The Role of the Strictly Conserved Positively Charged Residue Differs among the Gram-positive, Gram-negative, and Chloroplast YidC Homologs*

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Background: B. subtilis YidC1 requires the strictly conserved arginine for function.

Results: The conserved positively charged residue is critical for S. mutans YidC2 function but not for E. coli or chloroplast homologs.

Conclusion: The role of the positively charged residue differs among YidC family members and for insertion of various substrates.

Significance: Understanding the importance of the conserved charge is crucial for defining the YidC insertion mechanism.

Recently, the structure of YidC2 from Bacillus halodurans revealed that the conserved positively charged residue within transmembrane segment one (at position 72) is located in a hydrophilic groove that is embedded in the inner leaflet of the lipid bilayer. The arginine residue was essential for the Bacillus subtilis SpoIIIJ (YidC1) to insert MifM and to complement a SpoIIIJ mutant strain. Here, we investigated the importance of the conserved positively charged residue for the function of the Escherichia coli YidC, Streptococcus mutans YidC2, and the chloroplast Arabidopsis thaliana Alb3. Like the Gram-positive B. subtilis SpoIIIJ, the conserved arginine was required for functioning of the Gram-positive S. mutans YidC2 and was necessary to complement the E. coli YidC deletion strain and to promote insertion of a YidC-dependent membrane protein synthesized with one but not two hydrophobic segments. In contrast, the conserved positively charged residue was not required for the E. coli YidC or the A. thaliana Alb3 to functionally complement the E. coli YidC deletion strain or to promote insertion of YidC-dependent membrane proteins. Our results also show that the C-terminal half of the helical hairpin structure in cytoplasmic loop C1 is important for the activity of YidC because various deletions in the region either eliminate or impair YidC function. The results here underscore the importance of the cytoplasmic hairpin region for YidC and show that the arginine is critical for the tested Gram-positive YidC homolog but is not essential for the tested Gram-negative and chloroplast YidC homologs.

The YidC/Oxa1/Alb3 family of proteins facilitates the insertion, folding, and assembly of proteins in all kingdoms of life (1–3). Bacterial YidC can function either independently or cooperatively with the Sec machinery in membrane protein insertion (4–6). The bacterial YidC promotes the insertion of proteins into the cytoplasmic membrane, whereas the mitochondrial Oxa1 and chloroplast Alb3 homologs catalyze the insertion/folding of proteins into the inner membrane of mitochondria and the thylakoid membrane of chloroplasts, respectively. Intriguingly, the chloroplast Alb3, mitochondrial Oxa1, and the Streptococcus mutans YidC2 homologs can functionally substitute for YidC in Escherichia coli (7–9). Conversely, bacterial YidC can substitute for the mitochondrial Oxa1 (when the extended C-terminal matrix domain of Oxa1 is attached to YidC) and support insertion of Oxa1 substrates into the inner membrane of the mitochondria (10).

All YidC proteins have an evolutionarily conserved core domain of five TM3 segments, containing the insertase activity of the proteins (11–13). First, the Gram-positive bacterial YidC and the organelar Oxa1 and Alb3 proteins contain only five TM domains. These proteins are synthesized with the respective presequences, which are proteolytically removed following membrane insertion. Second, the E. coli YidC has an extra TM segment at its N terminus and a large periplasmic domain following it (14), but these regions are not essential for YidC activity (15). The region containing the C-terminal five TM segments is essential for the membrane insertase activity of the E. coli YidC (15).

Until recently, there was very little structural information available on YidC. In 2008, x-ray crystallography showed that the large periplasmic domain of the E. coli YidC adopted a super-ß-sandwich domain commonly seen in sugar-binding proteins (16, 17). Using cryo-electron microscopy methods, Kohler et al. (18) showed that YidC was bound to the ribosome nascent chain complex as a dimer, whereas Seitl et al. (19) showed that YidC can bind a ribosome nascent chain as a monomer. This latter study and Kedrov et al. (20) confirmed that the functional unit of YidC is a monomer.

