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Proteomic analysis of secreted membrane vesicles of archaeal *Sulfolobus* species reveals the presence of endosome sorting complex components

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Abstract The crenarchaea *Sulfolobus acidocaldarius*, *S. solfataricus* and *S. tokodaii,* release membrane vesicles into the medium. These membrane vesicles consist of tetraether lipids and are coated with an S-layer. A proteomic analysis reveals the presence of proteins homologous to subunits of the eukaryotic endosomal sorting complex required for transport (ESCRT). Immunodetection of one of these homologs suggest a cell surface localization in intact cells. These data suggest that the membrane vesicles in *Sulfolobus* sp. emerge from a specific budding process with similarity to the endosomal sorting pathway.

Keywords Archaea · *Sulfolobus* · Cell envelope · Proteome

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Introduction

The release of cell surface-derived membrane vesicles is a common process in bacteria and eukaryotes. In gram-negative bacteria, several functions have been attributed to the release of outer membrane vesicles such as DNA and protein secretion, cell to cell communication and a stress response to cell envelope damage. For example, the α-hemolysin toxin of *Escherichia coli* is released in association with outer membrane vesicles (Balsalobre et al. 2006), while denatured proteins at the cell envelope are expelled from the cell via outer membrane vesicles (McBroom and Kuehn 2007). Outer membrane vesicles produced by *Pseudomonas aeruginosa* are equipped with antimicrobial activity and have been implicated in quorum sensing allowing individual cells to act as a group (Mashburn and Whiteley 2005). Furthermore, vesicles have been shown to be an important constituent of *P. aeruginosa* biofilms, although their role in the formation of the biofilm itself is uncertain (Schooling and Beveridge 2006). Vesicles present in the biofilm are proteolytic active and bind antibiotics thereby possibly forming an extra barrier of defense. In gram-positive bacteria there are fewer reports on vesicle release that also have been studied to a lesser detail (Dorward and Garon 1990). Vesicles covered with starch-degrading enzymes are released by *Thermoanaerobacterium thermosulfurogenes* EM1 under phosphate limiting growth conditions (Mayer and Gottschalk 2003; Specka et al. 1991).

Despite the importance of the bacterial extracellular vesicles the mechanisms of vesicle biogenesis are not well understood. In *E. coli*, it was noted that the increase of vesicle release in response to bulk periplasmic protein overexpression involved a stress response independent and distinct from previously known stress responses (McBroom and Kuehn 2007). However, the components of this novel stress response are unknown. Current models of vesicle formation in gram-negative bacteria are based on the creation of an unstable outer membrane resulting in the rather unspecific and spontaneous release of outer membrane blebs (Mashburn-Warren and Whiteley 2006).

In eukaryotes there appear to be two major pathways for the release of extracellular vesicles. In the first pathway, vesicles are pinched off directly from the plasma membrane. Under stress conditions this non-lethal membrane blebbing allows cells to discard damaged cellular organelles, macromolecules and lipids (Marin-Castano et al. 2005). Furthermore, the release of cellular microparticles, sometimes referred to as microvesicles, is part of the plasma membrane remodeling process when the asymmetric distribution of the phospholipids between the two leaflets is lost (Freyssinet 2003). Initially, the microparticles were regarded as "cell dust" but now it is clear that they can be either beneficial or detrimental (Freyssinet 2003). In the second major pathway, intraluminal vesicles of multivesicular bodies (MVB) are exported out of the cell. The MVB fuses with the plasma membrane upon which the intraluminal vesicles are released as exosomes (Keller et al. 2006). Exosomes are small vesicles with diameters typically between 30 and 100 nm and are found in various bodily fluids, such as urine, blood, saliva and snake venom (Johnstone et al. 1987; Ogawa et al. 2008a, b; Pisitkun et al. 2004). Exosomes are involved in cell to cell communication in general, but have also been reported to mediate the transport of mRNA between cells (Valadi et al. 2007). In eukaryotes, most of the studies concern the release of vesicles in mammals although the amoebe *Dictyostelium discodium* and the pathogenic fungus *Cryptococcus neoformans* have been reported to also produce extracellular vesicles (Rodrigues et al. 2007; Tatischeff et al. 1998).

In the third domain of life, i.e., the archaea, little is known about vesicle release. An electron microscopic investigation of *Sulfolobus acidocaldarius* and *Ignicoccus* suggests the release of vesicles through a further uncharacterized budding-off process (Grimm et al. 1998; Nather and Rachel 2004). *Ignicoccus* seems not only to release vesicles, but similar structures are also observed to accumulate in the space between inner and outer membrane (Rachel et al. 2002). These vesicles may originate from the outer membrane in manner similar to that in gram-negative

bacteria. Vesicles isolated from the growth medium of *S. islandicus* were shown to contain a protein factor which inhibits the growth of other *Sulfolobus* spp. (Prangishvili et al. 2000). These vesicles contain the crystalline surface-layer (S-layer) protein which indicates that they are indeed derived from the cell surface. However, the composition of these vesicles has never been studied.

Here we show that the release of vesicles is a widespread phenomenon among the thermoacidophilic members of the *Sulfolobus* genus. Furthermore, proteomic analysis of the vesicles reveals the presence of homologs of eukaryote endosomal protein sorting complex proteins suggesting that vesicle release is a specific process.

Experimental procedures

Strains and growth conditions

S. acidocaldarius strain DSM639, *S. tokodaii* strain DSM16993 and *S. solfatarcius* P2 strain DSM1617 were from DSMZ (Braunschweig, Germany) and grown at pH 3.0 in Brocks medium (Brock et al. 1972) supplemented with 0.3% (w/v) glucose and 0.1% (w/v) casamino acids. Pre-cultures (50 ml) were grown at 80°C until the late log-phase, from which 3 ml were transferred into 800 ml cultures that were grown in 3 L flasks, shaking at $\sim$160 rpm at 80°C till early log (OD_{600} of 0.5–0.7) or stationary phase. For the lipid determinations, a 2 L culture of *S. solfataricus* was grown until stationary phase.

