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Edge-strand of BepA interacts with immature LptD on the β -barrel assembly machine to direct it to on- and off-pathways

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Abstract

The outer membrane (OM) of gram-negative bacteria functions as a selective permeability barrier. *Escherichia coli* periplasmic Zn-metallopeptidase BepA contributes to the maintenance of OM integrity through its involvement in the biogenesis and degradation of LptD, a β -barrel protein component of the lipopolysaccharide translocon. BepA either promotes the maturation of LptD when it is on the normal assembly pathway (on-pathway) or degrades it when its assembly is compromised (off-pathway). BepA performs these functions probably on the β -barrel assembly machinery (BAM) complex. However, how BepA recognizes and directs an immature LptD to different pathways remains unclear. Here, we explored the interactions among BepA, LptD, and the BAM complex. We found that the interaction of the BepA edge-strand located adjacent to the active site with LptD was crucial not only for proteolysis but also, unexpectedly, for assembly promotion of LptD. Site-directed crosslinking analyses indicated that the unstructured N-terminal half of the β -barrel-forming domain of an immature LptD contacts with the BepA edge-strand. Furthermore, the C-terminal region of the β -barrel-forming domain of the BepA-bound LptD intermediate interacted with a “seam” strand of BamA, suggesting that BepA recognized LptD assembling on the BAM complex. Our findings provide important insights into the functional mechanism of BepA.

Introduction

The cell envelope of diderm bacteria is composed of two membranes, namely the inner (cytoplasmic) membrane (IM) and the outer membrane (OM). The intermembrane space, known as periplasmic space, contains a peptidoglycan layer. The OM is the outermost layer of a cell directly facing the external milieu and acts as a selective permeability barrier that prevents the penetration of toxic compounds including antibiotics (Nikaido, 2003). The cell surface localization as well as the functional impotence of the OM make its components suitable drug targets.

Outer membrane proteins (OMPs), generally exhibiting a β -barrel fold formed by more than 8 β -strands, play important roles in maintaining the structural and functional integrity of the OM (Konovalova et al., 2017). Therefore, irregularities in OMP biogenesis result in elevated cellular sensitivity to toxic compounds (Hart et al., 2019; Oh et al., 2011). After synthesis in the cytoplasm and following translocation across the IM to the periplasm through the SecYEG translocon, OMPs are delivered to the OM by periplasmic chaperones such as DegP, Skp, and SurA, and are finally integrated into the OM (Konovalova et al., 2017; Plummer and Fleming, 2016; Ricci and Silhavy, 2019). The OM assembly of OMPs is mediated by the β -barrel assembly machinery (BAM) complex consisting of a β -barrel OMP, BamA, and four lipoproteins, namely BamB, BamC, BamD, and BamE (Plummer and Fleming, 2016; Ricci and Silhavy, 2019; Tomasek and Kahne, 2021). Among the BAM complex subunits, BamA and BamD are the only essential components, although recent studies have shown that certain BamA mutations render all other BAM subunits dispensable (Hart et al., 2020). The BAM complex has a silk-hat-like structure (Bakelar et al., 2016; Gu et al., 2016; Han et al., 2016; Iadanza et al., 2016; Tomasek et al., 2020); the OM-embedded C-terminal β -barrel domain of BamA forms the “crown” and the N-terminal periplasmic polypeptide transport-associated (POTRA) domains of BamA form the “brim” together with the BamB/C/D/E lipoproteins.

Lipopolysaccharide (LPS), another major OM constituent localized in the outer leaflet of the OM, is also important for the maintenance of the structure and function of the OM (Sperandeo et al., 2017). LPS is synthesized on the cytoplasmic side of the IM and flipped across the IM to the periplasm. After maturation, it is transported to the OM by the LPS transport (Lpt) proteins (Sperandeo et al., 2017). A heterodimer of LptD, a β -barrel OMP, and LptE, a lipoprotein, plays roles in the final step to insert LPS into the OM (Dong et al., 2014; Qiao et al., 2014; Wu et al., 2006). The OM assembly process of LptD is unique in that it is accompanied by

rearrangement of intramolecular disulfide bonds. The mature form of LptD possesses two disulfide bonds formed by non-consecutive pairs of Cys residues (C31-C724 and C173-C725) (Ruiz et al., 2010). It has been shown, however, that an assembly intermediate of LptD having disulfide bonds formed by consecutive pairs of the Cys residues (C31-C173 and C724-C725) (LptD^C; LptD with Consecutive disulfide bonds) is first generated and isomerized to LptD^{NC} (LptD with Non-Consecutive disulfide bonds) during its assembly/maturation, which is triggered by the association of LptD with LptE (Chng et al., 2012; Narita et al., 2013). The LptD^C to LptD^{NC} conversion should occur at a later step in the OM assembly because LptD presumably associates with LptE on the BAM complex (Chimalakonda et al., 2011; Chng et al., 2012; Lee et al., 2016; Narita et al., 2013).

BepA (formally called YfgC), a bi-functional periplasmic protein that plays an important role in maintaining OM integrity (Narita et al., 2013), belongs to the M48 family zinc metalloproteases that include prokaryotic and eukaryotic proteases (such as Ste24, Oma1, and HptX) involved in membrane quality control (Rawlings et al., 2018). We have previously shown that BepA is involved in the biogenesis and quality control of LptD. While BepA promotes the LptD^C to LptD^{NC} conversion (chaperone-like function) (Narita et al., 2013), it also degrades the stalled or misassembled LptD^C molecules that are generated due to an *lptD* mutation (*lptD4213*) or decreased availability of or weakened interaction with LptE (protease function) (Narita et al., 2013; Soltes et al., 2017). The BepA protein consists of an N-terminal M48 metalloprotease domain and a C-terminal tetratricopeptide repeat (TPR) domain that are associated closely to form a compact structure (**Figure 1A**) (Bryant et al., 2021; Shahrizal et al., 2019). Our previous study suggested that the TPR domain of BepA directly interacts with LptD and with the BAM complex with its TPR domain inserted into the interior of the periplasmic part (brim) of the BAM complex (Daimon et al., 2017; Narita et al., 2013). A mutational study has suggested that these interactions are important for BepA functions (Shahrizal et al., 2019). Recent studies have also shown that the His-246 residue of BepA that coordinates the zinc ion at the proteolytic active site acts as an ON/OFF switch (His switch) for the proteolytic activity of BepA (Bryant et al., 2021; Daimon et al., 2020). The dual functions of BepA should be appropriately regulated because the unregulated expression of the proteolytic activity of BepA caused by an H246 mutation leads to the degradation of the normally assembling LptD intermediate (Daimon et al., 2020). However, information on the molecular mechanism of this regulation and the modes of the BepA–LptD interaction in each BepA function remains elusive.

Here, we investigated the mechanism by which BepA established interaction with LptD in promotion of its assembly and degradation. Our results showed that a conserved β -strand (edge-strand) located adjacent to the BepA active site directly contacts with LptD and plays important roles in substrate proteolysis, like many other proteases. In addition, we unexpectedly found that the edge-strand-mediated interaction with a substrate is also required for the chaperone-like function of BepA, which should be enabled by the His switch-mediated repression of the proteolytic activity. Crosslinking experiments demonstrated that BepA could interact with an LptD molecule assembling on the BAM complex. Based on these observations, we propose a model explaining the edge-strand/His switch-mediated functional regulation of BepA in LptD assembly/degradation.

Results

Interaction of the BepA edge-strand with LptD is crucial not only for proteolysis but also for assembly promotion of LptD by BepA

Zinc-metalloproteases usually possess a β -strand, called edge-strand, located close to their proteolytic active sites (Akiyama et al., 2015; López-Pelegrín et al., 2013; Stöcker and Bode, 1995). While the edge-strand is known to play a critical role in substrate proteolysis by directly interacting with a substrate polypeptide by the strand addition mechanism and converts it into an extended conformation for its presentation to the active site and proteolysis, it has not been well-characterized in M48 proteases. The solved structures of *Escherichia coli* BepA (Bryant et al., 2021; Shahrizal et al., 2019) show that it has a β -strand (β 2) that is conserved among the M48-peptidases and is located adjacent to the active site (**Figure 1A** and **Figure 1-figure supplement 1**), suggesting that this strand presumably acts as an edge-strand. To examine the role of the β 2 strand in BepA functions, we constructed BepA mutants by introducing Pro at each position in β 2 (from Asn-105 to Phe-110; **Figure 1A**). We then investigated the effects of β 2 mutations on the proteolytic activity of BepA against overproduced LptD. When LptD is overproduced from a multi-copy plasmid, it mainly accumulates in the form of LptD^C possibly due to the limited availability of its partner protein, LptE (Daimon et al., 2020, 2017). This species probably represents a “normal” assembly intermediate as it is associated with the BAM complex (See below) and can be converted to the mature form (LptD^{NC}) when LptE is co-expressed (Miyazaki et al., 2018). As reported previously (Daimon et al., 2020, 2017), overproduced LptD was degraded by co-expressed wild-type BepA to generate discrete degradation products (**Figure 1B**). To examine the possible roles of the β 2 strand in the function of BepA, we introduced a Pro substitution into the β 2 strand, as a Pro residue would affect the secondary structure, and thus the function, of this strand. We found that the expression of a few BepA mutants (N105P, A106P, F107P, and A108P) led to a significantly decreased generation of the LptD degradation products (**Figure 1B**). These mutants accumulated at a level comparable to that of wild-type BepA, suggesting that they did not exert drastic effects on the overall structure of BepA. Furthermore, some of these mutations compromised the degradation of BamA in a Δ *surA* strain (Daimon et al., 2017) and the self-cleavage of BepA-His₁₀ (BepA possessing a C-terminal His₁₀-tag) within the His₁₀-tag (Narita et al., 2013) (**Figure 1-figure supplement 2A and 2B**). These results strongly suggest that the β 2 strand is important for the proteolytic activity of BepA.

