

Identification of a Multicomponent Complex Required for Outer Membrane Biogenesis in *Escherichia coli*

Tao Wu,^{1,2,5} Juliana Malinverni,^{3,5} Natividad Ruiz,³ Seokhee Kim,^{1,2} Thomas J. Silhavy,³ and Daniel Kahne^{1,2,4,*}

¹Department of Chemistry

Princeton University

Princeton, New Jersey 08544

²Department of Chemistry and Chemical Biology

Harvard University

Cambridge, Massachusetts 01238

³Department of Molecular Biology

Princeton University

Princeton, New Jersey 08544

⁴Department of Biological Chemistry and Molecular Pharmacology

Harvard Medical School

Boston, Massachusetts 02115

Summary

Gram-negative bacteria have an outer membrane (OM) that functions as a barrier to protect the cell from toxic compounds such as antibiotics and detergents. The OM is a highly asymmetric bilayer composed of phospholipids, glycolipids, and proteins. Assembly of this essential organelle occurs outside the cytoplasm in an environment that lacks obvious energy sources such as ATP, and the mechanisms involved are poorly understood. We describe the identification of a multiprotein complex required for the assembly of proteins in the OM of *Escherichia coli*. We also demonstrate genetic interactions between genes encoding components of this protein assembly complex and *imp*, which encodes a protein involved in the assembly of lipopolysaccharides (LPS) in the OM. These genetic interactions suggest a role for YfgL, one of the lipoprotein components of the protein assembly complex, in a homeostatic control mechanism that coordinates the overall OM assembly process.

Introduction

The outer membrane (OM) is an essential organelle of Gram-negative bacteria and is an asymmetrical bilayer that functions as a selective permeability barrier (Nikaido, 2003). The outer leaflet of the OM is rich in lipopolysaccharides (LPS), while the inner leaflet consists of phospholipids (Figure 1). In addition, the OM contains two major classes of proteins, lipoproteins and β barrel proteins such as LamB. After translocation from the cytoplasm, OM lipoproteins are sorted and subsequently targeted to the periplasmic face of this membrane via the LolA/LolB pathway (Yokota et al., 1999). However, the mechanisms for targeting and assembling the other OM components are largely unknown. Moreover, it has been difficult to envision the mechanics of

the assembly processes since they occur in an extracytoplasmic cellular compartment (periplasm) that is devoid of any obvious energy source such as ATP.

Several assembly factors involved in OM biogenesis have been recently identified, including Imp (Braun and Silhavy, 2002) and YfgL in *E. coli* (Ruiz et al., 2005) as well as Imp (Bos et al., 2004) and Omp85 in *Neisseria meningitidis* (Genevrois et al., 2003; Voulhoux et al., 2003). In *E. coli*, Imp is an essential OM β barrel protein that exists in its native state as part of a higher-order complex that is required for the proper assembly of the OM (Braun and Silhavy, 2002). Cells depleted of Imp mislocalize newly synthesized lipids (LPS and phospholipids) and OM proteins (OMPs) to a novel high-density membrane fraction. Unlike *E. coli*, *N. meningitidis* does not require LPS for viability (Steeghs et al., 1998), and *imp* is not essential in this organism (Bos et al., 2004). Exploiting this fact, Bos et al. (2004) have recently demonstrated that Imp is required for LPS assembly in the OM in this organism.

The *imp4213* allele of *E. coli* encodes a mutant Imp protein that confers OM permeability defects, resulting in sensitivity to bile salts and many antibiotics including vancomycin and its glycolipid derivative chlorobiphenyl vancomycin (CBPV) (Sampson et al., 1989; Eggert et al., 2001). As described in the linked paper in this issue of *Cell* (Ruiz et al., 2005), we discovered that null mutations in *yfgL*, a gene encoding a putative OM lipoprotein, specifically correct a subset of the permeability phenotypes conferred by the *imp4213* allele. Although the *imp4213* allele does not alter the steady-state levels of OMPs, strains carrying null mutations in *yfgL* have lower levels of OMPs such as OmpA and LamB, thereby implicating YfgL in membrane biogenesis (Ruiz et al., 2005).

Here we show that YfgL exists in a heterooligomeric complex with at least three other proteins of previously unknown function: YaeT, YfiO, and NlpB. Although no reports specifically link YfiO, NlpB, or their homologs to OM assembly, a homolog of YaeT, designated Omp85, has been shown to be essential and required for OM biogenesis in *Neisseria meningitidis* (Voulhoux et al., 2003; Genevrois et al., 2003). However, the role of Omp85 in *Neisseria* is contested, with one report by Genevrois et al. (2003) arguing that Omp85 serves to incorporate LPS and phospholipids into the OM, while another report by Voulhoux et al. (2003) contends that Omp85 is responsible for OM protein assembly. The latter conclusion is further supported by the evidence that mitochondria and chloroplasts also contain Omp85 homologs, Tob55 and Toc75, respectively, that exist in complexes that have been shown to be required for the insertion of β barrel proteins into the OMs of these cellular organelles (Paschen et al., 2003; Gentle et al., 2004). If YaeT were required for targeting either lipids or OMPs to the OM in *E. coli*, logic would then predict that *yaeT* would be an essential gene, since both components are required to assemble the OM and this structure is essential in *E. coli*. However, conflicting reports have appeared regarding the essential nature of

*Correspondence: kahne@chemistry.harvard.edu

⁵These authors contributed equally to this work.

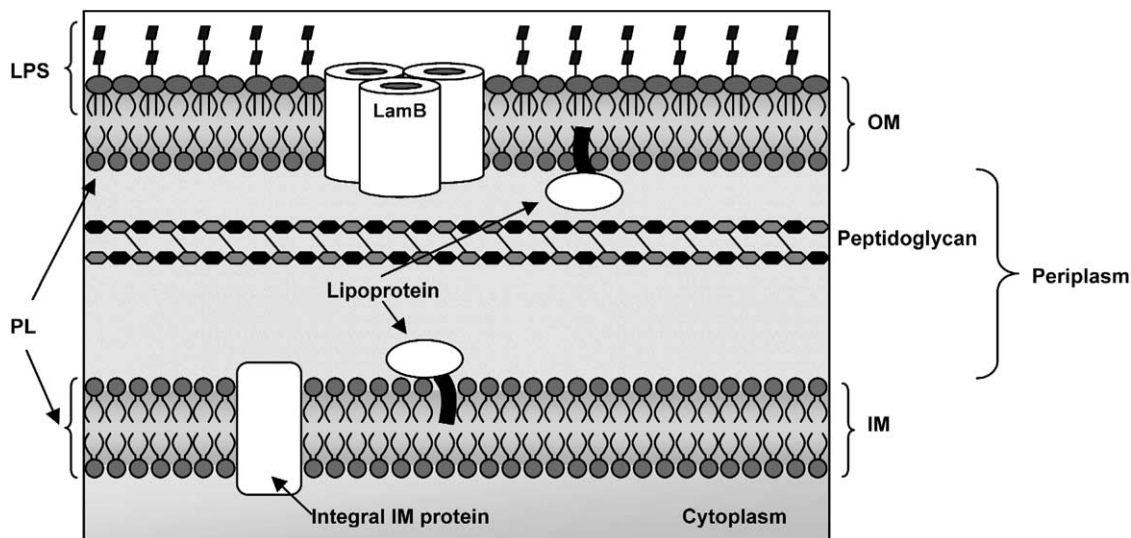


Figure 1. Outer Membrane of Gram-Negative Bacteria

LPS, lipopolysaccharide; PL, phospholipids; IM, inner membrane; OM, outer membrane.

the *yaeT* gene in this organism (Gerdes et al., 2003; Dartigalongue et al., 2001).

