JOURNAL OF BACTERIOLOGY, July 1992, p. 4205–4211
0021-9193/92/134205-07$02.00/0

Vol. 174, No. 13

The Stable Maintenance System *pem* of Plasmid R100: Degradation of PemI Protein May Allow PemK Protein To Inhibit Cell Growth

SUGURU TSUCHIMOTO,[1] YUKINOBU NISHIMURA,[2] AND EIICHI OHTSUBO[1]*

Institute of Applied Microbiology, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113,[1] and Faculty of Science, Toho University, Miyama 2-2-1, Funabashi, Chiba 274,[2] Japan

Received 12 August 1991/Accepted 23 April 1992

We constructed plasmids carrying heat-inducible *pemI* and *pemK* genes, which were fused with the collagen-*lacZ* sequence in frame. The PemK-collagen-LacZ (PemK*) protein produced from the fusion gene upon heat induction inhibited the growth of cells and killed most of the cells in the absence of the PemI protein but did not do so in the presence of the PemI protein. This supports our previous assumption that the PemK protein inhibits cell division, leading to cell death, whereas the PemI protein suppresses the function of the PemK protein. We also constructed the plasmid carrying the heat-inducible *pem* operon which consists of the intact *pemI* gene and the *pemK* gene fused with collagen-*lacZ*. The simultaneously induced PemI and PemK* proteins did not inhibit the growth of cells. However, the temperature shift to 30°C after induction of both proteins at 42°C caused inhibition of cell growth and death of most cells. This suggests that the PemI protein is somehow inactivated upon the arrest of de novo synthesis of the PemI and PemK* proteins, allowing the PemK* protein to function. We observed that the PemI-collagen-LacZ (PemI*) protein was degraded faster than the PemK* protein, perhaps by the action of a protease(s). In fact, the *lon* mutation, which caused no apparent degradation of the PemI* protein, did not allow the PemK* protein to function, supporting the suggestion described above. Instability of the PemI protein would explain why the cells which have lost the *pem*$^+$ plasmid are preferentially killed.

The naturally occurring plasmid R100 is stably maintained in dividing bacterial cells in spite of its low copy number (1 to 2 per chromosome) (19, 25). The *pem* region, which is located near the region essential for autonomous replication of R100, is responsible in part for the stable maintenance of R100 (28). The growth of cells which have lost the plasmid carrying *pem* is inhibited, while the plasmid-containing cells grow normally (27, 28). Upon growth inhibition by the action of *pem*, some cells cannot resume cell division and die (27).

The *pem* region consists of two genes, *pemI* and *pemK* (28), which code for proteins of 9.3 and 11.9 kDa, respectively (2, 23). These two genes are transcribed from a promoter located upstream of *pemI* (26). The PemK protein, the *pemK* gene product, is supposed to inhibit the growth of host cells which have lost the *pem*$^+$ plasmid, and the PemI protein, the *pemI* gene product, is supposed to suppress the function of the PemK protein (28).

Plasmid R1, which is closely related to R100, has the homolog of the *pem* region, ParD, which has been found independently to have the same sequence as that of *pem* (3). A mutation in the *kis* gene (corresponding to *pemI*) in ParD caused the death of cells by the action of the *kid* gene (corresponding to *pemK*) product (4).

Here we report the results of an analysis of the functions of the PemI and PemK proteins, leading to an understanding of the mechanism of postsegregational growth inhibition in the *pem* system at the genetic and molecular levels.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* K-12 strains and plasmids used are listed in Tables 1 and 2, respectively. Most of the pDOM plasmids were constructed as described below.

Media. The liquid medium used was L broth (1.0% peptone [Kyokuto], 0.5% yeast extract [Difco], 0.5% NaCl, 0.2% glucose [pH 7.0]). Agar plates contained 1.5% agar (Eiken Chemical Co.). For mutagenesis in constructing mutant plasmids, 2× YT broth (17) was used.

Enzymes and reagents. The restriction endonucleases used were *Bam*HI, *Eco*RI (Takara Shuzo Co.), *Bcl*I, *Eag*I, and *Nae*I (New England Biolabs). Phage T4 DNA ligase, *E. coli* DNA ligase, phage T4 DNA polymerase, Klenow fragment of *E. coli* DNA polymerase I, and RNase I (Takara Shuzo Co.) were also used, as recommended by the manufacturers.

The molecular size standards for proteins were purchased from Bio-Rad Laboratories. Other reagents were obtained from Sigma Chemical Co. or Wako Pure Chemical Industries.

Plasmid construction. To construct plasmids pDOM101 to pDOM104 (see Fig. 1), a parental plasmid, pDOM100, was first constructed by replacing the *Bam*HI-*Eco*RI segment in the multiple cloning site of pUC119 with the *Bam*HI-*Eco*RI fragment (nucleotides 2835 to 3917) of pHO100 (28). pDOM101 and pDOM102 were obtained by introducing a *Bam*HI site in the N-terminal regions of the *pemI* and *pemK* coding frames in pDOM100, respectively, by site-directed mutagenesis by the method of Kunkel et al. (14). Similarly, pDOM103 and pDOM104 were obtained by introducing a *Bcl*I site at the termination codon of the *pemI* and *pemK* coding frames in pDOM100, respectively.

