1 Supplementary information file

2 Biopearling of interconnected outer membrane vesicle chains by a marine 3 flavobacterium

4

5 Tanja Fischer[1], Martin Schorb[2], Greta Reintjes[3], Androniki Kolovou[2], Rachel Santarella-6 Mellwig[2], Stephanie Markert[4], Erhard Rhiel[5], Sten Littmann[6], Dörte Becher[7], Thomas 7 Schweder[4], Jens Harder[1*]

8

9 [1]Department of Microbiology, Max Planck Institute for Marine Microbiology, Bremen, 10 Germany

11 [2]Electron Microscopy Core Facility, EMBL Heidelberg, Heidelberg, Germany

12 [3]Department of Molecular Ecology, Max Planck Institute for Marine Microbiology, Bremen, 13 Germany

14 [4]Pharmaceutical Biotechnology, University of Greifswald, Greifswald, Germany

15 [5]Institute for Chemistry and Biology of the Marine Environment, Carl von Ossietzky 16 University of Oldenburg, Oldenburg, Germany

17 [6]Department of Biogeochemistry, Max Planck Institute for Marine Microbiology, Bremen, 18 Germany

19 [7]Department for Microbial Proteomics, University of Greifswald, Greifswald, Germany

20

21 [*] Address correspondence to Jens Harder, jharder@mpi-bremen.de

22

23 **This supplementary information file contains:**

24 Supplementary methods

25 Supplementary tables S1-S7

26 Supplementary figures S1-S12

27

28 Supplementary methods:

29 Preparation of outer membranes

30 For outer membrane preparation following Shetty *et al*. (2011), the cell pellet was
31 resuspended in 5 ml of KCl/Tris buffer (100 mM KCl, 50 mM Tris, pH 7.5) and sonicated for
32 6 min on ice (6 pulses of 1min, 30 W, Bandelin Sonoplus HD70, Bandelin, Berlin, Germany).
33 The lysate was centrifuged at 10 000x *g* for 10 min in a Beckman 70.1 Ti rotor (Beckman L-
34 70 ultracentrifuge, Beckman Coulter, Brea, USA) and the supernatant was filtered through a
35 0.2 µm filter. The filtrate was centrifuged at 40 000x *g* (Beckman 70.1 Ti rotor) for 60 min.
36 The pellet was resuspended in 1 ml 2% (w/v) sarkosyl, incubated at 21 °C for 30 min and
37 centrifuged at 40 000x *g* (Beckman 70.1 Ti rotor) for 90 min. The OM pellet was dissolved in
38 the aforementioned KCl/Tris buffer. After Møller *et al.* (2005) a whole cell pellet was
39 resuspended in 6 ml buffer (20 mM Tris/HCl, 16 mM EDTA, pH 8) and disrupted by
40 sonication (4 pulses of 1 min, 30 W). Undisrupted cells were removed by centrifugation at
41 8000x *g* for 10 min (Beckman 70.1 Ti rotor) and the supernatant was centrifuged at 20 590x *g*
42 for 1.5 h. The pellet containing both membranes was resuspended in 1 ml 0.5% (w/v) sarkosyl
43 in 20 mM Tris/HCl (pH 7.2) and kept on ice for 20 min until centrifugation at 20 590x *g* for
44 1 h. This step was repeated, before the OM pellet was washed twice in the aforementioned
45 KCl/Tris buffer. OM isolation after Koßmehl *et al.* (2013) was performed by resuspending
46 whole cells in 1 ml lysis buffer (100 mM Tris/HCl (pH 7.5), 10% glycerol, 0.5 mM DDT) and
47 250 units DNase I and disrupting the cells using a One shot (Constant systems, Daventry,
48 UK). Cell debris was removed by centrifugation 4390x *g* for 15 min. The supernatant
49 received 20 ml of ice-cold 100 mM Na_2CO_3 and was incubated stirring on ice for 1.5 h. The
50 total membrane fraction was collected by ultracentrifugation at 200 000x *g* for 1 h (Beckman
51 70.1 Ti rotor). The membrane pellet was resuspended in 1 ml lysis buffer (see above) and
52 ultracentrifuged at 104 000x *g* for 1 h. The pellet was resuspended in 600 µL 1% sarkosyl

(w/v) in the KCl/Tris buffer and incubated 40 min at 1400 rpm and 22 °C. The suspension was ultracentrifuged at 104 000x *g* for 1 h. The OM pellet was resuspended in 200 µL IEF buffer (7 M urea, 2M thiourea, 4% w/v CHAPS, 30 mM Tris pH 8) to solubilize OM proteins. Insoluble material was removed by centrifugation at 20 000x *g* for 30 min. According to McCaig *et al.* (2013), a cell pellet was resuspended in 20 ml of the above mentioned KCl/Tris buffer and the suspension was centrifuged at 5000x *g* for 30 min. The pellet was resuspended in 10 ml 20 mM Tris/HCl (pH 8) and centrifuged at 10 000x *g* for 20 min. Then, the pellet was resuspended in 1 ml of a mixture of 20 mM Tris, 20% (w/v) sucrose, 15 mM EDTA and 200 µg lysozyme and incubated on ice for 40 min before 26 mM $MgCl_2$ x 6 H_20 and 200 units DNase I were added and incubated 20 min on ice. Spheroblasted bacteria were pelleted at 10 000x *g* for 20 min, afterwards resuspended in 1 ml KCl/Tris buffer and sonicated (4 pulses, 1 min each, 30 W). The suspension was centrifuged at 8000x *g* for 10 min to remove unbroken cells. The supernatant was ultracentrifuged at 100 000x *g* (Beckman 70.1 Ti rotor) for 1 h to pellet the membranes. The membrane pellet was resuspended in 20 mM Tris/HCl pH 8 with 0.5% sarkosyl (w/v) to solubilize the cytoplasmic membrane. The suspension was ultracentrifuged at 100 000x *g* for 1 h to pellet the OM. After method 1 of Thein *et al.* (2010) a whole cell pellet was resuspended in 500 µL 0.2 M Tris/HCl (pH 8), 1 M sucrose, 1 mM EDTA and 100 µL of lysozyme (5 mg/ml). The mixture was vortexed and incubated for 5 min at 21 °C, and then 2 ml water was added and incubated for 20 min to generate spheroblasts. A solution of 3 ml 50 mM Tris/HCl pH 8, 2% sarkosyl, 10 mM $MgCl_2$ and 150 units DNase I were added and incubated for 20 min. Ultracentrifugation at 85 000x *g* (Beckman 70.1 Ti rotor) for 30 min yielded the OM pellet, which was washed in 750 µL 50 mM Tris/HCl (pH 8), 2% w/v sarkosyl, 10 mM $MgCl_2$ and centrifuged as aforementioned. The pellet was washed three times with 500 µL water and the final OM pellet was resuspended in 500 µL of the KCl/Tris buffer. Following method 2 of Thein *et al.* (2010) whole cells were resuspended in 6 mL 0.1 M Tris/HCl (pH 7.3) and 150 units DNase I. Cells were lysed by French™ pressing (SLM Aminco R FA-078, SLM instruments, Urbana, USA) three times at 8.6 MPa and the cell lysate was centrifuged at 4390x *g* for 15 min. Seven mL supernatant were mixed with ice-cold 0.1 M Na_2CO_3 (pH 11) to a final volume of 70 mL and stirred for 1 h at 4 °C. The OM was obtained by ultracentrifugation at 120 000x *g* (Beckman 70.1 Ti rotor) for 1 h. The OM fraction was washed three times by an ultracentrifugation at 85 000x *g* for 20 min and suspended in 500 µL water. Finally the OM was suspended in 500 µL KCl/Tris buffer. OM after Kulkarni *et al.* (2014) was obtained by cell lysis: a cell pellet was dissolved in 5 mL of 20% w/v sucrose and 30 mM Tris (pH 8.0), 0.3 mg lysozyme and 50 units DNase I. The

87 suspension was sonicated for 6 min on ice (6 pulses of 1min, 30 W, Bandelin Sonoplus
88 HD70). Cell debris was removed by centrifugation (4390x *g*, 10 min). The supernatant was
89 applied on a sucrose gradient (20-70% w/v) and centrifuged at 158 000x *g* for 5 h (Beckman
90 70.1 Ti rotor).
91
92 **Preparation of vesicle fractions**
93 Vesicles were prepared by several techniques. Following Kulkarni *et al.* (2014), cultures were
94 shaken by hand for 10 s and then cells were pelleted in 50mL-tubes for 15 min at 4390x *g* and
95 4 °C. The supernatant was filtered through a 0.2 µm syringe filter and ultracentrifuged at
96 142 019x *g* for 2 h at 4 °C in a Beckman 70.1 Ti rotor. Pelleted vesicles were resuspended in
97 the KCl/Tris buffer. According to Shetty *et al.* (2011) cultures were shaken for 30 s and cells
98 removed by centrifugation with 4390x *g* for 20 min. The supernatant was filtered twice
99 through a 0.2 µm filter and was centrifuged for 60 min at 15 000x *g* (Beckman 70.1 Ti rotor).
100 The vesicles were obtained by ultracentrifugation at 140 000x *g* for 2 h. For the method
101 according to Møller *et al.* (2005), vesicles were removed from a cell pellet by resuspension in
102 the KCl/Tris buffer. After removal of the cells by centrifugation at 6000x *g* for 15 min
103 (method1) or 8000x *g*, 10 min (Beckman 70.1 Ti rotor) (method 2), the supernatant was
104 filtered through a 0.2 µm filter and centrifuged at 20 590x *g* for 100 min (Beckman 70.1 Ti
105 rotor). The pellet was washed twice with the KCl/Tris buffer. Vortexing for 30 s was the
106 mechanical stress to resolve vesicles according to Remis *et al.* (2014), followed by cell
107 removal (5000x *g*, 10 min), filtration through a 0.2 µm filter and ultracentrifugation at
108 140 000x *g* for 1 h (Beckman 70.1 Ti rotor). McCaig *et al.* (2013) included after cell pelleting
109 a clarification at 7500x *g* for 20 min (Beckman 70.1Ti rotor) before filtration through a
110 0.2 µm filter and ultracentrifugation at 100 000x *g* for 1h (Beckman 70.1 Ti rotor). The pellet
111 was washed twice with 1 mL of the KCl/Tris buffer.
112
113 **Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**
114 SDS-PAGE was conducted to visualize protein patterns of different samples of outer
115 membrane and vesicle preparations. Proteins were separated in a 12% separation gel (1.75 mL
116 water, 1.25 mL 4x separation buffer (1.5 M TRIS pH 8.8, 0.4% w/v SDS), 2 mL 30%
117 acrylamide/bis-acrylamide solution 19:1, 50 µL 10% (w/v) ammoniumpersulfate (APS), 5 µL
118 tetramethylethylenediamine (TEMED)) and a 4% stacking gel (1.41 mL water, 0.75 mL 4x
119 stacking buffer (0.5 M Tris pH 8.8, 0.4% w/v SDS), 0.33 mL 30% acrylamide/bis-acrylamide
120 solution 19:1, 30 µL 10% (w/v) APS, 3 µL TEMED) using the Bio-Rad (Bio-Rad, Munich,

