Unravelling potential virulence factor candidates in *Xanthomonas citri*. subsp. *citri* by secretome analysis

Rafael M Ferreira, Leandro M Moreira, Jesus A Ferro, Marcia R R Soares, Marcelo L Laia, Alessandro M Varani, Julio C F de Oliveira, Maria Ines T Ferro

Citrus canker is a major disease affecting citrus production in Brazil. Its mainly caused by *Xanthomonas citri* subsp. *citri* strain 306 pathotype A (Xac). We analysed the differential expression of proteins secreted by wild type Xac and an asymptomatic mutant for *hrpB4* (*ΔhrpB4*) grown in Nutrient Broth (NB) and a medium mimicking growth conditions in the plant (XAM1). This allowed the identification of 55 secreted proteins, of which 37 were secreted by both strains when cultured in XAM1. In this secreted protein repertoire, the following stand out: Virk, Polyphosphate-selective porin, Cellulase, Endoglucanase, Histone-like protein, Ribosomal proteins, five hypothetical proteins expressed only in the wild type strain, Lytic murein transglycosylase, Lipoprotein, Leucyl-tRNA synthetase, Co-chaperonin, Toluene tolerance, C-type cytochrome biogenesis membrane protein, Aminopeptidase and two hypothetical proteins expressed only in the *ΔhrpB4* mutant. Furthermore, Peptidoglycan-associated outer membrane protein, Regulator of pathogenicity factor, Outer membrane proteins, Endopolygalacturonase, Chorismate mutase, Peptidyl-prolyl cis-trans isomerase and seven hypothetical proteins were detected in both strains, suggesting that there was no relationship with the secretion mediated by the type III secretory system, which is not functional in the mutant strain. Also worth mentioning is the Elongation factor Tu (EF-Tu), expressed only the wild type strain, and Type IV pilus assembly protein, Flagellin (FliC) and Flagellar hook-associated protein, identified in the wild-type strain secretome when grown only in NB. Noteworthy, that FliC, EF-Tu are classically characterized as PAMPs (Pathogen-associated molecular patterns), responsible for a PAMP-triggered immunity response. Therefore, our results highlight proteins potentially involved with the virulence. Overall, we conclude that the use of secretome data is a valuable approach that may bring more knowledge of the biology of this important plant pathogen, which ultimately can lead to the establishment of new strategies to combat citrus canker.

1 **Unravelling potential virulence factor candidates in *Xanthomonas citri*. subsp. *citri* by**

2 **secretome analysis**

4 Rafael M. Ferreira[1#*], Leandro M. Moreira[2#], Jesus A. Ferro[1], Marcia R. Soares[3], Marcelo L.

5 Laia[4], Alessandro M. Varani[1*], Julio C.F. de Oliveira[5], Maria Ines T. Ferro[1]

7 [1]Departmento de Tecnologia, Universidade Estadual Paulista "Júlio de Mesquita Filho",

8 Jaboticabal, São Paulo, Brazil.

9 [2]Departamento de Ciências Biológicas - Núcleo de Pesquisas em Ciências Biológicas-NUPEB,

10 Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil.

11 [3]Departamento de Bioquímica, Universidade Federal do Rio de Janeiro, Instituto de Química,

12 Rio de Janeiro, Rio de Janeiro, Brazil

13 [4]Departamento de Engenharia Florestal, Universidade Federal dos Vales do Jequitinhonha e

14 Mucuri, Diamantina, Minas Gerais, Brazil

15 [5]Departamento de Ciências Biológicas, Universidade Federal de São Paulo, Diadema, São Paulo,

16 Brazil

22 *Corresponding author: Rafael M. Ferreira, marini64@gmail.com and Alessandro M. Varani,

23 amvarani@fcav.unesp.br

24 # These authors contributed equally to this work.

26 **Running Title:** Secretome analysis of *Xanthomonas citri*.

29   **Abstract**

30   Citrus canker is a major disease affecting citrus production in Brazil. Its mainly caused by

31   *Xanthomonas citri* subsp. *citri* strain 306 pathotype A (Xac). We analysed the differential

32   expression of proteins secreted by wild type Xac and an asymptomatic mutant for *hrpB4*

33   (*ΔhrpB4*) grown in Nutrient Broth (NB) and a medium mimicking growth conditions in the plant

34   (XAM1). This allowed the identification of 55 secreted proteins, of which 37 were secreted by

35   both strains when cultured in XAM1. In this secreted protein repertoire, the following stand out:

36   Virk, Polyphosphate-selective porin, Cellulase, Endoglucanase, Histone-like protein, Ribosomal

37   proteins, five hypothetical proteins expressed only in the wild type strain, Lytic murein

38   transglycosylase, Lipoprotein, Leucyl-tRNA synthetase, Co-chaperonin, Toluene tolerance, C-

39   type cytochrome biogenesis membrane protein, Aminopeptidase and two hypothetical proteins

40   expressed only in the *ΔhrpB4* mutant. Furthermore, Peptidoglycan-associated outer membrane

41   protein, Regulator of pathogenicity factor, Outer membrane proteins, Endopolygalacturonase,

42   Chorismate mutase, Peptidyl-prolyl cis-trans isomerase and seven hypothetical proteins were

43   detected in both strains, suggesting that there was no relationship with the secretion mediated by

44   the type III secretory system, which is not functional in the mutant strain. Also worth mentioning

45   is the Elongation factor Tu (EF-Tu), expressed only the wild type strain, and Type IV pilus

46   assembly protein, Flagellin (FliC) and Flagellar hook-associated protein, identified in the wild-

47   type strain secretome when grown only in NB. Noteworthy, that FliC, EF-Tu are classically

48   characterized as PAMPs (Pathogen-associated molecular patterns), responsible for a PAMP-

49   triggered immunity response. Therefore, our results highlight proteins potentially involved with

50   the virulence. Overall, we conclude that the use of secretome data is a valuable approach that

51   may bring more knowledge of the biology of this important plant pathogen, which ultimately can

52   lead to the establishment of new strategies to combat citrus canker.

53

54

55

56

57

58

59

60 **Introduction**

61 Proteins secreted by bacteria are known to have key functions, such as the provision of nutrients,

62 cell-cell communication, detoxification, inhibition of potential competitors, etc. (Tseng, Tyler &

63 Setubal, 2009). The extracellular proteins of pathogenic bacteria also play a critical role in their

64 pathogenicity and adaptation to a compatible host (Kamoun et al., 1993; Qian et al., 2013).

65 Six types of secretory systems (SS), which have been previously described, are critical

66 for exporting proteins to the external environment or for directly contacting the host (Tseng,

67 Tyler & Setubal, 2009). SS can be classified into two groups: I) one-step secretion systems,

68 wherein secreted proteins are exported across the inner and outer membranes in a single step,

69 include the type I (T1SS), type III (T3SS), Type IV (T4SS), and type VI (T6SS) secretion

70 systems; or II) two-step secretion systems, wherein proteins are first exported into the

71 periplasmic space via the general secretion (Sec) or two-arginine (Tat) pathways, and then

72 translocated across the outer membrane via the type II (T2SS), type V (T5SS), or less commonly,

73 the T1 or T4SS systems (Saier, 2006).

74 Secreted proteins (secretomes) from different organisms cultivated in different

75 physiological conditions have been analysed, which often validates biological information

76 derived from genomic sequencing projects. Previous studies have focused on the secretomes of

77 specialized secretion apparatuses, which include the Tat (Pickering, Yudistira & Oresnik, 2012),

78 T3SS (Deng et al., 2010) and T2SS secretomes (Sikora et al., 2011). In other studies, the overall

79 goal was to characterize the secretome irrespective of the secretion apparatus used (Zijnge,

80 Kieselbach & Oscarsson, 2012). More recently, comparative proteomics, which aims to compare

81 the secretomes of different bacterial strains cultivated in the same physiological conditions (Xu

82 et al., 2013) or the secretomes of the same strain cultivated in different physiological conditions

83 or hosts (Villeth et al., 2009), has gained traction.

84 The secretomes of plant pathogenic bacteria have also been studied (Kazemi-Pour,

85 Condemine & Hugouvieux-Cotte-Pattat, 2004; Nissinen et al., 2007; Schumacher et al., 2014),

86 including bacteria of the genus *Xanthomonas* (Evans et al., 2007; Wang et al., 2013).

87 *Xanthomonas citri* subsp. *citri* (*Xac*), strain 306 pathotype A, which causes canker A, affects

88 different citrus species. The study of its general proteome have contributed to a greater

89 understanding of its pathogenicity and adaptation (Yamazaki, Hirata & Tsuyumu, 2008; Soares

90 et al., 2010; Facincani et al., 2014; Moreira et al., 2014). Since *Xac* is a quarantine

microorganism that is the cause of numerous losses to the citrus industry, characterization of its genome, proteome, and secretome are important for understanding the mechanisms of its interaction with compatible hosts.

In *Xac*, as well as in other bacteria of the same genus, the T3SS has been the best studied SS. Through this apparatus, specific effector proteins of virulence are secreted (Büttner & He, 2009), which mediate the typical symptoms of the disease in their respective susceptible plant hosts. Previous studies have shown that mutations in genes that are part of or regulate this apparatus completely prevent or substantially reduce the classical phenotypes of the disease (Büttner et al., 2002). Random mutations in *Xac* were generated previously by our group using a EZ-Tn5 <Kan> transposition cassette, which were used to identify several genes that might be associated with its virulence (Laia et al., 2009). Of these, one mutation in particular resulted in non-pathogenicity; the mutation was in *hrpB4* (*ΔhrpB4*-XAC0410), located within the *hrp* (*Hypersensitive Response and Pathogenicity*) gene cluster and coding for the T3SS apparatus (Figure 1A). HrpB4 is the inner membrane protein that associates with T3SS, thus a structural protein that plays a role on T3SS apparatus assembly (Figure 1B). Interestingly, in addition to being unable to induce virulence (Figure 1C), *ΔhrpB4* also fails to develop within the plant despite growing normally in an energy-rich culture medium. At the same time, because *ΔhrpB4* was unable to grow *in planta*, it would not be possible to obtain the secreted proteins in infective conditions in concentrations capable of being analysed by proteomics.

