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## The Secretome landscape of *Escherichia coli* O157:H7: Deciphering the cell-surface, outer membrane vesicle and extracellular subproteomes

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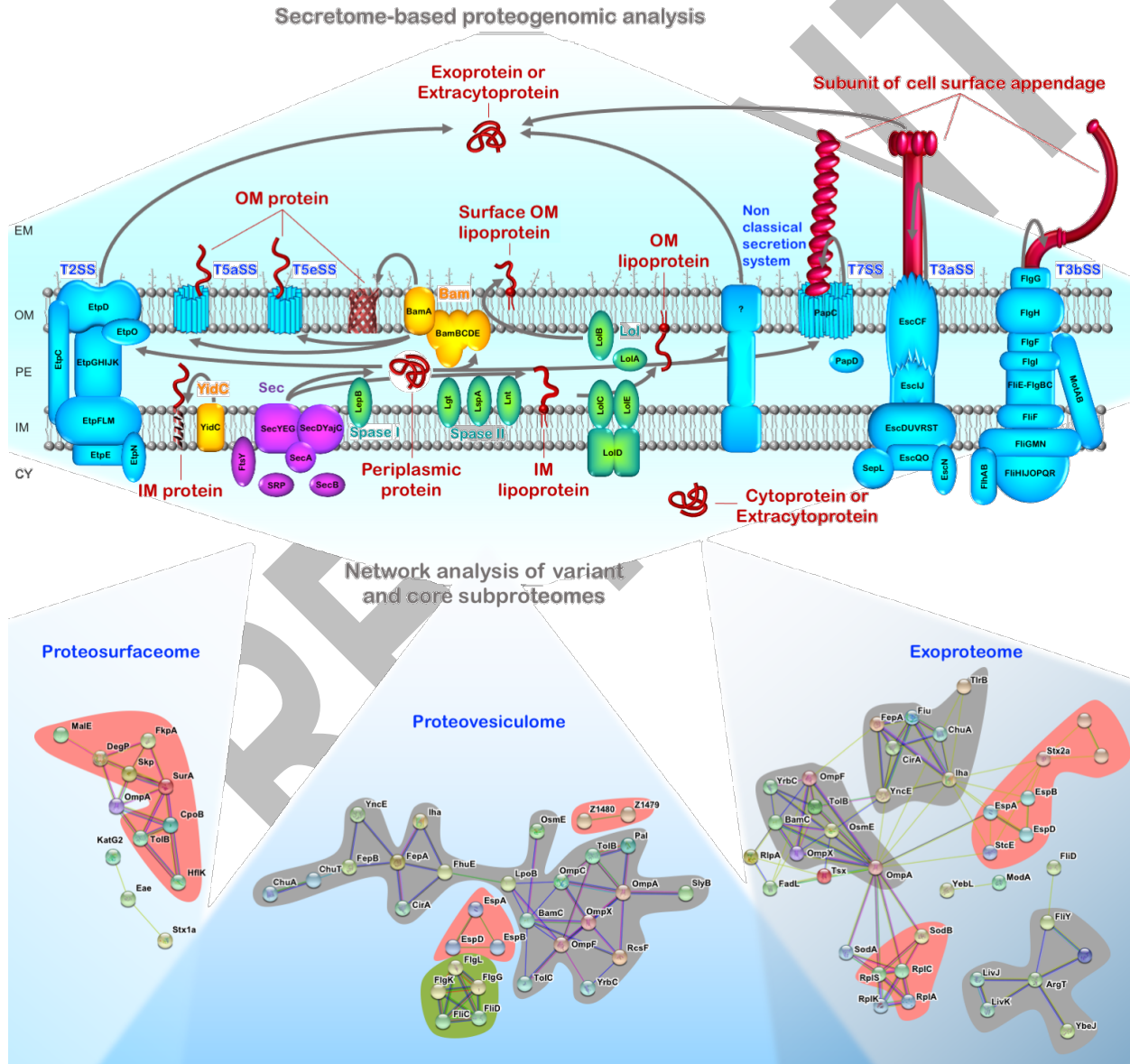
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1 **The Secretome Landscape of *Escherichia coli* O157:H7: Deciphering the Cell-Surface, Outer**  
 2 **Membrane Vesicle and Extracellular Subproteomes**

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6 **GRAPHICAL ABSTRACT**

**Deciphering the extracytoplasmic subproteomes in *E. coli* O157:H7**



8        **The Secretome Landscape of *Escherichia coli* O157:H7:**  
9        **Deciphering the Cell-Surface, Outer Membrane Vesicle and**  
10        **Extracellular Subproteomes**

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24 **Abstract**

25 Among diarrheagenic *E. coli* (DEC), enterohaemorrhagic *E. coli* (EHEC) are the most virulent  
26 anthropozoonotic agents. The ability of bacterial cells to functionally interact with their  
27 surrounding essentially relies on the secretion of different protein effectors. To experimentally  
28 determine the repertoire of extracytoproteins in *E. coli* O157:H7, a subproteomic analysis was  
29 performed not only considering the extracellular milieu but the cell surface and outer membrane  
30 vesicles. Following a secretome-based approach, the proteins trafficking from the interior to the  
31 exterior of the cell were depicted considering cognate protein transport systems and subcellular  
32 localisation. Label-free quantitative analysis of the proteosurfaceome, proteovesiculome and  
33 exoproteome from *E. coli* O157:H7 grown in three different nutrient media revealed differential  
34 protein expression profiles and allowed defining the core and variant subproteomes. Network  
35 analysis further revealed the higher abundance of some protein clusters in chemically defined  
36 medium over rich complex medium, especially related to some outer membrane proteins, ABC  
37 transport and Type III secretion systems. This first comprehensive study of the EHEC secretome  
38 unravels the profound influence of environmental conditions on the extracytoplasmic proteome,  
39 provides new insight in the physiology of *E. coli* O157:H7 and identifies potentially important  
40 molecular targets for the development of preventive strategies against EHEC/STEC.

41 **Keywords:** Shiga toxin-producing *E. coli* (STEC); Subproteomic analysis; Secretome; Outer  
42 membrane vesicle; Cell-surface proteins; Extracellular proteins

## 43 **Introduction**

44 *Escherichia coli* are an inhabitant of warm-blooded animals intestines and typically coexist with  
45 the host as a commensal with mutual benefit for both organisms [1]. However, some highly  
46 adapted *E. coli* strains have acquired specific virulence attributes, which allow them causing a  
47 broad spectrum of diseases. Based on clinical syndromes, these pathogenic strains can be  
48 discriminated into extraintestinal pathogenic *E. coli* (ExPEC) responsible for urinary tract  
49 infections, sepsis and/or meningitis [2] and intestinal pathogenic *E. coli* (InPEC), especially the  
50 diarrheagenic *E. coli* (DEC) subset. DEC represent a leading cause for traveller's diarrhoea but  
51 also pediatric disease in developing countries, and an emerging source of diarrhoea in  
52 industrialised countries [3]. Enterohaemorrhagic *E. coli* (EHEC), which belong to the group of  
53 shigatoxin encoding *E. coli* (STEC), are the most severe etiologic agent for *E. coli* diarrhoea in  
54 humans, with a wide spectrum of diseases ranging from aqueous or bloody diarrhoea to severe  
55 cases of haemorrhagic colitis to haemolytic uremic syndrome (HUS), the leading cause of chronic  
56 renal failure in children [4, 5]. Over the 400 distinct serotypes of STEC reported to date [6], only  
57 a handful of serogroups are reported in human outbreaks [7], included EHEC O157:H7 considered  
58 as one of the most severe [8].

59 While shigatoxin (Stx) is regarded as the main virulence factor for EHEC, it is neither  
60 essential in DEC nor sufficient in EHEC. Actually, the ability of EHEC to colonise the intestinal  
61 epithelial surface would also be a major factor in tuning the virulence level and thus the severity  
62 of the infection, *e.g.* the attaching and effacing lesions (A/E lesions) [9, 10]. Most of the genes  
63 necessary to form A/E lesions are encoded by a 35-kb chromosomal pathogenicity island (PAI)  
64 known as the locus for enterocyte effacement (LEE), particularly genes encoding a Type III,  
65 subtype a, secretion system (T3aSS) and the cognate effector proteins [11, 12]. Besides infection,

66 some virulence factors could play a role as niche factors in various environmental conditions  
67 encounters by bacterial cells along the food chain [13, 14], from soil, plants, animal carriage, food  
68 matrices to human gastrointestinal tract when considering EHEC [15, 16]. While the main secreted  
69 virulence factors are now well characterised in EHEC O157:H7 [17-19], additional genes are most  
70 probably induced in the course of an infection [20] but their expression at a protein level and in  
71 other environmental conditions remain elusive. In lipopolysaccharidic (LPS) diderm bacteria (*i.e.*  
72 archetypal Gram-negative bacteria) [21], nine protein secretion systems, from Type I to Type IX  
73 (T1SS-T9SS) have been uncovered to date but, surprisingly enough, some proteins can be found  
74 as localised extracellularly without any obvious cognate secretion pathways. Such transport *via*  
75 unknown protein trafficking route is referred as non-classical secretion (NCS) [22, 23], where  
76 different molecular mechanisms can be at play, *e.g.* controlled lysis, piggybacking or outer  
77 membrane vesicles (OMVs) [24-26]. As secretion refers to the active transport from the interior to  
78 the exterior of the cell compartment [21], secreted proteins can have four main possible fates in  
79 LPS-diderm bacteria, *i.e.* to (i) remain associated with the bacterial outer membrane (OM), (ii) be  
80 released into the extracellular milieu, (iii) be vectorised in OMVs, or (iv) be injected into a target  
81 cell (either a eukaryotic or bacterial cell) [27, 28]. Environmental growth conditions are known to  
82 influence the secretion of some classically and non-classically secreted proteins in *E. coli* O157:H7  
83 [29], however, the occurrence at a global secretome scale remains to be explored. Considering the  
84 poor correlation between mRNA and protein abundance, which is even more exacerbated when  
85 dealing with secreted proteins [30-32], proteomics is a pre-request to functional analyses.

