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Neisseria gonorrhoeae PilA Is an FtsY Homolog

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The *pilA* gene of *Neisseria gonorrhoeae* was initially identified in a screen for transcriptional regulators of *pilE*, the expression locus for pilin, the major structural component of gonococcal pili. The predicted protein sequence for PilA has significant homology to two GTPases of the mammalian signal recognition particle (SRP), SRP54 and SRα. Homologs of SRP54 and SRα were subsequently identified in bacteria (Ffh and FtsY, respectively) and appear to form an SRP-like apparatus in prokaryotes. Of the two proteins, PilA is the most similar to FtsY (47% identical and 67% similar at the amino acid level). Like FtsY, PilA is essential for viability and hydrolyzes GTP. The similarities between PilA and the bacterial FtsY led us to ask whether PilA might function as the gonococcal FtsY. In this work, we show that overproduction of PilA in *Escherichia coli* leads to an accumulation of pre-β-lactamase, similar to previous observations with other bacterial SRP components. Low-level expression of *pilA* in an *ftsY* conditional mutant can complement the *ftsY* mutation and restore normal growth to this strain under nonpermissive conditions. In addition, purified PilA can replace FtsY in an in vitro translocation assay using purified *E. coli* SRP components. A PilA mutant that is severely affected in its GTPase activity cannot replace FtsY in vivo or in vitro. However, overexpression of the GTPase mutant leads to the accumulation of pre-β-lactamase, suggesting that the mutant protein may interact with the SRP apparatus to affect protein maturation. Taken together, these results show that the gonococcal PilA is an FtsY homolog and that the GTPase activity is necessary for its function.

Neisseria gonorrhoeae is a major sexually transmitted pathogen that infects only humans. This gram-negative diplococcus normally infects cells of the urogenital tract to cause urethritis and cervicitis. Pili are the primary virulence factor for the gonococcus, a fimbrial adhesin that is absolutely required for infection in human volunteers (9, 49). Pilin is the major protein component of pili and is expressed from the *pilE* gene in *N. gonorrhoeae* MS11A (29). Pilin undergoes both phase and antigenic variation, which are likely important for evasion of the host immune system (45). In an effort to identify transcriptional regulators of *pilE* in *N. gonorrhoeae*, Taha et al. (54) isolated the *pilA* and *pilB* genes. PilA and PilB were proposed to be members of the two-component family of prokaryotic proteins which transduce environmental signals to cytoplasmic regulators via phosphorylation (33). The amino-terminal portion of PilA was predicted to contain a DNA-binding motif, and it has been demonstrated that PilA binds DNA in a sequence-specific manner (3, 51).

The carboxy-terminal part of PilA was shown to have significant homology to the G (GTP-binding) domains of the 54-kDa subunit of the eukaryotic signal recognition particle (SRP54) and of the SRP docking protein, SRα (50). The homology between PilA and these proteins is most striking in the G domains, and purified PilA has significant GTPase activity (4). This activity has an absolute requirement for $MgCl_2$ and a strict specificity for GTP.

The eukaryotic SRP is a ribonucleoprotein (RNP) complex of a 7S RNA and six different polypeptides of 9, 14, 19, 54, 68, and 72 kDa and is required for the targeting and insertion of the signal sequence of exported proteins into the endoplasmic reticulum membrane (reviewed in references 38 and 60). The SRP54 protein is associated with the 7S RNA in the complex and binds to the hydrophobic signal sequence of the nascent protein as it emerges from the ribosome. The complex is then targeted to the endoplasmic reticulum membrane, where it is bound by the docking protein. The SRP is next released from the nascent protein-ribosome-translocon complex in a GTP-dependent manner, and translation resumes concomitantly with translocation.

There is ample evidence for the existence and function of a bacterial SRP. The bacterial SRP54 and SRα homologs were initially identified by sequence homology and are called Ffh (or P48) and FtsY, respectively (5, 41). The bacterial SRP appears to be much simpler than the eukaryotic counterpart, consisting of Ffh, FtsY, and a 4.5S RNA (encoded by the *ffs* gene). The *ffh*, *ftsY*, and *ffs* genes are essential in *Escherichia coli* (10, 15, 34). Ffh interacts with 4.5S RNA (24, 30, 35, 40) and also with the hydrophobic signal sequences of presecretory proteins (6, 25). FtsY is the membrane-associated docking protein of the apparatus and has been shown to interact with the Ffh-4.5S RNA complex (30, 41).

Ffh and FtsY are both GTPases, and GTPase activity is required for the function of each in protein targeting (21, 36, 37, 42). Depletion or overexpression of any of the three components (Ffh, 4.5S RNA, or FtsY) leads to the accumulation of a number of preproteins (21, 24, 34, 35, 40, 47). Depletion of the 4.5S RNA or Ffh can also result in the induction of a heat shock response (7, 35). These observations suggest that the relative levels of each component of the SRP are critical for proper function of the apparatus.

The Ffh and SRP54 proteins have three domains: an M domain, which interacts with the signal peptide of nascent proteins as well as to the 7S RNA (4.5S in *E. coli*); a G domain, which has the GTPase activity required for the interaction of the complex with the docking protein and its subsequent re-

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lease; and an N domain of highly conserved, yet unknown function (60). FtsY and SRα also have three domains: the G domain, which has the GTPase activity and is homologous to the G domain of SRP54 and Ffh; an N domain of unknown function that is homologous to the N domain of SRP54 and Ffh; and a unique amino-terminal domain (60). The amino-terminal domain of FtsY is postulated to be important for membrane localization of the protein (37, 62).

Using a screening approach that takes advantage of the fact that the relative levels of each of the components of the bacterial SRP system are critical for function and survival of the organism (17, 24), Ulbrandt et al. (56) identified a number of proteins that utilize the SRP for localization. Eight of those identified were shown to encode polytopic cytoplasmic membrane proteins. Consistent with this, de Gier et al. (12) also demonstrated that the depletion of either Ffh or 4.5S RNA reduces the efficiency of insertion of the cytoplasmic membrane protein leader peptidase (Lep) and another group, MacFarlane and Müller (26) demonstrated that the depletion of Ffh or 4.5S RNA prevents the functional membrane insertion of lactose permease (LacY). Thus, it is becoming clear that the bacterial SRP is important for the proper localization of inner membrane proteins.

