Identification of ZipA, a Signal Recognition Particle-Dependent Protein from *Neisseria gonorrhoeae*

Ying Du and Cindy Grove Arvidson*

Department of Microbiology and Molecular Genetics, Michigan State University,
East Lansing, Michigan 48824-1101

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A genetic screen designed to identify proteins that utilize the signal recognition particle (SRP) for targeting in *Escherichia coli* was used to screen a *Neisseria gonorrhoeae* plasmid library. Six plasmids were identified in this screen, and each is predicted to encode one or more putative cytoplasmic membrane (CM) proteins. One of these, pSLO7, has three open reading frames (ORFs), two of which have no similarity to known proteins in GenBank other than sequences from the closely related *N. meningitidis*. Further analyses showed that one of these, SLO7ORF3, encodes a protein that is dependent on the SRP for localization. This gene also appears to be essential in *N. gonorrhoeae* since it was not possible to generate null mutations in the gene. Although appearing unique to *Neisseria* at the DNA sequence level, SLO7ORF3 was found to share some features with the cell division gene *zipA* of *E. coli*. These features included similar chromosomal locations (with respect to linked genes) as well as similarities in the predicted protein domain structures. Here, we show that SLO7ORF3 can complement an *E. coli* conditional *zipA* mutant and therefore encodes a functional ZipA homolog in *N. gonorrhoeae*. This observation is significant in that it is the first ZipA homolog identified in a non-rod-shaped organism. Also interesting is that this is the fourth cell division protein (the others are FtsE, FtsX, and FtsQ) shown to utilize the SRP for localization, which may in part explain why the genes encoding the three SRP components are essential in bacteria.

The prokaryotic signal recognition particle (SRP) is a complex of two proteins, FtsY and Ffh, and a 4.5S RNA that targets a subset of proteins to the cytoplasmic membrane (CM) cotranslationally (13, 32, 36, 49, 59). The use of a cotranslational targeting system by this subset of proteins is thought to be advantageous in that it avoids their aggregation in the cytoplasm, which might occur if they were targeted posttranslationally. All of the SRP components are essential for viability in *Escherichia coli* (9, 17, 37), suggesting that this targeting system is critical for the proper localization of proteins involved in essential cell processes.

This is certainly true for the sexually transmitted pathogen, *Neisseria gonorrhoeae*. The gonococcal FtsY ortholog PilA was shown to be essential to the gonococcus (55) before PilA was shown to be part of the SRP (5). Since *N. gonorrhoeae* is an important human pathogen, the most intensively studied proteins of this bacterium are proteins involved in interactions with host cells. These virulence factors are mostly outer membrane (OM) components such as pili (53), PII or Opa (27), PI or porin (25), lipooligosaccharide (44), and iron utilization proteins (7, 11, 12). A few, such as the immunoglobulin A1 protease, are secreted (50). Relatively little is known, however, of other membrane-associated proteins in *Neisseria* or their functions, especially those of the CM. CM proteins include many transporters for nutrients, as well as enzymes involved in the maturation of outer membrane components. Also included are efflux pumps, which prevent otherwise harmful materials

from accumulating within the cytoplasm. This class of proteins can be very important for pathogens as a mechanism to exclude harmful antimicrobial agents and are important targets for vaccine and drug development. Other CM proteins include those involved in energy generation and conservation, respiration, cell division, and protein translocation. The CM also contains proteins involved in signal transduction, which is necessary for the organism to sense its environment, and components necessary to respond to such signals. One of the goals of our research has been to identify and characterize putative CM proteins of *N. gonorrhoeae*, with the ultimate goal of identifying unique proteins that might be useful as targets for drug development.

Using a screening approach that takes advantage of the fact that the relative levels of each of the components of the prokaryotic SRP are critical for function and survival of the organism (59), we have identified several genes of *N. gonorrhoeae* that encode proteins that utilize the SRP for localization. Sequence analysis of these genes revealed one that is apparently unique to *Neisseria* spp., having no close matches in the GenBank database. Further examination of this gene, however, suggested that it might be structurally and functionally related to the cell division protein, ZipA, of *E. coli*.

MATERIALS AND METHODS

DNA manipulations. *E. coli* recombinant DNA manipulations were performed as described previously (42). Cloning vectors used were pET24a (Novagen, Madison, Wis.), pACYC184, pHSS6 (48), pWSK129 (63), and pWSKlacIOPE1. pWSKlacIOPE1 was constructed by ligating a 2.8-kb *lacIOPErm* fragment of pHSXerm (46) into the *NotI* site of pWSK29 (63). Restriction enzymes and T4 DNA ligase (New England Biolabs, Beverly, Mass.) were used according to the manufacturer's recommendations. PCR was done by using a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, Calif.) and *Taq* DNA polymerase

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824-1101. Phone: (517) 355-6463, ext. 1573. Fax: (517) 353-8957. E-mail: arvidso3@msu.edu.

