YidC is involved in the biogenesis of anaerobic respiratory complexes in the inner membrane of *Escherichia coli*

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YidC of *Escherichia coli* belongs to the evolutionarily conserved Oxa1/Alb3/YidC family. Members of this family have all been implicated in membrane protein biogenesis of aerobic respiratory and energy transducing proteins. YidC is essential for the insertion of subunit c of the F₁F₀ ATP synthase and subunit a of cytochrome *o* oxidase. The aim of this study was to investigate whether YidC plays a role during anaerobic growth of *E. coli*, specifically when either nitrate or fumarate are used as terminal electron acceptors or under fermentative conditions. The effect of YidC depletion on the growth, enzyme activities and protein levels in the inner membrane was determined. YidC is essential for all anaerobic growth conditions tested and this is not due to the decreased levels of F₁F₀ ATP synthase in the inner membrane only. The results suggest a role for YidC in the membrane biogenesis of integral membrane proteins.
parts of the anaerobic respiratory chain.

In *Escherichia coli* about 30% of all encoded proteins are located in the inner and outer membranes. The cytoplasmic or inner membrane contains essential energy transducing complexes such as components of the electron transport chain, as well as solute transporters. Most integral inner membrane proteins are inserted cotranslationally via the general secretory pathway otherwise known as the Sec system. In this pathway ribosome-bound nascent chains are targeted by the bacterial signal recognition particle (SRP) to the SecYEG translocase via the SRP receptor FtsY. The membrane insertion of these proteins proceeds by a co-translational “threading mechanism” (1) in which the accessory protein YidC might play an important role in the clearance of transmembrane segments (TMSs) from the SecYEG channel (2). A small subset of integral membrane proteins are targeted directly to YidC where they are integrated into the membrane in a Sec-independent manner. YidC belongs to the evolutionarily conserved Oxa1/Alb3/YidC family. Oxa1 (Oxidase assembly) from yeast was the first member of this family to be described. It was originally identified as an essential factor for the biogenesis of respiratory complexes in the mitochondrion (3;4), more specifically the insertion of subunits of the cytochrome *bc*$_1$ oxidase and ATP synthase. Alb3 is located in the thylakoid membranes of *Arabidopsis* chloroplasts (5), and involved in the biogenesis of light harvesting complexes. Members of the family have all been implicated in membrane protein biogenesis of respiratory and energy transducing proteins. In *E. coli* it has been shown that YidC is essential for the insertion of subunit c of the F$_1$F$_0$ ATP synthase (F$_0$c) (6) and subunit a of cytochrome *o* oxidase (CyoA) (7;8).

While the effect of YidC depletion on the insertion of respiratory proteins has been studied in aerobically grown *E. coli* cells, the same has not been done for anaerobically grown cells. In the absence of oxygen, *E. coli* is able to use other organic (fermentation) and inorganic (anaerobic respiration) molecules as terminal electron acceptors. Nitrate is commonly used as an alternative electron acceptor to oxygen and the protein complex nitrate reductase (NarGHI) replaces the cytochrome *o* oxidase. The α and β subunits, NarG and NarH respectively, are located in
the cytoplasm and attach to the membrane via the \( \gamma \) subunit NarI to form the nitrate reductase complex (9). *E. coli* can also use fumarate as a terminal electron acceptor. The structure of fumarate reductase (FrdABCD) is similar to that of nitrate reductase except that the integral membrane part consists of two smaller subunits, namely FrdC and D (10). Although another translocation pathway, the Tat system (Twin Arginine Translocation) has been shown to target the peripheral \( \alpha \beta \) subcomplex to the integral membrane \( \gamma \) subunit of some anaerobic respiratory complexes, for example the DMSO reductase (DmsABC) (11), this is not the case for all terminal reductases such as the fumarate reductase (11). Also, how the integral membrane \( \gamma \) subunits of these complexes are inserted into the membrane is not known.