Isolation of extracellular membrane vesicles

Cultures were cooled down on ice for 20 min, followed by low spin centrifugation for 10 min at 4°C and 12,000 $\times g$ to remove cells. Subsequently, vesicles were taken from the supernatant by high spin centrifugation at 4°C for 45 min at 125,000 $\times g$. The high speed centrifugation pellets were collected in residual supernatant fluid and centrifuged for 1 min at 16,000 $\times g$ to remove remaining residual cell debris. The cleaned-up vesicle fractions were then subjected to a final ultracentrifugation at 376,000 $\times g$ for 45 min at 4°C to pellet the vesicles. Alternatively, the cleaned-up vesicle fractions were applied on a CsCl-gradient, typically ranging from 0.5 to 1.2 g/ml, in sodium-acetate buffer (20 mM Na-acetate, 0.5 mM $CaCl_2$, pH 3.0) and spun for 16 h at 288,000 $\times g$. Vesicles were collected from the CsCl-gradient and washed three times with 400 µl sodium-acetate buffer by ultrafiltration on a microcon YM3 filter (Millipore) yielding a final volume of 30–40 µl. Protein content of the isolated vesicles was quantified using a DC protein assay (Biorad, USA). Vesicles were stored at −20°C.

Isolation of cytoplasmic membranes

Cell pellets obtained during the isolation of extracellular membrane vesicles (see above) were suspended in 5 ml of a buffer containing 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl. Cells were lysed by French press treatment at 14 kPsi, and cell debris was removed from the lysate by centrifugation at 5,000 $\times g$ for 10 min at 4°C. Membranes were collected by ultracentrifugation at 376,000 $\times g$ for 1 h at 4°C, and finally resuspended in 1.6 ml of 20 mM Tris-HCl, pH 7.5, and 150 mM NaCl and stored at −20°C.

In gel trypsin digestion and MALDI-TOF/TOF mass spectrometry

Isolated vesicles were heated at 90°C for 5 min in sample buffer and then subjected to SDS-PAGE using 12% poly-acrylamide gels. Proteins were visualized with Coomassie brilliant blue G250 after which the protein bands were cut out from the gel. Gel pieces were washed with 500 µl demineralized water, destained twice with 500 µl 30% acetonitrile (ACN) in 25 mM NH_4HCO_3, and once with 400 µl 50% ACN in 25 mM NH_4HCO_3 and subsequently dried in a speedvac. Gel pieces were soaked in 15 µl freshly prepared 10 mM DTT for 45 min at 55°C, followed by the addition of 10 µl of 55 mM iodoacetamide, both in 50 mM NH_4CO_3. Incubation was continued for 30 min in the dark. Next gel pieces were washed for 30 min with 400 µl 50% ACN in 50 mM NH_4HCO_3 and dried in a speedvac. An aliquot of 20 µl of 10 ng/µl trypsin (MS-grade, Promega, Leiden, The Netherlands) was added to the dried gel pieces in 9% ACN in 40 mM NH_4HCO_3 and incubated overnight at 37°C. Surplus overlay solution was collected after which peptides were extracted with 20 µl 1% formic acid, followed by extractions with 20 µl 50% ACN in 1% formic acid and 10 µl 100% ACN. Fractions of the peptide extraction were pooled, dried in a speedvac and dissolved in 4 µl 0.1% trifluoroacetic acid (TFA). Samples were finally mixed in a 1:1 ratio with a solution of 5 mg/ml α-cyano-4-hydroxycinnamic acid and 1 pmol of the calibration peptide ACTH 18-39 (Sigma Aldrich, mass is 2,464.1989 Da) in 70% ACN/30% (or 0.1%) TFA spotted on a stainless steel target plate and analyzed in positive ion mode with MS and MS/MS on a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Spectra were calibrated internally based on the 841.5021 Da trypsin autolysis product and the added calibration peptide.

LC-MS sample preparation

Isolated membrane vesicles (12–15 µg of protein) were suspended in 500 µl denaturing buffer (50 mM Na_2CO_3, pH 9.0, 6 M Urea, 1% DDM and 1 M thiourea).and sonicated for 10 min in a water bath prior to overnight incubation at 30°C. To remove the denaturing buffer, samples were spun on a 3 kDa cut off filter (YM3, Millipore) for 1 h, after which 450 µl 40 mM NH_4HCO_3 was added and the sample was spun again for 1 h. This was repeated at least five times, after which the sample was concentrated to ∼30 µl in 40 mM NH_4HCO_3. Next, the sample was diluted to 40 µl yielding final concentrations of 40 mM NH_4HCO_3, 10 ng/µl trypsin and 9% ACN. Proteins were digested overnight at 37°C and then dried in a speedvac. Dried peptides were dissolved in 5 µl 0.1% TFA and were separated on a Zorbax 300 SB C18 reverse phases capillary column (75 µm × 150 mm, 3.5 µm particle size) mounted on an Ettan MDLC nanoflow liquid chromatography system (GE Healthcare). The elution buffers used in the gradient were Buffer A (0.065% TFA) and B (84% acetonitrile, 0.050% TFA). The nanoLC gradient was from 20 to 80% B and elution was for 45 min with a flow rate 250 nL min^{-1}. MS data acquisition was performed in positive ion mode on the 4700 Proteomics Analyzer. During MS acquisition, peptides with signal-to-noise intensities above 25 were selected for MS/MS fragmentation.

