Isolation of Cold-Sensitive yidC Mutants Provides Insights into the Substrate Profile of the YidC Insertase and the Importance of Transmembrane 3 in YidC Function

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YidC, a 60-kDa integral membrane protein, plays an important role in membrane protein insertion in bacteria. YidC can function together with the SecYEG machinery or operate independently as a membrane protein insertase. In this paper, we describe two new yidC mutants that lead to a cold-sensitive phenotype in bacterial cell growth. Both alleles impart a cold-sensitive phenotype and result from point mutations localized to the third transmembrane (TM3) segment of YidC, indicating that this region is crucial for YidC function. We found that the yidC(C423R) mutant confers a weak phenotype on membrane protein insertion while a yidC(P431L) mutant leads to a stronger phenotype. In both cases, the affected substrates include the P3 coat protein and ATP synthase F,F, subunit c (F,C), while CyoA (the quinol binding subunit of the cytochrome bo3 oxidase complex) and wild-type procoat are slightly affected or not affected in either cold-sensitive mutant. To determine if the different substrates require various levels of YidC activity for membrane insertion, we performed studies where YidC was depleted using an arabinose-dependent expression system. We found that 3M-PC-Lep (a construct with three negatively charged residues inserted into the middle of the procoat-Lep [PC-Lep] protein) and P3 P2 (a construct with the Lep P2 domain added at the C terminus of P3 coat) required the highest amount of YidC and that CyoA-N-P2 (a construct with the amino-terminal part of CyoA fused to the Lep P2 soluble domain) and PC-Lep required the least, while F,C required moderate YidC levels. Although the cold-sensitive mutations can preferentially affect one substrate over another, our results indicate that different substrates require different levels of YidC activity for membrane insertion. Finally, we obtained several intragenic suppressors that overcame the cold sensitivity of the C423R mutation. One pair of mutations suggests an interaction between TM2 and TM3 of YidC. The studies reveal the critical regions of the YidC protein and provide insight into the substrate profile of the YidC insertase.

The Sec machinery is the main translocase used to localize proteins into the inner membrane in bacteria (for a review, see references 5, 13, and 25). While most Escherichia coli proteins utilize the Sec pathway for membrane insertion, the integration of the M13 procoat protein and the P3 coat protein had been shown to be Sec independent and was believed to occur spontaneously in bacteria (6, 8). The ability of these proteins to insert spontaneously into the membrane was challenged, however, by the discovery in 2000 that the integral membrane protein YidC facilitated the insertion of proteins into the lipid bilayer that did not require the Sec machinery, including phage coat proteins (17).

YidC is an evolutionarily conserved protein that is also found in mitochondria and chloroplasts (6, 12, 26). In mitochondria and bacteria, YidC plays a critical role in the assembly of the energy-transducing complexes (23). Depletion of YidC leads to a marked reduction in the assembly of the F,F, ATPase and cytochrome bo oxidase (24). The conserved function of YidC members is highlighted by the fact that the chloroplast YidC homolog Alb3 and the mitochondrial homolog Oxal can function in bacteria (10, 21), and the bacterial YidC can function in mitochondria (15).

In membrane protein biogenesis, YidC can function alone or together with SecYEG to mediate membrane protein insertion in bacteria. The YidC-only pathway is operational in the insertion of a subset of membrane proteins, including the phage coat proteins and ATPase F,F, subunit c (F,C). In vivo depletion of YidC blocks the membrane insertion of P3 coat and F,C into the inner membrane (3, 20, 27, 28). In addition, insertion of these proteins can occur into YidC-containing proteoliposomes (19, 22). YidC contacts the hydrophobic domains of the substrates during the membrane protein insertion process (3, 20), consistent with its function as a membrane protein insertase in the YidC-only pathway. The role of YidC in the Sec pathway is not fully defined. However, the combined data suggest that YidC can function in the insertion, integration, folding, and assembly of membrane proteins (6).

