A hydrophilic microenvironment required for the channel-independent insertase function of YidC

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The recently solved crystal structure of YidC suggests that it mediates membrane protein insertion by means of an intramembrane cavity rather than a transmembrane pore. This novel concept of protein translocation prompted us to characterize the native, membrane-integrated state of YidC with respect to the hydrophobic nature of its transmembrane (TM) region. Here, we show that the cavity-forming region of SpoIIIJ, a YidC homolog, is indeed open to the aqueous milieus of the Bacillus subtilis cells and that the overall hydrophilicity of the cavity, along with the presence of an arginine residue on several alternative sites of the cavity surface, is functionally important. We propose that YidC functions as a proteinaceous amphiphile that interacts with newly synthesized membrane proteins and reduces energetic costs of their membrane traversal.

membrane protein insertion | MiF | SpoIIIJ | YidC

Introduction

Biogenesis of membrane proteins, a fundamental cellular process essential for all living organisms, includes insertion of a newly synthesized membrane protein into the membrane followed by its folding and assembly with other cellular components. In the Sec-dependent pathway in bacteria, membrane insertion is mediated by the SecYEG protein-conducting channel in the plasma (cytoplasmic) membrane (1-3) whereas acquisition of the native conformation is facilitated by the conserved YidC-Oxa1/Ah3 family of membrane proteins (4-7). In a Sec-independent pathway, YidC facilitates insertion of a class of membrane proteins independently of SecYEG. Thus, YidC is a dual-function protein that serves as a chaperone or an insertase in membrane protein biogenesis (4-7).

Bacillus subtilis possesses two YidC homologs, SpoIIIJ (YidC1) and YidC2 (YqjG). These proteins in combination with their substrate MiF have provided us a unique in vivo experimental system to study YidC. While SpoIIIJ and YidC2 share growth-essential functions, indicated from the synthetic lethal phenotype of their deletion (8, 9), SpoIIIJ is constitutively expressed and YidC2 is induced upon dysfunction of SpoIIIJ (10, 11) in a manner repressible autogenously (12). MiF is encoded from the upstream open reading frame of yidC2 and plays an essential role in this cross-feedback and autogenous regulation by undergoing regulated elongation arrest in its translation (12-14). The ribosome stalling at miF leads to exposure of the Shine-Dalgarno (SD) sequence of yidC2 to enhance its translation. Importantly, elongation arrest of miF is released upon the YidC-dependent membrane insertion of the nascent MiF polypeptide, enabling the yidC2 translation to be up-regulated when cellular YidC activity declines. In this manner, MiF enables the cell to maintain the capacity of the YidC pathways of membrane protein biogenesis under changing intracellular and extracellular conditions (11, 12). This regulatory system also enables us to monitor the in vivo activities of YidC proteins; expression of a yidC2-lacZ translational fusion gene and, hence, the β-galactosidase activity, will increase in response to a decrease in the SpoIIIJ activity (11).

Although both SecYEG and YidC could facilitate membrane protein insertion, their modes of actions are fundamentally different. For instance, while SecYEG can mediate membrane insertion of proteins with multiple TM segments as well as those having large extracytoplasmic (periplasmic) domains, YidC, as an insertase, is specialized in insertion of small membrane proteins that possess a single or two TM segment(s) and (a) short extracytoplasmic region(s) (7). Crystal structures of archaeal and bacterial SecYB and SecYE(G) complexes reveal an hourglass-shaped transmembrane pore formed by the TM segments of SecY. The pore can also open laterally to the lipid phase of the membrane, allowing release of a TM segment of substrates out of the translocon pore to establish membrane protein integration (15-17). Although earlier electronmicroscopic studies of E. coli YidC and S. cerevisiae Oxa1 led to a proposal that YidC forms a homo-dimer, which creates a channel-like structure at the subunit interface (18), recent evidence suggests that a monomer of YidC interacts with the ribosome that is translating a membrane protein (19, 20). The crystal structures of YidC from Bacillus halodurans at resolution up to 2.4 Å (21) revealed that the five TM segments of YidC forms a cavity presumably in the lipid bilayer. This cavity appears to be open to the lipidic phase and the cytoplasm but not to the extracytoplasmic environment (Fig. 1A and B), arguing against the dimeric insertion pore model. Strikingly, the concave surface of the cavity is enriched in hydrophilic amino acid residues, including a conserved arginine. Genetic