121 Germany) system. Prior to loading on the gel, 20 µL of sample was mixed with 6.5 µL
122 loading buffer (0.2 M Tris/HCl pH 6.8, 10% w/v SDS, 10 mM DTT, 20% w/v glycerol,
123 0.05% w/v bromophenol blue), heated to 95°C for 5 minutes and centrifuged shortly (13817x
124 *g*, 1 min). The PageRuler Unstained Protein Ladder (Fermentas, Burlington, Canada) was
125 used to calibrate the size of the protein bands. Gel electrophoresis was performed for 20
126 minutes at 70 V to obtain a uniform front and a voltage of 110 V was applied until the front
127 reached the end of the gel. The gel was stained for 1.5 h with Coomassie staining solution (0.
128 025% (w/v) Coomassie G 250; 10% (v/v) glacial acetic acid; 40% (v/v) ethanol) and excess
129 color was removed with 20% ethanol for several hours. The gel was washed in water for 1h
130 before it was documented using a scanner (Image Scanner, Amersham Biosciences).
131

132 **Matrix-assisted laser desorption/ionization - time of flight - mass spectroscopy (MALDI-**
133 **TOF-MS)**
134 Protein bands of interest were cut out of an SDS-gel and placed into an Eppendorf tube
135 containing 20 µL PCR-water. The proteins in the gel were digested with trypsin and applied
136 onto a MALDI-target (Ettan Spot Handling Workstation, GE Healthcare, Little Chalfont,
137 UK). The analysis using tandem mass spectrometry (MS/MS) was conducted with a 4800
138 MALDI-TOF/TOF Analyzer (Applied Biosystems, Darmstadt, Germany). Masses from 900
139 to 3700 Da were detected. The detected peptides were matched onto the *in silico* predicted
140 peptides from the genome of strain Hel3_A1_48. Open reading frames were predicted by
141 RAST (Aziz *et al.*, 2008). The sequences of the detected ORFs were blasted with PSIBLAST
142 (Altschul *et al.*, 1990) using the BLOSUM 80 matrix against the NCBI database of non-
143 redundant protein sequences and cross-checked with the pfam database (Punta *et al.*, 2012).
144

145 ## Supplementary tables:

146 **Supplementary Table S1:** MALDI-TOF-MS protein identification of strain Hel3_A1_48
147 OM [A] and vesicle/appendage preparations [B]. Numbers on the left are corresponding to the
148 numbered, excised and analyzed protein bands of the SDS-PAGE (Fig. S10). Annotation
149 according to NCBI and Blast.

Band	Protein ID	Annotation
A		
1	AOR25966	TonB-dependent receptor
2	AOR26448	TonB-dependent receptor
3	AOR25288	Protein with peptidoglycan binding domain similar to OmpA
4	AOR25711	Transporter
5	AOR25254	OM-channels superfamily, putative beta-barrel porin-2
6	AOR26266	TonB-dependent receptor
7	AOR25738	Protein with unknown function
8	AOR25929	Phosphopyruvate hydratase (enolase)
9	AOR26723	OM-channels superfamily, putative beta-barrel porin-2
10	AOR25530	Elongation factor Tu
11	AOR26932	Molecular chaperone Dnak, HSP 70
B		
1	AOR25966	TonB-dependent receptor
2	AOR26448	TonB-dependent receptor
4	AOR25711	Transporter
5	AOR25254	OM-channels superfamily, putative beta-barrel porin-2
6	AOR26266	TonB-dependent receptor
7	AOR25738	Protein with unknown function
9	AOR26723	OM-channels superfamily, putative beta-barrel porin-2
12	AOR25680	T9SS C-terminal target domain-containing protein with lamin tail domain
13	AOR25736	Gliding motility protein GldJ
14	AOR26642	Gliding motility protein GldK
15	AOR26575	DUF5017 domain protein
16	AOR26645	Gliding motility protein GldN
17	AOR25720	Hypothetical protein
18	AOR26233	DUF2490 domain-containing protein, outer membrane beta barrel superfamily
19	AOR26075	TonB-dependent receptor
20	AOR26302	Protein with peptidoglycan binding domain similar to OmpA
21	AOR26574	TonB-dependent receptor
22	AOR26571	TonB-dependent receptor
23	AOR25658	Peptidase M1 family containing bacterial aminopeptidase N

150

151

152

153 **Supplementary Table S2:** Subcellular localization of proteins of strain Hel3_A1_48 based
154 on PSORTb predictions using the amino acid sequences encoded in the genome and the
155 numbers of identified proteins in the four different fractions analyzed in this study. VE vesicle
156 fraction exponential phase, VS vesicle fraction stationary phase, ME membrane fraction
157 exponential phase, MS membrane fraction stationary phase.

	Genome	VE	VS	ME	MS
Total proteins	1866	374	285	1310	1075
Cytoplasm	895	136	73	748	642
Cytoplasmic membrane	319	34	14	190	136
Extracellular	29	14	13	21	14
Outer membrane	74	59	58	66	65
Periplasm	17	8	9	14	12
Unknown	532	123	118	271	206

158

159

160

161 **Supplementary Table S3**: Abundant proteins of strain Hel3_A1_48. Relative protein
162 abundance (NSAF%) values for proteins of each of the four fractions are listed. The sum of
163 NSAF% values for all proteins in each fraction, for proteins with an NSAF% ≥ 0.4 per
164 fraction and the localization of these proteins (NSAF% ≥ 0.4) according to PSORTb 3.0.2 are
165 shown. Other percentages were calculated based on the abundant proteins (NSAF% ≥ 0.4)
166 representing 100%. VE vesicle fraction exponential phase, VS vesicle fraction stationary
167 phase, ME membrane fraction exponential phase, MS membrane fraction stationary phase.

	VE	**%**	**VS**	**%**	**ME**	**%**	**MS**	**%**
All proteins	96.11		99.27		99.98		99.82	
Proteins NSAF% ≥ 0.4	59.83	100	74.34	100	28.35	100	32.00	100
Cytoplasm	8.23	13.8	6.46	8.7	11.87	41.9	7.25	22.7
Cytoplasmic membrane	6.60	11.0	8.11	10.9	3.70	13.1	4.97	15.5
Extracellular	1.60	2.7	1.25	1.7	0	0	0.64	2
Outer membrane	21.94	36.7	27.42	36.9	5.82	20.5	6.82	21.3
Periplasm	0	0	1.35	1.8	0	0	0.41	1.3
Unknown	21.46	35.9	29.76	40.0	6.96	24.6	11.92	37.3

168

169

170 **Supplementary Table S4:** Selected marker proteins for subcellular localizations: Detected
171 proteins from the proteome were analyzed with PSORTb to predict their localization. From
172 that, a minimum of 10 proteins for each localization were picked and literature search was
173 performed to confirm the localization. The sums of the NSAF% values of the marker proteins
174 for each localization were calculated for all four samples. They were used to calculate the
175 ratios for the abundance of proteins from each localization in the four samples (presented in
176 table S7). VE vesicle fraction exponential phase, VS vesicle fraction stationary phase, ME
177 membrane fraction exponential phase, MS membrane fraction stationary phase.

Accession Number	Annotation	VE	VS [NSAF%]	ME	MS	Localization PSORTb
AOR25932	DNA-directed RNA polymerase subunit alpha	0.142	0	0.254	0.225	Cytoplasmic
AOR26673	Glutaminyl-tRNA synthetase	0.106	0.170	0.129	0.229	Cytoplasmic
AOR25523	DNA-directed RNA polymerase subunit beta	0.083	0	0.225	0.218	Cytoplasmic
AOR25522	DNA-directed RNA polymerase subunit beta prime	0.077	0.010	0.335	0.215	Cytoplasmic
AOR26332	Phenylalanyl-tRNA synthetase subunit alpha	0.048	0	0.114	0.093	Cytoplasmic
AOR26899	Histidyl-tRNA synthetase	0.044	0	0.068	0.105	Cytoplasmic
AOR26378	Methionyl-tRNA formyltransferase	0.031	0	0.028	0.088	Cytoplasmic
AOR26094	Glycyl-tRNA synthetase	0.027	0	0.038	0.112	Cytoplasmic
AOR25945	50S ribosomal protein L5	0.271	0	0.601	0.339	Cytoplasmic
AOR25961	30S ribosomal protein S7	0.267	0	0.271	0.151	Cytoplasmic
AOR26257	30S ribosomal protein S2	0.219	0	0.568	0.323	Cytoplasmic
AOR25943	30S ribosomal protein S8	0.214	0	0.187	0.160	Cytoplasmic
AOR26083	50S ribosomal protein L21	0.212	0	0.509	0.329	Cytoplasmic
AOR25935	30S ribosomal protein S13	0.201	0	0.204	0.100	Cytoplasmic
AOR25756	50S ribosomal protein L20	0.190	0.038	0.242	0.224	Cytoplasmic
AOR25526	50S ribosomal protein L1	0.181	0	0.456	0.223	Cytoplasmic
AOR25933	30S ribosomal protein S4	0.147	0	0.684	0.421	Cytoplasmic
AOR25525	50S ribosomal protein L10	0.135	0	0.120	0.120	Cytoplasmic
AOR25947	50S ribosomal protein L14	0.120	0	0.191	0.062	Cytoplasmic
AOR25515	50S ribosomal protein L19	0.118	0.039	0.263	0.114	Cytoplasmic
AOR26255	50S ribosomal protein L13	0.115	0	0.168	0.139	Cytoplasmic
AOR25242	30S ribosomal protein S1	0.096	0	0.264	0.103	Cytoplasmic
AOR26010	50S ribosomal protein L25	0.094	0	0.116	0.180	Cytoplasmic
AOR25957	50S ribosomal protein L3	0.091	0	0.211	0.249	Cytoplasmic
AOR25942	50S ribosomal protein L6	0.078	0	0.266	0.276	Cytoplasmic
AOR25941	50S ribosomal protein L18	0.073	0	0.123	0.125	Cytoplasmic
AOR25954	50S ribosomal protein L2	0.038	0	0.198	0.084	Cytoplasmic
Sum of NSAF%		**3.419**	**0.257**	**6.834**	**5.007**	Cytoplasmic
AOR26407	ATP F0F1 synthase subunit B	0.194	0.096	0.460	0.768	Cytoplasmic membrane

