Many proteins form multimeric complexes that play crucial roles in various cellular processes. Studying how proteins are correctly folded and assembled into such complexes in a living cell is important for understanding the physiological roles and the qualitative and quantitative regulation of the complex. However, few methods are suitable for analyzing these rapidly occurring processes. Site-directed in vivo photo-cross-linking is an elegant technique that enables analysis of protein–protein interactions in living cells with high spatial resolution. However, the conventional site-directed in vivo photo-cross-linking method is unsuitable for analyzing dynamic processes. Here, by combining an improved site-directed in vivo photo-cross-linking technique with a pulse–chase approach, we developed a new method that can analyze the folding and assembly of a newly synthesized protein with high spatiotemporal resolution. We demonstrate that this method, named the pulse–chase and in vivo photo-cross-linking experiment (Pixie), enables the kinetic analysis of the formation of an Escherichia coli periplasmic soluble protein complex (PhoA). We also used our new technique to investigate assembly/folding processes of two membrane complexes (SecD–SecF in the inner membrane and LptD–LptE in the outer membrane), which provided new insights into the biogenesis of these complexes. Our Pixie method permits analysis of the dynamic behavior of various proteins and enables examination of protein–protein interactions at the level of individual amino acid residues. We anticipate that our new technique will have valuable utility for studies of protein dynamics in many organisms.

Many cellular proteins function as multimeric complexes that play important roles in various aspects of cellular physiology. Some complexes have a simple structure comprising only two or a few subunits, whereas others have a more complex structure that contains numerous different subunits (such as ribosomes, proteasomes, and respiratory chain complexes). These complexes are required to be formed in a well-coordinated manner as unbalanced biogenesis and/or mis-assembly of subunits would yield non-functional or even aberrant complexes (or subunits) that could disrupt the cellular functions, which may ultimately result in cell death (1–3). In some cases, the activity of a complex is modulated through an exchange of a subunit(s) (4–6), indicating that the subunit assembly process can contribute to the functional regulation of a complex. Hence, knowledge of subunit maturation (folding) and assembly in a living cell facilitates understanding of the cellular function of the relevant complex. However, these processes proceed very rapidly in vivo; hence, it is generally difficult to follow them with high spatiotemporal resolution.

Site-directed in vivo photo-cross-linking is a state-of-the-art technique that enables analysis of protein–protein interactions (PPI) in a living cell. It uses an evolved pair of tRNAs and its partner aminocacyl tRNA synthetase to introduce an unnatural amino acid in the position of an amber codon in a target protein. With this technique, it is possible to incorporate a photocross-linkable amino acid analog such as p-benzoyl-l-phenylalanine (pBPA) into a protein of interest in a site-specific manner in vivo (7). Upon UV irradiation, proteins containing pBPA generate covalently cross-linked products with nearby molecules (proteins, nucleic acids, and lipids) that are proximal to the pBPA site in the target protein (8–10). Because a photoactive amino acid analog can be introduced to essentially any position in the target protein, it is possible to analyze protein folding and interaction with high spatial resolution (at the level of an amino acid residue). Moreover, this method enables the detection of not only static but also transient interactions (3, 11–13). Thus, the site-directed in vivo photo-cross-linking approach would be superior to conventionally-used biochemical techniques such as affinity isolation (pulldown or immunoprecipitation (IP)) and chemical cross-linking to investigate PPIs in a cell. Although numerous studies have reported the effectiveness of this approach in analyzing in vivo PPI, certain issues persist. In this method, long UV irradiation (typically over several minutes) is generally required to generate detectable levels of the cross-linked products, which deter precise monitoring of rapidly-proceeding processes. Moreover, long UV irradiation raises a concern regarding UV-induced damage to cellular factors including proteins, which could lead to detection of some artificial cross-linking. The current site-directed in vivo photo-cross-linking technique is thus not suitable for analysis of subunit maturation (folding) and assembly in a living cell.

The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Figs. S1–S8, Table S1, and supporting Refs. 43–48.

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A. Schematic representation of the PiXie method. This method enables the analysis of the dynamic interaction of newly synthesized protein (yellow) with its partner protein(s) (blue) and/or a cellular factor(s) (green) involved in its maturation. B. A general protocol for PiXie. Cross-linked products are indicated by XL and a green arrow. C. Actual procedures of UV irradiation and chase termination by TCA-treatment in PiXie. See the details in the main text and “Experimental procedures.”

In study, we improved this technique to make it applicable to investigation of the in vivo protein dynamics with high spatio-temporal resolution by combining a pulse–chase approach with a strong UV irradiator. We demonstrate that this new technique, named PiXie (pulse–chase and in vivo photo-cross-linking experiment; Fig. 1A), can be successfully used to investigate the folding and/or the complex formation of newly synthesized soluble and membrane proteins in a living cell.