Significance

How membrane proteins are guided into the membrane is a fundamental question of cell biology. Translocons are known to create a polypeptide-conducting, transmembrane channel having a lateral gate to allow lipid phase partitioning of the substrate. Here, we show that YidC guides a class of membrane proteins in a channel-independent fashion. Our experiments using intact Bacillus subtilis cells show that SpoIIIJ, a YidC homolog, forms a water-accessible cavity in the cell membrane and that the cavity’s overall hydrophilicity as well as the presence of an arginine residue at one of several alternative places on the cavity is functionally important. Probably, extracellular part of substrate is first attracted to the YidC cavity rather than a transmembrane pore. This novel concept of protein translocation prompted us to characterize the native, membrane-integrated state of YidC with respect to the hydrophobic nature of its transmembrane (TM) region. Here, we show that the cavity-forming region of SpoIIIJ, a YidC homolog, is indeed open to the aqueous milieus of the Bacillus subtilis cells and that the overall hydrophilicity of the cavity, along with the presence of an arginine residue on several alternative sites of the cavity surface, is functionally important. We propose that YidC functions as a proteinaceous amphiphile that interacts with newly synthesized membrane proteins and reduces energetic costs of their membrane traversal.

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analyses of a B. subtilis YidC homolog, SpoIIIJ, and its substrate membrane protein, MifM, revealed that the positive charge of the conserved arginine (Arg73 in SpoIIIJ) as well as negatively charged residues in the extracytoplasmic and transmembrane regions of MifM are essential for insertion of MifM into the membrane (21). From these results we proposed that SpoIIIJ mediates insertion of a class of membrane proteins such as MifM by a channel-independent mechanism, in which electrostatic attraction between the SpoIIIJ cavity and the substrate initiates the reaction (21). The other B. subtilis YidC homolog, YidC2 (YqjG), also functions with similar mechanism for insertion of MifM (12). The importance of the cavity was also supported by photo-crosslinking experiments showing that the inner surface of the cavity of SpoIIIJ interacts with substrate protein in vivo (21). Together with the crystal structure of Escherichia coli YidC (22), it is suggested that having a hydrophilic and positively charged cavity is a feature shared by the SpoIIIJ family members.

Since the unprecedented hydrophilic arrangement of YidC bears crucial importance in our understanding of membrane protein biogenesis, its occurrence in the native membrane must be verified using intact living cells. Here, we explored the hydrophilic nature and the functional requirements of the cavity-forming transmembrane region of SpoIIIJ in intact cells. The YidC cavity indeed proved to be accessible by water and its hydrophilicity, including an arginine residue somewhere in the cavity, important functionally. That YidC creates an aqueous microenvironment in the membrane gives a strong support to the channel-independent mode of its action.

**Results**

**Water-accessibility of the SpoIIIJ intramembrane cavity**
YidC forms an intramembrane cavity that is open laterally, presumably toward the lipid phase of the membrane and the cytoplasm, whereas it is inaccessible from the extracytoplasm (Fig. 1A and B). The inner surface of the cavity contains several hydrophilic amino acid residues, including the essential arginine, raising a possibility that YidC forms a hydrophilic local environment in the otherwise hydrophobic lipid bilayer. To experimentally verify this unusual hydrophilic arrangement, we examined water accessibility of YidC TM residues using intact living cells and the NEM (N-ethylmaleimide)-reactivity assay. NEM is membrane-permeable and alkylates the thiol group of a cysteine residue of protein in a water-dependent reaction (23-25), enabling us to assess water availability of a specific site of the target protein in intact cells by strategically placing a cysteine residue.

