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Cloning, Mapping, and Characterization of the *Escherichia coli prc* Gene, Which Is Involved in C-Terminal Processing of Penicillin-Binding Protein 3

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The *prc* gene, which is involved in cleavage of the C-terminal peptide from the precursor form of penicillin-binding protein 3 (PBP 3) of *Escherichia coli*, was cloned and mapped at 40.4 min on the chromosome. The gene product was identified as a protein of about 80 kDa in maxicell and in vitro systems. Fractionation of the maxicells producing the product suggested that the product was associated with the periplasmic side of the cytoplasmic membrane. This was consistent with the notion that the C-terminal processing of PBP 3 probably occurs outside the cytoplasmic membrane: the processing was found to be dependent on the *secY* and *secA* functions, indicating that the *prc* product or PBP 3 or both share the translocation machinery with other extracytoplasmic proteins. DNA sequencing analysis of the *prc* gene region identified an open reading frame, with two possible translational starts 6 bp apart from each other, that could code for a product with a calculated molecular weight of 76,667 or 76,432. The *prc* mutant was sensitive to thermal and osmotic stresses. Southern analysis of the chromosomal DNA of the mutant unexpectedly revealed that the mutation was a deletion of the entire *prc* gene and thus that the *prc* gene is conditionally dispensable. The mutation resulted in greatly reduced heat shock response at low osmolarity and in leakage of periplasmic proteins.

It is well known that many proteins destined for extracytoplasmic locations are initially synthesized as precursor forms and processed into mature forms by proteolytic cleavage of N-terminal signal peptides during transfer across the cytoplasmic membrane. Penicillin-binding protein 3 (PBP 3) of *Escherichia coli* is a cytoplasmic membrane protein that is essential for the formation of a septum of the murein sacculus (60, 61, 66). The bulk of the molecule, except for the N-terminal membrane anchor region, protrudes into the periplasmic space, where it acts on murein (7). This protein is coded for by the *ftsI* gene (66), and its primary product with a molecular weight of 63,850 is processed into a mature form showing a higher electrophoretic mobility corresponding to about 60 kDa (43). It was first suspected that the maturation of PBP 3 involved processing of the N-terminal signal peptide (40). The N-terminal region seemed to serve for insertion into the membrane: artificial removal of the N-terminal 40 residues resulted in accumulation of the protein in the cytoplasm (3). In the N-terminal region there is a pentapeptide with an amino acid sequence similar to the consensus for bacterial lipoproteins; this pentapeptide was shown, for a minor fraction of the molecules, to actually undergo the lipid modification and probably the processing (22). However, further investigations with gene manipulation to produce hybrid and truncated PBP 3 molecules (20) and with peptide mapping and amino acid sequence analysis of purified precursor and mature forms (42) revealed that cleavage of the C-terminal 11 residues, rather than that of the N-terminal signal peptide, is responsible for the maturation detected as a change in electrophoretic mobility.

We fortuitously found a mutant, JE7304, that was defective in the processing of PBP 3; we utilized it to elucidate the mode of the processing (20, 42). The processing mutation in JE7304 was named *prc* (processing involving the C-terminal cleavage). JE7304 showed thermosensitive growth on a salt-free L-agar plate. This suggests that the *prc* gene is involved in some essential cellular process, which may or may not be related to the cell division function of PBP 3. To gain a better understanding of this unique processing reaction and its function in cell growth and division, we cloned, mapped, and sequenced the *prc* gene in the present study. The gene product was identified, and its localization was investigated. Characterization of the *prc* mutant suggested that this gene is involved in protection of the bacterium from thermal and osmotic stresses.

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MATERIALS AND METHODS

Bacterial strains and culture media. The bacterial strains used were derivatives of *E. coli* K-12 and are described in Table 1. Bacteria were grown in L broth (41) containing 0.5% NaCl and 10 mM glucose, in buffered L broth-glucose-thymine (BLGT) medium (20), or in minimal medium E (70). Cell growth was monitored by using a Klett-Summerson

TABLE 1. Bacterial strains

Strain	Relevant genotype or comment	Source or reference
JE5606	*ponA1104*(Ts) *dacB12 dacA1191*	20
JE7304	JE5606 *prc-7304*[a]	20; this work
W3110	Wild type	Laboratory collection
JE7913	JE7304 *prc*$^+$	P1(W3110) × JE7304
DH1, DH5	*recA endA hsdR17*	18
JM109	*recA endA hsdR17* (F′ *lacI*^{q})	75
CSR603	*recA uvrA phr*	53
R594	Prototroph	Laboratory collection
MC4100	*sec*$^+$	9; K. Ito
MM18	MC4100 [λpΦ(*malE-lacZ*) (Hyb) 72-47]	4; K. Ito
IQ85	MC4100 *secY24*(Ts) *zhd-33*::Tn*10*	55
IQ86	MC4100 *zhd-33*::Tn*10*, isogenic to IQ85	55
MM52	MC4100 *secA51*(Ts)	47; K. Ito
MM113	MC4100 *secA*(Am) *supF*(Ts)	32; D. B. Oliver
RF101	*polA12*(Ts) *zig*::Tn*5*	17
P4X8	HfrP4X	Laboratory collection
JE7914	P4X8 *polA12*(Ts) *zig*::Tn*5*	P1(RF101) × P4X8
LC102	F$^-$ *leu ile xyl str argG his trp gal purE lac*	Laboratory collection
JE7915	LC102 *polA12*(Ts) *zig*::Tn*5*	P1(RF101) × LC102
FB191	*eda-51*::Tn*10*	57
JE7916	JE7304 *eda-51*::Tn*10*	P1(FB191) × JE7304
K27	*fadD88*	48; N. Otsuji
JE7917	K27 *eda-51*::Tn*10*	P1(FB191) × K27
JE7918	K27 *fadD*$^+$, isogenic to JE7919	P1(JE7304) × K27
JE7919	K27 *fadD*$^+$ *prc-7304*	P1(JE7304) × K27
JE7920	K27 *fadD*$^+$ *prc-7304 eda-51*::Tn*10*	P1(JE7916) × JE7919
JE7921	K27 *prc-7304 eda-51*::Tn*10*	P1(JE7920) × K27
JE7922	W3110 *eda-51*::Tn*10*, isogenic to JE7923	P1(JE7916) × W3110
JE7923	W3110 *eda-51*::Tn*10 prc-7304*	P1(JE7916) × W3110
JE7924	Wild type, isogenic to JE7925	P1(JE7304) × JE7922
JE7925	W3110 *prc-7304*	P1(JE7304) × JE7922

[a] *prc-7304* is a deletion mutation that covers *prc* and probably the *ptsLPM* operon [Δ(*prc-ptsLPM*?)*7304*; see Results].

colorimeter with a no. 54 filter. For plates, media were solidified with 1.5% agar. The thermosensitivity of the *prc* mutant was tested on salt-free 1/2L agar medium, in which the content of Bacto-Tryptone (Difco) and yeast extract of L-agar medium was reduced by half and neither NaCl nor glucose was included. Eda and FadD phenotypes were tested on minimal agar media (65) with 20 mM glucuronic acid (57) and 0.1% oleic acid (dissolved with 0.4% Brij 35) (48), respectively, as the sole carbon sources. The Pel phenotype was tested by cross-streaking against phage λ*vir* on EMB agar medium (41) containing 1% mannose. An RNase indicator plate was as described previously (51). K medium and sulfate-free Hershey medium were used in maxicell experiments (53). For selection and cultivation of transformants, an appropriate antibiotic was added to the medium at a concentration of 50 μg/ml (ampicillin), 25 μg/ml (chloramphenicol), 50 μg/ml (kanamycin), or 12.5 μg/ml (tetracycline) for high-copy-number plasmids, and at half the concentration for low-copy-number plasmids and for plasmids integrated into chromosomes.

Plasmids. The *prc*$^+$-carrying plasmids pHR53 and pHR61 and some of their derivatives constructed in this study are illustrated in Fig. 1. A *ftsI*$^+$-carrying plasmid, pMS316, was described previously (24). Plasmid pHR12 carries the kanamycin resistance (*neo*) gene of pKC7 (originally derived from Tn*5*) flanked by the pUC18/19 multiple cloning site (75) on either side in the same orientation, and pUC-4K (69) carries the *kan* gene (originally from Tn*903*) inserted into the center of the symmetrical pUC7 multiple cloning site. The *neo* and *kan* fragments were cut out with appropriate restriction enzymes and inserted into pHR53 to construct pHR74-77 and pHR80. In pHR74 and pHR76 the *neo* genes were inserted into the same positions as those in pHR75 and pHR77, respectively, but in the opposite orientation. The 3.5-kb fragment between the *Eco*RI$_1$ site and the *Eco*RI site within the multiple cloning site fragment of pHR76 was inserted into the *Eco*RI site of pACYC184 (11) in either orientation to construct pHR87 and pHR88. Plasmid pHR140 was constructed by ligating the 2.6-kb *Eco*RI$_1$-*Pst*I$_3$ fragment with the larger *Eco*RI-*Pst*I fragment of pBR322 (2). In pHR126 a synthetic translation terminator (39) was sandwiched between two *Bam*HI-*Hin*dIII regions of the pUC18/19 multiple cloning site in an inverted orientation. A synthetic translation terminator fragment was cut out with appropriate restriction enzymes and inserted to pHR140 to construct pHR154 through pHR156. The expression vector p18LCP1 has the thermosensitive λ *c*I857 repressor gene and the p_L promoter cloned into the *Eco*RI-*Bam*HI region of pUC18; the p_L promoter is oriented so that the transcription proceeds into the *Bam*HI-*Hin*dIII region of the multiple cloning site (74). Plasmids pHR143/144, pHR164, and pLPRC21/3R were constructed by inserting the 2.9-kb *Eco*RI fragment of pHR53 containing the *prc* gene into the *Eco*RI sites of pMF3 (38), the *Eco*RI site of pBR322, and the *Xba*I site of p18LCP1, respectively (for the *Xba*I site of p18LCP1, the termini of the fragments were converted to blunt ends by treatment with *E. coli* DNA polymerase Klenow fragment). After the cleaved sites were made blunt ended with T4 DNA polymerase, the 1.3-kb *Hae*III fragment containing the *cat* gene of pACYC184 was inserted into the *Eco*T14I site of pHR164 to construct pHR182. Plasmid pHR145 was constructed by inserting the 3.4-kb *prc*$^+$-containing fragment between the *Eco*RI$_1$ site and the *Eco*RI site within the vector of pHR61 into the *Eco*RI site of pMF3, with the *Eco*RI$_1$ site joined to the *blaZ* fragment of pMF3.

