Archaeal and Bacterial SecD and SecF Homologs Exhibit Striking Structural and Functional Conservation

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The majority of secretory proteins are translocated into and across hydrophobic membranes via the universally conserved Sec pore. Accessory proteins, including the SecDF-YajC Escherichia coli membrane complex, are required for efficient protein secretion. E. coli SecDF-YajC has been proposed to be involved in the membrane cycling of SecA, the cytoplasmic bacterial translocation ATPase, and in the stabilizing of SecG, a subunit of the Sec pore. While there are no identified archaeal homologs of either SecA or SecG, many archaea possess homologs of SecD and SecF. Here, we present the first study that addresses the function of archaeal SecD and SecF homologs. We show that the SecD and SecF components in the model archaeon Haloferax volcanii form a cytoplasmic membrane complex in the native host. Furthermore, as in E. coli, an H. volcanii ΔsecFD mutant strain exhibits both severe cold sensitivity and a Sec-specific protein translocation defect. Taken together, these results demonstrate significant functional conservation among the prokaryotic SecD and SecF homologs despite the distinct composition of their translocation machineries.

All organisms need to transport proteins across hydrophobic membranes. Many of these proteins are passed through the endoplasmic reticular membrane of eukaryotes and the cytoplasmic membrane of bacteria using the universally conserved Sec pathway (35). Substrates translocated via this pathway contain N-terminal Sec signal sequences that target these proteins to a heterotrimeric membrane protein complex termed the translocon or Sec pore (47). While the essential pore components Sec61α/Sec61γ and SecY/E in eukaryotes and bacteria, respectively, are universally conserved, the third subunit of these complexes, the eukaryotic Sec61β and bacterial SecG, are distinct and dispensable (4, 13, 17, 25, 34). Similarly, many of the additional components required for Sec translocation are distinct in bacteria and eukaryotes. For example, bacteria require the cytoplasmic ATPase SecA for protein translocation, while in yeast, translocation across the endoplasmic reticular membrane relies on the luminal ATPase Kar2p (12, 41, 49). Furthermore, many homologs of components associated with the eukaryotic Sec pore, such as Sec62/63 (39), have no known homologs in bacteria (4). Conversely, no eukaryotic homologs of the bacterial SecD, SecF, and YajC proteins, which together form a SecYEG-associated heterotrimeric complex, have been identified (4, 8, 15).

The universally conserved subunits of the archaeal Sec pore share more amino acid similarities with the eukaryotic homologs than with the bacterial homologs (4, 17). Consistent with this observation, archaea also contain a homolog of the eukaryotic Sec61β, rather than the bacterial SecG protein, and lack a SecA homolog (20, 34, 46). However, archaeal homologs of the bacterial SecD and SecF components have been identified in many euryarchaea (4, 11, 36). This finding is particularly surprising, as it has been proposed that the Escherichia coli SecDF-YajC complex is required for efficient SecA membrane cycling (7, 8, 10) and functionally interacts with SecG (19). The absence of SecA and SecG homologs in the archaeal domain precludes the involvement of these proteins in archael SecDF function.

In this work, we have begun to address the role of archaeal SecD and SecF. In particular, we wished to determine if these homologs of bacterial accessory secretory proteins might function in protein translocation in a system where the core Sec translocation machinery is distinct from that of bacteria. We have cloned and sequenced the secFD operon of the model archaeon Haloferax volcanii. Like many bacteria and all sequenced archaean species, H. volcanii lacks a yajC homolog (4, 11). The membrane proteins that the secFD operon encodes have predicted membrane topologies that are identical to those of the corresponding E. coli SecD and SecF proteins (33). Here, we show that like their E. coli homologs (33), the H. volcanii proteins form a cytoplasmic membrane complex in their native host. Furthermore, consistent with the E. coli secDF-yajC null mutant phenotype (14, 31, 33), we demonstrate that an H. volcanii ΔsecFD deletion strain is viable but confers severe cold sensitivity and perturbs Sec-dependent protein translocation. Our data suggest that the H. volcanii SecFD complex assists translocating or translocated Sec substrates to assume stable, folded conformations. The results presented here are consistent with the archaeal SecF complex functioning late in protein translocation, as previously proposed for the E. coli complex (22, 33), raising the question...
of whether bacterial SecD and SecF exhibit a function independent of SecA and SecG.

