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Identification of YidC Residues That Define Interactions with the Sec Apparatus

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In bacteria, a subset of membrane proteins insert into the membrane via the Sec apparatus with the assistance of the widely conserved essential membrane protein insertase YidC. After threading into the SecYEG translocon, transmembrane segments of nascent proteins are thought to exit the translocon via a lateral gate in SecY, where YidC facilitates their transfer into the lipid bilayer. Interactions between YidC and components of the Sec apparatus are critical to its function. The first periplasmic loop of YidC interacts directly with SecF. We sought to identify the regions or residues of YidC that interact with SecY or with additional components of the Sec apparatus other than SecDF. Using a synthetic lethal screen, we identified residues of YidC that, when mutated, led to dependence on SecDF for viability. Each residue identified is highly conserved among YidC homologs; most lie within transmembrane domains. Overexpression of SecY in the presence of two YidC mutants partially rescued viability in the absence of SecDF, suggesting that the corresponding wild-type YidC residues (G355 and M471) participate in interactions, direct or indirect, with SecY. *Staphylococcus aureus* YidC complemented depletion of YidC, but not of SecDF, in *Escherichia coli*. G355 of *E. coli* YidC is invariant in *S. aureus* YidC, suggesting that this highly conserved glycine serves a conserved function in interactions with SecY. This study demonstrates that transmembrane residues are critical in YidC interactions with the Sec apparatus and provides guidance on YidC residues of interest for future structure-function analyses.

In all organisms, membrane proteins participate in critical fundamental processes, including signal transduction, transcription regulation, the transport of ions and macromolecules, proteolysis, motility, metabolism, energy generation, and energy transduction. Specialized cellular machineries enable the proper insertion and folding of membrane proteins in the lipid bilayer. In bacteria, a subset of membrane proteins insert into the membrane via the Sec apparatus. The Sec apparatus is a multiprotein complex that facilitates the insertion of certain membrane proteins, and it is the means by which many extracytoplasmic proteins are translocated across the cytoplasmic membrane.

The core of the Sec apparatus in bacteria consists of a heterotrimer of the multipass membrane protein SecY and the single-pass transmembrane proteins SecE and SecG (1–3). The central hourglass-shaped hydrophilic channel is formed by SecY and is flanked by SecE and SecG. SecE, which is essential, is wrapped around the central channel, forming extensive contacts with SecY (1). Loss of SecE leads to instability of SecY (4, 5). SecG is nonessential for viability, and although not required for protein translocation, it makes the process more efficient (6–8).

SecD and SecF form a heterodimeric complex that functions in an accessory manner to the central translocation apparatus. The SecDF complex has two large loops on the periplasmic side of the membrane that engage the preprotein as it emerges from the translocon (9, 10). It has been postulated that cyclical conformational changes in these periplasmic loops leads to the stepwise translocation that is observed for nascent proteins (11–13).

For certain membrane proteins, proper biogenesis depends on YidC, which functions as a chaperone for these proteins, facilitating their folding, insertion into the bilayer, and proper topology (14–16). YidC is widely conserved, with homologs in other bacteria, archaea, mitochondria, and chloroplasts (17, 18). YidC is an integral membrane protein that is present in significant stoichiometric excess to SecYEG (19), suggesting that one pool of the protein is associated with the Sec apparatus and another pool is not. This is consistent with the observation that some YidC substrates are inserted via the Sec translocon, whereas others are inserted via a Sec-independent pathway (18, 20–22).

Genome-wide studies indicate that of the approximately 900 cytoplasmic membrane proteins in *Escherichia coli*, 17 to 32% are dependent on YidC for biogenesis (23–26). For Sec-dependent YidC substrates, YidC is thought to facilitate the partitioning of nascent transmembrane segments from the SecY channel into the lipid bilayer as well as the proper bundling of transmembrane segments (27, 28). Monomeric YidC associated with the Sec translocon is positioned at the lateral gate of SecY (29), which has been proposed to serve as the exit site of transmembrane segments from the translocation channel (1, 30).

The critical role of YidC in the biogenesis of these Sec-dependent membrane proteins suggests that interactions between YidC and components of the Sec apparatus are critical to its function. Direct interactions between YidC and the Sec translocon have been documented for two translocon components, the accessory protein SecF and the channel protein SecY (29, 31). YidC binds SecF via its large periplasmic loop; residues 215 to 265 of this loop are required and sufficient for the interaction with SecF (31). The regions or residues of YidC that interact with SecY have been unknown. In addition, it is unknown whether YidC interacts with additional components of the Sec apparatus. In this study, we

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Source or reference
E. coli strains		
MC4100	Δ(*fruK-yeiR*)*725* (*fruA25*) *relA1 rpsL150 rbsR22* Δ(*fimB fimE*) 632(::IS1) *deoC1*	54
YidC depletion (AG223)	MC4100 *attλ*::(*araC* P_{BAD}-*yidC aadA*) Δ*yidC*::FRT	25
SecDF depletion (JP352)	MC4100 Δ*ara714 tgt*::*kan* P_{BAD}-*secDF*	Gift of J. Beckwith
YidC SecDF depletion	AG223 Δ*ara714 tgt*::*kan* P_{BAD}-*secDF*	This study
DH5α	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169*, *hsd*R17(r_K^- m_K^+) λ^-	Laboratory stock
DH10B	F^- *endA1 recA1 galE15 galK16 nupG rpsL* Δ*lacX74* Φ80*lacZ*ΔM15 *araD139* Δ(*ara*, *leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ^-	Laboratory stock
Plasmids		
pRC7	Unstable miniF plasmid, Amp^r	Gift of T. Bernhardt
pRC7-*yajCsecDF*	pRC7-*yajCsecDF*, Amp^r	This study
pACYC184	Cloning vector, Cm^r Tet^r	Laboratory stock
p-*yidDC*	pACYC184-*yidDC*, Cm^r	This study
p-*yidD*	pACYC184-*yidD*, Cm^r	This study
p-*yidC*	pACYC184-*yidC*, Cm^r	This study
pACYC177	Cloning vector, Amp^r Kan^r	Laboratory stock
pACYC177-$yidC_{Sa}$	Cloning vector carrying *S. aureus yidC*, Kan^r	This study
p-$yidC_{Sa}$	pACYC184-$yidC_{Sa}$, *S. aureus yidC*, Cm^r	This study
p-GFP	pDSW207, cloning vector carrying *gfp*, Amp^r	Gift of J. Beckwith
p-SecY-GFP	pDSW208-*secY*, cloning vector carrying *secY*::*gfp*, Amp^r	Gift of J. Beckwith

performed a screen designed to identify residues of YidC that interact with the Sec apparatus independent of the YajC-SecDF complex. We identified mutations in YidC that lead to dependence on SecDF for viability and provide evidence that the highly conserved residue G355 serves a conserved function in interactions, either direct or indirect, with SecY.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. All experimental *E. coli* strains are derivatives of MC4100. Strains were maintained in Luria broth (LB) at 37°C unless otherwise noted. Strains that carried *yidC* under the control of an arabinose promoter were maintained in LB medium supplemented with 1% arabinose for overnight cultures and 0.2 or 1% arabinose for exponential-phase growth. The SecDF single depletion strain was maintained in LB medium supplemented with 0.2% arabinose. Where appropriate, glucose was added to a final concentration of 0.2%, and antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1} (for the miniF plasmid and its derivatives, 15 μg ml^{-1}); chloramphenicol, 25 μg ml^{-1}; spectinomycin, 25 μg ml^{-1}; and kanamycin, 50 μg ml^{-1}.