Genetic methods. An Hfr cross and transduction with P1*kc* were carried out by standard procedures (41). Adsorption of λ particles to bacteria was examined as described by Scandella and Arber (54) with λ*vir* as a tester phage and W3110 as an indicator strain, and adsorption and DNA injection were monitored by transfection to ampicillin resistance with in vitro packaged pHC79-derived cosmids (68).

DNA techniques. Procedures for construction, isolation, and analysis of plasmids and for transformation were based on those of Maniatis et al. (37). Chromosomal DNA was prepared as described previously by lysis of bacterial cells with lysozyme and proteinase K (58). Pulsed-field gel electrophoresis (59) and Southern blotting (37) were carried out essentially as described previously. The 2.9-kb *Eco*RI fragment of pHR53 containing the *prc* gene was labeled with [α-^{32}P]dCTP by a Multiprime DNA labeling system (Amersham) and used as a probe. The nucleotide sequences on both strands were determined with an M13 sequencing kit (Takara Shuzo) with [α-^{32}P]dCTP labeling. Computer analysis of the sequence was performed with Genetyx (Software

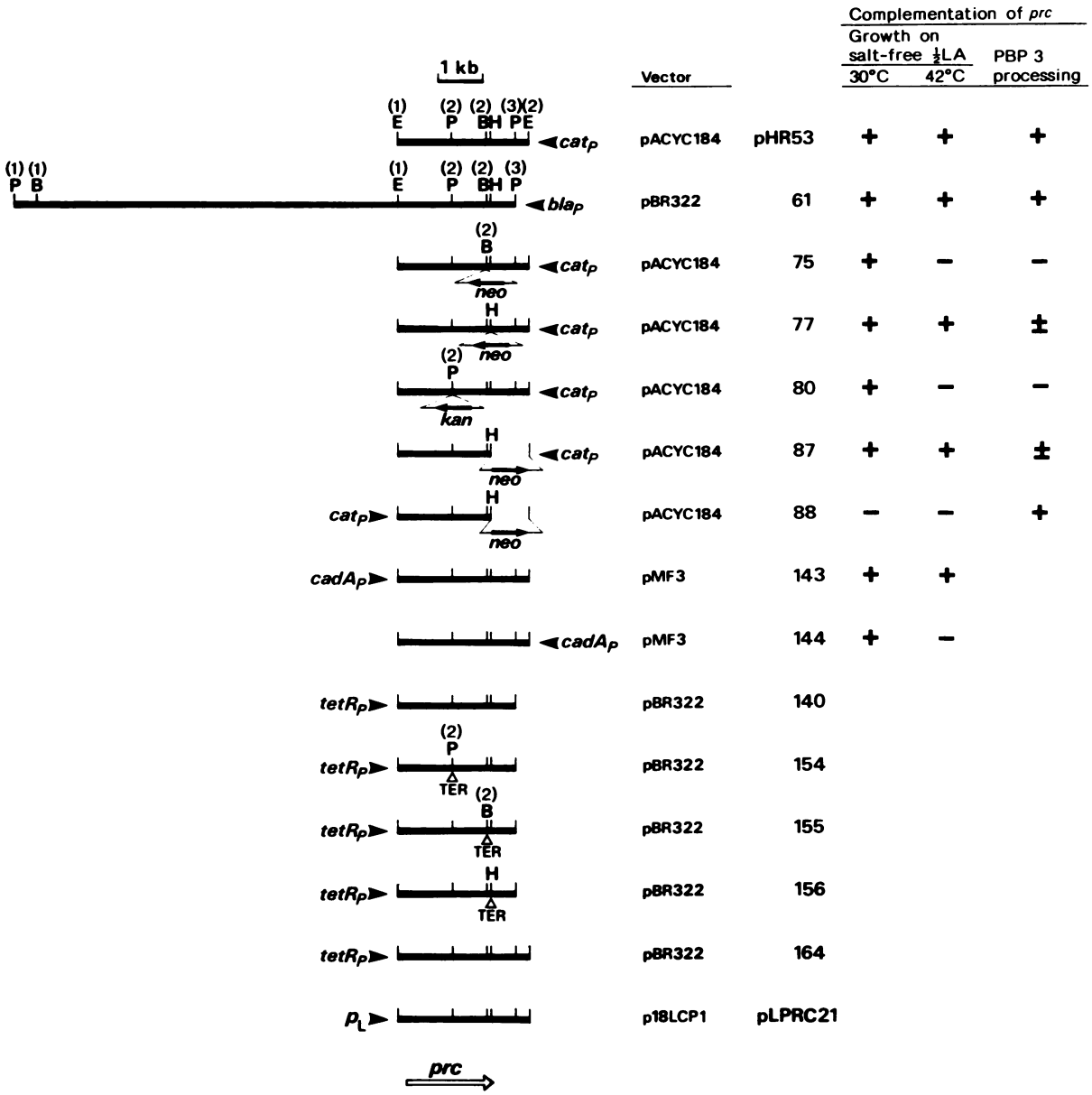

FIG. 1. Plasmids carrying the *prc* gene. Only the cloned chromosomal regions are shown. Arrowheads denote the directions of the promoters on vectors. Closed arrows and open triangles denote the insertions of DNA fragments containing kanamycin resistance genes (*neo* derived from pHR12 and *kan* derived from pUC-4K) and of a synthetic translation terminator (TER), respectively. The location and orientation of the *prc* gene are indicated by an open arrow at the bottom. To examine complementation, the *prc* mutant transformed with the plasmid indicated was incubated on a salt-free 1/2L agar plate at 30 and 42°C for growth tests. Complementation of the processing defect was examined by PBP assays of the transformants with SDS–6 M urea–7.2% polyacrylamide gel electrophoresis. ±, partial correction of the processing defect. Complementation was not tested where not indicated. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and P, *Pst*I. The multiple sites are numbered within parentheses for convenience.

Development Co.) and Dnasis (Hitachi Software Engineering) programs.

Radioactive labeling and analyses of proteins. Maxicells were prepared and labeled as described by Sancar et al. (53). In vitro synthesis of protein was performed with a prokaryotic DNA-directed translation kit (Amersham). [^{35}S]methionine was used for labeling. For protein fractionation, maxicells were suspended in 10 mM sodium phosphate buffer (pH 6.8), disrupted by sonication with a Cellruptor sonicator (Cosmo Bio), and centrifuged for 40 min at 100,000 × *g*. Conversion to spheroplasts (5) and fractionation with 0.1 N NaOH (52) were as described previously. Dithiothreitol (0.2 mM) was included in buffers throughout the fractionation procedures. Analyses of the processing kinetics of PBP 3 and OmpA were carried out as described before (20, 22). For examination of the heat shock response, cells growing exponentially at 30°C in medium E supplemented with 20 mM glucose, 2 μg of thiamine per ml, and 20 μg each of 18 amino acids per ml (no methionine or cysteine) were washed and resuspended in sodium phosphate buffer (100 or 2 mM)

containing 20 mM glucose, 0.2 mM $MgCl_2$, and 18 amino acids and then pulsed-labeled with [^{35}S]methionine for 5 min at 30°C and for 5 min at 5 min after transfer to 44°C. Labeled proteins were precipitated with 5% trichloroacetic acid and dissolved in a sample buffer for electrophoresis. Assays for PBPs with benzyl[^{14}C]penicillin (Amersham; 54 mCi/mmol) were performed essentially as described previously (66). Samples were analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis (66) with 10% polyacrylamide unless otherwise specified. ^{14}C-labeled methylated proteins (Amersham) were used as molecular weight markers. Radioactive protein bands were detected by fluorography with diphenyloxazole-dimethyl sulfoxide solution (6), Enlightning (New England Nuclear), or a fluorographic solution (20) that was slightly modified as follows: a stock solution was made by dissolving 4 g of diphenyloxazole and 30 g of naphthalene in 50 ml of xylene, 150 ml of ethanol, and 50 ml 2-(2-ethoxyethoxy)ethyl acetate; three parts of the stock solution were mixed with seven parts of 80% acetic acid immediately before use.

Observation of morphology of bacterial cells. Cells were treated with chloramphenicol, stained for nucleoids with 4′,6-diamidino-2-phenylindole, and observed with an incident fluorescence microscope equipped with phase-contrast optics (28, 29).