**Fig. 1.** Experimental design of NEM-reactivity assay to assess water accessibility to the SpoIIIJ cavity. (A) Ribbon diagram representations of the crystal structure of B. halodurans YidC2 (PDB ID: 3WO6). Shown are the side views (left and center) and a top view (right). Arg72 (corresponding to Arg73 in B. subtilis SpoIII; Fig. S1) is shown by magenta spheres. TM and C1 indicate the transmembrane segments (with numbers) and the first cytoplasmic region, respectively. (B) A surface model (upper) and cut-away molecular surface representation (lower) of B. halodurans YidC2. Orange dot-lined circles encircle the intramembrane cavity. Arg72 (Arg73 in SpoIIIJ) is shown in magenta. (C) A schematic representation of the membrane integration topology of B. subtilis SpoIIIJ and the sites where a unique cysteine was introduced for NEM-reactivity assay. TM1-TM5, C1-C3 and E1-E3 show the transmembrane, the cytoplasmic, and the extracytoplasmic regions, respectively. (D) The work flow of the assay. Intact cells were treated with NEM. Proteins were then extracted with SDS and subjected to PEGylation of the remaining thios under denaturing conditions. Finally, SpoIIIJ species were visualized after SDS-PAGE. (E) Electrophoretic separation of the PEG-modified and unmodified SpoIIIJ species. Positions of cysteine introduced into SpoIIIJ are shown at the bottom. Each sample received four different treatments as indicated by + and −. DTT in excess was included in alternate samples at the PEGylation step to give unmodified controls. PEGylated SpoIIIJ forms multiple slow-migrating bands due to heterogeneity of the Mal-PEG preparation. The bands near the 42 kDa position are non-specific.

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Fig. 2. Water accessibility profiles of the transmembrane regions of SpoIIIJ as assessed by NEM-reactivity. (A) NEM modification efficiencies (mean ± s.d., n ≥ 3) of cysteines at the indicated positions. Red columns show the efficiencies of > 50% and blue columns show lower (< 50%) modification efficiencies. Asterisks indicate SpoIIIJ derivatives that were unable to assess because of the lack of PEGylation even without NEM treatment. Striped columns represent non-functional SpoIIIJ mutants. (B) A schematic representation of the sites of cysteines and their NEM modification efficiencies. The sites of higher and lower modification as well as nonreactive sites are color-coded as in A. The numbered four positions are located in the extracytoplasmatic half of the membrane but NEM-modified efficiently. The sites of non-functional cysteine substitutions are striped. (C) Ribbon (left) and surface (right) representations of the front and back views of the B. halodurans YidC2 structure with the color-coded water accessibility.

Fig. 3. Functional importance of hydrophilicity of the SpoIIIJ cavity. (A) Efficiencies of MifM insertion into the membrane by the SpoIIIJ variants. Upper panel shows β-galactosidase activities (mean ± s.d., n = 3) of the spoIIIJ mutant strains harboring the yidCZ-lacZ reporter gene, which inversely correlate with the efficiencies of MifM insertion. Lower panel shows cellular accumulation of SpoIIIJ derivatives determined by anti-SpoIIIJ immunoblotting. (B) NEM modification efficiencies of cysteine introduced either at the 213rd or 228th position of the wild type and SpoIIIJ cavity mutants indicated at the bottom. (C) Growth-supporting abilities of the SpoIIIJ cavity mutants. Complementation assay of B. subtilis was carried out using strains lacking the yidC2 gene and having a rescue plasmid encoding IPTG-inducible spoIIIJ-FLAG. The chromosome contained the indicated spoIIIJ alleles shown on the left. Cultures were serially diluted (from 10⁻¹ to 10⁻⁶) and spotted onto LB agar plates containing 0 (right panel) or 1 mM (left panel) IPTG, which were then incubated for 17.5 hours at 37°C.

We first constructed the cysteine-less SpoIIIJ (SpoIIIJ- C134A), which proved to be functional as shown by the low β-galactosidase activity of the yidC2-lacZ reporter (Fig S2), whose expression level inversely correlates with the efficiency of membrane insertion of MifM, a YidC substrate (11). We then introduced a cysteine residue into selected single positions of the cysteine-less SpoIIIJ. Mutant proteins were expressed from the native chromosomal locus under the control of the native spoIIIJ promoter. Intact cells were then treated with NEM directly without any cell disruption (Fig 1D).

Proteins extracted from NEM-treated cells are solubilized, denatured in SDS and then subjected to the counter modification with maleimide-PEG (Mal-PEG), an alkylating reagent of ~5 kDa (Fig 1D). An NEM-unmodified fraction of the target protein, still having free thiol, is now modified by Mal-PEG and mobility-shifted, whereas the NEM-modified fraction of the protein resists the counter modification and does not show any appreciable mobility shift. The efficiency of NEM-modification was assessed by the extent of counter modification with Mal-PEG (26).