To characterize the secretome of *Xac*, we cultured the wild-type strain and the *ΔhrpB4* mutant in two different culture media: nutrient broth (NB) and the defined medium, XAM1 (Wengelnik K, Van den Ackerveken G, 1996). The latter medium was chosen based on previous results showing that XAM1 medium simulates conditions experienced by *Xac* during plant infections (Facincani et al., 2014). The mutant strain, in which the T3SS is non-functional, was chosen to test the dependence of the secretome on the T3SS. In addition, since several authors have previously reported a relationship between xanthan gum production and the pathogenicity of the microorganism (Katzen et al., 1998), assays to determine the production of xanthan gum and the dry weight of cells were performed at each condition.

122 **Materials and methods**

123 **Growth conditions**

124 Stocks of wild-type *Xac* and the *ΔhrpB4* mutant strain were stored in phosphate-buffered saline

125 at room temperature. Both were cultured in Nutrient Agar plates prior to the preparation of the

126 pre-inoculum. All maintenance cultures were maintained at 28°C, and kanamycin was added at

127 100 μg/mL for solid culture media and 50 μg/mL for liquid culture media. Wild-type *Xac* and

128 *ΔhrpB4* were cultured in a 15-mL liquid Nutrient Broth (NB) pre-inoculum, maintained at 200

129 rpm and 28°C, until the cultures reached $OD_{600nm} = 1.0$ (approximately 10^8 CFU/mL). Cultures

130 were centrifuged at $3,100 \times g$ for 15 min and the supernatant was discarded. The pellet was

131 resuspended in water and added to the culture medium. Both wild-type *Xac* and *ΔhrpB4* were

132 grown in 500 mL NB culture medium (200 rpm, 28°C) until the culture reached an OD_{600nm} of at

133 most 1.4 (approximately 10^9 CFU/mL). At OD_{600nm} less than 1.4, growth is still logarithmic,

134 which prevents the release of proteins into the culture medium due to cell lysis, thus enabling us

135 to accurately profile the secretome (Alexander Watt et al., 2005). XAM1 media (7.5 mM

136 $(NH_4)_2SO_4$, 33 mM KH_2PO_4, 60 mM K_2HPO_4, 1.7 mM sodium citrate [$C_6H_5Na_3O_7 \bullet 2 H_2O$], 0.9

137 mM $MgSO_4$, 9.9 mM fructose, 9.9 mM sucrose and 0.03% of Casamino acids in a final volume

138 of 1 L) was used to induce virulence (Wengelnik K, Van den Ackerveken G, 1996). Each

139 bacterium was grown in 2 L (4× 500 mL) of this medium, to which 1 mg/mL of Bovine Serum

140 Albumin (BSA) was added. Bacteria were grown at 28°C with shaking at 200 rpm until the

141 culture reached an OD_{600} of at most 0.57, wherein growth was still logarithmic.

142 Wild-type *Xac* or *ΔhrpB4* were grown for 16 or 23 h in NB liquid culture media until it

143 reached an OD_{600} of 1.0 or 1.4, respectively. Both strains were also grown in XAM1 liquid media

144 for 24 h, which is equivalent in time to the early stages of infection *in vivo*. Wild-type bacteria

145 reached an OD_{600} of 0.57, and the mutant achieved an OD_{600} of 0.24. Higher cell densities in

146 XAM1 liquid media could not be achieved even with longer cultivation periods (>24 h). Thus, to

147 achieve the highest density of bacteria without compromising their structural integrity, a

148 cultivation time of 24 h was chosen, which corresponded with the initial stage of infection. The

149 difference in growth between the two culture media is due to the fact that the first is an energy-

150 rich medium and the second a defined medium.

151 Several replicates were used for each condition to obtain the total pool of proteins used

152 for this study.

153

Centrifugation and filtration of samples

155 Cultures were centrifuged in two 60 min steps at 4°C. In the first step, the samples were

156 centrifuged at 3,100 × g, the bacterial pellet was discarded and the supernatant containing

157 secreted proteins was transferred to clean tubes. In the second step, the (first) supernatant was

158 centrifuged at 11,000 × g and again the supernatant was recovered. For strains cultivated in NB

159 liquid medium, the supernatant was recovered and then filtered using 0.2-μm (Millipore^{TM})

160 nitrocellulose membranes shortly after the second centrifugation.

161   For bacteria cultured in XAM1 medium, the supernatant could not be filtered due to the

162 elevated production of xanthan gum, which caused changes in the membrane filtration profile.

163 All samples were frozen using liquid nitrogen and lyophilized to complete dryness. Each dried

164 sample corresponding to 500 mL of the supernatant was resuspended in 50 mL of double-

165 distilled autoclaved water (ddH$_2$O). To precipitate xanthan gum produced by cultures grown in

166 XAM1 medium, absolute ethyl alcohol (1:3) was added to the resuspended sample (in ddH$_2$O),

167 and the sample was centrifuged at 3,000 × g for 5 min at 30°C. These samples were then filtered

168 through 0.2 μm (Millipore^{TM}) nitrocellulose membranes and again lyophilized and resuspended

169 in 50 mL of ddH2O.

170

Precipitation of proteins and SDS-PAGE electrophoresis

172 Proteins in the filtered supernatant were precipitated with trichloroacetic acid (TCA), with

173 modifications (Hirose et al., 2000). Briefly, TCA was added to the resuspended sample to a final

174 concentration of 10%, and each sample was vigorously mixed and stored at 4°C for 16 h.

175 Samples were centrifuged at 20,000 × g for 20 min at 4°C. The supernatant was discarded and

176 the precipitate washed three times with cold acetone to remove residual TCA. Precipitated

177 proteins were then lyophilized to complete dryness and dissolved in 25 mM NH$_4$HCO$_3$ buffer,

178 500 mM urea, pH 8.0. All samples were quantified and normalized. The proteins were then

179 hydrolysed in solution.

180

Trypsinization of samples and mass spectrometry

182 Proteins were reduced by adding 1 μg of DTT per 50 μg of protein and incubating for 1 h at

183 37°C. Proteins were then alkylated by adding 5 μg of iodoacetamide per 50 μg of protein and

184   incubating for 1 h at room temperature in the dark. Proteins were trypsinized by adding 20 μg of

185   Promega Sequencing Grade Modified Trypsin (Madison. USA) (1:50) and incubating for 20 h at

186   37°C. The hydrolysis reaction was terminated by adding 2 μL of formic acid. The samples were

187   then loaded into a Waters® nanoACQUITY UPLC® capillary chromatography system (Waters,

188   Milford, MA). The digested proteins were desalinized using a Waters Opti-Pak C18 trap column.

189   The volume of the injected sample was 10 μL, and liquid chromatography was performed on a

190   reverse-phase C18 column Ease 150 mm × 2.1 mm (Waters, Milford, MA). Proteins were eluted

191   with a flow rate of 0.3 μL/min using a linear gradient ranging from 5% to 50% acetonitrile

192   containing 0.1% formic acid.

193   The capillary liquid chromatography system (nLC) was coupled to a mass spectrometer with an

194   electrospray ionization (ESI) source and quadrupole/time-of-flight in series analysers (Q-TOF

195   Micro from Waters, Milford, MA) (UPLC-MS/MS), which permitted the direct analysis of

196   eluted peptides in the acetonitrile gradient in Q-TOF. For ESI, a voltage of 3000 V and a

197   temperature of 80°C was applied in the capillary. A MassLynx data system (Version 4.1, Waters)

198   was used to control the instrument and acquire data, and the experiments were performed by

199   scanning mass/charge (m/z) ratios between 200 and 2000 using a first scan time of 1 s, which

200   was applied during the entire chromatographic process. The averages of the mass spectra

201   corresponding to each sign of the total ion current chromatogram (TIC) were calculated,

202   allowing an accurate determination of the molecular mass. Exact mass values were obtained

203   using a LockSpray™ source (Waters, Milford, MA). This reference mass was used to correct the

204   mass of the analyte (sample) throughout the study. Phosphoric acid was used as the reference in

205   this study, which has an m/z ratio of 588.8692.

206   The acquisition of data dependent on MS/MS were performed on precursors with charge states 2

207   or 3 over a mass range of m/z 50–2000 and at an interval below 2 m/z. At most, 3 ions were

208   selected for MS/MS analysis of a single MS. The masses of Na^+ and K^+ were automatically

209   excluded. The collision induced dissociation (CID) based MS/MS spectra were obtained by

210   using argon as the collision gas at a pressure of 13 PSI and a collision voltage that ranged from

211   18 to 45 V, depending on the mass of the precursor. The scanning ratio was 1 s.

