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Supplementary Information for

Reversible auto-inhibitory regulation of *Escherichia coli* metallopeptidase BepA for selective β-barrel protein degradation

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Supplementary Information Text

Supplementary Results

Isolation of the mutations that suppress self-degradation of BepA(Δα9) and BepA(H246A)

We overexpressed and purified wild-type and the mutant forms of the BepA proteins with His_{10}-tag at their C-terminus by metal-affinity chromatography. SDS/PAGE analysis of the eluted fraction of BepA(Δα9) and BepA(H246A) showed that the purified preparations contained less amounts of full-length BepA compared to wild-type BepA, with several faster-migrating proteins (Fig. 2*A*, lanes 5 and 11). Some of these faster-migrating species (such as 31.5 kDa and 15 kDa bands) possibly represent self-degradation products because their amounts increased upon incubation of the purified preparations with concomitant decrease in the amount of the full-length protein (Fig. 2*A*). N-terminal sequence analysis of the major fragment of about 30 kDa (Fig. 2*A*, blue arrowheads) showed that this fragment was a C-terminal part of BepA generated by cleavage between Ala-181 and Leu-182. To reduce the self-degradation, which was supposed to improve the yield of full-length BepA, we conducted random mutagenesis against the codons for A181 and L182 and screened for the mutants with decreased degradation (see *SI Appendix, Supplementary Materials and Methods* for details). We found that a pair of mutations (A181E/L182T) significantly suppresses the *in vivo* self-degradation of BepA(Δα9) and BepA(H246A) (Fig. 2*A*, lanes 7 and 13). The purified preparations of BepA(Δα9) and BepA(H246A) carrying the A181E/L182T mutations (represented as BepA(Δα9)* and BepA(H246A)*, respectively) contained primarily full-length proteins (Fig. 2*A*, lanes 7 and 13), although incubation of these proteins for 8 h at 37°C still resulted in decrease in the amount of the full-length proteins and concomitant generation of faster migrating species. This conversion should also result from self-degradation because it was not observed with the BepA(Δα9)* and BepA(H246A)* derivatives additionally having the active site mutation (H136R) (Fig. 2*A*), or when incubation was performed in the presence of a metal chelating reagent such as 1,10-phenanthroline or EDTA, inhibitors of zinc metallopeptidases (*SI Appendix*, Fig. S5*A*). N-terminal sequence analysis of a major self-degradation product of BepA(Δα9)* (Fig. 2*A*, green arrowhead) indicated that it was cleaved between Ala-171 and Met-172.

Comparison of the □-casein degradation by BepA(Δα9) and BepA(H246A) with or without the A181E/L182T mutations showed that the A181E/L182T mutations had little effect on the proteolytic activity of the Δα9 and the H246A mutants (*SI Appendix*, Fig. S5*D*). Also, time course-analysis showed that □-casein degradation was observed even after most of BepA(Δα9)* was self-cleaved at the A171/M172 site (*SI Appendix*, Fig. S6*B*), suggesting that the self-cleaved BepA(Δα9)* remained proteolytically active.

Supplementary Materials and Methods

Bacterial strains, plasmids and media

Strain SN896(DE3) was constructed by lysogenizing λ(DE3) into SN896. Derivatives of pUC-bepA or pCDF-bepA-his_{10} encoding a mutant form of BepA were constructed by site-directed mutagenesis using pairs of complementary primers.

Derivatives of pSTD-bepA were constructed by site-directed mutagenesis or by subcloning an EcoRI-HindIII fragment of the pUC-bepA derivatives into the same sites of pSTD689. To construct pCDF-bepA(A181E/L182T)Δ(239-247)::GSGSGS-his_{10}, the codons for Ala-181 and Leu-182 of the *bepA* gene on pCDF-bepAΔ(239-247)::GSGSGS-his_{10} were mutagenized by site-directed mutagenesis using a pair of primers with randomized sequences for these codons. The plasmid library thus obtained was introduced into SN896(DE3) and the transformants were screened for the elevated accumulation of full-length BepA by immunoblotting. One of them that showed the highest accumulation level was stored and the DNA sequence of the *bepA* region was determined.

Unless indicated otherwise, cells were grown in L medium (10 g/L Bacto Tryptone, 5 g/L Bacto Yeast Extract and 5 g/L NaCl; pH was adjusted to 7.2 using NaOH) or M9 medium (without $CaCl_2$).

Ampicillin (50 μg/mL) and spectinomycin (each of 50 μg/mL) were added as appropriate for selecting transformants and for growing plasmid-bearing strains.

