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* Δ secFD 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 Sec61 α /Sec61 γ and SecY/SecE in eukaryotes and bacteria, respectively, are universally conserved, the third subunit of these complexes, the eukaryotic Sec61 β 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 ho-

mologs than with the bacterial homologs (4, 17). Consistent with this observation, archaea also contain a homolog of the eukaryotic Sec61 β , 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 archaeal 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 archaeal 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* Δ secFD 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 SecFD complex functioning late in protein translocation, as previously proposed for the *E. coli* complex (22, 33), raising the question

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or reference
Strains		
DH5 α	<i>E. coli</i> ϕ 80 <i>dlacZ</i> Δ M15 (<i>argF-lac</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1</i>	Lab collection
	<i>gyrA relA1 phoA</i>	
DL739	<i>E. coli</i> MC4100 <i>dam-13::Tn9</i>	3
WFD11	<i>H. volcanii</i> Δ pHV2	5
DS70	<i>H. volcanii</i> Δ pHV2	48
H98	DS70 Δ <i>pyrE2</i> Δ <i>hdrB</i>	1
H99	DS70 Δ <i>pyrE2</i> Δ <i>hdrB</i> Δ <i>trpA</i>	1
RK19.1	WFD11 containing pRK19.1	This study
WR-Hv-NP15	WFD11 containing pNP15	37
NH-Hv10	DS70 Δ <i>pyrE2</i> Δ <i>hdrB</i> Δ <i>trpA</i> Δ <i>secFD::(fdx-trpA)</i>	This study
NH-Hv11	H98 containing pRK19.1	This study
NH-Hv12	NH-Hv10 containing pRK19.1	This study
NH-Hv13	H98 containing pMLH3	This study
NH-Hv14	NH-Hv10 containing pMLH3	This study
NH-Hv19	H98 containing pNH-AM	This study
NH-Hv20	NH-Hv10 containing pNH-AM	This study
NH-Hv33	H98 containing pNHGly-DM	This study
NH-Hv34	NH-Hv10 containing pNHGly-DM	This study
NH-Hv35	H98 containing pNHGlyDTED-DM	This study
NH-Hv36	NH-Hv10 containing pNHGlyDTED-DM	This study
NH-Hv43	H98 containing pFG-pIV	This study
NH-Hv44	NH-Hv10 containing pFG-pIV	This study
Plasmids		
pBluescript II KS(+)	Cloning vector	Stratagene
pGB70	Amp ^r pUC19 carrying <i>pyrE2</i> under the control of P _{fdx}	2
pTA106	Amp ^r pBluescript II SK(+) carrying <i>trpA</i> under the control of P _{fdx}	1
pTA131	Amp ^r pBluescript II SK(+) carrying <i>pyrE2</i> under the control of P _{fdx}	1
pTA230	Amp ^r <i>H. volcanii</i> - <i>E. coli</i> shuttle vector carrying <i>pyrE2</i> under the control of P _{fdx}	1
pMLH3	(Amp ^r Nb ^r Mev ^r) <i>H. volcanii</i> - <i>E. coli</i> shuttle vector	18
pNP15	(Amp ^r Nb ^r) <i>H. volcanii</i> - <i>E. coli</i> shuttle vector	30
pRK19.1	(Amp ^r Nb ^r) <i>H. volcanii</i> - <i>E. coli</i> shuttle vector carrying the <i>secFD</i> operon with a SecD C-terminal PGHHHHHHH epitope tag	This study
pNHsecKO5	Amp ^r pBluescript II KS(+) containing 800 bp of upstream DNA from the <i>secFD</i> locus	This study
pNHsec5'3'	Amp ^r ; 800 bp of downstream DNA from the <i>secFD</i> locus cloned into pNHsecKO5	This study
pNHsecFD::trpA	Amp ^r P _{fdx} - <i>trpA</i> fragment from pTA106 cloned into pNHsec5'3'	This study
pNHsecDFKO	Amp ^r ; 1.6 kb of <i>secFD</i> 5'- and 3'-flanking DNA cloned into pTA131	This study
pNH-AM	(Amp ^r Nb ^r) P _{fdx} - <i>amy-myc</i> cloned into pMLH3 (Nb ^r), replaces Mev ^r cassette	This study
pFG-pIV	(Amp ^r Nb ^r) P _{R1/6} - <i>pIV-myc</i> cloned into pNB38CBD (Nb ^r), replacing CBD	This study
pNHGly-DM	(Amp ^r Ura ⁺) P _{CWG} - <i>hdrB-myc</i> cloned into pTA230	This study
pNHGlyDTED-DM	(Amp ^r Ura ⁺) P _{CWG} -(DTED)- <i>hdrB-myc</i> cloned into pTA230, mutant signal sequence	This study
pKD9	(Amp ^r Nb ^r) P _{fdx} - <i>arb-myc</i> cloned into pMLH3, replaces Mev ^r cassette	Lab collection, K. Dilks, unpublished

