Interaction of the *Escherichia coli* Lipoprotein NlpI with Periplasmic Prc (Tsp) Protease

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Received October 3, 2003; accepted November 7, 2003

Escherichia coli spr (suppressor of prc) mutants and nlpI mutants show thermosensitive growth. The thermosensitivity of the spr mutants was suppressed by the nlpImutations. Expression of the fusion genes encoding hexa-histidine-tagged NlpI (NlpI-His) and purification of the tagged NlpI showed that NlpI-His bound with Prc protease and IbpB chaperone. NlpI-His with the amino acid substitution of G103D did not bind with either of these proteins, while NlpI-His variants (NlpI-284-His, NlpI-Q283-His, and NlpI-G282-His) lacking 10 to 12 residues from the carboxy terminus bound with both proteins. The tagged NlpI lacking 11 amino acid residues from the carboxy terminus was processed by Prc, but that lacking 12 residues was not. The thermosensitivity of the nlpI mutant was corrected by the production of the former NlpI variant, but not by production of the latter. Expression of the truncated NlpI that lacked 10 or 11 residues from the carboxy terminus corrected the thermosensitivity of the prc nlpI double mutant, while expression of the full-length NlpI did not. Thus, it was suggested that NlpI was activated by Prc protease processing.

Key words: nlpI, spr, prc, lipoprotein, peptidoglycan.

The product of the *Escherichia coli prc* gene is a protease located in the periplasm (1). This protease is responsible for processing the carboxy (C)-terminal region of penicillin-binding protein (PBP) 3 (2, 3), which is known to be a lipoprotein (4) having peptidoglycan-synthesizing activity (5). It is also known as Tsp protease because it degrades incomplete polypeptide chains with a particular C-terminal tag specified by 10Sa RNA (6).

The *prc* mutant does not grow at 42°C (1). This thermosensitivity is suppressed by a mutation of the *spr* gene (7) that encodes a putative lipoprotein having a signal amino acid sequence for modification by lipid (8, 9). Mutants of *spr* alone also show thermosensitive growth, and this thermosensitivity is suppressed by overproduction of PBP 7 (7), which is reported to be a *meso*-diaminopimelate-Dalanine DD-endopeptidase (10). Spr is supposed to have peptidoglycan-hydrolyzing peptidase activity because its amino acid sequence shows significant homology to γ -Dglutamyl-L-diamino acid endopeptidase II of *Bacillus sphaericus* (11, 12).

We here isolated thermoresistant revertants from the spr strain and found that a mutation in nlpI, the gene for another lipoprotein (13), suppressed the thermosensitivity of the spr mutant. We detected interaction of the hexa-histidine-tagged NlpI with other proteins including the Prc (Tsp) protease.

MATERIALS AND METHODS

Bacterial Strains—The bacterial strains used are derivatives of *E. coli* K-12 and are described in Table 1. JE7951 is the *spr-1* derivative of W3110. To produce thermoresistant suppressor mutants, several colonies of JE7951 (*spr-1*) isolated on an L agar plate were grown overnight in L broth separately, and each culture was plated on a salt-free 1/2L agar plate (1) and incubated at 42° C overnight. One colony from each plate was picked up, purified at 30°C, and then the thermoresistance was confirmed on a salt-free 1/2L agar plate. The *recJ* strain, KM354, was used to construct the gene disruptant (14).

Plasmids-A plasmid, pHR596 (H. Hara and Y. Nishimura, unpublished), was constructed from a 0.57kb fragment carrying the wild-type *spr* gene and the vector pTrc99A (15). A gene library was constructed from 2-6 kb of partially digested Sau3AI fragments of the chromosomal DNA of W3110 and the vector pCL1920 (16) digested with BamHI. pNI1 was selected from the gene library as a plasmid that corrected the thermosensitive growth of Rev1 harboring pHR596 (Fig. 1). pNI1 was digested with ScaI and ligated with the chloramphenicolresistant fragment (cm) from pKRP10 (17) to construct pNI2 (Fig. 1). pNI3 was constructed from the 6.8-kb KpnI fragment of pNI1 through self-ligation (Fig. 1). The 1.4kb PstI fragment of pNI1 was cloned into the same site of pCL1920 to construct pNI4 (Fig. 1). The wild-type and mutant *nlpI* genes were cloned into the vector pBAD *Myc*-His C (Invitrogen) to construct the fusion genes. To construct pNI5 encoding the hexa-histidine-tagged NlpI, the *nlpI* in the chromosomal DNA of W3110 was amplified by PCR using primers containing the NcoI site

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Strain	Relevant characteristics	Source or reference
W3110	wild type	Laboratory stock
JE7951	spr-1	(7)
JE7934	$prc\Delta$::neo	(7)
JE7456	str xyl argG	Laboratory stock
CAG12072	<i>zgj-203</i> ::Tn <i>10</i>	(26)
TOP10	$recA1 \ araD139 \ \Delta(araA-leu) \ 7697$	Invitrogen
Rev1	spr-1 nlpI1	Spontaneously from JE7951
Rev2	spr-1 nlpI2	Spontaneously from JE7951
Rev3	spr-1 nlpI3	Spontaneously from JE7951
Rev4	spr-1 nlpI4	Spontaneously from JE7951
Rev5	spr-1 nlpI5	Spontaneously from JE7951
Rev6	spr-1 nlpI6	Spontaneously from JE7951
TAR1	str xyl argG zgj-203::Tn10	$CAG12072~(P1) \times JE7456$
TAR2	<i>argG zgj-203</i> ::Tn <i>10</i>	TAR1 $(P1) \times W3110$
TAR3	<i>spr-1 argG zgj-203</i> ::Tn <i>10</i>	TAR1 (P1) \times JE7951
KM354	recJ pTP223 gam bet exo	(14)
MU2	nlpI::cm	Derived from KM354 by transformation with linearized nlpI::cm DNA
MU24	nlpI::cm	$MU2~(P1)\times W3110$
MU25	nlpI1	$Rev1(P1) \times TAR2$
MU26	nlpI2	$Rev2(P1) \times TAR2$
MU27	nlpI3	$Rev3(P1) \times TAR2$
MU124	spr-1 nlpI::cm	$MU2~(P1) \times JE7951$
MU125	spr-1 nlpI1	$Rev1(P1) \times TAR3$
MU126	spr-1 nlpI2	$Rev2(P1) \times TAR3$
MU127	spr-1 nlpI3	$Rev3(P1) \times TAR3$
PN1	prc∆::neo nlpI::cm	$MU24~(P1)\times JE7934$
HSB1	ibpB::cm	Derived from KM354 by transformation with linearized <i>ibpB::cm</i> DNA
HSB2	ibpB::cm	$HSB1 (P1) \times W3110$
HSB3	spr-1 ibpB::cm	$HSB1 (P1) \times JE7951$