Plasmids pDOM105, pDOM106, and pDOM108 (see Fig. 1) were constructed as follows. Plasmids pDOM101 to

TABLE 1. *E. coli* K-12 strains used

Strain	Markers	Source or reference
MV1184	F' *traD36 proAB lacI*^{q}*Z* ΔM15/ Δ(*lac-proAB*) *ara strA thi*(ϕ80 *lacZ*ΔM15) Δ(*srl-recA*)*306*::Tn*10*	29
BW313	*HfrKL* 16*PO*/45[*lysA*(61-62)] *dut-1 ung-1 thi-1 relA1*	13, 14
GM33	F$^-$ *dam-3*	16
MC1000	F$^-$ *ara* Δ(*ara-leu*) Δ*lacX74 galU galK str*	5
GC4670	F$^-$ *lon*::Tn*10 thr leu lacY*	S. Casaregola

pDOM104 were digested by *Bam*HI or *Bcl*I, and then by *Nae*I or *Eag*I, whose cleavage sites are present in the *pemI* and *pemK* coding frames, respectively. The *Bam*HI-*Eag*I fragment of pDOM101 (Fig. 1, fragment a) and the *Eag*I-*Bcl*I fragment of pDOM104 (Fig. 1, fragment a) were mixed and cloned into the *Bam*HI site of pJG200 to obtain pDOM105. Similarly, the *Bam*HI-*Eag*I fragment of pDOM102 (Fig. 1, fragment a) and the *Eag*I-*Bcl*II fragment of pDOM104 (Fig. 1, fragment b) were mixed and cloned into the *Bam*HI site of pJG200 to obtain pDOM106; the *Bam*HI-*Eag*I fragment of pDOM101 (Fig. 1, fragment a) and the *Eag*I-*Bcl*II fragment of pDOM103 (Fig. 1, fragment b) were mixed and cloned into the *Bam*HI site of pJG200 to obtain pDOM108.

Plasmid pPS3006K was constructed by ligation of the *Pvu*II-digested DNA of plasmid pPS3006, a pSC101 derivative carrying *lon*$^+$ (obtained from A. L. Goldberg), with the *Hin*cII fragment containing the kanamycin resistance gene (*kan*) which had been cloned in pUC19.

Kinetics of cell growth and production of β-galactosidase. The liquid culture of strain MC1000 or GC4670 was incubated overnight at 30°C in L broth containing 100 μg of ampicillin per ml and diluted appropriately in L broth containing 100 μg of ampicillin per ml. After incubation at 30°C until turbidity had reached an OD_{660} of 0.2 to 0.3, the temperature was shifted to 42°C. The turbidity of the culture was measured at 600 nm with a spectrophotometer (Spectronic 20A, Shimadzu Co.). β-Galactosidase (LacZ) activity per unit of turbidity of the culture was measured by the method of Miller (21).

Viable cell and total cell counts. Viable cells were measured as the number of colony formers on L agar plates containing 100 μg of ampicillin per ml after incubation of the plates at 30 or 42°C for 1 to 4 days. Total cells were counted on a counting chamber (Thoma, Atago) with a phase-contrast microscope.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described by Laemmli (15). Proteins in the gel were stained with Coomassie brilliant blue (6).

RESULTS

The PemK protein inhibits cell growth, leading to cell death. To study functions of the PemI and PemK proteins, we constructed plasmids carrying the heat-inducible *pemI* and *pemK* genes, which were fused in frame with the collagen-*lacZ* coding region of an expression vector pJG200 carrying p_R promoter of bacteriophage λ, which is under the control of a thermolabile repressor (Fig. 1). Plasmids pDOM108 and pDOM106 carry the fusion genes (here called *pemI** and *pemK**, respectively), which encode the tripartite proteins PemI-collagen-LacZ and PemK-collagen-LacZ (here called PemI* and PemK*, respectively) (Fig. 1 and Table 2). The PemI* protein induced in the cells of MC1000 harboring pDOM108(*pemI**$^+$) did not inhibit cell growth, whereas the PemK* protein induced in the cells harboring pDOM106 (*pemK**$^+$) inhibited cell growth (Fig. 2A, panel a). This supports our previous assumption that the PemK protein is an inhibitor of cell growth, while the PemI protein is not (27). The LacZ activity in the cells harboring pDOM106(*pemK**$^+$) was much less than that in the cells harboring pDOM108 (*pemI**$^+$) (Fig. 2A, panel b). This shows that the PemK* protein which was produced in only a small amount after heat induction can effectively inhibit cell growth.

The inhibition of cell growth by the PemK protein is assumed to cause killing of cells (28). To prove this, the number of viable cells was determined by plating and incubating the cells at 42 or 30°C after inducing the PemK* protein at 42°C for 10 min in the cell harboring pDOM106(*pemK**$^+$).