86 Growth conditions are known to deeply influence the physiology of *E. coli* O157:H7, for  
87 instance in the ability to adhere to different extracellular matrix components [33], the adherence  
88 to intestinal epithelial cells [34, 35] or the virulence with the LEE expression [36, 37]. However,

89 there is still a gap of knowledge in apprehending the whole proteins secreted by *E. coli* O157:H7.  
90 For some times, the investigations of the extracellular proteome (exoproteome) were often  
91 restricted to the use of chemically defined medium due to the limit imposed by two-dimensional  
92 electrophoresis (2-DE) approaches, especially with the isoelectric focusing (IEF) in the first  
93 dimension, and the access to the cell-surface proteome (proteosurfaceome) proven difficult  
94 especially due to the hydrophobicity of outer membrane proteins (OMPs). Nowadays, the  
95 development of shotgun off-gel proteomics has clearly exceeded in-gel 2-DE approaches to  
96 circumvent these limitations, together with cell-surface biotin labelling to efficiently investigate  
97 the proteosurfaceome [38, 39]. The present study aims at deciphering the secretome landscape of  
98 *E. coli* O157:H7 by using a range of growth media most likely or known to induce changes in the  
99 protein secretion profiles, namely (i) M9 as a minimal medium forcing the bacterial cell to activate  
100 its full anabolic potential, (ii) DMEM (Dulbecco's modified Eagle's medium) as a chemically  
101 defined medium known to induce the expression of several virulence factors, and (iii) BHI (brain  
102 heart infusion) as a rich complex medium classically use for the growth of microbial pathogens.  
103 To get a whole picture of proteins secreted by *E. coli* O157:H7, three subproteomes were  
104 considered and characterised by relative quantification analysis, namely the proteosurfaceome  
105 (proteome of the cell surface), exoproteome (proteome of the extracellular milieu) and  
106 proteovesiculome (proteome of the membrane vesicles).

## 107 **Material and Methods**

### 108 **Bacterial growth conditions**

109 *Escherichia coli* O157:H7 strain EDL933 was used for this study [40]. Pre-cultures were grown  
110 aerobically in M9, DMEM (Dulbecco's modified eagle medium, Gibco), or BHI (brain-heart  
111 infusion, Becton-Dickinson) liquid media at 37°C under orbital shaking (150 rpm) in triplicates.  
112 After 1:100 (v/v) dilution, bacterial cultures were grown in the same conditions until late  
113 exponential phase and sampled at about 3, 5 and 8 h from BHI, DMEM and M9, respectively.  
114 Samples corresponded to the same OD<sub>600 nm</sub> (0.5) and bacterial cells were harvested by  
115 centrifugation (4 000 g, 5 min, 4°C) and the culture supernatant recovered.

### 116 **Isolation of bacterial cell-surface, outer membrane vesicle, and extracellular** 117 **proteins**

118 Bacterial cell-surface proteins were isolated following biotinylation and protein affinity  
119 purification as previously described in details [39] using sulfo-NHS-SS-biotin (sulfo-succinimidyl  
120 biotin-amidoethyl dithio-propionate) for protein labelling and neutravidin for purification. Briefly,  
121 bacterial cells were washed with 10 mM PBS pH 8.0 containing 1 mM phenylmethylsulfonyl  
122 fluoride (PMSF) and centrifuged as above. Pelleted cells were weighed to ensure the same amount  
123 of wet cells (100 mg) was labelled with sulfo-NHS-SS-biotin (1.5 mM) for 1 min at room  
124 temperature. After quenching the excess of biotinylation reagent with glycine buffer (500 mM)  
125 and centrifugation, cells were resuspended in Triton X-100 lysis buffer (1 %, 1 mM PMSF, 10 mM  
126 PBS pH 8). Cells were disrupted using FastPrep-24 classic (MP Biomedicals) with two steps of  
127 20 s at 6 m/s, cell debris discarded by centrifugation (20 000 g, 30 min, 4°C) and exactly the same  
128 volume of each biotinylated protein samples (500 µl) was used for affinity purification using  
129 neutravidin agarose resin (Pierce Thermo Scientific, 400 µl). After washing (10 mM PBS, pH 8,



130 1 % NP-40), labelled proteins were eluted (2 % SDS, 20 % glycerol, 5 %  $\beta$ -mercaptoethanol,  
131 50 mM dithiothreitol, 62.5 mM Tris-HCl).

132 Recovery of OMV and extracellular proteins were adapted from previously described protocols  
133 [41]. Briefly, the same volume of culture supernatant corresponding to the same amount of  
134 bacterial cells was first filtered through a 0.45  $\mu$ m membrane under vacuum (to eliminate residual  
135 cells), and ultrafiltrated on a polyethersulfone (PES) membrane at a 500 kDa cut-off (to  
136 concentrate the supernatant); the ultrafiltrate was kept and corresponded to the extracellular  
137 fraction, whereas the retentate was further processed to get the OMV fraction. In the ultrafiltrate,  
138 0.2 mM PMSF was added to inhibit protease activity as well as sodium deoxycholate  
139 (0.2 mg.mL<sup>-1</sup>) to support protein precipitation with trichloroacetic acid (10 % w/v). After  
140 centrifugation (20 000 g, 30 min, 4°C), the precipitate was washed with ice-cold acetone and all  
141 samples were solubilised in the same volume of PBS. The retentate was further ultracentrifuged  
142 (100 000 g, 3 h, 4°C) and all samples were resuspended in the same volume of PBS. Protein  
143 concentration was determined by the method of Bradford using bovine serum albumin as the  
144 standard [34].

#### 145 **Protein preparation for LC-MS/MS analysis**

146 To avoid bias in the comparison of protein abundance by relative quantification analyses, the  
147 bacterial cell surface, extracellular milieu and OMV fractions, respectively, were adjusted  
148 respective to the same amount of biomass in the different growth conditions as indicated above.

149 To eliminate interfering molecules (such as salts) and concentrate proteins in a single band of  
150 polyacrylamide gel, each sample was submitted to a short SDS-PAGE (sodium dodecyl sulfate–  
151 polyacrylamide gel electrophoresis) migration (T=12.5 %, C= 3.3%) in a BioRad Mini Protean II  
152 unit. After the addition of the same volume of Laemmli buffer (2 % SDS, 25 % glycerol, 5 %  $\beta$ -

153 mercaptoethanol, 0.005 % bromphenol blue and 62.5 mM Tris-HCl, final) and heating at 95°C for  
154 5 min, 15 µl of protein extracts from each subcellular fraction were migrated and just allowed to  
155 concentrate at the junction between stacking and resolving gel after a few minutes migration  
156 (25 mA/gel at 100 V). Gel was then stained with Coomassie Brilliant Blue G250 and the single  
157 bands in each lane were excised. The proteins in the gel were reduced in 100 mM ammonium  
158 bicarbonate with 45 mM dithiothreitol for 45 min at 50°C, and alkylated with 100 mM  
159 iodoacetamide for 20 min at room temperature in the dark. The gel was washed once in 25 mM  
160 ammonium bicarbonate in 5 % acetonitrile for 30 min and twice in 25 mM ammonium bicarbonate  
161 in 50 % acetonitrile for 30 min each. The gel was dehydrated with 100 % acetonitrile, then the  
162 samples were hydrolysed with 400 ng of trypsin (Promega) for 5 h and the peptides extracted with  
163 100 % acetonitrile. After air drying, the hydrolysed samples were resuspended in the same volume  
164 of a 0.1 % trifluoroacetic acid aqueous solution before injection (3 µl) and nanoHPLC separation  
165 (Ultimate 3000, Dionex) with a C18 column (LC Acclaim PepMap, ThermoScientific, 75 µm,  
166 15 cm) with acetonitrile solution gradient (99.9 % acetonitrile, 0.1 % water) from 10 to 40 %  
167 during 50 min (as validated for samples with at least less than 500 different proteins). The peptides  
168 separated on the nanoHPLC column were nanoelectrosprayed *via* a nanoESI source in the mass  
169 spectrometer (LTQ Velos IonTrapOrbitrap, ThermoScientific) used in data dependant mode with  
170 full MS in the range 400-1 400 m/z with a resolution set up at 30 000 in the orbitrap. For MS/MS,  
171 top ten mode was used (full scan MS followed by ten MS/MS analysis on major peptides in the  
172 full scan) with 2 repeat counts, dynamic exclusion set up at 80 s, and CID in ion trap set up at 37 %  
173 in collision energy. Each condition was performed in triplicates (biological replicates) with two  
174 runs per sample (technical repeats).

