A novel membrane protein insertion pathway in Escherichia coli

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SecDFyajC is not required for the maintenance of the proton motive force

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Summary

SecDFyajC of *Escherichia coli* is required for efficient export of proteins *in vivo*. However, the functional role of SecDFyajC in protein translocation is unclear. We evaluated the postulated function of SecDFyajC in the maintenance of the proton motive force. As previously reported, inner membrane vesicles (IMVs) lacking SecDFyajC are defective in the generation of a stable proton motive force when energized with succinate. This phenomenon is, however, not observed when NADH is used as an electron donor. Moreover, the proton motive force generated in SecDFyajC-depleted vesicles stimulated translocation to the same extent as seen with IMVs containing SecDFyajC. Further analysis demonstrates that the reduced proton motive force with succinate in IMVs lacking SecDFyajC is due to a lower amount of the enzyme succinate dehydrogenase. The expression of this enzyme complex is repressed by growth on glucose media, the condition used to deplete SecDFyajC. These results demonstrate that SecDFyajC is not required for proton motive force-driven protein translocation.

Introduction

In bacteria, the so-called 'Sec system' provides the general route for proteins to cross the cytoplasmic membrane and for membrane proteins to insert into this membrane (reviewed in 60). The Sec system is composed of eight different proteins that form

two complexes, a "core" complex formed by SecY, SecE, SecG (SecYEG) and the ATPase SecA, and a complex containing SecD, SecF, YajC and YidC (187). Upon binding of SecA and a precursor protein, the SecYEG complex assembles into a protein-conducting channel. Cycles of ATP binding and hydrolysis by SecA drives translocation of the precursor protein through the channel. The proton motive force (PMF) stimulates the protein movement by providing additional directionality to the process and can completely drive translocation at late stages (59, 210). YidC forms a tetrameric complex with SecD, SecF and YajC (187) and is involved in the insertion of proteins into the cytoplasmic membrane (205, 213). The functions of SecD, SecF and YajC in protein translocation are less clear. *In vitro* protein translocation reconstituted with SecYEG proteoliposomes does not require SecDFyajC (28), whereas cells lacking SecD and/or SecF have a severe export defect and are barely viable (191). Based on the observation that SecDFyajC-depleted IMVs are impaired in the generation of a PMF (11), it has been suggested that SecDFyajC stimulates protein translocation by maintenance of the PMF. However, the mechanism by which SecDFyajC functions in this process has remained elusive.

In this report we examined the role of SecDFyajC in maintenance of a PMF. We show that the previously observed deficiency of *E. coli* inner membrane vesicles lacking SecDFyajC to generate a stable PMF is due to the reduced expression of the electron transport chain component succinate dehydrogenase that is repressed when cells are grown on glucose media, the condition that is used to deplete SecDFyajC.

Results

Depletion of the SecDFyajC complex in an E. coli unc⁻ strain

To examine how SecDFyajC couples the PMF to protein translocation we constructed *E. coli* strain NN103 that lacks the F_1F_0 ATPase (*unc*⁻) and in which the *secDF* operon is under control of the arabinose promoter. In this strain SecDFyajC can be depleted by growing the cells for several generations in the presence of glucose whereas the *unc* mutation makes that IMVs from this strain are not able to generate a PMF by ATP hydrolysis. In the absence of the F_1F_0 ATPase, the growth defect due to SecDFyajC depletion starts two generations earlier than in the parental *unc*⁺ strain (7

versus 9 generations, respectively). IMVs were isolated from cells grown on LB containing 0.2% arabinose and from cells that have been grown for 6 generations on LB 0.2% glucose media. After six generations on glucose media the amounts of SecD (data not shown) and SecF (Fig. 1B) were below a detectable level. The two membrane preparations have comparable polypeptide profiles (Fig. 1A) and similar amounts of SecY, SecA and YidC (Fig. 1B).