AOR25548	Cell division transporter, ATP-binding protein FtsE	0.100	0	0.090	0.092	Cytoplasmic membrane
AOR25815	Cell division protein FtsH	0.063	0	0.244	0.163	Cytoplasmic membrane
AOR26062	Cell division protein FtsX	0.051	0	0.068	0.063	Cytoplasmic membrane
AOR26972	Cell division protein FtsK	0.025	0	0.113	0.099	Cytoplasmic membrane
AOR25746	Biopolymer transporter ExbB	0.033	0	0.065	0.072	Cytoplasmic membrane
AOR26532	Cytochrome C	0.031	0.016	0.094	0.073	Cytoplasmic membrane
AOR26856	Lipid A export ATP-binding/permease protein MsbA	0.041	0	0.179	0.125	Cytoplasmic membrane
AOR25922	Methionine ABC transporter ATP-binding protein	0	0.017	0.075	0.117	Cytoplasmic membrane
AOR26714	ABC transporter, transmembrane region	0	0	0.043	0.012	Cytoplasmic membrane
AOR25703	ABC transporter, ATP-binding protein	0	0	0.044	0.011	Cytoplasmic membrane
AOR25921	ABC transporter, permease	0	0	0.045	0.019	Cytoplasmic membrane
AOR25559	ABC transporter, permease	0	0	0.040	0.057	Cytoplasmic membrane
AOR25371	ABC transporter, ATP-binding protein	0	0	0.036	0.017	Cytoplasmic membrane
AOR25233	Lipoprotein-releasing system ATP-binding protein LolD	0	0	0.117	0.130	Cytoplasmic membrane
AOR26251	Cytochrome oxidase subunit III	0	0	0.147	0.104	Cytoplasmic membrane
Sum of NSAF%		**0.539**	**0.130**	**1.861**	**1.923**	Cytoplasmic membrane
AOR26993	T9SS C-terminal target domain-containing protein	0.620	0.317	0.117	0	Extracellular
AOR25487	T9SS C-terminal target domain-containing protein	0.510	0.538	0.124	0.104	Extracellular
AOR25970	Peptidase, S8 family	0.393	0.355	0.125	0.046	Extracellular
AOR25255	Peptidase, M28 family	0.211	0.200	0.066	0.052	Extracellular
AOR26853	Endonuclease I	0.201	0.140	0.027	0.016	Extracellular
AOR25557	Peptidase, M28 family	0.104	0.085	0.020	0.033	Extracellular
AOR25712	T9SS C-terminal target domain-containing protein	0.051	0.025	0.013	0.006	Extracellular
AOR26095	T9SS C-terminal target domain-containing protein	0.048	0	0.005	0	Extracellular
AOR26355	Peptidase, S8 family	0.047	0.078	0.037	0.019	Extracellular
AOR26579	T9SS C-terminal target domain-containing protein	0	0	0.047	0.030	Extracellular
AOR26771	Peptidase M22	0	0	0.011	0	Extracellular
AOR25374	T9SS C-terminal target domain-containing protein	0	0	0.004	0	Extracellular
Sum of NSAF%		**2.185**	**1.738**	**0.596**	**0.305**	Extracellular
AOR25897	TonB-dependent receptor	0.056	0.082	0.015	0.013	Outer membrane

AOR25517	TonB-dependent receptor	0.048	0.043	0.014	0.016	Outer membrane
AOR26137	TonB-dependent receptor	0.048	0.174	0.060	0.090	Outer membrane
AOR26630	TonB-dependent receptor	0.044	0.039	0.036	0.052	Outer membrane
AOR25546	TonB-dependent receptor	0.043	0.062	0.051	0.055	Outer membrane
AOR25619	TonB-dependent receptor	0.036	0.018	0.008	0.011	Outer membrane
AOR25452	TonB-dependent receptor	0.027	0.107	0.017	0.032	Outer membrane
AOR26017	TonB-dependent receptor	0.020	0.033	0.003	0.003	Outer membrane
AOR25900	TonB-dependent receptor	0.010	0.011	0.005	0.003	Outer membrane
AOR25966	TonB-dependent receptor	1.852	3.622	1.712	1.805	Outer membrane
AOR26448	TonB-dependent receptor	1.001	2.390	0.641	1.386	Outer membrane
AOR26574	TonB-dependent receptor	0.622	0.656	0.297	0.393	Outer membrane
AOR26504	TonB-dependent receptor	0.521	0.648	0.277	0.310	Outer membrane
AOR26266	TonB-dependent receptor	0.409	0.296	0.320	0.248	Outer membrane
AOR25782	TonB-dependent receptor	0.307	0.232	0.049	0.126	Outer membrane
AOR26268	TonB-dependent receptor	0.225	0.221	0.090	0.117	Outer membrane
AOR26075	TonB-dependent receptor	0.194	0.131	0.071	0.081	Outer membrane
AOR26163	TonB-dependent receptor	0.179	0.251	0.106	0.103	Outer membrane
AOR26960	TonB-dependent receptor	0.164	0.234	0.100	0.115	Outer membrane
AOR26571	TonB-dependent receptor	0.140	0.321	0.101	0.178	Outer membrane
AOR25403	TonB-dependent receptor	0.091	0.143	0.072	0.092	Outer membrane
AOR26302	Cell envelope biogenesis protein OmpA	0.972	2.576	1.343	1.389	Outer membrane
Sum of NSAF%		**7.011**	**12.29**	**5.390**	**6.620**	Outer membrane
AOR26842	Superoxide dismutase [Cu-Zn]	0.073	0.930	0.112	0.321	Periplasmic
AOR25498	Metallophosphatase	0	0	0.016	0.017	Periplasmic
AOR25512	PDZ domain-containing protein	0.209	0.136	0.092	0.044	Periplasmic
AOR25581	LysM peptidoglycan-binding domain-containing protein	0.339	0.099	0.065	0.016	Periplasmic
AOR25888	Beta-glucosidase	0.011	0.051	0.012	0.018	Periplasmic
AOR26084	Insulinase family protein	0.231	0.416	0.106	0.158	Periplasmic
AOR26305	Superoxide dismutase	0	0	0.123	0.414	Periplasmic
AOR26519	Glycosylasparaginase	0	0	0.006	0	Periplasmic
AOR26592	Cytochrome-c peroxidase	0.091	0.029	0.016	0	Periplasmic

AOR26730	Alkaline phosphatase	0.036	0.027	0.039	0.042	Periplasmic
AOR26806	S9 family peptidase	0	0	0.034	0.009	Periplasmic
AOR26842	Superoxide dismutase family protein	0.073	0.930	0.112	0.321	Periplasmic
AOR26862	ABC transporter substrate-binding protein	0	0.008	0.017	0.012	Periplasmic
Sum of NSAF%		**1.065**	**2.625**	**0.748**	**1.372**	Periplasmic

178

179

180

181 **Supplementary Table S5:** Ratios between the four fractions for the different localizations
182 were calculated based on NSAF% values of specific marker proteins (see supplementary table
183 S6) for the localizations. VE vesicle fraction exponential phase, VS vesicle fraction stationary
184 phase, ME membrane fraction exponential phase, MS membrane fraction stationary phase.

PSORTb	VE/VS	ME/VE	MS/VS	ME/MS
Cytoplasm	13.32	2.00	19.50	1.36
Cytoplasmic membrane	4.15	3.45	14.80	0.97
Extracellular	1.26	0.27	0.18	1.96
Outer membrane	0.57	0.77	0.54	0.81
Periplasm	0.41	0.70	0.52	0.55

185
186
187
188
189

190 **Supplementary Table S6:** Detection of type IX secretion system (T9SS) components:
191 Proteins of the T9SS in the genome of strain Hel3_A1_48 and detection in the proteomic
192 analysis. Localization was predicted by PSORTb 3.0.2 and the manual localization is based
193 on the model proposed by McBride & Nakane (2015). VE vesicle fraction exponential phase,
194 VS vesicle fraction stationary phase, ME membrane fraction exponential phase, MS
195 membrane fraction stationary phase.

Accession Number	Annotation	VE	VS	ME	MS	Localization PSORTb	Localization model
			[NSAF%]				
AOR25736	Gliding motility protein GldJ	3.687	3.168	0.182	0.196	OuterMembrane	OM + periplasm
AOR26642	Gliding motility protein GldK	3.347	2.605	0.198	0.189	Cytoplasmic	OM + periplasm
AOR26645	Gliding motility protein GldN	2.615	2.859	0.156	0.145	Cytoplasmic	OM + periplasm
AOR25738	PorV	2.179	3.286	0.288	0.212	OuterMembrane	OM
AOR25874	SprT, similar to PorT family protein	0.975	0.432	0.051	0.060	Unknown	OM
AOR26643	Gliding motility protein GldL	0.874	0.256	1.545	2.432	Unknown	CM + cytoplasm+ periplasm
AOR26184	T9SS membrane protein PorP/SprF	0.592	1.131	0.110	0.064	OuterMembrane	OM
AOR26556	Outer membrane protein beta-barrel domain (porT family protein)	0.436	0.576	0.064	0.011	OuterMembrane	OM
AOR25784	T9SS membrane protein PorP/SprF	0.325	0.140	0.020	0	Unknown	OM
AOR25737	PorU	0.188	0.216	0.042	0.040	Unknown	OM
AOR26644	Gliding motility protein GldM	0.128	0.031	0.392	0.320	Unknown	CM + periplasm
AOR26400	SprE, similar to TPR domain protein	0.088	0.037	0.038	0.027	OuterMembrane	OM + periplasm
AOR26544	Gliding motility-related protein SprA	0.055	0.032	0.078	0.076	Unknown	OM
AOR26618	Gliding motility protein GldB	0.037	0.105	0.037	0.052	Cytoplasmic	OM + periplasm
AOR26578	T9SS membrane protein PorP/SprF	0	0.472	0.166	0.041	Unknown	OM
AOR25315	Gliding motility protein GldI	0	0	0.022	0	Unknown	OM + periplasm
AOR26597	Gliding motility protein GldD	0	0	0.007	0	Unknown	OM + periplasm
AOR26185	SprB (adhesin)	0	0	0	0	OuterMembrane	OM

	similar to PKD domain protein						
AOR26428	Gliding motility lipoprotein GldH	0	0	0	0	CytoplasmicMembran e	OM + periplasm
/	RemA (adhesin)						
/	SprC						
/	SprD						
/	GldG						
/	GldF						
/	GldA						
		13.16	11.84	3.067	3.612		

196

197

198 **Supplementary Table S7:** Detection of proteins of strain Hel3_A1_48 with a T9SS C-
199 terminal secretion tail (TIGR04183) or Bac_Flav_CTERM (TIGR04131) domain in the
200 genome and the proteomic analysis. NSAF% values of the fractions are displayed, if the
201 protein was identified in at least two replicates in one of the samples during proteomic
202 analysis. VE vesicle fraction exponential phase, VS vesicle fraction stationary phase, ME
203 membrane fraction exponential phase, MS membrane fraction stationary phase.

