Both the secAcsR11 and ΔsecG::kan mutations cause cold-sensitive growth, although the growth defect due to the latter mutation occurs in a strain-specific manner. Overexpression of pgsA encoding phosphatidylglycerophosphate synthase suppresses the growth defects of the two mutants. We investigated the mechanism underlying the pgsA-dependent suppression of the two mutations using purified mutant SecA and inverted membrane vesicles (IMVs) prepared from pgsA-overexpressing cells. The acidic phospholipid content increased by about 10% upon pgsA overexpression. This increase resulted in the stimulation of proOmpA translocation only when mutant SecA or SecG-depleted IMVs were used. The translocation-coupled ATPase activity of SecA was significantly defective with the mutant SecA or SecG-depleted IMVs, but it recovered to a near normal level when the acidic phospholipid level was increased. The stimulation of ATPase activity was observed only at low temperature. The steady-state level of membrane-inserted SecA was low with the mutant SecA or SecG-depleted IMVs, and it decreased further upon the increase in the acidic phospholipid content. However, the level of SecA insertion markedly increased upon the inhibition of SecA deinsertion by the addition of β,γ-imido adenosine 5′-triphosphate (AMP-PNP), especially with IMVs containing increased levels of acidic phospholipids. These results indicate that the increase in the level of acidic phospholipids stimulates the SecA cycle in the two mutants by facilitating both the insertion and deinsertion of SecA.

Protein translocation across the Escherichia coli cytoplasmic membrane is catalyzed by a machinery comprising six Sec factors (A, D, E, F, G, and Y) with the help of the secretin-specific molecular chaperone SecB (1–6). Membrane insertion and deinsertion of SecA coupled to ATP binding and hydrolysis, respectively, have been proposed to be the direct driving force for protein translocation (7, 8). SecG, a small membrane protein, also undergoes a membrane topology inversion cycle, which is assumed to be coupled to and to stabilize the SecA cycle (9). Indeed, SecA insertion significantly decreases upon SecG depletion (10–12).

A secG null mutant exhibits cold-sensitive growth, albeit in a strain-specific manner (13, 14). Overexpression of pgsA, which encodes phosphatidylglycerophosphate synthase (15, 16), suppresses the cold-sensitive phenotype of the secG null mutant (17). Moreover, overexpression of pgsA encoding a biosynthetic sn-glycerol-3-phosphate dehydrogenase, which is involved in phospholipid synthesis (18, 19), also suppresses the cold-sensitive property of the secG null mutant (20). These results suggest that the absence of the SecG function is compensated for by the manipulation of the phospholipid composition in membranes, although the underlying mechanism is not fully understood. We previously found that among six cold-sensitive mutants, i.e. secAcsR11, secDcs57, secEcs501, secFcs62, secYcs39, and ΔsecG::kan, only the first and last ones restored growth at low temperature upon the overexpression of pgsA (12). We then found that a secAcsR11-ΔsecG::kan double mutation causes synthetic lethality that is no longer suppressed by pgsA overexpression (12). These results revealed the functional interaction between SecA and SecG. However, it is not clear how the pgsA overexpression specifically restores the growth of the two mutants. The translocation of proOmpA in KN425, the ΔsecG::kan derivative of W3110 M25, was found to be stimulated when the strain harbored a multicopy plasmid carrying pgsA (17). However, because the cold-sensitive growth of the ΔsecG mutant is strain-specific, it remains uncertain whether the stimulation of protein translocation by pgsA overexpression is general or strain-specific. Furthermore, it is not known whether or not pgsA overexpression also stimulates protein translocation in the secAcsR11 mutant. In vitro protein translocation into SecG-depleted inverted membrane vesicles (IMVs)1 (12, 21) or with mutant SecA possessing the R11 mutation (12) is defective even at 37 °C in the absence, but not the presence, of PMF. PMF thus overcomes the translocation defect caused by the SecG depletion or the cold-sensitive SecA at 37 °C, both of which retard the SecA cycle (10–12). We recently found that acceleration of the SecA cycle underlies the PMF-dependent stimulation of protein translocation (22). From these observations, it seems likely that pgsA overexpression also stimulates the SecA cycle, thereby suppressing the translocation defect caused by the SecG-depletion or cold-sensitive SecA mutation.

