Toward a better understanding of the mechanisms of symbiosis: a comprehensive proteome map a nascent insect symbiont

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Symbiotic bacteria are common in insects and can affect various aspects of their hosts' biology. Although the effects of insect symbionts have been clarified for various insect symbiosis models, due to the difficulty of cultivating them *in vitro*, there is still limited knowledge available on the molecular features that drive symbiosis. *Serratia symbiotica* is one of the most common symbionts found in aphids. The recent findings of free-living strains that are considered as nascent partners of aphids provide the opportunity to examine the molecular mechanisms that a symbiont can deployed at the early stages of the symbiosis (*i.e.*, symbiotic factors). In this work, a proteomic approach was used to establish a comprehensive proteome map of the free-living *S. symbiotica* strain CWBI-2.3^T. Most of the 720 proteins identified are related to housekeeping or primary metabolism. Of these, 76 were identified as candidate proteins possibly promoting host colonization. Our results provide strong evidence that *S. symbiotica* CWBI-2.3^T is well-armed for invading insect host tissues, and suggest that certain molecular features usually harbored by pathogenic bacteria are no longer present. This comprehensive proteome map provides a series of candidate genes for further studies to understand the molecular cross-talk between insects and symbiotic bacteria.

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Abstract

Symbiotic bacteria are common in insects and can affect various aspects of their hosts'

biology. Although the effects of insect symbionts have been clarified for various insect

symbiosis models, due to the difficulty of cultivating them *in vitro*, there is still limited

knowledge available on the molecular features that drive symbiosis. *Serratia symbiotica* is

one of the most common symbionts found in aphids. The recent findings of free-living strains

that are considered as nascent partners of aphids provide the opportunity to examine the

molecular mechanisms that a symbiont can deployed at the early stages of the symbiosis (*i.e.*,

symbiotic factors). In this work, a proteomic approach was used to establish a comprehensive

proteome map of the free-living *S. symbiotica* strain CWBI-2.3^{T}. Most of the 720 proteins

identified are related to housekeeping or primary metabolism. Of these, 76 were identified as

candidate proteins possibly promoting host colonization. Our results provide strong evidence

that *S. symbiotica* CWBI-2.3^{T} is well-armed for invading insect host tissues, and suggest that

certain molecular features usually harbored by pathogenic bacteria are no longer present. This

comprehensive proteome map provides a series of candidate genes for further studies to

understand the molecular cross-talk between insects and symbiotic bacteria.

Introduction

40

41   Insecta is the most diverse class of animals described so far, and many of them are intimately

42   associated with symbiotic bacteria (Buchner, 1965). Some symbionts are obligate partners for

43   their host and fulfill an essential nutrient function (Baumann, 2005; Vigneron et al., 2014),

44   whereas others are considered as facultative symbionts by being only beneficial in the context

45   of various ecological interactions (Oliver et al., 2010). Regarding these relationships between

46   insects and bacteria, a particular attention has been paid in the recent decades to the symbionts

47   harbored by members of the *Aphididae* family (Baumann, 2005; Oliver et al., 2010). In order

48   to get essential amino acids, these sap-feeding insects rely on their nutritional obligate

49   symbiont *Buchnera aphidicola* which is confined in a specialized organ called the bacteriome

50   (Moran, McCutcheon & Nakabachi, 2008). In addition to *B. aphidicola*, aphids can also

51   harbor a wide range of facultative symbionts that are not essential but can bring benefits to

52   their hosts according to environmental conditions, such as protection against parasites and

53   heat-stress, and adaptation to host plants (Oliver et al., 2010).

54   Ultimately derived from free-living bacterial ancestors, symbiotic bacteria share

55   similar mechanisms with opportunistic pathogens to facilitate successful colonization of a

56   particular niche. These include quorum sensing, the two-component regulatory systems and

57   the use of secretion systems (Hentschel, Steinert & Hacker, 2000). These mechanistic

58   similarities for host colonization are particularly prominent in facultative symbionts (Dale &

59   Moran, 2006; Silver et al., 2007; Degnan et al., 2009; Watson et al., 2010) considering their

60   short-time association with insects in comparison to obligate symbionts that lead to the loss of

61   many genes during the acquisition of the endosymbiotic lifestyle (Moran, McCutcheon &

62   Nakabachi, 2008). And unlike obligate symbionts that display a strict intracellular lifestyle,

63   facultative ones exhibit a more flexible tissue tropism, ranging from intracellular localization

64   within specialized host cells (*e. g.* bacteriocytes) to extracellular lifestyle in host gut or other

65   tissues (Salem et al., 2015). This more flexible tissue tropism is expected to facilitate

66   horizontal transfers of facultative symbionts beside their maternal transmission (Sandström et

67   al., 2001). Host acquisition of facultative partners from various environmental sources

68   indicates that they have retained the ability to infect alternative hosts and are more prone to

69   survive outside their primary host in comparison to obligate symbionts (Renoz et al., 2015;

70   Salem et al., 2015).

71   The establishment and persistence of these facultative partnerships is determined by

72   complex cross-talks between symbionts and their respective insect hosts that eventually lead

73   to a stabilized association between both partners. This so-called "symbiotic partnership"

74   involves colonization, invasion, and host modulation factors, such as quorum sensing system,

75   iron chelators, secretion and transport mechanisms (Hentschel, Steinert & Hacker, 2000).

76   Considering the evolutionary perspective, symbiotic bacteria may derive from opportunistic

77   pathogens through several steps that include the loss of virulence factors that may harm the

78   host while keeping functional the genes allowing host colonization in the context of mutual

79   benefits (Ochman & Moran, 2001). Yet, while the effects of symbionts have been clarified in

80   a number of insect symbiosis models (Oliver et al., 2010; Feldhaar, 2011), the molecular

81 features determining the establishment and the persistence of these symbiotic bacteria in their

82 host remain elusive. This is mainly due to the difficulty of isolating them from their insect

83 hosts and cultivating them *in vitro* because of their host dependence (Kikuchi, 2009).

84 Recently, culturing attempts have been successful for several strains of *Serratia symbiotica*

85 (Sabri et al., 2011; Foray et al., 2014), one of the most common facultative symbiont found in

86 aphids (Oliver et al., 2010). This symbiont species exhibits a wide diversity of strains that

87 vary in their degree of reliance on hosts, ranging from co-obligate and strictly intracellular

88 strains (Manzano-Marín & Latorre, 2016) to free-living extracellular strains residing in the

89 insect gut (Renoz *et al.* in prep). Genomic features of the strain CWBI-2.3^T, one of these so-

90 called "free-living" *S. symbiotica*, suggest that these strains are involved in the nascent phases

91 of the symbiosis (Manzano-Marín & Latorre, 2016), and could be acquired directly from

92 environmental sources to form emerging symbiotic associations with aphid partners (Renoz et

93 al., 2015).