We next analyzed whether the $\beta 2$ strand directly interacts with a substrate by using a site-directed *in vivo* photo-crosslinking approach (Chin and Schultz, 2002; Miyazaki et al., 2020a). We expressed derivatives of BepA-His₁₀ harboring a photoreactive amino acid analog, *p*-benzoyl-L-phenylalanine (*p*BPA), at each position in the $\beta 2$ strand in a Δ bepA strain and examined their complementation activity regarding erythromycin (EM) sensitivity of the cell and self-cleavage of BepA-His₁₀. The results showed that certain mutants (F107*p*BPA, F109*p*BPA, and F110*p*BPA) were as functional as the wild-type, but others exhibited neither significant complementation activity nor self-cleavage (**Figure 1-figure supplement 3A and 3B**). For the photo-crosslinking experiments conducted in this study, we used BepA variants harboring the E137Q mutation in the H¹³⁶ExxH motif for two reasons. First, this mutation would repress the possible degradation of LptD by the BepA derivatives. Second, our previous study has shown that this mutation stabilizes the interaction of BepA with LptD (Daimon et al., 2017). Following UV irradiation of cells expressing each of the BepA mutants, BepA-His₁₀ and its crosslinked products were purified from the membrane fractions by affinity isolation using Ni-NTA agarose and subjected to immunoblotting analysis (**Figure 1C**). The N105*p*BPA, A106*p*BPA, F107*p*BPA, and A108*p*BPA derivatives of BepA generated, in a UV-dependent manner, evident crosslinked products that reacted with both anti-BepA and anti-LptD antibodies. Taken together, these results imply that the $\beta 2$ strand of BepA directly interacts with LptD and plays an important role in their degradation. The structure, intramolecular disposition, as well as involvement in substrate interaction and proteolysis of the $\beta 2$ strand strongly supports the hypothesis that it indeed acts as the edge-strand to recognize and present a substrate to the active site. We have thus referred to the $\beta 2$ strand as “the edge-strand” hereafter. BepA N105*p*BPA and F107*p*BPA derivatives also generated the unidentified crosslinked products (**Figure 1C, open arrowheads**), suggesting that the edge strand would interact with some other substrates. The differential effects of the mutations on the degradation of LptD and BamA and the self-cleavage of the C-terminal tag (**Figure 1B and Figure 1-figure supplement 2A and 2B**) might reflect their different interaction properties and/or affinities for the BepA edge-strand. Our previous study also suggested substrate-specific interaction of an edge-strand for another metallopeptidase, RseP (Akiyama et al., 2015).

BepA not only degrades the stalled or misassembled LptD (when LptD is on the normal (on-) pathway) but also promotes the maturation of LptD through facilitating its assembly with the partner protein LptE (chaperone-like activity) (on the off-pathway) (Narita et al., 2013). Therefore, we examined the effects of the edge-strand Pro mutations on the chaperone-like activity of BepA. LptD possesses two intramolecular disulfide bonds.

While the mature (fully assembled) LptD has non-consecutive disulfide bonds, an assembly intermediate form of LptD (LptD^C) that accumulates in the absence of functional BepA possesses consecutive disulfide bonds. Consistent with our previous results (Narita et al., 2013), expression of the wild-type BepA in a $\Delta bepA$ strain markedly decreased the accumulation of LptD^C. On the contrary, only a partial decrease in accumulation was observed with the expression of the E137Q mutant. We found that the A106P mutant was defective in reducing LptD^C accumulation, suggesting that a mutation in the edge-strand could affect the chaperone-like function (**Figure 1-figure supplement 4A**). Subsequently, we examined the effects of two edge-strand mutations—A106P and F1107P—that markedly compromised LptD degradation on the chaperone-like function of BepA using pulse-chase experiments. Expression of the wild-type BepA in $\Delta bepA$ cells significantly accelerated the conversion of LptD^C to LptD^{NC}, whereas expression of the protease-dead E137Q mutant demonstrated weakened conversion (**Figure 1D**). The acceleration of the LptD^C to LptD^{NC} conversion by the F107P mutant was weaker than that by the wild-type BepA (almost the same or slightly stronger as compared with the E137Q mutant), and no detectable acceleration was observed with the A106P mutant (**Figure 1D**). These results unexpectedly revealed that the edge-strand was also important for the chaperone-like activity of BepA.

BepA derivatives with a Cys substitution (note that BepA intrinsically does not possess a Cys residue), instead of a Pro substitution, at the positions of Asn-105, Ala-106, or Phe-107 in the $\beta 2$ strand exhibited almost normal chaperone-like and proteolytic functions (**Figure 1-figure supplement 4B and 4C**). This supports the idea that the secondary structure of the edge-strand, but not the individual amino acid residues, is important for its function. Note that, although it is possible that a Pro mutation also affects a local structure around the edge-strand, the A106P mutant, which was defective in LptD and BamA degradation, still exhibited significant self-cleavage of the probably unstructured C-terminal tag (**Figure 1-figure supplement 2B**), suggesting that this mutation, and possibly the other Pro mutations, specifically affected the edge-strand structure, but not the active site or the protein's overall structures.

BepA interacts with the N-terminal half of the β -barrel-forming domain of the LptD assembly intermediate

While BepA interacts with LptD to promote either its proper OM assembly or proteolytic elimination depending on the situation (Narita et al., 2013), the details of the BepA–LptD interaction, including the region(s) in LptD to which BepA binds, remain largely unknown. Thus, we performed a systematic photo-crosslinking

analysis to identify the BepA-contact region in the LptD assembly intermediate LptD^C. We performed photo-crosslinking experiments in cells ectopically co-expressing an LptD derivative containing a photo-reactive amino acid analog *p*BPA [LptD(*p*BPA)] and a protease-dead variant of BepA, BepA(E137Q). We first introduced *p*BPA at each of the 50 positions (approximately every 15 residues) in the mature part of LptD and performed photo-crosslinking analysis. Cells expressing LptD(*p*BPA)-His₁₀ and BepA(E137Q) were grown and UV-irradiated, and the total cellular proteins were analyzed by immunoblotting with anti-BepA and anti-His antibodies. Under this condition, expressed LptD-His₁₀ was considerably accumulated as LptD^C (**Figure 2-figure supplement 1**) irrespective of co-expression of BepA(E137Q). We detected clear crosslinking with BepA mainly in the N-terminal half of the LptD β -barrel-forming domain (**Figure 2A**). We then performed a detailed photo-crosslinking analysis for the 20 additional sites in the N-terminal half of the LptD β -barrel-forming domain (**Figure 2B**) and found that BepA was crosslinked at several of these sites. The residues at the BepA-cross-linkable sites were oriented both inward and outward in the mature LptD β -barrel (**Figure 2C and 2D**). Moreover, the residue Gln-393 at which the strongest crosslinking was observed was oriented inward. These results suggest that LptD while interacting with BepA would not assume a higher-order structure like closed β -barrel (See Discussion). We selected a few LptD(*p*BPA) derivatives that had been crosslinked with BepA as representatives and examined their functionality. They supported the growth of LptD-depleted cells when expressed from a plasmid, indicating that they were functional (**Figure 2-figure supplement 2**). The above-mentioned crosslinking results thus likely reflect a functional interaction of LptD with BepA in the normal assembly pathway.

BepA edge-strand directly interacts with the Tyr-331 residue in the β 7 strand of the LptD β -barrel domain

We investigated further to identify the region of LptD that interacts with the BepA edge-strand. First, we examined the effects of the BepA edge-strand Pro mutations (F107P and A106P) on the LptD(*p*BPA)–BepA crosslinking. The F107P mutation significantly decreased the efficiency of the crosslinking of BepA(N105*p*BPA) and BepA(A106*p*BPA) with LptD (**Figure 3-figure supplement 1**). Additionally, the A106P mutation exhibited a similar effect on the crosslinking of BepA(N105*p*BPA) with LptD. Based on these effects on crosslinking, we inferred that these mutations affected the interaction of the edge-strand with LptD. Subsequently, we selected several LptD(*p*BPA) derivatives that showed relatively strong crosslinking with BepA and examined the effect of F107P and A106P mutations in the BepA edge-strand on the crosslinking of LptD(*p*BPA) with BepA. We found

that these mutations altered LptD–BepA crosslinking in a site-specific manner. Further, the amount of crosslinked products markedly decreased for LptD(Y331*p*BPA), but not for other mutants (**Figure 3A**). These results strongly suggest that the region around Tyr-331 in the β 7 strand of the LptD β -barrel domain (**Figure 2D**) is crosslinked with the edge-strand of BepA.

To further confirm the direct interaction of the BepA edge-strand with the LptD β 7 strand, we conducted site-specific disulfide crosslinking experiments. For this analysis, we used the above-described single Cys derivatives of BepA harboring a Cys residue at the position of Asn-105, Ala-106, or Phe-107, and derivatives of LptD having a Cys substitution at either of the six positions, including Tyr-331 at which introduction of *p*BPA showed clear crosslinking with BepA (**Figure 3A**). The wild-type LptD protein harbors intrinsic four Cys residues that form two disulfide bonds essential for the LptD function; therefore, each of these Cys-substituted LptD mutants possessed five Cys residues in total. We confirmed that these Cys-substituted LptD derivatives accumulated normally and retained their function (**Figure 3-figure supplement 2**). Cells expressing a combination of BepA Cys mutants and LptD Cys mutants were grown, and total proteins were acid-denatured and dissolved in SDS containing N-ethylmaleimide (NEM; NEM was included to block free Cys residues). Then, LptD-His₁₀ and its crosslinked products were affinity-isolated using the C-terminal His₁₀-tag, treated with or without 2-mercaptoethanol (ME), and analyzed by SDS-PAGE and anti-BepA immunoblotting. We observed that certain combinations of BepA and LptD derivatives showed a high-molecular-mass band in electrophoresis results. Among them, the combination of BepA(N105C) and LptD(Y331C) showed the most intense band that exhibited reaction with the anti-BepA antibody (**Figure 3B** and **Figure 3-figure supplement 2B**, no ME). These high-molecular-mass bands were not observed with the wild-type LptD (no additional Cys) and disappeared upon treatment with ME, suggesting that they were disulfide-crosslinked products (**Figure 3B** and **Figure 3-figure supplement 2B**, + ME). These results are consistent with the photo-cross-linking experiments (**Figure 3A**) and indicate that the edge-strand of BepA can directly bind to several regions in the N-terminal half of the LptD β -barrel-forming domain, which includes the β 7 strand containing Tyr-331.

