Increased expression of the bacterial glycolipid MPIase is required for efficient protein translocation across membranes in cold conditions

Katsuhiro Sawasato¹, Sonomi Suzuki², Ken-ichi Nishiyama¹,²

From ¹the United Graduate School of Agricultural Sciences, Iwate University, Morioka, Iwate 020-8550, Japan; ²Department of Biological Chemistry and Food Sciences, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan.

Running title: MPIase increases in the cold

*To whom correspondence should be addressed: Ken-ichi Nishiyama: Department of Biological Chemistry and Food Sciences, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan; nishiyama@iwate-u.ac.jp; Tel. +81-19-621-6471; Fax. +81-19-621-6243.

Keywords: glycolipid, low temperature, MPIase (membrane protein integrase), protein translocation, protein integration, lipochaperone, cold sensitivity, membrane fluidity, cell stress

ABSTRACT

Protein integration into and translocation across biological membranes are vital events for organismal survival and are fundamentally conserved among many organisms. MPIase (membrane protein integrase) is a glycolipid that drives membrane protein integration into the cytoplasmic membrane in *Escherichia coli*. MPIase also stimulates protein translocation across the membrane, but how its expression is regulated is incompletely understood. In this study, we found that the expression level of MPIase significantly increases in the cold (< 25°C), whereas that of the SecYEG translocon does not. Using previously created gene-knockout *E. coli* strains, we also found that either the *cdsA* or *ynbB* gene, both encoding rate-limiting enzymes for MPIase biosynthesis, is responsible for the increase in the MPIase expression. Furthermore, using pulse-chase experiments and protein integration assays, we demonstrated that the increase in MPIase levels is important for efficient protein translocation, but not for protein integration. We conclude that MPIase expression is required to stimulate protein translocation in cold conditions and is controlled by *cdsA* and *ynbB* gene expression.

Protein integration into and protein translocation across biological membranes are vital events for all organisms to survive. The mechanisms underlying protein integration and protein translocation are
conserved in all organisms at the fundamental level. In a model organism, *Escherichia coli*, both protein integration and protein translocation proceed with the aid of SecYEG translocon (1-4). It is also known that several membrane proteins of small size or with a C-terminal transmembrane stretch are integrated independently of the SecYEG translocon (5,6). A lot of mutants defective in protein integration and protein translocation have been isolated, most of which are cold-sensitive. Moreover, protein translocation even in the wild type cells includes a cold-sensitive step (7). Although it is assumed that the alteration in membrane fluidity that accompanies a temperature change is involved in the cold sensitivity of these reactions, the mechanism underlying the cold sensitivity is totally unknown.

We have identified and characterized an integration factor named MPIase, essential for protein integration by means of a reconstitution system (8-10). MPIase has turned out to be a glycolipid that drives membrane protein integration (9). MPIase also stimulates protein translocation (10,11). MPIase interacts with SecYEG to transform its dimer orientation into the “side-by-side” structure (11), whereas SecYEG without MPIase forms a dimer referred to as the “back-to-back” structure, in which SecE lies at the dimer interface (11-13). The translocation activity of the side-by-side structure of the SecYEG dimer is ~10-fold higher as compared with that of the back-to-back structure (11). SecG repeats the cycle of topology inversion coupled with a structural change of SecA to stimulate protein translocation (14,15). MPIase is essential for the topology inversion of SecG (11). Protein translocation in ΔsecG strains is severely impaired in the cold like that in most sec mutants (16).

Recently, we demonstrated that MPIase is essential for membrane protein integration and stimulates protein translocation in vivo as well as in vitro (17). We identified CdsA and YnbB as rate-limiting enzymes for MPIase biosynthesis (17). CdsA is well known as a CDP-diacylglycerol (CDP-DAG) synthase involved in phospholipid biosynthesis (18,19). YnbB is paralogous to CdsA (20). CdsA is essential for cell viability, while YnbB is not (21). We demonstrated that these enzymes synthesize GlcNAc-PP-DAG (Compound I), the first intermediate during MPIase biosynthesis, by incorporating GlcNAc-P into CDP-DAG (17). CdsA synthesizes CDP-DAG from phosphatidic acid and CTP, subsequently GlcNAc-P is transferred on CdsA, and then CDP-DAG is converted to compound I on CdsA (17). Overproduction of CdsA and YnbB results in an increase in the MPIase level correlated with the extent of the overproduction of these enzymes. Depletion of CdsA caused MPIase depletion with a severe defect in the processing of M13 procoat and pOmpA. On the other hand, disruption of the *ynbB* gene has only a minor effect on the MPIase level, suggesting that CdsA mainly contributes to
the synthesis of MPIase.

In this study, we found that the expression level of MPIase increases specifically at low temperature, whereas that of SecYEG does not change (16). Either the *cdsA* or *ynbB* gene is involved in the increase in the MPIase level in the cold. When the MPIase level is repressed, protein translocation is impaired in the cold. On the other hand, protein integration is not affected under these conditions. Thus, the increase in the MPIase level is important to stimulate protein translocation at low temperature.

