Outer membrane vesicles can contribute to cellulose degradation in *Teredinibacter turnerae,* a cultivable intracellular endosymbiont of shipworms

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Data availability

The authors confirm that the data supporting this study's findings are available within the article and its supplementary materials. The mass spectrometric raw files are accessible at https://massive.ucsd.edu under accession MassIVE MSV000094250 and at https://www.proteomexchange.org under accession PXD050419.

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Abstract

Teredinibacter turnerae is a cultivable cellulolytic Gammaproteobacterium (Cellvibrionaceae) that commonly occurs as an intracellular endosymbiont in the gills of wood-eating bivalves of the family Teredinidae (shipworms). The genome of *T. turnerae* encodes a broad range of enzymes that deconstruct cellulose, hemicellulose, and pectin and contribute to lignocellulose digestion in the shipworm gut. However, the mechanism by which symbiont-made enzymes are secreted by *T. turnerae* and subsequently transported to the site of lignocellulose digestion in the shipworm gut is incompletely understood. Here, we show that *T. turnerae* cultures grown on carboxymethyl cellulose (CMC) produce outer membrane vesicles (OMVs) that contain a variety of proteins identified by LC-MS/MS as carbohydrate-active enzymes with predicted activities against cellulose, hemicellulose, and pectin. Reducing sugar assays and zymography confirm that these OMVs retain cellulolytic activity, as evidenced by hydrolysis of CMC. Additionally, these OMVs were enriched with *TonB*-dependent receptors, which are essential to carbohydrate and iron acquisition by free-living bacteria. These observations suggest potential roles for OMVs in lignocellulose utilization by *T. turnerae* in the free-living state, in enzyme transport and host interaction during symbiotic association, and in commercial applications such as lignocellulosic biomass conversion.

Introduction

The biological degradation of lignocellulose, a primary component of wood and all plant biomass, is a critical process in the global carbon cycle and has potential applications in renewable energy and chemical production through biomass conversion (Ragauskas *et al.*, 2014; Østby *et al.*, 2020). Lignocellulose is a complex composite material composed primarily of microfibrils of cellulose, the most abundant organic polymer on Earth (Dahmen *et al.*, 2019), embedded in a matrix of hemicellulose and lignin. Pectins may also be present in smaller quantities. However, due to its complexity, lignocellulose degradation requires a suite of specialized enzymes to convert its component polymers into accessible nutrients. This complexity precludes most organisms from utilizing lignocellulose and creates obstacles to bioconversion to renewable fuels or fine chemicals (Cragg *et al.*, 2015).

One of the unique biological systems where efficient wood digestion occurs is found within shipworms, a group of marine bivalve mollusks of the family Teredinidae and the primary degraders of woody plant material in mangrove forests and in association with driftwood and marine woodfalls (Voight, 2015; Cragg *et al.*, 2020). Shipworms harbor endosymbiotic bacteria in bacteriocytes confined to the gland of Deshayes, a tissue located within their gills (Distel *et al.*, 1991). The genomes of these bacteria, primarily from the genus *Teredinibacter*, encode a wide variety of enzymes targeting lignocellulose (Distel *et al.*, 2002; Yang *et al.*, 2009; O'Connor *et al.*, 2014; Altamia, Shipway, *et al.*, 2020; Altamia *et al.*, 2021) and express them within this gland (O'Connor *et al.*, 2014; Sabbadin *et al.*, 2018). The resulting bacterial enzymes are also found in the shipworm's cecum (O'Connor *et al.*, 2014; Sabbadin *et al.*, 2018; Altamia and Distel, 2022), a specialized organ that is the primary location of wood digestion for the shipworm and is nearly devoid of microbes (Betcher *et al.*, 2012). This physical separation

84 between the bacterial symbionts in the gill and wood digestion in the gut requires a transport
85 mechanism for symbiont-made cellulolytic enzymes. Recently, a system of ducts called the ducts
86 of Deshayes was shown to serve as an extracellular transport path for bacterial cellulases from
87 the gills to the mouth of shipworms (Altamia and Distel, 2022). However, the mechanism by
88 which enzymes produced by the intracellular bacteria move across multiple membranes to an
89 external transport path is still unknown.

90 Bacteria use a variety of mechanisms to degrade lignocellulosic substrates in their
91 environments. For example, polysaccharide-degrading bacteria, including the common rumen
92 bacteria *Bacteroides*, *Clostridia*, and *Fibrobacter,* use the type IX secretion pathway (T9SS) to
93 secrete large molecular-weight proteins, including multidomain carbohydrate-active enzymes
94 (CAZymes) (Gharechahi *et al.*, 2023). These enzymes may be released into the environment or
95 bound to the cell envelope (McGavin *et al.*, 1990; Cai *et al.*, 1999; Yan and Wu, 2013). In
96 addition to the secretion of soluble or membrane-bound enzymes, bacteria may export
97 CAZymes by producing outer membrane vesicles (OMVs). OMVs are spherical buds that
98 originate from the bacterial outer membrane and can contain various cellular components such
99 as lipopolysaccharides, proteins, small molecules, and nucleic acids (Schwechheimer and Kuehn,
100 2015; Huang *et al.*, 2022).

101 Recent studies have shown that OMVs can be highly enriched in CAZymes and retain
102 activity capable of degrading cellulose and other plant biomass (Arntzen *et al.*, 2017; Ichikawa
103 *et al.*, 2019; Salvachúa *et al.*, 2020). The majority of research on OMVs has been done on
104 pathogenic bacteria due to the capacity of OMVs to carry and deliver virulence factors and
105 toxins into host cells (O'Donoghue and Krachler, 2016; Lynch and Alegado, 2017; Caruana and

106 Walper, 2020). However, the role of OMVs in marine and other environments has been
107 increasingly recognized, likely playing crucial roles in nutrient processing and ecological
108 interactions (Frias *et al.*, 2010; Biller *et al.*, 2014, 2022; Fischer *et al.*, 2019; Fadeev *et al.*, 2023).
109 *Teredinibacter turnerae*, unlike obligate intracellular symbionts, is also capable of free-living
110 growth (Waterbury *et al.*, 1983) and may directly acquire carbon from cellulose or other plant
111 biomass in the marine environment (Naka and Haygood, 2023). Investigating the functionality of
112 OMVs in *T. turnerae* could be pivotal to understanding their complex symbiotic interaction and
113 the ecological impact of their dual lifestyle.