BepA interacts with an LptD intermediate associating with the seam region of BamA on the BAM complex

We further investigated the mode of the interaction of the BepA-associated LptD with the BAM complex. It has been recently shown that an assembly intermediate of LptD (LptD4213) interacts with the seam

region formed by the N- and C-terminal β -strands (β 1 and β 16, respectively) in the BamA barrel domain and forms a hetero-complex, in which the C-terminal β -signal of the LptD4213 was associated with the β 1 strand of the BamA seam (Lee et al., 2019). It has been suggested that the interaction of the β -signal with the BamA β 1 strand generally facilitates the folding of the β -barrel domain of a substrate OMP (See **Figure 4D** and **5B**) (Tomasek and Kahne, 2021). We first examined the interaction of the BepA-associated LptD intermediate with BamA and BamD by conducting *in vivo* photo-crosslinking experiments using the LptD derivatives with *pBPA* at the position of Glu-749 or Tyr-726 in addition to the position of Tyr-331. *pBPA* at Glu-749 and Tyr-726 residue, both of which are located near the β -signal of LptD, have been reported to be crosslinked with BamA and BamD, respectively, during the LptD assembly (**Figure 4D**) (Lee et al., 2019, 2018). Complementation assay results showed that either of the LptD derivatives containing one or two *pBPA* at Glu-749 and Tyr-726 were functional (**Figure 4-figure supplement 1**). After UV-irradiation of the cells expressing LptD-His₁₀ and the *pBPA* derivatives, LptD-His₁₀ and its crosslinked products were affinity-isolated from the membrane fractions and analyzed by immunoblotting. The single *pBPA* derivatives indeed generated crosslinked products with the expected factors as: LptD(Y331*pBPA*) with BepA, LptD(D749*pBPA*) with BamA, and LptD(Y726*pBPA*) with BamD (**Figure 4A** and **4B**). With the double *pBPA* derivatives LptD(Y331/D749*pBPA*) and LptD(Y331/Y726*pBPA*), new crosslinked products with higher molecular sizes were observed in addition to the ones observed with single *pBPA* mutants (**Figure 4A** and **4B**). These results showed that the higher molecular-sized products represented BepA–LptD(Y331/D749*pBPA*)–BamA and BepA–LptD(Y331/Y726*pBPA*)–BamD crosslinked products and that BepA interacts with an assembly intermediate of LptD on the BAM complex.

We further conducted photo-crosslinking experiments using LptD locked on the BAM complex by using an SH-crosslinker, 1,4-bismaleimidobutane (BMB). It has been previously shown (Lee et al., 2019) that a cysteine placed near the N-terminal β -strand of the BamA β -barrel (S439C) was crosslinked with a cysteine introduced near the β -signal of LptD (E733C) via BMB treatment (**Figure 4D**; **Figure 4-figure supplement 2A**). We introduced a Y331*pBPA* mutation into LptD(E733C) and confirmed that the resultant mutant was functional (Fig. S10B). When the LptD(Y331*pBPA*/E733C) mutant was expressed in a strain having a chromosomal *bamA*(S439C) mutant gene, LptD–BepA crosslinked products were detected upon UV irradiation, whereas LptD–BamA crosslinked products were detected upon BMB treatment. When cells were first treated with BMB and then UV-irradiated, a higher mass product that reacted with both anti-BepA and anti-BamA antibodies was generated.

259 The generation of this product depended on both BMB-treatment and UV-irradiation (**Figure 4C**). These results
 260 are fully consistent with the above photo-crosslinking results and further demonstrated that BepA could interact
 261 with an LptD assembly intermediate associating with the seam site of BamA on the BAM complex.

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Discussion

The involvement of BepA in the maintenance of structural and functional integrity of the OM was first suggested on the basis of increased sensitivities of the $\Delta bepA(yfgC)$ strain to several antibiotics and chemicals, which was similar to the characteristics of strains with a disruption of genes encoding proteins engaged in outer membrane biogenesis (Narita et al., 2013; Ruiz et al., 2005; Tamae et al., 2008). We have previously shown that BepA is involved in the biogenesis and quality control of LptD, probably on the BAM complex (Daimon et al., 2020, 2017; Narita et al., 2013). However, the mechanism by which BepA recognizes and interacts with LptD remains elusive. This information is important to understand the mechanism of BepA to distinguish between the normal (on-) and off-pathway intermediates of LptD that are either assembled into the OM or degraded, respectively. To gain insight into the BepA–LptD interaction, we examined the role of the conserved edge-strand of BepA in its function. Our results showed that the BepA edge-strand participates not only in the proteolytic activity but also, unexpectedly, in the chaperone-like activity through its direct interaction with LptD (**Figure 1**). The results of the photo- and disulfide-crosslinking analyses indicated that the N-terminal half of the LptD β -barrel-forming domain interacts with the BepA edge-strand (**Figure 2** and **Figure 3**). Moreover, we showed that BepA demonstrated interaction with an LptD assembly intermediate whose C-terminal region was associated with the seam strand of BamA on the BAM complex (**Figure 4**). These observations provide useful insights into the BepA functions involved in the biogenesis and quality control of LptD.

Our previous results have shown that BepA interacts with the BAM complex via its C-terminal TPR domain partly inserted into the periplasmic ring-like structure of the BAM complex (Daimon et al., 2017; Shahrizal et al., 2019). In this study, we observed that BepA showed crosslinking with the N-terminal half of the LptD β -barrel-forming domain but not with the C-terminal half. This observation was consistent with the localization of LptD on the BAM complex; the N-terminal region was localized at/near the periplasmic surface of the BAM complex, and the C-terminal region was inserted deep into the BAM complex's interior. The results of the Cys crosslinking experiments showed that several positions, including Tyr-331, in the N-terminal half of the LptD β -barrel-forming domain interacted with the edge-strand. The recently solved structures of BepA (Bryant et al., 2021; Shahrizal et al., 2019) showed that the active site region including the edge-strand was located inside the BepA molecule, leading to the suggestion that structural changes in BepA including the movement of the $\alpha 6$ and $\alpha 9$ loops covering the active site/edge-strand were necessary to enable access of a substrate to the active site/edge-

strand region (**Figure 5-figure supplement 1**). However, even after such structural changes, the edge-strand should be located at a recessed position. This suggests that the regions around the BepA-crosslinked positions in the LptD assembly intermediate do not form an extensive β -sheet structure as found in the mature LptD to gain access to the edge-strand of BepA (**Figure 5-figure supplement 1**). Furthermore, *p*BPA at positions of both the inward-pointing and outward-pointing residues in the mature β -barrel domain of LptD was crosslinked with BepA. Collectively, these results support the hypothesis that the BepA-interacting region of the LptD intermediate is largely unstructured. The unstructured nature of these proteins is in fact helpful to accommodate in or around the narrow space inside the BAM complex. The TPR domain of BepA has also been shown to contact with LptD to promote its biogenesis and degradation (Daimon et al., 2017). Currently, we have no information available on the part of LptD that interacts with the TPR domain. The TPR domain may act either together with the edge-strand at the same step or independently at other steps during LptD assembly and degradation.

Further, our results suggest that the proper interaction of LptD with the edge-strand of BepA is important for the promotion of its assembly as well as degradation by BepA (**Figure 1** and **Figure 5A**). This finding was unexpected as it raised a question of how the degradation of the normally assembling LptD intermediate can be avoided despite its interaction with the edge-strand near the protease active site. In the BepA structures, the conserved His-246 residue is coordinated to a zinc ion in the active site to block the activation of a water molecule necessary for the catalysis of the proteolytic reaction (Bryant et al., 2021; Shahrizal et al., 2019). We have recently reported that His-246 acts as a switch to regulate the proteolytic activity of BepA (Daimon et al., 2020). This His-Switch-mediated repression of the protease activity would enable the interaction of LptD intermediates at the protease active site of BepA without degradation. Indeed, the derepressed BepA(H246A) mutant degrades an LptD intermediate on the normal assembly pathway, instead of promoting its biogenesis (Daimon et al., 2020). One possible model for the discrimination of LptD intermediates by BepA for assembly or degradation would be as follows (**Figure 5A**). In both assembly and degradation pathways of LptD, an unknown signal(s), such as a specific interaction of BepA with the substrate and/or the BAM complex, induces a structural change in BepA (dislocation of the $\alpha 6$ loop covering the active site) to expose its active site and to enable the interaction between the LptD polypeptide and the edge-strand, whereas His-246 continues to repress the degradation of LptD by BepA. This interaction is necessary for both proper assembly and degradation of LptD. The transient interaction of normally assembling LptD with the "protease activity-repressed" state of BepA would

provide sufficient time for the progression of the maturation processes of LptD (including association with LptE), resulting in the final release of mature LptD from BamA. In contrast, during the prolonged stay of stalled or misassembled LptD molecules, BepA undergoes a further structural change including the movement of the $\alpha 9$ loop, stochastically or induced by a specific signal(s), to remove His-246 from the active site zinc, leading to the degradation of the molecules that cannot be subjected to maturation successfully. Our previous results of pulse-chase experiments showed that, although the conversion of LptD^C to LptD^{NC} was relatively slow, it started immediately after the synthesis of LptD and proceeded rather constantly (Narita et al., 2013). In contrast, degradation of stalled LptD in an LptE-depleted strain apparently occurred in a biphasic manner; little degradation of stalled LptD was observed for several minutes after synthesis ((Narita et al., 2013), indicating the presence of a lag period for degradation of stalled LptD to occur. These observations are consistent with the above model. Notably, it has been reported that BepA expression can result in the degradation of a mutant form of LptD (LptD4213) that probably mimics a late-state assembly intermediate of LptD (Lee et al., 2016) and possesses a substantial degree of a higher order (β -barrel-like) structure. The mechanism by which LptD4213 is degraded by BepA remains unclear, but its N-terminal region may interact with BepA.

