The *Haloferax volcanii* FtsY Homolog Is Critical for Haloarchaeal Growth but Does Not Require the A Domain

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Received 30 November 2004/Accepted 11 March 2005

The targeting of many Sec substrates to the membrane-associated translocation pore requires the cytoplasmic signal recognition particle (SRP). In *Eukarya* and *Bacteria* it has been shown that membrane docking of the SRP-substrate complex occurs via the universally conserved SRP receptor (Sra/β and FtsY, respectively). While much has been learned about the archaeal SRP in recent years, few studies have examined archaeal Sra/FtsY homologs. In the present study the FtsY homolog of *Haloferax volcanii* was characterized in its native host. Disruption of the sole chromosomal copy of *ftsY* in *H. volcanii* was possible only under conditions where either the full-length haloarchaeal FtsY or an amino-terminally truncated version of this protein lacking the A domain, was expressed in *trans*. Subcellular fractionation analysis of *H. volcanii* *ftsY* deletion strains expressing either one of the complementing proteins revealed that in addition to a cytoplasmic pool, both proteins cofractionate with the haloarchaeal cytoplasmic membrane. Moreover, membrane localization of the universally conserved SRP subunit SRP54, the key binding partner of FtsY, was detected in both *H. volcanii* strains. These analyses suggest that the *H. volcanii* FtsY homolog plays a crucial role but does not require its A domain for haloarchaeal growth.

All living organisms can be classified into three domains: the *Eukarya*, the *Bacteria*, and the *Archaea* (45). While the archaea are morphologically similar to the bacteria, at the molecular level they exhibit a combination of unique as well as bacterial and eukaryotic characteristics (18, 31). Recent whole-genome analyses of numerous archaea have expanded our understanding of this domain and provided insight into phylogenetic relationships, putative metabolic pathways, and cellular processes (3, 14, 37, 38). Furthermore, a more complete understanding of the *Archaea* has proven to have important implications for our understanding of cellular functions conserved in *Eukarya* and *Bacteria* (21, 34). This has been demonstrated in studies examining archaeal protein translocation via the universally conserved Sec pathway, which is thought to be responsible for the translocation of the majority of proteins into and across the endoplasmic reticular (ER) or cytoplasmic membranes in eukaryotes and prokaryotes, respectively (19, 35). Specifically, the notable achievement of the crystal structure of an archaeal Sec translocation pore, the first such structure obtained from any organism, has greatly contributed to the understanding of this universally conserved pore in all organisms (42). However, in addition to homologs of the universally conserved Sec components, archaea possess a mixture of homologs of bacterial and eukaryotic translocation components (29, 31). This observation, as well as the fact that homologs of translocation ATPases from *Bacteria* or *Eukarya* are lacking (e.g., SecA and Kar2p/BiP, respectively), suggest unique features of the archaeal protein translocation pathway (29, 31). Studying the significance of the similarities and differences among the Sec pathways of *Archaea*, *Bacteria*, and

Eukarya will further our understanding of translocation in all the domains.

Recent in vivo and in vitro analyses of the protein translocation mechanisms of *Haloferax volcanii*, a halophilic archaeon amenable to genetic and biochemical analyses, have complemented genomic and structural analyses of the archaea (19, 35). The composition of the archaeal SRP is more similar to that of eukaryotes than the composition of bacterial SRP (10, 29, 35). In contrast, the composition of the eukaryotic SRP receptor (SR), a component of the ER membrane that docks the SRP nascent chain to the membrane, shows less similarity to its archaeal homolog (29). The eukaryotic SR is a heterodimer composed of Sra and a membrane protein, Srβ, that anchors the Sra subunit to the ER membrane. In contrast, bacteria and archaea seem only to contain a homolog of the Sra subunit, called FtsY (29, 32).

Despite the lack of a prokaryotic Srβ homolog, FtsY is found in both cytoplasmic and membrane-associated pools in the bacterial and archaeal species examined to date (6, 20, 25). FtsY is a member of the universally conserved SRP subfamily of GTPases whose members are characterized by the presence of a four alpha-helical bundle (termed the N domain) and immediately followed by the catalytic GTPase region (termed the G domain) (8). Both of these domains are involved in recognition of the universally conserved protein component of the SRP, SRP54 (termed Ffh in bacteria), and play critical roles in SRP-dependent protein translocation (9, 12). Many, though not all, examples of FtsY include an additional amino-terminal domain, named the “A” domain (9, 11, 12, 15). This name originated from the largely acidic character of the first example of FtsY to be described, that from *Escherichia coli*. Although not universal, many FtsY examples also have acidic A domains, including FtsY homologs from the haloarchaea (A. Haddad and M. Pohlschröder, unpublished observations).

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In the absence of obvious membrane spanning regions in FtsY, it had been thought that the amino-terminal A domain mediated the interaction of FtsY with the membrane (7, 23, 24). However, this view recently has been challenged by several observations. First, sequence alignments reveal that in contrast to the strongly conserved N and G domains, the A domain is poorly conserved (29). Moreover, several bacterial and archaeal FtsY homologs lack an A domain (9, 11, 12). Second, the essential role of the A domain has been reevaluated in light of recent observations in *E. coli*, demonstrating that expression of FtsY lacking an A domain functionally complements disruption of the full-length protein (11). The amino-terminally truncated construct also associated with the *E. coli* cytoplasmic membrane, albeit less efficiently than full-length FtsY (11). Finally, in vitro analysis of purified proteins interacting with *H. volcanii* inverted vesicles showed that both the full-length haloarchaeal FtsY homolog and a version of FtsY lacking the A domain were able to associate with these membrane preparations (19). Interestingly, additional in vitro experiments suggested that the A domain may be involved in recruiting the SRP to the haloarchaeal cytoplasmic membrane (19).