Proteins with T9SS C-terminal domain: TIGR04183		**VE**	**VS**	**ME**	**MS**
			[NSAF%]		
AOR25680	LTD-domain protein	0.693	1.875	0.406	0.465
AOR26993	T9SS C-terminal target domain-containing protein	0.620	0.317	0.117	0
AOR26484	T9SS C-terminal target domain-containing protein	0.536	0.284	0.060	0.017
AOR25487	Fibronectin type 3 domain protein	0.510	0.538	0.124	0.104
AOR25970	Peptidase, S8 family	0.393	0.355	0.125	0.046
AOR25407	T9SS C-terminal target domain-containing protein	0.350	0.785	0.080	0.094
AOR25787	Endonuclease I	0.270	0.246	0.019	0.012
AOR26027	T9SS C-terminal target domain-containing protein	0.203	0.239	0.033	0.022
AOR26853	Endonuclease I	0.201	0.140	0.027	0.016
AOR25737	Peptidase, C25 family	0.188	0.216	0.042	0.040
AOR25692	Zinc-dependent metalloprotease	0.083	0.021	0.006	0
AOR25405	Endoglucanase, GH5 (cellulase)	0.065	0.042	0.007	0.004
AOR25421	Phosphoenolpyruvate synthase	0.037	0.060	0.010	0.002
AOR25447	T9SS C-terminal target domain-containing protein	0.029	0	0.003	0.004
AOR26020	Glycoside hydrolase, GH5 family, CBM6	0.020	0.008	0	0
AOR25397	Carbohydrate binding domain protein	0.016	0	0	0
AOR26873	DOMON-domain containing protein	0	0	0.023	0.030
AOR25723	T9SS C-terminal target domain-containing protein	0	0	0	0.013
AOR25893	T9SS C-terminal target domain-containing protein	0	0	0	0
AOR25438	T9SS C-terminal target domain-containing protein	0	0	0	0
AOR26291	T9SS C-terminal target domain-containing protein	0	0	0	0
AOR26016	PKD domain protein	0	0	0	0
AOR26883	Peptidase, M43 family, (metalloprotease),	0	0	0	0
AOR25889	Glycosyl hydrolase family	0	0	0	0
AOR25401	Glycoside hydrolase family, GH 30	0	0	0	0
AOR25645	DUF1501 domain-containing protein	0	0	0	0
AOR26026	Sortilin domain containing protein	0	0	0	0

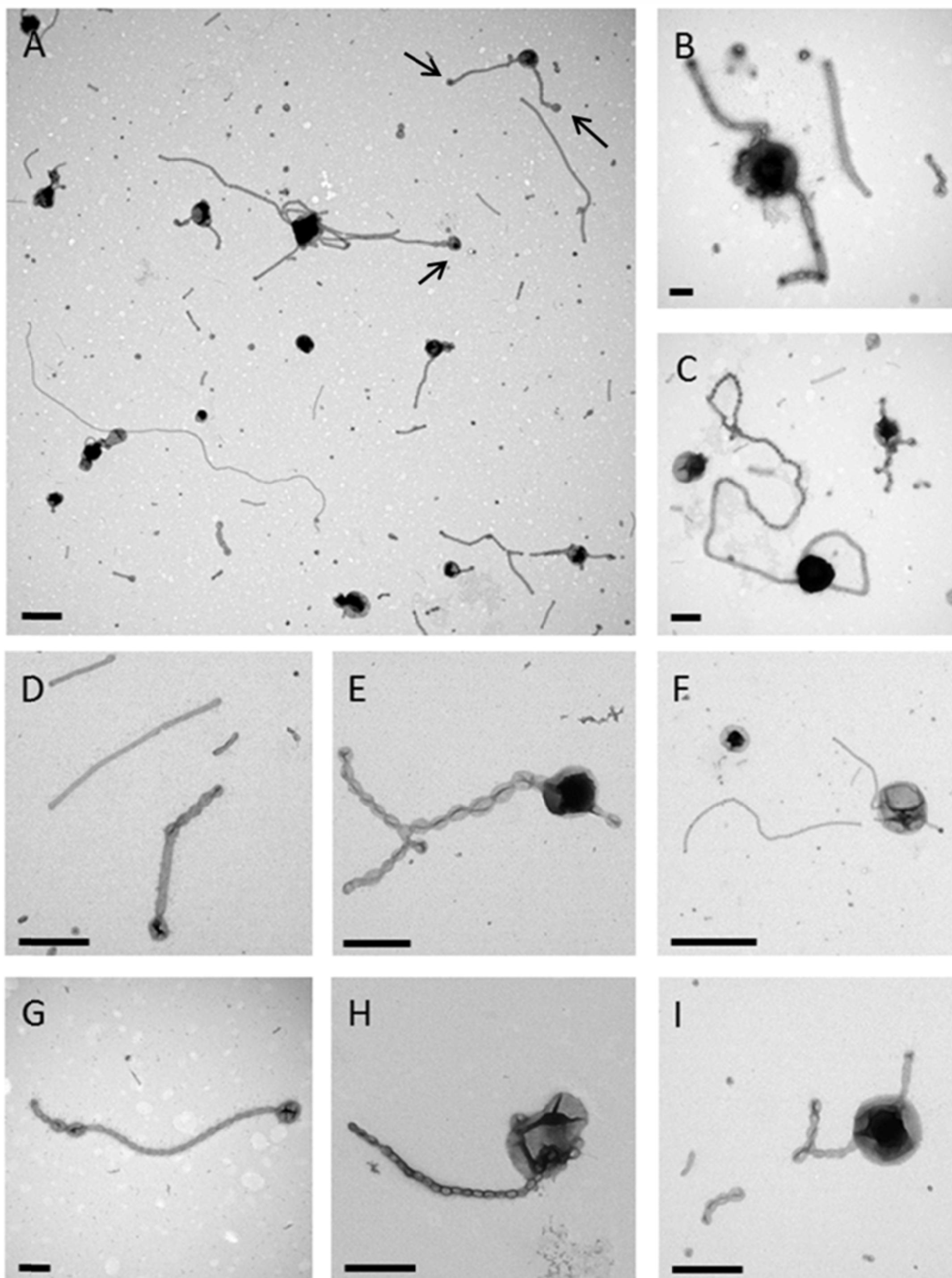
204

Proteins with T9SS C-terminal domain: TIGR04131		**VE**	**VS**	**ME**	**MS**
			[NSAF%]		
AOR26617	Gliding motility-associated C-terminal domain	0.057	0.040	0.010	0.007
AOR25712	Zinc-dependent metalloprotease	0.051	0.025	0.013	0.006
AOR26095	L-type lectin	0.048	0	0.005	0
AOR25373	Gliding motility-associated C-terminal domain	0.033	0.030	0.011	0.009
AOR25785	PKD domain-containing protein	0.027	0	0.003	0.003
AOR26729	Gliding motility-associated C-terminal domain	0	0.056	0.011	0.029

AOR26579	Fibronectin type 3 domain-containing protein	0	0	0.047	0.030
AOR25374	Gliding motility-associated C-terminal domain	0	0	0.004	0
AOR26185	PKD and CUB domain-containing protein	0	0	0	0

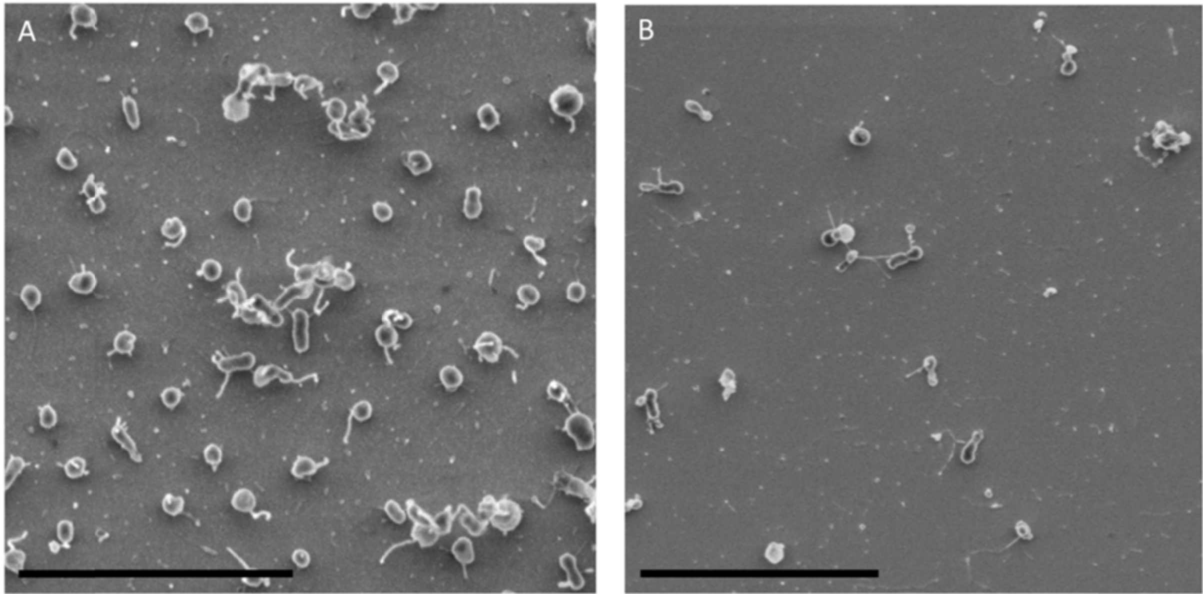
205
206
207
208
209
210
211
212
213
214
215
216
217