Results

Development of the PiXie method

Conventional in vivo photo-cross-linking requires long UV irradiation of samples, which deters analysis of rapidly proceeding processes that occur in a living cell, with high temporal resolution. We found that the use of a strong UV irradiator (SP-9) enables drastic shortening of the UV irradiation time without significant reductions in cross-linking efficiency (Fig. 2; details are described below), thereby overcoming the aforementioned issues. We compared the efficiency of photo-cross-linking using SP-9 with that of photo-cross-linking using B-100AP, a UV irradiator used in our previous studies in which a long irradiation time (3–10 min) was needed to obtain a significant amount of the cross-linked products (3, 11–13). We used Escherichia coli alkaline phosphatase (PhoA) as a model protein. PhoA is a periplasmic homodimeric enzyme. Our previous biochemical study reported that after synthesis, PhoA is transported to the periplasm across the inner membrane (14) and folded into a partially protease-resistant monomeric form with two intramolecular disulfide bonds, which finally assemble into an enzymatically active dimeric form (14–16). PhoA(A427pBPA), possessing pBPA at the Ala-427 position located in the PhoA subunit interface (Fig. 3A), was synthesized in a pBPA-dependent manner (Fig. S1B) and exhibited an enzymatic activity comparable with that of wild-type (WT) PhoA (Fig. S1C). PhoA(A427pBPA) generated a clear cross-linked product of about 100 kDa upon UV irradiation with B-100AP (Fig. S1D). Experiments with two differentially-tagged PhoA derivatives showed that the ~100-kDa band was detected using antibodies for both of these tags, indicating that it represented a cross-linked PhoA dimer (Fig. S1E). When B-100AP was used, a UV irradiation time of >3–6 min was needed to obtain ~40% cross-linking efficiency. In contrast, only a 1-s irradiation was sufficient to obtain similar results with SP-9 (Fig. 2, A–C).

Drastic shortening of UV irradiation time enabled combining the in vivo photo-cross-linking technique with a pulse–chase experiment for real-time analysis of dynamic PPIs in protein biogenesis. We established a method, named PiXie (Fig. 1A), that enables in vivo analysis of the interaction and folding kinetics of newly synthesized proteins. An outline of the PiXie method is as follows (Fig. 1, B and C). (i) Cells expressing a pBPA-incorporated target protein were pulse-labeled with [35S]Met. The chase began with the addition of excess non-labeled Met. (ii) An aliquot of the pulse-labeled cell culture (typically 350 μl) was rapidly dispensed into individual wells of a temperature-controlled microtiter plate. (iii) At each time point after the initiation of chase, cells were UV-irradiated for 1 s using the SP-9 irradiator to induce the cross-linking (the timing and length of irradiation were precisely controlled by a computer program). (iv) Immediately after UV irradiation, ice-cold trichloroacetic acid (TCA) was added to instantly denature proteins and terminate the chase. (v) The target proteins were isolated through IP or pulldown, and their cross-linking patterns were analyzed by using SDS-PAGE and phosphorimaging.

To validate the effectiveness of the PiXie method, we applied this method to analyze the maturation (dimer formation) kinetics of PhoA. We previously studied the maturation process of PhoA by using biochemical assays based on the differential sensitivities of PhoA in the different maturation steps to a protease and a reducing agent (16). This previous study provided an estimate of the PhoA maturation time; it took 4–8 min for chromosomally-encoded PhoA to dimerize at 15°C. In this study, we analyzed the maturation kinetics of chromosomally-encoded PhoA(A427pBPA) by using the PiXie method. At 15°C, the amount of the cross-linked PhoA dimer increased during the chase period and plateaued in 6–8 min (Fig. 3B and Fig. S2). We examined cross-linking of three additional PhoA derivatives containing pBPA in the dimeric interface.
and Tyr-87) or on the external surface of the dimer (at Trp-220) (Figs. S1A and S4A). All derivatives displayed near normal PhoA activities (Fig. S1). When PhoA(L37pBPA), PhoA (Y87pBPA), and PhoA(A427pBPA) were expressed from a plasmid in E. coli, they formed a cross-linked dimer (Figs. S1D and S4B) with similar kinetics (Fig. S4C). In contrast, no cross-linked dimer was generated when PhoA(W220pBPA) was used (Fig. S1D). These results suggest that the observed cross-linking does not reflect the random collisions of immature PhoA subunits, but rather the productive association of folded PhoA subunits. Several pBPA-containing proteins used in this study, including PhoA(L37pBPA) and PhoA(Y87pBPA), exhibited a rather low cross-linking efficiency, probably partly because of the relatively long distance between introduced pBPA and the “target” site and/or to low flexibility of the pBPA at each position. Furthermore, we observed a significant increase in the rate of the cross-linked dimer formation at 30 °C rather than at 15 °C (Fig. S3). Because the temperature at the time of UV irradiation did not affect the efficiency of the cross-linking reaction itself (Fig. S3), the observation indicates that the maturation (dimer formation) of PhoA would be faster at an elevated temperature. These results indicate that the PiXie method is useful for high-temporal resolution analysis of the dynamic assembly process of a newly synthesized protein in vivo.