Historically, attempts to identify factors involved in the OM biogenesis in *E. coli* have been met with limited success due in part to the difficulty in designing appropriate genetic selections or screens. Furthermore, it has been difficult to interpret the phenotypes resulting from defects in OMP and LPS assembly, since the optimal translocation of LPS and OMPs appears to share some degree of codependency (Braun and Silhavy, 2002). For example, various stages of OMP folding are enhanced in the presence of phospholipids (Kloser et al., 1998) and LPS (Pages et al., 1990; Ried et al., 1990), while the translocation of LPS to the OM likely requires at least one OMP component based on results obtained with *Neisseria* (Bos et al., 2004).

This work describes additional factors responsible for OM assembly in *E. coli*. We propose based on genetic interactions between *yfgL* and *imp* reported in the accompanying paper (Ruiz et al., 2005), as well as genetic interactions described here, that the activities of all of these OM assembly factors function in distinct capacities but are inextricably linked to ensure the coordinated assembly of both LPS and OMPs.

Results

YfgL Is an OM Lipoprotein

We have proposed a role for YfgL in OM biogenesis (Ruiz et al., 2005), but sequence analysis makes no functional predictions. It does, however, predict that YfgL is an OM lipoprotein. To test whether YfgL undergoes the covalent posttranslational modification typical of lipoproteins, wild-type *E. coli* and a strain overproducing a functional (data not shown) His-tagged YfgL were grown in the presence of radiola-

beled palmitate. As shown in Figure 2A, a labeled protein of 40 kDa was detected in the autoradiogram and shown to be His-tagged YfgL by Western blotting with both anti-YfgL and anti-His-tag antibodies (Figures 2B and 2C, respectively). Radiolabeled YfgL was not detected in samples from the wild-type strain, although Western blot analysis using the anti-YfgL antibody (Figure 2B) confirmed the presence of this protein. The expression levels of *yfgL* in the wild-type strain are apparently too low for detection by this labeling method. These results support the prediction that YfgL is a lipoprotein.

Lipoproteins are secreted into the periplasm, where they are anchored to either the inner or outer membrane. The “+2 rule” for lipoprotein sorting can often predict to which membrane a given lipoprotein will anchor (Yamaguchi et al., 1988; Gennity and Inouye, 1991). The amino acid in the +2 position of YfgL is serine, suggesting YfgL is directed to the periplasmic face of the OM. To test this, we fractionated the inner and outer membranes of wild-type *E. coli* using sedimentation equilibrium. Western blot analyses using an antibody specific for YfgL, as well as antibodies specific for inner (55 kDa) and outer (*imp*) membrane proteins, revealed that YfgL cofractionates with OMPs as predicted (Figures 2D and 2E). Thus, YfgL is an OM lipoprotein. The amino-terminal lipid moiety of YfgL likely embeds the protein in the bilayer with the bulk of the protein remaining in the periplasm.

YfgL Forms a Complex with YaeT, YfiO, and NlpB

The genetic interactions we have described between *yfgL* and *imp4213* in the preceding paper (Ruiz et al., 2005) prompted biochemical studies to determine if these two proteins interact directly. For this purpose, we used a plasmid that produces a carboxy-terminal His-tagged YfgL. Under the conditions employed in our

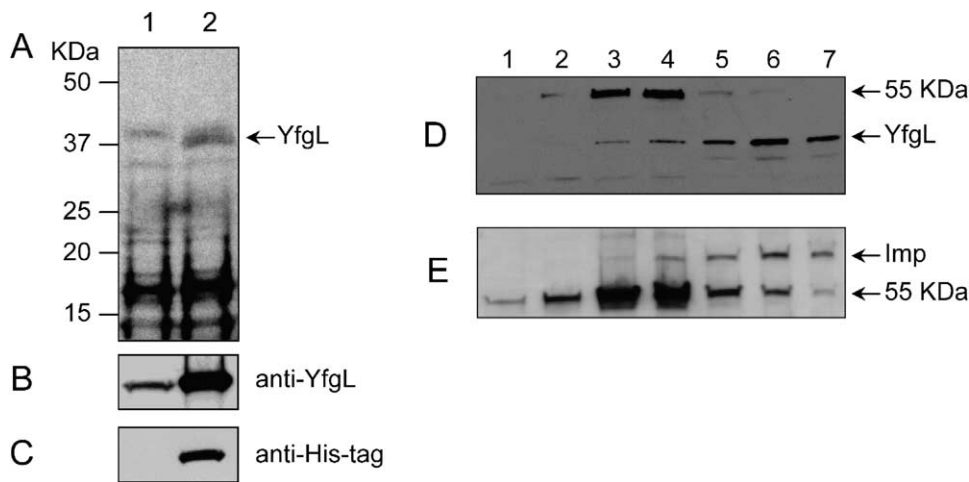


Figure 2. YfgL Is an OM Lipoprotein

(A) Autoradiography showing H^3 -palmitate labeled proteins. Lane 1: MC4100, the wild-type strain; lane 2: TW009 (pTW004) strain, a *yfgL* null strain containing a plasmid that has a C-terminal His-tagged *yfgL* controlled by an arabinose-inducible promoter, grown in presence of 0.01% arabinose.

(B and C) Western blot of the nitrocellulose membrane that was used to generate the autoradiography in (A), probed with anti-YfgL antibody (B) and with anti-His-tag antibody (C).

(D and E) Western blot of samples from membrane fractionation of MC4100 cells. The total membrane fraction isolated from MC4100 cells was loaded onto a sucrose gradient and centrifuged to equilibrium. Fractions 1–7 were collected from the top to the bottom of the gradient and subjected to Western blot analysis with anti-YfgL antibody (D) or anti-Imp antibody (E), which also recognizes an unknown 55 kDa inner membrane protein (Braun and Silhavy, 2002). The fraction number of each sample is shown above the lanes in (D).

experiments, the levels of His-tagged YfgL produced from this plasmid owing to leaky expression from the T7 promoter are similar to the levels of YfgL produced from the chromosomal gene.