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

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. 5-Fluoroorotic acid (5-FOA) was purchased from Toronto Research Chemicals. The anti-Myc monoclonal antibody (1-9E10) was obtained from the Genetics Core Facility, University of Pennsylvania.

Strains and growth conditions. Archaeal and bacterial strains and plasmids used in this study are listed in Table 1. *H. volcanii* strains were routinely grown at 45°C in 18% modified growth medium (MGM) as described in the Halohandbook (9), unless otherwise specified. MGM was supplemented with novobiocin (0.3 μ g/ml) when required. For the *pyrE2* counterselection scheme, Ura⁺ transformants were selected on CA medium (2) supplemented with thymidine (40 μ g/ml) and counterselected on CA medium containing uracil (50 μ g/ml), thymidine (40 μ g/ml), and 5-FOA (150 μ g/ml). *E. coli* strains were grown at 37°C in NZCYM medium supplemented with ampicillin (200 μ g/ml) when necessary (40).

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* cosmid library. A *secD*-positive cosmid (cosmid 51) 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 pRK19.1 (SecD•6xHis). Using the forward primer SecF-pF1 and the reverse primer HvSecDh•6xHisR1, the *H. volcanii* *secFD* operon, including 340 bp of upstream DNA, was amplified, such that a PGHHHHHHH 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, pRK19.1, was transformed into the Dam⁻

E. coli strain DL739 (3) to passively demethylate the plasmid DNA. Purified plasmid was then transformed into an *H. volcanii* strain (WFD11) as described previously (9), yielding strain RK19.1. Expression of the epitope-tagged SecD was confirmed by immunoblotting of whole-cell lysates using an anti-pentahistidine antibody.

Generation of *secFD* deletion constructs. A total of 800 bp of 5' sequence flanking the *H. volcanii secFD* coding sequences was amplified by PCR from wild-type chromosomal DNA using the forward primer HvSecF5F-XhoI and the reverse primer HvSecF5R-Hind3. The amplified product was digested with XhoI and HindIII and cloned into XhoI-HindIII-digested, calf intestinal phosphatase (CIP)-treated pBluescript II KS(+) to generate plasmid pNHsecKO5. A corresponding 3'-flanking region was amplified from wild-type *H. volcanii* chromosomal DNA using the forward primer HvSecD3F-EcoRI and the reverse primer SecD-down1. 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, pNHsec5'3', was excised, purified, and ligated into CIP-treated XhoI-XbaI-cut pTA131, generating pNHsecFDKO. Finally, the HindIII-EcoRI (P_{fdx} -*trpA*) fragment of pTA106 was excised and ligated into CIP-treated HindIII-EcoRI-digested pNHsecFDKO, yielding pNHsecFD::trpA.