**PiXie method can be used to analyze the subunit folding and assembly of an IM protein complex**

Furthermore, we applied the PiXie method to analyze protein complex formation in the IM. SecD and SecF, multispansing IM proteins, assemble into a complex (SecD/F) and function in concert with translocon (SecY/E/G) to facilitate protein translocation across the IM (17). Based on the crystal structure of Deinococcus radiodurans SecD (Fig. 4A) (18), we introduced pBPA into the interface for association with SecF (at Ser-533 and Lys-563) and the first periplasmic region (P1 domain; at Arg-268) of hemagglutinin (HA)-tagged SecD to monitor the SecD–SecF assembly and the SecD P1 domain folding, respectively. Full-length products of these SecDpBPA derivatives accumulated in the presence of pBPA (Fig. S5A). These proteins were functional because they reversed protein export defects caused by the secD1(Cs) mutation that decreased the expression level of the chromosomally encoded SecD, when they were co-expressed from a plasmid together, along with SecF (Fig. S5B). Each of SecD(S533pBPA) and SecD(K563pBPA) generated an ~85-kDa band upon UV irradiation (Figs. 4B and Fig. S5C and S6). This band reacted with both anti-HA and anti-SecF antibodies, indicating that it represents a SecDxSecF cross-linked product (Fig. S5C). Additionally, SecD(R268pBPA) generated a band migrating slightly more slowly than SecD (Figs. 4B and Fig. S5E). The results of the previous disulfide cross-linking experiments (17) and additional in vivo photo-cross-linking experiments using a SecD derivative having pBPA at Arg-418, proximal to Arg-268, strongly suggested that the slowly migrating band is an intramolecular cross-linked product (Fig. S5, D and E; see the legend for details).

The results of the PiXie analysis using the aforementioned pBPA-containing SecD derivatives showed that both the intramolar and the inter-molecular cross-linked products were detected at very early time points during the chase; however, the formation of the former significantly preceded that of the latter (Fig. 4B). Formation of these cross-linked products was prominently inhibited by the chromosomal secY125(Cs) mutation that impairs the protein translocation across and integration into the membrane (Fig. S7) (19), suggesting that the cross-linked products represented a functional protein or a protein complex that had been formed via the normal biogenesis pathway. These results strongly suggest that folding of the periplasmic P1
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Figure 3. PiXie for the PhoA dimerization. A, crystal structure of the E. coli PhoA dimer (PDB code 3tg0). One subunit is shown in pink, and the other in gray. The Ala-to-pBPA substitution at position 427 is indicated by a blue sphere. B, time course of PhoA dimerization at 15°C and 30°C. RM1860 (phoR phoA(A427amb)) or EVOL-pBPA cells were first grown at 30°C in the presence of 0.02% arabinose until the early log phase and then the cultures were shifted to 15°C or kept at 30°C. After growth further at each temperature for 1 h, cells were pulse-labeled with [35S]Met for 30 s. Following UV irradiation for 1 s at the indicated time points during the chase, total cellular proteins were immediately acid-precipitated. SDS-solubilized proteins were subjected to IP with anti-PhoA antibodies and 10% SDS-PAGE analysis. Cross-linked products are indicated by XL and blue arrowheads. Asterisks indicate non-specific bands (upper). The CE was calculated using Equation 2 described under “Experimental procedures” and the relative values were plotted with CE at 12 min set to 1. Mean ± standard deviation values from four independent experiments are shown (lower). The absolute cross-linking efficiency (mean value) of PhoA(A427pBPA) at 12 min was 31.9% for 15°C and 44.0% for 30°C.

PiXie can be used to capture an assembly intermediate of an outer membrane protein complex

Finally, we investigated the assembly process of the OM LptD–LptE complex that translocates lipopolysaccharide (LPS) to the outer leaflet of the OM. LptD is a β-barrel OM protein, whereas LptE is a lipoprotein with an N-terminal lipid moiety that associates with the OM. In the functional LptD–LptE complex, the protein domain of LptE is inserted within the interior of the LptD barrel (20, 21). In LptD biogenesis, an intermediate form (LptDC) containing two consecutive intramolecular disulfide bonds (Cys-31–Cys-173 and Cys-724–Cys-725) was first formed and then converted to the mature form (LptDCN) having non-consecutive intramolecular disulfide bonds (Cys-31–Cys-724 and Cys-173–Cys-725) (3, 22). LptE facilitates the LptDC–LptDCN conversion possibly through direct interaction with LptDCN (3, 22, 23); however, this has not been demonstrated directly in the wild-type LptD maturation. Thus, we examined the assembly of the LptD–LptE complex using the PiXie method.