Nucleotide sequence accession number. The nucleotide sequence of *prc* has been submitted to DDBJ and will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession no. D00674.

RESULTS

Cloning of the *prc* gene. The *prc* mutant JE7304 showed thermosensitive growth at 42°C on a salt-free L-agar plate, although its defect in the processing of PBP 3 was not conditional: no mature form was detectable even under the conditions permissive for growth (20). A phage P1*kc* lysate prepared on a wild-type strain, W3110, was used to infect JE7304, and thermoresistant transductants were selected on a salt-free 1/2L agar plate. (On this plate thermoresistant revertants or suppressor mutants, which appeared at relatively high frequency, formed smaller colonies and were distinguishable from transductants.) Twelve transductants were tested for the electrophoretic mobility of PBP 3; all showed normal processing of PBP 3, suggesting that the mutation causing the thermosensitive growth was closely linked or identical to *prc*. Therefore we tried cloning the *prc* gene by selecting recombinant plasmids that corrected the thermosensitive growth of JE7304.

Chromosomal DNA of one of the thermoresistant transductants, JE7913, was digested with *Eco*RI or *Pst*I, and the fragments were inserted into the corresponding site of pACYC184 or pBR322, respectively. The recombinant plasmids were introduced into JE7304 by transformation, and tetracycline-resistant transformants were selected at 42°C on salt-free 1/2L agar plates. Four pACYC184 derivatives and four pBR322 derivatives that complemented the growth defect of JE7304 were isolated. All but one pACYC184 derivative (pHR52) corrected the processing defect as well, and they contained common restriction fragments (a 2.9-kb *Eco*RI fragment for the pACYC184 derivatives and 9.7- and 1.4-kb *Pst*I fragments for the pBR322 derivatives) with or without other distinctive fragments. (Plasmid pHR52 contained a single 2.3-kb *Eco*RI fragment.) One of the pACYC184 derivatives, pHR53, and one of the pBR322 derivatives, pHR61, containing the common fragments only were chosen for further experiments. Restriction maps of these plasmids indicated that the cloned fragments were derived from the same chromosomal region and that the 2.6-kb fragment between the *Eco*RI$_1$ and *Pst*I$_3$ sites contained the gene that can complement the *prc* defect (Fig. 1). Two *Pst*I fragments cloned in pHR61 were contained in all of the pBR322 derivatives, suggesting that the *Pst*I$_2$ site was within the *prc* gene. This was confirmed by inserting a DNA fragment containing the *kan* gene from pUC-4K into the *Pst*I$_2$ site. The resultant plasmid, pHR80, did not correct the processing defect of JE7304. The *Bam*HI$_2$ site was similarly shown to be within *prc* by inserting a DNA fragment containing the *neo* gene from pHR12 (pHR75 [Fig. 1] and pHR74 with the opposite *neo* orientation). All of these insertions also abolished the ability to complement the growth defect of JE7304, indicating that *prc* is responsible for the thermosensitive growth.

When *neo* was inserted into the *Hin*dIII site (pHR77 [Fig. 1] and pHR76 with the opposite *neo* orientation) or the 0.9-kb *Hin*dIII-*Eco*RI$_2$ fragment was replaced with *neo* (pHR87), the thermosensitive growth was complemented but the processing defect was only partially corrected. Inverting the 3.5-kb *Eco*RI fragment of pHR87, which contains the 2.0-kb *Eco*RI$_1$-*Hin*dIII chromosomal fragment and *neo*, restored the ability to completely correct the processing defect (pHR88). The essential region for complementing the processing defect was contained in the *Eco*RI$_1$-*Hin*dIII fragment. In pHR88, the *Eco*RI$_1$ site was placed to the proximal side within the *cat* gene of pACYC184. Transcription from the *cat* promoter may have affected the expression of the *prc* gene. We infer that the *prc* gene is transcribed from left to right and to a little beyond the *Hin*dIII site in Fig. 1 and that truncation at the *Hin*dIII site in pHR87 partially impairs the ability to complement the processing defect, which may be suppressed by elevated expression by the readthrough transcription from the *cat* promoter in pHR88. The sequencing analysis actually located the *Hin*dIII site very close to the 3′ end of the *prc* coding region.

Nucleotide sequence of the *prc* gene. We determined the nucleotide sequence of a 3,178-bp chromosomal fragment, the *Eco*RI fragment cloned in pHR53 and 218 bp in the contiguous 9.5-kb *Pst*I-*Eco*RI fragment in pHR61 (Fig. 1); the entire sequence is shown in Fig. 2. Only one major open reading frame (ORF) of significant length was identified. The ORF started with an AUG initiation codon either at nucleotides 287 through 290 or at nucleotides 293 through 296 and ended with a UAA termination codon at nucleotides 2333 through 2336. A *Pst*I site occurred in the middle, and *Bam*HI and *Hin*dIII sites were in the distal region, as inferred above. The ORF is transcribed counterclockwise on the chromosome. It could code for a polypeptide of 682 or 680 amino acids with a predicted molecular weight of 76,677 or 76,432. We do not know at present which of the two AUG codons is used as the initiator; each AUG codon is preceded by a potential ribosome binding site (56, 62). Immediately downstream of the termination codon is a transcriptional terminator including an inverted repeat and a poly(T) track (49). A search for sequences with homology to the consensus promoter sequence (21) identified two promoterlike sequences, one about 150 bp upstream of the *Eco*RI$_1$ site and another just downstream of the *Eco*RI$_1$ site. The latter was not sufficient to express *prc* as a single copy: when we recloned the 2.9-kb *Eco*RI$_1$-*Eco*RI$_2$ fragment containing the latter promoterlike sequence into a mini-F plasmid pMF3 (HR143 and pHR144; Fig. 1), only pHR143, which has the *Eco*RI$_1$ site joined to the promoter of the *cadA* gene in the vector (originated from the staphylococcal *blaZ* fragment [38, 45,

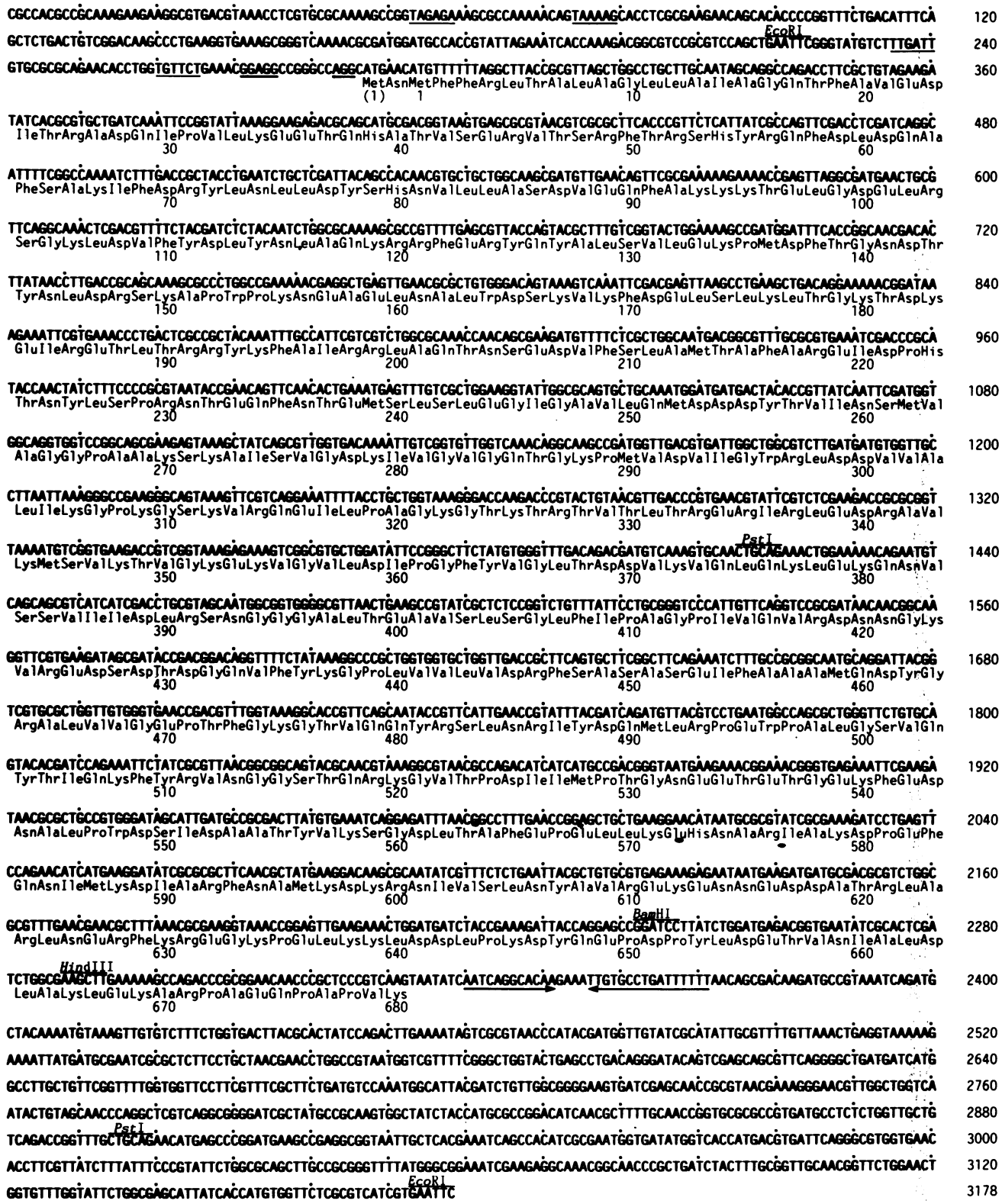

FIG. 2. Nucleotide sequence of the *prc* gene and the deduced amino acid sequence of the gene product. The antisense strand is presented. Sequences with homology to the consensus promoter sequence are underscored with thin lines, and potential ribosome-binding sites underlined with heavy lines. A transcription terminator is indicated by arrows. It is not known whether translation starts at nucleotide 287 or at nucleotide 293. The amino acid residues are numbered starting with Met for the latter ATG codon.

