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DegP primarily functions as a protease for the biogenesis of β-barrel outer membrane proteins in the Gram-negative bacterium *Escherichia coli*

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DegP (also designated as HtrA) and its homologs are found in prokaryotic cells and such eukaryotic organelles as mitochondria and chloroplasts. DegP has been found to be essential for the growth of Gram-negative bacteria under heat shock conditions and arguably considered to possess both protease and chaperone activities. The function of DegP has not been clearly defined. Using genetically incorporated non-natural amino acids as photo-crosslinkers, here we identified the β-barrel outer membrane proteins (OMPs) as the major natural substrates of DegP in *Escherichia coli* cells. We also demonstrated that DegP primarily functions as a protease, at both low and high temperatures, to eliminate unfolded OMPs, with hardly any appreciable chaperone activity in cells. We also found that the toxic and cell membrane-damaging misfolded OMPs would accumulate in DegP-lacking cells cultured under heat shock conditions. Together, our study defines the primary function of DegP in OMP biogenesis and offers a mechanistic insight into the essentiality of DegP for cell growth under heat shock conditions.

Structured digital abstract

- DegP physically interacts with ompA by pull down (1, 2)
- DegP physically interacts with ompX, ompA, ompW, ompF, nmpC and ompC by pull down (View interaction)
- DegP physically interacts with ompC and ompF by pull down (View interaction)
- DegP physically interacts with ompC and ompA by pull down (View interaction)
- DegP physically interacts with ompC, malE, fkpA, ompW, ompA, ompF, nmpC and ompX by cross-linking study (View interaction)

Introduction

Gram-negative bacterial cells and such eukaryotic organelles as mitochondria and chloroplasts are surrounded by a special envelope that consists of the inner and outer membranes and a periplasmic or intermembrane space between them [1–3]. Integrated into such outer membrane are the β-barrel outer membrane proteins (OMPs) that are exclusively composed of an even number of β-strands [4,5]. The biogenesis

Abbreviations
OMP, outer membrane protein; *p*Bpa, *p*-benzoyl-L-phenylalanine.

of OMPs in Gram-negative bacteria is a complex cellular process, in which the nascent polypeptide chains synthesized in the cytoplasm have to be translocated across the hydrophobic inner membrane and the hydrophilic periplasmic space, before being folded and inserted into the outer membrane [6,7]. The quality control for the biogenesis of OMPs in cells involves not only molecular chaperones that promote their folding and/or prevent their misfolding but also proteases that degrade their misfolded forms. The quality control factors SurA, DegP and Skp have been characterized to function in the periplasmic space for OMP biogenesis, all of them having been reported to exhibit molecular chaperone activity while, additionally, DegP also possesses protease activity [8–14]. Earlier genetic studies implicate SurA as playing a primary role while Skp and DegP playing a minor role for OMP biogenesis [8,9]. Our recent biochemical study demonstrated that DegP binds to unfolded OMPs at a far slower rate but with a higher affinity than SurA and Skp do [10], indicating that its role in OMP biogenesis might differ from the other two. Moreover, we recently identified FkpA as a key quality control factor for OMP biogenesis under heat shock conditions [11].

In retrospect, DegP was found to be essential for *Escherichia coli* cells to grow at high temperatures [15,16], thus being also designated as HtrA (high temperature requirement). Additionally, DegP homologs present in the mitochondria and chloroplasts of eukaryotic cells were also reported to be essential for the growth of yeasts and plants under stress conditions [12,13]. Moreover, the deficiencies of DegP homologs were related to neurodegeneration diseases in humans [14]. Intriguingly, DegP has been considered to exhibit both protease and chaperone activities [17–22]. Over the years, DegP has been reported to be able to degrade model substrate proteins [23,24], inner membrane proteins [25], periplasmic proteins [17,26,27] and assembly-defective mutant OMPs [28–30]. On the other hand, the chaperone activity of DegP was claimed based on its capacity to promote the refolding of periplasmic protein MalS or model substrate protein citrate synthase, as well as the observation that the growth defect of *degP*$^{-}$ mutant cells at high temperature could be partially rescued by an overexpression of the protease-deficient DegP(S210A) mutant protein [17]. It was also observed by us that DegP(S210A) is able to suppress the aggregation of model substrate protein lysozyme [31] and to promote the refolding of periplasmic protein PhoA [32].

Regarding the function of DegP, critical issues not yet resolved include the following. What substrate proteins does DegP work upon in cells? Does DegP function as both protease and molecular chaperone on these natural substrate proteins? What is the molecular and cellular basis underlying the essentiality of DegP for cells to grow under high temperature conditions? In an attempt to address these issues, here we identified OMPs as the major substrate proteins of DegP by performing *in vivo* photo-crosslinking mediated by non-natural amino acid probes that were genetically incorporated into DegP in combination with mass spectrometry analysis. We then demonstrated that DegP primarily functions as a protease, with hardly any appreciable chaperone activity, for the quality control of OMP biogenesis in cells. Our results also suggest that the accumulation of misfolded OMPs in *degP*$^{-}$ mutant cells leads to cell membrane damage, which is largely responsible for the phenotype of growth defects.

Results

Identification of OMPs as the major substrate proteins of DegP in *E. coli* cells

The substrate proteins of DegP in cells were identified by employing our recently developed non-natural amino acid DiZPK, 3-(3-methyl-3H-diazirine-3-yl)-propaminocarbonyl-N$^{\varepsilon}$-L-lysine, as the photo-crosslinker [32]. For the purpose of capturing its substrate proteins, residues near the enzyme active site, i.e. around the interior surface of the DegP trimeric unit, were specifically selected for DiZPK replacements, by referring to the reported structures of the DegP 12-mer and 24-mer [18,31,33].

Our immunoblotting results, as displayed in Fig. S1, demonstrated the formation of photo-crosslinked products for each DiZPK variant of DegP(S210A)His$_6$ except that introduced at position F198 (i.e. the F198DiZPK). Such photo-crosslinked products apparently reflect both DegP's self-oligomerization (e.g. as dimers and trimers, whose positions are indicated in Fig. S1) and the interaction of DegP with other proteins. Among all these DiZPK variants, the M42DiZPK protein apparently yielded the maximum number of photo-crosslinked products, as indicated by the number of protein bands detected by the antibody against the His-tag (Fig. 1A, lane 2), and was thus chosen for further studies. Given that the patterns of the photo-crosslinked products for each DiZPK variant were largely comparable when the cells were cultured either of 37 °C or 44 °C (Fig. S1), only the samples of 37 °C were subjected to purification using Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography in the presence of 8 M urea that effectively

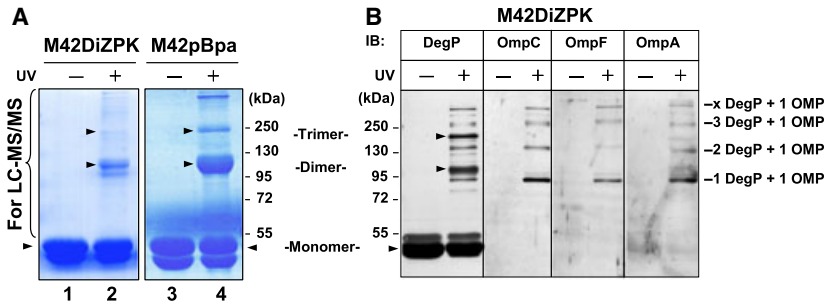

Fig. 1. Identification of DegP's substrate proteins in cells by photo-crosslinking. (A) Coomassie Blue staining results of the photo-crosslinked protein products of M42DiZPK and M42pBpa variants of DegP(S210A)His_6 after being purified by Ni-NTA affinity chromatography and separated by SDS/PAGE. The photo-crosslinked protein products (i.e. those having a size larger than DegP monomer) were collected and subjected to protein identification by mass spectrometry (LC-MS/MS). (B) Immunoblotting results verifying the presence of selected OMPs in the photo-crosslinked products of the M42DiZPK variant of DegP(S210A)His_6, using antibodies against the indicated proteins. The overlapped bands represent the crosslinked complexes containing one OMP molecule plus one or multiple copies of DegP subunits, as indicated on the right. Positions of DegP monomers, dimers and trimers are indicated.

removes the indirectly bound proteins. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis revealed that the proteins crosslinked with DegP, as displayed in Table 1, were largely OMPs.