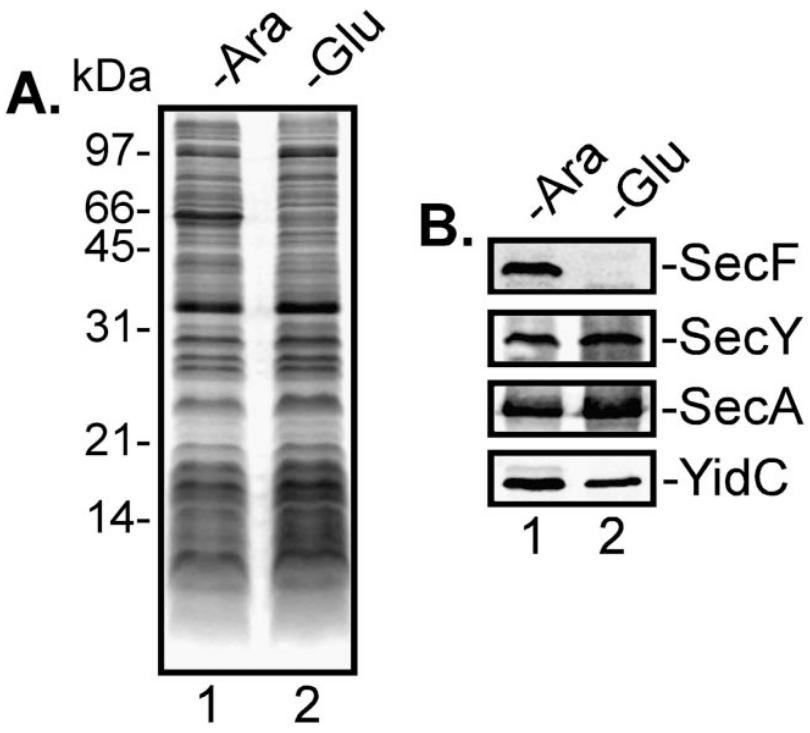

Fig. 1: SecF is below a detectable level after six generations on media containing 0.2% glucose. IMVs were prepared from strain NN103 grown until an OD_{660} of 0.6 in LB with 0.2 % arabinose (lane 1) or from the same cells that subsequently had been grown for 6 generations on LB media containing 0.2 % glucose (lane 2). (**A**) IMVs were analysed by SDS-PAGE and CBB staining or (**B**) by immunostaining using antibodies against SecF, SecY, YidC, and SecA. Positions of molecular weight markers (in kDa) are indicated.

Inner membrane vesicles depleted for SecDFyajC are able to maintain a proton motive force

Previously, Arkowitz and Wickner have shown that IMVs lacking SecDF are unable to maintain a stable PMF in the presence of succinate (11). Indeed, when we measured the two components of the PMF, i.e., the transmembrane pH gradient (ΔpH)

and electrical potential (Δψ), by the change in fluorescence of the pH- and potential-sensitive dyes 9-amino-6-chloro-2-methoxyacridine (ACMA) and oxonol V, respectively, the ΔpH and Δψ generated with 5 mM succinate was reduced in SecDFyajC-depleted IMVs as compared to SecDFyajC-containing IMVs (see for ΔpH measurements, Figs. 2A and 2B). Surprisingly, when instead of succinate, NADH was used as an electron donor, the generated ΔpH and Δψ in SecDFyajC-containing and -depleted IMVs was identical (See Figs. 2C and 2D for ΔpH measurements).

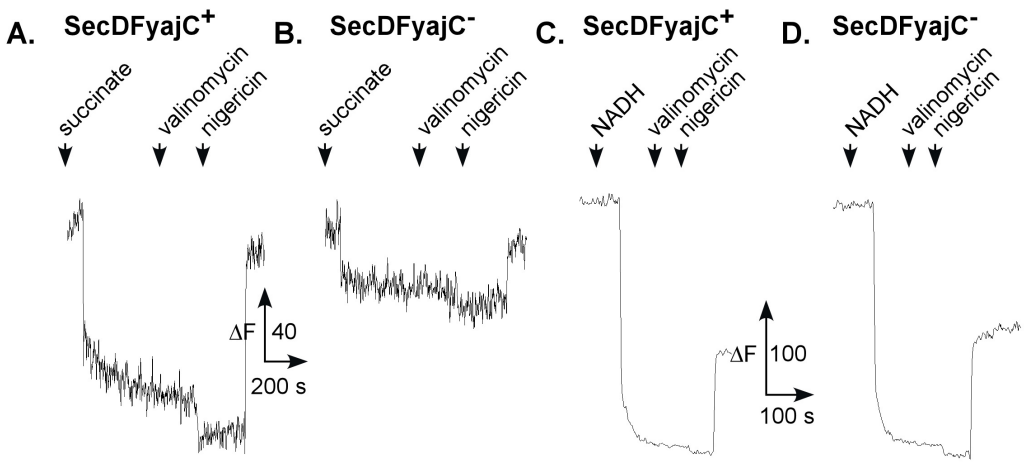

Fig. 2: Inner membrane vesicles depleted for SecDFyajC are able to maintain a proton motive force. The generation of ΔpH by IMVs from strain NN103 grown on arabinose or glucose media (to deplete SecDFyajC) was determined by monitoring the fluorescence quenching of ACMA. Where specified, 5 mM succinate (A, B) or 1.25 mM NADH (C, D) were added to the vesicles to generate a proton motive force. Valinomycin (1 μM) was used to convert the generated Δψ into a ΔpH. Subsequently, the ΔpH was dissipated by the addition of 0.25 μM nigericin.