Chromosomal deletion of the *secFD* operon. A refinement of the *pyrE2* counterselection scheme first developed by Bitan-Banin and colleagues for *H. volcanii* (2) was used to replace the *H. volcanii* chromosomal *secFD* operon with the P_{fdx} -*trpA* cassette, which constitutively expresses the *H. volcanii* tryptophan synthase alpha chain under the control of the *H. volcanii* ferredoxin promoter P_{fdx} (1). Unmethylated pNHsecFD::trpA DNA was purified from a DL739/pNHsecFD::trpA transformant and transformed into *H. volcanii* strain H99. Since the pNHsecFD::trpA plasmid does not contain an *H. volcanii* origin of replication, Ura⁺ transformants that had undergone single recombination events at the chromosomal *secFD* locus resulting in plasmid integration were selected on CA plus thymidine agar plates lacking uracil. Resolution of the cointegrate thus formed will either revert the chromosomal locus to the wild type or generate a disrupted $\Delta secFD::trpA$ 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 $\Delta secFD::trpA$ but that had lost the plasmid were selected by plating onto minimal agar medium containing 5-FOA and uracil but lacking tryptophan. Disruption of the chromosomal locus in the resulting $\Delta secFD::trpA$ strain was confirmed by PCR using a forward primer specific to an *H. volcanii* chromosomal region upstream of the 800-bp 5' targeting region in pNHsecFD::trpA, SECUP5F, and a reverse primer, trpCAB3F EcoRI, specific to the *trpA* cassette. A second PCR using the forward primer SecF-F5 and the reverse primer HvSecF-OUT5R confirmed the deletion of the chromosomal locus.

Generation of reporter constructs. pNH-GlyDM was generated by amplifying the cell wall glycoprotein (CWG) promoter region and the 5' end of the gene (the portion encoding the signal sequence) using forward primer Gly-bigUP5F and reverse primer Gly-wtssRev. The *hdrB-myc* fragment was amplified from plasmid pNH-GDM (N. Hand, unpublished data) using forward primer Gly-wtssFor and reverse primer MycTag-end3R. The glycoprotein promoter fragment was fused to the *gly-hdrB-myc* fragment by overlap PCR using the gel-purified PCR products as template "megaprimers" and Gly-bigUP5F and MycTag-end3R as forward and reverse amplification primers. The resulting overlap PCR product was gel purified, digested with EcoRI and HindIII, and ligated into CIP-treated EcoRI-HindIII-cut pTA230.

pNH-GlyDTED-DM was generated in a fashion similar to that for pNH-GlyDM, with the exception that the mutagenic primers GlyDTEDmutF and GlyDTEDmutR were used in place of Gly-wtssFor and Gly-wtssRev, respectively.

The pNH-AM construct was generated by PCR amplifying the *Natronococcus* sp. strain Ah-36 α -amylase gene from plasmid pAMY-RR (37) using the forward HvFdxAmy and reverse AmyMyc3R primers. In an overlap PCR, the P_{fdx} fragment (amplified by PCR from pGB70 [2] using forward primer pGB70upEcoRI and reverse primer FdxRev) was fused to the 5' end of the HvFdxAmy-AmyMyc3R PCR product by using the two purified fragments as template "megaprimers" and pGB70upEcoRI and AmyMyc3R as forward and reverse amplification primers. The resulting overlap PCR product was gel purified, digested with Acc65I and HindIII, and ligated into CIP-treated Acc65I-HindIII-cut pMLH3.

H. volcanii proteinase IV was PCR amplified from chromosomal DNA using primers pIV-For and pIV-Rev. The resulting 1.1-kb PCR product was gel purified, digested with NcoI and XbaI, and ligated into CIP-treated NcoI-XbaI-cut

pNB38CBD (29), yielding the plasmid pFG-pIV, in which proteinase IV-Myc is expressed from the plasmid-encoded synthetic haloarchaeal promoter PrR16.

Localization of reporter constructs in cell cultures. *H. volcanii* cell lysate and culture supernatant samples of cultures expressing Gly-DM, GlyDTED-DM, Arb-Myc, and Amy-Myc were prepared by pelleting the cells from 2 ml of culture. A total of 1.8 ml of the culture supernatant was removed to a separate centrifuge tube, and the remainder of the supernatant was discarded. The cell pellets were resuspended in 1.8 ml of MGM, and 0.2 ml of 100% (vol/vol) trichloroacetic acid (TCA) was added to both the cell and the culture supernatant samples to precipitate the proteins. Protein pellets were washed twice with 80% acetone, air dried, and resuspended in 1× NuPAGE sample buffer. Cell pellets of cell-associated proteinase IV homolog (pIV)-Myc-expressing cells were treated the same way, while the supernatant of these cultures was discarded. Substrates were detected with anti-Myc monoclonal antibodies (1:1,000). Western blots were performed as described previously (37).