## 175 **Data processing and bioinformatic analysis**

176 The proteins identification was performed from *E. coli* O157:H7 strain EDL933 database  
177 (KEGG Genome T00044, 5449 protein entries) using Mascot search engine. Peptides were  
178 validated for a Mascot score permitting to obtain a false discovery rate (FDR) at below 1 % and  
179 proteins were identified when at least two peptides matched significantly (score higher than 20) in  
180 the database with search parameters set to 10 ppm for peptide and 0.5 Da for fragment mass  
181 tolerance. Comparison of protein abundance was performed following the standard workflow  
182 provided by Progenesis QI for proteomics applying the label-free relative quantification method  
183 using Hi-N mode (Nonlinear Dynamics, Waters); basically, the MS1 survey scans are used for ion  
184 abundance quantification where peptide ions falling within the robust estimation limits are used to  
185 calculate the normalisation factor and selected as normalisation reference to compare peptidic  
186 signals between runs. For each subproteome, differential protein quantification was considered  
187 significant for fold abundance greater than 1.5 and *p*-value lower than 0.05. FDR (false discovery  
188 rate) approach was applied to overcome the issue of multiple hypothesis testing and *q*-values  
189 (adjusted *p*-values) were further calculated.

190 Secretomic analysis was performed for LPS-dermal bacteria [42] using different software  
191 tools to predict protein transport pathways, subcellular localisations and protein categories. *In*  
192 *silico* predictions of protein subcellular localisation (SCL) were accessed using PSORTb v.3.0.2  
193 [43] combined with SignalP v5.0 to identify export signal peptides [44] and SecretomeP v2.0 for  
194 prediction of non-classically secreted proteins [23] as well as KEGG (Kyoto encyclopedia of genes  
195 and genomes) [45] and literature survey for protein secretion pathways [14, 46]. Identified proteins  
196 predicted as located at the cytoplasm were filtered for moonlight activity using MoonProt v2.0  
197 [47]. Combining these different predictions with literature survey and Uniprot information [48],

198 protein transport route was predicted for each protein, including export system across the  
199 cytoplasmic membrane, secretion system across the outer membrane, translocational and/or post-  
200 translocational maturation systems (Figure 1). The information of gene ontology (GO) for  
201 molecular function and biological process was retrieved from UniprotKB and QuickGo [49, 50]  
202 and enrichment of overrepresented GO terms was performed using Panther up to the ancestor level  
203 2 [51]. Network analysis to identify protein-protein links was performed using STRING v11.0 [52,  
204 53].

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## 205 **Results**

### 206 **Mapping the protein secretion routes following secretomic analysis of the** 207 **proteosurfaceome, proteovesiculome and exoproteome of *E. coli* O157:H7**

208 To experimentally determine the repertoire of proteins with an extracytoplasmic localisation and  
209 transported from the interior to the exterior of the cell in *E. coli* O157:H7, a subproteomic analysis  
210 was performed not only considering the extracellular milieu (exoproteome) but also the proteins  
211 present at the cell surface (proteosurfaceome) and in outer membrane vesicles (proteovesiculome).  
212 Besides, three growth media most likely to induce differential protein expression profiles in *E. coli*  
213 O157:H7 were used, namely (i) a bacteria-culture minimal medium (M9), (ii) a bacteria-culture  
214 rich complex medium (BHI) and (iii) a tissue-culture chemically defined medium (DMEM). In  
215 order to map the transport routes of the total 154 distinct proteins experimentally identified in these  
216 conditions, a secretomic analysis was performed (Figure 1 and supplementary material Table S1).

217 Scanning the amino acid sequence of these extracytoproteins, 69 proteins were predicted  
218 to possess a N-terminal signal peptide (SP) and thus exported across the inner membrane (IM) by  
219 the Sec system (Figures 1, 2 and supplementary material Table S1). Among them, 14 harboured a  
220 SP of type II (SPII) and were therefore predicted to be supported by the lipoprotein maturation  
221 pathway, that is processing by the lipoprotein diacylglyceryl transferase (Lgt), cleaved by a signal  
222 peptidase of type II (SPase II) and then the lipoprotein N-acyl transferase (Lnt) [54]; half of them  
223 were predicted to be anchored to the IM and thus to be inner membrane lipoproteins (IMLs),  
224 whereas the other half were predicted to be outer membrane lipoproteins (OMLs) translocated to  
225 the OM across the periplasm by the Lol (lipoprotein outer membrane localisation) pathway [55]  
226 and two of them would be surface exposed lipoproteins (SOMLs) following translocation through  
227 Bam ( $\beta$ -barrel assembly machinery) [56, 57]. Among the 55 proteins possessing a SPI and thus

228 cleaved by a SPase I, (i) 33 were predicted as simply exported into the periplasm, *i.e.* some  
229 periplasmic proteins (PPs), (ii) one as anchored to the IM by an  $\alpha$ -helical transmembrane domain  
230 through the insertase YidC [58], *i.e.* as an IMP (inner membrane protein), and (ii) 16 as anchored  
231 to the OM by  $\beta$ -barrel upon translocation through the insertase Bam [59], *i.e.* some OMPs (outer  
232 membrane proteins). The remaining proteins with a SPI were predicted as secreted across the OM  
233 through the Type II secretion system (T2SS), T5aSS, T7SS or by NCS, and were either some  
234 exoproteins (soluble extracellular proteins) or subunit of cell-surface appendage (SCSA). Of note,  
235 no protein exported across the IM by the alternative Tat (twin-arginine translocation) system and  
236 thus exhibiting the cognate SP could be here identified.

237       Regarding the proteins lacking a SP, 8 were predicted as SCSA and thus cell-surface  
238 exposed following secretion and assembly by the T3aSS to form the injectisome needle or by the  
239 T3bSS to form flagella (Figure 1 and supplementary material Table S1); two were IMPs inserted  
240 *via* YidC to the IM. Following the secretome concept, the presence of the cognate transport systems  
241 and post-translocation maturation pathways was checked out (Figure 2); for each of the above  
242 proteins actively translocated, exported and/or secreted, the proteins associated to the different  
243 transport routes were indeed encoded in the genome of *E. coli* O157:H7 EDL933. The remaining  
244 74 proteins lacking a SP were primarily predicted as cytoplasmic but 65 % of them (48 out of 74)  
245 could also have an extracytoplasmic localisation upon NCS. Among these extracytoplasmic  
246 cytoproteins (ECPs), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is known to be found  
247 in the extracellular milieu and at the cell surface of EHEC where it can moonlight by binding to  
248 plasminogen and fibrinogen [60]; in this species, GAPDH was further demonstrated to be  
249 piggybacked through the T3aSS [61]. Additional glycolytic enzymes known to be cell-surface  
250 exposed and to moonlight as adhesins in different bacterial species were here identified, including

251 enolase, pyruvate kinase or triosephosphate isomerase [62]. Some chaperones and elongation  
252 factors are also known to be cell-surface exposed, such as the elongation factor TU then  
253 participating to bacterial aggregation in *E. coli* [63].

254 From the 154 distinct proteins identified, 42, 58 and 127 were found as part of the  
255 proteosurfaceome, proteovesiculome and exoproteome, respectively (supplementary material  
256 Table S1). While 46 proteins were found in common between the proteovesiculome and  
257 exoproteome, essentially some PPs and OMPs (37 and 24 %, respectively), only 14 proteins from  
258 the proteovesiculome (essentially some PPs, 79 %) and 25 proteins from the exoproteome  
259 (essentially some ECPs and PPs, 44 and 40 %, respectively) were common to the  
260 proteosurfaceome. With regards to the secretome analysis (Figure 2), the large majority of these  
261 proteins experimentally identified were predicted as extracytoplasmic, namely 91 % (38 out of  
262 42), 98 % (57 out of 58) and 80 % (102 out of 127) for the proteosurfaceome, proteovesiculome  
263 and exoproteome, respectively (supplementary material Table S1). In the proteosurfaceome and  
264 proteovesiculome, the majority of the proteins (60 and 90 %, respectively) were predicted as  
265 associated with the cell envelope, namely in the IM, periplasm, OM, or cell surface (Figure 2 and  
266 supplementary material Table S1); 60 and 79 % of these proteins were exported *via* Sec for the  
267 proteosurfaceome and proteovesiculome, respectively, and were mainly predicted as PPs or  
268 associated to the OM *via* Bam as OMPs or OMLs (Figure 2). While 39 % of the proteins were  
269 predicted as exported *via* Sec in the exoproteome, only a few exoproteins were identified as  
270 secreted by T2SS or T3SS but rather through NCS (Figure 2).