In 2014, there was a breakthrough in the YidC/Oxa1/Alb3 field with the determination of the crystal structure of the Bacillus halodurans YidC2 at a 2.4 Å resolution (21). The struc-

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1 The abbreviations used are: TM, transmembrane segment; PC-Lep, Procoat Lep.
Strictly Conserved Positive Charge among YidC Homologs

EXPERIMENTAL PROCEDURES

Materials, Strains, and Plasmids—Ampicillin, chloramphenicol, and lysozyme were purchased from Sigma. Proteinase K was purchased from Life Technologies. Isopropyl 1-thio-β-D-galactopyranoside was from Research Products International Corp. PMSF was purchased from United States Biochemical (Affymetrix). Phosphate-buffered saline (PBS) (pH 7.2) was purchased from Thermo Scientific. Tran-35S-label, a mixture of (Affymetrix). Phosphate-buffered saline (PBS) (pH 7.2) was purchased from United States Biochemical Corp. PMSF was purchased from United States Biochemical.

Bacterial Growth and Pulse Labeling—E. coli JS7131 was transformed with pMS119 encoding either Pf3-23Lep, PC-Lep, or PreCyoA-N-P2 and then further transformed with pACYC184 encoding YidC or the appropriate homolog (H1Alb3 or 247YidC2). The cells were grown in LB media in the presence of ampicillin (final concentration, 100 µg/ml) and chloramphenicol (final concentration, 25 µg/ml). Depletion of the chromosomally encoded YidC was achieved by growth in the presence of 0.2% glucose for 3 h at 37 °C. The cells were subsequently washed with M9 medium and then transferred into this medium, which lacks Met, and grown for 30 min. Expression of YidC-dependent membrane proteins was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside for 5 min, followed by pulse-labeling with [35S]methionine (70 µCi/ml) for 1 min.

Signal Peptide Processing and Protease Mapping—To analyze membrane insertion of PC-Lep and PreCyoA-N-P2, a signal peptide cleavage assay was used. After [35S] labeling, an equal volume of 20% TCA was added to precipitate total proteins. The precipitate was washed with ice-cold acetone and resuspended in Tris-SDS (pH 8.0) buffer. The samples were then subjected to immunoprecipitation to examine membrane insertion by signal peptide processing of PC-Lep to C-Lep or PreCyoA-N-P2 to CyoA-N-P2. Antiserum to leader peptidase was used to immunoprecipitate PC-Lep and C-Lep or PreCyoA-N-P2 and CyoA-N-P2.

Protease accessibility was employed to assay membrane insertion of Pf3-23Lep. Following radiolabeling, cells were collected by centrifugation at 4 °C and resuspended in 40% sucrose and 33 mM Tris-HCl (pH 8.0). To prepare spheroplasts, lysozyme (5 µg/ml) and 1 mM EDTA (pH 8.0) were added, and the sample was kept on ice for 20 min. Where indicated, proteinase K was added (0.5 mg/ml final concentration) and incubated on ice for 1 h, followed by the addition of PMSF to quench the proteinase K reaction. Immunoprecipitation of Pf3-23Lep was performed using leader peptidase antiserum,
and OmpA was precipitated with OmpA antiserum, as described previously. The samples were analyzed by SDS-PAGE and phosphorimaging.

Quantitation of the Membrane Insertion Data—The translocation efficiencies were determined by quantitation of the appropriate bands using ImageJ, developed at National Institutes of Health. The percent of translocation across the membrane was determined by calculating the percent of the signal peptidease processed band appearing on the gels. For the Pf3-derived proteins the protease-protected bands were analyzed.

For Pf3-23Lep, one of the seven methionines in the protein sequence was lost upon proteinase K cleavage, so Equation 1 was used to determine the percent of translocation.

\[
\text{% translocation} = \frac{7/6 \text{ cleaved Pf3-23Lep}}{7/6 \text{ cleaved Pf3-23Lep} + 1/6 \text{ uncleaved Pf3-23Lep}} \times 100 \quad (\text{Eq. 1})
\]

For PreCyOA-N-P2, one out of seven methionine residues were lost upon signal peptidease cleavage; thus Equation 2 was used to determine the percentage of the protein translocated.

\[
\text{% translocation} = \frac{7/5 \text{ processed PreCyOA-N-P2}}{7/5 \text{ processed PreCyOA-N-P2} + 7/5 \text{ uncleaved PreCyOA-N-P2}} \times 100 \quad (\text{Eq. 2})
\]

The % translocation for all the insertion studies is presented in the figures with the data.