94 *S. symbiotica* strain CWBI-2.3^T offers a unique opportunity to analyze what remains of

95 the molecular machinery that is involved at the beginning of an evolutionary transition from

96 an independent lifestyle in the environment to a more intimate lifestyle. In the present study,

97 we used a gel-free proteomic approach applied to symbiont cell cultures in order to decipher

98 the molecular features that a free-living *S. symbiotica* holds and can express to colonize its

99 host, without being a pathogen (Renoz et al., 2015). Taking advantage of the *S. symbiotica*

100 CWBI-2.3^T genome sequence (Foray et al., 2014), we report here the characterization of the

101 membrane and cytosolic proteomes of this symbiont. 720 different proteins have been

102 identified, and classified by their putative function. Of these, 76 were identified as putative

103 symbiotic factors. Ours results provide strong evidence the *S. symbiotica* CWBI-2.3[T] is well-

104 armed to invade the insect tissues, and suggest that certain molecular features usually found in

105 pathogenic bacteria are no longer present. This comprehensive proteome map of an insect

106 facultative symbiont provides a first step and a solid basis to implement genetic modification

107 experiments to tackle the mechanisms used by symbiotic bacteria to settle in a novel insect

108 host.

Materials and Methods

1. Bacterial strain and growth conditions

111 A free-living *S. symbiotica* strain, CWBI-2.3[T], isolated from a natural *Aphis fabae* collected in

112 Belgium in 2009 was routinely maintained on 863 agar at 20°C, containing 10% glucose, 10%

113 yeast extract, 10% casein peptone, and 17% agar. A draft version of *S. symbiotica* CWBI-2.3[T]

114 available under the GenBank accession number CCES01000000. This draft contains 3,664

115 predicted protein-coding sequences (Foray et al., 2014).

116 To prepare proteins extracts, triplicate cultures were grown in 400 mL of 863 medium

117 under vigorous agitation at 20°C (Sabri et al., 2011) and were collected during the earlier

118 exponential growth phase (OD_{600} of 0.4-0.6). Cells were harvested by centrifugation at 3200 g

119 for 10 min at 20°C. The resulting pellets were washed with cooled sterile PBS (pH 7.4) and

120 stored at -80°C until protein extraction.

2. Protein extraction

122   Bacterial cells were resuspended in 500 µL of homogenization buffer (100 mM TEAB, 1mM

123   PMSF, and 2 mg.mL^{-1} each of leupeptin, aprotinin, antipain, pepstatin, and chymostatin).

124   Samples were sonicated three times at high intensity for 5 min at 4°C using a bath sonicator

125   (Bioruptor, Diagenode). Cellular wastes were removed by a 2000 rpm centrifugation of 5 min

126   at 4°C. The supernatant was then centrifuged at 4°C for 30 min at 40,000 rpm to separate

127   microsomal fraction from soluble fraction. The protein concentration was determined by

128   Bradford assay (Bradford, 1976) using a commercial dye reagent (Bio-rad) and using IgG

129   gamma globulin as standard. Both soluble and microsomal fractions were subjected to

130   chloroform/methanol precipitation, as previously described (Wessel & Flügge, 1984).

131   **3. Reduction/alkylation/digestion**

132   Proteins were suspended in 100 µL of 50 mM NH$_4$HCO$_3$ containing 0.1% RapiGest (Waters)

133   for soluble fraction and 0.5% RapidGest for crude membrane by vortexing at room

134   temperature for 30 min and by sonicating at high intensity for 5 min at 4°C using a bath

135   sonicator (Biotuptor, Diagenode). Disulfide bonds were reduced by incubation for 1 h at 60°C

136   with 25 mM tris(2-carboxyethyl)phosphine. Then, cysteine residues were blocked in 200 mM

137   methyl-methanethiosulfonate (MMTS) for 15 min at room temperature in the dark.

138   Solubilized crude membrane proteins were first diluted five times with 50 mM TEAB to reach

139   a concentration of 0.1% (v/v) in RapiGest. Proteins digestions was performed overnight at

140   37°C using sequencing-grade-modified trypsin (Promega) at a protease/protein ratio of 1/20

141   and RapiGest subsequently lysed by incubating the protein sample in 1% trifluoroacetic acid

142 (TFA) for 1 h at 37 °C. After centrifugation of the sample at 54,000 rpm for 45 min at 4 °C,

143 the supernatant was vacuum dried (SpeedVac SC 200, Savant).

4. 1-D LC separation

145 Samples (20 µg) were resuspended in loading buffer (2% ACN, 0.1% TFA) and subjected to

146 reverse-phase chromatography on a C18 PepMap 100 column (Acclaim® pepMap 100-75 µM

147 i.d. × 5 mm-C18-3 µm-100 Å, LC Packing) for 180 min at a flow rate of 300 nL.min^{-1} using a

148 linear gradient from 8% (v/v) ACN in water/0.1% (v/v) TFA to 76% (v/v) ACN in

149 water/0.1% (v/v) TFA. The eluted peptides were spotted onto a MALDI plate together with

150 ionization matrix (4 mg.mL^{-1} of CHCA, 70% ACN, 0.1% TFA) (Probot, LC Packings).

5. MALDI-MS/MS and database search analysis

152 Mass spectrometry analyses were performed on an AB 4800 MALDI TOF/TOF analyzer

153 using a 200 Hz solid-state laser operating at 355 nm (Szopinska et al., 2011). MS spectra were

154 obtained using a laser intensity of 3200 and 2000 laser shots per spot (100 shots/sub-

155 spectrum) in the *m/z* range of 800 to 4000, whereas MS/MS spectra were obtained by

156 automatic selection of the 15 most intense precursor ions per spot using a laser intensity of

157 4000 and 2000 laser shots per precursor (100 shots/sub-spectrum). Collision-induced

158 dissociation was performed with an energy of 1 kV with air as the collision gas at a pressure

159 of 1.10^6 Torr.

160 Data were collected using the ABSciex 4000 Series Explorer^{TM} software. MS data

161 from the soluble and the microsomal fractions were pooled and analyzed as a single data set

162 using ProteinPilot software v.4.0 with the Paragon[TM] search algorithm (Shilov et al., 2007)

163 (AB SCIEX). The MS data were searched against the *S. symbiotica* CWBI-2.3[T] database

164 (containing 3397 protein sequences downloaded on Augustus 08th 2015, GenBank accession

165 number: CCES00000000). The searched options in ProteinPilot were "thorough search",

166 "MMTS" as the cysteine modification, "trypsin" as the digestion enzyme. All reported

167 proteins were identified with 95% or greater confidence, as determined by ProteinPilot unused

168 scores (>1.3). This corresponds to a stringent threshold of false discovery rate, lower than 1%.