BepA possibly facilitates the association of LptD with LptE on the BAM complex as the phenotypes caused by the disruption of BepA, including drug sensitivity and retarded disulfide rearrangement in LptD, can be suppressed by the overproduction of LptE (Narita et al., 2013). We have previously detected two species of LptD^C; one is not associated with LptE and observed only in an extremely early phase of its membrane assembly, and the other is associated with LptE and formed at a later phase (Miyazaki et al., 2018). It is likely that the LptD^C molecule that was simultaneously crosslinked with BepA and BamA was not associated with LptE, as it was accumulated in the LptE-limiting condition; therefore, it would represent the former species of LptD^C mentioned above. We assume that this LptD intermediate may possess a partially folded structure in which the BamA-associating C-terminal region of the barrel domain has a certain degree of a higher order (β -sheet) structure, but the BepA-associating N-terminal region is largely unstructured. Such a state of LptD may be favorable for the association of LptE with LptD (**Figure 4D**). Interestingly, Tyr-331 of LptD that contacts with the edge-strand of BepA is located at the end of the LptE-surrounding region of the LptD β -barrel in the mature LptD/E complex (**Figure 2D**). The interaction with the edge-strand of BepA may pin the partially folded structure of LptD transiently and facilitate the association between LptD and LptE. It might also assist the formation of the β -sheet structure in the β barrel

forming domain of LptD. Although it is possible that BepA actively promotes the LptD–LptE association by interacting with both LptD and LptE, it is also possible that BepA plays a passive role by maintaining an appropriate structure of LptD for association with LptE without showing direct interaction with LptE.

The biogenesis of LptD has been well studied. LptD has been a focus of OM protein research because it provides important information on its essential cellular function and it can be used as a model for OM protein insertion into the OM by the BAM complex. Based on the results obtained in the previous and current studies, we propose a model of the BepA-assisted biogenesis of LptD (**Figure 5B**): (i) After synthesis in the cytoplasm, LptD is translocated to the periplasm through the SecYEG translocon, during or just after which the Cys residues are oxidized by DsbA to form LptD^C (Chng et al., 2012; Narita et al., 2013). It is then targeted to the BAM complex with the aid of periplasmic chaperones including SurA (Schwalm et al., 2013; Vertommen et al., 2009); (ii) The C-terminal β -signal region of LptD is inserted into the BAM complex possibly through the periplasmic ring-like structure formed by the BamA POTRA domains and the lipoprotein subunits of the BAM complex, and it establishes interaction with BamD and BamA (Lee et al., 2019); (iii) Although details of the exact juncture at which interaction occurs are unknown, BepA interacts with the largely unstructured N-terminal half of the LptD β -barrel-forming domain whose C-terminal β -signal region is associated with the seam region of BamA. This helps the association of LptE with LptD; (iv) Then, the folding (β -sheet formation) of the unstructured N-terminal region of the LptD β -barrel domain occurs to form a premature form of LptD with a substantially folded β -barrel domain and LptE within it, like LptD4213 (Lee et al., 2019, 2016); (v) The β -barrel domain of LptD β -barrel is finally released from BamA and closed to form the mature LptD/E complex. The isomerization of two disulfide bonds in LptD (LptD^C to LptD^{NC}) should occur at a later step after the association of LptD and LptE (Lee et al., 2016; Miyazaki et al., 2018). The LptD intermediates that are stalled at certain steps in the above-mentioned processes as a result of misfolding are eliminated by the action of several peptidases including DegP (in the periplasm) and BepA /YcaL (on the BAM complex) (Soltes et al., 2017).

Our study reports several new findings on the interaction of BepA with LptD and BamA on the BAM complex where BepA plays a crucial role in the biogenesis and degradation of LptD. Further, we proposed a model explaining the role of BepA in these processes. Nonetheless, there are many questions that warrant further investigation. It would be especially important to elucidate the mechanism by which the substrate gains access to the active site buried inside the BepA molecule and the manner in which the switching of BepA from the state

375 with chaperone-like function to that with protease function occurs. The possible movement of the $\alpha 6$ and $\alpha 9$ loops
 376 should be directly examined. It is possible that there are signals that arise from the BAM complex and/or from
 377 (stalled) LptD to induce this structural/functional conversion of BepA. Further study, including structural and
 378 biochemical analysis of the BepA–LptD–BAM complex, is necessary to substantiate our model and to elucidate
 379 the molecular details of BepA functions. Cell surface proteins are suitable drug targets as they are more easily
 380 accessible from the external milieu than cytoplasmic proteins. These studies can provide a basis for the
 381 development of new drugs targeted to BepA/LptD/BAM.

Materials and methods

Key resources table

Reagent type or resource	Designation	Source reference	Identifiers	Additional information
Strains	<i>E. coli</i>	This study	N/A	Supplementary file 1
Virus	P1 bacteriophage	Laboratory stock	CGSC12133	
Recombinant DNA	Plasmids	This study	N/A	Supplementary file 2
Oligonucleotides		This study	PCR primers	described in the below
Antibodies	1 st antibodies	This study	N/A	listed in the below
Antibody	Goat Anti-Rabbit IgG (H + L)-HRP Conjugate	Bio-Rad Laboratories	1706515	
Chemical compound	H-p-Bz-Phe-OH	Bachem	F2800	
Chemical compound	Methionine, L-[³⁵ S] Translation Grade	American Radiolabeled Chemicals	ARS 01014	
Chemical compound	nProtein A Sepharose™ 4 Fast Flow	GE Healthcare	17528004	
Chemical compound	Ni-NTA Agarose	QIAGEN	30250	
Critical Commercial Assays	ECL™ Western Blotting Detection Reagents	GE Healthcare	RPN2106	
Critical Commercial Assays	ECL™ Prime Western Blotting Detection Reagents	GE Healthcare	RPN2232	
Software, Algorithm	Microsoft Excel	Microsoft	N/A	
Software, Algorithm	Bio-imaging Analyzer BAS-1800, BAS-5000	Fujifilm/GE Healthcare	N/A	
Software, Algorithm	Image Qaunt LAS 4000 mini	Fujifilm/GE Healthcare	N/A	
Software, Algorithm	Multi Gauge	Fujifilm/GE Healthcare	N/A	

Bacterial strains, Plasmids and Primers

Escherichia coli K12 strains and plasmids used in this study are listed in Tables S1 and S2, respectively. Details of the strain and plasmid construction and media are described in Construction of Mutant Strains and Plasmids Construction, respectively.

Media and Bacterial Cultures

E. coli cells were grown in L rich medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 5 g/L NaCl; pH adjusted to 7.2 with NaOH) or M9 synthetic medium (without CaCl₂; (Miller, 1972)) supplemented with maltose (final 0.2%), glycerol (final 0.4%), all amino acids (except Met and Cys; final concentration of 20 µg/mL each). 50 µg/mL ampicillin (Amp), 20 µg/mL chloramphenicol (Cm), 25 µg/mL kanamycin (Km), 25 µg/mL tetracycline (Tet) and 50 µg/mL spectinomycin (Spc) were added as appropriate for growing plasmid-bearing cells and selection of transformants and transductants. Bacterial growth was monitored with Mini photo 518R (660 nm; TAITEC Co., Saitama, Japan).

Antibodies

Penta-His HRP conjugate was purchased from QIAGEN (Hilden, Germany). Anti-BepA (Narita et al., 2013), anti-LptD (Narita et al., 2013) and anti-BamA (Gunasinghe et al., 2018) antibodies were described previously.

Construction of Mutant Strains

RM2091 (JE6631, *purC80::Tn10*) was constructed by transferring the *purC80::Tn10* marker, which is located near the *bepA* gene, from CAG18470 (Nichols et al., 1998) into JE6631 (Miyazaki et al., 2018), respectively, by P1 transduction. RM2243 (AD16, *bepA(E137Q) purC80::Tn10*) was constructed as follows. pRM330 (a plasmid carrying *bepA(E137Q)*, see below) was introduced into RM2091 to yield cells with pRM330 integrated into the chromosome by homologous recombination in the *bepA* region. They were then grown on an L-agar plate containing 5% sucrose to select cells that had lost the integrated plasmid. The plasmid-cured cells were screened for those having the chromosomal *bepA(E137Q)* allele at the *bepA* locus. The *bepA(E137Q)* allele was finally transferred to AD16 (Kihara et al., 1995) by joint P1 transduction with the *purC80::Tn10* marker. One of such strains was named RM2243. RM3654 (AD16, Δ *bepA*, *bamA*⁺ *zae502::Tn10*) and RM3655 (AD16, *bamA(S439C) zae502::Tn10*) were constructed as follows, pRM845 (a plasmid carrying *bamA(S439C)*, see below) was introduced into YH188 (JE6631, *zae502::Tn10*) (Hizukuri and Akiyama, 2012) to yield cells with pRM845 integrated into the chromosome by homologous recombination in the *bamA* region. They were then grown on an L-agar plate containing 5% sucrose to select cells that had lost the integrated plasmid. The plasmid-cured cells were screened for those having the chromosomal *bamA(S439C)* allele at the *bamA* locus. The *bamA(S439C)* allele was transferred to SN56 (Narita et al., 2013) by joint P1 transduction with the *zae502::Tn10* marker. Strains having

the *bamA*⁺ allele and the *bamA*(S439C) allele were picked up and named RM3654 and RM3655, respectively. RM2831 (HM1742, *kan araC-P_{araBAD}-lptD*) were constructed by essentially the same procedure as the construction of strains with a chromosomal C-terminal his₁₀-tagged gene (Miyazaki et al., 2020b). First, a *kan araC-P_{araBAD}-lptD* fragment having a sequence identical to the upstream or downstream region of the *lptD* start codon at the respective ends of the fragment, was PCR-amplified from pRM741 (a plasmid carrying a *kan* cassette at the upstream of an *araC-P_{araBAD}*) using a pair of primers, *ara-lptD-f* (5'-TTGTCACGCGCAACGTTACCGATGATGGAACAATAAAATCAACGTCATATGAATATCCTCCTTAG-3') and *ara-lptD-r* (5'-GGTGGCAATCATGGTGGCCAGGAGAGTGGGGATACGTTTTTTCATGGTGAATTCCTCCTGCTAG-3'). Then, the chromosomal *lptD* locus of the *E. coli* DY330 strain was replaced by this fragment using the λ-Red recombination system (Yu et al., 2000). The *kan araC-P_{araBAD}-lptD* was finally transferred to HM1742 by P1 transduction.