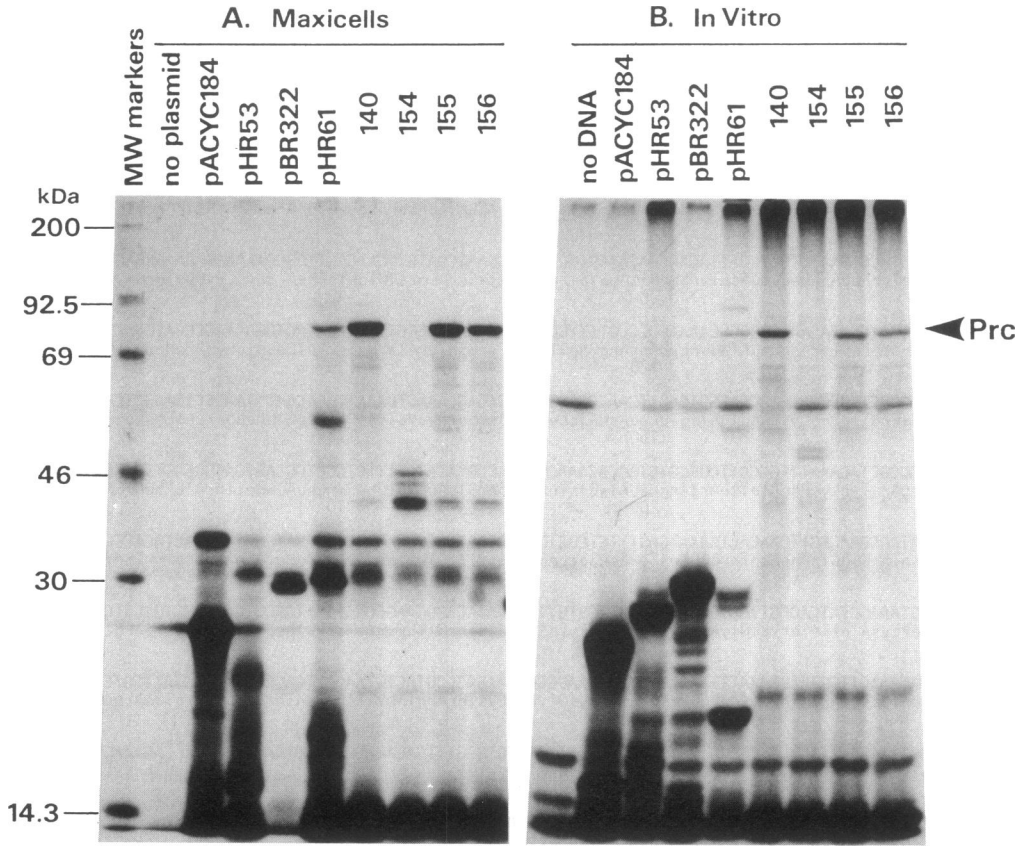

FIG. 3. Identification of the *prc* gene product. Proteins were labeled with [^{35}S]methionine in maxicells (A) and in an in vitro system (B) under the direction of the plasmids indicated above the lanes and analyzed by SDS-gel electrophoresis. An arrowhead points to the position of the *prc* product (Prc). We cannot explain the discrepancies, especially in lower parts of lanes, between the protein band patterns from the maxicell and in vitro systems with the same plasmid.

46]), corrected the thermosensitive growth of JE7304, and pHR144, which has the insert in the opposite orientation, did not. The product, as deduced from the identified ORF, was considerably hydrophilic and contained two hydrophobic regions near the N terminus and toward the middle of the polypeptide. It exhibited no significant homology to any proteases, peptidases, or other proteins in the EMBL protein data base (SWISS-PROT, release 9.0).

Identification of the *prc* product. To identify the *prc* gene product, proteins were synthesized in maxicells and in an in vitro-coupled transcription-translation system in the presence of [^{35}S]methionine under the direction of pHR53 and pHR61 and analyzed by SDS-gel electrophoresis. Protein bands migrating at the positions of about 80 and 55 kDa were detected for pHR61 but not for pHR53 in maxicells (Fig. 3A) and in an in vitro system (Fig. 3B). In both of these plasmids *prc* was placed next to the promoter of the drug resistance genes of the vectors (*cat* in pHR53 and *bla* in pHR61) in the opposite orientation. Moreover, the 2.9-kb *Eco*RI fragment in pHR53 did not seem to contain a strong promoter. Therefore we recloned the 2.6-kb *Eco*RI$_1$-*Pst*I$_3$ fragment containing *prc* into pBR322 to construct pHR140 (Fig. 1), in which the *prc* orientation matched the direction of transcription from the promoter of the *tet* repressor gene (*tetR*p, originated from the ancestral plasmid [2]). A remarkable overproduction of the 80-kDa protein was observed with pHR140. Next, we inserted a synthetic translation terminator (39), instead of the *kan* and *neo* fragments used in the earlier experiments, into restriction sites within the *prc* ORF (Fig. 1). Insertion of the terminator into the *Pst*I$_2$ site within *prc* (pHR154) eliminated the 80-kDa protein. When the terminator was inserted into the *Bam*HI$_2$ site (pHR155) and into the *Hin*dIII site (pHR156), which are both close to the distal end of the *prc* ORF (Fig. 2), products of almost a normal size were observed. These results indicate that the 80-kDa protein is the *prc* product. No difference in electrophoretic mobility was observed between the product synthesized in vitro and that synthesized in maxicells, when tested in SDS gels of several different polyacrylamide concentrations, and no processing seems to occur in the *prc* product.

Localization of the *prc* product in a cell. The cellular location of the *prc* product was investigated in maxicells labeled with [^{35}S]methionine under the direction of pHR164 (carrying *prc*, *bla*, and *tet*) or of pHR182 (carrying *prc*, *bla*, *tet*, and *cat*). When the maxicells of CSR603(pHR164) were disintegrated by sonication and fractionated by centrifugation, the *prc* product was observed in both the soluble fraction and the insoluble membrane fraction (Fig. 4A). The membrane fraction was further treated with 1% sodium *N*-lauroylsarcosinate (Sarkosyl) (16), and the *prc* product was found in the solubilized cytoplasmic membrane fraction. The *bla* product (β-lactamase) and the *tet* product (Tet protein) were recovered in the soluble fraction and in the cytoplasmic membrane fraction, respectively, which is con-

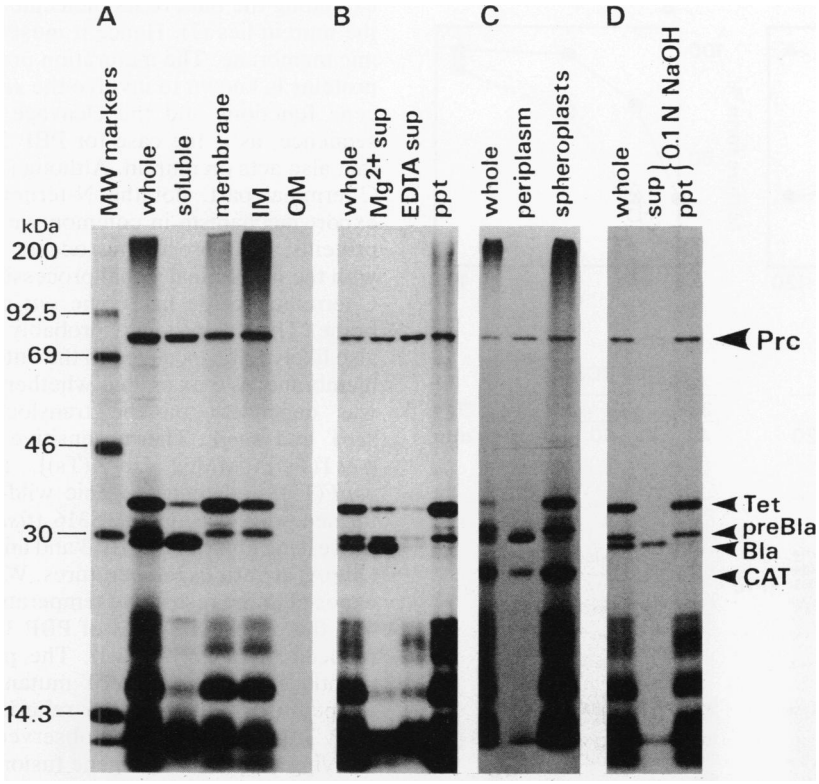

FIG. 4. Localization of the *prc* gene product in maxicells. Maxicells carrying pHR164 (A, B, and D) or pHR182 (C) were labeled with [^{35}S]methionine and fractionated as described below, and the whole maxicells (whole) and the fractions were analyzed by SDS-gel electrophoresis. (A) The maxicells were sonicated in a phosphate buffer and separated by centrifugation into the supernatant (soluble) and the precipitate (membrane), and the latter was further separated by centrifugation, after treatment with 1% Sarkosyl, into the supernatant (IM) and the precipitate (OM). (B) The maxicells were sonicated and centrifuged in the presence of 5 mM $MgCl_2$, and the supernatant (Mg^{2+} sup) was removed. The precipitate was resuspended in the presence of 10 mM EDTA and separated by centrifugation into the supernatant (EDTA sup) and the precipitate (ppt). (C) The maxicells were incubated with lysozyme in a hypertonic buffer and separated by centrifugation into the supernatant (periplasm) and the precipitate (spheroplasts). (D) The maxicells were treated with 0.1 N NaOH and separated by centrifugation into the supernatant (sup) and the precipitate (ppt). Equivalent amounts of samples were electrophoresed in each panel, except for the whole maxicells in B and C, which were run in smaller amounts. A large arrowhead points to the position of the *prc* product (Prc). Small arrowheads indicate the products of drug resistance genes: Tet protein, β-lactamase (Bla) and its precursor form (preBla), and the *cat* gene product (CAT).