**Results**

The expression level of MPIase specifically increases at low temperature

We examined the effect of temperature on the expression level of MPIase. Wild-type cells (EK413) were cultivated at 37°C until they reached the early log phase and then were transferred to 20°C or 42°C. After the cultivation has been continued for 3 h, the MPIase level was determined by quantitative immunoblotting using anti-MPIase antisera. The expression level of MPIase was slightly decreased at 42°C compared with at 37°C (Fig. 1A). On the other hand, the MPIase level significantly increased at 20°C (Fig. 1A). The expression level of SecY remained unchanged at each temperature examined (Fig. 1A). The SecA level slightly increased at 20°C compared with at 37°C, consistent with the previous report (22). Next, we examined the expression level of MPIase at each temperature from 15°C to 48°C. We observed that the MPIase level increased as the temperature decreased (Fig. 1B and C). Below 25°C, the MPIase level was the same (Fig. 1B and C). The expression level of MPIase in the cold was ~7-fold higher as compared with that at 48°C (Fig. 1C). These results indicate that the expression level of MPIase specifically increases at low temperature. Next, we carried out the temperature shift experiments, monitoring the MPIase level. EK413 cells, grown at 15°C, were shifted to 42°C. After the heat shock, the cells hardly grew until 30 min and started to grow after 1 h. The MPIase level decreased as the cultivation time at high temperature (Figs. 1D and E), suggesting that the excess amounts of MPIase should have been degraded in the cells to fit the new environment through the system for stress response/adaptation. It is not likely that MPIase was degraded by non-enzymatic reaction under high temperature, and that MPIase was diluted because of cell proliferation at high temperature, considering that the cells hardly grew until 30 min.

The *cdsA* and *ynbB* genes are involved in the increase in the MPIase level

Since CdsA and YnbB are the rate-limiting enzymes for MPIase biosynthesis (17), these enzymes might be involved in the increase in the MPIase level at low temperature. In the previous study, we constructed a *ynbB* knockout (KS21), a *cdsA* knockout (KS22), and a *ynbB/cdsA* double
knockout (KS23) (17) (Table 1). CdsA is essential for cell viability, while YnbB is not as KS21 grew as well as EK413 at 37°C (17). In KS22 and KS23, CdsA is expressed from the complementary plasmid pAra-CdsA, in which the cdsA gene is under the control of the arabinose promoter. These strains can grow as well as EK413 in the presence of inducer arabinose at 37°C, while they cannot grow in the absence of arabinose because of MPIase depletion (17). We examined the effect of disruption of the cdsA or ynbB gene on the chromosome on the increase in the MPIase level at low temperature. We observed an increase in the MPIase level in KS21 and KS22/pAra-CdsA at 20°C as well as EK413. On the other hand, the increase in the MPIase level was significantly repressed in KS23/pAra-CdsA (Fig. 2A and B). These results indicate that either the cdsA or ynbB gene on the chromosome is involved in the increase in the MPIase level at low temperature. Under these conditions, phospholipid biosynthesis was not affected, since no PA accumulation was observed (17). The expression level of CdsA increased at low temperature, in correlation with the increase in the amount of MPIase at 20°C (Fig. 2C). Consistent with these observations, Icho et al. reports that a plasmid with 5.8 kb DNA fragment containing the cdsA gene directed the overproduction of CdsA at 30°C but not at 42°C (19).

An increase in the MPIase level is important for efficient protein translocation at low temperature

MPIase stimulates protein translocation by affecting the dimer orientation of SecYEG and is essential for topology inversion of SecG (11). To examine the effect of repression of the increase in the MPIase level on protein translocation at low temperature, processing of the signal (or leader) sequence of OmpA was monitored by means of pulse-chase experiments. In the previous study, no accumulation of pOmpA was observed at 37°C in KS21, KS22/pAra-CdsA or KS23/pAra-CdsA (17), although the MPIase levels in these strains are slightly less than that in EK413 (Fig. 2A and B), indicating that these strains have a sufficient amount of MPIase to stimulate protein translocation at 37°C. In contrast, we observed slight accumulation of pOmpA at 20°C in KS21 and KS22/pAra-CdsA (Fig. 3A and B). Moreover, a severe defect in translocation of pOmpA was observed in KS23/pAra-CdsA (Fig. 3A and B), while it was not as much as under MPIase-depleted conditions (ΔYnbB/ΔCdsA) (Fig. 3A and B). The expression levels of SecYEG and SecD were similar in all strains (Fig. 3C), indicating that the defect in protein translocation observed in KS23/pAra-CdsA, supplemented with arabinose, was caused by repression of the increase in the MPIase level. These results indicate that the increase in the MPIase is important for efficient protein translocation at low temperature.

Reduction of the MPIase level causes a
severe defect in protein translocation at low temperature

Tam41p is a mitochondrial CDP-DAG synthase (23). When Tam41p lacking the mitochondrial targeting signal was expressed in E. coli, it synthesized CDP-DAG but not compound I (17). Expression of Tam41p reduced PA, since CdsA and YnbB compete with Tam41p for PA. We re-examined the effects of Tam41p overproduction in detail at the MPIase level and protein translocation at 37°C. Tam41p was mildly expressed from a low copy plasmid in which the TAM41 gene was placed under the tet promoter (pTet-Tam41p). Introduction of pTet-Tam41p into EK413 resulted in a slight reduction in the MPIase level with slight accumulation of pOmpA (Fig. 4A and B). Overproduction of Tam41p from pTac-Tam41p caused a significant reduction in the MPIase level (Fig. 4A) with a severe defect in pOmpA translocation of the same extent as that on MPIase-depletion (Fig. 4B and C).

