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The *sim* Gene of *Escherichia coli* Phage P1: Nucleotide Sequence and Purification of the Processed Protein

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The *sim* gene of bacteriophage P1 causes exclusion of a superinfecting P1 phage. We determined the nucleotide sequence of a 1.9-kb DNA fragment that, in plasmids, causes Sim phenotype. There are two open reading frames within this region for proteins of 82 and 259 amino acids. A 1.3-kb fragment containing the larger open reading frame was inserted into an expression vector. Induced cells carrying the hybrid plasmid, termed pBD5, were not infected by phage P1 and produced a 24-kDa protein and, to a smaller extent, a 25-kDa protein. The 24-kDa protein was purified. Comparison of its amino-terminal amino acid sequence with the nucleotide sequence indicated that it is processed from a precursor protein by removal of a hydrophobic leader peptide of 20 amino acids. *In vivo* processing depends on *sec*A gene function and is necessary for Sim interference with P1 infection. The data are discussed with respect to the function of the *sim* gene in superinfection exclusion.

INTRODUCTION

The *sim* gene of bacteriophage P1 was detected during attempts to clone the *c*1 repressor gene (Devlin *et al.*, 1982). Carried by a multicopy vector, the *sim* gene confers immunity to cells against infection with wild-type P1 and also *c*1, *c*4, and *vir* mutants. Therefore the extended immunity was called *super*immunity (*sim*). The *sim* gene is localized on the P1 *Eco*RI-9 DNA fragment within the *imm*I region (Bächi and Arber, 1977; Yarmolinsky, 1987).

In a previous study (Kliem and Dreiseikelmann, 1989), we showed that cells carrying a multicopy plasmid with the *sim* gene (pMK4) are not really immune. Such cells are not lysogenized, can adsorb phage, but fail to synthesize phage-specific proteins following infection. The *sim* gene product does not interfere with the circularization and replication of phage P1 DNA or with the maturation or release of progeny phages. We concluded that the *sim* gene product blocks some early step following infection but not transformation, probably at a stage between adsorption of the phage and circularization of the injected DNA. Thus the *sim* gene does not confer immunity but rather a superinfection exclusion (Kliem and Dreiseikelmann, 1989). Genes which may have a similar function have been described in other bacteriophages, such as *sie*A in phage P22 (Susskind *et al.*, 1974) and *imm*T in phage T4 (Anderson and Eigner, 1971). The actual mechanism for superinfection exclusion is not yet known. The genes have not been sequenced and the gene products have not been purified or characterized. It has been speculated that the *imm*T gene product may be a nuclease or may activate a periplasmic endonuclease of *E. coli* (Anderson and Eigner, 1971).

Plasmids with the cloned *sim* region of P1 express the Sim phenotype and produce three proteins in a minicell system (Kliem and Dreiseikelmann, 1989). We have now sequenced the *sim* region and have identified and purified a 24-kDa protein which seems to be the protein responsible for the superinfection exclusion.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli JM101 Δ *lac pro, thi, sup*E44 [F′ *tra*D36, *pro*AB, *lac*I^{q}Z M15] was used for transformation with pUC13 and M13mp8 and M13mp9 RF derivatives (Yanisch–Perron *et al.*, 1985). *E. coli* C600 F$^-$, *thi*-1, *thr*-1, *leu*B61, *lac*Y1, *sup*E44, *ton*A21 was used for transformation with pJF118EH. Expression of genes from pPLc2819 derivatives was performed in *E. coli* K12 Δ H1 Δ *trp*, Sm^r, *lac*Z, Δ *bio-uvr*B, Δ *trp*EA2, (λ Nam7-Nam53cI857 Δ H1) (Remaut *et al.*, 1981). *E. coli* MM52 F$^-$, Δ (*lac*)U169, *ara*D136, *rel*A, *rsp*L, *thi, sec*A51$_{ts}$ was a gift from J. Tommassen (Oliver and Beckwith, 1981).

