Complete Secretion of Activable Bovine Prochymosin by Genetically Engineered L Forms of Proteus mirabilis

CHRISTIAN KLESSEN, KARL-HERRMANN SCHMIDT, JOHANNES GUMPERT, HANS-HELMUT GROSSE, AND HORST MALKE*

Central Institute of Microbiology and Experimental Therapy, Academy of Sciences of the German Democratic Republic, Jena 6900, German Democratic Republic

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To circumvent problems encountered in the synthesis of active chymosin in a number of bacteria and fungi, a recombinant DNA L-form expression system that directed the complete secretion of fully activable prochymosin into the extracellular culture medium was developed. The expression plasmid constructions involved the in-frame fusion of prochymosin cDNA minus codons 1 to 4 to streptococcal pyrogenic exotoxin type A gene (speA') sequences, including the speA' promoter, ribosomal binding site, and signal sequence and five codons of mature SpeA. Secretion of fusion prochymosin enzymatically and immunologically indistinguishable from bovine prochymosin was achieved after transformation of two stable protoplast type L-form strains derived from Proteus mirabilis. The secreted proenzyme was converted by autocatalytic processing to chymosin showing milk-clotting activity. In controlled laboratory fermentation processes, a maximum specific rate of activable prochymosin synthesis of \(0.57 \times 10^{-3}\) h\(^{-1}\) was determined from the time courses of biomass dry weight and product formation. Yields as high as \(40 \pm 10\) \(\mu\)g/ml were obtained in the cell-free culture fluid of strain L99 carrying a naturally altered expression plasmid of increased segregational stability. The expression-secretion system described may be generally useful for production of recombinant mammalian proteins synthesized intracellularly as aberrantly folded insoluble aggregates.

Chymosin, an aspartyl proteinase found in the abomasum of unweaned calves (7), has been a prime target in many countries for production in genetically engineered microorganisms because of its indispensable role in cheese manufacturing and its limited availability. Notwithstanding tremendous efforts put into establishing chymosin-producing bacteria (2, 5, 14, 30–32) and fungi (3, 9, 27, 28, 34), poor yields and aberrantly folded protein have been major obstacles to industrial utility. The stratagem commonly used for microbial expression of chymosin involves the linking of prochymosin cDNA (11, 29) to an appropriate host promoter and the 5' end of a structural gene to provide a ribosomal binding site together with an in-frame translation initiation codon. The synthesized prochymosin is then cleaved autocatalytically under acidic conditions to form the proteolytically active mature enzyme (6). Expression systems designed for cytoplasmic prochymosin production invariably lead to insoluble protein aggregates which are largely unaccessible. Therefore, exploiting secretion pathways favoring disulfide bond formation and correct protein folding appears to be mandatory for efficient microbial chymosin synthesis. However, for a variety of reasons, many of which are not well understood, there are limitations on the efficiency of prochymosin secretion by bacteria (Y. J. Vos, P. J. Lemson, A. L. M. Simonetti, and P. M. Andreoli, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, O-5, p. 262) and fungi (34).

Our strategy for microbial chymosin production involved both the construction of novel expression-secretion vectors and the use of cell wall-deficient bacteria as novel hosts. The expression unit we constructed is composed of prochymosin cDNA linked to the transcriptional, translational, and secretory control elements of the streptococcal pyrogenic exotoxin type A gene, speA' (38). The host cells used are stable protoplast type L forms derived from Proteus mirabilis (8, 10). Bacterial L forms have not served before as hosts for the production of recombinant proteins, but they promise to offer new assets, especially by secreting protein directly into the medium, owing to the absence of a periplasmic space. We describe here the quantitative release of recombinant prochymosin into the L-form culture medium and the conversion of this proenzyme to enzymatically active chymosin.

