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Loss of Outer Membrane Proteins without Inhibition of Lipid Export in an Escherichia coli YaeT Mutant*

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Escherichia coli yaeT encodes an essential, conserved outer membrane (OM) protein that is an ortholog of Neisseria meningitidis Omp85. Conflicting data with N. meningitidis indicate that Omp85 functions either in assembly of OM proteins or in export of OM lipids. The role of YaeT in E. coli was investigated with a new temperature-sensitive mutant harboring nine amino acid substitutions. The mutant stops growing after 60 min at 44 °C. After 30 min at 44 °C, incorporation of [35S]methionine into newly synthesized OM proteins is selectively inhibited. Synthesis and export of OM phospholipids and lipopolysaccharide are not impaired. OM protein levels are low, even at 30 °C, and the buoyant density of the OM is correspondingly lower. By Western blotting, we show that levels of the major OM protein OmpA are lower in the mutant in whole cells, membranes, and the growth medium. SecA functions as a multicopy suppressor of the temperature-sensitive phenotype and partially restores OM proteins. Our data are consistent with a critical role for YaeT in OM protein assembly in E. coli.

The envelope of Gram-negative bacteria is composed of an inner membrane (IM)1 (1, 2) and an outer membrane (OM) (3, 4), separated by the periplasmic space and peptidoglycan (5, 6). The OM has a lipid and protein profile distinct from that of the IM (4). The OM contains phospholipids on its inner surface and lipopolysaccharide (LPS) on its outside surface (3, 4, 7, 8). The most abundant OM proteins are the trimeric porins, but many additional minor proteins, including some enzymes, are also present (3, 4). All OM protein structures studied to date are characterized by an inside-out β-barrel architecture not found in IM proteins (9). The only exceptions are the lipoproteins, which associate with the OM through their N-terminal lipid-modified cysteine residues (10–12).

The lipids and proteins of the OM are synthesized on the cytoplasmic surface of the IM, transported across the IM and periplasm, and assembled into the OM (2–4, 7, 13). Several mechanisms have been identified for transporting proteins across the IM, including the Sec pathway (14–16), the twin arginine transport system (17, 18), and the type I secretion pathway (19, 20). The transport of lipoproteins from the outer face of the IM to the OM requires the LolABCDE machinery (11, 12). Recent genetic and structural studies of the IM protein MsbA, an essential ABC transporter, have established its role as a flipase or pump required for the export of LPS and phospholipids (21–25).

The mechanisms by which nascent proteins, phospholipids, and LPS are assembled into the OM following transport across the periplasm remain poorly characterized. OM proteins are synthesized with signal peptides that are cleaved at the periplasmic surface of the IM (26). OM protein folding in the periplasm prior to insertion into the OM is facilitated by chaperones, such as SurA and HlpA, and may be accelerated by LPS (27–30). Subsequent steps are uncertain. Some OM proteins, like FhuA, have high affinity LPS-binding sites (31).

OM proteins are generally not required for growth (4). However, in Neisseria meningitidis, Omp85 is a minor, essential OM protein (32, 33). Depletion experiments have led to contradictory conclusions regarding Omp85 function. Genevris et al. (33) showed that transport of lipids to the OM is selectively blocked, as in MsbA mutants (22). However, Voulhoux et al. (32) demonstrated that OM protein folding and oligomerization are defective. Both groups employed a construct with a chromosomal deletion of omp85 and an intact copy of the omp85 gene behind an inducible promoter on a plasmid. This strategy required a prolonged (>6 h) incubation to deplete pre-existing Omp85. Recently, Kahn and co-workers (34) discovered that YaeT exists in a heterooligomeric OM complex with three lipoproteins of unknown function: YigL, NipB, and YF0. Their findings confirm that YaeT is essential for viability in Escherichia coli and demonstrate a role for YaeT in OM biogenesis. However, lipid trafficking, global outer membrane protein composition, and buoyant density were not examined.

YaeT is the E. coli ortholog of Omp85 (35). We have now examined the function of YaeT. We confirm that yaeT is essential in E. coli, as in other bacteria (32, 33, 36, 37). The yaeT gene in E. coli is situated upstream of lpxA, lpxR, and lpxD (7) and downstream of uppS (38), cdsA (39), and yaeL (40) (Fig. 1A). We have constructed a yaeT deletion mutant covered by a plasmid that expresses a temperature-sensitive (ts) allele of yaeT that stops growing after 60 min at 44 °C. The levels of major OM proteins are greatly reduced, and the OM buoyant density is reduced. The level of new OM proteins is low at the permissive temperature and is almost undetectable after only 30 min at 44 °C, but lipid synthesis and export are relatively unaffected. Moderate overexpression of SecA, a component of the pre-protein translocase (41, 42), largely restores OM proteins and growth to normal rates at 44 °C. Our data are consistent with a critical role for YaeT in the assembly of OM proteins in E. coli.
**EXPERIMENTAL PROCEDURES**