DNA sequencing

The nucleotide sequence was determined with the chain termination method (Sanger *et al.*, 1977). Various

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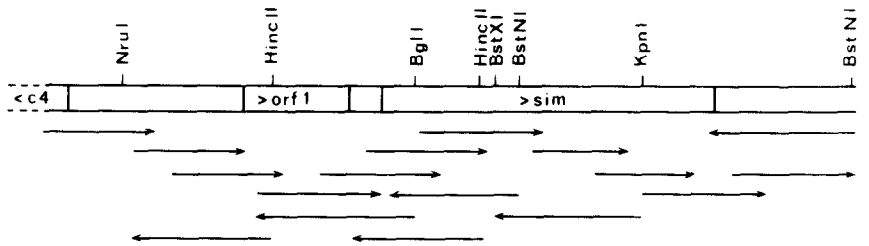

FIG. 1. Strategy for determination of the nucleotide sequence of a 1.9-kb DNA fragment from the *sim* region. Sequence of the upper strand was mainly determined with the aid of deletion derivatives produced by limited digestion with exonuclease III and VII (Yanisch-Perron *et al.*, 1985). The lower strand was sequenced after subcloning of different DNA restriction fragments.

DNA inserts were ligated into M13mp8 and mp9 RF-DNA (Messing and Vieira, 1982). DNA synthesis at the M13 single strand was performed with a T7 DNA polymerase system purchased from U.S. Biochemical Corporation (Tabor and Richardson, 1987).

SDS–PAGE

Polyacrylamide slab gels (14 or 17.5% acrylamide, 1 mm thick, 8 cm long) were prepared as described (Laemmli, 1970). Electrophoresis was performed for about 2 hr at 20 mA. Gels were stained with Coomassie brilliant blue G-250. The protein marker mix from Pharmacia contained phosphorylase b (mol wt 94,000), BSA (mol wt 67,000), ovalbumin (mol wt 43,000), carboxyanhydrase (mol wt 30,000), trypsin inhibitor (mol wt 20,100), α-lactoalbumin (mol wt 14,400).

Isolation of the 24-kDa protein

Growth of the cells and induction. K12 Δ H1 Δ *trp* (pBD5) was grown in 1 liter TBY medium at 30° to a density of 3×10^8 cells/ml. Temperature was shifted to 42° for 20 min and incubation was continued for 2 hr at 37°. Cells were harvested by centrifugation. The cells (wet wt 4 g) were resuspended in 5 ml cooled buffer A (20 m*M* Tris–HCl, pH 7.8, 40 m*M* NaCl, 0.1 m*M* EDTA, 7 m*M* β-mercaptoethanol).

Crude extract. Cells were disrupted by a French Press at a pressure of 18,000 psi. The lysate was centrifuged for 1 hr at 30,000 rpm in a TST55.5 rotor (Kontron) at 4°.

Streptomycin sulfate step. Streptomycin sulfate (30% solution) was slowly added to the crude extract to a final concentration of 3%. The mixture was stirred for 30 min at 0° and centrifuged for 30 min at 8000 rpm in a A8.24 rotor (Kontron).

Ammonium sulfate precipitation. Solid ammonium sulfate was added to the supernatant to 60% saturation. The mixture was stirred in ice for 30 min. After 2 hr at 0° the precipitate was collected by centrifugation for 30 min at 8000 rpm in a A8.24 rotor. The precipitate was resuspended in 4 ml buffer B (20 m*M* sodium phosphate, pH 7.8, 0.1 m*M* EDTA, 7 m*M* β-mercaptoethanol) and dialyzed for 2 hr against the same buffer.

Hydroxyapatite chromatography. The dialyzed fraction was loaded onto a hydroxyapatite column (diameter 1.5 cm, height 2.5 cm), previously equilibrated with buffer B. The column was washed until the OD_{280} was 0.05. Adsorbed proteins were eluted with a linear gradient (100 ml) of 20 to 200 m*M* sodium phosphate in buffer B. The flow rate was 10 ml/hr. Fractions of 1 ml were collected. The 24-kDa protein was identified by SDS–PAGE. Fractions containing this protein, which eluted between 40 and 80 m*M* sodium phosphate, were pooled and dialyzed against buffer A.

Mono Q ion-exchange chromatography. The dialyzed sample was loaded on an equilibrated Mono Q column (HR5/5, Pharmacia) and chromatographed by FPLC. The 24-kDa protein was eluted at a NaCl concentration of 300 to 400 m*M* NaCl with a linear gradient from 0.05 to 1 *M* NaCl in buffer A.

