition and fusion of *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and mu. J. Mol. Biol. **104**:541–555.
6. **Case, C. C., B. Bukau, S. Granett, M. R. Villarejo, and W. Boos.** 1986. Contrasting mechanisms of *envZ* control of *mal* and *pho* regulon genes in *Escherichia coli*. J. Bacteriol. **166**:706–712.
7. **Chamberlin, M., J. McGrath, and L. Waskell.** 1970. New RNA polymerase from *Escherichia coli* infected with bacteriophage T7. Nature (London) **228**:227–231.
8. **Davanloo, P., A. H. Rosenberg, J. J. Dunn, and F. W. Studier.** 1984. Cloning and expression of the gene for bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **81**:2035–2039.
9. **Donch, J., Y. S. Chung, and J. Greenberg.** 1969. Locus for radiation resistance in *Escherichia coli* strain B/r. Genetics **61**:363–370.
10. **Earhart, C. F., M. Lundrigan, C. L. Pickett, and J. R. Pierce.** 1979. *Escherichia coli* K-12 mutants that lack major outer membrane protein *a*. FEMS Microbiol. Lett. **6**:277–280.
11. **Erola, S. V., F. A. Burr, and B. Burr.** 1986. The suitability of restriction fragment length polymorphisms as genetic markers in maize. Theor. Appl. Genet. **71**:765–771.
12. **Garrett, S., R. K. Taylor, and T. J. Silhavy.** 1983. Isolation and characterization of chain-terminating nonsense mutations in a porin regulator gene, *envZ*. J. Bacteriol. **156**:62–69.
13. **Garrett, S., R. K. Taylor, T. J. Silhavy, and M. L. Berman.** 1985. Isolation and characterization of Δ*ompB* strains of *Escherichia coli* by a general method based on gene fusions. J. Bacteriol. **162**:840–844.
14. **Gayda, R. C., G. W. Henderson, and A. Markovitz.** 1979. Neuroactive drugs inhibit trypsin and outer membrane protein processing in *Escherichia coli* K-12. J. Bacteriol. **136**:369–380.
15. **Gayda, R. C., and A. Markovitz.** 1978. Cloned DNA fragment specifying major outer membrane protein *a* in *Escherichia coli* K-12. J. Bacteriol. **136**:369–380.
16. **Gordon, G., R. C. Gayda, and A. Markovitz.** 1984. Sequence of the regulatory region of *ompT*, the gene specifying major outer membrane protein *a* (3b) of *Escherichia coli* K-12: implications for regulation and processing. Mol. Gen. Genet. **193**:414–421.
17. **Hall, M. N., and T. J. Silhavy.** 1981. The *ompB* locus and the regulation of the major outer membrane porin proteins of *Escherichia coli* K12. J. Mol. Biol. **146**:23–43.
18. **Hollifield, W. C., Jr., E. H. Fiss, and J. B. Nielands.** 1978. Modification of ferric enterobactin receptor protein from the outer membrane of *Escherichia coli*. Biochem. Biophys. Res. Commun. **83**:739–746.
19. **Ichihara, S., N. Beppu, and S. Mizushima.** 1984. Protease IV, a cytoplasmic membrane protein of *Escherichia coli*, has signal peptide peptidase activity. J. Biol. Chem. **259**:9853–9857.
20. **Ikeda, R. A., and C. C. Richardson.** 1987. Enzymatic properties of a proteolytically nicked RNA polymerase of bacteriophage T7. J. Biol. Chem. **262**:3790–3799.
21. **Ikeda, R. A., and C. C. Richardson.** 1987. Interactions of a proteolytically nicked RNA polymerase of bacteriophage T7 with its promoter. J. Biol. Chem. **262**:3800–3808.
22. **King, G. C., C. T. Martin, T. T. Pham, and J. E. Coleman.** 1986. Transcription of T7 RNA polymerase is not zinc-dependent and is abolished on amidomethylation of cysteine-347. Biochemistry **25**:36–40.
23. **Kotani, H., Y. Ishizaki, N. Hiraoka, and A. Obayashi.** 1987. Nucleotide sequence and expression of the cloned gene of bacteriophage SP6 RNA polymerase. Nucleic Acids Res. **15**: 2653–2664.
24. **Kowit, J. D., W. N. Choy, S. P. Champe, and A. L. Goldberg.** 1976. Role and location of "protease I" from *Escherichia coli*. J. Bacteriol. **128**:776–784.
25. **Leytus, S. P., L. K. Bowles, J. Konisky, and W. F. Mangel.** 1981. Activation of plasminogen to plasmin by a protease associated with the outer membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **78**:1485–1489.