Protein identification and mass spectrometry analysis

Proteins were identified by matching the peptide masses and MS/MS spectra against the individual *Sulfolobus* sp. databases. The *S. solfataricus* database was obtained from http://www-archbac.u-psud.fr/projects/sulfolobus/, the *S. tokodaii* database was obtained from http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=st and the *S. acido-caldarius* database was obtained from http://gib.genes.nig.ac.jp/single/index.php?spid=Saci_DSM639. Data obtained from the in gel trypsin digestion were filtered manually for mismatches with well known trypsin autolysis products and human keratin contaminants. False positives were removed manually when the MS and MS/MS spectra obtained from one spot indicated multiple proteins. Spectra were examined visually to exclude the matching of non-peptide masses. In case of the LC-MS well known trypsin autolysis products and human keratin contamination were removed from the peak list of the MS spectra, after which the peak list and MS/MS spectra were matched against the individual *Sulfolobus* databases. Identifications with a probability of $p > 0.95$ were deemed significant, provided at least one unique peptide mass was matched.

Lipid analysis

Membrane vesicle and cell fractions where freeze-dried overnight, and subsequently acid hydrolyzed by refluxing with 5% HCl in methanol for 3 h. The pH of the cooled

solution was adjusted to pH 5 with 1 N KOH in MeOH (96%). Bi-distilled water was added to a final ratio of H$_2$O:MeOH (1:1) and this mixture was washed 3× with dichloromethane. The dichloromethane layer was dried using a combined sodium sulfate and sodium carbonate column to yield the acid hydrolysis extract. A C$_{46}$ GDGT internal standard (1 μg) was added to the extract (Huguet et al. 2006). GDGTs were analyzed using an LC/MS procedure slightly modified from as described before (Schouten et al. 2007). Analyses were performed using an Agilent (Palo Alto, California, USA) 1100 series LC-MSD SL equipped with an autoinjector and Chemstation chromatography manager software. Separation was achieved on a Prevail Cyano column (2.1 × 150 mm, 3 μm; Alltech, Deerfield, IL, USA), maintained at 30°C. Injection volumes were 10 μL. GDGTs were eluted isocratic with 99% A and 1% B for 5 min, followed by a linear gradient to 1.8% B in 45 min, where A = hexane and B = propanol. Flow rate was 0.2 mL/min. After each analysis the column was cleaned by back flushing hexane/propanol (90:10, by volume) at 0.2 mL/min for 10 min. Detection was achieved using APCI-MS of the eluent. Conditions for APCI-MS were as follows: nebulizer pressure 60 psi, vaporizer temperature 400°C, drying gas (N$_2$) flow 6 L/min and temperature 200°C, capillary voltage 3 kV, corona 5 μA (∼3.2 kV). GDGTs were detected by single ion monitoring of their $[M + H]^+$ ions (dwell time 237 ms) Concentrations of GDGTs were determined by integration of their peak areas relative to that of the C$_{46}$ GDGT internal standard and corrected for ionization (Huguet et al. 2006).

Electron microscopy

Samples were negatively stained with 2% uranyl acetate on glow discharged carbon coated copper grids. Electron microscopy was performed on a Phillips CM120 electron microscope equipped with a Gatan SP 4 k slow-scan CCD camera.

Immunogold labeling

For on-section immunolabeling trypton grown cells were cryoimmobilized by high-pressure freezing according as described (Hohenberg et al. 1994). In short, cells were sucked into cellulose microcapillaries of 200 μm diameter and 2 mm-long capillary tube segments were transferred to aluminum platelets of 150 μm depth containing 1-hexadecene. The platelets were sandwiched with platelets without any cavity and then frozen with a high-pressure freezer (Bal-Tec HPM 010, Balzers, Liechtenstein). The frozen capillary tubes were freed from extraneous hexadecene under liquid nitrogen and transferred to 2 ml microtubes with screw caps (Sarstedt #72.694) containing

the substitution medium precooled to −90°C. Samples were freeze-substituted (using a Leica AFS unit) in 0.5% uranyl acetate in anhydrous acetone at −90°C for 32 h, warmed up to −60°C within 3 h, kept at −60°C for 4 h, warmed up to −40°C within 2 h and kept there for 4 h. After washing with ethanol the samples were transferred into a mixture of ethanol-Lowicryl K11 M, then infiltrated with the polar methacrylate resin Lowicryl K11 M (Polysciences, Eppelheim, Germany) and polymerized by UV irradiation at −40°C. Ultrathin sections were labeled with a rabbit antibody against the AAA family ATPase SSO0909 and protein A-10 nm gold complexes (gift from Dr. York Stierhof, ZMBP Tübingen, Germany). Rabbit Anti-Sso0909 antibodies were obtained using recombinant Sso0909 as described by (Cooper and Patterson 2008). These sections were finally stained with 1% aqueous uranyl acetate and lead citrate and imaged in a Philips CM10 electron microscope at 60 kV using a 30 μm objective aperture.

Results

Morphology of extracellular membrane vesicles

Three different *Sulfolobus* spp., i.e., *S. solfataricus*, *S. acidocaldarius* and *S. tokodaii* were grown in minimal medium supplemented with glucose and casamino acids until the early log-phase and stationary phase. Vesicles were obtained from cell cleared growth medium that was further processed by ultracentrifugation. Examination of resuspended pellets by EM revealed the presence of morphologically similar membrane vesicles-like structures in all three *Sulfolobus* spp. (Fig. 1). The pellet fraction from *S. acidocaldarius* also contained several protein filaments, including flagella that were not observed in the other two studied species (not shown). The diameter of the vesicles ranged from about 90 to 230 nm, and they were surrounded by a protein layer, presumably formed by the surface-layer (S-layer) protein. Only low numbers of vesicles could be obtained during the early exponential growth phase (<15 μg of protein/l), while amounts increased up to the stationary phase (∼30 μg of protein/l). Both morphology and size distribution of the membrane vesicles did not differ significantly between exponential growing and stationary phase cultures.