212

213   **Processing of the generated spectra and signal peptide prediction**

214 All data were processed with ProteinLynx Global Server (version 2.0, Waters), which
215 automatically corrected values of m/z spectrum of MS and MS/MS according to the mass of the
216 Lockspray reference ion. Proteins were identified using the *Xac* database from NCBI (BioProject
217 PRJNA297) and UniProt databases, and the MASCOT program (Version: 2.2.1 - Licensed to
218 Brazilian Synchrotron Light Laboratory). The values of monoisotopic masses of the MS/MS
219 spectra (MS/MS Ion Search) were used, which considered cysteine carbamidomethylation as a
220 fixed modification and oxidation of methionine as a variable modification. In the hydrolysis by
221 trypsin, the possible loss of one cleavage site was considered and the tolerance of masses of
222 peptides and fragments was set to ± 0.05 Da.

223 The amino acid sequences of identified proteins were submitted to analysis by PrediSi
224 (Prediction of Signal peptides, [www.predisi.de)](www.predisi.de) (Hiller et al., 2004). Identified proteins that
225 lacked signal peptides according to PrediSi analysis were submitted to SecretomeP 2.0 for
226 further analysis (Bendtsen et al., 2005). Comparisons between proteins from different organisms
227 were performed using the bioinformatics tool BLASTP.

228

229 **Identification of candidate PIP-boxes in the regulatory regions of genes**
230 Several genes regulated by HrpX possess a consensus nucleotide sequence, TTCGC-N_{15}-
231 TTCGC, which has been termed the plant-inducible promoter box (PIP-box) (da Silva et al.,
232 2002). The presence of a PIP-box or PIP-box-like sequence suggests that the candidate gene is
233 regulated by HrpX and encodes an effector protein of the T3SS pathway. PIP-box analysis was
234 performed following methods described by Silva (da Silva et al., 2002).

235 Candidate PIP-box sequences and their genomic locations were identified by searching
236 the complete *Xac* genome for sequences that matched the following PIP-box sequence patterns:
237 1. Forward orientation (uncomplemented) - TTCGN-N_{15}-TTCGN or TTCGN-N_{16}-TTCGN or 2.
238 Reverse orientation (complemented) – NCGAA-N_{15}-NCGAA or NCGAA-N_{16}-NCGAA. Each
239 candidate PIP-box sequence was further analysed to determine whether it was part of a promoter,
240 which should be between 10 and 1000 nucleotide bases long and precede the start codon of a
241 gene.

242

243 **Xanthan gum production**

244 For xanthan gum production, three (triplicate) 250-mL Erlenmeyer flasks containing 100 mL of

245 Gum medium (25 g/L glucose, 3g/L yeast extract, 2 g/L K_2HPO_4, 0,1 g/L $MgSO_{4.}7H_2O$, pH 7.0

246 with 4 M HCl) were inoculated with 2.5 mL of each bacterial strain grown in NB liquid medium

247 (OD_{600} of 0.3) and incubated at 29°C for 96 h in a rotary shaker at 178 rpm (Shu & Yang, 1990).

248   After 96 h, cultures were centrifuged at 9,666 × g for 40 min. The bacterial pellets were

249 stored at -20°C and the supernatants were transferred to 500-mL beakers. Xanthan gum was

250 recovered from the supernatants by isopropanol precipitation. KCl (4 g) was added to each

251 beaker, followed by stirring at room temperature for 15 min. Two volumes of cold isopropyl

252 alcohol were added and the xanthan gum from each beaker was removed and placed in plastic

253 containers, which were previously weighed, with the aid of a glass rod and a sieve. After 48 h at

254 37°C, the containers, plus sample, were weighed again and the amount of xanthan gum

255 calculated. The mass of the bacterial pellet from each culture was also measured. For this, each

256 pellet was resuspended with 1 mL MilliQ autoclaved water and transferred to a pre-weighed

257 beaker and weighed again after 24 h at 70°C. Three replicates were used for each condition and

258 Statistical Analysis was performed (Unpaired Student t test).

259

260 **Results and Discussion**

261 Fifty-five unique proteins were identified in total on the secretomes of wild-type *Xac* and the

262 mutant *ΔhrpB* cultured in different growth media. Of these, the majority (37 = 67.3%) were

263 secreted by both strains only when cultured in XAM1 medium, whereas 12 (21.8%) were

264 secreted only when cultured in NB (Figure 2A). Considering that the XAM1 medium simulates

265 conditions experienced by *Xac* during plant infections or when *Xac* is in contact with the plant

266 meristem, the former set of proteins are considered the repertoire of proteins required for

267 pathogenicity and adaptation during infections. Regardless of the growth media, 19 proteins

268 (34.5%) were secreted solely by the wild-type strain, whereas 14 (25.5%) were secreted solely

269 by the mutant strain (Figure 2A). These differences may be directly attributed to the non-

270 functionality of the T3SS system in the mutant *ΔhrpB4*. It is also possible that the functional loss

271 of the T3SS apparatus, which may be important for maintaining cellular homeostasis and for

272 adaptation in plant tissues, has pleiotropic effects on the expression/secretion of certain proteins.

273   The Venn diagram shown in Figure 2C underscores the differences and overlap in

274 proteins secreted under the different conditions tested. Analysis of proteins according to their

275   MASCOT annotated functions revealed that seven were associated with adaptation and virulence
276   (category VII), and 12 were hypothetical proteins (category VIII) (Figure 2B). The following
277   sections describe in details these main findings.

278

279   **Proteins secreted by both strains grown in XAM1 medium**

280   Table 1 lists the 13 proteins secreted in common by both strains. Of these, four are hypothetical
281   proteins (XAC2562, XAC0677, XAC0223 and XAC0232), three of which contain a signal
282   peptide sequence (Table 1). Among the other nine proteins with annotated functions (Pcp,
283   Cysteine protease, RpfN, Slp, OmpP6, Peh-1, PheA and Prolyl-peptidyl cis-trans isomerase -
284   PPIase), six have a signal peptide sequence, and only the gene encoding for Peh-1 is downstream
285   of a PIP-box sequence (Table 1). All of these membrane proteins were previously identified in
286   the formation of outer membrane vesicles (OMVs) when *X. campestris* pv. *campestris* was
287   grown in XVM2 medium (Sidhu et al., 2008), which is similar to XAM1 in that it also simulates
288   conditions experienced by the microorganism during infections. In general, proteins secreted by
289   both wild-type *Xac* and *ΔhrpB4* in these conditions likely represent a core set of proteins
290   required to survive and adapt to the plant interior. Importantly, the loss of T3SS function in the
291   *hrpB4* mutant did not affect the secretion of these proteins; therefore, it is likely that these
292   proteins are secreted by the other secretory systems, such as Sec, Tat, T2SS, and T5SS. The
293   presence of signal peptide sequences in at least 69% of these proteins reinforces their targeted
294   secretion.

295   Proteins with annotated functions described bellow appear to have a fundamental role in
296   either the virulence of the genus *Xanthomonas* or their adaptation to the plant. The Pcp protein,
297   encoded by the gene XAC1466, is a peptidoglycan-associated outer membrane lipoprotein
298   homologous to the SlyB protein in *Xanthomonas campestris*. This protein is directly regulated by
299   a two-component system in most bacteria (PhoPQ), which is present in many bacterial pathogens
300   of both animals and vegetables (Perez et al., 2009). This two-component system, which is
301   encoded by the genes XAC4023 and XAC4022, is also expressed in *Xac* and has been shown to
302   be active during the early stages of infection (Moreira et al., 2014). Proteins homologous to Pcp
303   are also essential for maintaining the integrity of the cell envelope in *Pseudomonas putida*
304   (Rodríguez-Herva, Ramos-González & Ramos, 1996), and more recently, were associated with
305   OMV formation in *P. aeruginosa* (Wessel et al., 2013).

306   The Peh-1 protein, encoded by the gene XAC0661, is an endopolygalacturonase that
307   facilitates plant cell wall degradation (Liu, Chatterjee & Chatterjee, 1994). Breakdown of the
308   plant cell wall also releases sources of carbon to nourish the pathogen during infections. This
309   protein is secreted by T2SS, and has been reported to be regulated by HrpG/X (Wang, Rong &
310   He, 2008), which is consistent with the presence of the plant-inducible promoter (PIP-box)
311   sequence located upstream of the gene. HrpG and HrpX are dual regulatory elements that
312   regulate the expression of genes involved in the formation and secretion of T3SS proteins (Guo
313   et al., 2011). In transcriptome studies, the *hrpX* gene was previously shown to be expressed in
314   *Xac* when grown in XAM1 medium, and remains active even in the *ΔhrpB4* strain (Laia et al.,
315   2009), which is consistent with its secretion by both strains in our study. Mutants of
316   *Xanthomonas campestris* in which the gene encoding the PghAxc protein (homologous to Peh-1
317   in *Xac*) was inactivated showed attenuated virulence when sprayed onto *Arabidopsis* hosts,
318   demonstrating the importance of this protein in the early stages of infection (Wang, Rong & He,
319   2008).

320   A protein with cysteine protease functions, encoded by the XAC2853 gene, was also
321   secreted by both strains grown in XAM1 medium. This protein is homologous to CysP2 in *X.*
322   *oryzae* pv. *oryzae*, has a defective PIP-box sequence upstream of the gene in the *Xac* genome, is
323   secreted by T2SS, and is regulated by HrpG/X (Furutani et al., 2004; Yamazaki, Hirata &
324   Tsuyumu, 2008). Proteins with this function are considered important virulence factors because
325   they hydrolyse peptides in the host cell. Inoculation of a citrus host *in vivo* with a mutant
326   deficient in a cysteine protease, encoded by the gene XAC2853, resulted in less virulence
327   (Soares-Costa et al., 2012). In studies involving *Pseudomonas*, the virulence of the pathogen was
328   directly correlated with the secretion of the AvrRpt2 protein, which has cysteine protease
329   functions, by T3SS (Axtell et al., 2003; Cui et al., 2013).