To determine whether enzymes of the electron transport chain are affected by the depletion of SecDFyajC, the NADH oxidase and succinate dehydrogenase activity was measured. Whereas IMVs lacking SecDFyajC have a slightly higher NADH oxidase activity as compared to IMVs containing SecDFyajC, the succinate dehydrogenase activity was 50% reduced (Fig. 3A). Moreover, immunoblot analysis shows that the amount of SdhB, one of the three subunits of the succinate dehydrogenase complex, is reduced in IMVs lacking SecDFyajC (Fig. 3B). Similar

results were obtained with the parental *unc*$^{+}$ strain JP325 (MC4100, *tgt::kan*-P_{BAD}-*yajCsecDF*), indicating that the observed effects are neither caused by the *unc* mutation nor that they are strain-dependent.

Taken together, our results demonstrate that IMVs lacking SecDFyajC are able to maintain a proton motive force and indicate that the effect observed by Arkowitz and Wickner (11) is due to a reduced expression of the enzyme succinate dehydrogenase.

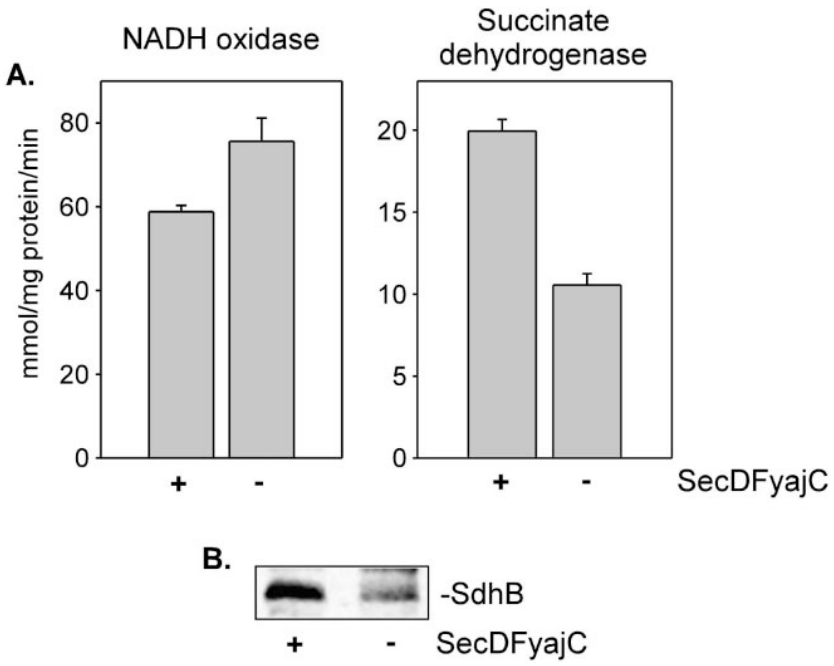

Fig. 3: Inner membrane vesicles lacking SecDFyajC show a reduced succinate dehydrogenase activity. (**A**) The NADH oxidase and succinate dehydrogenase activity of IMVs from *E. coli* strain NN103 containing and lacking SecDFyajC were determined as described in the Material and Methods. The results shown are the mean of 5 independent experiments. (**B**) IMVs (10 μg) from *E. coli* strain NN103 grown on arabinose or glucose media were analysed by SDS-PAGE, blotted onto PVDF membrane and immunostained with an antibody against FrdAB (this antibody cross-reacts with SdhB).

The proton motive force stimulates translocation of proOmpA into inner membrane vesicles lacking SecDFyajC

In vitro translocation of proOmpA is strictly dependent on ATP hydrolysis and stimulated by the PMF (Fig. 4; 267). To determine if the PMF in IMVs lacking SecDFyajC is able to stimulate translocation of saturating amounts proOmpA, the effect of the PMF on proOmpA translocation was determined. Without a PMF, the

ATP-dependent translocation of proOmpA into SecDFyajC-containing and -depleted IMVs is comparable (Fig. 4). Translocation is stimulated 2 to 3-fold when a PMF is generated by the addition of NADH. This result demonstrates that the PMF-stimulated proOmpA translocation is independent of the presence of SecDFyajC.