Subcellular fractionation of *H. volcanii* and SecD•6xHis localization. A 10-ml culture of *H. volcanii* strain RK19.1 was grown to mid-log phase (optical density at 600 nm, ~0.5) as described previously, and the cells were harvested at 6,500 × g for 10 min at 4°C. The supernatant was discarded, the cell pellet was washed once with 10 ml of ice-cold basal salts solution (9), and the cell suspension was centrifuged as described above. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of basal salts solution supplemented with protease inhibitors (1 μg/ml pepstatin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Cells were lysed by four cycles of freeze-thaw in liquid nitrogen. Thirty units of RQ1 DNase I was added to the cell lysate, and the mixture was incubated at 37°C for 1 h. Cellular debris was removed by two consecutive spins at 20,800 × g for 5 min at 4°C. The supernatant was then subjected to ultracentrifugation at 80,000 rpm (average relative centrifugal force, 264,000 × g) for 30 min at 4°C. The supernatant was collected as the cytoplasmic fraction, the membrane pellet was resuspended in 1 ml of basal salts solution, and both fractions were recentrifuged. Cytoplasmic proteins were precipitated from the cytoplasmic fraction by precipitation with TCA (10% [vol/vol] final). The membrane pellet and acetone-washed cytoplasmic protein pellet were resuspended in 65 μl of 10 mM Tris, pH 8.0, to which 25 μl of 4× lithium dodecyl sulfate sample buffer and 10 μl of NuPAGE sample reducing agent were added. Protein samples were denatured at 70°C for 10 min and electrophoresed on 4 to 12% Bis-Tris NuPAGE gels. SecD was identified by Western blot analysis using anti-pentahistidine monoclonal antibodies (1:1,000).

Large-scale purification of *H. volcanii* cytoplasmic membranes. Two 2-liter cultures of *H. volcanii* strain RK19.1 were grown with shaking in MGM (plus novobiocin) to late log phase. Cells were pelleted at 6,500 × g for 10 min at 4°C. The resulting cell pellet was resuspended in 48 ml of chilled PB(2M)S containing 10 mM EDTA. PB(2M)S was prepared by adding an additional 2 mol of NaCl per liter to standard 1× phosphate-buffered saline (40). The cell suspension was frozen once at -80°C and thawed at 37°C. RQ1 DNase I was added to a final concentration of 1 unit/ml. The cell suspension was then passed through a French press twice at 20,000 psi. Both cellular debris and unbroken cells were removed from the lysate by centrifugation at 18,900 × g for 10 min at 4°C. The supernatant was carefully transferred to precooled ultracentrifugation tubes. Membranes were pelleted from the cleared lysate by ultracentrifugation at 50,000 rpm (average relative centrifugal force, 184,000 × g) for 1 h at 10°C in a Beckman type 70Ti rotor. The supernatant was collected as the cytoplasmic fraction. The membrane pellets were resuspended in a total of 4.8 ml PB(2M)S buffer by gentle trituration through an 18-gauge needle. Four milliliters of the membrane suspension was washed once by adjusting the volume to 46 ml with PB(2M)S and recentrifuged as described above. Membrane pellets were resuspended in a total of 5 ml PB(2M)S buffer. As a control, membranes were purified in parallel from strain WRHv-NP15.

