JOURNAL OF BACTERIOLOGY, Apr. 2010, p. 1929–1936 Vol. 192, No. 7
0021-9193/10/$12.00 doi:10.1128/JB.01328-09

Role of the C-Terminal Cytoplasmic Domain of FlhA in Bacterial Flagellar Type III Protein Export▽

Tohru Minamino,[1,2,3]* Masafumi Shimada,[1] Mayuko Okabe,[2,4]† Yumiko Saijo-Hamano,[1,2] Katsumi Imada,[1,2] May Kihara,[4] and Keiichi Namba[1,2]*

Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan[1]; Dynamic NanoMachine Project, ICORP, JST, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan[2]; PRESTO, JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan[3]; and Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520-8114[4]

Received 7 October 2009/Accepted 15 January 2010

For construction of the bacterial flagellum, many of the flagellar proteins are exported into the central channel of the flagellar structure by the flagellar type III protein export apparatus. FlhA and FlhB, which are integral membrane proteins of the export apparatus, form a docking platform for the soluble components of the export apparatus, FliH, FliI, and FliJ. The C-terminal cytoplasmic domain of FlhA ($FlhA_C$) is required for protein export, but it is not clear how it works. Here, we analyzed a temperature-sensitive *Salmonella enterica* mutant, the *flhA*(*G368C*) mutant, which has a mutation in the sequence encoding $FlhA_C$. The G368C mutation did not eliminate the interactions with FliH, FliI, FliJ, and the C-terminal cytoplasmic domain of FlhB, suggesting that the mutation blocks the export process after the FliH-FliI-FliJ-export substrate complex binds to the FlhA-FlhB platform. Limited proteolysis showed that $FlhA_C$ consists of at least three subdomains, a flexible linker, $FlhA_{CN}$, and $FlhA_{CC}$, and that $FlhA_{CN}$ becomes sensitive to proteolysis by the G368C mutation. Intragenic suppressor mutations were identified in these subdomains and restored flagellar protein export to a considerable degree. However, none of these suppressor mutations suppressed the protease sensitivity. We suggest that $FlhA_C$ not only forms part of the docking platform for the FliH-FliI-FliJ-export substrate complex but also is directly involved in the translocation of the export substrate into the central channel of the growing flagellar structure.

The bacterial flagellum, which is responsible for motility, is a supramolecular complex of about 30 different proteins, and it consists of at least three substructures: the basal body, the hook, and the filament. Flagellar assembly begins with the basal body, followed by the hook and finally the filament. Many of the flagellar component proteins are translocated into the central channel of the growing flagellar structure and then to the distal end of the structure for self-assembly by the flagellar type III protein export apparatus (11, 16, 22). This export apparatus consists of six integral membrane proteins, FlhA, FlhB, FliO, FliP, FliQ, and FliR, and three soluble proteins, FliH, FliI, and FliJ (18, 21). These protein components show significant sequence and functional similarities to those of the type III secretion systems of pathogenic bacteria, which directly inject virulence factors into their host cells (11, 16).

FliI is an ATPase (4) and forms an $FliH_2$-FliI complex with its regulator, FliH, in the cytoplasm (20). FliI self-assembles into a homo-hexamer and hence exhibits full ATPase activity (1, 8, 17). FliH and FliI, together with FliJ and the export substrate, bind to the export core complex, which is composed of the six integral membrane proteins, to recruit export substrates from the cytoplasm to the core complex (14) and facilitate the initial entry of export substrates into the export gate (23). FliJ not only prevents premature aggregation of export substrates in the cytoplasm (13) but also plays an important role in the escort mechanism for cycling export chaperones during flagellar assembly (3). The export core complex is believed to be located in the central pore of the basal body MS ring (11, 16, 22). In fact, it has been found that FlhA, FliP, and FliR are associated with the MS ring (5, 9). The FliR-FlhB fusion protein is partially functional, suggesting that FliR and FlhB interact with each other within the MS ring (29). The export core complex utilizes a proton motive force across the cytoplasmic membrane as the energy source to drive the successive unfolding of export substrates and their translocation into the central channel of the growing flagellum (23, 27). Here we refer to the export core complex as the "export gate," as we have previously (8, 16, 23, 24).

FlhA is a 692-amino-acid protein consisting of two regions: a hydrophobic N-terminal transmembrane region with eight predicted α-helical transmembrane spans ($FlhA_{TM}$) and a hydrophilic C-terminal cytoplasmic region ($FlhA_C$) (12, 15). $FlhA_{TM}$ is responsible for the association with the MS ring (9). $FlhA_C$ interacts with FliH, FliI, FliJ, and the C-terminal cytoplasmic domain of FlhB (6, 12, 21, 24) and plays a role in the initial export process with these proteins (28). It has been shown that the V404M mutation in $FlhA_C$ increases not only the probability of FliI binding to the export gate in the absence of FliH (14) but also the efficiency of substrate translocation

* Corresponding author. Mailing address: Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-4625. Fax: 81-6-6879-4652. E-mail for Tohru Minamino: tohru@fbs.osaka-u.ac.jp. E-mail for Keiichi Namba: keiichi@fbs.osaka-u.ac.jp.

† Present address: Department of Immunology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.