SDS-PAGE and Immunoblotting Experiments

Cells of SN56 and SN56/pTWV-lptD-his$_{10}$ additionally carrying a vector (pUC18 or pSTD689) or their derivatives encoding wild-type or a mutant form of BepA were grown to an early log phase at 37°C in M9 medium supplemented with 2 μg/ml thiamine, 0.2% maltose, 1 mM IPTG and either 19 amino acids other than Met or 18 amino acids other than Met and Cys. Proteins were precipitated with 5% trichloroacetic acid, washed with acetone, solubilized in SDS sample buffer with or without 2-mercaptoethanol and separated by 7.5% or 10% SDS-PAGE. Then, they were blotted onto a PVDF membrane filter (Merck Millipore; Billerica, MA, USA). The filter was blocked with 5% skimmed milk, and probed with anti-BepA, anti-LptD, or anti-DegP antiserum followed by HRP-conjugated anti-rabbit goat antibody. The proteins recognized by the antibodies were visualized using ECL Prime Western Blotting Detection Reagents (GE Healthcare) and a lumino-image analyzer (LAS-4000mini; Fujifilm).

Pulse-Chase and Immunoprecipitation Experiments

SN56 carrying pUC18 or its derivative encoding wild-type or a mutant form of BepA were grown at 30 or 37°C to an early log phase in M9 medium supplemented with 18 amino acids other than Met and Cys, 2 μg/ml thiamine, and 0.2% maltose. Then the cells were induced with 1 mM (final conc.) IPTG for the appropriate time periods, labeled with 370 kBq/ml [^{35}S]-Met (American Radiolabeled Chemicals) for 3 min at 30 or 37°C, and chased with 0.07% (final conc.) cold Met. At the indicated time points, a portion of the cultures were withdrawn and mixed with the same volume of 10% trichloroacetic acid to precipitate proteins. The proteins were dissolved in 50 μl of 50 mM Tris·HCl (pH 8.1) containing 1% SDS and 1 mM EDTA, boiled for 5 min, and diluted with 1 ml of Triton buffer containing 50 mM Tris·HCl (pH 8.1), 150 mM NaCl, 2% (wt/vol) Triton X-100, and 0.1 mM EDTA. After removal of insoluble materials by centrifugation at 20,000 × g for 5 min, the supernatant was subjected to immunoprecipitation with anti-LptD antiserum and Dynabeads Protein A (Invitrogen). Proteins recovered with the antibody were eluted from beads by boiling for 5 min in SDS sample buffer without 2-mercaptoethanol, separated by 7.5% SDS/PAGE, and visualized with with BAS-1800 (Fujifilm). Where specified, the eluted samples were further treated with 10% (final conc.) 2-mercaptoethanol before SDS/PAGE. Proteins were separated by 7.5% SDS/PAGE, and visualized with with BAS-1800 (Fujifilm). Band intensities were quantified by using MultiGauge software (Fujifilm).

Purification of BepA.

SN896(DE3) cells carrying pCDF-bepA-his$_{10}$ derivatives were grown in L medium at 30°C. When the culture OD (at 600 nm) reached 0.2, expression of the BepA derivatives was induced with 75 μM IPTG for 2 h. Cells were then harvested, washed once with 5 mM Tris·HCl (pH 8.0), and resuspended in 5 mM Tris·HCl (pH 8.0) containing 300 mM sucrose, 10 μg/ml DNase I and cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche). They were converted into spheroplasts by addition of 50 μg/ml lysozyme and 1 mM EDTA followed by incubation for 20 min at 4°C. After addition of 2 mM $MgCl_2$, the spheroplasts and insoluble materials were removed by successive centrifugations at 10,000 × g for 5 min and at 100,000 × g for 30 min to obtain the periplasmic fraction. The periplasmic fraction was applied to a TALON metal affinity resin (Clontech) column. The column was successively washed with buffer A [5 mM Tris·HCl (pH 8.0), 50 mM NaCl] and buffer A containing 5 mM imidazole, and finally eluted with buffer A containing 250 mM imidazole. The buffer of eluted fraction was exchanged to 5 mM Tris·HCl (pH 8.0) containing 10% glycerol by passage through a Sephadex G-25 desalting column (PD-10; GE Healthcare), followed by concentration by the Amicon® Ultra centrifugal filters (Millipore). Protein concentration of purified proteins was determined using the Bio-Rad Bradford protein assay.