169 The "unused" score is a measurement of the protein identification confidence taking into

170 account peptides from spectra that have not already used by higher scoring proteins.

171   All identified proteins were analyzed by PSORTb version 3.0.2

172 (http://www.psort.org/psortb/; (Gardy et al., 2005)) for *in silico* analysis of their localization

173 and were assigned to functional categories based on clusters of orthologous group of proteins

174 (COG; http://www.ncbi.nlm.nih.gov/COG/).

6. Phenotypic assays

6.1.Detection of siderophores

177 Siderophore system are enable invasive bacteria to scavenge iron under limiting conditions in

178 symbiosis (Braun & Hantke, 2011; Han et al., 2013). Here, Chromazurol (CAS, (Schwyn &

179 Neilands, 1987)) agar was employed to test the production of siderophores by *S. symbiotica*

180 CWBI-2.3[T]. On CAS agar plates a color change from blue to orange indicates siderophore

181   producing bacteria due to Fe^{3+} removal from the dye. *Bacillus pumilus* has been used as

182   negative control and *Bacillus subtilis* has been used as positive control.

183   **6.2.Swimming motility testing of *S. symbiotica* CWBI-2.3^T**

184   Motility is a bacterial function that can be required for host colonization (Lee et al., 2015).

185   Swimming motility testing was conducted according to a previously described procedure with

186   a slight modification (Picot et al., 2004). Briefly, Semisolid Motility Test medium was used to

187   detect motility. The agar concentration is sufficient to form a soft gel without hindering

188   motility. Precultures of bacterial isolates were separately prepared in 863 medium at early

189   stationary phase (OD_{600} of 0.4-0.6), and layered on plates with 863 medium containing 1.7%

190   agar. The plates were incubated at 20°C until colonies developed. Swimming motility was

191   evaluated using plates prepared with 863 medium supplemented with 0.2% agar. A single

192   colony was inoculated by puncture in the middle of the plates. The motility was estimated by

193   measuring the diameter of the halo (in centimeters) four days after inoculation. *S. marcescens*

194   Db11 and *Staphylococcus xylosus* have been used as positive and negative controls,

195   respectively. For every result, the value of mobility was determined on three independent

196   replicates. Results were expressed as mean ± standard error of the mean (SEM).

Results and discussion

1. Proteome map

199   The reference proteome map was built through the analysis of soluble and microsomal

200   fraction from three independent *S. symbiotica* CWBI-2.3^T cultures using a gel-free approach.

201 Due to their low abundance and poor solubility, membrane proteins are generally poorly

202 represented on two-dimensional gels (Geert Baggerman, 2006). We therefore decided to rely

203 on a gel-free approach. Soluble and membrane proteins were separated by ultracentrifugation

204 and the microsomal fraction stripped at alkaline pH to remove soluble proteins (Wu et al.,

205 2003). Overall, spectral data were pooled and analyzed as a single data set. This led to the

206 identification of 720 different proteins (Supporting Information Table S1), corresponding to

207 19.7% of all predicted proteins of the recently sequenced strain *S. symbiotica* CWBI-2.3[T]

208 (Foray et al., 2014).

209 The *in silico* prediction of the cellular localization identified 64% of cytoplasmic,

210 3.5% of OMPs, 2.4% of periplasmic, 17% of inner membrane and less than 1% of

211 extracellular proteins. It was not possible to predict the exact cellular localization for 12.4%

212 of the identified proteins (either multiple localization sites or unknown localization).

213 The *S. symbiotica* CWBI-2.3[T] annotated proteins were classified into 23 clusters of

214 orthologous group (COG) categories (Figure. 1). Most of the proteins belong to the following

215 categories: translation (15.1%), amino acids transport and metabolism (7.9%), cell

216 wall/membrane/envelope biogenesis (7.1%), replication/recombination and repair (6.1%) and

217 carbohydrate transport and metabolism (6.0%). The putative symbiotic factors that have been

218 identified in this study (summarized in Table 1) were classified according to their assumed

219 function. Putative symbiotic traits are involved in: (i) motility and chemotaxis, (ii) adhesion,

220 invasion and biofilm formation, (iii) iron uptake, (iv) protection against reactive oxygen

221 radicals, (v) secretion and transport mechanisms, and (vi) hydrolytic enzymes.
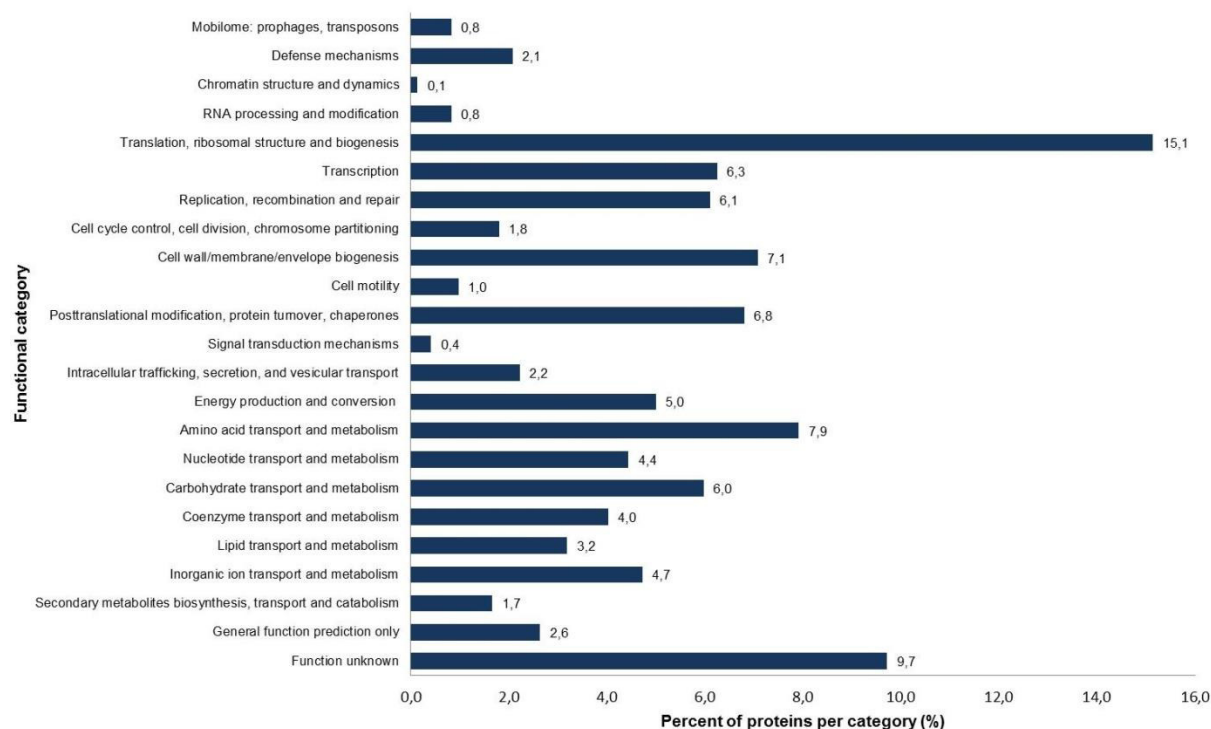
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Figure 1. Percentage of identified proteins according to different function categories based on

the NCBI COG functional annotation.