In *E. coli* only 3 natural substrates of YidC have been identified namely F\(_o\)c, CyoA and more recently the ion channel MscL (12). The identification of additional inner membrane proteins which rely solely on YidC for their insertion would give insight into the insertion mechanism of YidC and a possible evolutionarily conserved function of the Oxa1/Alb3/YidC family. The aim of this study was to investigate whether YidC plays a role in the membrane biogenesis of anaerobic respiratory complexes. More specifically the complexes essential for growth when either nitrate or fumarate is used as a terminal electron acceptor or under fermentative conditions. Our data demonstrate that YidC is essential for all growth conditions tested and that this is not only because of decreased levels of F\(_i\)F\(_o\) ATP synthase in the inner membrane. The results suggest a role for YidC in the membrane biogenesis of integral membrane subunits of the anaerobic respiratory chain.
Experimental procedures

Bacterial strains and plasmids. The YidC depletion strain *E. coli* FTL10 [MC4100-A, Δ*yidC*, *attB*:(*araC*+, *P*$_{BAD}$, *yidC*+) Kan$^R$] (13) was a generous gift of Frank Sargent (University of East Anglia, Norwich, United Kingdom). Strains *E. coli* SF100 [F* lacX74 galE galK thi rpsL (strA) *AphoA(pvuII), ΔompT] and NN100 [SF100, *lpp D(uncB-C) zid::Tn10] (14) were used to test dependence of anaerobically grown cells on the F$_1$F$_0$ATPase. Plasmid pTrc99A (15) was used to construct plasmid pET589 containing *E. coli yidC* (16). *E. coli* FTL10 was transformed with plasmids pTrc99A and pET589 to construct “YidC-” and “YidC+” depletion strains when grown on glucose containing media.

Materials. Potassium nitrate was purchased from Fluka and zinc powder, lapachol and sodium fumarate from Sigma-Aldrich. Texas Red was purchased from Invitrogen. Antiserum against YidC was raised in chickens against purified His-tagged YidC (Agrisera AB, Sweden). Antisera against fumarate reductase subunits FrdA and FrdB, the nitrate reductase complex (NarGHI), subunit c of the F$_1$F$_0$ ATP synthase (F$_{0}$c), leader peptidase (LepB), phage shock protein A (PspA) and subunit K of the NADH dehydrogenase I (NuoK) were generous gifts from Joel Weiner (University of Alberta, Canada), Axel Magalon (LCB-IBSM CNRS, Marseille, France), Gabriele Deckers-Hebestreit (University of Osnabruck, Germany), Jan Tommassen (Utrecht University, The Netherlands), William Wickner (Dartmouth Medical School, USA), and Takao Yagi (The Scripps Research Institute, USA), respectively. The D-glucose HK Assay Kit was purchased from Megazyme and the Nitrate Test from Merck. Restore™ Western Blot Stripping Buffer was purchased from Pierce. Alkaline phosphatase conjugated anti-chicken, anti-mouse and anti-rabbit IgG were purchased from Sigma-Aldrich.

Bacterial growth and processing. Cells were grown anaerobically at 37°C on basal anaerobic growth medium supplemented with 0.1% yeast extract (Difco) as previously described (17). The following carbon sources, electron acceptors and trace metals were added as indicated: 0.5% (w/v) glucose, 0.5 % (v/v) glycerol, 1 % (w/v) potassium nitrate, 10 mM sodium fumarate, 1 μM sodium selenite and 1 μM sodium molybdate. Sodium selenite and sodium...
molybdate were only added when potassium nitrate was used as an electron acceptor.

To deplete cells of YidC, E. coli FTL10 harbouring either plasmid pET589 (“YidC+”) or pTrc99A (“YidC-”) were grown aerobically at 37°C overnight in basal anaerobic growth medium containing 0.5% arabinose. Cells were then harvested, washed in basal anaerobic growth medium and resuspended in basal anaerobic growth medium containing glucose or glycerol and potassium nitrate or sodium fumarate as indicated and growth was continued to an OD$_{600}$ of 0.6. This corresponds to late exponential phase. The cells were then diluted 2-fold with the same medium and the procedure was repeated until the cessation of the “YidC-” strain (adapted from 28). Inner membrane vesicles (IMVs) were prepared from these cells as previously described (18). All further analysis was performed using these IMVs. The glucose and nitrate contents of the growth media were assayed after growth in the second dilution in centrifuge-cleared supernatant.

E. coli strains SF100 and NN100 were first grown overnight aerobically in basal anaerobic growth medium containing glucose. The cells were then washed in basal anaerobic growth medium and inoculated into the same medium containing the combinations of carbon sources, electron acceptors and trace metals used under YidC depleting conditions. The OD$_{600}$ was measured after 8 hours of growth at 37 °C under anaerobic conditions.