Purification of SecD•6xHis-containing protein complexes by Ni-NTA metal affinity chromatography. For each strain, 5 ml of purified *H. volcanii* cytoplasmic membrane suspension [in PB(2M)S] was solubilized for 1 h at room temperature with continuous inversion on a rolling platform by the addition of 5 ml of Ni-nitrilotriacetic acid (NTA) native adjustment buffer [PB(2M)S, 20% glycerol, 20 mM imidazole, 0.4% DDM]. Batch binding of the His tag to the Ni-NTA resin {preequilibrated with native column binding buffer [PB(2M)S, 10% glycerol, 10 mM imidazole, 0.2% DDM]} was performed with continuous inversion for 1 h at room temperature. The Ni-NTA beads were washed with 2 ml of native column binding buffer (see above). A 2-ml wash was performed with native column wash buffer [PB(2M)S, 10% glycerol, 40 mM imidazole, 0.2% DDM]. Bound proteins were then eluted with native elution buffer [PB(2M)S, 10% glycerol, 250 mM imidazole, 0.2% DDM] in four fractions of 0.25 ml each and precipitated with 10% TCA for separation by polyacrylamide gel electrophoresis.

FIG. 1. The SecD•6xHis 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•6xHis, 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-pentahistidine antibody. Migrations of molecular mass markers are indicated by arrows (in kilodaltons).

Polyacrylamide 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 spectrometry. Coomassie-stained protein bands of interest were excised from the gel. The proteins were prepared for in-gel trypsin digestion, subsequent extraction, and mass spectroscopic analysis of the tryptic peptides according to a modified version (24) of the protocol described previously by Mortz and colleagues (23). 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 spectroscopic 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.

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 translationally 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, respec-

FIG. 2. In vivo copurification of *H. volcanii* SecF with SecD•6xHis. Membrane proteins were purified from dodecyl maltoside-solubilized membranes of the SecD•6xHis-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).

tively) 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 the SecD•6xHis 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 anti-pentahistidine antibodies revealed that the SecD•6xHis protein, which migrated at an apparent molecular mass of ~55 kDa, was exclusively detected in the membrane fraction of these cells (Fig. 1B).

Eluates of Ni-NTA-purified membrane fractions from *H. volcanii* cells carrying a plasmid-borne insert expressing SecF and SecD•6xHis (RK19.1) and the control strain, WR-Hv-NP15 (containing a novobiocin-resistant shuttle vector with no

SecF- and SecD•6xHis-encoding insert), were analyzed by denaturing 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 ~30 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). Western blot analysis using antipeptidohistidine 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-ion spectra were compared to a database of conceptual, *in silico* trypsin-digested polypeptides corresponding to the available sequences from the partial *H. volcanii* proteome, generously 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, consistent with a SecDF heterodimer and a SecD homodimer, respectively.

When the samples were heated to 70°C prior to gel electrophoresis, 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 deletion strain is viable, we employed a knock-out strategy (1) that allows for the recovery of deletion mutations of nonessential genes, even if the resulting mutants exhibit a strong growth defect. Briefly, the secFD coding sequences were replaced by the prototrophic *trpA* marker under the control of the *H. volcanii* P_{fdx} promoter (P_{fdx}-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 tryptophan, demanding the retention of the P_{fdx}-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 obtain 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 negligible 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 demonstrate 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 haloarchaeal 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 expressing 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 ΔsecFD 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 P_{fdx} promoter (data not shown). If the mode of translocation was posttranslational, it is possible that the instability 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 expressed 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

FIG. 3. Deletion of the *secFD* operon confers a severe cold-sensitive growth defect. Growth of *H. volcanii* *secFD*⁺ and Δ *secFD* strains was measured in triplicate by plotting the optical density at 600 nm (OD₆₀₀) against time (in hours). In all three panels, growth of *secFD*⁺ strains is indicated by solid lines, and that of the Δ *secFD* strains is indicated by dashed lines (error bars indicate ± 1 standard deviation). (A and B) Growth of *secFD*⁺ (H98) and Δ *secFD* (NH-Hv10) strains at (A) 45°C and (B) 30°C. (C) Growth of *secFD*⁺ (NH-Hv11) and Δ *secFD* (NH-Hv12) strains expressing *SecFD*•6xHis (on plasmid pRK19.1) at 30°C.

FIG. 4. The *H. volcanii* Δ *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 Δ *secFD* *H. 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.

might also observe lower levels of translocated membrane-associated *H. volcanii* Sec substrates. Therefore, we next analyzed cellular protein levels of a predicted *H. volcanii* 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 Δ *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 *H. volcanii* 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 Δ *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. 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 Δ *secFD* (NH-Hv44) strains were determined by Western blot analysis of cell lysates from *H. 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.