Next, we examined the effect of Tam41p expression on the increase in the MPIase level at low temperature. Tam41p was mildly expressed from pTet-Tam41p. While the expression level of MPIase in EK413/pTet-Tam41p increased at 20°C, the level was half that in EK413 with the empty vector (Fig. 5A), indicating that Tam41p inhibits the increase in the MPIase level at low temperature. Tam41p expression also reduced the MPIase level at low temperature in KS21, KS22/pAra-CdsA and KS23/pAra-CdsA (Fig. 5B and C). In particular, the MPIase level in KS22 was as low as that in KS23 (Fig. 5B and C). In contrast to the results at 37°C (Fig. 4B and C), the inhibition of the increase in the MPIase level on Tam41p expression caused a severe defect in protein translocation at low temperature (Fig. 5D and E). It is noteworthy that the translocation of pOmpA in KS22/pAra-CdsA and KS23/pAra-CdsA with Tam41p expression was impaired to the same extent as under MPIase-depleted conditions (∆YnbB/∆CdsA) at 20°C (Fig. 5D and E), although these strains both have a detectable amount of MPIase (Fig. 5B and C), indicating that the amount of MPIase in these strains is not sufficient to stimulate protein translocation at 20°C. The expression of Tam41p has no effect on the expression level of SecYEG or SecD (Fig. 5F), indicating that the retardation of pOmpA translocation at low temperature was caused by the decrease in the MPIase level. From these results, we concluded that the amount of MPIase needed to stimulate protein translocation increases at low temperature.

Increase in the expression level of MPIase is not necessary for efficient protein integration at low temperature

MPIase is essential for protein integration both in vivo and in vitro (9,17). When MPIase was depleted, M13 procoat was never processed into the mature form (17). To examine the effect of repression of the increase in the MPIase level on protein
integration at low temperature, we examined M13 procoat integration in KS23/pAra-CdsA with Tam41p. Accumulation of M13 procoat was not observed even when the cells were cultivated at 20°C, while M13 procoat accumulation was observed under MPIase-depleted conditions (Fig. 6A). Pulse-chase experiments revealed that M13 procoat was efficiently processed into M13 coat in KS23/pAra-CdsA with Tam41p (Fig. 6B). These results indicate that the reduction in the level of MPIase in KS23/pAra-CdsA is sufficient for protein integration at low temperature.

Next, we further examined the effect of repression of the increase in MPIase by means of the in vitro integration assay. It is reported that YidC is also required for M13 procoat integration (24). The expression level of YidC was similar in each strain (Fig. 6C). To avoid complexity due to signal (or leader) processing, we used M13 procoat H5 with an uncleavable signal (or leader) sequence (25). M13 procoat H5 (Fig. 6D) and 3L-Pf3 coat (Fig. 6E) were efficiently integrated at 20°C into INV prepared from KS23/pAra-CdsA and KS23/pAra-CdsA with Tam41p expression, cultivated at 20°C, as well as ones prepared from EK413, consistent with the results of in vivo analysis. These results again demonstrated that the increase in the MPIase is not necessary for efficient protein integration at low temperature. Taking these results together, we conclude that the increase in the MPIase level is necessary for efficient protein translocation at low temperature, but it is not necessary for protein integration.

The effect of repression of the increase in the MPIase level on cell growth at low temperature

In the previous study, we demonstrated that MPIase is essential for cell viability (17). We examined the effect of repression of the increase in the MPIase on cell growth at low temperature. The results are summarized at Table 2. KS23/pAra-CdsA grew as well as other strains on LB plates although pOmpA translocation was impaired in this strain at low temperature. On M9 plates, the colony size of this strain was slightly smaller than those of other strains. The expression of Tam41p inhibited the increase in the MPIase level at low temperature with a severe defect in pOmpA translocation of the same extent as under MPIase-depleted conditions (Fig. 5D and F), but M13 procoat was efficiently integrated (Fig. 6). We examined the effect of Tam41p expression on cell growth at low temperature. EK413 cells harboring pTet-Tam41p grew as well as EK413 cells on LB agar plates. On the other hand, the growth of KS21, KS22, and KS23 was slightly affected by Tam41p expression. The growth defect was prominent on M9 plates. Tam41p expression caused a slight growth defect in EK413 and KS21. Furthermore, the growth of KS22 and KS23 was significantly affected. All these results indicate that the increase in the MPIase level is not essential for cell growth.
MPIase increases in the cold

growth at low temperature on rich and minimum media.

Discussion
In this study, we found that the expression level of MPIase significantly increased at low temperature. It has been thought that there is a cold-sensitive step in protein export itself (7), including protein translocation across the cytoplasmic membrane of E. coli. This cold-sensitivity is accounted for by the low fluidity of biological membranes in the cold (26). However, the mechanisms underlying the cold-sensitivity and the strategy for overcoming the stress are totally unknown. The increase in unsaturated fatty acids in the cold to increase the membrane fluidity (27) does not fully represent the strategy. Moreover, the expression levels of export factors including SecYEG, SecD and YidC did not change. Only the MPIase level was upregulated in a cold environment. We found that the failure in increasing the MPIase level in the cold caused a severe defect in protein translocation, indicating that a higher level of MPIase is necessary for protein translocation in the cold. Therefore, the increase in the MPIase level is a stress response to cold stress. On the other hand, such an increase in MPIase is not necessary for the Sec-independent protein integration that is absolutely MPIase-dependent (9,17).

The cdsA or ynbB gene was found to be responsible for the increase in the MPIase level in the cold. Recently, we reported that both CdsA and YnbB are biosynthetic enzymes not only for phospholipids, but also for MPIase (17). The finding that Tam41p, a mitochondrial CDP-DAG synthase (23), which can biosynthesize phospholipids but not MPIase in E. coli (17), inhibited MPIase biosynthesis, supports these functions of CdsA and YnbB. Since the reaction catalyzed by CdsA and YnbB is rate-limiting for MPIase biosynthesis, the expression levels of these proteins correspond to that of MPIase, i.e., depletion of CdsA/YnbB causes MPIase depletion and overproduction of CdsA/YnbB causes MPIase overproduction (17). When the MPIase level increased in the cold, the CdsA level also increased.