**Protein determination and western blotting.** Protein concentrations were determined with the DC Protein assay (Biorad) using bovine serum albumin as a standard. SDS-PAGE and immunoblot analyses were carried out according to methods previously described (19;20). Gels for immunoblot analyses were loaded with equal amounts of total inner membrane protein. Signal capture and quantification were performed using LumiAnalyst 3.1 (Roche Molecular Biochemicals). Immunoblot analyses were repeated at least in triplicate in independent experiments.

**Enzyme assays.** ATPase activity of IMVs was measured at 4°C using malachite green as described (21). Nitrate reductase and fumarate reductase activities of IMVs were measured spectrophotometrically at 25°C using a Hitachi U-1100 spectrophotometer. The activities were measured by following the oxidation of reduced lapachol at 481nm in the presence of nitrate or fumarate (22). The reactions were
performed in an anaerobic cuvette with a rubber stopper under nitrogen. The extinction coefficient of lapachol was taken to be 2.66 mM\(^{-1}\)cm\(^{-1}\) and specific activity was expressed as nanomoles nitrate or fumarate reduced per minute per milligram IMV.

**Translocation assay.** The presence of a functional Sec translocase was verified with IMVs using Texas Red-labeled proOmpA as a substrate (23). Proteinase K was added after the translocation reaction to digest any untranslocated material.

**RESULTS**

**Depletion of YidC leads to the cessation of growth under anaerobic conditions.** To examine if YidC is needed for protein biogenesis of respiratory and energy transduction proteins expressed under anaerobic conditions, we examined the growth of *E. coli* FTL10 (13) under anaerobic conditions in the presence of various electron acceptors and carbon sources. Cells harboring either pET589 (“YidC+”) or pTre99A (“YidC-”) were grown as described in the Material and Methods until the cessation of the YidC- strain (adapted from 28). The following combinations of electron acceptors and carbon sources where used: glucose only; nitrate and glycerol; nitrate and glucose; fumarate and glycerol; fumarate and glucose. Under YidC-depleting conditions, the YidC- strain ceased to grow under all conditions tested (Table 1). An aliquot of growth medium was sampled once cells had reached an OD of 0.6 after the cells had been diluted once, cleared of cells by centrifugation and assayed for D-glucose and nitrate. This confirmed that the cells were utilizing the glucose and nitrate in the growth medium (data not shown).

**The inner membrane protein profile is largely unchanged following YidC depletion.** Examination of the inner membrane protein profile revealed that few changes occurred upon YidC depletion (supplemental Fig. 1A). The levels of the inner membrane protein leader peptidase (LepB), which is not strictly dependant on YidC for its insertion, are unaffected by YidC depletion as examined by immunoblot analysis (supplemental Fig. 1B). The levels of YidC were monitored by immunoblot analysis using antibodies against YidC. The intensity of the bands present on the immunoblots against YidC...
were quantified, the blots were stripped and reprobed with antibodies against LepB which was used as a control membrane protein. The intensity of the bands of YidC were then expressed as the ratio of the YidC and the LepB bands in order to compare relative levels of YidC in the membrane (Fig. 1A). This process was repeated for all immunoblots in the study. This showed that the levels of YidC in IMVs isolated from strains grown in the same growth medium for a comparable amount of time, decreases substantially in the YidC- strains.

Depletion of YidC interferes with the membrane assembly of F₀c. Subunit c of the F₁F₀ ATP synthase (F₀c) is a well-studied substrate of YidC. The levels of F₀c in IMVs were found to decrease upon YidC depletion under all anaerobic growth conditions tested (Fig. 1B). The total ATPase activities of IMVs were tested to confirm that the F₁F₀ ATP synthase was indeed not fully functional. It has been shown that 95% of the total ATPase activity of wild type IMVs can be attributed to the F₁F₀ ATP synthase (van de Laan, M., unpublished data). Under YidC-depleting conditions, the ATPase activity of YidC- IMVs isolated from cells under all growth conditions is reduced with respect to the YidC+ IMVs (Fig. 1C). The reduction in ATPase activity after YidC depletion was greater amongst cells grown in combination with nitrate or fumarate and glucose than those where glycerol was the carbon source.