330   RpfN, which is characterized as a virulence factor regulator and encoded by the gene
331   XAC2504, has an OprB domain (carbohydrate-selective porin). The OprB domain is a specific
332   porin channel for glucose transport, but can also mediate the transport of other monosaccharides
333   to the inner membrane (Wylie & Worobec, 1995). *Pseudomonas aeruginosa* mutants defective
334   for this protein demonstrated a reduced ability to utilize various types of monosaccharides,
335   indicating its importance in the import of different sugars into the cell (Nikaido, 2003).
336   According to SecretomeP 2.0 analysis, this protein is not predicted to be secreted by the Sec

337   pathway but rather by a non-classical route. The role of this protein in the pathogenicity of *Xac* is
338   not well understood, but according to Dow and colleagues (Dow et al., 2000),
339   endopolygalacturonase levels were increased in *Xanthomonas campestris* mutants, in which this
340   gene was inactivated. Moreover, Moreira and colleagues suggested that the absence of the *rpfN*
341   gene and mutations in the PTS system associated with the internalization of fructose could
342   explain the fastidious growth of *X. fuscans* B strain, which causes canker B in citrus plants
343   (Moreira et al., 2010).

344   Slp, which is encoded by the gene XAC1113, is an outer membrane lipoprotein. Studies
345   of homologues of this protein in the *Xanthomonas* genus are limited. However, in studies of the
346   transcriptome of *Xylella fastidiosa*, which causes CVC (Citrus Variegated Chlorosis) in citrus
347   plants, a homologue of the *Xac slp* gene was robustly expressed in growth conditions that
348   induced the formation of biofilm (De Souza et al., 2004). Biofilms are fundamental to the
349   induction of virulence and are produced on a large scale in bacteria of the genus *Xanthomonas*
350   when grown in conditions that induce virulence (Dow et al., 2003; Rigano et al., 2007). Thus, the
351   methodological difficulties of protein extraction from strains grown in XAM1 medium could be
352   explained in part by the secretion of Slp proteins. In *E. coli*, changes to the composition of
353   lipoproteins in the outer membrane directly affect surface adhesion, reinforcing the importance
354   of these proteins to biofilm formation (Otto et al., 2001).

355   PheA, which is encoded by the gene XAC3467 and is secreted by both strains grown in
356   XAM1 medium, is a chorismate mutase. PheA appears to be fundamentally important for plant-
357   pathogen interactions because it catalyses the conversion of chorismate to prephenate, which is a
358   precursor for the aromatic amino acids tyrosine (Tyr) and phenylalanine (Phe) (Kast et al., 2000).
359   Chorismate is a substrate of the shikimate pathway, which regulates plant cell growth,
360   development, structure, and pathogen defence. It can be converted into indole-acetic acid (IAA),
361   salicylic acid (SA) and tryptophan (Bekal, Niblack & Lambert, 2003), culminating in the
362   activation of plant defence systems (Weaver & Herrmann, 1997) (Figure 3). Inactivation of the
363   chorismate mutase gene in *Ustilago maydis*, the fungal agent that causes corn smut, drastically
364   reduced symptoms associated with the disease (Djamei & Kahmann, 2012), demonstrating the
365   importance of this protein to host-pathogen interactions. Finally, in bacteria of the genus
366   *Xanthomonas* (*Xanthomonas oryzae* pv. *Oryzae* XKK.12), this protein has been reported to be
367   directly involved in the process of virulence (Degrassi et al., 2010).

368   Peptidyl-prolyl cis-trans isomerase (PPIase) is a protein encoded by the gene XAC1585.

369   Similar to Slp, Peh-1, and PheA, this protein was identified in the extracellular matrix of both

370   strains when grown in induction medium, confirming data described by Yamazaki et al.

371   (Yamazaki, Hirata & Tsuyumu, 2008). However, a paralogous protein was also secreted only by

372   the mutant *ΔhrpB4* grown in NB medium (supplementary Table 1). The PPIases play a pivotal

373   role in catalysing the proper folding of many prokaryotic and eukaryotic proteins that are

374   implicated in a variety of biological functions ranging from cell cycle regulation to bacterial

375   infection (Pissavin & Hugouvieux-Cotte-Pattat, 1997; Zang et al., 2007). In *Pseudomonas*

376   *syringae* and *Xanthomonas*, proteins orthologous to PPIase were identified in the secretome

377   (Kaffarnik et al., 2009). In *Xylella fastidiosa*, which causes CVC, this protein is part of the

378   composition of mature biofilms (Silva et al., 2011), and is differentially expressed when *Xylella*

379   is subjected to thermal stress (Da Silva Neto et al., 2007).

380   The last protein with an annotated function secreted by both strains only when grown in

381   XAM1 medium is a proteinase (XAC0552). The gene encoding this protein has been identified

382   only in species that cause citrus canker; its genomic organization has been maintained in these

383   species. In addition to containing a peptidase_S8 domain in its carboxyl-terminal region, it

384   contains a Pro_kuma_activ domain (Pro-kumamolisin - PF09286.6) in its amino-terminal region,

385   whose cleavage results in the activation of the remaining peptide (Comellas-Bigler et al., 2004).

386   Although this domain has not been examined in the context of bacterial pathogen proteins, in the

387   phytopathogenic fungi *Mycosphaerella graminicola*, expansion of the number of peptidases

388   containing this domain may be associated with enhanced pathogenesis of wheat hosts (Goodwin

389   et al., 2011).

390   Hypothetical proteins identified in this condition are encoded by the genes: XAC0223,

391   XAC2562, XAC0677, and XAC0232. Of these, a protein worth noting is encoded by the

392   XAC0223 gene (Figure 3). This protein contains domains associated with the external membrane

393   and a porin domain (Park & Ronald, 2012; Bahar et al., 2014). Orthologues of this gene in *X.*

394   *campestris* pv. *raphani 756C* and *X. oryzae* pv. *oryzicola* have been reported to be activators of

395   XA21-mediated immunity. Ax21 is secreted by a type I secretion system and in association with

396   outer membrane vesicles (Bahar et al., 2014), and can act in a quorum-sensing system, motility,

397   biofilm formation and virulence (Han et al., 2011). In *X. oryzae* pv. *oryzicola*, Ax21 was

398   identified in the secretome, and deletion of the Ax21 gene resulted in reduced biofilm formation

399 and extracellular polysaccharide production (Qian et al., 2013). Although classically the rice
400 XA21 receptor directly recognizes Ax21 (Song et al., 1995), recent data have shown that other
401 receptors, such as by Fls2, which is associated with a response in the presence of flagellin in
402 *Arabidopsis*, can also recognize Ax21 secreted by *Xanthomonas* (Danna et al., 2011). This
403 assumes that there is a dynamic between pathogens and microbe-associated molecular patterns in
404 plants.

405   Although information about the functions of proteins encoded by the XAC2562,
406 XAC0677, and XAC0232 genes is limited, domains associated with the function of lipoproteins
407 were found in all of these proteins, which would justify their presence in the secretome.

408

409 **Proteins secreted only by the wild-type strain grown in XAM1 medium**
410 The data so far indicate that the production and secretion of important proteins associated with
411 virulence and adaptation still occur in the Δ*hrpB4* mutant. However, the impact of the mutation
412 to the *hrpB4* gene, which is associated with a loss of function of the T3SS system, is revealed by
413 the identification of 14 unique proteins secreted only by wild-type *Xac* when cultured in XAM1
414 medium. Of these, nine had an annotated function (OprO, VirK, ribosomal proteins RplW, RplR
415 and RpsE, HupB and cellulase Egl/Egl, EngXca), whereas the other five were hypothetical
416 proteins (XAC0678, XAC3605, XAC1497, XAC3380 and XAC2682). Ten of the total amount
417 of these proteins have a signal peptide sequence and only one hypothetical protein has a PIP-box
418 sequence upstream of the start of the gene (Table 2). Although lacking annotated functions, all of
419 these hypothetical proteins have a signal peptide sequence, suggesting that their secretion is
420 critical for certain biological processes. Among the proteins with annotated functions, five are
421 worth discussing further.

422   The OprO protein, encoded by the gene XAC3472, is an outer membrane porin specific
423 for polyphosphates that can be regulated by the two-component regulatory systems, PhoPQ and
424 PhoBR. In *Pseudomonas aeruginosa*, OprO is an anion-specific porin with a higher affinity for
425 pyrophosphates than orthophosphates, and was expressed only under energy-poor growth
426 conditions in phosphate during the stationary phase of bacterial growth (Siehnel, Egli &
427 Hancock, 1992). Importantly, XAM1 medium lacks phosphate, similar to the mesophyll of citrus
428 hosts. In recent work, Moreira and colleagues demonstrated that OprO, like other phosphate
429 internalization systems, is expressed in *Xac* only during infection periods (Moreira et al., 2014).