Why is a higher level of MPIase necessary for protein translocation but not for Sec-independent integration in the cold? The reason would be the difference in the mode of action of MPIase between protein translocation and protein integration. MPIase stimulates protein translocation by affecting the dimer structure of SecYEG (11). In the absence of MPIase, SecYEG forms a ‘back-to-back’ dimer, in which SecE lies at the dimer interface, as revealed by the crystal structure (13) and crosslinking studies (12). On the other hand, MPIase transforms SecYEG into the ‘side-by-side’ structure, as SecG but not SecE is crosslinked in the presence of MPIase (11). The side-by-side SecYEG is ~10 fold more active than the back-to-back SecYEG (11). The cycle of topology inversion of SecG (14,15) occurs
only when SecYEG forms the side-by-side dimer (11), indicating that the side-by-side structure is necessary for efficient protein translocation. Since SecYEG undergoes dynamic structural changes during the catalytic cycle of translocation including SecG inversion (11,14,15), it is plausible that the translocation reaction does not proceed easily under conditions where the membrane fluidity is low in the cold. To maintain the side-by-side structure of SecYEG and subsequently to support the dynamic changes of SecYEG during protein translocation in the cold, a higher level of MPIase would be necessary. Consistent with this notion, the extent of the defect in protein translocation observed in the ΔsecG mutants (16) is quite similar to that in the MPIase-depleted strains (17). Moreover, a similar extent of the translocation defect has been observed in the secAcsR11 mutant, in which SecG inversion is inhibited in the cold (15,28). On the other hand, in Sec-independent integration, MPIase directly interacts with substrate membrane proteins (9) possibly working like a ‘lipochaperone’ or a ‘glycochaperone’, and then integrates them into membranes, at least at the initial step of integration (9,29). An increase in the MPIase level would not be necessary for this process.

Although the colony size was smaller when MPIase did not increase in the cold, the growth defect was not so significant. In E. coli, protein translocation can occur post-translationally (1,2). If the precursor proteins accumulated in the cytosol could be translocated in the meantime, essential functions in the cell envelope would express resultantly. In such cases, cell with defects in protein translocation would grow, albeit slowly. Consistently, the growth phenotype of SecG-deficient strains is weak in the cold, while the defects in protein translocation are significant (16). On the other hand, membrane proteins must be integrated into the cytoplasmic membrane co-translationally (4). Therefore, defects in protein integration should directly lead to cell death. We also reported that MPIase is involved in Sec-dependent integration (10). The weak growth phenotype, observed in the absence of the increase in the MPIase level in the cold, suggests that the dynamic structural change of SecYEG is not necessary for Sec-dependent as well as Sec-independent integration.

We could not exclude the possibility that the increase in the MPIase level was caused by a decrease in the rate of degradation of it in the cold, however, this is not likely for the following reasons. (i) The expression level of CdsA indeed increased in the cold (Fig. 2C), which should have caused the increase in the MPIase level, since CdsA is the rate-limiting enzyme for MPIase biosynthesis (17). (ii) The increase in the MPIase level was significantly repressed in the ynbB/cdsA knockout, when cdsA was expressed from the arabinose promoter. Also, the increase in the MPIase level in the cold was inhibited by Tam41p. If the decrease in the degradation rate was responsible for the
increase, the MPIase level should increase under such conditions. (iii) Protein translocation was severely inhibited when MPIase was not increased in the cold. Therefore, cells should positively make an effort to increase the MPIase level in the cold to stimulate protein translocation.

The mechanisms underlying the stimulation of protein translocation by MPIase are totally unknown. While MPIase is co-precipitated with SecYEG, indicating that MPIase directly interacts with SecYEG (11), the stoichiometry, the interacting partner(s), and the domains for interaction are unclear. The finding that the amount of MPIase necessary for the stimulation increases in the cold would be a clue for solving how MPIase stimulates protein translocation.

**Experimental procedures**

**Materials**

The *E. coli* strains and plasmids used in this study are listed in Table 1. Anti-MPIase antibody was raised in rabbits against the purified MPIase, chemically crosslinked to keyhole limpet hemocyanin (9). Antibodies against SecE (30), SecG (14), SecD (31), SecB (32), and OmpA (33) were raised in rabbits using the respective purified proteins, while those against CdsA (Glu75–Ser92) (17), M13 procoat (Ala24–Tyr44) (17), YidC (Lys382–Gln402) (17), and SecY (Ser426–Arg443) (34) were raised against the respective synthetic peptides. All the antibodies were obtained through commercially available custom services. The antibody against Tam41p was a gift from Dr. Y. Tamura (Yamagata Univ.). [35S] EXPRESS Protein Labeling Mix, a mixture containing [35S] Met and [35S] Cys (~37 TBq/mmol), was obtained from Perkin Elmer, Inc.

**Detection of MPIase by immunoblotting**

Cell culture (0.5 mL) was treated with 5%, and then precipitates were recovered by centrifugation (10,000 x g, 5 min), followed by washing with acetone. They were applied to the SDS gel (35). After electrophoresis, proteins/glycolipids were transferred to PVDF membrane as described (30). After the membrane was dried, it was soaked in 0.1% poly(isobutyl methacrylate) in hexane for 75 s at room temperature. The dried membrane was then soaked in 10% horse serum in TBS buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.5 mM KCl) for 30 min, followed by incubation with anti-MPIase antibody for 2 h. The immunodecorated band was then visualized, as described (36).

