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Biochemistry

Isolation of the *Escherichia coli* leader peptidase gene and effects of leader peptidase overproduction *in vivo*

(membrane biogenesis/phage M13 coat protein)

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ABSTRACT **The only covalent modifications known to accompany protein insertion into membranes or protein secretion are glycosylation and the proteolytic removal of an NH_2-terminal leader (signal) sequence. This latter reaction is catalyzed by leader peptidase, a constitutive, membrane-bound proteinase. We now report the identification of a plasmid-bearing strain of *Escherichia coli* that overproduces leader peptidase 4- to 6-fold. This strain grows normally and shows an unaltered polypeptide composition of inner and outer membranes. The leader peptidase gene has been subcloned and transferred from this plasmid to the multicopy plasmid pBR322, yielding a new plasmid (pTD101). Strains transformed by pTD101 have a 30-fold increase in leader peptidase. We have studied the effect of leader peptidase overproduction on the insertion of newlymade M13 phage coat protein into the plasma membrane of infected cells. The overproducer strain, when infected by M13 phage, shows a dramatic acceleration in the conversion of procoat (a cytoplasmic precursor form) to coat (an integral, transmembrane protein). Thus the leader peptidase that converts M13 procoat to coat *in vitro* can catalyze this reaction *in vivo* as well.**

During the last decade, biochemical techniques have been used to study membrane assembly and secretion (reviewed in ref. 1). Recent genetic studies with microorganisms (2, 3) have cast new light on these processes but have not yet allowed identification of new enzymatic activities. We have purified and studied leader peptidase (4, 5), an *Escherichia coli* membrane enzyme that cleaves precursor forms of membrane and secreted proteins. This is the only known isolated enzyme of membrane biogenesis. We have therefore sought to isolate its gene as an aid to studying the role of leader peptidase in protein localization.

Clarke and Carbon (6) have prepared (and generously provided) a collection of over 2000 *E. coli* strains, most of which bear a segment of the *E. coli* genome cloned in the multicopy plasmid ColE1. Because these segments are each approximately 1/250th the size of the total genome and were generated by random shear of the DNA, each gene might be expected to be represented on several plasmids in the collection. In each strain, the many copies of the few plasmid-borne genes lead to the selective overproduction of their respective proteins. In some cases the overproduced protein might be fatal to the cell, and the respective plasmid would not appear in the collection.

Because most precursor proteins of *E. coli* are processed within seconds of their synthesis, it seemed likely that overproduction of leader peptidase would not be fatal. Many genes have been identified in the Clarke and Carbon collection by the plasmid's ability to complement chromosomal mutants (6), but no such mutants are available for leader peptidase. We therefore sought to assay directly for overproduction of the enzyme, exploiting the insensitivity of leader peptidase to serine protease inhibitors (5) and the ability of leader peptidase to posttranslationally cleave M13 procoat to coat protein (7). We now describe an assay for leader peptidase in crude extracts, the isolation and manipulation of its gene, and the effects of enzyme overproduction on membrane assembly.