The strong similarities between PilA and the bacterial FtsY proteins led us to reexamine the function of PilA as a transcriptional regulator and consider the possibility that PilA may play a role in protein maturation. In this report, we show that PilA does not regulate transcription of a *pilE-lacZ* fusion in *E. coli*, regardless of the conditions used. We present evidence to suggest that PilA plays a role in protein maturation. We show that overproduction of PilA in *E. coli* causes the accumulation of a presecretory protein and that this accumulation appears to be independent of SecY. We also demonstrate that *pilA* can complement an *ftsY* conditional mutation in *E. coli* and restore normal growth to this strain under nonpermissive conditions and that a PilA mutant defective in GTPase activity can no longer perform this function. Finally, we show that PilA can partially replace FtsY in an in vitro translocation assay. We conclude from these data that PilA is the gonococcal FtsY.

MATERIALS AND METHODS

DNA manipulations. *E. coli* recombinant DNA manipulations were performed as previously described (28). Cloning vectors used were pACYC184 (11), pWSK129 (61), pTacTerm (32), and the pBluescript series (Stratagene, La Jolla, Calif.). Restriction enzymes (New England Biolabs, Beverly, Mass.) and T4 DNA ligase (Boehringer Mannheim, Indianapolis, Ind.) were used according to the manufacturers' recommendations. PCR was done using a Perkin-Elmer 9600 thermocycler (PE Applied Biosystems, Foster City, Calif.) and *Taq* DNA polymerase (Boehringer). DNA sequence determination was done by the Core Facility of the Department of Molecular Microbiology and Immunology at the Oregon Health Sciences University using an ABI 377 automated fluorescence DNA sequencer (PE Applied Biosystems).

Growth and construction of bacterial strains. The *E. coli* strains used were JM109, CJ236 (18), CA201 (3), and N4156::pAra14-FtsY (24). *E. coli* were routinely grown in Luria broth supplemented as necessary with carbenicillin (Cb) at 100 mg/liter (plasmids) or 40 mg/liter (chromosomal), kanamycin (Kn) at 60 mg/liter, chloramphenicol (Cm) at 25 mg/liter, spectinomycin (Sp) and streptomycin sulfate (St), each at 25 mg/liter, or erythromycin (Em) at 300 mg/liter. Minimal medium used was that of Vogel and Bonner (58) supplemented with 0.2% Casamino Acids.

N. gonorrhoeae strains used were derivatives of MS11A (P^+Tr) (44) and were maintained in a humidified 5% CO_2 atmosphere on GC agar (Difco) with supplements (19). Em was used at 3 mg/liter, and Kn was used at 100 mg/liter. *N. gonorrhoeae* transformation was performed as described previously (46). *E. coli-N. gonorrhoeae* shuttle plasmids, and their manipulations have been described previously (31).

Plasmid constructions. pNG4-26 contains the *pilA* and *pilB* genes under control of their native promoters and was constructed by ligating a 4.2-kb *Cla*I-*Sma*I fragment from pNG1711 (29) into *Cla*I-*Eco*RV-digested pACYC184. pTPA129 is a low-copy-number plasmid with the PolA-independent pSC101 origin of replication (11) and was constructed by inserting the *Eco*RI-*Hin*dIII fragment from pTPA5 containing P_{tac}-*pilA*-*rrnB*$_t$ into *Eco*RI-*Hin*dIII-digested pWSK129 (61).

In order to replace the wild-type copy of *pilA* in *N. gonorrhoeae* with the mutated *pilA* genes, derivatives of pNG1711 (29) were constructed. pNG1711 contains a 9.9-kb *Bcl*I fragment from *N. gonorrhoeae* MS11A in the *Bam*HI site of pBR322 and contains the *pilE1*, *opaE*, *pilA*, and *pilB* genes, in that order. The *pilA* and *pilB* genes are divergently transcribed, and their 5′ ends overlap such that the *pilA* promoter lies within the *pilB* open reading frame, and the putative *pilB* promoter overlaps the *pilA* start codon (54). A 2.2-kb fragment containing the *opaE* and *pilE1* genes, which are located 3′ of *pilA* on this plasmid, were replaced with a 1.2-kb Em^r cassette (55) to facilitate the selection of transformants, to create pNG1711Erm. This leaves 2.5 kb of homology 3′ to *pilA* on the gonococcal chromosome and 5.2 kb of flanking homology in the 5′ direction.

To introduce *pilA* in multicopy into *N. gonorrhoeae*, a 4.4-kb *Hpa*I-*Sma*I (*pilA*-Em^r) fragment from pNG1711Erm was ligated into the *Sma*I site of the shuttle plasmid pMGC18.1 to create pGC400.

Site-directed mutagenesis of *pilA*. Site-directed mutagenesis was performed by the Kunkel method (20) using the Mutagene kit (Bio-Rad Laboratories, Richmond, Calif.). Briefly, an 804-bp *Not*I-*Sal*I fragment from pTPA5 (3) containing the 3′ end of the *pilA* gene was cloned into pBluescript II SK(−) to generate the mutagenesis template. Oligonucleotides corresponding to the plus strand introducing the nucleotide changes (indicated in bold) were used to prime synthesis of the second strand (G308A, 5′ CCGCCG**C**CCGCCTGC 3′; K370A, 5′ GCT TATCGTTAC**AGCGCTC**GACGGC 3′). DNA from transformants was isolated and screened for the presence of the mutation by restriction analysis. The G→C change to introduce the G308A mutation destroys the recognition site for *Eag*I. Two additional nucleotides were changed in addition to the T→G change to introduce the K370A mutation in order to create a recognition site for *Hae*II. The DNA sequence of the entire 804-bp insert was determined to rule out the possibility of second site mutations introduced by the mutagenesis procedure. *Not*I-*Sal*I fragments containing the mutations were subcloned back into *Not*I-*Sal*I-digested pTPA5 to create pTPA308 or pTPA370. To facilitate transfer of these mutations to the *N. gonorrhoeae* genome, the derivatives pNG1711ErmG308A and pNG1711ErmK370A were constructed as described above.