Table 1. Bacterial strains

(underlined), 5'-TGGGCCATGGAGCCTTTTTTGCG-3' and 5'-GTCACCATGGGCTGGTCCGATTCTGCC-3'. The amplified DNA was digested with NcoI, then inserted into the same site of pBAD Myc-His C. To construct pNI6 encoding the hexa-histidine-tagged NlpI with the amino-acid substitution G103D, the mutated *nlpI* in the chromosomal DNA of Rev3 (spr-1 nlpI3) was amplified by PCR using the primers described above, and the amplified DNA was digested with the NcoI and inserted into the same site of pBAD Myc-His C. To construct pNI7 encoding the truncated and hexa-histidine-tagged NlpI, the fragment containing the truncated *nlpI* was amplified by PCR using the forward primer 5'-TGGGCCATGGAGC-CTTTTTTGCG-3' and the reverse primer 5'-GGTCCAT-GGGGTCCTGGCCC-3'. The amplified DNA was digested with NcoI, then inserted into the same site of pBAD Myc-His C. pNI8 and pNI9 were constructed in the same way as pNI7 except that 5'-CATCCATGGCCTGGCCCAGG-3' or 5'-CTT<u>CCATGG</u>GGCCCAGGAGC-3', respectively, was used as the reverse primer in PCR. To construct pNI10 encoding NlpI, the *nlpI* in the chromosomal DNA of W3110 was amplified by PCR using the forward primer containing the NcoI site (underlined) 5'-TGGGCCAT-GGAGCCTTTTTTGCG-3' and the reverse primer containing the BamHI site (underlined) 5'-TGGATCCGGCT-CAAAGTAGG-3'. The amplified DNA was digested with NcoI and BamHI and inserted into the same sites of pTrc99A. pNI12, pNI13, and pNI14 were constructed in the same way as pNI10 except that 5'-CCAGGGATCCT-TAGTCCTGGCC-3', 5'-GTCAGGATCCTACTGGCCCAG-

3', or 5'-ATCT<u>GGATCC</u>TAGCCCAGGAG-3', respectively, was used as the reverse primer containing the *Bam*HI site (underlined) in PCR. The amplified DNA was digested with *NcoI* and *Bam*HI and then inserted into the same sites of pTrc99A.

Expression and Purification of Hexa-Histidine-Tagged NlpI Protein—E. coli cells were transformed with the recombinant plasmids. The transformants were grown in L broth at 42°C for 4 h, then incubated in L broth containing 0.01% arabinose at 42°C for 4 h for expression of the fusion genes. Cells from 400 ml of medium were collected, washed once with ice-cold 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl (buffer A), suspended in 4 ml of the buffer A containing 10 mM imidazole and 1% Triton X-100, and disrupted by sonication with cooling by ice-water. The mixture was centrifuged for 15 min at 17,700 ×g to obtain a clear lysate. Four milliliters of the supernatant was mixed with 1 ml of nickelnitrilotriacetic acid (Ni-NTA) resin and incubated with rotation for 2 h at 4°C. The mixture was loaded onto an open column. The column was washed with 4 ml each of buffer A containing 20 mM imidazole and then containing 40 mM imidazole. The bound proteins were eluted with 1.2 ml of buffer A containing 100 mM imidazole, and fractions of 0.4 ml were collected.

Mass Spectrometry Analysis—An electrospray ion trap mass spectrometer (LCQ DECA XP ion trap mass system, Thermo Electron) coupled online with a capillary HPLC (Magic 2002, Michrom BioResources) was used to acquire MS/MS spectra. A 0.2×50 mm MAGICMS C18

	l kb	Compler <i>nlpI</i> ⁻ :Ts	nentation of <i>prc [–] nlpI [–]</i> :Te
pNI1	$\begin{array}{c c} pnp & nlpI & deaD \\ \hline P & P & K & S & P \end{array} `` \triangleleft P_{lac}$	+	
pNI2		_	
pNI3	▲ P _{lac}	-	
pNI4	▲ P _{lac}	+	
pNI5 (NlpI-His)		+	—
pNI6 (G103D-His)	$\mathbf{P}_{BAD} \blacktriangleright \begin{array}{c} 293 \\ \blacksquare \\ GAC \\ CAG \\ $	_	
pNI7 (D284-His)	$\mathbf{P}_{BAD} \blacktriangleright \underbrace{=}_{\text{GC CAG GAC C}}$	+	—
pNI8 (Q283-His)	$\mathbf{P}_{BAD} \blacktriangleright \underbrace{=}_{GGC CAG G}$	+	_
pNI9 (G282-His)	$\mathbf{P}_{BAD} \blacktriangleright \frac{283}{\text{GGC C}}$		-
pNI10 (NlpI)	$P_{trc} \rightarrow$	+	_
pNI12 (D284)	$P_{trc} \rightarrow$	+	+
pNI13 (Q283)	$\mathbf{P}_{trc} \blacktriangleright \frac{284}{\operatorname{GGC} \operatorname{CAG} \operatorname{TAG}}$	+	+
pNI14 (G282)	$\mathbf{P}_{uv} \blacktriangleright \frac{283}{\operatorname{GGC TAG}}$	_	—

Fig. 1. Plasmids carrying the region of the nlpI gene and complementation of the thermosensitive (Ts) growth defect. Arrowheads denote the directions of the promoters on the vector pCL1920, pBAD Myc-His C, and pTrc99A. The PBAD promoter regulates the expression of the downstream nlpI fusion genes encoding hexa-histidine-tagged proteins. The asterisk marks a mutation site of nlpI3. The hatched boxes indicate the sequences of 134 nucleotides in the vector pBAD Myc-His C encoding a part of the Myc epitope and the hexa-histidine tag. Restriction sites: P, Pst; K, KpnI; S, ScaI.

column (5 mm particle diameter, 200 Å pore size) with mobile phases of A (water:acetonitrile:formic acid, 98:2: 0.1) and B (water:acetonitrile:formic acid, 10:90:0.1) was used with a gradient of 5–65% in 40 min.

RESUSTS AND DISCUSSION

Isolation of Suppressor Mutants and Determination of Suppressor Mutation Allele—Six thermoresistant revertants were isolated independently from the *spr-1* mutant, JE7951. The introduction of an *spr*⁺-carrying plasmid pHR596 into each of the revertants made each strain thermosensitive in growth at low osmolarity again, indicating that the suppressor mutations alone caused thermosensitivity as the *spr* mutations alone did.

The thermoresistant revertant Rev1 harboring pHR596 (spr^+) was transformed with a gene library constructed from the chromosomal DNA of W3110, and the thermoresistant transformants were selected on salt-free 1/2L agar at 42°C. The plasmid pNI1 carrying a fragment corresponding to the 71.2 min region of the *E. coli* chromo-

some was found in a thermoresistant transformant (Fig. 1). The introduction of plasmid pNI4 containing the intact nlpI encoding a lipoprotein (13) into Rev1 harboring pHR596 made the cell thermoresistant, but the introduction of either plasmid pNI2 or pNI3 containing the disrupted nlpI did not (Fig. 1). Thus, the suppressor mutation in Rev1 was supposed to be within the nlpI gene. The other strains harboring pHR596, namely, Rev2, Rev3, Rev4, Rev5, and Rev6, were also made thermoresistant by introduction of pNI1.

The DNA fragments containing nlpI of these six strains were amplified by PCR, and the nucleotide sequences were determined. In every strain, there was a mutation in the coding region of nlpI (Fig. 2). There were nonsense mutations in nlpI1, nlpI4, and nlpI6, frame-shift mutations in nlpI2 and nlpI5, and a missense mutation in nlpI3 that caused the amino-acid substitution of G103D.