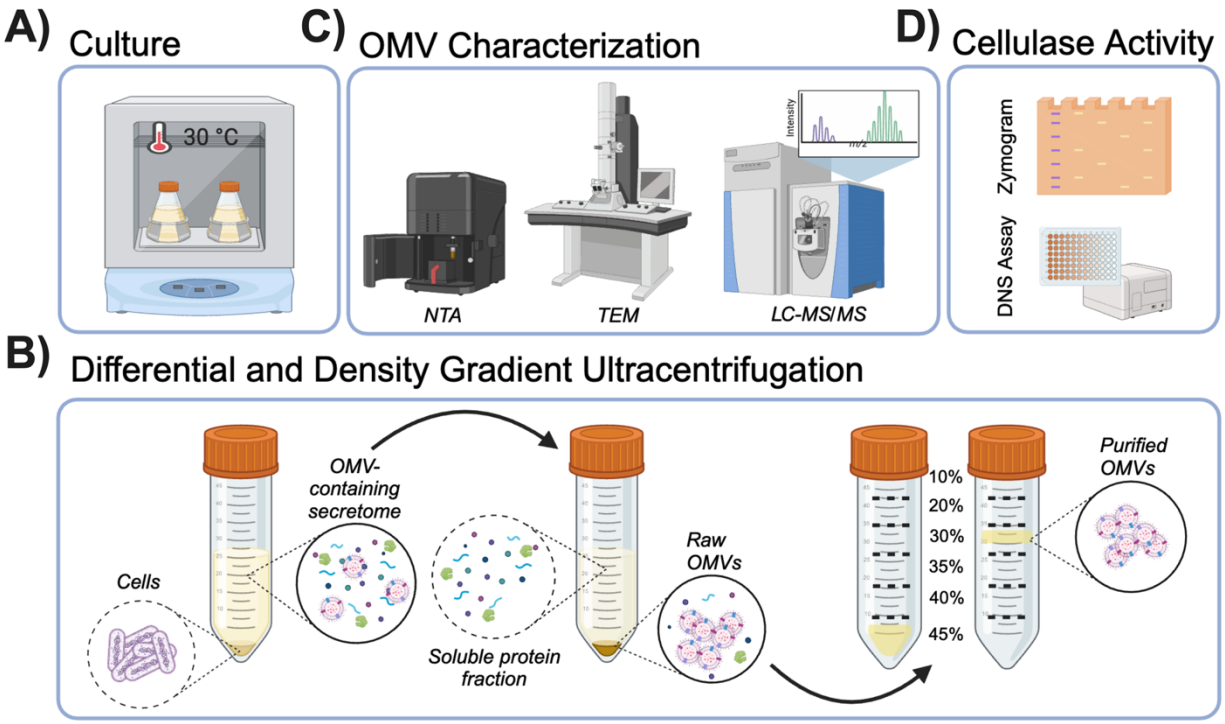
114 Here, we purify and characterize the protein composition of OMVs produced by *T.*
115 *turnerae* during growth on water-soluble carboxymethyl cellulose (CMC). Proteins putatively
116 involved in interactions with lignocellulose were identified by LC-MS/MS analysis and
117 comparison to the Carbohydrate Active Enzyme Database (CAZy). Additionally, activity assays
118 were used to show that OMVs produced by *T. turnerae* are capable of cellulose hydrolysis.
119 These observations suggest that OMVs contribute to cellulose utilization by *T. turnerae* and may
120 play essential roles in their metabolism in free-living and symbiotic states.

121 Experimental Procedures

122 Strain isolation, selection, and growth conditions

123 *T. turnerae* is an endosymbiont species with widespread occurrence among shipworm
124 species (Distel *et al.*, 2002; Altamia *et al.*, 2014; Altamia, Lin, *et al.*, 2020). Two strains were
125 selected: *T. turnerae* T7901 (ATCC 39867), which was previously isolated from *Bankia gouldi* and
126 was the first shipworm symbiont brought into pure culture (Waterbury *et al.*, 1983; Distel *et al.*,
127 2002; Yang *et al.*, 2009); and *T. turnerae* SR01903, a closely related strain of *T. turnerae* (Altamia
128 *et al.*, 2014). *T. turnerae* SR01903 was isolated from the gills of a single specimen of *Lyrodus*

129 *pedicellatus* found in naturally occurring driftwood collected by hand in shallow water in the
130 Indian River Lagoon, Merit Island, FL. (N 28.40605 W 80.66034) on January 24, 2020. Gills were
131 removed by dissection and homogenized in 1.0 ml of SBM medium (Waterbury *et al.*, 1983) in
132 an autoclave-sterilized glass dounce homogenizer. Homogenate was streaked onto a culture
133 plate containing 1.0% Bacto agar shipworm basal medium (SBM) at pH 8.0 supplemented with
134 0.2% w/v powdered cellulose (Sigmacell Type 101; Sigma-Aldrich) and 0.025% NH_4Cl. Plates
135 were incubated at 30 °C until individual colonies could be observed. An individual colony was
136 then picked and subjected to multiple rounds of restreaking from single colonies to ensure
137 clonality. To identify the isolate, the 16S rRNA gene was PCR amplified, sequenced, and
138 compared to published *T. turnerae* genomes. The complete genome of *T. turnerae* SR01903 was
139 then sequenced (SRA number SRR28421271) and submitted to Genbank (Submission number
140 SUB14332655, Bioproject PRJNA1090931, assembly accession number pending; assembly and
141 annotation files provided in additional files for review but not for publication). For the
142 experiments described herein, strains were initially propagated in 6 mL cultures in shipworm
143 basal medium (Waterbury *et al.*, 1983) supplemented with 0.025% NH_4Cl and 0.2%
144 carboxymethyl cellulose for 4 days before being diluted 1/250 in fresh media and harvested
145 after 2 days (OD600 0.2-0.3). All cultures were incubated in a shaker incubator at 30 °C and 100
146 rpm. An overview of the procedures conducted in this research is presented in Figure 1.



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148 *Figure 1. Methods used to isolate and characterize outer membrane vesicles from T. turnerae. Diagram showing: (A) bacterial*
149 *culture, (B) OMV isolation based on differential and density gradient separation, (C) OMV visualization and size by TEM and NTA,*
150 *proteome analysis by LC-MS/MS, and (D) detection of cellulase activity in purified OMV preparations by zymography and*
151 *reducing sugar (DNS) assay. Created with BioRender.com*

152 ## Isolation of Outer Membrane Vesicles (OMVs)

153 Bacterial cells were separated from the culture supernatant by centrifugation at 5,000 x
154 g for 20 minutes at 4 °C. The supernatant was then transferred to a fresh tube, and
155 centrifugation was repeated to remove residual bacterial cells. The final supernatant was
156 carefully collected without disturbing the remaining pellet and filtered through a 0.22 µm
157 polyethersulfone filter as an additional precaution to remove cells. The putative OMVs were
158 then pelleted from the filtrate by ultracentrifugation at 120,000 x g (T-647.5 rotor, Sorvall) for 90
159 minutes at 4 °C. For purification, the resulting pellet was resuspended in 0.1 M phosphate-
160 buffered saline (PBS) and fractionated by bottom-up density gradient ultracentrifugation using
161 Optiprep™ (iodixanol density gradient medium) as follows. The resuspended OMV-containing
162 pellet was mixed with 60% iodixanol solution to a final density of 45% (w/v) and placed at the

163 bottom of an ultracentrifuge tube. A discontinuous density gradient was generated using a
164 syringe and G21 needle to deposit layers of 40%, 35%, 30%, 20%, and 10% iodixanol with a final
165 layer of 0.25 mL 0.1 M PBS on top. The resulting gradient was then subjected to centrifugation
166 for 16 hours at 150,000 x g and 4 °C (SW55 Ti rotor, Beckman-Coulter). Sample banding was
167 visually observed at the interface of the 30% and 20% fractions (Supporting Information: Figure
168 S1). All fractions were collected, and iodixanol was removed by passive diffusion dialysis (1,000
169 kDa MWCO) in exchanging buffer of 50 mM ammonium bicarbonate pH 8.3 at 4 °C. OMV
170 samples were removed from the dialysis bag and concentrated under vacuum to 100 µL
171 (SPD121P SpeedVac, Thermo Scientific). Particle visualization and characterization proceeded
172 with the single 30% fraction.

173 Transmission electron microscopy (TEM)

174 Purified OMVs were diluted and absorbed onto 200 mesh formvar-treated and carbon-
175 coated copper grids for 60 seconds. Samples were then fixed for 5 minutes in 4% glutaraldehyde
176 in 0.1 M sodium cacodylate, and grids were stained with 1% aqueous uranyl acetate for 60
177 seconds and left to dry. OMVs were imaged in an FEI Tecnai T12 (Thermo Fisher) transmission
178 electron microscope at 80 KV with an AMT bottom-mount camera.