We wished to further characterize the archaeal FtsY homolog in vivo. In the present study, we have employed a recently developed gene knockout strategy to demonstrate in vivo that the *H. volcanii* FtsY homolog is crucial for growth (2). Furthermore, we show that a haloarchaeal FtsY construct lacking the amino-terminal A domain is able to complement a chromosomal *ftsY* deletion and that in both strains the SRP is targeted to the membrane. Thus, although the archaeal SRP-dependent pathway shares similarities with that of both eukaryotes and bacteria, the archaeal FtsY appears in vivo to be more similar to the *E. coli* FtsY.

MATERIALS AND METHODS

Reagents. Reagents, including antibiotics, were purchased from Sigma-Aldrich (St. Louis, MO) except where noted. 5-Fluoroorotic acid (5-FOA) was from Toronto Research Chemicals (North York, Canada). The *H. volcanii* cosmid library was generously provided by R. L. Charlebois (Department of Biology, University of Ottawa) (5). Anti-dihydrofolate reductase (DHFR) antibodies were a kind gift from M. Mevarech (Tel Aviv University, Tel Aviv, Israel). Antibodies against the *H. volcanii* FtsY homolog were generously provided by J. Eichler (Ben Gurion University of the Negev, Beersheva, Israel) (19). Antibodies recognizing the *H. volcanii* SRP54 homolog were described previously (35). Protease inhibitors (pepstatin and leupeptin) were from Roche Applied Science (Indianapolis, IN). The enhanced chemiluminescence (ECL+) detection system was from Amersham Pharmacia (Piscataway, NJ). All enzymes were from New England Biolabs (Beverly, MA) unless otherwise indicated. Anti-penta-His antibodies and DNA purification systems (QIAquick and QIAprep) were from QIAGEN (Valencia, CA). RQ1 DNase I was from Promega (Madison, WI). NuPage gel system and reagents were from Invitrogen (Carlsbad, CA). Blotting membranes (polyvinylidene difluoride [PVDF]-plus) were from GE Osmonics Inc. (Minnetonka, MN).

Strains and growth conditions. Archaeal strains used in this study are listed in Table 1. Unless otherwise indicated, *H. volcanii* strains were routinely grown with aeration at 45°C in rich medium (RM) supplemented with novobiocin (0.3 µg/ml) or 5-FOA (150 µg/ml) when required (22). For gene integration, Casamino acid medium (CA) was used and supplemented with novobiocin as above, when needed (2). *E. coli* strains MP12 or XL1-Blue (Stratagene, La Jolla, CA), were routinely grown at 37°C in NZCYM supplemented with ampicillin (100 µg/ml) as required (30, 36).

Cloning and sequence analysis of the *H. volcanii* *ftsY* homolog. Standard homology searches using several bacterial *ftsY* sequences as queries, identified a single *ftsY* homolog present in a partial genome sequence of *H. volcanii* (<http://zdna2.umbi.umd.edu/Public/hvo/>). A single cosmid from an *H. volcanii* cosmid library (cos105) (5) was found to contain the *ftsY* open reading frame (ORF) via

TABLE 1. *H. volcanii* strains and plasmids used in this study

Strain or plasmid	Relevant property(ies)	Reference or source
Strains		
WFD11	ΔpHV2	4
WR480	Ura mutant, 5-FOA ^r ; Δ <i>pyrE2</i>	2
AHco1	Ura ⁺ , 5-FOA ^s ; WR480-derived pAH-k1 cointegant	This study
AHwt20	Ura mutant, Nb ^r , 5-FOA ^r ; Δ <i>pyrE2</i> , Δ <i>ftsY</i> ; AH-co1 derived harboring pAH-x1	This study
AHwt21	Ura mutant, Nb ^r , 5-FOA ^r ; Δ <i>pyrE2</i> ; AH-co1 derived harboring pAH-x1	This study
AHng09	Ura mutant, Nb ^r , 5-FOA ^r ; Δ <i>pyrE2</i> , Δ <i>ftsY</i> ; AH-co1 derived harboring pAH-x2	This study
AHng10	Ura mutant, Nb ^r , 5-FOA ^r ; Δ <i>pyrE2</i> ; AH-co1 derived harboring pAH-x2	This study
AHng15	Ura mutant, Nb ^r , 5-FOA ^r ; Δ <i>pyrE2</i> , Δ <i>ftsY</i> ; AH-co1 derived harboring pAH-x3	This study
Plasmids		
pNP15	Amp ^r , Nb ^r ; <i>H. volcanii</i> shuttle vector	27
pGB70	Amp ^r , <i>H. volcanii</i> shuttle vector containing <i>pyrE2</i>	2
pAH-k1	pGB70; <i>ftsY</i> gene targeting vector	This study
pAH-x1	pNP15 containing <i>ftsY</i> · 6×His	This study
pAH-x2	pNP15 containing <i>ftsYNG</i> · 6×His	This study
pAH-x3	pNP15 containing <i>ftsYG</i> · 6×His	This study

PCR using oligonucleotide primers flanking the ORF (*ftsY*-For and *ftsY*-Rev). The sequence of the ORF was confirmed using standard automated sequencing methods. Additional sequence information flanking the *ftsY* ORF was determined via primer walking.