430   VirK, encoded by the gene XAC0435, is another protein secreted exclusively by the wild-
431   type strain when cultured in XAM1 medium. This protein has a signal peptide for its secretion
432   through the Sec pathway into the periplasmic space. In *E. coli*, VirK was characterized as a
433   periplasmic protein required for the efficient secretion of plasmid-encoded toxins (Tapia-
434   Pastrana et al., 2012). An orthologous gene found in *Ralstonia solanacearum* is regulated by
435   HrpG. Microarray studies of the *Xac* transcriptome demonstrated that the expression of VirK was
436   increased by more than two-fold when grown in XVM2 medium compared to when grown in
437   non-inducing energy-rich culture medium (Astua-monge et al., 2005). The same study reported
438   that a number of genes encoding secreted proteins were robustly expressed by XVM2 medium.
439   Among the enzymes involved in cell wall degradation is cellulase (Egl), which is encoded by the
440   genes XAC0029 and XAC0028. Both proteins were also secreted only by wild-type *Xac* in
441   XAM1 medium; this was also true for the cellulase, EngXca. These proteins are predicted to be
442   transported via the Sec pathway to the periplasm, and then secreted into the extracellular region
443   by T2SS. Their secretion only by wild-type bacterium indicates that inactivation of T3SS in the
444   mutant could suppress T2SS-secreted enzymes that degrade the cell wall of the host.
445   Furthermore, EngXca has been specifically reported to be controlled by *rpfF* in *Xanthomonas*
446   *campestris*. Poplawsky and colleagues (Poplawsky et al., 1998) and Siciliano and colleagues
447   (Siciliano et al., 2006) reported that *Xac* mutants, in which the *rpfF* and *rpfC* genes were
448   inactivated, exhibited significantly decreased production of cell wall degrading enzymes and
449   xanthan gum.

450   During the preparation of samples, the supernatant of the *ΔhrpB4* strain cultured in
451   XAM1 medium could be easily filtered through a 0.2 μm membrane, whereas the supernatant of
452   the wild-type strain cultured in XAM1 medium could not be easily filtered. This is likely because
453   the excess production of xanthan gum by wild-type bacteria obstructed the pores of the
454   membrane. According to our studies, wild-type *Xac* is able to produce nearly 10 times more
455   xanthan gum than the pathogenic deficient mutant *ΔhrpB4* (mg gum/mg bacteria). Whereas wild-
456   type *Xac* produces 46.21 ± 6.56A mg gum/mg bacteria, *ΔhrpB4* produces only 5.21 ± 1.38B mg
457   gum/mg bacteria (p value = 0.0004). These data underscore the importance of these genes to the
458   formation of biofilm, as well as the importance of the hrpB4 gene in maintaining the activities of
459   the T3SS and T2SS secretory systems.

460   The XAC0678 gene encodes a hypothetical protein but by sequence homology, it is
461   predicted to encode a lipoprotein membrane protein. This hypothetical protein has a consensus
462   PIP-box sequence upstream of the start of the gene, which indicates its probable regulation by
463   HrpX. Furthermore, this protein is predicted to have a signal peptide for the Sec pathway, which
464   could instruct its export into the periplasmic space.

465

466   **Proteins secreted only by the mutant *ΔhrpB4* grown in XAM1 medium**
467   Ten proteins were secreted only by the mutant strain grown in XAM1 medium, 9 of which had
468   annotated functions (Mlt, VacJ, OmpW, lei, GroES, YrbC, CycK, and PepN) and only one was
469   predicted to be a hypothetical protein (XAC1761). Seven proteins had a signal peptide sequence,
470   and a PIP-box sequence was not present upstream of any of these genes (Table 3).
471   Among these proteins, one worth noting is Mlt, which is a lytic murein transglycosylase.
472   *Xac* has two copies of the *mlt* gene, XAC3225 and XACb0007, which are located on the
473   chromosome and on the pXAC64 plasmid, respectively. The chromosomal copy of the gene is
474   flanked by genes that encode the T3SS, XopE3 and XopAI effector proteins and the plasmid
475   copy is flanked by the gene encoding XopE2 (Moreira et al., 2010). Mlt is highly similar to the
476   hopAJ1 protein of *Pseudomonas syringae*, which was previously annotated as a T3SS helper
477   protein (Oh et al., 2007). Mutations in the chromosomal copy reduced the ability of the mutant to
478   cause canker (Laia et al., 2009).
479   The proteins encoded by the genes XAC3664 and XAC1479 are predicted to be outer
480   membrane proteins. Although previously annotated as a hypothetical protein, the protein
481   encoded by the gene, XAC1761, is predicted to be a lipoprotein based on its homology with
482   sequences from other organisms.

483

484   **Proteins secreted only by the wild-type strain grown in NB medium**
485   Only 4 proteins were secreted by the wild-type strain grown in NB medium. Of these, 3
486   are directly associated with motility: PilY1, which is a Type IV pilus assembly protein, FliC
487   (cover filament) and FliD (filament), which are structural proteins of the flagellum (Table 4). In
488   addition, a hypothetical protein encoded by the gene XAC3314 was also secreted under these
489   conditions. Of these four proteins, only PilY1 has a signal peptide sequence and a PIP-box
490   sequence was not present upstream of any of these genes. PilY1 contains a DUF239 domain;

491   these are found in a family of plant and bacterial proteins (PF03010). In addition, orthologous

492   proteins were only found in Xac29, XacW, and Xcv, in Xoo MAFF and Xoo PXO, a protein

493   similar to transposase. Mutant *Pseudomonas* strains, in which the *pilY1* gene was inactivated,

494   exhibited robust suppression of the swarming motility (Kuchma et al., 2010). In *Xylella*

495   *fastidiosa,* a mutation in the *pilY1* gene led to a reduction, but not a complete loss, of type IV

496   pilus apparatus assembly and twitching motility (Li et al., 2007). In Xoo, *pilY1*, in combination

497   with other genes involved in adhesion and biofilm formation, was upregulated six days after

498   infection in the plant (Soto-Suárez et al., 2010).

499   FliC and FliD are protein subunits that form the structure of flagella. FliC, also known as

500   flagellin, forms the flagellar filament itself, and is therefore abundantly synthesized by

501   flagellated bacteria. FliC is directly associated with the innate immune response of plants, since

502   it has the ability to bind to specific receptors, such as Toll-like receptor 5 (TLR5) (Hayashi et al.,

503   2001). More recently, part of the N-terminal portion of this protein, corresponding to 22 amino

504   acids (flg22) and conserved across a wide range of pathogens, was shown to activate plant

505   defence mechanisms (Navarro et al., 2004). This 22-amino acid protein fragment is recognized

506   by specific receptors such as by the kinase FLS2, which in turn activate the MAPK signalling

507   cascade (mitogen-activated protein kinase), which is capable of regulating the expression of over

508   900 genes in the *Arabidopsis thaliana* genome (Asai et al., 2002).

509

510   **Protein secreted only by the wild-type strain in both growth media**

511   The only protein detected exclusively in the secretome of the wild-type strain when grown in

512   either growth media was the EF-Tu protein encoded by the gene XAC0957 (Supplementary

513   Table 1).

514   EF-Tu is also characterized as a PAMP, like FliC (Nürnberger & Kemmerling, 2006).

515   Although EF-Tu has a fundamental role in protein synthesis, a diversity of other functions have

516   been attributed to this protein. In *Burkholderia pseudomallei,* EF-Tu was characterized as a

517   membrane-associated protein that is secreted by the formation of outer membrane vesicles

518   (OMV), and is therefore an important target in the development of vaccines against this pathogen

519   (Nieves et al., 2010). In *E. coli*, EF-Tu acts as a chaperone molecule by binding to denatured

520   proteins after stress conditions (Caldas, El Yaagoubi & Richarme, 1998). In *Agrobacterium*

521   *tumefaciens*, EF-Tu can act as an inducer in *Arabidopsis*, whose specific receptor is the EFR

522 protein (Zipfel et al., 2006). EF-Tu has also been reported to be critical for biofilm formation in

523 *Xylella* (Silva et al., 2011) and, more recently, in *Xac* (Zimaro et al., 2013). Considering all of

524 these functions attributed to this protein and the inability of the *ΔhrpB4* mutant to secrete this

525 protein under all conditions tested, we hypothesize that the secretion of this protein may be

526 associated with T3SS in *Xanthomonas*. Besides its participation in the production of biofilms,

527 EF-Tu may have another key role in the virulence process, and may represent a potential

528 moonlighting protein (Jeffery, 1999; Henderson & Martin, 2011).

529

530 **All other secreted proteins detected in our study**

531 In addition to the proteins described above, 13 other secreted proteins were detected in this study

532 (Supplementary Table 1). Because these proteins were detected in specific but restricted

533 conditions, we could not exclusively associate them with a specific growth media or specific

534 bacterial strain. The majority (8) of these have enzymatic functions (amylase, proteases, and

535 isomerase). Two membrane-associated proteins (OMPs), and two hypothetical proteins,

536 XAC0753 and XAC0868, were also identified. The latter two proteins have domains with

537 peptidase activity, although XAC0868 has a more representative ricin B lectin domain.

538 Interestingly, the protein encoded by XAC0868 was the only protein detected in all four

539 conditions tested and exists only in *Xanthomonas* strains that infect citrus plants. A model

540 highlighting proteins related to Xac adaptation was devised (Figure 4).

541

542 **Pathogenicity of *Xac* compared to other plant pathogens**

543 To better understand the importance of secreted proteins in the virulence of plant-specific

544 pathogens of the *Xanthomonadaceae* family, a comparative analysis of proteins identified in this

545 study with proteins identified in six other proteomic studies was performed (Table 5). Despite

546 the diversity of model organisms studied and methodologies used, orthologous proteins were

547 identified in these studies.