MATERIALS AND METHODS

Plasmids and bacterial strains. The plasmids used are described in Table 1. Escherichia coli JM101 (39) or Streptococcus sanguis Challis (obtained from Jon Ranhand) served as primary hosts for established and newly constructed plasmids. The strains LVI and L99 were stable protoplast type L forms derived by penicillin induction from P. mirabilis VI. They have been maintained in this institute by serial transfer in liquid antibiotic-free medium of normal osmolarity for more than 25 and 5 years, respectively. These organisms do not produce any organized constituents of the peptidoglycan layer and no longer revert to the parental bacterial form under any known conditions (10, 26, 35).

Media and growth conditions. E. coli cells were grown aerobically in LB medium (21). The Challis strain of S. sanguis was grown without agitation in Todd-Hewitt broth (Difco Laboratories). Mass cultures of P. mirabilis LVI and L99 were grown aerobically at 37°C in shaken flasks (250 rpm; 30 ml of culture in a 100-ml flask) or in an aerated (90 liters of air per h) laboratory fermentor (2 liters of culture in a 4.5-liter vessel). The fermentor cultures were stirred with an edgeless disk (diameter, 7 cm) equipped on both sides with an undulatory profile to minimize shearing and to prevent the relative oxygen saturation of the medium from reaching values smaller than 5%. Maximum stirring at 800 rpm led to an oxygen transfer rate of 0.25 g per liter per h. In either cultivation, the medium was freshly prepared beef extract supplemented with the following nutrients (per liter):
pSM6 Em'; streptococcal cloning vector
pGK13 Em' Cm'; streptococcal cloning vector
pUC1833 Ap' pUC8 carrying the S' end of the streptococcal pyrogenic exotoxin type A gene (*speA*'), including its promoter (P) and ribosomal binding site and codons 1 to 41
pKMS3 Ap' *Skel* p*speA*') prochymosin positive (Ap*'); pUC9 carrying a tripartite in-frame fusion of *speA* codons 1 to 35, prochymosin cDNA codons 6 to 339, and streptokinase coding sequence (sk) minus codons 1 to 39
pKM9106 Ap' lacPOZ* 'prochymosin positive (Ap*'); pUC9 carrying a tripartite in-frame fusion of *speA* codons 1 to 35 and prochymosin cDNA minus codons 1 to 5
pKM1836 Ap' prochymosin positive *speA* 'prochymosin positive (Ap*'); pUC18 carrying an in-frame fusion of *speA* codons 1 to 35
pKM636 Ap' Em' prochymosin positive *speA* 'prochymosin positive (Ap*)
pKM6361 Ap' Em' prochymosin positive *speA* 'prochymosin positive (Ap*) deletion variant of pKM636
pKM1336 Cm' Em' prochymosin positive *speA* 'prochymosin positive (Ap*'); pGK13 carrying the prochymosin expression secretion cassette as a HindIII fragment