Lipid content of extracellular membrane vesicles

Quantitative lipid analysis revealed that the membrane vesicles where enriched in archaeal tetraether lipids [glycerol dialkyl glycerol tetraethers (GDGTs) and a glycerol trialkyl glycerol tetraether (GTGT)] (Table 1) as
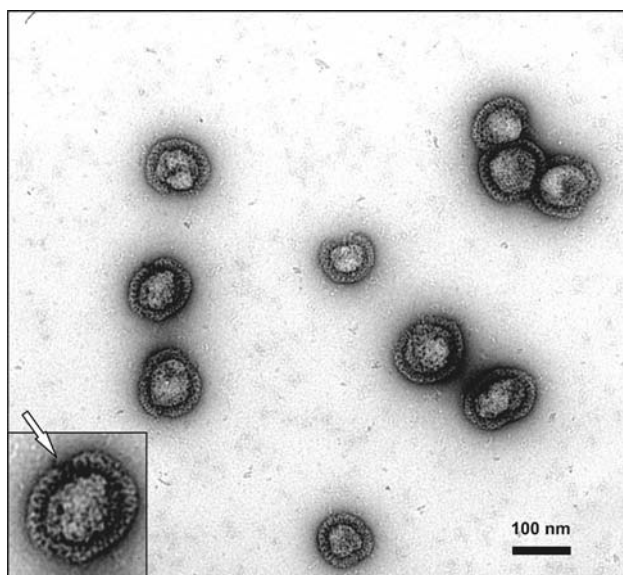
Fig. 1 Electron microscopy of uranyl acetate stained membrane vesicles from *S. solfataricus* showing the proteinaceous surface-layer (S-layer) surrounding the vesicles. Insert, membrane vesicle at higher magnification. The *white arrow* indicates the stalk (*in white*) through which the S-layer is attached to the membrane in cells. The spaces between the stalks are also visible (*in black*)

compared to the tetraether lipid content of intact cells. The combined content of GDGT-5 (contains 5 cyclopentanes) and GDGT-6 (contains six cyclopentanes) drops from 27% for whole cells (cytoplasmic membranes) to about 13% in the membrane vesicles. These data indicate that the membrane vesicles originated from the cytoplasm membrane, although the ratios of the various lipid components differ from that of the cytoplasm membrane.

Proteomic analysis of extracellular membrane vesicles

Membrane vesicles of the stationary phase grown cells were further purified on a CsCl-gradient and analyzed by SDS-PAGE. Proteins were either directly identified by excision from the gel followed by mass spectrometry or by LC-MS after in liquid trypsin digestion of the vesicle fractions (Fig. 2a, Table 2). The protein band pattern in SDS-PAGE indicates that the membrane vesicles have a specific protein composition (Fig. 2a), markedly different from that of isolated cytoplasm membranes (compare with Fig. 2b). The LC-MS analysis of the vesicle membrane fraction yielded a high number of identified proteins, i.e., 29, 48 and 29 in *S. acidocaldarius*, *S. solfataricus* and *S. tokodaii*, respectively (Table 2). In *S. tokodaii* no ribosome proteins were identified while in *S. acidocaldarius*, and *S. solfataricus* only a few ribosomal proteins were found suggesting that the cytosolic contamination is low. In the next section, we will only discuss the proteins that could be identified on a coomassie-stained SDS-PAGE and the

proteins that were identified in the vesicle fractions of all three *Sulfolobus* spp. (the ORF numbers are indicated in bold in Table 2).

Surprisingly, the membrane vesicles contained two archaeal ESCRT-III like proteins that were identified in all three *Sulfolobus* spp. In *S. solfataricus* a third archaeal ESCRT-III like protein (SSO0619) was found. In eukaryotes, Endosomal Sorting Complex Required for Transport proteins (ESCRT) functions in protein sorting in the endosomal transport pathway (Babst et al. 2002). Eukaryote membrane proteins destined for degradation are sorted to MVB via several ESCRT complexes with ESCRT-III being the last complex before inward budding into the lumen of the endosome leads to the creation of the MVB. The MVB can then fuse with the lysosome or vacuole, but may also fuse with the plasma membrane leading to the release of its intraluminal vesicles as exosomes (Williams and Urbe 2007). Three archaeal ESCRT-III like proteins, i.e., SSO0881, Saci_1416 and ST1237 appeared relatively abundant as they were visible on a coomassie blue stained SDS-PAGE (Fig. 2a).

The membrane vesicles also contained an AAA family ATPase, i.e., SSO0909, Saci_1372 and ST1215 (Ogura and Wilkinson 2001). Sequence analysis revealed that the ATPases contain an N-terminal microtubule interacting and trafficking (MIT) sub-domain and a putative C-terminal Vps4-oligomerization domain. As both domains are typically found in eukaryotic Vps4 proteins (Stuchell-Brereton et al. 2007; Vajjhala et al. 2006), the identified ATPases likely represent an archaeal ortholog of the eukaryotic Vps4 as was suggested recently (Hobel et al. 2008; Obita et al. 2007). In eukaryotes, Vps4 releases ESCRT-III sub-units from the endosomal membrane in an ATP-dependent manner (Babst et al. 1998). In SDS-PAGE, only a 14 kDa fragment of the 42 kDa *S. solfataricus* protein could be detected.