548   Yamazaki et al. (Yamazaki, Hirata & Tsuyumu, 2008) studied the role of the HrpG gene

549 in the regulation of the synthesis of *Xac* proteins secreted by T2SS in energy-rich culture

550 medium. In these studies, a total of 11 proteins were identified, six of which were extracellular

551 enzymes. Zimaro and colleagues compared the proteome of planktonic *Xac* cells and *Xac* biofilm

552 cells, and identified 53 differentially expressed proteins, of which 31 were upregulated in

553   biofilms. Silva and colleagues identified 456 proteins potentially related to biofilm formation in
554   *Xylella fastidiosa* (Silva et al., 2011). Wang and colleagues analysed the *Xanthomonas oryzae*
555   pv. *oryzae* secretome grown in culture medium and in plants, and identified 109 unique proteins.
556   Proteins involved in pathogenicity and the induction of host defence were identified only in the
557   secretome of bacteria in contact with the plant (Yamazaki, Hirata & Tsuyumu, 2008). Using the
558   same model study, Gonzalez and colleagues identified 324 different proteins secreted by Xoo in
559   contact with the xylem of the host plant. Of these, 64 were suggested to be associated with the
560   process of virulence (González et al., 2012). Sidhu and colleagues (Sidhu et al., 2008) identified
561   31 proteins associated with OMV, half of which were associated with the induction of virulence
562   in *Xanthomonas campestris* pv. *campestris*.

563   This comparative analysis revealed the correlation of a number of proteins with the life
564   cycle or the infection model of the associated pathogen. For instance, endopolygalacturonase and
565   proteinases, which seem to be crucial to the virulence process, are induced by mesophilic strains.
566   Proteins associated with motility and chemotaxis have been identified in virtually every
567   proteomics study included in our analysis, which is consistent with the importance of these
568   proteins in the formation of cellular aggregates, biofilms or even for inducing a response in the
569   host plant (Malamud et al., 2011; Dunger et al., 2014). Mlt was detected only in this study,
570   indicating its importance to the induction of virulence in *Xac*. Interestingly, EF-Tu was
571   independently identified in five other studies, including in the Xf strain, whereas GroEL was
572   detected in four other studies, and flagellin (FliC), VirK and peptidyl-prolyl cis-trans isomerase
573   (PPI) were identified in three other studies. It is important to note that FliC is absent in the
574   *Xylella* genome since it does not have flagellum. The possibility that GroEL, FliC, Ef-Tu and PPI
575   can act as moonlighting proteins in bacteria of the family Xanthomonadaceae is highlighted
576   (Henderson & Martin, 2011).

577

578   **Conclusions**
579   Our results indicate that the secretome analysis is a valuable approach to highlight proteins
580   potentially involved with the virulence in *Xanthomonas*. Fifty-five unique proteins were
581   identified in total on the secretomes of wild-type *Xac* and the mutant *ΔhrpB* cultured in different
582   growth media. Of these, 37 have a predicted signal peptide for secretion via the general secretory
583   pathway and are potentially related to the pathogenicity and adaptation during infections and

584 plant-pathogen interactions. However, of the 18 proteins lacking a signal peptide, 13 were
585 predicted to be secreted by non-classical routes. This demonstrates that the majority (50) of
586 proteins detected in this study were neither contaminant proteins nor proteins released by cell
587 lysis. Interestingly, the percentage of secreted proteins related to the pathogenicity, virulence and
588 adaptation increased nearly two-fold (from 15 to 29%) when considering only proteins secreted
589 by wild-type *Xac* bacterium compared to those secreted by its mutant *ΔhrpB4*. These results
590 corroborate that the *hrpB4* gene act as T3SS central player, impacting the emergence of
591 pathogenicity in *Xanthomonas*. For instance, the expression/secretion of several T2SS proteins
592 appears to be regulated by T3SS activity. Our results indicate that this regulation also occurs in
593 *Xac*. The profile of proteins secreted exclusively by wild-type *Xac* and the increased amount of
594 xanthan gum produced in XAM1 medium suggests that DSFs (diffusible signalling factors)
595 responsible for controlling these factors may be related to the lack of expression of the HrpB4
596 protein, since the *ΔhrpB4* mutant, which is deficient in the production of this protein, was unable
597 to secrete several of these factors. Further analysis are necessary to confirm that hypothesis.

598   Overall, the secretome method allows the testing of not only one, but several mutants at a
599 time. This may be a powerful tool to shed light into the biology of *Xanthomonas*. Indeed,
600 analyses are underway with different mutants generated under different mutagenic
601 methodologies to evaluate others gene targets that might be related to the disease.

602

603 **Acknowledgements**
604 We thank all the members of the Laboratory of Molecular Biology of the Department of
605 Technology (UNESP - Universidade Estadual Paulista – Jaboticabal). We would also like to
606 thank Brazilian Synchrotron Light Laboratory.

607

608 **References**
609

610 Alexander Watt S., Wilke A., Patschkowski T., Niehaus K. 2005. Comprehensive analysis of the
611   extracellular proteins from Xanthomonas campestris pv. campestris B100. *Proteomics*
612   5:153–167.

613 Asai T., Tena G., Plotnikova J., Willmann MR., Chiu W-L., Gomez-Gomez L., Boller T.,
614   Ausubel FM., Sheen J. 2002. MAP kinase signalling cascade in Arabidopsis innate
615   immunity. *Nature* 415:977–983.

616 Astua-monge G., Freitas-astua J., Roncoletta J., Carvalho S a., Machado M a., Bacocina G. 2005.
617   Expression Profiling of Virulence and Pathogenicity Genes of Xanthomonas axonopodis pv
618   . citri Expression Profiling of Virulence and Pathogenicity Genes of Xanthomonas
619   axonopodis pv . citri. 187:1201–1205.

620 Axtell MJ., Chisholm ST., Dahlbeck D., Staskawicz BJ. 2003. Genetic and molecular evidence
621   that the Pseudomonas syringae type III effector protein AvrRpt2 is a cysteine protease.
622   *Molecular Microbiology* 49:1537–1546.

623 Bahar O., Pruitt R., Luu DD., Schwessinger B., Daudi a., Liu F., Ruan R., Fontaine-Bodin L.,
624   Koebnik R., Ronald P. 2014. The Xanthomonas Ax21 protein is processed by the general
625   secretory system and is secreted in association with outer membrane vesicles. *PeerJ* 2:e242.

626 Bekal S., Niblack TL., Lambert KN. 2003. A chorismate mutase from the soybean cyst nematode
627   Heterodera glycines shows polymorphisms that correlate with virulence. *Molecular plant-*
628   *microbe interactions : MPMI* 16:439–446.

629 Bendtsen JD., Kiemer L., Fausbøll A., Brunak S. 2005. Non-classical protein secretion in
630   bacteria. *BMC microbiology* 5:58.

631 Büttner D., Nennstiel D., Klüsener B., Bonas U. 2002. Functional Analysis of HrpF , a Putative
632   Type III Translocon Protein from Xanthomonas campestris pv . vesicatoria Functional
633   Analysis of HrpF , a Putative Type III Translocon Protein from Xanthomonas campestris pv
634   . vesicatoria. *Jour* 184:2389–2398.

635 Büttner D., He SY. 2009. Type III Protein Secretion in Plant Pathogenic Bacteria. *Plant*
636   *Physiology* 150:1656–1664.

637 Caldas TD., El Yaagoubi A., Richarme G. 1998. Chaperone properties of bacterial elongation
638   factor EF-Tu. *Journal of Biological Chemistry* 273:11478–11482.

639 Comellas-Bigler M., Maskos K., Huber R., Oyama H., Oda K., Bode W. 2004. 1.2 Å crystal
640   structure of the serine carboxyl proteinase pro-kumamolisin: Structure of an intact pro-
641   subtilase. *Structure* 12:1313–1323.

642 Cui F., Wu S., Sun W., Coaker G., Kunkel B., He P., Shan L. 2013. The Pseudomonas syringae
643   type III effector AvrRpt2 promotes pathogen virulence via stimulating Arabidopsis
644   auxin/indole acetic acid protein turnover. *Plant physiology* 162:1018–29.

645 Danna CH., Millet Y a., Koller T., Han S-W., Bent AF., Ronald PC., Ausubel FM. 2011. The
646   Arabidopsis flagellin receptor FLS2 mediates the perception of Xanthomonas Ax21
647   secreted peptides. *Proceedings of the National Academy of Sciences of the United States of*
648   *America* 108:9286–9291.

649 Degrassi G., Devescovi G., Bigirimana J., Venturi V. 2010. Xanthomonas oryzae pv. oryzae

650   XKK.12 contains an AroQgamma chorismate mutase that is involved in rice virulence.
651   *Phytopathology* 100:262–270.

652   Deng W., De Hoog CL., Yu HB., Li Y., Croxen M a., Thomas N a., Puente JL., Foster LJ.,
653   Finlay BB. 2010. A comprehensive proteomic analysis of the type III secretome of
654   Citrobacter rodentium. *Journal of Biological Chemistry* 285:6790–6800.

655   Djamei A., Kahmann R. 2012. Ustilago maydis: Dissecting the Molecular Interface between
656   Pathogen and Plant. *PLoS Pathogens* 8:11–14.

657   Dow JM., Feng JX., Barber CE., Tang JL., Daniels MJ. 2000. Novel genes involved in the
658   regulation of pathogenicity factor production within the rpf gene cluster of Xanthomonas
659   campestris. *Microbiology (Reading, England)* 146 (Pt 4:885–91.

660   Dow JM., Crossman L., Findlay K., He Y-Q., Feng J-X., Tang J-L. 2003. Biofilm dispersal in
661   Xanthomonas campestris is controlled by cell-cell signaling and is required for full
662   virulence to plants. *Proceedings of the National Academy of Sciences of the United States of
663   America* 100:10995–11000.