**Materials**—Tryptone and yeast extract were from Difco. Radioisotopes and Enhance were purchased from PerkinElmer Life Sciences. Restriction enzymes were purchased from New England Biolabs. T4 DNA ligase, shrimp alkaline phosphatase, and custom-made primers were synthesized by Invitrogen. The gene for Pfu turbo was purchased from Stratagene. Protein concentrations were determined with the BCA Protein Assay Reagent from Pierce, using bovine serum albumin as the standard (43). All other chemicals were purchased from either Sigma or Mallinckrodt.

**Plasmid Construction**—Standard recombinant DNA techniques were utilized in the construction of plasmids (44). W3110 chromosomal DNA was obtained using the Easy DNA kit from Invitrogen. Plasmids were isolated using QiAprep spin miniprep kit from Qiagen. The Qiaex II gel extraction kit from Qiagen was used to extract DNA from agarose gels. Restriction enzymes, T4 DNA ligase, and shrimp alkaline phosphatase were used according to the manufacturer’s instructions. Competent cells were prepared according to Inoue et al. (45). Plasmids and strains are listed in Table 1.

**E. coli yaeT** was amplified from W3110 genomic DNA by PCR with Pfuitube, according to the manufacturer’s instructions. The forward primer was YaeT fNdeI, and the reverse primer was YaeT BamHI (Table II). These primers were used at a final concentration of 2.5 ng/µl in a 100-µl PCR mixture containing 100 ng of genomic DNA and 5 units of Pfuitube polymerase. The reaction conditions were as follows: 94°C denaturation for 1 min followed by 25 cycles of 94°C (denaturation) for 1 min, 55°C (annealing) for 1 min, and 72°C (extension) for 2.5 min. This was followed by a 10-min run-off at 72°C. The gel-purified PCR product was digested with NdeI and BamHI and then ligated into an NdeI/BamHI-treated vector, pACYC184 (47). Plasmid pWTD25 was constructed by cloning the XbaI/BamHI fragment of pWTD25 (containing a 5′ ribosomal binding site) into Xbal/BamHI- and shrimp alkaline phosphatase-treated pET23a vector (Novagen), yielding pWTD25. Plasmid pWTD27 was constructed by cloning the XbaI/BamHI fragment of pWTD25 (containing a 5′ ribosomal binding site) into Xbal/BamHI- and shrimp alkaline phosphatase-treated vector pET23a (47). Plasmid pWTD31 was constructed by cloning the XbaI/BamHI fragment of pWTD25 into Xbal/BamHI- and shrimp alkaline phosphatase-treated vector pWSK29 (47).

Plasmids were sequenced at the Duke University sequencing facility. When appropriate, antibodies were added as follows: chloramphenicol, 30 µg/ml; kanamycin, 25 µg/ml; and ampicillin, 90 µg/ml. In experiments depicted, the overnight culture temperature was set at 30 °C, and the growth temperature was set at 42 °C for plate grown bacteria and 44 °C for liquid grown bacteria.

**Replacement of the yaeT Gene**—Replacement of yaeT with the kan gene from pUC4K was done using λ-red-mediated recombination (48). The kan gene was amplified by PCR from plasmid DNA using primers YaeT koA and YaeT koB (Table II). The gel-purified DNA was treated with DpnI to remove contaminating methylated plasmid DNA and was used to transform E. coli strain DH5α/pET23d by electroporation. Following a 2-h outgrowth in LB broth (49) at 30 °C, the plates were plated on LB agar plates containing kanamycin and incubated overnight at 30 °C. A P_Lys 1-lysate made from DH5α(yaeT::kan)pWTD27 was used to transduce W3110/pWTD27 to KmR (50). The resulting strain was designated WD401/pWTD27.

**Isolation of ts yaeT Mutation**—The error-prone PCR—PCR mutagenesis was performed using Pfu turbo DNA polymerase from Stratagene. The target DNA was pWTD25, which was used at a final concentration of 300 or 50 pg/µl. Primers were YaeT BamHI and T7 promoter (Table II). A 50-µl reaction mixture contained the Mutazyme reaction buffer (1×), 200 µM dNTPs, 125 ng of primers, and 2.5 units of Mutazyme DNA polymerase. The reaction conditions were as follows: a 94°C denaturation step for 1 min was followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 20 s. The final DNA product was ligated into BamHI/XbaI-digested plasmid pWTD25. Cells were transformed with the plasmid DNA containing mutagenized yaeT. Following an overnight incubation at 37 °C, ~1000 AmpR colonies were scraped and pooled into 5 ml of LB broth. These cells were diluted into 50 ml of LB broth containing ampicillin, grown overnight, and used as the recipient cells for a P_Lys transduction using a lysate prepared from WD401/pWTD27. KmR colonies (which were also AmpR) because of the presence of the vector pWSK25) were screened by streaking on LB/ampicillin plates and incubating overnight at 30 and 42 °C. A representative construct with the desired ts phenotype was designated WD401/pWTD30-9.