▽ Published ahead of print on 29 January 2010.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli BL21(DE3)/pLysS	Overproduction of proteins	Novagen
Salmonella strains		
SJW1103	Wild type for motility and chemotaxis	31
SJW1364	*flhA*	10
SJW1368	Δ(*cheW-flhD*); master operon mutant	25
SJW2228	*flhA*(*G368C*)	28
SJW2228iH	*flhA*(*G368C*) Δ*fliH*	This study
MMA2228-1	*flhA*(*G368C*/*L359F*)	This study
MMA2228-3	*flhA*(*G368C*/*G364R*)	This study
MMA2228-7	*flhA*(*G368C*/*R370S*)	This study
MMA2228-9	*flhA*(*G368C*/*P550S*)	This study
MMHI001	Δ*fliH* Δ*fliI*	17
MMHI0117	Δ*fliH* Δ*fliI flhB*(*P28T*)	23
MMHI0117-28	Δ*fliH* Δ*fliI flhB*(*P28T*) *flhA*(*G368C*)	This study
Plasmids		
pGEX-6p-1	Expression vector	GE Healthcare
pTrc99A	Expression vector	GE Healthcare
pTrc99AFF4	Expression vector	26
pGKK1702	pGEX-6p-1/GST-FliI	8
pMM102	pTrc99A/His-FlhA_C	21
pMM102(G368C)	pTrc99A/His-FlhA_C(G368C)	This study
pMM104	pET19b/His-FlhA_C	21
pMM104(G368C)	pET19b/His-FlhA_C(G368C)	This study
pMM104(G368C/L359F)	pET19b/His-FlhA_C(G368C)	This study
pMM104(G368C/G364R)	pET19b/His-FlhA_C(G368C/G364R)	This study
pMM104(G368C/R370S)	pET19b/His-FlhA_C(G368C/R370S)	This study
pMM104(G368C/P550S)	pET19b/His-FlhA_C(G368C/P550S)	This study
pMM130	pTrc99AFF4/FlhA	9
pMM130(L359F)	pTrc99AFF4/FlhA(L359F)	This study
pMM130(G364R)	pTrc99AFF4/FlhA(G364R)	This study
pMM130(R370S)	pTrc99AFF4/FlhA(R370S)	This study
pMM130(P550S)	pTrc99AFF4/FlhA(P550S)	This study
pMM306	pTrc99A/His-FliH	21
pMM310	pET19b/His-FliH	21
pMM1702	pTrc99A/His-FliI	21
pMM309iI	pTrc99AFF4/FliH+His-FliI	20
pMMJ1001	pGEX-6p-1/GST-FliJ	This study
pMMHB1001	pGEX-6p-1/GST-FlhB_C	24
pTIH001	pGEX-6p-1/GST-FliH	T. Ibuki, unpublished data

through the export gate in the absence of FliH and FliI (23). Recently, it has been shown that FlhA_C is also required for substrate recognition (7). These observations suggest that an interaction between FlhA_C and FliI is coupled with substrate entry, although it is not clear how.

In order to understand the mechanism of substrate entry into the export gate, we characterized a temperature-sensitive *Salmonella enterica* mutant, the *flhA*(*G368C*) mutant, whose mutation blocks the flagellar protein export process at 42°C (28). We show here that this mutation severely inhibits translocation of flagellar proteins through the export gate after the FliH-FliI-FliJ complex binds to the FlhA-FlhB platform of the gate and that the impaired ability of the *flhA*(*G368C*) mutant to export flagellar proteins is restored almost to wild-type levels by intragenic second-site mutations that may alter the interactions between subdomains of FlhA_C for possible rearrangement for the export function.

MATERIALS AND METHODS

Bacterial strains, plasmids, transductional crosses, DNA manipulations, and media. The bacterial strains and plasmids used in this study are listed in Table 1. P22-mediated transduction was carried out with p22HT*int* as described previously (30). An *fliH* null mutation (Δ*fliH*) was introduced into SJW2228 using the λ Red homologous recombination system (2). DNA manipulations, site-directed mutagenesis, and DNA sequencing were carried out as described previously (28). L broth (LB) and soft tryptone agar plates were prepared as described previously (18, 21). Ampicillin and chloramphenicol were added to LB to final concentrations of 100 and 30 μg/ml, respectively.

Motility assays on soft agar plates. Fresh colonies were inoculated onto soft tryptone agar plates and incubated at 30°C or 42°C.

Preparation of whole-cell and culture supernatant fractions and immunoblotting. *Salmonella* cells were grown at 30°C or 42°C with shaking until the optical density at 600 nm (OD_{600}) was ca. 1.2 to 1.4. Cellular and culture supernatant fractions were prepared as described previously (18). Cell pellets were resuspended in SDS loading buffer normalized using the cell density to obtain the same number of cells. The proteins in the culture supernatants were precipitated with 10% trichloroacetic acid, suspended in a Tris-SDS loading buffer, and heated at 95°C for 5 min. After SDS-PAGE, immunoblotting with polyclonal anti-FlhA_C and anti-FlgD antibodies was carried out as described previously (18). Detection was performed with an enhanced chemoluminescence (ECL) immunoblot detection kit (GE Healthcare).

Purification of GST, GST-FlhB_C, GST-FliH, GST-FliI, and GST-FliJ. Soluble fractions prepared from SJW1368(Δ*cheW*-Δ*flhD*) expressing glutathione *S*-transferase (GST), GST-FlhB_C, GST-FliH, GST-FliI, and GST-FliJ were loaded onto a glutathione Sepharose 4B column (GE Healthcare). After washing with phosphate-buffered saline (PBS) (containing [per liter] 8 g of NaCl, 0.2 g of KCl, 3.63 g of $Na_2HPO_4 \cdot 12H_2O$, and 0.24 g of KH_2PO_4; pH 7.4), proteins were

eluted with 50 mM Tris-HCl (pH 8.0), 10 mM reduced glutathione. Fractions containing GST, GST-FlhB$_C$, GST-FliH, GST-FliI, or GST-FliJ were pooled and dialyzed overnight against PBS at 4°C with three changes of PBS.

Pull-down assay. Soluble fractions prepared from cultures of SJW1103 (wild type) carrying pMM102 or pMM102(G368C) were incubated at 42°C and loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen) whose bed volume was 1 ml. After the column was washed with 20 ml of a binding buffer (20 mM Tris-HCl [pH 8.0], 500 mM NaCl) containing 10 mM imidazole and then with 5 ml of binding buffer containing 60 mM imidazole at 42°C, proteins were eluted with binding buffer containing imidazole at 42°C; during elution the imidazole concentration was increased stepwise to 100, 200, 300, and 600 mM. Eluted fractions were analyzed by both Coomassie brilliant blue (CBB) staining and immunoblotting with polyclonal anti-FliI, anti-FliH, and anti-FliJ antibodies.

Purified His-FlhA$_C$ and His-FlhA$_C$(G368C) were incubated with purified GST, GST-FlhB$_C$, GST-FliH, GST-FliI, or GST-FliJ at 42°C for 1 h and loaded onto a glutathione Sepharose 4B column (bed volume, 1 ml) preincubated at 42°C. After the column was washed with 20 ml PBS at 42°C, proteins were eluted with 50 mM Tris-HCl (pH 8.0), 10 mM reduced glutathione at 42°C. Eluted fractions were analyzed by both CBB staining and immunoblotting with polyclonal anti-FlhA$_C$ antibody.

Purification of His-FlhA$_C$ and mutant variants of this construct and limited proteolysis. His-FlhA$_C$ and mutant variants were purified by Ni-NTA affinity chromatography as described previously (21) and dialyzed overnight against 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT).

His-FlhA$_C$ and the mutant variants (0.5 mg/ml) were incubated with the Glu-C endoproteinase (Roche Diagnostics) at a protein-to-protease ratio of 300:1 (wt/wt) in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM DTT at 42°C. After incubation was started at 42°C, aliquots were collected at 0, 5, 15, 30, 60, 120, and 180 min, and trichloroacetic acid was added to a final concentration of 10%. The molecular masses of proteolytic cleavage products were determined by using a mass spectrometer (PerSeptive Biosystems) as described previously (28).

RESULTS

Dominance properties of FlhA$_C$(G368C). The temperature-sensitive *flhA*(*G368C*) mutation substantially reduced motility (Fig. 1A, left panel) and flagellar protein export (Fig. 1A, right panel) at 42°C, in agreement with a previous report (28). To investigate how this mutation affects the flagellar protein export process, we analyzed the effect of the G368C mutation on the interaction of FlhA$_C$ with the soluble components of the export apparatus. As FlhA$_C$ exerts a strong inhibitory effect on wild-type motility by titrating away other essential proteins involved in flagellar protein export (21, 28), we first analyzed the motility of wild-type cells carrying a plasmid that overexpresses His-FlhA$_C$(G368C) on soft tryptone agar plates containing 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 42°C (Fig. 1B). The motility of wild-type cells overexpressing His-FlhA$_C$(G368C) was much poorer than that of the vector control and even poorer (although slightly) than that of the wild type overexpressing His-FlhA$_C$. Immunoblotting with polyclonal anti-FlhA$_C$ antibody revealed that the cellular level of His-FlhA$_C$(G368C) was the wild-type level (data not shown). These results suggest that His-FlhA$_C$(G368C) is able to interact with and sequester the soluble components of the export apparatus and/or export substrates.

To identify which components are sequestered by overproduction of His-FlhA$_C$ or His-FlhA$_C$(G368C), we carried out pull-down assays by using Ni-NTA affinity chromatography at 42°C (Fig. 1C). FliI copurified with both His-FlhA$_C$ and His-FlhA$_C$(G368C). No FliI was seen in the elution fractions of the vector control or a *fliI* null mutant overexpressing His-FlhA$_C$ or His-FlhA$_C$(G368C) (data not shown). In agreement with this, pull-down assays performed by using GST affinity chromatography showed that a slightly larger amount of His-FlhA$_C$(G368C) copurified with GST-FliI (Fig. 2, second row). These results indicate that the interaction between FliI and FlhA$_C$ is not eliminated by the G368C mutation. However, neither FliH nor FliJ copurified (data not shown). Since it has been shown that FlhA$_C$ binds to these proteins (6, 12, 21, 24), it was possible that FliH and FliJ were dissociated by stringent washes during purification.

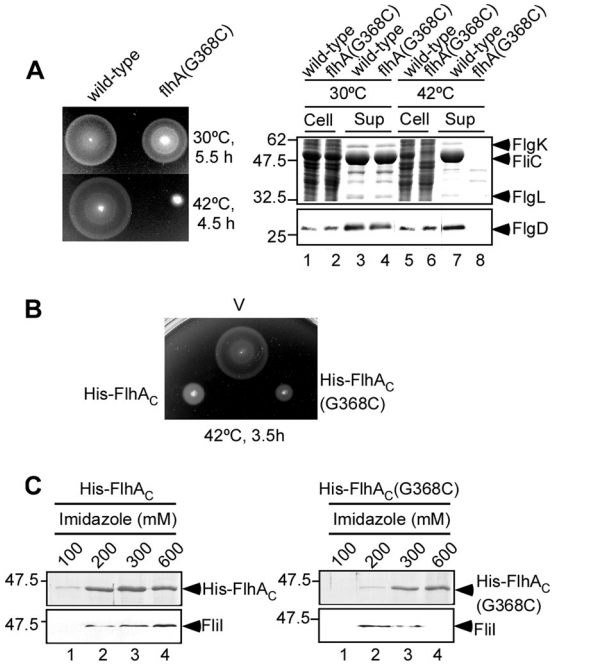

FIG. 1. Characterization of the temperature-sensitive *flhA*(*G368C*) mutant. (A) (Left panel) Swarming motility of SJW1103 (wild type) and SJW2228 [*flhA*(*G368C*)] on soft agar plates at 30°C and 42°C. (Right panel) Secretion assays. Secretion of FliC, FlgK, and FlgL was analyzed by Coomassie brilliant blue (CBB) staining (upper panel). Secretion of FlgD was measured by immunoblotting with a polyclonal anti-FlgD antibody (lower panel). Cell, whole-cell proteins; Sup, culture supernatant fractions. The positions of molecular mass markers (in kDa) are indicated on the left. (B) Dominant negative effect on wild-type swarming motility of SJW1103 transformed with pTrc99A (V), pMM102 (His-FlhA$_C$) or pMM102(G368C) [His-FlhA$_C$(G368C)] on soft agar plates containing 0.1 mM IPTG at 42°C. (C) Pull-down assay. The soluble fractions prepared from SJW1103 overproducing His-FlhA$_C$ (left panel) or His-FlhA$_C$(G368C) (right panel) were loaded onto an Ni-NTA agarose column. After washing, proteins were eluted with a buffer containing 100 mM, 200 mM, 300 mM, and 600 mM imidazole. Pull-down assays were done at 42°C. Eluted His-FlhA$_C$ was analyzed by CBB staining, while eluted FliI was detected by immunoblotting with a polyclonal anti-FliI antibody.

Bypass effects of FliI on motility of the *flhA*(*G368C*) Δ*fliH* double mutant. Since the FliH null mutation is suppressed by overproduction of FliI or by mutations in FlhA and FlhB, FliH is thought to be involved in the effective docking of FliI with the export substrate with the FlhA-FlhB platform of the export gate (14). Therefore, we investigated whether the G368C mutation allows FliI overproduction to bypass the FliH defect at 42°C. An *flhA*(*G368C*) Δ*fliH* double mutant was transformed with a plasmid encoding His-FliI, and then the motility of the resulting transformants was analyzed at 30°C and 42°C (Fig. 3A). Overproduction of FliI improved the motility of the dou-

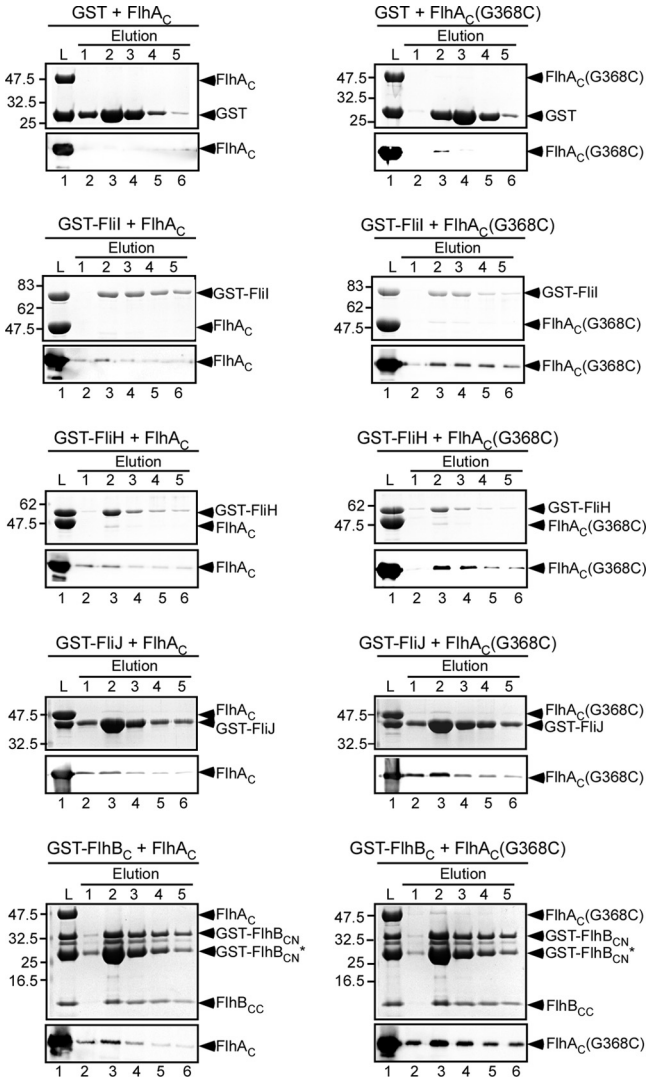

FIG. 2. Interactions of FlhA$_C$ with FliH, FliI, FliJ, and FlhB$_C$. Purified His-FlhA$_C$ (FlhA$_C$) (left panels) or His-FlhA$_C$(G368C) [FlhA$_C$(G368C)] (right panels) was mixed with purified GST (first row), GST-FliI (second row), GST-FliH (third row), GST-FliJ (fourth row), or GST-FlhB$_C$ (fifth row) and incubated at 42°C for 1 h. Then the mixtures (lanes L) were loaded onto a GST column preincubated at 42°C. After washing with PBS at 42°C, proteins were eluted with 50 mM Tris-HCl (pH 8.0), 10 mM reduced glutathione. The eluted proteins were analyzed by both CBB staining (upper panels) and immunoblotting with polyclonal anti-FlhA$_C$ antibody (lower panels). Although GST-FlhB$_C$ is cleaved between Asp-269 and Pro-270, GST-FlhB$_{CN}$ and FlhB$_{CC}$ associate with each other even after cleavage (19). The arrowheads indicate the positions of GST, GST-FliI, GST-FliH, GST-FliJ, GST-FlhB$_{CN}$, FlhB$_{CC}$, His-FlhA$_C$, and His-FlhA$_C$(G368C). The arrowhead labeled GST-FlhB$_{CN}$* indicates a degraded product just under the main band.

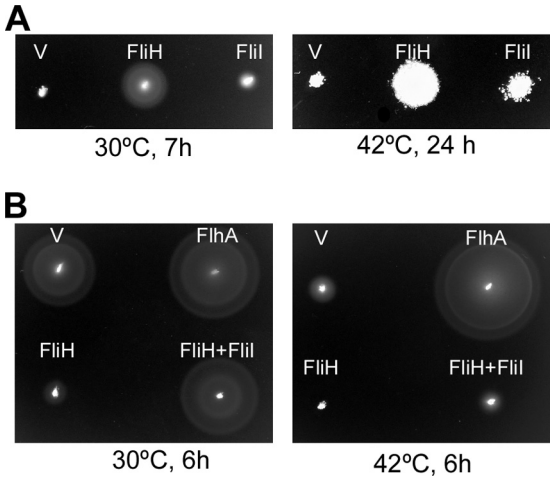

FIG. 3. *In vivo* interaction of FlhA(G368C) with FliH and FliI. (A) Bypass effect of FliI on the motility of SJW2228iH [*flhA*(*G368C*) Δ*fliH*]. The swarming motility of the double mutant transformed with pTrc99A (V), pMM310 (FliH), and pMM1702 (FliI) was determined on soft agar plates at 30°C and 42°C. (B) Multicopy effect of FliH on the motility of the *flhA*(*G368C*) mutant. A swarming motility assay was performed with the *flhA*(*G368C*) mutant carrying pTrc99AFF4 (V), pMM130 (FlhA), pMM306 (FliH), or pMM309iI (FliH+FliI) at 30°C and 42°C.

ble mutant to some extent, suggesting that the G368C mutation does not interfere with the association between FliI and the platform at 42°C.

Interaction between FlhA$_C$ and FliH. It has been shown that the extreme N-terminal region of FliH is required not only for the interaction with the C-ring protein FliN but also for effective association with the export gate (24). Therefore, we tested if the G368C mutation interferes with the association between FliH and the export gate. Since the presence of free FliH eliminates the export process when FliI is missing, probably due to the binding of FliH to the export gate that inhibits substrate entry into the gate (21, 23, 24), we examined the multicopy effect of FliH on the motility of the *flhA*(*G368C*) mutant on soft agar plates at 30°C and 42°C (Fig. 3B). FliH overproduction strongly inhibited the motility of the *flhA* mutant, and this inhibition of motility was substantially relieved by co-overproduction of FliI (Fig. 3B) but not by co-overproduction of FliN alone or by co-overproduction of FliN plus FliM (data not shown). These results suggest that FliH can bind to the export gate even in the presence of the G368C mutation at 42°C.

Pull-down assays performed by using GST affinity chromatography have shown that there is an interaction between FlhA$_C$ and FliH (24). Therefore, we also carried out pull-down assays using GST affinity chromatography at 42°C (Fig. 2). We used GST as a negative control. His-FlhA$_C$ copurified with GST-FliH but not with GST (Fig. 2, third and first rows, respectively). A very small amount of His-FlhA$_C$(G368C) was detected in the elution fractions derived from GST alone (Fig. 2, row 1), presumably due to its nonspecific binding to the column. However, the amount of His-FlhA$_C$(G368C) that coeluted with GST-FliH was 20-fold larger than the amount that coeluted with GST when the amounts were normalized using the amounts of GST and GST-FliH. These results indicate that the G368C mutation does not decrease the FlhA$_C$-FliH interaction at 42°C.

Interaction of FlhA$_C$ with FliJ and the C-terminal cytoplasmic domain of FlhB. FlhA$_C$ also interacts with FliJ and the C-terminal cytoplasmic domain of FlhB (FlhB$_C$) (6, 12, 21). To investigate whether the G368C mutation interferes with these interactions, we carried out pull-down assays using GST affinity

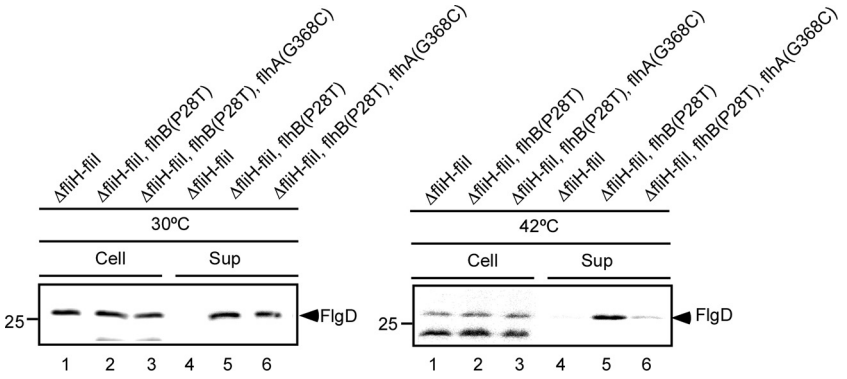

FIG. 4. Assay of secretion of FlgD: immunoblotting with polyclonal anti-FlgD antibody of whole cells and culture supernatants of MMHI001 (Δ*fliH* Δ*fliI*), MMHI0117 [Δ*fliH* Δ*fliI flhB*(*P28T*)], and MMHI0117-28 [Δ*fliH* Δ*fliI flhB*(*P28T*) *flhA*(*G368C*)].

chromatography at 42°C (Fig. 2). Interactions of $FlhA_C$ with GST-FliJ and GST-$FlhB_C$ were clearly detected even in the presence of the G368C mutation (Fig. 2, fourth and fifth rows, respectively). Interestingly, the amount of His-$FlhA_C$(G368C) that coeluted with GST-FliJ or GST-FlhB was much larger than the amount of His-$FlhA_C$ that coeluted (Fig. 2, fourth and fifth rows, respectively), indicating that the mutation somehow increases the binding affinity of $FlhA_C$ for both FliJ and $FlhB_C$.

Effect of the G368C mutation on substrate translocation through the export gate. We found that the G368C mutation in $FlhA_C$ does not interfere with the interactions with FliH, FliI, FliJ, and $FlhB_C$ even at 42°C (Fig. 1 and 2), raising the possibility that this mutation directly blocks translocation of an export substrate through the export gate. Since the P28T mutation of FlhB greatly increases the export efficiency in the absence of FliH and FliI, probably by increasing the probability of entry of flagellar proteins into the export gate (23), we analyzed the levels of secretion of the hook capping protein FlgD by a Δ*fliH* Δ*fliI flhB*(*P28T*) *flhA*(*G368C*) mutant (Fig. 4). At 30°C, the level of FlgD secretion by this mutant was similar to the level of FlgD secretion by the Δ*fliH* Δ*fliI flhB*(*P28T*) mutant (Fig. 4, left panel). In contrast, the G368C mutation reduced the levels of secretion considerably at 42°C (Fig. 4, right panel). These results suggest that this mutation significantly reduced the efficiency of protein translocation into the central channel of the growing flagellar structure.

Isolation of pseudorevertants from a culture of the *flhA*(*G368C*) mutant. To understand how the G368C mutation interferes with flagellar protein export at 42°C, pseudorevertants were isolated from an *flhA*(*G368C*) mutant overnight culture by streaking the culture on soft agar plates, incubating the plates at 42°C for 2 days, and looking for motility halos emerging from the streaks. A total of 16 motile colonies were purified from such halos. The motility of these pseudorevertants was considerably better than that of the parent strain at 42°C, although it was not as good as that of the wild-type strain (Fig. 5A). In agreement with this, the levels of flagellar proteins secreted by these pseudorevertants were recovered to a significant degree (data not shown).

P22-mediated transduction experiments showed that all of the suppressor mutations were cotransduced with the parental G368C mutation, indicating that they were located near the first-site mutation (data not shown). Therefore, we sequenced the *flhBAE* operon of the pseudorevertants, and the mutations were all missense mutations in FlhA; these mutations included L359F (isolated nine times), G364R (isolated three times), R370S (isolated twice), and P550S (isolated twice) (Fig. 5B). The L359F mutation is in the flexible linker region formed by residues 328 to 361, while the other three mutations are in the relatively stable $FlhA_C$38K fragment (28).

Limited proteolysis by endoproteinase Glu-C. To test if the G368C mutation causes a conformational change in $FlhA_C$ at 42°C, we purified His-$FlhA_C$(G368C), carried out limited proteolysis at 42°C with an endoproteinase, Glu-C, which cleaves the C-terminal side of Glu residues, and determined the molecular masses of the fragments by matrix-assisted laser desorption ionization–time of flight mass spectrometry. The time course of degradation, as monitored by SDS-PAGE, is shown in Fig. 5C. In agreement with a previous report (28), most of wild-type $FlhA_C$ was degraded into a stable 38-kDa fragment ($FlhA_C$38K) (Fig. 5C, top left panel), which consisted of residues 352 to 692 (Fig. 5B). A very small amount of a 20-kDa fragment ($FlhA_C$20K) was also seen (Fig. 5C, top left panel). In contrast, the $FlhA_C$38K fragment derived from the G368C mutant variant was very unstable and quickly degraded into the $FlhA_C$20K fragment through several intermediate fragments (Fig. 5C, top right panel), indicating that the G368C mutation reduced the structural stability of $FlhA_C$ considerably. Based on the molecular mass (20,236 Da), $FlhA_C$20K was identified as a fragment consisting of amino acid residues 509 to 692 with a deduced molecular mass of 20,252 Da (Fig. 5B).

To examine if the structural instability of the $FlhA_C$38K fragment caused by the G368C mutation is reversed by the second-site mutations, we purified four intragenic suppressor mutant variants of His-$FlhA_C$ and carried out limited proteolysis at 42°C. As shown by the data for the G368C/G364R and G368C/P550S mutants (Fig. 5C, bottom left and right panels, respectively), the $FlhA_C$38K fragments were still highly susceptible to the protease compared with the wild type and were as unstable as the His-$FlhA_C$(G368C) fragment. These results indicate that the second-site mutations do not restore the structural stability of $FlhA_C$38K, suggesting that the loss-of-function phenotype of the *flhA*(*G368C*) mutant at a restrictive temperature (42°C) is not an unavoidable consequence of the structural instability caused by the G368C mutation.

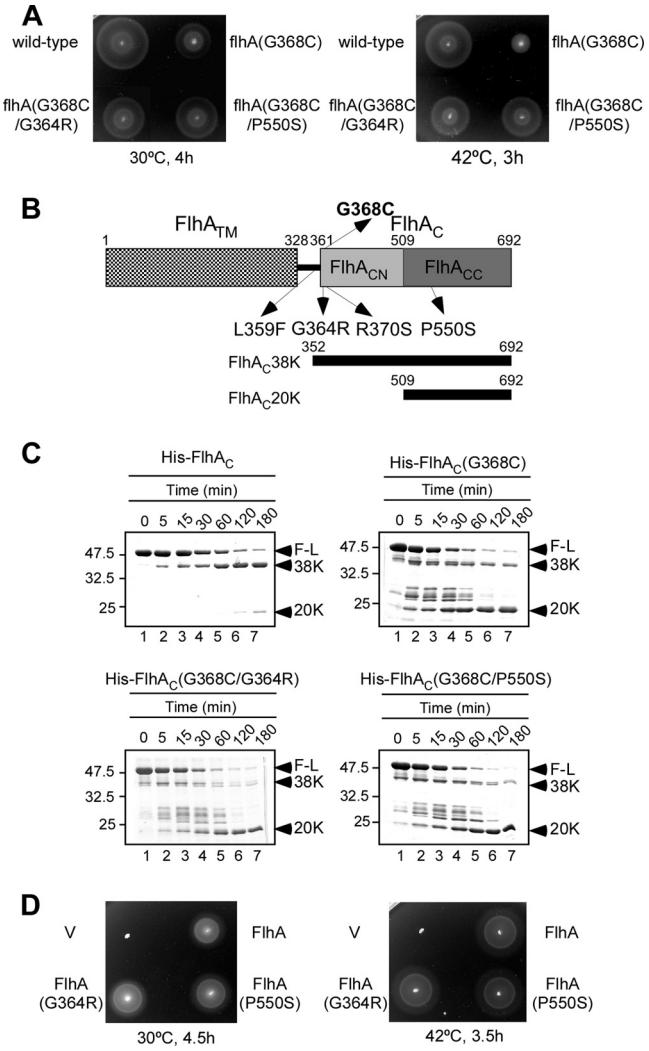

FIG. 5. Characterization of pseudorevertants isolated from the *flhA*(G368C) mutant. (A) Swarming motility assay performed with the wild type, the *flhA*(*G368C*) mutant, and *flhA*(*G368C*) mutant pseudorevertants, including MMA2228-3 [*flhA*(*G368C*/*G364R*)] and MMA2228-9 [*flhA*(*G368C*/*P550S*)], at 30°C and 42°C. (B) Locations of the G368C mutation and intragenic suppressor mutations in stable fragments of FlhA produced by limited proteolysis. FlhA consists of an N-terminal transmembrane region (FlhA$_{TM}$) and a C-terminal cytoplasmic domain (FlhA$_C$) (15). The present study established that FlhA$_C$ can be divided into three subdomains: the linker (black bar), FlhA$_{CN}$, and FlhA$_{CC}$. The sites of mutations in FlhA of a temperature-sensitive *flhA*(*G368C*) mutant and pseudorevertants of this mutant are indicated by arrows. The N and C termini of FlhA are labeled 1 and 692, respectively. The regions corresponding to FlhA$_C$38K and FlhA$_C$20K are also indicated. (C) Limited proteolysis of His-FlhA$_C$, FlhA$_C$(G368C), and two suppressor variants of FlhA$_C$(G368C), His-FlhA$_C$(G368C/G364R) and His-FlhA$_C$(G368C/P550S), by the Glu-C endoproteinase at 42°C. The time course of digestion was monitored by SDS-PAGE and CBB staining. The arrowheads indicate the positions of full-length FlhA$_C$ (F-L) and its 38-kDa (38K) and 20-kDa (20K) cleavage products. (D) Swarming motility assay performed with the mutants with only second-site mutations. SJW1364 (*flhA*) was transformed with pTrc99A (V), pMM130 (wild type) (FlhA), pMM130(G364R) [FlhA(G364R)], or pMM130(P550S) [FlhA(P550S)] and incubated on soft agar plates at 30°C and 42°C.

Motility of the second-site *flhA* mutants. To test the effect of each second-site mutation by itself on motility, we carried out site-directed mutagenesis and analyzed the motility of the second-site *flhA* mutants on soft tryptone agar plates. The motility of all of the mutants was normal at both 30°C and 42°C (Fig. 5D), as shown by the data for the *flhA*(*G364R*) and *flhA*(*P550S*) mutants, indicating that the second-site mutations alone displayed no phenotype. These results suggest that the mutated residues are not important for flagellar protein export.

DISCUSSION

The G368C mutation blocks substrate entry into the export gate. The flagellar type III protein export process requires the following three steps: delivery of export substrates to the export gate located in the central pore of the basal body MS ring, insertion of the natively disordered N-terminal segment into the presumably narrow pore of the export gate, and successive unfolding and translocation of the compactly folded domains of export substrates by the export gate (16). The FliH$_2$-FliI complex, possibly together with FliJ, delivers export substrates to the export gate, which is composed of six integral membrane proteins, including FlhA and FlhB (14). The interaction between the FliH-FliI complex and the FlhA-FlhB platform allows the unfolded N-terminal segment of an export substrate to be efficiently inserted into the export gate (23).

FlhA$_C$ is directly involved in the substrate delivery process along with FliH, FliI, FliJ, and FlhB$_C$ (6, 21, 24, 28). It has also been shown that the V404M mutation in FlhA$_C$ increases the export efficiency in the absence of the FliH and FliI, suggesting that FlhA$_C$ is involved in the process of substrate insertion into the export gate (23). However, it was not clear how FlhA$_C$ functions in this process. Here, we characterized the *flhA*(*G368C*) mutant; in this mutant the secretion activity of flagellar proteins was considerably reduced at a restrictive temperature (42°C) but not at a permissive temperature (30°C) (Fig. 1A). FlhA$_C$(G368C) was still able to interact with FliH, FliI, FliJ, and FlhB$_C$ (Fig. 1 and 2). Introduction of the *flhA*(*G368C*) mutation into the Δ*fliH* Δ*fliI flhB*(*P28T*) mutant strain, which can efficiently export flagellar proteins even in the absence of FliH and FliI (23), resulted in a significant decrease in the level of secretion of flagellar proteins at 42°C but not at 30°C (Fig. 4), indicating that the G368C mutation directly inhibits the translocation of export substrates into the central channel of the growing flagellar structure. This suggests that FlhA$_C$ not only forms part of the docking platform of the export gate for efficient substrate delivery but also is directly involved in the translocation process. Since the P28T mutation of FlhB allows efficient entry of flagellar proteins and translocation of these proteins into the export gate even in the absence of FliH and FliI (23), the G368C mutation seems to block the export process after docking of the FliH-FliI-FliJ-export substrate complex to the FlhA-FlhB platform of the export gate.

The G368C mutation causes partial disorder in the FlhA$_C$ structure. Limited proteolysis of wild-type FlhA$_C$ gave rise to a relatively stable FlhA$_C$38K fragment, which consists of amino acid residues 352 to 692 of FlhA (Fig. 5B and 5C). FlhA$_C$38K consists of at least two subdomains, FlhA$_{CN}$ and FlhA$_{CC}$, which consist of residues 352 to 508 and residues 509 to 692, respectively (Fig. 5B). In contrast to wild-type FlhA$_C$38K, FlhA$_C$38K(G368C) was susceptible to the protease, and it was

easily degraded into a more stable $FlhA_C$20K fragment identified as $FlhA_{CC}$ (Fig. 5C, top right panel), indicating that the G368C mutation induces structural disorder or partial unfolding in $FlhA_{CN}$. Thus, $FlhA_{CN}$ appears to be unstable due to the G368C mutation. This observation is consistent with the crystal structure of $FlhA_C$ that shows a relatively independent domain arrangement of $FlhA_{CN}$ and $FlhA_{CC}$ (28a).

Gly-368 is buried in the $FlhA_{CN}$ domain in the crystal structure. However, since $FlhA_C$(G368C) tends to form a dimer in the absence of a reducing reagent (data not shown), Cys-368 seems to be surface exposed a significant fraction of the time. This is consistent with the observation obtained by limited proteolysis that the G368 mutation destabilizes $FlhA_{CN}$ to expose cleavage sites in $FlhA_{CN}$ to solvent.

The second-site mutations do not affect the protease sensitivity of $FlhA_{CN}$. Suppressor analysis of the G368C mutant identified one suppressor mutation in the flexible linker region, two suppressor mutations in $FlhA_{CN}$, and one suppressor mutation in $FlhA_{CC}$. The improved motility of the suppressor mutants on soft agar plates was due to restoration of flagellar protein export almost to wild-type levels (Fig. 5A). The level of motility of the mutant with each second-site mutation was essentially the same as the wild-type level (Fig. 5D). Thus, there is no phenotype that is associated with the suppressor mutations alone, implying that the residues of the second-site mutations are not directly involved in flagellar protein export. Limited proteolysis revealed that the suppressor mutant variants of $FlhA_C$ are partially disordered at 42°C, similar to $FlhA_C$(G368C) (Fig. 5C), indicating that the structural instability of $FlhA_{CN}$ caused by the first-site mutation is not reversed by the second-site mutations. Therefore, the structural instability does not appear to be directly responsible for the loss-of-function phenotype of the *flhA*(*G368C*) mutant, although this is rather unexpected for a temperature-sensitive mutation.

The L359F and P550S mutations, which are located in the flexible linker and $FlhA_{CC}$, respectively, suppress the G368C mutation located in $FlhA_{CN}$ at 42°C (Fig. 5), even though they are located in different domains and are relatively far from one another in the crystal structure of $FlhA_C$ (Saijo-Hamano et al., submitted). This suggests that the second-site mutations compensate for the first-site mutation by altering the interactions among the linker, $FlhA_{CN}$, and $FlhA_{CC}$. As discussed above, the G368C mutation inhibits the export process after docking of the FliH-FliI-FliJ-export substrate complex with the $FlhA_C$-$FlhB_C$ platform of the export gate. Therefore, we propose that the interactions of the FliH-FliI-FliJ-export substrate complex with the $FlhA_C$ domain may need to induce a relative conformational arrangement of the three subdomains of $FlhA_C$ to allow export substrates to be translocated into the central channel of the growing flagellar structure. Since this process occurs on the cytoplasmic side of the export gate, it is likely that the conformational rearrangement of $FlhA_C$ is responsible for substrate entry into the gate.

ACKNOWLEDGMENTS

We acknowledge T. Ibuki for his kind gift of pTIH001 and M. Kinoshita for technical assistance.

This work was supported in part by Grants-in-Aid for Scientific Research 16087207 and 21227006 to K.N., by grants from the Targeted Proteins Research Program to T.M. K.N., and K.I. from the Ministry of Education, Science and Culture of Japan, and by U.S. Public Health Service grant AI12202 to Robert M. Macnab, who was a professor at Yale University and died suddenly on 7 September 2003.

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