(Roche Molecular Biochemicals, Indianapolis, Ind.). Oligonucleotide primers were purchased from Sigma-Genosys (The Woodlands, Tex.) or the Michigan State University Macromolecular Structure, Sequencing, and Synthesis Facility and the sequences are available on request. The QIAquick PCR purification kit (Qiagen, Valencia, Calif.) was used to purify PCR products. DNA sequence determination of pSLO plasmids was done by the Core Facility of the Department of Molecular Microbiology and Immunology at Oregon Health Sciences University. All other plasmids were sequenced by the Michigan State University Macromolecular Structure, Sequencing, and Synthesis Facility. DNA sequences were analyzed by using the Oxford Molecular Group DNA analysis programs MacVector and Omega (Accelrys, San Diego, Calif.).

Transposon mutagenesis. Transposon mutagenesis of plasmid DNA was performed by in vitro transposition with EZ::TN transposase (Epicentre Technologies, Madison, Wis.), and the modified transposon TnErmUP contained on the plasmid pMODErmUP (H. S. Seifert, unpublished). The TnErmUP transposon was prepared by PCR amplification from pMODErmUP with the oligonucleotide primers FP-1 and RP-1 (Epicentre). After the transposition reaction, plasmid DNA was transformed into *E. coli* DH5 α , and transformants were selected for erythromycin resistance (Em^r). The position of the transposon insertion was determined by PCR with oligonucleotide primers homologous to the ends of the transposon (SqFP and SqRP; Epicentre) and to the ends of the pSLO7 insert, followed by restriction analysis.

Southern blot analysis. Chromosomal DNA from *N. gonorrhoeae* transformants was isolated and digested with *Cla*I. Fragments were separated on 1% agarose gels, and DNA was blotted to nylon membranes as described previously (4). DNA probes for hybridization were generated by the random priming method with the digoxigenin DNA-labeling and detection kit (Roche).

Growth of bacterial strains. *E. coli* strains used were DH5 α , BL21(λ DE3) (52), S17-1 λ pir, HDB29 (59), N4156::pAra14-FtsY (30), CH3/pCH32, and CH5/pCH32 (21). *E. coli* were routinely grown in Luria broth (LB) supplemented as necessary with ampicillin at 100 mg/liter, chloramphenicol at 20 mg/liter, kanamycin at 50 mg/liter, spectinomycin or streptomycin at 25 mg/liter, or erythromycin at 300 mg/liter. *N. gonorrhoeae* MS11A (P⁺tr [45]) was maintained in a humidified 5% CO₂ atmosphere on GC agar (Difco Laboratories, Sparks, Md.) with supplements (26). *N. gonorrhoeae* transformation was performed as described previously (47). Erythromycin was used at 3 mg/liter for *N. gonorrhoeae*.

Protein isolation and analysis. Bacterial cells were fractionated into the periplasm, cytoplasm, and cytoplasmic and outer membranes as described previously (4), and protein concentrations were determined by the Bradford method (Bio-Rad Laboratories, Richmond, Calif.). Proteins were separated on 15% polyacrylamide gels and transferred to nitrocellulose membranes electrophoretically. Detection of His₆-tagged proteins was done by using Ni-nitrilotriacetic acid (NTA)-horseradish peroxidase (HRP) conjugate (Qiagen) in Tris-buffered saline. Membranes were incubated in 10% (wt/vol) nonfat dry milk in Tris-buffered saline to block and then with Ni-NTA-HRP in 2% milk. HRP was detected with the chemiluminescent detection agent SuperSignal (Pierce, Rockford, Ill.) used according to the manufacturers' directions.

Construction of the SLO screen strain. *E. coli ffh* was amplified from genomic DNA by PCR and cloned into pBluescript II SK(−) to create pBluFfh. pBluFfh was then digested with *Hpa*I to remove an internal 777-bp fragment that was replaced with a 1,222-bp fragment encoding resistance to erythromycin (Em^r [57]). This is the same deletion as was used for construction of the *ffh::kan-1* allele (37). The entire *ffh::erm* fragment was then cloned into the suicide plasmid pKAS32 (51), which contains the π -dependent R6K origin of replication (33), an RP4 origin of transfer for conjugation, and the *rpsL* gene, which renders the host strain (S17-1 λ pir) sensitive to streptomycin (Sm^s) which is dominant to streptomycin-resistant (Sm^r) alleles. The resulting strain S17-1 λ pir/pSFfh::erm was mated with HDB29 (Sm^r), and transconjugants were selected for Em^r and Sm^r. pSFfh::erm cannot replicate in HDB29, forcing the *ffh::erm* allele to recombine with the *ffh::kan-1* allele on the chromosome of HDB29. Selection for Sm^r ensures that a double crossover event (replacement of *ffh::kan-1* with *ffh::erm*) and not a single crossover event (integration of pSFfh::erm into the chromosome) occurs. HDB29 also contains an *ffh* gene under the control of P_{trc} on a separate replicon, although if the *ffh::erm* allele were to recombine at this locus, there would be no intact *ffh* gene and these recombinants would not survive, since *ffh* is essential to *E. coli* (37). Multiple Em^r transconjugants were obtained and screened for kanamycin sensitivity and carbenicillin sensitivity to ensure that the *ffh::kan-1* allele was no longer present and that the entire plasmid, pSFfh::erm, had not integrated into the chromosome. The resulting strain was called CGA29 (*ffh::erm* λ D69 *Hind*III::lacI^q P_{trc}*ffh*).