225

Protein designation	Accession number	Predicted localization
Motility and chemotaxis		
Flagellar L-ring protein	CDS58522.1	Outer membrane
Flagellar P-ring protein	CDS58521.1	Periplasmic
Basal-body rod modification protein FlgD	CDS58526.1	Extracellular
Flagellar basal-body rod protein FlgG	CDS58523.1	Extracellular
Flagellar biosynthesis protein FlhA	CDS58536.1	Inner membrane
Flagellar brake protein YcgR	CDS58313.1	Cytoplasmic
Flagellar transcriptional regulator FlhC	CDS58540.1	Cytoplasmic
Flagellar transcriptional regulator FlhD	CDS58541.1	Cytoplasmic
Probable HTH-type transcriptional regulator LrhA	CDS57959.1	Cytoplasmic

Type IV pilus biogenesis and competence protein PilQ	CDS55476.1	Outer membrane

Adhesion, invasion, biofilm formation

Transcriptional regulatory protein PhoP	CDS58188.1	Cytoplasmic
Sensor protein PhoQ	CDS58187.1	Inner membrane
Phosphate-specific transport system accessory protein PhoU	CDS56284.1	Cytoplasmic
Transcriptional regulatory protein CpxR	CDS56277.1	Cytoplasmic
Transcriptional regulatory protein OmpR	CDS56155.1	Cytoplasmic
Osmolarity sensor protein EnvZ	CDS56154.1	Inner membrane
Acyl-homoserine-lactone synthase SwrI	CDS56499.1	Cytoplasmic
cAMP-activated global transcriptional regulator CRP	CDS56109.1	Cytoplasmic
DNA-binding protein H-NS	CDS58425.1	Cytoplasmic
RNA polymerase sigma factor RpoS	CDS55654.1	Cytoplasmic
Outer membrane protein A	CDS57861.1	Outer membrane
FKBP-type peptidyl-prolyl cis-trans isomerase FkpA	CDS56095.1	Periplasmic

Iron acquisition

Biopolymer transport protein ExbB	CDS55934.1	Inner membrane
Biopolymer transport protein ExbD	CDS55935.1	Inner membrane
Ferric uptake regulation protein	CDS57198.1	Cytoplasmic
Periplasmic chelated iron-binding protein YfeA	CDS58219.1	Periplasmic
Chelated iron transport system membrane protein YfeB	CDS58218.1	Inner membrane
Bacterial non-heme ferritin	CDS58146.1	Cytoplasmic
DNA protection during starvation protein	CDS57357.1	Cytoplasmic
Putative peroxiredoxin bcp	CDS57424.1	Unknown
Succinate dehydrogenase flavoprotein subunit	CDS57210.1	Inner membrane
Succinate dehydrogenase iron-sulfur subunit	CDS57211.1	Inner membrane
Fe(3+) dicitrate transport protein FecA	CDS55609.1	Outer membrane

Protection against reactive oxygen radicals

Superoxide dismutase [Mn]	CDS56813.1	Probable multiple localization sites
Catalase	CDS57987.1	Probable multiple localization sites
Thiol peroxidase	CDS58350.1	Periplasmic
Peroxiredoxin Bcp	CDS57424.1	Unknown
Delta-aminolevulinic acid dehydratase HemB	CDS56723.1	Cytoplasmic
Porphobilinogen deaminase HemC	CDS56648.1	Cytoplasmic
Uroporphyrinogen-III C-methyltransferase HemX	CDS56650.1	Inner membrane
Glutamate-1-semialdehyde 2,1-aminomutase HemL	CDS55589.1	Cytoplasmic
Protein HemY	CDS56651.1	Inner membrane
Putative thioredoxin domain-containing protein	CDS58953.1	Cytoplasmic
Glutaredoxin YdhD	CDS58265.1	Unknown
Glutathione reductase	CDS56214.1	Cytoplasmic
Electron transport complex subunit C	CDS58281.1	Cytoplasmic
Thioredoxin reductase	CDS57678.1	Cytoplasmic
Hydrogen peroxide-inducible genes activator	CDS56327.1	Cytoplasmic
Thioredoxin	CDS56739.1	Cytoplasmic
Chaperone protein ClpB	CDS59016.1	Cytoplasmic
Chaperone protein DnaK	CDS55519.1	Cytoplasmic
Protein GrpE	CDS58735.1	Cytoplasmic
Chaperone protein HtpG	CDS58965.1	Cytoplasmic
Fe/S biogenesis protein NfuA	CDS56165.1	Cytoplasmic

Secretion and transport mechanisms

Protein translocase subunit SecA	CDS55578.1	Cytoplasmic
Protein-export protein SecB	CDS56816.1	Cytoplasmic
Protein translocase subunit SecD	CDS58907.1	Inner membrane
Protein translocase subunit SecF	CDS58906.1	Inner membrane
Protein-export membrane protein SecG	CDS56777.1	Inner membrane
Protein translocase subunit SecY	CDS56065.1	Inner membrane
Sec-independent protein translocase protein TatA	CDS56720.1	Inner membrane

SecYEG protein translocase auxillary subunit	CDS58908.1	Inner membrane
Membrane protein insertase YidC	CDS56458.1	Inner membrane
Type II secretion system protein G	CDS55483.1	Inner membrane
Similar to Syringopeptin synthetase C (fragment)	CDS58510.1	Probable multiple localization sites
Multidrug efflux pump subunit AcrA	CDS58973.1	Inner membrane
Outer membrane protein TolC	CDS55974.1	Outer membrane
Hydrolytic enzymes		
Chaperone protein ClpB	CDS59016.1	Cytoplasmic
ATP-dependent Clp protease ATP-binding subunit ClpX	CDS58879.1	Cytoplasmic
ATP-dependent Clp protease proteolytic subunit	CDS58880.1	Cytoplasmic
Lon protease	CDS58878.1	Cytoplasmic
Periplasmic serine endoprotease DegP	CDS55594.1	Periplasmic
Periplasmic pH-dependent serine endoprotease DegQ	CDS55928.1	Periplasmic
Zinc metallopeptidase	CDS58788.1	Inner membrane
ATP-dependent zinc metalloprotease FtsH	CDS56781.1	Inner membrane
Chitodextrinase (modular protein)	CDS55463.1	Unknown

226 **Table 1.** Identification of putative symbiotic factors in *S. symbiotica* that might be required

227 for establishing an association with its insect host.