664   Dunger G., Guzzo CR., Andrade MO., Jones JB., Farah CS. 2014. Xanthomonas citri subsp. citri
665   type IV Pilus is required for twitching motility, biofilm development, and adherence.
666   *Molecular plant-microbe interactions : MPMI* 27:1132–47.

667   Evans FF., Raftery MJ., Egan S., Kjelleberg S. 2007. Profiling the secretome of the marine
668   bacterium Pseudoalteromonas tunicata using amine-specific isobaric tagging (iTRAQ).
669   *Journal of Proteome Research* 6:967–975.

670   Facincani AP., Moreira LM., Soares MR., Ferreira CB., Ferreira RM., Ferro MIT., Ferro J a.,
671   Gozzo FC., De Oliveira JCF. 2014. Comparative proteomic analysis reveals that T3SS, Tfp,
672   and xanthan gum are key factors in initial stages of Citrus sinensis infection by
673   Xanthomonas citri subsp. citri. *Functional and Integrative Genomics* 14:205–217.

674   Furutani A., Tsuge S., Ohnishi K., Hikichi Y., Oku T., Tsuno K., Inoue Y., Ochiai H., Kaku H.,
675   Kubo Y. 2004. Evidence for HrpXo-Dependent Expression of Type II Secretory Proteins in
676   Xanthomonas oryzae pv . oryzae. *Society* 186:1374–1380.

677   González JF., Degrassi G., Devescovi G., De Vleesschauwer D., Höfte M., Myers MP., Venturi
678   V. 2012. A proteomic study of Xanthomonas oryzae pv. oryzae in rice xylem sap. *Journal
679   of Proteomics* 75:5911–5919.

680   Goodwin SB., M'Barek S Ben., Dhillon B., Wittenberg AHJ., Crane CF., Hane JK., Foster AJ.,
681   van der Lee T a J., Grimwood J., Aerts A., Antoniw J., Bailey A., Bluhm B., Bowler J.,
682   Bristow J., van der Burgt A., Canto-Canché B., Churchill ACL., Conde-Ferràez L., Cools
683   HJ., Coutinho PM., Csukai M., Dehal P., de Wit P., Donzelli B., van de Geest HC., van
684   Ham RCHJ., Hammond-Kosack KE., Henrissat B., Kilian A., Kobayashi AK., Koopmann

685   E., Kourmpetis Y., Kuzniar A., Lindquist E., Lombard V., Maliepaard C., Martins N.,
686   Mehrabi R., Nap JPH., Ponomarenko A., Rudd JJ., Salamov A., Schmutz J., Schouten HJ.,
687   Shapiro H., Stergiopoulos I., Torriani SFF., Tu H., de Vries RP., Waalwijk C., Ware SB.,
688   Wiebenga A., Zwiers LH., Oliver RP., Grigoriev I V., Kema GHJ. 2011. Finished genome
689   of the fungal wheat pathogen Mycosphaerella graminicola reveals dispensome structure,
690   chromosome plasticity, and stealth pathogenesis. *PLoS Genetics* 7.

691 Guo Y., Figueiredo F., Jones J., Wang N. 2011. HrpG and HrpX play global roles in
692   coordinating different virulence traits of Xanthomonas axonopodis pv. citri. *Molecular*
693   *plant-microbe interactions : MPMI* 24:649–661.

694 Han SW., Sriariyanun M., Lee SW., Sharma M., Bahar O., Bower Z., Ronald PC. 2011. Small
695   protein-mediated quorum sensing in a Gram-negative bacterium. *PLoS ONE* 6.

696 Hayashi F., Smith KD., Ozinsky A., Hawn TR., Yi EC., Goodlett DR., Eng JK., Akira S.,
697   Underhill DM., Aderem A. 2001. The innate immune response to bacterial flagellin is
698   mediated by Toll-like receptor 5. *Nature* 410:1099–1103.

699 Henderson B., Martin A. 2011. Bacterial virulence in the moonlight: Multitasking bacterial
700   moonlighting proteins are virulence determinants in infectious disease. *Infection and*
701   *Immunity* 79:3476–3491.

702 Hiller K., Grote A., Scheer M., Münch R., Jahn D. 2004. PrediSi: Prediction of signal peptides
703   and their cleavage positions. *Nucleic Acids Research* 32:375–379.

704 Hirose I., Sano K., Shioda I., Kumano M., Nakamura K., Yamane K. 2000. Proteome analysis of
705   Bacillus subtilis extracellular proteins: A two-dimensional protein electrophoretic study.
706   *Microbiology* 146:65–75.

707 Jeffery CJ. 1999. Moonlighting proteins. *Trends in Biochemical Sciences* 24:8–11.

708 Kaffarnik F a R., Jones AME., Rathjen JP., Peck SC. 2009. Effector proteins of the bacterial
709   pathogen Pseudomonas syringae alter the extracellular proteome of the host plant,
710   Arabidopsis thaliana. *Molecular & cellular proteomics : MCP* 8:145–156.

711 Kamoun S., Young M., Glasscock C., Tyler BM. 1993. Extracellular Protein Elicitors from
712   Phytophthora: Host-Specificity and Induction of Resistance to Bacterial and Fungal
713   Phytopathogens. *Molecular plant-microbe interactions : MPMI* 6:15–25.

714 Kast P., Grisostomi C., Chen I a., Li S., Krengel U., Xue Y., Hilvert D. 2000. A strategically
715   positioned cation is crucial for efficient catalysis by chorismate mutase. *Journal of*
716   *Biological Chemistry* 275:36832–36838.

717 Katzen F., Ferreiro DU., Oddo CG., Ielmini MV., Becker A., Pühler A., Ielpi L. 1998.
718   Xanthomonas campestris pv. campestris gum mutants: Effects on xanthan biosynthesis and

719   plant virulence. *Journal of Bacteriology* 180:1607–1617.

720   Kazemi-Pour N., Condemine G., Hugouvieux-Cotte-Pattat N. 2004. The secretome of the plant
721   pathogenic bacterium Erwinia chrysanthemi. *Proteomics* 4:3177–3186.

722   Kuchma SL., Ballok a. E., Merritt JH., Hammond JH., Lu W., Rabinowitz JD., O'Toole G
723   a. 2010. Cyclic-di-GMP-mediated repression of swarming motility by Pseudomonas
724   aeruginosa: The pilY1 gene and its impact on surface-associated behaviors. *Journal of*
725   *Bacteriology* 192:2950–2964.

726   Laia ML., Moreira LM., Dezajacomo J., Brigati JB., Ferreira CB., Ferro MIT., Silva ACR., Ferro
727   J a., Oliveira JCF. 2009a. New genes of Xanthomonas citri subsp. citri involved in
728   pathogenesis and adaptation revealed by a transposon-based mutant library. *BMC*
729   *microbiology* 9:12.

730   Li Y., Hao G., Galvani CD., Meng Y., De La Fuente L., Hoch HC., Burr TJ. 2007. Type I and
731   type IV pili of Xylella fastidiosa affect twitching motility, biofilm formation and cell-cell
732   aggregation. *Microbiology* 153:719–726.

733   Liu Y., Chatterjee a., Chatterjee a K. 1994. Nucleotide sequence and expression of a novel
734   pectate lyase gene (pel- 3) and a closely linked endopolygalacturonase gene (peh-1) of
735   Erwinia carotovora subsp. carotovora 71. *Applied and Environmental Microbiology*
736   60:2545–2552.

737   Malamud F., Torres PS., Roeschlin R., Rigano L a., Enrique R., Bonomi HR., Castagnaro AP.,
738   Marano MR., Vojnov A a. 2011. The Xanthomonas axonopodis pv. citri flagellum is
739   required for mature biofilm and canker development. *Microbiology* 157:819–829.

740   Moreira LM., Almeida Jr NF., Potnis N., Digiampietri LA., Adi SS., Bortolossi JC., da Silva
741   AC., da Silva AM., de Moraes FE., de Oliveira JC., de Souza RF., Facincani AP., Ferraz
742   AL., Ferro MI., Furlan LR., Gimenez DF., Jones JB., Kitajima EW., Laia ML., Leite Jr
743   RP., Nishiyama MY., Rodrigues Neto J., Nociti LA., Norman DJ., Ostroski EH., Pereira Jr
744   HA., Staskawicz BJ., Tezza RI., Ferro JA., Vinatzer BA., Setubal JC. 2010. Novel insights
745   into the genomic basis of citrus canker based on the genome sequences of two strains of
746   Xanthomonas fuscans subsp. aurantifolii. *BMC Genomics* 11.

747   Moreira LM., Facincani AP., Ferreira CB., Ferreira RM., Ferro MIT., Gozzo FC., de Oliveira
748   JCF., Ferro JA., Soares MR. 2014. Chemotactic signal transduction and phosphate
749   metabolism as adaptive strategies during citrus canker induction by Xanthomonas citri.
750   *Functional & Integrative Genomics*.

751   Navarro L., Navarro L., Zipfel C., Zipfel C., Rowland O., Rowland O., Keller I., Keller I.,
752   Robatzek S., Robatzek S., Boller T., Boller T., Jones JDG., Jones JDG. 2004. The
753   Transcriptional Innate Immune Response to g22. Interplay and Overlap with Avr Gene-

754   Dependent Defense Responses and Bacterial Pathogenesis. *Plant Physiology* 135:1113–
755   1128.

756 Nieves W., Heang J., Asakrah S., zu Bentrup KH., Roy CJ., Morici L a. 2010. Immunospecific
757   Responses to Bacterial Elongation Factor Tu during Burkholderia Infection and
758   Immunization. *PLoS ONE* 5:1–12.

759 Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited.
760   *Microbiology and molecular biology reviews : MMBR* 67:593–656.