Construction of strains and plasmids. The *tgt*::*kan* P_{BAD}-*secDF* allele from JP352 was introduced by standard P1 transduction into strain MC4100 Δara714 and the YidC depletion strain AG223 to make the MC4100 SecDF depletion strain and the YidC SecDF double depletion strain, respectively. *tgt* is immediately upstream of the *yajC-secDF* operon; therefore, it is tightly linked. The dependence of each of these strains on arabinose for viability was verified.

For construction of plasmids pACYC184-*yidD*, pACYC184-*yidC*, and pACYC184-*yidDC*, the target gene or genes were amplified as SphI-HindIII fragments by PCR with Pfx polymerase (Invitrogen) and using *E. coli* MC4100 as a template. The amplified fragments were ligated into the SphI and HindIII sites of pACYC184. The expression of *yidD* or *yidDC* genes from the pACYC184-derivative plasmids should be driven from the native promoter of the *yidDC* operon. No known promoter has been reported for *yidC* in the fragment that was cloned, but our results indicate that there is a promoter immediately upstream of the open reading frame of the *yidC* gene in the cloned fragment. To generate the unstable miniF plasmid pRC7-*yajCsecDF*, the entire *yajC secDF* operon, including its native promoter, was amplified as a SalI-HindIII fragment and ligated into the SalI and HindIII sites in pRC7. To generate pACYC184 carrying *Staphylococcus aureus yidC*, the coding sequence for *yidC*, as well as 57 nucleotides upstream of the start codon, was amplified as an ScaI-PstI fragment by PCR, using *S. aureus* strain SH1000 as the template, and cloned into the ScaI and PstI sites of pACYC177. The same DNA fragment was subsequently subcloned as a fragment with BamHI and blunted PstI ends into the BamHI and EcoRV sites of pACYC184. The mutations in the plasmid pACYC184-*yidC* (D121N/Y153H/G355C) were subcloned by replacing the HindIII-PstI, HindIII-KpnI, or PstI-KpnI fragment of the mutant plasmid with the analogous fragment of the pACYC184-*yidC* (wild-type) plasmid; this approach was used to generate plasmids containing all possible combinations of single and double mutations. Sequence analysis was performed to verify that each construct was correct. The sequences of the primers used in this study are available from the authors upon request.

Construction of YidC mutant libraries and screening for mutants that lead to dependence on SecDF for viability. Two YidC mutant libraries were generated using a GeneMorph II EZClone domain mutagenesis kit according to the manufacturer's instructions. For the first library, in which mutations were generated within the entire *yidC* open reading frame (encoding residues 1 to 548), the mutant megaprimers were generated by amplifying the entire *yidC* coding sequence, using pACYC184-*yidC* as the template. The plasmid (2.4 μg) was used as the template, so that the initial target amount (the amplified DNA) was approximately 750 ng, which was designed to lead to a low frequency of mutations. For the second library, in which mutations were restricted to the coding sequence for residues 265 to 548, the mutant megaprimers were generated by amplifying the coding sequence for *yidC* residues 265 to 548, using pACYC184-*yidC* as the template. A 4.68-μg sample of the plasmid was used as the template, so that the initial target amount was approximately 750 ng, again designed to lead to a low frequency of mutations. DpnI-treated DNA from these EZClone reactions was transformed into highly competent DH10B cells (Invitrogen) by electroporation instead of the XL-10 gold competent cells provided by the kit, because pACYC184-*yidC* carries chloramphenicol resistance, as does XL-10 gold. The transformation reactions were plated on LB plates containing chloramphenicol, and after overnight incubation at 37°C, colonies were pooled directly from the transformation plates. The library of plasmids was isolated from the pooled colonies. For the full-length YidC library, 11,302 colonies were

recovered and pooled, and for the library of YidC residues 265 to 548, 6,498 colonies were recovered and pooled; in each case, this represents greater than 2-fold coverage of the target sequence. To determine the randomness of the mutations, plasmid DNA was prepared and sequenced for about 20 colonies from each library, randomly selected from the primary transformation plates.

The reporter strain *E. coli* AG223 *tgt*::*kan* P_{ara}-*yajCsecDF* pRC7-*yajCsecDF* was made ultracompetent using the method of Inoue et al. (32). An amount of each YidC mutant library that would allow isolation of independent colonies was transformed multiple times into the competent cells and plated onto LB media containing chloramphenicol, glucose, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Blue colonies were reisolated on LB media containing chloramphenicol, ampicillin (15 μg ml^{-1}), and 1% arabinose. The mutant plasmids were isolated and retransformed into the reporter strain to confirm the observed phenotype and were sequenced to identify any mutations in the *yidC* gene. For each library, screening was carried out to near saturation.

Analysis of strain viability. To test whether a plasmid was able to complement the depletion of SecDF, YidC, or SecDF and YidC, the plasmid was introduced into the target depletion strain by transformation in the presence of arabinose, and the transformed strain was maintained in the presence of arabinose. Overnight cultures grown in the presence of arabinose were diluted to an optical density at 600 nm (OD_{600}) of 4.0. Five or 10 μl of 10-fold dilutions from 10^{-2} to 10^{-7} was spotted onto media containing or lacking 1% arabinose and incubated overnight at 37°C. The volume of dilution spotted was consistent through any individual experiment. Where indicated, glucose was added to a final concentration of 0.2% to the media that lacked arabinose.

Complementation of YidC mutants by SecY overexpression. AG223 *tgt*::*kan* P_{ara}-*yajCsecDF* strains carrying pACYC184-*yidC* mutant plasmids and pDSW207 or pDSW208-*secY*, which encode green fluorescent protein (GFP) and SecY-GFP, respectively, were generated and maintained in the presence of 1% arabinose. Overnight cultures, grown in the presence of 1% arabinose, were diluted and spotted as described above on plates containing only antibiotics, antibiotics plus isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 5 μM, or antibiotics plus 1% arabinose. To prepare whole-cell proteins of these strains, overnight cultures grown in the presence of 1% arabinose were back diluted into media without arabinose and grown to mid-exponential phase; proteins were then prepared using standard procedures.

Western blot analysis. Overnight cultures from single colony isolates were back diluted and grown to mid-exponential phase. Whole-cell protein preparations were prepared and transferred to nitrocellulose using a semidry transfer apparatus, and Western blot analysis was performed using standard procedures. Antiserum to YidC (gift of M. Mueller) was used at a dilution of 1:5,000. Peroxidase-conjugated goat anti-rabbit secondary antibody was used at a dilution of 1:5,000. Chemiluminescent signals were produced using ECL substrate (Fisher) and were captured on radiographic film.