RESULTS

SLO screen of a gonococcal gene bank. Previous studies have shown that multicopy plasmids expressing SRP-dependent *E. coli* proteins confer a lethal phenotype in strains producing limiting amounts of Ffh (59). In *E. coli* strain CGA29, *ffh* is under the control of the IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible *trc* promoter. LacI repression of *ffh* is not complete in this strain, and a small amount of Ffh is made in the absence of IPTG. Ulbrandt et al. (59) showed that this small amount of Ffh is sufficient for survival of the bacterium under normal conditions. However, if a multicopy plasmid expressing a protein that utilizes the SRP is present in this strain, it can no longer survive unless the level of Ffh in the cell is increased by the addition of IPTG to the growth medium. They termed this effect SLO, for synthetic lethality upon overproduction. Since the SRP proteins from *E. coli* and *Neisseria* spp. are similar and since we have shown that *N. gonorrhoeae* PilA can functionally replace FtsY in *E. coli* (5), we reasoned that gonococcal proteins that utilize the SRP might also confer a SLO phenotype to the conditional Ffh strain in this system.

The *N. gonorrhoeae* strain MS11A gene library pCBB (4) was introduced into CGA29, and transformants were selected on plates containing 10 μ M IPTG. Transformants were next cultured on duplicate plates with or without IPTG and examined to identify those with little or no growth under Ffh-limiting conditions. In a screen of 1008 CGA29pCBB transformants, 14 were found to be sensitive to growth under Ffh-limiting conditions. These were divided into two groups based on the severity of their growth defects. Five isolates were moderately affected, with colonies noticeably smaller than a vector-only control on plates lacking IPTG. Nine isolates were severely affected, with little or no growth in the absence of IPTG.

Plasmid DNA was isolated from each strain and retransformed into CGA29 to confirm that the SLO phenotype was plasmid linked. Of the 14 original isolates, 4 were eliminated because the inserts were unstable. The SLO phenotypes of the remaining 10 were the same as the original isolates (6 severe and 4 moderate) and the DNA sequences of each of the plasmids were determined. First, the sequences were scanned for the presence of open reading frames (ORFs) by using the DNA analysis programs MacVector and Omega. The deduced protein sequence for each ORF was then analyzed by using PSORT (34), which predicts the cellular localization of a protein. The protein sequences were also used to search the GenBank database by using BLAST (2). And finally, the sequences of each insert were used as query in a BLAST search of the annotated *N. gonorrhoeae* strain FA1090 database (<http://www.stdgen.lanl.gov>).

A summary of the sequence analyses of the six severe SLO clones is shown in Table 1. These results showed that each of the plasmids encoded at least one putative inner or CM protein. Since the bacterial SRP targets a subset of proteins whose final destination is the CM (13, 41, 36, 49, 59), these results indicate that this heterologous screen does identify proteins from a *Neisseria* gene bank that interact with the *E. coli* SRP. Of the four plasmids conferring a moderate SLO phenotype, three encoded at least one putative CM protein and only one appeared to be a false positive, encoding two putative cyto-

TABLE 1. Sequence analysis of severe SLO clones

SLO clone	Insert size (bp)	BLAST result (sequence [bp on plasmid/bp of ORF])	PSORT location
7	2,121	AmpD Conserved hypothetical protein (5' 914/945 ^a)	Cytoplasm Periplasm
16	4,307	Unknown ^b ORF (5' 642/1284 ^a) Cytosine permease (5' 883/1224) TldD (CsrA suppressor) Histidine permease Glutamine permease	CM CM Cytoplasm CM CM
24	2,953	Protein disulfide isomerase (3' 312/492) Ubiquinone oxidoreductase ABC-type exporter	CM or OM CM Periplasm
48	2,273	Phosphoglucosyltransferase (5' 463/1383) Proline <i>cis/trans</i> isomerase Na ⁺ /H ⁺ antiporter	Cytoplasm Cytoplasm CM
53	2,366	ABC transporter permease (5' 694/1932) DsbC (protein disulfide isomerase) DNA replication protein (5' 658/2199)	CM Periplasm Cytoplasm
87	2,161	Hypothetical protein Glutamate permease (5' 618/1213)	CM or OM CM

^a Partial sequence.^b Not in GenBank.

plasmic proteins (data not shown). The frequency obtained in this preliminary screen is 0.9% (9 of 1008), a finding similar to the 1 to 2% estimated by Ulbrandt et al. for SRP-dependent proteins in *E. coli* (59). Of the six severe SLO clones, one (SLO7) appeared to encode a putative CM protein that was not similar to any sequences in the GenBank database, suggesting that it was unique to *Neisseria*.