TABLE 2. Plasmids used

Plasmid[a]	Parental plasmid	Description	Source or reference
pJG200	pBR322	With p_R promoter, cI857, the tripartite gene consisting of ATG of *cro*, the collagen linker, and *lacZ*	9
pHO100	pSM1 and pMB8::Tn*3*	A composite plasmid carrying *pem*	23
pHS12	pSC101	A high-copy-number mutant	1
pDOM11	pHS12	With the region of nucleotides 2835 to 3497 of pHO100 (containing *pemI* and its promoter)	28
pDOM100	pUC119	With the region of nucleotides 2835 to 3917 of pHO100 (containing *pem* and its promoter)	This work
pDOM101	pDOM100	With a *Bam*HI site at the N-terminal region of *pemI*	This work
pDOM102	pDOM100	With a *Bam*HI site at the N-terminal region of *pemK*	This work
pDOM103	pDOM100	With a *Bcl*I site at the termination codon of *pemI*	This work
pDOM104	pDOM100	With a *Bcl*I site at the termination codon of *pemK*	This work
pDOM105	pJG200	With *pemI* and *pemK**	This work
pDOM106	pJG200	With *pemK**	This work
pDOM108	pJG200	With *pemI**	This work
pPS3006	pSC101	With *lon*	A. L. Goldberg
pPS3006K	pPS3006	With *lon*	This work

[a] pHS12 and pDOM11 carry *tet*. pPS3006K carries *kan*. All of the other plasmids carry *amp*. *pemK** and *pemI** indicate tripartite genes of *pemK* and *pemI* fused with the collagen-*lacZ* sequence, respectively (Fig. 1, and see the text).