Sequence analysis. After selecting representative sequences from each class of *Proteobacteria*, using the Concise protein database (http://www.ncbi.nlm.nih.gov/genomes/prokhits.cgi), the alignment of YidC homologs in Gram-negative bacteria was performed using ClustalX (http://www.clustal.org/clustal2/). The alignments were visualized using Ugene (http://ugene.unipro.ru/documentation/manual/sequence_view/view_components.html). Topology predictions for *S. aureus* YidC were obtained for gi 150375006 (GenBank accession number BAF68266.1) or YIDC_STAAE using the Uniprot annotation (http://www.uniprot.org/uniprot/A6QIT4) and the algorithms LipoP (http://www.cbs.dtu.dk/services/LipoP/), Phobius (http://phobius.sbc.su.se/), Philius (http://www.yeastrc.org/philius/pages/philius/runPhilius.jsp), Topcons (http://topcons.cbr.su.se/), Memsat3, and MemsatSVM (http://bioinf.cs.ucl.ac.uk/psipred/). The alignment of *E. coli* YidC and *S. aureus* YidC was obtained by protein BLAST.

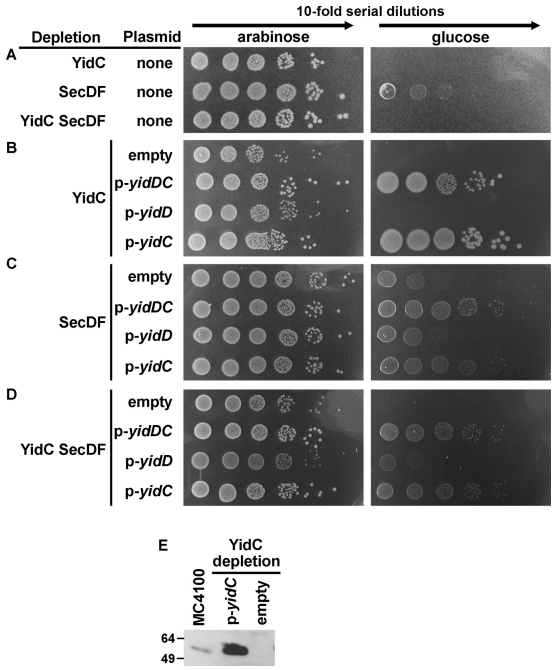
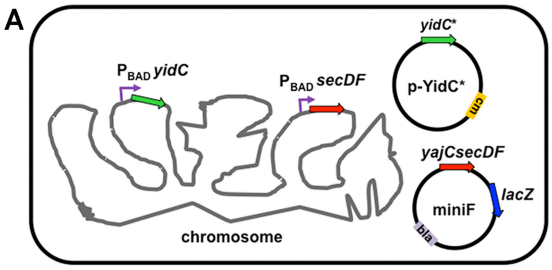


FIG 1 Bacterial viability upon YidC overexpression in YidC, SecDF, and YidC SecDF depletion strains. (A) Depletion of YidC, SecDF, or both leads to loss of viability. (B to D) *yidC* and *yidDC*, but not *yidD*, complement growth of a YidC depletion strain (B), SecDF depletion strain (C), and YidC SecDF double depletion strain (D). Serial 10-fold dilutions of strains carrying pACYC184, pACYC184-*yidDC*, and pACYC184-*yidC*, but not pACYC184-*yidD* or pACYC184, were grown on media containing (left) or lacking (right) arabinose. (E) Level of YidC produced from multicopy YidC (pACYC184-*yidC*) used in this study. Proteins in lanes 2 and 3 were prepared from YidC depletion strains grown under depletion conditions (0.2% glucose).

RESULTS

Generation and characterization of *yidC secDF* double depletion strain. We generated a strain of *E. coli* in which both YidC and SecF can be depleted by placing chromosomal *yidC* and chromosomal *secDF* under the control of arabinose promoters. Immediately upstream of and cotranscribed with *yidC* is the small nonessential open reading frame *yidD*, which encodes a factor that participates in membrane protein insertion (33). Immediately upstream of and cotranscribed with *secF* are *yajC* and *secD*, which encode accessory proteins to the Sec translocon. *secDF* is essential, but viability in strains in which SecDF is depleted can be rescued by overexpression of *yidC* (34). SecDF depletion does not lead to a reduction in the level of YidC (29). In our double depletion strain, *yidD* and *yajC* are transcribed from the native promoter at the native chromosomal loci, whereas *yidC* and *secDF* are under the control of the arabinose promoter and integrated into the chromosome.

As expected, depletion of YidC and SecDF, which occurs upon growth in the absence of arabinose, leads to loss of viability (Fig. 1A). Of note, the decrease in viability is less severe for the SecDF depletion strain than for either the YidC depletion strain or the YidC SecDF double depletion strain, indicating that under the growth conditions used, the requirement for YidC is more pronounced than the requirement for SecDF. Growth of each depletion strain was rescued by introduction of either *yidC* or *yidDC* on the multicopy plasmid pACYC184 (Fig. 1B to D). Multicopy ex-



B

Arabinose	Colony color	miniF-*yajCsecDF*	p-*yidC**
+	[a] blue	[a]	N/A
-	white	-	functionally WT
-	blue	+	mutants of interest

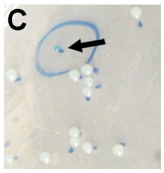


FIG 2 Design of screen to identify residues of YidC required for viability in the absence of SecDF. (A) Experimental set-up for screen. (B) Experimental conditions and potential phenotypes, in the absence of ampicillin, such that miniF plasmid is unstable. Footnote a indicates that, when grown in the presence of arabinose, ampicillin is required to maintain the miniF-*yajCsecDF* plasmid. Colony color is determined on media containing X-Gal. WT, wild type. Functionally WT refers to the ability of multicopy YidC to interact sufficiently with the Sec apparatus to maintain viability in the absence of SecDF. (C) Photograph of a blue colony (arrow) on a plate with white colonies.

pression of *yidD* provided a slight growth advantage above vector alone to the YidC SecDF double depletion strain but did not rescue the viability of the other depletion strains (Fig. 1B to D). The ability of multicopy *yidC* and *yidDC* to rescue viability of strains lacking SecDF is consistent with the previous observation that overexpression of *yidC* rescues growth of SecDF depletion (34). Multicopy *yidC*, which was used for subsequent experiments reported here, produced severalfold more YidC than the parent wild-type *E. coli* strain (Fig. 1E).

Screen to identify residues of YidC required for viability in the absence of SecDF. Based on the findings described above, we reasoned that in the absence of SecDF, the interaction of YidC with the Sec apparatus is weakened; nonetheless, some sites of interaction remain, such that overexpression of YidC results in suppression of functional defects due to the absence of SecDF. These non-SecDF sites of interaction likely include interactions with SecY. Thus, we predicted that mutations in YidC that result in a defective interaction with SecY would be synthetic lethal with depletion of SecDF.

Therefore, to identify YidC residues that interact with SecY, we looked for mutations in YidC that prevented loss of SecDF from cells (Fig. 2). We constructed two plasmid libraries of *yidC* mutants. For the first library, mutations were generated within the entire *yidC* open reading frame (encoding residues 1 to 548). Since YidC residues 215 to 265 are sufficient for interaction with SecF (31), a second YidC library was created in which mutations were restricted to the coding sequence for residues 265 to 548. Sequencing of 20 blindly selected clones of each library demonstrated a random distribution of mutations which, in the first library, were present in greater than 70% of library clones and at a frequency of slightly more than one mutation per library clone, but in the second library they were present at a lower frequency.