Construction of *ftsY* deletion strains. The *ftsY* ORF in *H. volcanii* was deleted using a recently developed counter-selection scheme (2). Briefly, a targeting construct is created using genomic regions flanking the ORF to be targeted that are cloned in tandem into a nonreplicative plasmid upstream of the *pyrE2* gene cassette. The targeting construct is subsequently transformed into *H. volcanii* strain WR480, a uracil auxotroph; thus, selection in the absence of uracil will generate a strain in which the targeting plasmid is integrated within either the upstream or downstream genomic regions flanking the targeted ORF (i.e., cointegant or "pop-in"). The cointegant strain is then grown without selection for a limited number of generations, allowing for recombination to take place, and subsequently plated on 5-FOA to select against the presence of the *pyrE2* gene cassette. Under these conditions, recombination eliminating the *pyrE2* gene occurs in one of two ways: (i) it removes the targeted gene, or (ii) it resolves the original plasmid. PCR was used to generate 860-bp DNA fragments that contained 800 bp of flanking genomic DNA (either upstream or downstream) along with 60 bp of the *ftsY* ORF. These were amplified from *H. volcanii* chromosomal DNA using primers designed to amplify the upstream (*ftsYUP860*-For and *ftsYUP860*-Rev) and downstream (*ftsYDN860*-For and *ftsYDN860*-Rev) flanking regions. The PCR product representing the upstream fragment was first digested with EcoRI and KpnI, agarose gel-purified (QIASPIN) and ligated to plasmid DNA (pGB70) that had been digested with EcoRI and KpnI and treated with calf intestinal phosphatase (CIP). Resulting plasmids, harboring the upstream *ftsY* fragment were digested with KpnI, CIP treated, and subsequently ligated to the PCR product representing the *ftsY* downstream fragment that had been digested with KpnI and gel purified. The resulting plasmid containing the *ftsY* downstream flanking sequence in the desired orientation, here named pAH-k1, was first passed through a *dam* mutant *E. coli* host (DL739) prior to transforming *H. volcanii* strain WR480, as previously described (17). Transformants were plated on CA medium to select for cointegant strains (e.g., strain AH-co1). Independent colonies were then grown in liquid RM for 48 h at 40°C to allow for recombination at the *ftsY* locus and subsequently plated on RM supplemented with 5-FOA. Genotypes of isolated clones were determined by colony PCR using primers flanking the targeted region on the chromosome (*ftsY*screen-For and *ftsY*screen-Rev).

Expression of *FtsY* · 6×His derivatives in *H. volcanii*. The *ftsY* ORF was modified by introducing a nonapeptide sequence encoding a six-histidine epitope tag (pro-pro-gly-his₆) in frame at the carboxy terminus using PCR amplification from the *H. volcanii* cosmid library (*ftsYWT*-For and *ftsY* · 6×His-Rev). The amplification product was first purified from the reaction mixture (QIASPIN),

TABLE 2. Oligodeoxynucleotide primers used in this study

Primer	Sequence	Location
<i>ftsY</i> -F	5'-ATTTTACATATGGCGCTCCTCCAGAGC	5' end of <i>ftsY</i> ; forward primer
<i>ftsY</i> -R	5'-GGGGGGAAGCTTTTACTCGTCTTCACCGAG	3' end of <i>ftsY</i> ; reverse primer
<i>ftsYUP860</i> -F	5'-ATTTAAGAATTCGTGGGTGCGCATCGAAGACG	800 bp upstream to <i>ftsY</i> ; forward primer
<i>ftsYUP860</i> -R	5'-ATTTAAGGTACCGGCGGTCTCTTCAACGTCG	60 bp within 5' end of <i>ftsY</i> ; reverse primer
<i>ftsYDN860</i> -F	5'-ATTTAAGGTACCCACATCGAGAAGTTTCGACC	800 bp downstream to <i>ftsY</i> ; forward primer
<i>ftsYDN860</i> -R	5'-ATTTAAGGTACCCGCGGGTCGCCGACATCC	60 bp within 3' end of <i>ftsY</i> ; reverse primer
<i>ftsY</i> screen-F	5'-CGACATCGGCACCATCAACTAC	899 bp upstream to <i>ftsY</i> ; forward primer
<i>ftsY</i> screen-R	5'-CGCACCTCCGAAGCAACC	941 bp downstream to <i>ftsY</i> ; reverse primer
<i>ftsY</i> WT-F	5'-GGGGGGCCATGGAGCAGATGCAGGGCGAGG	5' end of <i>ftsY</i> ; forward primer
<i>ftsY</i> NG-F	5'-CATGCGCCGCGCCGCCGCTTCG	93 bp within <i>ftsY</i> ; forward primer
<i>ftsY</i> G-F	5'-CATGGAGGTCATCAGCGTCGGG	715 bp within <i>ftsY</i> ; forward primer
<i>ftsY</i> · 6xHis-R	5'-ATTGGGGTACCTTAGTGATGGTGATGCGGGCCGCCCTCGTCTTCAACGAGCAGG	3' end of <i>ftsY</i>

digested with NcoI and KpnI, gel purified, and finally ligated to pNP15 plasmid DNA (Table 1), which had previously been digested with NcoI and KpnI, treated with CIP, and gel purified (pAH-x1). This created plasmid pAH-x1. Amino-terminal truncations of the *H. volcanii* FtsY homolog were designed removing either the predicted A domain [amino acids 2 to 164, to create FtsY(NG) · 6xHis], or both the A and N domains [amino acids 2 to 238, to create FtsY(G) · 6xHis]. These constructs were created identically to the full-length construct to generate plasmids pAH-x2 and pAH-x3, respectively, except that different forward primers were used in the initial PCR [for FtsY(NG) · 6xHis, the *ftsY*NG-For primer was used and for FtsY(G) · 6xHis, the *ftsY*G-For primer was used]. Each plasmid was passed through a dam mutant *E. coli* host, transformed into the *H. volcanii* cointegrant strain AH-co1, and subsequently plated on CA medium supplemented with novobiocin. In this way, both the *ftsY* targeting vector (pAH-k1), already integrated at the chromosomal *ftsY* locus, and the plasmid designed to supply the *ftsY* · 6xHis variants *in trans* were maintained. Independent colonies from strains harboring each plasmid were then grown in liquid RM containing novobiocin for 48 h to allow for recombination at the *ftsY* locus and subsequently plated on medium containing novobiocin and 5-FOA. 5-FOA-resistant strains were genotyped using PCR as described above. To confirm the expression of the plasmid encoded FtsY constructs, whole-cell lysates were prepared from isolated transformants by growth in liquid culture (10 ml) with novobiocin to an OD₆₀₀ of between 0.6 and 0.8. One ml of cells were pelleted at 4,500 × g and lysed directly in 90 µl to 120 µl of NuPage lithium dodecyl sulfate (LDS) sample buffer supplemented with reductant, according to the manufacturer's instructions, and incubated for one hour at 37°C prior to polyacrylamide gel electrophoresis (PAGE).