**E. coli Genomic DNA Library Construction**—Genomic DNA from E. coli W3110 (50 µg) was partially digested with 2 units of Sau3A1 for 30 min at 37 °C in 0.2 ml of Sau3A1 buffer. Then 6 µl of 0.5 mM EDTA, pH 8.0, was added to stop the reaction, and the mixture was incubated at 65 °C for 20 min to inactivate the enzyme. The DNA was resuspended on a 1% agarose gel, and fragments corresponding to 2–6.5 kb were recovered. Plasmid pACYC184 was opened with BamHI, gel-purified, and treated with shrimp alkaline phosphatase. For the ligation reaction, 200 ng of partially digested genomic DNA was incubated at 16 °C overnight with 200 ng of treated plasmid and 2 units of T4 DNA ligase in a total volume of 25 µl of ligation buffer. E. coli XLI-Blue cells were transformed with the ligation mixture, and CmR transfectants were selected. Ligation reactions were performed according to the manufacturer’s instructions. Colonies were picked, and the ends of the inserts were sequenced with primers designed for the BamHI site of pACYC184 (pACYC184/BamHIA and pACYC184/BamHIB, Table II).

**Labeling, Membrane Preparation, and Sucrose Gradients**—Cells were grown in 25 ml of LB broth at 30°C, 50 °C, and then diluted 4-fold into fresh, pre-warmed LB broth at 44 °C. After 30 min of growth, 50 µl of cells were labeled with 4 µCi/ml [35S]methionine/cysteine (1175 Ci/mmol) for 10 min. Cells were cooled rapidly on ice and harvested by centrifugation. Spheroplasts were prepared at 0°C by lysozyme/EDTA treatment and broken by mild sonication (21, 51). The cell lysate was cleared by centrifugation at 4000 x g for 4 °C for 20 min. Membranes were prepared by two sequential ultracentrifugation steps at 100,000 x g for 60 min with a final membrane step in 10 mM Tris, pH 7.8, 250 mM sucrose, at 4 °C (21, 51). Membranes were separated at 4°C on a 30–60% (w/w) isopycnic sucrose gradient prepared in 10 mM Tris acetate, pH 7.8, and 0.5 mM EDTA, which was centrifuged at 155,000 x g for 18 h in a Beckman SW41 rotor (22, 51). OM phospholipase A, 1M NADH oxidase, total protein, and radioactivity were measured for each 0.5 ml fraction (21, 22).

**Western Blotting**—Western blot analysis was performed according to the method of Sorensen et al. (53). Anti-pro-OmpA and anti-SecA antibodies were generously provided by Dr. William Wickner (Dartmouth University) and Dr. Timothy Yahr (University of Iowa) and were used at titers of 1/20,000 and 1/10,000, respectively. Horseradish peroxidase-conjugated anti-rabbit IgG from Pierce was used at a titer of 1/20,000.

**The ECL Plus kit from Amersham Biosciences was used for detection.**
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RESULTS

YaeT Is Essential in E. coli—To study YaeT function in E. coli, we attempted to disrupt the chromosomal copy of yaeT by replacing it with a kanamycin resistance cassette (kan). Experiments without a covering plasmid were unsuccessful. Therefore, we amplified yaeT from E. coli W3110 genomic DNA (Tables I and II) and cloned it into the vector pMAK705, which carries a ts origin of replication (46). The resulting hybrid plasmid, pWTD27, was transformed into E. coli DY330 (Table I), and the chromosomal copy of yaeT was then replaced with the kan cassette by homologous recombination at 30 °C with linear DNA (48). Genomic DNA was isolated from four random kanamycin-resistant (KmR) clones. PCR, using primers outside of yaeT, demonstrated that yaeT was replaced with the smaller kan gene (Fig. 1B). A P1<sub>φ</sub> lysate produced from one of these KmR DY330 derivatives was used to transduce W3110/pWTD27 (Table I). One of these KmR clones with yaeT on a ts plasmid was purified and designated WD401/pWTD27. We grew both W3110/pWTD27 and WD401/pWTD27 to mid-log phase at 30 °C and diluted them 10-fold into fresh LB broth, pre-warmed to 44 °C. Growth was continued at 44 °C, and the cultures were back-diluted 10-fold whenever A<sub>600</sub> reached 0.3–0.4. The WD401/pWTD27 culture stopped growing after 3–4 h at 44 °C and gradually lost viability (Fig. 1C). The results are consistent with recent findings that yaeT is essential in E. coli (34).