We generated a derivative of the YidC SecDF depletion strain that carried *secDF* and *lacZ* on a miniF plasmid that is unstable in the absence of antibiotic selection (ampicillin). Upon introducing the plasmid libraries of *yidC* mutants into this strain, we screened for maintenance of the unstable miniF-*yajCsecDF lacZ* plasmid under growth conditions that result in depletion of chromosomal YidC and SecDF (no arabinose). Multiple rounds of screening were performed with each of the two YidC mutant plasmid libraries. For the first YidC mutant plasmid library, 10,000 plasmids were screened, and for the second, more than 5,000 plasmids were screened, constituting near saturation of screening for each library. Colonies that maintained the miniF-*yajCsecDF lacZ* plasmid represented about 1% of all colonies and were identified by blue colony color on plates that contained X-Gal and lacked ampicillin (Fig. 2B and C). In general, the mutant YidC proteins were associated with slower growth of the isolates, since most blue colony isolates were smaller than the white colonies on the same plate (Fig. 2C).

Identification of mutations in multicopy YidC that lead to dependence on SecDF for viability. Seven clearly distinct YidC mutants were isolated from the blue colonies; all but one of these was isolated from multiple independent transformations of the libraries (Table 2). Four mutants contained nucleotide mutations that lead to a single amino acid change, whereas three contained nucleotide mutations that lead to two or more amino acid changes. As expected, all of the YidC mutants that were isolated from blue colonies were functional with respect to enabling cell

TABLE 2 YidC mutants that grew as blue colonies in SecDF reporter strain

Mutation(s)		No. of independent isolations	Rescue of viability of depletion strains			Domain of mutation(s)[a]
Amino acid change(s)	Nucleotide substitution(s)		YidC	SecDF	YidC SecDF	
D121N, Y153H, G355C	G361A, T457C, G1063T	8	Yes	No	No	PL1 (D121, Y153), TM2 (G355)
V328D	T983A	2	Yes	No	No	PL1
P431S	C1291T, A1419G[b]	3	Yes	No	No	TM3
L420M, M498T, Y517C	C1258A, T1493C, A1550G, C1564T[b]	9	Yes	No	No	TM3 (L420), TM5 (M498), TM6 (Y517)
V295I, F363S	G883A, T1088C	2	Yes	Yes	Yes	PL1 (V295), TM2 (F363)
R366H	G1097A, G1206A[b]	1	Yes	Yes	Yes	TM2
M471I	G1413A	4	Yes	Yes	No	TM4

[a] TM, transmembrane segment; PL, periplasmic loop.
[b] Synonymous mutation.

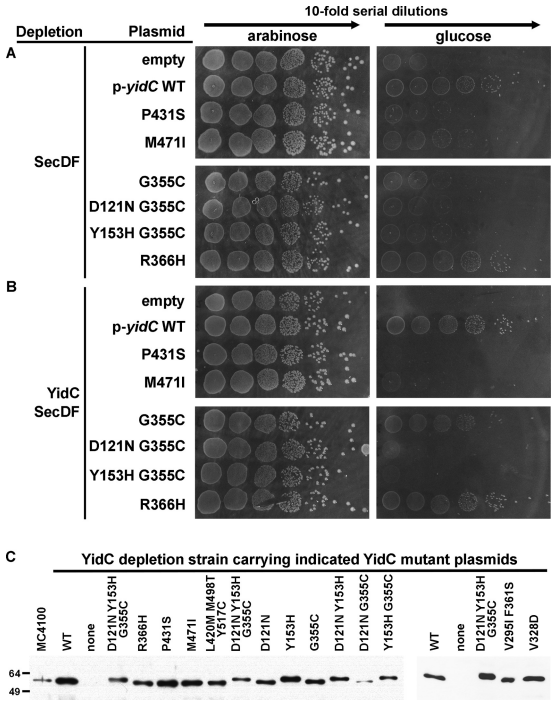


FIG 3 Characterization and mapping of selected mutations identified in screen. (A) Rescue of YidC, SecDF, or YidC SecDF depletion. Viability was determined by spotting serial 10-fold dilutions on media containing (left) or lacking (right) arabinose. (B) Western blot analysis of levels of YidC mutant protein synthesis. All strains were grown in the absence of arabinose.

viability, in that they rescued growth of the YidC depletion strain when streaked onto depletion media (media lacking arabinose and containing 0.2% glucose) (Table 2).

We predicted that the maintenance of the miniF-*yajCsecDF lacZ* plasmid that we observed for the seven YidC mutants would correlate with an inability of the YidC mutant plasmid to rescue growth of the SecDF depletion strain or the YidC SecDF double depletion strain under depletion conditions. To test this, each YidC mutant plasmid was introduced into each of these depletion strains under nondepleting conditions, and viability under depletion conditions was then examined by restreaking single colonies onto depletion media. Four of the seven mutants (D121N/Y153H/G355C, V328D, P431S, and L420M/M498T/Y517C) gave the expected true-positive phenotype of being unable to rescue growth of either the SecDF depletion strain or the YidC SecDF double depletion strain under depletion conditions (Table 2 and Fig. 3A and data not shown). Two YidC mutants (V295I/F363S and R366H) rescued growth of both of these depletion strains, yet during the initial screen they maintained the miniF plasmid that carries *secDF* and *lacZ* (Table 2 and Fig. 3A and data not shown); maintenance of the miniF plasmid was due to the plasmids encoding the YidC mutants, since the phenotype was reproduced by reintroducing these plasmids into the reporter strain. The explanation for this phenotype remains unclear.

One of the mutants (M471I) rescued growth of the SecDF depletion strain but not that of the double depletion strain (Table 2 and Fig. 3A). Of note, the selection for maintenance of the miniF plasmid that carries *secDF* and *lacZ* was made in the absence of both chromosomally encoded SecDF and chromosomally encoded YidC. Thus, this mutant is able to rescue the growth defect of SecDF depletion only when native YidC is present.

A potential explanation for why mutants of YidC are unable to rescue viability in the absence of SecDF is the decreased stability of the mutant protein relative to the wild-type protein, such that the cells have insufficient YidC for rescue of SecDF depletion. We eliminated this possibility by Western blot analysis of the mutant proteins. YidC mutant D121N/Y153H/G355C displayed lower levels of production of mutant YidC than the plasmid-borne wild-type YidC, albeit higher levels than native YidC in the parent *E. coli* strain MC4100 (Fig. 3C). Since the level of YidC in this mutant was comparable to that of the R366H mutant, which rescued growth of both the SecDF depletion strain and the YidC SecDF double depletion strain (Fig. 1B to D), it seems unlikely that this level of reduction in YidC production is responsible for the phenotype of the D121N/Y153H/G355C mutant.

To define which of the mutations in the YidC mutant D121N/Y153H/G355C was responsible for its dependence on SecDF for viability, we generated derivatives that contained each mutation alone or each possible combination of pairs of the three mutations. Among these derivatives, only those mutants that contained the G355C mutation gave blue colonies when placed in the reporter strain (the strain that was used for the initial screen) (Table 3). In addition, only the G355C-containing mutants were unable to rescue viability of the SecDF and YidC SecDF depletion strains (Table 3 and Fig. 3A and B). Thus, the dependence of the D121N/Y153H/G355C mutant on SecDF for viability is attributable to the G355C mutation. Why the G355C mutation was not isolated on its own in the screen is unclear.

Identified residues are conserved. YidC is positioned at the lateral gate of SecY, which consists of SecY transmembrane segments 2b, 3, 7, and 8 (1, 29). These SecY transmembrane segments are each highly conserved (1), and as determined by cross-linking analysis, each forms contacts with YidC (29). These observations raised the possibility that conserved residues within YidC transmembrane segments participate in YidC-SecY interactions.