271 **Growth media induce shifting in extracytoprotein abundance and allow**  
272 **identifying core and variant proteosurfaceomes, proteovesiculomes and**  
273 **exoproteomes of *E. coli* O157:H7**

274 To evaluate the influence of the different culture media on proteins transported from the interior  
275 to the exterior of *E. coli* O157:H7 cells, a label-free quantitative proteomic analysis was applied  
276 to determine the relative protein abundance in these three subproteomes, namely the  
277 proteosurfaceome, proteovesiculome and exoproteome. Shifting in protein abundance was  
278 analysed individually for each subproteome comparing all three culture media simultaneously  
279 (Figure 3 and Tables 1, 2 and 3). Proteins demonstrating significant differences in their abundance  
280 were further referred as differential proteins and constituted the variant subproteome; at the  
281 opposite, proteins found in the three growth media with no significant differences in their  
282 abundance were referred as common proteins and constituted the core subproteome.

283 In the proteosurfaceome, 55 % (23 out of 42) of total identified proteins in this subproteome  
284 showed a change in their relative abundance (Figure 3 and Table 1); this variant proteosurfaceome  
285 was essentially constituted of PPs exported by Sec system. The majority of the differential proteins  
286 (78%, 18 out of 23) presented the highest abundance when bacteria were grown in DMEM; in  
287 contrast, the lowest abundance of differential proteins was observed in BHI (Table 1). Regarding  
288 the core proteosurfaceome, the identified proteins were essentially ECPs transported by NCS  
289 (supplementary material Table S1). Concerning the proteovesiculome, 84 % (49 out of 58) of the  
290 identified proteins constituted the variant proteome as they showed a differential abundance in a  
291 media-dependent manner (Figure 3 and Table 2); the differential proteins were essentially  
292 exported *via* Sec to the periplasm and corresponded to PPs or further translocated to the OM as  
293 OMPs, OMLs, SOMLs or SCSAs. M9 appears as the culture condition where differential proteins



294 were higher abundant, *i.e.* 78 % (38 out of 49) comparing to the other two growth media. On the  
295 contrary, only around 34 % (43 out of 127) of the identified proteins constituted the variant  
296 exoproteome (Figure 3 and Table 3); these differential proteins were essentially PPs exported *via*  
297 Sec, OMLs and OMPs translocated to the OM, or SCSAs and exoproteins secreted across the OM.  
298 The core exoproteome corresponded in the vast majority (75 %, 62 out of 83) to cytoproteins and  
299 also ECPs transported by NCS (supplementary material Table S1). As for the proteovesiculome  
300 (but in contrast to the proteosurfaceome), M9 condition was again the culture condition for the  
301 exoproteome in which most differential proteins were identified (59 %, 26 out of 44), whereas the  
302 condition showing the lowest differential abundance was the BHI (Figure 3 and Table 3).

303 To gain further insight in these subproteomes, differential and common proteins were  
304 categorised according to the prediction of gene ontology (GO) classes respective to their  
305 subcellular localisation (SCL), biological processes and /or molecular function (Figure 4).  
306 Concerning the SCL prediction, the variant proteosurfaceome and proteovesiculome presented  
307 similar profiles but differing from the exoproteome (Figure 4A). Changes in proteins abundance  
308 were more perceptible on proteins predicted as located in the periplasm (GO: 0042597) and at the  
309 OM (GO: 0036406) with both SCL representing more than 50 % of all differential proteins.  
310 Proteins predicted as extracellular (GO: 0005576) represented more than 10 % of all differential  
311 proteins in both the proteosurfaceome and proteovesiculome (Figure 4A). For the exoproteome,  
312 proteins predicted as located in the periplasm represented the majority of the differential proteins  
313 (74 %), whereas both exoproteins and OMPs represented less than 20 %. Proteins predicted as  
314 cytoplasmic (GO: 0005737) were not identified in the variant subproteomes (Figure 4A). In  
315 contrast, some cytoplasmic proteins (cytoproteins) were identified in all the core subproteomes

316 (Figure 4A); in the exoproteome, 63 % of the common proteins were ECPs trafficking through  
317 NCS, some of them predicted with moonlighting activities (supplementary material Table S1).

318 To categorise which molecular functions and biological processes were affected in the three  
319 studied subproteomes, a enrichment of GO terms was performed. To facilitate the data presentation  
320 in Figure 4, GO terms were elevated to the ancestor level 2 (supplementary material Table S1).  
321 While only a few proteins had no attributed molecular function (supplementary material Table S1),  
322 proteins associated to binding (GO: 0005488) represented the majority of the identified proteins  
323 in variant and core subproteomes, *i.e.* around 50 % (Figure 4B). Catalytic activity (GO: 0003824)  
324 and transporter activity (GO: 0005212) represented similar percentage of identified proteins in the  
325 different subproteomes, whereas structural molecule activity (GO: 0005198) represented around  
326 10 % in the variant proteovesiculome and exoproteome but was completely absent in  
327 proteosurfaceome. The majority of the common proteins in all core subproteomes were associated  
328 with binding and catalytic activity (GO: 0005488 and 0003824). A significant number of proteins  
329 related with transport activities (GO: 0005212) were identified in the variant subproteomes but not  
330 in the core subproteomes.

331 The enrichment for GO terms related to biological process presented a completely different  
332 trend between subproteomes (Figure 4C). Proteins identified in proteosurfaceome were essentially  
333 associated with cellular adaptation to the environment. While differential proteins were associated  
334 with localisation (GO: 0051179), cellular process (GO: 0009987), over 60 % of common proteins  
335 were essentially related to metabolic pathways (GO: 0008152) in the proteosurfaceome and  
336 exoproteome or localisation in the proteovesiculome. Remarkably, only proteins identified in the  
337 variant subproteomes were associated with processes involved in interactions with other organisms  
338 (GO: 0051704), cellular organisation (GO: 0071840) and biological adhesion (GO: 0022610). In

339 addition, most differential proteins from the variant exoproteome were associated to response to  
340 stimulus (GO: 0050896). In the core exoproteome, 65 % of the common proteins were associated  
341 with metabolic process (GO: 0008152), whereas more than the half of the core proteovesiculome  
342 was represented by common proteins related to localisation process (GO: 0051179).

343 **Network analysis of the variant and core proteosurfaceomes,**  
344 **proteovesiculomes and exoproteomes of *E. coli* O157:H7 highlighted functional**  
345 **protein clusters related to some outer membrane proteins, ABC transport and**  
346 **Type III secretion systems**

347 To investigate the links between the proteins identified in each of the variant and core  
348 subproteomes of *E. coli* O157:H7 EDL933, a network analysis was performed to profile potential  
349 associations and several functional clusters could be highlighted (Figure 5).

350 In the variant proteosurfaceome, several differential proteins were positively influenced in  
351 their abundance in DMEM over the two other growth media M9 and BHI, especially several  
352 molecular periplasmic chaperones, namely DegP, Skp, SurA, HflK and FkpA (Figure 5A,  
353 Table 1). Some proteins associated to cell division as part of the Tol-Pal system were also  
354 highlighted in DMEM, namely TolB and CpoB (Figure 5A and Table 1). In the variant  
355 proteovesiculome, some flagellar proteins secreted and assembled through the T3bSS were clearly  
356 pinpointed with a higher abundance (Figure 5A and Table 2); as such, FlgG, FlgL, FliD, FliC and  
357 FlgK were up to 40 fold more abundant in BHI compared to DMEM or M9. Interestingly, in both  
358 the variant proteovesiculome and exoproteome, it appeared that some proteins secreted through  
359 the T3aSS showed highest abundances when bacteria were grown in DMEM, especially EspA,  
360 EspB and EspD showing up to 50-fold higher in abundance in DMEM compared to BHI or M9  
361 (Figure 5A). Similarly, phage major capsid proteins Z1480 and Z1479 were highlighted in this

362 growth condition. In both the proteovesiculome and exoproteome, several porins (namely OmpA,  
363 OmpF and OmpX) were noticeably highlighted as up to 10-fold more abundant in M9 compared  
364 to DMEM or BHI (Figure 5A). For the exoproteome, another clearly defined functional cluster  
365 with higher protein abundance in M9 was related to some ABC transporters, namely with  
366 periplasmic binding proteins LivJ, LivK, FliY, GlnH and YbeJ (Figure 5A). Regarding the variant  
367 exoproteome and besides the virulence factor StcE secreted by a T2SS, a group of ribosomal  
368 subunits transported through NCS and potentially moonlighting, *i.e.* RplC, RplS and RplA, was  
369 highlighted to cluster with high abundance in DMEM (Figure 5A).