Isolation of a ts Mutation in yaeT—We were concerned about the relatively long time (4–6 h) before loss of viability of WD401/pWTD27 at 44 °C (Fig. 1C). This delay presumably depends on diluting pre-existing YaeT to levels that do not support viability. We therefore isolated a ts allele of yaeT by selecting for ampicillin resistance (Amp<sup>R</sup>) at 30 °C. Approximately 1000 Amp<sup>R</sup> W3110 colonies were pooled and transduced to Km<sup>R</sup> using a P1<sub>φ</sub> lysate from WD401/pWTD27, in which the kan cassette replaces the chromosomal copy of yaeT. The desired Amp<sup>R</sup>Km<sup>R</sup> colonies were then screened for ts growth on plates. One colony of −50 failed to grow at 42 °C. A representative strain was designated WD401/pWTD30-9 (Fig. 2A). Plasmid DNA from this organism was sequenced, identifying nine amino acid substitutions distributed randomly throughout the YaeT protein (Table III). When tested in liquid medium (Fig. 2B), WD401/pWTD30-9 stopped growing after 60 min at 44 °C (Fig. 2B). The mutant strain was no more sensitive at 30 °C than wild type to several antibiotics or the detergent SDS as determined by disk diffusion assay (data not shown). The mutant grew as well as wild type on M9 minimal medium at 30 °C and remained temperature-sensitive at 42 °C (data not shown). We were unable to identify a single amino acid substitution responsible for the ts phenotype of the altered YaeT protein encoded by pWTD30-9.

Analysis of Lipid Export in Cells Expressing Wild-type and Mutant YaeT—in N. meningitidis, the YaeT ortholog, Omp85, was proposed to be essential for the transport of lipids to the OM (33). To analyze transport of newly synthesized phospholipids and lipid A in our construct, we labeled the control and mutant cells with <sup>32</sup>P for 10 min following 30 min of growth at 44 °C. Under these conditions, <sup>32</sup>P incorporation was not significantly reduced in the mutant when compared with wild type. Cells were then treated with lysozyme/EDTA and broken by sonic irradiation (22, 51). Membranes were separated into inner and outer fractions by isopycnic sucrose gradient centrifugation (22, 51).

Assays of the marker enzymes, phospholipase A (OM) and NADH oxidase (1M) (51, 57), in the control strain, WD401/pWTD30, demonstrated that the membranes were well resolved (Fig. 3A, upper panel). Newly synthesized phospholipids were distributed evenly between IMs and OMs (Fig. 3A, lower panel). Most of the newly synthesized lipid A was associated with the OM (which banded as a doublet in this strain). The results for the YaeT mutant WD401/pWTD30-9 differed (Fig. 3B). The IMs of the mutant banded at about the same density as the IMs of the wild-type, and the activity of NADH oxidase was normal. However, the YaeT mutant OMs were much lighter than wild type, and the activity of the OM phospholipase A was reduced. Despite the decrease in OM buoyant density in the mutant cells, newly synthesized phospholipids were exported normally. There was slight accumulation of lipid A in the IM, but most of it still reached the altered OM (Fig. 3B, lower panel). These results suggest that, in contrast to MshA (22), YaeT does not play a direct role in the export of newly synthesized lipids to the OM. The reduction in the OM density of the mutant (Fig. 3B) indicates that profound changes in OM composition must be taking place. However, because the OM peak in the mutant migrates at the middle of the gradient where small amounts of fused IM-OMs are found in the wild.

### Table I

| Strain | Relevant genotype | Source or Ref. |
|--------|-------------------|----------------|
| E. coli W3110 | Wild-type, F<sup>+</sup>, A<sup>−</sup> | E. coli Genetic Stock Center, Yale University |
| DY330 | W3110 Δ lacU169 gal490 lac8157 Δ(cro-bioA) | 48 |
| WD401 | W3110 ΔyaeT::kan | This work |
| XL1-Blue MR | ΔmcrABC recA1 endA1 gyrA96 relA1 supE44 thi1 lac | Stratagene |