PCR screen of *N. gonorrhoeae* transformants. Em^r transformants were passed to GCB (Em) plates, and the remainder of the colony was placed into 20 μl of 0.05 N NaOH–0.25% sodium dodecyl sulfate (SDS) and heated to 50°C for 10 min. This was diluted 10-fold with distilled H_2O, and 1 μl was used as a template for PCR. Oligonucleotide primers homologous to the 5′ and 3′ ends of *pilA* were used to generate a 1,349-bp product. The product was ethanol precipitated, and a portion of it was digested with *Eag*I or *Hae*II as necessary. Digested DNAs were separated by electrophoresis on 1.8% agarose gels in Tris-borate-EDTA buffer (28).

Protein preparation and analysis. Wild-type and mutant PilA proteins were purified as described previously (4). Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, Ill.) or by the Bradford method (Bio-Rad), depending on the presence of reducing agents and detergents in the samples. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (22) and stained with Coomassie blue or transferred to nitrocellulose for immunoblots. Western blot analysis was done in phosphate-buffered saline with nonfat dry milk at 10% (wt/vol) for blocking and 2% for antibody incubations. Generation of antibodies to PilA has been described previously (3). Antibodies to β-lactamase were purchased from 5 Prime→3 Prime, Inc. (Boulder, Colo.). Antibodies to SecY were provided by William Wickner. Secondary antibodies were goat anti-rabbit antibody conjugated to alkaline phosphatase or horseradish peroxidase and used according to the manufacturer's instructions (Pierce).

In vitro protein translocation assays. Coupled translation-translocation assays were conducted as described previously (37). Where indicated, reaction mixtures contained two equivalents of salt-washed canine pancreatic microsomal membranes (KRMs) or trypsin-treated KRMs (TKRMs); 50 nM canine SRP or *E. coli* Ffh-4.5S RNP; and 150 to 500 nM FtsY, PilA, or PilA G308A. At the end of the reaction, samples were trichloroacetic acid precipitated and analyzed by SDS-PAGE on 10 to 15% polyacrylamide gradient gels, followed by PhosphorImager analysis.

Enzyme assays. GTPase assays were done as described previously (4). [γ-^{32}P]GTP (6,000 Ci/mmol) was supplied by New England Nuclear. β-Galactosidase assays were done as described previously (16).

RESULTS

PilA does not regulate a *pilE-lacZ* transcriptional fusion. PilA was initially isolated as part of a putative two-component regulatory system based on its ability to control expression of a *pilE*-CAT (chloramphenicol acetyltransferase) fusion in *E. coli* (54). In an attempt to confirm this result, a *pilE-lacZ* transcriptional fusion was constructed. pNGP3-1 contains the *pilE* promoter and associated upstream regulatory regions on a 301-bp fragment inserted upstream of the promoterless *lacZ* gene (3).

This fragment extends from −241 to +60 with respect to the *pilE* transcriptional start point (29) and contains the same *pilE* 5′ sequences as the *pilE*-CAT fusion used by Taha et al. (54). The *pilE-lacZ* fusion was transferred to the bacteriophage λRS45 to create λNGP3-145, which was then used to lysogenize *E. coli* CA201, placing the *pilE-lacZ* fusion in single copy on the bacterial chromosome (48). CA201λNGP3-145 lysogens were transformed with the plasmid pACYC184 or with pNG4-26 (*pilA*$^+$ *pilB*$^+$). β-Galactosidase assays of these strains were done, and the repression ratios (*pilA pilB*$^+$/*pilA*$^+$ *pilB*$^+$) were 0.92 ± 0.07 (mid-log phase) and 0.89 ± 0.26 (stationary phase) (values are averages of three or more independent determinations ± standard errors). Taha et al. (54) showed that *pilA* and *pilB* together resulted in approximately fivefold repression of a *pilE*-CAT fusion (repression ratio of 5.0). In contrast, we observed essentially no repression of our *pilE-lacZ* fusion when cultures were assayed at either the mid-logarithmic or stationary phase of growth.

Since Taha et al. (54) also reported that the presence of *pilA* alone resulted in a fivefold increase in *pilE*-CAT expression, λNGP3-145 was used to lysogenize the PilA expression strain, CA201/pMS421/pTPA5 (3). pTPA5 contains *pilA* under control of a *tac* promoter, which is controlled by the Lac repressor expressed by the plasmid pMS421. Lysogens containing pTPA5 or the vector control, pTacTerm, were grown in minimal medium to mid-log phase and induced with 500 μM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. β-Galactosidase assays were done, and the activation ratio was 0.78 ± 0.05 (with λNGP3-145) (values are averages of three or more independent determinations ± standard errors). Again, no activation of *pilE-lacZ* expression was observed.

To determine if the small amount of repression observed was significant, a deletion derivative of λNGP3-145 was constructed. λNGP1-445 contains a *pilE-lacZ* fusion from which 175 bp of the 5′ end of the *pilE* promoter fragment have been deleted. While PilA has been shown to bind to the *pilE* promoter fragment from λNGP3-145 in a gel retardation assay, the deletion derivative on λNGP1-445 was not bound by PilA in this same assay system (3). β-Galactosidase activities of λNGP1-445 lysogens in the presence and absence of *pilA* were nearly identical, resulting in an activation ratio of 0.88 ± 0.13. Taken together, our results show no apparent regulation of a *pilE-lacZ* transcriptional fusion by *pilA* in the presence or absence of *pilB* in *E. coli*.

To address the effects of PilA on *pilE* expression in *N. gonorrhoeae*, we made use of a *pilE-lacZ* transcriptional fusion described by Boyle-Vavra and Seifert (8). This strain (MS11Cm-Lac2) is a derivative of MS11A that contains a *pilE-lacZ* transcriptional fusion at the *pilE2* expression locus, leaving the *pilE1* expression locus intact. This strain is thus still pilated and therefore transformable. Since *pilA* is apparently essential for the gonococcus (54), it is not possible to compare expression of *pilE* in *pilA*$^+$ and *pilA* strains. To circumvent this, we introduced a multicopy plasmid expressing *pilA* (pGC400) into the *pilE-lacZ* fusion strain and measured β-galactosidase activity. Western blot analysis of the pGC400-containing strain with anti-PilA sera showed it to have at least fivefold more PilA in the cell than the wild-type strain (data not shown). *pilE-lacZ* expression in a strain containing excess PilA was not significantly different from that of a strain containing the vector only (pMGC18.1) (31). Specifically, expression levels in β-galactosidase units (nanomoles of ONPG hydrolyzed per minute per milligram of protein) were 24,062 ± 3,014 for pMGC18.1 (*pilA*) and 26,996 ± 2,603 pGC400 (*pilA*$^+$) (averages of three or more independent determinations ± standard errors). This suggests again, that PilA is not affecting *pilE* expression in the gonococcus.