To construct various nlpI mutants with a defined genetic background, TAR2 (argG zgj-203::Tn10) and TAR3 (spr-1 argG zgj-203::Tn10) were transduced with phage P1 lysates prepared on Rev1, Rev2, and Rev3, respectively. ArgG⁺ transductants were selected on Davis minimal agar plates (18) at 30°C. The transductants, MU25 (nlpI1), MU26 (nlpI2), and MU27 (nlpI3), derived from the spr⁺ strain TAR2 showed thermosensitive growth. The cotransduction frequencies of $argG^+$ and the thermosensitive allele were 64% (32/48), 54% (26/48), and 37% (18/48) in the transduction with P1 lysate from Rev1, Rev2 and Rev3, respectively. The thermosensitivity of these strains was corrected by introducing pNI1 $(nlpI^+)$. On the other hand, the transductants MU125 (spr-1 nlpI1), MU126 (spr-1 nlpI2), and MU127 (spr-1 nlpI3), derived from the spr mutant TAR3, showed thermoresistant growth. The cotransduction frequencies of $argG^+$ and the thermoresistant allele were 72% (35/48), 87% (42/48), and 81% (39/48) in the transduction with P1 lysate from Rev1, Rev2, and Rev3, respectively. A strain carrying nlpI disrupted with the chloramphenicol-resistant cassette was constructed from the recJ strain KM354 by transformation with the linearized pNI2 (nlpI::cm). The disrupted nlpI was also introduced into W3110 (wildtype) and JE7951 (*spr-1*) by P1 transduction. MU24 (*nlpI*: :cm) derived from W3110 was thermosensitive, while MU124 (spr-1 nlpI::cm) derived from JE7951 was thermoresistant. Thus it was confirmed that the *nlpI* mutation did suppress the thermosensitive growth of the spr mutation.

Construction of the Gene Encoding Hexa-Histidine-Tagged NlpI and Correction of the nlpI Mutation—There are five tetratricopeptide repeat (TPR) motifs (13, 19) in the amino acid sequence of NlpI (Fig. 2). These motifs are known to mediate intermolecular protein-protein interactions that lead to the formation of protein complexes (20, 21). In order to identify the proteins that interact with NlpI, *nlpI* fusion genes encoding hexa-histidinetagged proteins were constructed, because purification of hexa-histidine-tagged proteins in non-denaturing conditions has been reported to be a convenient method to detect protein-protein interactions (22). The NlpI lacking the C-terminal glutamine and followed by 41 extra residues containing a hexa-histidine tag (NlpI-His) was encoded by the fusion gene in pNI5 (Fig. 1). The mutated NlpI with the amino acid substitution of G103D (NlpI-