**Pulse-chase experiments and immunoprecipitation**

An overnight culture was inoculated at 1:100 into M9 medium supplemented with 18 amino acids (10 μM each) other than Met and Cys. Where specified, 0.2% arabinose was added to induce CdsA. When the growth reached the early log phase at 37°C, cells were
transferred to 20°C, and cultivation was continued for 2 h. [35S] EXPRESS Protein Labeling Mix was added at ~100 kBq/ml to allow labeling for 1.5 min. Labeling was terminated by adding cold Met and Cys (12 mM each). After the specified chase time, 500 μL of the culture was withdrawn and cellular proteins were precipitated with 5% TCA. The TCA precipitates were washed with acetone, and then solubilized in 50 μL of 50 mM Tris-HCl (pH 7.5), 1% SDS, 1 mM EDTA. The samples were boiled for 3 min, and then diluted with 1 mL of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride. The insoluble materials were removed by centrifugation (10,000 x g 5 min at 4°C). Two μL of antiserum against either OmpA or M13 coat was added to the supernatant, and then the mixture was incubated overnight at 4°C. The mixture received 20 μL of protein A Sepharose (GE Healthcare), and then was kept at 4°C for 60 min. After brief centrifugation, the resin was washed with 500 μL of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, and 500 μL of 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, successively. The washed resin was suspended in 20 μL of the sample buffer used for SDS-PAGE and then boiled for 3 min. The immunoprecipitated materials were analyzed by SDS-PAGE and visualized with a Phosphorimager (GE Healthcare).

**Assaying of protein integration in vitro**

Substrate membrane protein was in vitro synthesized by means of a pure system, i.e., a reconstituted translation system composed of purified components (8,37), a gift from Prof. T. Ueda (the Univ. of Tokyo). The reaction mixture (20 μL) containing INV (0.2 mg protein/mL), plasmid DNA, and [35S] methionine and cysteine (~10 MBq/mL) was incubated for 60 min at 20°C. After the reaction had been terminated by chilling on ice, the mixture was divided into two parts. One part (3 μL) was treated with 5% trichloroacetic acid to precipitate proteins. The other part (15 μL) was mixed with an equal volume of proteinase K (1 mg/mL), followed by incubation for 20 min at 25°C. After TCA (5%) had been mixed in, samples were incubated for 5 min at 56°C to inactivate proteinase K, followed by recovery of proteins by centrifugation (10,000 x g for 5 min at 4°C). Radioactive materials were separated by SDS-PAGE and then detected by autoradiography.

**Other methods**

SDS-PAGE was carried out using 12.5% acrylamide-0.27% N,N'-bismethyleneacrylamide containing 6 M urea (10) for M13 (pro)coat and 3L-Pf3 coat or not containing it (35) for SecE, SecG, SecB and MPIase. Gels composed of 10% acrylamide-0.13% N,N'-bismethyleneacrylamide (38) were used to analyze OmpA, SecD and SecA, 13.5% acrylamide-0.33% N,N'-bismethyleneacrylamide (38) to analyze...
CdsA, and 15% acrylamide-0.33% N,N’-bismethyleneacrylamide (38) to analyze SecY. The radioactive bands were quantitated by mean of ImageQuant TL software (GE Healthcare). Bands on immunoblots were quantitated by mean of a CS analyzer (ATTO). Proteins were quantitated using bovine serum albumin as a standard (39).
**Acknowledgements:** We thank Prof. T. Ueda (The Univ. of Tokyo) for the pure system optimized for the integration assays, Dr. Y. Tamura (Yamagata Univ.) for the anti-Tam41p antibody, and Ms M Saikudo and Ms M. Saikudo for the technical assistance. K.S. was supported by a SUNBOR scholarship (Suntory Foundation for Life Sciences).

**Conflicts of interest:** The authors declare that they have no conflicts of interest with the contents of this article.
References

1. du Plessis, D. J., Nouwen, N., and Driessen, A. J. (2011) The Sec translocase. Biochim. Biophys. Acta 1808, 851-865
2. Beckwith, J. (2013) The Sec-dependent pathway. Res. Microbiol. 164, 497-504
3. Facey, S. J., and Kuhn, A. (2004) Membrane integration of E. coli model membrane proteins. Biochim. Biophys. Acta 1694, 55-66
4. Xie, K., and Dalbey, R. E. (2008) Inserting proteins into the bacterial cytoplasmic membrane using the Sec and YidC translocases. Nat Rev Microbiol 6, 234-244
5. Kiefer, D., and Kuhn, A. (2018) YidC-mediated membrane insertion. FEMS Microbiol. Lett. 365
6. Nishiyama, K., and Shimamoto, K. (2014) Glycolipozyme membrane protein integrase (MPIase): recent data. Biomol. Concepts 5, 429-438
7. Pogliano, K. J., and Beckwith, J. (1993) The Cs sec mutants of Escherichia coli reflect the cold sensitivity of protein export itself. Genetics 133, 763-773
8. Nishiyama, K., Maeda, M., Abe, M., Kanamori, T., Shimamoto, K., Kusumoto, S., Ueda, T., and Tokuda, H. (2010) A novel complete reconstitution system for membrane integration of the simplest membrane protein. Biochem. Biophys. Res. Commun. 394, 733-736
9. Nishiyama, K., Maeda, M., Yanagisawa, K., Nagase, R., Komura, H., Iwashita, T., Yamagaki, T., Kusumoto, S., Tokuda, H., and Shimamoto, K. (2012) MPIase is a glycolipozyme essential for membrane protein integration. Nat. Commun. 3, 1260
10. Nishiyama, K., Ikegami, A., Moser, M., Schiltz, E., Tokuda, H., and Muller, M. (2006) A derivative of lipid A is involved in signal recognition particle/SecYEG-dependent and -independent membrane integrations. J. Biol. Chem. 281, 35667-35676
11. Moser, M., Nagamori, S., Huber, M., Tokuda, H., and Nishiyama, K. (2013) Glycolipozyme MPIase is essential for topology inversion of SecG during preprotein translocation. Proc. Natl. Acad. Sci. U. S. A. 110, 9734-9739
12. Kaufmann, A., Manting, E. H., Veenendaal, A. K., Driessen, A. J., and van der Does, C. (1999) Cysteine-directed cross-linking demonstrates that helix 3 of SecE is close to helix 2 of SecY and helix 3 of a neighboring SecE. Biochemistry 38, 9115-9125
13. Breyton, C., Haase, W., Rapoport, T. A., Kuhlbrandt, W., and Collinson, I. (2002) Three-dimensional structure of the bacterial protein-translocation complex SecYEG. Nature 418, 662-665
14. Nishiyama, K., Suzuki, T., and Tokuda, H. (1996) Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. Cell 85, 71-81
15. Suzuki, H., Nishiyama, K., and Tokuda, H. (1998) Coupled structure changes of SecA
and SecG revealed by the synthetic lethality of the secAcSRI1 and ΔsecG:kan double mutant. *Mol. Microbiol.* **29**, 331-341