218 **Supplementary figures:**



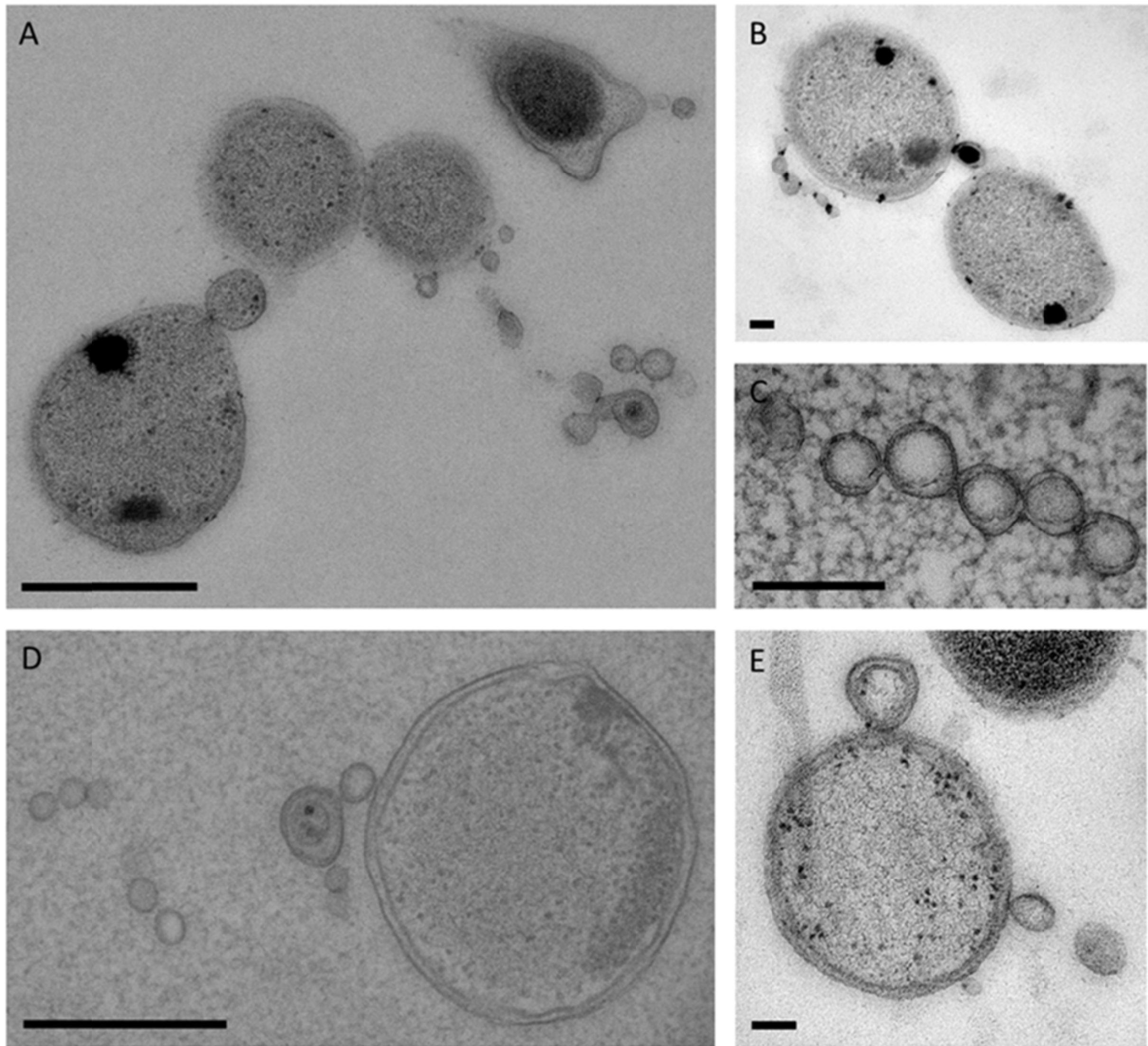
219

220 **Supplementary Fig. S1:** TEM micrographs of strain Hel3_A1_48 in stationary growth phase.
221 Cells grown in HaHa_100V medium at 21°C to stationary phase were negatively stained with
222 1% uranyl acetate. Arrows in [A] are pointing to larger vesicles at the distal end of the
223 appendage (also in [D, G]). Other images show extracellular vesicle chains [A-I] and
224 membrane tubes [B, D]. Bars correspond to 250 nm [B, G], 500 nm [C, D, E, H, I], 1µm [A]
225 or 2 µm [F].



Supplementary Fig. S2: SEM (SE) micrographs of strain Hel3_A1_48. Cells grown in HaHa_100V medium at 21°C to late exponential [A] or stationary phase [B] were settled on a silica wafer, dehydrated by an ethanol-series and preserved using critical point drying. Bars correspond to 4 µm [A] or 5 µm [B].

234



235 **Supplementary Fig. S3:** TEM micrographs of 70 nm thin sections of strain Hel3_A1_48.
236 Cells grown in HaHa_100V medium at 21°C were pelleted and fixed by high-pressure
237 freezing, freeze-substitution and sectioning into 70 nm thin sections. TEM images showed
238 cells with and without the storage compound [A, B], OM extensions [A-E], OMVs [A-E], O-
239 IMVs [A, D, E] and vesicles with a storage compound [B]. Bars correspond to 100 nm [B, E],
240 200 nm [C] and 500 nm [A, D].

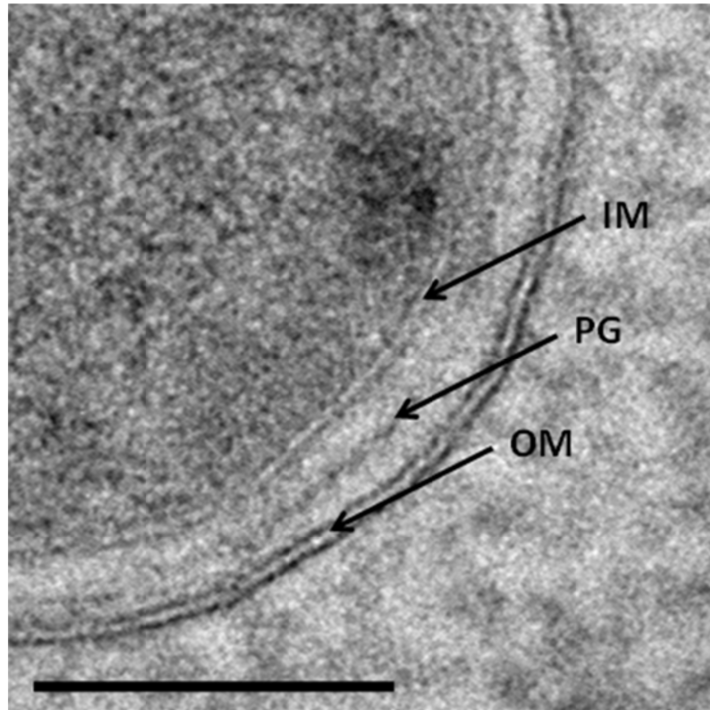
241

242

243

244

245



246 **Supplementary Fig. S4:** TEM micrograph of a thin section of a strain Hel3_A1_48 cell. The
247 Gram-negative membrane system consists of the inner membrane (IM), the thin
248 peptidoglycan layer (PG) and the outer membrane (OM). The picture shows an extended
249 periplasm of around 19-21 nm in width. Bar corresponds to 100 nm.