sistent with their known location (Fig. 4A). The precursor form of β-lactamase (pre-β-lactamase) was recovered in the cytoplasmic membrane fraction. When the maxicells harboring pHR182 were converted to spheroplasts and centrifuged, the *prc* product was fractionated both to the supernatant periplasmic fraction containing β-lactamase and to the pelleted spheroplasts containing Tet, pre-β-lactamase, and the cytoplasmic *cat* gene product (Fig. 4C). The fraction of broken spheroplasts in this treatment seems to be small, because only a small amount of the *cat* product was detected in the supernatant. These results may indicate that the *prc* product is distributed in both the periplasm and the cytoplasmic membrane. However, it does not appear that any major processing occurs in the *prc* product. Some proteins are known to show Mg^{2+}-dependent binding to the cytoplasmic membrane (27, 29). The maxicells were sonicated and centrifuged in the presence of 5 mM $MgCl_2$, and then the pelleted membrane fraction was washed with a buffer containing 10 mM EDTA and centrifuged again. The *prc* product did not show a clear Mg^{2+}-dependent fractionation pattern (Fig. 4B). When the maxicells were fractionated by the method of Russel and Model (52), which consists of treatment with 0.1 N NaOH followed by centrifugation, the *prc* product was pelleted together with Tet protein and pre-β-lactamase and β-lactamase was left in the supernatant (Fig. 4D). It seems probable that the *prc* product is associated with the periplasmic side of the cytoplasmic membrane and that it is partially released into soluble fractions during fractionation procedures. It should be noted that overproduction of the product and the artificial maxicell system may have affected the localization.

Overproduction of the *prc* product. Plasmid pHR88, in which the *prc* gene truncated at the *Hin*dIII site was placed under the *cat* promoter, allowed the complete processing of PBP 3 in JE7304, but it did not complement the thermosensitive growth. In fact, JE7304 harboring pHR88 did not grow on a salt-free 1/2L agar plate even at 30°C (Fig. 1). We could not obtain a plasmid with the 2.9-kb *Eco*RI fragment of pHR53 in the inverted orientation. We supposed that hyperexpression of the *prc* function may be deleterious to cell growth, and we constructed plasmid pLPRC21 (Fig. 1), in which the *prc* gene was placed under the control of the λ p_L promoter and the thermosensitive *c*I857 repressor. When the culture temperature was shifted up to 42°C, a strain harboring this plasmid stopped growing in 30 min (Fig. 5A), and production of a large amount of the *prc* product was induced

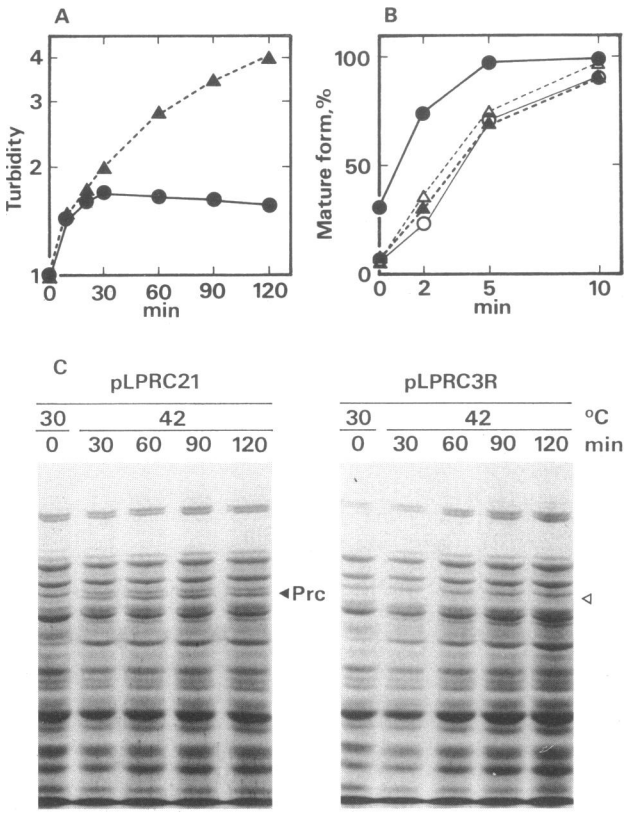

FIG. 5. Overproduction of the *prc* gene product and its effects. (A) Growth curves of DH5(pLPRC21) (●) and DH5(pLPRC3R) (▲). Exponential cultures in BLGT medium containing ampicillin were transferred from 30 to 42°C at time zero, and the turbidity relative to the zero-time value (about 45 Klett units) is plotted. (B) Effect of the overproduction on the processing of PBP 3. DH5(pLPRC21) (○, ●) and DH5(pLPRC3R) (△, ▲) were grown in minimal medium and subjected to pulse-chase experiments for the analysis of processing kinetics at 30°C (○, △) or after 30 min of incubation at 42°C (●, ▲). The percentage of the mature form was plotted against the time of chase. (C) SDS-gel electrophoresis of total cellular proteins of R594(pLPRC21) and R594(pLPRC3R). Exponential cultures in L broth containing ampicillin were transferred from 30 to 42°C at time zero, and aliquots withdrawn at indicated times were analyzed by SDS–8% polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue. The *prc* product (Prc) was induced in R594(pLPRC21) (closed arrowhead) but not in R594(pLPRC3R) (open arrowhead).

as shown in the Coomassie brilliant blue-stained SDS gel (Fig. 5C). Under these conditions, cells were subjected to a pulse-chase experiment and analyzed for the processing kinetics; the processing rate of PBP 3 was found to be much increased (Fig. 5B). With plasmid pLPRC3R, which had the *prc* gene in the orientation opposite to that in pLPRC21, neither growth inhibition, overproduction of the *prc* product, nor a processing rate increase was observed (Fig. 5). These results indicate that the *prc* hyperexpression is deleterious to cell growth and suggest that the *prc* product is a processing enzyme, although we cannot completely eliminate other possibilities (e.g., it may be an activator of the processing enzyme).

Accumulation of the precursor form of PBP 3 in mutants defective in general protein transport. PBP 3 participates in septal murein synthesis and appears to be an ectoprotein, extending the bulk of its molecule into the periplasm where the murein lies (7). Hence it must pass across the cytoplasmic membrane. The maturation process of extracytoplasmic proteins is known to involve the *secY* (26, 55) and *secA* (47) gene functions and the cleavage of an N-terminal signal sequence, as is the case for PBP 5 (23, 50), an ectoprotein that also acts on murein. Although PBP 3 is processed in the C-terminal part, not the N-terminal part, it may use the export mechanism in common with other extracytoplasmic proteins. The processing occurs rather slowly compared with the N-terminal signal processing (22), possibly after the C-terminal region has come out of the cytoplasmic membrane. The *prc* product, probably a processing enzyme, is also likely to be localized to the outer side of the cytoplasmic membrane. We examined whether the processing of PBP 3 was dependent on the translocation process involving *secY* and *secA*. Thermosensitive secretion mutants IQ85 [*secY*(Ts)], MM52 [*secA*(Ts)], and MM113 [*secA*(Am) *supF*(Ts)] and their isogenic wild-type strains were transformed with plasmid pMS316 (*ftsI*$^+$) and analyzed for the processing kinetics of PBP 3 and an outer membrane protein, OmpA, at various temperatures. When the mutant cells were exposed to the restrictive temperatures (37 and 42°C) for 2 to 4 h, the processing rates of PBP 3 and OmpA were greatly reduced (data not shown). The processing of PBP 3 was slightly slower in the *secY* mutant even at the permissive temperature (30°C). Similar retardation in the processing of PBP 3 and OmpA was observed when a strain MM18 carrying the *malE-lacZ* gene fusion (23, 25) was induced by maltose. These results indicate that PBP 3, the *prc* product, or both share the translocation machinery with other extracytoplasmic proteins.

Mapping of *prc*. Plasmid pHR53 was introduced and multiplied at 30°C in a *polA*(Ts) HfrP4X strain, JE7914, that could support the replication of this pACYC184 derivative at 30°C but not at the restrictive 42°C (11). Then the transformants were grown at 42°C in the presence of tetracycline, and a stable drug-resistant clone was obtained in which the plasmid should be integrated into the chromosome by homologous recombination around *prc*. An Hfr cross with a multiply auxotrophic *polA*(Ts) *str* strain, JE7915, as the recipient was conducted at 42°C with selection for Tet^r Str^r. Examination of the recombinants for the auxotrophic and sugar-fermenting phenotypes suggested that the tetracycline resistance gene (and thus the integrated *prc*$^+$ plasmid) was located around *his* at 44 min on the chromosome map.