We then performed additional experiments to verify the interaction between DegP and the OMPs. First, our immunoblotting analysis demonstrated that the same bands, representing a crosslinked product, could be detected not only by antibodies against OMPs (e.g. OmpA, OmpF or OmpC; the quality analysis results of these antibodies are shown in Fig. S2) but also by antibodies against DegP(S210A)His_6 (Fig. 1B). Given that only one non-natural amino acid photo-crosslinker was incorporated into each DegP polypeptide (subunit), three of the four dominant protein bands that were commonly detected with antibodies against OmpC, OmpF or OmpA (as indicated in Fig. 1B) apparently represent crosslinked products containing one OMP molecule and one, two or three DegP subunits, as judged by their sizes. The other band (of the largest size) may represent a crosslinked product containing one OMP subunit and an unknown number, but certainly larger than three, of DegP subunits (thus labeled as 'x DegP + 1 OMP' in Fig. 1B). Again, self-crosslinked DegP dimers and trimers were clearly visible in the anti-DegP(S210A)His_6 blotting analysis (as indicated in Fig. 1A,B), with larger oligomeric forms of DegP also probably present but hardly detectable here. Since DegP was reported to exist as 12- and 24-mers in the presence of unfolded model substrate proteins [18,31,33] and as 3- or 6-mers in the presence of phospholipid membranes [34], it would be highly desirable to find the actual oligomeric status of DegP when it functions in cells.

Table 1. Putative substrate proteins of DegP identified by mass spectrometry. Mascot score, as defined before [47], is a probability-based index. In brief, a higher score indicates a better match between the peptide information obtained from mass spectrometry analysis and the peptide information of a protein in the database; it does not reflect the abundance of a protein in the samples. In our case, a value higher than 37 is considered to be statistically significant ($P < 0.05$). The co-purification samples were purified from cell lysates using Ni-NTA affinity chromatography under non-denaturing conditions. IB, immunoblotting; ND, not determined.

			Mascot score			
Protein	Description	Mass (kDa)	DiZPK	*p*Bpa	Co-purification	IB
OmpC	Outer membrane porin protein	38	795	465	838	+
NmpC	Outer membrane porin protein	36	517	56	264	ND
OmpF	Outer membrane porin protein	37	422	–	313	+
OmpA	Outer membrane porin protein	35	125	379	133	+
OmpX	Outer membrane porin protein	16	155	–	52	+
OmpW	Outer membrane porin protein	21	91	109	208	+
FkpA[a]	Peptidyl-prolyl *cis–trans* isomerase, chaperon	26	72	–	–	–
MalE[a]	Maltose-binding protein	48	–	53	–	–

[a]Non-OMP, periplasmic proteins.

Second, by utilizing another non-natural amino acid, *p*-benzoyl-L-phenylalanine (*p*Bpa), as the photo-cross-linker (incorporated at the same site, M42), we also captured most of the OMPs that were captured using the DiZPK probe (Fig. 1A, lane 4; Table 1). Third, all the OMPs identified by using DiZPK were also identified by a more conventional (non-crosslinking) co-purification approach with DegP(S210A)His_6. A Coomassie Blue stained gel of such co-purified proteins is shown in Fig. S3 and the co-purified proteins identified by mass spectrometry are also listed in Table 1. Besides OMPs, two periplasmic proteins, FkpA and MalE, were also identified, but only via one of the two photo-crosslinkers and not by conventional co-purification or by immunoblotting (as indicated in Table 1). Given that we recently identified FkpA as a quality control factor for OMP biogenesis under heat shock conditions [11] and that both FkpA and MalE were found to exhibit chaperone activities under *in vitro* conditions [35,36], the meaning of the interaction between DegP and these two non-OMP proteins (FkpA and MalE) deserves further investigation.

It is noteworthy that five of our identified OMPs somehow represent the most abundant five out of a total of about 20 as indicated by our protein abundance information pooled in Table S1 [37–40]. It follows that the other OMPs failed to be identified in this study probably due to their low abundance. For instance, we noticed that among the non-identified OMPs, the most abundant one is FepA, but it usually exists at a level five-fold lower than that of OmpC, being the least abundant OMP identified in our study (Table S1). It should also be noted that many periplasmic or inner membrane proteins that exist in cells with an abundance much higher than the six OMPs we identified here (Table S1) were not identified as proteins interacting with DegP in our study. Taken together, these data strongly suggest that OMPs apparently represent the major substrate proteins of DegP in living cells.

Our additional analysis demonstrates that only unfolded forms of these identified OMPs, except OmpA, could be effectively retained by DegP(S210A) in cells cultured at either 37 °C or 44 °C. This is shown by an SDS/PAGE analysis (results displayed in Fig. 2A) and a semi-native SDS/PAGE analysis (results displayed in Fig. S4A) of the co-purified proteins. Such unfolded OMPs bound to DegP(S210A), after being isolated, were found to be accessible for degradation by exogenously added wild-type DegP

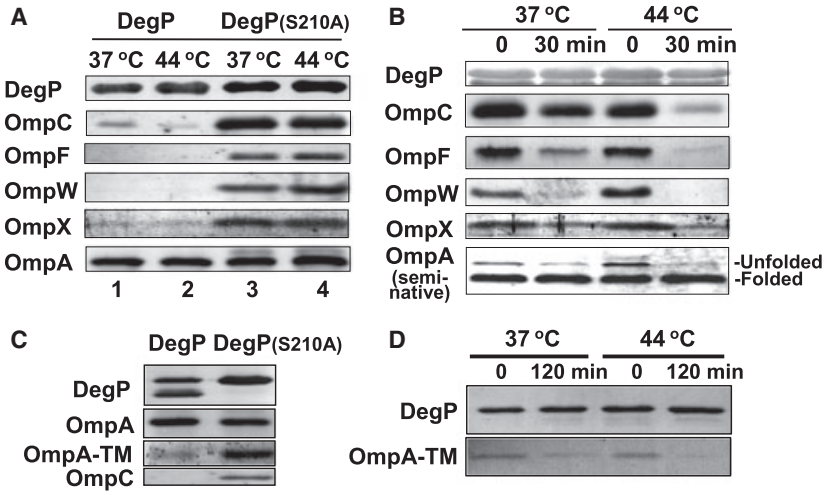

Fig. 2. OMPs were retained only by the protease-deficient DegP(S210A) but not by the wild-type DegP. (A) Immunoblotting results of the proteins co-purified with the wild-type DegP and protease-deficient DegP(S210A) proteins expressed in *degP*$^-$ mutant cells cultured at either 37 °C or 44 °C. The samples were separated on denaturing SDS/PAGE and blotted against antibodies of the indicated representative OMPs. (B) Immunoblotting results analyzing the levels of OMP proteins after their forms captured by DegP(S210A) were incubated with an equal amount of exogenously added wild-type DegP (with a concentration equal to that of DegP(S210A), 0.5 mg·mL^{-1}) at either 37 °C or 44 °C for 30 min. The samples were separated via denaturing (OmpW, OmpX, OmpC and OmpF) or semi-native (OmpA) SDS/PAGE. (C) The transmembrane domain of OmpA (OmpA-TM) co-purified with the wild-type DegP and protease-deficient DegP(S210A) proteins expressed in *degP*$^-$ mutant cells cultured at 37 °C, analyzed similarly as in (A). (D) The level of OmpA-TM after its form captured by DegP (S210A) was incubated with an equal amount of exogenously added wild-type DegP at either 37 °C or 44 °C for 120 min, analyzed similarly as in (B). The double bands of DegP seen in (B) and (C) have been commonly observed as its autocleavage products that are believed to be generated in the cells.