Protein sequencing. The protein was transferred to a membrane (Immobilon, Millipore) and the amino-terminal amino acids were determined with an automated protein sequencer (Knauer model 810, Berlin). Separation of the PTH amino acids was performed on-line on a PTH-C_{18} column (Applied Biosystems, 220 × 2.1 mm) at a flow rate of 0.24 ml/min using a sodium acetate/THF/acetonitrile eluent system as described (Hunkapiller, 1985).

RESULTS

Nucleotide sequence analysis of the *sim* region

In a preceding paper (Kliem and Dreiseikelmann, 1989) we have shown that the *sim* gene product interferes with P1 infection by blocking an early step following phage adsorption. In minicells three proteins with apparent molecular weights of about 25, 24, and 15 kDa were detected originating from the 2.1-kb *Eco*RI–*Pvu*II *sim* region. In order to sequence the gene(s) re-

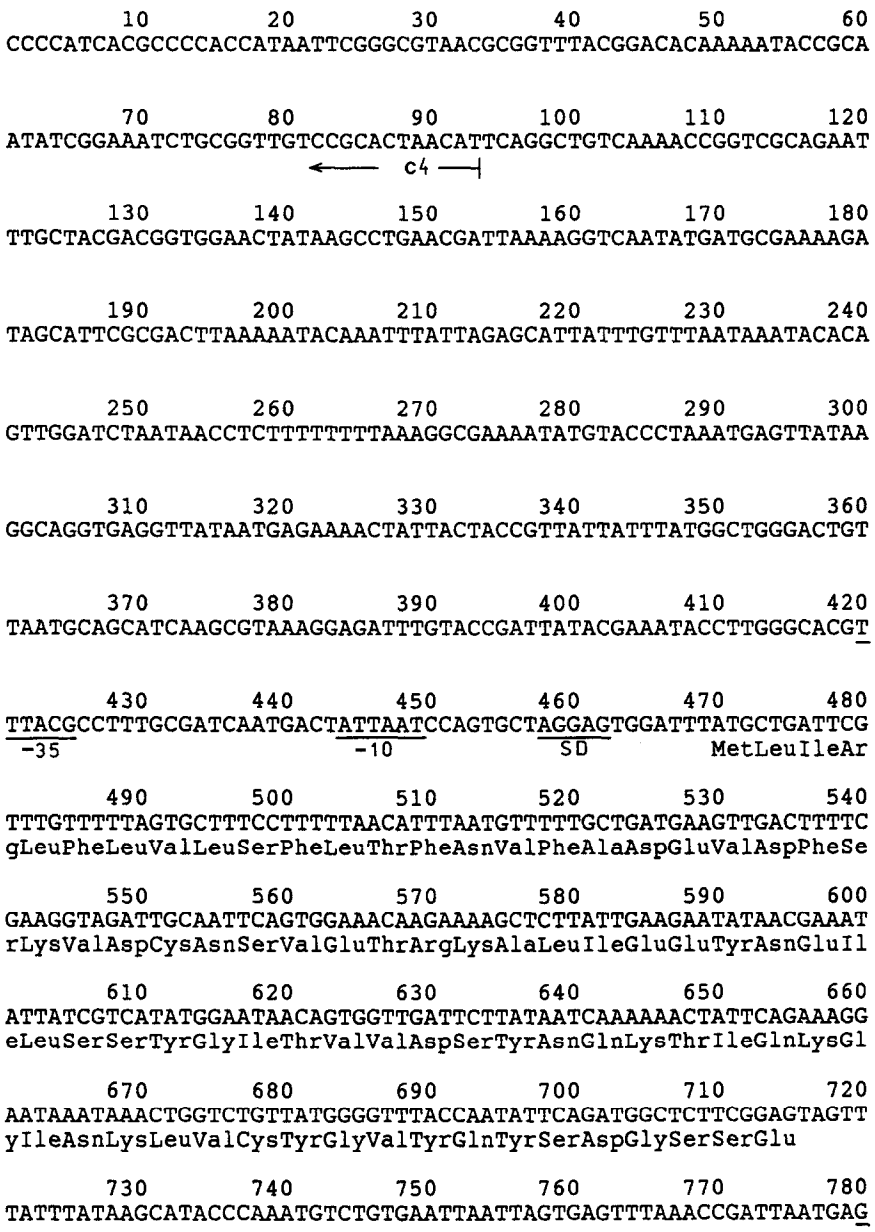

FIG. 2. Nucleotide sequence of a 1.9-kb DNA fragment of the *sim* region. The two open reading frames which could encode proteins are shown with their deduced amino acid sequences. A possible promoter and the Shine–Dalgarno (SD) sequences are marked.

sponsible for the superinfection exclusion phenotype within this region, we inserted various subfragments of the *Eco*RI–*Pvu*II fragment into M13mp8 and mp9 RF-DNA (Bächi and Arber, 1977). The sequencing strategy is summarized in Fig. 1. The nucleotide sequence determined by dideoxy sequencing is shown in Fig. 2.

At the 5′ end of the nucleotide sequence there is part of the *c*4 gene reading from right to left (position 93–1). The sequence agrees with published data (Baumstark and Scott, 1987). Two other open reading frames with the opposite orientation as the *c*4 gene (positions 1524–1381 and 1272–1132), and one with the same orientation (position 290–445), are evident. However these lack apparent transcriptional (promoter) and translational (ribosome binding site) signals. Two open reading frames which could possibly