16. Nishiyama, K., Hanada, M., and Tokuda, H. (1994) Disruption of the gene encoding p12 (SecG) reveals the direct involvement and important function of SecG in the protein translocation of *Escherichia coli* at low temperature. *EMBO J.* **13**, 3272-3277

17. Sawasato, K., Sato, R., Nishikawa, H., Iimura, N., Kamemoto, Y., Fujikawa, K., Yamaguchi, T., Kuruma, Y., Tamura, Y., Endo, T., Ueda, T., Shimamoto, K., and Nishiyama, K. I. (2019) CdsA is involved in biosynthesis of glycolipid MPIase essential for membrane protein integration in vivo. *Sci Rep* **9**, 1372

18. Sparrow, C. P., and Raetz, C. R. (1985) Purification and properties of the membrane-bound CDP-diglyceride synthetase from *Escherichia coli*. *J. Biol. Chem.* **260**, 12084-12091

19. Icho, T., Sparrow, C. P., and Raetz, C. R. (1985) Molecular cloning and sequencing of the gene for CDP-diglyceride synthetase of *Escherichia coli*. *J. Biol. Chem.* **260**, 12078-12083

20. Sato, R., Sawasato, K., and Nishiyama, K. I. (2019) YnbB is a CdsA paralogue dedicated to biosynthesis of glycolipid MPIase involved in membrane protein integration. *Biochem. Biophys. Res. Commun.* **510**, 636-642

21. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 2006 0008

22. Murakami, A., Nakatogawa, H., and Ito, K. (2004) Translation arrest of SecM is essential for the basal and regulated expression of SecA. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 12330-12335

23. Tamura, Y., Harada, Y., Nishikawa, S., Yamano, K., Kamiya, M., Shiota, T., Kuroda, T., Kuge, O., Sesaki, H., Imai, K., Tomii, K., and Endo, T. (2013) Tam41 is a CDP-diacylglycerol synthase required for cardiolipin biosynthesis in mitochondria. *Cell Metab.* **17**, 709-718

24. Samuelson, J. C., Chen, M., Jiang, F., Moller, I., Wiedmann, M., Kuhn, A., Phillips, G. J., and Dalbey, R. E. (2000) YidC mediates membrane protein insertion in bacteria. *Nature* **406**, 637-641

25. Kuhn, A., and Wickner, W. (1985) Conserved residues of the leader peptide are essential for cleavage by leader peptidase. *J. Biol. Chem.* **260**, 15914-15918

26. Wickner, W., Driessen, A. J., and Hartl, F. U. (1991) The enzymology of protein translocation across the *Escherichia coli* plasma membrane. *Annu. Rev. Biochem.* **60**, 14
MPiase increases in the cold

101-124
27. Marr, A. G., and Ingraham, J. L. (1962) Effect of temperature on the composition of fatty acids in *Escherichia coli*. *J. Bacteriol.* **84**, 1260-1267
28. Suzuki, H., Nishiyama, K., and Tokuda, H. (1999) Increases in acidic phospholipid contents specifically restore protein translocation in a cold-sensitive secA or secG null mutant. *J. Biol. Chem.* **274**, 31020-31024
29. Nishikawa, H., Sasaki, M., and Nishiyama, K. (2017) Membrane insertion of Fo subunit of FoF1 ATPase depends on glycolipoyzme MPiase and is stimulated by YidC. *Biochem. Biophys. Res. Commun.* **487**, 477-482
30. Nishiyama, K., Mizushima, S., and Tokuda, H. (1992) The carboxyl-terminal region of SecE interacts with SecY and is functional in the reconstitution of protein translocation activity in *Escherichia coli*. *J. Biol. Chem.* **267**, 7170-7176
31. Matsuyama, S., Fujita, Y., and Mizushima, S. (1993) SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. *EMBO J.* **12**, 265-270
32. Shimizu, H., Nishiyama, K., and Tokuda, H. (1997) Expression of gpsA encoding biosynthetic sn-glycerol 3-phosphate dehydrogenase suppresses both the LB- phenotype of a secB null mutant and the cold-sensitive phenotype of a secG null mutant. *Mol. Microbiol.* **26**, 1013-1021
33. Tani, K., Tokuda, H., and Mizushima, S. (1990) Translocation of ProOmpA possessing an intramolecular disulfide bridge into membrane vesicles of *Escherichia coli*. Effect of membrane energization. *J. Biol. Chem.* **265**, 17341-17347
34. Nishiyama, K., Kabuyama, Y., Akimaru, J., Matsuyama, S., Tokuda, H., and Mizushima, S. (1991) SecY is an indispensable component of the protein secretory machinery of *Escherichia coli*. *Biochim. Biophys. Acta* **1065**, 89-97
35. Hussain, M., Ichihara, S., and Mizushima, S. (1980) Accumulation of glyceride-containing precursor of the outer membrane lipoprotein in the cytoplasmic membrane of *Escherichia coli* treated with globomycin. *J. Biol. Chem.* **255**, 3707-3712
36. Sugai, R., Takemae, K., Tokuda, H., and Nishiyama, K. (2007) Topology inversion of SecG is essential for cytosolic SecA-dependent stimulation of protein translocation. *J. Biol. Chem.* **282**, 29540-29548
37. Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001) Cell-free translation reconstituted with purified components. *Nat. Biotechnol.* **19**, 751-755
38. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
39. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275