250

251

252

253

254

255

256

257

258

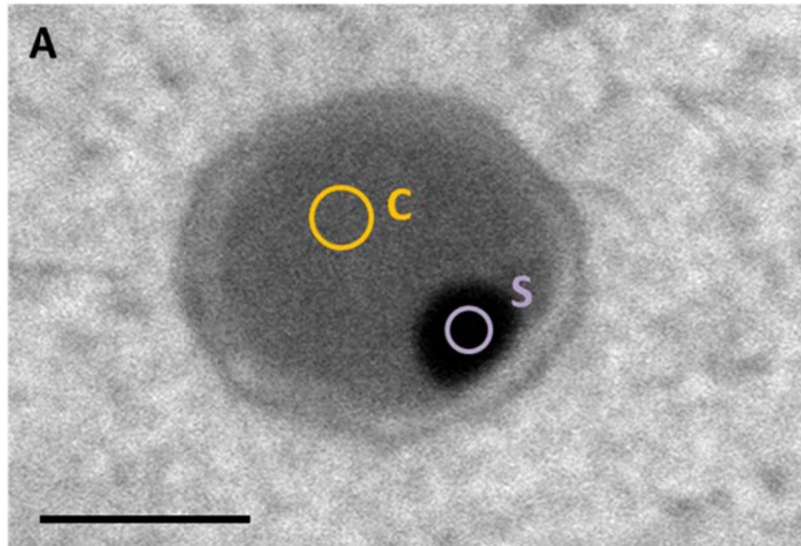
259

260

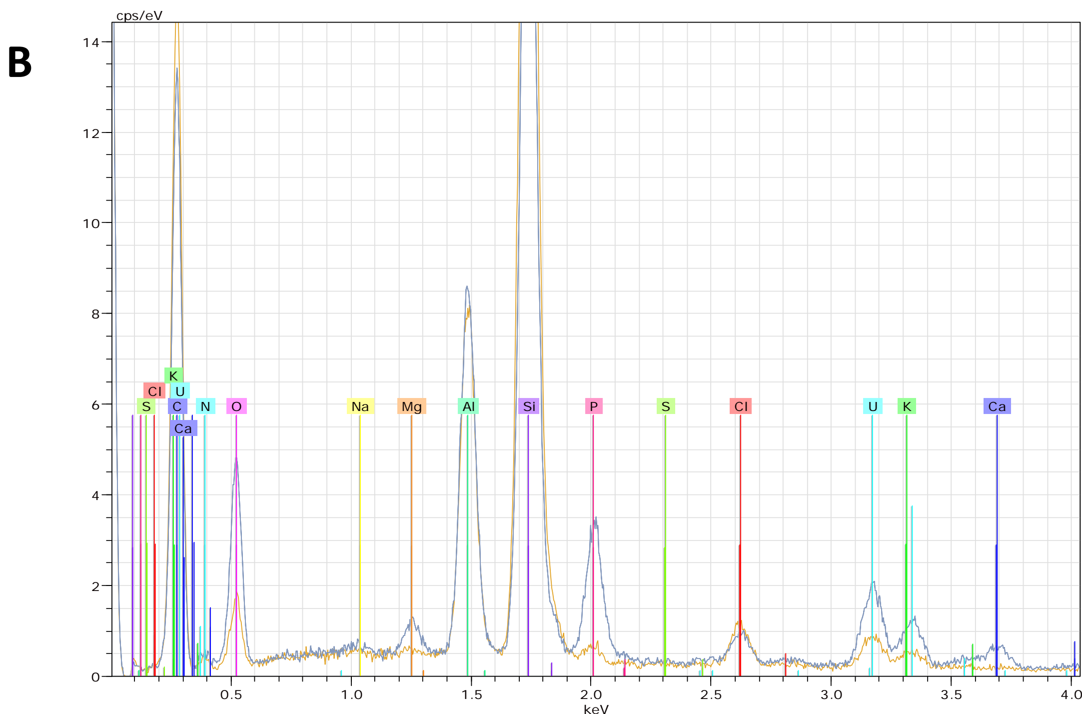
261

262

263



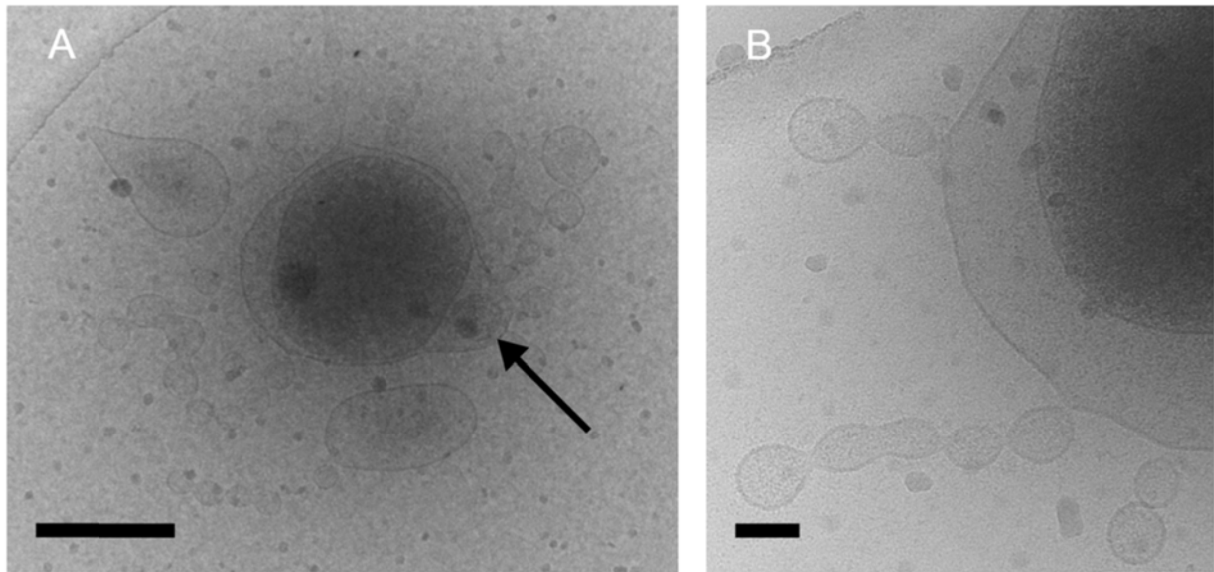
264
265
266
267
268
269
270
271
272
273
274



275 **Supplementary Fig. S5:** Energy-dispersive X-ray (EDX) measurement of 300 nm thin
276 sections of strain Hel3_A1_48. The STEM image [A] shows a cell with **a** storage compound
277 (S) inside and a reference sample site in the cell (C) (magnification: 160 000 x, accelerating
278 voltage (HV): 10.0 kV, working distance: 9.5 mm; bar corresponds to 200 nm). The EDX
279 spectra [B] of the sample sites S [purple spectrum] and C [orange spectrum] revealed an
280 elevated concentration of phosphorus, oxygen, magnesium, calcium, potassium and uranium
281 (uranium from contrasting, aluminum from sample holder, silicon from STEM detector) in the
282 storage compound.

283

284



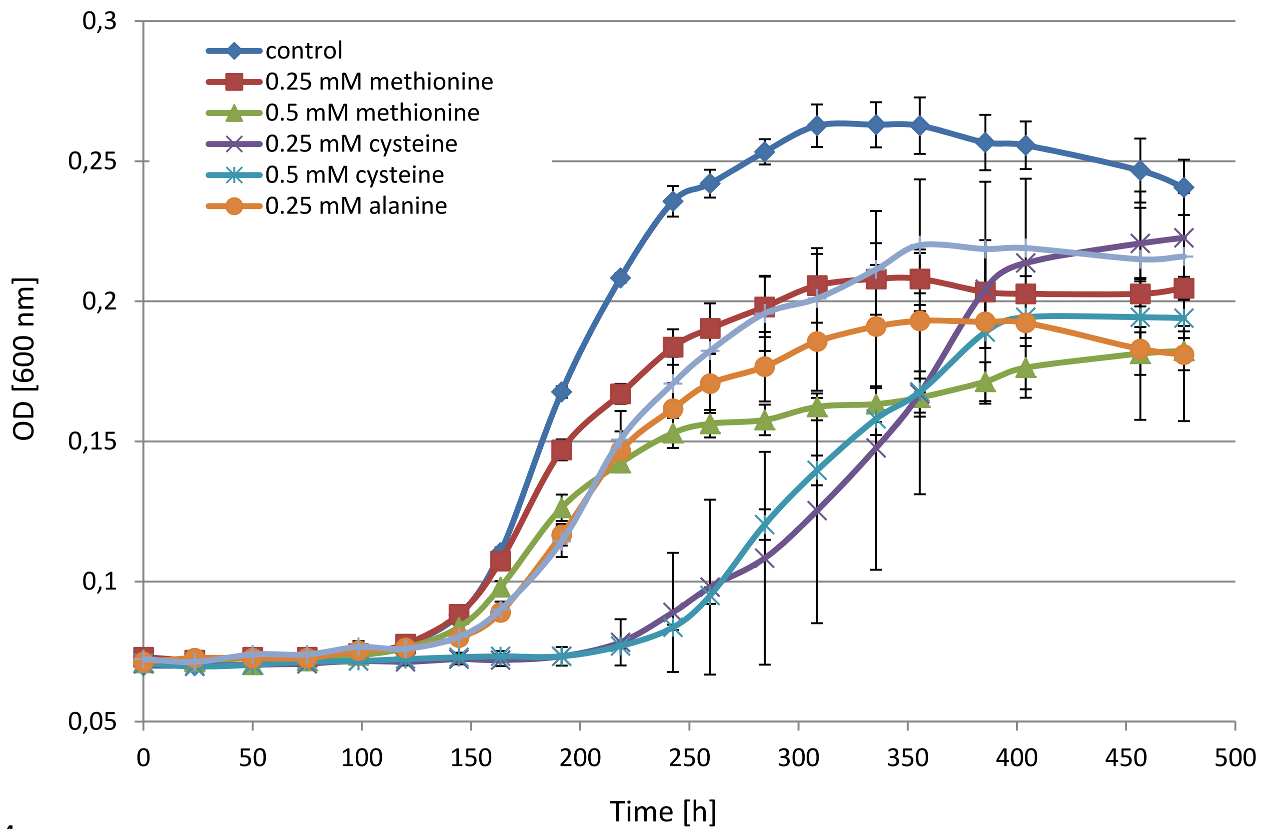
285 **Supplementary Fig. S6:** Cryo-EM micrographs of stationary phase cells of strain
286 Hel3_A1_48. Cells grown in HaHa_100V medium at 21°C were directly frozen on the grid in
287 liquid ethane. Arrow in [A] points towards the IMV, still enclosed by the outer membrane of
288 the cell. [B] shows the large periplasm of the cell and associated outer membrane vesicle
289 chains. Bars represent 500 nm [A] and 100 nm [B].

290

291

292

293



294

295 **Supplementary Fig. S7:** Growth curves of strain Hel3_A1_48 in HaHa_100V medium and
296 medium supplemented with different concentrations of the amino acids methionine, cysteine
297 and alanine. Cells were grown at 21 °C on a tilting shaker with 55 rpm in 15 ml tubes and the
298 optical density was measured at 600 nm.