MATERIALS AND METHODS

Reagents. The *H. volcanii* cosmid library was kindly provided by R. Charlebois (University of Ottawa, Ontario, Canada). Dodecyl maltoside (DDM) was purchased from Anatrace. The anti-Myc monoclonal antibody (1-9E10) was purchased from BD Biosciences. 5-Fluoroorotic acid (5-FOA) was purchased from Toronto Research Chemicals. The cosmid library was kindly provided by R. Charlebois (University of Ottawa, Ontario, Canada). Dodecyl maltoside (DDM) was purchased from Anatrace. The anti-Myc monoclonal antibody (1-9E10) was purchased from BD Biosciences. 5-Fluoroorotic acid (5-FOA) was purchased from Toronto Research Chemicals. The anti-Myc monoclonal antibody (1-9E10) was purchased from BD Biosciences. 5-Fluoroorotic acid (5-FOA) was purchased from Toronto Research Chemicals.

TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
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<tr>
<td>DHA sHs</td>
<td><em>Escherichia coli</em> d80DlacZ:ΔM15 (argF-lac)U169 recA1 endA1 hsdR17(k+ mK+ supE44 thi-1) gyrA relA1 phoA</td>
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<tr>
<td>DL739</td>
<td><em>H. volcanii</em> ΔpHV2</td>
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<tr>
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<td><em>H. volcanii</em> ΔpHV2</td>
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<tr>
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<td>WFD11 containing pRK9.1</td>
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<td>NH-Hv44</td>
<td>NH-Hv10 containing pFG-pIV</td>
<td>This study</td>
</tr>
</tbody>
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Plasmids

- pBluescript II KS(+) Cloning vector
- pGB70 Amp' pUC19 carrying pyrE2 under the control of Pgsd
- pTA106 Amp' pBluescript II KS(+) carrying trpA under the control of Pgsd
- pTA131 Amp' pBluescript II KS(+) carrying pyrE2 under the control of Pgsd
- pTA230 Amp' *H. volcanii-E. coli* shuttle vector carrying pyrE2 under the control of Pgsd
- pMLH3 (Amp' Nb' Mev') *H. volcanii-E. coli* shuttle vector
- pNP15 (Amp' Nb') *H. volcanii-E. coli* shuttle vector
- pRK9.1 (Amp' Nb') *H. volcanii-E. coli* shuttle vector carrying the secFD operon with a SecD300-bp fragment
- pNHsecKO5 Amp' pBluescript II KS(+) containing 800 bp of upstream DNA from the secFD locus
- pNHsecK5'3' Amp'; 800 bp of downstream DNA from the secFD locus cloned into pNHsecK05
- pNHsecFD::trpA Amp' Pgsd-trpA fragment from pTA106 cloned into pNHsecK5'3'
- pNHsecDFK0 Amp'; 1.6 kb of secFD 5' - and 3'-flanking DNA cloned into pTA131
- pNH-AM (Amp' Nb') Pgsd-amy-myc cloned into pMLH3 (Nb'), replaces Mev' cassette
- pFG-pIV (Amp' Nb') Pgsd-p IV-myc cloned into pBBS3CBD (Nb'), replacing CBD
- pNHGly-Dm (Amp' Ura') PCG-hdrB-myc cloned into pTA230
- pNHGlyDTE-DTE-Dm (Amp' Ura') PCG-wd-hdrB-myc cloned into pTA230, mutant signal sequence
- pKD9 (Amp' Nb') Pgsd-amy-myc cloned into pMLH3, replaces Mev' cassette

*a* CBD, cellulose binding domain of the *Clostridium thermocellum* cellulosome.

Cloning and sequencing of the *H. volcanii* secFD operon. A ~300-bp fragment of the secD open reading frame from *H. volcanii* chromosomal DNA was generated by PCR using the degenerate forward primer SecD-F2 and the degenerate reverse primer SecD-R4 (sequences of all oligonucleotide primers used in this study are available upon request). The amplified fragment was blunted with T4 DNA polymerase and cloned into pBluescript II KS(+) (Stratagene), and its DNA sequence was determined (Genetics Core Facility, University of Pennsylvania). Using this sequence information, *H. volcanii*-specific secD primers were designed. Primers SecD2 and SecD3 were used to screen an *H. volcanii* chromosomal DNA library. A 300-bp fragment of the secFD operon was identified by PCR. This cosmid was used as a template for DNA sequencing to identify the DNA sequence of the entire secFD operon by primer walking.