Plasmids Construction

pSTD689-derived plasmids carrying a *bepA* mutants were constructed from pRM290 (pSTD689-*bepA*) (Daimon et al., 2017) by site-directed mutagenesis. Derivatives of pRM291 (pSTD689-*bepA*(E137Q)) (Daimon et al., 2017) carrying an additional Cys mutation and derivatives of pUC-*bepA*(E137Q)-*his₁₀* (pUC18-*bepA*(E137Q)-*his₁₀*) (Narita et al., 2013) carrying an *amber* mutation were constructed by site-directed mutagenesis. pUC18-*bepA*(Pro)-*his₁₀* and pUC18-*bepA*(amb)-*his₁₀* plasmids were constructed from pUC-*bepA*-*his₁₀* (pUC18-*bepA*-*his₁₀*) (Narita et al., 2013) by site-directed mutagenesis. To construct pUC18-*bepA*(Pro, amb, E137Q)-*his₁₀* plasmids, a mutation for the individual Pro substitutions was introduced into each of the pUC18-*bepA*(amb, E137Q)-*his₁₀* plasmids by site-directed mutagenesis. pNB91 (pMW118-*bepA*(E137Q)) was constructed by subcloning an EcoRI-HindIII *bepA*(E137Q) fragment prepared from pUC-*bepA*(E137Q) (pUC18-*bepA*(E137Q)) (Narita et al., 2013) into the same sites of pMW118. pRM807 (pMW118-*bepA*(A106P, E137Q)) and pRM808 (pMW118-*bepA*(F107P, E137Q)) were also constructed by subcloning an EcoRI-HindIII *bepA* fragment from each of pSTD639-*bepA* plasmids into the same sites of pMW118.

Plasmids carrying *lptD*(amb)-*his₁₀* were constructed from pRM309 (pRM294-*lptD*-*his₁₀*) (Miyazaki et al., 2018) by site-directed mutagenesis. pRM821 (pRM294-*lptD*(Y331amb, D749amb)-*his₁₀*) and pRM822

(pRM294-*lptD*(Y331*amb*, Y726*amb*)-*his10*) were constructed from pRM626 (pRM294-*lptD*(Y331*amb*)-*his10*) by site-directed mutagenesis. pRM829 (pRM294-*lptD*(E733C)-*his10*) and pRM831 (pRM294-*lptD*(Y331, E733C)-*his10*) were constructed from pRM309 and pRM626, respectively, by site-directed mutagenesis. Plasmid carrying *lptD*(Cys)-*his10* were constructed as follows. Each mutation for the Cys substitutions was introduced to pRM309 by site-directed mutagenesis. The BamHI-HindIII *lptD*(Cys)-*his10* fragment of the resulting plasmids was subcloned into the same site of pTWV228 (Takara Bio inc., Shiga, Japan) to generate pTWV228-*lptD*(Cys)-*his10*. pRM320 (pUC118-*bepA*-*yfgD*) was constructed by PCR amplification of the *bepA*-*yfgD* fragment from the genome of MC4100 using a pair of primers, *bepA*-f (5'-GCGCGCGGATCCATTTGAGTGGGCTAATCTTCG-3') and *yfgD*-r (5'-GCGCGCGTCGACCGAACTACGCGAAGTTAATCC-3'), and, cloning it into the BamHI-SalI site of pUC118 (Takara Bio) after digestion with these enzymes. For the construction of pRM324, the *bepA*(E137Q) mutation was introduced into pRM320 by site-directed mutagenesis. pRM330 (pK18mobsacB-*bepA*(E137Q)-*yfgD*) was constructed by subcloning the BamHI-SalI *bepA*(E137Q)-*yfgD* fragment from pRM324 into the same sites of pK18mobsacB (Schäfer et al., 1994).

pRM823 (pUC118-*bamA*) was constructed by *in vitro* recombination using In-Fusion HD cloning kit (Takara Bio) of a EcoRI-BamHI fragment from pUC118 and a *bamA* fragment prepared by PCR amplification from the genome of MC4100 using a pair of primers, *bamA*-f (5'-GCGCGAATTCAGGAAGAACGCATAATAACG-3') and *bamA*-r (5'-GCGCGGATCCTTACCAGGTTTTACCGATG-3'). For the construction of pRM836, the *bamA*(S439C) mutation was introduced into pRM823 by site-directed mutagenesis. pRM845 (pK18mobsacB-*bamA*(S439C)) was constructed by subcloning the EcoRI-BamHI *bamA*(S439C) fragment from pRM823 into the same sites of pK18mobsacB.

Immunoblotting analysis

Acid-denatured proteins were solubilized in SDS-sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5 mg/mL bromophenol blue) with or without 10% β -ME, separated by SDS-PAGE and electro-blotted onto a PVDF membrane (Merck Millipore; Billerica, MA). The membrane was first blocked with 5% skim milk in PBST (Phosphate Buffered Saline with Tween 20), and then incubated with Penta-His HRP conjugate (1/2,000

or 1/3,000 dilution)), anti-BepA (1/10,000), anti-LptD (1/50,000) or anti-BamA (1/20,000). After washing with PBST, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1/5,000) (Goat Anti-Rabbit IgG (H + L)-HRP Conjugate; Bio-Rad Laboratories, Inc., Hercules, CA) in PBST (This step was omitted for the detection using Penta-His HRP conjugate). Proteins were visualized with ECLTM Western Blotting Detection Reagents (GE Healthcare UK Ltd, *Amersham Place* Little Chalfont, England) or ECLTM Prime Western Blotting Detection Reagents (GE Healthcare) and LAS4000 mini lumino-image analyzer (GE Healthcare).

Pulse-chase analysis for assay of the LptD disulfide-isomerization

Cells were first grown at 30°C in M9-medium supplemented with 2 µg/mL thiamine, 0.4% glycerol, 0.2% maltose, all amino acids (except Met and Cys) with or without 0.05% arabinose until early log phase. After induction with 1 mM IPTG for 15 min, cells were pulse-labeled with 370 kBq/mL [³⁵S]Methionine for 1 min. At appropriate time points after addition of excess nonradioactive Met (final conc. 250 µg/mL), total cellular proteins were precipitated with 5% TCA, washed with acetone, solubilized in SDS-buffer (50 mM Tris-HCl (pH 8.1), 1% SDS, 1 mM EDTA) and diluted 33-fold with Triton-buffer (50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2% Triton X-100, 0.1 mM EDTA). After clarification, samples were incubated with anti-LptD antibodies and nProtein A SepharoseTM 4 Fast Flow (GE healthcare) at 4°C over-night with slow rotation. Proteins bound to the antibody/ProteinA-Sepharose were recovered by centrifugation, washed with Triton buffer and then with 10 mM Tris-HCl (pH 8.1) and eluted by incubation at 98°C for more than 5 min in SDS-sample buffer. The samples were divided into two portions and one was treated with 10% β-ME. The proteins were separated by SDS-PAGE, and visualized with BAS1800 phosphoimager. Relative LptD^{NC} were calculated by the following equation: Relative LptD^{NC} = [LptD^{NC}(X min)]/[LptD^C(X min) + LptD^{NC}(X min)], where LptD^{NC} and LptD^C are the intensities of the respective bands.

***In vivo* photo-crosslinking analysis**

For the experiments in Figure 1D and Figure 3-figure supplement 1, cells were grown at 30°C in L medium containing 0.5 mM *p*BPA and 0.02% arabinose until early log phase and induced with 1 mM IPTG for 1 h. A half volume of the cell cultures was put on a petri dish and UV-irradiated at 4°C for 10 min using B-100AP UV lamp (365 nm; UVP, LLC., Upland, CA), at the distance of 4 cm. The other half was kept on ice as non-UV-irradiated

samples. Membrane fractions were prepared by sonical cell disruption and the following ultracentrifugation, and solubilized with SDS-buffer (50 mM Tris-HCl (pH 8.1), 1% SDS, 1 mM EDTA). After 33-fold diluted with Triton-buffer (50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2% Triton X-100, 0.1 mM EDTA) and clarification, samples were subjected to pull-down with Ni-NTA Agarose (QIAGEN). The isolated proteins were solubilized in SDS-sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5 mg/mL bromophenol blue) containing ME and analyzed by SDS-PAGE and immunoblotting analysis.

For the experiments in Figure 2A, 2B and 3A, cells were grown at 30°C in L medium containing 0.5 mM *p*BPA and 0.02% arabinose until early log phase and induced with 1 mM IPTG for 3 h. While a half volume of the cell cultures was UV-irradiated for 10 min, the other half was kept on ice as non-UV-irradiated samples, as above. Total cellular proteins were precipitated with 5% TCA, washed with acetone, and solubilized in SDS-sample buffer containing ME. The samples were subjected to SDS-PAGE and immunoblotting analysis.