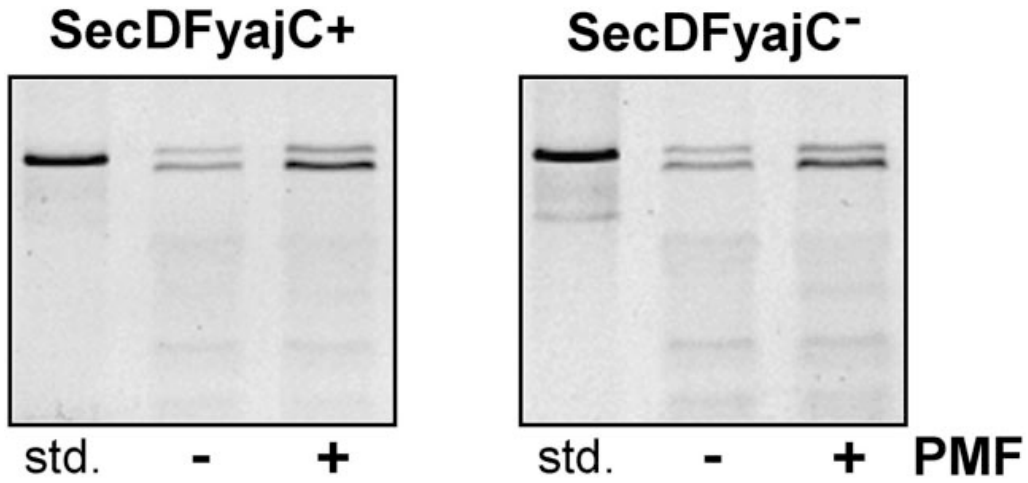

Fig. 4: *In vitro* translocation of proOmpA into inner membrane vesicles depleted for SecDFyajC in the absence and presence of a proton motive force. ProOmpA translocation was assayed at 0.5 mM ATP in the absence and presence of 5 mM NADH to generate a proton motive force. Reactions were carried out as described in Material and Methods.

Discussion

The mechanism by which SecDFyajC functions in precursor protein translocation is an unresolved question in the bacterial translocation field despite the fact that the genes have been identified in the late eighties. It has been suggested that SecDFyajC stimulates protein translocation by maintenance of the PMF as SecDFyajC-depleted IMVs were found to be impaired in the generation of the PMF (11). We now show that this observation is due to a reduced expression of succinate dehydrogenase, one of the enzymes of the electron transport chain. Depletion of SecDFyajC was achieved in our studies by growing cells harbouring the *secDF* operon under control of the arabinose promoter for several generations on glucose-containing medium. Glucose is known to repress several enzymes of the tricarboxylic acid cycle including succinate

dehydrogenase (*sdhCDAB*) operon, whereas the control cells that are grown on arabinose do not exhibit this repressing effect (189, 203). When instead of succinate, NADH is used to generate a PMF, no difference is observed between SecDFyajC-depleted and -containing IMVs. Moreover, the generated PMF in IMVs lacking SecDFyajC gives a 2- to 3-fold stimulation of proOmpA translocation, which is similar to SecDFyajC-containing IMVs. It has been reported that the effect of SecDFyajC on PMF-dependent proOmpA translocation only becomes apparent at low ATP concentrations (11, 62). However, even at 2 μM ATP addition of NADH gives a 2- to 3-fold stimulation of translocation in IMVs lacking SecDFyajC (data not shown). Summarizing, our data demonstrate that SecDFyajC is not involved in coupling protein translocation to the PMF, which is in agreement with the observation that the PMF stimulates translocation of proOmpA into proteoliposomes containing only SecYEG (28).

Our study shows that extreme care has to be taken when different growth conditions are used to deplete proteins *in vivo*. Pleiotropic effects do not only arise from the depletion of essential proteins but also the growth on glucose may result in the alteration of the expression levels of other proteins that may cause drastic changes in cell physiology.