179 Nanoparticle tracking analysis (NTA)

180 NTA was performed using the ZetaView® PMX-220 Twin (Particle Metrix) configured with
181 488 nm and 640 nm lasers with long wave-pass cut-off filters (500 nm and 660 nm, respectively)
182 and a sensitive CMOS camera 640 x 480 pixels. Samples were diluted in 2 mL of 0.1 µm filtered
183 deionized water (18 MΩ/cm) to obtain a particle concentration between 1 x 10^7 and 1 x 10^8
184 particles/mL. The instrument was set to a sensitivity of 80, a shutter speed of 100, and a frame
185 rate of 30 frames per second. Each sample was measured at 11 different positions throughout

186 the sample cell, with 1 cycle of reading at each position to have a minimum of 1,000 traces. If
187 the number of traces was below 1,000 counts, some additional sample was flushed inside the
188 sample cell, and the acquisition was repeated. Post-acquisition parameters were set to a
189 minimum brightness of 20, a maximum size area of 1,000 pixels, a minimum size area of 10
190 pixels, and tracelength of 15 frames. Automated cell quality control was checked using high-
191 quality deionized water. Camera alignment and focus optimization were performed using
192 polystyrene Nanosphere™ 100 nm size standard beads. Data analysis was performed with
193 ZetaView® 8.05.14 software provided by the manufacturer. Automated reports of the particle
194 recordings across the 11 positions were manually checked, and any outlier position was
195 removed to calculate particle concentration and distribution.

196 Proteomic analysis

197 OMVs were lysed and denatured in a solution containing 5% sodium dodecyl sulfate
198 (SDS), 100 mM Tris (pH=8), 20 mM chloroacetamide, and 10 mM tris (2-carboxyethyl) phosphine
199 hydrochloride and incubated at 90 °C for 10 minutes. Proteins were aggregated and isolated
200 using Sera-Mag™ carboxylate-modified SpeedBeads (SP3 beads) and digested in 100 mM
201 NH_4HCO_3 containing Trypsin (final concentration 6 ng/µL) overnight at 37 °C and 1,200 rpm.
202 Digested samples were loaded onto C18 tips, and peptides were separated online using an 88-
203 minute LC gradient (Evosep LC system). MS analysis was performed on a Q-Exactive HF-X Hybrid
204 Orbitrap mass spectrometer (Thermo Scientific). One full high-resolution MS spectrum was
205 acquired with a resolution of 45,000, an AGC target of 3×10^6 with a maximum ion time of 45
206 ms, and a scan range of 400-1,500 m/z, followed by 20 HCD MS/MS scans with a resolution of
207 1,5000, an AGC target of 1×10^5 with a maximum ion time of 120 ms, NCE of 27, and isolation
208 window of 2 m/z. The resulting MS/MS spectra were searched against the strain-specific and

209 universal contaminant protein databases using Sequest within Proteome Discoverer 1.4.

210 Identifications were filtered to include only high-confidence peptides based on a better than 1%

211 false discovery rate (FDR) against a decoy database. Proteins were linked to peptides, and only

212 proteins with >2 peptide spectral matches (PSM) with two unique peptides were kept

213 (Supporting Information: Dataset S1).

214 Functional characterization of OMV proteins

215 Identified proteins were annotated against the Clusters of Orthologous Groups (COG) of

216 proteins database (Galperin *et al.*, 2021), and their subcellular locations were predicted using

217 CELLO v2.5 (http://cello.life.nctu.edu.tw/) (Yu *et al.*, 2006) and PSORTdb v4.0

218 (https://db.psort.org/) (Lau *et al.*, 2021). Enrichment and functional associations between

219 proteins were determined by STRINGdb v11 (https://string-db.org/) (Szklarczyk *et al.*, 2019) and

220 ShinyGO v0.8 (http://bioinformatics.sdstate.edu/go/) (Ge *et al.*, 2020). Settings were selected to

221 use all coding sequences in the T7901 genome as background and a minimum FDR stringency of

222 1×10^{-5}. We then established which OMV proteins are predicted to exhibit carbohydrate-active

223 properties using the annotated T7901 genome in the Carbohydrate Active Enzymes Database

224 (CAZy, https://cazy.org).

225 Enzymatic activity assays

226 The cellulolytic activity of proteins in OMV preparations was visualized and size

227 fractionated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE, 1.5 mm thickness, 9%

228 acrylamide, and 0.2% CMC final concentration). For zymographic analysis, 14 µg of protein

229 sample determined by Pierce 660 assay (Thermo Scientific) using bovine serum albumin as

230 standard were heat denatured by boiling for 3 minutes in SDS without a reducing agent to

231 facilitate recovery of activity after refolding and added to each lane. After electrophoresis, the

232 SDS-PAGE gel was transferred to 500 mL of refolding buffer (20% isopropanol, 0.1% Triton X-100
233 in 1x PBS, pH 7.4) and gently shaken for 1 hour at room temperature. The gel was then
234 incubated in fresh 1x PBS buffer, pH 7.4, for 16 hours at room temperature before being stained
235 with 0.1% Congo Red in 1x PBS buffer, pH 7.4 for 1 hour. The zymogram was destained with 1 M
236 NaCl overnight to visualize regions where cellulase activity removed CMC. Additionally, the
237 production of reducing ends was measured by the colorimetric 3,5-dinitrosalicylic acid (DNS)
238 method (Ghose, 1987). Reactions were carried out in 96-well plates containing 50 µL 1% CMC,
239 30 µL 50 mM citrate buffer, and 20 µL purified OMV suspension and incubated for 4 hours at 37
240 °C. Next, 900 µL of DNS was added to each reaction, and reactions were heated at 99 °C for 10
241 minutes. Absorbance was measured at 540 nm, and sugar concentration was calculated by
242 comparison to a glucose standard curve. The enzyme activity unit was defined as the amount of
243 enzyme that liberated 1 µmol of reducing sugar per minute.

244 Results

245 OMV isolation and characterization

246 OMVs were isolated from cell-free supernatants of two closely related strains, T7901 and
247 SR01903, by density gradient ultracentrifugation. The presence of OMVs was confirmed by NTA
248 and TEM (Figure 2 A-D). The mean concentration of particles and average particle diameter size
249 determined by NTA were 1.67 x 10^9 particles/mL and 162.5 $\pm$ 49.7 nm for T7901 and 1.30 x 10^9
250 particles/mL and 206.6 $\pm$ 61.7 nm for SR01903. TEM images (Figure 2 C-D) show that the
251 putative OMV-enriched fractions from strains T7901 and SR01903 contain particles with size
252 distribution and morphology typical of bacterial OMVs.