370 By definition, proteins of the core subproteomes were not influenced in their abundance by  
371 the growth media, nonetheless, connectivity network analysis revealed several functional clusters  
372 (Figure 5B). In the core proteosurfaceome, a clear cluster of proteins involved in protein  
373 biosynthesis and folding was highlighted, namely with the elongation factor TufA and Fusa, the  
374 ribosomal proteins RlpA, RlpE and RpsC, molecular chaperone DnaK and GroEL, as well as FtsH  
375 involved in the quality control of membrane proteins. In addition, an association network of several  
376 proteins primarily related to the central catabolism was disclosed, especially with several  
377 glycolytic enzymes, namely the glyceraldehyde-3-phosphate dehydrogenase GapA, the fructose-  
378 biphosphate aldolase FbaA, the pyruvate dehydrogenase components AceE and AceF, as well as  
379 the pyruvate formate lyase PflB (Figure 5B). While it was scarce for the core proteovesiculome, a  
380 dense protein-protein association network was observed for the core exoproteome. In the latter,  
381 two main functional clusters regrouping primarily cytoproteins could be underscored, one  
382 involving numerous ribosomal proteins (namely RplE, RplD, RpoA, RpsC, RpsM, RplX, RpsR,  
383 RplQ and RplJ) and one involving several enzymes from central metabolic pathways, especially  
384 glycolytic enzymes (*e.g.* Eno, GpmA, Pgi, LpdA, GapA, FbaA or AceE) (Figure 4B).

## 385 **Discussion**

386 While numerous proteomic analyses have been dedicated *E. coli* strain K12, whose secretome and  
387 extracytoproteome is relatively well characterised [64-69], much fewer investigations have been  
388 dedicated to the secretome of EHEC strains [70]. Bearing in mind that domesticated laboratory  
389 strains of *E. coli* should not be considered as archetypical models [71], proteomic analyses of the  
390 secretome appears much more relevant in commensal or pathogenic strains of *E. coli* to apprehend  
391 the ecophysiology of this bacterial species. Respective to EHEC O157:H7, whole-cell or  
392 cytoplasmic proteome has been studied on numerous occasions, however, only a handful of reports  
393 have dedicated to the exoproteome [72-74] and, surprisingly enough, none to the proteosurfaceome  
394 or proteovesiculome. From the first investigation following a 2DE approach, 29 exoproteins were  
395 identified in *E. coli* O157:H7 strain EDL933 grown in LB [72]; using a LC/LC-MS/MS approach,  
396 the latest study allowed to reproducibly identify 65 exoproteins from bacteria grown in M9 [74].  
397 Compared to the total of 127 exoproteins presently identified from this same *E. coli* O157:H7  
398 strain using three very different growth media, our work outperformed previous investigations but  
399 more importantly our subcellular fraction procedure allowed to discriminate free soluble  
400 extracellular proteins in the culture supernatant (true exoproteome) from proteins vectorised by  
401 OMVs (proteovesiculome), which are generally mixed up in other studies of the so-called  
402 exoproteome, also often misleadingly called secretome [21].

403 From the premise that secreted proteins reflect the lifestyle of bacterial cells in given  
404 environmental conditions, the secretome of *E. coli* O157:H7 EDL933 was investigated using  
405 distinct growth culture media most likely to induce differential protein expression profiles.  
406 Following the secretome concept aiming at apprehending comprehensively the protein trafficking  
407 from the interior to the exterior of the a cell considering both the secreted proteins and their cognate

408 secretion systems, the extracytoplasmic proteome of *E. coli* O157:H7 EDL933 was for the first time  
409 experimentally and comprehensively deciphered considering the proteosurfaceome,  
410 proteovesiculome and exoproteome. From (i) the export across or insertion in the IM, (ii) the post-  
411 translocational maturations in the periplasm, and (iii) the secretion across, anchoring to or insertion  
412 in the OM, the different systems involved in protein translocation and transport were identified by  
413 proteogenomic analyses. This analytical approach further allowed to attribute each of the  
414 extracytoplasmic proteins experimentally identified with the respective transport pathways and  
415 thus provided a map of the protein trafficking routes. Besides, this secretome-based analysis  
416 permitted to ascribe each of the experimentally identified proteins to a category and different SCL.

417 While secreted proteins generally require a N-terminal SP, *e.g.* proteins first exported by the  
418 Sec system and then further secreted by the T2SS, T5SS or T7SS (Figure 1), some other proteins  
419 lack such a SP, *e.g.* proteins secreted *via* a T3SS. From sequence similarity, such secreted proteins  
420 could be deduced based on homology and a secretion pathway could then be attributed. Besides  
421 these known secretion systems, however, no secretion system could be attributed to some  
422 identified exoproteins. Such proteins could be transported by NCS, that is by unknown secretion  
423 systems or mechanisms [22, 23]. It can be wise to further discriminate NCS from unconventional  
424 protein trafficking (UCT), in the sense that NCS refers to an active protein transport mechanism  
425 through a conducting channel, whereas UCT refers to mechanisms unrelated to secretion *per se*  
426 but participating to protein trafficking from inside to outside the cell compartment. Different NCS  
427 mechanisms can be at play, such as piggybacking as demonstrated in *E. coli* O157:H7 for the  
428 GAPDH, which associates to T3aSS effectors and is thus non-specifically secreted *via* this system  
429 [61]. Among ECPs, it is interesting to note that a number is well-known to associate to other  
430 proteins, especially the molecular chaperone in the course of protein folding and quality check, or

431 ribosomal proteins in the course of protein synthesis, especially upon co-translational  
432 translocational. Including glycolytic enzyme known to moonlight at the bacterial cell surface in  
433 numerous bacterial species [62], the hypothesis that such ECPs could be secreted by piggybacking  
434 would clearly necessitate further in-depth investigations. One of the most well-known UCT  
435 mechanisms involve OMVs. Upon blebbing of the OM, OMVs carrying periplasmic and OM-  
436 associated proteins are released in the culture supernatant; it cannot be excluded that some of them  
437 burst out liberating their luminal content (with periplasmic proteins) as well as OM-associated  
438 proteins in the extracellular milieu. Contrary to most exoproteomic analyses that just recover the  
439 culture supernatant, the present investigation took a great care to differentiate the exoproteome  
440 from the proteovesiculome by separating the culture supernatant into two fractions. As suggested  
441 by the identification of phage capsid proteins, some ECPs could be released upon activation of  
442 lysogenic bacteriophage entering into a lytic cycle. As an UCT mechanism, bacterial cell lysis  
443 could also be induced in the course of control cell lysis by siblings, *i.e.* autolysis [75] or following  
444 apoptosis upon holin activation [76].

445 Investigating the proteosurfaceome, exoproteome and proteovesiculome, a comprehensive  
446 picture of the experimental extracytoplasmic proteome landscape of *E. coli* O157:H7 EDL933 was  
447 obtained for the first time. Proteomic quantitative analysis revealed significant changes in all  
448 subproteomes. Remarkably, 76 % of the total proteins identified in the extracellular milieu  
449 changed their abundance when comparing the three culture media investigated. Actually, proteins  
450 found in the exoproteome appeared with the highest abundance in minimal chemically defined  
451 medium M9. While M9 has sometimes been considered as inhibiting protein secretion [77], it must  
452 be stressed that minimal media are well-known in bacterial physiology to trigger the full anabolic  
453 potential of a cell. Indeed, in M9 neither macromolecules (*e.g.* lipids, polysaccharides, proteins,

454 DNA) nor elementary bricks (*e.g.* fatty acids, glucides, amino acids, nucleotides) are present, thus  
455 the cell needs to synthesise them all *de novo*. While BHI is clearly a rich medium providing a  
456 whole set of biochemical macromolecules that needs first to be degraded (depolymerised) before  
457 being use by the cell, DMEM can be viewed as an in-between since macromolecules are absent but  
458 some elementary bricks (namely amino acids or some vitamins) are readily available for the cell;  
459 in the latter and contrary to growth in M9, the cell does not necessarily trigger the basic  
460 biosynthetic pathways to synthesise them but at least some polymerisation and assembly pathways.  
461 In agreement with the fact that protein secretion is an essential anabolic cellular function, changes  
462 in the extracytoproteome (including the proteosurfaceome, proteovesiculome and exoproteome)  
463 were clearly demonstrated in M9 compared to the rich media. In agreement with active proteins  
464 trafficking in the cell, the biological processes related to transport, tethering or maintenance of  
465 molecules in a specific location (GO: 0051179) were the most affected by culture conditions in  
466 these subproteomes. Considering the biochemical nature of molecules to be transported differs  
467 between rich complex and chemically defined media (*i.e.* use of partially depolymerised  
468 compounds versus biosynthesis of elementary bricks, respectively) to support bacterial  
469 metabolism and growth, the differential bacterial physiology in these conditions could explain that  
470 a significant number of proteins related to transport activities (GO: 0005212) were identified in  
471 the variant subproteomes rather than in the core subproteomes. This investigation allowed  
472 providing the first dynamic picture of extracytoplasmic proteins differentially or similarly  
473 expressed in response to different culture conditions respective to their predicted SCL and  
474 biological processes. All-in-all and by modulating the levels of protein expression in the  
475 proteosurfaceome, proteovesiculome and exoproteome, *E. coli* O157 appeared to adapt its secretion  
476 profile depending on the environmental conditions.