To identify proteins interacting with YfgL, we performed coimmunoprecipitation experiments using a monoclonal anti-His-tag antibody. As shown in Figure 3A, lane 2, this antibody precipitated a protein of 40 kDa, which we identified as YfgL by Western blot using both anti-YfgL and anti-His-tag antibodies (data not shown). In addition, three coprecipitating proteins of approximate molecular masses of 90, 35, and 25 kDa were detected in silver-stained SDS-PAGE (Figure 3A, lane 2). The specificity of the coprecipitation is demonstrated by the fact that neither YfgL nor any of the three proteins are precipitated from a strain that does not produce His-tagged YfgL (Figure 3A, lane 1).

To our surprise, the 90 kDa protein was not Imp, as confirmed by Western blot with anti-Imp antibody. To determine the identity of this protein, the first ten amino acids were determined by Edman degradation. This sequence of amino acids obtained is an exact match to a sequence found in the *E. coli* protein YaeT. In YaeT, this sequence is located immediately downstream of a putative 20 amino acid signal sequence. Signal sequence processing would leave this ten amino acid sequence at the amino terminus of mature YaeT.

Several attempts to sequence the 35 and 25 kDa proteins by Edman degradation failed. This suggested that the amino terminus of these proteins might be blocked or modified. These two proteins were then submitted for sequence analysis by mass spectroscopy. The results showed that the 35 kDa protein is NlpB and the 25 kDa protein is YfiO. NlpB is an OM lipoprotein of

unknown function (Bouvier et al., 1991). The amino-terminal amino acid sequence of YfiO contains a predicted signal sequence and a lipobox. The +2 rule predicts that YfiO is an OM lipoprotein. YfiO has significant sequence homology with ComL, a peptidoglycan-linked OM lipoprotein in *Neisseria gonorrhoeae* (37% sequence identity), which was shown to be involved in transformation competence (Fussenegger et al., 1996). The fact that both of these proteins are lipoproteins explains why Edman degradation failed.

In order to confirm the interaction between YfgL, YaeT, YfiO, and NlpB, we constructed plasmids that express carboxy-terminal His-tagged versions of YaeT, YfiO, and NlpB. In strains producing His-tagged YaeT, immunoprecipitation using the monoclonal anti-His-tag antibody coprecipitated YfgL, as verified by Western blot using anti-YfgL antibody (data not shown). We currently do not have antibody directed against YfiO or NlpB, so we could not confirm the presence of these proteins in the complex. However, when immunoprecipitation was performed in strains producing either the His-tagged YfiO or the His-tagged NlpB, both YfgL and YaeT were coprecipitated as verified by Western blot using the anti-YaeT and anti-YfgL antibodies (Figure 3B). Thus, we conclude YaeT, YfgL, YfiO, and NlpB are all present in a large protein complex.

YaeT Is Essential in *E. coli*

Our initial attempts to delete the *yaeT* gene in *E. coli* using standard genetic techniques were unsuccessful, prompting us to develop a strain in which YaeT could be depleted (Figure 4A). This depletion strain contains a large, internal, nonpolar deletion of the chromosomal gene, and it carries a wild-type *yaeT* gene under the

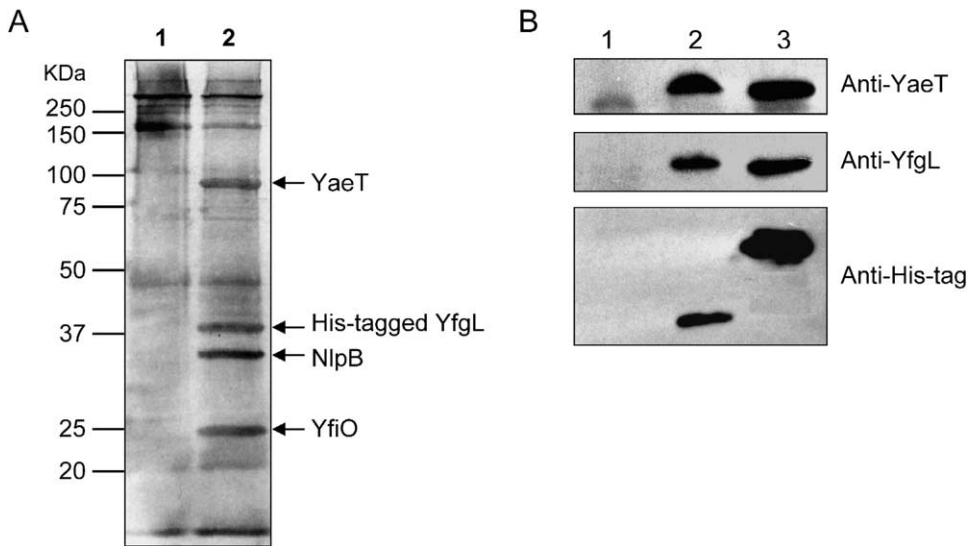


Figure 3. YfgL Forms a Complex with YaeT, YfiO, and NlpB

(A) Silver-stained SDS-polyacrylamide gel of samples from cell lysates of MC4100 (lane 1) and NR721 (pTW006) strain (lane 2) immunoprecipitated with anti-His-tag antibody.

(B) Western blot of samples obtained by immunoprecipitation using anti-His-tag antibodies of cell lysates from the wild-type MC4100 (lane 1); NR814 (pTW007), a *yfiO::kan* strain that expresses YfiO-His from pTW007 (lane 2); and NR815 (pTW008), a *nlpB* null strain that expresses NlpB-His from pTW008 (lane 3).

control of the *araBAD* promoter at the λ attachment site (Experimental Procedures). The resulting *yaeT* depletion strain (JCM166) could only form colonies on solid medium when supplemented with arabinose.

We monitored growth of JCM166 in liquid culture in the absence or presence of arabinose by monitoring optical density (OD_{600}) over time. In rich medium containing arabinose, JCM166 grew at rates comparable to wild-type (Figure 4B). In the absence of inducer, the strain grew at near normal rates for three or four generations, and then growth slowed to a stop (Figure 4B). As YaeT was depleted, cell death occurred as measured by colony forming units/ml (data not shown). Therefore, YaeT is essential in *E. coli* as predicted by Gerdes et al. (2003).

Genetic Support for a Functional YaeT/YfgL/YfiO/NlpB Complex

As shown above, YfiO and NlpB form a complex with YfgL and YaeT. YaeT is essential and so is YfiO (Onufryk et al., 2005), but YfgL and NlpB are not (Eggert et al., 2001; Ruiz et al., 2005; Bouvier et al., 1991). Transposon insertions in both *nlpB* and *yfiO* were obtained from the *E. coli* Genome Project at the University of Wisconsin-Madison. The insertion in *nlpB*, *nlpB::kan*, interrupts the gene at codon 41 (out of 346) and thus is likely a null mutation. The insertion in *yfiO*, *yfiO::kan*, interrupts the gene at codon 227 (out of 246). This mutation is not a null mutation, since strains carrying this mutation are viable. However, the YaeT complex purified from strains carrying *yfiO::kan* lacks detectable amounts of YfiO, and the phenotypes conferred by this mutation are recessive (data not shown). Therefore, we conclude that *yfiO::kan* greatly reduces but does not abolish YfiO function.