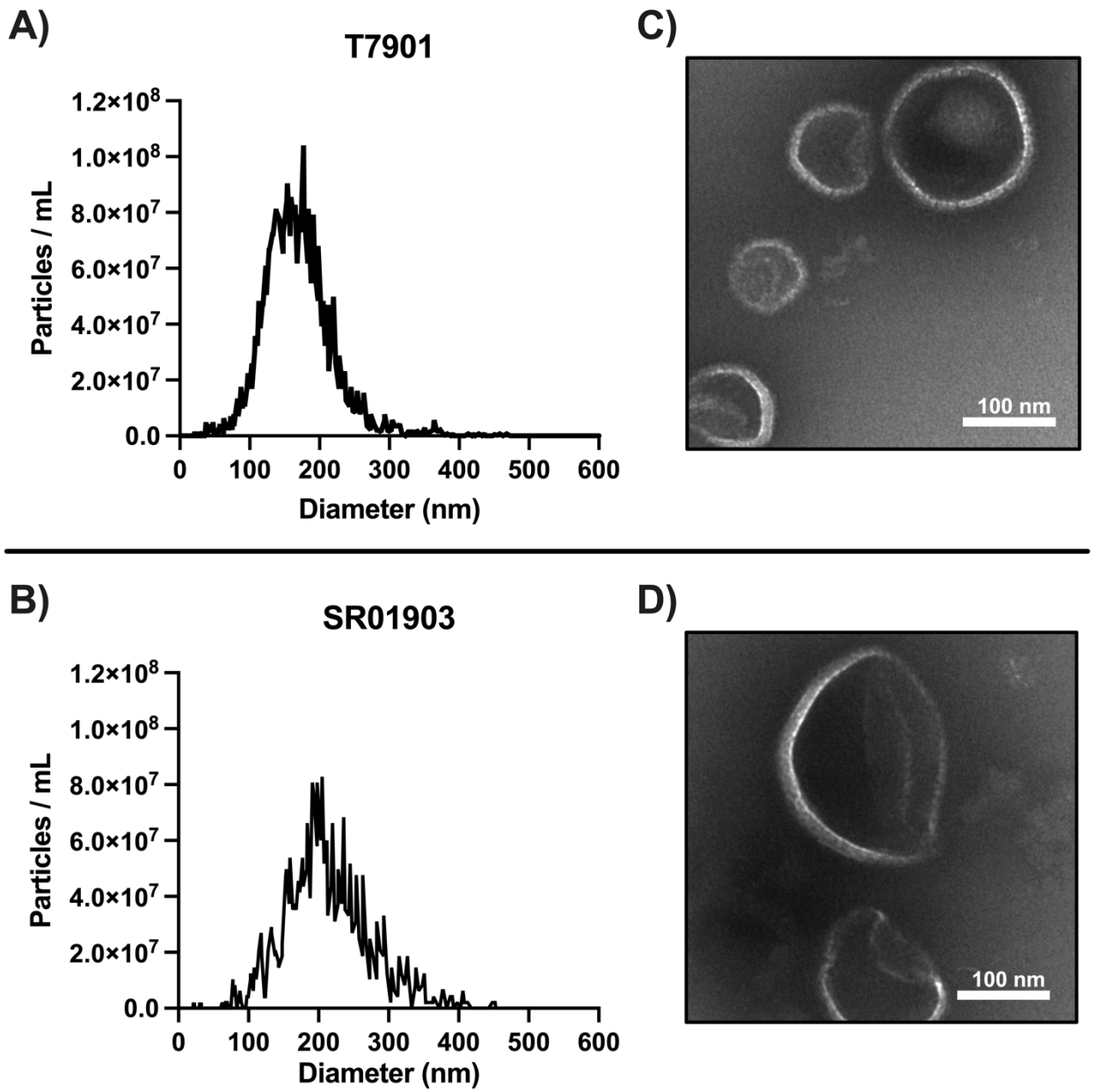

Figure 2. Size, concentration, and visualization of OMVs isolated from T. turnerae culture supernatants after growth on carboxymethyl cellulose (CMC). (A-B) Size distribution and particle concentration for strains T7901 and SR01903, respectively. OMVs from SR01903 were slightly larger in average size than OMVs from T7901. *(C-D) Isolated OMVs from strains T7901 and SR01903 were imaged using transmission electron microscopy. Scale bar = 100 nm.*

OMV proteome analysis

The protein contents of OMVs from both strains were analyzed by LC-MS/MS. Peptide-generated data were used to search against T7901 and SR01903 annotated protein sequences to identify OMV proteins and their relative abundance. A cut-off of two or more unique

262 predicted peptides per identified protein was used, resulting in the identification of 472 and 569
263 proteins for T7901 and SR01903, respectively. The Top 100 most abundant proteins identified in
264 T7901 and SR01903 represented 81% and 74% of the total peptide content, respectively. Among
265 those proteins, 71 were identified in both strains, indicating that the two strains produce a
266 common core set of OMV proteins when grown under the examined culture condition. After
267 protein identification, PSORTdb v4.0 (Lau *et al.*, 2021) and CELLO v2.5 (Yu *et al.*, 2006) were
268 used to predict the subcellular location of all identified proteins. Outer membrane proteins
269 comprised most (~75% and ~60%, respectively) of the OMV protein content of both T7901 and
270 SR01903 (Figure 3 A). OMV proteins were clustered into orthologous groups (COG); 65% and
271 60% of OMV proteins for T7901 and SR01903 were assigned a single cluster, respectively. Most
272 OMV proteins assigned to clusters in both strains were categorized as Cell
273 Wall/Membrane/Envelope Biogenesis, Inorganic Ion Transport and Metabolism, and
274 Carbohydrate Transport and Metabolism (Figure 3 B).