Next, we carried out a Southern hybridization experiment, in which *Not*I fragments of the whole chromosome of W3110, separated by pulsed-field gel electrophoresis (59, 68), were probed with the radiolabeled 2.9-kb *Eco*RI fragment cloned in pHR53 (data not shown). Positive hybridization was obtained with a fragment derived from a region around 40 min (Fig. 6A), which was consistent with the result of the Hfr cross experiment. Comparison with the physical map of *E. coli* chromosome (30) of this region showed that the restriction patterns of the cloned fragments of pHR53 and pHR61 (Fig. 1) fit the pattern around the map coordinate 1,933 kb or 40.4 min (Fig. 6A).

This map position of the cloned gene was confirmed to be the same as that of the thermosensitivity mutation in JE7304 by P1 transduction experiments with strains JE7304 (*prc*), FB191 (*eda-51*::Tn*10*), K27 (*fadD88*), and their derivatives; some representative experiments are summarized in Fig. 6B. The *prc* allele was selected and scored on a salt-free 1/2L agar plate at 42°C. The results indicate that *prc* is located

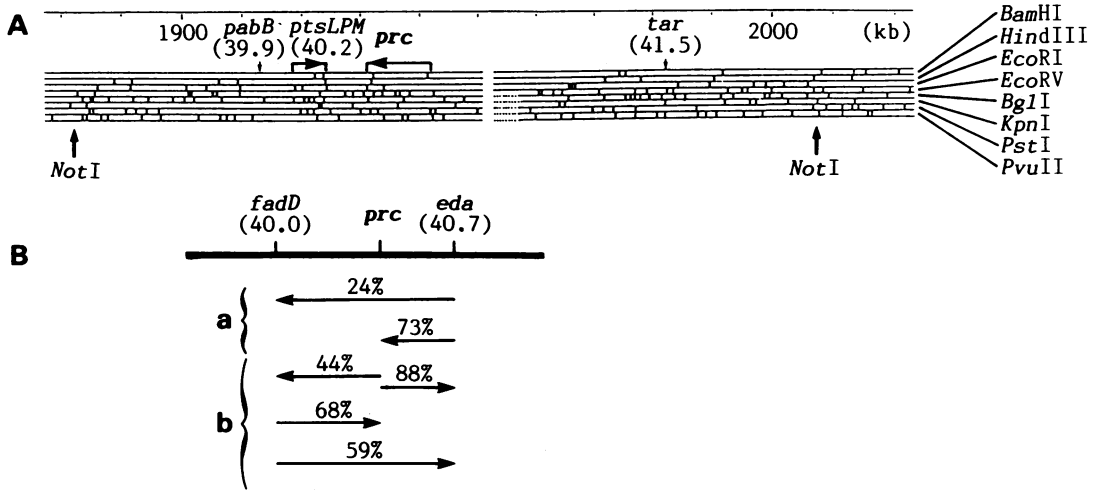

FIG. 6. Mapping of the *prc* gene. (A) Alignment of the restriction map of the *prc* gene (Fig. 1) to the physical map of the *E. coli* chromosome (30). The *ptsLPM* operon (14) was also localized, although we found a few disagreements in the restriction patterns. The regions of the matching patterns are indicated by square brackets, and the locations and orientations of the genes are indicated by arrowheads on the brackets. Large arrows indicate approximate end points of the *Not*I fragment to which the 2.9-kb *Eco*RI fragment containing *prc* hybridized. The numbers in parentheses are the map positions (in minutes) according to the latest version of the *E. coli* genetic map (1); these positions are a little different from those indicated in the physical map of Kohara et al. (30). (B) P1 transductional analysis of the map position of the *prc* gene. Arrows are drawn from selected markers to unselected markers, and the cotransduction frequencies are given over the arrows. The donor and recipient strains are JE7917 and JE7919 in part a and JE7918 and JE7921 in part b. In part a the donor contained a Tn*10* insertion, which would affect the cotransduction frequencies.

between *fadD* and *eda*, which are positioned at 40.0 and 40.7 min, respectively, on the genetic map (1).

Properties of the *prc* mutant. During cloning and mapping procedures we noticed that JE7304 was Mal$^+$ and yet phage λ resistant. Further analyses showed that it was normal in adsorption of λ particles but defective in DNA injection and was Man$^-$, indicating that it carried a mutation in the *ptsLPM* operon (also called *pel* and *manXYZ*) (1, 12, 15), which maps at 40.2 min between *fadD* and *eda*. Comparison of the restriction map of this operon (14) with the physical map of the *E. coli* chromosome (30) located the operon at the map coordinate 1,922 kb (Fig. 6A), 11 kb from *prc*. However, transductional analysis showed that these two mutations in JE7304 were linked at a frequency of 100%. We suspected that JE7304 had a deletion covering these genes in whole or in part. This was more likely than two independent mutations, considering that JE7304 had spontaneously appeared from JE5606, which was *prc*$^+$ *ptsLPM*$^+$. We carried out a Southern hybridization experiment, in which the radiolabeled 2.9-kb *Eco*RI fragment containing the *prc* gene was used to probe chromosomal DNA digested by a variety of restriction enzymes. No DNA hybridizable with the *prc*$^+$ fragment was detected in the *prc* mutant, although the amounts of chromosomal DNA applied were the same as those in the isogenic wild type, which revealed the expected hybridization patterns (Fig. 7). It was concluded that the *prc* mutation found in JE7304 is a deletion that covers the entire *prc* gene. We do not know the endpoints of the deletion. It probably extends counterclockwise in the chromosome to or beyond the *ptsLPM* region but not to *fadD* or *eda*.

The *prc* mutation in JE7304 was transferred to a wild-type strain, W3110, by cotransduction with *eda* and was characterized in comparison with the isogenic wild type. The *prc* mutation in the otherwise wild-type genetic background caused an inability to form colonies at 42°C on a salt-free L-agar plate and a defect in the processing of PBP 3; these defects were corrected by *prc*$^+$-carrying plasmids. In salt (0.5% NaCl)-containing L media (liquid and agar solidified) that also contained 10 mM glucose, no growth defect was detectable at 30 and 42°C. In a salt-free liquid L medium, growth of the *prc* mutant continued after a shiftup to 42°C but stopped at a lower final cell density (Fig. 8A). Micro-

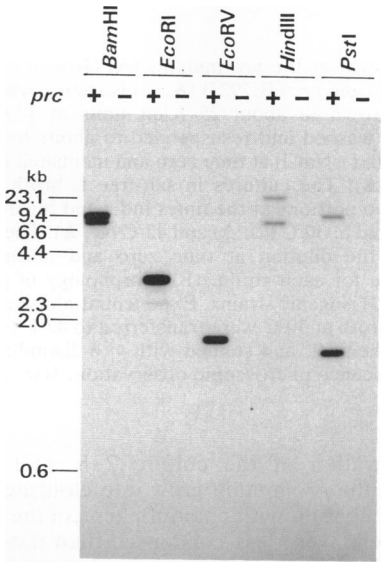

FIG. 7. Southern blot analysis of the chromosome of the *prc* mutant. Chromosomal DNA preparations from *prc*$^+$ (JE7924) and *prc* (JE7925) isogenic strains were digested with the restriction enzymes indicated above, electrophoresed on 0.8% agarose, transferred to nitrocellulose, and probed with ^{32}P-labeled 2.9-kb *Eco*RI fragments containing the *prc* gene.