<table>
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<tr>
<th>Plasmid</th>
<th>Relevant properties</th>
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<tr>
<td>pSM6</td>
<td>Em'; streptococcal cloning vector</td>
</tr>
<tr>
<td>pGK13</td>
<td>Em' Cm'; streptococcal cloning vector</td>
</tr>
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<td>pUC1833</td>
<td>Ap' pUC8 carrying the S' end of the streptococcal pyrogenic exotoxin type A gene (<em>speA</em>'), including its promoter (P) and ribosomal binding site and codons 1 to 41</td>
</tr>
<tr>
<td>pKMS3</td>
<td>Ap' <em>Skel</em> p<em>speA</em>') prochymosin positive (Ap*'); pUC9 carrying a tripartite in-frame fusion of <em>speA</em> codons 1 to 35, prochymosin cDNA codons 6 to 339, and streptokinase coding sequence (sk) minus codons 1 to 39</td>
</tr>
<tr>
<td>pKM9106</td>
<td>Ap' lacPOZ* 'prochymosin positive (Ap*'); pUC9 carrying a tripartite in-frame fusion of <em>speA</em> codons 1 to 35 and prochymosin cDNA minus codons 1 to 5</td>
</tr>
<tr>
<td>pKM1836</td>
<td>Ap' prochymosin positive <em>speA</em> 'prochymosin positive (Ap*'); pUC18 carrying an in-frame fusion of <em>speA</em> codons 1 to 35</td>
</tr>
<tr>
<td>pKM636</td>
<td>Ap' Em' prochymosin positive <em>speA</em> 'prochymosin positive (Ap*)</td>
</tr>
<tr>
<td>pKM6361</td>
<td>Ap' Em' prochymosin positive <em>speA</em> 'prochymosin positive (Ap*) deletion variant of pKM636</td>
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DNA manipulation. Large-scale plasmid isolation was carried out by CsCl-ethidium bromide density gradient equilibrium centrifugation by the method of Maniatis et al. (25). The method of Holmes and Quigley (12) was used for miniprepARATION of *E. coli* plasmids. For plasmid screening in the L-form strains, the protocol of R. Geuther (unpublished data) was followed. Approximately 10⁸ cells harvested by centrifugation from liquid cultures or from solid medium were suspended in 100 μl of solution 1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA). Two hundred microliters of solution 2 (0.2 N NaOH, 1% sodium dodecyl sulfate [SDS]) was added, and the mixture was incubated for 20 min at 50°C. After being cooled on ice, 100 μl of phenol-chloroform (5 g of phenol, 5 ml of chloroform, 1 ml of water, 5 μg of 8-hydroxyquinoline) was added, and the mixture was vortexed and centrifuged. The DNA in the aqueous phase was precipitated by the addition of 30 μl of 3 M sodium acetate and 350 μl of isopropanol. After centrifugation, the sediment was dissolved in 200 μl of distilled water and 10 μl of 100 mM spermidine was added. After incubation for 10 min at room temperature and centrifugation, the sediment was dissolved in 200 μl of solution 3 (0.3 M sodium acetate, 20 mM magnesium chloride) before nucleic acids were precipitated by the addition of 500 μl of ethanol. The DNA was dried in a vacuum and dissolved in 30 μl of 10 mM Tris hydrochloride [pH 7.6]–1 mM EDTA before being used in 5- to 10-μl volumes for restriction analysis. Agarose gel electrophoresis was carried out by standard procedures (25), and DNA restriction fragments were isolated from low-melting-point agarose by phenol extraction (17).

Identification of recombinant chymosin by Western immuno blotting. One-milliliter volumes of cell-free culture filtrates were adjusted to pH 8.0 with crystalline Tris base, and SDS was added to a final concentration of 1%. Proteins were denatured by boiling them for 5 min in a water bath, and 50-μl samples were subjected to SDS-10% polyacrylamide gel electrophoresis by the method of Laemmli (19). The proteins were transferred to nitrocellulose by electroblotting (18), and the membranes were blocked with nonfat dry milk (13) and subjected to the peroxidase technique for immunodetection of chymosin. The primary antibody was anti-calf chymosin raised in rabbits, and the secondary antibody was goat anti-rabbit immunoglobulin G conjugated with peroxi dase. The blots were developed with diaminobenzidine (37). Non-chymosin-reactive rabbit serum was included as a control in the immunodetection experiments. In addition, Bacto-Peptone, 10 g; yeast extract (Difco), 5 g; sucrose, 20 g; NaCl, 5 g (LFS medium). For growth on agar, the liquid media were solidified with 1.5% agar (Difco). If required, selective antibiotics were used at the following final concentrations (per milliliter): erythromycin, 5 to 15 μg for *S. sanguis* and the L forms and 50 μg for *E. coli*; ampicillin, 50 to 100 μg; chloramphenicol, 5 μg. The time course (t) of growth and product formation was monitored by determining optical density at 540 nm (OD₅₄₀), biomass dry weight (X), and extracellular prochymosin (P). Cubic spline functions were then used for curve fitting to derive specific rates of growth and prochymosin synthesis, as defined by the equations μ = 1/X(dX/dt) and kₚ = 1/X(dp/dt), respectively.