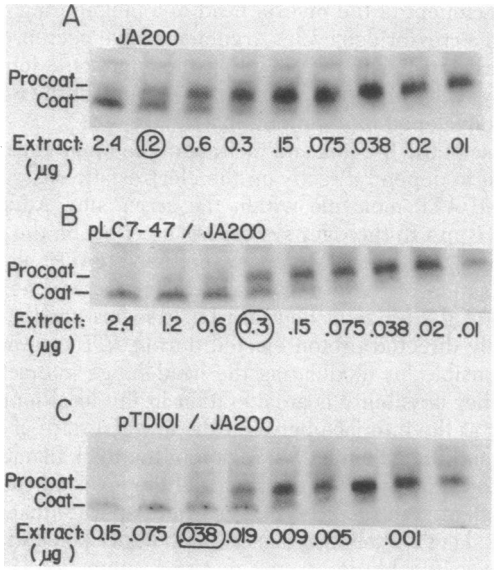

FIG. 1. Leader peptidase activity in crude extracts of *E. coli* JA200 (*A*) and this strain with plasmid pLC7-47 (*B*) (6) or (*C*) pTD101. Cultures (1 ml) of each strain were grown at 37°C in L broth to OD_{600} = 0.5 and centrifuged, and the cells were resuspended in 150 μl of 10 mM Tris·HCl (pH 8.1)/10 mM EDTA/1% Triton X-100. Cells were disrupted by brief sonication at 0°C and the indicated amount of cell extract protein was assayed for leader peptidase by posttranslational conversion of procoat to coat in the presence of mixed proteinase inhibitors. The levels of extract yielding procoat and coat bands of equal intensity (4) are circled.

MATERIALS AND METHODS

Bacteria and Viruses. A collection of strains bearing random fragments of *E. coli* DNA in the multicopy plasmid ColE1 (6) was the kind gift of L. Clarke and J. Carbon. They also provided strain JA200 (F^+/C600 Δ*trpE5*, *recA*), which was used for subcloning experiments. Strain HJM114 (ref. 8; F′ *lac pro*/∇*lac pro*) and its ampicillin-resistant transformant with plasmid pTD101 were used for pulse–chase experiments. Wild-type M13, M13 with an amber mutant in gene 7 (M13 am7-H2), and a pseudorevertant of an amber mutant of M13 in the coat protein

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Abbreviation: kb, kilobase pair(s).

(gene 8), termed M13 am8-H1R6 (9–11), were the generous gift of David Pratt.

Media. L broth, L plates, and M9 minimal medium (with 0.5% glucose and thiamin at 1 μg/ml) were as described by Miller (12).

Analysis of DNA. Phage T4 ligase was from New England BioLabs. Restriction enzymes were from Bethesda Research Laboratories (Rockville, MD) and were used as described (13), Fragments of DNA were separated on horizontal 0.75% agarose slab gels in Tris/EDTA/borate buffer (14) and were visualized under ultraviolet light after soaking for 20 min in ethidium bromide (0.2 μg/ml). Ligation and transformation were as described by DeVries *et al.* (15) and Collins *et al.* (16), respectively.

Leader Peptidase Overproducer. Individual strains from the collection of Clarke and Carbon (6) were grown at 37°C in 1.3 ml of L broth supplemented with colicin E1. One milliliter of culture (OD_{600} = 0.5) was centrifuged for 4 min in the Brinkmann microcentrifuge at 4°C and resuspended in 50 μl of lysis buffer [20% sucrose/10 mM EDTA/10 mM Tris·HCl (pH 8.1)/1% Triton X-100/1 mg of lysozyme per ml/5 μg of DNase per ml/1 μg of RNase per ml]. After 30 min at 23°C, cell lysate (10 μl of a portion diluted 1/60) was assayed for leader peptidase (4) in the presence of several inhibitors of common proteinases (1 mM each phenylmethylsulfonyl fluoride, *N*-carbobenzyloxy-L-phenylethyl chloromethyl ketone, and L-tosylamido-2-phenylethyl chloromethyl ketone).

Other Methods. $NaDodSO_4$ gel electrophoresis and fluorography were done as described (17). Protein was assayed by the method of Bradford (18), using bovine serum albumin as a standard.