Construction of pRK9.1 (secD-express). Using the forward primer SecF-pF1 and the reverse primer HsecD-HisH1, the *H. volcanii* secFD operon, including 340 bp of upstream DNA, was amplified, such that a PGHHHHHH oligopeptide was added in frame to the C terminus of the secD open reading frame. The PCR fragment was blunted with T4 DNA polymerase, gel purified, and cut with BamHI. This 2.7-kb fragment was ligated into the shuttle vector pMLH3 (18) that had been cut with XhoI, blunted with Klenow, and then cut with BamHI. The resulting plasmid, pRK9.1, was transformed into the Dam-
PCR using a forward primer specific to an H. volcanii fied, digested with NcoI and XbaI, and ligated into CIP-treated NcoI-XbaI-cut primers pIV-For and pIV-Rev. The resulting 1.1-kb PCR product was gel puri- digested with Acc65I and HindIII, and ligated into CIP-treated Acc65I-HindIII-“megaprimers” and pGB70upEcoRI and AmyMyc3R as forward and reverse AmyMyc3R PCR product by using the two purified fragments as template pNHsecFD::trpA, yielding pNHsecFD::trpA.

Chromosomal deletion of the secFD operon. A refinement of the pnrE2 counter- tereselection scheme first developed by Bitan-Banin and colleagues for H. volcanii (2) was used to replace the H. volcanii chromosomal loci. To allow resolution of the cointegrate thus formed will either revert the chromosomal loci to the wild type or generate a disrupted ΔsecFD::trpA chromosomal locus. To allow resolution of the cointegrate, single colonies of cointegrate strains were inoculated into liquid minimal medium lacking tryptophan but containing uracil and grown to early log phase. Cells from these cultures were pelleted, and those cells that retained ΔsecFD::trpA but that had lost the plasmid were selected by plasmid curing with 5% FOA and uracil lacking tryptophan. Disruption of the chromosomal locus in the resulting ΔsecFD::trpA strain was confirmed by PCR using a forward primer specific to an H. volcanii chromosomal locus upstream of the 800-bp 5′ targeting region in pNHsecFD::trpA, SECUPUFS, and a reverse primer, trpCA3BFS EcoRI, specific to the trpCA cassette. A second PCR using the forward primer SecF-F5 and the reverse primer HvSecF5R-Hind3. The amplified product was digested with XhoI and ligated into CIP-treated megaprimers and pNHsecKO5. The resulting 800-bp 5′ sequence flanking the H. volcanii secFD coding sequences was amplified by PCR from wild-type H. volcanii chromosomal DNA using the forward primer HvSecF5-F and the reverse primer HvSecF5R-Hind3. The amplified product was digested with XhoI and HindIII and cloned into XhoI-HindIII-digested pACYC184. The amplified fragment was then digested with EcoRI and XbaI, purified using a QIAquick spin column, and ligated into CIP-treated EcoRI-XbaI-cut pNHsecKO5. The XhoI-XbaI fragment from the resulting plasmid, pNH- sec3′, was excised, purified, and ligated into CIP-treated XhoI-XbaI-cut pTA131, generating pNHsecFDKO. Finally, the HindIII-EcoRI (Pm BamHI fragment of pTA106 was excised and ligated into CIP-treated HindIII-EcoRI-digested pNHsecFDKO, yielding pNHsecFD::trpA.

Generation of deletion constructs. A total of 800 bp of 5′ sequence flanking the H. volcanii secFD coding sequences was amplified by PCR from wild-type H. volcanii chromosomal DNA using the forward primer HvSecF5-F and the reverse primer HvSecF5R-Hind3. The amplified product was digested with XhoI and HindIII and ligated into CIP-treated EcoRI-XbaI-cut pNHsecKO5. The XhoI-XbaI fragment from the resulting plasmid, pNH- sec3′, was excised, purified, and ligated into CIP-treated XhoI-XbaI-cut pTA131, generating pNHsecFDKO. Finally, the HindIII-EcoRI (Pm BamHI fragment of pTA106 was excised and ligated into CIP-treated HindIII-EcoRI-digested pNHsecFDKO, yielding pNHsecFD::trpA.

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RESULTS

Cloning and sequence analysis of the H. volcanii secFD operon. Since the genome sequence was unavailable at the onset of this study, the H. volcanii secD and secF genes were identified using a degenerate PCR, and the coding and flanking DNA regions were cloned and sequenced (NCBI accession number AF395892). As in E. coli, the H. volcanii secD and secF genes are located in a single operon and appear to be transcriptionally coupled (14). Unlike E. coli, however, the haloarchaeal secF gene is located upstream of secD, and no homolog of yajC is present. Indeed, yajC is absent from many bacterial species and has no identified archaeal homolog (4, 11).