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1	ATG	AAC	GCC:	FTT:	TTTO	GCG	CTG	GŪG	TTT	CGT	TGC	GAC	AGC	ACT	TAC	<u>GCT</u>	TGC.	AGG	ATG	CAG	TAA'	LAC.	TTCC
1	М	К	Р	F	L	R	W	С	F	v	А	т	А	\mathbf{L}	т	L	А	G	С	S	N	т	S
	G	V	K	V	K	S	S	F	X Y	с н	r c	N	R	L	Y	5	R	K	5	top)		
70	TGG	CGI	[AA]	AG:	[GA	AGT(сст	CGC	GGT	ACC	ATT	GCA	ACC	GAC	TTT	ACA	GCA	GGA	AGT	GAT	TCT(GGC.	ACGT
24	W	R	K	s	Е	v	L	А	v	Р	L	Q	Ρ	т	L	Q	Q	Е	v	I	L	А	R
139	ATG	GA	ACA	AAT(CCTI	rgco	CAG	TCG	GGC	TTT	AAC	CGA	TGA	CGA	ACG	CGC	ACA	GCT	TTT	ATA	TGA	GCG	CGGA
47	М	Е	Q	I	L	A	ន	R	A	L	т	D	D	Е	R	<u>A</u>	Q	L	L	Y	E	R	G
208	GTG	тто	TAT	FGA	PAG	сто	CGG	TCT	GAG	GGC	АТТ	AGC	GCG	таа	CGA	ттт	TTC	GCA	AGC	GCT	GGC	ААТ	CCGA
70	v	Τ.	Î	D	s	Τ.	G	T.	R	A	Т.	A	R	N	D	F	s	0	A	T.	A	т	R
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277	CCG	GAT	TAT	GCC	rga <i>i</i>	AGT2	ATT	CAA	TTA	фтт	AG	CAT	АТА	TTT	AAC	GCA	GGC	AGG	CAA	TTT	TGA'	TGC	TGCC
93	Ρ	D	М	Р	Е	v	F	N	Y	L	G	I	Y	\mathbf{L}	т	Q	А	G	Ν	F	D	А	А
346	TAT	GAA	AGCC	STT.	L'GA'I	erc:	rgr	ACT	TGA	GCT	TGA	TCC	AAC	TTA	CAA	CTA	CGC	GCA	CTT	GAA.	rcG	CGG	GATC
110	<u>Y</u>	E	A	Ľ	D	S	V	Г	Е	Г	D	P	T	Y	N	Y	A	н	Г	N	R	Ģ	1
415	GCA	TT	ATA:	TTAC	CGGC	CGG	TCG	TGA	CAA	GTT	AGC	GCA	AGA	TGA	TCT	GCT	GGC	GTT	TTA	TCA	AGA	CGA	TCCC
415 139	GCA A	TT#	ATA: Y	TTAC Y	CGGC G	GGG	TCG R	TGA D	CAA K	GTT L	AGC	GCA O	AGA D	TGA D	TCT L	GCT L	GGC A	GTT F	TTA Y	TCAJ O	AGA D	CGA' D	TCCC P
415 139	GCA A	TT# L	ATA: Y	TTAC	GGGC G	G G	TCG R	TGA D	CAA K	GTT L	AGC	GCA Q	AGA D	TGA D		GCT L		GTT F	TTA Y	TCA. Q	AGA D	CGA D	TCCC P
415 139	GCA <u>A</u>	L TT	ATA: Y	PTAC Y	GGGC G	GGG	TCG R	TGA D	K K	GTT L	AGC	GCA Q 1	AGA D :T	TGA D (st	TCT L op)	GCT L 5:	GGC A C	GTT F sto	TTA Y (9	TCAJ Q	AGA D	CGA D	TCCC P
415 139 484	GCA <u>A</u> AAT	L L	ATA: Y	TTAC	GGGG G CCG1	GGG	TCG R TCT	TGA D GTG	K K GCT	GTT L	AGC	GCA Q 1 CGC		TGA D (st GCA	TCT L <i>op)</i> GAA	GCT L 5: GCT	GGC A C I(R CGA	GTT F sto TGA	TTA Y p) GAA	TCA Q GCA	AGA D GGC'	CGA D TAA	TCCC P AGAA
415 139 484 162	GCA <u>A</u> AAT N	L L GAT	Y Y TCC: P	TTAC Y TTTC F	CGGC G CCG1 R	GGG G TAG	ICG R ICT L	TGA D GTG	GCI	GTT L TTA Y	AGC A TCT	GCA Q 1 CGC A	AGA D ;T ¢ CGA E	TGA D (st GCA Q	TCT <u>L</u> <i>OP)</i> GAA K	GCT L 5: GCT L	GGC A C I(R CGA D	GTT F sto TGA E	TTA Y p) GAA K	TCA Q GCA	AGA D GGC' A	CGA D TAA K	TCCC P AGAA E
415 139 484 162	GCA <u>A</u> AAT <u>N</u>	L L GAJ D	Y Y TCC: P	TTAC Y TTTC F	CGGC G CCGT R	GGG G TAG S	ICG R ICT L	TGA D GTG	CAA K GCT L	GTT L TTA Y	AGC A TCT	GCA Q 1 CGC A	AGA D ;T CGA E	TGA D (st GCA Q	TCT L op) GAA K	GCT L 5: GCT L	GGC A C I(R CGA D	GTT F sto TGA E	TTA Y p) GAA K	TCA Q GCA Q	AGA D GGC' A	CGA D TAA K	TCCC P AGAA E
415 139 484 162 553	GCA A AAT N GTG	TTZ L GAT D	Y Y ICC: P SAA/	TTAC Y TTTC F	CGGC G CCG7 R GCAC	CGG G TAG S	TCG R TCT L CGA	TGA D GTG W AAAA	K K K K K K K K K K K K K K K K K K K	GTT L TTA Y	AGC A TCT TAA	Q Q 1 CGC A	AGA D T COA E	TGA D (st GCA Q GTG	TCT L op) GAA K GGG	GCT 5: GCT L ATG	GGC A C I(R CGA D GAA	GTT F sto TGA E CAT	TTA Y D) GAA K TGT	TCA Q GCA Q CGA	AGA D GGC' A GTT	CGA D TAA K CTA	TCCC P AGAA E CCTG
415 139 484 162 553 185	GCA A AAT N GTG V	GAT D TTC	Y Y ICC: P SAAA	TTAC Y TTTC F ACAC Q	CGGC G CCG7 R GCAC H	CGG G TAG S CTTC F	ICG R ICT L CGA E	TGA D GTG W AAA K	GCI L ATC	GTT L TTA Y GGA D	AGC A TCT TAA K	GCA Q 1 CGC A .GGA E	AGA D T C Q ACA Q	TGA D (st GCA Q GTG W	TCT L OP) GAA K GGG G	GCT 5: GCT L ATG	GGC A C I(R C GAA D GAA	GTT F TGA E CAT I	TTA Y GAA K TGT V	TCA Q GCA Q CGA E	AGA D GGC A GTT F	CGA D TAA K CTA Y	TCCC P AGAA E CCTG L
415 139 484 162 553 185	GCA A AAT N GTG V	GAT D TTC	Y Y CCC P GAA/ K	TTAC Y TTTC F ACAC Q	GGGG G CCG7 R GCAC H	CGG? G TAG? S CTTC F	ICG R ICT L CGA E	TGA D GTG M AAA K		GTT L TTA Y GGA D	AGC A TCT TAA K	GCA Q 1 CGC A GGA E	AGA D T CGA E ACA Q	TGA D (st GCA Q GTG W	TCT L OP) GAA K GGG G	GCT 5: GCT L ATG	GGC A C I(R C GAA D GAA	GTT F Sto TGA E CAT I	TTA Y D) GAA K TGT V	TCA Q GCA Q CGA E	AGA D GGC A GTT F	CGA D TAA. K CTA Y	TCCC P AGAA E CCTG L
415 139 484 162 553 185 622	GCA A AA1 N GTG V GGC	GAT D TTC L AAC	Y Y ICC: P GAA/ K	TTTO F ACAO Q TAGO	GGGG G CCG7 R GCAC H	CGG? G FAG? S CTTC F	ICG R ICT L CGA E AAC	TGA D GTG M AAA K GTT	GCT L ATC S	GTT L TTA Y GGA	AGC A TCT TAA K AAG	GCA Q 1 CGC A GGA E GCT	AGA D T C Q ACA Q CAA	TGA D (st GCA Q GTG W GGC	TCT L OP) GAA K GGG G GGA	GCT 5: GCT L ATG W CGC	GGC A C C GA D GAA N AAC	GTT F TGA E CAT I GGA	TTA Y GAA K TGT V TAA	CGAC	AGA D GGC' A GTT F	CGA D TAA K CTA Y GCT	AGAA E CCTG L CGGCT
415 139 484 162 553 185 622 208	GCA A AAT N GTG V GGC G	GAT D TTC L AAC	Y Y P CC: P SAAA K CAT: I	TTTO F ACAO Q TAGO S	GGGG G R GCAC H CGAZ E	CGG G TAG S CTTC F ACAJ Q	ICG R ICT L CGA E AAC T	GTG M AAA K GTT L	GCT L LATC S AAT M	GTT L TTA Y GGA D CGGA E	AGC A TCT TAA K AAG R	GCA Q 1 CGC A GGA E GCT L		TGA D (st GCA Q GTG W GGC	TCT L OP) GAA K GGG G GGA D Stol	GCT 5: GCT L ATG W CGC	GGC A C C C GA D GAA N AAC T	GTT F TGA E CAT I GGA D	TTA Y GAA K TGT V TAA N	TCA Q GCA Q CGA E CAC T	AGA D GGC' A GTT F CTC S	CGA D TAA K CTA Y GCT L	AGAA E CCTG L CGCT A
415 139 484 162 553 185 622 208	GCA A AAT N GTG V GGC G	E CGAT D CGAT D CGAT D CGAT C CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	Y P CCC P GAAA K I	TTAC Y F ACAC Q TAGO S	GGGG G CCG7 R GCAC H CGAZ E	CGG G F CTTC F Q	TCG R TCT L CGA E AAC T	TGA D GTG M AAAA K GTT L	GCT L ATC S AAT M	GTT L TTA Y GGA D CGGA E	AGC TCT TAA K AAG R	GCA Q 1 CGC A GGA E GCT L C	AGA D T CQA E ACA Q CAA K m:G	TGA D (st GCA Q GTG W GGC	TCT L op) GAA K GGGG G GGA D Stop	GCT 5: GCT L ATG W CGC	GGC A C C GAA D GAA N AAC T	GTT F TGA E CAT I GGA D	TTA Y GAA K TGT V TAA N	TCA Q GCA Q CGA E CAC T	AGA D GGC' A GTT F CTC S	CGA D TAA. K CTA Y GCT L	AGAA E CCTG L CGCT A