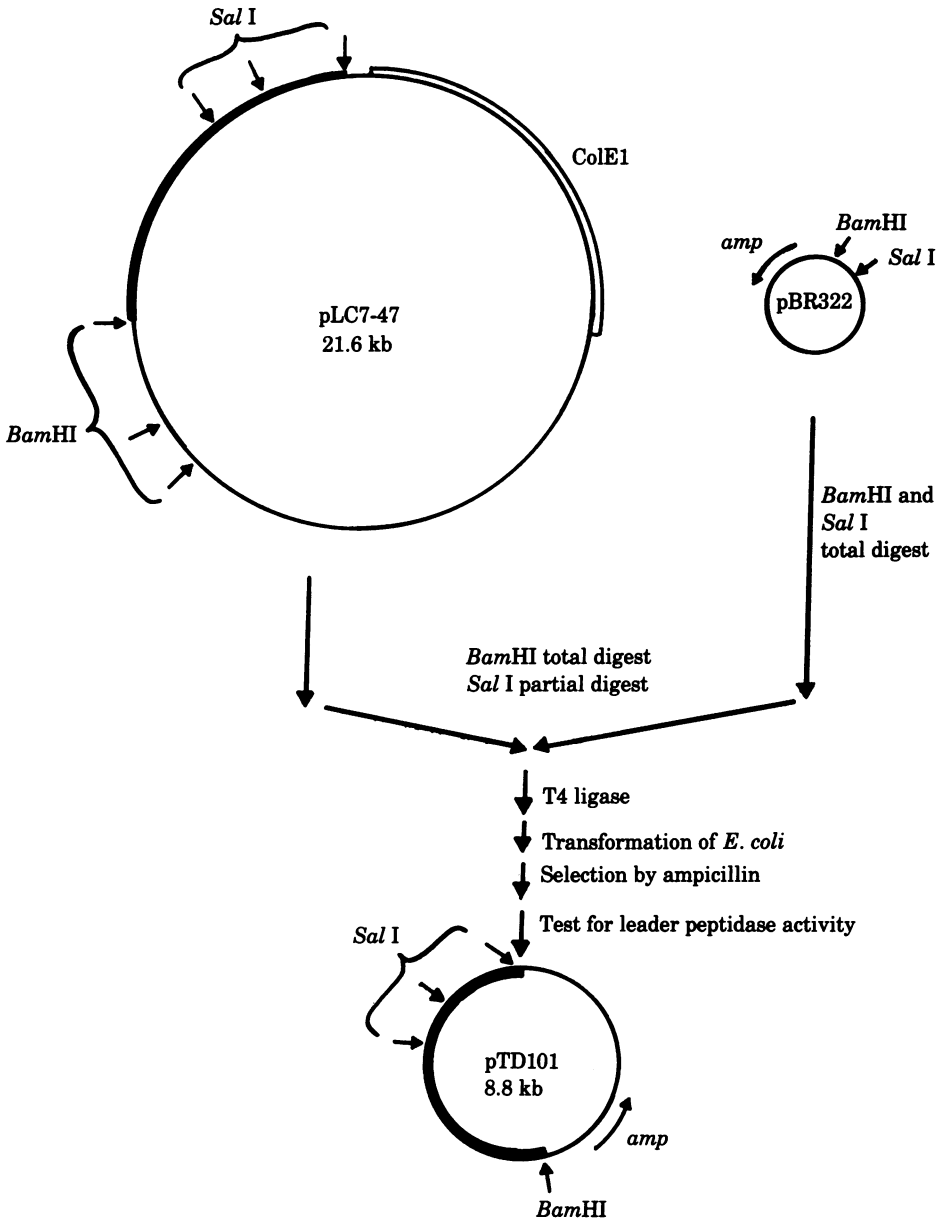

FIG. 2. Construction of the plasmid pTD101. *E. coli* DNA that was inserted into pTD101 is indicated by the thick dark line. Three micrograms of pLC7-47 was digested completely with *Bam*HI and partially with *Sal* I. The digestion products were extracted with phenol, precipitated with ethanol, and resuspended in a small volume of ligation buffer (15). pBR322 (0.8 μg) was digested to completion by *Bam*HI and *Sal* I and the fragments were isolated in the same manner. The products of each digestion were ligated [in a 60-μl reaction mixture for 16 hr at 16°C (15)] and used to transform (16) *E. coli* JA200. Ampicillin-resistant clones were tested for the overproduction of leader peptidase.