If the three lipoproteins are present in a complex, we would expect that mutations that reduce or abolish any of them would confer similar phenotypes. We reported previously that *yfgL* null mutations alter the OM permeability barrier. This is evidenced by the increased antibiotic sensitivity exhibited by strains lacking only YfgL (Ruiz et al., 2005). We found that *yfiO::kan* strains exhibited phenotypes similar to the *yfgL* mutants. In contrast, strains lacking only NlpB showed only slight defects in OM permeability. These results support our conclusions that all three lipoproteins are functionally related and that YfgL and YfiO are more important for function than NlpB.

Alterations in OM permeability are a crude test of function; any mutation that alters the synthesis or assembly of any of the OM components can cause permeability defects. A more precise test of the functional relationship between members of an essential multi-component complex would employ double mutants, the logic of which is outlined below.

The three lipoproteins are components of the essential YaeT complex. As noted above, strains carrying either the *yfgL::kan*, the *nlpB::kan* or the *yfiO::kan* mutations are viable. However, if the association of these lipoproteins with YaeT and each other is functionally significant, then pairwise combinations of these mutations may impair function enough to cause detectable changes in cell growth. Such synthetic relationships between mutations in genes whose products form multiprotein complexes have been observed in yeast (e.g., Kaiser and Schekman [1990], Scidmore et al. [1993]).

To test for synthetic phenotypes, we attempted to construct pairwise combinations of lipoprotein double mutants by introducing either the *yfiO::kan* or the *nlpB::kan* allele into a strain carrying *yfgL8* by P1 trans-

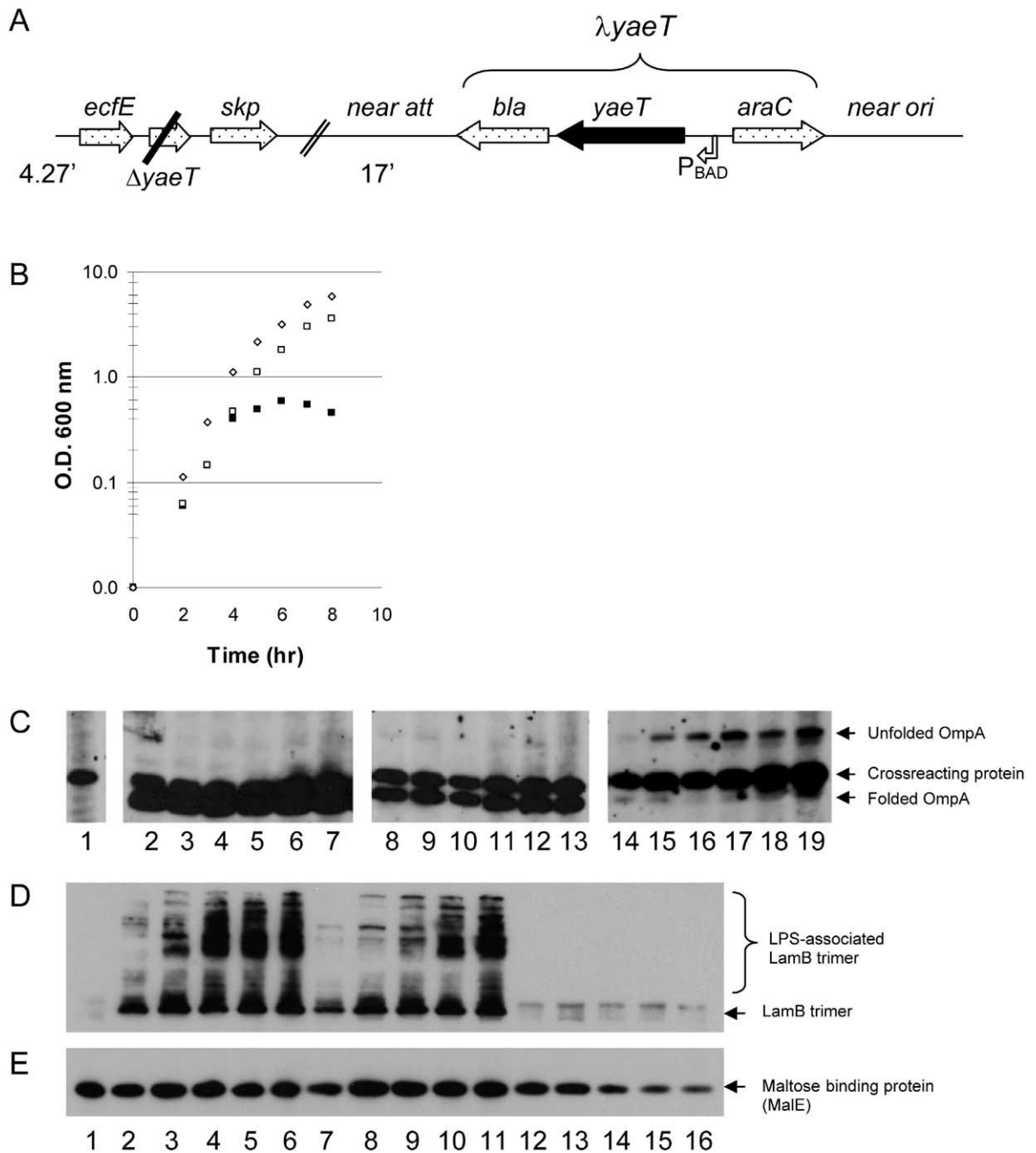


Figure 4. YaeT Assembles OMPs

(A) Genetic organization of the *yaeT* depletion strain JCM166. The chromosomal loci are indicated in minutes.

(B) The YaeT depletion strain JCM166 was grown in the presence (□) or absence (■) of arabinose and compared with wild-type *yaeT* strain JCM158 (◇).

(C and D) The folding patterns of trimeric LamB or monomeric OmpA were analyzed at 30°C. (C) Lane 1, Δ *ompA* strain SP292 at 8 hr; samples were taken from 3 to 8 hr from each of the following cultures: wild-type JCM158 (lanes 2–7), YaeT depletion strain JCM166 grown in the presence of arabinose (lanes 8–13), and in the absence of arabinose (lanes 14–19). (D) Lane 1, Δ *lamB* strain MCR106 at 8 hr; samples were taken from 4 to 8 hr from each of the following cultures: JCM158 (lanes 2–6), JCM166 grown with arabinose (lanes 7–11), and JCM166 grown without arabinose (lanes 12–16).

(E) Samples were additionally monitored for periplasmic MalE as a control.

duction selecting for resistance to kanamycin on rich media at 37°C. Recall that *yfgL8* is a loss-of-function *yfgL* allele (Ruiz et al., 2005).