We report here that the increase in the acidic phospholipid content on pgsA overexpression stimulates the SecA cycle specifically in the secAcsR11 and ΔsecG::kan mutants.

EXPERIMENTAL PROCEDURES

Bacterial Strains—E. coli PR520 (MC4100 secAcsR11 Δgal-bio) (23), PR518 (MC4100 secAcsR11 Δgal-bio DsecG::kan) (12), KN370 (C600 ΔsecG::kan recD1009) (13), K003 (HfrH Tn518 ara-13 tyr met RNaseI) Lpp ΔuncB-C::Tn10) (24), and KN553 (K003 ΔsecG::kan) (9) were used.

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Materials—SecA (25), SecB (26), and proOmpA (27) were purified from cells overproducing the respective proteins. Cold-sensitive SecA possessing the R11 mutation was overproduced in HS1 harboring pH57 (12), which carries the secAcsR11 allele under the control of the ara regulon, and purified as reported (25). Antibodies against SecE (28), SecF (29), SecG (30), and SecY (31) were raised in rabbits against synthetic peptides corresponding to the Ser2-Lys18 region of SecE, the Ala2-Arg21 region of SecF, the Gln25-Asn112 region of SecG, and the Met1-Arg22 region of SecY, respectively. Anti-SecD antibodies were raised against the purified protein as reported (29). [35S]proOmpA was synthesized in vitro in the presence of Tran35S-label (4 MBq/mmol) and partially purified means of gel filtration to remove small molecules (32). ATP, AMP-PNP, and creatine kinase were purchased from Roche Molecular Biochemicals. Proteinase K was from Merck. Succinate and creatine phosphate were from Sigma. Na232P (629 GBq/mg I) and Tran35S-label (37 TBq/mmol) as [35S]methionine were from ICN. [35S]Orthophosphoric acid was from NEN Life Science Products.

Overexpression of pgsA—A DNA fragment containing the pgsA gene was amplified by polymerase chain reaction with a pair of primers (5'-CCGAAGATGATATTTACCGAGCTGTTT-3' and 5'-GCCGGAATTCC-AAGCCCGAAAAGACATC-3') and pJKV43 carrying pgsA (17), followed by digestion with BamHI and BglII. The resultant fragment (680 base pairs) was inserted into the BamHI-BglII site of pUC19BglII (9), a derivative of pUC19, to construct the pgsA overproducer, pUPA. Preparation of proOmpA to Mature OmpA—E. coli PR520, PR518, KN570, and KN553 harboring pUC19 or pUPA1 were labeled at 30 °C for 1.5 min with Tran35S-label (1.08 μCi), followed by a chase for the specified times after the addition of nonradioactive methionine plus cysteine, each at 12 mM. As a control, MC4100 cells harboring no plasmid were also subjected to pulse-chase experiments. The processing of 35S-labeled proOmpA (25 μg/ml) and [35S]methionine were from ICN.

[35S]Orthophosphoric acid was from NEN Life Science Products.