RESULTS

Small logarithmically growing cultures of individual strains from the collection of Clarke and Carbon (6) were mixed with a solution of sucrose, lysozyme, EDTA, DNase, RNase, and Triton X-100 and lysed by incubation for 30 min at 23°C. An equal aliquot of each lysate was then assayed for its ability to convert M13 procoat to coat protein posttranslationally. Approximately 1/10th of the procoat was converted to coat protein by lysate from most strains tested. Increasing the amount of lysate (and, hence, leader peptidase) 4-fold led to an obvious increase in coat protein production, and, therefore, this is the level of overproduction that was detectable by routine assay. Strain 7-47 of the collection clearly registered an overproduction of leader peptidase. This was confirmed by quantitative assay (4) of the specific activity of the enzyme (Fig. 1 *A* and *B*). All the leader peptidase from strain 7-47 was recovered in the expected fractions from the leader peptidase purification protocol that we have described (4). This shows that pLC7-47 does not code for an activator of leader peptidase but rather for the enzyme itself. The growth behavior of 7-47 was comparable to that of other strains in the collection, and the inner and outer membranes of 7-47 had virtually identical protein profiles as strain 7-48, its neighbor in the collection (data not shown). This suggests that overproduction of leader peptidase is not normally harmful to the uninfected cell.

The plasmid in the overproducing strain, pLC7-47, was 21.6 kilobase pairs (kb), composed of 15 kb of *E. coli* DNA linked to 6.6 kb of ColE1 DNA. This plasmid was digested with restriction enzymes and the fragments were inserted into the plasmid pBR322 as indicated in Fig. 2. One plasmid with such an insertion, pTD101, which derived 4 kb of DNA from pBR322 and 4.8 kb of *E. coli* DNA from pLC7-47, caused a 30-fold overproduction of leader peptidase in transformed cells (Fig. 1*C*). A restriction map of the plasmid is presented in Fig. 3*A*. Plasmids with specific deletions have been derived from pTD101 and assayed for the overproduction of leader peptidase (Fig. 3*B*). These studies suggest that the leader peptidase gene is in the 2.3-kb region between the *Bam*HI site and the proximal *Bgl* II site. A second region of 0.7 kb between the second *Bgl* II site and the *Pvu* II site also appears to be necessary for leader peptidase expression. This region could code only for a protein of 233 amino acids—i.e., approximately 26,000 daltons. Because the leader peptidase polypeptide is 39,000 daltons (ref. 4; unpublished data), this region is probably not coding for leader peptidase *per se*. Further studies will be necessary to determine its role in leader peptidase expression.

We have examined the effects of leader peptidase overproduction on the maturation of M13 coat protein from its biosynthetic precursor, procoat. We have previously shown (19–21) that the conversion of procoat to coat is posttranslational and slow in cells infected by M13 with an amber mutation in gene 7, a gene required to catalyze virion assembly. The conversion of procoat to coat in cells infected by M13 am7 is dramatically accelerated by the overproduction of leader peptidase (Fig. 4*A*). There is also a slow posttranslational conversion of procoat to coat (11) in cells infected by M13 am8-H1R6 (9), a virus with three amino acid residue changes from M13 in its coat protein (10) at positions 2 (Glu to Leu), 6 (Pro to Ser), and 11 (Asn to Asp). This slow conversion is also accelerated *in vivo* by the overproduction of leader peptidase (Fig. 4*B*), in agreement with the finding of Russel and Model (11) that this mutant procoat is a poor substrate for conversion to coat *in vitro* by detergent extracts of *E. coli* plasma membranes. Taken together, these data provide firm evidence that the leader peptidase we have isolated (4) and whose gene we have cloned is the one respon-