LptE(R150pBPA) and LptD(L533pBPA) are functional proteins and can be cross-linked to LptD and LptE, respectively (Fig. 5A) (24). We confirmed the cross-linking of these pBPA-containing derivatives using a strain expressing LptE(R150pBPA) or LptD(L533pBPA), together with the wild-type partner protein (LptD and LptE, respectively), from a plasmid (Fig. S8A). LptDC containing non-consecutive disulfide bonds and LptDCN containing consecutive disulfide bonds are easily discernable, as the former migrates slower than, but the latter migrates faster than, the fully reduced form of LptD (LptDred) in a non-reducing SDS-PAGE (3). In the strains expressing these pBPA-containing derivatives, the kinetics of the LptD disulfide bond isomerization was nearly equal to that in the strain expressing wild-type LptD and LptE, indicating that the introduction of pBPA weakly affected the biogenesis of LptD–LptE complex (Fig. S8B). PiXie was performed using these LptD and LptE derivatives (Fig. 5B and Fig. S8C). When the samples were analyzed by a non-reducing SDS-PAGE, two LptD–LptE cross-linked products (LptDxE) were detected; however, in reducing conditions, only one cross-linked product was observed, suggesting that the rapidly-migrating and the slowly-migrating cross-linked products contained LptDC and LptDCN, respectively, and migrated to the same position when the disulfide bonds were cleaved via reduction (note that LptE does not have disulfide bonds). Concurrently, the proportion of the upper cross-linked product and LptDCN in total LptD in the UV-irradiated samples was almost equal to the proportion of LptDCN in the non-UV-irradiated samples (Fig. S8D). We thus assigned the upper cross-linked band as an LptDCN–LptE cross-linked product and the lower band as an LptDC–LptE cross-linked product. The LptD–LptE cross-linking was hardly detected at a 1-min chase. In the later chase period, the LptDC–LptE cross-linked product peaked; however, its level decreased with time with a concomitant increase in the level of the LptDCN–LptE cross-linked product (Fig. 5B and Fig. S8C). These results strongly suggest that LptDC, the assembly intermediate of LptD, directly interacts with LptE possibly at the BAM complex in the OM (25) and is then converted to LptDCN. In addition, we observed that only a small proportion of LptDC was cross-linked with LptE just after pulse-labeling (at the 1-min chase point); however, the proportion significantly increased during the chase period (Fig. 5C), strongly suggesting that LptDC can assume two different states (LptE-cross-linkable and LptE-non-cross-linkable) during its biogenesis. These results demonstrate that an immature protein complex can be captured using PiXie.

Discussion

In this study, we developed a method, named PiXie, by combining an in vivo photo-cross-linking approach with a pulse-chase approach to analyze the rapid and dynamic processes in protein biogenesis that occur in a living cell. This method used a strong UV irradiator that could drastically shorten the UV irradiation time necessary for efficient cross-linking. To estab-
lphas PiXie, we used PhoA, a periplasmic alkaline phosphatase, as a model protein. Comparison of the previously used UV irradiator with the new one showed that only 1/250 irradiation time was required to obtain comparable cross-linking with the new UV irradiator. Furthermore, we then examined the dimerization kinetics of PhoA using PiXie, which showed that $t_{3/2}$ for PhoA dimerization using PiXie was 2–4 min at 15 °C (Fig. 3), which is concurrent with findings of previous biochemical studies (16). At 30 °C, the dimerization rate was significantly accelerated ($t_{3/2}$ at 30 °C was around 1 min). These results showed that the PiXie method can be used to investigate the dynamic maturation process of a newly synthesized protein.

To further validate the effectiveness of PiXie, we applied this technique to investigate the assembly process of two membrane protein complexes differently localized, SecD/SecF in the IM and LptD/LptE in the OM. PiXie for SecD/SecF showed that the intramolecular cross-linking in the periplasmic P1 domain of SecD occurred earlier than the SecD–SecF cross-linking, suggesting that at least the folding of the P1 domain is uncoupled with and proceeds prior to the SecD–SecF association (Fig. 4). This indicates either that the membrane domains of SecD and/or SecF fold more slowly than the extra-membrane domain or that the association of the folded membrane domains is a slower process than the folding of the extra-membrane domain.

In the analysis of LptD/LptE, we found that LptDC was cross-linked with LptE during the formation of the “mature” LptD–LptE complex (Fig. 5), consistent with previous results obtained with a mutant LptD protein that was postulated to mimic the bona fide assembly intermediate (23). Our results suggest that LptDC, an LptD folding intermediate, can assume two different states; LptDC was not efficiently cross-linked with LptE at an early step of biogenesis; however, it could be cross-linked at later steps. One possibility would be that the former LptDC species exists in an LptE-free state in the periplasm or at the BAM complex in the OM, but the latter one is associating with LptE. Alternatively, both of the LptDC species might be proximal to LptE but have different conformations and/or spatial arrangements, which results in differential LptE-cross-linking. Furthermore, our results showed that, during the 10–30-min chase, nearly half of LptDC was cross-linked with LptE with a 1-s UV irradiation. If association with LptE directly triggers immediate conversion of LptDC to LptDNc, LptDC should be converted far more rapidly to LptDNc than was observed. This raises the possibility that the interaction between LptDC–LptE, as detected by cross-linking, is not sufficient for the conversion of LptE-associated LptDC to LptDNc and that certain other factor(s) promote this conversion. These results indicate that PiXie is suitable for analysis of the protein dynamics not only in soluble compartments but also in a membrane.