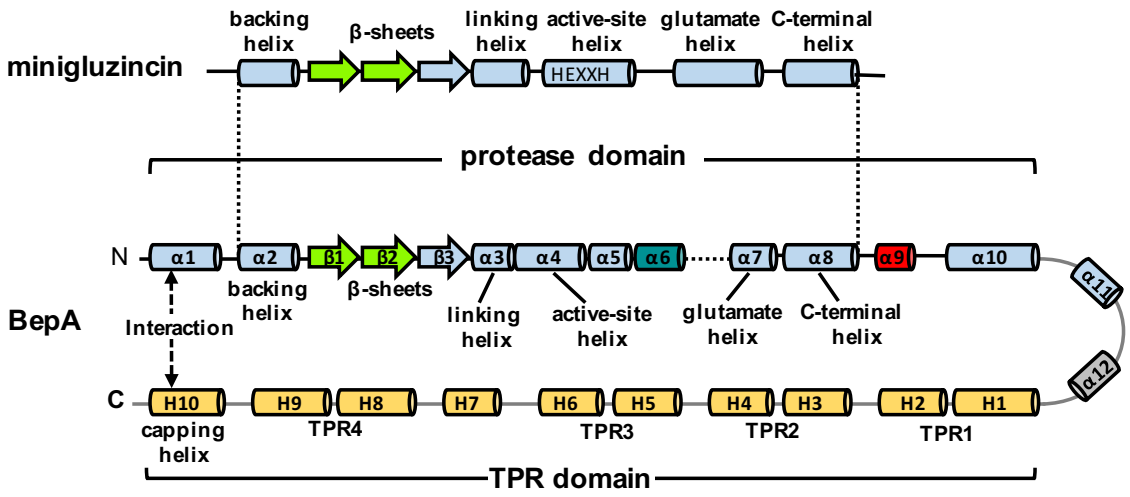

Fig. S1. Comparison of the secondary structure arrangements between minigluzincin and BepA. The secondary structure arrangements of the BepA (15) and minigluzincin (23) are schematically depicted. Arrows and rods indicate β-strands and α-helices, respectively, and colored as in Fig. 1*A*.

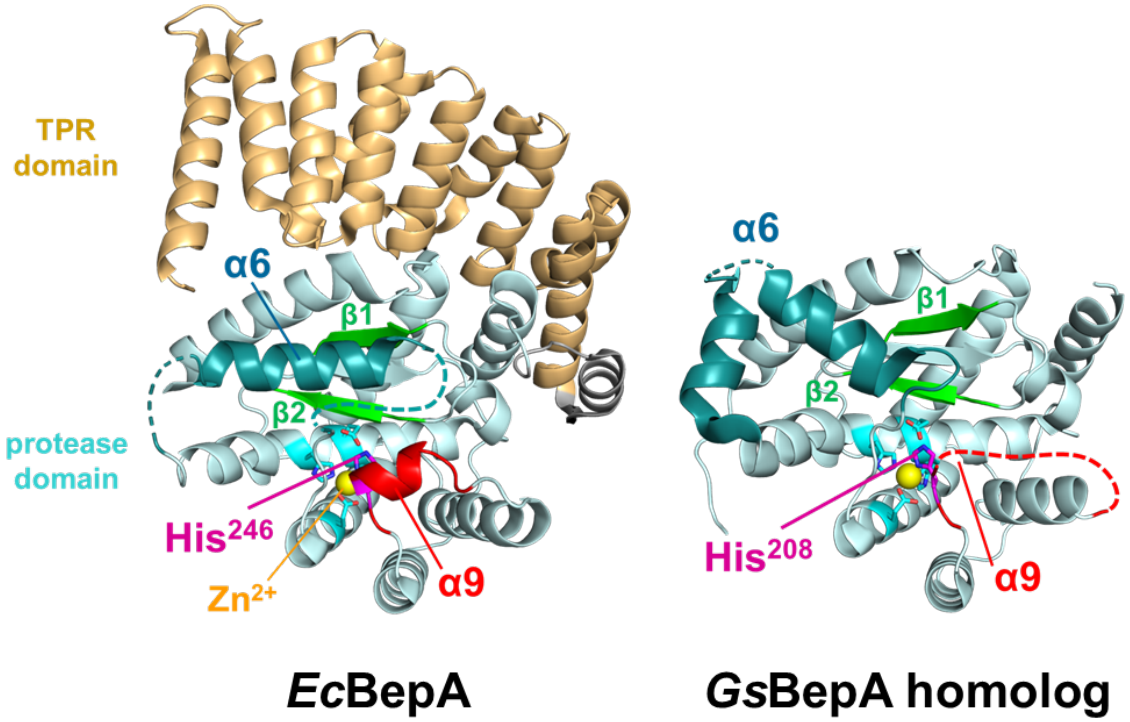

Fig. S2. Structures of *E. coli* BepA (PBD ID: 6AIT) and *G. sulfurreducens* BepA homolog (PDB ID 3C37). The regions corresponding to the α9/H246 loop (red), α6 loop (teal), β1/ β2 (light green), zinc atom (yellow) and His-246 residue (magenta) of *E. coli* BepA are colored as in Fig. 1*A*.