A protein with a von Willebrand factor type A (vWA) domain was also present in the membrane vesicles, while in vesicles of *S. tokodaii*, a second vWA domain containing protein (ST0828) was found. vWA domains were originally found in the blood coagulation protein von Willebrand factor where it functions in the formation of protein aggregates (Whittaker and Hynes 2002). In eukaryotes von Willebrand factor is stored in secretory vesicular compartments, so-called Weibel-Palade bodies, where it also recruits other proteins to be secreted (Bierings et al. 2007; Blagoveshchenskaya et al. 2002).

Membrane vesicles derived from all three species also contained a protein containing a forkhead-associated (FHA) domain. The gene encoding this protein together with the genes of the above mentioned vWA domain containing proteins cluster together. FHA-domain containing proteins function in protein–protein interaction and

Table 1 Lipid composition of *S. solfataricus* cells and secreted membrane vesicles. *GDGT* glycerol dialkyl glycerol tetraethers, *GTGT* glycerol trialkyl glycerol tetraether. Numbers indicate number of cyclopentane moieties

Lipid species	Membrane vesicles	Whole cells	
	ng/mg dry weight		
GTGT-0	1.3	1.1	
GDGT-0	3.0	4.3	
GDGT-1	5.5	4.6	
GDGT-2	20	14	
GDGT-3	89	48	
GDGT-4	404	217	
GDGT-4 isomer	7.0	9.0	
GDGT-5	73	89	
GDGT-6	8.4	21	
GDGT-7	0	0	
Total amount GDGTs	610	407	
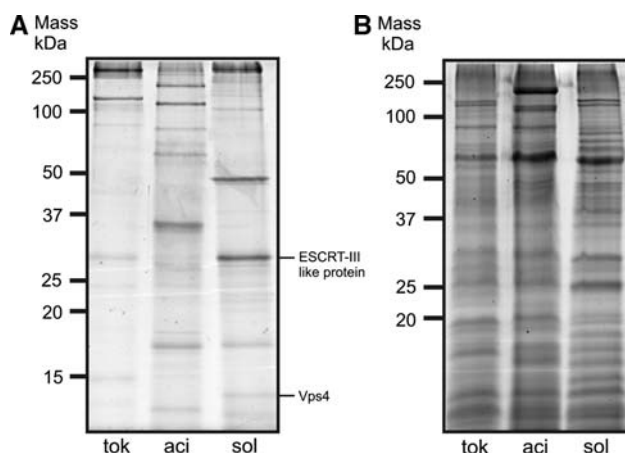
Fig. 2 SDS-PAGE of the CsCl-gradient enriched membrane vesicle fraction (**a**) and cytoplasmic membranes (**b**) of *S. solfataricus* (sol), *S. acidocaldarius* (aci) or *S. tokodaii* (tok). The buoyancy density of the fractions was <0.7 g/ml. The position of the archaeal ESCRT-III like proteins are indicated. Vps4 is only visible in the *S. solfataricus* fraction

play a role in a variety of processes, such as DNA damage response or signaling pathways required for virulence (Curry et al. 2005; Kolas et al. 2007).

As expected from the EM, SDS-PAGE and mass spectroscopy analysis, the membrane vesicles of all three *Sulfolobus* spp. contain S-layer proteins. In addition, the membrane vesicles contain a protein homologous to thiosulfate sulfur transferase. These proteins catalyze the transfer of thiosulfate to sulfur acceptors like cyanide, and function in the protection of the respiratory chain from inhibition by cyanide and/or H_2S (Billaut-Laden et al. 2006). Finally, a dipeptide-binding protein (DppA) was identified in these vesicles which together with the S-layer proteins, are the proteins found in all three species that are synthesized as a precursor with a N-terminal signal peptide which directs the protein to the secretion machinery. DppA is a cell envelop protein involved in substrate uptake.

Two additional proteins were identified in the SDS-PAGE of only the *S. acidocaldarius* membrane vesicles. These are a flottilin (Saci_1749) and a pyridine nucleotide-

Table 2 Proteins identified by mass spectrometry in membrane vesicle fraction of *Sulfolobus solfataricus*, *S. acidocaldarius* and *S. tokodaii*. Proteins identified in all three species are in bold

Functional group	Annotation	ORF number	In gel	LC-MS
Transport	Oligopeptide-binding protein	**SSO2619**	+	+
		Saci_1038	+	+
		ST2539	+	+
		Saci_1760	+	+
		ST0706	+	+
		SSO3043		+
		SSO2669		+
		SSO1273		+
	Sugar-binding protein	Saci_1165	+	+
		SSO0999		+
		SSO3053		+
	ABC transporter ATP-binding protein	ST0700		+
		Saci_1765		+
	Na+/proline symporter	SSO2476		+
	Putative amino acid transporter	SSO3189		+
		ST1607		+
	Predicted transporter	SSO3184		+
		ST2360		+
	Shikimate transporter	ST2618		+
		Saci_2282		+
Energy and metabolism	Protease	SSO2088		+
		ST2611		+
		SSO2551		+
		ST2492		+
	Putative thiazole biosynthetic enzyme	ST0361		+
		Saci_0854		+
	Glutamate/leucine dehydrogenase	SSO1907		+
		ST2241		+
	Acetyl-coenzyme A synthase	Saci_0306		+
		SSO3203		+
	V-type ATPase subunit b	ST1437		+
		Saci_1549		+
	Predicted ATPase	SSO2277		+
		ST2240		+
	Carbon monoxide dehydrogenase	SSO3009		+
		Saci_2271		+
	NAD(FAD)-dependent dehydrogenase	Saci_0331	+	+
	Glutaredoxin-related protein	SSO0192		+
	Metal-dependent hydrolase	SSO0099		+
	L-lactate dehydrogenase	SSO2585		+
	Succinate dehydrogenase	Saci_0982		+
	Cytochrome/quinol oxidase subunit II	Saci_2089		+
	V-type ATPase subunit I	SSO0559		+
	Malate synthase	SSO1334		+
	Thioredoxin reductase	SSO2222		+
	PaaI_thioesterase	SSO2140		+
	Rubyerithrin	Saci_2272		+
	Cytochrome b558/566, subunit A	ST1664	+	+