Analysis of the ORFs of pSLO7. Sequence analysis of the pSLO7 insert showed it contains three putative ORFs that we designated ORF1, ORF2, and ORF3 (Fig. 1). Only the 5' 914 bp of the 945-bp ORF1 are on pSLO7, and this ORF encodes a hypothetical protein with no similarities in the GenBank database, other than a conserved hypothetical protein from *N. meningitidis*. PSORT analysis of the predicted protein sequence of ORF1 suggests it is localized to the periplasm. ORF2 encodes a protein with 49% identity and 61% similarity to AmpD of *E. coli*. AmpD is thought to be a regulator of β -lactamase induction (58) and is probably involved in the recycling

TABLE 2. SLO screen of pSLO7::TnErmUP insertion mutants

Plasmid	Genotype	Growth ^a	
		With IPTG	Without IPTG
pHSS7	Vector control	+	+
pSLO7	Wild-type insert	+	—
pSLO7::TnErmUP#3	<i>orf1</i> ⁺ <i>orf2</i> <i>orf3</i> ⁺	+	—
pSLO7::TnErmUP#11	<i>orf1</i> <i>orf2</i> ⁺ <i>orf3</i> ⁺	+	—
pSLO7::TnErmUP#10	<i>orf1</i> ⁺ <i>orf2</i> ⁺ <i>orf3</i>	+	+
pSLO7::TnErmUP#26	<i>orf1</i> ⁺ <i>orf2</i> ⁺ <i>orf3</i>	+	+
pSLO7ORF3	<i>orf3</i> ⁺	+	—

^a +, Growth; —, no growth.

of products of peptidoglycan turnover (23). PSORT analysis of the putative AmpD homolog predicts it to localize to the cytoplasm. ORF3, like ORF1, is only partly on pSLO7 (5' 642 bp of 1,284 bp). Also like ORF1, ORF3 appears to encode a protein unique to *Neisseria* and encodes a protein with no similarities in the GenBank database, aside from hypothetical proteins in *N. meningitidis*. PSORT analysis of the predicted protein encoded by ORF3 suggests that it localizes to the CM.

Transposon mutagenesis of pSLO7. In order to determine which of the ORFs of pSLO7 was responsible for the SLO phenotype, each ORF was disrupted by in vitro transposition (19) by using the transposon TnErmUP contained on the plasmid pMODErmUP (kindly provided by H. Seifert). pMODErmUP is a modification of pMOD-2<MCS> (Epicentre) in which the *ermC* gene (62) and a *Neisseria* uptake sequence (NUS [18]) have been inserted between the mosaic ends recognized by the EZ::TN transposase (Fig. 1B). After transformation of the transposition mix, kanamycin-resistant *Em*^r transformants were selected, and the positions of the TnErmUP insertions determined by PCR and restriction analysis (Fig. 1). Each of these plasmids was then transformed into CGA29, and transformants were scored for growth on plates with or without IPTG (Table 2). Plasmids with insertions in ORF1 (pSLO7::TnEm#11) or ORF2 (pSLO7::TnEm#3) only grew in the presence of IPTG, i.e., confer an SLO phenotype. However, CGA29 harboring either of the ORF3 insertion plasmids (pSLO7::TnEm#10 or pSLO7::TnEm#26) grew well in the presence or absence of IPTG, no longer conferring an SLO phenotype. This indicates that the remaining ORFs on this plasmid, ORF1 and ORF2, do not encode SRP-dependent proteins and suggests that ORF3 is responsible for the SLO phenotype of pSLO7. To confirm this, since pSLO7 contains a truncated ORF3 gene, the entire predicted ORF3 gene, along with associated upstream expression sequences, was PCR amplified from MS11A genomic DNA and cloned into pHSS6 (48). The resulting plasmid, pSLO7ORF3, conferred an SLO phenotype when transformed into CGA29 and grown without IPTG (Table 2), thus confirming that ORF3 is responsible for the SLO phenotype.

Effects of SRP depletion on cellular localization of SLO7ORF3. In order to detect small amounts of the SLO7ORF3 protein in cellular protein extracts, a plasmid was constructed such that an epitope tag (His₆) was added to the C terminus of the full-length ORF3 protein. We were unable to detect a full-length SLO7ORF3-His₆ when overexpressed (data not shown); therefore, a truncated version was constructed. The 5' 840 bp of the SLO7ORF3 coding region was

A. pSLO7 insert

B. TnErmUP

FIG. 1. Schematic diagram of the pSLO7 insert and the TnErmUP insertions. (A) pSLO7 insert. Arrows of ORFs indicate the direction of transcription. The "▼" symbols indicate the positions of the TnErmUP (3E, 10E, 11E, and 26E) insertions. (B) TnErmUP transposon. Small arrows indicate oligonucleotide primers used to prepare the transposon for in vitro transposition (FP-1 and RP-1) and to map transposon insertions (SqRP and SqFP).

FIG. 2. Effect of SRP depletion on localization of SLO7ORF3'-His₆ in *E. coli*. N4156::pAra14-FtsY transformed with pSLO7ORF3'-His₆ was grown for 7 h with (FtsY replete) or without (FtsY depleted) arabinose. The cells were fractionated, and 100 µg of cytoplasm or Sarkosyl-extracted membrane (CM) proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. SLO7ORF3'-His₆ was detected with Ni-NTA-HRP, followed by chemiluminescent detection of HRP.