***H. volcanii* translation inhibition.** *H. volcanii* strain AHwt20 was treated with the translational inhibitor puromycin sulfate as described by Ring et al. with minor modifications (33). Briefly, 50 µl of a late log phase AHwt20 culture grown in RM supplemented with novobiocin was transferred to 10 ml of Hv-min medium, supplemented with uracil (50 µg/ml), novobiocin (0.3 µg/ml), and methionine assay medium (250 µg/ml [Difco 242310]) (1). Once grown to late log phase the culture was diluted 1:100 in Hv-min with supplements and grown to mid-log phase, at which time it was divided into two cultures, which were incubated for an additional 20 min, one in the absence the other in the presence of 70 µl/ml of puromycin sulfate. Cytoplasmic and membrane fractions were then prepared from a 10-ml aliquot of each culture and analyzed by sodium dodecyl sulfate (SDS)-PAGE and Western blot analysis. The efficacy of the puromycin treatment was confirmed via metabolic labeling similar to the method described previously (33). Briefly, aliquots of the cultures, following incubation in the presence or absence of inhibitor, were labeled with [³⁵S]methionine (>1,000 Ci/mmol; NEG-709A; Office of Radiation Safety, University of Pennsylvania) for 5 min, precipitated with 10% (vol/vol) trichloroacetic acid (TCA), and then subjected to SDS-PAGE and visualized via phosphorimaging (Molecular Dynamics).

Subcellular fractionation of *H. volcanii*. *H. volcanii* strains grown in RM supplemented with novobiocin were harvested at mid- to late-log phase (OD₆₀₀ of 0.5 to 0.8) at 4,500 × g at 4°C for 10 min. Cells (5 ml) were washed once in basal salt medium (BSM; 3.5 M NaCl, 15 mM MgSO₄ · 7H₂O, 50 mM KCl, and 50 mM Tris [pH 7.2]), and cell pellets were resuspended in 0.5 ml BSM containing protease inhibitors (pepstatin, 1 µg/ml, and leupeptin, 2 µg/ml). Cells were lysed via sonication on ice at setting "50" using a Dynatech Sonic Dismembrator Model 150 equipped with a microtip probe using eight pulses of 3 s in 5-s intervals. Cellular debris was removed via two successive spins at top speed in a microcentrifuge at 4°C. The supernatant was then spun at 85,000 rpm for 30 min

at 4°C (Beckman TLA 100.2) to separate the cytoplasmic and membrane fractions. The cytoplasmic fraction was then precipitated with 10% (vol/vol) trichloroacetic acid (TCA) for 30 min on ice. Precipitated protein was collected at top speed (20,800 × g) in a microcentrifuge at 4°C for 30 min, washed with cold 80% (vol/vol) acetone, and centrifuged again as before. Following removal of the acetone, the pellets were allowed to air dry briefly prior to resuspension in protein sample buffer. The membrane pellet was washed once with cold BSM containing protease inhibitors and subsequently resuspended in 75 µl of double-distilled H₂O containing protease inhibitors. Both membrane and cytoplasmic fractions were solubilized in NuPage LDS sample buffer for one hour at 37°C prior to PAGE. For analysis of the *H. volcanii* SRP54 homolog, SDS sample buffer was used and electrophoresis was otherwise identical.

Western blot analysis. Proteins were resolved using the bis-Tris NuPage gel electrophoresis systems (Invitrogen). Proteins were transferred to Magna nylon membranes using a Bio-Rad trans-blot SD semidry transfer cell for 30 min at 15 V, according to the manufacturer's instructions. Primary antibody (anti-FtsY, 1:2,000; anti-penta His, 1:2,000; anti-SRP54, 1:1,000; and anti-DHFR1, 1:7,000) and secondary antibody incubations (horseradish peroxidase conjugated [HRP] sheep anti-mouse IgG, 1:8,000, or donkey anti-rabbit immunoglobulin G [IgG] [both from Amersham Biosciences], 1:10,000) were performed in 3% (wt/vol) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween-20 for one hour at room temperature. Washes were with PBS containing 0.1% (vol/vol) Tween-20. Membranes were processed with the ECL Plus Western Blotting detection system (Amersham Biosciences) according to the manufacturer's instructions. Immunoreactive bands were visualized by exposure to Super Rx Fuji Medical X-ray film (Fuji Photo Film, Co., Ltd., Tokyo, Japan). For quantification purposes, developed films were scanned and individual bands were quantified using the public domain software Image J (1.30v; Wayne Rasband, National Institutes of Health; <http://rsb.info.nih.gov/ij>). Band intensities were determined using integration of the area underlying each band peak.

Nucleotide sequence accession number. The nucleotide sequence for the ORF encoding the *H. volcanii* *ftsY* homolog has been deposited in GenBank under accession number AY187867.