The H. volcanii SecD and SecF homologs were analyzed using TMHMM (21, 43) to predict their membrane topologies. Consistent with the experimentally derived topologies of E. coli SecD and SecF (33), the H. volcanii homologs of these proteins were predicted to encode integral membrane proteins with six transmembrane segments (Fig. 1A). Like in E. coli, the amino and carboxy termini of these proteins are predicted to be located in the cytoplasm, and substantial extracytoplasmic domains are present between transmembrane helices 1 and 2 of both proteins. The difference in molecular masses between SecD and SecF (predicted to be 55 kDa and 30.4 kDa, respectively) is mainly due to the larger size of this extracytoplasmic loop in the SecD homolog.

Haloarchaeal SecD and SecF homologs form a heteromeric protein complex. SecD, SecF, and YajC form a heterotrimeric complex in E. coli that has been shown to interact with the Sec pore (7). To examine the interaction of the haloarchaeal SecD and SecF homologs with each other and potentially with other components, we constructed a vector, pRK19.1, that expresses the H. volcanii secFD operon. In this construct, a His6 tag was added to the C terminus of SecD to allow for Ni-NTA purification studies of membrane preparations from H. volcanii.

Subcellular fractionations of an H. volcanii strain expressing SecD•His protein were carried out to determine whether this tagged protein was expressed and stably localized to the cytoplasmic membrane in its native host. Western blot analysis of cytoplasmic and membrane fractions using antibodies specific for SecD or SecF revealed that SecD•His was detected exclusively in the membrane fraction of H. volcanii cells expressing SecD•His (Fig. 1B). The presence of SecD•His was confirmed in cell lysates by mass spectrometry–time of flight analysis. Migrations of molecular mass markers are indicated by arrows (in kilodaltons).

Polycrylamide gel electrophoresis of protein samples. Ni-NTA elution fractions were prepared for electrophoresis by adding NuPAGE sample buffer and reducing agent according to the manufacturer’s instructions. Protein samples were denatured at 70°C for 10 min and electrophoresed on 4 to 12% bis-Tris NuPAGE gels, except in the “seminative” sample shown in Fig. 2, where samples were warmed to 37°C for 1 h and separated on 3 to 8% Tris-acetate NuPAGE gels. Proteins were visualized by staining with Bio-Safe Coomassie G-250 according to the manufacturer’s instructions.

Mass spectroscopy. Coomassie-stained protein bands of interest were excised from the gel. The proteins were prepared for in-gel trypsin digestion, subsequent extraction, and mass spectrometric analysis of the tryptic peptides according to a modified version (24) of the protocol described previously by Mortz and colleagues (25). The conceptual in silico-translated proteome of preliminary sequence data for the H. volcanii DS2 genome, obtained from Jonathan A. Eisen at the Institute for Genomic Research, was used as a data set for mass spectrometric analysis.

Nucleotide sequence accession numbers. DNA sequences corresponding to the H. volcanii secFD operon have been deposited in the NCBI database under accession number AF395892.

FIG. 1. The SecD•His protein localizes to the H. volcanii cytoplasmic membrane. (A) Predicted topology of H. volcanii SecD and SecF using TMHMM (extracytoplasmic [ext] and cytoplasmic [cyt] sides of the membrane, respectively). (B) Cytoplasmic (cyt) and membrane (mem) fractions prepared from H. volcanii strain RK19.1, expressing SecD•His, and the control strain, WR-Hv-NP15, containing a novobiocin-resistant shuttle vector not expressing this tagged protein, were subjected to Western blot analysis using an anti-penta-histidine antibody. Migrations of molecular mass markers are indicated by arrows (in kilodaltons).

FIG. 2. In vivo copurification of H. volcanii SecF with SecD•His. Membrane proteins were purified from dodecyl maltoside-solubilized membranes of the SecD•His-expressing strain RK19.1 and the control strain, WR-Hv-NP15, using Ni-NTA affinity chromatography. Eluates were subjected to reducing, “seminative” denaturing conditions. Samples were separated on a 3 to 8% Tris-acetate gradient gel, and the proteins were visualized by Coomassie stain. The identities of the bands indicated were verified by matrix-assisted laser desorption ionization–time of flight analysis. Migrations of molecular mass markers are indicated by arrows (in kilodaltons).
SecF- and SecD•6xHis-encoding insert), were analyzed by de-
naturing gel electrophoresis. When protein samples were
heated to 37°C and separated on Tris-acetate gradient gels,
protein bands specific to RK19.1 were identified by Coomassie
staining, migrating at apparent molecular masses of ~50 kDa
and ~55 kDa, consistent with the predicted molecular masses of
SecF and SecD•6xHis, respectively (Fig. 2). In addition, we
observed bands at ~80 kDa and ~100 kDa as well as several
higher-molecular-mass bands of lower intensity (Fig. 2). West-
ern blot analysis using antipentahistidine antibodies suggested
that these bands corresponded to SecD•6xHis-containing
complexes not denatured under the mild conditions used (data
not shown).