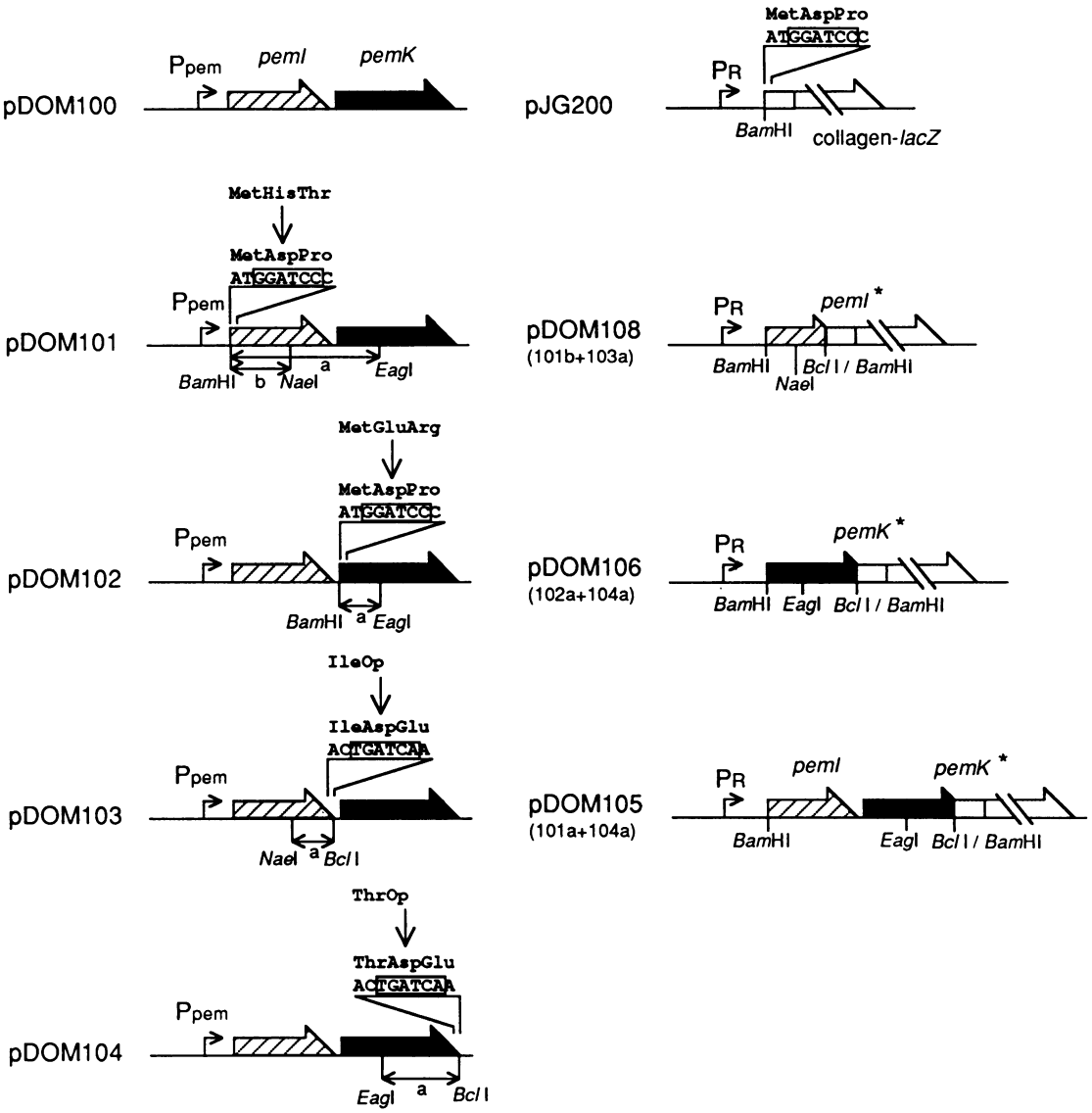

FIG. 1. Structures of plasmids. Hatched and solid arrows indicate *pemI* and *pemK* coding regions, respectively. The collagen-*lacZ* segments in fusion genes are indicated by open arrows connected to open boxes. The promoter of *pem* genes (p_{pem}) and the λ p_R promoter are also shown. In pDOM101 to pDOM104, nucleotide and amino acid sequences in the mutated regions are shown. The recognition sequences for *Bam*HI and *Bgl*II introduced by site-directed mutagenesis are boxed. Thin horizontal lines with two arrowheads (labeled a or b) indicate relevant restriction fragments. In vector plasmid pJG200, nucleotide and amino acid sequences in the N-terminal region of collagen-*lacZ* and the *Bam*HI site used to clone fragments are indicated. Cutting sites for relevant restriction endonucleases are also shown. Restriction fragments used to construct pDOM108, pDOM106, and pDOM105 are shown in parentheses.

Induction of the PemK* protein caused a marked reduction in the number of colony formers: no colonies were formed when plates were incubated at 42°C (a temperature at which the synthesis of the PemK* protein continued [Table 3] and colonies were also not formed when plates were incubated at 30°C (a temperature at which the synthesis of PemK* was arrested), but a few small colonies appeared after incubation of the plates for several days at 30°C (Table 3). Heat induction of the PemI* protein in the cells harboring pDOM108(*pemI*$^{*+}$) and of the collagen-LacZ protein in the cells harboring pJG200(*pem*$^{-}$), however, caused no reduction in the number of colony formers (Table 3). These results indicate that inhibition of cell growth by the PemK* protein leads to the death of most cells; only a few cells could resume dividing after the arrest of de novo synthesis of PemK*.

The PemI protein suppresses the growth inhibition by the PemK protein. To prove our previous assumption that the PemI protein suppresses the function of the PemK protein (28), we introduced plasmid pDOM11, a pHS12 derivative carrying the intact *pemI* gene which is constitutively expressed from the *pem* promoter (Table 2), into the cell harboring pDOM106(*pemK*$^{*+}$) and examined the growth of cells and LacZ activity upon heat induction of the PemK* protein. The PemK* protein induced in the cells harboring pDOM106(*pemK*$^{*+}$) and pDOM11(*pemI*$^{+}$) did not inhibit cell growth (Fig. 2B, panel a), but the same protein induced in the cells harboring pDOM106(*pemK*$^{*+}$) and pHS12(*pem*$^{-}$) instead of pDOM11 inhibited cell growth (Fig. 2B, panel a). The LacZ activity in the cells harboring pDOM106(*pemK*$^{*+}$) and pDOM11(*pemI*$^{+}$) was higher than that in those harbor-

TABLE 3. Viable cells in total cells

Plasmid(s)	No. of colony formers[a]		
	A	B	C
In MC1000 (*lon*$^+$)			
pDOM106(*pemK**$^+$)	<6.1 × 10^{-6}	<6.1 × 10^{-4} (0.16)	1.1
pDOM108(*pemI**$^+$)	1.0	1.2	1.0
pJG200(*pem*$^-$)	1.0	1.0	0.81
pDOM106(*pemK**$^+$) + pDOM11(*pemI*$^+$)	1.1	0.70	0.75
pDOM106(*pemK**$^+$) + pHS12(*pem*$^-$)	<1.2 × 10^{-5}	2.9 × 10^{-4} (0.52)	1.1
pDOM105(*pemI*$^+$ *pemK**$^+$)	0.96	<6.2 × 10^{-4} (0.0017)	0.99
In GC4670 (*lon*)			
pDOM106(*pemK**$^+$)	<1.5 × 10^{-5}	2.1 × 10^{-4} (0.041)	1.0
pDOM108(*pemI**$^+$)	1.0	0.82	1.1
pJG200(*pem*$^-$)	1.0	0.82	1.1
pDOM105(*pemI*$^+$ *pemK**$^+$)	0.22 (0.48)	0.82	1.1

[a] Numbers indicate viable cells (colony formers) in total cells. (A) Cells were plated and incubated at 42°C overnight after induction of a Pem protein(s) at 42°C for 30 min; (B) cells were plated and incubated at 30°C overnight after heat induction of a Pem protein(s) at 42°C for 30 min; and (C) cells were plated and incubated at 30°C overnight without heat induction. In the case that small colonies were formed during incubation of plates for several days, the number of viable cells calculated from those colonies is shown in parentheses.

ing pDOM106(*pemK**$^+$) and pHS12(*pem*$^-$) (Fig. 2B, panel b). These results show that the PemI protein supplied from pDOM11(*pemI*$^+$) suppresses growth inhibition by the PemK* protein.

Induction of the PemK* protein in the cells harboring pDOM106(*pemK**) and pDOM11(*pemI*$^+$) did not cause a reduction in the number of colony formers, whereas induction of the PemK* protein in the cells harboring pDOM106(*pemK**$^+$) and pHS12(*pem*$^-$) caused a marked reduction in the number of colony formers (Table 3). This confirms that the PemI protein suppresses inhibition of cell growth and thus also suppresses killing of cells by the PemK protein.