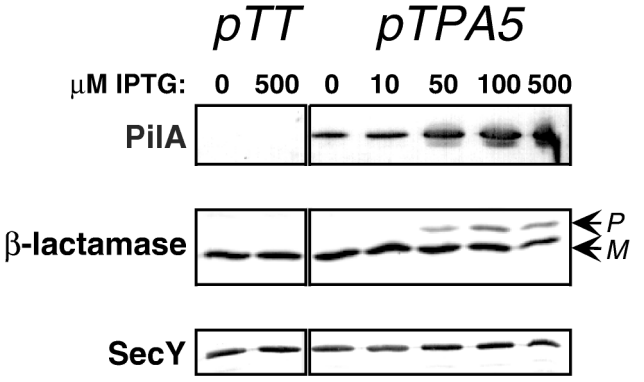

FIG. 1. Immunoblot analysis of proteins from strains overproducing PilA. *E. coli* CA201/pMS421 harboring pTacTerm (pTT) or pTPA5 was grown in 5 ml of minimal medium at 30°C to mid-logarithmic phase and induced for 2 h with various concentrations of IPTG as indicated. Cells were pelleted and resuspended in equal amounts of SDS-PAGE loading buffer. A total of 5 μl of protein was loaded per lane of 12.5% polyacrylamide gels. Coomassie blue-stained gels of samples showed that roughly equal amounts of protein were present in each sample (not shown). Antibodies used are indicated to the left of the corresponding gel. M, mature β-lactamase (signal sequence cleaved); P, pre-β-lactamase.

Effect of PilA overproduction on protein maturation. Analysis of the predicted PilA protein sequence reveals that it has significant homology to the α subunit of the mammalian SRP docking protein (SRα) and its bacterial homolog, FtsY (54). The processing of some presecretory proteins has been shown to be inhibited upon overexpression of FtsY (21, 24, 47). To examine the effect of PilA overproduction on protein processing, *E. coli* strains containing *pilA* controlled by the *tac* promoter were grown and induced with various amounts of IPTG. Immunoblot analysis with antisera raised against purified PilA (3) showed that significant amounts of PilA are produced even in the absence of IPTG, indicating that repression of *pilA* is not complete in this system (Fig. 1). Increasing amounts of IPTG resulted in increasing amounts of PilA produced. The negative control, *E. coli* containing the expression vector pTacTerm, did not react with the PilA antisera. Immunoblot analysis with antibodies to β-lactamase (encoded on pTacTerm) showed that increasing amounts of PilA result in the accumulation of increasing amounts of pre-β-lactamase (Fig. 1). In contrast, no pre-β-lactamase was observed in samples containing the vector grown with or without IPTG induction. This suggests that excess PilA is somehow affecting the processing of β-lactamase into its mature form.

Since the accumulation of pre-β-lactamase in the presence of PilA could be due to a general effect on protein translocation, we examined these extracts for the levels of SecY, one of the components of the SecYEG translocon that is utilized by both the general secretory pathway (GSP) and SRP secretion pathways in bacteria (57). Figure 1 shows no difference in the levels of SecY in our experiments. This indicates that the accumulation of pre-β-lactamase is not due to a difference in the amount of SecY in the cell.

***pilA* complements an *E. coli* conditional *ftsY* mutant.** As *ftsY* is essential for cell viability in *E. coli* (15), complementation analysis was performed on an *ftsY* conditional lethal mutant. N4156::pAra14-FtsY contains the *ftsY* gene under control of the tight *araB* promoter in place of the native *ftsY* on the chromosome (24). This strain requires arabinose for growth and does not grow in its absence. This strain is also *polA*,