To characterize this assay system, we first replaced Glu169 in the second extracytoplasmic (E2) loop with cysteine. SpoIIIJ- C134A/E169C thus constructed was expected to have a fully water-accessible cysteine (Fig 1C). When NEM was omitted from the first reaction, the protein was efficiently modified with Mal-PEG with concomitant disappearance of the unmodified species (Fig 1E, lanes 1-4). The cysteine-less SpoIIIJ did not show this mobility shift and the intensity of the unmodified band remained unchanged (Fig 1E, lane 6). By contrast, NEM treatment of the E169C-expressing cells almost completely blocked the Mal-PEG modification even after denaturation (Fig 1E, lane 8), indicating that the cysteine at this position was fully accessible by NEM and water as expected.

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We examined a total of 74 single-cysteine mutant derivatives of SpoIIIJ to cover the TM regions of SpoIIIJ (Fig. 1C). Images of immunoblotting in the NEM-reactivity assay are shown in Fig. 1E (lanes 9-28) for selected target positions and in Fig. S3 for all the positions examined. Cysteines at positions 185, 187, 188 and 189 were fully reactive with NEM, as judged from the lack of counter modification with Mal-PEG (Fig 1E, lanes 10 vs 12, lanes 18 vs 20, lanes 22 vs 24 and lanes 26 vs 28, respectively). By contrast, cysteine at position 186 was not markedly reactive with NEM, as judged from the evident counter modification (lanes 14 vs 16).

In summary, our systematic in vivo NEM-modification assay suggests that the SpoIIIJ cavity creates an aqueous environment in the living cell membrane. Functional assays showed that most of the mutant SpoIIIJ derivatives were functional, although some others were less functional (Fig. S2). Although we included the non-functional SpoIIIJ mutants (Fig. S2 and those shown in striped colors in Fig. 2A and B) in our analysis, omitting them does not essentially affect our conclusion.

Functional importance of general hydropphilicity of the YidC cavity

We next addressed whether the hydropophilicity of the cavity is important for the YidC function. In our previous genetic studies, single alanine substitutions for the conserved hydrophilic residues in the cavity did not deteriorate SpoIIIJ functions, except for Arg73 (21). We reason that single alanine substitutions may be insufficient to reduce the overall hydropophilicity of the cavity. Therefore we selected six hydrophilic residues in the cavity (Gln140, Thr184, Gln187, Gln188, Gly231, Asn232) that were efficiently modified by NEM (Fig. 2) for their simultaneous replacement with either alanine (SpoIIIJ-6A) or leucine (SpoIIIJ-6L) to make the cavity more hydroporphic. As a control, we constructed a mutant, in which the six residues were replaced either by hydrophilic aspartagine or glutamine (SpoIIIJ-5N1Q, having mutations Q140N, T184N, Q187N, Q188N, G231N and N232Q). NEM-reactivity of cysteine introduced either at the 213th or 228th position was significantly lowered by the mutation. 5N1Q mutant, in which the six residues were replaced either by hydrophilic aspartagine or glutamine (SpoIIIJ-5N1Q, having mutations Q140N, T184N, Q187N, Q188N, G231N and N232Q). NEM-reactivity of cysteine introduced either at the 213th or 228th position was significantly lowered by the mutation.

We then assessed the insertase activity of the SpoIIIJ mutants using the yidC2-lacZ reporter. Whereas cell expressing wild type spoIIIJ had a β-galactosidase activity of 5.3 units (Fig. 3A, column 1), the spoIIIJ-deletion strain (ΔspoIIIJ) had 25.7 units of it (Fig 3A, column 2). β-galactosidase activity of cells expressing spoIIIJ-6A was 15.0 units and that of spoIIIJ-6L-expressing cells was 15.2 units, showing defects in SpoIIIJ function. By contrast, cells expressing spoIIIJ-5N1Q had only 3.5 units, showing full functionality of SpoIIIJ. Immunoblotting showed that cellular abundance of the mutant SpoIIIJ derivatives were functional, although some others were less functional (Fig. S2). Although we included the non-functional SpoIIIJ mutants (Fig. S2 and those shown in striped colors in Fig. 2A and B) in our analysis, omitting them does not essentially affect our conclusion.