Biochemicals. Restriction enzymes, T4 DNA ligase, and Klenow fragments were obtained from Boehringer Mannheim Biochemicals and used as supplied by the supplier. Chymosin was prepared to electrophoretic uniformity by commercial calf rennet (Christoff Hansen Laboratory, Copenhagen, Denmark) by affinity chromatography on histidyl-Sepharose (1). Chymosin thus purified is called affinity-purified chymosin in this paper. Antiserum against affinity-purified chymosin was produced in rabbits by the intramuscular route as described by Louvard et al. (22).

Transformation procedures. Transformation of competent *E. coli* cells with plasmid DNA was carried out by the calcium chloride procedure essentially as described by Dagert and Ehrlich (4). For plasmid transformation of the *P. mirabilis* L-form strains, cells were grown in LFS medium with vigorous aeration for 18 h at 37°C. After centrifugation at 5,000 × g, the cell pellets were suspended in modified LFS medium containing 0.4 M sucrose to a density of about 10⁹ cells per ml. Samples (0.1 ml) of the cell suspensions were treated with 10 to 30 μl of plasmid DNA (300 μg/ml) and 0.15 ml of 30% polyethylene glycol in 0.4 M sucrose. The mixtures were placed on ice for 10 min and incubated further for 10 min at 37°C with gentle shaking. After the addition of 0.9-ml volumes of modified LFS medium and incubation for an additional 3 h at 37°C, 0.1-ml samples were plated on LFS agar containing 5 μg of erythromycin per ml. The plates were incubated for 5 to 10 days, after which the transformant colonies were transferred to fresh agar plates. Transformation frequency is expressed as the number of erythromycin-resistant colonies per microgram of plasmid DNA. Growth in liquid medium was initiated overnight with transformant cells, and serial passages in subcultures continued until adaptation to growth in liquid medium was attained.
samples derived from isogenic strains lacking prochymosin sequences capable of being expressed served as negative controls.

Milk-clotting assay of chymosin. Chymosin production by engineered L-form strains was quantitated by measuring the milk-clotting activity of cell-free culture supernatant fluids and cytoplasmic protein fractions obtained from osmotically shocked cell pellets. For colony screening, aqueous extracts of overgrown agar blocks taken from primary or secondary transformation plates served as samples for qualitative detection of chymosin production. Samples were activated by acidification to pH 2.5 with 1 N HCl and incubation for 3 h at room temperature (6). They were then neutralized to pH 6.2 with 1 N NaOH and assayed in 7-μl portions in well plates of 1-mm thickness containing 1% agarose, 12% (wt/vol) dried skim milk, 0.1 M sodium acetate, and 10 mM calcium chloride (pH 6.2). The plates were incubated for 18 h at 37°C and read by comparing the sizes of the milk-clotting zones produced by the samples with those produced by a series of chymosin standard solutions made from affinity-purified calf chymosin.