Although the *yfiO::kan* allele could be easily intro-

duced into the wild-type strain MC4100, we could not introduce the *yfiO::kan* allele into MC4100 strains carrying *yfgL8*. This synthetic lethality was further demonstrated by the fact that linkage between *yfiO::kan* and

the nearby marker *nadB::Tn10* was disrupted if the recipient strain carried *yfgL8*. When we used a strain carrying *yfiO::kan* and *nadB::Tn10* as the donor in a cross with the wild-type strain MC4100 and selected for tetracycline-resistant transductants, 29 out of 110 transductants (i.e., 26%) also inherited the *yfiO::kan* allele. However, with an isogenic recipient carrying the *yfgL8* allele, none of the transductants (0/200) inherited the *yfiO::kan* allele. These results confirm that *yfiO::kan* and *yfgL8* are a synthetic lethal pair.

It is important to note that the synthetic lethality exhibited by the *yfiO::kan* and *yfgL8* double mutants is conditional. Transductants that inherit both mutations can be obtained if they are selected at 30°C in minimal media. These transductants do not grow on any media at 37°C. On rich media at 30°C, they grow very poorly. We interpret this to mean that defects in these two lipoproteins can be tolerated only under conditions in which cell growth is slowed substantially.

In contrast, the *nlpB::kan* allele could be introduced into strains carrying *yfgL8* at the same frequency as into wild-type following selection for kanamycin resistance at 37°C. However, the *nlpB::kan yfgL8* double mutants exhibited irregular and heterogeneous colony morphology. Thus, although a strain carrying this pair of alleles is viable, it does not behave like either single mutant or wild-type. Despite the fact that this synthetic phenotype is modest, we think it significant because it parallels the permeability phenotypes (see above) and OM defects (see below) exhibited by the mutants defective in each lipoprotein individually; mutations in *yfgL* or *yfiO* confer stronger phenotypes than *nlpB* mutations.

The synthetic phenotypes exhibited by pairwise combinations of mutations in the genes encoding the lipoproteins provide genetic evidence supporting the existence and functionality of the YaeT/YfgL/YfiO/NlpB complex. Based on the severity of the synthetic phenotypes, we further conclude that YfgL and YfiO are more important for complex function than NlpB.

The YaeT Complex Is Required for OM Protein Assembly in *E. coli*

To determine whether or not YaeT affects OMP assembly in *E. coli*, we monitored the folding and targeting of two OMPs, OmpA and LamB. The periplasmic maltose binding protein (MalE) was used as an internal control. Both OmpA and LamB assume folded structures when targeted to the OM that are stable during SDS PAGE analysis. Samples containing the proteins must be heated to disrupt these structures. Folded OmpA is a monomer that runs faster than the fully denatured protein (Schweizer et al., 1978). Folded LamB is trimeric and runs slower than the denatured monomer. LamB trimers often migrate as a ladder owing to the association of LPS molecules (Misra et al., 1991). Mistargeting should result in decreased levels of folded OmpA and LamB and increased levels of the unfolded proteins in unheated samples (Voulhoux et al., 2003). The unfolded forms of both proteins would be susceptible to degradation by periplasmic proteases.

As shown in Figure 4C, OmpA was present in the folded conformation in our wild-type strain (JCM158)

throughout its growth cycle. As expected, OmpA also folded normally in our YaeT depletion strain in the presence of arabinose, which maintains YaeT production (Figure 4C). However, after 4 hr of growth in the absence of arabinose, at a time when YaeT depletion started to impair growth (Figure 4B), there was a dramatic decrease in the amount of folded OmpA. Although some of the protein was apparently degraded, we could detect unfolded OmpA, and the levels of the unfolded protein increased with time (Figure 4C). These results demonstrate directly that OmpA folding is compromised by YaeT depletion.

Similar results were obtained with LamB (Figure 4D). The depletion strain grown in the presence of arabinose showed levels of LamB trimers comparable to those of wild-type (Figure 4D, compare lanes 2–6 with lanes 7–11). In contrast, the depletion strain grown in the absence of arabinose had greatly diminished levels of LamB trimers (Figure 4D, lanes 12–16). Overall steady-state levels of LamB, as determined by analyzing the abundance of heat-denatured monomeric LamB, were also much lower in samples grown without arabinose than in samples prepared from cells grown in the presence of arabinose (data not shown). We believe that the low levels of LamB reflect degradation of the unfolded species, since levels and signal sequence processing of the coregulated, periplasmic MalE are largely unaffected by YaeT depletion (Figure 4E) at early time points. At later time points, when cell death is apparent, levels of MalE decreased as well, but the effect is much less severe than for LamB. Therefore, these data are consistent with the hypothesis that depletion of YaeT results in a general defect in the folding and/or the assembly of all OM β barrel proteins.

We have shown previously that strains lacking YfgL have decreased steady-state levels of both LamB and OmpA (Ruiz et al., 2005), and this result is now understandable given that YfgL is in a complex with YaeT. Since the other two lipoproteins are functionally related, we also compared the levels of LamB and OmpA in *yfiO* and *nlpB* mutants to those found in wild-type. Cells from overnight cultures had reduced levels of LamB and OmpA in both *yfgL* and *yfiO* single mutants when compared to wild-type cells (Figure 5). However, the defects appeared somewhat more severe in the *yfgL* mutant. Interestingly, the levels of YfgL are not altered in the *yfiO* mutant, despite the fact that both proteins are part of a multiprotein complex (data not shown). Strains lacking NlpB did not show clear defects in the levels of LamB and OmpA. Again, this is consistent with the fact that *yfgL* and *yfiO* confer stronger phenotypes than *nlpB* (see above).

Genetic Interactions of *yfiO* and *nlpB* with *imp4213*

In the accompanying paper, we demonstrated that *yfgL* null alleles specifically suppress some of the OM permeability defects of strains carrying *imp4213*. In addition, *imp4213* and *yfgL* null alleles are a conditionally synthetic lethal pair; double mutants cannot be obtained unless growth is slowed (Ruiz et al., 2005). Since YfgL is in a complex with YfiO and NlpB, we tested whether either *yfiO::kan* or *nlpB::kan* also show synthetic interactions with *imp4213*.

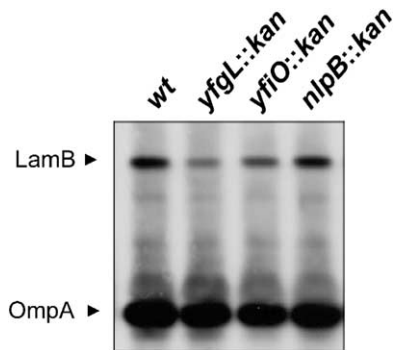


Figure 5. Reduced Levels of LamB and OmpA in *yfgL::kan* and *yfiO::kan* Strains

Cells from overnight cultures were processed following a gentle lysis procedure (see Experimental Procedures). Samples were boiled prior to electrophoresis and subject to Western blot analysis for LamB and OmpA. Genotypes are shown above the lanes. “wt” refers to wild-type. From left to right, the strains used were MC4100, NR721, NR814, and NR815.