The four bands indicated in Fig. 2 as well as corresponding
regions in the control lane were excised, digested with trypsin,
and subjected to matrix-assisted laser desorption ionization-
time of flight mass spectroscopic analysis. The resulting mass-
onion spectra were compared to a database of conceptual, in
silico trypsin-digested polypeptides corresponding to the avail-
able sequences from the partial H. volcanii proteome, gener-
ously provided by Jonathan Eisen (The Institute for Genomic Research). This analysis confirmed the identity of the 55-kDa
band as SecD•6xHis and the 30-kDa band as SecF, while no
peptides corresponding to either protein were detectable in the
control samples. Similarly, we confirmed the presence of SecD
and SecF tryptic peptides in the ~80-kDa band and obtained
exclusively SecD tryptic fragments in the ~100-kDa band, con-
sistent with a SecDF heterodimer and a SecD homodimer,
respectively.

When the samples were heated to 70°C prior to gel electro-
phoresis, only monomeric forms of SecD•6xHis and SecF were
identified (data not shown).

The H. volcanii secFD operon is not essential for growth. It
has been shown that while mutations in the E. coli secDF-yajC
operon result in a general protein secretion defect, this operon
is not essential for viability (14, 31, 33). To determine if an H.
volcanii secFD operon deletion strain is viable, we employed a knock-
out strategy (1) that allows for the recovery of deletion muta-
tions of nonessential genes, even if the resulting mutants ex-
hibit a strong growth defect. Briefly, the secFD coding sequences were replaced by the prototrophic trpA marker un-
der the control of the H. volcanii Pgly promoter (Pgly-trpA) in a
ΔtrpA H. volcanii strain (H99) using a suicide vector, pNHsecFD::trpA. Both the integration and resolution of
pNHsecFD::trpA were carried out in media lacking trypto-
phan, demanding the retention of the Pgly-trpA cassette. Using
this approach, Trp prototrophic colonies were recovered. PCR
screening of these colonies confirmed that the ΔsecFD::trpA construct was at the native locus in this strain and that the
secFD coding sequences had been deleted. The ability to ob-
tain ΔsecFD::trpA mutant NH-Hv10 demonstrated that the
secFD operon is not essential for the growth of H. volcanii
under the conditions tested.

Deletion of the H. volcanii secFD operon leads to a severe
cold-sensitive growth phenotype. While the H. volcanii
ΔsecFD::trpA mutant strain (NH-Hv10) was viable, growth was
severely impaired on solid medium at 45°C (standard growth temperature for H. volcanii) compared to an H. volcanii
secFD+ trpA+ strain (H98) (data not shown). However, when
grown in liquid medium at 45°C, deletion of the secFD operon
did not appear to perturb the growth of H. volcanii (Fig. 3A).
Interestingly, growth of the mutant strain at 30°C was negligi-
ble compared to that of H98 in liquid medium as well as on
plates (Fig. 3B and data not shown). The growth phenotypes
were not apparent when wild-type H. volcanii secF and
secD•6xHis were provided in trans on plasmid pRK19.1,
strongly suggesting that these phenotypes were due to the loss of SecF and SecD function (Fig. 3C and data not shown).
The slower growth of the transformed H. volcanii strains relative to
strains lacking the plasmid (Fig. 3B and C; note the different
scales) possibly reflects the burden of the presence of the
plasmid or an effect of the antibiotic novobiocin, which was
used to maintain the plasmid. Taken together, the results dem-
onstrate that, like in E. coli (14), the deletion of these genes results
in a severe cold-sensitive growth phenotype.

Deletion of the H. volcanii secFD operon results in a Sec-
specific protein export defect. To ascertain whether the halo-
archaeal SecD and SecF membrane proteins are involved in
Sec-dependent protein translocation, the secretion of both Sec
substrates and Sec-independent twin-arginine translocation
(Tat) substrates was assayed in secFD+ and ΔsecFD H. volcanii
strains.

(i) Effects of ΔsecFD on Sec substrates. Due to the fact that
most H. volcanii Sec substrates are predicted to remain cell
associated upon translocation, we constructed a reporter
protein, Gly-DM. In this construct, which is expressed from
the CWG promoter, the CWG Sec signal sequence is fused to
the N terminus of Myc-tagged H. volcanii dihydrofolate reductase
II. While technical difficulties have prevented us from carrying
out pulse-chase experiments, the use of this reporter construct
allowed us to monitor levels of proteins translocated into the
culture supernatant by Western blot analysis.