RESULTS

The *H. volcanii* *ftsY* homolog is critical for growth. Our *in vivo* characterization of the *H. volcanii* FtsY homolog began with the disruption of the *ftsY* locus using a counterselection strategy recently developed for this organism (2). Similar to approaches developed for use in *Saccharomyces cerevisiae*, this scheme takes advantage of the ability to positively or negatively select for the activity of orotate phosphoribosyl transferase (ORPTase, a component of the uracil biosynthetic pathway) by either requiring growth in the absence of uracil (for positive selection) or growth in the presence of the toxic compound 5-FOA (for counterselection). Briefly, in a Δ *pyrE2* strain (WR480, Table 1) a nonreplicative plasmid containing *pyrE2* (the gene encoding the major *H. volcanii* ORPTase) was first targeted to the *ftsY* locus via homologous recombination with

FIG. 1. Disruption of the *H. volcanii* chromosomal *ftsY* locus requires expression of either FtsY or FtsYNG in *trans*. (A) Schematic diagram of the *H. volcanii* *ftsY* chromosomal locus targeted for disruption. The boxed region represents the *H. volcanii* *ftsY* locus (1,371 bp) targeted for deletion using flanking genomic information (emboldened lines). Arrows flanking this area of recombination denote positions of oligonucleotide primers used to determine the genotype of the resulting strains. (B) Representations of the epitope-tagged derivatives that were expressed in *trans* during gene targeting. Letters within the boxes designate the respective domains. The small white boxes at the carboxy terminus of each construct (following the G domain) represent the location of the 6× His tag. (C) Amplicons generated using the PCR with primers denoted in (A) from *H. volcanii* strains as indicated (Δ denotes *ftsY* deletion; wt denotes wild-type *ftsY* locus). (D) Western blot analysis of *H. volcanii* whole-cell lysates prepared from strains analyzed in (C), using an antibody recognizing the *H. volcanii* FtsY homolog.

cloned *ftsY* flanking sequences. Plasmid cointegrants were selected by uracil prototrophy. Resolution of the cointegrated plasmid was selected in a subsequent step by growth on media containing both uracil and 5-FOA. This second recombination event can either regenerate the intact gene or generate a knock-out at the chromosomal locus.

As confirmed by PCR, we were able to generate *H. volcanii* cointegrant strains that had integrated the targeting plasmid adjacent to the *ftsY* locus (AH-co1, Table 1). However, PCR analysis of 27 5-FOA-resistant colonies arising from two independent counterselections of AH-co1 revealed that all clones had retained *ftsY* intact (Fig. 1A and data not shown). These results strongly suggest that FtsY is crucial for *H. volcanii* growth under these conditions. It is possible, however, that the retention of chromosomal *ftsY* in these strains occurred because the integrated plasmid was unable to recombine in a manner that would allow for its deletion. To eliminate this pos-

sibility, AH-co1 was transformed with a plasmid designed to express full-length FtsY that has a six-histidine epitope tag at the carboxy terminus (FtsY · 6×His, pAH-x1, Table 1 and Fig. 1B) creating strain AH-co1x1. When strain AH-co1x1 was exposed to 5-FOA, recombinants containing either the intact or disrupted *ftsY* gene were recovered [Fig. 1C, (AHwt20) and (AHwt21), respectively]. A total of 29 clones were screened with 6 retaining intact *ftsY* loci (data not shown). This result confirms that the haloarchaeal FtsY homolog is important for *H. volcanii* growth and, moreover, that *ftsY* · 6×His encodes a functional protein.

The A domain of the *H. volcanii* FtsY homolog is dispensable for growth. In vitro membrane binding assays suggested that the amino-terminal A domain of the *H. volcanii* FtsY homolog is required for the association of the SRP with the archaeal cytoplasmic membrane (19). However, the recently resolved heterodimeric structure from cocrystals of the *Thermus aquaticus* FtsY homolog, which lacks a notable A domain, with the *T. aquaticus* SRP subunit, Ffh, identified potential Ffh interaction sites in the N domain (9, 12). To test the importance of the A domain for in vivo function of the haloarchaeal FtsY, we asked whether expression of an amino-terminally truncated *H. volcanii* FtsY construct, lacking the A domain [*ftsYNG* · 6×His], was able to complement an *H. volcanii* chromosomal *ftsY* deletion. The boundary between the A and N domains of the *H. volcanii* FtsY homolog was determined by amino acid alignments of a number of prokaryotic homologs with the *E. coli* FtsY whose domains had been defined based on amino acid composition (29).

To ascertain the importance of the *H. volcanii* FtsY A domain for its function, we repeated the complementation strategy similar to the one described above, testing the functionality of the tagged full-length FtsY by transforming the cointegrant strain AH-co1 with a plasmid expressing *ftsYNG* · 6×His (Fig. 1B and Table 1, pAH-x2). Using PCR screening, genotypic analysis of 5-FOA-resistant colonies revealed strains possessing either a disrupted (AHng09) or an intact (AHng10) *ftsY* locus (Fig. 1C; 2 out of 24 clones screened contained disrupted *ftsY* loci). These results strongly suggested that *ftsYNG* · 6×His provided in *trans* could functionally complement a chromosomal *ftsY* deletion. To confirm the absence of wild-type FtsY in strain AHng09, immunoblot analysis of whole-cell lysates was performed, using an antibody that recognizes the *H. volcanii* FtsY NG domain (Fig. 1D). Western blots clearly showed the lack of a specific band detecting the full-length FtsY in strain AHng09, suggesting that the endogenous *ftsY* was not expressed in this strain. Furthermore, it identified a specific immunoreactive band at 32 kDa, the predicted molecular mass of FtsYNG · 6×His in strain AHng09. Additionally, comparison of the growth rates of AHwt20 and AHng09 under several different conditions (in both rich and minimal media at 45°C and 30°C [the lower temperature is known to reveal a temperature-sensitive phenotype linked with certain secretion defects]) revealed no differences between these strains (28) (data not shown).