For experiments in Figure 4A and 4B, cells were grown at 30°C in L medium containing 0.5 mM *p*BPA and 0.02% arabinose until early log phase and induced with 1 mM IPTG for 3 h. While a half volume of the cell cultures was UV-irradiated for 10 min, the other half was kept on ice as non-UV-irradiated samples, as above. Membrane fractions were prepared as above and solubilized in SDS-buffer. After dilution with Triton-buffer and clarification, samples were subjected to pull-down with Ni-NTA Agarose. The isolated proteins were solubilized in SDS-sample buffer containing ME and analyzed by SDS-PAGE and immunoblotting analysis.

BMB crosslinking combined with photo-crosslinking

Cells were grown at 30°C in L medium containing 0.5 mM *p*BPA and 0.02% arabinose until early log phase and induced with 1 mM IPTG for 3 h. BMB crosslinking was performed essentially according to the previously described procedures (Lee et al., 2019). After quenching the BMB crosslinking, cells were treated with or without UV-irradiation for 30 min at 4°C. Total cellular proteins were precipitated with 5% TCA, washed with acetone, and suspended in SDS-sample buffer containing 12.5 mM NEM for blocking the free Cys residues. After dilution with Triton-buffer and clarification, samples were subjected to pull-down with Ni-NTA Agarose. The isolated proteins were suspended in SDS-sample buffer containing ME and analyzed by SDS-PAGE and immunoblotting analysis.

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Figure 1

Figure 1. The edge-strand of BepA is crucial for functional interaction with LptD

(A) Crystal structure of BepA (PDB code: 6AIT). The peptidase and the TPR domains of BepA are shown in gray and orange, respectively. The edge-strand, the proteolytic active site (the HExxH motif and the third zinc ligand, Glu-201) and the regulatory His-246 residue (His switch) in the peptidase domain are shown in red, blue, and green, respectively, and the coordinated zinc atom is shown in yellow. An enlarged view of the active site region is shown in right. (B) Protease activities of the BepA edge-strand mutants. Cells of SN56 (Δ bepA) carrying pTWV228-*lptD-his*₁₀ and either pSTD689 or pSTD689-*bepA* plasmids were grown at 30°C in L-medium until early log phase and induced with 1 mM IPTG for 1 h. Total cellular proteins were acid-precipitated and analyzed by 7.5 or 10% Laemmli SDS-PAGE and immunoblotting with the indicated antibodies. (C) *In vivo* photo-crosslinking analysis of the BepA edge-strand. Cells of SN56 carrying pEVOL-pBpF and pUC18-*bepA(E137Q, amb)-his*₁₀ plasmids were grown at 30°C in L medium containing 0.02% arabinose and 0.5 mM pBPA until early log phase, and induced with 1 mM IPTG for 1 h to express the indicated BepA(pBPA) variants. The cultures were divided into two portions, each of which was treated with or without UV-irradiation for 10 min at 4°C. Proteins of the total membrane fractions were subjected to pull-down with Ni-NTA agarose. Purified proteins were analyzed by 7.5% Laemmli SDS-PAGE and immunoblotting with the indicated antibodies. Open triangles indicate unknown crosslinked products. (D) Chaperone-like activities of the BepA edge-strand mutants. Cells of SN56 carrying pSTD689 or a pSTD689-*bepA* plasmid were grown at 30°C in M9-based medium until early log phase, induced with 1 mM IPTG for 15 min, pulse-labeled with ³⁵S-Met for 1 min and chased for the indicated periods. At each time point, total cellular proteins were acid-precipitated, subjected to IP with an anti-LptD antibody, and analyzed by 7.5% Laemmli SDS-PAGE followed by phosphorimaging. The ratio of the band intensities of LptD^{NC} to that of total LptD (LptD^C + LptD^{NC}) at the each time point was quantitated and the mean values were plotted with S.D. (n = 2 technical replicates). The result shown is a representative of two independent experiments that were conducted using the same transformants (i.e. two technical replicates).

Figure 3

Figure 3. BepA edge-strand directly contacts with the Tyr-331 residue in the N-terminal half region of the LptD β-barrel forming domain

(A) Effect of the BepA edge-strand mutations on the crosslinking between BepA and the LptD derivatives having pBPA in the N-terminal half region of the LptD β-barrel-forming domain. Cells of SN56 ($\Delta bepA$) carrying pEVOL-pBpF, pMW118-*bepA*(E137Q, *mut*) and pRM294-*lptD*(*amb*)-*his*₁₀ were grown, induced to express a BepA and a LptDpBPA derivative and subjected to photo-crosslinking analysis as described in Figure 2. (B) Disulfide crosslinking between the Cys residues in the edge-strand of BepA and the N-terminal half region of the LptD β-barrel-forming domain. Cells of SN56 ($\Delta bepA$) carrying a combination of plasmids encoding WT or a Cys-introduced mutant of BepA and LptD-His₁₀ as indicated were grown in L-medium and induced with 1 mM IPTG for 3 h to express BepA (Cys) and LptD(Cys)-His₁₀. Total cellular proteins were acid-precipitated, solubilized with SDS-buffer containing NEM (for blocking free thiol groups) and subjected to pull-down with Ni-NTA agarose. The purified proteins were treated with or without 2-mercaptoethanol (ME) and analyzed by 7.5% Laemmli SDS-PAGE and immunoblotting with the indicated antibodies. The result shown is a representative of two technical replicates.

Figure 4

Figure 4. BepA interacts with a LptD intermediate assembling on the BAM complex

(A, B) *In vivo* photo-crosslinking of an LptD mutant having pBPA at two positions with BepA. Cells of RM2243 (*bepA(E137Q)*) carrying pEVOL-pBpF, pMW118-*bepA(E137Q)* and pRM294-*lptD(amb)-his₁₀* plasmids were grown at 30°C in L medium containing 0.5 mM pBPA until early log phase and induced with 1 mM IPTG for 3 h to express the indicated LptD(pBPA) variants. The cultures were divided into two portions, each of which was treated with or without UV-irradiation for 30 min at 4°C. Proteins of the total membrane fractions were subjected to pull-down with Ni-NTA agarose. Purified proteins were analyzed by 7.5% Laemmli SDS-PAGE by immunoblotting with the indicated antibodies. (C) Simultaneous crosslinking of LptD having Y331pBPA and E733C with the BepA edge-strand and the seam region of BamA(S439C). Cells of RM3655 (*bamA(S439C)*, Δ *bepA*)/pEVOL-pBpF/pMW118-*bepA(E137Q)* carrying pRM294-*lptD(E733C)-his₁₀* or pRM294-*lptD(Y331amb, E733C)-his₁₀* were grown and induced as in A. After treatment with or without BMB and the following quenching of BMB by addition of excess cysteine, the cultures were divided into two portions, each of which was treated with or without UV-irradiation for 10 min at 4°C. Total cellular proteins were acid-precipitated, solubilized with SDS-buffer containing NEM and subjected to pull-down with Ni-NTA agarose. Purified proteins were analyzed by 7.5% Laemmli SDS-PAGE and immunoblotting with the indicated antibodies. The anti-BamA immunoblotting showed that the amount of the BepAxLptDxBamA crosslinked product was much lower than that of the LptDxBamA crosslinked product. Although the anti-BepA antibodies apparently cross-reacted weakly with the LptDxBamA crosslinked products (asterisks), the higher signal intensity of the BepAxLptDxBamA crosslinked product band as compared with the intensity of the LptDxBamA band indicate that the detection of the former band with the anti-BepA antibodies cannot be ascribed to this cross-reactivity. In A–C, we confirmed that the amounts of the isolated non-crosslinked LptD-His₁₀ derivatives was roughly equal by CBB staining or anti-His immunoblotting (Figure 4-figure supplement 3). The result shown is a representative of two technical replicates. (D) A schematic cartoon of the interaction of the LptD assembly intermediate with BepA and BamA/D on the BAM complex.

Figure 5

Figure 5. Model for the substrate recognition and discrimination by BepA

(A) A schematic cartoon of the substrate recognition by BepA at its active site. See the text for details. (B) An overview of the proposed LptD assembly process and BepA-mediated discrimination of the assembling and stalled LptD species. See the text for details.

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Figure 1-figure supplement 1

Figure 1-figure supplement 1. Sequence alignment of BepA homologs and *E. coli* M48 family peptidases

(A) Alignment of the edge strand amino acid sequences of BepA homologs of *E. coli*, *Klebsiella pneumoniae*, *Xenorhabdus nematophila*, *Yersinia pestis*, *Vibrio cholerae*, *Aeromonas salmonicida*, *Pseudomonas aeruginosa* and *Legionella parisiensis*. (B) Alignment of the edge strand amino acid sequences of BepA and other *E. coli* M48 family peptidase (HtpX, LoiP, and YcaL). The alignments were conducted by using the Clustal Omega program. Conserved residues are marked in red.

Figure 1-figure supplement 2

Figure 1-figure supplement 2. Effects of the BepA edge strand Pro mutations on the BamA degradation and the BepA self-cleavage

(A) Degradation of BamA in a Δ *surA* strain expressing the BepA edge strand Pro mutants. Cells of SN259 (Δ *bepA*, Δ *surA*) carrying either pSTD689 or pSTD689-*bepA* plasmids were grown at 30°C in M9-based medium supplemented with 1 mM IPTG for 4 h. Total cellular proteins were acid-precipitated and analyzed by 7.5 or 10% Laemmli SDS-PAGE and immunoblotting with the indicated antibodies. (B) C-terminal self-cleavages of the BepA edge strand mutants. Cells of SN56 (Δ *bepA*) carrying either pUC18 or pUC18-*bepA-his₁₀* plasmids were grown at 30°C in L medium until early log phase and induced with 1 mM IPTG for 1 h. Total cellular proteins were acid-precipitated and analyzed by 7.5% Laemmli SDS-PAGE and immunoblotting with an anti-BepA antibody. The result shown is a representative of two independent experiments that were conducted using the same transformants (i.e. two technical replicates).