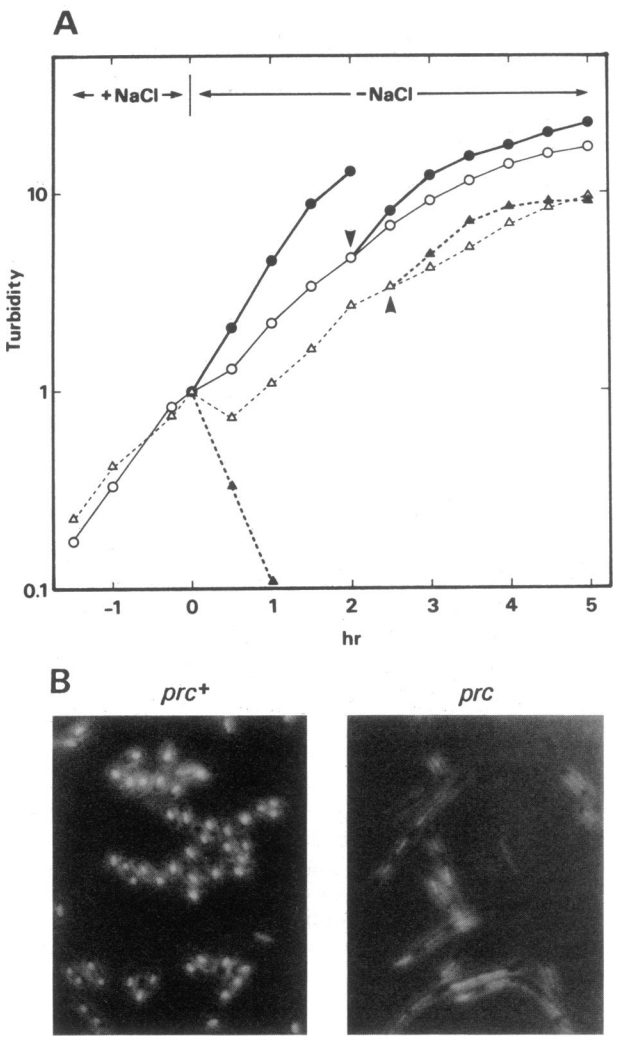

FIG. 8. Growth of the *prc* mutant. (A) Growth curves of *prc*$^+$ (JE7922) (●, ○) and *prc* (JE7923) (▲, △) isogenic strains. Exponential cultures grown to about 25 Klett units at 30°C in L broth (+NaCl) were washed and resuspended to about 10 Klett units in salt-free L broth (−NaCl) at time zero and incubated at 30°C (○, △) and 42°C (●, ▲). The cultures in salt-free L broth at 30°C were divided into two portions at the times indicated by arrowheads and further incubated at 30°C (○, △) and 42°C (●, ▲). The turbidity was corrected for the dilution at time zero and normalized to the zero-time value for each strain. (B) Morphology of *prc*$^+$ (JE7922) and *prc* (JE7923) isogenic strains. Exponential-phase cultures grown in salt-free L broth at 30°C were transferred to 42°C for 2 h, treated with chloramphenicol, and stained with 4′,6-diamidino-2-phenylindole for fluorescence microscopic observation. Bar, 10 μm.

scopic observation of the culture 2 h after the shiftup revealed that the *prc* mutant grew into elongated cells (Fig. 8B). After incubation with chloramphenicol the nucleoids of the mutant cells were less condensed than those of normal cells. When the *prc* mutant grown in salt-containing L broth was transferred to salt-free L broth and cultured at 30°C, the turbidity decreased transiently, indicating partial cell lysis (Fig. 8A). The simultaneous temperature shiftup with the transfer to salt-free broth (Fig. 8A; at time zero) was lethal, leading to complete cell lysis.

We examined the heat shock response of the *prc* mutant in high- and low-osmolar milieux. Cells of *prc* and *prc*$^+$ strains were suspended in 100 and 2 mM sodium phosphate buffers containing glucose, Mg^{2+}, and 18 amino acids (no methionine and cysteine) and pulse-labeled with [^{35}S]methionine at 30°C and after transfer to 44°C. At high osmolarity (in 100 mM phosphate) heat-inducible synthesis of heat shock proteins GroEL and DnaK was observed equally with the *prc* mutant and with the wild type, whereas at low osmolarity (in 2 mM phosphate) the mutant showed little or no induction for GroEL and partial induction for DnaK (Fig. 9). During the labeling period of 5 to 10 min after the shiftup, the overall rate of protein synthesis was not affected: comparable amounts of radioactivity were incorporated into trichloroacetic acid-insoluble materials in the mutant and wild-type cells. Thus, the *prc* mutant has a defect in the heat shock response. This defect was corrected by a mini-F plasmid, pHR145, carrying *prc*$^+$ (Fig. 9).

The *prc* mutation affected the ability of a cell to retain periplasmic proteins as well. When grown on an RNase test agar plate (51), the *prc* mutant formed a pink halo, indicating that RNase was significantly released into the culture medium (data not shown). The leakage of the enzyme was corrected by a plasmid, pHR53, carrying *prc*$^+$. Since the test agar contained 0.5% NaCl and the culture temperature was 30°C, the leakage was unlikely to be due to cell lysis; probably the periplasmic RNase I was released to form a halo. Quantitative assays of enzyme activities with respect to those released into the culture media and those retained by the cells revealed that about 11% of periplasmic alkaline phosphatase was lost from the *prc* cells into the medium. The losses of cytoplasmic β-galactosidase from the *prc* mutant cells and of either of these enzymes from the wild-type were 1 to 3%.

DISCUSSION

We cloned, mapped, and sequenced the *prc* gene, whose inactivation confers thermosensitive growth on a salt-free L-agar plate. The *prc* mutation, first found in JE7304 (20), turned out to be a large deletion that covers the entire coding region of the gene, indicating that the *prc* function is dispensable for growth at low temperatures and in high-osmolar milieux. Shiftdown of culture medium osmolarity caused partial lysis of the *prc* mutant cells even at 30°C, and the simultaneous temperature shiftup prevented the recovery from lysis. The *prc* function thus seemed to be involved in protection of a cell from thermal and osmotic stresses. The *prc* gene was originally identified as a gene involved in a unique processing reaction that cleaves the C-terminal 11 residues from the precursor form of PBP 3 (20, 42). Although the predicted amino acid sequence for the *prc* product showed no significant similarity to any other protease or peptidase, we suppose that the product is directly involved in proteolytic processing because its overproduction led to faster kinetics of PBP 3 processing. The *prc* product probably resides on the outer side of the cytoplasmic membrane, where it can process the C-terminal region of PBP 3 that protrudes into the periplasmic space (7). Loss of the *prc* function resulted in leakage of periplasmic proteins. In some of these respects *prc* is like *degP* (*htrA*). *degP* is also a conditionally dispensable gene that is required for bacterial survival against high-temperature stress but not at low temperatures (35, 64). At restrictive temperatures the insertion mutant eventually lyses. It encodes a proteolytic enzyme that functions in the periplasm (36, 64). Cavard et al.

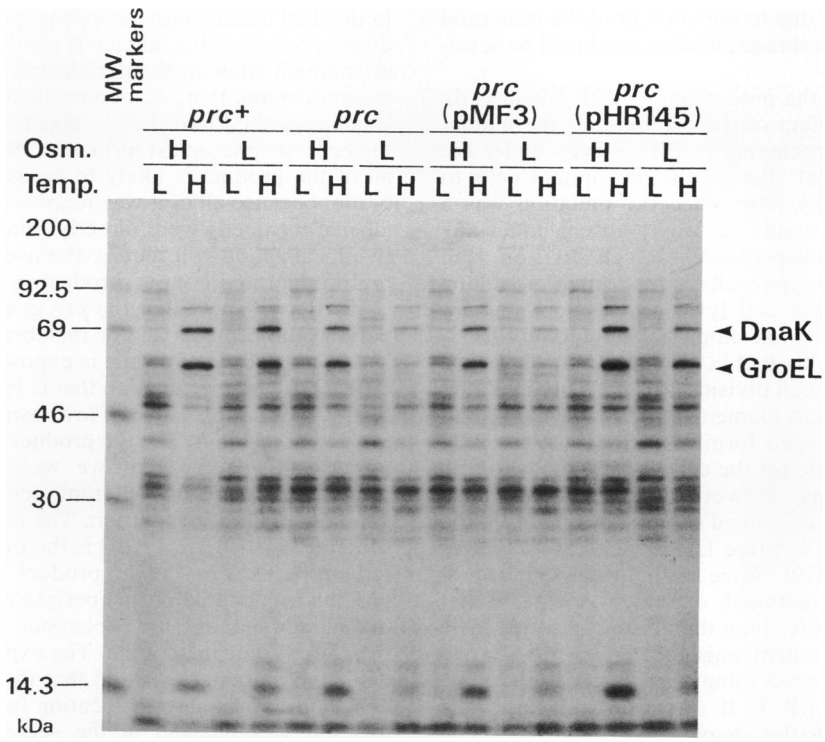

FIG. 9. Heat shock response of the *prc* mutant. Cells of JE7924 (*prc*$^+$), JE7925 (the isogenic *prc* mutant), and JE7925 carrying pMF3 and pHR145 (*prc*$^+$) were pulse-labeled at high (100 mM phosphate; Osm. H) and low (2 mM phosphate; Osm. L) osmolarities with [^{35}S]methionine for 5 min at 30°C (Temp. L) and for 5 min at 5 min after an upshift to 44°C (Temp. H). The labeled proteins were analyzed by SDS-gel electrophoresis. The positions of DnaK and GroEL proteins are indicated by arrowheads.

(10) reported that *degP* strains leaked cellular proteins and that the presence of a *pldA* mutation restored the nonleaky phenotype. We recently found that the leaky phenotype (but not the thermosensitivity) of the *prc* strains was also suppressed by a *pldA* mutation (19). However, *prc* is obviously distinct from *degP*, as revealed by mapping and sequencing analyses.

Because a variety of defects caused by the *prc* deletion mutation could be corrected by plasmids carrying *prc*$^+$ as the only ORF of a significant size, it seemed most probable that they were due to the *prc* null phenotype, although the deletion was found to cover a large chromosomal region around *prc*. An alternative possibility was that the defects were due to the loss of another nearby gene(s) by this deletion and corrected by the cloned *prc* gene. Very recently Kornitzer et al. reported a new heat shock protein gene, *htpX*, just downstream of *prc* (31). However, they found that cells with this gene insertionally disrupted had no apparent phenotype. Furthermore, when we replaced the *Pst*I$_2$-*Bam*HI$_2$ fragment within the cloned *prc* ORF with the *neo* gene fragment and crossed the construct back into the chromosome, the resultant strain showed thermosensitive growth on a salt-free L-agar plate and a defect in the processing of PBP 3, just as the original *prc* mutant did (19). Detailed characterization of this strain will be required for the final conclusion. The possibility was also excluded that *prc* could be deleted only with the concomitant deletion of a nearby gene(s).