415 139 484 162 553 185 622 208 691	GCA A AAT N GTG V GGC G GAG	TTF L GGAT D GTTC L CAAC N	Y P CCC P GAAN K L	TTAC Y TTTC F ACAC Q TAGO S	CGGC G R GCAC H CGAZ E	CGG(G F CTTC F Q	TCG R TCT L CGA E AAC T	TGA D GTG M AAAA K GTT L CTT	GCTA GCT L ATC S ZAAT M	GTT L TTA Y GGA D GGA E	AGC A TCT E TAA K AAG R	GCA Q 1 CCGC A GGA E GGCT L C C TTAA	AGA D ;T CGA CAA K m:G	TGA D (st GCA Q GTG W GGC A A ()	TCT L op) GAA K GGGG G GGA D Stop CCT	GCT L 5: GCT L ATG ATG CGC A P)	GGC A C C C C C C C C C C C C C	GTT F Sto TGA E CAT I GGA D GGG	TTA Y GAA K TGT V TAA N GGA	TCA Q GCA Q CGA E CAC T	AGA(D GGC' A GTT F CTC(S GGA(CGA D TAA K CTA Y GCT L CAG	AGAA E CCTG L CGCT A
415 139 484 162 553 185 622 208 691 231	GCA A AATT N GTG V GGC G GAG E	TTF L GGAJ D STTC L SAAC N	Y P CCC: P GAAA K I CAT: I	TTAC Y F ACAC Q FAGO S CAG7 S	CGGC G CCGJ R GCAC H E CGAZ E	CGG' G F CTTC F Q AAACO T	TCG R TCT L CGA E AAAC T CAA	TGA D GTG M AAA K GTT L CTT F	CAA K GCT L ATC S VAAT M	GTT L TTTA Y GGGA D CGGA E TTTT []	AGC A TCT TCT TAA K AAGG R AGG	GCA Q 1 CGCC A GGA E GGT L C TAA K	AGA D T T T T T T T T	TGA D (st GCA Q GTG GTG GTG (¹ C GTA	TCT L op) GAA K GGG G GGA D Stop CCT L	GCT 5: GCT L ATG W CGC A S	GGC A C C C GAA N AAAC T T T L	GTT F Sto TGA E CAT I GGA D GGG G	TTA <u>P</u>) GAA K TGT V TAA N GGA D	TCAJ Q GCA(Q CGA(E CAC(T TTTT(L	AGA(D GGC' A GTT F CTC(S GGA(D	CGA D IAA. K CTA Y GCT L CAG	AGAA E CCTG L CGCT A CGCC
415 139 484 162 553 185 622 208 691 231	GCA A AAT GTG V GGC G GGC G GAG E	ATTA L CGAT D TTC L CAT H	Y Y P GAAN K CAT: I	TTAG Y FTTC F ACAC Q FAGO S CAG? S	CGGC G R GGCAC H CGAZ E	CGG' G FAG' S CTTO F ACAJ Q AACO T	TCG R TCT L CGA E AAC T CAA	TGA D GTG M AAA K GTT L CTT F	GCTA SCTA M CTA Y	GTT L TTA Y GGA D CGGA E TTT L	AGC A TCT E TAA K AAG R AGG G	GCA Q 1 CGC A GGA E GGCT L CI CI TAA K	AGA D :T CGA E ACA Q CAA K m:G CAA K m:G	TGA D (st GCA Q GTG W GGC A A (: C TA	TCT L op) GAA K GGG GGA D Stoj CCT L	GCT 5: GCT L ATG W CGC A CGC A S	GGCC C C C C GAA N AAAC T T T L	GTT F sto TGA E CAT I GGA GGG GGG G	TTA Y p) GAA K TGT V TAA N GGA D	TCAJ Q GCA(Q CGA(E CAC(T TTT(L	AGA D GGC' A STT F CTC S GGA D	CGA D TAA. K CTA Y GCT L CAG S	AGAA E CCTG L CGCT A CGCC
415 139 484 162 553 185 622 208 691 231 760	GCA A AATI N GTG V GGC G GGC G GAG E ACG	GAT D GAT D CAAC N CAAC N H GCZ	Y Y CCC: P SAAN K CAT: I CAT: L	TTTO Y F ACAO Q FAGO S CAG: S	CGGC G R GCCGT R GCCAC H CGAZ E CAAZ	CGG' G FAG' S CTTC F Q AACCA T AACCTC	TCG R ICT L CGA E AAAC T CAA N GGC	TGA D GTG M AAA K GTT L CTT F GGT	CAA K GCT L LATC S ZAAT M ZCTA Y TGC	GTT L TTTA Y GGA CGGA E TTTT L CAA	AGC A TCT TAA K AAGG R AGG G CAA	GCA Q 1 CGC A GGA E GCT L C1 TAA K CGT	AGA D :T CGA E ACA Q CAA K m:G GTA Y TCA	TGA D (st GCA Q GTG W GGCC A GTCA Q TAA	TCT L op) GAA K GGGG G GGA D stoj CCT L CTT	GCT 5: GCT L ATG W CGC A P) AAG S TGT	GGCC C C C C GAA N GAA T T T C GAA T T T GA	GTT F sto TGA E CAT I GGA D GGG G GCA	TTA Y D) GAA K TGT V TAA N GGA D CCG	TCAJ Q GCA(Q CGA(E CAC(T T TTT(L ATA(AGA D GGC' A GTT F CTCC S GGA D CGC.	CGA D TAA. K CTA Y GCT L CAG S ATT	ICCC P AGAA E CCTG L CGCT A CGCC A CGCC A GTTG
415 139 484 162 553 185 622 208 691 231 760 254	GCA A AATT N GTG V GGC G GAG E ACG T	GCZ	Y P GAAN K CAT: I CAT: L ACTO L	TTAC Y TTTC F ACAC Q FAGC S CAG: S STTC F	CGGC G R GCCGT R GCCAC H CGAZ E CAAZ K	CGG' G FAG' S CTTC F ACAJ Q AACCA T ACTC L	ICG R ICT L CGA E AAC T CAA SGCC	TGA D GTG M AAA K GTT L CTT F GGT V	CAA K GCT L CAAT M CTA Y TGC A	GTT L TTA Y GGGA E TTTT L CCAA N	AGC A TCT E TAA K AAGG G CAA	GCA Q 1 CGC A GGA E GCT L C1 TAA K CGT V	AGA D T CGA CAA CAA CAA CAA CAA CAA CAA CAA CAA	TGA D (st GCA Q GTG W GGCC A A (' CTA CTAA N	TCT L op) GAA K GGGG G GGA D Stop CCTT L CTT _F	GCT L 5: GCT L ATG CGC A P AAG S TGT V	GGCC A C C C C C A D C C A A C C A D C C A D C C A D C C C C	GTT F TGA CAT I GGA G GGG G GCA H	TTA Y GAA TGT V TAA N GGA D CCG R	TCAJ Q GCA(Q CGA(E CAC(T T TTT(L ATA(Y	AGA(D GGC' A GTT(F CTC(S GGA(D CGC; A	CGA D IAA. K CTA Y GCT L CAG S ATT L	AGAA E CCTG L CGCT A CGCC A CGCC A CGCC L
415 139 484 162 553 185 622 208 691 231 760 254	GCA A AATT N GTG V GGC G GGC G GAG E ACG T	GCA1 H GCA1 C CA1 C CA1 C CA1 C CA1 C CA1 C CA1 C CA1 C CA1 C CA1 C CA1 C CA1 C CA1 C CA1 C C CA1 C C C C	Y Y CCC P SAAM K CAT I CAT C L	TTTC F ACAC Q FAGO S CAG: S GTTC F	CGGC G CCGT R GCAC H CGAZ E CAAZ K	CGG' G S CTTC F Q AACAA Q AACCO L	TCG R TCT L CGA E AAAC T CAA N GGCC	TGA D GTG M AAA K GTT L CTT F GGT V	CAA K GCT L ATC S CTA M Y TGC A	GTT L TTTA Y GGGA D CGGA E TTTT L CCAA N	AGC A TCT TAA K AAG R AGG CAA N	GCA Q 1 CGC A GGA E C C C C C C C C C C C C C	AGA D T T T T T T T T	TGA D (st GCA Q GTG W GGCC A (st C TA A N TAA	TCT L op) GAA K GGGG GGA Stop CCT L CTT -F	GCT L 5: GCT L ATG CGC A P AAG S TGT V	GGCC A C C C GAA N AAAC T T T C T C GAA T T T C T C GAA S A C C A D C C C C C C C C C C C C C C C	GTT F TGA E CAT I GGA G G G G G G CA H	TTA Y D) GAA K TGT V TAA N GGA D CCG R	TCAJ Q GCA(Q CGA(E T T TTT(L ATA(Y C	AGA(D GGC' A STT(F S GGA(D CGC) A	CGA D IAA. K CTA Y CTA L CAG S L L	AGAA E CCTG L CGCT A CGCC A CGCC A GTTG L
415 139 484 162 553 185 622 208 691 231 760 254 829	GCA A AATT N GTG V GGC G GGC G GAG E ACG T GAA	CGAT D CTTC L CAAC N CAAC H CCAT H CCAT	Y Y CCC: P SAAA K CAT: I CCT: L ACT: L	TTAC Y TTTC F ACAC Q TAGO S S TTC F GCTC	CCGGC CCGT R GCCAC H CGAZ E CAAZ K CCTCC	CGG? G F S CTTC F ACCA Q AACCO L GGGGO	TCG R TCT L CGA E AACC T CAA N GGC A CCA	TGA D GTG M AAA K GTT L CTT F GGT V GGA	CAA K GCTI L ATC S ZAAT M Y TGC A	GTTT L TTTA Y GGGA D CGGA E TTTT L CAA N AGA	AGC AGC TCT TTAA K AAGG R AGG CAA N TGA	GCA Q 1 CCGC A GGA E GGCT L C C TAAA K CCGT V CCT	AGA D T T T T T T T T	TGA D (st GCA Q GTG W GGCC A (: CTA TAA N AGA	TCT L op) GAA K GGGG GGA CCT L CTT - F ATC	GCT L 5: GCT L ATG CGC A P AAG S TGT V GGA	GGCC A C C C GAA N AACC T T C C GAA N T T C T C C GAA N C C GAA N C C C C C C C C C C C C C C C C C C	GTT F TGA CAT I GGA D GGG G G G G G CA H GCA	TTA Y D GAA K TGT V TAA N GGA N CCCG R ATA	TCAJ Q GCA(Q CGA(E T T TTT(L ATA(Y G G	AGA(D GGC' A STTC F S S GGA(D CGC) A	CGA D TAA. K CTA Y CTA L CAG S L	AGAA E CCTG L CGCT A CGCC A CGCC A STTG L