When the relative amounts of the Gly-DM in the cell and
supernatant fractions of the secFD+ and ΔsecFD strains ex-
pressing this reporter were determined, we only identified a
band corresponding in size to the mature protein, the majority
of which was present in the supernatant fraction (Fig. 4A). In
agreement with an involvement of the archaeal SecD and SecF
homologs in protein translocation, the intensity of the Gly-DM
immunoreactive band in the culture supernatant of the ΔsecD
strain was significantly reduced. However, accumulation of a
Gly-DM precursor in this strain was not observed. Similar
results were also obtained when Gly-DM was expressed from
the constitutive Pgly promoter (data not shown). If the mode of
translocation was posttranslational, it is possible that the in-
stability of a putative cytoplasmic precursor of the construct
might account for the difference in the levels of the secreted
form. To test this possibility, we constructed a version of the
Gly-DM reporter with a mutant, inactive signal sequence (Fig.
4B). This GlyDTED-DM construct, which was also expressed
from the CWG promoter, was detected in similar amounts in
the cellular fractions of secFD+ and ΔsecFD strains, indicating
that this N-terminally tagged construct was efficiently ex-
pressed and stable in the cytoplasm of both strains. Hence, the
low abundance of the secreted construct expressed with the
wild-type signal sequence (Gly-DM) in the ΔsecFD mutant
strain was likely due to the instability of a translocating or
translocated form of this construct.

If the protein stability of Sec substrates was affected at a
stage during or after translocation through the Sec pore, we
might also observe lower levels of translocated membrane-associated parasitic Sec substrates. Therefore, we next analyzed cellular protein levels of a predicted parasitic Sec substrate, the proteinase IV homolog (pIV). Due to the lack of antibodies directed against this membrane-associated proteinase, we used a plasmid-encoded Myc-tagged pIV construct under the control of the native pIV promoter. Consistent with the effect of the \( \Delta \text{secFD} \) mutation on the secreted Gly-DM construct, cellular levels of pIV-Myc were found to be significantly lower in the mutant background (Fig. 5). Attempts to transform parasitic with a plasmid encoding the pIV-Myc reporter with a mutated signal sequence were unsuccessful, possibly due to the toxicity of the proteinase in the cytoplasm (M. Pohlschröder, unpublished data). Thus, while the effect of the \( \Delta \text{secFD} \) mutation on secreted pIV-Myc supports a putative involvement of SecD and SecF in stabilizing translocating or translocated Sec substrates, we cannot exclude the possible instability of cytoplasmic pIV-Myc precursor.

**FIG. 3.** Deletion of the secFD operon confers a severe cold-sensitive growth defect. Growth of \( H. \text{volcanii} \) secFD\(^{+}\) and \( \Delta \text{secFD} \) strains was measured in triplicate by plotting the optical density at 600 nm (OD\(_{600}\)) against time (in hours). In all three panels, growth of secFD\(^{+}\) strains is indicated by solid lines, and that of the \( \Delta \text{secFD} \) strains is indicated by dashed lines (error bars indicate ±1 standard deviation). (A and B) Growth of secFD\(^{+}\) (H98) and \( \Delta \text{secFD} \) (NH-Hv10) strains at (A) 45°C and (B) 30°C. (C) Growth of secFD\(^{+}\) (NH-Hv11) and \( \Delta \text{secFD} \) (NH-Hv12) strains expressing SecFD\(\times 6\)His (on plasmid pRK19.1) at 30°C.

**FIG. 4.** The \( H. \text{volcanii} \) \( \Delta \text{secFD} \) strain confers a Sec translocation defect. (A) Protein levels of Gly-DM and GlyDTED-DM in cell (c) and culture supernatant (s) fractions from secFD\(^{+}\) and \( \Delta \text{secFD} \) \( H. \text{volcanii} \) cultures expressing these constructs were determined by Western blot analysis (using anti-Myc antibodies). Predicted positions of precursor (p) and mature (m) reporter proteins are indicated. Standardized equivalents of cell and supernatant fractions were analyzed. Strain H98 was used as a negative control (control). (B) CWG signal sequence (wild-type) and mutated CWG signal sequence (mutant). The residues that are altered in this GlyDTED construct are underlined and colored gray.