FIG. 6. The $\Delta secFD$ translocation defect is Sec specific. Protein levels of arabinanase-Myc (Arb) and α -amylase-Myc (Amy) in cell (c) and culture supernatant (s) fractions from $secFD^+$ and $\Delta secFD$ *H. 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.

Interestingly, Western blot analysis of *H. volcanii* cell lysates of both the $secFD^+$ and $\Delta secFD$ 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.

(ii) **Effects of $\Delta secFD$ on Tat substrates.** The *Natronococcus amalyticus* α -amylase and the *H. volcanii* arabinanase contain typical Tat signal sequences (37; K. Dilks, unpublished data). Thus, if the translocation defect observed in the $\Delta secFD$ strain was specific to Sec substrates, the secretion of these proteins expressed in $secFD^+$ and $\Delta secFD$ strains should be comparable. As predicted, no significant differences in the levels of either substrate were observed (Fig. 6). Thus, these results demonstrate that the $\Delta secFD$ mutation causes a protein export defect that is specific to the Sec pathway.

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 SecE, 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 domains, 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 β) 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 β 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 β 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 SecD•6xHis, 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 SecD•6xHis 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 $\Delta secFD$ strain grew similarly to an *H. volcanii* $secFD^+$ 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* $\Delta secDF$ 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 $secFD^+$ 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 $secFD^+$ 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 GlyDTE-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 *H. volcanii* pIV homolog, which contains a typical Sec signal sequence, was barely detectable when expressed with a C-terminal Myc tag in the $\Delta secFD$ strain, while it was readily detectable in the $secFD^+$ strain (Fig. 5). Attempts to transform *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 *H. volcanii* $\Delta secFD$ 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 *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 $secFD^+$ and the $\Delta secFD$ 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 $secFD^+$ and $\Delta secFD$ mutant strains. Most importantly, these results suggest that the $\Delta secFD$ 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 *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 lipid composition of prokaryotic $secFD$ 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 *E. coli* (27, 42). Consistent with this observation, depletion of the *Bacillus subtilis* YidC-related proteins SpoIIIJ and YqjG 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 *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 *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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REFERENCES

1. Allers, T., H. P. Ngo, M. Mevarech, and R. G. Lloyd. 2004. Development of additional selectable markers for the halophilic archaeon *Haloflex volcanii* based on the *leuB* and *trpA* genes. Appl. Environ. Microbiol. **70**:943–953.
2. Bitan-Banin, G., R. Ortenberg, and M. Mevarech. 2003. Development of a gene knockout system for the halophilic archaeon *Haloflex volcanii* by use of the *pyrE* gene. J. Bacteriol. **185**:772–778.
3. Blyn, L. B., B. A. Braaten, and D. A. Low. 1990. Regulation of pap pilin phase variation by a mechanism involving differential dam methylation states. EMBO J. **9**:4045–4054.
4. Cao, T. B., and M. H. Saier, Jr. 2003. The general protein secretory pathway: phylogenetic analyses leading to evolutionary conclusions. Biochim. Biophys. Acta **1609**:115–125.
5. Charlebois, R. L., W. L. Lam, S. W. Cline, and W. F. Doolittle. 1987. Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaeobacterium. Proc. Natl. Acad. Sci. USA **84**:8530–8534.
6. Dilks, K., M. I. Giménez, and M. Pohlschröder. 2005. Genetic and biochemical analysis of the twin-arginine translocation pathway in halophilic archaea. J. Bacteriol. **187**:8104–8113.
7. Duong, F., and W. Wickner. 1997. Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme. EMBO J. **16**:2756–2768.
8. Duong, F., and W. Wickner. 1997. The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling. EMBO J. **16**:4871–4879.