G103D-His) and with the C-terminal modified region as the above protein was encoded in pNI6. The fusion genes in pNI7, pNI8, and pNI9 encoded the truncated NlpI lacking the C-terminal 10, 11, and 12 amino acids, respectively, followed by the same extra sequences as the above fusion proteins. The products encoded in pNI7, pNI8, and pNI9 were designated NlpI-D284-His, NlpI-Q283-His, and NlpI-G282-His, respectively. The thermosensitive growth of the *nlpI* disruptant, MU24, was corrected by the introduction of pNI5 (encoding NlpI-His), but not by the introduction of pNI6 (encoding NlpI-G103D-His) (Fig. 1). Thus the production of NlpI-His corrected the defect that was caused by the lack of the wildtype NlpI, although the production of NlpI-G103D-His did not. The introduction of pNI7 (encoding NlpI-D284-His) or pNI8 (encoding NlpI-Q283-His) corrected the thermosensitivity of MU24, but introduction of pNI9 (encoding NlpI-G282-His) did not. This result showed that the C-terminal 11 amino acid residues of the nascent NlpI protein were not necessary for the function of NlpI but that the lack of 12 residues in NlpI impaired the function.

Detection of Proteins that Bind with the Hexa-Hstidine-Tagged NlpI Variants—The C-terminal hexa-histidinetagged proteins from the cells were purified by Ni-NTA metal-affinity chromatography and analyzed by SDS polyacrylamide gel electrophoresis (Fig. 3A). The most intense band in each sample, located at a position correFig. 2. The nucleotide sequence of the nlpI gene and its predicted amino acid sequence. The *nlpI* gene encodes a 34-kDa polypeptide containing 294 amino acids including a signal sequence consisting of 18 amino acids from the Nterminus. The amino acids comprising the lipobox sequence are boxed. The mutation sites of *nlpI1*, 2, 3, 4, 5, and 6 and the resulting amino acid substitutions are indicated by arrowheads and italic letters, respectively. The insertion of a cm cassette next to the 727th T forms a stop codon, TGA, just at the junction site. The amino acid residues in TPR1 (from A-62 to M-95), TPR2 (from P-96 to Y-129), TPR3 (from N-130 to D-163), TPR4 (from R-166 to G-199), and TPR5 (from S-234 to N-267) are underlined, and the residues that conform to the consensus TPR motif are boxed.

sponding to a mass of 34 kDa to 38 kDa, was supposed to be the hexa-histidine-tagged NlpI variant. There were many other bands in the lanes of the hexa-histidinetagged NlpI variants (Fig. 3A). Most of them seemed to bind with the NlpI variants, as they did not appear in the sample of the empty vector. Among these coeluted proteins, 16-, 32-, and 76-kDa proteins were analyzed because they were not found in the sample of NlpI-G103D-His that carried the substitution of the conserved glycine in the TPR domain, and thus were supposed to be related with the specific interaction through the TPR domain with NlpI. The 16-, 32-, and 76-kDa proteins were extracted from the gel and digested with trypsin, then analysed by LC-MS/MS. Peptide mass mapping by LC-MS/MS analysis of the peptides, provided identification of the peptides accounting for approximately 19.7, 35.0, and 39.4% of IbpB (23), NlpI itself, and the Prc sequence, respectively (Table 2). The 16-kDa band was not found in the sample of the *ibpB* null mutant, HSB2, harboring pNI5 (encoding NlpI-His), and the 76-kDa band was not found in the sample of the *prc* null mutant, JE7934, harboring pNI5 (Fig. 4). These results confirmed the identities of the 16-kDa and 76-kDa proteins as IbpB and Prc, respectively. These proteins were also detected in the samples of NlpI-D284-His, NlpI-Q283-His, and NlpI-G282-His (Fig. 3A). These results suggest that the

C-terminal region of NlpI was not necessary for the bind-

ing of these proteins with NlpI.



Fig. 3. SDS-polyacrylamide gel electrophoresis of the eluate containing hexa-histidine-tagged NlpI variants. (A) The fusion genes were expressed and the resulting products were purified by Ni-NTA metal-affinity chromatography as described in "MATERIALS AND METHODS". The fractions from TOP10/pBAD Myc-His C (lane 1), TOP10/pNI5 (encoding NlpI-His) (lane 2), TOP10/pNI6 (encoding NlpI-G103D-His) (lane 3), TOP10/pNI7 (encoding NlpI-D284-His) (lane 4), TOP10/pNI8 (encoding NlpI-Q283-His) (lane 5), and TOP10/ pNI9 (NlpI-G282-His) (lane 6) were analyzed using 12% polyacrylamide gel and stained with Coomassie Brilliant Blue. Asterisks indicate hexa-histidine-tagged proteins, and large arrowheads indicate 76-, 32-, and 16-kDa protein bands. Small arrowheads indicate putative degradation products. (B) The fractions eluted from the Ni-NTA column were stored for 16 days at 4°C and then incubated at 37°C for 14 h. The incubated fractions were analyzed using 12% polyacrylamide gel and stained with Coomassie Brilliant Blue (lane 1, TOP10/pNI5; lane 2, TOP10/ pNI6). The proteins on the gel were transferred to a polyvinylidene fluoride membrane by semi-dry blotting and detected by Ni-NTA AP Conjugate (QIAGEN), as described in the supplier's instructions (lane 3, TOP10/pNI5; lane 4, TOP10/pNI6). Asterisks indicate hexahistidine-tagged proteins, and the arrowhead indicates the putative degradation product.

Processing of the Hexa-Histidine-Tagged NlpI Variants with Prc Protease—The 32-kDa band was found in the sample of the *nlpI* null mutant, MU24, harboring pNI5 (encoding NlpI-His), but was not found in the sample of the *prc* null mutant, JE7934, harboring pNI5 (Fig. 4). These results suggest that the 32-kDa protein was not derived from the product of the chromosomal *nlpI* gene, but instead was produced by proteolysis of NlpI-His. In order to confirm the role of Prc protease, the partially purified fractions from W3110 (*prc*⁺)/pNI5 and JE7934 (*prc*\Delta::*neo*)/pNI5 were incubated at 37°C for 12 h (Fig. 5). A time-dependent increase in the intensity of the 32-kDa

Table 2.	Peptides	identified	by	LC-MS/MS.