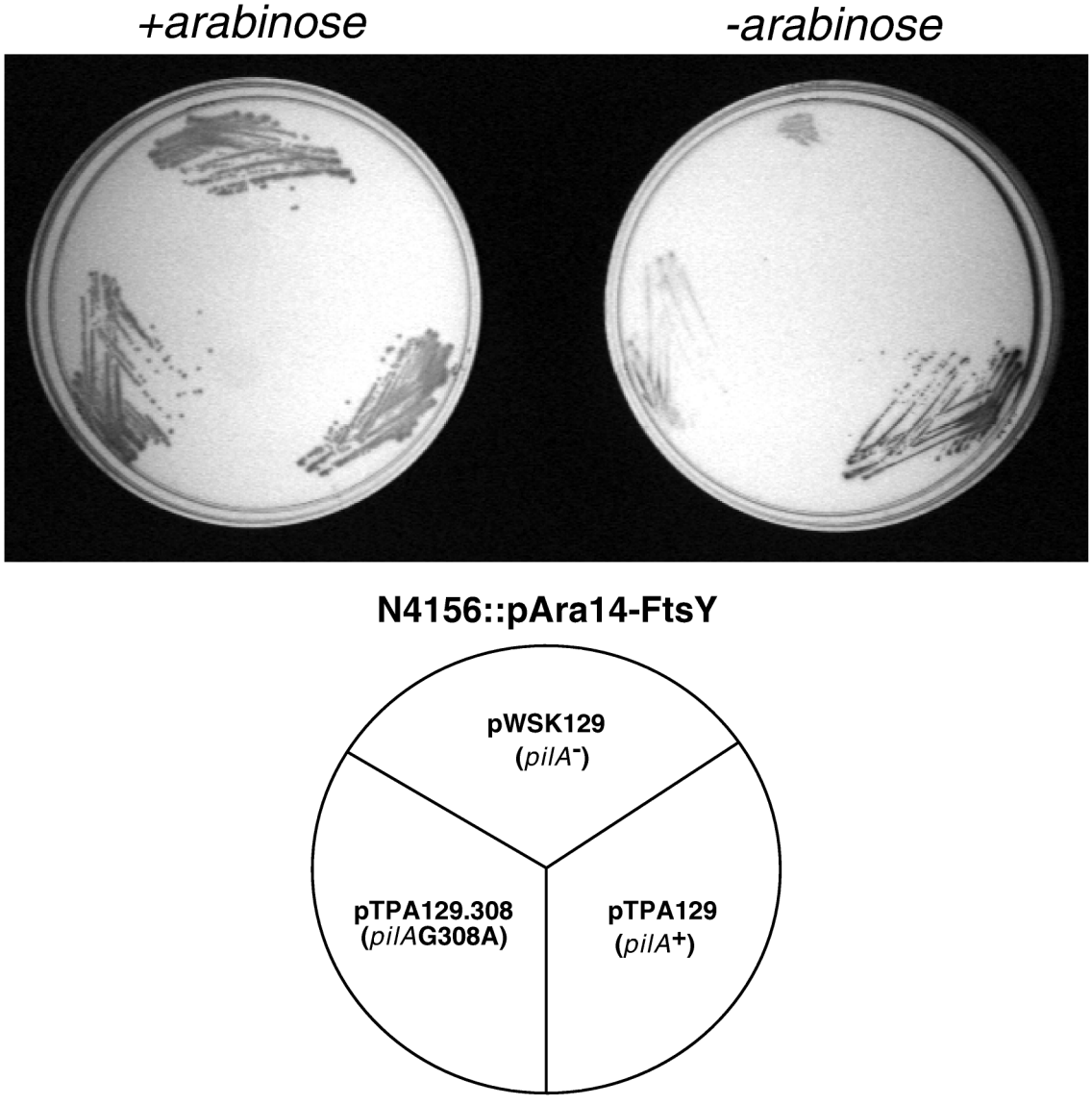

FIG. 2. Complementation of *E. coli ftsY* with *N. gonorrhoeae pilA. E. coli* N4156::pAra14-FtsY was transformed with pWSK129 (vector), pTPA129 (pWSK129 + wild-type *pilA*), and pTPA129.308 (pWSK129 + *pilA* G308A) and plated on minimal glucose plates with (+) or without (−) arabinose. Plates were incubated overnight at 37°C and photographed using a Bio-Rad Gel Doc 1000 system with back lighting.

therefore, a PolA-independent *pilA* plasmid (pTPA129) was constructed (see Materials and Methods). N4156::pAra14-FtsY cells were transformed with pTPA129 or the vector control, pWSK129. Transformants were selected on Luria broth $Cb^{40}Kn^{60}$ara plates and then struck on minimal medium containing glucose alone or with 0.2% arabinose (Fig. 2). Strains containing pTPA129 and pWSK129 grew well in the presence of arabinose. However, only the pTPA129 (*pilA*$^+$) transformant was able to grow in the absence of arabinose. Growth curves of these strains in liquid media of the same composition showed similar results (data not shown). These results demonstrate that *pilA* can complement *ftsY* in *E. coli*.

Since depletion of FtsY has been shown to result in the accumulation of pre-β-lactamase (24, 47) we wished to determine if PilA could also complement this defect. As indicated above, N4156::pAra14-FtsY does not grow in the absence of arabinose. However, when cells grown in arabinose are washed to remove the arabinose and placed into arabinose-free liquid medium, they grow, albeit significantly more slowly than those grown in the presence of arabinose, and they eventually die off (24, 47). This suggests that the cells are utilizing the FtsY that remains in the cell from before the shift to nonpermissive conditions, which is eventually used up. N4156::pAra14-FtsY cells containing pWSK129 or pTPA129 were shifted to growth with or without arabinose for 6 h, and total proteins were subjected to SDS-PAGE. Figure 3 shows immunoblots of these gels obtained by using antisera to PilA and β-lactamase. Immunoblot analysis with PilA antisera showed similar amounts of PilA produced in the presence or absence of arabinose, indicating that FtsY depletion does not affect PilA expression. Immunoblot analysis with β-lactamase antisera shows an accumulation of pre-β-lactamase in the pWSK129-containing strain under nonpermissive conditions (no arabinose). In contrast, there is no accumulation of pre-β-lactamase in pTPA129-con-

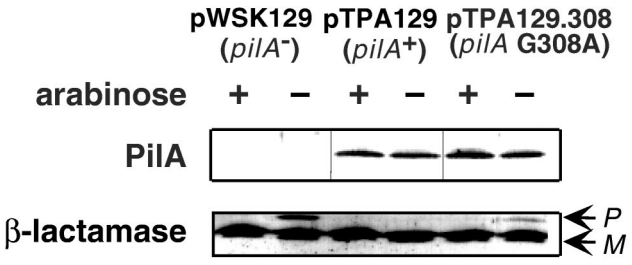

FIG. 3. Immunoblot analysis of proteins from N4156::pAra14-FtsY transformed with pWSK129 (vector), pTPA129 (pWSK129 + wild-type *pilA*), and pTPA129.308 (pWSK129 + *pilA* G308A) grown in minimal glucose medium with (+) or without (−) arabinose. Cultures grown overnight in minimal medium containing arabinose were washed twice with arabinose-free medium and used to inoculate duplicate tubes of media with or without arabinose. Cultures were grown at 37°C for 6 h. Cells were harvested, and proteins were resuspended in equal amounts of SDS-PAGE loading buffer. Equal amounts of protein were loaded in each lane of 12.5% polyacrylamide gels. Coomassie blue-stained gels of samples showed that roughly equal amounts of protein were present in each lane (not shown). Antibodies used are indicated to the left of the corresponding gel. M, mature β-lactamase (signal sequence cleaved); P, pre-β-lactamase.

taining cells. Thus, PilA is also able to complement the protein maturation defect of FtsY-depleted cells.

PilA functions in an in vitro protein translocation assay. We next determined whether PilA could catalyze protein translocation in vitro. It has been demonstrated recently that the *E. coli* Ffh-4.5S RNP and FtsY can replace mammalian SRP and the α subunit of the SRP receptor, respectively, in a heterologous protein translocation system (37). In this system, mammalian microsomal membranes are depleted of endogenous SRα by mild protease treatment, rendering them inactive in targeting and translocation. Activity can be restored, however, by addition of both *E. coli* SRP and SRP receptor (FtsY). We therefore tested whether PilA could substitute for FtsY in this system, monitoring signal sequence cleavage of a model secretory protein, preprolactin (pPL) (Fig. 4). Significant translocation activity was observed in the presence of trypsinized membranes (TKRMs), the Ffh-4.5S RNP and FtsY (~75% processing of pPL at 150 nM FtsY) (Fig. 4, lane 5) as reported previously (37). When PilA was added instead of FtsY, modest but reproducible translocation activity was observed (~20% processing at 150 nM PilA) (Fig. 4, lane 6). This level of activity represents a maximum, as no increase in processing was observed when more PilA was added (up to 500 nM; data not shown). This reduced level of activity is likely to result from the fact that the N-terminal domain of PilA is smaller and less negatively charged compared to FtsY, features which have been shown to be important in this assay. These results complement the in vivo observations above and support the conclusion that PilA is indeed an FtsY homolog.