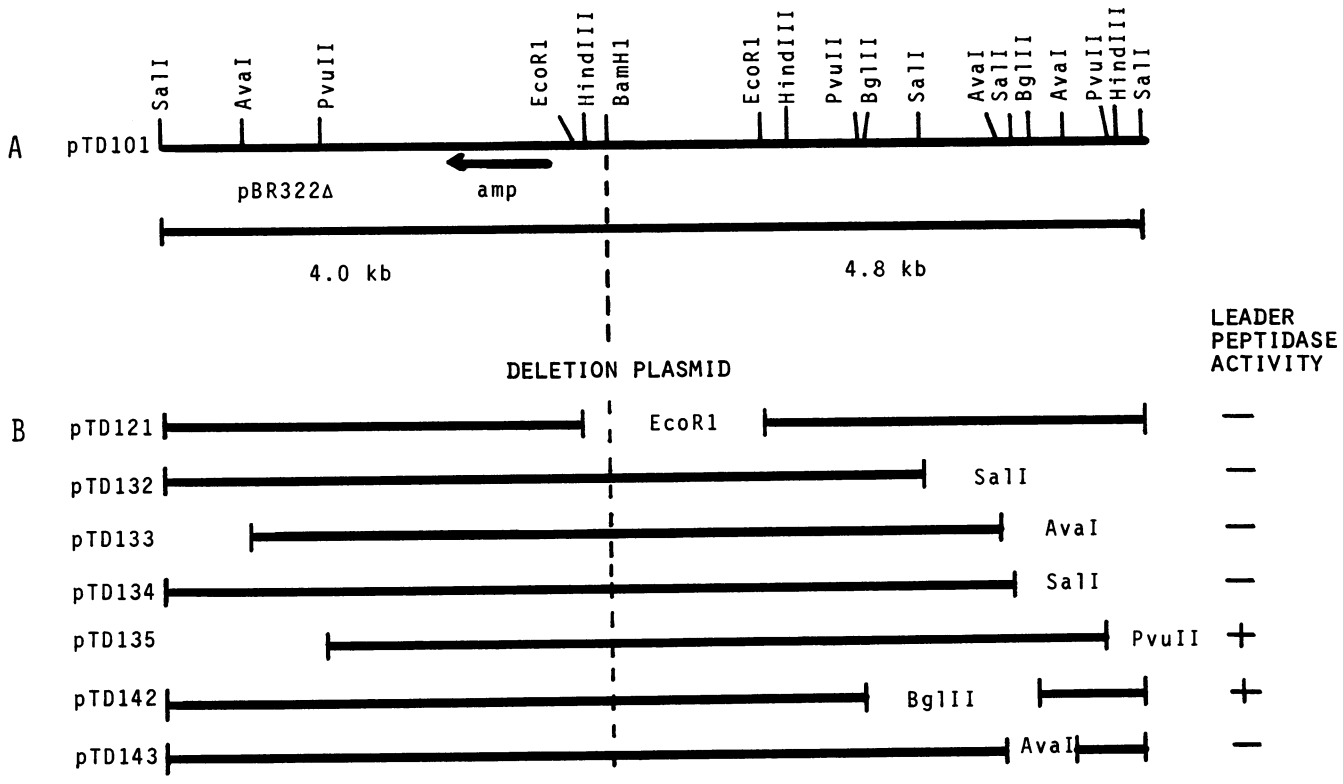

FIG. 3. (*A*) Restriction map of pTD101. (*B*) Various deletion derivatives of pTD101 were prepared by digestion with the indicated restriction enzyme followed by ligation and transformation. Strains with plasmids pTD135 and pTD142 have the same level of leader peptidase as strain pTD101/JA200, while strains with the other plasmids in *B* had the same leader peptidase levels as strain JA200.