encode proteins are indicated along with their amino acid sequences in Fig. 2. One open reading frame starts at position 470 and ends at position 715. Upstream of it there is a possible promoter with a typical −10 and −35 sequence and a Shine–Dalgarno consensus sequence. The open reading frame would encode a protein of 82 amino acids. The second open reading frame (position 776–1547) has two start codons (position 776 and 791) but

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       790        800        810        820        830        840
TGAATGGAAAATGAAATTATTTAATGTAATAACATTTTGTTGTGCTATTTTTGCTGGAAG
SD        MetLysLeuPheAsnValIleThrPheCysCysAlaIlePheAlaGlySe

       850        860        870        880        890        900
CGCGATAGCTGATAATAAATTGCCAGATTGGCTTTCTACCTCAAAGAAAGATTATGATTT
rAlaIleAlaAspAsnLysLeuProAspTrpLeuSerThrSerLysLysAspTyrAspLe

       910        920        930        940        950        960
AGTAAGGGCATTCTATTTGTCTGGATTTGCTTCGAAAGCAATGAACAATCAATTTGGTTA
uValArgAlaPheTyrLeuSerGlyPheAlaSerLysAlaMetAsnAsnGlnPheGlyTy

       970        980        990       1000       1010       1020
TCATTTGCCATCTGAGTTGGTTAATGATTTTAAAGATAATGAATTTGCTGCTCAGGAAAA
rHisLeuProSerGluLeuValAsnAspPheLysAspAsnGluPheAlaAlaGlnGluLy

      1030       1040       1050       1060       1070       1080
ATGGAACACAATTCCAATTGTGTATGGTGAAATAAAATCCATAAGAATGGTGAATAATAA
sTrpAsnThrIleProIleValTyrGlyGluIleLysSerIleArgMetValAsnAsnLy

      1090       1100       1110       1120       1130       1140
ACCAATTGTAGAATTATTTACTCCAGGGGAAAATGCAACGCCTATAAATTATATCAAATT
sProIleValGluLeuPheThrProGlyGluAsnAlaThrProIleAsnTyrIleLysLe

      1150       1160       1170       1180       1190       1200
GAAAATATTGGATTCAAAGCAAGACTCTCTGTTAAAACTAAAAAAAAGGGGATGATATATA
uLysIleLeuAspSerLysGlnAspSerLeuLeuLysLeuLysLysGlyAspAspIleTy

      1210       1220       1230       1240       1250       1260
TGCAGTGTGCTCCGGTGCTAATTTTAGCTTAGTGCCAATTCTGAGCAACTGCACTCCAGC
rAlaValCysSerGlyAlaAsnPheSerLeuValProIleLeuSerAsnCysThrProAl