Construction of a PilA GTPase mutant. PilA contains the four consensus GTPase elements of the GTPase superfamily (1), all of which are absolutely conserved. We have previously shown that, like *E. coli* FtsY (21), PilA has a GTPase activity that is GTP specific and requires Mg^{++} (4). The consensus sequence for box 3 is DXXG (14) and is DTAG in PilA. The glycine residue of this motif is thought to interact with the three glycine residues of box 1 to form the P loop which interacts with the phosphate group of GTP (43). Amino acid residue Gly308 of PilA (DTA**G**) was changed to Ala by site-directed mutagenesis to create pTPA308. PilA G308A was purified from CA201/pMS421/pTPA308 cells as described for the wild-type PilA (4) and analyzed for GTPase activity. The kinetic parameters for the PilA mutant were determined by assays in which the substrate (GTP) concentration was varied. The results showed that the K_m of this mutant for GTP was 11.5 ± 0.8 μM, similar to that of the wild type (9.6 ± 0.4 μM), but the V_{max} was 186 ± 53 pmol min^{-1} mg^{-1}, 10-fold less than that of the wild-type enzyme (1,900 ± 276). Thus, PilA G308A is severely affected in its ability to hydrolyze GTP.

Effects of the PilA G308A mutation on protein maturation. CA201/pMS421/pTPA308 cells were induced with various amounts of IPTG, and total proteins were subjected to SDS-PAGE and Western blot analysis using antibodies to β-lactamase. An accumulation of pre-β-lactamase was observed with increasing levels of PilA G308A (data not shown), as was observed for the wild-type PilA (Fig. 1). This suggests that, like wild-type PilA or FtsY, the PilA G308A mutant may be able to interact with the SRP and disrupt protein maturation (21, 24, 47).

To determine if the PilA G308A mutant could complement the *ftsY* mutation in *E. coli*, a pWSK129 derivative expressing the PilA G308A mutation (pTPA129.308) was transformed into N4156::pAra14-FtsY. Resulting transformants were struck on minimal medium containing glucose alone or glucose with arabinose. Figure 2 shows that unlike the wild-type PilA, PilA G308A cannot replace the *E. coli* FtsY. N4156::pAra14-FtsY cells containing pTPA129.308 were grown in liquid medium in the presence or absence of arabinose and the proteins were separated by SDS-PAGE. Immunoblots using PilA antisera showed that, similar to the wild-type PilA, significant amounts of the mutant G308A were made even in the absence of IPTG, and the levels were similar when grown in the presence or absence of arabinose (Fig. 3). Immunoblots with β-lactamase antisera showed an accumulation of pre-β-lactamase under nonpermissive conditions, although not as great as that observed for the vector control under nonpermissive conditions. This indicates that the mutant may be slightly able to alleviate the protein maturation defect, although not nearly as well as the wild-type PilA.

We next asked whether the PilA G308A mutant could replace the wild-type PilA in our in vitro protein translocation assay. As seen in Fig. 4 (lane 7), no stimulation of translocation activity in the presence of PilA G308A was observed. Indeed, only background levels of processing were observed, even with 500 nM PilA G308A (data not shown). These results demonstrate that this mutant protein is completely inactive in this assay. Taken together, they show that the GTPase activity of PilA is required for its function in vivo as well as in vitro.

The GTPase activity of PilA is also important in the gonococcus. We next attempted to replace the wild-type copy of *pilA* in *N. gonorrhoeae* with mutated *pilA* genes. Plasmid DNA from pNG1711Erm (encodes wild-type PilA), pNG1711 ErmG308A (PilA G308A), or pNG1711ErmK370A (PilA K370A, a mutant only slightly affected for GTPase activity [2a]) was linearized and used to transform *N. gonorrhoeae* strain MS11A as previously described (46). A 1,349-bp fragment containing the *pilA* gene was PCR amplified from Em^r transformants. These products were screened for the presence of the mutation by restriction analysis (see Materials and Methods). In the 5.2-kb region of sequence homology where a crossover event must occur to yield Em^r transformants with pNG1711ErmG308A, there is a 0.4-kb region where a crossover event would result in the transformant having a wild-type *pilA* gene. A crossover event in the remaining 4.8 kb would result in the introduction of the G308A mutation. If all sequences in this region are equal for homologous recombination, one would expect 7.8% of Em^r transformants to have the wild-type genotype and 92.2% to have the mutant genotype. However, of 74 Em^r transformants screened (from three inde-