299

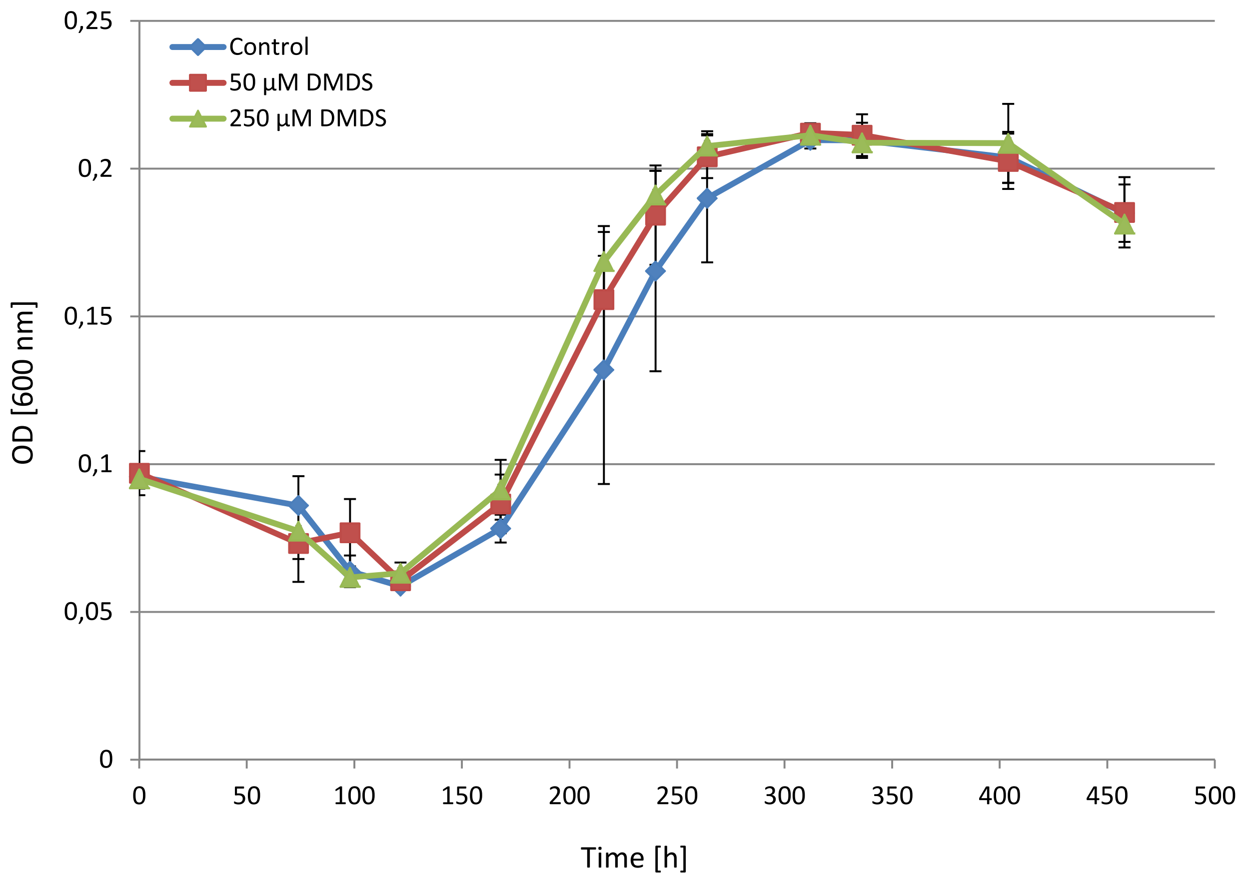
300

301

302

303

304



305
306 **Supplementary Fig. S8:** Growth curves of strain Hel3_A1_48 in HaHa_100V medium and
307 medium supplemented with different concentrations of DMDS. Cells were grown at 21 °C in
308 gastight 1 L Duran bottles with 900 ml headspace and the optical density was measured at
309 600 nm.

310

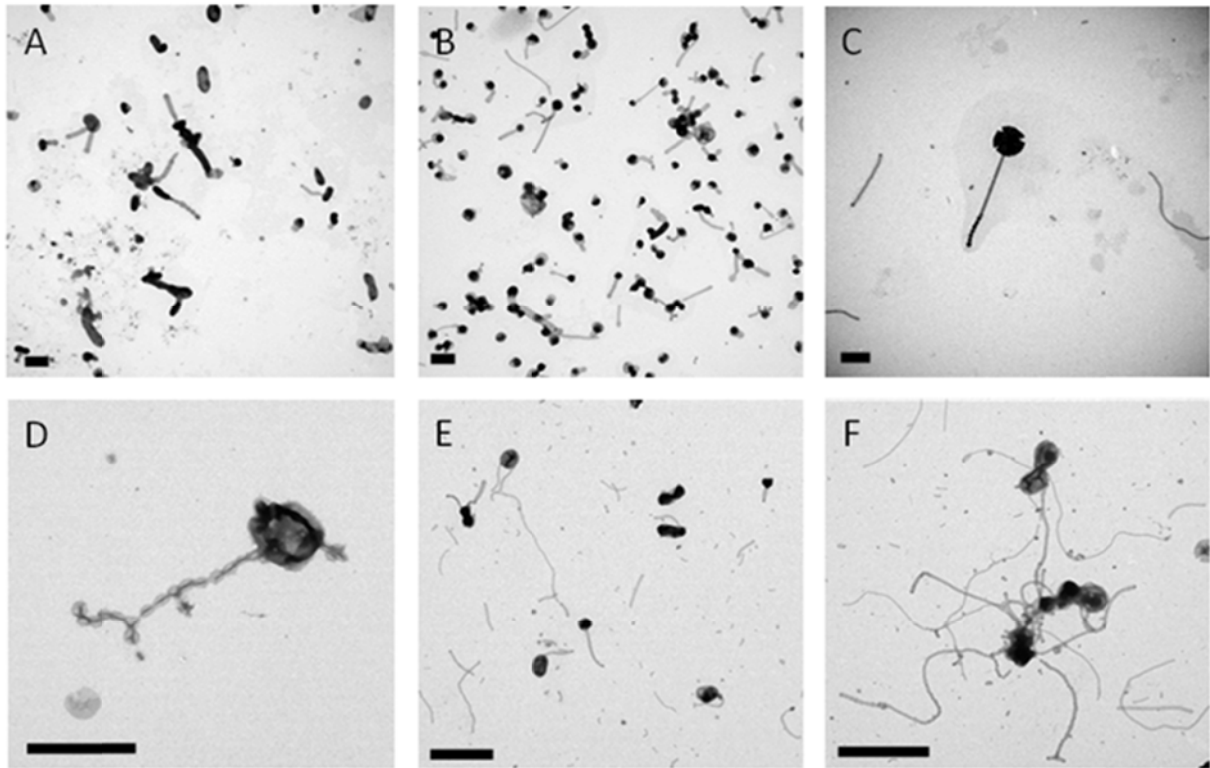
311

312

313

314

315



316 **Supplementary Fig. S9:** TEM micrographs of strain Hel3_A1_48 cells in stationary growth
317 phase. Cells were grown at 21°C in HaHa_100V medium with supplemented cysteine [A],
318 methionine [B], DMDS [C] or with HaHa_minV only on glucose and casamino acids [D],
319 plus additional sodium carbonate [E], with HaHa_minV only on casamino acids [F] or plus
320 additional sodium carbonate [G]. They were negatively stained with 1% uranyl acetate for
321 TEM. Bars correspond to 500 nm [C], 1 µm [A, B, D] or 2 µm [E, F].