We failed to introduce *yfiO::kan* into strains carrying *imp4213* whether we selected for kanamycin-resistant transductants in rich (LB) or minimal glycerol media at 37°C, suggesting that these mutations are a synthetic lethal pair. We provided more convincing evidence for this synthetic lethality by showing disruption of linkage between *yfiO::kan* and *nadB::Tn10*. While *yfiO::kan* and *nadB::Tn10* are 26% linked when introduced into wild-type, 0/108 tetracycline-resistant transductants carried *yfiO::kan* if the recipient was an *imp4213* mutant. However, the double mutant can be obtained if transductants are selected on minimal media at 30°C, demonstrating that *imp4213* and *yfiO::kan* are a conditionally synthetic lethal pair.

On the other hand, we were able to introduce the *nlpB::kan* allele into strains carrying *imp4213*. Still, the double mutants displayed heterogeneous morphology, suggesting a modest synthetic phenotype. Here again, phenotypes conferred by *nlpB* are weaker than those conferred by *yfgL* and *yfiO*.

Since null mutations in *yfgL* suppress some of the OM defects caused by *imp4213* (Eggert et al., 2001; Ruiz et al., 2005), we tested whether the *yfiO::kan* and the *nlpB::kan* mutations also suppress *imp4213*. However, we found that neither the *yfiO imp4213* nor the *nlpB imp4213* double mutant could suppress the sensitivity to bile salts and antibiotics caused by the *imp4213* allele.

Discussion

In almost all Gram-negative bacteria, OM assembly requires the organized insertion of lipids, LPS, and OMPs into a highly asymmetric bilayer (Nikaido, 2003). The assembly of this complex membrane structure presents many challenges for the bacterium. The OM components are synthesized in the cytoplasm or the inner membrane and must be transported to their destination by some means. Assembly takes place outside the cell wall, in an environment that is exposed to the external

milieu and lacking in any obvious energy sources such as ATP. In addition, it is unclear how Gram-negative bacteria coordinate the assembly of lipids and OMPs in order to avoid disturbances in barrier permeability while the cell is growing and dividing.

In the accompanying paper, we showed that some of the OM permeability defects caused by *imp4213* are corrected by null mutations in *yfgL* (Ruiz et al., 2005), which we confirm here encodes for an OM lipoprotein. Individually, mutations that compromise the function of Imp or YfgL disrupt but together improve OM integrity, establishing a genetic connection between *yfgL* and *imp* (Ruiz et al., 2005). We hypothesized that Imp and YfgL serve distinct but related functions in the assembly of the OM of *E. coli*, and we accordingly set out to test for possible interactions between the two proteins. We have thus far been unable to identify physical contacts between YfgL and Imp but have shown that YfgL exists as part of a multiprotein complex that includes YaeT, an essential β barrel protein; the OM lipoprotein NlpB (Bouvier et al., 1991); and the putative OM lipoprotein YfiO. Thus, *yfgL* is genetically connected to *imp*, which specifies an OM protein involved in LPS assembly (Bos et al., 2004), but YfgL is physically connected to YaeT, which assembles β barrel proteins in the OM. To reconcile these genetic and biochemical results, we suggest that YfgL may play multiple roles in OM assembly.

We report three types of genetic interactions involving *imp*, *yfgL*, *yfiO*, and *nlpB*. First, insertion mutations in the genes for the lipoproteins exhibit pairwise synthetic phenotypes ranging from lethality to slow growth and heterogeneous colony morphology. Second, mutations in the genes for the lipoproteins exhibit synthetic phenotypes with *imp4213*. Finally, null mutations in *yfgL* are specific suppressors of some of the permeability phenotypes caused by *imp4213* (Ruiz et al., 2005).

As stated in the Results, the synthetic phenotypes exhibited by mutations in the genes for the lipoproteins provide strong genetic support for the presence of all of these proteins in a functional complex with YaeT. Defects in each of these lipoproteins can be tolerated, but pairwise combinations of these mutations exacerbate the defect.

The genetic interactions we have documented between mutations in *yfgL*, *yfiO*, and *nlpB* and *imp4213* suggest a functional connection between the gene products. The most straightforward explanation for these genetic interactions is that all of these proteins exist in a large complex. The biochemical evidence presented here demonstrates that YaeT would be part of this complex as well. What is lacking is biochemical support for the presence of Imp in such a complex. Since it is possible that this large Imp-containing complex does not survive cellular fractionation, this possibility must remain open. It is worth noting that the idea for a large OM assembly machine is not new. More than 30 years ago, Manfred Bayer (1968) reported zones of inner membrane-OM “fusion,” and, ever since, such zones have been eponymously termed “Bayer bridges.” Smit and Nikaido (1978) and Muhlradt et al. (1973) reported that newly synthesized OM components appeared on the surface of cells at sites corresponding to Bayer bridges, but this observation was never con-

firmed either genetically or biochemically. Controversy about sample preparation (Kellenberger, 1990; Bayer, 1991) and reports of soluble periplasmic assembly intermediates for OMPs (e.g., Halegoua and Inouye [1979], Metcalfe and Holland [1980], Freudl et al. [1985]) have caused this model to fall into disfavor. Perhaps Bayer bridges deserve more careful consideration.

Since we cannot find Imp in a complex with YaeT and the three lipoproteins, we must also consider the possibility that these two OM assembly machines are separate functional entities. If so, the genetic interactions we document must reflect some other feature of OM biogenesis.

Let us assume that the assembly machineries for LPS (Imp) and OMPs (YaeT complex) are separate entities. Then, it is not unreasonable to propose that the synthetic phenotypes affecting cell growth and viability that we observe between *imp4213* and individual mutations in the genes for the lipoproteins might simply reflect a cumulative defect in OM integrity. The OM is essential in *E. coli*, so severe assembly defects would likely be lethal. Indeed, most of these phenotypes are suppressed by slowing cell growth, which also slows OM assembly, thus masking this cumulative defect.

What is more difficult to explain is the highly specific ameliorative effect of a *yfgL* null mutation on the permeability phenotypes conferred by *imp4213*. If all three lipoproteins are in a complex, why can only *yfgL* null mutations suppress *imp4213*? Given the fact that the phenotypes conferred by *nlpB* mutations are always weaker than those conferred by *yfgL* and *yfiO*, it is perhaps not surprising that *nlpB* mutations are not *imp4213* suppressors. However, it is not clear why *yfiO* mutations do not suppress *imp4213*, since *yfgL* and *yfiO* mutants have similar phenotypes. It is possible that the defects caused by *yfiO::kan* are not as severe as those caused by the lack of YfgL and, therefore, not strong enough to suppress *imp4213*. Indeed, strains lacking YfgL may have a somewhat greater defect in OMP targeting. Alternatively, it may be that *yfgL* is the only suppressor of *imp4213* because YfgL performs a unique function.