Figure 1-figure supplement 3

Figure 1-figure supplement 3. Functionality of the BepA derivatives having pBPA in the edge strand

(A) Complementation activity of the BepA(pBPA) derivatives. Cells from 30 µL of overnight cultures of SN56 ($\Delta bepA$) carrying pEVOL-pBpF and either pUC18 or pUC18-*bepA(amb)-his₁₀* plasmids were washed, suspended in saline, and serially diluted with saline (to about 10⁹ cells/mL). 2.5 µL each of the diluted cells were spotted on L-agar plate or L-agar plates supplemented with 20 µg/mL erythromycin (EM) and 1 mM IPTG with or without 0.5 mM pBPA. Plates were incubated at 30°C for 22 h. (B) Self-cleavage of the BepA(pBPA) derivatives. Cells of the same strains in A were grown at 30°C in L medium supplemented with 0.02% arabinose and 0.5 mM pBPA until early log phase and induced with 1 mM IPTG for 1 h. Total cellular proteins were acid-precipitated and analyzed by 7.5% Laemmli SDS-PAGE and immunoblotting with an anti-BepA antibody. The result shown is a representative of two technical replicates.

Figure 1-figure supplement 4

Figure 1-figure supplement 4. The chaperone-like and proteolytic activities of the BepA edge strand mutants

(A, B) The accumulation of the BepA mutants and LptD^C in the Δ *bepA* cells expressing the BepA mutants. Cells of AD16 (*bepA*⁺) carrying pSTD689 (vector) or SN56 (Δ *bepA*) carrying either pSTD689 or pSTD689-*bepA* plasmids were grown at 30°C in L medium until early log phase and induced with 1 mM IPTG for 1 h. Total cellular proteins were acid-precipitated, and analyzed by 10 or 7.5% Laemmli SDS-PAGE under a reducing (+ME) or non-reducing (no ME) condition and immunoblotting with anti-BepA (upper panels) or anti-LptD (lower panel) antibodies. (C) Protease activities of the BepA edge strand Cys mutants against overproduced LptD. Cells of SN56 (Δ *bepA*) carrying pTWV228-*lptD-his*₁₀ and either pSTD689 or pSTD689-*bepA* plasmids were grown at 30°C in L-medium until early log phase and induced with 1 mM IPTG for 1 h. Total cellular proteins were analyzed as in Figure 1B. The result shown is a representative of two technical replicates.

Figure 2-figure supplement 1

Figure 2-figure supplement 1. Over-expressed LptD molecules mainly accumulate as LptD^C

Cells of RM2243(*bepA(E137Q)*)/pEVOL-pBpF carrying either pMW118 or pMW118-*bepA(E137Q)* and either pRM294 or pRM294-*lptD-his₁₀* were grown in L medium containing 0.02% arabinose and 0.5 mM pBPA until early log phase at 30°C and induced with 1 mM IPTG for 3 h. Total cellular proteins were acid-precipitated and analyzed by 7.5 or 10% Laemmli SDS-PAGE under a reducing (+ME) or non-reducing (no ME) condition and immunoblotting with the indicated antibodies. The result shown is a representative of two biological replicates.

Figure 2-figure supplement 2

Figure 2-figure supplement 2. Complementation activity of the LptD(pBPA) derivatives

Cells of RM3588 (P_{ara} -*lptD*) carrying pEVOL-pBpF and either pRM294 or pRM294-*lptD(amb)-his₁₀* plasmids were grown at 30°C in L-medium supplemented with 0.1% arabinose for 2.5 h. Cells were washed, suspended in saline, and serially diluted with saline (to about 10⁹ cells/mL). 2.5 µL each of the diluted cells were spotted on L-agar plate containing 0.1% arabinose or L medium-based agar plates supplemented with 1 mM IPTG with or without 0.5 mM pBPA. Plates were incubated at 30°C for 22 h. The result shown is a representative of two technical replicates.

Figure 3-figure supplement 1

Figure 3-figure supplement 1. Effects of the BepA edge strand mutations on the crosslinking of the BepA edge strand to LptD

Cells SN56 (Δ bepA) carrying pEVOL-pBpF and pUC18-bepA(E137Q, mut., amb)-his₁₀ plasmids were grown in L medium containing 0.02% arabinose and 0.5 mM pBPA until early log phase at 30°C and induced with 1 mM IPTG for 1 h to express the indicated BepA (pBPA) variants. The cultures were divided into two portions, each of which was treated with or without UV-irradiation for 10 min at 4°C. Proteins of the total membrane fractions were subjected to pull-down with Ni-NTA agarose. Purified proteins were analyzed by 7.5% Laemmli SDS-PAGE and immunoblotting with the indicated antibodies. For analysis of the cellular accumulation of each BepA derivative, total cellular proteins of cells of the same cultures were acid-precipitated, and analyzed by 10% Laemmli SDS-PAGE and immunoblotting with the indicated antibodies. Open triangles indicate unknown crosslinked products. The result shown is a representative of two technical replicates.

Figure 3-figure supplement 2

Figure 3-figure supplement 2. Disulfide crosslinking between BepA and LptD

(A) Complementation activity of the LptD derivatives with an engineered Cys residue. Cells of RM3588 (*P_{ara}-lptD*) carrying pRM294 or pRM294-*lptD(Cys)-his₁₀* plasmids were grown at 30°C in L medium supplemented with 0.1% arabinose for 2.5 h. Cells were washed, suspended in saline, and serially diluted with saline (to about 10⁹ cells/mL). 2.5 μL each of the diluted cells were spotted on L-agar plate containing 0.1% arabinose or 1 mM IPTG. Plates were incubated at 30°C for 22 h. (B) BepA-LptD disulfide crosslinking. Cells of SN56 (*ΔbepA*) carrying a combination of plasmids encoding WT or a Cys-mutant form of BepA and LptD-His₁₀ as indicated were grown in L-medium and induced with 1 mM IPTG for 3 h to express BepA(Cys) and LptD(Cys)-His₁₀. Total cellular proteins were acid-precipitated, solubilized with SDS buffer containing NEM and subjected to pull-down with Ni-NTA agarose. The purified proteins were treated with or without 2-mercaptoethanol (ME) and analyzed by 7.5% Laemmli SDS-PAGE and immunoblotting with the indicated antibodies. The result shown is a representative of two technical replicates.

Figure 4-figure supplement 1

Figure 4-figure supplement 1. Complementation activity of the LptD derivatives having *pBPA* at one or two positions

Cells of RM3588 (*P_{ara}-lptD*) carrying pEVOL-pBpF and either pRM294 or pRM294-*lptD(amb)-his₁₀* plasmids were grown and analyzed as in Figure 2-figure supplement 2. The result shown is a representative of two technical replicates.

Figure 4-figure supplement 2

Figure 4-figure supplement 2. BMB crosslinking between LptD(Y331pBPA/E733C) and BamA(S439C)

(A) BMB crosslinking. Cells of RM3654 (*bamA*⁺, Δ *bepA*)/pEVOL-pBpF/pMW118-*bepA*(E137Q) or RM3655 (*bamA*(S439C), Δ *bepA*)/pEVOL-pBpF/pMW118-*bepA*(E137Q) carrying each pRM294-*lptD-his₁₀* derivative were grown at 30°C in L medium containing 0.5 mM pBPA until early log phase and induced with 1 mM IPTG for 3 h to express the indicated LptD variants. Cell cultures were treated with TCEP to reduce possible artificially-formed disulfide bonds for E733C in LptD and S439C in BamA. The cultures were divided into two portions, each of which was treated with or without BMB. After quenching of BMB by addition of excess cysteine, total cellular proteins were acid-precipitated, solubilized with SDS-buffer containing NEM and subjected to pull-down with Ni-NTA agarose. The purified proteins were analyzed by 7.5% Laemmli SDS-PAGE and immunoblotting with the indicated antibodies. (B) Complementation activity of LptD derivatives. Cells of RM3588 (*P_{ara}-lptD*) carrying pEVOL-pBpF and pRM294 or each pRM294-*lptD-his₁₀* derivative were grown and analyzed as in Figure 2-figure supplement 2. The result shown is a representative of two technical replicates.

Figure 4-figure supplement 3

Figure 4-figure supplement 3. The amount of affinity purified LptD-His₁₀ derivatives

Affinity isolated proteins in Figure 4A-C were separated by 7.5% Laemmli SDS-PAGE and subjected to CBB staining (A and B for Figure 4A and B, respectively) or anti-His immunoblotting (C for Figure 4C). The result shown is a representative of two technical replicates.

Figure 5-figure supplement 1

Figure 5-figure supplement 1. $\alpha 6$ and $\alpha 9$ -loop regions shield the intramolecular active site and edge strand of BepA
The space-filling models of wild type BepA (*left*), BepA without the $\alpha 6$ -loop (*middle*) and BepA without the $\alpha 6$ - and $\alpha 9$ -loops (*right*). The peptidase and the TPR domains of the BepA crystal structure (PDB code: 6AIT) are shown in gray and orange, respectively. The $\alpha 6$ -loop, the $\alpha 9$ -loop, the proteolytic active site (the HExxH motif and the third zinc ligand, Glu-201) and the edge strand in the peptidase domain are shown in magenta, green, blue and red, respectively.