PCR amplified from *N. gonorrhoeae* strain MS11A genomic DNA and ligated into pET24a, placing a truncated (280 amino acids) ORF3 with a C-terminal His₆ tag under the control of a T7 promoter. The resulting plasmid, pSLO7ORF3'-His₆, was transformed into *E. coli* strain BL21(ΔDE3) (52) and induced with IPTG. Cells were then fractionated into periplasmic, cytoplasmic, and membrane fractions as described previously (4). The membrane fraction was extracted with 0.2% Sarkosyl to separate soluble (CM) and insoluble (OM) proteins (21). Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose, and the ORF3'-His₆ protein was detected by using an Ni-NTA-HRP conjugate. ORF3'-His₆ was found in both membrane fractions but not the cytoplasm or periplasm, suggesting that SLO7ORF3 localizes to the CM.

To determine whether the localization of the ORF3'-His₆ protein was dependent on the SRP in *E. coli*, the pSLO7ORF3'-His₆ construct was subcloned onto a derivative of pWSK129 (63), placing an IPTG-inducible *lac*-promoter upstream of ORF3. This construct, pWSKORF3'-His₆, was transformed into the conditional SRP *E. coli* strain, N4156::pAra14-FtsY (30), in which the production of the SRP receptor, FtsY, is controlled by the *araBAD* promoter. This strain absolutely requires L-arabinose for growth, and culturing in the absence of this sugar results in the depletion of FtsY and eventually kills the cell. However, before the cells die, the depletion of FtsY causes an accumulation in the cytoplasm of proteins that are dependent on the SRP. N4156::pAra14-FtsY/pWSKORF3'-His₆ was grown overnight in medium containing 0.2% L-arabinose. The culture was washed with medium lacking glucose with or without arabinose at a starting concentration of $\sim 10^7$ CFU/ml. After growth for 7 h at 37°C, the cultures were harvested and cells fractionated as described above. ORF3'-His₆ protein was detected with Ni-NTA-HRP, and the results showed that under conditions of FtsY depletion (medium lacking L-arabinose) ORF3'-His₆ accumulates in the cytoplasm (Fig. 2), indicating that under these conditions ORF3'-His₆ is not targeting to the membrane as it does under FtsY-replete (i.e., with arabinose) conditions. We conclude from this result that SLO7ORF3 is directly or indirectly dependent on the SRP for CM localization.

ORF3 is essential for *N. gonorrhoeae*. To determine whether any of the genes on pSLO7 were essential to *N. gonorrhoeae*, plasmids containing transposon insertions in each of the puta-

tive ORFs were used to transform *N. gonorrhoeae* strain MS11A (45). *Neisseria* spp. are naturally competent for transformation and will take up DNA at high frequencies as long as the DNA contains an NUS (18). Since there are two NUSs in pSLO7 (both in ORF2), as well as one on TnErmUP, it was predicted that the transposon insertion mutant plasmids would be taken up efficiently by MS11A. MS11A was transformed with linearized pSLO7::TnErmUP plasmid DNA and selected for Em^r. If a gene is essential to the gonococcus, it would be predicted that no Em^r transformants would be obtained in such an experiment. Table 3 summarizes the results of three independent transformation experiments. Experiments 1 and 3 utilized 0.2 µg of DNA, and experiment 2 was done with 0.6 µg of plasmid DNA. In each experiment, high frequencies of transformation were observed for plasmids with insertions in ORF1 and ORF2 (2.1×10^{-5} to 4.6×10^{-4} Em^r/total CFU), but very few Em^r transformants were obtained with plasmids containing insertions in ORF3. This strongly suggested that ORF3 is essential to the gonococcus. pSLO7::TnErmUP#10, which contains an insertion just after the predicted start codon of ORF3, yielded a total of six Em^r transformants: two in experiment 2 and four in experiment 3. In order to determine whether these transformants had a disruption of the ORF3 gene, Southern blot analyses were performed on DNA from two of the ORF3 Em^r transformants and one each of the ORF1 and ORF2 Em^r transformants (data not shown). When the TnErmUP transposon was used as a probe, a single band was observed for each of the mutants, as expected. When pSLO7 was used as a probe, two bands were observed in the putative ORF3 mutants, whereas only a single band (the same mobility as that observed with the transposon probe) was observed for the ORF1 and ORF2 mutants. The mobility of this band was slightly slower than the band observed in MS11A (wild type), a finding consistent with an insertion of 1.3 kb, the size of the transposon. The two bands observed for the putative ORF3 mutants suggested a duplication event, such that these isolates are heterodiploids containing a mutated, as well as a wild-type copy of ORF3. This supports the conclusion that ORF3 is essential to the gonococcus.

Analysis of the putative ORF3 gene product. The ORF3 sequence on the *N. gonorrhoeae* strain FA1090 chromosome is located between *ligA*, the gene for DNA ligase (28) and *ampD*. Comparison to the *ligA* region of the *E. coli* K-12 genome (8) revealed that the cell division gene, *zipA* (21), is found in the same position with respect to *ligA* as ORF3 in *Neisseria*.