Plasmids

| Plasmids | Description | Source |
|----------|-------------|-------|
| pUC4K | kan<sup>R</sup> | Amersham Biosciences |
| pET23a | Expression Vector; T7 lac promoter, amp<sup>R</sup> | Novagen |
| pMAK705 | Ts replicon, cm<sup>R</sup> | 46 |
| pWSK29 | Low copy vector, lac promoter, amp<sup>R</sup> | 47 |
| pACYC184 | Low copy vector, tet<sup>R</sup>, cm<sup>R</sup> | New England Biolabs |
| pWTD25 | pET23a containing E. coli yaeT (Ndel/BamH1 sites) | This work |
| pWTD27 | XbaI/BamHI fragment of pWTD25 cloned into pMAK705 | This work |
| pWTD30 | XbaI/BamHI fragment of pWTD25 cloned into pWSK29 | This work |
| pWTD30-9 | YaeT (ts) cloned into XbaI/BamHI sites of pWSK29 | This work |
| pWTD32 | XbaI/BamHI fragment of pWTD31 cloned into pACYC184 | This work |
| pWTD33 | XbaI/BamHI fragment of pWTD31 cloned into pWSK29 | This work |
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**Table II**

| Name            | Sequence                                      |
|-----------------|-----------------------------------------------|
| YaeTNdel        | 5’-GGA CAT ATC GGC ATG AAA AAG TTG CTC-3’     |
| YaeTBamHI       | 5’-GGA TCG TCA CAA GAT TTT ACC GAT G-3’      |
| SecANdel        | 5’-GGA AAT TCG TAA CAA ATG TGT TAA CTA AAG-3’|
| SecABamHI       | 5’-GGA TCA TTA TGG CAG GGC ATG G-3’          |
| YaeTkoA         | 5’-GAT TGC TCT CGG TTA GAG TTA GCT AAG AAC GCA TAA TAA CTA ACC GGA AAA CG-3’ |
| YaeTkoB         | 5’-GAT TGC TCT CGG TTA GAG TTA GCT AAG AAC GCA TAA TAA CTA ACC GGA AAA CG-3’ |
| pACYC184/BamHIA  | 5’-CTA TCG ACT ACG CGA TCA TG-3’             |
| pACYC184/BamHIB  | 5’-CGG TGA TCT CGG CGA TTA GT-3’             |

**FIG. 1.** Demonstration that yaeT is essential in E. coli. A, schematic representation of the region of the E. coli chromosome flanking yaeT and replacement of yaeT with the kanamycin resistance gene (kan). B, PCRs with genomic DNA from DY330/pWT2D27 (lane 1) and four random kanamycin-resistant derivatives (lanes 2–5), using primers flanking the chromosomal region around yaeT (5’-GAT TGC TCT CGG TTA GAG-3’ and 5’-CTA AAG TCA TCG TTA CAC TAC-3’). C, growth curve of W3110/pWT2D27 (circles) and WD401/pWT2D27 (squares). Mid-log phase cells were grown at 30 °C in LB broth containing chloramphenicol and diluted 10-fold into the same broth, pre-warmed to 44 °C, but lacking chloramphenicol. Cultures were diluted 10-fold whenever A_{600} reached 0.3–0.4. The plotted A_{600} is the cumulative growth yield.

**FIG. 2.** A ts missense mutation in yaeT. A, growth on LB/ampicillin plates of the control strain WD401/pWT3D0 and mutant strain WD401/pWT3D0-9 at 30 and 42 °C. B, growth of WD401/pWT3D0 (circles) and WD401/pWT3D0-9 (squares) in LB medium after a shift to 44 °C.

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**Analysis of Membrane Protein Composition and Export in the YaeT Mutant**—We next determined the quantity and localization of newly synthesized membrane proteins in wild-type and mutant cells by a 10-min labeling with [35S]methionine/cysteine (Fig. 5, middle panel). Consistent with the reduced buoyant density of the OMs, labeling of IM proteins was relatively unaffected (Fig. 4B, middle panel). Greatly reduced levels or the complete absence of all major OM proteins was also seen by staining the gels with Coomassie Blue (Fig. 4, A versus B, lower panels).

At the permissive temperature (30 °C), the OMs of the mutant were again much lighter than wild type (Fig. 5, B versus A, top panels), but the activity of the OM phospholipase A marker was not reduced. Significant OM protein synthesis was measurable at 30 °C by short term labeling with [35S]methionine/cysteine (Fig. 5, middle panels). Some OM proteins were detected by Coomassie Blue staining (Fig. 5, lower panels). The results indicate that the point mutations present in W401/pWT3D0-9 result in a partial defect in OM protein assembly that is nonetheless compatible with cell growth at 30 °C. Analogous partial defects in protein export at the permissive temperature can occur in conditional mutants in the SecY and SecA components of the protein secretion apparatus (58).

To examine protein processing in our YaeT mutant construct, levels of the major OM protein, OmpA, were determined in each sucrose gradient fraction of Fig. 4 using a polyclonal antibody to pro-OmpA (18). In the wild type, most of the OmpA was localized near the bottom of the gradient together with the OM phospholipase A marker (Figs. 4A and 6A). In the mutant,
the levels of OmpA were significantly lower (Fig. 6A), confirming the results of Fig. 4, and the protein was detected near the middle of the gradient together with the residual phospholipase A (Fig. 4B). There was no detectable accumulation of pro-OmpA in the YaeT mutant (either at 30 or 44 °C), in contrast to the accumulation observed upon inhibition of SecA with sodium azide (Fig. 6B). OmpA levels were also decreased when analyzing whole cell lysates by Western blot (Fig. 6B). Levels of OmpA in the growth media were also lower in the mutant strain than in the wild-type strain (data not shown). Loss of lipid export to the OM caused by inactivation of MsbA in the ts mutant WD2 (21, 22) likewise did not result in the accumulation of pro-OmpA (Fig. 6B).