Materials and Methods

Strains and plasmids

The *unc* deletion of *Escherichia coli* K003 (*lpp* D(*uncB-C*) *zid::Tn10*); 267) was introduced into *E. coli* SF100 (F^-, Δ*lac*X74, *gal*K, *thi*, *rps*L, *str*A Δ*pho*A(pvuII), Δ*omp*T) by P1 transduction (166) resulting in strain NN100. *E. coli* JP325 (*ara*Δ714, Δ[*argF-lac*]U169, *rps*L150, *rel*A1, *thi*, *flb*5301, *deo*C1, *pts*F25, *recA*::*cat*, *tgt::kan-ara*C^+-P_{BAD}*::yajCsecDF*) (67) was a generous gift of Jon Beckwith (Harvard University, Boston, USA). The *tgt::kan-ara*C^+-P_{BAD}*::yajCsecDF* mutation was transduced to *E. coli* NN100 giving NN103.

Replacement of Ser66 in the mature region of OmpA was done using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) and plasmid pET2345 (cys-less *ompA*; 51) as template. The *BamHI/HindIII* fragment from the resulting

plasmid (pBluescript-*ompA* S66C) was cloned into pJF118 E/H giving pNN207. In the resulting plasmid, the mutant *E. coli ompA* gene is under *tac* promoter control.

Materials

SecA (30), SecB (258) and proOmpA S66C (41) were purified as described. Labelling of the unique cysteine in proOmpA with fluorescein-5-maleimide (Molecular Probes) has been described elsewhere (51). Proteinase K, sodium succinate, reduced form of nicotinamide adenine dinucleotide (NADH), ATP, valinomycin, nigericin, phenylmethylsulfonyl fluoride (PMSF), dichlorophenolindophenol and phenazine ethosulfate were purchased from Sigma. 9-amino-6-chloro-2-methoxyacridine (ACMA) and oxonol V were from Molecular Probes. IMVs were prepared as described (124). Antibodies against *E. coli* fumarate reductase (FrdAB) that cross-react with the SdhB subunit of succinate dehydrogenase were generously provided by Joel Weiner (University of Alberta, Edmonton, Canada).

Growth conditions

To deplete SecDFyajC, cells of *E. coli* strain NN103 were grown until an OD_{660} of 0.6 on LB containing 0.2% arabinose, harvested, washed in medium without arabinose and resuspended in LB containing 0.2% glucose until an OD_{660} of 0.3. Cells were further grown to an OD_{660} of 0.6 and diluted two-fold with LB containing 0.2% glucose and this procedure was repeated for 6 generations. During this process, kanamycin was present at a concentration of 25 μg/ml.

Determination of ΔpH and Δψ

The generation of transmembrane pH gradient (ΔpH) and electrical potential (Δψ) in IMVs was monitored by the fluorescence quenching of ACMA and oxonol V, respectively. The reaction mixture (total volume 2 ml) contained buffer A (50 mM HEPES/KOH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mg/ml BSA, 2 mM DTT), IMVs (with NADH and succinate, 50 and 200 μg of membrane protein was used, respectively) and 1 μM ACMA or oxonol V. The solution was kept at 30°C. To generate a PMF, NADH (1.25 mM final concentration) or succinate (5 mM final concentration) was added to the reaction mixture. The fluorescence emission of ACMA was measured at 474 nm with excitation at 409 nm (slit: 3 nm) and that of

oxonol V at 634 nm with excitation at 599 nm (slit: 10 nm) using a Perkin Elmer LS50B Luminescence Spectrometer.

NADH oxidase and succinate dehydrogenase activity

NADH oxidase activity of IMVs was determined at 30°C using 10 μg/ml membrane protein and 200 μM NADH in buffer A. Reactions were initiated by addition of NADH and monitored at 340 nm. Succinate dehydrogenase activity of IMVs was determined at 30°C using 75 μg/ml membrane protein and 5 mM succinate in buffer B (50 mM HEPES/KOH pH 7.8, 1 mM KCN, 0.25 μM EDTA, 50 μM dichlorophenolindophenol, 1.5 mM phenazine ethosulfate; 155). Reactions were initiated by addition of succinate and monitored at 600 nm.

In vitro translocation

Translocation of proOmpA-fluorescein (1.25 μg/ml) into IMVs was performed in buffer A with SecA (10 μg/ml), SecB (35 μg/ml), 0.5 mM ATP, 10 mM phosphocreatine and 50 μg/ml creatine kinase. After warming the mixture for 2 min at 37°C, the reaction was initiated by addition of IMVs (200 μg/ml) and terminated after 5 min by chilling on ice. To generate a PMF, 5 mM NADH was added. Samples were treated with proteinase K (0.1 mg/ml) for 30 min on ice, precipitated with 5% TCA, washed with ice-cold acetone and analysed by 12% SDS-PAGE and protease protected proOmpA was visualized with a Roche Lumi-Imager F1.

Acknowledgements

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