protein under *in vitro* conditions, as shown by the SDS/PAGE analysis displayed in Fig. 2B. It should be pointed out that OmpA, being distinguishable from the other OMPs for possessing a periplasmic domain, was captured by DegP(S210A) in both folded and unfolded forms (Fig. S4B). Uniquely, it is able to interact with the wild-type DegP in its folded form (Fig. S4B), as similarly observed by others [18]. Notably, we found that, with the periplasmic domain removed, the transmembrane part of OmpA, designated OmpA-TM, behaved much like the other OMPs. For instance, this truncated form of OmpA could be captured by DegP(S210A) only in its unfolded form (Fig. S4C), it was no longer retained by wild-type DegP (Fig. 2C), and its unfolded form captured by DegP(S210A) could be effectively degraded by exogenously added wild-type DegP (Fig. 2D). One conceivable explanation of these observations is that the unfolded OmpA proteins, similar to the other unfolded OMPs, are taken as substrates of DegP, while the folded OmpAs might function as an interaction partner to regulate, for example, the activity of DegP. Together, these observations strongly imply that DegP is able to capture unfolded OMPs in cells and effectively degrade them.

DegP primarily functions as a protease towards cellular OMPs

We then investigated how the presence and absence of DegP would affect the folding status of OMPs in *E. coli* cells. The DegP protein was reported to switch from a chaperone at low temperatures to a protease at high temperatures by one group [17] but was equally efficient as a protease at all temperatures by another [24], both based on *in vitro* studies. We thus analyzed the folding status of OMPs in *degP*$^-$ mutant cells cultured at three typical temperatures, 44 °C, 37 °C or 28 °C. For this, we made use of the unique property of the β-barrel structures of OMPs, which are extremely stable and are not disrupted even in the presence of SDS unless boiled. As such, the folded forms of the OMPs can be effectively distinguished from the unfolded ones because they will migrate at different positions when analyzed using semi-native SDS/PAGE, wherein the samples are analyzed in the presence of SDS but without being boiled [39].

The results of such semi-native SDS/PAGE analysis demonstrate that the levels of folded OMPs (as represented by OmpA, OmpC and OmpF) in *degP*$^-$ cells were significantly higher when cultured at 44 °C (Fig. 3A), less so at 37 °C (Fig. 3D) and were largely the same at 28 °C (Fig. 3E) compared with those in wild-type cells. Meanwhile, unfolded OMPs were found to be accumulated to a significant level in *degP*$^-$ cells only when cultured at 44 °C (Fig. 3A, lane 2) but not at 37 °C (Fig. 3D, lane 2) nor at 28 °C (Fig. 3E, lane 2). The reason that a lower level of folded OMPs was detectable in the wild-type cells than in the *degP*$^-$ cells might be due to the occurrence of DegP-mediated degradation of nascent OMPs in the former (but not in the latter), particularly at higher culturing temperatures. It should be noted that such accumulation of unfolded OMPs did not seem to be a result of cell death, since our colony forming units counting results (Fig. S5) clearly indicate that the *degP*$^-$ cells were alive after being cultured at 44 °C for up to 6 h. Additionally, the accumulation of unfolded OMPs (as represented by OmpF) could also be observed when the *degP*$^-$ cells were cultured at 42 °C for 1 h, as shown by our semi-native SDS/PAGE results displayed in Fig. S6. For comparison, the levels of folded OMPs were found to be decreased but without any accumulation of unfolded OMPs in *skp*$^-$ *surA*$^-$ cells (Skp and SurA are both well-characterized periplasmic chaperones) cultured at 44 °C (Fig. S7A, lane 2), presumably due to the degradation of unfolded OMPs by DegP in such mutant cells.

To find out whether the accumulation of unfolded OMPs in the *degP*$^-$ cells cultured at heat shock temperatures simply resulted from the lack of DegP as a protease, we performed a similar analysis on the folding status of OMPs with a mutant strain in which the chromosomal wild-type *degP* gene was replaced by the protease-defective *degP(S210A)* mutant gene. The level of the DegP protein, in the form of DegP(S210A), in this mutant strain will be exactly the same as that in the wild-type strain except that it lacks the protease activity. Similar to the *degP*$^-$ cells, this strain was also found to be unable to grow at 44 °C either in liquid medium (growth curve presented in Fig. 3B) or in solid medium (representative colony plating results displayed in Fig. 3C). Likewise, unfolded OMPs were found to accumulate in such cells and the level of folded OMPs was also highly comparable with that of the *degP*$^-$ cells, as shown by our semi-native SDS/PAGE results displayed in Fig. 3A. Together, these observations strongly suggest that DegP does not exhibit any appreciable chaperone activity in promoting folding or preventing misfolding, but exhibits efficient protease activity towards unfolded OMPs under the heat shock conditions of 44 °C. In support of this conclusion, the complementary overexpression of DegP(S210A) in the *degP*$^-$ mutant cells, when cultured at 44 °C, neither decreased the levels of unfolded OMPs nor increased the levels of folded OMPs as shown by our semi-native SDS/PAGE analysis (Fig. S7A, lane 5).