Finally, we asked whether a gene that encodes a version of FtsY that lacks both the A and the N domains (*ftsYG* · 6×His) from the same expression vector that was used for the previous assays (pAH-x3, Table 1 and Fig. 1B) could complement a chromosomal *ftsY* deletion. Genotypic analysis of 5-FOA-re-

FIG. 2. The *H. volcanii* FtsY A domain is dispensable for its membrane association. Western blot analysis of cytoplasmic and membrane fractions prepared from a nontransformed *H. volcanii* strain (WFD11) and Δ ftsY strains expressing different FtsY constructs. Standardized equivalents of cytoplasmic (c) and membrane (m) fractions were analyzed using (A) anti-*H. volcanii* FtsY antibodies or (B) antibodies recognizing the *H. volcanii* cytoplasmic protein DHFR1.

sistant colonies derived from these experiments (a total of 24 colonies were analyzed) indicated that, although the protein was properly expressed, the *ftsY* locus in each one of these strains remained intact (Fig. 1 C and D). These results not only confirm that the highly conserved N domain is required for the *H. volcanii* FtsY function but also demonstrate that disruption of the chromosomal *ftsY* locus in *H. volcanii*, complemented by *ftsYNG* · 6×His, was not due to secondary effects introduced by the expression vector.

Membrane-associated FtsY is detected in the presence and absence of the A domain. Both in vivo and in vitro analyses suggest that in most of the prokaryotic systems examined to date, FtsY is present in the cytosol as well as associated with the membrane (6, 19, 25). Consistent with these results, immunoblot analysis of *H. volcanii* membrane and cytoplasmic fractions, using an α -FtsY antibody, detected this protein in both fractions in wild-type strain WFD11 (Fig. 2A).

Having constructed a strain expressing FtsYNG in the absence of the chromosomal *ftsY* gene (Fig. 1), we were able to directly test the contribution of the haloarchaeal FtsY A domain to membrane binding in vivo without potential contributions from the endogenous protein. Immunoblot analysis of subcellular fractions from Δ ftsY strains expressing full-length (AHwt20) and A-domain-truncated FtsY variants (AHng09) both showed similar relative amounts of the proteins in the membrane and the cytoplasm (Fig. 2A). It should be noted that, while the strain expressing the FtsYNG construct (AHng09) reproducibly maintained a membrane associated pool of this protein, the total amount of this pool varied among experiments (AHwt20 had consistent levels of cytoplasmic and membrane-associated FtsY) (Fig. 2A and data not shown). To confirm the purity of the membrane fractions used for the subcellular localization studies, we also tested the cellular fractions for the presence of the cytoplasmic protein, dihydrofolate reductase 1 (DHFR1). Immunoblot analysis, using α -DHFR1 antibodies, identified a band of the expected size only in the cytoplasmic fraction, confirming that the identification of membrane-associated FtsY was not due to contamination with cytoplasmic proteins (Fig. 2B). These in vivo results suggest that the *H. volcanii* FtsY homolog is present in both cytoplasmic and membrane fractions and that, under our assay conditions, the A domain of this protein is not necessary for the association of FtsY with the membrane.

Cellular localization of SRP in *H. volcanii* wild-type and Δ ftsY strains. (i) The *H. volcanii* SRP54 homolog can be detected in both the haloarchaeal cytoplasm and membrane. Cellular localization studies suggest that subpopulations of SRP54, the universally conserved SRP component that interacts with the SR, is associated with the ER membrane (43). Consistent with these results, a subpopulation of the *Acidianus ambivalens* SRP54 homolog and the *Halobacterium halobium* 7SRNA homolog have been shown to be associated with the archaeal cytoplasmic membranes (16, 25). However, while in vitro membrane binding assays also point to a membrane-associated pool of the *H. volcanii* SRP54 homolog (19), previous results from *H. volcanii* cellular fractionations only identified this SRP subunit in the cytoplasm (41). Because of the widely accepted role of FtsY in facilitating membrane association of the SRP complex, we first reexamined the localization of the *H. volcanii* SRP54 homolog in the AHwt20 strain. Western blot analysis of cytoplasmic and membrane fractions revealed the presence of an immunoreactive band migrating at 52 kDa in both fractions (Fig. 3A). To determine whether the immunoreactive bands were specific to the *H. volcanii* SRP54 homolog, an *H. volcanii* strain expressing a his-tagged SRP54 (strain WR6c) was analyzed by Western blot (35). Analysis of cytoplasmic and membrane fractions from this strain revealed identical immunoreactive bands using both anti-penta-his and anti-SRP54 antibodies (data not shown). These results confirm that the bands are specific to the *H. volcanii* SRP54 homolog and demonstrate that, similar to organisms of other domains, a pool of the archaeal SRP54 is stably associated with the cytoplasmic membrane.