477 In allowing the transport of protein effectors, secretion systems are closely related to the  
478 main virulence features in pathogenic bacteria. The present work revealed that DMEM induced  
479 major changes in the proteosurfaceome comparing to the other two growth media. As expected  
480 from the up-regulation of the LEE in DMEM [77, 78], the abundance of proteins related with the  
481 injectisome (T3aSS) was higher in this medium. Interestingly, in this condition the abundance of  
482 proteins related to flagellum (T3bSS) was lower, which could suggest some kind of counter-  
483 regulation of these two types of T3SS. While such regulation trend has never been reported for  
484 *E. coli* O157:H7 EDL933 and would require further in-depth investigations, in *Yersinia* and  
485 *Shigella*, flagellar genes are induced at temperatures below 30°C but injectisome genes are  
486 transcribed at 37°C [79-82]. In *E. coli* O157:H7, the induction of the LEE in DMEM would result  
487 from the presence of sodium bicarbonate, which enhances the transcription of *ler* [36]. The effect  
488 of NaHCO<sub>3</sub> on the expression of other genes and secretion of proteins remain unknown but would  
489 undoubtedly require further investigations. Actually, differential expression of other virulence  
490 factors in *E. coli* O157:H7 has been reported in the literature but generally relies on transcriptional  
491 analysis [36, 37, 77, 78, 83-88] or on adhesion phenotype to various cell models [34, 83, 89, 90].  
492 While transcriptomics is useful for initial information about the genetic expression in some given  
493 conditions, the correlation between mRNA level and protein abundance is known to be poor and  
494 at most indicates that a protein is likely expressed when mRNA is present [30]. As such,  
495 proteomics remains the ultimate method of choice to ascertain that proteins are effectively  
496 expressed, especially for secreted proteins since the regulation level is not only transcriptional but  
497 can occur at pre-transcriptional, post-transcriptional, post-translational and/or post-translocational  
498 levels. Besides the influence of the nutrient composition, the bacterial growth rate *per se* could  
499 influence protein expression and secretion, such as the flagellar biosynthesis [57]; a rigorous

500 investigation of the influence of the bacterial growth rate in a given medium, thought, cannot be  
501 performed in batch culture but in chemostat cultures [91, 92]. Additional environmental  
502 parameters, especially temperature and pH, could also be tested to assay their effects on the  
503 expression of the secreted proteins and thus broaden our knowledge of the secretome landscape.  
504 In fact, the experimental identification of extracytoplasmic proteins is necessary to better  
505 understand the physiology and ecophysiology of *E. coli* O157:H7 in various environments.  
506 Extracytoproteins have also practical importance for the development of preventive strategies  
507 against EHEC and/or DEC contamination along the food chain and ultimately human infection.  
508 This can include the development of antagonists targeting important virulence or colonisation  
509 factors but also the identification of potential antigens for vaccine development [93, 94].

510 **Author's contributions**

511 RM and MD conceptualise the overarching aims of the research study. RM, IC, SL, CC and MD  
512 conceived and designed the experiments. RM, IC, VA and CC performed the experiments and data  
513 acquisition. RM, IC, VA, SL, CC, MH, VL, MP, AP and MD analysed and interpreted the data.  
514 MD had management as well as coordination responsibility for the execution of the research work.  
515 MP and MD contributed to the acquisition of the financial supports and resources leading to this  
516 publication. RM, IC, VA, SL, CC, MH, VL, MP, AP and MD wrote the article, including drafting  
517 and revising critically the manuscript for important intellectual content.

518

519 RM: Conceptualisation, Methodology, Formal analysis, Investigation, Writing - original draft,  
520 Writing - review & editing, Visualisation. IC: Conceptualisation, Methodology, Formal analysis,  
521 Investigation, Writing - original draft, Writing - review & editing, Visualisation, Supervision. VA:  
522 Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualisation.  
523 SL: Conceptualisation, Formal analysis, Writing - original draft, Writing - review & editing,  
524 Visualisation. CC: Conceptualisation, Validation, Formal analysis, Investigation, Resources,  
525 Writing - original draft, Writing - review & editing. MH: Formal analysis, Resources, Writing -  
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527 analysis, Resources, Writing - review & editing, Supervision, Funding acquisition. AP: Formal  
528 analysis, Writing - review & editing, Supervision. MD Conceptualisation, Resources, Formal  
529 analysis, Investigation, Writing - original draft, Writing - review & editing, Visualisation,  
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531

532 **Competing interests**

533 MP and AP are permanent employees of the GSK group of companies. All other authors have  
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## 551 **Supplementary material**

552 **Table S1 Total proteins identified in the proteosurfaceome, proteovesiculome and**  
553 **exoproteome of *E. coli* O157:H7 EDL933**

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## 803 **Figures legends**

### 804 **Figure 1 Decision tree for predicting the subcellular localisation of proteins in a LPS-diderm** 805 **bacterium based on the biology of protein secretion.**

806 The protein sequences are sequentially analysed in a workflow for (i) signal peptide (SP), (ii) the  
807 type of SP, (iii) the presence of transmembrane (TM)  $\alpha$ -helices responsible for IM retention, (iv)  
808 the delivery of lipoprotein to the Lol system, (v) the presence of the presence of TM  $\beta$ -barrel  
809 responsible for OM retention, and (vi) the systems involved in translocation into or across the OM.  
810 In the end, proteins are discriminated first into different protein categories and then for their final  
811 subcellular localisation (SCL).

812 SP I: SP of type I, SP II: SP of type II, SP IV: SP of type IV, Tat-SP: twin-arginine translocation  
813 SP, Lol: localisation of lipoproteins, Bam:  $\beta$ -barrel assembly machinery, T2SS: Type II secretion  
814 system, T3aSS: Type III, subtype a, secretion system, T3bSS: Type III, subtype b, secretion  
815 system, T5aSS: Type V, subtype a, secretion system, T5eSS: Type V, subtype e, secretion system,  
816 T7SS: Type VII secretion system, NCS: non-classical secretion, CP: cytoplasmic protein, IMP: inner  
817 membrane protein, IML: inner membrane lipoprotein, PP: periplasmic protein, OML: outer  
818 membrane lipoprotein, OMP: outer membrane protein, SOML: surface outer membrane  
819 lipoprotein, SCSA: subunit of cell surface appendage, EP: exoprotein, ECP: extracytoplasmic  
820 cytoplasmic protein, CY: cytoplasm, IM: inner membrane, PE: periplasm, OM: outer membrane, CS: cell  
821 surface, EM: extracellular milieu.

### 822 **Figure 2 Secretome based analysis of the proteins identified in the different extracytoplasmic** 823 **subproteomes of *E. coli* O157:H7 EDL933**

824 Schematic representation of the secretion routes for the proteins identified in the  
825 proteosurfaceomes, proteovesiculomes and exoproteomes (Figure 1 and supplementary material

826 Table S1). The different categories of proteins are depicted in red and the number of proteins  
827 identified in the proteosurfaceome, proteovesiculome and exoproteome is indicated in black, grey  
828 and white, respectively. Components of the different protein transport systems were identified in  
829 *E. coli* O157:H7 EDL933 and reported with Uniprot number; export systems are indicated in  
830 violet, insertases in yellow, posttranslocational maturation pathway in green, and protein secretion  
831 systems in blue.

832 Sec: secretion, SPase I: signal peptidase of type I, SPase II: lipoprotein maturation pathway  
833 involving the signal peptidase of type II, Lol: localisation of lipoproteins, Bam:  $\beta$ -barrel assembly  
834 machinery, T2SS: Type II secretion system, T3aSS: Type III, subtype a, secretion system, T3bSS:  
835 Type III, subtype b, secretion system, T5aSS: Type V, subtype a, secretion system, T5eSS: Type  
836 V, subtype e, secretion system, T7SS: Type VII secretion system, NCS: non-classical secretion,  
837 CPs: cytoproteins, IMPs: inner membrane proteins, IMLs: inner membrane lipoproteins, PPs:  
838 periplasmic proteins, OMLs: outer membrane lipoproteins, OMPs: outer membrane proteins,  
839 SOMLs: surface outer membrane lipoproteins, SCSAs: subunits of cell surface appendages, EP:  
840 exoproteins, ECPs: extracytoplasmic cytoproteins, CY: cytoplasm, IM: inner membrane, PE:  
841 periplasm, OM: outer membrane, CS: cell surface, EM: extracellular milieu.