The Sec translocon is not affected by YidC depletion in E. coli FTL10. There are many reports in the literature that show that YidC depletion does not affect the translocation of proteins via the Sec translocon or the Tat-pathway (13;24;25). Using IMVs isolated from anaerobically glucose-grown cells, the translocation of Texas Red-labeled proOmpA still occurred in YidC- IMVs (supplemental Fig. 1C). The processing of proOmpA is, however, reduced which can mostly be attributed to a disturbance of the proton motive force (PMF) since a similar effect is seen when the ionophores nigericin and valinomycin are added. No protection of Texas Red-labeled proOmpA from the externally added protease is observed when ATP is absent. These data confirm for anaerobically grown cells that the YidC-depletion does not affect the levels of the functional Sec-translocon.
Upon YidC depletion the induction of PspA is reduced in anaerobically grown cells compared to aerobically grown ones. In aerobically grown *E. coli* cells, YidC depletion leads to a massive upregulation of the Psp (Phage Shock Protein) operon. This can be monitored by immunoblot analysis using antibodies against the membrane associated PspA protein. We found that upon YidC depletion under anaerobic growth conditions the PspA induction was greatly reduced when compared to that seen under aerobic growth conditions (Fig. 2).

Growth defect following YidC depletion is not only due to the decrease of functional $F_{1}F_{0}$ ATP synthase in the membrane. In order to test whether the cessation of growth under YidC depleting conditions observed under anaerobic conditions can be attributed to the decrease in the levels of functional $F_{1}F_{0}$ ATP synthase in the membrane, *E. coli* NN100 and SF100 strains were grown in the same growth medium examined under YidC depleting conditions. *E. coli* strain NN100 is a derivative of strain SF100 and contains a deletion of the *uncB-C* genes resulting in a strain with no functional $F_{1}F_{0}$ ATP synthase (14). Cells were grown anaerobically at 37 °C and the OD was measured after 8 hours of growth (Fig. 3). As expected, the growth of NN100 cells in medium containing only glucose was severely impaired. These cells were, however, able to grow anaerobically in all other growth medium tested. The presence of the $F_{1}F_{0}$ ATP synthase is therefore essential only under fermentative conditions, and not needed for anaerobic growth with the electron acceptors tested. Growth with nitrate as the electron acceptor of NN100 reached a higher OD than SF100 cells grown under the same conditions, suggesting that a partial dissipation of the PMF occurs in the presence of a functional $F_{1}F_{0}$ ATP synthase that reduces the growth.

Activity and protein levels of nitrate reductase in membranes decreases upon YidC depletion. Since the decreased levels of functional $F_{1}F_{0}$ ATP synthase in the membrane are not responsible for the cessation of growth observed, the depletion of YidC must affect the assembly of some other membrane-located complex(es) needed for anaerobic respiration when nitrate or fumarate are the electron acceptors, and/or other essential membrane-localized functions. We
therefore investigated the protein levels and enzyme activities of other membrane-located complexes essential for growth when nitrate or fumarate are the electron acceptors, namely the nitrate and fumarate reductases and the NADH dehydrogenase I. In the absence of oxygen, *E. coli* can use alternative electron acceptors to form an electron transport chain. In the presence of selenium, molybdenum and high levels of nitrate, the expression of the nitrate reductase containing operon *narGHIJ* is induced rather than *napFDAGHBC* (26). Using an antibody against the entire complex, immunoblot analysis revealed that the levels of NarGHI decrease upon YidC depletion (Fig. 4A). The activity of the nitrate reductase was also assayed by the oxidation of a hydroxylated naphthoquinol menaquinol analogue, reduced lapachol, under anaerobic conditions. Under YidC depleting conditions the reduction of nitrate by IMVs is reduced (Table 2). The activity of nitrate reductase was higher in IMVs isolated from nitrate/glycerol grown cells in comparison with nitrate/glucose grown. For nitrate/glycerol grown cells nitrate reductase activity was 2.4 fold reduced in the YidC- strain and for nitrate/glucose grown cells the reduction was greater, 3.5 fold.

**Activity and protein levels of fumarate reductase in membranes decreases upon YidC depletion.** Using the same strategy as for the nitrate reductase complex, the effect of YidC depletion on another membrane located terminal reductase, fumarate reductase, was examined. Immunoblot analysis of IMVs was carried out using antibodies directed against the α and β subunits of the reductase (FrdA and B, respectively) (Fig. 4B). The data show that the levels of FrdA and FrdB decrease upon YidC depletion. Also, fumarate reductase activity of IMVs as assayed by the oxidation of reduced lapachol was found to decrease upon YidC depletion (Table 2). As with nitrate reductase, the activity of fumarate reductase was higher in IMVs isolated from fumarate/glycerol grown cells in comparison with fumarate/glucose grown. For fumarate/glycerol grown cells fumarate reductase activity was 1.7 fold reduced in the YidC- strain and for fumarate/glucose grown cells the reduction was greater, 2.8 fold.