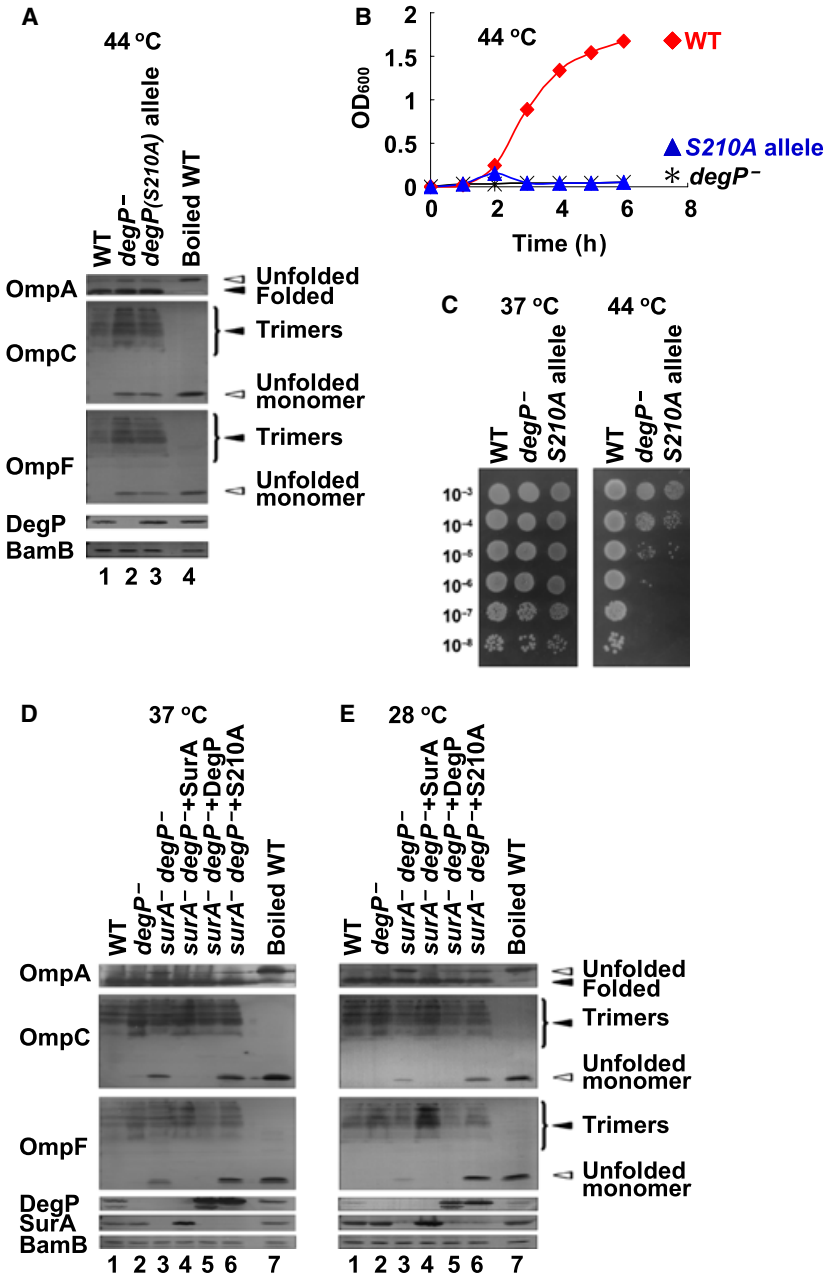

Fig. 3. DegP functions primarily as a protease towards OMPs in cells. (A) Immunoblotting results indicating the folding status of OMPs when the chromosomal *degP* gene was deleted (*degP*$^-$) or mutated to *degP(S210A)* in cells. Cells were cultured at 44 °C for 6 h (after being pre-cultured overnight at 37 °C) before their lysates were subjected to separation by semi-native SDS/PAGE. Positions of unfolded and folded OMPs are indicated on the right. (B), (C) Growth properties of cells of the indicated genotypes in liquid LB medium (B) or on agar LB plates (C), showing the growth defects of *degP*$^-$ and *degP(S210A)* cells at 44 °C. (D), (E) Immunoblotting results indicating the folding status of OMPs in *surA*$^-$ *degP*$^-$ double deletion cells, as well as those having complementary expression of the indicated proteins, cultured at 37 °C (D) or 28 °C (E). Here the outer membrane lipoprotein BamB was monitored to indicate equal sample loading. Positions of unfolded OMPs were determined by analyzing the cell lysate samples that were heated at 100 °C for 10 min before loading (A, lane 4; D, E, lanes 7).

We next examined whether DegP is able to exhibit any chaperone activities towards OMPs at the lower growth temperatures of 37 °C or 28 °C. For this purpose, we utilized the *surA*$^-$ *degP*$^-$ double deletion strain which was reported to accumulate unfolded OMPs at both 37 °C and 28 °C [9], as also confirmed by us using semi-native SDS/PAGE (Fig. 3D,E, lanes 3). Meanwhile, the overexpression of DegP(S210A) in this *surA*$^-$ *degP*$^-$ strain was found not to prevent but to promote an accumulation of unfolded OMPs (as represented by OmpA, OmpC and OmpF; Fig. 3D,E, lanes 6). A similar effect was also observed when the protease-defective DegP(S210A) protein was overexpressed in the *degP*$^-$ cells and cultured at 44 °C (Fig. S7A). In comparison, overexpression of the wild-type DegP in both the *degP*$^-$ *surA*$^-$ double mutant strain (Fig. 3D,E, lanes 5) and the *degP*$^-$ strain (Fig. S7A) effectively removed (cleaved) the unfolded OMPs. On the other hand, levels of the folded OMPs were largely comparable in the *surA*$^-$ *degP*$^-$ double mutant cells with or without overexpressing the protease-deficient DegP(S210A) (Fig. 3D,E, lanes 6). For comparison,

the expression of SurA, a well-characterized primary chaperone for OMP folding, led to a significant disappearance of unfolded OMPs (at both 37 °C and 28 °C) and a significant increase of folded OMPs (especially at 28 °C) (Fig. 3D,E, lanes 4). The chaperone function of SurA for OMP biogenesis at 37 °C was then further verified by our data displayed in Fig. S7B, showing that levels of the folded OMP in the *surA*$^{-}$ cells (lane 2) were significantly lower than those in the wild-type (lane 1) or even the *surA*$^{-}$ *degP*$^{-}$ cells (lane 3, apparently due to the lack of DegP protease activity). It should be pointed out that previous studies have demonstrated that the folding and assembly of OMPs will not occur in the periplasmic space and will only occur when they are inserted into the outer membrane with the help of the assembly/folding-promoting Bam/YaeT/Omp85 complex [35,36,41]. Together, once again, these observations strongly suggest that DegP primarily functions as a protease, instead of a chaperone, in OMP biogenesis.

Our conclusion of DegP functioning primarily as a protease in cells is also strongly supported by the following *in vitro* observations. First, the addition of DegP(S210A) was found to be unable to prevent the aggregation of unfolded OmpF proteins even at a 20 to 1 molecular ratio (Fig. 4A, right panel) while, as a positive control, the addition of SurA significantly prevented the aggregation of OmpF (Fig. 4A, left panel). The addition of the wild-type DegP protein, on the other hand, efficiently degraded the unfolded OmpF proteins (Fig. 4A, middle panel). Second, the addition of DegP(S210A) was found to be unable to promote the refolding/reassembly of unfolded OmpF (Fig. 4B; time course data in lanes 9-12; dosage effect data in lanes 21-24) in our *in vitro* OMP folding system, either at 37 °C or 44 °C, while the addition of SurA significantly promoted the refolding/reassembly of unfolded OmpF (Fig. 4B; lanes 1–4; lanes 13–16) at the same temperatures. Similarly, the addition of the wild-type DegP efficiently degraded the unfolded OmpF without

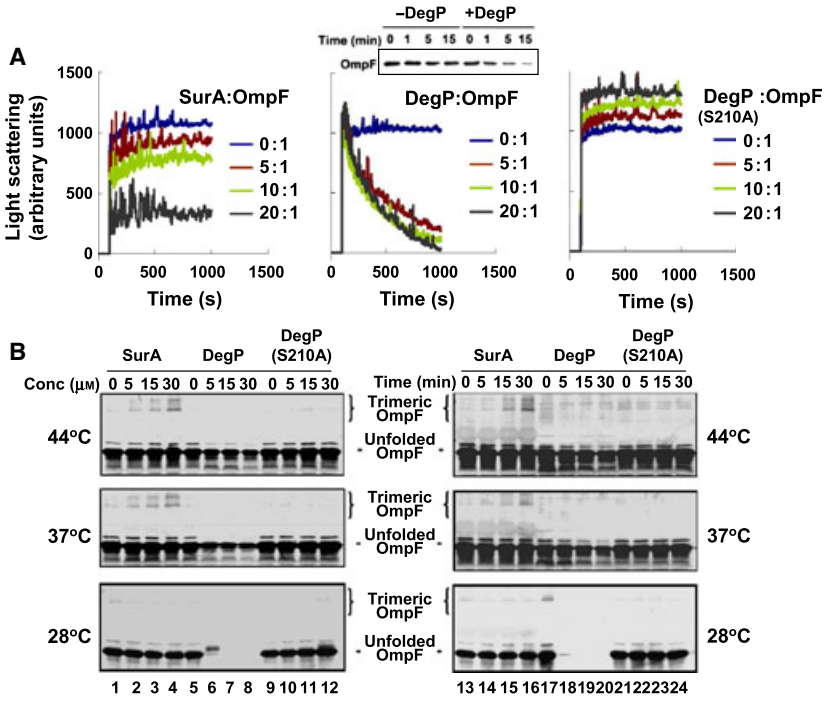

Fig. 4. The wild-type DegP and protease-deficient DegP(S210A) mutant proteins neither prevent aggregation nor promote refolding of unfolded OMPs under *in vitro* conditions. (A) The extent of aggregation, as monitored by light scattering at 500 nm, of the chemically denatured OmpF (0.5 μM) when diluted in the presence of increasing concentrations of SurA (left), wild-type DegP (middle) or DegP(S210A) (right). Shown as an inset are the immunoblotting results indicating the levels of OmpF in the sample containing a wild-type DegP : OmpF ratio of 5 : 1 at the indicated time points. (B) On the left are the immunoblotting results indicating the level of refolded-assembled OmpF from chemically denatured OmpF in the presence of isolated outer membrane fraction and increasing concentrations of SurA, wild-type DegP or DegP(S210A). On the right are similar results indicating the time-dependent levels of refolded-assembled OmpF in the presence of a fixed concentration (30 μM) of SurA, wild-type DegP or DegP(S210A). All these experiments were performed at 28 °C, 37 °C or 44 °C, with the samples being separated with semi-native SDS/PAGE. It should be noted that wild-type DegP degraded OmpF significantly in such *in vitro* assay, especially at 28 °C.