      1270       1280       1290       1300       1310       1320
AACAGACGTCATTGATGCTGCACTCTCTTTTTCTGGTGAATATATGTTCCCTGCTTTTGA
aThrAspValIleAspAlaAlaLeuSerPheSerGlyGluTyrMetPheProAlaPheAs

      1330       1340       1350       1360       1370       1380
TTCTTTTTCACCTACTAAGCAAAACGTCAAATATATATTCACAAATCAAGATCCTGTTCA
pSerPheSerProThrLysGlnAsnValLysTyrIlePheThrAsnGlnAspProValGl

      1390       1400       1410       1420       1430       1440
GATGATGAATTTTATAGGGTACCTATCCTTAGTCGATACAACGAAGGATAAGAATAAAAT
nMetMetAsnPheIleGlyTyrLeuSerLeuValAspThrThrLysAspLysAsnLysMe

      1450       1460       1470       1480       1490       1500
GGATATGGTTCGTAAGTGTACGCCTTGGAAGCCGGAATGTTCACAACAATTCGTTGATGT
tAspMetValArgLysCysThrProTrpLysProGluCysSerGlnGlnPheValAspVa

      1510       1520       1530       1540       1550       1560
AATGGAAGGATTTGATAGCATCATGTATAAATATGAGGGAGAATTTAAAAACTACATAGA
lMetGluGlyPheAspSerIleMetTyrLysTyrGluGlyGluPheLysAsnTyrIleGl

      1570       1580       1590       1600       1610       1620
ATTAAAATAGTGTTTGAAAGGATAGTCAATTTAAGTTTTAACACCAACGCCCATTAAAGG
uLeuLys

      1630       1640       1650       1660       1670       1680
GCTTTTATTGTTTTACTCAAAACAACCTGATTAGTGTATAACCATATCATATCCCTCATT

      1690       1700       1710       1720       1730       1740
CCACCTACACTGATTACCCCCAGACAACAATATTCCTACTCAATGAACAAATGACTACTC

      1750       1760       1770       1780       1790       1800
GTAGAATCGGTTAACACACCAGATTCTACGAGGTTTCAATGACACCACGACAATTACTCG

      1810       1820       1830       1840       1850       1860
AAGACGTCAAAACCCGCTTCACACCTTTGATTGCGGATGAACCGGCCTTACTGGAATCCC

      1870       1880       1890       1900
TGCTAAGAAAAGCATTGGGAACCTACCAGGATAGGGCGGG
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FIG. 2—*Continued*

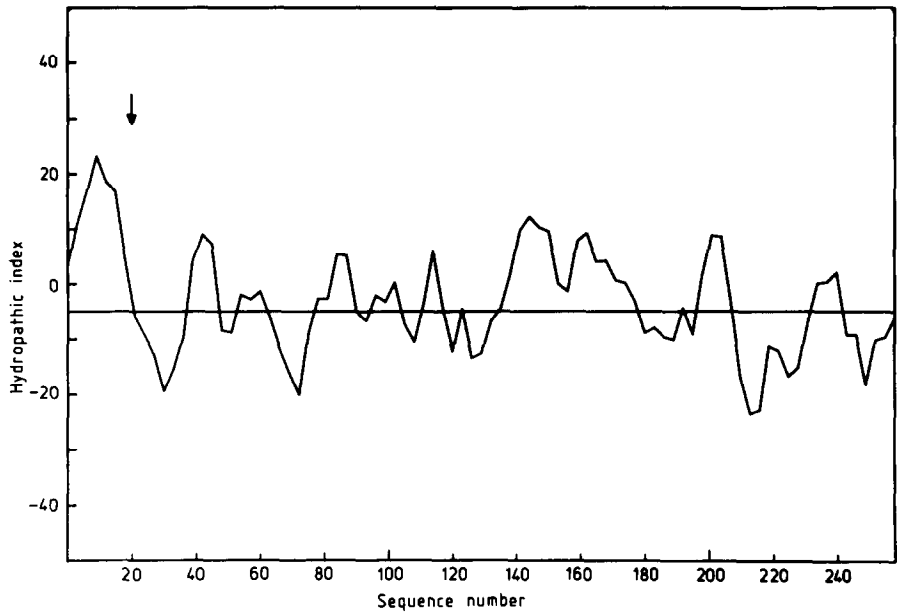

FIG. 3. Hydropathic plot of the hypothetical Sim protein. The hydropathic plot was performed as described at a span setting of nine residues (Kyte and Doolittle, 1982).

only the AUG at 791 has a possible ribosome binding site, allowing for a protein of 259 amino acids. A promoter is not evident. At the end of the open reading frame there is an inverted repeat followed by numerous thymine residues, which may represent a transcriptional terminator (position 1606–1634).