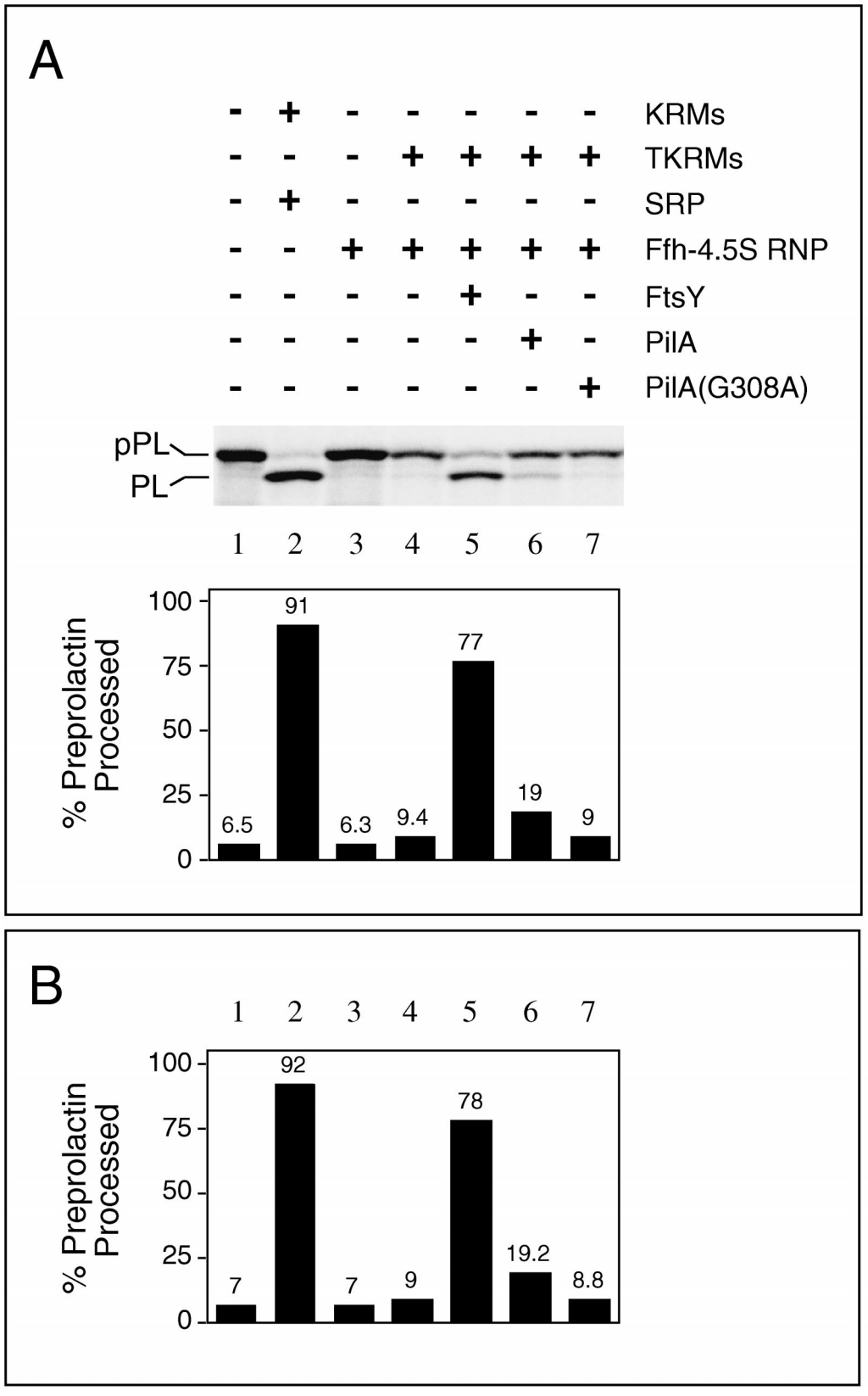

FIG. 4. Translocation of preprolactin (pPL) catalyzed by PilA. (A) (Upper) SDS-PAGE and PhosphorImager analysis of ^{35}S-labeled nascent proteins, where pPL and PL indicate precursor and signal sequence-cleaved forms of prolactin, respectively. (Lower) Quantitation of the data, depicted as the percentages of the signal sequence cleaved. Preprolactin was synthesized in a wheat germ translation extract in the presence of the indicated components, as described in Materials and Methods. FtsY, PilA, and PilA G308A were each present at 150 nM final concentrations. (B) Quantitation of results from an independent experiment, where lane identities are identical to those in panel A.

pendent transformations), all contained the wild-type *pilA* gene. In contrast, in the 5.2-kb region of sequence homology where a crossover event must occur to yield Em^r transformants with pNG1711ErmK370A, there is a 0.2-kb region where a crossover event would result in the transformant having a wild-type *pilA* gene. A crossover event in the remaining 5.0 kb would result in the introduction of the K370A mutation. This predicts a frequency of 3.8% wild-type and 96.2% mutant transformants. Of 47 Em^r transformants screened (from three independent transformations), 37 contained the wild-type *pilA* gene and 10 contained the K370A mutation. This corresponds to a frequency of 79% wild-type transformants and 21% mutant transformants. The fact that we were able to replace the wild-type *pilA* with a gene encoding the K370A mutation, albeit at a lower frequency than expected, but not the G308A mutation indicates that the GTPase activity of PilA is important in the gonococcus.

DISCUSSION

We recently compared the *pilA* sequence with sequences in the nearly completed genome sequence of *N. gonorrhoeae* FA1090 (13) using the BLAST program (2). This search yielded only two genes with significant homology. Comparison of these sequences with those of the *E. coli ftsY* and *ffh* genes suggests that they are the gonococcal SRP homologs. The homology between *ffh* and *pilA* is located exclusively in the G domains, while the homology between *ftsY* and *pilA* occurs throughout the entire protein, suggesting that *pilA* and *ftsY* are true homologs. The striking homology between PilA and the G domains of the SRP proteins initially led us to examine PilA for GTPase activity (4). These experiments showed that, like FtsY (21), PilA is GTPase.

To examine the relationship between the GTPase and purported regulatory activities of PilA, we attempted to confirm the transcriptional regulation experiments using a *pilE-lacZ* fusion. Regardless of the conditions used, we were unable to demonstrate any significant effect of PilA on *pilE-lacZ* expression. The *pilE-lacZ* fusion used in this study contained a fragment with the same endpoints of the *pilE* upstream region used by Taha et al. (54). Thus, the only difference between our systems was the reporter system used. The reason for this discrepancy in results is not clear, although it is worth noting that Taha et al. (52) have also reported PilA control of *pilC* in *N. meningitidis* with a *lacZ* reporter system, which implies that both the *lacZ* and CAT systems give similar results in their hands.

Our inability to demonstrate transcriptional regulation by PilA and the striking similarities between PilA and FtsY led us to hypothesize that PilA might actually be the gonococcal FtsY homolog. We first examined the effect of overexpression of PilA on maturation of the secreted protein, β-lactamase. Maturation of this protein has been shown to be inhibited upon overexpression of components of the bacterial SRP (21, 24, 47, 56). *E. coli* strains containing *pilA* under the control of the *tac* promoter were grown and induced with various amounts of IPTG. Immunoblot analysis of total proteins from these strains showed an accumulation of pre-β-lactamase with increasing concentrations of PilA (Fig. 1). Immunoblot analysis of duplicate samples showed no difference in SecY levels in these samples. SecY, a major component of the membrane-bound translocation apparatus, is thought to be utilized for both the GSP and SRP secretion systems (57). Thus, the accumulation of pre-β-lactamase in these samples is likely due to a defect prior to the translocation step. This is consistent with the interpretation that excess PilA perturbs the SRP system by titrating out other components of the apparatus. Others have shown that the relative levels of each of the components of the *E. coli* SRP are critical for optimal function (17, 24, 34, 47). Using a slightly different system, Taha et al. (53) looked at the effect of PilA on β-lactamase processing in *E. coli* and also observed an accumulation of the preprotein in the presence of excess PilA. They concluded that the effect was nonspecific and likely a secondary effect of transcriptional regulation of cell growth rate by PilA.