9. Dyall-Smith, M. 21 December 2005, posting date. The halo handbook (v4.9). [Online.] <http://www.microbiol.unimelb.edu.au/people/dyallsmith/HaloHandbook/>.
10. Economou, A., J. A. Pogliano, J. Beckwith, D. B. Oliver, and W. Wickner. 1995. SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. *Cell* **83**:1171–1181.
11. Eichler, J. 2003. Evolution of the prokaryotic protein translocation complex: a comparison of archaeal and bacterial versions of SecDF. *Mol. Phylogenet. Evol.* **27**:504–509.
12. Fekkes, P., and A. J. Driessen. 1999. Protein targeting to the bacterial cytoplasmic membrane. *Microbiol. Mol. Biol. Rev.* **63**:161–173.
13. Finke, K., K. Plath, S. Panzner, S. Prehn, T. A. Rapoport, E. Hartmann, and T. Sommer. 1996. A second trimeric complex containing homologs of the Sec61p complex functions in protein transport across the ER membrane of *S. cerevisiae*. *EMBO J.* **15**:1482–1494.
14. Gardel, C., S. Benson, J. Hunt, S. Michaelis, and J. Beckwith. 1987. *secD*, a new gene involved in protein export in *Escherichia coli*. *J. Bacteriol.* **169**:1286–1290.
15. Gardel, C., K. Johnson, A. Jacq, and J. Beckwith. 1990. The *secD* locus of *E. coli* codes for two membrane proteins required for protein export. *EMBO J.* **9**:3209–3216.
16. Haddad, A., R. W. Rose, and M. Pohlshörder. 2005. The *Haloferax volcanii* FtsY homolog is critical for haloarchaeal growth but does not require the A domain. *J. Bacteriol.* **187**:4015–4022.
17. Hartmann, E., T. Sommer, S. Prehn, D. Görlich, S. Jentsch, and T. A. Rapoport. 1994. Evolutionary conservation of components of the protein translocation complex. *Nature* **367**:654–657.
18. Holmes, M., F. Pfeifer, and M. Dyall-Smith. 1994. Improved shuttle vectors for *Haloferax volcanii* including a dual-resistance plasmid. *Gene* **146**:117–121.
19. Kato, Y., K. Nishiyama, and H. Tokuda. 2003. Depletion of SecDF-YajC causes a decrease in the level of SecG: implication for their functional interaction. *FEBS Lett.* **550**:114–118.
20. Kinch, L. N., M. H. Saier, Jr., and N. V. Grishin. 2002. Sec61beta—a component of the archaeal protein secretory system. *Trends Biochem. Sci.* **27**:170–171.
21. Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305**:567–580.
22. Matsuyama, S., Y. Fujita, and S. Mizushima. 1993. SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. *EMBO J.* **12**:265–270.
23. Mortz, E., O. Vorm, M. Mann, and P. Roepstorff. 1994. Identification of proteins in polyacrylamide gels by mass spectrometric peptide mapping combined with database search. *Biol. Mass Spectrom.* **23**:249–261.
24. Naumann, B., E. J. Stauber, A. Busch, F. Sommer, and M. Hippler. 2005. N-terminal processing of Lhca3 is a key step in remodeling of the photosystem I-light-harvesting complex under iron deficiency in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **280**:20431–20441.
25. Nishiyama, K., M. Hanada, and H. Tokuda. 1994. Disruption of the gene encoding p12 (SecG) reveals the direct involvement and important function of SecG in the protein translocation of *Escherichia coli* at low temperature. *EMBO J.* **13**:3272–3277.
26. Nouwen, N., and A. J. Driessen. 2005. Inactivation of protein translocation by cold-sensitive mutations in the *yajC-secDF* operon. *J. Bacteriol.* **187**:6852–6855.
27. Nouwen, N., and A. J. Driessen. 2002. SecDFyajC forms a heterotetrameric complex with YidC. *Mol. Microbiol.* **44**:1397–1405.
28. Nouwen, N., M. Piwowarek, G. Berrelkamp, and A. J. Driessen. 2005. The large first periplasmic loop of SecD and SecF plays an important role in SecDF functioning. *J. Bacteriol.* **187**:5857–5860.