Because OmpA depletion is seen in whole cell lysates as well as membranes, and OmpA is not found in the growth medium, we conclude that unassembled outer membrane proteins are neither accumulating in the periplasm of the mutant nor secreted from the cell. The combined data suggest that the loss of OM proteins seen in the YaeT mutant at 44 °C probably occurs after translocation across the IM and leader peptidase cleavage. It might be due to breakdown of OM proteins that have not been assembled properly into the OM.

A Multicopy Suppressor of WD401/pWTD30-9—To study possible interactions of YaeT with other proteins, we screened a pACYC184-based E. coli genomic DNA library for multicopy suppressors of the ts growth phenotype of WD401/pWTD30-9. The mutant strain was transformed with the E. coli library, and plasmid DNA was isolated from chloramphenicol resistant (CmR) colonies that grew at 42 °C. Because the mutant strain also contained pWTD30-9 (conferring AmpR), E. coli XL1-Blue

| Nt no. | Codon (wt) | Codon (mut) | aa no. | aa (wt) | aa (mut) | Conserved | Predicted locationa |
|-------|------------|-------------|--------|---------|----------|-----------|-------------------|
| 31    | CTG        | CCG         | 11     | Leu     | Pro      | Yes       | Signal sequence   |
| 86    | ATT        | ATA         | 29     | Ile     | Ile (silent) |          |                   |
| 234   | CGT        | AGT         | 79     | Arg     | Ser      | No        | Periplasm         |
| 1044  | GTG        | ATG         | 349    | Val     | Met      | Yes       | Periplasm         |
| 1168  | AAT        | AGT         | 390    | Asn     | Ser      | No        | Periplasm         |
| 1252  | GTA        | GGA         | 418    | Val     | Gly      | Yes       | Periplasm         |
| 1518  | AAG        | GAG         | 507    | Lys     | Glu      | No        | Outside           |
| 1647  | TAC        | CAC         | 550    | Tyr     | His      | No        | Outside           |
| 1899  | CGC        | GGC         | 634    | Arg     | Gly      | No        | OM/bilayer        |
| 2218  | GAT        | GGT         | 740    | Asp     | Gly      | Yes       | Outside           |

a Based upon topological model of N. meningitidis OmpA proposed by Voulhoux et al. (32).

![Fig. 3. Efficient export of newly synthesized lipids from the IM to the OM in the YaeT mutant at 44 °C. Control (A) and mutant (B) cells were grown at 30 °C in 25 ml of LB/ampicillin to A600 \(\sim \) 1.0. Cells were diluted 5-fold into fresh broth pre-warmed to 44 °C. After 30 min at 44 °C, 4 μCi/ml of \(^{32}\)P was added, and growth was continued for 10 min. IMs and OMs were separated on a 30–60% isopycnic sucrose gradient, and 18–20 fractions (0.5 ml each) were collected. OM phospholipase A activity (×), IM NADH oxidase activity ( ), and total protein concentration ( ) were measured and expressed as nmol/min/ml × 50, nmol/s/ml, and mg/ml, respectively. Portions of each fraction were treated with mild acid to release lipid A from LPS (21, 22). Lipids were extracted and analyzed by thin layer chromatography. PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol.](image-url)
cells were first transformed with the plasmid DNA recovered from the temperature-resistant transformants and selected for CmR. Most suppressor plasmids obtained in this manner contained the wild-type \textit{yae}T gene, as determined by restriction enzyme digestion. However, sequencing of the inserts demonstrated that 3 of 44 hybrid plasmids harbored the \textit{secA} gene, which encodes the IM ATPase involved in signal sequence-dependent pre-protein translocation (16). The \textit{secA} gene was amplified from one such plasmid and cloned into both pACYC184 and pWSK29 to give plasmids pWTD32 and pWTD33, respectively (Table I). The former overexpresses SecA about 5-fold and the latter about 50-fold, as judged by Western blotting (data not shown).