Table 2 continued

Functional group	Annotation	ORF number	In gel	LC-MS
	Serine/Threonine protein kinase	ST1565		+
	α-amylase	SSO1172	+	+
	Phenylacetyl-CoA ligase	ST2420		+
Cell surface	Large S-layer protein	SSO0389	+	+
		Saci_2355	+	
	Small S-layer protein	ST2194		+
	Flagellin	Saci_1178		+
Transcription, translation and turnover	DNA/RNA-binding protein Alba	Saci_1322		+
	TBP interacting protein TIP49	SSO2450		+
	50S Ribosomal protein L10	SSO0344		+
	30S Ribosomal protein S3a	SSO0746		+
	30S Ribosomal protein S3	SSO0411		+
	Hsp20	SSO2427		+
	Elongation factor 1α	SSO0216		+
		Saci_0685		+
	Thermosome β	SSO0282		+
		Saci_0666		+
	Thermosome α	SSO0862		+
		Saci_1401		+
	Vps4 homolog	**SSO0909**	+	+
		ST1215		+
		Saci_1372		+
	AAA family ATPase	ST0209		+
		Saci_0773/Saci_0838		+
		ST2584		+
Structural	von Willebrand factor type A domain	**SSO1091**		+
		ST0830		+
		Saci_1211		+
		ST0828		+
	Forkhead-associated domain	**SSO1088**		+
		ST0829		+
		Saci_1210		+
Stress	Sulfur transferase	**SSO1817**		+
		ST2564		+
		Saci_2198		+
	Superoxide dismutase	SSO0316		+
Other	Archaeal ESCRT-III like proteins	**SSO0881**	+	+
		ST1237	+	+
		Saci_1416	+	+
		SSO0451		+
		ST0168		+
		Saci_0451	+	+
		SSO0619		+
	Flotillin	Saci_1749	+	+
	Hypothetical protein	Saci_1272	+	+
		ST1599		+
		SSO2748		+
		ST1712		+

Table 2 continued

Functional group	Annotation	ORF number	In gel	LC-MS
		SSO1175		+
		SSO2633		+
		SSO2602		+
		Saci_2171		+
		Saci_1920		+
		ST1876		+
		Saci_1803		+

disulfide oxidoreductase (Saci_0331). In eukaryotes flotti-lins are often found to be lipid-raft associated (Neumann-Giesen et al. 2004), but the archaeal protein lacks the conserved cysteines which in rat represent the sites of modification to anchor the protein to the membrane. The pyridine nucleotide-disulfide oxidoreductase contains a NADH and FADH-binding domain.

Immunolabeling and localization of the Vps4 homolog in *S. solfataricus*

In eukaryotes, the ESCRT-III proteins and the AAA family ATPase Vps4 have been proposed to act in concert in the creation and constriction of membrane "necks" that arise during MVB vesicle formation (Kieffer et al. 2008). Assuming that the archaeal ESCRT-III like proteins and the archaeal Vps4 homolog function in a similar way, they would be predicted to function at the membrane. To gain insight in the cellular localization of the archaeal Vps4 ATPase, *S. solfataricus* cells were immunogold labeled with an antibody directed against Vps4 homolog, SSO0909. The labeling observed was mostly at the periphery of the cell (Fig. 3) indicating that the ATPase localizes to the membrane. Only weak labeling in the cytoplasm was observed.

Discussion

Here we report that *Sulfolobus* sp. release membrane vesicles with a unique protein composition. The phenomenon of membrane vesicle release has previously been reported for *S. islandicus* (Prangishvili et al. 2000), but the protein and lipid composition of these vesicles were not determined. Those vesicles yielded a diffraction pattern which indicates a crystalline element with a periodicity of 22 nm corresponding to the lattice constant of the *Sulfolobus* S-layer. We now show that the membrane vesicle-like structures indeed contain the major S-layer protein and that they are enriched in the typical archaeal tetraether cytoplasm membrane lipids. Moreover, analysis of their protein composition reveals the presence of proteins that are
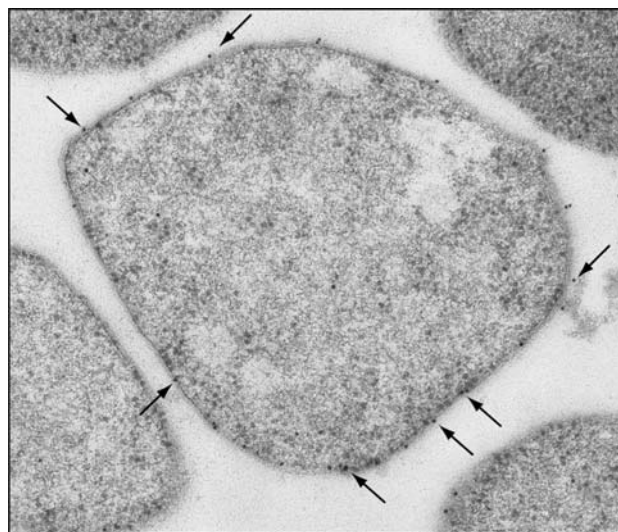
Fig. 3 Immunogold labeling of uranyl acetate stained *S. solfataricus* cells with an antibody directed against the Vps4 homolog, SSO0909. *Arrows* indicate gold particles visible as *black dots* that localize the cell periphery

homologous to components of the endosomal sorting pathway of eukaryotes. In all three *Sulfolobus* spp. at least two proteins were identified that are homologous to eukaryotic ESCRT-III proteins together with an archaeal Vps4-like ATPase.