Table S1. Strains used in this study

Strain	Genotype	References
AD16	$\Delta pro-lac\ thi^+ lac^R Z\Delta M15 Y^+ pro^+$	(Kihara et al., 1995)
SN56	AD16, $\Delta bepA$	(Narita et al., 2013)
SN259	AD16, $\Delta bepA\ \Delta surA::kan$	(Narita et al., 2013)
RM2243	AD16, $bepA(E137Q)\ purC80::Tn10$	This study
RM3654	AD16, $\Delta bepA\ bamA^+ zae502::Tn10$	This study
RM3655	AD16, $\Delta bepA\ bamA(S439C)\ zae502::Tn10$	This study
JE6631	$Hfr::str\ thi\ polA1$	(Mori et al., 2002)
RM2091	JE6631, $\ purC80::Tn10$	This study
YH188	JE6631, $ zae502::Tn10$	(Hizukuri and Akiyama, 2012)
MC4100	$F^- araD139\ \Delta(argF-lac)U169\ rpsL150\ relA1\ flbB5301\ deoC1\ ptsF25\ rbsR$	(Casadaban, 1976)
CU141	MC4100/ $F^+ lac^R lacZ^+, Y^+, A^+$	(Akiyama et al., 1994)
HM1742	CU141 ara^+	(Mori and Ito, 2006)
RM3588	HM1742, $kan\ araC-P_{araBAD-lptD}$	This study
DY330	W3110, $\Delta lacU169\ gal490\ \lambda c1857\ \Delta(cro-bioA)$	(Yu et al., 2000)
RM3444	DY330, $kan\ araC-P_{araBAD-lptD}$	This study

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Table S2. Plasmids used in this study

Plasmid	Vector	Encoded protein and description	Reference or source
pEVOL-pBpF		p15A-derivative encoding an evolved <i>M. jannaschii</i> aminoacyl-tRNA synthetase/ suppressor tRNA pair for incorporation of <i>pBPA</i> ; Cm ^R	(Young et al., 2010)
pSTD689		pACYC184-derived expression vector; P _{lac} , Spc ^R	(Kanehara et al., 2003)
pRM290	pSTD689	BepA	(Daimon et al., 2017)
pRM291	pSTD689	BepA(E137Q)	(Daimon et al., 2017)
pRM512	pSTD689	BepA(N105P)	This study
pRM513	pSTD689	BepA(A106P)	This study
pRM514	pSTD689	BepA(F107P)	This study
pRM515	pSTD689	BepA(A108P)	This study
pRM516	pSTD689	BepA(F109P)	This study
pRM517	pSTD689	BepA(F110P)	This study
pRM833	pSTD689	BepA(N105C)	This study
pRM786	pSTD689	BepA(N105C, E137Q)	This study
pRM834	pSTD689	BepA(A106C)	This study
pRM787	pSTD689	BepA(A106C, E137Q)	This study
pRM835	pSTD689	BepA(F107C)	This study
pRM788	pSTD689	BepA(F107C, E137Q)	This study
pUC18		Expression vector; P _{lac} , Amp ^R	Takara Bio
pUC-bepA(E137Q)	pUC18	BepA(E137Q)	(Narita et al., 2013)
pUC-bepA-his ₁₀	pUC18	BepA-His ₁₀	(Narita et al., 2013)
pUC-bepA(E137Q)-his ₁₀	pUC18	BepA(E137Q)-His ₁₀	(Narita et al., 2013)
pYN333	pUC18	BepA(N105P)-His ₁₀	This study
pYN334	pUC18	BepA(A106P)-His ₁₀	This study
pYN335	pUC18	BepA(F107P)-His ₁₀	This study
pYN336	pUC18	BepA(A108P)-His ₁₀	This study
pYN337	pUC18	BepA(F109P)-His ₁₀	This study
pYN338	pUC18	BepA(F110P)-His ₁₀	This study
pYN339	pUC18	BepA(N105 ^{amb})-His ₁₀	This study
pRM536	pUC18	BepA(N105 ^{amb} , E137Q)-His ₁₀	This study
pRM778	pUC18	BepA(N105 ^{amb} , A106P, E137Q)-His ₁₀	This study
pRM779	pUC18	BepA(N105 ^{amb} , F107P, E137Q)-His ₁₀	This study
pYN340	pUC18	BepA(A106 ^{amb})-His ₁₀	This study
pRM537	pUC18	BepA(A106 ^{amb} , E137Q)-His ₁₀	This study
pYN345	pUC18	BepA(A106 ^{amb} , F107P, E137Q)-His ₁₀	This study
pYN341	pUC18	BepA(F107 ^{amb})-His ₁₀	This study
pRM538	pUC18	BepA(F107 ^{amb} , E137Q)-His ₁₀	This study

pYN346	pUC18	BepA(F107 <i>amb</i> , A106P, E137Q)-His ₁₀	This study
pYN342	pUC18	BepA(A108 <i>amb</i>)-His ₁₀	This study
pRM539	pUC18	BepA(A108 <i>amb</i> , E137Q)-His ₁₀	This study
pYN343	pUC18	BepA(F109 <i>amb</i>)-His ₁₀	This study
pRM540	pUC18	BepA(F109 <i>amb</i> , E137Q)-His ₁₀	This study
pYN344	pUC18	BepA(F110 <i>amb</i>)-His ₁₀	This study
pRM541	pUC18	BepA(F110 <i>amb</i> , E137Q)-His ₁₀	This study
pMW118		pSC101-derived expression vector; P _{lac} , Amp ^R	Nippon Gene
pNB91	pMW118	BepA(E137Q)	This study
pRM807	pMW118	BepA(A106P, E137Q)	This study
pRM808	pMW118	BepA(F107P, E137Q)	This study
pTWV228		pBR322-derived expression vector; P _{lac} , Amp ^R	Takara Bio
pRM267	pTWV228	LptD-His ₁₀ (using the native SD sequence)	(Daimon et al., 2017)
pRM816	pTWV228	LptD-His ₁₀ ^{SD} (using a strong SD sequence)	This study
pRM810	pTWV228	LptD(M287C)-His ₁₀ ^{SD}	This study
pRM811	pTWV228	LptD(Y331C)-His ₁₀ ^{SD}	This study
pRM812	pTWV228	LptD(E391C)-His ₁₀ ^{SD}	This study
pRM813	pTWV228	LptD(Q393C)-His ₁₀ ^{SD}	This study
pRM814	pTWV228	LptD(H416C)-His ₁₀ ^{SD}	This study
pRM815	pTWV228	LptD(V430C)-His ₁₀ ^{SD}	This study
pRM294		pBR322-derived expression vector; P _{lac} , Spc ^R	(Miyazaki et al., 2018)
pRM309	pRM294	LptD-His ₁₀ ^{SD} (using a strong SD sequence)	(Miyazaki et al., 2018)
pNB22	pRM294	LptD(R40 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB23	pRM294	LptD(N55 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB24	pRM294	LptD(T70 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB25	pRM294	LptD(D85 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB26	pRM294	LptD(P100 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB27	pRM294	LptD(N115 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB28	pRM294	LptD(T130 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB29	pRM294	LptD(R145 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB30	pRM294	LptD(N160 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB31	pRM294	LptD(P175 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB49	pRM294	LptD(D190 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB50	pRM294	LptD(V205 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB51	pRM294	LptD(V220 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB52	pRM294	LptD(Y235 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB53	pRM294	LptD(N250 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM618	pRM294	LptD(T258 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM619	pRM294	LptD(I259 <i>amb</i>)-His ₁₀ ^{SD}	This study

pNB54	pRM294	LptD(H265 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM620	pRM294	LptD(E273 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB55	pRM294	LptD(S280 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM621	pRM294	LptD(L286 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM622	pRM294	LptD(M287 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB56	pRM294	LptD(S295 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB57	pRM294	LptD(R310 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM623	pRM294	LptD(Y314 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB58	pRM294	LptD(W325 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM624	pRM294	LptD(F327 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM625	pRM294	LptD(D330 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM626	pRM294	LptD(Y331 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB59	pRM294	LptD(F340 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB60	pRM294	LptD(A355 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM627	pRM294	LptD(K358 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB61	pRM294	LptD(A370 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM628	pRM294	LptD(T371 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB62	pRM294	LptD(T385 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM629	pRM294	LptD(E391 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM630	pRM294	LptD(Q393 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB63	pRM294	LptD(Q400 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB64	pRM294	LptD(V415 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM631	pRM294	LptD(H416 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM632	pRM294	LptD(R429 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB65	pRM294	LptD(V430 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM633	pRM294	LptD(H431 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB66	pRM294	LptD(G445 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM634	pRM294	LptD(H457 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM635	pRM294	LptD(Y458 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB67	pRM294	LptD(Q460 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB68	pRM294	LptD(D475 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM636	pRM294	LptD(V478 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM637	pRM294	LptD(N479 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB69	pRM294	LptD(K490 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB70	pRM294	LptD(T505 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB71	pRM294	LptD(R520 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB72	pRM294	LptD(S535 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB73	pRM294	LptD(D550 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB74	pRM294	LptD(R565 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB75	pRM294	LptD(G580 <i>amb</i>)-His ₁₀ ^{SD}	This study

pNB76	pRM294	LptD(I595 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB77	pRM294	LptD(G610 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB78	pRM294	LptD(G625 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB79	pRM294	LptD(S640 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB80	pRM294	LptD(N655 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB81	pRM294	LptD(K670 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB82	pRM294	LptD(V685 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB83	pRM294	LptD(V700 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB84	pRM294	LptD(S715 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB32	pRM294	LptD(Y726 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB85	pRM294	LptD(V730 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB86	pRM294	LptD(H745 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM809	pRM294	LptD(D749 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB87	pRM294	LptD(G760 <i>amb</i>)-His ₁₀ ^{SD}	This study
pNB88	pRM294	LptD(S775 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM821	pRM294	LptD(Y331 <i>amb</i> , D749 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM822	pRM294	LptD(Y331 <i>amb</i> , Y726 <i>amb</i>)-His ₁₀ ^{SD}	This study
pRM829	pRM294	LptD(E733C)-His ₁₀ ^{SD}	This study
pRM831	pRM294	LptD(Y331 <i>amb</i> , E733C)-His ₁₀ ^{SD}	This study
pUC118		Expression vector; P _{lac} , Amp ^R	Takara Bio
pRM320	pUC118	<i>bepA-yfgD</i>	This study
pRM324	pUC118	<i>bepA(E137Q)-yfgD</i>	This study
pRM823	pUC118	<i>bamA</i>	This study
pRM836	pUC118	<i>bamA(S439C)</i>	This study
pK18mobsacB		pBR322-derived vector carrying <i>sacB</i> for chromosomal gene replacement; Km ^R	(Schäfer et al., 1994)
pRM330	pK18mobsacB	<i>bepA(E137Q)-yfgD</i>	This study
pRM845	pK18mobsacB	<i>bamA(S439C)</i>	This study

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