RESULTS

Construction of prochymosin secretion vectors. The strategy used for the construction of prochymosin secretion vectors involved the in-frame insertion of the prochymosin cDNA downstream of the speA signal peptide processing site. Of the three starting plasmids characterized previously, pUC1833 (20) provided the speA promoter; pKMS3 (15) yielded the ribosomal binding site, the speA signal sequence-coding region, and the 5' end of prochymosin cDNA; and the complete remainder of the latter came from pKM9106 (15). pUC1833 was cleaved with XbaI and PstI to yield the 3.4-kilobase (kb) recipient molecule for the insertion of the 0.3-kb XbaI-BglII fragment from pKMS3 and the 1.1-kb BglII-PstI fragment from pKM9106 (Fig. 1). The three gel-purified fragments were joined in a directional cloning experiment to form pKM1836, containing the entire prochymosin expression-secretion cassette in the form of a 2.2-kb HindIII fragment. In this construct, codons 1 to 35 came from speA, codons 36 to 38 were polylinker codons, and codons 39 to 399 corresponded to the complete prochymosin cDNA except for 15 nucleotides encoding the N-terminal pentapeptide (Fig. 1). Since ampicillin resistance cannot serve as a selective marker in L forms, pKM1836 was fused with the streptococcal vector pSM6 encoding erythromycin resistance, as shown in Fig. 1. Plasmid pSM6 (5.6 kb), which has a single EcoRI site, was derived previously from the group A streptococcal macrolide-lincosamide-streptogramin B resistance plasmid pSM10419 (22.4 kb) by a series of consecutive deletions which did not affect antibiotic resistance and replication functions (20). A second plasmid selectable with erythromycin was obtained by inserting the 2.2-kb HindIII fragment from pKMS3 into pGK13 (Fig. 1). The new constructs were transformed into E. coli JM101 with double selection for ampicillin plus erythromycin and chloramphenicol plus erythromycin, respectively, to yield clones carrying pKM636 (10.5 kb) or pKM1336 (7.1 kb) capable of replication and expressing erythromycin resistance in the P. mirabilis L-form strains.

Secretion of prochymosin and its activation to chymosin. The expression plasmids pKM636 and pKM1336 were transformed into P. mirabilis LVI and L99 with transformation frequencies ranging from 3 × 10³ to 10⁷ erythromycin-resistant colonies per μg of plasmid DNA. Erythromycin-resistant L-form colonies were screened for chymosin production by the milk-clotting assay. Simultaneously, the plasmids of the transformant clones were analyzed by digestion with restriction enzymes. Any of the transformants containing unaltered plasmids showed distinct milk-clotting activity when aqueous extracts of samples containing cells and the surrounding agar medium were subjected to the assay. A limited number of randomly chosen transformants derived from each plasmid and host strain were adapted to growth in liquid medium and characterized further.

To confirm the identity of the product, cell-free culture supernatant samples were applied to SDS-polyacrylamide gel electrophoresis, and immunologically reactive protein...
was identified by Western blotting. Single positive bands were detected at identical positions for samples derived from strains carrying pKM1336 and pKM636 (Fig. 2). The molecular weight of the reactive bands ($M_r = 41,000$) agreed with that deduced from the amino acid sequence of the expected fused protein without the 30-amino-acid signal sequence. As expected, authentic chymosin bands were detected at positions corresponding to an $M_r$ of 36,000. Samples derived from isogenic strains lacking the prochymosin secretion cassette showed no immunologically reactive bands (Fig. 2). Furthermore, the cytoplasmic protein fractions obtained by osmotic shock of cells carrying the expression plasmids were free of any detectable immunologically reactive materials.

In order to test the biological activity of the prochymosin-reactive protein, cell-free culture fluids were examined after acidification and neutralization in well plates for milk-clotting activity by using authentic chymosin to calibrate the assay. Negative controls included samples derived from strains carrying plasmids free of the prochymosin expression cassette as well as unactivated samples from the producer strains. Representative results are illustrated in Fig. 3. The minimum amount of purified commercial calf chymosin required to clot milk in the assay was about 3 ng per well. *P. mirabilis* L99 and LVI were each tested for prochymosin production with both pKM636 and pKM1336. The enzyme was present at about 30 and 15 µg/ml in culture supernatant fluids from shaken cultures of L99 or LVI, respectively, containing either pKM636 or pKM1336. The protein had an absolute requirement for activation before generating clotting activity and was not seen in control samples from cultures grown with insert-free vectors.