Overexpression of pgsA—A DNA fragment containing the pgsA gene was amplified by polymerase chain reaction with a pair of primers (5'-CCGAAGATGATATTTACCGAGCTGTTT-3' and 5'-GCCGGAATTCC-AAGCCCGAAAAGACATC-3') and pJKV43 carrying pgsA (17), followed by digestion with BamHI and BglII. The resultant fragment (680 base pairs) was inserted into the BamHI-BglII site of pUC19BglII (9), a derivative of pUC19, to construct the pgsA overproducer, pUPA. Preparation of proOmpA to Mature OmpA—E. coli PR520, PR518, KN570, and KN553 harboring pUC19 or pUPA1 were labeled at 30 °C for 1.5 min with Tran35S-label (1.08 μCi), followed by a chase for the specified times after the addition of nonradioactive methionine plus cysteine, each at 12 mM. As a control, MC4100 cells harboring no plasmid were also subjected to pulse-chase experiments. The processing of 35S-labeled proOmpA to OmpA was analyzed by SDS-PAGE and fluorography after immunoprecipitation with an anti-OmpA antibody (13). Prophospholipid Compositions—K003 or KN553 harboring pUPA1 or its vector, pUC19, were labeled with [35S]orthophosphate (337 TBq/mmol) for 1 h at 37 °C. Lipids were extracted with chloroform-methanol (1:1) and then analyzed by thin layer chromatography with Silicagel 60 (Merck) and chloroform, methanol, H2O, 30% NH4OH (120:75:6:2) as the developing solvent. Phospholipids were identified on the chromatogram by autoradiography. The spots of phospholipids were then scraped off to determine radioactivity.

Preparation of Inverted Membrane Vesicles—IMVs were prepared from E. coli K003, KN553, and PR520 as described (33) and washed with 4 mM urea as reported (34).

Protein Translocation into Urea-washed IMVs—The reaction mixture, comprising 4 μM urea-treated IMVs (0.2 mg/ml), SecA at a specified concentration, SecB (50 μg/ml), 1 mM ATP, 5 mM succinate, 5 mM creatine phosphate, 5 mM potassium phosphate (pH 7.5), was preincubated at 20 °C for 2 min. The reaction was initiated by the successive addition of prewarmed nonradioactive proOmpA (25 μg/ml) and [35S]proOmpA (2.0 × 104 cpm/ml). Aliquots (25 μl) of the reaction mixture were withdrawn at various times and mixed with proteinase K (1 mg/ml) to terminate the reaction. After proteinase K digestion on ice, the IMVs were recovered by centrifugation (170,000 × g for 30 min) at 4 °C, and then suspended in the original volume of Buffer A. The membrane suspension was mixed with an equal volume of a solution comprising 2 mM ATP, 10 mM succinate, 5 mM creatine phosphate, 10 μg/ml creatine kinase, and 0.1 mg/ml SecB in Buffer A. After preincubation for 2 min at 20 °C, proOmpA (25 μg/ml) was added to initiate the translocation reaction. At the indicated times, aliquots (100 μl) were withdrawn and treated with proteinase K (1 mg/ml) on ice for 15 min. The proteinase K-resistant 30-kDa band detected on autoradiography after SDS-PAGE was densitometrically quantitated and expressed as a percentage of the total amount of radiolabeled SecA.

Other Methods—Densitometric quantitation was carried out with an ATTO densitograph. The membrane potential (inside positive) and ΔpH (inside acidic) were examined at 20 °C by monitoring the fluorescence quenching of oxonol V (1 μM) and quinacrine (1 μM), respectively, as described (33). Protein was determined by the method of Lowry et al. (36). SDS-PAGE was performed according to Laemmli (37).

RESULTS

Effect of pgsA Overexpression on Protein Translocation at Low Temperature—The translocation of proOmpA was examined at 20 °C in the specified strains harboring pUC19 (open symbols) or pUPA1 (closed symbols), as described under "Experimental Procedures." The amounts of proOmpA and OmpA on the fluorogram were densitometrically determined. The percentage of proOmpA compared to the total amount of OmpA materials was calculated. The numbers of methionine and cysteine residues in proOmpA (eight) and OmpA (seven) were used in the calculation. A, KN370 (triangles) and PR520 (squares), B, PR518 (circles), KN553 (triangles), and MC4100 harboring no plasmid (diamonds).