Identified	Sequence	Sequence
protein	positions	
IbpB	12-18	QWIGFDK
	122–131	NEPEPIAAQR
	132 - 142	IAISERPALNS
NlpI	47-54	MEQILASR
	55-61	ALTDDER
	62-68	AQLLYER
	69–78	GVLYDSLGLR
	83 - 92	NDFSQALAIR
	137 - 145	GIALYYGGR
	221 - 242	ADATDNTSLAEHLSETNFYLGK
	243 - 258	YYLSLGDLDSATALFK
	259 - 272	LAVANNVHNFVEHR
Prc	27 - 34	ADQIPVLK
	57-67	QFDLDQAFSAK
	72 - 93	YLNLLDYSHNVLLASDVEQFAK
	95 - 103	KTELGDELR
	96 - 103	TELGDELR
	107 - 119	LDVFYDLYNLAQK
	125 - 134	YQYALSVLEK
	135 - 148	PMDFTGNDTYNLDR
	170 - 176	FDELSLK
	219 - 230	EIDPHTNYLSPR
	280 - 288	IVGVGQTGK
	298-306	LDDVVALIK
	355 - 372	VGVLDIPGFYVGLTDDVK
	381 - 391	QNVSSVIIDLR
	424 - 437	VREDSDTDGQVFYK
	426-437	EDSDTDGQVFYK
	438-446	GPLVVLVDR
	465 - 475	ALVVGEPTFGK
	521 - 540	GVTPDIIMPTGNEETETGEK
	559 - 571	SGDLTAFEPELLK
	580-588	DPEFQNIMK
	639–645	KLDDLPK
	646-666	DYQEPDPYLDETVNIALDLAK
	670–680	ARPAEQPAPVK

band was observed in the sample of W3110/pNI5, but not in the sample of JE7934/pNI5 (Fig. 5). Moreover, the intensity of the 32-kDa band became much greater than that of the 38-kDa band of NlpI-His after storage of the sample from TOP10/pNI5 at 4°C for 16 days and then incubation at 37°C for 14 h (Fig. 3B). No increase in the intensity of the 32-kDa band was observed in the sample from TOP10/pNI6 (encoding NlpI-G103D-His) that had not been copurified with Prc protease (Fig. 3B). These results indicate that Prc protease degraded NlpI-His to the 32-kDa protein. Western blotting showed that the 32kDa protein did not contain a hexa-histidine tag (Fig. 3B); thus the processing of NlpI-His by Prc protease should have occurred in the C-terminal region. The proteolysis of NlpI-His by Prc protease seemed to occur even at 4°C. The faint 32-kDa band in the sample of 0 h incubation (Fig. 5 lane 1) is supposed to be caused by the proteolysis that occurred during and/or after the elution from the Ni-NTA column.

The 32-kDa protein was detected in samples of NlpI-D284-His and NlpI-Q283-His, but was not detected in a sample of NlpI-G282-His (Fig. 3A). This suggests that the



Fig. 4. SDS-polyacrylamide gel electrophoresis of the eluate containing NlpI-His from various strains. The fusion genes were expressed and the resulting products were purified as in Fig. 3. The fractions from W3110 (wild-type)/pBAD *Myc*-His C (lane 1), W3110/pNI5 (encoding NlpI-His) (lane 2), HSB2 (*ibpB::cm)*/pNI5 (lane 3), MU24 (*nlpI::cm)*/pNI5 (lane 4), and JE7934 (*prcA::neo*)/pNI5 (lane 5) were analyzed using 12% polyacrylamide gel and stained with Coomassie Brilliant Blue. Asterisks indicate NlpI-His and arrowheads indicate bands of Prc, NlpI, and IbpB.

amino acid residues upstream of Q283 in NlpI are necessary for processing by the Prc protease. There were also several bands corresponding to the mass of 29 kDa to 31 kDa in the samples of NlpI-D284-His and NlpI-Q283-His. All of them were shown to contain the sequence of NlpI by peptide mass mapping (data not shown). These proteins are also supposed to be the products of the proteolysis of hexa-histidine-tagged NlpIs by Prc protease, because these bands were not found in the samples from the JE7934 ($prc\Delta$::neo) (data not shown). The amino-acid residues downstream of Q285 in NlpI might be necessary for the strict specificity of the cleavage site in hexa-histidine-tagged NlpI by Prc protease.

Functional Interaction of NlpI with Prc Protease-Among the hexa-histidine tagged NlpI variants, only those that could be processed by Prc protease corrected the thermosensitivity of MU24 (nlpI::cm) (Fig. 1). To investigate whether the processed form of NlpI was active or not, we constructed plasmids, pNI12, pNI13, and pNI14, that encoded NlpI lacking the C-terminal 10 (NlpI-D284), 11 (NlpI-Q283), and 12 (NlpI-G282) residues, respectively. The introduction of pNI10 (encoding full-length NlpI), pNI12 (encoding NlpI-D284), or pNI13 (encoding NlpI-Q283) to the *nlpI* disruptant MU24 corrected the thermosensitive growth, and that of pNI14 (encoding NlpI-G282) did not. This result showed that synthesis of the full-length NlpI or the truncated NlpI lacking fewer than 12 residues in MU24 (*nlpI*::*cm prc*⁺) resulted in formation of the functional form of NlpI, while that lacking 12 residues did not. On the other hand, the thermosensitivity of the *nlpI prc* double mutant, PN1, was not corrected by the introduction of either pNI10 (encoding full-length NlpI) or any plasmid encoding a hexa-histidine-tagged NlpI variant. Only the introduc-



Fig. 5. Time course of the degradation of NlpI-His. The fusion genes were expressed and the resulting products were purified as in Fig. 3. The fractions from W3110 (wild-type)/pNI5 (encoding NlpI-His) (lanes 1–5) and JE7934 ($prc\Delta:neo$)/pNI5 (lanes 6, 7) were incubated at 37°C for 0 h (lanes 1, 6), 1 h (lane 2), 3 h (lane 3), 6 h (lane 4), and 12 h (lanes 5, 7). The gel-loading buffer was added at the indicated time, and then the mixture was heated at 95°C for 5 min. 12% polyacrylamide gel was used and stained with Coomassie Brilliant Blue. Asterisks indicate hexa-histidine-tagged NlpI, and the large arrowhead indicates degradation products. The small arrowhead indicates Prc protease.

tion of a plasmid encoding truncated NlpI, pNI12 (encoding NlpI-D284) or pNI13 (encoding NlpI-Q283), corrected the thermosensitivity of PN1 ($prc\Delta::neo\ nlpI::cm$). These results showed that the synthesis of only a truncated NlpI resulted in formation of the functional form of NlpI in PN1 ($prc\Delta::neo\ nlpI::cm$), suggesting that the removal of the C-terminal residues was necessary for the NlpI protein to be functional. Further work is required to clarify the exact site of the processing and the regulation of the processing in the cell.