A combination of a site-directed in vivo photo-cross-linking technique with a pulse–chase approach would be a powerful approach to investigate protein dynamics in a living cell. Indeed, a similar approach has been successfully employed previously to analyze an OM protein biogenesis in E. coli (26). In that study, however, cells were withdrawn at intervals during the chase period and UV-irradiated on ice for 4 min for cross-linking. Hence, some biological processes or reactions, including PPIs, may not have been instantaneously halted only by rapid chilling of cells on ice. In addition, UV exposure in minutes could elevate the sample temperature even on ice, thereby resulting in the progression of the biological processes or reactions. The method reported in this study circumvents the aforementioned issues and would thus be of significant utility to study in vivo dynamics of newly synthesized proteins in native conditions. Similarly, a strong UV irradiator would also be useful for an in vivo photo-cross-linking experiment even when it is not combined with a pulse–chase approach. For example, it

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**Figure 4. PiXie analysis of the SecD/SecF assembly.** A, a crystal structure of the D. radiodurans SecD/SecF complex (PDB code 5xap). SecD and SecF are shown in orange and gray, respectively. The positions corresponding to R268, S533, and K563 in E. coli SecD (18) where pBPA was introduced are indicated by blue (R268), red (S533), or green (K563) spheres. B, kinetics of the SecD P1 domain folding and SecD–SecF association (left), HM1246/pEvol-pBpF/pBAD18-ha-secD(amb) + secF cells were grown at 37 °C until the early log phase, and then the culture was shifted to 30 °C. After grown further for 30 min, cells were induced to express HA-SecDpBPA with 0.02% arabinose for 20 min and labeled with [35S]Met for 30 s. Following UV irradiation for 1 s at the indicated time points during the chase, total cellular proteins were immediately acid-precipitated (UV irr. −); the data are the same as those for the no UV-irradiated, 12 min-chased samples in Fig. S6 and shown for comparison. Proteins were SDS-solubilized and subjected to IP with anti-HA antibodies and 10% SDS-PAGE analysis. Intra indicates a product of HA-SecD with intramolecular cross-linkage. SecDxF indicates cross-linked products between HA-SecD and SecF. The cross-linked products are also indicated by blue arrowheads. (right) The CE was calculated using Equations 3 and 4 described under “Experimental procedures” and the relative values were plotted with CE at 12 min set to 1. The inset is an enlarged view of the region for 0- to 4-min chase. Mean ± standard deviation values from three independent experiments are shown. The absolute cross-linking efficiency (mean value) of each SecDpBPA variants at 12 min is 25.1% (R268), 20.3% (S533) and 15.1% (K563), respectively.
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In this study, we focused on and analyzed the subunit association of the protein complexes, and PiXie could be used to capture more short-lived transient PPIs. It would be possible to determine when and how a newly synthesized target protein interacts with cellular proteins, including molecular chaperones, foldases, translocons, and proteases that mediate protein maturation and metabolic processes such as folding, targeting, export, and degradation. However, identification of novel cross-linked partners that normally interact with the newly synthesized target protein only transiently would not be easy (unless the identities of these proteins can be deduced from other information). Moreover, the low amount of the cross-linked products that such proteins would generate associated with the small volume (less than 500 μl) of cultures that can be used for UV irradiation by the new UV irradiator would deter purification of a sufficient amount of the cross-linked products for mass spectrometric analysis, as performed previously (12, 24). If this issue could be overcome, for instance, through improvement of analytical techniques and/or instruments, including the UV irradiator, it would be possible to identify interacting proteins systematically and understand the life cycle of the target protein from the perspective of PPI by using PiXie.

In this study, we used pBPA as a photoreactive amino acid analog, because it is one of the most commonly used and easily available. pBPA is undoubtedly useful; however, it might affect the function of target proteins and exhibit low cross-linking efficiency in some cases, because of its properties, including a large size and low flexibility. The use of other photo-reactive amino acid analogs could overcome these issues. To date, additional useful photo-cross-linkers with different properties that can be genetically incorporated into a protein have been developed, including aryl azide-based pAzPA, trifluoromethyl phenyldiazirine-bearing TfmdPhe, and pyrrolysine-based and diazirine-containing DiZPK and its derivative DiZSeK (27–30). The use of photoreactive amino acid analogs appropriate for the protein of interest and for experimental purposes would further enhance results in the PiXie analysis.

The PiXie method can be improved through several means. For instance, use of a more powerful UV irradiator could further shorten the irradiation time and enable detection of weaker and/or more short-lived interactions. Furthermore, automation of each step of the procedures would help to obtain more accurate and reproducible results. A combination of such improvements would significantly increase the temporal resolution of analysis using this method. As the site-directed in vivo photo-cross-linking approach can be used in not only prokaryotic cells, but also in eukaryotic cells, including yeast and mammalian cells (31, 32), PiXie could be applied for detailed analysis of various cellular processes in many organisms.