228 ## 2. Motility and chemotaxis

229 Motility is a bacterial function often required for initiating host colonization, symbiont

230 transmission and symbiosis establishment (Ruby, 2008; Lee et al., 2015). Motility is mostly

231 driven by flagella made of filament, basal body and motor (Ridgway, Silverman & Simon,

232 1977). We identified four basal body proteins (FlgH, CDS58522.1; FlgI, CDS58521.1; FlgD,

233 CDS58526.1; FlgG, CDS58523.1), one motor protein (FlhA, CDS58536.1), but no

234   filamentous proteins. We also identified the protein YcgR (CDS58313.1), a member of the

235   T3SS family that acts as a flagellar brake, regulating swimming and swarming by interaction

236   with motor proteins (Paul et al., 2010). We detected FlhC (CDS58540.1) and FlhD

237   (CDS58541.1) that form the major transcriptional regulator FlhDC of flagellum biosynthesis

238   and whose expression depends on flagellar secretion apparatus component FlhA

239   (CDS58536.1) that participates in the secretion of the hook and filament proteins (Bange et

240   al., 2010). Finally, we identified LrhA (CDS57959.1), a transcriptional regulator of *flhDC*

241   (Lehnen et al., 2002). In the light of these results, swimming motility was tested to know

242   whether the flagellar apparatus of *S. symbiotica* CWBI-2.3$^{\text{T}}$ was functional. The motility

243   assay (Figure. 2) showed that *S. symbiotica* CWBI-2.3$^{\text{T}}$ is not endowed with swimming

244   motility in the conditions used in this study. Genes involved in cell motility are among the

245   more highly reduced in the evolutionary transition from a free-living to an endosymbiotic

246   lifestyle (Manzano-Marín et al., 2012). In obligate intracellular symbionts and many

247   facultative ones, a significant number of genes responsible for flagellar assembly have been

248   partially or totally lost during their evolutionary transition (Moya et al., 2008; Degnan et al.,

249   2010), and only proteins export within the flagella assembly pathway have been kept (Toft &

250   Fares, 2008). Consequently, these symbionts have become nonmotile. This seems to be the

251   case for *S. symbiotica* CWBI-2.3$^{\text{T}}$. Nevertheless, it cannot be excluded that *S. symbiotica* use

252   other kinds of motility such as twitching since we identified PilQ (CDS55476.1), a secretin

253   that is essential for the biogenesis of type IV pili and that can be involved in twitching

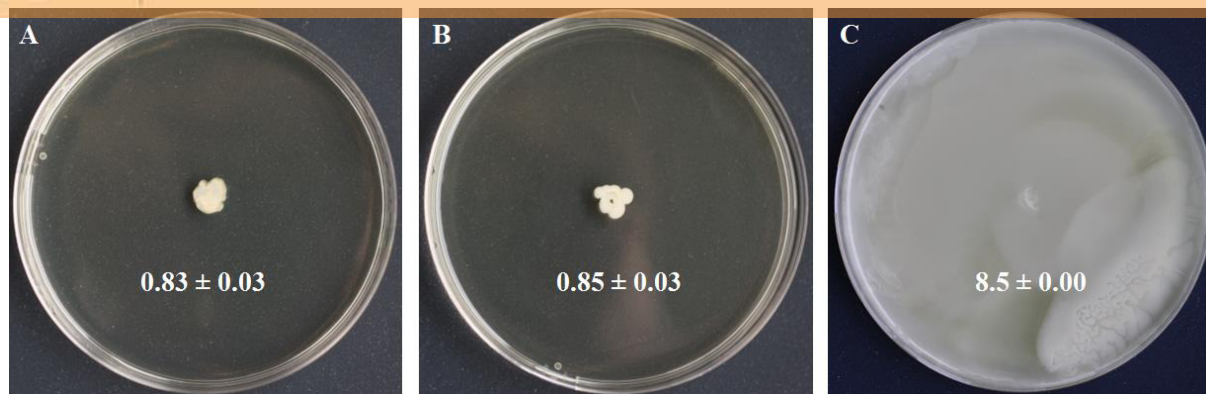254   motility, adhesion to host epithelial cells and in protein secretion (Lim et al., 2008).

Figure 2. Swimming motility testing of *S. symbiotica* CWBI-2.3^{T} as described in Materials

and methods. *S. symbiotica* is not endowed with swimming motility (A). *S. xylosus* was used

as negative control (B) and *S. marcescens* Db11 was used as positive control (C).

Motility is generally pointed as a bacterial function working together with chemotaxis,

the capacity of motile bacteria to sense and respond to changes in the concentration of

chemicals in environment. By mediating bacterial migration into the host tissues, chemotaxis

enables bacteria to reach and maintain their preferred niches for colonization (Caetano-

Anollés, Crist-Estes & Bauer, 1988; Kremer et al., 2013). In our study, we did not identify

chemotaxis (Che) proteins and chemoreceptor proteins (methyl-accepting chemotaxis protein

[MCP]). The only chemotaxis related proteins that was identified is LrhA (CDS57959.1), that

has been observed to be a key transcriptional regulator of flagella, motility and chemotaxis

genes in *E. coli* (Lehnen et al., 2002). The *che* genes are absent from the genome sequence of

S. symbiotica CWBI-2.3^{T} as well as from other *S. symbiotica* strains, while they are found in

the other *Serratia* species and other insect facultative symbionts (Toh et al., 2006). This

absence of *che* genes in *S. symbiotica* could also be a consequence of its progressive

271 evolutionary transition from a free-living to an endosymbiotic life-style that involves the loss

272 of flagellar proteins, fimbrial, pili and chemotaxis-related proteins dispensable in a stable

273 environment (*i.e.* the insect tissues in which bacterial symbionts reside) (Manzano-Marín et

274 al., 2012).

3. Proteins involved in adhesion, invasion and biofilm formation

3.1. The two-component signaling systems

277 The two-component signaling systems (TCS) are among the most universal mechanism by

278 which bacteria sense their environment and respond accordingly (Groisman, 2001). They have

279 been pointed as essential in the formation of biofilms that can protect invasive bacteria against

280 antimicrobial agents, such as antibiotics and host immune effectors (Ramey et al., 2004; Kim

281 et al., 2014). *S. symbiotica* CWBI-2.3[T] encodes the PhoP/Q regulon (CDS58188.1 and

282 CDS58187.1) that is associated with virulence in various bacterial pathogens (Groisman,

283 2001; García-Calderón, Casadesús & Ramos-Morales, 2007) and that regulates the activation

284 of secretion system genes that are required for host cell invasion and intracellular survival

285 (Dale & Moran, 2006). The mutualistic symbiont of tsetse flies, *Sodalis glossinidus* carrying a

286 *phoP* mutant fails to infect insect hosts (Pontes et al., 2011), suggesting that the PhoP/Q

287 system is necessary for host colonization and the establishment of a symbiotic association.