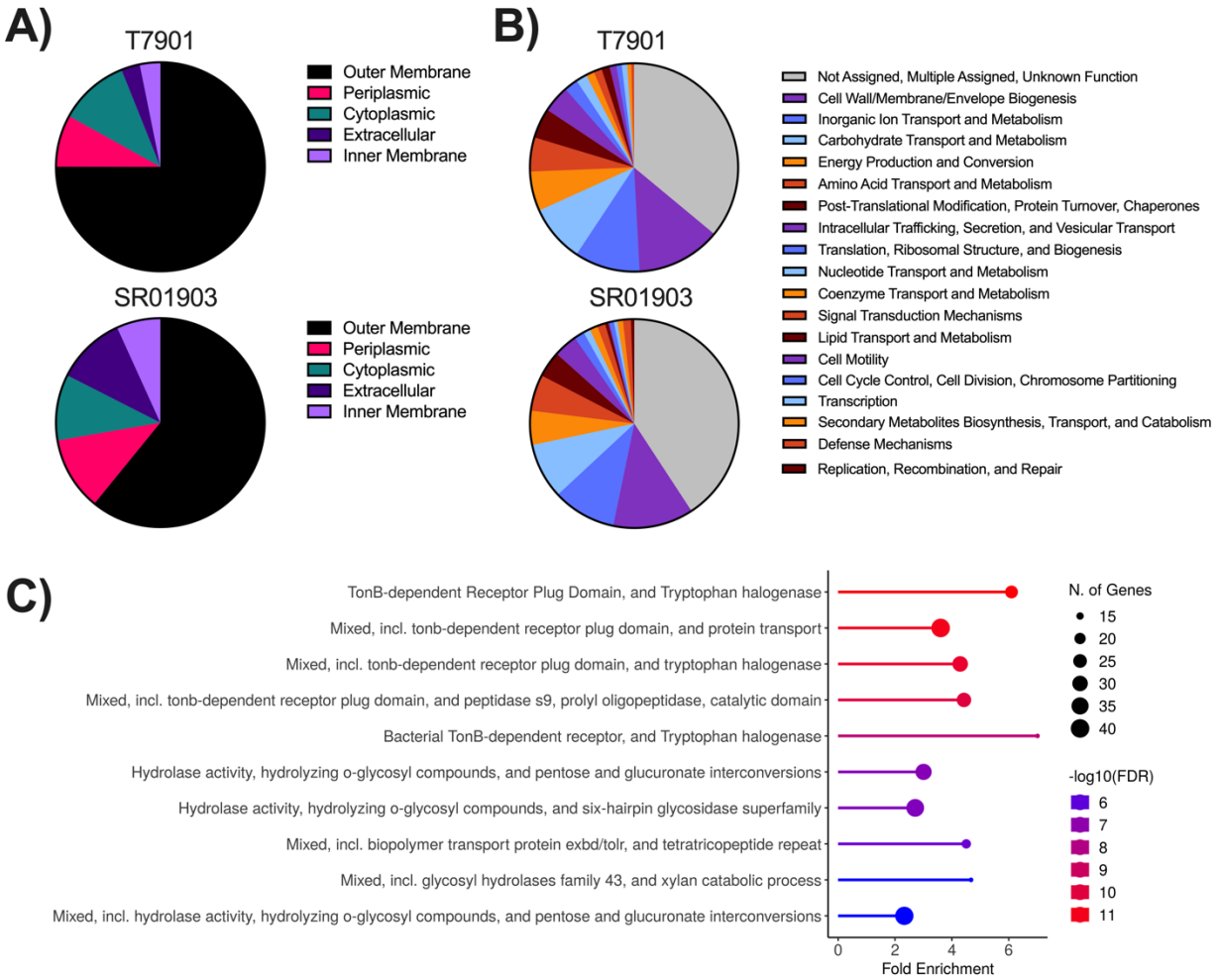

Figure 3. T. turnerae OMV proteome profiles. (A) Relative protein abundance grouped by predicted subcellular location and (B) Clusters of Orthologous Groups (COG) functional categories of identified proteins. (C) Top 10 STRINGdb pathways (ranked by FDR) found in OMVs of T7901. Similar top pathways were found in OMVs of SR01903.

TonB-dependent receptors and glycosyl hydrolase activity are enriched in OMVs

To highlight functional associations, OMV protein content was analyzed using STRING (Szklarczyk *et al.*, 2019) enrichment. We found significant enrichment of pathways involving *TonB*-dependent receptors (TBDR) and glycosyl hydrolase activity (Figure 4 C). The most abundant proteins were TBDRs, which constituted ~47% and ~44% of the OMV protein content in T7901 and SR01903, respectively. TBDRs are known to be deployed for the uptake of sugars from complex carbohydrates (Blanvillain *et al.*, 2007; Pollet *et al.*, 2021). Additionally, *TonB* knockouts of *T. turnerae* were shown to have lost the ability to grow on cellulose (Naka and

287 Haygood, 2023), which suggests that the *TonB* system in *T. turnerae* is critical to cellulose
288 utilization.

289 Efficient utilization of lignocellulose for growth often requires a cocktail of multiple
290 enzymes targeting inner (endo-acting) and outer (exo-acting) polysaccharide glycosidic bonds
291 and various bonds found in heteroxylans. In total, 39 and 48 predicted CAZymes were present,
292 representing 30 and 29 different catalytic CAZy families and 8.3% and 8.4% of identified OMV
293 proteins in T7901 and SR01903, respectively. Most identified CAZy modules belong to glycoside
294 hydrolase families (GH) predicted to be involved in cellulose and hemicellulose degradation
295 (Figure 4 A) and that include several hydrolytic activities: endo-glucanases (GH5, GH8, GH9,
296 GH10, GH11, GH26, GH44, GH45, GH51, GH62), exo-glucanases (GH5, GH9, GH26GH43GH26,
297 GH43), and beta-glucosidases (GH2, GH3, GH5, GH39). In addition, multiple enzymes were
298 predicted to include non-catalytic carbohydrate-binding modules (CBMs), which can enhance
299 enzyme activity by increasing accessibility to the substrate (Hervé *et al.*, 2010).

300 Cellulase activity

301 Many OMV proteins were predicted to have or be associated with cellulolytic activity.
302 We confirmed these by testing OMV suspensions for hydrolytic activity against CMC by
303 zymography and reducing sugar DNS assay. In zymograms, clear zones indicating endoglucanase
304 activity (Teather and Wood, 1982) were visualized between 95-130 kDa for both strains after 16-
305 hour incubation (Figure 4 B). The production of reducing sugars from CMC was measured using
306 DNS assay, and when normalized to total protein content, OMVs produced from SR01903 had
307 the equivalent cellulolytic activity as a commercial soluble cellulase enzyme cocktail. Notably,
308 OMVs produced from T7901 showed ~10x the activity of the commercial cellulase cocktail
309 (Figure 4 C).