**Protein levels of the NADH dehydrogenase I subunit K.** A knockdown of the human Oxa1l in HEK293 cells results in a
reduced activity and protein content of the $F_1F_0$ ATP synthase and complex I, the NADH:ubiquinone oxidoreductase (27). E. coli expresses two types of NADH dehydrogenases. NADH dehydrogenase I consists of 13 subunits located at the inner membrane, encoded by the genes nuoA-N. Seven of the subunits are integral membrane proteins with the number of TMSs ranging from 3 to 14. The membrane located protein NADH dehydrogenase II encoded by the gene ndh, is 47 kDa and YidC depletion under aerobic conditions has no effect on its activity (25). Under anaerobic conditions in the presence of nitrate, both types can participate in respiration (28) and therefore NADH dehydrogenase activity assays cannot be used as a measure for the YidC-depletion effect on these complexes. NADH dehydrogenase I is expressed under all anaerobic conditions, but is important only during growth in the presence of fumarate (28). NADH dehydrogenase I bears more similarity to human complex I than NADH dehydrogenase II. Owing to its small size (10.8 kDa), subunit K (NuoK), a subunit with 3 TMS, is a possible YidC substrate. Immunoblot analysis of IMVs was carried out using antibodies directed against NuoK (Fig. 4C). The data show that the levels of NuoK decrease upon YidC depletion.

DISCUSSION

The data presented in this study demonstrate that under YidC depleting conditions, E. coli is unable to grow anaerobically with either nitrate or fumarate as electron acceptors. The levels of various essential protein complexes were examined in order to determine possible reasons for the cessation of growth. Although some changes in the membrane protein profile were observed, the overall profile remained mostly unchanged under YidC-depleting conditions confirming the notion that most membrane proteins insert independently of YidC. It has been previously shown that neither the Sec-translocase or the Tat complex are influenced by YidC depletion in E. coli FTL10 (13)(25). One report suggested that SecE is weakly dependent on YidC (24), but the translocation of secretory proteins is only mildly affected by YidC depletion in aerobically grown cells (2). Under our test conditions of anaerobic growth the Sec translocase also remained intact, and the levels of LepB, a Sec translocon substrate are unaffected by YidC-depletion. Translocation was only marginally affected which can mostly be attributed to a reduced capacity of
YidC membranes to generate a PMF. In anaerobically glucose-grown (fermenting) cells, the latter can be attributed to the decreased levels of F0c in the membrane and thus of the functional F1F0 ATP synthase that is solely responsible for the generation of a PMF under those conditions (29).

Since YidC is essential for the insertion of F0c and CyoA, YidC-depletion under aerobic growth conditions results in a massive upregulation of PspA (25). PspA is upregulated in response to stress conditions that result in the dissipation of the PMF (30). Research in this area has been largely focused on aerobically grown E. coli cells but one proteomic study has shown that under osmotic stress the PspA protein is not induced when cells are grown anaerobically (31). Our data is in agreement with this study as the induction of PspA under the anaerobic growth conditions tested is greatly reduced when compared to aerobically grown cells. There are conflicting reports as to the mechanism by which the proteins contained in the psp operon enable the cell to cope with the dissipation of the PMF. One study in E. coli suggested that PspA and PspG switch the cell to anaerobic respiration and fermentation thereby adjusting the energy usage and PMF generation of the cell (32). However, other microarray studies on Yersinia enterocolitica and E. coli showed only an upregulation of the genes contained in the psp operon upon secretin overproduction and did not reveal a switch to anaerobic metabolism related to the induction of the Psp operon (33;34). The mechanism by which Psp proteins operate is therefore still largely unclear under aerobic as well as anaerobic growth conditions.

Under anaerobic conditions and in the presence of nitrate, electron transfer from formate (from formate dehydrogenase) to nitrate is coupled to the generation of a PMF (9;35). With fumarate reduction, the fumarate reductase is directly responsible for proton translocation (10). A reduction of the levels of fumarate and nitrate reductases and of the F1F0 ATP synthase in the YidC depletion strain will result both in a loss of the PMF and of PMF-dependent ATP synthesis. Since these anaerobically grown cells can still generate ATP by the metabolism of glucose or glycerol at substrate level (36;37), the F1F0 ATP synthase is not essential for ATP synthesis under these anaerobically respiring conditions. Indeed, the unc- strain E. coli NN100 was able to grow with either nitrate or fumarate as an electron acceptor.