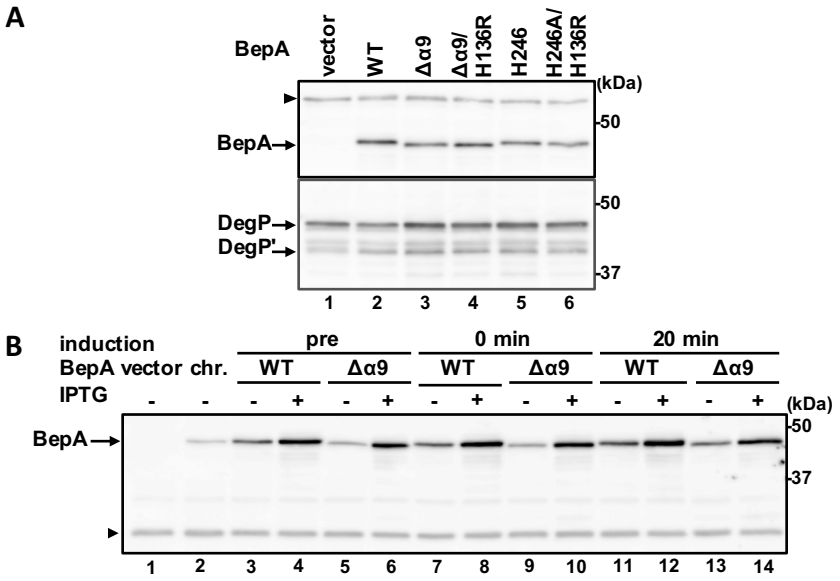

Fig. S3. Accumulation levels of BepA and DegP under the conditions of the pulse-chase experiments shown in Fig. 1. (*A*) Cells were grown and treated as in Fig. 1*D* except that they were not pulse-labeled. A portion was withdrawn at the time point corresponding to just before pulse-labeling or at 60 min chase for analysis of BepA and DegP, respectively. Proteins were acid-precipitated and subjected to SDS/PAGE and immunoblotting analysis with anti-BepA (upper panel) or anti-DegP (lower panel) antiserum. DegP' indicates the degradation products of DegP. The migration positions of molecular mass markers are shown. (*B*) Cells were grown and treated as in Fig. 1*E* except that they were not pulse-labeled. A portion was withdrawn at the time point corresponding to just before (IPTG -) or 15 minutes after (IPTG +) the induction for each culture. Proteins were acid-precipitated and subjected to SDS/PAGE and immunoblotting analysis with anti-BepA antiserum. Arrowheads indicate a non-specific band serving as a loading control. The representative results of two independent replicates are shown.

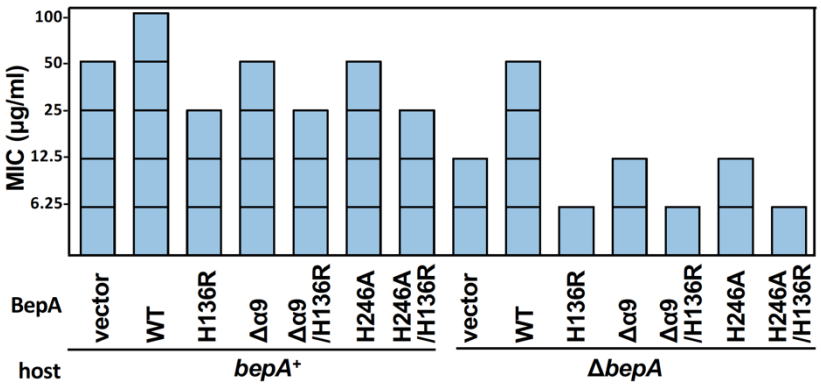

Fig. S4. Effects of expression of the Δα9 and the H246A BepA mutants on the erythromycin sensitivity of the cells. Strains AD16 (Δ*bepA*) and SN56 (*bepA*$^{+}$) carrying a plasmid encoding wild type or the indicated mutant form of BepA were grown to stationary phase in L medium with 0.1% glucose at 30°C. Under this condition, BepA would be expressed at a similar level to chromosomal BepA (1). The minimum inhibitory concentrations (MICs) were determined by spotting 5 μl of the 10^3-fold-diluted cultures on L-0.1% glucose agar plates supplemented with the various concentrations of erythromycin and incubating the plates at 30°C for 24 h, as reported previously (2). The representative results of two independent experiments are shown.