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mutation in the Shine-Dalgarno sequence (sdm3-spoIII) showed
the normal reporter expression (Fig. 3A, sdm3). Thus, the spoIII-
64 and the spoIII-6L mutations impair the activity of SpoIII to
insert MiFM into the membrane to support cell growth (8, 9).

Deletion of yidC2 makes spoIIIJ essential for cell viability (8,
9), allowing us to examine functionality of the SpoIIIJ mutant
derivatives in supporting growth of B. subtilis. We used plasmid
expressing spoIIIJ-FLAG under the IPTG-inducible promoter
to assess the growth phenotypes of spoIIIJ mutations on the
chromosome that was also deleted for yidC2, in the absence of
IPTG the chromosomal spoIIIJ (with a mutation to be tested) was
the sole source of YidC. We observed severe growth defects for
strains having the spoIIIJ-6A or the spoIIIJ-6L mutation in the
absence of IPTG. By contrast, the spoIIIJ-SN1Q and the sdm3-
spoIIIJ cells grew normally even in the absence of IPTG. These
results show that the hydrophilicity of the cavity is required for the
growth-supporting function of SpoIIIJ. Taken together with the
results obtained from the lacZ reporter assay, the SpoIIIJ cavity
must be hydrophilic to function normally.

Flexible positional requirements for the essential positive
charge within the YidC cavity

The SpoIIIJ cavity contains an arginine residue that is func-
tionally essential, leading us to propose a charge attraction model
for the initiation and translocation of MiFM-like substrates (21). In
this case, substrate recognition may not be based on strict struc-
tural complementarity and electrostatic interaction may allow
certain positional flexibility. We addressed whether the arginine
residue can be relocated to different positions on the cavity,
by constructing a series of SpoIIIJ mutants with the original
Arg73 replaced with alanine and having a unique arginine at
various positions within the TM segments of SpoIIIJ. Western
blotting experiments showed that the arginine-relocating muta-
tions sometimes destabilized the SpoIIIJ protein (Fig. S4). Most
of the unstable protein had an arginine residue outside the cavity
(shown in blue in Fig. S4, A and B), which may have caused severe
hydrophobic mismatches.

We used the yidC2-lacZ reporter assay and the growth
complementation assay to assess functionality of the mutant
forms of SpoIIIJ. While many Arg-relocated mutants gave
elevated β-galactosidase activity, comparable to the activity ob-
served with the spoIIIJ-deleted cells (Fig 4B, mutants shown in
black columns) as well as with cells carrying the spoIIIJ-R734
mutation (21), several mutants expressed β-galactosidase at levels
significantly lower than the above-mentioned class of mutants.
The latter SpoIIIJ variants can still support MiFM insertion even
though they have lost the crucial arginine at the original position
and instead contain a relocated arginine at a different position
(Fig. 4B). Six of them (termed Class I that includes I72R, I76R,
Q140R, L144R, W228R and G231R; shown in red in Fig 4B and
C) had β-galactosidase activities of lower than 15 units, indicative
of nearly full functionality in inserting MiFM into the membrane.

Remaining five mutants (termed Class II that includes T69R,
I137R, T184R, I213R and M235R) had β-galactosidase activities
ranging from 15 to 25 units, indicative of partial functionality.

The class I mutations were found only in TM1, TM2 and TM5,
whereas the class II mutations were found in all the five TM
segments. Locations of these residues on the crystal structure of
B. halodurans YidC2, revealed that they, except I72, project their
side chains toward the inside of the cavity (Fig. 4C). The side
chain of I72 projects toward TM2 but still seems to be accessible
from the cavity interior. TM2 and TM5 are both geometrically
close to TM1, where Arg73 originally resided, possibly explaining
why the class I mutations occurred only in TM1, TM2 and TM5.

Growth complementation assay showed that all the Class I and
the class II R73A/I213R mutations fully supported cell growth
in the absence of YidC2. The class II mutations other than
R73A/I213R resulted in poor growth (Fig. S5). Thus, the abilities
of the SpoIIIJ variants to support cell growth correlated well
with their insertase activities. These systematic analyses strongly
support the idea that the concave surface of the cavity must be
positively charged to maintain SpoIIIJ’s function and the position
flexibility is allowed about exact positions of the positive charge,
being consistent with the charge attraction model.