The individual residues which, when mutated, conferred dependence on SecDF for viability were distributed throughout most of the length of YidC (Fig. 4A). All but one, V328D, are within transmembrane segments. G355C maps to transmembrane segment 2, and P431S maps to transmembrane segment 3. The mutation that led to dependence on SecDF only in the ab-

TABLE 3 Analysis of mutations present in YidC D121N/Y153H/G355C

Mutation(s)			Rescue of viability of depletion strains		
Amino acid change(s)	Nucleotide substitution(s)	LacZ phenotype[a]	YidC	SecDF	YidC SecDF
D121N, Y153H, G355C	G361A, T457C, G1063T	Blue	Yes	No	No
D121N, Y153H	G361A, T457C	White	Yes	Yes	Yes
Y153H, G355C	T457C, G1063T	White	Yes	Yes	Yes
D121N, G355C	G361A, G1063T	Blue	Yes	No	No
D121N	G361A	White	Yes	Yes	Yes
Y153H	T457C	White	Yes	Yes	Yes
G355C	G1063T	Blue	Yes	No	No

[a] Colony color on plates that contain X-Gal and glucose but lack ampicillin and arabinose.

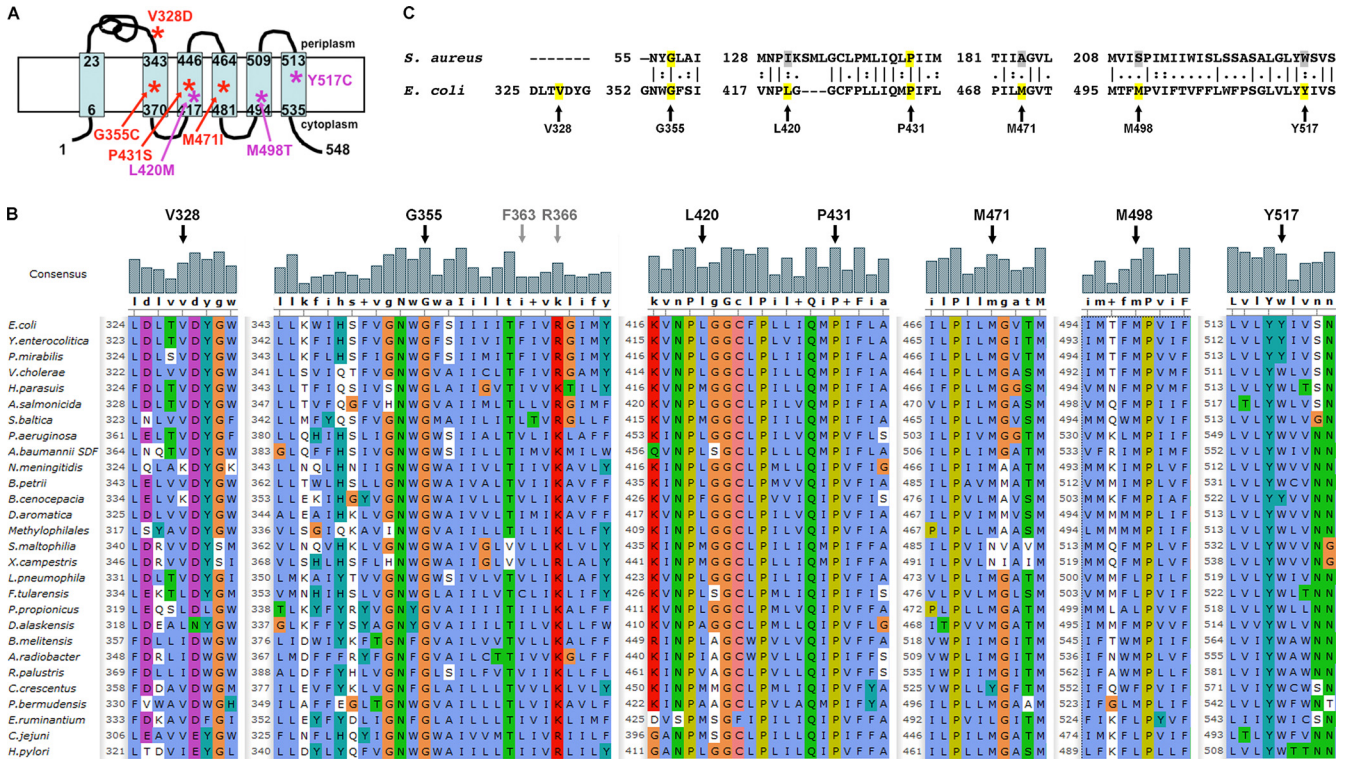


FIG 4 Conservation of residues identified in screen. (A) Diagram of topology of *E. coli* YidC, with mutations mapped. (Adapted from material originally published in the *Journal of Biological Chemistry*. Saaf A, Monne M, de Gier JW, von Heijne G. Membrane topology of the 60-kDa Oxa1p homologue from Escherichia coli. 1998; *Journal of Biological Chemistry* **273:**30415–30418 [53]. Copyrighted by the American Society for Biochemistry and Molecular Biology.) Single mutants are shown in red lettering, and the residues of the triple mutant L420M/M498T/Y517C are shown in magenta lettering. (B) Conservation of those residues identified in the screen that led to dependence on SecDF for viability among YidC homologs of Gram-negative bacteria. Also aligned are F363 and R366 (gray lettering and arrows), which did not clearly lead to dependence on SecDF for viability. Amino acid residues G, P, S, and T are shown in orange; H, K, and R in red; F, W, and Y in blue; and I, L, M, and V in green. (C) Conservation of residues identified in screen between YidC homologs of *E. coli* and *S. aureus*. Highlighted in yellow are the residues that, when mutated in *E. coli*, led to dependence on SecDF for viability, as well as the aligned residues in *S. aureus* which show identity.

sence of chromosomally encoded YidC, M471I, lies in transmembrane segment 4. Although we have not examined the three mutations of the L420M/M498T/Y517C YidC mutant individually in this study, each also lies within a transmembrane segment (transmembrane segments 3, 5, and 6, respectively).

Among YidC homologs in a wide range of Gram-negative bacteria, each of the residues that, when mutated, was synthetic lethal with SecDF depletion in *E. coli* is highly conserved (Fig. 4B). G355, P431, and Y517 are invariant among the sequences we examined. Nearly all of the others are substituted only very rarely, and when they are, the substituted amino acid is a conservative change, with the exception that V328 is occasionally replaced with lysine.

For a subset of the identified YidC mutations, overexpression of SecY rescues growth in the absence of SecDF. Functional defects due to alterations in protein-protein interactions can often be compensated for by overexpression of the interacting partners. We used this principle to genetically test whether any of the mutations in YidC that conferred dependence on SecDF for viability are in residues that normally participate in interactions with SecY. Mild overexpression of *secY* was attained by placing the gene under the control of a leaky P_{Trc} promoter on a multicopy vector and then growing strains carrying this vector in the presence of 5 μM or no IPTG.