It was shown that the protein levels and reducing activities of
both the nitrate and the fumarate reductases were diminished upon YidC depletion. This could be responsible for the growth cessation observed. The α and β subunits of the nitrate reductase, NarG and NarH respectively are cytoplasmically located and attach to the integral membrane γ subunit NarI to form the nitrate reductase complex (9). Neither NarG, H or I have any predicted Tat targeting sequences or signal peptide cleavage sites (TATFIND 1.4 (38) SignalP 3.0 (39)). NarG has, however, a vestige motif at its N-terminus which appears to be a remnant of the Tat (Twin arginine translocation) signature (40). It has been suggested that NarJ which is contained in the same operon as NarGHI is a Tat-dependent chaperone which delivers the NarG and NarH complex to the membrane inserted NarI (41). In the same study it was reported that the growth rate of cells in nitrate-containing media was impaired in the Δtat E. coli strain. This, however, conflicts with a previous study which reported that anaerobic growth with nitrate was not impaired in a ΔtatABCD E. coli strain (11). Neither studies determined if mislocalization of the Tat-dependent formate dehydrogenase would have any effect on growth in nitrate-containing medium.

The Tat-pathway has been implicated in the membrane location of anaerobic respiratory which have an αβγ structure but where the αβ subunits are periplasmically located (11;42), for example the NiFe hydrogenase-2 isoenzyme. The β subunit of this enzyme has one TMS which is inserted by the Tat-translocase itself and which is YidC-independent (13;43). The αβ subunits still attach and retain activity in the absence of the integral membrane γ subunit owing to this C-terminally located TMS of the β subunit (43). In the nitrate reductase neither NarG or NarH have a TMS and therefore they may need NarI to functionally attach to the membrane.

Fumarate reductase too has an αβγ structure with FrdA and FrdB forming an cytoplasmically-located αβ subcomplex which attaches to the membrane via the integral membrane proteins FrdC and D. The decrease in the levels of FrdA and FrdB associated with the inner membrane is suggestive of a role for YidC in the insertion of FrdC and FrdD since FrdA and FrdB cannot assemble at the bacterial membrane without first the correct assembly of integral membrane parts FrdC and FrdD (44). FrdC and FrD are both small (15 and 13 kDa, respectively) 3 TMS-containing proteins that have their N-termini located in the
cytoplasm and their C-termini in the periplasm. Unlike some other anaerobic respiratory components, fumarate reductase has been shown to be independent of the Tat system for its membrane location and activity (11). It has recently been reported that Oxa11 from human embryonic kidney cells does not appear to be responsible for the insertion and/or assembly of complex II, the succinate:ubiquinone oxidoreductase whose structure is analogous to fumarate reductase (27).

The levels of NuoK of the NADH dehydrogenase I were also examined following YidC depletion. These too, were shown to decrease in the YidC- strain. If NuoK levels were decreased owing to YidC dependence for its insertion, it would have a detrimental NADH dehydrogenase activity as most of the active peripheral arm of the complex is also absent if the NuoK subunit is (46). This could contribute to or be responsible for the cessation of growth under YidC depleting conditions in the presence of fumarate. Interestingly, a recent crosslinking study indicates an association between YidC and NuoCD (47), lending further support to the notion that NADH dehydrogenase I assembly may be YidC-dependent.

Although it has been shown in this study and by others that neither the Sec-translocase or the Tat complex are influenced by YidC depletion in E. coli FTL10, we cannot exclude other effects of YidC depletion that may affect the membrane targeting and insertion of the systems analyzed in this study. The other identified YidC only substrate, MscL, is essential only under osmotic stress conditions. Obviously, a defect in the membrane assembly and folding of other membrane proteins as for instance the transport systems involved in the uptake of nitrate and fumarate may further contribute to the growth defect in cells depleted from YidC (48). Future studies will focus on the insertion and assembly of the integral membrane components of the nitrate and fumarate reductase complexes as well as those of the NADH dehydrogenase I.