SecA overexpression from pWTD32 restored the growth of WD401/pWTD30 at the nonpermissive temperature (Fig. 7A). SecA overexpression from pWTD32 also restored OmpA levels to about half of wild-type levels during a 60-min shift to 44 °C (Fig. 7B, blot 3 versus blot 1). More importantly, SecA overexpression had no visible effect on levels of the OM proteins in the wild-type background (Fig. 7B, blot 2 versus blot 1), suggesting the SecA effect is specific for the mutant background. During the 60-min shift to 44 °C, the apparent growth rates of WD401/pWTD30-9/pWTD32 and WD401/pWTD30-9/pACYC184 were indistinguishable from each other and their wild-type counterparts (not shown). Attempts to disrupt the chromosomal \textit{yae}T gene in the setting of secA overexpression...
from either pWTD32 or pWTD33, in the absence of pWTD30-9, were unsuccessful.

**DISCUSSION**

*E. coli* YaeT is an essential, conserved OM protein found in all Gram-negative genomes. We have now analyzed the role of YaeT in *E. coli* OM biogenesis by isolating a new temperature-sensitive missense mutant that rapidly loses function under nonpermissive conditions. The ts growth of our construct correlates with the presence of nine amino acid substitutions (Table III), scattered throughout the YaeT protein. A single critical amino acid substitution does not appear to be responsible for the phenotype, as judged by site-directed mutagenesis of the wild-type gene. Remarkably, the secA gene functions as a multicopy suppressor of this YaeT mutant, suggesting a genetic interaction between SecA and YaeT. The function of SecA has been studied extensively over the past 2 decades (16). It is an essential component of the *E. coli* pre-protein translocation case (16).

By using our *yaeT* construct, we analyzed both lipid and protein export from the IM to the OM following 30 min of growth at 44 °C (Figs. 3 and 4). At the time point we chose to label the cells, the growth rate of the mutant was indistinguishable from that of the control strain. In the published reports of Omp85 function in *N. meningitidis*, cellular events were studied at 6 h (33) or even 20 h (32) after the shift to noninduced conditions. In the latter case, the cells had actually stopped growing. Our experiments show that loss of YaeT function at 44 °C has little or no effect on the synthesis and transport of phospholipids and LPS from the IM to the OM (Fig. 3). Most of the labeled lipid A is found in the OM (or at the density where most OM marker is found), and the other phospholipids are equally distributed between the OM and IM. Our data are therefore inconsistent with the results of Genevrois *et al.* (33), who reported that depletion of Omp85 from *N. meningitidis* results in accumulation of all classes of lipids in the IM, similar to what is seen in MsbA mutants (21, 22). However, *N. meningitidis* is the only known Gram-negative organism that does not require LPS for survival (59), suggesting that significant differences in envelope assembly may exist. Although Omp85 and YaeT are 32% identical and 50% similar at the amino acid level, they could play slightly different roles in their respective organisms.
An *E. coli* yaeT Mutation Suppressed by secA

Fig. 6. Western blotting of OmpA in the YaeT mutant. A, equal volumes of the even-numbered fractions from Fig. 4 were separated by 15% SDS-PAGE and analyzed by Western blotting, using a polyclonal antibody to *E. coli* pro-OmpA. B, pro-OmpA Western blot of whole cell lysates of W3110 treated with 2 mM sodium azide for 0–10 min demonstrated accumulation of pro-OmpA. The WD401/pWTD30 control and WD401/pWTD30-9 YaeT mutant cells in mid-log phase at 30 °C were each shifted for 40 min to 44 °C prior to analysis. W3110 and WD2/msbA2 were likewise shifted for 40 min at 44 °C prior to blotting. Each lane contains 200 ng of protein. WT, wild-type; Mut, mutant.

The OMs isolated from our YaeT mutant migrated differently from the wild type on sucrose gradients (Figs. 3–5). The mutant OMs were found in the more buoyant regions, about halfway between normal OMs and IMs. This effect may be caused by selective loss of OM proteins, which may be due to reduced OM protein synthesis and/or accelerated turnover. However, we cannot altogether rule out the possibility that this may be due to a large increase in fused IM-OM particles, which also migrate at this density in sucrose gradients of wild-type membranes. Nonetheless, the results in Figs. 4–6 are consistent with a striking reduction in both newly synthesized and pre-existing OM proteins in the mutant, especially after 30 min at 44 °C. Our results are therefore consistent with those of Voulhoux et al. (32) and Wu et al. (34) who showed that depletion of YaeT/Omp85 from *N. meningitidis* or *E. coli*, respectively, results in the accumulation of incorrectly assembled OM proteins. Moreover, because OM protein depletion is seen in both membranes and whole cell lysates, and OM proteins are not found secreted into the growth medium, we conclude that misfolded or unassembled proteins are not accumulating in the periplasm of the mutant.