To demonstrate that the acid activation of prochymosin secreted from the L-form cells occurred by a proteolytic cleavage process, culture supernatant fluid from L99(pKM636) was analyzed before and after acidification by SDS-polyacrylamide gel electrophoresis and immunoblotting. The 41-kilodalton prochymosin band present before activation disappeared after about 8 h of incubation to give rise to a smaller immunologically reactive protein having the molecular weight of chymosin (36 kilodaltons) (Fig. 4). This result shows that the L-form cells synthesized and secreted prochymosin in an inactive form which was fully activable by a cleavage process which generated milk-clotting activity.

*P. mirabilis* L99(pKM636), which for unknown reasons produced significantly higher amounts of prochymosin than LVI for both expression plasmids, was used in fermentation processes to study the kinetics of prochymosin production during growth. When the fermentation started with an inoculum of 200 ml taken from 6-h shaken cultures, biomass dry weight production and prochymosin secretion were related until hour 16 (Fig. 5). The maximum prochymosin yield (17.6 mg/liter) was obtained later than the maximum dry mass (4.2 g/liter), indicating the continuation of prochymosin synthesis in quasi-stationary-phase cells. Under the conditions of this experiment, the maximum specific rates of growth (0.183/h) and prochymosin synthesis (0.57 × 10^-3/h) were obtained at hour 6 of fermentation, when the biomass doubling time was
FIG. 5. Time course of dry mass formation ( ◦ ) and activable prochymosin synthesis (□) by P. mirabilis L99(pKM636) cultivated in a laboratory fermentor as specified in Materials and Methods. By linear regression analysis, the correlation between dry mass and OD600 is given by the equation \( X(\text{g/l}) = 0.51 \times \text{OD}_{600} - 0.051 \) (\( r = 0.9; P = 0.05 \)). Similarly, the equation \( N(\text{m}^3/l) = 2.48 \times 10^8 \times \text{OD}_{600} - 1.87 \times 10^4 \) (\( r = 0.98 \)) holds for the correlation between cell number (N) and OD.

3.78 h and the pH value of the culture tended to increase above pH 7. To obtain stable prochymosin yields at the end of the fermentation process, we found it necessary to avoid pH values greater than 6.5 as soon as the biomass had ceased increasing, i.e., at hour 16 of fermentation. At no time in the course of fermentation was any intracellular prochymosin detectable, indicating, together with the immunological evidence mentioned above, that product secretion was virtually complete.

Deletion variant of pKM636 with increased stability and productivity. In the course of the initial posttransformation screening experiments designed to establish stable strains, a relatively high proportion of clones that harbored altered plasmids and exhibited highly variable chymosin production was observed. In a typical experiment, 41 erythromycin-resistant and prochymosin-producing L99(pKM636) clones were isolated from the primary transformation plates and adapted to growth in liquid medium containing erythromycin. During the adaptation phase, eight clones lost the capability of producing activable prochymosin because of structural alterations of pKM636 which affected the prochymosin expression cassette, as evidenced by restriction analysis of their plasmids. The remaining 33 clones secreted prochymosin at levels ranging from 1 to 40 \( \mu \text{g/ml} \). Of these, the most productive clone was studied further. Its plasmid DNA was isolated and used to transform E. coli JM101 to ampicillin resistance. The ampicillin-resistant transformants were simultaneously erythromycin resistant but carried a deletion variant of pKM636 designated pKM636I (6.8 kb). The deletion \( \Delta \text{KM636I} \) (indicated in Fig. 1) removed a 3.7-kb stretch of DNA from the pSM6 component of pKM636 not involved in autonomous replication, antibiotic resistance, and prochymosin production. When present in P. mirabilis L99, pKM636I directed the synthesis of prochymosin at levels as high as 40 ± 10 \( \mu \text{g/ml} \) in adapted shaken cultures. In addition, in the absence of erythromycin, pKM636I proved to be more stably inherited than pKM636, from which it was derived (Table 2).