(ii) The A domain of the *H. volcanii* FtsY homolog is not required for the membrane association of the haloarchaeal SRP. Recent in vitro assays showed that, while *H. volcanii* wild-type FtsY could promote SRP54 binding to haloarchaeal inverted vesicles, an amino-terminally truncated FtsYNG construct was unable to promote this binding (19). These results are not consistent with our findings that an *H. volcanii* FtsYNG construct can complement a haloarchaeal chromosomal *ftsY* knockout. However, it should be noted that the construct used in the in vitro studies (19) contains a larger amino terminal truncation than the one used in these studies (see Fig. 4 and Discussion). To show that the A domain is indeed dispensable

FIG. 3. The *H. volcanii* SRP54 does not require the FtsY A domain for its membrane association. Cytoplasmic (c) and membrane (m) fractions prepared from the AHwt20 and AHng09 Δ ftsY strains were subjected to Western blot analysis using (A) an antibody recognizing the *H. volcanii* SRP54 homolog or (B) antibodies recognizing the *H. volcanii* cytoplasmic protein DHFR1.

FIG. 4. Sequence conservation and divergence of FtsY homologs. (A) Boxed representation of FtsY homolog domains. Shading of boxes represents increasing conservation from light to dark. Blackened areas labeled G1 to G4 represent highly conserved motifs in the G domain involved in nucleotide binding and catalysis (9, 12). The N domain is defined as a four alpha-helical bundle whose presence is universally conserved in the SRP family of GTPases (8). The A domain is the least conserved portion of these proteins, with little to no conservation in primary sequence among those representatives that possess such a domain (11, 29). (B) Amino acid alignments of portions of the *Thermus aquaticus* and *E. coli* FtsY homologs are adapted from that of Egea et al. (9). The amino terminus of the *H. volcanii* FtsYNG · 6×His construct used in this study was aligned to these sequences based on the strongly conserved “ALLEADV” motif present within the N domain (the underlined portion of amino acid sequences in boldface type) (9, 12). The arrow above the *H. volcanii* sequence denotes the first amino acid position of the A domain truncation used in in vitro analyses (19). The sequence boundary for the *E. coli* protein was extended from amino acid 201 in Egea et al. (9) to amino acid position 196 to include those amino acids in the *E. coli* protein that were recently shown necessary to complement a deletion of the chromosomal *ftsY* in this organism (11).

for SRP54 membrane-association in vivo, Western blot analysis of cytoplasmic and membrane fractions from the *H. volcanii* Δ *ftsY* strain complemented by *ftsYNG* (AHng09) was performed. Consistent with the complementation data, ratios of cytoplasmic and membrane-associated SRP54 were similar to those observed in the strain expressing full-length FtsY (Fig. 3A).

Inhibition of *H. volcanii* protein synthesis leads to the release of membrane-bound SRP54 but not of FtsY. *H. halobium* translation inhibition with puromycin, a translation inhibitor that releases the nascent polypeptide chain from the ribosome, results in the release of a subset of membrane-associated 7S RNA subunit of the haloarchaeal SRP from the cytoplasmic membrane (16). To determine whether translation inhibition has an effect on *H. volcanii* SRP and FtsY membrane association, cellular localization of the *H. volcanii* SRP54 and FtsY homologs were determined following puromycin treatment. Western blot analyses using anti-SRP54 antibodies showed that, following translation inhibition, the ratio of free to membrane-associated SRP54 increased nearly threefold (Fig. 5A). These results suggest that a subpopulation of the membrane-associated SRP is associated with the ribosome nascent chain complex (RNC). Conversely, similar analyses using anti-FtsY antibodies indicated that ratios of cytoplasmic to membrane-bound FtsY did not change significantly upon translation inhibition. This suggests that FtsY membrane association is not merely due to the membrane-bound SRP-RNC complex (Fig. 5B).

DISCUSSION

It is becoming increasingly clear that the study of archaeal cellular processes has the potential to significantly advance the

understanding of not only archaeal but also bacterial and eukaryotic cell biology. Here we report the in vivo characterization of the *H. volcanii* protein FtsY, a homolog of the bacterial and eukaryotic SRP receptor. Our studies with the chromosomal Δ *ftsY* strains strongly suggest that FtsY is crucial for growth under the conditions used in this study (see Materials and Methods). Attempts to delete the chromosomal *ftsY* gene were only successful when functional *H. volcanii* *ftsY* constructs were expressed in *trans*. While these data suggest that the

FIG. 5. Inhibition of protein synthesis results in decreased membrane-associated SRP54 without a change in membrane-associated FtsY. Western blot analysis of membrane (m) and cytoplasmic (c) fractions prepared from *H. volcanii* strain AHwt20 grown in the absence (–) or presence (+) of puromycin for 20 min prior to fractionation. Complete cessation of protein synthesis under these conditions were confirmed using metabolic protein labeling (see Materials and Methods and data not shown). Western blots are shown using antibodies recognizing *H. volcanii* SRP54 (A) or FtsY (B).

protein is essential for growth under the conditions tested, we cannot rule out that viable but severely compromised *ftsY* knockout strains (obtained during the nonselective step prior to the counterselection) were outcompeted and thus not recovered in our assays. Testing this possibility directly by depletion of FtsY from a Δ *ftsY* chromosomal strain is currently not possible since, to date, no tightly controlled promoters have been identified in *H. volcanii*.

Interestingly, we observed that in addition to the wild-type *ftsY*, a gene encoding an *H. volcanii* FtsY construct lacking the N-terminal A domain (*ftsYNG* · 6×*His*) could complement the chromosomal *ftsY* knockout. In contrast, a *ftsY* construct expressing only the G domain of this protein could not complement an *ftsY* deletion. Taken together, these results demonstrate that both the N and G domains of the *H. volcanii* FtsY homolog are essential for its function, while the A domain is dispensable under the growth conditions tested in this study. The fact that a truncated FtsY consisting of only the G domain was insufficient for complementation is not surprising given the universal conservation of both the N and G domains in all GTPases of the 5RP family. The current view of the organization of these two domains is that they act as a single functional unit (44).