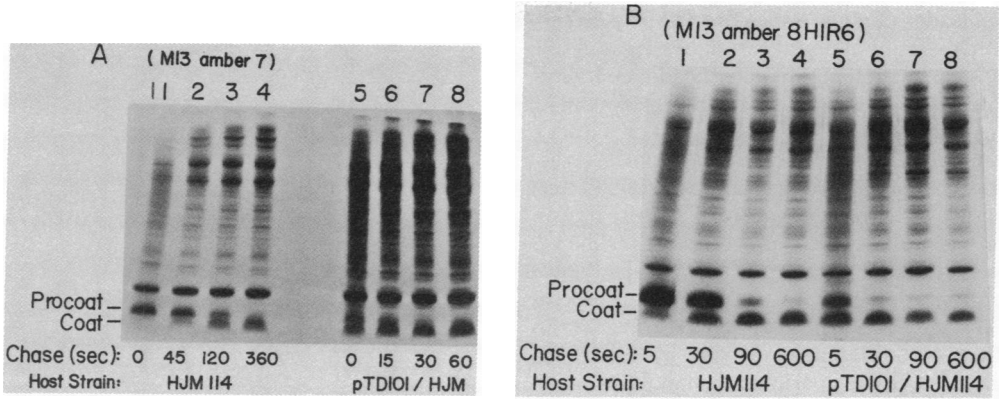

FIG. 4. Effect of leader peptidase overproduction on the conversion of procoat to coat. Cells were infected by M13 derivative viruses, pulse-labeled for 15 sec with either [^{3}H]proline (*A*) or [^{35}S]methionine (*B*), and chased with excess nonradioactive amino acid as described (17). After the indicated intervals of chase, aliquots were harvested and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and fluorography. (*A*) Wild-type *E. coli* strain HJM114 (lanes 1–4) or overproducer strain pTD101/HJM114 (lanes 5–8) was infected by M13 amber 7 virus. (*B*) As in *A*, but infection was with M13 am8-H1R6 (9–11). Pulse-labeling of uninfected control cultures showed, as we have previously reported (19), that a small amount of the apparent procoat, corresponding to the amount seen in lanes 4, 7, and 8, was in fact lipoprotein.

sible for the proteolytic processing of M13 procoat.

In addition to these effects of overproduction of leader peptidase on procoat processing, the level of leader peptidase has major effects on the growth, viability, and virus production of cells infected by M13. *E. coli* strain HJM114 and strain pTD101/HJM114 (leader peptidase overproducer) grow at comparable rates (Fig. 5*A*). Upon infection with wild-type M13, the culture of the overproducing strain continues to increase in turbidity but shows a pronounced lag in the increase of colony-forming units (Fig. 5*A*) and in the production of progeny virus (Fig. 5*B*). Microscopic examination revealed that this was accompanied by a marked delay in the completion of septation. In contrast, when the overproducer strain was infected by M13 with an amber mutation in gene 7 (Fig. 5*C*), there was enhanced cell growth and partial resistance to killing compared to the wild-type bacterial strain infected by this same virus. However, the overproduction of leader peptidase did not bypass the requirement for M13 gene 7 for the assembly of functional virions.

DISCUSSION

The isolation of the gene for leader peptidase has facilitated the isolation of this enzyme from the overproducer strain and may provide a convenient starting point for studies of its genetics. The DNA sequence of plasmid pTD101, which bears this gene, should reveal the amino acid sequence of the 39,000-dalton leader peptidase polypeptide. In earlier studies (4), we isolated leader peptidase by assaying its conversion of M13 procoat to coat. Our identification of pLC7-47 as bearing the leader peptidase gene was based on this same assay. Studies of M13-infected overproducer cells (Fig. 4) have shown that the leader peptidase that we have studied is the one that interacts with M13 procoat protein *in vivo*. Thus our studies of the reconstitution of assembly with purified components (22, 23) have indeed employed the correct components.

Study of leader peptidase and its gene may shed light on several central questions of secretion and membrane biogenesis.