**TABLE 2. Segregational stability of pKM636I in L99 compared with pKM636**

<table>
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<tr>
<th>No. of passages*</th>
<th>Erythromycin-resistant CFU (%)</th>
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<tr>
<td></td>
<td>pKM636I</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
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<tr>
<td>5</td>
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<td>8</td>
<td>55</td>
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<td>10</td>
<td>20</td>
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* Cells adapted to growth in liquid medium containing erythromycin were washed and serially passaged at one-tenth the original density for 18 h at 37°C in shaken flasks containing antibiotic-free LFS medium. Samples were differentially plated onto erythromycin-containing or antibiotic-free LFS agar to determine the percentage of erythromycin-resistant colonies. The latter were simultaneously tested for prochymosin production and were always found to produce activable prochymosin in the milk-clotting assay.

b Ten serial passages correspond to about 50 cell mass doublings.

**DISCUSSION**

The results presented here show that biologically active chymosin can be produced in bacterial L forms. The identity of the protein was confirmed by demonstrating milk-clotting activity, reactivity with chymosin-specific antibodies in immunoblotting experiments, and processing of the proenzyme to a polypeptide having the molecular mass of authentic chymosin. The results imply that the transcriptional and translational control signals derived from a streptococcal gene (speA) to express prochymosin cDNA were functional in the heterologous host. Most importantly, since secretion is necessary for recombinant prochymosin to be activable, the present experimental design provided the first procarboxylic system for chymosin production to enable the complete release of prochymosin into the extracellular culture medium. Although the precise signal sequence processing was not determined, the data obtained by SDS-polyacrylamide gel electrophoresis indicate that the 30-amino-acid SpeA signal peptide was processed. The hybrid preprochymosin encoded in the expression vector has a calculated molecular mass of 44.5 kilodaltons. Removal of the signal peptide would result in a polypeptide of 368 amino acids with an observed molecular mass of 41 kilodaltons. Of these, the seven N-terminal amino acid residues originated from speA' and the polylinker and replaced the N-terminal four amino acids of prochymosin. As shown before by others (2, 14, 27, 31) and confirmed here, the short N-terminal extension of foreign protein does not severely affect the autocatalytic processing activity of prochymosin. However, the autocatalytic activation rate might well be affected by the nature of the N-terminal extension, since the fused prochymosin expressed in P. mirabilis L forms required time periods for complete activation that were longer than those known to be necessary for the activation of authentic prochymosin (6).

In engineering L forms to produce chymosin, we did not primarily aim at providing an alternative source of the enzyme for the cheesemaking industry. In view of the strict regulations concerning food additives, microorganisms more compatible with food processing may be more satisfactory, although they may pose their own problems. Rather, we developed the recombinant DNA L-form expression system...
as a possible alternative for the synthesis of mammalian gene products that, for one reason or another, have proved difficult to produce microbiologically. To assess the utility of the system, chymosin was chosen as a model because low product yields, incomplete secretion, aggregate formation, incorrect folding, breakdown by host proteases, and complexing with cell wall constituents are among the principal problems encountered in hitherto established microbial systems for its production. The L-form system compares favorably with these systems in any respect and may be advantageous for the production of other recombinant proteins synthesized intracellularly as insoluble aggregates. In the design of expression vectors to be used in L forms, ampicillin resistance is excluded as a selective marker. However, erythromycin resistance genes of the macrolide-lincosamide-streptogramin B resistance type from streptococci have proved to be perfectly suitable. As shown previously (23, 24), these genes are well expressed in gram-negative bacteria, and furthermore, L forms are intrinsically much more sensitive to macrolides than are their normal bacterial forms (36), enabling low concentrations of the antibiotics to be used for selection. Problems of plasmid stability remain and are presumably caused at least in part by the fundamentally altered cell surface structure and unequal cell division processes. However, structural alteration, if allowed to evolve under selective pressure and monitored so that it does not affect the desired functions, may rapidly lead to variants of the expression vectors that, as shown here for pKM6361, are more stably inherited.

ACKNOWLEDGMENTS

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LITERATURE CITED