The expression of multicopy *secY* led to partial rescue of viability of the YidC SecDF depletion strain under depletion conditions in the presence of YidC M471I and, to a lesser extent, of YidC D121N/Y153H/G355C and YidC G355C (Fig. 5, arrows). The latter result also is consistent with the G355C mutation being the phenotypically important substitution in YidC D121N/Y153H/G355C. Neither of the other two YidC mutants that are dependent on SecDF for viability (P431S and L420M/M498T/Y517C) displayed improved growth with expression of multicopy *secY* (Fig. 5). Moreover, YidC R366H, which is independent of SecDF for viability, showed no change in growth in the presence or absence of multicopy *secY* (Fig. 5). The ability of *secY* overexpression to enhance growth in the presence of YidC M471I or YidC G355C indicates that these mutations alter interactions of YidC with SecY either directly or indirectly.

***Staphylococcus aureus* YidC complements depletion of YidC, but not of SecDF, in *E. coli*.** YidC is widely conserved, with homologs in other bacteria, archaea, mitochondria, and chloroplasts (17, 18). YidC homologs present in Gram-positive bacteria are predicted to have the same general organization as that of YidC in *E. coli* and other Gram-negative bacteria, except that the first large periplasmic loop is foreshortened (35). *In silico* analysis predicts that YidC from the Gram-positive bacterium *S. aureus* is a lipoprotein, as has been previously suggested for the *B. subtilis* homolog SpoIIIJ and the *Streptococcus mutans* homologs YidC1

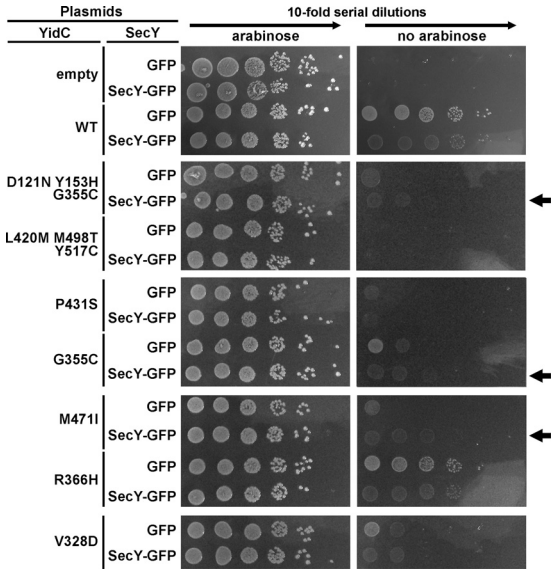


FIG 5 Multicopy expression of SecY rescues growth of a subset of YidC mutants in the absence of SecDF. Viability determined by spotting serial 10-fold dilutions on media containing (left) or lacking (right) arabinose. Arrows indicate three YidC mutants in the presence of which multicopy expression of SecY rescues growth under conditions of SecDF depletion.

and YidC2 (36, 37). Diacylglycerol modification of the thiol group of Cys-20 (Fig. 6A, asterisk) and processing of the modified preprotein between Gly-19 and Cys-20 would leave the modified Cys-20 as the mature amino terminus, with the lipid moiety anchored in the outer leaflet of the cytoplasmic membrane (38). Topology predictions of *S. aureus* YidC using multiple alignment algorithms predict that *S. aureus* YidC has 4 or 5 transmembrane segments, depending on whether a third 2- to 4-residue-long periplasmic loop is recognized at residue 227 to residue 228 or 230 (Fig. 6A). With the exception of MemsatSVM, other prediction algorithms listed in Materials and Methods and the Uniprot annotation agree with the Topcons consensus in this region. Given reasonable alignment of the sequence of YidC from *S. aureus* with that of *E. coli* in this region (Fig. 6B), we predict that *S. aureus* YidC has 5 transmembrane segments after the lipoprotein signal sequence, with a short periplasmic loop between transmembrane segments 4 and 5. Alignment of *S. aureus* YidC with *E. coli* YidC shows regions of substantial identity and similarity, interspersed with regions of divergence (Fig. 6C).

Among the residues of *E. coli* YidC that we found led to dependence on SecDF for growth, G355 and P431 are identical in *S. aureus* YidC, whereas V328 does not align due to substantial divergence in the region of the first periplasmic loops (Fig. 6C). L420 and Y519 show conservative substitutions (in *S. aureus* YidC, isoleucine and tryptophan, respectively), and M498 is weakly similar (in *S. aureus* YidC, a serine) (Fig. 6C).

Since in *E. coli* YidC those residues that interact with SecF lie within the first periplasmic loop (31), which is largely absent from *S. aureus* YidC, it was of interest to examine whether *S. aureus* YidC could complement the *E. coli* YidC depletion strain and whether any observed complementation depended on SecDF. *S. aureus yidC* rescued the viability of the *E. coli* YidC depletion strain under depletion growth conditions (Fig. 7A), indicating functional conservation with respect to those YidC functions that are essential for viability. However, unlike *E. coli yidC*, *S. aureus yidC* carried on a multicopy vector did not enable growth in the absence of SecDF (Fig. 7B). *S. aureus yidC* led to partial rescue of viability of the YidC SecDF double depletion strain under depletion conditions (Fig. 7C). Consistent with incomplete rescue of growth of the YidC SecDF double depletion strain, the double depletion strain carrying YidC of *S. aureus* and *yajC secDF* and *lacZ* on a miniF plasmid grown under depletion conditions produced blue colonies (Fig. 7D). These findings suggest that the absence of YidC and the absence of SecDF lead to loss of viability in ways that do not completely overlap, and that overexpression of *S. aureus* YidC can rescue the former but not the latter, whereas overexpression of *E. coli* YidC can rescue both.

DISCUSSION

Transmembrane segments (TMs) of nascent membrane proteins targeted to the Sec translocon are sequentially inserted into the SecY translocon and then partitioned into the lipid bilayer via a lateral gate in SecY. YidC, docked at the SecY lateral gate (29), interacts with a subset of transmembrane segments as they exit the translocon. YidC is believed to enable folding of the proteins with correct topology into an intrinsically stable conformation (27, 39, 40), as well as stabilization of unbalanced transmembrane segments as they are folded (25). Folded membrane proteins are then released from YidC into the lipid bilayer.

Based on several observations, we hypothesized that in *E. coli* transfer of nascent cytoplasmic membrane proteins from the Sec apparatus into the cytoplasmic membrane depends on at least two sets of functional interactions between YidC and components of the Sec apparatus. The first observation is that YidC, SecY, and SecDF components of the membrane protein insertion machinery are each essential for viability (10, 18, 41, 42). Second, overexpression of YidC rescues the lethality of SecDF depletion (34 and this study). Third, residues 215 to 265 of the large first periplasmic loop of YidC interact with the SecF component of the SecDF complex, but this region of YidC is not essential for its function (43). The site on SecF of this interaction has not been mapped, but it almost certainly lies within its periplasmic segment.

We identified four classes of mutations in YidC that lead to maintenance of a miniF plasmid that carried the *yajC-secDF* complex for growth: (i) a mutation that leads to dependence on SecDF for viability and for which viability in the absence of SecDF is partially rescued by multicopy expression of SecY (G355C); (ii) mutations that lead to dependence on SecDF for viability and for which viability in the absence of SecDF is not rescued by multicopy of SecY (V328, P431S, and L420M/M498T/Y517C); (iii) a mutation that is able to rescue the growth defect of SecDF depletion only when native YidC is present (M471I), suggesting that production of wild-type YidC from the chromosome stabilizes or enables M471I YidC to function normally, and for which viability in the absence of SecDF is partially rescued by multicopy of SecY; and (iv) mutations which, for reasons that are unclear, lead to maintenance of a miniF plasmid that carries *yajC-secDF* but enable growth of a SecDF depletion strain under depletion conditions (V295I/F363S and R366H). With the exception of M471, all of these residues lie within highly conserved regions in the YidC/Alb3/OxaI family of proteins (28).