322

323

324

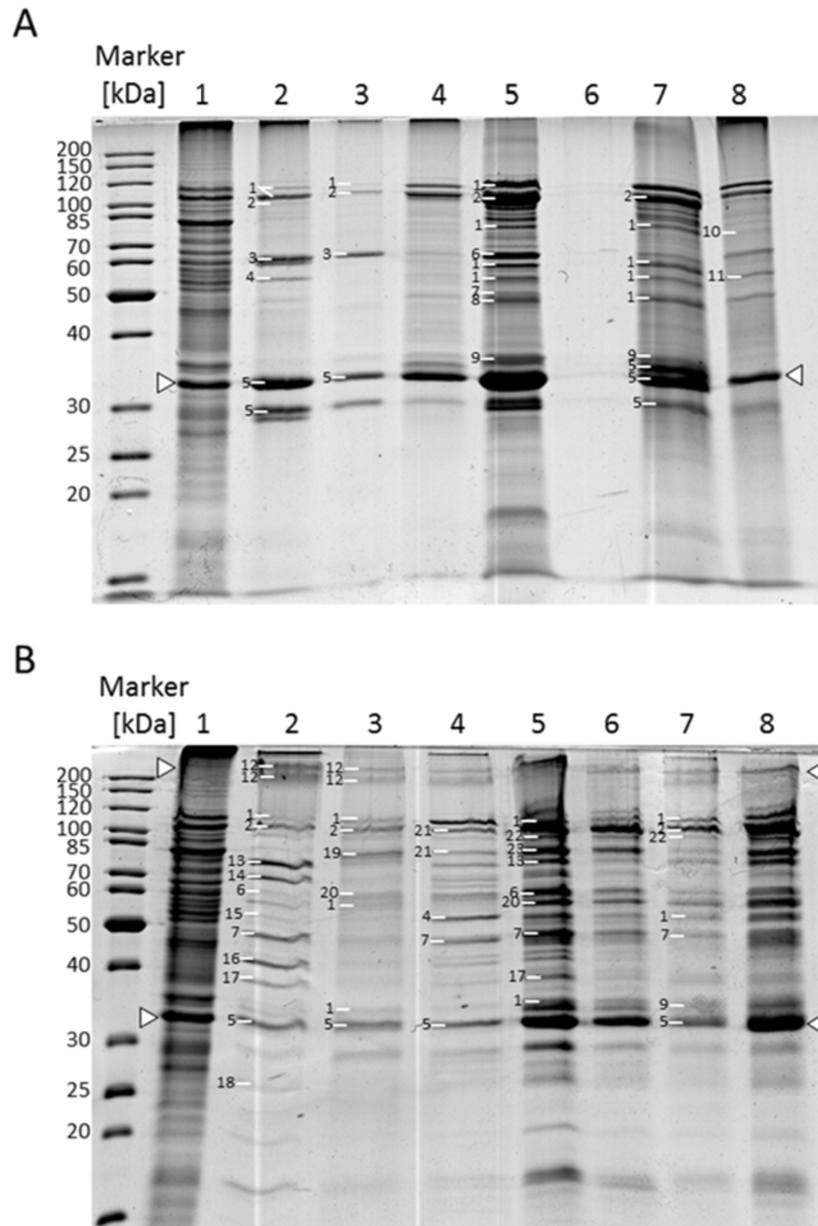
325

326

327

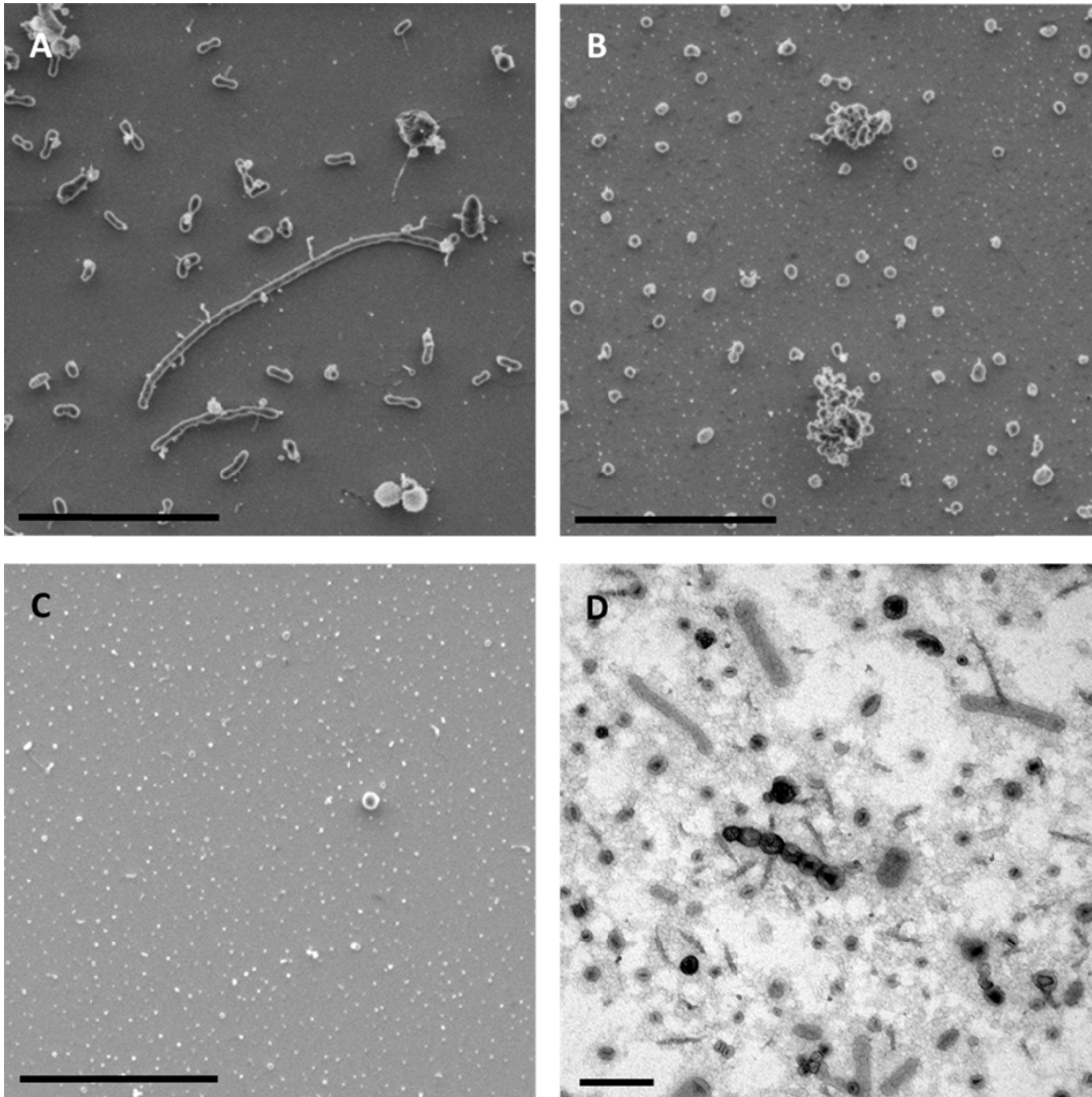
328

329



Supplementary Fig. S10: SDS-PAGE images of strain Hel3_A1_48 outer membrane (OM) preparations [A] and vesicle/appendage preparations [B]. [A1, B1] show whole cell extracts. OM fractions were prepared according to Shetty *et al.* (2011) [A2], Møller *et al.* (2005) [A3], Koßmehl *et al.* (2013) [A4], McCaig *et al.* (2013) [A5], Thein *et al.* (2010) method 1 [A6], Thein *et al.* (2010) method 2 [A7] and Kulkarni *et al.* (2014) [A8]. The vesicle/appendage fraction was performed as described by Kulkarni *et al.* (2014) with exponential phase cells [B2], Kulkarni *et al.* (2014) with stationary phase cells [B3], Shetty *et al.* (2011) [B4], Møller *et al.* (2005) method 1 [B5], Møller *et al.* (2005) method 2 [B6], Remis *et al.* (2014) [B7] and McCaig *et al.* (2013) [B8] (see methods for details). Marked bands were excised and analyzed by MALDI-TOF-MS. Results are presented in Suppl. Table S1. The triangles denote a 35kDa porin (AOR25254) and a lamin tail domain-containing protein with a C-terminal T9SS target domain (AOR25680) that had a predicted weight of 61 kDa and appeared to have a molecular weight of over 200 kDa in SDS PAGE gel.

344



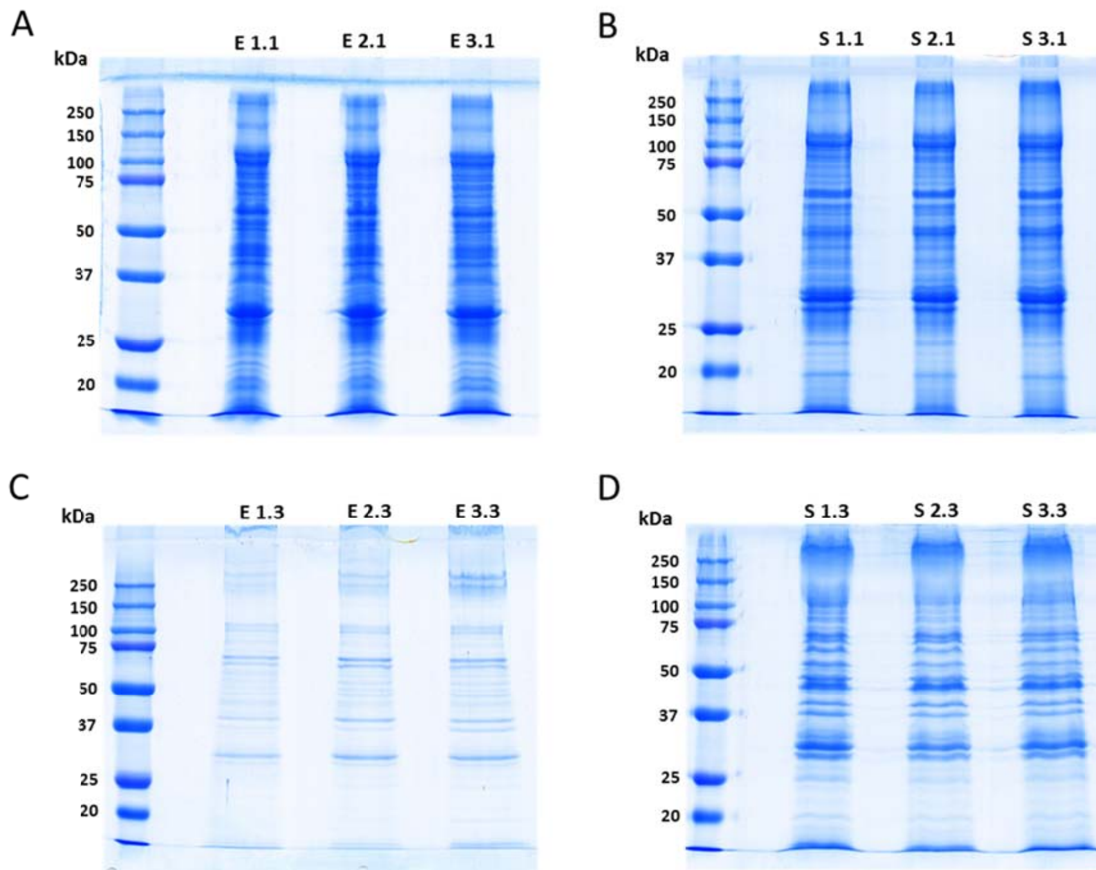
345 **Supplementary Fig. S11:** SEM (SE) and TEM images of the cell and vesicle/appendage
346 fractions obtained by differential centrifugation and filtration for proteomics. The differential
347 centrifugation yielded a pellet (4390x *g*, 10 min) with whole cells and aggregates [A]. Small
348 cells were pelleted with 27 440x *g* for 15 min [B]. The supernatant was filtered through a
349 0.2 µm filter and the filtrate was then centrifuged again using 142 019x *g* for 2 h. The pellet 3
350 consisted then mainly of single vesicles, vesicle chains and tubes [C, D]. Bars in SEM [A-C]
351 and TEM images [D] correspond to 5 µm [A, B], 4 µm [C] and 250 nm [D].

352 Due to a larger biomass pellet 1 was used for the crude membrane preparation.

353

354

355



356

357 **Supplementary Fig. S12:** SDS-PAGE images of strain Hel3_A1_48 protein samples used for
358 proteomic analysis by LC-MS/MS. Crude membrane fractions of exponential phase cells [A]
359 and of stationary phase cells [B] and the vesicle/appendage fraction of the exponential phase
360 [C] and of the stationary phase [D] were each prepared from three cultures.

361

362

363

364

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. Journal of molecular biology. 1990 Oct 5; 215(3):403-10.

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F. The RAST Server: rapid annotations using subsystems technology. BMC genomics. 2008 Dec; 9(1):75.

McBride MJ, Nakane D. 2015. Flavobacterium gliding motility and the type IX secretion system. Curr Opin Microbiol 28:72-77.

Koßmehl S, Wöhlbrand L, Drüppel K, Feenders C, Blasius B, Rabus R. Subcellular protein localization (cell envelope) in *Phaeobacter inhibens* DSM 17395. Proteomics. 2013 Oct; 13(18-19):2743-60.

Kulkarni HM, Jagannadham MV. Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. Microbiology. 2014 Oct 1; 160(10):2109-21.

McCaig WD, Koller A, Thanassi DG. Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. Journal of bacteriology. 2013 Mar 15; 195(6):1120-32.

Møller JD, Barnes AC, Dalsgaard I, Ellis AE. Characterisation of surface blebbing and membrane vesicles produced by *Flavobacterium psychrophilum*. Diseases of aquatic organisms. 2005 May 20; 64(3):201-9.

Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A. The Pfam protein families database. Nucleic acids research. 2011 Nov 29; 40(D1):D290-301.

Remis JP, Wei D, Gorur A, Zemla M, Haraga J, Allen S, Witkowska HE, Costerton JW, Berleman JE, Auer M. Bacterial social networks: structure and composition of

398 *Myxococcus xanthus* outer membrane vesicle chains. Environmental microbiology. 2014
399 Feb; 16(2):598-610.
400
401 Shetty A, Chen S, Tocheva EI, Jensen GJ, Hickey WJ. Nanopods: a new bacterial structure
402 and mechanism for deployment of outer membrane vesicles. PloS one. 2011 Jun 7;
403 6(6):e20725.
404
405 Thein M, Sauer G, Paramasivam N, Grin I, Linke D. Efficient subfractionation of gram-
406 negative bacteria for proteomics studies. Journal of proteome research. 2010 Oct 28;
407 9(12):6135-47.