promoting any refolding (Fig. 4B, lanes 5–8 and 17–20). It should be pointed out that, at 28 °C, neither SurA (Fig. 4B, lanes 1–4 and 13–16) nor DegP(S210A) (lanes 9–12 and 21–24) promoted the refolding/reassembly of OmpF; again, the wild-type DegP (lanes 5–8 and 17–20) effectively degraded the unfolded OmpF. Despite the low efficiency, our *in vitro* refolding system apparently mimics certain aspects of OMP biogenesis in living cells. All the protein samples used here (SurA, DegP, DegP(S210A) and OmpF) were purified to almost homogeneity and the outer membrane fraction used here lacked OmpF, as demonstrated by SDS/PAGE analysis (Fig. S8). The small sharp band above that of unfolded OmpF monomer (Fig. 4B) may represent the precursor form of the endogenous OmpF, some of which might form coaggregates with the recombinant OmpF (which lacks the signal peptide) during overexpression. Autocleavage of DegP, as has been commonly observed by us [10] and others [18,42], has been found to occur in cells and is believed to be biologically significant [42]. Taken together, both our *in vivo* (Figs 3 and S7) and *in vitro* (Fig. 4) observations presented here demonstrate that DegP is able to function as an efficient protease, instead of a chaperone, towards unfolded OMPs at all temperatures.

The accumulated misfolded OMPs in DegP-lacking cells are linked to membrane damage and growth defects

The results described above strongly hint that the accumulation of misfolded OMPs in such non-growing mutant cells as $degP^-$ and the chromosomal *degP (S210A)* allele, as well as in the $surA^-$ $degP^-$ double deletion cells [9], may lead to cell growth defects. In supporting this claim, we observed a proportional correlation between the increase of unfolded OMP levels (Fig. 5A) and the decrease of the growth rate (Fig. 5B) for $degP^-$ cells upon temperature elevation from 36.5 °C to 44.5 °C, a range within which the growth rate of the wild-type cells was found to be largely invariable (it exhibits a similar pattern of growth at 37 °C and 44 °C, when plated on Petri dishes, as shown in Fig. 3C).

To unveil the potential damage elicited by such unfolded OMPs, we decided to examine their presence on the outer and inner membranes. For this purpose, the outer and inner membranes were separated by sucrose gradient centrifugation of the total membrane components that were isolated from cell lysates by ultracentrifugation [9,40]. As a technical validation, we first tried to separate the outer and inner membranes of the wild-type cells. As shown by the SDS/PAGE analysis results displayed in Fig. 5C, separation of the two membranes was effective; as expected, the outer membrane proteins were detected largely in the high density fractions (as indicated by the presence of such outer membrane marker proteins as OmpA, OmpC, OmpF and BamB in lanes 6 and 7) while the inner membrane proteins were detected in the low density fractions (as indicated by the presence of such inner membrane marker proteins as PpiD and SecE in lanes 2–6).

The SDS/PAGE analysis results displayed in Fig. 5D indicate that, remarkably, for the $degP^-$ mutant cells cultured at 44 °C, both unfolded and folded OMPs (as represented by OmpA, OmpC and OmpF) as well as the representative inner membrane proteins were all found to be greatly enriched in the high density fraction (e.g. in lane 7). These results might be explained as such that either the outer and inner membranes 'sticking' together as mediated by the misfolded OMPs or the inner membrane proteins were mislocalized to the outer membrane. We also noticed that the folded OMPs of the non-growing $degP^-$ cells migrated as smears (Fig. 5D, lanes 6 and 7) but as sharp bands in the wild-type cells (Fig. 5C, lanes 6 and 7), with the former as a whole also migrating at a lower mobility (compare lanes 6 and 7 in Fig. 5C and D). Apparently, the structural states of the folded OMPs in the outer membrane of $degP^-$ cells were somehow altered. This might be explained as due to the presence of inner membranes in the high density fraction of outer membranes, thus preventing the adequate solubilization of folded OMPs by SDS, a case similar to what is usually observed when analyzing total cell lysates containing both outer and inner membrane fractions by semi-native SDS/PAGE (Figs 3, 5A and S7).

To investigate whether there are any potential defects in the structure of the cell envelope of the $degP^-$ mutant cells cultured at 44 °C, we performed light microscope live-cell fluorescence imaging as well as thin-section transmission electron microscopy analysis. For the fluorescence imaging, the red fluorescent protein was expressed in the periplasmic space and the green fluorescent protein in the cytosol of the cells, thus visualizing each by their unique fluorescent colors. The live-cell fluorescence imaging results of the $degP^-$ mutant cells, displayed in Fig. 6B, demonstrate that the cell envelope structure became abnormal. This is indicated by the formation of periplasm-containing red-colored vesicles (Fig. 6B, arrowheads 2 and 4) and the presence of a hardly visible periplasmic space (Fig. 6B, arrowheads 3), in contrast to similar analysis results of the wild-type cells (Fig. 6A) that were also cultured at the heat shock temperature of 44 °C.