Effect on cell growth by simultaneous induction of PemI and PemK proteins and subsequent repression of de novo synthesis of the proteins. To study the effect of the simultaneous induction of the PemI and PemK proteins on cell growth, we constructed a plasmid carrying the intact *pemI* gene and the *pemK** gene which were under the control of the p_R promoter (Fig. 1). The growth of the cells harboring the plasmid pDOM105(*pemI*$^+$ *pemK**$^+$) was not inhibited upon induction of the PemI and PemK* proteins and further incubation at 42°C (Fig. 3A), and the LacZ activity in the lysate of cells harboring pDOM105 increased to a high level (Fig. 3B). These results indicate that the PemI protein suppressed growth inhibition by the PemK* protein, even though the PemK* protein is produced in a large amount. Note that the LacZ activity was higher in these cells (Fig. 3B) than in cells harboring both pDOM106(*pemK**$^+$) and pDOM11(*pemI*$^+$) (Fig. 2B, panel b). A possible explanation of this result is that the PemI protein produced coordinately with the PemK* protein causes more effective suppression of the function of the PemK* protein than the PemI protein supplied in trans from a coresident *pemI*$^+$ plasmid.

Importantly, however, the growth of the cells harboring pDOM105(*pemI*$^+$ *pemK**$^+$) was inhibited upon temperature shift and further incubation of the cell culture at 30°C after induction of the PemI and PemK* proteins at 42°C for 10 min (Fig. 3A and B). This shows clearly that the arrest of de novo synthesis of both PemI and PemK* proteins after heat induction causes inhibition of cell growth.

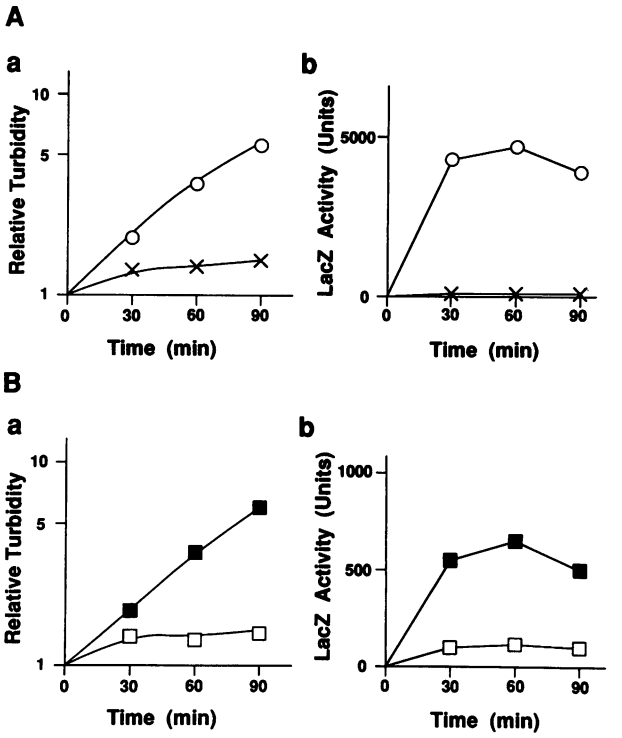

FIG. 2. Kinetics of cell growth and production of PemI* and PemK*. (A) Kinetics of the culture of strain MC1000 harboring pDOM108(*pemI**$^+$) (○) or pDOM106(*pemK**$^+$) (×). (a) Relative turbidity to indicate cell growth; (b) LacZ activity to indicate the production of the PemK* or PemI* protein. (B) Kinetics of the culture of the strain MC1000 harboring pDOM106(*pemK**$^+$) and pHS12(*pem*$^-$) (□) or pDOM106(*pemK**$^+$) and pDOM11(*pemI*$^+$) (■). (a) Relative turbidity to indicate cell growth; (b) LacZ activity which refers to the *pemK** gene activity. All values in panels A and B are plotted against incubation time after the temperature shift from 30 to 42°C.

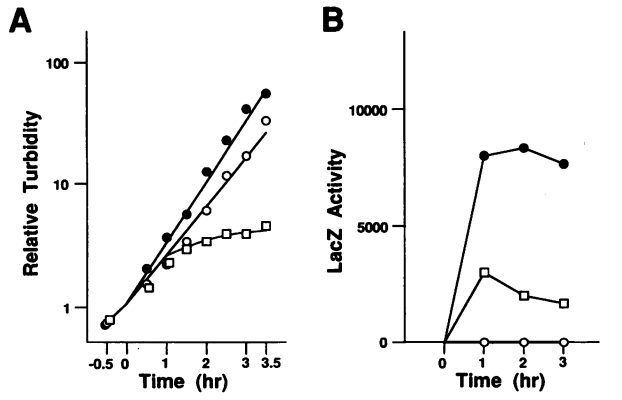

FIG. 3. Kinetics of the growth of cells of strain MC1000 harboring pDOM105(*pemI*$^+$ *pemK**$^+$) and of the production of the PemK* protein. Relative turbidity (A) and LacZ activity (B) are plotted against time of incubation at 42°C (●) or at 30°C (□) after induction of both PemI and PemK* proteins at 42°C for 10 min or of incubation at 30°C without induction (○).