Complementation Assay—E. coli JS7131 cells bearing the respective pACYC184 plasmids were grown overnight in Luria broth (LB) with 0.2% arabinose and 25 μg/ml chloramphenicol. The following morning, the cells were pelleted, washed with PBS, and resuspended in lysis buffer. The samples were analyzed by SDS-PAGE using a 10% polyacrylamide gel and electroblotted onto nitrocellulose membranes. Polyclonal antiserum against E. coli YidC and S. mutans YidC2 was generated against C-terminal peptides. The antibody against S. mutans YidC2 was a gift from Jeanine Brady. Antibody produced against a C-terminal peptide from Pisum sativum Alb3, which reacts with A. thaliana Alb3, was a gift from Ralph Henry.

RESULTS

Arginine 366 in the E. coli YidC Is Not Required for Function—The crystal structure of B. halodurans YidC2 revealed a hydrophilic groove that was exposed to the cytoplasm and the inner bilayer leaflet (Fig. 1A) (21). An arginine (at position 72 on the B. halodurans YidC2) was present within the groove, and this arginine in the B. subtilis SpoIII (YidC1) is essential for membrane protein insertion of the single spanning MiFM protein. Remarkably, this positive charge is invariant in all YidC homologs in bacteria, mitochondria, and chloroplasts, even though little of the overall YidC sequence is conserved between the various organisms (11, 12), which suggests the positively charged residue is important for YidC function. Fig. 1B shows a sequence alignment of YidC proteins in E. coli, S. mutans, B. halodurans, and B. subtilis and the sequence of the A. thaliana chloroplasts Alb3 protein. The sequences show the strictly conserved positively charged residue in TM1 (corresponding to TM2 in E. coli YidC) and the location of the N-terminal (CH1) and the C-terminal (CH2) helices of the helical hairpin structure in the cytoplasmic loop 1 of YidC homologs. Although the conserved arginine is important for the B. subtilis SpoIII, it appeared that the arginine was not important for the E. coli YidC because a quintuple serine mutant (changing residues 366–370 to serine residues) could rescue the growth of the YidC depletion strain and promoted the insertion of the procoat protein (15).

To further examine the importance of the arginine at position 366 of the E. coli YidC, we mutated this residue and tested the activity of the YidC mutant (Fig. 2). First, we tested the ability of various YidC mutants to complement JS7131, the YidC depletion strain. JS7131 has the chromosomal copy of yidC inactivated and another copy of the gene introduced into the chromosome under the control of the araBAD promoter (22). Growth of JS7131 is arabinose-dependent because YidC, which is essential for cell growth, is expressed when the cells are grown in LB medium with arabinose, but it is not expressed when grown in LB medium with glucose. We cloned the YidC arginine mutants into pACYC184 under the control of the endogenous yidC promoter, as described previously (7), and we investigated whether the mutated YidC proteins could complement the growth defect of JS7131 when the chromosomal YidC was depleted. The overnight cultures of JS7131 bearing the respective YidC arginine 366 mutants were back-diluted 1:100 and grown in LB media for 3 h at 37 °C. The cultures were then serially diluted and spotted on LB agar plates supplemented with either arabinose or glucose. Fig. 2A shows that all of the
mutants fully complemented the YidC depletion strain (see plates with glucose) with the exception of the glutamic and aspartic acid substitution mutants.

To confirm that the activity of YidC was truly unimpaired, we examined whether the YidC mutants were functional for the membrane insertion of 2 YidC-dependent proteins. First,
we analyzed the model YidC-dependent protein Pf3-23Lep (see Fig. 1, C and D) that has the leader peptidase (Lep) residues 23 to 323 fused after the TM segment of the Pf3 coat; an Arg was placed at the C terminus of TM2 of Lep to prevent the translocation of Lep TM2 (23). Translocation of the N-tail of Pf3-23Lep can be monitored using proteinase K digestion (Fig. 2B, left panel). When YidC R366A or R366L was expressed under chromosomal YidC depletion conditions, Pf3-23Lep was inserted, which illustrates that these mutants facilitate insertion. However, the negatively charged YidC366D and R366E mutants were unable to facilitate insertion of the Pf3-23Lep protein.