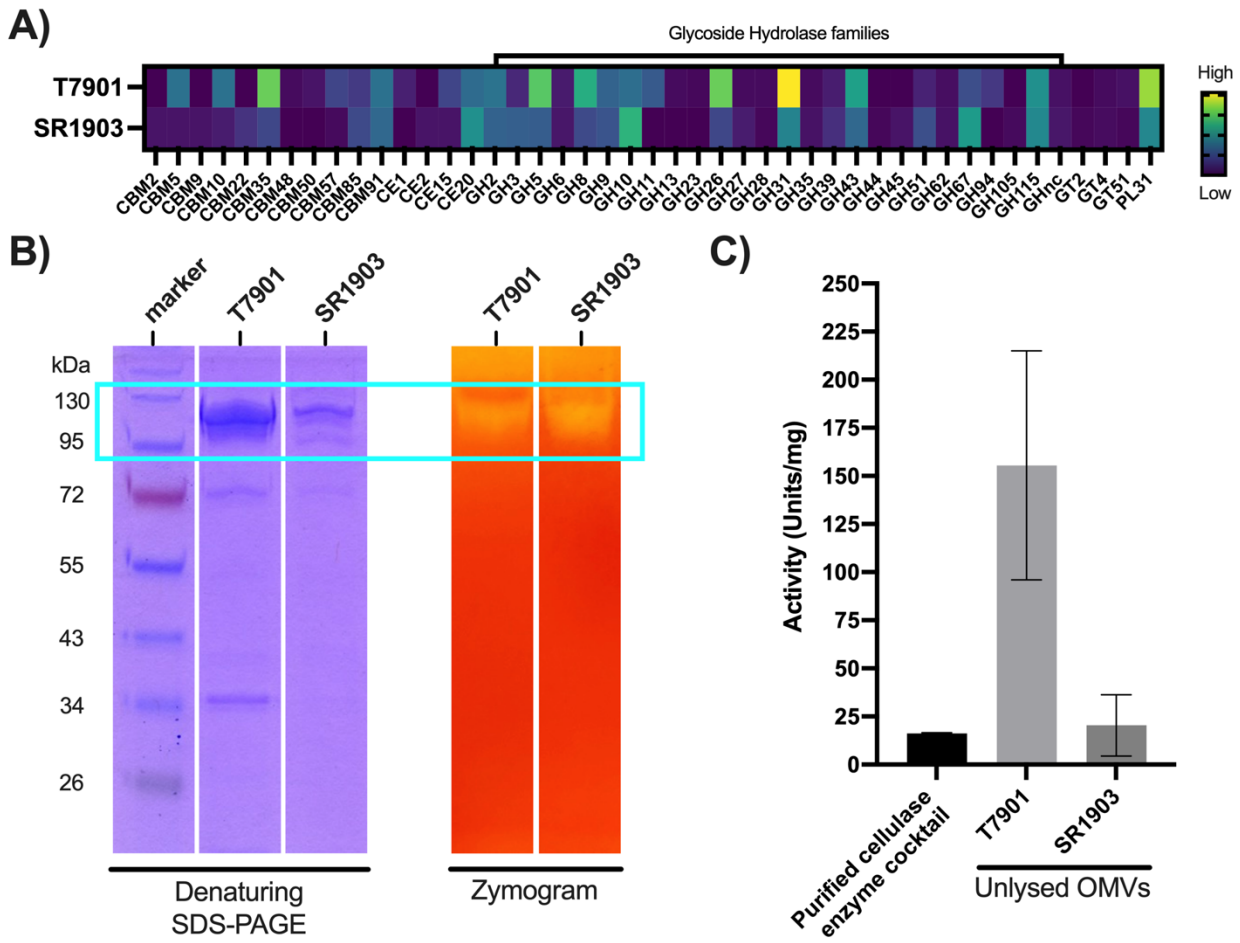

Figure 4. Carbohydrate-active modules and observed cellulase activity of T. turnerae OMVs. (A) Heatmap profile of the relative abundance of the predicted carbohydrate-active enzymes found in OMVs. (B) Denaturing SDS-PAGE (left) and zymogram (right; CMC substrate) of isolated OMVs. (C) Histogram showing cellulase activity (CMC substrate) in Units/mg (as determined by DNS assay) for isolated OMVs and a purified cellulase enzyme cocktail (Cellulase R-10, Goldbio CAS# 9012-54-8). Bars indicate means (error bars: standard deviations of three replicates).

Discussion

T. turnerae secretes a broad array of carbohydrate-active enzymes within the host's gills and when grown in pure culture (Yang *et al.*, 2009; O'Connor *et al.*, 2014; Sabbadin *et al.*, 2018). Genome sequence suggests these enzymes may be translocated across the inner membrane by Sec and Sec-independent (twin-arginine, Tat) pathways and through the outer membrane by the Type II (T2SS) generalized secretory pathway. The *T. turnerae* genome also encodes the complete Type VI secretion system (T6SS), which mediates many types of bacteria-bacteria and

323 bacteria-host interactions (Yang *et al.*, 2009). However, the role of OMVs in the transport of
324 cellulolytic enzymes by *T. turnerae* has not been investigated. Here, we show that *T. turnerae*
325 strains T7901 and SR01903 produce OMVs that contain diverse carbohydrate-active enzymes,
326 including glycoside hydrolases, carbohydrate esterases, and polysaccharide lyase with predicted
327 activity against cellulose, hemicellulose, and pectin. We further show that these OMV
328 preparations can hydrolyze carboxymethyl cellulose (CMC) with specific activities comparable to
329 or greater than that observed for a commercial purified cellulase enzyme cocktail (Figure 4 C).
330 In purified OMVs isolated from *T. turnerae* strains T7901 and SR01903, we detected
331 representatives of 11 carbohydrate-binding module (CBM) families (CBM2, 5, 9, 10, 22, 35, 48,
332 50, 57, 85, and 91). Each of these families is known to bind cellulose or hemicellulose
333 components, except for CBM50, which typically binds peptidoglycan or chitin. Additionally,
334 catalytic modules representing 23 glycoside hydrolase (GH) families, four carbohydrate esterase
335 (CE) families, and one polysaccharide lyase (PL) were detected. All of these GH families (GH2, 3,
336 5, 8, 9, 10, 11, 13, 23, 26, 28, 31, 35, 39, 43, 44, 45, 51, 62, 67, 94, 105, and 115) target bonds
337 found within cellulose, hemicellulose or pectin, except GH23, which primarily acts on
338 peptidoglycan. Finally, the four carbohydrate esterase (CE) families detected (CE1, 2, 15, and 20)
339 target bonds found in hemicellulose, or bonds covalently linking hemicellulose to lignin. This
340 combination of modules indicates a complete lignocellulose deconstruction system, including
341 potential endo- and exo-activities against cellulose and hemicellulose backbones, debranching
342 of hemicellulose sidechains, and hydrolysis of the resulting oligomers and monomers.
343 The fact that this wide array of activities was found in OMVs produced by cells of *T.*
344 *turnerae* strains T7901 and SR01903 grown with cellulose as a sole carbon source indicates that

345 the expression of genes active against other lignocellulose components was either constitutive
346 or co-regulated with expression of genes targeting cellulose. The ability of *T. turnerae* to express
347 these activities in the absence of induction by their specific target substrates is consistent with
348 the observation that shipworm endosymbionts express a wide range of lignocellulolytic
349 enzymes when growing within the gill bacteriocytes of the host where they have no direct
350 contact with lignocellulose (O'Connor *et al.*, 2014; Sabbadin *et al.*, 2018). Notably, six of the
351 catalytic module families detected in *T. turnerae* OMVs (CE11, 15, and GH9, 10, 11, and 45) were
352 also detected in the cecum content of *Bankia setacea* in a previous investigation (O'Connor *et*
353 *al.*, 2014); however, it should be noted that this shipworm species hosts four *Teredinibacter*
354 species, *T. waterburyi* (Altamia, Shipway, *et al.*, 2020), *T. franksiae*, *T. haidensis*, and *T. purpureus*
355 (Altamia *et al.*, 2021) but does not harbor *T. turnerae* (O'Connor *et al.*, 2014).
356 While diverse CAZymes are present in the OMV proteome of *T. turnerae*, they comprise
357 less than 10% of the total protein content of OMVs produced under the conditions examined
358 here. Interestingly, the most abundant proteins observed were identified as TonB-dependent
359 receptors (TBDR), accounting for nearly half of the proteins detected. TBDR enrichment has
360 been previously reported in OMVs of several Gram-negative bacteria (Veith *et al.*, 2015;
361 Zakharzhevskaya *et al.*, 2017; Dhurve *et al.*, 2022; Fadeev *et al.*, 2023). TBDRs are outer
362 membrane-associated proteins that bind and mediate the energy-dependent movement of
363 siderophores and various nutrients, including carbohydrates that are too large to be taken up
364 via transmembrane diffusion across the outer membrane (Silale and Van Den Berg, 2023). For
365 example, in the marine bacterium *Alteromonas macleodii*, TBDR genes were selectively
366 expressed in carbon- and iron-limiting conditions (Manck *et al.*, 2020) and during

367 polysaccharide utilization (Neumann *et al.*, 2015). Recently, it was shown that two of *T.*
368 *turnerae's* four *TonB* genes are essential for growth in iron-limiting conditions and for growth
369 with cellulose as a sole carbon source. This dependence indicates that the *TonB* system is
370 essential for both iron acquisition and cellulose catabolism by *T. turnerae* (Naka and Haygood,
371 2023).

372 Interestingly, in a proteomic examination of the shipworm *Banka setacea*, a TBDR was
373 the only major symbiont-derived protein observed in the cecum content that was not
374 associated with the decomposition of lignocellulose (O'Connor *et al.*, 2014). This finding
375 suggests the presence of symbiont outer membranes in the cecum. However, the cecum of
376 shipworms has been shown by microscopic (Betcher *et al.*, 2012; Pesante *et al.*, 2021),
377 transcriptomic (Sabbadin et al., 2018), and proteomic (O'Connor *et al.*, 2014) methods to be
378 nearly devoid of intact bacterial cells. These observations argue against the transport of whole
379 bacterial cells from the gill to the cecum as suggested in (Pesante *et al.*, 2021), but might
380 suggest a role for OMVs in transporting lignocellulose degrading enzymes from the gill to the
381 gut in shipworms.