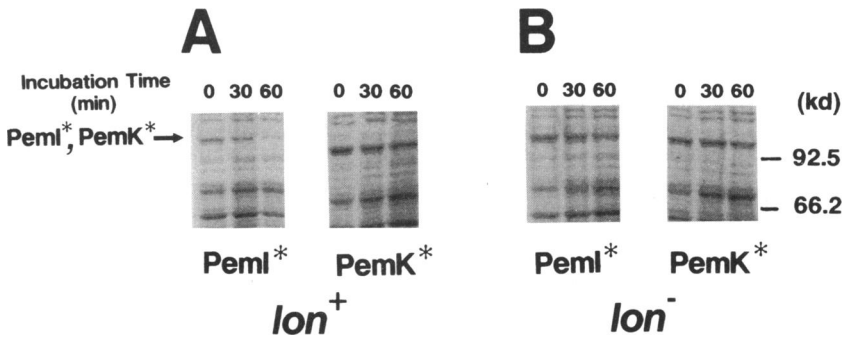

FIG. 4. Coomassie brilliant blue-stained polyacrylamide gels showing PemI* and PemK* proteins. (A) Proteins in cells of the *lon*$^+$ strain MC1000 harboring pDOM108(*pemI*$^{*+}$) (left) or pDOM105(*pemI*$^+$ *pemK*$^{*+}$) (right); (B) proteins in cells of the *lon* strain GC4670 harboring pDOM108(*pemI*$^{*+}$) (left) or pDOM105(*pemI*$^+$ *pemK*$^{*+}$) (right). The PemI* or PemK* protein was induced by a temperature shift from 30 to 42°C for 30 min. After the temperature was returned to 30°C, the extracts were made from the samples taken at 0, 30, and 60 min. The total proteins were prepared from the same volume of the culture and electrophoresed on an SDS-6% polyacrylamide gel. The band positions of the PemI* and PemK* proteins with almost the same sizes are indicated at the right sides of the gels. Molecular size standards used were β-galactosidase (LacZ) (116 kDa) and phosphorylase *b* (92.5 kDa), whose band positions are shown at the left sides of gels.

When cells harboring pDOM105(*pemI*$^+$ *pemK*$^{*+}$) were plated and incubated at 42°C after heat induction of both PemI and PemK* proteins, colonies were formed normally (Table 3). This supports the observation described above that continuous production of the PemI protein suppresses the growth inhibition by the PemK* protein which leads to cell death. However, when the cells were plated and incubated at 30°C after heat induction, no colonies were formed overnight, but a few small colonies appeared upon further incubation of the plates for several days (Table 3). This shows that the arrest of de novo synthesis of both PemI and PemK* proteins after heat induction causes not only inhibition of cell growth but also death of most cells. This and the results described above suggest that the PemI protein is somehow inactivated after the arrest of de novo synthesis of the PemI and PemK* proteins to allow the PemK* protein to function.

Instability of the PemI* protein. Proteins produced in the cells of strain MC1000 harboring pDOM105(*pemI pemK*$^{*+}$) or pDOM108(*pemI*$^{*+}$) at time zero or 30 or 60 min after heat induction were examined by SDS-polyacrylamide gel electrophoresis. A protein larger in size than LacZ (116 kDa), which is indicative of the PemI or PemK protein fused with the collagen-LacZ segment, was observed (Fig. 4).

We noticed that the PemI* protein, once overproduced, seemed to disappear during incubation at 30°C after heat induction, while the PemK* protein did not (Fig. 4A). It is likely that the PemI* protein is degraded rapidly in the cells of strain MC1000 by the action of a protease(s). To investigate this, we induced the Pem tripartite proteins in the cells of strain GC4670 with a mutation in the *lon* gene which is known to code for an ATP-dependent protease involved in degradation of some unstable proteins and all types of abnormal proteins (for a recent review, see reference 10). The PemI* protein, as well as the PemK* protein, was overproduced in strain GC4670 without apparent degradation of proteins (Fig. 4B). This and the results described above suggest that the PemI protein is degraded in the cells by the action of the Lon protease, either directly or indirectly.

Effect on cell growth by simultaneous induction of PemI and PemK proteins and subsequent repression of de novo synthesis of the proteins in the *lon* strain. The effect of the *lon* mutation on the growth of cells harboring pDOM105(*pemI*$^+$ *pemK*$^{*+}$) was tested by using GC4670 (*lon*) as a host. Simultaneous induction of PemI and PemK* proteins at 42°C for 10 min and subsequent repression of de novo synthesis of the proteins by the temperature shift to 30°C did not cause inhibition of the growth of the *lon* host cells harboring pDOM105 (Fig. 5). The same treatment, however, did cause inhibition of the growth of cells harboring pDOM105, when strain MC1000 (*lon*$^+$) was used as the host, as described earlier (Fig. 3A), or when plasmid pPS3006K carrying *lon*$^+$ was coresident in the host GC4670 (*lon*) used above (data not shown). Induction of the PemK* protein alone caused inhibition of the growth of both *lon* and *lon*$^+$ cells harboring pDOM106(*pemK*$^{*+}$) (Fig. 5), showing that the PemK* protein in the absence of the PemI protein can function even in the *lon* background. Note that the PemK* protein is certainly induced in the *lon* host, as it is in the *lon*$^+$ host, since