The results of Fig. 7B demonstrate that the OmpA made by the mutant cells, when grown at 30 °C, does not disappear when the cells are shifted to 44 °C for 60 min. This suggests that OmpA is stable to turnover if properly incorporated into the OM in the mutant. The absence of newly synthesized OM proteins (Fig. 4) in the mutant at 44 °C may be due to the degradation of improperly folded, nascent OM proteins in the setting of compromised YaeT function. Depletion of YaeT from *E. coli* results in an increase in misfolded OmpA (34). The reduction in OM proteins in our YaeT mutant also resembles the situation observed in RfaC mutants, which fail to make a complete LPS core because they lack heptosyl transferase I (61). Unlike the situation with YaeT, however, RfaC deletion mutants are viable under laboratory conditions (62). The reasons for the absence of OM proteins in RfaC mutants remain unknown.

Recently, another essential, minor OM protein, Imp/OstA, was reported in *E. coli* (63). Nonlethal point mutations in *imp* can give rise to organic solvent sensitivity (64) but deletions are not viable (63). *E. coli* cells depleted of Imp accumulate aberrant OMNs with an increased buoyant density (63). By using *N. meningitidis*, in which slow growth is possible without LPS or Imp (59, 65), Bos et al. (65) have recently provided indirect evidence that Imp is required for surface expression of LPS.

By using a technique termed chemical conditionality, Silhavy and co-workers (66) demonstrated that null mutations in the *yfgL* gene in the *imp4213* (a 23-codon deletion) background restore resistance to certain peptidoglycan synthesis inhibitors. The same group showed that YfgL is an OM lipoprotein and exists in a heterooligomeric OM complex with YaeT and two other lipoproteins of unknown function, YfgL and NlpB, but not Imp (34). These elegant studies have shed light on the process of OM biogenesis in Gram-negative bacteria and suggest a genetic link between OM protein export by YaeT and OM lipid export by Imp. The mechanisms of action of this OM machinery await further analysis.

By screening an *E. coli* genomic library, we found that moderate overexpression of SecA can bypass the ts phenotype of WD401/pWTD30-9 (Fig. 7). SecA is an essential component of the pre-protein translocase, along with the membrane components SecYEG (16, 67). SecA is a cytosolic ATPase that, upon recognition of pre-protein signal sequence, binds with high affinity to the SecYEG heterotrimeric membrane complex and drives protein export in an ATP-dependent manner (16, 30, 68, 69). SecA is thought to undergo major conformational changes in conjunction with ATP binding, membrane association, and pre-protein export. Although predicted to be a soluble cytosolic protein, some SecA is bound to the IM (42). Portions of the protein may be transiently exposed to the periplasm (70, 71). Although SecA is thought to interact with SecYEG to catalyze protein export, there is more SecA than SecYEG in *E. coli* membranes (72). Some types of protein translocation may even occur *in vitro* in the absence of SecY, -E or -G, provided that wild-type levels of SecA are present (73, 74). SecA has been reported to form a ring-like pore structure upon interaction with anionic phospholipids (60). These observations have led to the hypothesis that two SecA-dependent protein export pathways may exist, one that utilizes SecYEG and another that does not (60).

The ability of extra copies of SecA to restore growth at the
nonpermissive temperature to the YaeT is mutant is unclear. Extra SecA had no effect on levels of OmpA in a wild-type background (Fig. 7). Only when OM protein export was compromised in the mutant strain did extra copies of SecA increase levels of OM protein, especially OmpA. This rules out the possibility that SecA suppresses the growth defect by simply increasing the rate and amount of OM protein secretion. One explanation for this effect may be that the mutant YaeT is compromised in both its stability and its own translocation across the IM at 44 °C (the presence of a mutation in the predicted signal sequence supports this hypothesis (Table III)). Extra copies of SecA may increase export of sufficient defective YaeT to maintain growth at 44 °C.

YaeT consists of 810 amino acid residues and appears to have two domains (32, 33). The N-terminal region is hydrophilic and is predicted to face the periplasm. The C-terminal domain is likely to be embedded in the OM and is predicted to form an OM β-barrel. Extra copies of SecA may be able to stabilize the mutant YaeT protein at the nonpermissive temperature, if it is true that some portions of SecA are exposed to the periplasm (70, 71). Attempts to demonstrate a direct interaction of YaeT and SecA by chemical cross-linking and immunoprecipitation have not been successful.2 An intriguing possibility that we have not tested is that portions of the IM preprotein translocon may transiently contact portions of the YaeT multiprotein complex and that extra copies of SecA may stabilize these contacts.

In summary, we have reported a new temperature-sensitive YaeT mutant with severe defects in OM protein export at both permissive and nonpermissive temperatures. In contrast, only small effects were seen on OM lipid export. In addition, we have shown that moderate overexpression of the IM/cytoplasmic ATPase SecA results in partial restoration of OM proteins and complete suppression of growth defects at the nonpermissive temperature. These results support the hypothesis of an essential role for YaeT in OM biogenesis.

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