The thermosensitivity of the *prc* mutant at low osmolarity might be due to its defect in the heat induction of heat shock proteins. The defect was pronounced in the GroEL protein and less severe in the DnaK protein. The GroEL protein is considered to play a key role in supporting normal growth in association with GroES protein: a mutant (Δ*rpoH*) that lacks a heat shock σ factor, σ^{32}, which is required for the expression of heat shock proteins, grows only at 20°C or lower temperatures (76), and this defect was suppressed by high-level production of GroE proteins (33). When the Δ*rpoH* mutant was exposed to restrictive temperatures, inhibition of cell division was observed, followed by cell lysis (76). The mechanism by which the *prc* mutation alters a heat shock response is not understood at present. The *prc* product might be involved in the modulation of the σ^{32} function, but the involvement, if any, should be indirect, considering the cellular localization of these proteins. A decrease in σ^{32} would result in the uniformly reduced induction of all heat shock proteins, as shown in nonsense (73) and deletion (76) mutants of *rpoH*, and could not explain the differential induction of GroEL and DnaK proteins. Whether the *prc* mutation could affect the level of σ^{32} and the transcription of heat shock genes is now under investigation. It is also yet to be examined whether the *prc* expression itself is under heat shock control, although no sequence has been found that resembles the consensus for promoters regulated by σ^{32} (44) or by another heat shock σ factor, σ^{E} (13), in the sequenced region upstream of *prc*.

Leakage of periplasmic proteins has been reported in several mutations, including *E. coli lpo* (67), *Salmonella typhimurium lkyD* (71), and *E. coli lkyB* (*tolB*) (34), all of which are associated with defects in outer membrane components. *lpo* causes complete loss of murein lipoprotein, *lkyD* decreases the level of the murein-bound form of lipoprotein, and *lkyB* affects the expression of outer membrane proteins OmpC, OmpF, and LamB. Leaky phenotype of the

prc mutant also might be due to impairment of the structural integrity of the outer membrane, which could lead to sensitivity to osmotic stress.

It seems unlikely that the processing of PBP 3 is directly related to the growth defect of the *prc* mutant: the mutant showed no detectable processing of PBP 3 even under the permissive conditions that allowed the *prc* mutant cells to grow and divide normally (20); since the mutation was a deletion, the processing would be totally absent under any conditions. PBP 3 is indispensable for cell division, and inactivation of PBP 3 always results in the formation of long filaments (60, 61, 66); the cell lysis observed in the *prc* mutant might not be explained simply by a defect in PBP 3. When the mutant was cultivated in salt-free L broth at 30°C and then shifted to 42°C, cell division was partially inhibited and the cells grew into short filaments (Fig. 8B), which could suggest that the unprocessed form of PBP 3 was not fully active and was responsible for the cell division defect under the restrictive conditions. However, none of extragenic suppressor mutations we examined so far, which enabled the *prc* mutant to grow on a salt-free L-agar plate at 42°C, was mapped in the *ftsI* gene (19). Since the unprocessed form of PBP 3 was apparently functional, one might speculate that the unprocessed form rather than the processed form was a functional cellular component engaged in cell division in vivo and that so-called processing was degradation to restrict the function of PBP 3. If this were the case, the C-terminal 11 residues to be cleaved would be of crucial importance for cell division. However, a truncated PBP 3 in which the C-terminal 12 residues were missing was shown to be functional, complementing an *ftsI*(Ts) mutation (20).

The *prc* gene product was identified as a 76-kDa protein, unexpectedly large compared with the N-terminal processing enzymes Lep (36 kDa) and Lsp (18 kDa) (72). When this protein lacked the C-terminal 13 residues, the ability to complement the processing defect was impaired as shown for pHR76, pHR77, and pHR87. This C-terminal region might have an important, albeit not essential, role in the function or stability of the *prc* product. DNA sequence analysis revealed two possible translational starts, 6 bases apart from each other. To clarify which one is the actual initiator(s), we need to purify the product and determine its N-terminal amino acid sequence. Purification will also facilitate enzymological characterization. For the purpose of purification, we can now use a recombinant plasmid (pLPRC26) to overproduce the product and an in vitro complementation system (20) to assay the processing activity.

In the in vitro complementation system, the precursor form of PBP 3 present in the membrane fraction of a *prc* mutant was converted to the mature form by incubation in the presence of Triton X-100 with the membrane fraction of a prc^+ strain; i.e., the complementation activity could be recovered in the membrane. This was consistent with the observation in the present study that the *prc* gene product was associated with the cytoplasmic membrane. The predicted amino acid sequence contained two hydrophobic domains in the N-terminal and middle part, one or both of which could act as anchors to the membrane. Nevertheless, the *prc* product was also detected in the periplasmic fraction when maxicells containing the labeled product were fractionated. There was no indication of two molecular species in the *prc* product, except for the two AUG codons; both AUG codons might be used as start codons initiating two molecules, but the two molecules would differ only in the N-terminal Met-Asn residues and would be unlikely to result in the dual localization. It appears possible that the overproduced product in the maxicells nearly saturated the available attachment sites on the periplasmic side of the cytoplasmic membrane and that, as a consequence, some of the product was easily shed into the periplasm after sonic disruption of the cells or spheroplast formation. Without these procedures all of the product is likely to be associated with the membrane, because all of it was recovered in the pellet when the labeled maxicells were directly treated with NaOH. Definitive localization will require the use of antibody against the *prc* product in a nonoverproducing cell.

We thus suspect that the *prc* gene product acts on some periplasmic proteins and/or membrane proteins in which at least a part of the molecule is exposed to the periplasm. The target(s) of the *prc* function that is vital for cell physiology at high temperatures and in low osmolar milieux would be found among them. The *prc* product is probably a proteolytic processing enzyme, but we were unable to detect any protein band showing different electrophoretic mobility depending on the *prc* mutation. The target(s) might be a minor protein, as is PBP 3. PBP 3 is the only protein so far known to be processed by the *prc* product. Both PBP 3 and the *prc* product are located on the periplasmic side of the cytoplasmic membrane, and the processing probably occurs outside the cytoplasmic membrane. The experiments with secretion-defective mutants indicated that the processing was dependent on the protein-translocating function. When the translocation was blocked in the secretion mutants, the cells became abnormally elongated (47), possibly because PBP 3 uses the *sec* translocation machinery for its proper localization. The translocation of PBP 3 in this sense may be of primary importance to its function of septum formation. (A defect in translocation of other membrane-associated cell division proteins, such as the *ftsQ* gene product [8, 63], might also be responsible for the division defect.) Near the N terminus, PBP 3 (40, 43) and the *prc* product have a distinctly hydrophobic domain preceded by a segment containing one or several basic residues, which is characteristic of signal sequences (72). These N-terminal regions possibly serve as a signal for interaction with the translocation machinery, although they are not necessarily cleaved. As for PBP 3, it was shown that the molecule whose N-terminal 40 residues were artificially removed failed to translocate and accumulated in the cytoplasm (3).

The 2.9-kb *Eco*RI chromosomal insert in plasmid pHR53 contained the entire ORF for *prc*. It was connected in an orientation opposite to that of the *cat* promoter of the vector, and yet pHR53 was able to completely complement the *prc* mutation. Analysis of the DNA sequence identified a promoterlike sequence just downstream of the $EcoRI_1$ site. However, in maxicell and in vitro systems pHR53 did not direct the synthesis of the *prc* product to a detectable level, possibly owing to the weakness of this promoter or to the transcription from the *cat* promoter counteracting the transcription of *prc*. In contrast, the product was detectable with pHR61, although the *bla* promoter could be counteracting *prc* transcription. Transcription from somewhere in the upstream $PstI_1$-$EcoRI_1$ fragment might proceed beyond the $EcoRI_1$ site and contribute to the higher *prc* expression. There was another promoterlike sequence in the sequenced region upstream of the $EcoRI_1$ site. When recloned into a mini-F vector, the $EcoRI_1$-$EcoRI_2$ fragment was unable to complement the *prc* mutation unless placed under the *cadA* promoter, suggesting that the promoterlike sequence downstream of the $EcoRI_1$ site did not have enough promoter activity as a singly copy. Plasmid pHR61 probably expresses

prc by the cognate promoter(s), and the band of the product was much less intense than that of pHR140, in which *prc* is placed under the *tetR* promoter. We suppose that the *prc* gene is weakly expressed in vivo. Codon usage for this gene is also indicative of weak expression. When it was forced to be excessively expressed by extrinsic strong promoters, the λ p_L promoter in pLPRC21 and the *cat* promoter in pHR88, it showed a detrimental effect on the host cell. This effect and the pleiotropy of the *prc* mutation suggest that the *prc* function is important physiologically.

The phenotype of the *prc* mutant indicated that this gene is involved in protection of the bacterium from thermal and osmotic stresses, although the target(s) of such vital function is yet to be identified. The candidates for the target(s) include the products of a gene cloned in pHR52, which allowed the thermoresistant growth of JE7304 without correcting its processing defect and possibly carried a multicopy suppressor of *prc* (see Results), and of an extragenic suppressor(s) of *prc*. Thermoresistant revertants appeared from the *prc* mutant at a relatively high frequency. Since the *prc* mutation was proved to be a deletion, all of the revertants should contain an extragenic suppressor mutation(s). They were all defective in the processing of PBP 3. One of the suppressor mutations, when transferred into the prc^+ background, caused thermosensitive growth on a salt-free L-agar plate (19), suggesting its important physiological role. Characterization of these suppressors is now in progress.

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