842 **Figure 3 Heat maps for differential proteins in the proteosurfaceomes, proteovesiculomes**  
843 **and exoproteomes of *E. coli* O157:H7 EDL933 grown in M9, DMEM and BHI**

844 Shades of blue represent the relative abundance of variant proteins between the distinct growth  
845 media and for each subproteome. Dark blue indicates higher protein abundance in the given  
846 conditions compare to the other two, and *vice versa* for light blue.

847 **Figure 4 Distribution of common and differential proteins identified in the different**  
848 **extracytoplasmic subproteomes of *E. coli* O157:H7 EDL933 respective to the predicted SCL**  
849 **and biological process**

850 **A.** Percentage of common and differential proteins identified in the proteosurfaceomes,  
851 proteovesiculomes and exoproteomes respective to predicted SCL according the gene ontology  
852 (GO) terms for cellular component. Pink shades indicate proteins predicted as extracytoplasmic  
853 (ECY), *i.e.* are either located at the cell envelope (GO: 0030313), namely at the IM  
854 (GO: 0005886), in the periplasm (PE; GO: 0042597), at the OM (GO: 0019867), or at the cell  
855 surface (CS; GO: 0009986), or in the extracellular milieu (EM; GO: 0005576); it further includes  
856 proteins primarily predicted as cytoplasmic but also as present in the extracellular milieu  
857 (EM/CY). Violet colour stands for proteins solely predicted as cytoplasmic (CY; GO: 0005737).

858 **B.** Percentage of common and differential proteins identified in the proteosurfaceomes,  
859 proteovesiculomes and exoproteomes respective to the GO terms for molecular function. Identified  
860 molecular functions terms included (i) GO: 0005488 related to binding with the selective, non-  
861 covalent, often stoichiometric, interaction of a molecule with one or more specific sites on another  
862 molecule, (ii) GO: 0005215 related to transport with the directed movement of substances (such  
863 as macromolecules, small molecules, ions) into, out of or within a cell, or between cells, (iii) GO:  
864 0003824 related to catalytic activity with catalysis of a biochemical reaction at physiological  
865 temperatures, and (iv) GO: 0005198 related to structural activity with the action of a molecule that  
866 contributes to the structural integrity of a complex or its assembly within or outside a cell.

867 **C.** Percentage of common and differential proteins identified in the proteosurfaceomes,  
868 proteovesiculomes and exoproteomes respective to the GO terms for biological process. Identified  
869 biological process terms included (i) GO: 0071840 related to cellular organisation with

870 biosynthesis of constituent macromolecules, assembly, arrangement of constituent parts, or  
871 disassembly of a cellular component, (ii) GO:0051704 related to organism interaction with the  
872 involvement of another organism of the same or different species, (iii) GO:0022610 related to  
873 biological adhesion with the attachment of a cell or organism to a substrate, another cell, or other  
874 organism, (iv) GO: 0065007 related to regulation process with any process that modulates a  
875 measurable attribute of any biological process, quality or function, (v) GO: 0008152 related to  
876 metabolic process with the chemical reactions and pathways, including anabolism and catabolism,  
877 by which living organisms transform chemical substances, (vi) GO: 0050896 related to response  
878 to stimulus with any process that results in a change in state or activity of a cell, *e.g.* in terms of  
879 gene expression, protein synthesis, secretion or movement, as a result of a detected stimulus, (vii)  
880 GO: 0051179 related to molecular localisation with any process in which a substance or molecule  
881 is transported, tethered to or otherwise maintained in a specific location and (viii) GO: 0009987  
882 related to cellular process with any process that is carried out at the cellular level. Green bars stand  
883 for proteosurfaceome, blue bars for proteovesiculome and orange bars for exoproteome.  
884 Enrichment analysis was performed with Panther to the ancestor level 2 of GO [51].

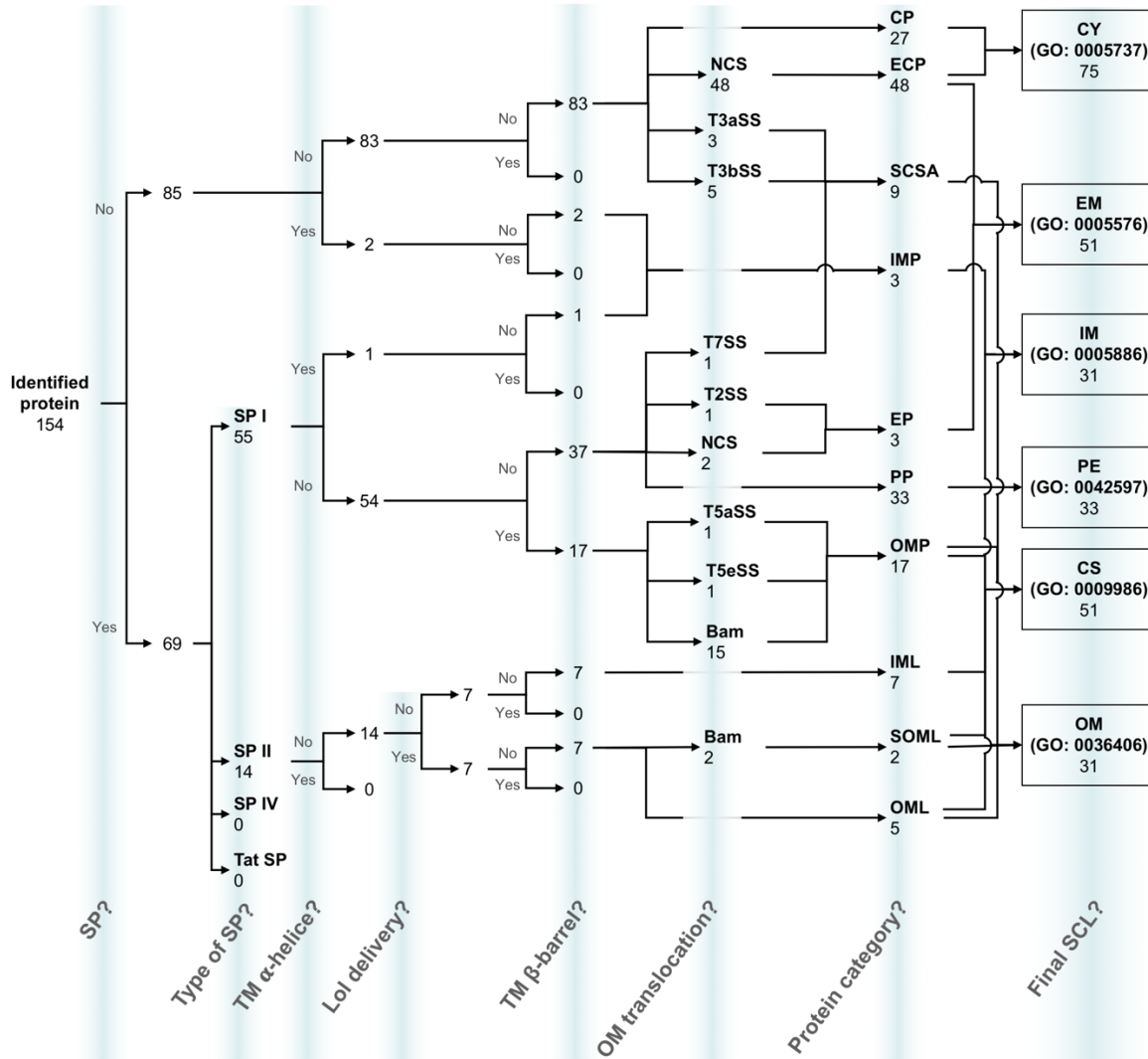
885 **Figure 5: Protein-protein interaction network of differential proteins and common proteins**  
886 **identified in the proteosurfaceomes, proteovesiculomes and exoproteomes of *E. coli* O157:H7**  
887 **EDL933 grown in M9, DMEM and BHI**

888 Interaction network for the variant subproteomes (A) and core subproteomes (B) with colour  
889 shades indicating higher protein abundance in DMEM (red), BHI (green) and M9 (grey) for the  
890 proteosurfaceome, proteovesiculome and exoproteome. Each network node (sphere) represents a  
891 protein produced by a single protein-coding gene locus. Edges (connecting lines) represent protein-  
892 protein associations, which are meant to be specific and meaningful, *i.e.* proteins that jointly

893 contribute to a shared function without necessarily meaning they are physically binding one with  
894 another.