The possible protein encoded by the latter open reading frame would have a molecular weight of 29,326 Da and may represent the 25-kDa protein previously described (Kliem and Dreiseikelmann, 1989). The apparent discrepancy between the molecular weight deduced from the nucleotide sequence and that determined by SDS–PAGE will be considered in the discussion. For simplicity we will maintain the 24- and 25-kDa protein nomenclature. A hydropathic plot shows that the 25-kDa protein resembles a precursor protein with a hydrophobic leader sequence (Fig. 3). The amino-terminal amino acid sequence has the characteristics of a hydrophobic signal sequence (Oliver, 1985): (i) the length of the hydrophobic region is 20 amino acids, (ii) the protein begins with a positively charged region (Lys 2, Asn 5), (iii) there are two helix destabilizing amino acids at the distal site of the hydrophobic amino acid sequence (Gly 16 and Ser 17), (iv) the hydrophobic leader ends with the characteristic Ala–X–Ala sequence (Ala 18–Ile 19–Ala 20). This suggests that the 24-kDa protein observed in minicells (Kliem and Dreiseikelmann, 1989) may originate by processing of the 25-kDa protein.

Isolation of the 24-kDa protein and determination of the amino-terminal amino acid sequence

The following experiments demonstrate that the protein predicted from the nucleotide sequence is indeed synthesized *in vivo* as a precursor (25 kDa) and is subsequently processed by proteolytic removal of a leader peptide to give the mature protein (24 kDa).

The 1320- and 1300-bp *Eco*RI–*Hin*dIII DNA fragments from pMK4d5 and pMK4d6 (Kliem and Dreiseikelmann, 1989) were inserted into the expression vector pPLc2819 (Remaut *et al.*, 1981). Cells carrying hybrid plasmid pBD4 or pBD5 were induced for 2 hr at 42°. Total protein from uninduced and induced cells was separated by SDS–PAGE. Overproduction of the 24- and the 25-kDa protein was observed (Fig. 4). Generally the 25-kDa protein was less abundant than the 24-kDa protein. The 24-kDa protein was purified from induced cells as described under Materials and Methods (Fig. 5). To eliminate minor contaminants from fractions obtained after chromatography on a Mono Q column these were further purified by electrophoresis on a preparative SDS polyacrylamide gel. The protein purified in this way gave a single band on a silver-stained SDS–PAGE loaded with 2 μg of protein (data not shown).

The purified protein was subjected to automated protein sequencing. The sequence of the first 15 amino acids was identical to that predicted from the nucleotide sequence analysis, but started with the aspartic acid residue at nucleotide position 851 corresponding to Asp 21. From this we conclude that the 24-kDa protein is the processed form of a precursor protein. The cleavage site is between Ala 20 and Asp 21. Processing would remove a hydrophobic leader sequence of 20 amino acids (see Fig. 3).

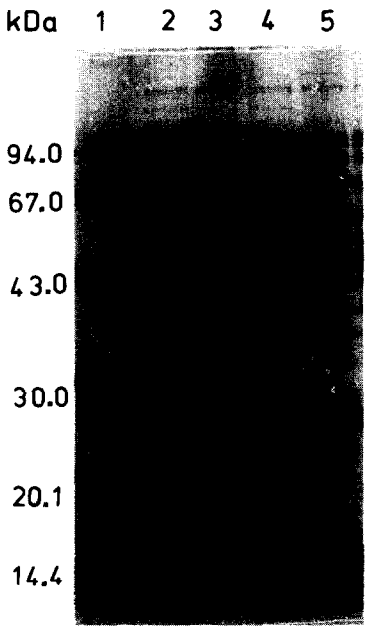

FIG. 4. Overproduction of a 25- and 24-kDa protein in cells with plasmids pBD4 and pBD5. SDS polyacrylamide gel (14%) of total protein from uninduced and induced cells. 1, marker proteins; 2, pBD4 without induction; 3, pBD4 with thermoinduction; 4, pBD5 without induction; 5, pBD5 with thermoinduction.