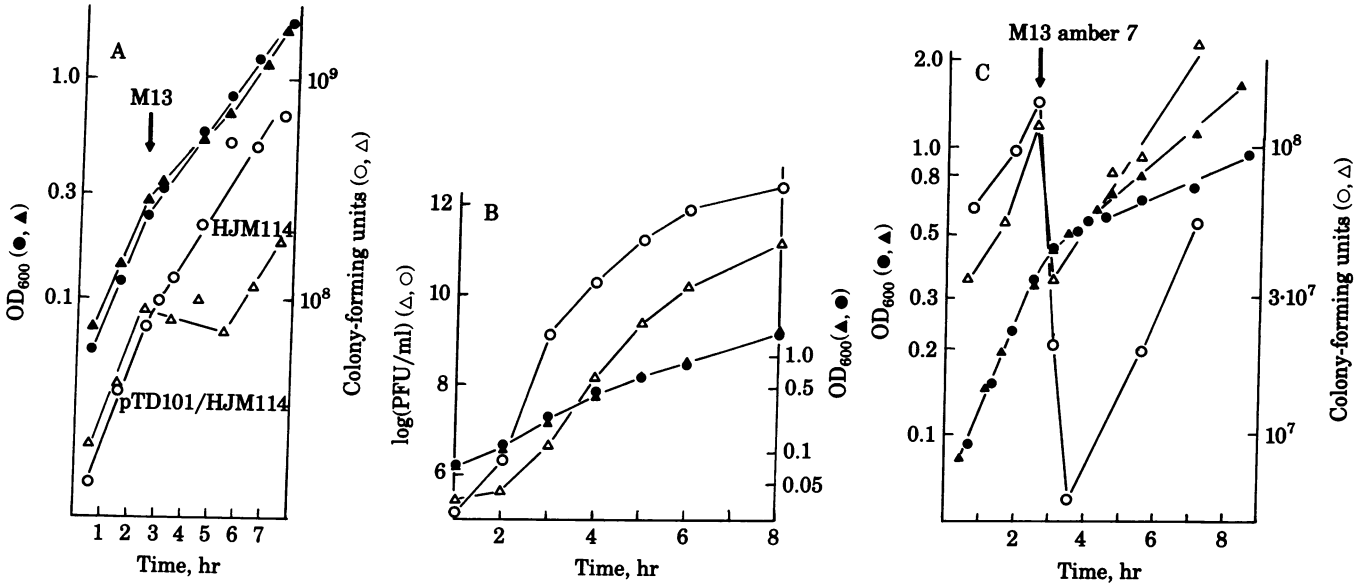

FIG. 5. Effects of overproduction of leader peptidase on M13-infected cells. HJM114 (○, ●) and pRD101/HJM114 (△, ▲) were grown in M9 medium at 37°C and infected by M13 (*A* and *B*) or by M13 am7 (*C*) at a multiplicity of 100. At the indicated times, OD$_{600}$, colony-forming units on L plates, and plaque-forming units (PFU) were assayed.

What is the basis of leader peptidase specificity? Many proteinases recognize either specific amino acid residues at the cleavage site or a single, specific protein substrate. Leader peptidase apparently falls into neither category. Though it cleaves M13 procoat between two alanines to yield coat protein plus leader peptide, it does not cleave between two other alanines at residues 9 and 10 of the coat protein. This cannot be ascribed simply to a lack of exposure of this region of procoat, because trypsin and chymotrypsin will readily cleave procoat after residues 8 and 11, respectively. Recent experiments (unpublished) have shown that removal of a few residues at the ends of procoat blocks its conversion by leader peptidase to coat. Despite this impressive specificity of the cleavage of procoat by leader peptidase, this enzyme also cleaves periplasmic and outer membrane precursor proteins (C. J. Daniels and D. Oxender, D. Perrin and M. Hofnung, personal communications).

This question of leader peptidase specificity, as well as questions such as whether the peptidase might even catalyze the transfer of polypeptidase across a bilayer, can be approached through a study of the biochemistry and genetics of leader peptidase. The studies reported here indicate that it converts procoat to coat *in vivo*. The molecular basis for the effects of leader peptidase overproduction on the viability, growth, and virus extrusion in M13 infected cells is not clear. Perhaps the rate of conversion of procoat to coat in infected wild-type cells is optimal, and either its acceleration by enzyme overproduction or is deceleration by a defect in virus gene 7 is harmful to the cell. In this general scheme, leader peptidase overproduction would partially restore the procoat processing rate in M13 am7-infected cell (Fig. 4*A*) and thus partially protect the cells from amber virus-induced death (Fig. 5*C*). Further studies of the function of the leader peptidase may be aided by the isolation of a temperature-sensitive, conditionally lethal mutant in its gene.

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