Similar results for the R366A and R366L mutants were observed with the YidC-dependent Procoat-Lep (PC-Lep) protein (24). PC-Lep corresponds to procoat with its C-terminal cytoplasmic region extended by 101 amino acids of the periplasmic domain of Lep (see Fig. 1, C and D) (25). PC-Lep is cleaved by signal peptidase (also known as leader peptidase, Lep) and converted to the mature form (denoted by C in Fig. 2B, right panel), termed coat-Lep during membrane insertion (Fig. 2B, top right panel). Membrane insertion of PC-Lep was observed when either the E. coli YidC R366A or R366L mutant was present under YidC depletion conditions indicating that
these YidC arginine mutants are functional. When YidC R366E or R366D was present, membrane insertion of PC-Lep was partially defective. As a negative control, we confirmed that PC-Lep accumulated in the precursor form (denoted by PC in Fig. 2B, right panel) in the cytoplasm under YidC depletion conditions when an empty plasmid was present in JS7131. We also showed by Western blot analysis that all of the YidC mutants were expressed and stable (Fig. 2C). The wild-type and Arg-366 YidC mutant proteins were readily detected on the Western blot. No band is seen at the respective position for the empty vector. The upper band on the Western blots, which is a non-specific band recognized by our antibody, serves as a loading control. The above complementation and membrane insertion results show that there are differences between the importance of the conserved arginine for the B. subtilis and E. coli YidC proteins because YidC requires a positively charged residue for its function in B. subtilis but not in E. coli.

C-terminal Half of the Predicted Hairpin Region of the E. coli YidC Is Important for Function—Previously, we reported that a deletion of the entire cytoplasmic loop 1 (residues 371–416) did not impair the activity of the E. coli YidC (15). In this published study, we showed that growth of JS7131 (the YidC depletion strain) containing pACYC184 YidC ΔC1 was observed when colonies were streaked out on an agar plate under glucose conditions. However, we wanted to reinvestigate the importance of the C1 region given the fact that this region forms a hairpin structure and is important for YidC function in B. subtilis (21).

To probe the function of the hairpin region in cytoplasmic loop 1 of the YidC protein, we made several deletions as follows: Δ371–416 to delete the entire cytoplasmic loop 1 (ΔC1); Δ389–398 to delete the N-terminal portion of the hairpin structure (ΔCH1); and Δ399–415 to delete the C-terminal region of the hairpin structure (ΔCH2). The deletion of the entire C1 loop inactivated the E. coli YidC because it did not complement the JS7131 strain under YidC depletion conditions (Fig. 3A, LB + glucose) and blocked membrane insertion of Pf3-23Lep and Procoat-Lep (Fig. 3B). Similarly, YidCΔCH2 with the C-terminal part of the helical hairpin structure deleted was not functional in rescuing JS7131 under YidC depletion conditions (Fig. 3A), and it was highly defective in membrane insertion of the YidC substrates Pf3-23Lep and PC-Lep (Fig. 3B). Although the YidC ΔCH1 mutant was fully capable of rescuing the YidC depletion strain grown in glucose, it was slightly impaired in its ability to promote membrane insertion of Pf3-23Lep and PC-Lep.

The helical hairpin structure appeared less important for the insertion of Pf3-23Lep and PC-Lep. The Gram-positive S. mutans YidC2 function, the precise sequence is not.

Strictly Conserved Positive Charge among YidC Homologs

Positively Charged Residue at Position 73 in the S. mutans YidC2 Is Critical for Its Function—We analyzed YidC2 from S. mutans to investigate whether other bacterial YidC homologs require the conserved positively charged residue for YidC function. The Gram-positive S. mutans has two YidC paralogs (YidC1 and YidC2) that play a role in membrane protein insertion (27). Previously, we showed that S. mutans 247YidC2 can complement the E. coli YidC depletion strain and function to insert proteins into the inner membrane of E. coli (7). 247YidC2 consists of residues 1–247 of the E. coli YidC fused to residues 25–310 of S. mutans YidC2 containing the conserved arginine at position 73 (Fig. 4D).