40. Chen, M., Xie, K., Nouwen, N., Driessen, A. J., and Dalbey, R. E. (2003) Conditional lethal mutations separate the M13 procoat and Pf3 coat functions of YidC: different YidC structural requirements for membrane protein insertion. *J. Biol. Chem.* **278**, 23295-23300
FOOTNOTES
This work was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science (Grant Numbers: 15KT0073, 16H01374, 16K15083, 17H02209 and 18KK0197 to K.N.).

The abbreviations used are: BPB, bromophenol blue; CDP-DAG, CDP-diacylglycerol; GlcNAC-P, N-Acetyl-D-glucosamine 1-phosphate; PA, phosphatidic acid; TCA, trichloroacetic acid
**Table 1. E.coli strains and plasmids used in this study.**

| Strain and plasmid | Relevant genotype and description | Reference |
|--------------------|-----------------------------------|-----------|
| EK413              | MC4100 ara⁺                      | (14)      |
| KS21               | EK413 ΔynbB                       | (17)      |
| KS22               | EK413 ΔcdsA::cat                  | (17)      |
| KS23               | EK413 ΔynbB ΔcdsA::cat            | (17)      |
| pAra-CdsA          | The *cdsA* gene was cloned into pKQ2 under the control of the arabinose promoter, spectinomycin resistant. | (17)      |
| pACYC184-Km        | The *cat* gene on pACYC184 was replaced with the *kan* gene. | (17)      |
| pTet-Tam41p        | The *TAM41* gene without the mitochondrial targeting signal was cloned into pACYC-Km under the control of the tet promoter. | (17)      |
| pTac-Tam41p        | The *TAM41* gene without the mitochondrial targeting signal was cloned into pUSI2 under the control of tac promoter. | (17)      |
| pMS119-PC          | The gene for M13 procoat is cloned under the control of the tac promoter, ampicillin resistant. | (40)      |
### Table 2. Growth of strains used in this study at 20°C.*

|          | EK413 | KS21 | KS22 | KS23 |
|----------|-------|------|------|------|
| cdxA     | +     | +    | Δ    | Δ    |
| ynbB     | +     | Δ    | +    | Δ    |
| pAra-CdxA| -     | -    | +    | +    |
| Arabinose| -     | -    | +    | +    |
| LB       | L     | L    | L    | L    |
|          | (+) Tam41p | L  | M    | M    |
| M9       | L     | L    | L    | M    |
|          | (+) Tam41p | M  | M    | S    | S    |