Processing of the 25-kDa protein is essential for its biological activity

The 1300-bp DNA fragment containing the *sim* gene was inserted into the expression vector pJF118EH with the *tac* promoter (Fürste *et al.*, 1986). The hybrid plasmid, called pBD6, was introduced into *E. coli* MM52

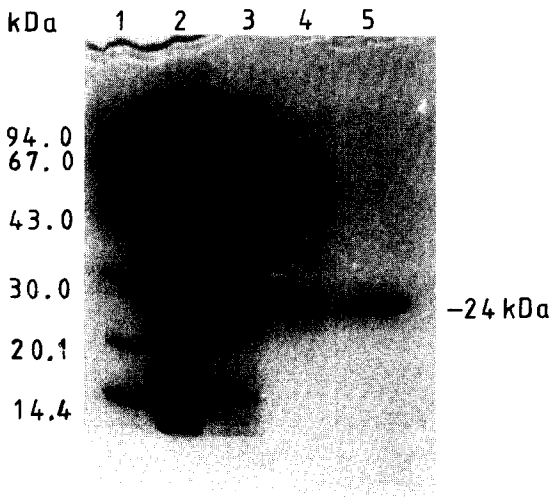

FIG. 5. Purification of the 24-kDa protein. SDS polyacrylamide gel (17.5%) with samples from crude extract (lane 2), ammonium sulfate precipitation (lane 3), hydroxyapatite fractions (lane 4), Mono Q fractions (lane 5), marker proteins (lane 1).

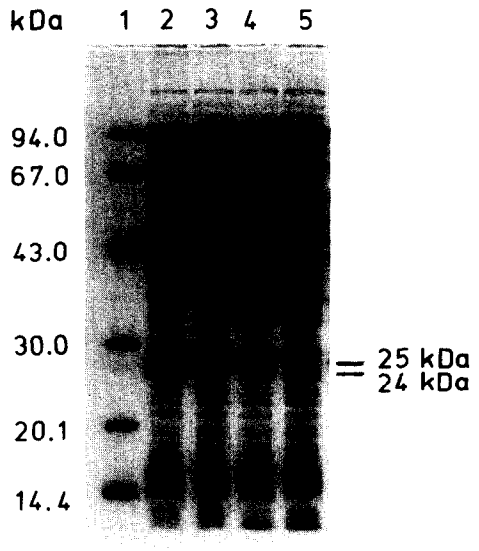

FIG. 6. Processing of the *Sim* protein in *secA*$_{ts}$ sells. SDS–polyacrylamide gel (14%) with total protein from *Escherichia coli secA*$_{ts}$ pBD6. Molecular weight standards (lane 1); 30°, induction with IPTG (lane 2); 30°, uninduced cells (lane 3); 42°, induction with IPTG (lane 4); 42°, uninduced cells (lane 5).

secA$_{ts}$. At the nonpermissive temperature, the precursor forms of many membrane proteins and periplasmic proteins are accumulated in *secA*$_{ts}$ cells (Oliver and Beckwith, 1981). Cells with pBD6 were induced with 4 m*M* IPTG at 30 and 42°, and total protein was analyzed by SDS–PAGE (Fig. 6). After induction at 30° the 24-kDa protein is the predominant protein as compared to the 25-kDa precursor. However, at 42° the reverse is observed—the 25-kDa protein accumulates and the processed form can hardly be detected. Therefore we conclude that the 25-kDa protein precursor is converted into the 24-kDa mature protein by proteolytic cleavage of the leader sequence depending on the *sec*A function.

Induced *secA*$_{ts}$ cells with pBD6 are not infected by P1 at the permissive temperature of 30°, but they are susceptible to the phage at the nonpermissive temperature of 42° (Table 1). Thus the *sec*A-dependent processing of the Sim protein is necessary for its function in blocking phage infection.