26. **Lundrigan, M., and C. F. Earhart.** 1981. Reduction in three iron-regulated outer membrane proteins and protein *a* by the *Escherichia coli* K-12 *perA* mutation. J. Bacteriol. **146**:804–807.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Manning, A., and P. Reeves.** 1977. Outer membrane protein 3b of *Escherichia coli* K-12: effects of growth temperature on the amount of the protein and further characterization on acrylamide gels. FEMS Microbiol. Lett. **1**:275–278.
29. **McDade, R. L., Jr., and K. H. Johnson.** 1980. Characterization of serologically dominant outer membrane proteins of *Neisseria gonorrhoeae*. J. Bacteriol. **141**:1183–1191.
30. **McIntosh, M. A., S. A. Chenault, and C. F. Earhart.** 1979. Genetic and physiological studies on the relationship between colicin B resistance and ferrienterochelin uptake in *Escherichia coli* K-12. J. Bacteriol. **137**:653–657.
31. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Mizuno, T., E. T. Wurtzel, and M. Inouye.** 1982. Osmoregulation of gene expression. II. DNA sequence of the *envZ* gene of the *ompB* operon of *Escherichia coli* and characterization of its gene product. J. Biol. Chem. **257**:13692–13698.
33. **Moffatt, B. A., J. J. Dunn, and F. W. Studier.** 1984. Nucleotide sequence of the gene for bacteriophage T7 RNA polymerase. J. Mol. Biol. **173**:265–269.
34. **Morris, C. E., J. F. Klement, and W. T. McAllister.** 1986. Cloning and expression of the bacteriophage T3 RNA polymerase gene. Gene **41**:193–200.
35. **Nossal, N. G., and L. A. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241**:3055–3062.
36. **Oliver, D. B., and J. Beckwith.** 1982. Regulation of a membrane

component required for protein secretion in *Escherichia coli*. Cell **30**:311–319.
37. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhymurium*. Isolation and characterization of cytoplasmic and outer membranes. J. Biol. Chem. **247**:3962–3972.
38. **Pacaud, M.** 1982. Identification and localization of two membrane-bound esterases from *Escherichia coli*. J. Bacteriol. **149**: 6–14.
39. **Pacaud, M.** 1982. Purification and characterization of two novel proteolytic enzymes in membranes of *Escherichia coli*. J. Biol. Chem. **257**:4333–4338.
40. **Palmer, S. M., and A. C. St. John.** 1987. Characterization of a membrane-associated serine protease in *Escherichia coli*. J. Bacteriol. **169**:1474–1479.
41. **Phillips, T. A., R. A. VanBogelen, and F. C. Neidhardt.** 1984. *lon* gene product of *Escherichia coli* is a heat-shock protein. J. Bacteriol. **159**:283–287.
42. **Rupprecht, K. R., G. Gordon, M. Lundrigan, R. C. Gayda, A. Markovitz, and C. Earhart.** 1983. *ompT*: *Escherichia coli* K-12 structural gene for protein *a* (3b). J. Bacteriol. **153**:1104–1106.
43. **Studier, F. W.** 1969. The genetics and physiology of bacteriophage T7. Virology **39**:562–574.
44. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**:237–248.
45. **Studier, F. W., and J. J. Dunn.** 1983. Organization and expression of bacteriophage T7 DNA. Cold Spring Harbor Symp. Quant. Biol. **47**:999–1007.
46. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
47. **Sunshine, M. G., M. Thom, W. Gibbs, R. Calendar, and B. Kelly.** 1971. P2 phage amber mutants: characterization by use of a polarity suppressor. Virology **46**:691–702.
48. **Swamy, K. H. S., and A. L. Goldberg.** 1982. Subcellular distribution of various proteases in *Escherichia coli*. J. Bacteriol. **149**:1027–1033.
49. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase-promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**: 1074–1078.
50. **Wandersman, C., F. Moreno, and M. Schwartz.** 1980. Pleiotropic mutations rendering *Escherichia coli* K-12 resistant to bacteriophage TP1. J. Bacteriol. **143**:1374–1383.
51. **Wanner, B. L., A. Sarthy, and J. Beckwith.** 1979. *Escherichia coli* pleiotropic mutant that reduces amounts of several periplasmic and outer membrane proteins. J. Bacteriol. **140**:229–239.
52. **Wolfe, P. B., P. Silver, and W. Wickner.** 1982. The isolation of homogeneous leader peptidase from a strain of *Escherichia coli* which overproduces the enzyme. J. Biol. Chem. **257**:7898–7902.
53. **Wood, W. B.** 1966. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting the restriction and modification of DNA. J. Mol. Biol. **16**:118–133.
54. **Zagursky, R. L., and M. L. Berman.** 1984. Cloning vectors that yield high levels of single-stranded DNA for rapid DNA sequencing. Gene **27**:183–191.