The similarity of the *Sulfolobus* vesicles to eukaryotic exosomes is striking. Both are extracellular membrane vesicles that contain a Vps4 protein. Furthermore, in eukaryotes components of the core ESCRT-III complex were identified in exosomes (Pisitkun et al. 2004). Together with the ESCRT-I and ESCRT-II protein complexes, ESCRT-III belongs to the group of class E vacuolar protein sorting (Vps) proteins (Babst et al. 2002). Vps class E proteins are essential for the formation of MVBs, which are late endosomal structures that either release their protein cargo into vacuoles/lysosomes upon fusion (Babst et al. 2002) or expel it from the cell as exosomes (Vella et al. 2007). In eukaryotes, the ATPase Vps4 interacts with ESCRT-III subunits to release the complex from the

endosomal membrane in an ATP-dependent manner (Babst et al. 1998, 2002; Shim et al. 2007). ESCRT-III and Vps4 are thought to be cooperatively involved in creating membrane buds (Kieffer et al. 2008). The discovery of a putative AAA family ATPase homologs to eukaryotic Vps4 proteins in conjunction with the archaeal ESCRT-III like proteins suggests that the mechanism of outward budding in *Sulfolobus* resembles the mechanism of inward budding at the endosomal membrane.

Compared to the cytoplasmic membrane, the membranes of the vesicles seem to contain a reduced content of lipids with cyclopentane rings. Since the majority of the vesicles are produced during the exponential growth phase, the observed difference between the vesicles and membranes of stationary phase grown cells could be a growth phase-dependent phenomenon rather than a significant difference in lipid composition. Overall, we therefore conclude that the lipid composition of the vesicles and cytoplasmic membranes is essentially similar providing strong support for the notion that these vesicles are derived from the cytoplasmic membrane. *Sulfolobus* cells are surrounded by a crystalline layer of surface protein and it is unclear if a mechanical or physical destabilization cytoplasmic membrane would be sufficient to also deform the S-layer allowing the vesicles to 'escape' from the cell envelop. The presence of the archaeal Vps4 ATPase in the vesicles and its localization at the periphery of the cell suggest it may be actively involved in formation of the vesicles, possibly by delivering the energy needed for budding of the cytoplasmic membrane and/or bulging of the S-layer to allow the expulsion of a S-layer coated membrane vesicles.

The core of eukaryotic ESCRT-III consists of four components with predicted highly similar secondary structures and similarity in their primary sequences (Babst et al. 2002; Shim et al. 2007). Bio-informatic analysis indicates that the archaeal ESCRT-III like proteins are dispersed across four groups (Obita et al. 2007) and perhaps the ESCRT-III core is evolutionary conserved between eukaryotes and archaea. The crystal structure of the human ESCRT-III protein CHMP3 revealed a core of five α-helices (Muziol et al. 2006), while a predicted sixth α-helix is located near the C-terminus (Shim et al. 2007). Based on α-helical predictions with the JPRED program, a core of five α-helices is also predicted for all *S. solfataricus* ESCRT-III like proteins (Fig. 4). However, the loop connecting the fifth and fourth α-helix is shorter in the *Sulfolobus* ESCRT-III like proteins compared to the human ortholog (Fig. 4). Alignment of human CHMP3 with *S. solfataricus* ESCRT-III like proteins indicates similarities in the range of 19–32%. The archaeal ESCRT-III like protein SSO0910 (not identified here) is part of an operon together with a protein of unknown function (SSO0911) and the identified Vps4 ATPase (SSO0909). To date this is the only archaeal ESCRT-III like protein that was shown to interact with an archaeal Vps4 homologue (SSO0909) (Obita et al. 2007). Interestingly, the archaeal ESCRT-III like protein identified in the vesicle fractions all lack the sixth C-terminal α-helix, which in eukaryotes functions as a regulatory domain (Shim et al. 2007). In eukaryotes, the removal of the regulatory domain results in permanent recruitment of ESCRT-III proteins onto endosomes. Perhaps due the absence of the sixth α-helix, the identified archaeal ESCRT-III like proteins are permanently membrane associated.

At the moment it is not possible to assign a definite function to the vesicles. The vesicles may be the end-product of a stress response in which the identified FHA-protein in conjunction with the vWA-containing proteins are part of a signaling cascade. In eukaryotes, such domains play an important role in the formation of protein aggregates (Whittaker and Hynes 2002) and protein-protein interactions (Smolka et al. 2006) and are involved in various types of stress responses (Kolas et al. 2007). Also of interest is the observation that the thiosulfate sulfur transferase, which catalyses the transfer of thiosulfate to sulfur acceptors like cyanide, is present in the membrane vesicle fractions of all three *Sulfolobus* sp. This protein is believed to protect the respiratory chain from inhibition by cyanide and/or H_2S and it may not only have a protective role on a cellular level but also at an extracellular level when associated with the vesicles (Billaut-Laden et al. 2006). Finally, the vesicles could also be involved in cell adhesion and/or biofilm formation based on the fact that vWA domain proteins are known to play pivotal roles in extracellular matrix formation and cell adhesion (Tuckwell 1999). Whether the vesicles are involved in cell to cell communication remains an open question. For instance, conjugation has been reported in *S. acidocaldarius* demonstrating that *Sulfolobus* cells can interact intimately (Schmidt et al. 1999). However, we could not detect any specific DNA associated with the membrane vesicles (unpublished results). In contrast, in a recent report on virus-like vesicles derived from Thermococcales it was suggested that these specifically contain DNA (Soler et al. 2008). In this respect it would be of interest to analyse the proteome of these vesicles and compare them to the vesicles derived from crenarchaeota.