The predicted SLO7ORF3 polypeptide (Fig. 3) is 428 amino acid residues long with a calculated molecular mass of 47.5

TABLE 3. Transformation of MS11 with pSLO7::TnErmUP insertion mutants

Plasmid (genotype)	Transformation frequency ^a		
	Expt 1	Expt 2	Expt 3
pSLO7::TnErmUP#3 (<i>orf2</i>)	4.6×10^{-4}	6.3×10^{-5}	7.0×10^{-5}
pSLO7::TnErmUP#11 (<i>orf1</i>)	3.6×10^{-5}	5.1×10^{-5}	2.1×10^{-5}
pSLO7::TnErmUP#10 (<i>orf3</i>)	$<1 \times 10^{-7}$	2.4×10^{-7}	3.9×10^{-7}
pSLO7::TnErmUP#26 (<i>orf3</i>)	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$	$<1 \times 10^{-7}$

^a Frequencies are reported as numbers of Em transformants per total number of CFU. The limit of detection in this assay was 1.0×10^{-7} ; a value of $<1 \times 10^{-7}$ therefore indicates that no transformants were obtained.

FIG. 3. Alignment of the amino acid sequence of the putative gonococcal ZipA homolog (*N. gonorrhoeae* [*N.g.*]) with the *E. coli* (*E.c.*) (21) and *H. influenzae* (*H.i.*) (14) ZipA sequences. The entire coding region of SLO7ORF3 is annotated in the STDGEN database (<http://www.stdgen.lanl.gov/>) as NG0236. CLUSTALW alignment was done by using the DNA analysis program Omega. Numbering is sequential for *N. gonorrhoeae*. The amino-terminal hydrophobic residues thought to anchor the protein in the CM are double underlined. The positively charged region (residues 26 to 50) believed to prevent further export of the protein through the CM is indicated by the bold underline. Proline (P) and glutamine (Q) residues in a region of high concentration of these residues are indicated in black boxes. Gray boxes indicate residues in the most conserved C-terminal region that are identical or functionally similar in at least two of the proteins indicated and show the high degree of similarity between SLO7ORF3 and these other two ZipA homologs.

kDa, values somewhat larger than *E. coli* ZipA at 328 residues and 36.4 kDa. *E. coli* ZipA has a hydrophobic N-terminal domain (residues 1 to 21) with no apparent signal peptidase cleavage site, followed by a stretch of basic residues, which reportedly prevents membrane translocation (3). This is followed by a large apparently cytoplasmic domain with no hydrophobic stretches that are long enough to span the membrane. It has been suggested that ZipA is anchored in the CM with the remainder of the protein in the cytoplasm (21). Comparison of the predicted SLO7ORF3 protein sequence and *E. coli* ZipA (by using pairwise BLAST) showed no significant similarities in the primary sequences. Interestingly, however, these two proteins do appear to share features of their predicted secondary protein structure, including the N-terminal hydrophobic region (residues 1 to 21), the following basic region (residues 26 to 50, net charge +6), and the remaining cytoplasmic regions. An interesting feature of *E. coli* ZipA is the unusually high number of proline (31%) and glutamine

(23%) residues, which are thought to form a rigid linker that holds the C-terminal domain in place extended from the membrane-anchored domain (21). SLO7ORF3 also has an atypically high number of proline (13%) and glutamine (8%) residues in a similar region of the predicted protein sequence (Fig. 3). Furthermore, a CLUSTALW alignment of the amino acid sequences of the C-terminal region of *E. coli* ZipA with the similar region of ORF3 and the putative ZipA of *H. influenzae* (shown in Fig. 3) shows that there is significant similarity in this region, leading us to hypothesize that SLO7ORF3 might encode an ortholog of ZipA.

Complementation of an *E. coli* conditional zipA mutant. The entire SLO7ORF3 region from *N. gonorrhoeae* strain MS11A was ligated into pET24a, placing a full-length ORF3 under the control of the T7 promoter. The resulting plasmid, pET-ORF3, was transformed into *E. coli* BL21(ΔDE3) (52) and analyzed for protein production upon induction with IPTG. SDS-PAGE analysis showed the induction of a protein of ~50

FIG. 4. In vivo complementation of *E. coli* *zipA* with *N. gonorrhoeae* SLO7ORF3. *E. coli* strains CH3 (*zipA*⁺)/pCH32 [*repA*(Ts) *zipA*⁺ *ftsZ*⁺] and CH5 (*zipA*::*aph*)/pCH32 [*repA*(Ts) *zipA*⁺ *ftsZ*⁺] transformed with pACYC184 (ORF3⁻) or pACYCLacORF3 (ORF3⁺) were streaked onto LB-chloramphenicol plates and grown at the permissive (30°C) or nonpermissive (37°C) temperature. Plates were photographed by using a UVP BioDoc-It system with back lighting.

kDa, similar to the predicted 47.5 kDa size of the ORF protein (data not shown).

In order to determine whether ORF3 could complement a conditional *zipA* mutant strain of *E. coli*, we obtained two strains, CH3 (*recA*::Tn10 *zipA*⁺) and CH5 (*recA*::Tn10 *zipA*::*aph*), both harboring the plasmid pCH32 [*repA*(Ts) *zipA*⁺ *ftsZ*⁺] from Piet deBoer (21). The *zipA* allele on the chromosome of CH5 has been insertionally inactivated (*zipA*::*aph*), such that this strain can only grow when harboring pCH32 and at the permissive temperature (30 to 32°C), since *zipA* is an essential gene. CH3 is a wild-type (*zipA*⁺) control that grows well at both the permissive (30 to 32°C) and the nonpermissive (37 to 42°C) temperature, even while harboring pCH32.