Previously, we isolated cold-sensitive (CS) and temperature-sensitive (TS) mutations in yidC as a means to more rapidly inactivate YidC in vivo to minimize secondary effects potentially caused by prolonged depletion of the protein (4). The conditional-lethal yidC mutants were identified from a small collection of mutants created by engineering protease cleavage sites into the hydrophilic regions of the YidC protein. The CS yidC mutants showed different effects on localization of specific membrane proteins. While the insertion of the P3 coat protein
was inhibited, the insertion of the M13 procoat protein was unaffected. One of the conclusions of the study was that there are different YidC structural requirements for insertion of Pf3 coat and M13 procoat since the CS mutants had different effects on the localization of these polypeptides (4).

Since the introduction of protease cleavage sites can result in significant structural changes to YidC, we have sought additional conditional mutants by random mutagenesis. In this paper, we report the isolation of new CS yidC mutants and their characterization to explore the idea that there are differ-

**FIG. 1.** CS phenotype and membrane topology of the YidC mutants. (A) CS phenotype of the yidC mutants. The JS7131 strain bearing the CS yidC(C423R), yidC(P431L), and wild-type yidC alleles on a low-copy-number pACYC184 plasmid was streaked on LB plates containing glucose (0.2%) or arabinose (0.2%) and incubated at 30°C for 2 days or at 37°C overnight. Each half of a plate contains three streaks from three individual colonies bearing the same construct. (B) The orientation of YidC in the plasma membrane of *E. coli* (16) and the locations of the CS mutations (amino acids 431 and 423) and suppressor mutation (residue 362) are indicated. (C) The amino acid sequences of YidC family members are indicated with the position of the CS mutation highlighted. In the family members, the CS mutations are predicted to be localized within the TM region, TM3 in *E. coli* and many gram-negative bacterial YidC proteins and TM2 in mitochondrial, chloroplasts, and most gram-positive bacterial YidC members. The top five sequences are YidC proteins from gram-negative bacteria; the next five sequences are from gram-positive bacteria; the YidC homologs in chloroplasts (Alb3) and mitochondria (Oxa1) are shown at the bottom. Cyt, cytoplasm; Peri, periplasm; IM, inner membrane.
ent YidC structural requirements for membrane insertion of different YidC-dependent polypeptides. The two isolated CS mutants contain single point mutations that alter amino acids within the third transmembrane domain (TM3) of YidC, indicating that this TM region plays an important role in its function. Of the two CS yidC mutants isolated, yidC(C423R), had a milder phenotype on the membrane insertion of YidC substrates while the phenotype of yidC(P431L) was notably stronger. While insertion of Pf3 coat and FoC were affected in both cases, localization of /H110023M-PC-Lep, a construct with three negatively charged residues inserted into the middle of the procoat-Lep (PC-Lep) protein, and a CyoA construct was affected only with the P431L mutant. Insertion of wild-type procoat was uninhibited in either mutant. To confirm these results, we performed an in vivo YidC depletion study to examine the role of YidC in the insertion of a number of well-established YidC substrates. This study revealed that at 30°C the proper localization of Pf3 coat and FoC required the highest levels of YidC, Fc,C required a moderate level, and a CyoA construct and procoat required only low levels of functional YidC. To begin to identify interactive domains of YidC, we further isolated intragenic suppressors of the C423R mutation. The data suggest an interaction between TM2 and TM3 of the insertase.

MATERIALS AND METHODS

Strains and plasmids. The genes encoding M13 procoat, PC-Lep, Fc,C, the amino-terminal part of CyoA fused to the Lep P2 soluble domain (CyoA-N-P2), and P3 coat with the Lep P2 domain added at the C terminus (P3-P2) are under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible tac promoter in the vector pMS119, which also carries lacIq. JS7131, a YidC depletion strain, is from our laboratory collection. E. coli strain MC1060 contains the wild-type yidC gene as a control. The CS yidC alleles were cloned in pACYC184 and analyzed in the JS7131 strain under YidC depletion conditions. The MYC-cs YidC strain was described previously (4).