In supporting our initial hypothesis, identification of each of the four mutations in YidC within the first two classes described above leads to dependence on SecDF for viability. Moreover, each

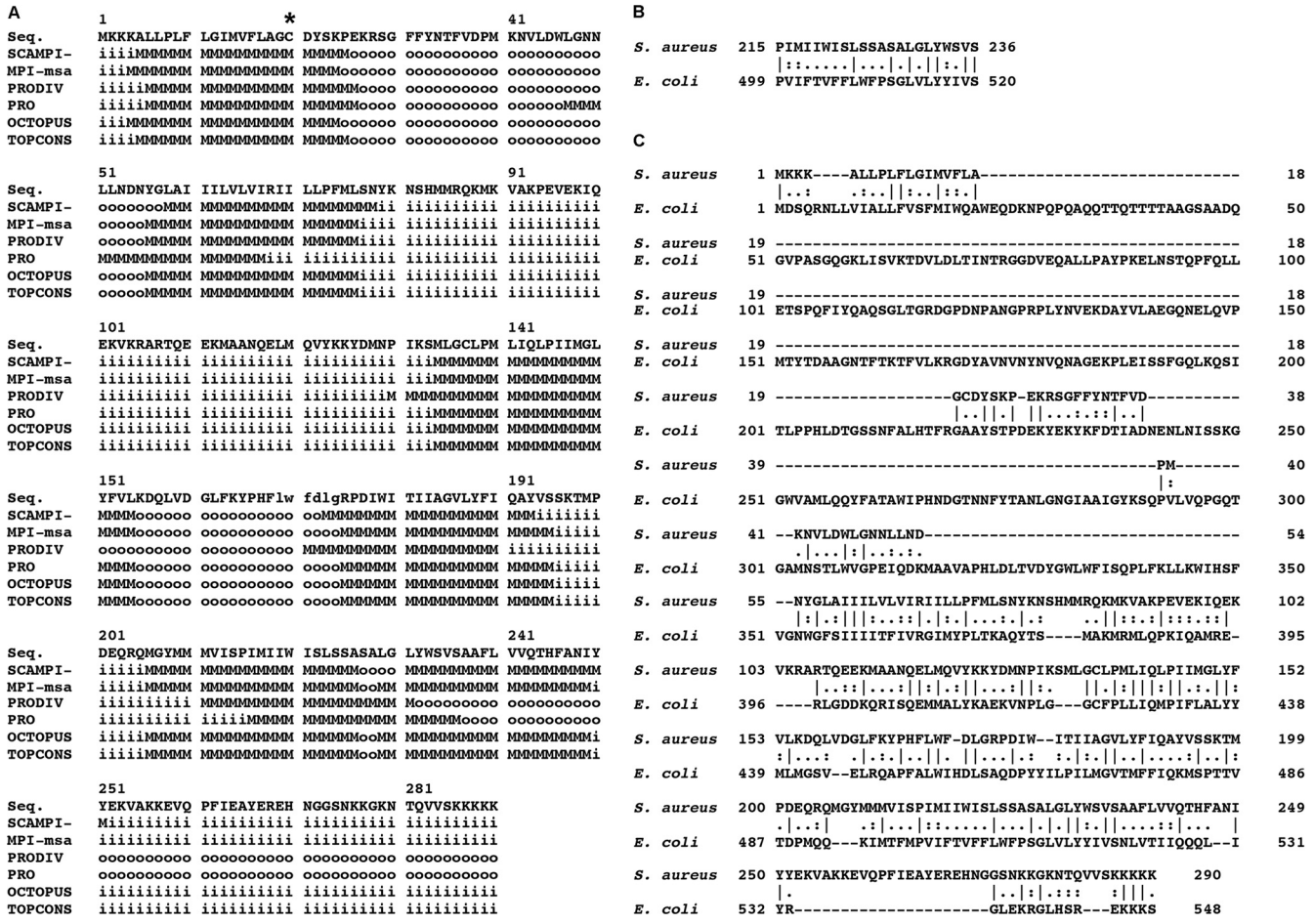


FIG 6 Comparison of *S. aureus* YidC to *E. coli* YidC. (A) Predicted topology of *S. aureus* YidC using multiple topology algorithms (see Materials and Methods). The asterisk indicates residue Cys-20, predicted to be modified by diacylglycerol moiety and, following processing, to be the mature amino terminus. (B) Alignment of the region of the potential third periplasmic loop of *S. aureus* YidC with the corresponding region of *E. coli* YidC using protein BLAST. (C) Alignment of *S. aureus* and *E. coli* YidC using protein BLAST.

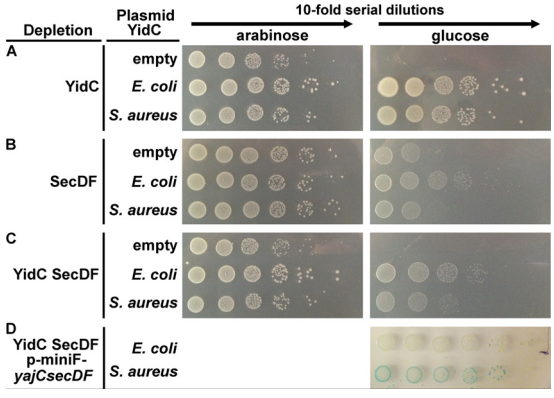


FIG 7 *S. aureus* YidC complements depletion of YidC but not of SecDF in *E. coli*. (A to C) *E. coli yidC* or *S. aureus yidC* complementation of growth of a YidC depletion strain (A), SecDF depletion strain (B), and YidC SecDF double depletion strain (C). (D) Loss or maintenance of miniF-*yajCsecDF lacZ* plasmid in strains depleted of YidC and SecDF and carrying either *E. coli yidC* or *S. aureus yidC*. Shown are serial 10-fold dilutions of the indicated strains grown on media containing arabinose (left) or glucose (right).

of the residues that we identified lies outside the region of the periplasmic loop of YidC that had previously been shown to interact with SecF, indicating that these residues are likely to lie on a surface of YidC distinct from the one that interacts with SecF and that potentially is in contact with other components of the apparatus.

The YidC residue we identified that is closest to the SecF interaction region of YidC is V328, which lies within a highly conserved helix at the extreme C-terminal end of the large first periplasmic loop (28, 44), adjacent to the periplasmic face of the cytoplasmic membrane (Fig. 4A). Most of the first periplasmic loop of YidC is dispensable for viability (43). However, the helix that contains V328 is essential for viability (43). Our identification of V328 as being required for stabilizing interactions with the Sec apparatus, combined with the essentiality of this short helix of the first periplasmic loop, raises the possibility that contacts made by V328 are key to critical functions of YidC.