While we were able to demonstrate that expression of FtsYNG in *trans* was sufficient to complement the loss of endogenous *ftsY*, the relative number of *ftsY* deletion recombinants and wild-type recombinants following counterselection was lower than that obtained when expressing the wild-type gene in *trans*. It is possible that the *H. volcanii* *ftsY* knockout strain, complemented by the expression of *ftsYNG* · 6×*His* (AHng09), is less fit than the wild-type strain and is therefore recovered less frequently. However, comparative analysis of the *H. volcanii* *ftsY* knockout strain complemented with full-length *ftsY* · 6×*His* (AHwt20) to that complemented with *ftsYNG* · 6×*His* (AHng09) suggest that the lack of the A domain does not severely affect its function. First, the growth rates between the two strains were the same at either 45°C or 30°C (data not shown); second, both FtsY · 6×*His* and FtsYNG · 6×*His* proteins associated with the *H. volcanii* cytoplasmic membrane; and finally, the *H. volcanii* homolog of SRP54, the critical binding partner of FtsY, was found to be associated with the cytoplasmic membrane in both the AHwt20 and AHng09 strains. While the plasmid-encoded *H. volcanii* FtsY variants were expressed under the control of a heterologous promoter, Western blot analysis using an antibody against the *H. volcanii* FtsY homolog suggest that the FtsY constructs and the chromosomally encoded FtsY are produced at comparable levels (Fig. 1D, compare endogenous FtsY seen in lanes 4 and 5 to the plasmid encoded protein seen in lane 1). Thus, it is unlikely that expression of any of the FtsY variants from their native promoter would significantly change these results.

Our data are in agreement with in vitro analyses of an *H. volcanii* FtsYNG construct, which demonstrated that the haloarchaeal FtsY A domain was dispensable for its association with *H. volcanii*-inverted membrane vesicles (19). However, these studies also suggested that, unlike the full-length FtsY, the *H. volcanii* FtsYNG construct was unable to promote association of the *H. volcanii* SRP54 homolog to the vesicles (19). Conversely, our data suggest that a subpopulation of

SRP54 is associated with the membrane. It should be noted, however, that the construct used in those studies lacked a portion of the N domain immediately adjacent to the highly conserved "ALLEADV" motif (Fig. 4), a motif that is thought to contribute to SRP54/FtsY binding (9, 12). In contrast, the truncation of the complementing FtsYNG construct characterized here, while lacking the A domain, includes 27 amino acids amino terminal to this motif (see Fig. 4B).

Our finding that the *H. volcanii* FtsY homolog is required for cellular growth but that the A domain is dispensable supports recent analyses of an amino-terminally truncated *E. coli* FtsY construct (11). These findings resurrect two important questions about this protein. First, "What is the function of the FtsY A domain?" As shown above, deleting this poorly conserved domain does not seem to be detrimental in either bacteria or archaea. In fact, several archaeal and bacterial orthologs seem to lack this domain, such as *Pyrobaculum aerophilum* strain IM2 and *Thermotoga volcanium*, respectively (9, 11, 12) (Haddad and Pohlschröder, unpublished). It had been long thought that the A domain was important for promoting association of FtsY with the cytoplasmic membrane (7, 23, 24). However, membrane binding of both the *E. coli* and *H. volcanii* FtsYNG constructs and complementation of Δ *ftsY* via expression of *ftsY* · 6×*His* clearly refute earlier proposals suggesting its involvement in membrane association. These results raise a second question: "How do FtsY homologs that do not possess obvious membrane anchors associate with the cytoplasmic membrane?" Unlike in the eukaryotes, where the FtsY homolog SR α interacts with the membrane protein Sr β , an Sr β homolog or other membrane proteins with which FtsY interact have not been identified (29). Furthermore, translation inhibition resulted in the release of a subpopulation of the membrane-bound SRP54 but not of FtsY from the *H. volcanii* cytoplasmic membrane. This result suggests that FtsY membrane association is not solely due to its association with translating SRP-ribosome nascent chain complexes. Finally, we have also performed copurification studies of *H. volcanii* membrane preparations utilizing the 6× His tag appended to each of the expressed FtsY variants in order to detect specific interacting components. Although the use of 6× His-tagged proteins has been successful in our laboratory in the copurification of the SRP components (35), we have not identified any additional proteins that stably interact with FtsY · 6×*His* (Haddad and Pohlschröder, unpublished). This is consistent with in vitro studies in *E. coli* suggesting that the primary interaction of FtsY with cytoplasmic membranes occurs with phospholipids, as well as genetic screens that have also not revealed interacting membrane protein components (7, 24, 39, 40). Interestingly, the ambiguity of a membrane anchor for the SR is not restricted to prokaryotes. While Sr α forms a heterodimer with the transmembrane protein Sr β , truncation of the yeast Sr β transmembrane anchor does not affect growth in the yeast *Saccharomyces cerevisiae* (26). Furthermore, in vitro protein translocation using purified components is unaffected when the transmembrane anchor within Sr β is removed (13). Thus, identifying mechanisms of bacterial and archaeal FtsY membrane association might also lead to a better understanding of the significance of the membrane localization of the eukaryotic SRP receptor.

ACKNOWLEDGMENTS

We thank Amy Decatur and members of the Pohlschröder laboratory, Kieran Dilks, and Nick Hand, for valuable comments on the manuscript. We also thank Jerry Eichler and Moshe Mevarech for generously providing antibodies. M. Mevarech also kindly provided the plasmid pGB70 and the strain WR480 that made our gene deletion studies possible. Mariska van den Hoeke provided important technical assistance.

Support was provided to R.W.R. by a predoctoral fellowship from the American Heart Association (reference no. 0110093U) and to M.P. by a National Science Foundation grant (reference no. MCB-0239215).

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