29. Ortenberg, R., and M. Mevarech. 2000. Evidence for post-translational membrane insertion of the integral membrane protein bacterioopsin expressed in the heterologous halophilic archaeon *Haloferax volcanii*. *J. Biol. Chem.* **275**:22839–22846.
30. Patenge, N., and J. Soppa. 1999. Extensive proteolysis inhibits high-level production of eukaryal G protein-coupled receptors in the archaeon *Haloferax volcanii*. *FEMS Microbiol. Lett.* **171**:27–35.
31. Pogliano, J. A., and J. Beckwith. 1994. SecD and SecF facilitate protein export in *Escherichia coli*. *EMBO J.* **13**:554–561.
32. Pogliano, K. J., and J. Beckwith. 1993. The Cs sec mutants of *Escherichia coli* reflect the cold sensitivity of protein export itself. *Genetics* **133**:763–773.
33. Pogliano, K. J., and J. Beckwith. 1994. Genetic and molecular characterization of the *Escherichia coli secD* operon and its products. *J. Bacteriol.* **176**:804–814.
34. Pohlshörder, M., K. Dilks, N. J. Hand, and R. W. Rose. 2004. Translocation of proteins across archaeal cytoplasmic membranes. *FEMS Microbiol. Rev.* **28**:3–24.
35. Pohlshörder, M., E. Hartmann, N. J. Hand, K. Dilks, and A. Haddad. 2005. Diversity and evolution of protein translocation. *Annu. Rev. Microbiol.* **59**:91–111.
36. Pohlshörder, M., W. A. Prinz, E. Hartmann, and J. Beckwith. 1997. Protein translocation in the three domains of life: variations on a theme. *Cell* **91**:563–566.
37. Rose, R. W., T. Bruser, J. C. Kissinger, and M. Pohlshörder. 2002. Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. *Mol. Microbiol.* **45**:943–950.
38. Rose, R. W., and M. Pohlshörder. 2002. In vivo analysis of an essential archaeal signal recognition particle in its native host. *J. Bacteriol.* **184**:3260–3267.
39. Rothblatt, J. A., R. J. Deshaies, S. L. Sanders, G. Daum, and R. Schekman. 1989. Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell Biol.* **109**:2641–2652.
40. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. Sanders, S. L., K. M. Whitfield, J. P. Vogel, M. D. Rose, and R. W. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell* **69**:353–365.
42. Scotti, P. A., M. L. Urbanus, J. Brunner, J. W. de Gier, G. von Heijne, C. van der Does, A. J. Driessen, B. Oudega, and J. Luijck. 2000. YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase. *EMBO J.* **19**:542–549.
43. Sonnhammer, E. L., G. von Heijne, and A. Krogh. 1998. A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **6**:175–182.
44. Tjalsma, H., S. Bron, and J. M. van Dijk. 2003. Complementary impact of paralogous Oxa1-like proteins of *Bacillus subtilis* on post-translational stages in protein secretion. *J. Biol. Chem.* **278**:15622–15632.
45. Ueguchi, C., and K. Ito. 1990. *Escherichia coli sec* mutants accumulate a processed immature form of maltose-binding protein (MBP), a late-phase intermediate in MBP export. *J. Bacteriol.* **172**:5643–5649.
46. Van den Berg, B., W. M. Clemons, Jr., I. Collinson, Y. Modis, E. Hartmann, S. C. Harrison, and T. A. Rapoport. 2004. X-ray structure of a protein-conducting channel. *Nature* **427**:36–44.
47. von Heijne, G. 1990. The signal peptide. *J. Membr. Biol.* **115**:195–201.
48. Wendoloski, D., C. Ferrer, and M. L. Dyall-Smith. 2001. A new simvastatin (mevinolin)-resistance marker from *Haloarcula hispanica* and a new *Haloferax volcanii* strain cured of plasmid pHV2. *Microbiology* **147**:959–964.
49. Young, B. P., R. A. Craven, P. J. Reid, M. Willer, and C. J. Stirling. 2001. Sec63p and Kar2p are required for the translocation of SRP-dependent precursors into the yeast endoplasmic reticulum in vivo. *EMBO J.* **20**:262–271.