Mutagenesis of yidC. New yidC mutants were isolated by transforming the plasmid pACYC184-yidC into the DNA repair-deficient strain XL1-Red (Stratagene). After transformants were cultured for several generations as described by the manufacturer, plasmid DNA was isolated, transformed into JS7131, and plated on LB agar plates supplemented with 0.2% arabinose. yidC mutants were identified by replica plating and culturing at 30°C and 42°C. Single mutations predicted to be responsible for the conditional phenotypes were confirmed by site-directed mutagenesis using a Quikchange mutagenesis kit (Stratagene). Mutations were verified by DNA sequencing.

YidC and PspA detection by Western blot analysis. JS7131 cells with pACYC184 bearing the CS yidC(C423R) and CS yidC(p431L) genes were grown in LB medium containing 0.2% glucose to an A600 of 0.4 at their permissive
temperatures, and then half of the culture was shifted to the nonpermissive temperatures. Following growth for 1, 2, or 3 h, cells were pelleted and resuspended in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading dye, and the samples were analyzed by 15% SDS-PAGE and visualized by phosphorimaging (27). YidC was detected by immunoblotting using antiserum that was prepared against a C-terminal peptide of YidC. Likewise, PspA antiserum was used to detect this polypeptide.

Assay for membrane insertion by in vivo signal peptide processing and protease mapping. The YidC depletion strain JS7131 cells expressing the CS YidC(C423) and YidC(P431L) proteins were grown in LB medium supplemented with glucose to an $A_{600}$ of 0.4 at the permissive temperatures, pelleted, resuspended in M9 minimal medium, and shifted to the nonpermissive temperature. Prior to labeling, 1 mM IPTG was added to the cultures for 5 min to induce the expression of the plasmid-encoded membrane protein substrates. The cells were then treated with [trans-$^{35}$S]methionine (100 μCi/ml cells) for 60 s to label the newly synthesized proteins. Where indicated (see text below), the cells were chased with cold methionine for various times. The insertion of M13, PC-Lep, −3M-PC-Lep, or CyoA-N-P2 was monitored by examining signal peptide processing. Following the addition of trichloroacetic acid to precipitate the radiolabeled proteins, the samples were subjected to SDS-PAGE to separate the precursor and mature forms of the protein. For analysis of membrane insertion of P3 P2 and F$_e$C, radiolabeled cultures were converted to spheroplasts and incubated in the presence or absence of proteinase K (PK; final concentration, 0.5 mg/ml) for 60 min at 0°C. The samples were precipitated with trichloroacetic acid and analyzed by 15% SDS-PAGE. Phosphorimaging was carried out as described previously (27). Signal peptide cleavage and protease mapping were performed for some substrates in the same fashion using MC1060 as a control. In addition, certain substrates were analyzed in the same fashion except that the YidC depletion strain JS7131 lacked the CS yidC alleles. The JS7131 strain expressing the substrates was grown in medium supplemented with 0.2% glucose or 0.2% arabinose and were labeled and analyzed by SDS-PAGE and phosphorimaging exactly as above.

RESULTS

Isolation and characterization of the CS mutants. Conditional lethal mutants of YidC were sought in order to identify regions important for the activity of the protein and to better characterize YidC-dependent membrane protein localization. The pACYC184 vector bearing the yidC gene was mutagenized using the mutator strain XL-1 RED and then used to transform the YidC depletion strain JS7131. In this strain, the sole copy of yidC is under the control of the araBAD promoter, permitting YidC to be depleted by growth in glucose medium (17). We screened for conditionally lethal mutants by plating transformants at either 30°C or 42°C on LB-glucose plates. Using this approach, we isolated two CS yidC mutants with alterations at C423R and P431L, with the phenotype shown in Fig. 1A. Both mutants showed a reduced growth at 30°C, whereas they grew well at 37°C (Fig. 1A, where chromosomal YidC is depleted on Glu plates). Both mutations are located within the third of six TM segments of YidC (Fig. 1B). The two altered residues are highly conserved among YidC homologs in gram-negative and gram-positive bacteria (Fig. 1C). The proline 431 residue is conserved in the mitochondrial and chloroplasts homologs of YidC but not the C423 residue.