We examined the phospholipid compositions of FS1576, MC4100, and K003 and their respective ΔsecG::kan derivatives, KN370, EK414, and KN553. The phospholipid compositions of these strains were essentially the same, suggesting that the strain-specific cold-sensitive growth of the ΔsecG::kan
mutant is not caused by a strain-dependent difference in the phospholipid composition. Moreover, PR518 and PR520 had essentially the same phospholipid compositions as these strains. K003 and its ΔsecG:kan derivative, KN553, lack F, F, -ATPase and have been frequently used to prepare IMVs for in vitro examination of protein translocation. We therefore analyzed the phospholipid compositions of these strains harboring pUC19 or pUPA1 (Table I). The pgsA overexpression caused an increase in the acidic phospholipid content (phosphatidylglycerol plus cardiolipin) of about 10% at the expense of phosphatidylethanolamine in both strains. Essentially the same results were obtained when the cells were incubated at 20 °C for 4 h after growth at 37 °C (data not shown).

In Vitro Translocation of proOmpA—The pgsA-dependent stimulation of proOmpA translocation was examined in vitro with IMVs prepared from K003 and KN553 harboring either pUC19 or pUPA1 in the presence of PMF using the purified wild type SecA (wtSecA) or cold-sensitive SecA (csSecA) at 20 °C (Fig. 2). Gltu at position 276 was found to be mutated to Ala in csSecA (12). This mutation is localized in the 267–340 region of SecA, which has been suggested to participate in precursor binding (39). When SecG-containing IMVs were used with wtSecA, the translocation activity was not affected by the pgsA overexpression (Fig. 2, compare open and closed squares). On the other hand, when csSecA was used instead of wtSecA, the pgsA overexpression significantly enhanced the proOmpA translocation into SecG-containing IMVs (Fig. 2, circles). Furthermore, the pgsA overexpression also increased the translocation into SecG-depleted IMVs, even if wtSecA was used (Fig. 2, upright triangles). The translocation activity in the absence of external SecA was only marginal (Fig. 2, inverted triangles). Taken together, these results indicate that the increase in the acidic phospholipid content due to pgsA overexpression stimulates proOmpA translocation both in vivo and in vitro when SecA carries the secAc::kan mutation or when the IMVs lack SecG. The pgsA overexpression also stimulated proOmpA translocation with csSecA or SecG-depleted IMVs in the absence of PMF (data not shown). However, the translocation activity under these conditions was lower than that determined with wtSecA and SecG-containing IMVs, indicating that PMF is also required for the recovery of protein translocation to a near normal level at 20 °C.

Enhanced generation of PMF could be the reason for the stimulation of proOmpA translocation by pgsA overexpression. We therefore examined the generation of a membrane potential (inside positive) and ΔpH (inside acidic) by monitoring the fluorescence quenching of oxonol V and quinacrine, respectively. All IMVs exhibited essentially the same extent of fluorescence quenching upon the addition of succinate (data not shown), indicating that the pgsA overexpression had little effect on the generation of Δψ and ΔpH, regardless of the presence or absence of SecG in the membrane.

The level of Sec proteins in IMVs was examined by SDS-PAGE and immunoblotting (Fig. 3). The pgsA overexpression did not affect the contents of Sec proteins in the membrane, indicating that the stimulation of proOmpA translocation in vitro is not caused by increases in the levels of Sec factors. Moreover, it should be noted that the lack of SecG had little effect on the contents of other Sec proteins in the membrane. Translocation ATPase—The ATPase activity of SecA increases upon the initiation of protein translocation (40). The increases in the ATPase activity on the addition of various amounts of proOmpA were examined with specified combinations of IMVs and SecAs at 20 °C (Fig. 4). Consistent with proOmpA translocation, when wtSecA was used with SecG-containing IMVs, the pgsA overexpression had no effect on the ATPase activity (Fig. 4, squares). However, when the assay was carried out with csSecA instead of wtSecA, the pgsA overexpression enhanced the ATPase activity over the concentration range of proOmpA examined (Fig. 4, circles). Essentially the same stimulation by the pgsA overexpression was observed