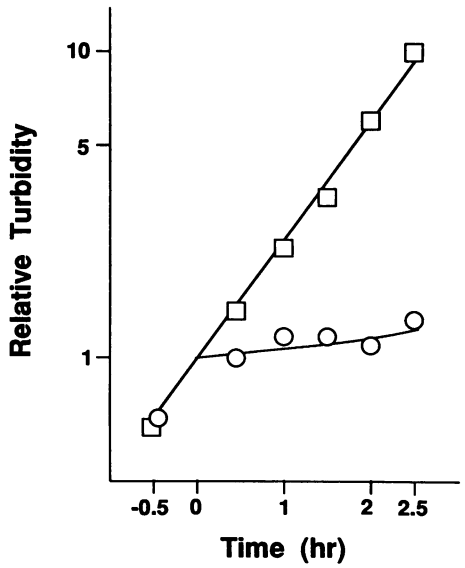

FIG. 5. Kinetics of the growth of cells of the *lon* strain GC4670 harboring pDOM105(*pemI*$^+$ *pemK*$^{*+}$) (□) or pDOM106(*pemK*$^{*+}$) (○). Relative turbidity was plotted against time of incubation at 30°C after induction by a temperature shift from 30 to 42°C for 10 min.

the LacZ activity in the *lon* cells harboring pDOM105(*pemI*$^+$ *pemK*$^{*+}$) at 30 or 60 min after the temperature shift was even higher than that in the *lon*$^+$ cells (data not shown). These results indicate that the PemI protein produced is not readily degraded in the *lon* background and thus can still suppress the inhibition of cell growth by the PemK* protein even upon subsequent repression of de novo synthesis of both PemI and PemK* proteins produced after heat induction.

When GC4670 (*lon*) cells harboring pDOM105(*pemI*$^+$ *pemK*$^{*+}$) were plated and incubated at 42°C after heat induction of both PemI and PemK* proteins, colonies were formed, as they were in the *lon*$^+$ host, although the number of colony formers decreased slightly (Table 3). However, when the cells were plated and incubated at 30°C after heat induction, colonies of these cells were formed (Table 3). When cells of GC4670 (*lon*) harboring pDOM106(*pemK*$^{*+}$) were incubated at 42 or 30°C after heat induction of the PemK* protein alone, however, colonies were not formed (Table 3). These results support the indication that the PemI protein, which is not readily degraded after the arrest of de novo synthesis of the PemI and PemK* proteins in the *lon* host, suppresses the growth inhibition by the PemK* protein which leads to cell death.

DISCUSSION

We have shown here that induction of the PemK* protein causes the inhibition of cell growth which leads to cell death, whereas the PemI protein suppresses the growth inhibition by the PemK* protein. We have also shown that simultaneous induction of the PemK* protein with the PemI protein did not cause inhibition of the growth of cells, but subsequent repression of de novo synthesis of both proteins caused inhibition of cell growth and the death of most cells in the *lon*$^+$ host but not in the *lon* host. These results and the observation that the PemI* protein is degraded faster than the PemK* protein in the *lon*$^+$ host, but not in the *lon* host, would support the idea that degradation of the PemI protein allows the PemK protein to function.

In the *pem* system acting under natural conditions, the function of the PemK protein appears postsegregationally in cells which have lost the *pem*$^+$ plasmid (27, 28). The synthesis of the PemI and PemK proteins is supposed to be arrested in such plasmid-free cells because of the disappearance of the *pem* region by segregation of the plasmid carrying it. Therefore, the *pem* action under natural conditions is likely to be explained by degradation of the PemI protein which allows the PemK protein to function.

The low-copy-number plasmid F has the *ccd* (*let*) region consisting of two genes, *ccdA* (*letA*) and *ccdB* (*letD*), responsible for the maintenance of this plasmid (20, 22). The nucleotide sequences of these genes are completely different from those of *pem*. However, the *ccdB* gene product inhibits cell division in cells which have lost the *ccd*$^+$ plasmid, while the *ccdA* gene product suppresses the inhibition in the plasmid-carrying cells (11, 12, 18). The mechanism proposed in the *pem* system may also be involved in the stable maintenance system of plasmid F.

Plasmid R1 has the *parB* locus consisting of *sok* and *hok* genes, which are responsible for the stable maintenance of the plasmid (8, 24). The Hok protein, the *hok* gene product, kills plasmid-free cells; the *sok* gene encodes an antisense RNA, which represses the expression of *hok*, located just downstream from *sok*. The Sok antisense RNA is supposed to be degraded much faster than the mRNA encoding the Hok protein in cells which have lost the *parB*$^+$ plasmid, allowing the Hok protein to kill the cells (7). This mechanism would be analogous to that of the *pem* system. In the *pem* system, however, the PemI protein suppresses the inhibition of cell growth by the PemK protein after translation from mRNA, while the Sok RNA suppresses the growth inhibition at the translational level.

ACKNOWLEDGMENTS

We thank D. Bastia for kindly sending us plasmid pJG200, A. L. Goldberg for plasmid pPS3006, and S. Casaregola for strain GC4670. We also thank M. Umeda for critical reading of the manuscript.