In conclusion, the released membrane vesicles are equipped with a unique composition with proteins predicted to function in extracellular matrix formation and/or adhesion, and possibly in detoxification. Moreover, the presence of homologs of eukaryotic ESCRT proteins as major constituents of these membrane vesicles suggests a specific mechanism of formation, possibly with similarity to the endosomal vesicle sorting pathway in eukaryotes. Future studies should address the mechanisms of vesicle release from the cell envelope of *Sulfolobus* and their

Fig. 4 Alignment of four *S. solfataricus* ESCRT-III like proteins and human CHMP3. The N-terminal halves were aligned with the multiple sequence alignment program CLUSTAL W (1.81), selected weight matrix BLOSUM, whereas the fifth and sixth helices were aligned manually. Conserved residues are indicated by an *asterisk*; similar residues are indicated by a *double dot*; and residues with weak similarity are indicated by a *single dot*. Similarity (sim) indicated in percentages. JPRED predicted α-helices are indicated in *italics* in the *S. solfataricus* ESCRT-III like proteins, whereas the underlined α-helices of the human CHMP3 protein were taken from the crystal structure, except for the sixth α-helix which was predicted by JPRED

```
SSO0619     ------------------------------------------------------------
SSO0881     ------------------------------------------------------------
SSO0451     MFPSVFIFTLLYSSSFHLRSKYITTLSVDTKMSYSGSSSSLMNVFGILLISDSNNLNSSH
SSO0910     ------------------------------------------------------------
CHMP3-HUMAN ------------------------------------------------------------

                                                            helix I
                       α α                     α α α α α α α α α α
SSO0619     --------------------------------------MKKKGK SLAELLIDVRIARNKV
SSO0881     ----MASSK VEDFVK NWGGKQEPSIGERIKnafkPQ---QPL RYRLVMANYRLRTMVSRL
SSO0451     YVFDLIRVD EVRYILAMLGKDFQKMWGYEENKFKIKGSKEP LKYKLVQAHYKISSMISRL
SSO0910     ------MFDK LPFIF NNEKRRK----------------AQLGKILTEISLKLKDQQTRL
CHMP3-HUMAN ---------MGL FGKTQE--------------------KP PKELVNEWSLKIRKEMRVV
                                                        :        :        :

                helix I                                    helix II
            α α α α α α α α α α     α α α α α α α α α α α α α α α α α α
SSO0619     QNIINRMQNKIG-TYNHIFMRNVASFP HLSKMVARESELLENVMDHLLTLEVILEILEIK
SSO0881     DVYISRLQERDRTLFEKVVESQMSKDTARAAMYANEIAEIRKISRQLITTQIALEQVQLR
SSO0451     DAYISRMQERDKILFERVVEAQMSKDTQRAAMYANEVAEIRKISRQLLTTQIALEQVQLR
SSO0910     EEAIRRLKDRDKELFEKVVRAQVEGDDAKAKIYAQEIADIRRIIKVIYTAFLAIEKVRLK
CHMP3-HUMAN DRQIRDIQREEEKVKRSVKDAAKKGQKDVCIVLAKEMIRSRKAVSKLYASKAHMNSVLMG
                :    *   :: .        . :              . : *.*    ..    : :     :: : :

                             helix III              helix IV
            α α α α α             α α α α α α α
SSO0619     IETIIYIGNIVTSAA SVVEAIKLLKESFN-LTP DISLLLDDIYSNFYVNVDLPKEIKIN-
SSO0881     LETITELGDV FNSLIPVLGVIKELRNAMKGV MPEISLELAELEEGLQEVVIEAGDFTGAP
SSO0451     LETVTELGDVFVN LIPVLGVIGELKSALRGIM PEVSLELTELGEGLQEIVTESGEFTGMG
SSO0910     LDTVQELQGVSLVLYPVAKILGDLKDQ IKGIA PEVAIALDSIISSVNGIAVETGAINDRG
CHMP3-HUMAN MKNQLAVLRVAGSLQKST EVMKAMQSLVK--IPEI QATMRELSKEMMKAGIIEEMLEDTF
                :.. :  :              : ::. ..    *:: : .:.  .            :

                          helix V
            α α α α α α α α                                      α
SSO0619     ------------V KEEARSVLLDAEKIAEKRK SETYY------------QVNT------
SSO0881     -------ANYGASS PEARKILEEASVVAEQRMKEK FPE-LPSFVTSTQKVSNQEQK----
SSO0451     -------SYAAASS PEARKILEEASVVAEQRMKEK FPD-LP-VGTGLASKSNS-------
SSO0910     -------VVPAVV DEQARQILDEAQKMAEVKVRELLPD-LPHPPVEQSSRASQAKPTI RK
CHMP3-HUMAN ESMDDQEEMEEEAEMEIDRILFEITAGALGKAPSKVTDALPEPEPPGAMAASEDEEEE EE
                                .:. :* ::. .*  :

               helix VI
            α α α α α α      α α      α α α α α α α                sim
SSO0619     --------------------------------------------- 168 aa     32%
SSO0881     --------------------------------------------- 221 aa     24%
SSO0451     --------------------------------------------- 284 aa     19%
SSO0910     ITERELLDYIVNNGGFLDIEHFSKVYGVEKQEVVKLLEALKNKGLIAVES 259 aa 21%
CHMP3-HUMAN ALEAMQSRLATLRS------------------------------- 222 aa     24%
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function in the cellular physiology. Herein we will target the genes encoding the ESCRT proteins for inactivation and assess their role in membrane vesicle formation.

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