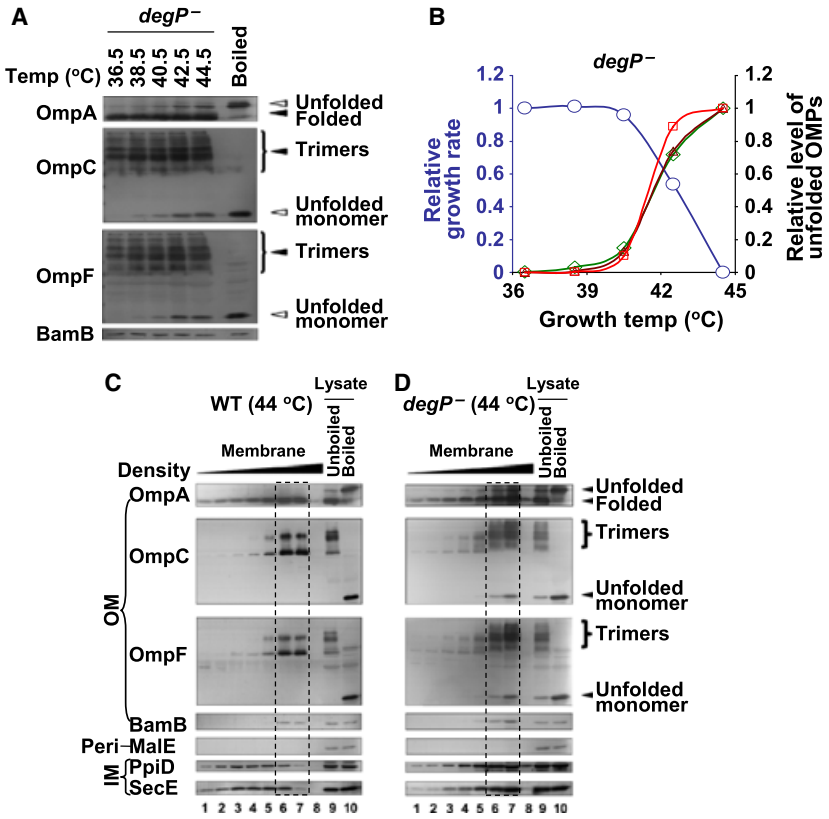

Fig. 5. The level of unfolded OMPs is inversely correlated with the cell growth rate and both unfolded OMPs and inner membrane proteins appear in the outer membrane fraction of *degP*⁻ cells. (A) Immunoblotting results indicating the folding status of OMPs in *degP*⁻ cells cultured at increasing temperatures. (B) Curves of the relative cell growth rate (blue) and the relative levels of misfolded OMPs (derived from the results displayed in panel A; OmpA, green; OmpC, brown; OmpF, red) of the *degP*⁻ cells plotted against the culturing temperatures. (C), (D) Immunoblotting results indicating the folding status of OMPs in the membrane fractions of wild-type (C) and *degP*⁻ cells (D) cultured at 44 °C. The inner and outer membranes were separated by sucrose gradient centrifugation and then subjected to semi-native SDS/PAGE analysis (the samples in lanes 1–8 correlate to the sucrose gradient centrifugation fractions of 5–12). The unboiled (lane 9) and boiled (lane 10) cell lysates were included as controls indicating the positions of the folded and unfolded OMPs. BamB, MalE, and PpiD (as well as SecE) were also monitored as indicators of the outer membrane, periplasmic and inner membrane proteins, respectively.

The thin-section transmission electron microscopy results of such *degP*⁻ mutant cells, as displayed in Fig. 6D, also unveiled similar cell envelope abnormalities. Here we observed periplasm-containing vesicles emerging on the surface of the cells (Fig. 6D, black arrowhead) and the periplasmic space being significantly narrowed (Fig. 6D, white arrowheads) in comparison with the results of the control wild-type cells displayed in Fig. 6C. These observations indicate that the outer membrane of such *degP*⁻ mutant cells was somehow impaired. This claim is also supported by our observation of a dramatic increase of the fluorescence intensity of the hydrophobic fluorescence probe 1-*N*-phenylnaphthylamine when added to such *degP*⁻ mutant cells (Fig. S9A), indicating a higher accessibility (thus impairments) of the outer membrane interior to the probe [43]. On the other hand, the claim is also supported by our observation of a considerable formation of foam when such *degP*⁻ mutant cells were resuspended in solution (Fig. S9B).

We also noticed that the inner membrane of such *degP*⁻ mutant cells did not seem to be impaired as judged from the following observations. First, the green fluorescent protein expressed in the cytoplasm was apparently well maintained in the cells (Fig. 6B). Second, we observed that the levels of the cytoplasmic protein GroEL are highly comparable in the wild-type and the *degP*⁻ cells cultured at 44 °C (Fig. S9C).

Discussion

In the present study, we first identified OMPs as the major natural substrate proteins of DegP in living *E. coli* cells. We then demonstrated that DegP primarily

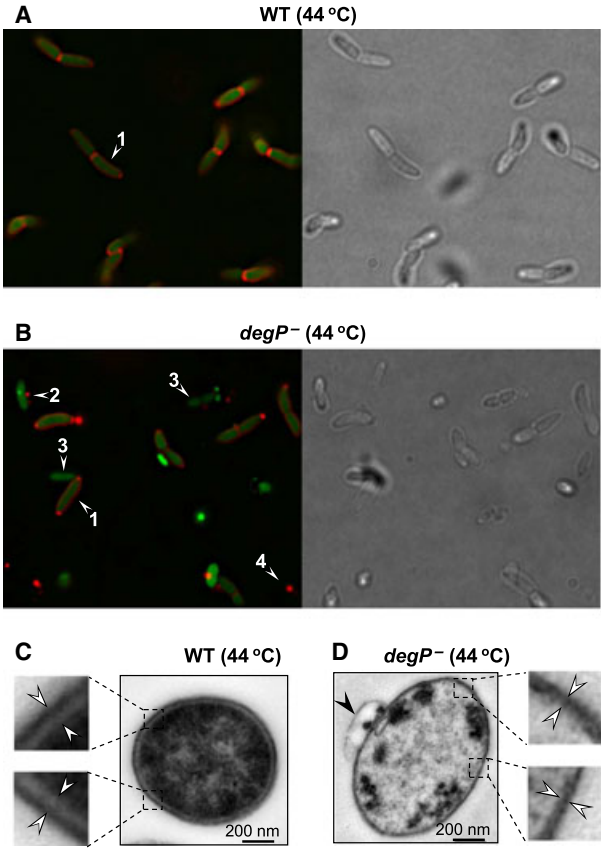

Fig. 6. The cell envelope is impaired in *degP*⁻ cells cultured at 44 °C. (A), (B) Live-cell fluorescence imaging micrographs of the periplasm and cytoplasm (as indicated by the specifically targeted red and green fluorescence proteins, respectively) in both wild-type (A) and *degP*⁻ mutant (B) cells cultured at 44 °C for 1 h. The corresponding white field micrographs are shown on the right. Arrows 1 and 3 respectively indicate intact and periplasm-deprived cells; arrows 2 and 4 respectively indicate a periplasm-containing vesicle being formed and having been released. (C), (D) Transmission electron micrographs of wild-type (C) and *degP*⁻ mutant (D) *E. coli* cells cultured at 44 °C. Two parts of each cell are enlarged for a better view of the periplasmic space (as indicated by pairs of white arrows). The black arrow in (D) indicates a presumed vesicle being formed.

exhibits protease activity, with no appreciable chaperone activity, for OMP biogenesis. Further, we showed that the accumulation of misfolded OMPs in DegP-lacking cells is linked to growth defects and membrane damage, thus providing a mechanistic insight into the long known intriguing phenomenon that DegP is required for the growth of *E. coli* cells at high temperatures [15,16].

The natural substrates of DegP in living cells have not yet been identified unequivocally. Our systematic *in vivo* photo-crosslinking and *in vitro* co-purification studies consistently identified OMPs as the predominant substrate proteins of DegP (Table 1). In support of our observation, earlier *in vitro* co-purification and co-crystallization data also indicated an interaction between DegP and OMPs [18]. In retrospect, a few non-OMP substrate proteins, mainly of periplasmic origin, have been reported to be degraded by DegP in cells, mostly based on genetic studies [17,25,26,44]. Briefly, the MalS and c-type cytochrome proteins were found to be removed in cells lacking the specific foldases of these two proteins but containing DegP [17,25]; the acylated precursor form of the colicin A lysis protein was identified in cells in which the signal peptidase was inhibited [44]; the CpxP protein was degraded by DegP only in cells expressing misfolded P pilus proteins PapE or PapG [27]. Apparently, possible reasons why these proteins were not identified among the proteins captured by DegP in our study include differences in the genetic backgrounds of the cells as well as the growth conditions.