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**FIGURE LEGENDS**

**Fig. 1.** Depletion of YidC leads to a decrease in the amount of functional F$_1$F$_0$ ATP synthase. IMVs were analyzed by immunoblotting using (A) anti-YidC and (B) anti-F$_o$c serums. Following electronic quantification of the YidC- and F$_o$c-labeled bands, the membranes were stripped and reprobed with anti-LepB serum. Levels of YidC and F$_o$c are expressed relative to levels of LepB. Data points are the mean of three separate measurements. The bars indicate the standard error of the mean. (C) Total ATPase activity of IMVs isolated from cells grown under all growth conditions was tested. Enzyme activities are expressed as nmol phosphate per milligram IMV per minute. Data points are the mean of three separate measurements. The bars indicate the standard error of the mean.

**Fig. 2.** PspA induction is reduced in anaerobically grown cells as compared to aerobically grown *E. coli* cells. (A) Immunoblot analysis of PspA levels in IMVs. Note that of the IMVs of aerobically grown 2-fold less IMVs were loaded on SDS-PAGE as compared to the other IMVs. (B) Levels of PspA are expressed relative to levels of LepB.

**Fig. 3.** *E. coli* strains SF100 (grey bars) and NN100 (white bars) were grown anaerobically basal anaerobic growth medium supplemented with 0.1% yeast extract to which combinations of carbon sources and electron acceptors were added. Cell cultures were grown for 8 hours after which OD measurements were made. Cells were grown in the presence of nitrate or fumarate with either glucose or glycerol as a carbon source. As a control experiment, cells were also grown in glucose. Data points are the mean of three separate growth experiments with the indicated standard error of the mean.

**Fig. 4.** Depletion of YidC leads to a decrease in the amount of protein complexes essential for anaerobic respiration (A) Immunoblot analysis of NarGHI complex levels in IMVs isolated from nitrate/glucose grown cells. Levels of NarG, H and I are expressed relative to levels of LepB. (B) Immunoblot analysis of FrdA (left panel) and FrdB (right panel) levels in IMVs. Levels of FrdA and FrdB are expressed relative to levels of LepB. (C) Immunoblot analysis of NuoK levels in IMVs. Levels of NuoK are expressed relative to levels of LepB.
TABLE 1. Growth media used and stage at which the growth of the YidC-strain ceased

| Growth conditions | Dilutions before growth ceased $^1$ |
|-------------------|-------------------------------------|
| Carbon source     | Electron acceptor                   |
| Glucose           | -                                   | 5          |
| Glycerol          | Nitrate                             | 3          |
| Glucose           | Nitrate                             | 4-5        |
| Glycerol          | Fumarate                            | 3          |
| Glucose           | Fumarate                            | 4          |

$^1$YidC- cells were grown anaerobically in the presence of the indicated carbon source and electron acceptor to an OD$_{660}$ of 0.6, diluted 2-fold with fresh medium and the procedure was repeated until the cessation of the YidC- strain as described in the Experimental Procedures section.
TABLE 2. Activity of the nitrate and fumarate reductases as assayed by lapachol oxidation of IMVs in the presence of electron acceptors nitrate or fumarate. IMVs were isolated from cells grown in basal anaerobic growth medium supplemented with 0.1% yeast extract to which nitrate or fumarate and glucose or glycerol were added.

| Growth conditions | Enzyme activity (nmol/min/mg of membrane protein) ¹ |
|-------------------|--------------------------------------------------|
| Electron acceptor | Carbon source | YidC | 127.3 ± 33.6 | 51.9 ± 9.5 |
| Nitrate           | Glycerol      | +   | 127.3 ± 33.6 |
|                   |               | -   | 51.9 ± 9.5   |
| Nitrate           | Glucose       | +   | 81.2 ± 25.5  |
|                   |               | -   | 23.2 ± 6.9   |
| Fumarate          | Glycerol      | +   | 15.2 ± 0.5   |
|                   |               | -   | 8.8 ± 0.8    |
| Fumarate          | Glucose       | +   | 5.5 ± 2.0    |
|                   |               | -   | 2.0 ± 0.5    |

¹ Enzyme activities are expressed as nmol nitrate or fumarate reduced per minute per milligram IMV. Results are the mean of three separate measurements with indicated standard error of the mean.
Figure 1
Figure 2
Figure 3
Figure 4
YidC is involved in the biogenesis of anaerobic respiratory complexes in the inner membrane of Escherichia coli
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