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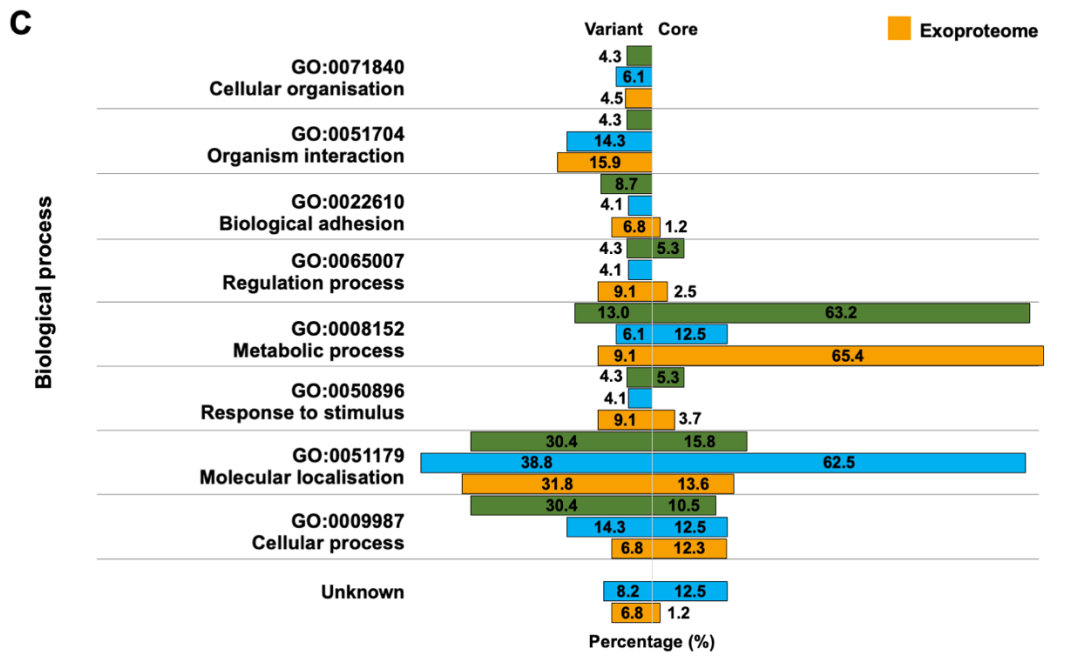
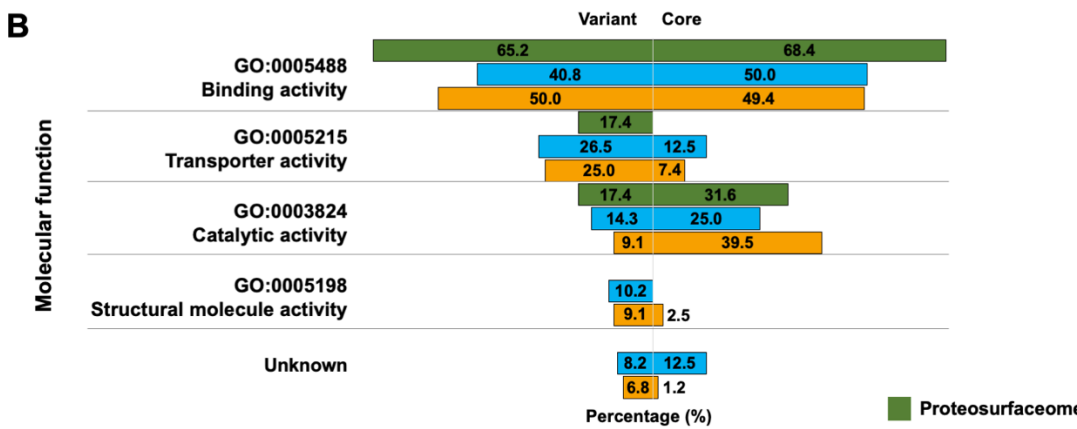
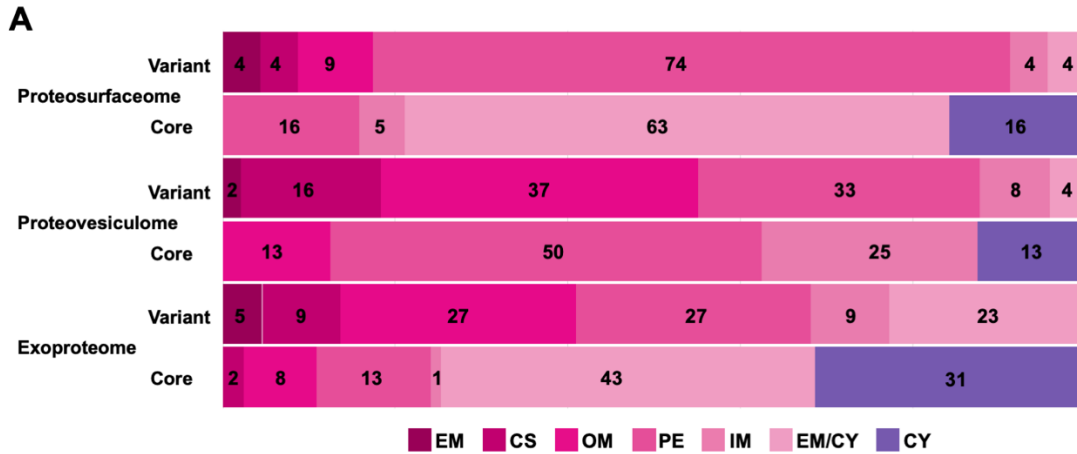


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897 **Figure 1**

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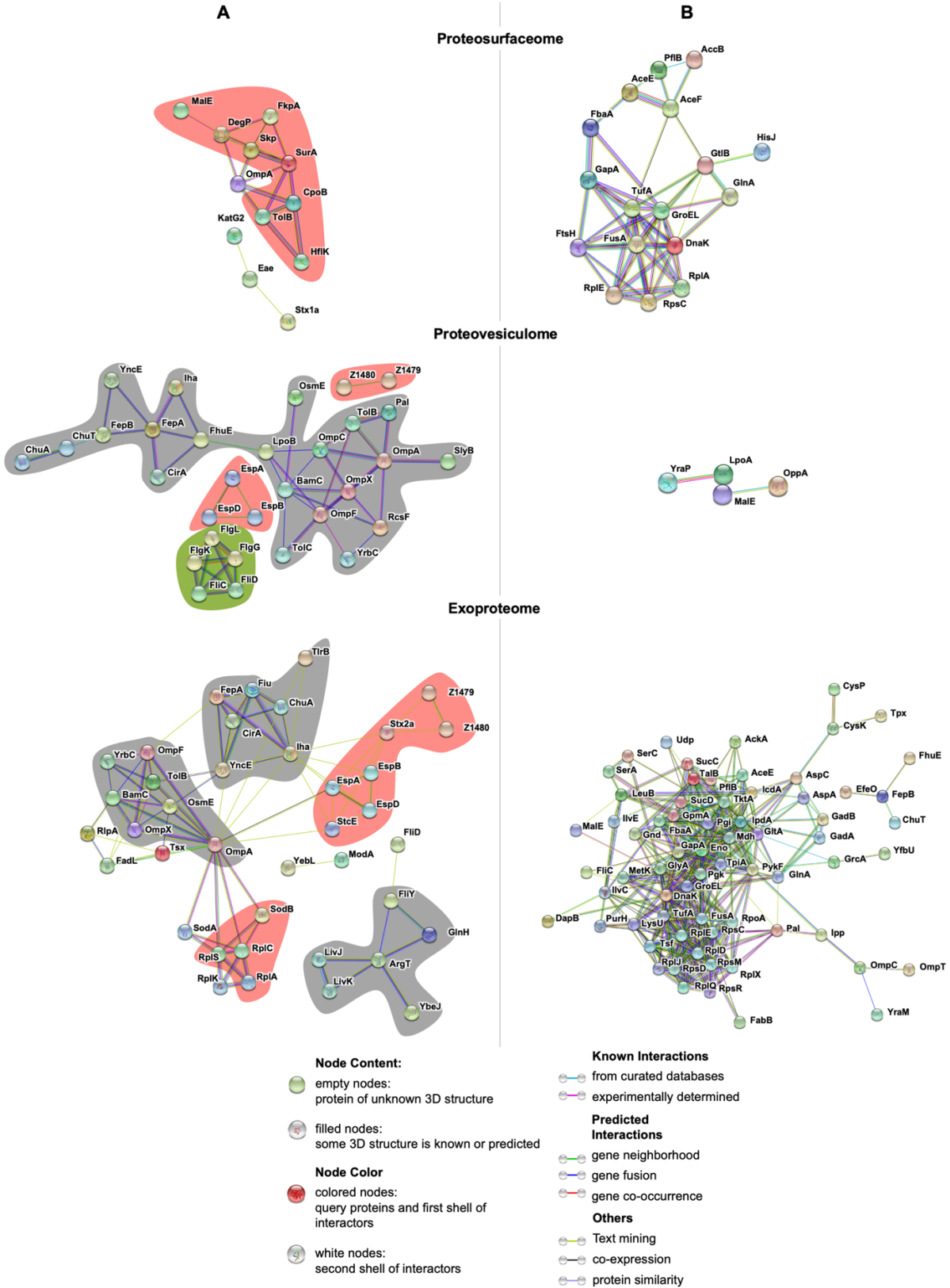




903

904 **Figure 4**





905

906 **Figure 5**