382 Outer membrane vesicles are critical in bacterial interactions with many animal and
383 plant hosts (Berleman and Auer, 2013; Lynch and Alegado, 2017). While most research has
384 focused on pathogenic interactions, OMVs may also contribute to beneficial host-symbiont
385 associations. For example, OMVs have been shown to mimic whole cells of *Vibrio fisheri* in the
386 induction of specific aspects of light organ development in the Hawaiian bobtail squid *Euprymna*
387 *scolopes* (Aschtgen *et al.*, 2016). OMVs have also been shown to be produced by
388 chemoautotrophic symbionts that occur in the trophosome of flatworms of the genus

389 *Paracatenula,* where they play a critical role in provisioning the host with carbohydrates
390 synthesized by the symbionts (Jäckle *et al.*, 2019). In the human gut microbiome, members of
391 *Bacteroides* have been shown to tailor OMV content to specific polysaccharides (Sartorio *et al.*,
392 2023), and OMVs produced by human and ruminant gut bacteria, including Bacteroides,
393 Fibrobacter, and Clostridium, communally degrade polysaccharides into nutrients available to
394 the hosts (Rakoff-Nahoum *et al.*, 2014; Arntzen *et al.*, 2017; Gharechahi *et al.*, 2023).
395 Outer membrane vesicles can also transport proteins across the plasma membranes of
396 eukaryotic organisms (O'Donoghue and Krachler, 2016; Schorey *et al.*, 2021; Toyofuku *et al.*,
397 2023). While previous research has focused mainly on the role of OMVs in introducing bacterial
398 molecules into eukaryotic cells, OMVs may also transport molecules produced by intracellular
399 bacteria out of their eukaryotic host. For example, *Salmonella enterica,* which grows in vesicles
400 within their host's cells, produces toxin-loaded OMVs that escape the infected host cells and
401 deliver their toxic cargo to uninfected neighboring cells (O'Donoghue and Krachler, 2016).
402 Similarly, OMVs produced by *Mycobacterium tuberculosis* are released from infected
403 macrophages, exporting Mycobacterial lipoproteins and lipoglycans that affect the immune
404 functions of neighboring uninfected host cells (Athman *et al.*, 2015). Thus, OMV-mediated
405 mechanisms could potentially explain the transport of cellulolytic proteins produced by *T.*
406 *turnerae* in their intracellular location within the host's bacteriocytes to an extracellular
407 location, allowing subsequent transport to the host's gut.
408 Packaging enzymes within OMVs or bound to OMV surfaces can have distinct advantages
409 over their secretion as soluble proteins or proteins bound to the cell surface. For example,
410 OMVs may protect proteins from degradation in the environment (Bonnington and Kuehn,

411 2014; Alves *et al.*, 2016; Zingl *et al.*, 2021), selectively concentrate proteins with specific
412 functions (Orench-Rivera and Kuehn, 2021; Sartorio *et al.*, 2023), and deliver proteins to remote
413 locations in sufficient quantity to produce a desired effect without the dilution that would be
414 experienced by soluble proteins (Toyofuku *et al.*, 2023). These factors may be especially
415 important for the degradation of complex substrates like lignocellulose, where the simultaneous
416 delivery of specific sets of proteins in the correct proportions and spatial orientation can yield
417 significant synergistic interactions that enhance efficiency (Park *et al.*, 2014).

418 However, little is known about OMV production and its function in environmental
419 lignocellulose degradation. Several investigations of lignocellulose-degrading microorganisms
420 from soil suggest OMVs play critical roles in terrestrial plant biomass degradation. For example,
421 when grown in the presence of lignin, *Pseudomonas putida* produces OMVs containing enzymes
422 functionally active against lignin aromatic components (Salvachúa *et al.*, 2020). Similarly,
423 *Trichoderma reesei*, a filamentous fungus, produces OMVs containing a variety of cellulases
424 when grown in the presence of cellulose (De Paula *et al.*, 2019).

425 In addition to their potential roles in shipworm symbiosis and the environmental
426 turnover of lignocellulose in terrestrial and aquatic environments, cellulolytic OMVs are of
427 significant interest in converting plant biomass into renewable liquid fuels or fine chemicals
428 (Thakur *et al.*, 2023). A significant challenge in biomass conversion is understanding how
429 cooperation among cellulases and associated enzymes can improve the design of efficient
430 enzyme cocktails tailored to individual feedstocks (Østby *et al.*, 2020). OMVs produced by *T.*
431 *turnerae* and other lignocellulolytic organisms may represent nature-based solutions that reveal
432 specific combinations, concentrations, and spatial organization of cooperating enzymes that

433 significantly enhance lignocellulose degradation in nature and so might inspire the design of

434 engineered lignocellulose deconstruction systems.

435

436 ## Figure legends

437 Figure 1. Methods used to isolate and characterize outer membrane vesicles from *T. turnerae*.

438 Diagram showing: (A) bacterial culture, (B) OMV isolation based on differential and

439 density gradient separation, (C) OMV visualization and size by TEM and NTA, proteome

440 analysis by LC-MS/MS, and (D) detection of cellulase activity in purified OMV

441 preparations by zymography and reducing sugar (DNS) assay. Created with

442 BioRender.com.

443 Figure 2. Size, concentration, and visualization of OMVs isolated from *T. turnerae* culture

444 supernatants after growth on carboxymethyl cellulose (CMC). (A-B) Size distribution

445 and particle concentration for strains T7901 and SR01903, respectively. OMVs from

446 SR01903 were slightly larger in average size than OMVs from T7901. (C-D) Isolated

447 OMVs from strains T7901 and SR01903 were imaged using transmission electron

448 microscopy. Scale bar = 100 nm.

449 Figure 3. T. turnerae OMV proteome profiles. (A) Relative protein abundance grouped by

450 predicted subcellular location and (B) Clusters of Orthologous Groups (COG) functional

451 categories of identified proteins. (C) Top 10 STRINGdb pathways (ranked by FDR) found

452 in OMVs of T7901. Similar top pathways were found in OMVs of SR01903.

453 Figure 4. Carbohydrate-active modules and observed cellulase activity of *T. turnerae* OMVs. (A)

454 Heatmap profile of the relative abundance of the predicted carbohydrate-active

455 enzymes found in OMVs. (B) Denaturing SDS-PAGE (left) and zymogram (right; CMC

substrate) of isolated OMVs. (C) Histogram showing cellulase activity (CMC substrate) in Units/mg (as determined by DNS assay) for isolated OMVs and a purified cellulase enzyme cocktail (Cellulase R-10, Goldbio CAS# 9012-54-8). Bars indicate means (error bars: standard deviations of three replicates).

Author Contribution Statement

Mark T. Gasser: Conceptualization; investigation; formal analysis; methodology; writing – original draft preparation. **Annie Liu:** Investigation; methodology. **Marvin A. Altamia:** Investigation; methodology. **Bryan R. Brensinger:** Investigation; methodology. **Sarah L. Brewer:** Investigation; methodology. **Ron Flatau:** Investigation; methodology. **Eric R. Hancock:** Investigation; methodology. **Sarah P. Preheim:** Supervision; writing – review and editing. **Claire Marie Filone:** Funding acquisition; supervision; writing – review and editing. **Dan L. Distel:** Funding acquisition; Conceptualization; supervision; writing – original draft preparation; writing – review and editing.