To examine whether YidC function was impaired at the nonpermissive temperature, we tested if the stress protein PspA was induced. Previously, PspA was shown to be highly expressed upon YidC depletion (24). This is apparently due to the fact that YidC is required for the assembly of the energy-transducing components F$_e$F$_o$ ATPase and cytochrome $b_{59}$ oxidase (24). It is well established that PspA is induced when the proton motive force is reduced (11, 14). We therefore investigated whether PspA was induced at the nonpermissive temperature by measuring its cellular level at both 30°C and 37°C (the permissive temperature for the CS mutants). The quantity of PspA was measured by immunoblot analysis. The upper panel in Fig. 2A shows that PspA was induced at the nonpermissive temperature for yidC(C423R), consistent with the idea that the YidC function is impaired at 30°C. Similar results were found with yidC(P431L) (Fig. 2A). In both cases, there is low-level expression of PspA at 37°C, indicating that YidC is not fully functional at the permissive temperature (Fig. 2A).

The cellular levels of YidC in the CS mutants were also measured to determine the stability of the protein at the nonpermissive temperature. Immunoblot analysis was performed with cell lysates prepared from the YidC depletion strain JS7131 expressing the CS yidC alleles on a plasmid at both the permissive and nonpermissive temperatures for various times. YidC was detected using antibody prepared against a C-terminal YidC peptide. As shown in Fig. 2B, the steady-state expression levels of yidC(C423R), and yidC(P431L) are similar at both the nonpermissive and permissive temperatures. In this

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<th>Protein</th>
<th>Membrane insertion with the indicated mutant$^a$</th>
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<tr>
<td></td>
<td>YidC C423R</td>
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<tr>
<td>PC-Lep</td>
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<td>Pi3 P2</td>
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<tr>
<td>−3M-PC-Lep</td>
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$^a$ +, insertion is greater than 80% at the nonpermissive temperature 30°C; +/−, insertion is between 80% and 20% insertion at 30°C; −, less than 20% membrane insertion at the nonpermissive temperature (30°C).
experiment, expression of the chromosomal copy of \textit{yidC} was repressed by growth in glucose (Fig. 2B, Glu lane).

To determine whether the C423R mutant was inactivated at the nonpermissive temperature, we first examined the localization of several substrates known to insert into the membrane by the YidC-only pathway (3, 20, 22, 27, 28). These include the Pf3 P2, which is a chimera of the Pf3 coat that is extended with the Lep P2 soluble domain at its carboxy terminus, and FoC, which contains a peptide tag (GVQDFTST) at its carboxy terminus and a His10 tag in the cytoplasmic region that allows the protein to be isolated using a metal chelate affinity resin (Table 1). Protease accessibility studies were used to measure the membrane insertion of Pf3 P2 and FoC. JS7131 bearing the plasmid expressing the mutant \textit{yidC} alleles was grown as described in Materials and Methods. Figure 3A shows that Pf3 P2 was digested by externally added protease at the permissive temperature (37°C) to a smaller, faster-migrating species, indicating that the amino-terminal tail of the protein translocated across the membrane. Insertion of Pf3 P2 was inhibited at the nonpermissive temperature (30°C), as about 50% of the protein was protected by protease digestion. In contrast, Pf3 P2 was completely inhibited when YidC was depleted in the depletion strain by growth in glucose for 3 h (Fig. 3A, right side). This suggests that the activity of CS YidC(C423R), though
lowered, still remains. Similar results were observed with FoC modified with the tag at the C terminus (Table 1). The addition of the tag to subunit c enabled us to monitor insertion of the C terminus as the C-terminal tag was cleaved by PK when insertion occurred (27). Whereas [35S]methionine-labeled FoC inserted efficiently at 37°C and was completely digested, as indicated by the appearance of a band with a lower molecular weight, its insertion was inhibited at the nonpermissive temperature 30°C (Fig. 3B, where the protected full-length FoC band appears).