To construct appropriate plasmids to test whether SLO7ORF3 could complement *zipA* in *E. coli*, ORF3 was subcloned from pET-ORF3 to a plasmid containing a *lac* promoter and the *lacI*^q gene, pWSKlacIOPE1 (see Materials and Methods). The entire *lacI*^q-*Plac*-SLO7ORF3 construct was then ligated into pACYC184. The resulting plasmid, pACYCLacORF3, was used to transform CH3/pCH32 and CH5/pCH32 (along with pACYC184 as a control). Transformants were then streaked on LB-chloramphenicol plates in duplicate and incubated at 30 or 37°C (Fig. 4). At 30°C, all four strains grew well, as expected. At 37°C the wild-type strain CH3/pCH32 harboring pACYC184 or pACYCLacORF3 grew well, but CH5/pCH32 harboring pACYC184 did not, which was also expected, since at the nonpermissive temperature ZipA would be depleted in this strain. However, CH5/pCH32 harboring pACYCLacORF3 did grow well at 37°C, the nonpermissive temperature for replication of the *zipA*⁺ plasmid, demonstrat-

ing that SLO7ORF3 can complement a *zipA*-null mutation in *E. coli*.

We next asked whether SLO7ORF3 could alleviate the filamentous phenotype of ZipA-depleted cells previously reported (21). Strains CH3/pCH32/pACYCLacORF3, CH3/pCH32/pACYC184, CH5/pCH32/pACYCLacORF3, and CH5/pCH32/pACYC184 were grown in liquid culture overnight at 30°C and then diluted 1:500 and shifted to 37°C and grown until an optical density at 600 nm (OD₆₀₀) of ~0.2 to 4 was reached. All strains except CH5/pCH32/pACYC184 reached this density in about 2 h. After 6 h of growth, CH5/pCH32/pACYC184 reached a maximum OD₆₀₀ of just under 0.2, a finding consistent with the inability of this strain to grow at the nonpermissive temperature. Samples of each culture were spotted onto glass microscope slides and Gram stained (Fig. 5). As expected, the *zipA*⁺ control strain CH3/pCH32 showed normal individual cells, with the occasional pair in the process of dividing. This was observed whether pACYC184 or pACYCLacORF3 was present, suggesting this level of ORF3 expression did not affect cell division in the presence of *E. coli* *zipA*. CH5/pCH32 harboring pACYC184, however, was extremely filamentous. Few, if any, individual cells were observed in any slide (four slides prepared from two different cultures were examined). Interestingly, CH5/pCH32 harboring pACYCLacORF3 showed an intermediate phenotype. Some filaments were observed, although none were as long as those of CH5/pCH32/pACYC184. Numerous individual cells, as well as short filaments of 2 to 5

FIG. 5. *N. gonorrhoeae* *zipA* partially alleviates filamentation phenotype of *E. coli* depleted for ZipA. *E. coli* strains harboring various *zipA* plasmids were cultured overnight at 30°C and then diluted 1:500 and shifted to 37°C and incubated for 2 h (A to C) or 6 h (D). Cultures were harvested, and the concentrations were adjusted to ~2 × 10⁹ CFU/ml. Then, 10 µl was spotted onto a microscope slide and Gram stained. (A) CH3 (*zipA*⁺)/pCH32 [*repA*(Ts) *zipA*⁺ *ftsZ*⁺]/pACYC-ORF3; (B) CH3 (*zipA*⁺)/pCH32 [*repA*(Ts) *zipA*⁺ *ftsZ*⁺]/pACYC184; (C) CH5 (*zipA*::*aph*)/pCH32 [*repA*(Ts) *zipA*⁺ *ftsZ*⁺]/pACYC-ORF3; (D) CH5 (*zipA*::*aph*)/pCH32 [*repA*(Ts) *zipA*⁺ *ftsZ*⁺]/pACYC184. Photomicrographs (courtesy of Matti Kiupel) were taken by using an Olympus Camedia E-10 digital camera attached to an Olympus BX40 microscope. Images were viewed under oil immersion at a magnification of ×1,000.

cells were visible, indicating that septation did occur but not as efficiently as in the wild type. This suggests that although SLO7ORF3 can complement the growth defect caused by ZipA depletion in *E. coli*, it only partially alleviates the defect in cell division. This may explain our inability to cure these strains of pCH32 (data not shown).

The growth of *E. coli* DH5 α containing pSLO7ORF3 (ORF3 expressed from its own promoter) had a slightly reduced growth rate, which could be explained by a defect in cell division. Examination of these cultures by light microscopy showed numerous filamentous cells, suggesting that overexpression of *N. gonorrhoeae* SLO7ORF3 blocks cell division (data not shown), a finding consistent with the phenomenon observed upon overexpression of *zipA* in *E. coli* (21). However, when SLO7ORF3 was overexpressed from an IPTG-inducible promoter (pACYCLacORF3) and pCH32 (*zipA*⁺ *ftsZ*⁺) was also present, this effect on growth was alleviated, presumably due to the excess FtsZ produced from this plasmid.