*Each strain was streaked onto an LB or M9 plate, followed by incubation at 20°C for 2 days. Where specified, arabinose was supplemented at 0.2%. L, M, and S indicate large, medium, and small colonies, respectively.
Figure 1. The expression level of MPIase increases in the cold. (A) An overnight culture of EK413 was 1:100 diluted in LB medium and then cultivated at 37°C. When cells reached the early log phase, they were shifted to 20°C or 42°C and cultivation was continued for another 3 h. Total cellular proteins were precipitated with TCA, followed by SDS-PAGE/immunoblotting using anti-MPIase (upper), anti-SecY (middle), and anti-SecA (lower) antisera. (B) EK413 cells were cultivated at the specified temperatures. The MPIase level was determined by immunoblotting and SecB was analyzed as a loading control. (C) The relative amount of MPIase, determined in (B), was plotted as a function of the temperature. Standard deviation was obtained from at least three independent experiments, and is indicated as error bars. The MPIase level at 37°C was set as 1.0. The *p values were determined against the MPIase level at 37°C. *p<0.05. (D) Temperature shift experiments. EK413 cells cultivated at 15°C were shifted to 42°C. At the indicated time after the shift, an aliquot was withdrawn to analyze the MPIase level. SecB was analyzed as a loading control. (E) The MPIase level, determined in (D), was plotted as a function of time after the shift. The level at 0 min was set as 3.0. Standard deviation was obtained from at least three independent experiments, and is indicated as error bars. The *p values were determined against the MPIase level at 0 min. *p<0.05, **p<0.01. Note that BPB run at 5 kDa, but not at the gel front in this gel system.
**Figure 2.** The *cdsA* and *ynbB* genes are involved in the increase in the MPIase level at low temperature. (A) Overnight cultures of the indicated cells were 1:100 diluted in LB medium. For KS22/pAra-CdsA and KS23/pAra-CdsA, 0.2% arabinose was added. When cells reached the early log phase at 37°C, they were shifted to 20°C, and cultivation was continued for another 3 h. The MPIase level was determined by immunoblotting. SecB was analyzed as a loading control. BPB run at 5 kDa. (B) The results in (A) and 2 replicates were determined, and the average values were indicated together with standard deviation. The MPIase level in EK413 at 37°C was set as 1.0. *p<0.05. (C) EK413 was cultivated as above, and then inner membrane vesicles were prepared. The expression levels of CdsA and SecY were determined by immunoblotting. The identity of CdsA had been confirmed using membrane vesicles prepared from CdsA-overproducing and CdsA-depleted strains (17).
Figure 3. The effect of repression of the increase in the MPIase level on protein translocation at low temperature. (A) Overnight cultures of the indicated cells were 1:100 diluted in M9 medium. For ‘EK413’ and ‘KS21’, these were cultivated at 37°C until the early log phase. For ‘KS22/pAra-CdsA’ and ‘KS23/pAra-CdsA’, 0.2% arabinose was added, and cultivation was carried out at 37°C until the early log phase. For ‘ΔYnbB/ΔCdsA’, the overnight culture of KS23/pAra-CdsA were washed three times with fresh M9 medium to remove arabinose before inoculation, followed by cultivation at 37°C in the absence arabinose until the growth became stopped. After these cells had been shifted to 20°C and cultivated for another 3 h, they were pulse-labeled for 30 s, and then chased for the indicated periods. OmpA and pOmpA were immunoprecipitated, followed by SDS-PAGE/autoradiography. (B) The results in (A) were quantitated and plotted as a function of the chase time. (C) The expression levels of SecYEG and SecD in the indicated cells were determined by immunoblotting. The identities of SecY (34) and SecG (16) had been confirmed by analyzing the cells overproducing respective proteins.
Figure 4. The effects of Tam41p expression on the MPIase level and protein translocation. (A) The indicated cells were cultivated in M9 minimal medium at 37°C. When cells reached the mid-log phase, 1 mM IPTG was added for EK413/pTac-Tam41p to induce Tam41p, and cultivation was continued for another 1 h. The expression levels of MPIase and Tam41p were determined by immunoblotting. BPB run at 5 kDa. (B) The indicated cells were cultivated as above, and then pulse-labeled for 30 s and chased for the indicated periods. For ‘ΔCdsA’, KS22/pAra-CdsA was cultivated in the absence of arabinose, prior to pulse-chase experiments. OmpA and pOmpA were immunoprecipitated, followed by SDS-PAGE/autoradiography. (C) The results in (B) were quantitated and plotted as a function of the chase time.
**Figure 5.** The effects of Tam41p expression on the increase in the MPIase level and protein translocation at low temperature. (A and B) The indicated cells were cultivated as described in Fig. 2A, and the total proteins were precipitated with TCA. The MPIase level was determined by immunoblotting. In (B), SecB was analyzed as a loading control. BPB run at 5 kDa. (C) The results in (B) and 2–4 replicates were determined, and the average values were indicated together with standard deviation. The MPIase level in EK413 (-) was set as 3.0. The *p* values were determined against the MPIase level in EK413 (-). *p*<0.05, **p*<0.01. (D) The indicated cells was cultivated as in Fig. 3A. Cells were pulse-labeled for 30 s and then chased for the indicated periods. OmpA and pOmpA were immunoprecipitated, followed by SDS-PAGE/autoradiography. (E) The results in (D) were quantitated and plotted as a function of the chase time. (F) The expression levels of SecYEG and SecD in the indicated cells were determined by immunoblotting.
Figure 6. The effects of repression of the increase in the MPIase level and Tam41p expression on the protein integration. (A) An overnight culture of KS23/pAra-CdsA/pTet-Tam41p harboring pMS119-PC was washed five times with LB medium, followed by cultivation in the presence and absence of 0.2% arabinose. When the growth ceased in the absence of 0.2% arabinose, cells were shifted to 20°C. After the cultivation had been continued for 2 h, 1 mM IPTG was added to induce M13 procoat, and the cultivation was continued for another 1 h. Total cellular proteins were precipitated with TCA, and then M13 (pro)coat was detected by immunoblotting. The identities of M13 procoat and coat had been confirmed using cells without pMS119-PC (17). (B) The indicated cells harboring pMS119-PC were cultivated as in Fig. 2A in M9 minimal medium supplemented with 0.2% arabinose. After 5 min induction with 1 mM IPTG, they were pulse-labeled for 90 s and then chased for the indicated periods. M13 (pro)coat was immunoprecipitated, followed by SDS-PAGE/autoradiography. (C) INV were prepared from the indicated cells cultivated at 20°C, followed by SDS-PAGE/immunoblotting using anti-YidC antisera. (D and E) M13 procoat H5 (D) and 3L-Pf3 coat (E) were synthesized in vitro by means of the pure system in the presence of INV. After 60 min incubation at 20°C, the reaction mixture was divided into two parts, one was analyzed as a translation control (20%) in the ‘-PK’ lanes, and the other was digested with protease K (PK) (+). At left, INV were not added (-). The integration activities are indicated at the bottom of each gel. The integration assay was repeated twice to obtain reproducibility.
Increased expression of the bacterial glycolipid MPIase is required for efficient protein translocation across membranes in cold conditions
Katsuhiro Sawasato, Sonomi Suzuki and Ken-ichi Nishiyama

J. Biol. Chem. published online April 1, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.008457

Alerts:

• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts