ATPase Activity and Multimer Formation of PilQ Protein Are Required for Thin Pilus Biogenesis in Plasmid R64*

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Plasmid R64 pilQ gene is essential for the formation of thin pilus, a type IV pilus. The pilQ product contains NTP binding motifs and belongs to the PulE-VirB11 family of NTPases. The pilQ gene was overexpressed with an N-terminal His tag, and PilQ protein was purified. Purified His tag PilQ protein displayed ATPase activity with a Vmax of 0.71 nmol/min/mg of protein and a Km of 0.26 mM at pH 6.5. By gel filtration chromatography, PilQ protein was eluted at the position corresponding to 460 kDa, suggesting that PilQ protein forms a homooctamer. To analyze the relationship between structure and function of PilQ protein, amino acid substitutions were introduced within several conserved motifs. Among 11 missense mutants, 7 mutants exhibited various levels of reduced DNA transfer frequencies in liquid matings. Four mutant genes (T234I, K238Q, D263N, and H328A) were overexpressed with a His tag. The purified mutant PilQ proteins contained various levels of reduced ATPase activity. Three mutant PilQ proteins formed stable multimers similar to wild-type PilQ, whereas the PilQ D263N multimer was unstable. PilQ D263N monomer exhibited low ATPase activity, while PilQ D263N multimer did not. These results indicate that ATPase activity of the PilQ multimer is essential for R64 thin pilus biogenesis.

Type IV pili have been identified in various Gram-negative pathogens such as Neisseria gonorrhoeae, Vibrio cholerae, Pseudomonas aeruginosa, and enteropathogenic Escherichia coli (1). The contribution of type IV pili to virulence lies primarily in their ability to promote the attachment of the pathogens to various receptors of host cells during colonization. Type IV pili are also required for bacterial locomotion known as twitching motility (2) as well as for the social gliding motility of myxobacteria (3). Proteins with extensive sequence similarity to type IV pilins were also found to be the essential components of protein secretion systems of Gram-negative bacteria and the DNA uptake systems of Gram-positive bacteria (4). Gram-negative bacteria such as Klebsiella oxytoca, Erwinia chrysanthemi, P. aeruginosa, and Xanthomonas campestris have protein secretion systems, called type II secretion systems or the general secretory pathway, which export extracellular proteins including pullulanase, pectinase, cellulase, and toxins outside of the outer membrane (5–7). The genes involved in pilus formation and protein export are related through amino acid sequence similarity among the structural subunits and conserved assembly proteins (4).

Of the various genes involved in the biogenesis of type IV pili and type II secretion systems, putative NTPases, including PulE of K. oxytoca (8), PilB of P. aeruginosa (9), OutE of E. chrysanthemi (6), and EpsE of V. cholerae (10), collectively known as the PulE-VirB11 family, are highly conserved. They are also found in type IV secretion systems including the conjugation systems of IncP plasmids RP4 and R751, and IncW plasmid R388 (11), the T-DNA transfer systems of agrobacterial Ti and Ri plasmids (12), and the Pil protein secretion system of Bordetella pertussis (13). The encoded proteins contain several conserved motifs: Walker box A and B involved in ATP binding (14), an Asp box, and a His box (see Fig. 4A). In addition to these motifs, a group of PulE-VirB11 family NT- Pases, related to type IV pilus biogenesis systems and type II secretion pathways, contain two CXXC motifs and an Arg box (8). Many of the PulE-VirB11 family NT-Pases have been shown to be essential for piliation, extracellular protein secretion, transformation, and conjugal transfer (15). Several PulE- VirB11 family NT-Pases have been purified and characterized (10, 16–18). Genetic studies have also been performed for the PulE-VirB11 family NT-Pases (8, 19–21). In addition, TrwD and TrbB proteins of plasmids R388 and RP4, respectively, and HP0525 protein in the cag pathogenicity island of Helicobacter pylori have been shown to exhibit NT-Pase activity (17, 22).

IncI plasmids R64 and ColI-P9 form two types of sex pili, a thick rigid pilus and a thin flexible pilus. The thin pilus is required only for conjugation in liquid media (23). A 16-kb DNA segment, including the traA-D and pilI-V genes, of the 54-kb R64 transfer region is required for the formation of thin pilus (24). R64 and ColI-P9 thin pili belong to the type IV pilus family based on the amino acid sequence similarities between various pil products and proteins related to the formation of other type IV pili. The products of the traBC and pilK-V genes were shown to be essential for the formation of thin pili on the cell surface (25, 26). R64 and ColI-P9 thin pili function in the formation of donor-recipient cell aggregates in liquid matings. Specificity of recipient cells is determined by seven C-terminal segments of PilV adhesins, which are switched through shuffling multiple inversion (27, 28). The R64 pilQ product shares amino acid sequence similarity with PilE-VirB11 family NT-Pases (24).

In this work, the R64 pilQ gene product, essential for thin pilus formation, was overproduced and purified. Purified PilQ protein formed homooctamers and displayed ATPase activity. Several amino acid substitutions were introduced into the pilQ gene within conserved motifs. Effects of pilQ mutations on thin pilus formation, ATPase activity, and multimer stability were examined.

1 The abbreviations used are: kb, kilobase pair(s); DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; MES, 2-morpholinoothanesulfonic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography; LB, Luria-Bertani.

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**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—The bacterial strains used in this work were *E. coli* JM83 (λlac-proAB) pRSL2h thi ara 800 diaZam515 (29), NF83 recA56 araΔlac-proAB pRSL2h φ80 diaZam515 (30), BL21 (DE3) dcm omprT hsdR fel ω (DE3) (31), and TN102 NaI12 (23). Plasmid vector pUC119 (32) was used for cloning. pET28b and pET11a (31) were used for overexpression of the *pilQ* gene. pKK641A’ (28) and pilQ genes for R64 thin pilus formation were used, and pilQ gene on pKK661, pKK641A’ pilQ1 (25) carried the *pilQ* frameshift mutation. pKK661 (28) was a PSSG576-derivative plasmid carrying pilQ genes for remaining R64 transfer functions including oriT. pKK702a (25) was a pUC119-derivative plasmid carrying R64 *pilQ* gene.

**Media**—Luria-Bertani (LB) medium was prepared as previously described (33). The solid medium contained 1.5% agar. Antibiotics were added to LB medium at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; and naldixid acid, 20 μg/ml.

**Conjugal Transfer**—*E. coli* NF83 or BL21 (DE3) cells harboring pKK661, pKK641A’ pilQ1 and *pilQ* plasmid, and *E. coli* TN102 cells were used as donor and recipient cells, respectively. Donor cells were grown to the log phase and mixed with recipient cells in the stationary phase. After shaking for 90 min at 37 °C, the mixture was plated at various dilutions onto LB plates containing chloramphenicol and naldixid acid. Since pKK661, pKK641A’ pilQ1 and *pilQ* plasmid complement each donor, cells harboring pKK661, pKK641A’ pilQ1 and pilQ plasmid transmitted pKK661 carrying oriT into recipient cells (23). Transfer frequency is presented as the ratio (expressed as percent) of the number of transconjugants to that of donor cells. In the case of BP4 (DE3) harboring pEQ11, LB medium containing 0, 10, 50, and 100 μM IPTG was used.

**Construction of Plasmids**—Construction of plasmids and other methods of DNA manipulation were performed as described previously (33).

To construct the T7 promoter overexpression system of the *pilQ* gene, an *NdeI* site was introduced at the initiation codon of the *pilQ* gene by PCR using appropriate primers and pKK702a DNA as a template. The *NdeI* fragment of the PCR product was inserted into the NdeI site of *BamHI* site of pET28b to give pEQ28. The Xhol-BamHI fragment of pEQ28 was inserted into the Xhol-BamHI sites of pUC119 and pET11a, respectively.

The *pilQ* mutants were constructed by the PCR-mediated site-directed mutagenesis method using pKK702b as a template (34). The resultant mutants are as follows: F2098S (TTT to TTC), 1219PT (ATA to ACA), T234H (ACC to ATC), K263Q (AAA to CAA), D265N (GAT to AAT), D304N (GAT to AAT), H328A (CAC to GCC), C375A (TGT to GCT), C423A (TGT to GCT), R432A (AGA to GCA), and I490V (ATC to GTC). To construct the Δ340 deletion mutant, the *NdeI*-Acrl fragment of pKK702b was introduced into the *HincII* site of pUC119 by blunt end ligations after treatment with Klenow fragment. To construct mutant *pilQ* overexpression plasmids, the Xhol-BamHI fragments of pKK702b with mutant *pilQ* genes were inserted into the Xhol-BamHI site of pET28b.

**Purification of PilQ Protein**—An overnight culture of BL21 (DE3) cells harboring pEQ28 was diluted 50-fold in LB medium (500 ml) containing kanamycin and incubated at 37 °C with shaking. When the *A* <sub>600</sub> of the culture reached ~0.5, IPTG was added to a final concentration of 1 mM. After 1 h, the induced cells were harvested by centrifugation, washed with acetone, and protein concentration and ATPase activity were determined by a protein assay kit (Bio-Rad) and by the TLC method, respectively. The following proteins were used as molecular mass standards: ferritin (450 kDa), catalase (240 kDa), and bovine serum albumin (68 kDa).

**ATP Digestion of Wild-type and Mutant PilQ Proteins**—To analyze ATP-induced conformational change of the PilQ protein, trypsin sensitivity of His tag PilQ protein was compared in the presence and absence of 1 mM ATP. Two μg of the purified His tag PilQ protein with or without mutations were digested on ice with 0.3 μg of trypsin in 20 μl of TES buffer (10 mM Tris-HCl, 100 mM NaCl, and 3 μM trypsin) for 30 min. The reaction was stopped by the addition of 1 μl of 4 μm phenylmethylsulfonyl fluoride and 5 μl of 20% trichloroacetic acid. The precipitates of acid-denatured proteins were collected by centrifugation, washed with acetone, and dissolved in SDS sample buffer. The samples were applied to SDS-PAGE (15% gel), and then tryptic fragments of PilQ protein were detected by Western blot analysis using anti-PilQ antibody.

**RESULTS**

**Overexpression and Purification of PilQ Protein**—To study the characteristics of the R64 pilQ product, the pilQ gene was cloned into pET28b to give pEQ28 (Fig. 1A). pEQ28 carried a modified pilQ gene in which a stretch of six histidine residues was attached at its N terminus. To examine the effects of the PilQ N-terminal His tag on PilQ activity, the complementation activity of pEQ28 for the *pilQ* frameshift mutation in R64 liquid matings was estimated. For that purpose, pEQ11, an empty derivative of pEQ28, was constructed. *E. coli* strain BL21 (DE3) harboring pKK661, pKK641A’ pilQ1 and was less than 0.0001%. When pKK702a carrying the wild-type pilQ gene on pUC119 was introduced into donor cells, the transfer
The purified His tag PilQ protein was used to immunize a rabbit. The purified anti-PilQ antibodies specifically reacted against a 58-kDa PilQ protein present in whole cell lysate from E. coli cells harboring pKK641A' or pKK702a.

Subcellular Localization of PilQ Protein—Subcellular fractionation experiments were performed to determine localization of PilQ protein within cells. E. coli cells harboring pKK641A' or pKK702a were separated into cytoplasmic, periplasmic, inner membrane, and outer membrane fractions. Proteins in every fraction were separated by SDS-PAGE and analyzed for PilQ protein by Western blotting using anti-PilQ antibody. PilQ protein was recovered almost exclusively in the cytoplasmic fraction of cells harboring pKK641A' or pKK702a (Fig. 2). These results indicate that the PilQ protein is a cytoplasmic protein.

**Detection of ATPase Activity**—To detect the ATPase activity of the purified His tag PilQ protein, thin layer chromatography (TLC) analysis was performed. When the purified PilQ protein was incubated with [γ-32P]ATP, release of a P, radiochromatographic spot was observed on a TLC plate in a time- and PilQ concentration-dependent manner (data not shown), indicating that the PilQ protein contains ATPase activity.

The effects of different reaction conditions on His tag PilQ ATPase activity were examined (Table II). When PilQ ATPase reactions were carried out under different pH conditions using MES (pH 5–7) and Tris (pH 7.5–9) buffers, maximum ATPase activity was observed around pH 6.5, while either increasing or decreasing the pH from 6.5 significantly lowered the ATPase activity. The PilQ ATPase required divalent cations since EDTA, a divalent cation chelator, strongly inhibited ATPase activity. The optimal concentration of Mg2+ required for ATPase activity was ~5 mM, whereas a similar concentration of Ca2+ resulted in lower activity. The optimal salt concentration was ~25 mM for both KCl and NaCl, while KCl produced slightly higher ATPase activity than NaCl. Therefore, further ATPase assays were performed with 5 mM MgCl2, 25 mM KCl and at pH 6.5. Kinetics of ATP-hydrolyzing activity of PilQ protein was estimated by the spectrophotometric method. As derived from the Eadie-Scatchard plot (data not shown), the apparent Ks for ATP was 0.26 mM, and the Vmax value was 0.71 nmol/min/mg of protein.

**Inhibition of ATP Hydrolysis with NTPs or dNTPs**—To examine whether His tag PilQ protein is able to bind to NTPs or dNTPs other than ATP, the inhibition of PilQ ATPase by NTPs or dNTPs was estimated. A standard ATPase assay with [γ-32P]ATP was performed in the presence of 5-fold excess of unlabeled NTPs or dNTPs. Unlabeled ATP apparently inhibited PilQ ATPase by 79% as expected (Fig. 3). PilQ ATPase inhibition by dATP and dGTP were 55% and 30%, respectively, while inhibition by GTP, CTP, UTP, dCTP, and dTTP was less than 20%. These results indicate that PilQ protein exhibits the highest affinity to ATP followed by dATP and dGTP. PilQ affinity to other NTPs or dNTPs is low. It is of great interest whether these NTPs or dNTPs are hydrolyzed by PilQ protein. [α-32P]dATP hydrolysis by PilQ protein was demonstrated by TLC analysis (data not shown).

**Construction of the Mutant pilQ Genes**—In the previous sections, we have demonstrated that R64 PilQ protein contains ATPase activity. Hence, it was of interest to test whether the ATPase activity of PilQ protein is required for R64 thin pilus biogenesis. For that purpose, several amino acid substitutions were introduced within the conserved motifs of the His tag pilQ genes, including the NTP-binding motifs, by the PCR method. In summary, 11 missense and 1 deletion mutants were obtained (Fig. 4A, Table III).

Intracellular production of mutant His tag PilQ protein was analyzed by Western blotting using anti-PilQ antibody (Fig. 4B, top panel). E. coli cells carrying missense pilQ genes produced mutant PilQ proteins at the same levels as that of the wild-type PilQ protein. Extracellular thin pilus production was analyzed using anti-pilin antibody (Fig. 4B, bottom panel). The culture media of E. coli cells harboring pKK641A' pilQ1 and mutant pilQ plasmids were ultracentrifuged to recover thin pili.
membrane.

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were carried out as detailed under "Experimental Procedures." Each of cells harboring pKK641A

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produced approximately twice, twice, 5-fold, and 10-fold as much PilQ protein as compared with those harboring pKK702a produced approximately twice as much PilQ protein as compared with those harboring pKK641A under the presence of 0, 10, 50, and 100 μM IPTG, respectively.

\[ \text{activity of mutant PilQ proteins for thin pilus biogenesis} \]

were estimated in the presence of various concentrations of IPTG, and is indicated as a percentage of transconjugants relative to donor cells. The effects of the addition of the mutant pilQ genes to donor cells harboring pKK661 and pKK641A

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were dominant negative over the wild-type pilQ gene—transfer frequency were studied to test whether the transdominant character of these mutants. All of the four mutant pilQ genes had little or no thin pili, whereas cells harboring the remaining missense pilQ mutants produced similar amounts of thin pili as the wild type.

The activity of mutant PilQ proteins for thin pilus biogenesis was also determined as transfer frequency in liquid matings. The effects of the addition of the mutant pilQ plasmids on the transfer frequency in liquid mating from donor cells harboring pKK661 and pKK641A

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were estimated. E. coli NF83 donor cells harboring pKK661, pKK641A

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pilQ1 were transmitted pKK661 into the recipient cells by conjugation at a frequency of 1.6%, while those harboring only pKK661 and pKK641A

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did not (Table III, pilQ activity). Different levels of recovery in the transfer frequency were observed by introducing various mutant pilQ genes into donor cells. Transfer frequencies were recovered to the wild-type levels by the pilQ I219T, D304N, R432A, and I490V mutants. For the pilQ

F209S, T234I, K238Q, H328A, C375A, and C423A mutants, various levels of recovery in transfer frequencies were observed. For the pilQ D263N and Δ340 mutants, no recovery was observed. These results are consistent with the results of extracellular thin pilus production (Fig. 4B) and indicate that the pilQ F209S, T234I, K238Q, D263N, H328A, C375A, C423A, and Δ340 mutant are defective in R64 thin pilus biogenesis and subsequently in R64 liquid matings.

Transdominance of the Mutant PilQ Genes over Wild-type pilQ Gene—Addition of pKK702h (mucopy wild-type His tag pilQ) to donor cells harboring pKK661 and pKK641A

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had little effect on the transfer frequency (Table III, Transdominance). The effects of introduction of multiple copies of mutant pilQ genes to donor cells harboring pKK661 and pKK641A

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on the transfer frequency were studied to test whether the His tag pilQ mutants were dominant negative over the wild-type pilQ gene. The introduction of pilQ T234I, K238Q, D263N, and H328A mutants into donor cells harboring pKK661 and pKK641A

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decreased the transfer frequency, indicating the transdominant character of these mutants. All of the four mutants exhibited reduced or no pilQ activity during liquid matings.

ATPase Activity of Mutant PilQ Proteins—The products of four mutant His tag pilQ genes (pilQ T234I, K238Q, D263N, and H328A), which exhibited reduced transfer frequency and transdominant character in liquid matings, were overproduced and purified by the same procedure as for wild-type His tag PilQ protein (Fig. 1B). ATPase activity of mutant PilQ proteins was determined. All of the mutant PilQ proteins exhibited reduced ATPase activity (Table III, ATPase activity). The ATPase activity of PilQ D263N was particularly low. Residual

TABLE I
Effects of overexpression of pilQ gene with His tag on the complementation of the pilQ1 mutation
Transfer frequency of pKK661 from E. coli BL21 (DE3) donor cells harboring pKK661, pKK641A

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pilQ1, and complementation plasmids in liquid mating was estimated in the presence of various concentrations of IPTG, and is indicated as a percentage of transconjugants relative to donor cells.

| Plasmid | Transfer frequency in the presence of various concentrations (μM) of IPTG |
|---------|---------------------------------------------------------------|
|         | 0       | 10     | 50     | 100    |
|         | μmol/min/mg protein % |
| pKK661, pKK641A ' | 1.2     | 0.97   | 0.8    | 0.93   |
| pKK661, pKK641A ' pilQ1 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| pKK661, pKK641A ' pilQ1, pKK702a | 1.0     | 0.51   | 0.54   | 0.5    |
| pKK661, pKK641A ' pilQ1, pEQ11 | 1.1     | 1.0    | 1.1    | 0.67   |

\(^a\) Overproduction of PilQ protein under various conditions was roughly estimated by Western blot analysis using anti-PilQ antibody. E. coli NF83 cells harboring pKK702a produced approximately twice as much PilQ protein as compared with those harboring pKK641A'. E. coli BL21 (DE3) cells harboring pKK702a produced approximately twice, twice, 5-fold, and 10-fold as much PilQ protein as compared with those harboring pKK641A' under the presence of 0, 10, 50, and 100 μM IPTG, respectively.

Fig. 2. Localization of R64 PilQ protein. Subcellular fractionation of cells harboring pKK641A' (upper panel) or pKK702a (lower panel) were carried out as detailed under "Experimental Procedures." Each fraction was analyzed by SDS-PAGE (12%), followed by Western blot analysis using anti-PilQ antibody. Lane W, whole cell lysate; lane C, cytoplasm; lane P, periplasm; lane I, inner membrane; lane O, outer membrane.

Fig. 3. Distinct substrate affinity of R64 PilQ protein. For detection of distinct substrate affinity, a standard ATPase assay with [γ-\text{32P}]-ATP as substrate was carried out with 5-fold molar excess of unlabeled NTPs or dNTPs. Effects of inhibitors were expressed as residual [γ-\text{32P}]ATP hydrolysis activity. The rate of ATP hydrolysis without NTPs or dNTPs was defined as 100%.

Detachment of pilQ F209S, T234I, K238Q, D263N, H328A, C375A, and C423A mutants, various levels of recovery in transfer frequencies were observed. For the pilQ D263N and Δ340 mutants, no recovery was observed. These results are consistent with the results of extracellular thin pilus production (Fig. 4B) and indicate that the pilQ F209S, T234I, K238Q, D263N, H328A, C375A, C423A, and Δ340 mutant are defective in R64 thin pilus biogenesis and subsequently in R64 liquid matings.
transfer frequencies in these mutants were roughly dependent on their residual ATPase activity. These results suggest that reduced ATPase activity in the pilQ T234I, K238Q, D263N, and H328A mutants results in their reduced transfer frequency. Thus, it may be concluded that the ATPase activity of PilQ protein is required for R64 thin pilus biogenesis and consequently for R64 liquid matings. The products of the other mutant pilQ genes (pilQ F209S, C375A, C423A, and Δ340) with reduced transfer frequency were also overproduced. However, these mutant proteins could not be purified due to their insolubility.

ATP Induced Conformational Change in Mutant PilQ Pro- teins—The PilQ T234I, K238Q, D263N, and H328A mutant proteins exhibited reduced ATPase activity. It is important to test whether these mutant proteins are defective in ATP binding. Conformational changes of wild-type and mutant PilQ proteins caused by ATP binding were examined by trypsin digestion. By trypsin digestion of wild-type PilQ protein, only two major fragments (36 and 29 kDa) were produced in the absence of ATP, while two additional minor fragments (32 and 23 kDa) were produced in the presence of 1 mM ATP (Fig. 5). Conformational change of PilQ protein by ATP-binding may result in distinct proteolytic fragment patterns. Distinct proteolytic patterns in the absence and presence of ATP were detected for PilQ T234I, D263N, and H328A proteins. However, for PilQ K238Q, the Walker box A mutant, the 29-kDa fragment was produced even in the absence of ATP. These results suggest that PilQ K238Q mutant is unable to bind to ATP, whereas PilQ T234I, D263N, and H328A mutants are able to bind to ATP despite residual ATPase activity.

Formation and Stability of the PilQ Multimer—Formation of hexameric ring structures of RP4 TrbB protein and H. pylori HP0525 protein, members of PulE-VirB11 family NTPases, have been reported in the presence of dATP and Mg²⁺ (40). In the Sephacryl S-200 gel filtration chromatography during PilQ purification, the wild-type and mutant His tag PilQ proteins

characteristics and positions of pilQ mutations. A, schematic representation of the C-terminal half of PilQ. At the top, several conserved motifs of R64 PilQ sequence are shown: Walker box A, Walker box B, Asp box, His box, two CXXC motifs, and Arg box. Highly conserved amino acids within these motifs were replaced by amino acids indicated by downward arrows. The number of amino acid residues in PilQ protein is indicated above the bar. B, intracellular mutant PilQ production (upper panel) and extracellular pilin production (lower panel) in various pilQ mutants. Thin pili were precipitated by ultracentrifugation of culture medium in which cells harboring pKK641A indicated at the left.

FIG. 4. Characteristics of the products of various mutant pilQ genes

| Plasmid          | pilQ activity | Transdominance | ATPase activity |
|------------------|---------------|----------------|----------------|
| pKK661, pKK641 | <0.0001       | 1.7            | NT             |
| pKK661, pKK641 | 1.6           | 1.5            | 1.02 (100)     |
| pKK661, pKK641 | 0.03          | 1.2            | NT             |
| pKK661, pKK641 | 1.5           | 1.2            | NT             |
| pKK661, pKK641 | 0.012         | 0.14           | 0.28 (27)      |
| pKK661, pKK641 | 0.002         | 0.03           | 0.075 (7)      |
| pKK661, pKK641 | <0.0001       | 0.009          | 0.013 (1.3)    |
| pKK661, pKK641 | 0.02          | 0.22           | 0.46 (45)      |
| pKK661, pKK641 | 0.08          | 1.4            | NT             |
| pKK661, pKK641 | 0.02          | 1.3            | NT             |
| pKK661, pKK641 | 1.4           | 1.4            | NT             |
| pKK661, pKK641 | 1.4           | 1.4            | NT             |
| pKK661, pKK641 | <0.0001       | 1.1            | 1.1            |

* NT, not tested.
were eluted at a position corresponding to a multimer larger than a monomer. The precise size and stability of PilQ multimers were determined by Superose 6 gel filtration chromatography in the presence or absence of 1 mM ATP. The wild-type His tag PilQ protein was eluted at a position corresponding to 460-kDa irrespective of the presence or absence of 1 mM ATP (Fig. 6A). Since the molecular mass of the PilQ monomer was shown to be 58 kDa, wild-type PilQ protein most likely forms a homooctamer in the presence and absence of 1 mM ATP. These results also indicate that the PilQ octamer is stable in the presence or absence of 1 mM ATP. The ATPase activity of each fraction roughly corresponds to its protein concentration. Three mutant PilQ proteins (T234I, K238Q, and H328A) formed stable multimers with reduced ATPase activity in the presence and absence of 1 mM ATP (data not shown). In contrast, PilQ D263N multimer was unstable especially in the absence of ATP, since PilQ D263N protein was eluted as a mixture of multimers and monomers (Fig. 6B). The PilQ D263N monomer exhibited weak ATPase activity, while its multimer did not. When concentrated PilQ D263N monomer fraction was rechromatographed on Superose 6, PilQ D263N protein was again eluted as a mixture of multimers and monomers, indicating that PilQ D263N protein is in equilibrium with multimer and monomer forms (data not shown). These results indicate that the region containing the PilQ D263N mutation is involved in stable multimer formation.

**DISCUSSION**

In the present work, we have overproduced the R64 pilQ gene encoding a PulE-VirB11 family NTPase with an N-terminal His tag. His tag PilQ protein was purified to near homogeneity (>98%). Purified PilQ protein displayed ATPase activity with a \( V_{\text{max}} \) of 0.71 nmol/min/mg of protein and a \( K_m \) of 0.26 mM at pH 6.5. The \( V_{\text{max}} \) and \( K_m \) values of PilQ ATPase are similar to those reported for PulE-VirB11 family NTPases such as R388 TrwD, RP4 TrbB, and *H. pylori* cag HP0525 (17, 22).

PilQ protein was shown to be located in the cytoplasmic fraction irrespective of the presence or absence of the other Pil proteins. These results differ from those of *K. oxytoca* PulE protein, which was shown to be located in the inner membrane (8). R388 TrwD was located in the cytoplasmic and outer membrane fraction (17). PilQ ATPase may function at or near the inner membrane, since it is involved in the formation of thin pilus, a cell envelope-associated appendage. Association of PilQ and the other Pil components may be weak.

Optimum pH for PilQ ATPase was different from those of R388 TrwD, RP4 TrbB, and *H. pylori* cag HP0525 (17, 22). The pH optimum of PilQ was located at approximately 6.5, while that of TrwD and HP0525 ATPase and TrbB dATPase...
was located at approximately 9.0–9.5. Differences in substrate affinities were found among PilQ, TrbB, HP0525, and TrwD NTPases. PilQ, HP0525, and TrwD exhibited the highest activity for ATP, while TrbB exhibited maximum activity for dATP. Preferred substrates for PilQ are ATP, dATP, and dGTP, whereas those for TrbB are dATP, GTP, and ATP, and those for HP0525 are ATP, dATP, CTP, and dCTP. Furthermore, TrbB protein displayed distinct optimum pH for ATPase and GTPase activities (22). The biological significance of differences in substrate affinity remains to be elucidated.

PilQ protein formed homooolomers either in the presence or absence of ATP. PilQ homooolomer formation was observed even in the absence of Mg2+ (data not shown). Formation of homohexameric ring structure was reported for TrbB, HP0525, and TrwD (22, 40). TrbB hexamer was formed in the presence of Mg2+ and dATP or dADP, while TrbB tetramer was formed in the absence of Mg2+ and dATP. In contrast, HP0525 hexamer was formed irrespective of the presence or absence of Mg2+ and dATP. The requirement of NTPs for multimimer formation of various PurE-VirB11 NTPases may depend on differences in their protein structure.

Mutation analyses have been performed on various PurE-VirB11 family NTPases (8, 19–21). In the present report, we have introduced several amino acid substitutions into the pilQ gene within the Walker box A, B, Asp box, His box, Arg box, and CXXC motifs. Two Walker box A mutants, PilQ T234I and PilQ K283Q, failed to produce thin pili and exhibited low transfer frequencies. Purified mutant proteins displayed reduced ATPase activities. PilQ K283Q affected ATPase activity more severely than PilQ T234I. A trypsin digestion assay revealed that PilQ K283Q lost ATP binding activity, while PilQ T234I retained it. The failure of ATP binding of PilQ K283Q may be due to the loss of positive charge required for the direct interaction with β and γ phosphates of ATP resulting from the substitution of lysine by glutamine. Similar Walker box A mutants of PurE and TrbB also displayed severe effects on pullulanase secretion and conjugation, respectively (8, 22). Walker box B mutant PilQ D304N had no effects on thin pilus formation and transfer frequency.

Asp box mutant PilQ D263N exhibited the most severe effects on thin pilus biogenesis and liquid matings. The purified PilQ D263N protein displayed very low ATPase activity. The PilQ D263N octamer was unstable, especially in the absence of ATP. His box mutant PilQ H328A failed to produce thin pili and exhibited low transfer frequency. The purified PilQ H328A protein displayed 43% ATPase activity.

Four mutant genes (pilQ T234I, K238Q, D263N, and H328A) exhibited dominant negative character over the wild-type allele. The dominant negative character of these mutants suggests that mixed octamers can be formed from the mutant and wild-type PilQ proteins. RP4 TrbB, H. pylori HP0525, and R388 TrwD form hexameric ring structures. Krause et al. (40) postulated that such ring structures may aid a repetitive step/process of NTP hydrolysis. Incorporation of mutant monomers into PilQ octamers may inhibit the function of the octamer as a whole. Hence, the observed transdominance of PilQ mutants over the wild-type allele supports the above postulation. In this regard, the finding that the monomer form of the PilQ D263N mutant exhibited residual ATPase activity, is of particular interest.

The four mutant proteins (PilQ T234I, K238Q, D263N, and H328A) displayed varying levels of residual ATPase activities (2–43% of wild-type). In contrast, the amounts of thin pili produced by these mutants were very low as were their transfer frequencies (less than 1.5% of the wild-type). This situation is different in RP4 TrbB mutants (22). Some TrbB mutants exhibiting very low dATPase activity still exhibited wild-type levels of conjugation activity. Dependence of the R64 thin pilus biogenesis system on PilQ is stronger than that of RP4 system on TrbB.

Since the two CXXC motifs and the Arg box are notably conserved among PulE-VirB11 family NTPases of type IV pilus biogenesis systems and type II secretion systems, mutations were introduced into these motifs in PilQ. Two CXXC motif mutants, PilQ C375A and C423A, failed to produce thin pili and exhibited low transfer frequencies. Overexpression of these mutants resulted in insoluble PilQ mutant proteins. Insolubility of these mutants may be due to the failure of correct folding. Cysteine residues in PilQ CXXC motifs may be responsible for intramolecular disulfide bond formation, as was suggested for K. oxytoca PulE (21). Lack of transdominance may be related to the insolubility or abnormal conformation of these mutant proteins, as they were unable to form mixed octamers with wild-type protein. Arg box mutant PilQ R432A carried pilQ activities similar to the wild type, although this arginine residue in the Arg box is highly conserved in many PurE-VirB11 family NTPases of type IV biogenesis systems and type II secretion systems.

Four additional mutants, PilQ F209S, I219T, I490V, and Δ340, have been constructed. PilQ F209S and Δ340 failed to produce thin pili and exhibited low or no transfer frequencies, respectively, whereas PilQ I219T and I490V were normal. Insolubility and lack of transdominance suggest incorrect folding of PilQ F209S and Δ340.

In conclusion, ATPase activity and multimimer formntion of PilQ protein are essential for R64 thin pilus biogenesis. However, further investigation is required to reveal the precise role(s) of R64 PilQ ATPase in the biogenesis of R64 thin pili.

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