### Table I

**Phospholipid compositions**

| Strain | Plasmid  | Phospholipid compositions<sup>a</sup> | % |
|--------|----------|--------------------------------------|---|
|        |          | Phosphatidylethanolamine | Phosphatidylglycerol | Cardiolipin |
| K003   | pUC19    | 69.7 ± 0.3 | 26.7 ± 0.2 | 3.6 ± 0.2 |
|        | pUPA1    | 59.8 ± 1.3 | 34.0 ± 2.0 | 6.3 ± 1.2 |
| KN553  | pUC19    | 64.0 ± 0.2 | 32.8 ± 0.5 | 3.3 ± 0.3 |
|        | pUPA1    | 52.1 ± 1.7 | 40.9 ± 1.8 | 6.0 ± 0.2 |

<sup>a</sup> The results are the averages of four experiments.
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FIG. 4. Effect of the increase in the acidic phospholipid content on the translocation-coupled ATPase activity of SecA. IMVs prepared from K003 (squares and circles), KN553 (upright triangles), or PR520 (inverted triangles) harboring pUC19 (open symbols) or pUPA1 (closed symbols) were used to examine the translocation-coupled ATPase activity with wtSecA (squares, triangles, and inverted triangles) or csSecA (circles) at 20 °C in the presence of various amounts of proOmpA, as described under “Experimental Procedures.”

when wtSecA was used with SecG-depleted IMVs (Fig. 4, upright triangles). To confirm further that such stimulation is specific to csSecA and ΔSecG, IMVs prepared from PR520 (secEcscsE501) were assayed for ATPase activity with wtSecA. The activity remained low irrespective of the pgsA overexpression (Fig. 4, inverted triangles). Apparent $K_m$ and $V_m$ values were determined under the respective conditions (Table II). Compared with the value obtained with wtSecA and SecG-containing IMVs, a significantly lower $V_m$ value was obtained when IMVs prepared from PR520 were used. The affinity for proOmpA slightly decreased with these IMVs. The pgsA overexpression had essentially no effect on the $K_m$ and $V_m$ values obtained with these IMVs. We previously reported that only the affinity for proOmpA decreases with csSecA or SecG-depleted IMVs at 37 °C (12). On the other hand, not only the affinity but also the $V_m$ value decreased with csSecA or SecG-depleted IMVs at 20 °C. The pgsA overexpression specifically restored the $V_m$ values.

Both the secAcR11 and ΔsecG:kan mutants grew normally at 37 °C in the absence of pgsA overexpression (12). We therefore determined whether or not the stimulation of ATPase activity by pgsA overexpression is dependent on temperature. ATPase activity, which increased upon the addition of a saturating amount of proOmpA, was measured at various temperatures with various combinations of two kinds of SecA and four kinds of IMVs. The stimulation of ATPase activity by pgsA overexpression was then estimated at each temperature for the specified combinations of SecAs and IMVs (Fig. 5). The stimulation of ATPase activity by the overexpression of pgsA was specific to a low temperature and was observed only when csSecA (Fig. 5, closed circles) or SecG-depleted IMVs (triangles) were used. In contrast, pgsA overexpression had no effect on ATPase activity determined with wtSecA and SecG-containing IMVs (Fig. 5, open circles).

The SecA Cycle Is Stimulated by pgsA Overexpression—The effect of pgsA overexpression on the SecA cycle was examined at 20 °C (Fig. 6). Membrane insertion of SecA was monitored as the level of the proteinase K-resistant 30-kDa fragment derived from $^{125}$I]SecA as reported (7). The 30-kDa fragment level was determined at various times after SecA insertion was initiated by the addition of proOmpA. The assays were performed in the presence of PMF, which was recently found to decrease the level of membrane-inserted SecA at 37 °C by stimulating deinsertion (22). PMF had the same effect at 20 °C, but the decrease in the 30-kDa fragment level upon the generation of PMF was slightly smaller than that at 37 °C (data not shown). When csSecA or SecG-depleted IMVs were used, the 30-kDa fragment level was only marginal. The 30-kDa fragment level observed with csSecA or SecG-depleted IMVs further decreased on the increase in the acidic phospholipid content. When AMP-PNP was added to inhibit SecA deinsertion, the 30-kDa fragment level markedly increased. The level obtained with csSecA or