288 *phoP/Q* is absent in the genome of several obligate and facultative symbionts (Hansen,

289 Vorburger & Moran, 2012) which is probably a consequence of the transition lifestyle from

290 opportunistic to obligate association that is accompanied by the loss of genes that no longer

291   provide an adaptive benefit in a stable intracellular symbiotic relationship (Dale & Moran,

292   2006).

293   We also identified PhoU (CDS56284.1) that controls biofilm formation under

294   phosphate limited conditions (Lamarche et al., 2008). CpxR (CDS56277.1), a member of the

295   two-component regulatory system CpxA/CpxR has also been detected. In *E. coli*, the

296   CpxA/CpxR system is required for settling biofilm communities and ensuring optimal cell-to-

297   cell interactions (Otto & Silhavy, 2002; Beloin et al., 2004). Once activated, this strategic

298   signaling pathway increases bacterial resistance to various environmental parameters, such as

299   high pH conditions and antibiotics (Dorel, Lejeune & Rodrigue, 2006). Finally, the two-

300   component regulatory system OmpR/EnvZ (CDS56155.1 and CDS56154.1) has also been

301   detected by our proteomic approach. In *E. coli*, this system can mediate signal transduction in

302   response to osmotic stress (Cai & Inouye, 2002) and can be involved in bacterial attachment

303   to host epithelium via curli fibrils and in the regulation of genes associated with the

304   invasiveness of pathogens and symbionts (Tabatabai & Forst, 1995).

305   **3.2.Quorum sensing**

306   Defined as the ability of bacteria to monitor cell density before expressing a phenotype (*e. g.*

307   biofilm formation or virulence) (Whitehead et al., 2001), quorum-sensing can play an

308   essential role in symbiont or pathogen-host interactions by allowing invasive bacteria to adapt

309   to the changing conditions found in their new niche and to resist various environmental

310   stresses (*e.g.* nutritional and oxidative stress) and associated with host-mediated responses.

311   With our proteomic approach, SwrI (CDS56499.1), an homologue of the autoinducer LuxI

312   that has been already depicted as a key effector for host colonization in several symbiosis

313   models (Visick et al., 2000; Pontes et al., 2008) was detected. This acyl-homoserine-lactone

314   synthase catalyzes the synthesis of *N*-acyl-L-homoserine lactones (AHLs) that accumulates

315   extracellularly as cell density increases. SwrR (the homologue of LuxR) has not been

316   identified. In several *Serratia* species, the SwrI/SwrR quorum sensing system regulates

317   diverse phenotypes such as swarming motility, the production of extracellular enzymes and

318   antibiotics, and the formation of biofilms (Van Houdt, Givskov & Michiels, 2007). Whether

319   *S. symbiotica* cells use cell-cell communication to monitor their population density via the

320   synchronization of their behavior, or to socially interact during host colonization, remains to

321   be investigated.

### 3.3.Other proteins potentially involved in host invasion

323   We identified a series of other effectors potentially involved in adhesion, invasion and biofilm

324   formation: Crp (CDS56109.1), which can repress biofilm formation (Jackson et al., 2002),

325   Hns (CDS58425.1), which reduces bacterial adhesion in anoxic conditions by modulating the

326   expression of flagellar genes (Landini & Zehnder, 2002), and the RNA polymerase sigma

327   factor RpoS (CDS55654.1), which has a regulatory role in biofilm development (Sheldon et

328   al., 2012). We identified the outer membrane protein A OmpA (CDS57861.1), known to

329   participate in various pathogenic processes such as adhesion, invasion, biofilm formation and

330   evasion of host defense (Smith et al., 2007; Martinez et al., 2014), and that is required for gut

331   colonization by *S. glossinidius* in tsetse flies (Maltz et al., 2012). Finally, our proteomics

332   analysis found the expression of the FKBP-type peptidyl-prolyl cis-trans isomerase FkpA

333   (CDS56095.1), a protein that has been depicted as being similar to the macrophage infectivity

334   potentiator (Mip) proteins of *Legionella pneumophila* and *Chlamydia trachomatis*

335   (Lundemose, Kay & Pearce, 1993), and that contributes to intracellular survival of some

336   members of the Enterobacteriacae (Horne et al., 1997).

4. Iron acquisition

338   Iron is a vital nutrient for growth of many bacterial species since it is used as a cofactor or as

339   a prosthetic group for essential enzymes involved in many cellular functions (Schaible &

340   Kaufmann, 2004). The availability of this element to bacteria within the host environment is

341   generally limited. The successful uptake of iron is necessary for bacterial growth and

342   virulence and a wide diversity of iron transport systems can be set up by invasive bacteria

343   depending on iron state in the host environment (Braun & Hantke, 2011). With our proteomic

344   approach, we successfully identified ExbB (CDS55934.1) and ExbD (CDS55935.1), two

345   components of the cytoplasmic membrane-localized TonB-ExbB-ExbD complex that is

346   required for full virulence and symbiosis in several invertebrates-bacteria interactions

347   (Watson et al., 2010). No siderophore was detected in our proteome; however, we identified

348   the transcriptional repressor Fur (CDS57198.1) that plays a key role in the regulation of

349   siderophore biosynthesis and iron transport (Escolar, Pérez-Martín & Lorenzo, 1999). A

350   functional siderophore system enables pathogens and symbionts to scavenge iron under

351   limiting conditions in symbiosis (Braun & Hantke, 2011; Han et al., 2013). By using a

352   universal chemical assay, we found that *S. symbiotica* CWBI-2.3^T is negative for siderophore

353 production (Figure. 3), indicating that the symbiont does not excrete siderophore in *in vitro*

354 conditions. The ABC transporter Yfe, that is required for host colonization by invasive

355 bacteria (Watson et al., 2010), was also detected with two identified proteins: YfeA

356 (CDS58219.1) and YfeB (CDS58218.1).