DISCUSSION

The prokaryotic SRP is a ubiquitous protein targeting system that targets a subset of proteins to the CM cotranslationally (13, 32, 36, 49, 59). All of the components of the SRP appear to be essential in bacteria (9, 17, 24, 35, 37, 65), suggesting that at least one of the proteins dependent on this system for localization is involved in an essential cell process. In this work, we set out to identify proteins from *N. gonorrhoeae* that utilize the SRP in an effort to identify essential CM proteins from this organism. Using a heterologous screening approach in *E. coli*, we identified several genes encoding proteins that appeared to utilize the SRP. Of these, one (SLO7ORF3) was determined to be a functional homolog of ZipA, an essential cell division protein in *E. coli*.

In *E. coli*, there are at least 10 components (FtsA, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, FtsZ, YbgQ, and ZipA) involved in the assembly of the septal ring, a membrane-associated cytoskeletal element that directs the formation of the division septum (10; reviewed in reference 40). In the initial stages of cell division FtsZ self-associates to accumulate at the prospective division site on the inner side of the CM, forming a structure called the Z ring (31), which then acts as a scaffold to which the other cell division proteins are recruited. ZipA is an essential protein that interacts with FtsZ (21), which while not required for Z ring formation, is required for recruitment of additional proteins to the Z ring (20, 29). Although ZipA is essential in *E. coli*, it appears to be less conserved in gram-negative bacteria and is the least-conserved cell division protein (40). Putative homologs of ZipA have been identified in several gram-negative bacteria (21, 39), mostly based on sequence homologies between the N-terminal and C-terminal domains of the protein, both of which are reported to be important for ZipA function (22, 29). Predicted proteins with significant homology to ZipA have not been identified in genome sequences of gram-positive bacteria, archaea, and some gram-negative bacteria, leading to the conclusion that it has either divergently evolved or other proteins serve its function in the cell (39).

Although the similarity of *N. gonorrhoeae* SLO7ORF3 to *E. coli* ZipA is low at the amino acid sequence level, there are significant similarities in key domains (Fig. 3). These include

(i) an N-terminal hydrophobic region (with no signal peptidase cleavage site) followed by a basic region (net positive charge of 5 to 8) that likely functions to anchor the protein in the CM and (ii) central region rich in proline and glutamine residues (21). A CLUSTALW alignment of this fragment with the *N. gonorrhoeae* ZipA shows 15% identity and 49% similarity at the amino acid level in this region (Fig. 3). Taken together, these observations and our data indicate that SLO7ORF3 does indeed encode the gonococcal cell division protein, ZipA. To the best of our knowledge, this is the first ZipA homolog identified in a non-rod-shaped bacterium. Thus, it will be interesting to more closely examine the role of ZipA in cell division in *N. gonorrhoeae*.

Of the 10 proteins shown to be involved in formation of the division septum in *E. coli*, genes encoding seven of these have been identified in *N. gonorrhoeae*: *ftsZ*, *ftsA*, *ftsQ*, and *ftsI* (15, 41) and *ftsK*, *ftsW*, and *ygbQ* (<http://www.stdgen.lanl.gov>). In addition to these, the *min* genes, which encode three proteins, MinC, MinD, and MinE, which are involved in positioning of the division septum have also been identified and characterized in *N. gonorrhoeae* (53). Studies of the function of these proteins in *N. gonorrhoeae* by Jo-Anne Dillon and coworkers indicates that these proteins play similar roles in cell division as in *E. coli* (38, 54). Genes encoding FtsE and FtsX have also been identified in *N. gonorrhoeae* (6), although it is not clear if they are involved in cell division in *Neisseria*. FtsL and FtsN are the only key cell division proteins remaining to be identified in *Neisseria*.

Numerous proteins have now been identified that utilize the SRP for targeting in bacteria (13, 32, 36, 49, 59, 60). These include an efflux pump (AcrB), several transport systems (LctP, LacY, KgtP, MalF, MtlA, and ProW), and some membrane-associated enzymes (PgsA, MdoH, and CdsA), none of which are essential for cell viability. In addition to these, however, are several proteins that may be involved in cell division. These include FtsQ (56, 61), which is essential in formation of the division septum (1, 16, 64), and FtsE and FtsX (59). While most SRP-dependent proteins are polytopic membrane proteins, two SRP-dependent cell division proteins (FtsQ and FtsE) are bitopic, having a single membrane-spanning domain, similar to ZipA.

There are several links between cell division and the SRP. Depletion of Ffh, the signal sequence binding SRP component, or cells expressing a mutated *ffh* gene results in defects in cell division in *E. coli* (37, 43). A point mutation in *ffs*, which encodes the 4.5S SRP RNA, of *Caulobacter crescentus* confers a temperature-sensitive defect in cell division (65). And finally, *ftsY*, which encodes the SRP docking protein, FtsY, in *E. coli* was initially identified as part of an operon (*ftsYEX*) containing genes that are temperature-sensitive for filamentation (*fts* [17]), and FtsY-depleted cells are filamentous, apparently defective in the completion of septation during cell division (30). All of these observations can be explained if one or more essential cell division proteins is dependent on the SRP for targeting. Since all of the other cell division proteins, with the exception of FtsZ and FtsA, are reported to localize to the CM, it will be interesting to determine whether any of these utilize the SRP.

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