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Supplemental Information

Figure_S1_SuppInfo.docx

Dataset_S1_SuppInfo.xlsx

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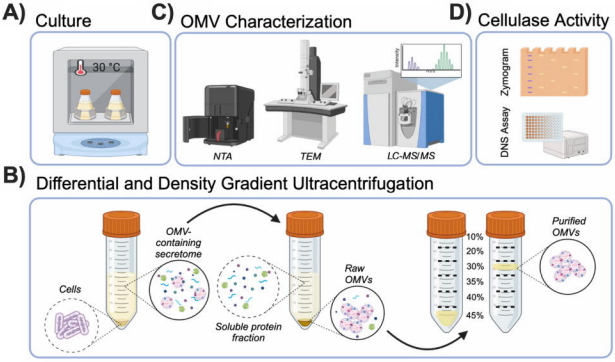
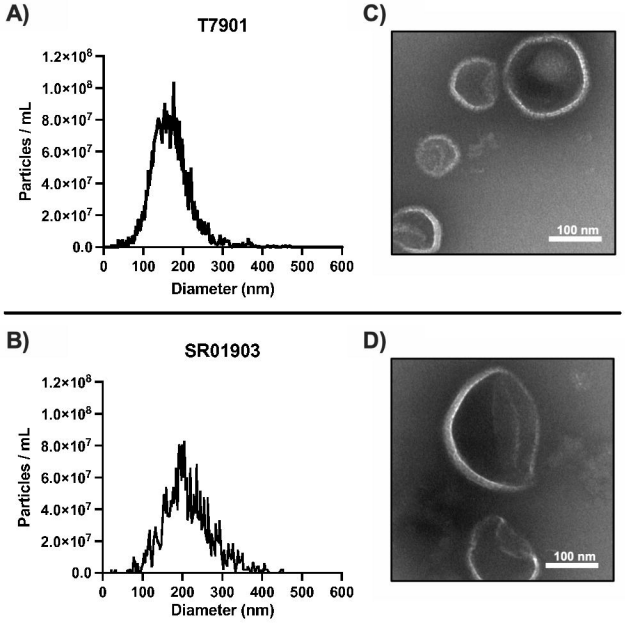
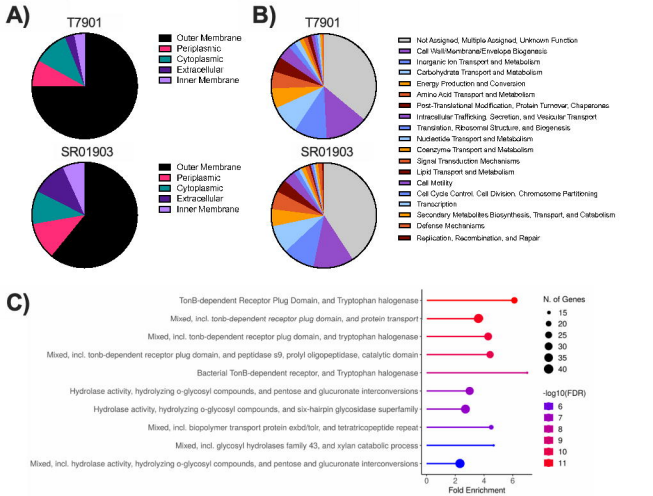
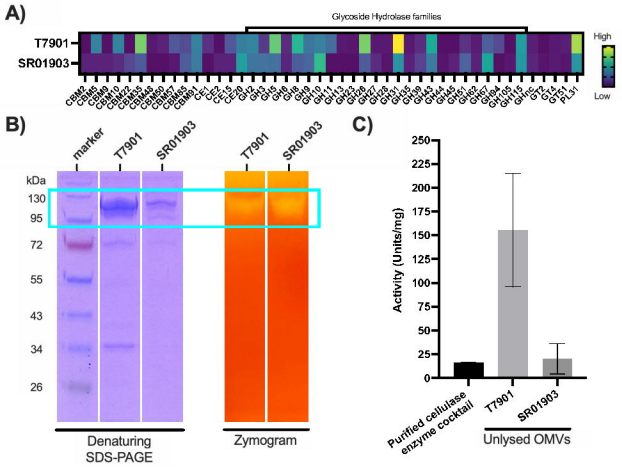

A)
Culture
30 °C
C)
OMV Characterization
NTA
TEM
LC-MS/MS
D)
Cellulase Activity
Zymogram
DNS Assay
B)
Differential and Density Gradient Ultracentrifugation
Cells
OMV-containing secretome
Soluble protein fraction
Raw OMVs
10%
20%
30%
35%
40%
45%
Purified OMVs


A)
T7901
Particles / mL
1.2×10^8
1.0×10^8
8.0×10^7
6.0×10^7
4.0×10^7
2.0×10^7
0.0
0
100
200
300
400
500
600
Diameter (nm)
C)
100 nm
B)
SR01903
Particles / mL
1.2×10^8
1.0×10^8
8.0×10^7
6.0×10^7
4.0×10^7
2.0×10^7
0.0
0
100
200
300
400
500
600
Diameter (nm)
D)
100 nm


A)
T7901
SR01903
Outer Membrane
Periplasmic
Cytoplasmic
Extracellular
Inner Membrane
B)
T7901
SR01903
Not Assigned, Multiple Assigned, Unknown Function
Cell Wall/Membrane/Envelope Biogenesis
Inorganic Ion Transport and Metabolism
Carbohydrate Transport and Metabolism
Energy Production and Conversion
Amino Acid Transport and Metabolism
Post-Translational Modification, Protein Turnover, Chaperones
Intracellular Trafficking, Secretion, and Vesicular Transport
Translation, Ribosomal Structure, and Biogenesis
Nucleotide Transport and Metabolism
Coenzyme Transport and Metabolism
Signal Transduction Mechanisms
Lipid Transport and Metabolism
Cell Motility
Cell Cycle Control, Cell Division, Chromosome Partitioning
Transcription
Secondary Metabolites Biosynthesis, Transport, and Catabolism
Defense Mechanisms
Replication, Recombination, and Repair
C)
TonB-dependent Receptor Plug Domain, and Tryptophan halogenase
Mixed, incl. tonb-dependent receptor plug domain, and protein transport
Mixed, incl. tonb-dependent receptor plug domain, and tryptophan halogenase
Mixed, incl. tonb-dependent receptor plug domain, and peptidase s9, prolyl oligopeptidase, catalytic domain
Bacterial TonB-dependent receptor, and Tryptophan halogenase
Hydrolase activity, hydrolyzing o-glycosyl compounds, and pentose and glucuronate interconversions
Hydrolase activity, hydrolyzing o-glycosyl compounds, and six-hairpin glycosidase superfamily
Mixed, incl. biopolymer transport protein exbd/tolr, and tetratricopeptide repeat
Mixed, incl. glycosyl hydrolases family 43, and xylan catabolic process
Mixed, incl. hydrolase activity, hydrolyzing o-glycosyl compounds, and pentose and glucuronate interconversions
0
2
4
6
Fold Enrichment
N. of Genes
15
20
25
30
35
40
-log10(FDR)
6
7
8
9
10
11


A)
Glycoside Hydrolase families
T7901
SR01903
High
Low
CBM2
CBM5
CBM9
CBM10
CBM22
CBM35
CBM46
CBM50
CBM57
CBM65
CBM81
CE1
CE2
CE15
CE20
GH2
GH3
GH5
GH6
GH8
GH9
GH10
GH11
GH13
GH23
GH26
GH27
GH28
GH31
GH35
GH39
GH43
GH44
GH45
GH51
GH62
GH67
GH94
GH105
GH115
GHnc
GT2
GT4
GT51
PL31
B)
marker
T7901
SR01903
T7901
SR01903
kDa
130
95
72
55
43
34
26
Denaturing SDS-PAGE
Zymogram
C)
Activity (Units/mg)
250
225
200
175
150
125
100
75
50
25
0
Purified cellulase enzyme cocktail
T7901
SR01903
Unlysed OMVs