TABLE 1

INFECTION OF *E. coli secA*$_{ts}$ CELLS WITH PHAGE P1

	Infective centers (ml)	
Strain	30°	42°
MM52 pJF118	3×10^9	8×10^{10}
MM52 pBD7	$<10^2$	5×10^{10}

In vitro properties of the Sim protein

It has been suggested for phage T4 that the *imm*T gene product may be a nuclease (Anderson and Eigner, 1971). The purified Sim protein (Mono Q fraction) had no detectable nuclease activity when tested with linear double-stranded DNA under a variety of conditions. For T4 it has recently been described (Obringer, 1988) that the *sp* gene product, which is encoded by a gene in the vicinity of the *imm*T gene, directly interacts with the phage tail. Phage infectivity is abolished by destruction of components of the injection apparatus. Incubation of purified Sim protein with phage P1 (15 to 60 min at room temperature; 20 m*M* Tris–HCl, pH 7.5, 10 m*M* NaCl, 5 m*M* $MgCl_2$, 5 m*M* $CaCl_2$) did not reduce the efficiency of plating of the lysate. This result suggests that the purified protein does not directly interact with some tail protein to inhibit the contact of phage particles with the receptor or the induction of a membrane channel for the injection of the DNA. The negative result does not exclude such interactions *in vivo*, which may need other factors.

DISCUSSION

The nucleotide sequence of the *sim* region contains two open reading frames. The smaller one is preceded by a typical promoter region and a Shine–Dalgarno sequence. Downstream of the smaller open reading frame a larger one follows with its own Shine–Dalgarno sequence. It is likely that both genes are transcribed from the same promoter as an operon. Plasmids pBD4 and pBD5 still express the *sim* phenotype, although the first open reading frame is deleted in these plasmids. This implies that the gene product of the small reading frame is not essential for the superinfection exclusion phenotype. However, deletion of a *Kpn*I–*Hin*dIII DNA fragment from plasmid pBD5, which removes about one-fifth of the distal part of the gene, resulted in the loss of the *sim* phenotype (data not shown). The product of the larger reading frame must therefore be the Sim protein. Its overproduction and processing in cells with pBD5 suffices to exclude infecting P1 phages from the cell.

The mechanism by which the Sim protein abolishes infection of cells by phage P1 is still not clear. We can now add the observation that the Sim protein may be a periplasmic or membrane-associated protein to our earlier results showing that the Sim protein blocks an early stage of P1 infection (Kliem and Dreiseikelmann, 1989). The following facts support this hypothesis: (i) the Sim protein is synthesized as a precursor with a hydrophobic leader sequence of 20 amino acid residues, (ii) only the processed form of the protein is biologically active, (iii) processing of the precursor is *sec*A dependent, since overproduction of the precursor is reduced at the nonpermissive temperature compared to the mature form at the permissive temperature. This is also observed for several other periplasmic and membrane proteins which are synthesized at a reduced level in *sec*A cells and accumulate the precursor forms (Liss and Oliver, 1986; Strauch *et al.*, 1986).

The Sim protein is a rather hydrophobic protein which may account for the discrepancy between the apparent molecular weight as determined by SDS–PAGE (25 kDa, sometimes up to 27 kDa depending on the choice of marker proteins) and the predicted molecular weight deduced from the nucleotide sequence (29.3 kDa). In the literature one can find contrasting statements on the mobility of hydrophobic proteins during electrophoresis. There are indications for lower (Garten *et al.*, 1975) as well as for higher mobilities (Heller, 1978) than expected after heating in detergent. The Sim protein, which was always incubated for 5 min at 100° before electrophoresis, had a higher mobility than expected.

Since the Sim protein was isolated from a soluble fraction (see Materials and Methods), we suggest that the major portion of the protein may be localized in the periplasmic space. However, we cannot exclude the possibility that part of the protein may also be localized in the membrane. If the Sim protein is a membrane protein, the membrane association would probably be a specific one, so that overproduction of the protein would lead to a saturation of potential membrane binding sites. Further studies employing isolated membranes and osmotic shock proteins from Sim-producing cells are necessary to decide the localization of the Sim protein and to eludicate the mechanism of superinfection exclusion.

ACKNOWLEDGMENTS

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