To test the importance of the conserved arginine for the S. mutans YidC2 function, we made a number of mutations within 247YidC2 and examined whether the YidC homolog can rescue the growth of the E. coli YidC depletion strain JS7131. The cultures expressing the 247YidC2 arginine mutants were spotted on LB agar plates with or without arabinose (Fig. 4A). The results show that YidC with Arg substituted with Ala, Leu, Glu, Gln, Asp, Met, Ile, Cys, or Ser could not complement the JS7131 strain when the chromosome-encoded YidC was depleted (Fig. 4A, Glc). Only the positively charged Lys residue substituted at position 73 complemented the YidC depletion strain (plates grown with glucose), indicating the positively charged residue is critical for the function of the S. mutans 247YidC2. To test whether the various mutants were impaired in membrane insertion, we examined the insertion of the YidC substrates Pf3-23Lep and PC-Lep (Fig. 4B). As can be seen, the 247YidC2 mutants R73Q and R73E were defective for the insertion of Pf3-23Lep into the membrane. We confirmed that the inability of the mutants to complement the YidC depletion was not due to lack of expression.
All the *S. mutans* 247YidC2 mutants were stably expressed (Fig. 4C). Remarkably, 247YidC2 R73Q with a neutral residue substituted for the arginine still mediated the insertion of PC-Lep insertion, although R73E with a negatively charged residue was slightly defective in mediating insertion of PC-Lep even though they did not complement growth.

**Positively Charged Residue at Position 152 in the A. thaliana Alb3 Is Not Critical for Its Function**—Because the functional importance of the conserved positively charged residue differed for the tested Gram-negative and Gram-positive homologs, we wanted to extend our analysis to an organellar YidC homolog. Therefore, we investigated the functional importance of the positively charged residue at position 152 of the chloroplast *A. thaliana* Alb3. We used the H1Alb3 construct because it could complement the *E. coli* YidC depletion strain JS7131 (7). H1Alb3 contains the first 57 amino acids of *E. coli* YidC fused to amino acids 59–462 of Alb3 via a valine linker (Fig. 5D). The *A. thaliana* Alb3 homolog differs from the Gram-negative and Gram-positive homologs.

**FIGURE 3.** C-terminal region of the helix hairpin region is important for the function of the *E. coli* YidC, but its precise sequence is not. **A**, complementation assay to examine the role of the helical hairpin structure for YidC function. The YidC depletion strain JS7131 was transformed with pACYC184-encoding YidC Ala-1, Ala-2, Ala-3, Ala-4, Δ371–416 (ΔC1), Δ389–398 (ΔCH1), and Δ399–415 (ΔCH2) mutants, and a spot test was used to examine complementation, as described in Fig. 2A, B. Ala-1, Ala-2, Ala-3, or Ala-4 was transformed with either pMS119 Pf3-23Lep or PC-Lep. Expression of the YidC substrate, labeling, and membrane insertion was performed as described in Fig. 2B. **Left panel** shows protease mapping of Pf3-23Lep. **Right panel** shows signal peptide processing of PC-Lep. **B**, YidC hairpin mutants were tested for their ability to insert Pf3-23Lep and PC-Lep. % Trans shows the percent translocation of Pf3-23Lep and PC-Lep. **C**, YidC mutants with deletions in the C1 loop were tested for their ability to insert preCyoA-N-P2, as described in 2B. **D**, Western blotting to detect expression of the *E. coli* YidC mutants. The percent translocation of Pf3-23Lep, PC-Lep, and PreCyoA-N-P2 was quantified as described under “Experimental Procedures.” P.K., proteinase K.
log contains a lysine instead of the conserved arginine residue in TM1.

The critical nature of Lys-152 of the A. thaliana Alb3 was examined by testing various substitution mutants of this residue in H1Alb3 and assaying the ability of these mutants to complement the YidC depletion strain (Fig. 5A). The results show that H1Alb3 with Lys-152 substituted with a glutamine or an arginine residue could rescue the growth defect of YidC depletion, as indicated by the growth under glucose conditions (Fig. 5A, Glc). In addition, membrane insertion of Pf3-23Lep and Procoat-Lep was efficient when the H1Alb3 K152Q was expressed under YidC depletion conditions (Fig. 5B). This shows that a positively charged residue is not strictly required for the H1Alb3. When a negatively charged residue was substituted for the positively charged residue in the hydrophilic groove, membrane insertion of Pf3-23Lep was affected, and the insertion of Procoat-Lep was inhibited, as was observed with E. coli YidC and S. mutans 247YidC. The results with the Alb3 homolog again show there are differing roles for the strictly conserved positively charged residues among the YidC homologs, as well as the importance of this positive charge for YidC family proteins to promote membrane insertion of different substrates.