| SecA | IMV$^*$ | pgsA overexpression | $K_m$ for proOmpA (nM) | $V_m$ (μmol/min/mg SecA) |
|------|--------|---------------------|-------------------------|-------------------------|
| Wild type K003 | – | – | 8.72 | 0.32 |
| | + | + | 6.27 | 0.31 |
| PR520 | – | – | 21.2 | 0.10 |
| | + | + | 13.7 | 0.10 |
| KN553 | + | + | 67.6 | 0.13 |
| | + | + | 70.8 | 0.32 |
| csSecA | K003 | – | 58.4 | 0.11 |
| | + | + | 46.6 | 0.25 |

$^*$ IMVs were prepared from the specified strains.

FIG. 5. Temperature dependence of the stimulation of ATPase activity by the acidic phospholipid increase. The translocation-coupled ATPase activity was examined by the addition of 0.2 μM proOmpA at various temperatures with wtSecA (triangles and open circles) or csSecA (closed circles) and IMVs prepared from K003 (open and closed circles) or KN553 (triangles) harboring pUC19 or pUPA1. The effect of the increase in the acidic phospholipid content on the ATPase activity was estimated at each temperature with the specified combinations of SecAs and IMVs. The activity ratio in the presence and absence of the acidic phospholipid increase is plotted as a function of temperature.

FIG. 6. Increase in acidic phospholipid stimulates the SecA cycle. The effect of the increase in the acidic phospholipid content on the level of SecA insertion was examined at 20 °C using csSecA (A) or SecG-depleted IMVs (B) in the presence of PMF, as described under “Experimental Procedures.” The control level of SecA insertion was examined with a combination of IMVs prepared from K003/pUC19 and wtSecA. The data are shown as circles (A and B). At 30 min, AMP-PNP (20 mM) was added (closed symbols) or not added (open symbols) to portions of the reaction mixture. A. IMVs prepared from K003 harboring pUC19 (triangles) or pUPA1 (squares) were used with csSecA. B. IMVs prepared from KN553 harboring pUC19 (triangles) or pUPA1 (squares) were used with wtSecA.
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SecG-depleted IMVs after AMP-PNP was appreciably higher with IMVs prepared from pgsA-overexpressing cells. These results indicate that the increase in the acidic phospholipid content affects the level of SecA insertion not only before but also after the inhibition of deinsertion. We conclude that pgsA overexpression stimulates the rates of both SecA insertion and deinsertion.

**DISCUSSION**

The importance of negatively charged lipids for protein translocation and, more specifically, the SecA function has been reported (41, 42). Moreover, nonbilayer lipids have also been found to be critically important for the translocation (43). These findings established the general importance of the phospholipid composition for protein translocation. On the other hand, the data shown here revealed that a rather small increase in the acidic phospholipid content very specifically complements the translocation defect caused by the secAcsR11 or secG:kan mutation. This specific effect was caused by neither enhancement of PMF generation nor elevation of the levels of Sec components. The increase in the acidic phospholipid content was found to enhance the ATP hydrolysis of SecA specifically when csSecA or SecG-depleted IMVs were used. Furthermore, this enhancement was specific to a low temperature. Taken together, these results most likely indicate that the increase in the acidic phospholipid content was required to accelerate the SecA cycle to near normal efficiency.

It is noteworthy that not only pgsA overexpression but also PMF was required for the recovery of protein translocation to a near normal level at 20 °C. PMF also accelerates the SecA deinsertion in an ATP hydrolysis-independent manner (22). In vitro protein translocation examined at 37 °C with csSecA or SecG-depleted IMVs was normal in the presence of PMF, indicating that the retardation of the SecA cycle by the mutations is less serious at 37 °C. On the other hand, because SecA insertion was more severely inhibited at 20 °C, not only PMF but also the increase in the acidic phospholipid content was required to accelerate the SecA cycle to near normal efficiency.

It seems likely that the efficiency of the SecA cycle is not affected by these mutations.

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