In contrast, the insertion of M13 PC-Lep and CyoA-N-P2 was unaffected at the nonpermissive temperature in the yidC(C423R) mutant. M13 PC-Lep is a protein that contains the Lep P2 domain at its carboxy terminus, allowing immunoprecipitation of the protein (Table 1). CyoA-N-P2 corresponds to the amino-terminal part of CyoA fused to the Lep soluble P2 domain (2) (Table 1). Both of these constructs insert into the membrane by the YidC pathway (2, 17). Membrane insertion of PC-Lep and CyoA-N-P2 was monitored by signal peptidase processing (Fig. 3C and D). JS7131 cells bearing the CS yidC mutants were grown in the presence of glucose for 3 h at the permissive or nonpermissive temperature under YidC depletion conditions. Pulse-chase experiments were performed as described in Materials and Methods. When M13 PC-Lep inserts across the membrane, it is processed by signal peptidase I and converted to the mature C-Lep protein. Figure 3C shows that PC-Lep was efficiently processed in the yidC(C423R) mutant at both the permissive and nonpermissive temperatures at a level indistinguishable from the wild-type MC1060 strain. Signal peptidase processing of CyoA-N-P2 in the yidC(C423R) mutant also indicated that this construct inserted efficiently across the membrane even at the nonpermissive temperature (Fig. 3D). Like procoat, CyoA-N-P2 inserts across the membrane by the YidC-only pathway (2). As a control, we showed that the membrane insertion of C-Lep (Fig. 3C) and CyoA-N-P2 (Fig. 3D) was inhibited by growing the JS7131 YidC depletion strain in glucose (Fig. 3, Glu lanes).

Next we examined whether membrane insertion of a Sec-dependent substrate was affected in the yidC(C423R) mutant. Previously, our laboratory showed that 3MP-PC-Lep absolutely requires YidC for membrane insertion (17). 3MP-PC-Lep contains three negatively charged residues inserted into the middle of the PC-Lep protein (1) (Table 1). This protein inserts across the membrane in a SecA-dependent manner (1) and in contrast to most Sec-dependent proteins is strictly dependent on YidC for membrane insertion (18). To examine membrane insertion of 3MP-PC-Lep, we monitored signal sequence processing. JS7131 cells expressing the yidC(C423R) mutant were pulse labeled for 1 min and chased for 0 and 2 min at the permissive or nonpermissive temperature under YidC depletion conditions. Figure 3E shows that the precursor form of 3MP-PC-Lep accumulated to a very small extent with increasing time at the nonpermissive temperature (30°C). As a control, we showed that insertion of 3MP-PC-Lep and the other substrates (P3 P2, Fc, PC-Lep, P-CyoA-N-P2) is efficient at 30°C in the MC1060 strain (Fig. 4). In addition, membrane insertion of PC-Lep and 3MP-PC-Lep is efficient in the parental strain JS7131 with pACYC184-yidC wt (where wt is wild type) (Fig. 4).

The second CS mutant, yidC(P431L), was also investigated to determine whether the affected substrates are similar to those found with the YidC(C423R) mutant. Like the YidC(C423R) mutant, insertion of newly synthesized 35S-labeled PC-Lep was not inhibited at the nonpermissive temperature (Fig. 5A). However,
there are observable differences with the other YidC substrates tested. The −3M-PC-Lep (Fig. 5B), Pf3 P2 (Fig. 5C), and FcPc (Fig. 5D) were strongly blocked at the nonpermissive temperature. In contrast, the membrane insertion of CyoA-N-P2 was inhibited only slightly (Fig. 5E), whereas its insertion was not inhibited in the yidC(P423R) mutant (Fig. 3D).

Notably, the insertion of the Sec-dependent −3M-PC-Lep was completely blocked even at the permissive temperature in the yidC(P431L) mutant. This is reminiscent of the CS mutant generated by the introduction of factor Xa and prescision protease sites after TM1 and before TM2, respectively (4). The −3M-PC-Lep is strictly YidC dependent in contrast to the insertion of most tested Sec-dependent proteins that are only moderately affected (18). To directly compare these mutants, we also investigated the MYC-cs yidC mutant in terms of its ability to promote insertion of FcPc and CyoA-N-P2. Figure 6 shows that insertion of the FcPc (Fig. 6A) and CyoA-N-P2 (Fig. 6B) was strongly impaired in the MYC-cs yidC mutant whereas PC-Lep was unaffected (Fig. 6C) (4). Taken together, the results indicate that the MYC-cs yidC mutant and the P431L mutant are defective at similar levels, and both exhibit a stronger phenotype than the C423R CS mutant.

FIG. 5. Membrane insertion of −3M-PC-Lep, FcPc, and CyoA-N-P2 is strongly inhibited in the CS YidC(P431L) mutant. (A) Signal peptide processing of PC-Lep in the YidC(P431L) mutant. JS7131 bearing both pACYC184-YidC(P431L) and pMS119-Pf3 P2 was grown and analyzed as described in Materials and Methods. (B) Signal peptide processing of −3M-PC-Lep in the YidC(P431L) strain. (C) Protease accessibility of Pf3 P2 in the CS YidC(P431L) mutant. (D) Protease accessibility of FcPc in YidC(P431L) mutant. (E) Signal peptide processing of preCyoA-N-P2 in the YidC(P431L) mutant. The control in panel A was prepared in strain JS7131 grown under YidC depletion conditions. The control in panel B was prepared in strain JS7131 grown under yidC-expressing conditions. The Pf3 P2 and CyoA-N-P2 controls in panels C and E, respectively, were produced in the JS7131 strain grown with or without glucose (Glu; YidC depletion) or arabinose (Ara; YidC expression).
Different substrates require different amounts of YidC for membrane insertion. The results with the CS yidC mutants indicate that the insertion of some substrates is profoundly affected at the nonpermissive temperature whereas others are not. One possibility is that the differences correlate with the level of dependence that the proteins have on YidC for membrane insertion. To test this, we examined the insertion at 30°C of several membrane proteins in JS7131 grown under YidC depletion conditions. YidC was depleted for various times by diluting an overnight culture of JS7131 strain into LB medium supplemented with glucose for various times, as summarized in Fig. 7. Immunoblot analysis was used to determine the level of YidC at different depletion times (Fig. 7A). YidC had to be depleted to a greater extent for inhibition in PC-Lep (Fig. 7B) and CyoA-N-P2 (Fig. 7E) insertion to occur, compared to insertion of Pf3 P2 (Fig. 7C), −3M-PC-Lep (Fig. 7D), and F_oC (Fig. 7F). It took an extended depletion time (greater than 275 min) to observe greater than 50% processing inhibition of PC-Lep and CyoA-N-P2 compared to a depletion time of 110 min (Fig. 7F). It took an extended depletion time (greater than 275 min) to observe greater than 50% processing inhibition of PC-Lep and CyoA-N-P2 compared to a depletion time of 110 min (Fig. 7F).

Identification of intragenic suppressor mutations and other CS mutations. We further predicted that the CS yidC mutants may be useful to understand how different regions of YidC may interact. Consequently, we isolated second-site mutations that overcame the cold sensitivity imparted by the C423R mutation (Fig. 8A). One such suppressor mutation resulted in introduction of a glutamic acid at position 362 (Fig. 1B shows the position in TM2). As expected, this suppressor mutant efficiently inserted Pf3 P2 and F_oC into the membrane at 30°C (Fig. 8B and C), as revealed by the generation of the shifted protected band at 30°C. This indicates that this YidC suppressor is functional for membrane insertion at 30°C. We also used site-directed mutagenesis to test whether a glutamic acid engineered at residue 426 could compensate for the C423R mutation, with the expectation that if the region around amino acid residue 423 is a helix, then it could form an ion pair, neutralizing the positively charged residue and thereby suppressing the CS phenotype. Figure 8A shows that residue 426 does, indeed, act as a suppressor, and the YidC(C423R 426E) strain grows well at 30°C (Fig. 8A, middle panel). Adding a negatively charged residue at position 419, 420, or 427, however, failed to suppress the cold sensitivity (data not shown).

Other CS mutants were obtained by introducing an arginine at other positions. As can be seen in Fig. 8D, an arginine introduced at position 422 or 424 also resulted in a CS phenotype, whereas arginines at 421 and 425 did not exhibit a CS phenotype. A lysine or glutamic acid at position 423 can also cause a CS phenotype while proline showed a wild-type phenotype. A lysine or glutamic acid at position 423 can also cause a CS phenotype while proline showed a wild-type phenotype. These later studies illustrate that many CS and corresponding suppressor mutants can be generated through extensive site-directed mutagenesis of yidC.

DISCUSSION

In this paper, we describe two CS yidC mutants that were isolated by targeted mutagenesis of the yidC gene. Interestingly, both the yidC(C423R) and yidC(P431L) CS mutants had alterations localized to TM3. The altered amino acids include a cysteine residue at position 423 and a proline at 431, both highly conserved within bacteria (Fig. 1C). The proline 431 residue is also conserved in the mitochondrial and chloroplast homologs of YidC but not the C423 residue. Our results reported here demonstrate that TM3 of E. coli YidC plays a critical role in YidC function and support previous structure-function studies (9). The phage shock protein A (PspA) protein was induced at the nonpermissive temperature in both CS yidC mutants (Fig. 2A), as has been observed previously when YidC is depleted (24). Induction of the phage shock response has been attributed to a reduction in the proton motive force (11, 14), partly as a result of inhibition in the insertion of the YidC substrate F_oC (20, 22, 28), leading to a defect in the assembly in the ATP synthase (24). Previously, it has been reported that an F_oC ATP synthase null mutation activates the phage shock protein response in E. coli (7). Indeed, both CS mutants described in this report resulted in an inhibition in the insertion of F_oC (Fig. 3B and 5D).

The CS mutants isolated in this work differentially affected the localization of a variety of membrane proteins at the non-
permissive temperature. Specifically, while insertion of Pf3 P2 and F0C were inhibited, −3M-PC-Lep was only mildly affected, and the insertion of P-CyoA-N-P2 and wild-type PC-Lep was unaffected in the C423R mutant (Fig. 3). In contrast, the P431L mutant exhibited a stronger phenotype as insertion of Pf3 P2, F0C, and −3M-PC-Lep was nearly completely inhibited at the nonpermissive temperature (Fig. 5). Also, in the P431L mutant insertion of preCyoA-N-P2 was inhibited while insertion of the PC-Lep remained unaffected. Similar results were observed with the MYC-cs mutant (Fig. 6) (4). A summary of the membrane insertion results with the tested substrates is shown in Table 1. The results fit well with the activity of the CS P431L mutant and the MYC-cs mutant being decreased to a larger extent than that of the YidC(C423R) mutant at the nonpermissive temperature.

We hypothesize the reason why insertion of some proteins
was affected at the nonpermissive temperature in the CS mutants has to do primarily with the level of YidC activity that is required for the different substrates for membrane insertion. Some substrates will be inhibited in membrane insertion if YidC activity is decreased by 50% whereas others will be unaffected. Another possible explanation, as proposed by Chen et al. (4) for the MYC-cs YidC mutant, is that different regions of YidC could be important for insertion of different substrates. This could explain why a mutation could affect insertion of Pf3 coat protein but have no effect with the procoat substrate (4).

To address this issue we examined the insertion of several YidC substrates as a function of depletion of wild-type YidC in vivo. As Fig. 7 shows, at 30°C the insertion of different substrates was affected differently as YidC was depleted from the cell. The insertions of Pf3 P2 and FcC were affected by a 110-min depletion time when the amounts of YidC were reduced approximately threefold. In contrast, insertion of FoC was not affected at the 110-min depletion time but was strongly affected when the depletion time was increased to 220 min, when YidC levels were further reduced. Strikingly, the insertion of the prototypic YidC substrate PC-Lep requires a sig-
nificant depletion of YidC to see inhibition. One possibility is that the defect we see on PC-Lep membrane insertion is due to an indirect effect after a prolonged YidC depletion. In vitro studies with YidC proteoliposomes will be required to test whether procoat is a YidC substrate. Similarly, the insertion of CyoA-N-P2 was affected only after the longest depletion time used. Taken together, these results show that PC-Lep and CyoA-N-P2 require the least YidC, P3 P2 and −3M-PC-Lep require the highest concentration of YidC for insertion, and the level of YidC for the F, C substrate falls in between PC-Lep and CyoA-N-P2 and the other substrates.

Based on the YidC depletion data at 30°C, it is predicted that the substrates P3 P2 and −3M-PC-Lep would be inhibited in membrane insertion in a CS YidC mutant with a lower activity before a CyoA-N-P2 construct and PC-Lep. This is what we observed. However, there are some inconsistencies. For example, the substrate −3M-PC-Lep is less affected than P3 P2, and F,C is affected in the CS YidC(C423R) mutant. This latter result is unexpected as YidC had to be depleted to a larger extent to see a block in F,C insertion compared to P3 P2 and −3M-PC-Lep (Fig. 7). This suggests that the CS mutation may cause not only the activity of YidC to be decreased but also minor perturbations in the structure that preferentially affect the insertion of one substrate over another.

In this work, we also isolated suppressor mutations of the YidC(C423R) mutant in order to obtain information on regions of the YidC protein that may interact with the C423 residue in TM3. We obtained suppressor mutations at residue 362 of the yidC gene using the mutator strain XL1-Red and a suppressor at residue 426 using site-directed mutagenesis. The C423R/T362E suppressor pair points to an interaction between the TM2 and TM3 regions of YidC (Fig. 8). This is an important observation because very little structural information exists about the YidC protein. The threonine-to-arginine suppressor mutation at residue 362 may be able to neutralize the C423R residue by a direct interaction, although there are other possible explanations. In addition, the identified suppressor mutation at 426 may be able to neutralize the positive charge of the arginine residue at 423 and would be expected to do so if this region of the protein forms a helix. Interestingly, site-directed mutagenesis studies revealed that a CS phenotype results from the introduction of not only an arginine at position 423 but also an arginine at positions 422 and 424 (Fig. 8D). Thus, it is very likely that additional CS yidC mutants could be isolated using extensive site-directed mutagenesis.

In conclusion, this work describes the isolation of two CS yidC mutants that are less active but remain stable at the nonpermissive temperature. The mutations responsible for the CS phenotype are found in the conserved TM3 segment of YidC, supporting an important role of this region in function of the insertase. Characterization of these CS mutants reveals that different substrates require different YidC activities for membrane insertion and that the perturbations caused by the CS mutation can preferentially affect one substrate over another. Future studies will focus on determining exactly how these mutations impair the activity of the enzyme at the lower temperature.

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