Mechanistic Studies to Uncover the Role of the Positive Charge in the S. mutans 247YidC2 and E. coli YidC Protein—The results we obtained with S. mutans 247YidC2 and the substrate protein Pf3-23Lep are consistent with the proposal of Kumazaki et al. (21) that the positively charged residue in the hydrophilic groove plays an important role for the insertion of the N-tail of the substrate by attracting the negatively charged residues of the substrate into the groove. If this is generally the case, the removal of the two negatively charged residues from the N-tail should inhibit insertion. We tested this proposal by mutating the Pf3-23Lep tail region by changing either the aspartic acid residue at position 7 or the aspartic acid residue at 18 or both acidic residues to asparagines. Fig. 6A (left panel) shows that the aspartic acid at position 18 is important for S. mutans YidC2-mediated membrane insertion as Pf3-23Lep18N and Pf3-23Lep7N18N were hindered in membrane insertion. Surprisingly, the negatively charged residue at position 7 was not important for membrane insertion. Nevertheless,
less, the results confirm that a negatively charged residue in the substrate N-tail contributes to the insertion process.

In contrast, the *E. coli* YidC was able to promote membrane insertion of Pf3-23Lep even without any negatively charged residues present in the N-tail, consistent with *E. coli* YidC not needing the positive charge in hydrophilic groove of YidC. Translocation of the N-tail was efficient when the negatively charged residue was substituted with an asparaginyl residue at either position 7 or 18 or at both positions (Fig. 6A, right panel). As a control, we confirmed that 7N18N Pf3-23Lep with a neutral N-tail requires YidC for insertion. Membrane translocation of the N-tail of 7N18N Pf3-23Lep was blocked when YidC was depleted (Fig. 6B). The results demonstrate that insertion of the N-tail does not depend on the attraction of the positively charged arginine 366 in the hydrophilic cavity of the *E. coli* YidC.

For all tested YidC homologs, changing the positively charged residue in the hydrophilic groove to a negatively charged residue significantly impacted the YidC function. The negatively charged mutants did not complement the growth defect of the YidC depletion strain nor did R366E facilitate the membrane insertion of Pf3-23Lep. One explanation for these results is that there is an electrostatic repulsion between the negatively charged residue in the N-tail and the negatively charged residue in the hydrophilic cavity of the YidC mutant. If this explanation is correct, the Pf3-23Lep lacking a negatively charged residue in the N-tail should be inserted efficiently by the negatively charged homologs. We tested this possibility with the *E. coli* YidC. Fig. 6C shows that YidC R366E did not facilitate the insertion of the uncharged Pf3-23Lep7N18N tail substrate. This result is not entirely consistent with the electrostatic repulsion model and suggests that a negative charged residue at 366 is inhibiting the function of YidC by some other mechanism.

**DISCUSSION**

In this report, we show that the conserved positively charged residue within the hydrophilic groove is critical for the Gram-positive *S. mutans* YidC2 to function in *E. coli* similar to the result recently reported for *B. subtilis* YidC1 (21). Conversely, we show that the positively charged residue is not essential for the function of the *E. coli* YidC and is not required for the chloroplast Alb3 to function in complementing the growth of the
Strikingly, we found the importance of the conserved positively charged residue depends on the properties of the YidC substrate. Although the positive charge in the \textit{S. mutans} YidC2 is essential for translocation of the N-tail of the single-span Pf3–23Lep, it is not critical for insertion of the M13 PC-Lep. PC-Lep was still inserted efficiently by the \textit{S. mutans} YidC possessing a neutral residue substituted for the conserved arginine, demonstrating that a positive charge in the groove is important for the \textit{S. mutans} YidC to insert only specific membrane proteins.