YfgL enhances the activity of YaeT to assemble OMPs. Is this the only function of YfgL? If so, then the ameliorative effect of *yfgL* null mutations on the *imp4213* phenotype must be a consequence of reduced OMP assembly. This is an attractive idea in the sense that defects in LPS assembly (*imp4213*) could be counterbalanced by defects in OMP assembly (*yfgL*). Although we cannot exclude this possibility, we do not favor it for the following reasons. The OMP assembly defect caused by *yfgL* null mutations is modest, and there are many documented ways to reduce OMPs—by regulatory mutation (*ompR*) or partial secretion defect (*secA*) for example (Hall and Silhavy, 1981; Oliver and Beckwith, 1981)—yet only *yfgL* mutations answered our repeated suppressor selections (Ruiz et al., 2005). Accordingly, this model requires that *imp4213* suppression is mediated by a very precise reduction in OMP levels that can only be obtained by the loss of YfgL.

The permeability barrier of the OM is not disrupted by changes in growth rate or media composition. Moreover, no environmental change has ever been reported that significantly alters the buoyant density of the OM.

A homeostatic control of OM composition would maintain the lipid/protein ratio and the highly asymmetric structure of this membrane despite these changes. It is possible that YfgL participates in this homeostatic control, and this is the explanation we favor for our genetic results; i.e., YfgL is multifunctional. We suspect that mutations that abolish production of YfgL answer our selections as suppressors of *imp4213* because they affect this homeostatic control in some specific way, not solely because they modestly reduce function of the YaeT/lipoprotein complex. But rigorous tests of this hypothesis must await a more detailed elucidation of the function of the Imp and YaeT/lipoprotein complexes and the identification of any other essential OM assembly factors.

Our analysis of OM biogenesis has been aided greatly by chemistry. Membrane permeability is not a specific phenotype in the absence of chemical probes. Once we appreciated that the extreme specificity we observed in mutant selections with subtle chemical changes to known antibiotics was a consequence of permeability, not target site selection, we could exploit our discovery to probe the basis of permeability. We believe that the deliberate use of antibiotics and other small molecule toxins with targets in the periplasm will prove invaluable in dissecting the functions of different components of the OM biogenesis machinery. Subtle changes in membrane composition caused by disabling various components of the assembly machinery lead to increased membrane permeability, but the effects are selective in that different defects are created in the membrane as different components are disabled. Toxic small molecules with different structures and physical properties are highly selective probes of different permeability defects in the OM, allowing for genetic selections to identify components of the biogenesis machinery.

Experimental Procedures

Microbiological Methods and Reagents

Strains (Table S1) were constructed by P1 transduction as described (Silhavy et al., 1984). The *yfiO::Tn5KAN-I-SceI* and *nlpB::Tn5KAN-I-SceI* (referred to as *yfiO::kan* and *nlpB::kan*, respectively) alleles were derived from strains FB22459 and FB20820, respectively (*E. coli* Genome Project at the University of Wisconsin-Madison <http://www.genome.wisc.edu/functional/tmmutagenesis.htm>). All media were prepared and cells were grown as described previously (Silhavy et al., 1984). Antibiotics were used at the following concentrations: tetracycline and kanamycin at 25 $\mu\text{g/ml}$, chloramphenicol at 20 $\mu\text{g/ml}$, ampicillin at 125 $\mu\text{g/ml}$ for maintaining plasmids, and 50 $\mu\text{g/ml}$ for alleles in single copy.

Antibiotic sensitivity was assessed by disks diffusion assays (Ruiz et al., 2005). Restriction enzymes and Taq DNA polymerase (New England Biolabs) were used as directed by the manufacturer.

Plasmid Construction

The primers used to construct the plasmids are listed in Table S2. *yfgL* was amplified by PCR using primers *yfgLNt* and *yfgLCt* and was introduced into the *NcoI* and *XhoI* site of pET21d(+) (Novagen) to create pTW003. To construct pTW004, C-terminally His-tagged *yfgL* was amplified from pTW003 using primers *yfgLNt* and *yfgL-BADC*, digested with *NcoI* and *XbaI*, and introduced into the *NcoI* and *XbaI* sites of pBAD24. To create pTW006, the *yfgL* gene was amplified from pTW003 using primers *yfgL-Nfi* and *yfgLCt*, and the PCR product was digested with *NdeI* and *XhoI* and introduced into the respective sites of pET23a(+) (Novagen).

In addition, C-terminally His-tagged *yfiO* and *nlpB* vectors were constructed. *yfiO* and *nlpB* were amplified from MC4100 using primers *yfiO*-N and *yfiO*-C, and *nlpB*-N and *nlpB*-C, respectively. Both *yfiO* and *nlpB* PCR products were treated with *Nde*I and *Xho*I and ligated to *Nde*I- and *Xho*I-digested pET23a(+), creating pTW007 and pTW008, respectively. All constructs were confirmed by DNA sequencing.

Plasmid pJCM1-*yaeT* was constructed by first digesting pAER1 (Rizzitello et al., 2001) with *Eco*RI and *Kpn*I to excise the 1508 bp *surA* fragment. The remaining 4879 bp hybrid pACYC177-pBAD18 vector fragment was purified. Next, a 2497 bp *yaeT* PCR product was amplified from JCM158 using primers *yaeT*-Fwd and *yaeT*-Rev. The resulting PCR product included 20 bp upstream of the *yaeT* translational start and 49 bp after the translational stop sequence. This product was digested with *Eco*RI and *Kpn*I and ligated to the purified hybrid vector. pJCM1-*yaeT* was confirmed to express functional YaeT in an arabinose-dependent manner, as the chromosomal *yaeT* allele of strains containing pJCM-*yaeT* can be replaced with the *yaeT::kan* allele via P1 transduction only in the presence of arabinose. The same fragment resulting from the *yaeT*-Fwd and *yaeT*-Rev amplification was similarly introduced into the pBAD18 vector using *Eco*RI and *Kpn*I enzymes to produce pBAD18-*yaeT*.