Discussion

Translocation of hydrophilic regions of a newly synthesized
polypeptide across the hydrophobic lipid bilayer is an energet-
ically challenging process in the membrane protein insertion
pathways. While the SecYEG translocon overcomes this difficulty
by forming a polypeptide-conducting channel that sequesters a
translocating polypeptide from the lipidic environment (15-17),
several lines of evidence (19, 20, 27), most notably the crystal
structure of B. halodurans YidC2 (21), suggest that YidC uses a
channel-independent mechanism.

The results of our systematic NEM-probing analysis of the
mono-cysteine derivatives of SpoIIIJ indicate that the SpoIIIJ
cavity provides an aqueous environment within the membrane
of living cells. Although cysteine substitution at certain positions,
such as in the midst of consecutive hydrophobic residues, could
itself have altered the local disposition of the polypeptide, we
envisioned that such cases were rare except for the non-functional
mutations. The overall conclusion obtained from our in vivo anal-
ysis agrees well with the crystal structures of YidC as well as the
results of molecular dynamics simulation of YidC, showing the
presence of water molecules in the cavity (21). The hydrophilic
residues on the concave surface of the cavity should contribute
to maintaining the local aqueous environment as simultaneous
substitution of non-polar alanine or leucine for the six selected
hydrophilic residues on the cavity significantly reduced the effi-
ciencies of NEM-modification of a cysteine introduced into the
cavity. Importantly, SpoIIIJ’s activities to insert MiFM as well as to
support cell growth are compromised significantly by the
spoIIIJ-6A and the spoIIIJ-6L mutations, corroborating the physiological
importance of the cavity hydrophilicity.

A role of the YidC cavity may be to provide a hydrophilic
environment in the otherwise hydrophobic lipid bilayer, thereby
reducing the energetic cost required for insertion of hydrophilic
regions of substrate into the membrane en route to the trans-
side. It is also conceivable that the hydrophilic mismatch at the
protein-lipid interface could elicit local structural rearrangements
of the lipid bilayer (28, 29) so as to affect substrate-membrane
interactions and thereby facilitate membrane insertion. We spec-
ulate that the YidC cavity is designed not simply as a hydrophilic
platform but to allow for the unusual arrangement of intramem-
brane aqueous space to be compatible with the thermodynamic
principle. While it is unknown how this is accomplished, the
notion is consistent with the observations that placement of an
arginine residue is possible within the cavity but not its outer
regions without severely destabilizing the protein (Fig S4).

The functional arginine does not strictly require a unique
positioning in the cavity, as we were able to relocate it from
the original 73rd position to several other positions within the
cavity without loss of function. Such positional flexibility appears
to be consistent with the electrostatic attracting force serving as
a primary driving force for insertion of substrate. As discussed
previously, the cytosolic C1 region with hairpin-like helices may
provide a substrate entry point (21). Therefore, arginines that
are closer to the C1 region may have higher functionality as an
insertase element, although such a positioning should also be
compatible with the subsequent step of translocation completion.

The hydrophilic surface within the membrane interior might
be also important for the chaperone functions of YidC in the
channel-independent insertion pathway. For instance, transmembrane
regions of membrane proteins may contain functionally impor-

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tant polar residues, which might be unstable in the lipidic environ-
ment upon release from translocon until assembling with a
partner transmembrane polypeptide also containing complemen-
tary polar residues (30, 31). It is tempting to speculate that the
hydrophilic cavity of YidC provides a transient docking surface
that binds a newly inserted TM segment before it finds a partner
of assembly, like regular aqueous phase chaperones do in the
inverted ways.

The YidC family contains divergent members in different
organisms, which differ in the modes of cooperation with other
factors including the signal recognition particle and the ribosome
(32–35). Moreover, each homolog can have multiple functions
and reaction mechanisms (7, 36–38). We envision that the peculiar-
ularity of having a hydrophilic cavity in the membrane may be
a common feature conserved in many of the family members.
Still, it is possible that the hydrophilic local environment is used
differently in different YidC homologs. For instance, the cavity
arginine in the E. coli YidC was reported to be dispensable for
the insertase activity for the Pf3 coat protein, which requires
the arginine when handled by Streptococcus mutans YidC2 (39).
Furthermore, the YidC homolog in the membrane protein will advance
our understanding of how living organisms manage to solve prob-
lems associated with the movement of macromolecules across
hydrophobic borders.

Experimental Procedures

Bacterial strains and plasmids

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