We here found that DegP primarily functions as a protease, with no appreciable chaperone function, for OMP biogenesis. Our data showing that the chaperone function of DegP is minimal include the following. First, deleting the *degP* gene did not lower the levels of folded OMPs in the cells (Fig. 3). Second, complementary expression of DegP(S210A) neither promoted OMP folding/assembly nor prevented OMP misfolding in *degP*⁻ cells (Figs 3 and S7). Third, DegP was neither able to promote refolding/reassembly nor to prevent the aggregation of the OMPs under our *in vitro* conditions (Fig. 4). Data supporting a protease function of DegP for OMPs include the following. First, only DegP(S210A), not wild-type DegP, was able to capture unfolded OMPs in the cells (Figs S4 and 2). Second, deletion of the *degP* gene led to the accumulation of unfolded OMPs in the cells (Fig. 3). Third, DegP was able to degrade the unfolded OMPs under *in vitro* conditions (Fig. 4). Notably, in the refolding assay, the apparent protease activity of DegP towards the free unfolded OmpF was found to be significantly higher at 28 °C than at 37 °C or 44 °C (Fig. 4B), but in the *in vitro* degradation assay it was found to be significantly lower at 37 °C than at 44 °C towards the unfolded OMPs bound to DegP(S210A) (Fig. 2B). To explain these apparently contradictory observations, we noticed that in earlier reports the effects of temperature on the protease activity of DegP were also inconsistent with respect to different substrate proteins, with the apparent activity found to be increased for casein and MalS but almost unchanged for insulin upon temperature elevation [17,24]. Taken together, these observations suggest that the tempera-

ture effect on the apparent protease activity of DegP is mainly exhibited on the substrate proteins instead of on DegP itself.

The chaperone activity of DegP was initially reported either using periplasmic protein MalS as the substrate in cells lacking DsbA (a protein needed for the correct formation of the disulfide bonds in MalS) or using citrate synthase as a substrate under *in vitro* conditions [17]. In a more recent report, DegP was proposed to act as a molecular chaperone towards OMPs mainly based on the observation that the wild-type DegP was able to capture folded OmpA and protease-resistant OmpC protomers [18]. Although we similarly observed binding of the folded OmpA to the wild-type DegP, our detailed analyses indicate that the observation of an interaction between the folded OmpA and wild-type DegP is apparently related to its possessing a periplasmic domain (Figs 2C,D and S4C). Clearly, this does not mean that the other OMPs behaved in a similar way (Fig. 2A,B). Furthermore, molecular chaperones are generally defined as ones binding to unfolded, but not folded, substrate (client) proteins. We also repeatedly detected stable OmpC to be co-purified with the wild-type DegP, but the amount could be ignored compared with the amount of OmpC to be co-purified with DegP(S210A) (Fig. 2A). This, together with the observation that the OmpC and other OMPs (except OmpA) co-purified with DegP(S210A) were largely unfolded and sensitive to protease degradation (Figs 2A,B and S4A), strongly indicates that the OMPs captured by the wild-type DegP were predominantly misfolded and thus subject to degradation in the cells. In sum, DegP, although it might exhibit chaperone activity towards periplasmic proteins and some model substrate proteins [17,31,32], primarily functions as a protease towards OMPs. In agreement with our conclusion, earlier studies by others [28,29] indicated that the overexpressed DegP (S210A), although rescued the growth of $degP^{-}$ omp^{-} mutant cells, could not correct the assembly of these mutant OMPs that could be effectively degraded by wild-type DegP [30]. Whether the DegP (HtrA) homologs located in the intermembrane space of eukaryotic mitochondria and chloroplasts also function in a similar manner merits further exploration.

The growth defect of $degP^{-}$ *E. coli* cells cultured at heat shock temperatures has long been known [15,16] but their molecular and subcellular defects remain uncharacterized. We here reveal that the essentiality of DegP under heat shock conditions may reside in the removal of the misfolded OMPs, whose accumulation onto the membranes apparently relates to growth defects, as schematically illustrated in Fig. 7. Our major evidence supporting this hypothesis is as follows. First, DegP primarily and effectively captures and degrades misfolded OMPs in *E. coli* cells. Second, the correlation between the accumulation of misfolded OMPs and growth defects of cells was repeatedly observed, not only in the $degP^{-}$ and $degP(S210A)$ allele cells cultured at 44 °C (Fig. 3A–C) but also in the $surA^{-}$ $degP^{-}$ cells cultured at 37 °C or 28 °C (Fig. 3D,E, Table S2). Third, and more important, the

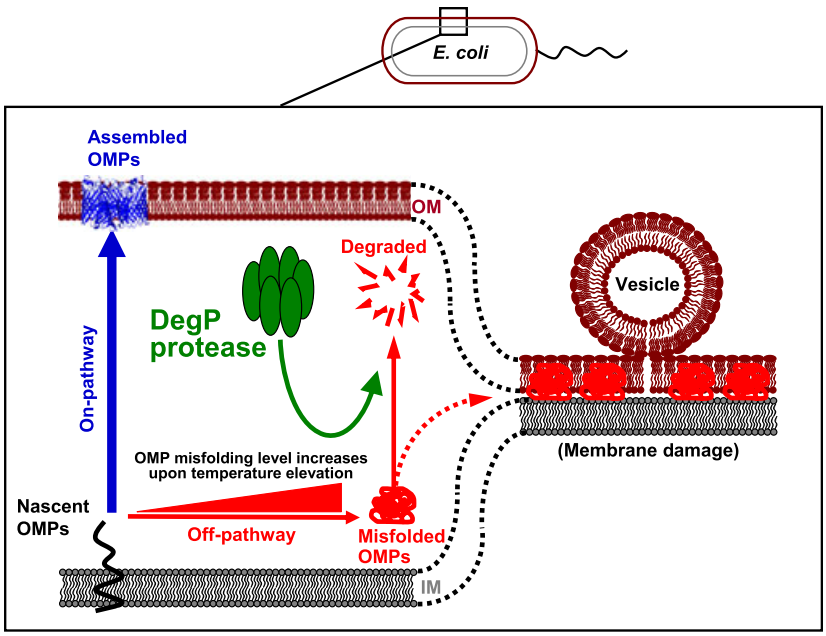

Fig. 7. Schematic diagram illustrating the essential protease function of DegP in OMP biogenesis. The nascent OMPs in the periplasm are destined for either the on-pathway (blue) for correct folding/assembly (as facilitated by molecular chaperones) or the off-pathway (red) towards misfolding (which will become more severe upon temperature elevation) and degradation by DegP. The lack of DegP would lead to the accumulation of toxic misfolded OMPs on the outer membrane (dashed red arrow), due to which the integrity of the outer membrane is impaired, accompanied by the formation of outer membrane vesicles, disappearance of the periplasmic space, sticking together of the outer and inner membranes and the eventual growth defect.

growth defects of the *degP*$^-$ cells were effectively rescued only by the overexpression (Fig. S10) but not the normal-level expression of DegP(S210A) (Fig. 3A–C). Most probably, the overwhelming level of this protease-defective degP protein in the cells would sequester and keep the misfolded OMPs in the periplasmic space, without refolding them, thus preventing their harmful accumulation on the membranes (Fig. S10B). In addition, our conclusion is also strongly supported by observations of others showing that expression of an assembly-defective mutant of OMPs in the *degP*$^-$ cells caused growth defects even when cultured at normal growth temperatures [28,29]. It follows that the essentiality of DegP (HtrA) homolog in yeast for growth under heat shock conditions may also be attributed to removal of the toxic misfolded mitochondrial β-barrel membrane proteins [12]. Last but not least, a potential antibacterial strategy might be developed to force the accumulation of toxic misfolded OMPs and thus to suppress the growth of pathogenic bacteria.

Experimental procedures

Plasmid construction, immunoblotting, protein purification, isolation of outer membrane for the *in vitro* folding assay, light scattering, outer membrane integrity assay

The details of the procedures for plasmid construction (primers used in this study were listed in Table S5), immunoblotting (antibodies used in this study were listed in Table S6), protein purification, isolation of outer membrane for the *in vitro* folding assay, light scattering and outer membrane integrity assay are described in the experimental procedures of the Supporting Information (Doc. S1). Other methods are described below.