Cell Fractionation

An overnight culture of MC4100 was used to inoculate 100 ml LB broth. The culture was subsequently grown at 37°C to an OD₆₀₀ of 1.0. Cells were pelleted (3000 × g, 4°C, 5 min) and resuspended in 6 ml cold 20 mM Tris-HCl (pH 8), and 300 μl lysozyme (2 mg/ml) was added to the suspension. After 2 min on ice, 12 ml of cold 1.5 mM NaEDTA (pH 8) was slowly added. Next, 180 μl PMSF (100 mM) was added, and cells were incubated on ice for 1 hr with occasional swirling. Spheroplasts were pelleted (18,000 × g, 5 min) and resuspended in 4 ml of 50 mM Tris-HCl (pH 8) with DNase and RNase at 20 μg/ml and 100 μl protease inhibitor cocktails (for use with bacterial cell extracts, Sigma). The suspension was passed through a french press (SLM Aminco, Spectronic Instruments) three times at 10,000 psi. Unbroken cells were removed by multiple centrifugation steps (1000 × g, 10 min, 4°C) until a pellet was no longer detectable. EDTA and lysozyme were added to the membrane suspension to final concentrations of 1 mM and 0.1 mg/ml, respectively. The lysate was cooled on ice/water for 30 min with occasional swirling. The final lysate was added to the top of a preliminary sucrose gradient containing 1.0 ml 25% (wt/wt) sucrose layered over 0.3 ml 65% (wt/wt) sucrose. Samples were centrifuged in a Beckman Optima L7 ultracentrifuge (55,000 rpm, 4°C, 2 hr). The top 3 ml of the samples was taken as cytoplasm fraction, the next 1 ml was discarded as cellular debris, and the bottom 1 ml of enriched membranes was collected from the bottom of the tube. This sample was mixed with 1.4 ml EDTA (5 mM) and loaded onto a secondary sucrose gradient with the following concentrations of sucrose from bottom to top: 0.5 ml 65% (wt/wt) sucrose, 0.5 ml 55% (wt/wt) sucrose, 1 ml 50% (wt/wt) sucrose, 2 ml 45% (wt/wt) sucrose, 2 ml 40% (wt/wt) sucrose, 2 ml 35% (wt/wt) sucrose, and 1.5 ml 30% (wt/wt) sucrose. Gradients were centrifuged for 17 hr at 36,000 rpm (4°C) in a Beckman Optima L7 ultracentrifuge. Collected fractions were subjected to electrophoresis through a 12% SDS-polyacrylamide gel followed by Western blot analysis using rabbit anti-YfgL and anti-Imp antisera (our laboratory stock) as described previously (Ruiz et al., 2005).

³H-Palmitate Labeling of YfgL

Cells were labeled with [9, 10 (n)-³H]-palmitic acid (Amersham) according to Snyder et al. (1995). Overnight cultures of MC4100 and TW009/pTW004 were diluted 1:100 into 10 ml LB broth containing 50 μCi ³H-palmitate and grown at 37°C to an OD₆₀₀ of 0.6. Then arabinose was added to a final concentration of 0.01%, and cultures were grown for 3 hr. One milliliter of each culture was pelleted, resuspended in 150 μl of SDS-sample buffer (Sigma), and boiled for 10 min. Sample (30 μl) was loaded onto a 12% SDS-polyacrylamide gel (Laemmli, 1970). After electrophoresis, proteins

were transferred onto a PVDF membrane. Labeled proteins were detected by autoradiography.

Immunoprecipitation

100 ml LB broth culture was inoculated with 1 ml overnight culture and grown to an OD₆₀₀ of 0.6. Cells were pelleted (5000 × g, 10 min, 4°C) and resuspended in 2 ml BugBuster reagent (Novagen). Next, 1 μl lysozyme (100 mg/ml stock), 1 μl Benzamide Nuclease (Novagen), and 100 μl protease inhibitor cocktail (Sigma) were added. The mixture was incubated at room temperature with gentle shaking for 20 min. Cell debris were pelleted (16,000 × g, 10 min, 4°C). The supernatant was saved as the total cell lysate. The IP experiment was performed according to the Sigma Protein G Immunoprecipitation Kit protocol. Proteins were eluted in 50 μl of Laemmli sample buffer (Bio-Rad), and 25 μl of sample was separated in a 12% SDS-polyacrylamide gel. Proteins were either visualized after staining with either Coomassie blue or the Silver Stain Plus kit (Bio-Rad) or transferred onto a PVDF membrane for Western blot analysis using anti-YfgL, anti-YaeT, and anti-5His antisera (Qiagen).

For sequencing, proteins were transferred onto PVDF membranes and stained with Coomassie blue, and bands were subject to sequencing by Edman degradation (ProSeq, Inc., MA). Protein sequencing by tandem mass spectrometry was done by Dr. Steven Gygi at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School.

Construction of a Diploid *yaeT* Strain

MC4100 contains an *araD139* mutation that renders the strain sensitive to arabinose. To use an arabinose-dependent expression system, we selected for spontaneous mutants on MacConkey agar supplemented with arabinose. We isolated JCM158, an arabinose-resistant mutant unable to catabolize the sugar. A *yaeT* allele under control of the arabinose-inducible promoter P_{BAD} was introduced into the JCM158 chromosome at the λ attachment site via the λInCh method (Boyd et al., 2000) using plasmid pBAD18-*yaeT*. The diploid *yaeT* strain was designated JCM160.

Construction of a YaeT Depletion Strain

The *yaeT::kan* product was amplified from pKD4 (Datsenko and Wanner, 2000) using primers *yaeT*-kan Fwd and *yaeT*-kan Rev. The resulting PCR product contained an internal kanamycin cassette with a 5' -*yaeT* flanking region situated 70 bp upstream of the *yaeT* translational start and a 3' flanking sequence located 2204 bp downstream of the *yaeT* translational start. Partial deletion of the *yaeT* locus was achieved using the recombineering method (Ellis et al., 2001) via the electroporation of *yaeT::kan* PCR product into DY378 containing pJCM-*yaeT* and subsequent selection in the presence of 0.025% arabinose and 25 μg/ml kanamycin. Recombination of the PCR product was targeted to the chromosomal *yaeT* locus, as the 5' flanking region of the *yaeT::kan* PCR product is not present in pJCM-*yaeT*. The resulting chromosomal *yaeT::kan* allele was introduced via P1 transduction into the chromosomal *yaeT* locus of JCM160 in the presence of arabinose. To prevent any possible polar effects caused by transcription of the kanamycin resistance cassette, the resistance gene was excised using Flp recombinase provided by pCP20 (Cherepanov and Wackernagel, 1995; Datsenko and Wanner, 2000). The resulting *yaeT* depletion strain was termed JCM166.

Gentle Lysis Preparation and Western Blot Analysis of LamB and OmpA

To maintain the folded structures of LamB and OmpA, protein samples were prepared using a gentle lysis protocol (Misra et al. 1991). The volumes of samples were normalized to the initial OD₆₀₀ of each culture, heated to 30°C (10 min), and subjected to SDS-PAGE (10% gel) overnight at 50V to avoid thermal denaturation of folded proteins (Laemmli, 1970). After transfer to a nitrocellulose membrane, the various folding states of LamB were hybridized with 1:30,000 rabbit antisera raised against LamB trimer and LamB monomer (Misra et al., 1991). Unfolded and folded monomeric OmpA both crossreact with each of the LamB antibodies. Rabbit antiserum raised against MBP was simultaneously added to a dilu-

tion of 1:15,000 (Misra et al., 1991). Donkey anti-rabbit IgG horseradish peroxidase conjugate (Amersham Pharmacia Biotech) was used as secondary antibody at a 1:6000 dilution. Bands were visualized using ECL (Amersham Pharmacia Biotech) and Biomax film (Kodak).

Supplemental Data

Supplemental Data include two tables and can be found with this article online at <http://www.cell.com/cgi/content/full/121/2/235/DC1/>.

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