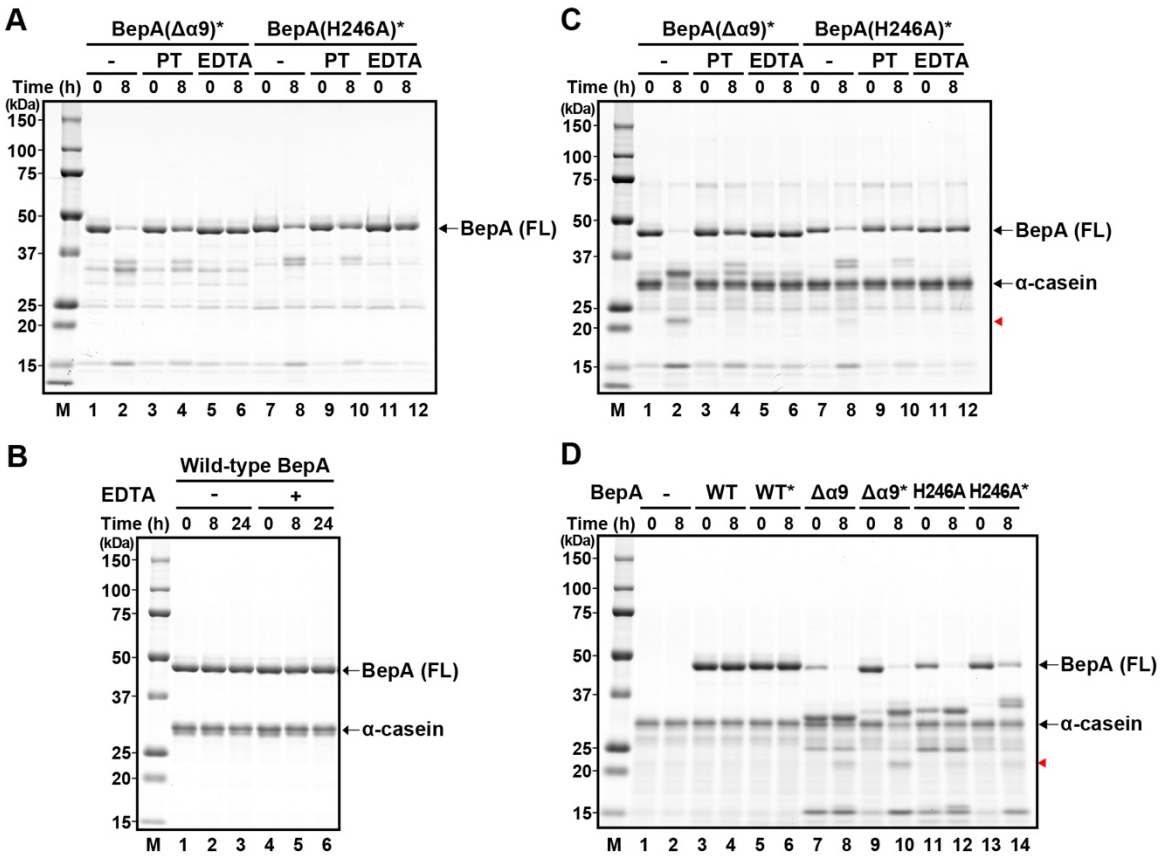

Fig. S5. Effects of metal chelators on the proteolytic activity of the Δα9 and the H246A mutants. (*A*) Effects of metal chelators on the self-cleavage activity of BepA mutants were analyzed by incubating the BepA(Δα9)* or the BepA(H246A)* protein, each carrying the A181E/L182T mutations, at 37°C in the absence (-) or presence of 250 μM 1,10-phenanthroline (PT) or 250 μM EDTA for 0 or 8 h, followed by SDS/PAGE and Coomassie Brilliant Blue G-250 (CBB) staining. (*B*) Wild-type BepA with a C-terminus His_{10}-tag was incubated with α-casein at 37°C in the absence (-) or presence (+) of 250 μM EDTA for 0, 8 or 24 h and analyzed by SDS/PAGE and CBB staining. (*C*) Effects of metal chelators on the caseinolytic activity of BepA(Δα9)* or BepA(H246A)* were analyzed as in *B* except that the reaction mixture contained α-casein. (*D*) Effects of the A181E/L182T mutations on the caseinolytic activity of BepA were analyzed by incubating wild-type (WT) or the mutant forms of the BepA proteins, either with or without the A181E/L182T mutation, with α-casein at 37°C for 0 or 8 h followed by SDS/PAGE and CBB staining. Red arrowheads indicate a degradation product of α-casein.