**Experimental procedures**

**Bacterial strains**

*E. coli* K12 strains used in this study are listed in Table S1. They were constructed as follows: RM1746 (MC4100, *phoR zaj-3053::Tn10*) was constructed by transferring *zaj-3053::Tn10*, which is located near the *phoR* and *phoA* genes, from CAG18091 (33) into SM138 (15) by P1 transduction. RM1792 (HM1742, *phoR zaj-3053::Tn10*) and RM1794 (JEE6631 (polA−), *phoR zaj-3053::Tn10*) were constructed by co-transduction of the *phoR* marker with *zaj-3053::Tn10* from RM1746 to
HM1742 (34) and JE6631 (35), respectively. RM1816 (HM1742, ΔphoR::kan) was constructed by transducing ΔphoR::kan from JW0390 (36) to HM1742. RM1856 (JE6631, phoA(A427amb) phoR zad-3053::Tn10) was constructed as follows: pRM229 (a plasmid carrying phoA(A427amb), see below) was introduced into RM1794 to yield cells with pRM229 integrated into the chromosome by homologous recombination. 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 phoA (A427amb) allele in place of the wild-type phoA gene. One of such strains was selected and named RM1856. RM1860 (phoA(A427amb) phoR zad-3053::Tn10) was constructed by co-transduction the phoA(A427amb) and the phoR mutations from RM1856 to RM1816 with zad-3053::Tn10. HM3314 (HM1246, secD1) was constructed by co-transduction of the secD1 mutation to HM1246 (37) with a linked Tn10. RM2035 (HM1246, secY125) was constructed by co-transduction of the secY125 mutation from SH465 (19) to HM1246 with zhd-33::Tn10.

Plasmids

Plasmids used in this study are listed in Table S1. Details of the plasmid construction are described below. pNM1 (pTTQ18-phoA) was constructed by PCR amplification of a phoA fragment from the genome of MC4100 using primers, phoA-for and phoA-ha-rev (5,-CGCGGATCCAGCGACGAGTCCAGAAGACATGTCGACTTCGACAGCTTATAGTC-3') and phoA-ha-atg (5,-CGCGGATCCAGCGACGAGTCCAGAAGACATGTCGACTTCGACAGCTTATAGTC-3') and, after digestion with BamHI and HindIII, cloning it into the same site of pTTQ18. For construction of the pTTQ18-phoA(amb) plasmids, an amber mutation was introduced into pNM1 by site-directed mutagenesis. pTWV228-phoA(amb) plasmids were constructed by subcloning the EcoRI-BamHI phoA(amb) fragment of pTTQ18-phoA(amb) into the same sites of pTTQ18. pRM247 (pTTQ18-phoA-his10) was constructed by PCR amplification of a phoA-his10 fragment from the genome of MC4100 using a pair of primers, phoA-for and phoA-his-rev (5',CGCGGATCCAGCGACGAGTCCAGAAGACATGTCGACTTCGACAGCTTATAGTC-3') and phoA-ha-rev (5',CGCGGATCCAGCGACGAGTCCAGAAGACATGTCGACTTCGACAGCTTATAGTC-3') and, after digestion with BamHI and HindIII, cloning it into the same site of pTTQ18. Construction of the pTTQ18-phoA(A427amb)-his10 plasmid, the A427amber mutation was introduced into pRM247 by site-directed mutagenesis. pRM248 (pTTQ18-phoA-ha) was constructed by PCR amplification of the phoA-ha fragment from the genome of MC4100 using a pair of primers, phoA-ha-for (5',CGCGGATCCAGCGACGAGTCCAGAAGACATGTCGACTTCGACAGCTTATAGTC-3') and phoA-ha-atg (5',CGCGGATCCAGCGACGAGTCCAGAAGACATGTCGACTTCGACAGCTTATAGTC-3') and, after digestion with PstI and HindIII, cloning it into the same sites of pTTQ18. For construction of pTTQ18-phoA(A427amb)-ha, the A427amber mutation was introduced into pRM248 by site-directed mutagenesis. pTTQ18-phoA(amb)-his10 + phoA(amb)-ha plasmids were constructed by subcloning the EcoRI-Sall phoA(amb)-his10 fragment of pTTQ18-phoA(amb)-his10 into the same sites of pTTQ18-phoA(amb)-ha. pRM225 (pK18mosbAC-iraP-phoA-psif) was constructed by PCR amplification of an iraP-phoA-psif fragment from the genome of MC4100 using a pair of primers, ch-phoA-for (5'-GCCTAGGATCCCCAGACATGAA-GTTGTG-3') and ch-phoA-rev (5'-CGCGGATCCAGCGACGAGTCCAGAAGACATGTCGACTTCGACAGCTTATAGTC-3') and phoA-ha-rev (5'-CGCGGATCCAGCGACGAGTCCAGAAGACATGTCGACTTCGACAGCTTATAGTC-3') and, after digestion with BamHI and Sall, cloning it into the same site of pK18mosbAC.