357
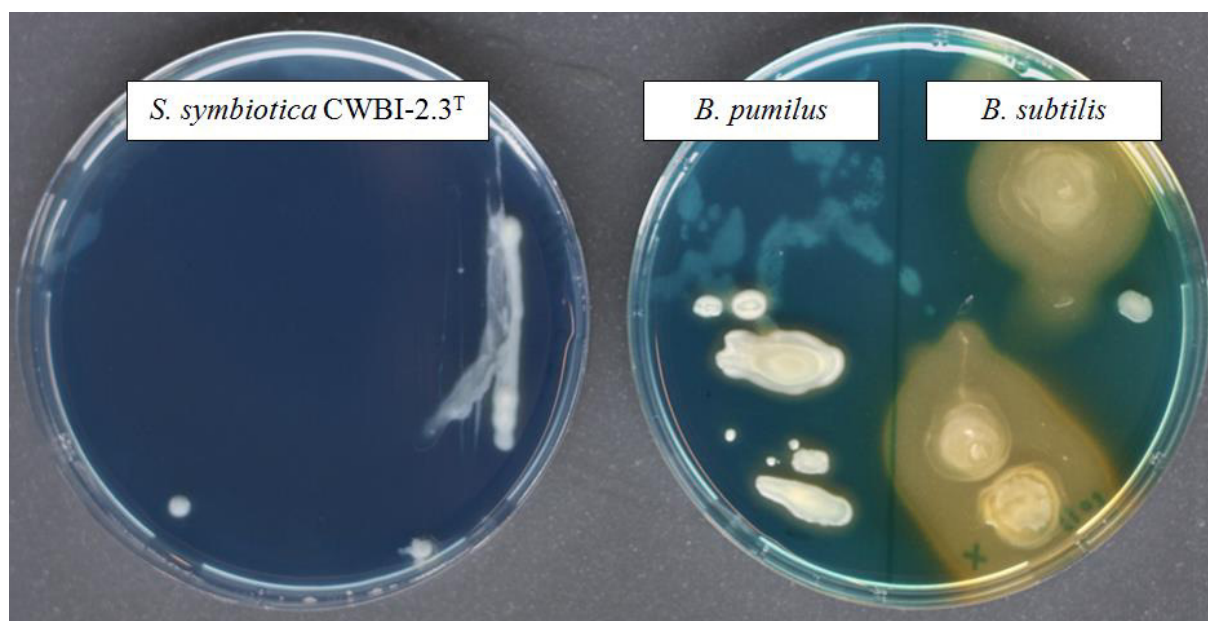
Figure 3. The screening of siderophore production *by S. symbiotica* CWBI-2.3T on CAS agar

359 plates after 48h of growth at 20°C as described in Materials and methods. *S. symbiotica* is

360 negative for siderophore production. *B. pumilus* was used as negative control and *B. subtilis*

361 was used as positive control.

362 The iron accumulated in the cytoplasm is often bound for specialized proteins such as

363 ferritin and bacterioferritin. We successfully identified two ferritins: the ferritin iron storage

364 protein Ftn (CDS58146.1), and Dps (CDS57357.1), which is a ferritin-like protein that can

365 protect DNA from oxidative damage by sequestering intracellular Fe^{2+}. Other iron-using

366 proteins have been identified: the thiol peroxidase Bcp (CDS57424.1), the two succinate

367 deshydrogenase SdhA (CDS57210.1) and SdhB (CDS57211.1), and the Fe(3+) dicitrate

368 transport protein FecA (CDS55609.1). In the context of symbiosis, proteins involved in iron

369 metabolism can have two functions: (i) enabling the bacterium to scavenge iron required for

370 its own metabolism; (ii) countering host defenses that include the production of ROS which

371 iron is a precursor.

372 **5. Protection against ROS**

373 The evolution of symbioses can be influenced by the oxidative homeostasis (*i. e.* the balance

374 between reactive oxygen species (ROS) and antioxidant molecules) (Moné, Monnin &

375 Kremer, 2014; Monnin et al., 2016). Indeed, ROS are toxic effectors that can participate to the

376 host immune defense to regulate symbiont populations. To cross the host oxidative

377 environment and protect themselves against ROS, invasive bacteria have developed a wide

378 range of defenses. Our proteome analysis indicates that *S. symbiotica* is equipped with several

379 proteins that are involved in the direct detoxification of ROS (Imlay, 2003). That includes the

380 superoxyde dismutase SodA (CDS56813.1), the catalase KatA (CDS57987.1), the thiol

381 peroxidase Tpx (CDS58350.1) and the peroxiredoxin Bcp (CDS57424.1). We also identified

382 five proteins (HemB, CDS56723.1; HemC, CDS56648.1; HemX, CDS56650.1; HemL,

383 CDS55589.1; HemY, CDS56651.1) that belong to a cluster of seven proteins involved in the

384 biosynthesis of protoporphyrin IX, the non-ferrous precursor of heme that is essential for the

385 functionality of the enzymes involved in cellular protection against toxic oxygen radicals.

386   The proteome of *S. symbiotica* CWBI-2.3[T] also includes several proteins involved in

387 disulfide reduction such as the putative thioredoxin YbbN (CDS58953.1), the glutaredoxin

388 YdhD (CDS58265.1), the glutathione reductase Gor (CDS56214.1), the electron transport

389 complex subunit C RsxC (CDS58281.1), the thioredoxin reductase TrxB (CDS57678.1), and

390 the thioredoxin TrxA (CDS56739.1). We also identified OxyR (CDS56327.1), the positive

391 regulator of hydrogen peroxide inducible genes (Bauer, Elsen & Bird, 1999).

392   In addition to this battery of defense mechanisms against oxidative stress, *S.*

393 *symbiotica* CWBI-2.3[T] also harbors a set of chaperones for protein refolding and maturation

394 such as ClpB (CDS59016.1), DnaK (CDS55519.1), GrpE (CDS58735.1), HtpG

395 (CDS58965.1) and NfuA (CDS56165.1) that are known to be involved in the repair of cell

396 components following ROS mediated damages (Takemoto, Zhang & Yonei, 1998; Kitagawa

397 et al., 2002; Hossain & Nakamoto, 2003; Angelini et al., 2008; Matuszewska et al., 2008).

398   **6. Secretion and transport mechanisms**

399 Protein secretion plays a central role in modulating the interactions between bacteria with

400 their environment, especially in pathogenic or symbiotic bacteria/host interactions. In our

401 study, we identified several components of the general secretion pathway (Sec) and the two-

402 arginine translocation pathway (Tat): the protein translocase subunit SecA (CDS55578.1), the

403 chaperone SecB (CDS56816.1), SecD (CDS58907.1), SecF (CDS58906.1), SecG

404 (CDS56777.1), SecY (CDS56065.1), and the sec-independent protein translocase, TatA

405 (CDS56720.1). These two pathways are responsible for the transport of many proteins such as

406   virulence factors and cell appendixes, across the outer membrane of Gram-negative bacteria

407   (Natale, Brüser & Driessen, 2008). YajC (CDS58908.1) and YidC (CDS56458.1), that

408   stabilize the insertion of SecA and its bound preprotein into the inner membrane, were also

409   identified and form with SecD and SecF the Sec protein secretion pathway.