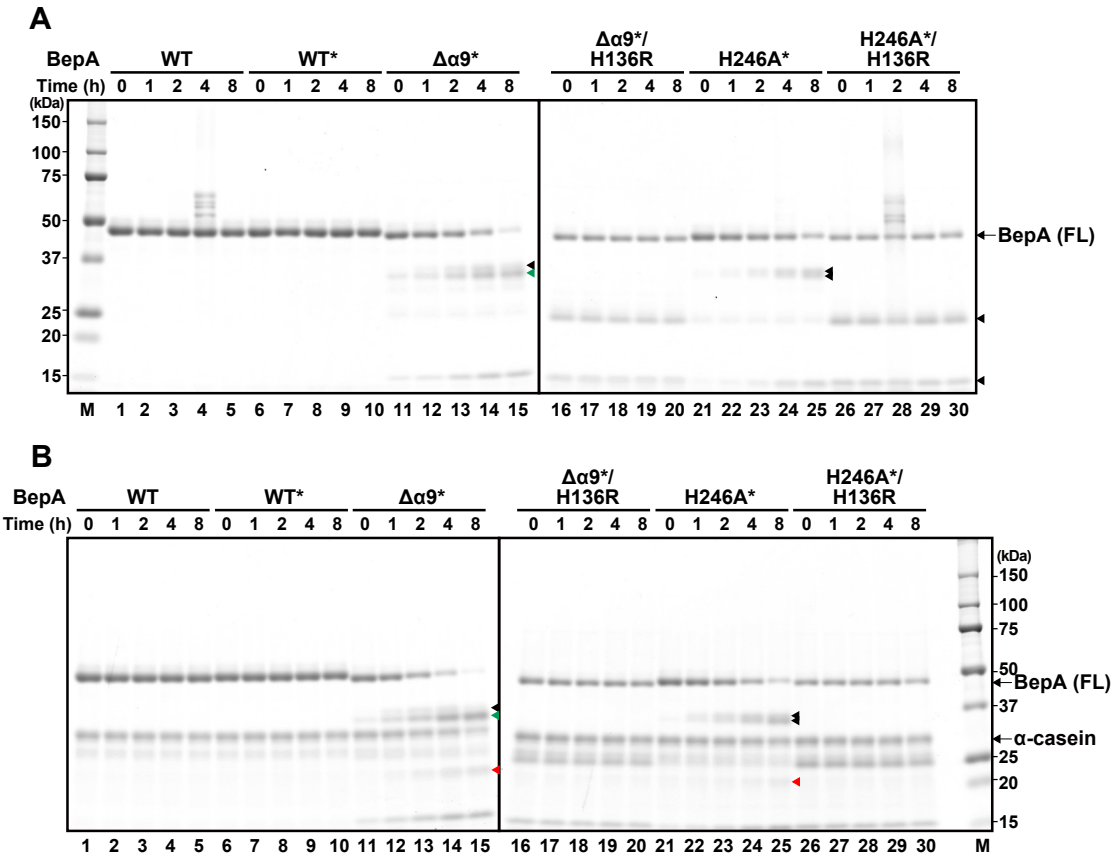

Fig. S6. Time course of α-casein degradation by the α9 and the H246 mutants of BepA. (*A*) Self-degradation of the Δα9 and the H246A mutants. Wild-type (WT) or the indicated mutant forms of BepA with a C-terminal His_{10}-tag were incubated at 37°C for 0, 1, 2, 4 or 8 h and analyzed by SDS/PAGE and CBB staining. BepA derivatives with the A181E/L182T mutations are indicated by asterisks. Full-length (FL) BepA derivatives are indicated. (*B*) Degradation of α-casein by the BepA derivatives. Wild-type (WT) or the indicated mutant forms of BepA, carrying the A181E/L182T mutations (indicated by asterisks) and a C-terminal His_{10}-tag, were incubated with α-casein at 37°C for 0, 1, 2, 4 or 8 h and analyzed by SDS/PAGE and CBB staining. Full-length (FL) BepA derivatives and α-casein are indicated. Proteolytic fragments of α-casein are indicated by red arrowheads. The C-terminal fragments of BepA derivatives generated by cleavage between Ala-171 and Met-172 are indicated by green arrowheads. Other proteolytic fragments of BepA derivatives are indicated by black arrowheads.