Studies of the role of the bacterial SRP indicate that it is important for the proper localization of inner membrane proteins (12, 26, 56). Interestingly, one of the proteins believed to be dependent upon the SRP is leader peptidase (Lep), which cleaves the hydrophobic signal sequences from secreted proteins following their translocation across the inner membrane. Thus, a perturbation of the bacterial SRP caused by depletion or overexpression of one of its components may affect the function of Lep, which may in turn affect the proper processing of secreted proteins. This may explain the accumulation of pre-β-lactamase observed by us and others (24, 47) upon overexpression of FtsY in *E. coli*.

We next demonstrated that PilA could substitute for FtsY in *E. coli* by complementation of a conditional *ftsY* mutant (N4156::pAra14-FtsY) (24). Figure 2 shows that strains expressing *pilA* can grow under conditions in which *ftsY* is not expressed (media lacking arabinose), indicating that PilA can replace FtsY in *E. coli*. Further evidence to support this is seen in Fig. 4. Using an in vitro translocation assay with purified *E. coli* Ffh-4.5S RNA, we observed that PilA could partially replace FtsY in this assay system. Under conditions where FtsY can effect processing of 75% of the substrate, PilA was able to direct the processing of nearly 20% of the same substrate. While this activity is low, it is reproducible and is significantly above background levels, indicating that the translocation observed is PilA mediated. The reduced level of activity observed in this assay may be due to the fact that the N-terminal domain of PilA is smaller and less negatively charged compared to FtsY, which has been shown to be important in this assay (37).

To determine whether the GTPase activity of PilA is required for its function, a mutation was introduced into box 3 of the conserved GTP-binding site of PilA. This mutant (PilA G308A) is 10-fold reduced in GTPase activity compared to the wild-type protein. In vivo, PilA G308A cannot replace FtsY in *E. coli* (Fig. 2) nor can it replace the wild-type PilA in *N. gonorrhoeae*. Additionally, PilA G308A cannot replace FtsY in an in vitro system (Fig. 4). However, overexpression of this mutant results in the accumulation of pre-β-lactamase, similar to that observed for the wild-type PilA (data not shown). This suggests that while PilA G308A cannot function as the docking protein for the SRP, it may be able to interact with other components of the SRP such that they can no longer function correctly in protein maturation. The corresponding mutation in box 3 of the *E. coli* FtsY (G385A) also results in a strong dominant lethal phenotype and affects translocation of some proteins as well (56).

The fact that our results indicate that PilA is part of a gonococcal SRP apparatus and not a transcriptional regulator raises a number of questions. First, why was PilA identified in a screen for transcriptional regulators of *pilE* in *E. coli*? We have been unable to repeat the transcriptional regulation results using a reporter system (*lacZ*) different from that used in the original screen (CAT). Both reporter enzymes are cytoplasmic, so it is unlikely that enzyme activities were directly affected by the SRP apparatus, although it is possible that the uptake of chloramphenicol by the transformants may have affected their survival on plates in the original screen (54).

However, this does not explain the results where CAT activity was measured in cell lysates. Thus, the answer to this question remains a mystery.

Second, why does PilA bind DNA in vitro? We showed that purified PilA binds to *pilE* promoter DNA in a sequence-specific manner using a gel retardation assay (3). Taha and Giorgini (51) obtained a similar result using crude preparations of PilA in a similar assay. Our results indicated that the binding was complex and required sequences at both ends of the promoter fragment. We concluded from this study that DNA looping may be involved in this interaction, and we hypothesize that PilA does not recognize a specific sequence per se but rather recognizes a secondary or tertiary structure formed by a particular DNA sequence. Indeed, the DNA sequences from −100 to +1 of the *pilE* promoter is 73% AT rich, which is consistent with bent DNA (3). We have screened a promoter library from *N. gonorrhoeae* MS11A for binding by PilA and identified 12 DNA fragments that compete with *pilE* DNA for PilA binding. These fragments are also directly bound by PilA in a gel mobility shift assay (2a). An alignment of these sequences with each other and the *pilE* promoter reveals no obvious consensus binding sequence (data not shown). It is possible that these fragments fold into a similar structure that results in an electrophoretic mobility shift in the presence of PilA.

FtsY physically interacts with the Ffh-4.5S RNA complex (30, 35) and may therefore come in contact with RNA, either the 4.5S RNA or the mRNA of the translation complex. DNA and RNA are structurally different from each other, although there are proteins which bind both RNA and DNA (23, 39). It is possible that secondary or tertiary structure is important for this binding. It is conceivable, although unlikely, that PilA actually binds RNA in vivo, and the in vitro DNA binding is an artifact of this activity.

A more attractive possibility is that PilA/FtsY somehow interacts with the chromosome. *ftsY* was initially identified as part of an operon containing genes which are temperature sensitive for filamentation (15), although no such mutations have ever been mapped to *ftsY*. FtsY-depleted cells are filamentous and are apparently defective in completion of septation during cell division (24). The same phenotype has been observed for cells depleted for Ffh or cells expressing a mutated in *ffh* gene (34, 42). Thus, the bacterial SRP may play a role in cell division and septation which may involve interactions between the components of the SRP and the chromosome.

What subset of gonococcal proteins might depend on PilA and the gonococcal SRP for maturation? Taha et al. (53) examined *N. gonorrhoeae* heterodiploids producing wild-type and truncated forms of PilA for the accumulation of Opa, a gonococcal outer membrane protein that is involved in epithelial cell invasion by gonococci (27, 59). They reported that these heterodiploids did not affect Opa signal sequence processing, although they did observe an induction of heat shock proteins in these strains. Thus, Opa likely does not utilize the gonococcal SRP. It will be interesting to determine which proteins of *N. gonorrhoeae* depend on the SRP system for proper localization.

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