Functional Interaction of NlpI with IbpB—IbpB is a 16-kDa heat-shock protein that shares 50% amino-acid homology with IbpA and binds to heterologous proteins to form inclusion bodies in E. coli cells (23, 24). To determine whether IbpB is necessary for the function of NlpI or not, the disrupted ibpB gene was introduced into the spr mutant. The resulting strain HSB3 (spr-1 ibpB::cm) showed thermosensitive growth, indicating that the disruption of *ibpB* did not suppress the growth defect due to the spr mutation. This suggested that formation of a complex between NlpI and IbpB did not relate to the suppression mechanism. The overproduction of NlpI-His in the ibpB mutant, HSB2, caused weak thermosensitivity, while in W3110 (wild-type), it did not, suggesting that IbpB reduced the toxic effect of the overproduced NlpI-His by forming the complex.

Mechanism by Which NlpI or Prc Mutation Suppresses the Thermosensitivity of Spr Mutant—The mutation in prc or nlpI suppressed the spr mutation. Spr protein is likely to degrade peptidoglycan because its amino acid sequence shows significant homology with the γ -Dglutamyl-L-diamino acid endopeptidase II of Bacillus sphaericus (11, 12). There are three genes, nlpC, ydhO, and yafL, in the E. coli chromosome that encode products homologous to Spr (25). We have evidence that NlpI depresses the expression of nlpC (A. Tadokoro, unpublished result). The mutation in nlpI is considered to increase the expression of nlpC and the other related genes that may substitute for Spr and thus maintain adequate peptidoglycan-degrading activity in the cell. The prc mutation may also increase the peptidoglycandegrading activity through the decrease in NlpI activity and/or other pathways. Further study is necessary to clarify the details of the mechanism.

We thank Dr. Hiroshi Hara for providing the strains and the plasmids and for valuable discussion. Thanks are also due to Dr. Tohru Nishihara for critically reading the manuscript.

REFERENCES

- Hara, H., Yamamoto, Y., Higashitani, A., Suzuki, H., and Nishimura, Y. (1991) Cloning, mapping, and characterization of the *Escherichia coli prc* gene, which is involved in C-terminal processing of penicillin-binding protein 3. J. Bacteriol. 173, 4799–4813
- Hara, H., Nishimura, Y., Kato, J., Suzuki, H., Nagasawa, H., Suzuki, A., and Hirota, Y. (1989) Genetic analyses of processing involving C-terminal cleavage in penicillin-binding protein 3 of *Escherichia coli*. J. Bacteriol. **171**, 5882–5889
- 3. Nagasawa, H., Sakagami, Y., Suzuki, A., Suzuki, H., Hara, H., and Hirota, Y. (1989) Determination of the cleavage site involved in C-terminal processing of penicillin-binding protein 3 of *Escherichia coli*. J. Bacteriol. **171**, 5890–5893
- 4. Hayashi, S., Hara, H., Suzuki, H., and Hirota, Y. (1988) Lipid modification of *Escherichia coli* penicillin-binding protein 3. J. Bacteriol. **170**, 5392–5395
- Tamura, T., Suzuki, H., Nishimura, Y., Mizoguchi, J., and Hirota, Y. (1980) On the process of cellular division in *Escherichia coli*: Isolation and characterization of penicillinbinding proteins 1a, 1b, and 3. *Proc. Natl Acad. Sci. USA* 77, 4499–4503
- Keiler, K.C., Waller, P.R.H., and Sauer, R.T. (1996) Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* 271, 990–993
- Hara, H., Abe, N., Nakakouji, M., Nishimura, Y., and Horiuchi, K. (1996) Overproduction of penicillin-binding protein 7 suppresses thermosensitive growth defect at low osmolarity due to an *spr* mutation of *Escherichia coli*. *Microb. Drug Resist.* 2, 63– 72
- 8. Ohfuchi, H. (1996) DDBJ/EMBL/GenBank databases. Loucus: D86610
- Wu, H.C. (1996) Biosynthesis of lipoproteins in Neidhardt Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhardt, F.C., Curtiss, R. III, Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M. Umbarger, H.E., eds.) 2nd ed., pp. 1005–1014, ASM Press, Washington, DC
- Romeis, T. and Höltje, J.V. (1994) Penicillin-binding protein 7/8 of *Escherichia coli* is a DD-endopeptidase. *Eur. J. Biochem.* 224, 597–604
- Bourgogne, T., Vacheron, M.J., Guinand, M., and Michel, G. (1992) Purification and partial characterization of the γ-Dglutamyl-L-di-amino acid endopeptidase II from *Bacillus* sphaericus. Int. J. Biochem. 24, 471–476

- Hourdou, M.L., Duez, C., Joris, B., Vacheron, M.J., Guinand, M., Michel, G., and Ghuysen, J.M. (1992) Cloning and nucleotide sequence of the gene encoding the γ-D-glutamyl-Ldiamino acid endopeptidase II of *Bacillus sphaericus*. FEMS Microbiol. Lett. **91**, 165–170
- Ohara, M., Wu, H.C., Sankaran, K., and Rick, P.D. (1999) Identification and characterization of a new lipoprotein, NlpI, in *Escherichia coli* K-12. J. Bacteriol. 181, 4318–4325
- 14. Murphy, K.C. (1998) Use of bacteriophage λ recombination functions to promote gene replacement in *Escherichia coli*. J. Bacteriol. 180, 2063–2071
- 15. Amann, E., Ochs, B., and Abel, K.J. (1988) Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene* **69**, 301–315
- Lerner, C.G. and Inouye, M. (1990) Low copy number plasmids for regulated low-level expression of cloned genes in *Eschirichia coli* with blue/white insert screening capability. *Nucleic Acids Res.* 18, 4631
- 17. Reece, K.S. and Phillips, G.J. (1995) New plasmids carrying antibiotic-resistance cassettes. *Gene* **165**, 141–142
- Davis, B.D. and Mingioli, E.S. (1950) Mutants of *Escherichia* coli requiring methionine or vitamin B12. J. Bacteriol. 60, 17– 28
- Borodovsky, M., Rudd, K.E., and Koonin, E.V. (1994) Intrinsic and extrinsic approaches for detecting genes in a bacterial genome. *Nucleic Acids Res.* 22, 4756–4767
- Das, A.K., Cohen, P.T.W., and Barford, D. (1998) The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. *EMBO J.* 17, 1192–1199
- Lamb, J.R., Tugendreich, S., and Hieter, P. (1995) Tetratrico peptide repeat interactions: to TPR or not to TPR? *Trends Biochem. Sci.* 20, 257–259
- 22. Juàrez, A., Nieto, J.M., Prenafeta, A., Miquelay, E., Balsalobre, C., Carrascal, M., and Madrid, C. (2000) Interaction of the nucleoid-associated proteins Hha and H-NS to modulate expression of the hemolysin operon in *Escherichia coli. Adv. Exp. Med. Biol.* 485, 127–131
- Jakob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) Small heat shock proteins are molecular chaperones. J. Biol. Chem. 268, 1517–1520
- Kitagawa, M., Miyakawa, M., Matsumura, Y., and Tsuchido, T. (2002) Escherichia coli small heat shock proteins, IbpA and IbpB, protect enzymes from inactivation by heat and oxidants. Eur. J. Biochem. 269, 2907–2917
- Anantharaman, V. and Aravind, L. (2003) Evolutionary history, structural features and biochemical diversity of the NlpC/ P60 superfamily of enzymes. *Genome Biol.* 4, R11
- 26. Singer, M. Baker, T.A., Schnitzler, G., Deischel, S.M., Goel, M., Dove, W., Jaacks, K.J., Grossman, A.D., Erickson, J.W., and Gross, C.A. (1989) A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol. Rev.* **53**, 1–24