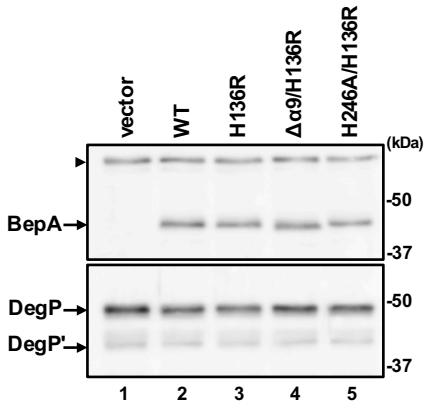

Fig. S7. Accumulation levels of BepA and DegP under the conditions of the pulse-chase experiments shown in Fig. 4. Cells were grown and treated as in Fig. 4 except that they were not pulse-labeled. A portion was withdrawn at the time point corresponding to just before pulse-labeling or at 20 min chase time for analysis of BepA (upper panel) and DegP (lower panel), respectively. Proteins were acid-precipitated and subjected to SDS/PAGE and immunoblotting analysis with anti-BepA or anti-DegP antiserum. Arrowhead indicates non-specific bands serving as a loading control. The representative results of two independent replicates are shown.

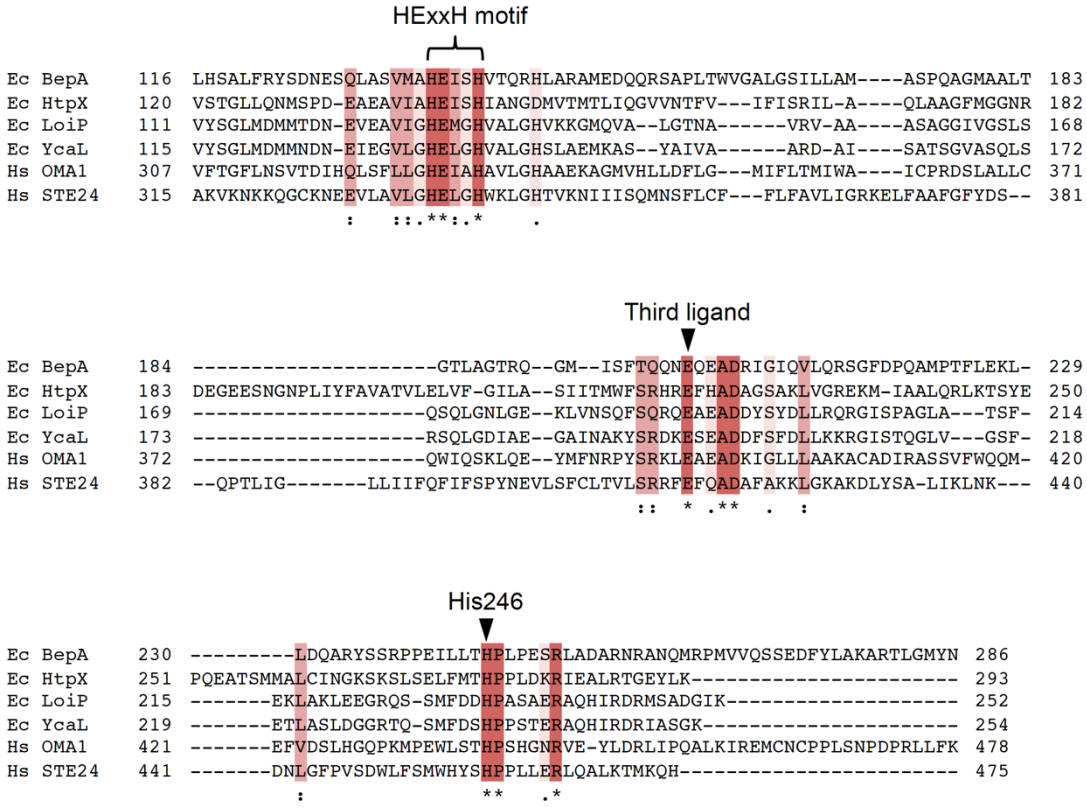

Fig. S8. Sequence alignment of M48 peptidase family proteins. Amino acid sequences of *Escherichia coli* BepA (Ec BepA; UniProt P66948), *E. coli* HtpX (Ec HtpX; UniProt P23894), *E. coli* LoiP (Ec LoiP; UniProt P25894), *E. coli* YcaL (Ec YcaL; UniProt P43674) and *Homo sapiens* OMA1 (Hs OMA1; UniProt Q96E52) and *H. sapiens* ZMPSTE24 (Hs STE24; UniProt O75844) are aligned by the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). Conserved residues are colored in red.