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. S.T. is the recipient of a JSPS Fellowship for Japanese Junior Scientists.

REFERENCES

1. **Armstrong, K. A., R. Acosta, E. Ledner, Y. Machida, N. Pancotto, M. McCormick, H. Ohtsubo, and E. Ohtsubo.** 1984. A 37×10^3 molecular weight plasmid-encoded protein is required for replication and copy number control in plasmid pSC101 and its temperature-sensitive derivative pHS1. J. Mol. Biol. **175:** 331–347.
2. **Armstrong, K. A., H. Ohtsubo, W. R. Bauer, Y. Yoshioka, C. Miyazaki, Y. Maeda, and E. Ohtsubo.** 1986. Characterization of the gene products produced in minicells by pSM1, a derivative of R100. Mol. Gen. Genet. **205:**56–65.
3. **Bravo, A., G. de Torrontegui, and R. Diaz.** 1987. Identification of components of a new stability system of plasmid R1, ParD, that is close to the origin of replication of this plasmid. Mol. Gen. Genet. **210:**101–110.
4. **Bravo, A., S. Ortega, G. de Torrontegui, and R. Diaz.** 1988. Killing of *Escherichia coli* modulated by components of the stability system ParD of plasmid R1. Mol. Gen. Genet. **215:**146–151.
5. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
6. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10:**2606–2617.
7. **Gerdes, K., K. Helin, O. W. Christensen, and A. Løbner-Olesen.** 1988. Translational control and differential RNA decay are key elements regulating postsegregational expression of the killer protein encoded by the *parB* locus of plasmid R1. J. Mol. Biol. **203:**119–129.
8. **Gerdes, K., P. B. Rasmussen, and S. Molin.** 1986. Unique type of plasmid maintenance function: postsegregational killing of plasmid-free cells. Proc. Natl. Acad. Sci. USA **83:**3116–3120.
9. **Germino, J., and D. Bastia.** 1984. Rapid purification of a cloned gene product by genetic fusion and site-specific proteolysis. Proc. Natl. Acad. Sci. USA **81:**4692–4696.
10. **Gottesman, S.** 1989. Genetics of proteolysis in *Escherichia coli*. Annu. Rev. Genet. **23:**163–198.
11. **Hiraga, S., A. Jaffé, T. Ogawa, H. Mori, and H. Takahashi.** 1986. F plasmid *ccd* mechanism in *Escherichia coli*. J. Bacteriol. **166:**100–104.
12. **Jaffé, A., T. Ogura, and S. Hiraga.** 1985. Effects of the *ccd* function of the F plasmid on bacterial growth. J. Bacteriol. **163:**841–849.
13. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
14. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Marinus, M. G., and N. R. Morris.** 1975. Pleiotropic effects of a DNA adenine methylation mutation (*dam-3*) in *Escherichia coli*

K12. Mutat. Res. **28**:15–26.
17. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–79.
18. **Miki, T., Z. T. Chang, and T. Horiuchi.** 1984. Control of cell division by sex factor F in *Escherichia coli*. II. Identification of genes for inhibitor protein and trigger protein on the 42.84-43.6 F segment. J. Mol. Biol. **174**:627–646.
19. **Miki, T., A. M. Eaton, and R. H. Rownd.** 1980. Cloning of replication incompatibility, and stability functions of R plasmid NR1. J. Bacteriol. **141**:87–99.
20. **Miki, T., K. Yoshioka, and T. Horiuchi.** 1984. Control of cell division by sex factor F in *Escherichia coli*. I. The 42.84-43.6 F fragment couples cell division of the host bacteria with replication of plasmid DNA. J. Mol. Biol. **174**:605–625.
21. **Miller, J. H.** 1972. Experiments in molecular genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Ogura, T., and S. Hiraga.** 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. USA **80**:4784–4788.
23. **Ohtsubo, H., T. B. Ryder, Y. Maeda, K. Armstrong, and E. Ohtsubo.** 1986. DNA replication of the resistance plasmid R100 and its control. Adv. Biophys. **21**:115–133.
24. **Rasmussen, P. B., K. Gerdes, and S. Molin.** 1987. Genetic analysis of the *parB*$^+$ locus of plasmid R1. Mol. Gen. Genet. **209**:122–128.
25. **Rownd, R. H., and D. D. Womble.** 1978. Molecular nature and replication of R factors, p. 161–193. *In* S. Mitsuhashi (ed.), R factor, drug resistance plasmid. University Park Press, Tokyo.
26. **Tsuchimoto, S.** 1990. Expression and function of *pem* genes, *pemI* and *pemK*, which are responsible for stable maintenance of plasmid R100. Ph.D. thesis. University of Tokyo, Tokyo, Japan.
27. **Tsuchimoto, S., and E. Ohtsubo.** 1989. Effect of the *pem* system on stable maintenance of plasmid R100 in various *Escherichia coli* hosts. Mol. Gen. Genet. **215**:463–468.
28. **Tsuchimoto, S., H. Ohtsubo, and E. Ohtsubo.** 1988. Two genes, *pemK* and *pemI*, responsible for stable maintenance of resistance plasmid R100. J. Bacteriol. **170**:1461–1466.
29. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.