For the construction of pRM229, the phoA(A427amb) mutation was introduced into pRM225 by site-directed mutagenesis. pBAD18-ha-secD(amb) + secF plasmids were constructed from pHM735 (pBAD18-ha-secD + secF) (17) by site-directed mutagenesis, pRM294, a derivative of pTWV228 having an spc marker instead of the amp marker, was constructed by in vitro recombination using In-Fusion HD cloning kit (Takara Bio Inc., Shiga, Japan) of a HindIII spc fragment from pHP45 (38) and a vector fragment that had been prepared by PCR amplification from pTWV228 using a pair of primers, pTWV-Spc-for (5'-TACAACGATAGCAGTGGTAACTGTACGACC-3') and pTWV-Spc-rev (5'-TACAACGATAGCAGTGGTAACTGTACGACC-3'), using Phusion DNA polymerase (New England Biolabs, Beverly, MA) and cloned into the same site of pRM294. pRM311 (pRM294-lptd-his10 + lptE-myrc) was constructed by subcloning the HindIII lptD-myc fragment of pRM273 (pTTQ18-lptE-mycrev) into the same site of pRM309. Derivatives of pRM311 containing an amber mutation in the lptD or lptE gene were constructed by site-directed mutagenesis.

Antibodies

Penta-His HRP conjugate was purchased from Qiagen (Hilden, Germany). The anti-PhoA antibody (anti-alkaline phosphatase (E. coli) antibody, 100 – 4134) was purchased from Rockland Immunochemicals Inc. (Limerick, PA), and anti-HA-tag antibodies (HA probe (F-7) and anti-Myc-tag antibody (c-Myc (9E10)) were purchased from Rockland Immunochemicals Inc. New York).

Media and bacterial cultures

Cells were grown in L medium (10 g/liter bacto-tryptone, 5 g/liter bacto-yeast extract, 5 g/liter NaCl, pH adjusted to 7.2) or M9 medium (without CaCl2). In addition, 50 µg/ml ampicillin, 20 µg/ml chloramphenicol, 25 µg/ml kanamycin, 25 µg/ml tetracycline, and 50 µg/ml spectinomycin were added, as appropriate, for cultivating plasmid-bearing cells and selection of transductants. Bacterial growth was monitored using mini photo 518R (660 nm; TAITEC Co., Saitama, Japan) or Klett-Summerson colorimeter (filter no. 54; Klett Manufacturing Co. Inc., New York).

Immunoblotting analysis

Proteins were separated by Laemmli SDS-PAGE and electroblotted onto a PVDF membrane (Merck Millipore; Billerica, MA). The membrane was first blocked with 5% skim milk in
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PBST (phosphate-buffered saline with Tween 20) and then incubated with anti-PhoA (at 1:10,000 dilution), anti-HA (1:5000), anti-Myc (1:5000), or anti-His (1:3000) antibodies. The membrane was washed with PBST and incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody in PBST. Proteins were visualized with ECL^TM^ Western blotting detection reagents (GE Healthcare UK Ltd., Little Chalfont, UK) or ECL^TM^ prime Western blotting detection reagents (GE Healthcare) and LAS3000 mini lumino-image analyzer (GE Healthcare) or LAS4000 mini lumino-image analyzer (GE Healthcare).

**UV irradiation conditions in vivo photo-cross-linking**

Cells with the target gene containing an amber mutation on a plasmid or the chromosome were grown in the medium containing 1 mM pBPA (H-p-Bz-Phe-OH F-2800; Bachem AG, Bubendorf, Switzerland), and induced as required to express the pBPA-incorporated target protein. For UV irradiation with B-100 AP UV lamp (365 nm; UVP, LLC, Upland, CA), a portion of cell cultures was placed on a Petri dish and UV-irradiated for an appropriate time at a distance of 4 cm at 4 °C. For UV irradiation with SP-9 (USHIO Inc., Tokyo, Japan), a 350-μl portion of cell cultures was placed into a well of a 24-well microtiter plate (AGC Techno Glass Co., Ltd., Shizuoka, Japan) set on a temperature-controllable and movable stage (MS Tech Co., Ltd., Kyoto, Japan). Cells were irradiated with UV light (365 nm) from SP-9 equipped with an SFH lens (USHIO) for an appropriate time at a distance of 5 cm. The time of UV-irradiation with SP-9 and the movement of the stage were controlled by a computer program (MS Tech Co. Ltd.).