**FIG. 5.** Deletion of the secFD operon perturbs proteinase IV levels. Levels of chromosomally encoded CWG (~98 kDa) and plasmid-encoded pIV-Myc (~35 kDa) in secFD\(^{+}\) (NH-Hv43) and \( \Delta \text{secFD} \) (NH-Hv44) strains were determined by Western blot analysis of cell lysates from \( H. \text{volcanii} \) cultures. Top and bottom panels correspond to identical lanes of a blot cut and probed separately with anti-CWG and anti-Myc antibodies, respectively. Standardized equivalents of cell lysates were analyzed.
Interestingly, Western blot analysis of *H. volcanii* cell lysates of both the secFD<sup>+</sup> and ΔsecFD strains revealed that the native CWG protein levels were not significantly different between these strains (Fig. 5). The lack of an observed difference in the levels of CWG may be due to glycosylation of this S-layer subunit upon translocation and its immediate incorporation into the cell wall structure, both factors that would increase its stability and thus might alleviate a requirement for SecDF. The stability of this essential protein is consistent with the absence of a significant growth phenotype at 45°C in liquid medium.

**DISCUSSION**

In *E. coli*, SecD and SecF are part of a heterotrimeric complex that associates and copurifies with the SecYEG pore (7). Although it is clear that bacterial SecD and SecF are required for efficient protein secretion, the precise function of these proteins has remained elusive. A number of putative models for SecDF function have been proposed; these include the stabilization of membrane-inserted SecA (7, 8, 10) and interaction with SecG, proposed to stabilize this component of the Sec pore (19). The membrane topologies of SecD and SecF, which indicate that both proteins have large conserved extracytoplasmic loops, are suggestive of an important function outside the cytoplasmic membrane bilayer (33). Binding of inactivating antibodies to the conserved SecD loop renders *E. coli* Sec substrates sensitive to proteases, suggesting that the SecDF-YajC complex plays a role late in translocation, perhaps facilitating the release of translocated proteins from the Sec pore (22). Furthermore, a recent deletion study of the major extracytoplasmic loops of *E. coli* SecD and SecF confirmed the functional importance of these domains (28).

SecD and SecF homologs have been identified in a number of euryarchaeal species as well as in the nanoarchaeon *Nanoarchaeum equitans* Kin4-M, although all archaeal species lack SecG and SecA homologs. It is certainly possible that the archaeal SecD and SecF homologs have evolved to interact with the SecG analog (Sec61<sup>B</sup>) or a putative unidentified SecA analog. However, while there is high sequence conservation among SecD and SecF homologs between the two prokaryotic domains (11), there is no significant sequence conservation between SecG and Sec61<sup>B</sup> or SecA and any putative archaeal analog of this ATPase. Thus, it is more likely that the function of archaeal SecD and SecF is independent of Sec61<sup>B</sup> and a SecA analog. This also raises the possibility that the bacterial SecDF has a SecG- and SecA-independent function.

In our analysis of membrane fractions from an *H. volcanii* strain overexpressing SecF and SecF<sup>Δ6xHis</sup>, we have demonstrated the interaction of these archaeal homologs (Fig. 2). We have not, thus far, found any evidence of copurification of the Sec61 complex, or any other putative interacting factors, with the SecF<sup>Δ6xHis</sup> construct. However, it is possible that such interactions with the archaeal SecDF complex are either transient or too weak to allow their copurification using the protocol we have developed. Like in *E. coli*, the copurification of these components may require very specific conditions (7). Thus, optimization of the high-salt purification protocol, possibly using chemical cross-linkers, will be necessary to investigate putative additional interactions between the *H. volcanii* SecDF complex and other known or yet- unidentified components.

To address the function of the archaeal SecDF complex, we deleted the *H. volcanii* secFD operon. While the ΔsecFD strain grew similarly to an *H. volcanii* secFD<sup>+</sup> strain in liquid medium at 45°C, it exhibited a severe growth defect at this temperature on solid medium. Furthermore, the deletion strain had a strong cold-sensitive growth phenotype at 30°C on both solid and in liquid media (Fig. 3A and B and data not shown). The growth defects of the mutant strain were shown to be specifically due to the lack of SecD and SecF, as it could be rescued by a plasmid expressing secFD (Fig. 3C and data not shown). Not only is the cold-sensitive phenotype reminiscent of that of the *E. coli* secDF null mutants, it is also suggestive of a protein export defect, as this process is inherently cold sensitive (14, 26, 32).