Table S1: Strains and plasmids used in this study

***E. coli* strains**	**Genotype**	**Reference**
AD16	Δ*pro-lac thi*/ F'*lacI*^{q} ZΔM15 *Y*$^{+}$ *pro*$^{+}$	(3)
SN56	AD16 Δ*bepA*::FRT	(4)
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	(5)
SN896	MC4100 Δ*bepA*::FRT	(4)
SN896(DE3)	SN896, λDE3	(4)
Plasmids	**Description**	**Reference or source**
pUC18	Expression vector; P$_{lac}$, *bla*	(6)
pUC-bepA	pUC18 derivative encoding BepA	(4)
pUC-bepA(H136R)	pUC-bepA derivative, H136R	(4)
pUC-bepA(E137Q)	pUC-bepA derivative, E137Q	(4)
pUC-bepAΔ(239-247)::GSGSGS	pUC-bepA derivative, Δα9	This study
pUC-bepA(H136R)Δ(239-247)::GSGSGS	pUC-bepA derivative, H136R, Δα9	This study
pUC-bepA(E137Q)Δ(239-247)::GSGSGS	pUC-bepA derivative, E137Q, Δα9	This study
pUC-bepA(H246A)	pUC-bepA derivative, H246A	This study
pUC-bepA(H136R/H246A)	pUC-bepA derivative, H136R/H246A	This study
pUC-bepA(E137Q/H246A)	pUC-bepA derivative, E137Q/H246A	This study
pSTD689	Expression vector; Plac, *aadA*	(7)
pSTD-bepA	pSTD689 derivative encoding BepA	(2)
pSTD-bepA(H136R)	pSTD-bepA derivative, H136R	This study
pSTD-bepAΔ(239-247)::GSGSGS	pSTD-bepA derivative, Δα9	This study
pSTD-bepA（H136R）Δ(239-247)::GSGSGS	pSTD-bepA derivative, H136R, Δα9	This study
pSTD-bepA(H246A)	pSTD-bepA derivative, H246A	This study
pSTD-bepA(H136R/H246A)	pSTD-bepA derivative, H136R/H246A	This study
pSTD-bepA(E103C)	pSTD-bepA derivative, E103C	This study
pSTD-bepA(E241C)	pSTD-bepA derivative, E241C	This study
pSTD-bepA(E103C/E241C)	pSTD-bepA derivative, E103C/E241C	This study
pTWV228	Expression vector; Plac, *bla*	(8)
pTWV-lptD-his$_{10}$	pTWV228 derivative encoding LptD-His$_{10}$	(2)
pCDFDuet-1	Expression vector; PT7 *aadA*	Novagen
pCDF-bepA-his$_{10}$	pCDFDuet-1 derivative encoding BepA-His$_{10}$	(4)
pCDF-bepA(A181E/L182T)-his$_{10}$	pCDFD-bepA-his$_{10}$ derivative, A181E/L182T	This study
pCDF-bepAΔ(239-247)::GSGSGS-his$_{10}$	pCDFD-bepA-his$_{10}$ derivative, Δα9	This study
pCDF-bepA(A181E/L182T)Δ(239-247)::GSGSGS-his$_{10}$	pCDFD-bepA-his$_{10}$ derivative, A181E/L182T, Δα9	This study
pCDF-bepA(H136R)(A181E/L182T)Δ(239-247)::GSGSGS-his$_{10}$	pCDFD-bepA-his$_{10}$ derivative, H136R/A181E/L182T, Δα9	This study
pCDF-bepA(H246A)-his$_{10}$	pCDFD-bepA-his$_{10}$ derivative, H246A	This study
pCDF-bepA(A181E/L182T)(H246A)-his$_{10}$	pCDFD-bepA-his$_{10}$ derivative, A181E/L182T/H246A	This study
pCDF-bepA(H136R)(A181E/L182T)(H246A)-his$_{10}$	pCDFD-bepA-his$_{10}$ derivative, H136R/A181E/L182T/H246A	This study

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