**Pixie**

To analyze dimerization of chromosomally-encoded PhoA described in Figs. 3A and Fig. S3, RM1860 (phoR phoA (A427amb))/pEVOL-pBpF cells were first grown at 30 °C in M9-medium supplemented with 2 μg/ml thiamine, 0.4% glycerol, 18 amino acids (except Met and Cys; final concentration of 20 μg/ml each), 1 mM pBPA, and 0.02% arabinose until the early log phase; thereafter, the cultures were shifted to 15 °C or 30 °C. After further culturing at each temperature for 1 h, cells were pulse-labeled with 370 kBq/ml [35S]Met (American Radiolabeled Chemicals, Inc., St. Louis, MO) for 30 s. After addition of excess nonradioactive Met (final conc. 250 μg/ml), a 350-μl portion of the cell cultures was quickly placed into wells of a 24-well microtiter plate on the temperature-controlled stage. The time of UV-irradiation for 1 s at each time points during the chase, total cellular proteins were immediately acid-precipitated. SDS-solubilized proteins were subjected to IP with anti-HA antibodies, 10% SDS-PAGE analysis and phosphor-imaging. Band intensities were quantified by using MultiGauge software and corrected for the number of methionine residues in HA-SecD and SecF. The CE for intramolecular cross-linking and SecD–SecF cross-linking were calculated using the following equations:

\[ CE = \frac{(XL) + (PhoA)}{(XL)/(PhoA))} \]  
\[ CE = \frac{(Intra)}{(Intra) + (HA-SecD))} \]  
\[ CE = \frac{(SecDx)(SecDxF)}{(SecDx) + (HA-SecD))} \]  

**For the analysis of LptD-LptE assembly described in Figs. 5 and S8C, plasmid pRM294-lptD-his+ lptE-myc or its derivative carrying an amber mutation was introduced into AD16/pEVOL-pBpF. Cells of the resulting strain were grown at 30 °C in M9-medium supplemented with 2 μg/ml thiamine, 0.4% glycerol, 0.2% maltose, 18 amino acids, and 1 mM pBPA until the early log phase, and then the cultures were shifted to 30 °C. After further growth at 30 °C for 30 min, cells were induced to express HA-SecDpBPA with 0.02% arabinose for 20 min and pulse-labeled with [35S]Met for 30 s. Following UV irradiation for 1 s at each time points during the chase, total cellular proteins were immediately acid-precipitated. Pre-
cipitated proteins were solubilized with 1% SDS and subjected to affinity-purification with Ni-NTA Agarose (Qiagen). Puriﬁed proteins were suspended into SDS-sample buffer with or without 2-mercaptoethanol (ME) and analyzed by 7.5% SDS-PAGE analysis and phosphor-imaging. Band intensities were quantiﬁed with MultiGauge software. The band intensities of LptD-LptE cross-linked products were corrected with the number of methionine residues in LptD-His10 and LptE-Myc. The CE for LptDNCxE and LptDCxE were calculated using the following equation:

\[
CE(\%) = \frac{((LptDNCx_E) \times (LptDCx_E))}{((LptDNCx_E) + (LptDCx_E) + (LptDNCxHis10) + (LptDCxHis10)) \times 100}
\] (Eq. 5)

wherein “(LptDNCx_E)”, “(LptDCx_E)”, “(LptDNCxHis10)”, and “(LptDCxHis10)” are the corrected intensities of the respective bands in the no ME gel. The ratio of LptDNCxE or LptDCxE to the total cross-linked products was calculated using the following equation:

\[
\text{ratio} = \frac{((LptDNCx_E) \times (LptDCx_E))}{((LptDNCx_E) + (LptDCx_E))}
\] (Eq. 6)

wherein “(LptDNCx_E)” and “(LptDCx_E)” are the intensities of the respective bands in no ME gel, and relative CE with the value at 12 min set to 1 was plotted. The ratio of LptDNCx Esper total LptD was calculated similarly using the following equation:

\[
\text{ratio} = \frac{((LptDNCx_E) \times (LptDCx_E))}{((LptDNCx_E) + (LptDCx_E)) + (LptDNCx_E) + (LptDCx_E)) \times 100}
\] (Eq. 7)

wherein “(LptDNCx_E)”, “(LptDCx_E)”, “(LptDNCxHis10)”, and “(LptDCxHis10)” are the intensities of the respective bands in no ME gel.

**PhoA enzyme assay**

The PhoA activity was assayed essentially as described previously using p-nitrophenyl phosphate (Sigma104; Sigma) as a substrate (42).

**Protein export assay**

To examine the export of MBP (periplasmic maltose-binding protein) and OmpA (outer membrane protein), total cellular proteins of [35S]Met-labeled cells were acid-precipitated, SDS-solubilized, and subjected to IP with anti-MBP and anti-OmpA antibodies. Proteins were separated by 10% SDS-PAGE and visualized with BAS1800 phosphorimager. Intensities of the mature and precursor proteins were quantiﬁed by using MultiGauge software and corrected with the number of methionine residues in each species. Protein export (%) was determined on the basis of the ratio of the corrected intensity of the mature band to the sum of the intensities of the precursor and mature bands.

**Author contributions**—R. M., H. M., and Y. A. conceived the idea and designed the experiments. R. M., N. M., and H. M. performed the experiments. R. M., N. M., H. M., and Y. A. analyzed the data, and R. M. and Y. A. wrote the manuscript.

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