To determine whether the growth phenotype, which was very similar to that of an *E. coli* ΔsecDF strain, was due to an involvement of the archaeal SecD and SecF in Sec translocation, the transport of Sec-dependent substrates was examined. Due to the predicted cell association of most secreted *H. volcanii* Sec substrates, we constructed a fusion protein in which the Sec signal sequence of the *H. volcanii* CWG was fused to Myc-tagged *H. volcanii* dihydrofolate reductase II. This construct, Gly-DM, was found to be efficiently translocated into the culture supernatant of a secFD<sup>+</sup> strain. In contrast, when this construct was expressed in the mutant strain, the mature protein was present in substantially reduced levels in the culture supernatant relative to the secFD<sup>+</sup> strain (Fig. 4), consistent with a translocation defect. However, a precursor of the...
fusion protein was not detected in the cellular fraction of either strain, raising the possibility that the precursor is unstable in the cytoplasm. This is unlikely, as the expression of a nonfunctional signal sequence mutant version of the construct GlyDTED-DM, which is retained in the cytoplasm as precursor protein, revealed similar cellular levels of the precursor in both strains (Fig. 4). These results strongly suggest that the observed decrease of the secreted Gly-DM construct in the supernatant fractions of the mutant strain is due to the instability of the translocating or translocated substrate. Consistent with these results, the membrane-associated \textit{H. volcanii} pIV homolog, which contains a typical Sec signal sequence, was barely detectable when expressed with a C-terminal Myc tag in the \textit{\Delta secFD} strain, while it was readily detectable in the \textit{secFD}+ strain (Fig. 5). Attempts to transform \textit{H. volcanii} with a plasmid encoding the pIV-Myc construct with a mutated signal sequence were unsuccessful, possibly due to the toxicity of cytoplasmic pIV.

Considering the severe translocation defect of the \textit{H. volcanii} \textit{\Delta secFD} strain grown at 45°C in liquid medium, it was surprising that this strain did not confer a stronger growth defect under the same conditions (Fig. 3). This may in part be due to the fact that the majority of secreted \textit{H. volcanii} proteins are predicted to be translocated via the Sec-independent Tat pathway (6, 37). However, haloarchaeal membrane proteins, as well as a subset of secreted proteins such as the essential CWG, still require a functional Sec pathway (16, 38). Consistent with the lack of a strong growth phenotype under the conditions described above, we did not observe significant differences in the levels of chromosomally expressed CWG between the strains (Fig. 5). The CWG may be inherently less sensitive to proteolytic degradation due to the nature of its incorporation into the S-layer and may be SecDF independent for this reason.

It should be noted that the C-terminal Myc tags used for the detection of both pIV and Gly-DM may have interfered with efficient folding of these substrates upon translocation, perhaps rendering them more protease sensitive. The high-salt environment that these organisms inhabit may present particular challenges to efficient extracytoplasmic protein folding. In fact, this environment may partly explain the rerouting of the majority of translocated haloarchaeal proteins to the Tat pathway, which translocates folded proteins (6, 37). Consistent with this hypothesis, two putative Tat substrates that were overexpressed with a C-terminal Myc tag were detected at similar levels in culture supernatants of the \textit{secFD}+ and \textit{\Delta secFD} mutant strains. Most importantly, these results suggest that the \textit{\Delta secFD} mutation causes a Sec-specific protein export defect.

Taken together, our data suggest that SecD and SecF are involved in allowing translocating or translocated Sec substrates to assume stable, folded conformations. The observed decrease in the levels of the Sec reporter substrate is reminiscent of previous work with \textit{E. coli} showing that spheroplasts preincubated with anti-SecD antibodies accumulate a Sec substrate in an extracytoplasmic trypsin-sensitive conformation (22). However, while the conserved domains in the large extracytoplasmic loops of SecD and SecF, as well as recent in vivo deletion studies demonstrating the importance of these loops (28), strongly suggest a function of these proteins late in translocation, it should be noted that similar phenotypes have also been reported for SecE and SecY mutants (32, 45). In future studies, it will be interesting to determine the role of prokaryotic \textit{secFD} knockout as well as other sec mutants.

A number of studies have also indicated that the membrane protein YidC, which is involved in Sec-dependent as well as Sec-independent membrane protein insertion, interacts with both the Sec pore and the SecDF-YajC complex in \textit{E. coli} (27, 42). Consistent with this observation, depletion of the \textit{Bacillus subtilis} YidC-related proteins SpoIIIJ and YgfJ is important for stabilizing certain secretory proteins (44). While we do not have any evidence to implicate YidC homologs as effectors of archaeal SecDF function, we are actively investigating the roles of the \textit{H. volcanii} YidC homologs.

The results presented here do not yet allow us to define the function of SecD and SecF. However, the remarkable congruence of the properties of these proteins in bacteria and archaea implies that the complexes that they form function in a similar manner. Thus, the observed effects on \textit{E. coli} SecA and SecG due to the loss of SecDF-YajC function may be either additional functions of the bacterial SecDF-YajC or secondary defects. In either case, the continuing analysis of archaeal SecD and SecF homologs may also reveal further insights into SecA- and SecG-independent functions of these proteins in bacteria.

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