Each of the other mutations in YidC that lead to dependence on SecDF for viability map to transmembrane segments: G355C to transmembrane segment 2, P431 to transmembrane segment 3, and L420M, M498T, and Y517C of the triple mutant within transmembrane segments 3, 5, and 6, respectively. In addition, the

mutation that displayed a mixed phenotype (M471I) lies in transmembrane segment 4. Deletion of residues 354 to 360 of transmembrane segment 2, residues 429 to 436 of transmembrane segment 3, or of transmembrane segments 3 and 4 and the intervening region each leads to loss of viability (43). However, changing residues W354, G355, and F356 to serine residues, residue P431 to a serine, residues N418, P419, L420, and G421 to serine residues, or Y517, I518, and V519 to serine residues did not lead to loss of viability (43); thus, G355 and P431 are essential only in the absence of SecDF. A YidC P431L mutant, which causes a cold-sensitive growth phenotype, displays defects in membrane insertion of Sec-independent, as well as Sec-dependent, YidC substrates (45). This suggests that P341 functions in membrane insertion independently of interaction with the Sec apparatus, which is consistent with our finding that expression of multicopy *secY* does not rescue viability of the YidC SecDF depletion in the presence of YidC P431S.

Each of the mutant proteins we identified in our screen functionally complements YidC depletion (Table 2). Nonetheless, three of the mutations that lie within transmembrane segments (G355C, F363S, and Y517C) are nonconservative substitutions. The ability of these mutant proteins to function in membrane protein insertion suggests that the mutations do not substantially alter the topology of the protein. The one other mutation that is nonconservative (V328D) lies within the large first periplasmic loop, where the change is unlikely to affect topology.

Given that YidC is docked at the lateral gate of SecY and can be cross-linked to SecY (29), we postulated that at least some of the mutations in YidC that lead to dependence on SecDF for disrupt or partially disrupt functional interactions with SecY. Moreover, given the high conservation of the SecY transmembrane segments that constitute the lateral gate (1), any YidC residue that interacts with SecY might also be conserved and be located within a transmembrane segment. At times, protein-protein interactions that have been altered by mutation can be functionally rescued by overexpression of the protein partners. Many of the amino acid changes we isolated are conservative changes, suggesting they lead to minor reductions in affinity of critical interactions. We find that for two of the YidC mutants in which the changes lie in conserved residues (G355C and M471I), overexpression of SecY leads to increased viability in the absence of SecDF (Fig. 5). As such, these residues in YidC may represent sites of interaction with SecY. A second possibility is that mutation of these residues leads to conformational changes in YidC that indirectly disrupt critical interactions with SecY. Finally, some evidence suggests that YidC functions as a dimer (34, 46–48), in which case the mutations we have identified might lead to disruption of YidC dimers that in turn alters critical interactions with SecY.

Two of the YidC mutants show no increase in viability with expression of multicopy SecY. Nevertheless, these mutations may disrupt a functional interaction with SecY if, for example, the mutation reduces the affinity of the interaction such that a basal level of expression of SecY from a multicopy plasmid is unable to rescue it. Alternatively, these mutations might disrupt a functional interaction with another component of the Sec apparatus.

The positioning of the residues we identified within transmembrane segments of YidC highlights the importance of functional interactions that are occurring within the membrane bilayer. These intramembrane interactions are particularly relevant to interactions of YidC with SecY. YidC is positioned at the lateral gate of SecY (29). The lateral gate consists of SecY transmembrane segments 2b, 3, 7, and 8 (1). Cross-linking analysis demonstrates that each of these four transmembrane segments of SecY interacts with YidC (29). The residues that are mutated in the two YidC mutants for which dependence on SecDF is partially rescued by multicopy expression of SecY (G355 and M471) are particularly strong candidates for participating in functional interactions with these transmembrane segments of SecY.

Consistent with our finding that multicopy expression of SecY rescues growth of strains carrying YidC G355C or YidC M471I in the absence of SecDF, SecY cross-links to YidC even in the absence of SecDF (29). That we observed only partial rescue of viability under these conditions might be explained in part by the interaction between SecY and YidC appearing to be weaker in the absence of SecDF, as determined by the intensity of bands identified by cross-linking analysis (29).

The Sec apparatus is a dynamic structure that changes conformation in conjunction with protein translocation (49–51). Interactions of the SecY lateral gate with YidC are lost in the presence of ribosomes and ribosome nascent chains (29), consistent with the proposal that this lateral gate opens and closes continuously (52). The loss of contacts that occurs in the presence of nascent membrane proteins likely accommodates the transfer of nascent transmembrane segments into the lipid bilayer. In principle, mutations in YidC that lead to dysfunctional interactions with SecY could lead either to a defect in binding SecY or a defect in releasing SecY during transfer of nascent transmembrane segments into the bilayer. Our selection criterion of dependence on SecDF would only lead to identification of mutations in the former category.

We find that *S. aureus* YidC functions in *E. coli* at least with respect to cell viability, as it rescues growth of *E. coli* cells that have been depleted of chromosomally encoded YidC. This was not unexpected, as *S. mutans* YidC homologs have previously been shown to partially function in *E. coli* (37). However, unlike *E. coli* YidC, when expressed from a multicopy plasmid, *S. aureus* YidC is unable to rescue the viability of SecDF depletion in *E. coli* (Fig. 7), suggesting that the interaction of *S. aureus* YidC with the Sec apparatus depends at least in part on SecDF. This is surprising, because whereas the overall topology of *S. aureus* YidC is similar to that of *E. coli* YidC, the domain that in *E. coli* YidC is required for interaction with SecDF (31) is substantially truncated in *S. aureus* YidC, such that the specific residues that are sufficient for *E. coli* YidC interaction with SecDF are completely absent (Fig. 6). Not only is the first periplasmic loop foreshortened relative to *E. coli* YidC but our findings also indicate that the extreme N terminus of *S. aureus* YidC is processed with lipid-modified Cys-20 at the mature N terminus anchored in the outer leaflet of the membrane. Thus, *S. aureus* YidC interacts with SecDF, either directly or indirectly, and the molecular nature of this interaction differs substantially from that of *E. coli* YidC with SecDF. It is noteworthy that *E. coli* YidC residue M471 is not conserved in *S. aureus* YidC; in *S. aureus*, this position contains an alanine. As M471 might be important for *E. coli* YidC interaction with SecY, *S. aureus* YidC may be impaired in its interaction with *E. coli* SecY, and as a consequence, it may be more dependent on an interaction with SecDF for function in this setting. An alternative explanation is that whereas the level of synthesis of *E. coli* YidC was sufficient to rescue viability in the absence of SecDF, the level of synthesis of *S. aureus* YidC was not; we think this explanation is unlikely, because

S. aureus YidC produced from the same vector rescues viability of depletion of chromosomally encoded YidC in *E. coli*.

The ability of *S. aureus* YidC to rescue growth in the absence of *E. coli* YidC indicates that *S. aureus* YidC is able to engage the *E. coli* Sec apparatus. Residues G355 and P431 of *E. coli* YidC, which were identified in our screen, are invariant in *S. aureus* YidC (Fig. 4B). This high degree of conservation provides additional support to the model that these residues are involved in conserved functional interactions of YidC with the Sec apparatus. It will be of interest to test whether these conserved residues are critical to functional interactions of *S. aureus* YidC with the *S. aureus* translocon, and specifically whether G57, which corresponds to G355 of *E. coli* YidC, participates in interactions with *S. aureus* SecY; this is the subject of future investigations.

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