Bacterial strains and culture conditions

All bacterial strains used in this study and their relevant genotype features are listed in Table S3. Cells were cultured at the indicated temperatures in LB broth supplemented with 25 μg·mL^{-1} chloramphenicol and/or 10 μg·mL^{-1} kanamycin, depending on whether or not a particular drug-resistant gene was carried in the cell. Subculturing was usually performed by 100-fold dilution of overnight-cultured cells and cultured for 6 h, except that JGS199 (*surA*$^-$ *degP*$^-$ pBAD*surA*) and JGS200 (*skp*$^-$ *surA*$^-$ pBAD*surA*) were cultured overnight in the presence of 0.2% L-arabinose (to induce the expression of SurA), washed twice with fresh LB, and 2000-fold and 10 000-fold diluted before being further cultured for 8 h to deplete SurA protein.

Expression and photo-crosslinking of the DiZPK- or Bpa-incorporated variants of DegP

Each of the in-frame amber mutation plasmids (Table S4), together with the pSUPAR-Mb-DiZPK-RS (encoding $tRNA^{DiZPK}$ and DiZPK-tRNA synthetase) or pSUP-Bpa-RS-6TRA helper plasmid (encoding $tRNA^{Bpa}$ and Bpa-tRNA synthetase), were transformed into *degP*$^-$ cells. The cells were cultured in LB broth at 37 °C to an A_{600} of 0.6 and induced to express the DiZPK- or Bpa-incorporated variants of DegP(S210A) by adding the inducer L-arabinose (to 0.02%) and DiZPK or *p*Bpa (both to a final concentration of 1 mM). The cells were then further incubated to an A_{600} of 1 before photo-crosslinking was performed by exposing the cells (on ice) to UV irradiation at 365 nm for 5 min using a Hoefer UVC-500 crosslinker, as described previously [32].

Purification and gel-based proteomic analysis of photo-crosslinked products of DegP variants

The photo-crosslinked products of DegP(S210A)-M42DiZPK or DegP(S210A)-M42pBpa were purified (as displayed in Fig. 1B) using Ni-NTA resin before being applied to gel-based proteomic analysis, according to the methods described before [32].

Co-purification of proteins interacting with DegP

Proteins interacting with the His-tagged wild-type DegP and protease-deficient DegP(S210A), expressed in *degP*$^-$ cells respectively from the low-copy vectors pACYC-pDegP-His_6 and pACYC-pDegP(S210A)-His_6, were co-purified using Ni-NTA resin. For this, cells were cultured overnight at 37 °C, 100-fold diluted and subcultured at 37 °C or 44 °C for 6 h, harvested and washed, and resuspended in buffer A (50 mM NaH_2PO_4 and 50 mM NaCl, pH 7.5) before being lysed by sonication. After centrifugation at 20 000 ***g*** for 30 min, the supernatants were loaded onto a 1 mL Ni-NTA agarose column (GE Healthcare, Uppsala, Sweden) and washed sequentially with 50 mL buffer A and 50 mL buffer A containing 50 mM imidazole before the bound proteins were eluted with 5 mL buffer A containing 500 mM imidazole. The eluted samples were then desalted using a PD-10 column (GE Healthcare) before being subjected to protein identification by mass spectrometry and immunoblotting analysis. The protein concentration was determined using a Bradford BCA Kit (Pierce, Rockford, IL, USA).

In vitro degradation of DegP(S210A)-bound OMPs by the externally added DegP

To test the ability to degrade the OMPs that DegP (S210A) bind in living cells, the high purity DegP (as

mentioned above) was added to 0.5 mg·mL^{-1} in buffer A containing the same concentration of the co-purified DegP(S210A)-OMP complexes. The mixture was then incubated at 37 °C or 44 °C for 30 min before being analyzed by SDS/PAGE and immunoblotting. To verify the degradation of OmpA-TM by DegP more convincingly, the incubation time was extended to 120 min.

Semi-native SDS/PAGE

Semi-native SDS/PAGE analysis of OMPs was performed by directly loading the samples with no heating treatment to maintain the folding status of the OMPs [39]. For preparing protein samples, cells cultured at the indicated temperatures for 6–8 h to the late log phase were immediately adjusted to an A_{600} of 1.0 with fresh LB broth. 1 mL of such cells was then pelleted and resuspended in 100 μL of buffer A (50 mM NaH_2PO_4 and 50 mM NaCl, pH 7.5), mixed with 50 μL of 6 × SDS sample loading buffer (TransGen) and incubated at 37 °C for 10 min [30] before being applied to SDS/PAGE. As controls, fully denatured OMPs were prepared by heating the samples at 100 °C for 10 min. All the electrophoreses were performed with 10% acrylamide gels. For the effective transfer and detection of the folded oligomers of OMPs that were wrapped by lipopolysaccharides, the gel was heated by steaming for 10 min before the proteins were transferred to the poly (vinylidene difluoride) membrane for immunoblotting analysis [29]. To indicate the position of the unfolded OMPs, a protein sample from the lysate of the wild-type cells boiled for 10 min was also analyzed.

Live-cell fluorescence imaging

For live-cell fluorescence imaging, wild-type and *degP*$^{-}$ mutant cells harboring pRay-sfGFP-SSmCherry plasmid were scratched by sterile loops from colonies formed on the agar plate, diluted into liquid LB medium to an appropriate concentration, transferred onto a glass-bottomed dish and covered with 2% (w/v) agarose gel slab prepared with LB medium before being incubated at 44 °C for 1 h. Live-cell imaging of such cells was then performed on a DV Elite microscope using optics that are specific for white field, green fluorescent protein or mCherry imaging.

Electron microscopy

The cells were cultured at 44 °C in LB broth for 6 h, harvested, adjusted to an A_{600} of 1.0 with fresh LB broth, treated with sucrose (with a final concentration of 20%) for 1 min, fixed with glutaraldehyde and formaldehyde (final concentrations of respectively 0.1% and 2%) for 1 h, and subjected to post-fixation and dehydration, as described previously [47]. The bacterial cells ultra-thin sectioned (on PowerTome-XL) of the indicated genotypes were negatively stained with 2% uranyl acetate before being imaged on a JEM-1400 transmission electron microscope operated at 80 kV with a magnification of 3000 or 25 000, according to methods described previously [46].

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Immunoblotting results, using the antibody against His-tag, of photo-crosslinked protein products of DegP(S210A)His_6 variants after being separated by SDS/PAGE.

Fig. S2. Specificities of the anti-OMP antibodies used in this study.

Fig. S3. Coomassie Blue staining results of proteins co-purified with DegP(S210A).

Fig. S4. The OmpC, OmpF and the transmembrane domain of OmpA were captured by DegP(S210A) largely in their unfolded states.

Fig. S5. The *degP*$^-$ cells cultured at 44 °C for 6 h are still alive.

Fig. S6. Misfolded OMPs already accumulate in the *degP*$^-$ cells under sub-lethal conditions.

Fig. S7. Chaperone function of SurA and protease function of DegP in OMP biogenesis.

Fig. S8. Coomassie Blue staining results of purified SurA, DegP, DegP(S210A) and OmpF, as well as the isolated outer membrane fraction (lacking OmpC and OmpF; at position indicated by the arrow) used in the *in vitro* refolding/reassembly assay.

Fig. S9. The membrane integrity is impaired in the *degP*$^-$ mutant cells cultured at 44 °C.

Fig. S10. Overexpressed DegP(S210A) prevents the toxic accumulation of misfolded OMPs onto the outer membrane but does not refold them.

Doc. S1. Experimental procedures.

Table S1. Abundance of OMPs and other representative envelope proteins in *E. coli* cells.

Table S2. Relation between misfolded OMPs and growth defect of strains analyzed in this study.

Table S3. *E. coli* strains used in this study.

Table S4. Plasmids used in this study.

Table S5. Primers used in this study.

Table S6. Antibodies used in this study.