One possible explanation why the positively charged residue may be less important for the M13 PC-Lep is that it inserts into the membrane with two hydrophobic domains simultaneously, driving membrane insertion as a helical hairpin (29). The hydrophobic interactions between PC-Lep and YidC, as well as the driving force of the membrane electrochemical potential, may be sufficient for translocation of the periplasmic loop of PC-Lep. In contrast, the hydrophobic driving force for Pf3–23Lep is insufficient and therefore needs the help of the positive charge in the \textit{S. mutans} YidC to attract the negative charges of the substrate peptide chain into the hydrophilic cavity for membrane translocation.

Remarkably, we found that a negatively charged residue substituted for the strictly conserved and positively charged residue inactivated YidC for all of the tested YidC homologs. When the negative charge was substituted for the positive charge in the hydrophilic groove, the \textit{E. coli} YidC, \textit{S. mutans} 247YidC2, and \textit{A. thaliana} H1Alb3 failed to rescue the cell growth and to promote the insertion of the single-span Pf3–23Lep protein under YidC depletion conditions. One possibility for the lack of insertion of the Pf3–23Lep substrate was due to the electrostatic
repulsion between the negative charge in the groove and the negative charges that are commonly found in the translocated regions of many YidC substrates. However, we found that the N-tail of the Pf3-23Lep substrate was still not membrane-inserted by YidC R366E when the tail lacked negative charges (Fig. 6C). Therefore, the introduction of the negatively charged residues into the hydrophilic cavity causes some other perturbation that inactivates the YidC mutant. It should be noted that it is unlikely that the mutation causes a big structural change as the negative charge in the groove did not affect the stability of the YidC homologs (Figs. 2C, 4C, and 5C). Also, the negatively charged mutants of 247YidC2 and H1Alb3 could insert PC-Lep, albeit less efficiently than the wild-type version of these proteins (Figs. 4B, and 5B).

Mechanistically, the positively charged residue in the S. mutans 247YidC2 protein could play a role in facilitating the translocation of the N-tail of Pf3-23Lep most likely by attracting the negatively charged residue. We found that the negatively charged residue at position 18 in the N-tail of Pf3-23Lep is important for translocation (Fig. 6A, left panel), consistent with the electrostatic interaction model put forward by Kumazaki and co-workers (21). However, the position of the negative charge within the N-tail of the substrate is important as substitution of the aspartic acid at position 7 to a neutral residue does not perturb translocation at all.

For the E. coli YidC, we found that the positive charge in the groove can be substituted with a neutral residue without an effect on its ability to promote membrane insertion, and we found that the negatively charged residues in the N-tail are not important for membrane insertion. This shows that the electrostatic attraction model does not apply for the E. coli YidC at least not for the Pf3-23Lep substrate. In the case of the E. coli YidC protein, the hydrophilic N-tail can be incorporated into the hydrophilic groove even without the assistance of the strictly conserved positive charge in the groove. It seems reasonable that the hydrophilic cavity would still provide a favorable environment for moving the N-tail lacking the negative charges into the membrane. A similar mechanism most likely is employed with the A. thaliana H1Alb3 to facilitate membrane insertion.

We also explored the functional importance of the cytoplasmic helical hairpin structure located at the entrance of the hydrophilic groove of YidC. Although removal of the N-terminal half of this hairpin segment did not affect YidC’s ability to complement the YidC depletion strain, it did have a small effect on membrane insertion of Pf3-23Lep and Procoat-Lep. Deletion of the C-terminal region of the hairpin or deletion of the entire hairpin structure completely eliminated YidC’s ability to complement the YidC depletion strain. Similarly, these deletions strongly impacted membrane insertion of Pf3-23Lep and PC-Lep and to a lesser extent insertion of preCyOA-N-P2. These studies demonstrate the dynamic helical hairpin region (21, 30) is important for YidC activity. However, its precise sequence is not critical as shown by the results of the alanine scanning mutagenesis.

In conclusion, our results show that the functional importance of the strictly conserved positively charged residue in YidC depends on the YidC homolog being examined. In general, the Gram-positive bacterial homologs require the positively charged residue, and the Gram-negative bacterial and chloroplasts Alb3 homologs do not. However, further analysis with more proteins needs to be performed because very few YidC proteins have been studied so far. Additionally, the C-terminal half of the helical hairpin of the E. coli YidC is important for YidC activity. Future studies will need to elucidate the precise function of this hairpin region in the membrane insertion pathway of YidC-dependent proteins.

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