410   We identified the Type II secretion system (T2SS) protein G (CDS55483.1),

411   suggesting that *S. symbiotica* CWBI-2.3^{T} could be provided by a secretion system only found

412   in proteobacteria and that can be found in symbiotic bacteria as well as pathogens

413   (Costechareyre et al., 2013). The T2SS secretes various virulence determinants and has been

414   shown to be important for virulence in many pathogens (Cianciotto, 2005). A recent study has

415   demonstrated that the T2SS is essential for gut colonization by the leech digestive tract

416   symbiont *Aeromonas veronii* (Maltz & Graf, 2011). The presence of T2SS is intriguing, since

417   most of the secretion systems described in insects symbionts belongs to type III and IV (Dale

418   & Moran, 2006; Degnan et al., 2009; Oliver et al., 2010). This T2SS has also been described

419   as an important virulence factor of a number of gram-negative bacterial plant pathogens (Jha,

420   Rajeshwari & Sonti, 2005). Its potential presence in *S. symbiotica* CWBI-2.3^{T} suggests that

421   the bacterium could be or could have been a plant pathogen. This hypothesis is supported by

422   the detection of a non-ribosomal peptide synthetase (CDS58510.1) that could be a fragment of

423   the syringopeptin synthetase C, an hydrolase that has been described as a virulence factor in

424   some plant pathogens (Scholz-Schroeder et al., 2001). Syringopeptin is a known necrosis-

425   inducing phytotoxin and therefore raises the question of the origin of symbionts; it is possible

426   that *S. symbiotica* was originally a plant pathogen that has been acquired by aphids feeding on

427 infected host plants, and then gradually domesticated by these insects. Experiments are being

428 conducted to investigate this hypothesis

429   Remarkably, two structural components of a multi-drug resistance efflux pump

430 belonging to the resistance nodulation division family were also identified: the perplasmic

431 protein AcrA (CDS58973.1) and the outer membrane channel protein TolC (CDS55974.1). *S.*

432 *symbiotica* CWBI-2.3[T] exhibits a resistance to vancomycin (Sabri et al., 2011). Whether these

433 antibiotics are indeed exported by an AcrAB-TolC efflux pump system remains to be tested.

434 ## 7. Hydrolytic enzymes

435 Invasive bacteria rely on proteolysis for a variety of purpose during the infection process.

436 Several enzymes with potential proteolytic and chitinolytic activity were identified in our

437 proteome analysis. ClpB (CDS59016.1), ClpX (CDS58879.1), ClpP (CDS58880.1), and the

438 Lon protease (CDS58878.1) are the main proteolytic players in the cytosol and can contribute

439 to bacterial virulence (Frees, Brøndsted & Ingmer, 2013). We also identify serine

440 endoproteases DegP (CDS55594.1) and DegQ (CDS55928.1). *S. symbiotica* CWBI-2.3[T] also

441 contains several metalloproteases such as YaeL (CDS58788.1) and FtsH (CDS56781.1).

442 Finally, we identified one chitodrexinase (CDS55463.1) involved in the pathway of the

443 degradation of chitin, a characteristic component of the cell walls of fungi and the

444 exoskeletons of arthropods. The identification of chitinases, which may possess antifungal

445 activity, may contribute to the protection of the aphid host against fungal pathogens as

446 observed in several cases of symbiosis (Łukasik et al., 2013). This question should be

447 addressed in further experiments.

Concluding remarks

449 The comprehensive proteome analysis of the free-living strain *S. symbiotica* CWBI-2.3[T]

450 resulted in the identification of 720 proteins corresponding to 19.7% of all theoretically

451 expressed proteins of the symbiont. Most of the identified proteins belong to housekeeping

452 and primary metabolism. Special attention was drawn to putative symbiotic factors and the

453 most striking were: (i) members of the two-components signaling systems that are no longer

454 expressed in obligate symbionts and several facultative ones, (ii) OmpA, which is a probable

455 key factor for symbiosis establishment, (iii) FkpA that could contribute to the intracellular

456 survival of *S. symbiotica*, (iv) iron transport mechanisms to access host iron resources, and (v)

457 several components of secretions systems which might be involved in the secretion of still

458 unknown effectors. In addition, *S. symbiotica* CWBI-2.3[T] is provided by a wide range of

459 protections against the hostile oxidative environment of the host. These results provide strong

460 evidence that *S. symbiotica* CWBI-2.3[T] is well-armed to invade, persist, and multiply in the

461 insect tissues. Interestingly, we did not find any pathogenic trait that would directly harm the

462 host such as toxins.

463 Swimming motility and chemotaxis, which can be determinant for host colonization,

464 are missing in the free-living *S. symbiotica* CWBI-2.3[T]. This could the consequence of a

465 gradual evolution from a free-living towards a host-dependent lifestyle that normally involves

466 a dramatic loss of symbiotic factors that are no longer useful in a stable environment. This

467 hypothesis should be addressed in further comparative genomic studies.

468   Phylogenetic analyses combined with fluorescence *in situ* hybridization approaches

469 applied on *S. symbiotica* strains harbored by various aphid species reveal that certain strains

470 naturally reside in the aphid gut (Renoz *et al.* in prep). Interestingly, *S. symbiotica* strain

471 CWBI-2.3[T] belongs to the same clade as these extracellular gut symbionts, suggesting that it

472 could be picked up directly from environmental sources, and thus initiate novel symbiotic

473 associations with the insect digestive tract as the first potential entry route. Molecular clues

474 suggest that *S. symbiotica* CWBI-2.3[T] could be, initially, a plant pathogen. The pathogenicity

475 of *S. symbiotica* to plants, as well as the role of plants as mediator of horizontal transfers of

476 the symbiont, are currently under investigation.

477   There is no doubt that free-living strains of *S. symbiotica* will constitute an

478 experimental tool for understanding the manifold strategies set up by symbiotic bacteria

479 during the nascent phase of a symbiosis in order to ensure their long-term establishment in a

480 novel insect host. This comprehensive proteome map of a facultative insect symbiont provides

481 a series of potential symbiotic factors for further studies to understand the molecular cross-

482 talk between aphids and *S. symbiotica* of ecological and evolutionary importance.

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495   Author contributions

496   François Renoz, Thierry Hance and Pierre Morsomme conceived and designed the

497   experiments; François Renoz, Hervé Degand and Anne-Marie Faber performed the

498   experiments; François Renoz, Antoine Champagne and Vincent Foray analyzed the data;

499   François Renoz wrote the manuscript; Antoine Champagne, Vincent Foray and Thierry Hance

500   made manuscript revision.

501   Conflicts of Interest

502   The authors declare no conflict of interest.

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