761 Nissinen RM., Ytterberg a. J., Bogdanove AJ., Van Wijk KJ., Beer S V. 2007. Analyses of the
762   secretomes of Erwinia amylovora and selected hrp mutants reveal novel type III secreted
763   proteins and an effect of HrpJ on extracellular harpin levels. *Molecular Plant Pathology*
764   8:55–67.

765 Nürnberger T., Kemmerling B. 2006. Receptor protein kinases--pattern recognition receptors in
766   plant immunity. *Trends in plant science* 11:519–522.

767 Ogata H., Goto S., Sato K., Fujibuchi W., Bono H., Kanehisa M. 1999. KEGG: Kyoto
768   encyclopedia of genes and genomes. *Nucleic Acids Research* 27:29–34.

769 Oh H-S., Kvitko BH., Morello JE., Collmer A. 2007. Pseudomonas syringae lytic
770   transglycosylases coregulated with the type III secretion system contribute to the
771   translocation of effector proteins into plant cells. *Journal of bacteriology* 189:8277–89.

772 Otto K., Norbeck J., Larsson T., Karlsson K a., Hermansson M. 2001. Adhesion of type 1-
773   fimbriated escherichia coli to abiotic surfaces leads to altered composition of outer
774   membrane proteins. *Journal of Bacteriology* 183:2445–2453.

775 Park C-J., Ronald PC. 2012. Cleavage and nuclear localization of the rice XA21 immune
776   receptor. *Nature Communications* 3:920.

777 Perez JC., Shin D., Zwir I., Latifi T., Hadley TJ., Groisman E a. 2009. Evolution of a bacterial
778   regulon controlling virulence and mg2+ homeostasis. *PLoS Genetics* 5.

779 Pickering BS., Yudistira H., Oresnik IJ. 2012. Characterization of the twin-arginine transport
780   secretome in Sinorhizobium meliloti and evidence for host-dependent phenotypes. *Applied*
781   *and Environmental Microbiology* 78:7141–7144.

782 Pissavin C., Hugouvieux-Cotte-Pattat N. 1997. Characterization of a periplasmic peptidyl-prolyl
783   cis-trans isomerase in Erwinia chrysanthemi. *FEMS Microbiology Letters* 157:59–65.

784 Poplawsky AR., Chun W., Slater H., Daniels MJ., Dow JM. 1998. Synthesis of Extracellular
785   Polysaccharide , Extracellular Enzymes , and Xanthomonadin in Xanthomonas campestris :
786   Evidence for the Involvement of Two Intercellular Regulatory Signals. *Molecular Paint-*
787   *Microbe Interactions* 11:68–70.

788 Qian G., Zhou Y., Zhao Y., Song Z., Wang S., Fan J., Hu B., Venturi V., Liu F. 2013. Proteomic
789   analysis reveals novel extracellular virulence-associated proteins and functions regulated by
790   the diffusible signal factor (DSF) in Xanthomonas oryzae pv. oryzicola. *Journal of*
791   *Proteome Research* 12:3327–3341.

792 Rigano L a., Siciliano F., Enrique R., Sendín L., Filippone P., Torres PS., Qüesta J., Dow JM.,
793   Castagnaro AP., Vojnov A a., Marano MR. 2007. Biofilm formation, epiphytic fitness, and
794   canker development in Xanthomonas axonopodis pv. citri. *Molecular plant-microbe*
795   *interactions : MPMI* 20:1222–1230.

796 Rodríguez-Herva JJ., Ramos-González MI., Ramos JL. 1996. The Pseudomonas putida
797   peptidoglycan-associated outer membrane lipoprotein is involved in maintenance of the
798   integrity of the cell envelope. *Journal of Bacteriology* 178:1699–1706.

799 Saier MH. 2006. Protein Secretion Systems in Gram-negative Bacteria. *Microbe* 1:414–419.

800 Schumacher J., Waite CJ., Bennett MH., Perez MF., Shethi K., Buck M. 2014. Differential
801   secretome analysis of Pseudomonas syringae pv tomato using gel-free MS proteomics.
802   *Frontiers in plant science* 5:242.

803 Shu CH., Yang ST. 1990. Effects of temperature on cell growth and xanthan production in batch
804   cultures of Xanthomonas campestris. *Biotechnology and bioengineering* 35:454–468.

805 Siciliano F., Torres P., Sendín L., Bermejo C., Filippone P., Vellice G., Ramallo J., Castagnaro
806   A., Vojnov A., Marano MR. 2006. Analysis of the molecular basis of Xanthomonas
807   axonopodis pv. citri pathogenesis in Citrus limon. *Electronic Journal of Biotechnology*
808   9:199–204.

809 Sidhu VK., Vorhölter F-J., Niehaus K., Watt S a. 2008. Analysis of outer membrane vesicle
810   associated proteins isolated from the plant pathogenic bacterium Xanthomonas campestris
811   pv. campestris. *BMC microbiology* 8:87.

812 Siehnel RJ., Egli C., Hancock RE. 1992. Polyphosphate-selective porin OprO of Pseudomonas
813   aeruginosa: expression, purification and sequence. *Molecular microbiology* 6:2319–2326.

814 Sikora AE., Zielke R a., Lawrence D a., Andrews PC., Sandkvist M. 2011. Proteomic analysis of
815   the Vibrio cholerae type II secretome reveals new proteins, including three related serine
816   proteases. *Journal of Biological Chemistry* 286:16555–16566.

817 Silva MS., De Souza A a., Takita M a., Labate C a., Machado M a. 2011. Analysis of the biofilm
818   proteome of Xylella fastidiosa. *Proteome Science* 9:58.

819 da Silva ACR., Ferro JA., Reinach FC., Farah CS., Furlan LR., Quaggio RB., Monteiro-Vitorello
820   CB., Van Sluys MA., Almeida NF., Alves LMC., do Amaral AM., Bertolini MC., Camargo
821   LEA., Camarotte G., Cannavan F., Cardozo J., Chambergo F., Ciapina LP., Cicarelli RMB.,

822   Coutinho LL., Cursino-Santos JR., El-Dorry H., Faria JB., Ferreira AJS., Ferreira RCC.,
823   Ferro MIT., Formighieri EF., Franco MC., Greggio CC., Gruber A., Katsuyama AM., Kishi
824   LT., Leite RP., Lemos EGM., Lemos MVF., Locali EC., Machado MA., Madeira AMBN.,
825   Martinez-Rossi NM., Martins EC., Meidanis J., Menck CFM., Miyaki CY., Moon DH.,
826   Moreira LM., Novo MTM., Okura VK., Oliveira MC., Oliveira VR., Pereira HA., Rossi A.,
827   Sena JAD., Silva C., de Souza RF., Spinola LAF., Takita MA., Tamura RE., Teixeira EC.,
828   Tezza RID., Trindade dos Santos M., Truffi D., Tsai SM., White FF., Setubal JC., Kitajima
829   JP. 2002. Comparison of the genomes of two Xanthomonas pathogens with differing host
830   specificities. *Nature* 417:459–463.

831   Da Silva Neto JF., Koide T., Gomes SL., Marques M V. 2007. The single extracytoplasmic-
832   function sigma factor of Xylella fastidiosa is involved in the heat shock response and
833   presents an unusual regulatory mechanism. *Journal of Bacteriology* 189:551–560.

834   Soares MR., Facincani AP., Ferreira RM., Moreira LM., de Oliveira JC., Ferro JA., Ferro MI.,
835   Meneghini R., Gozzo FC. 2010. Proteome of the phytopathogen Xanthomonas citri subsp.
836   citri: a global expression profile. *Proteome science* 8.

837   Soares-Costa a., Silveira RS., Novo MT., Alves MF., Carmona a. K., Belasque J., Henrique-
838   Silva F. 2012. Recombinant expression and characterization of a cysteine peptidase from
839   Xanthomonas citri subsp citri. *Genetics and molecular research : GMR* 11:4043–4057.

840   Song W., Wang G., Chen L., Kim H., Pi L., Hoisten T., Gardner J., Wang B., Zhai W., Zhu L.,
841   Fauquet C., Ronaldt P., Disas R., Gene R. 1995. A Receptor Kinase-Like Protein Encoded.
842   *Science*:2–4.

843   Soto-Suárez M., Bernal D., González C., Szurek B., Guyot R., Tohme J., Verdier V. 2010. In
844   planta gene expression analysis of Xanthomonas oryzae pathovar oryzae, African strain
845   MAI1. *BMC microbiology* 10:170.

846   De Souza A a., Takita M a., Coletta-Filho HD., Caldana C., Yanai GM., Muto NH., De Oliveira
847   RC., Nunes LR., Machado M a. 2004. Gene expression profile of the plant pathogen Xylella
848   fastidiosa during biofilm formation in vitro. *FEMS Microbiology Letters* 237:341–353.

849   Tapia-Pastrana G., Chavez-Dueñas L., Lanz-Mendoz H., Teter K., Navarro-García F. 2012. VirK
850   is a periplasmic protein required for efficient secretion of plasmid-encoded toxin from
851   enteroaggregative Escherichia coli. *Infection and Immunity* 80:2276–2285.

852   Tseng T-T., Tyler BM., Setubal JC. 2009. Protein secretion systems in bacterial-host
853   associations, and their description in the Gene Ontology. *BMC microbiology* 9 Suppl 1:S2.

854   Villeth GR., Reis FB., Tonietto A., Huergo L., De Souza EM., Pedrosa FO., Franco OL., Mehta
855   A. 2009. Comparative proteome analysis of Xanthomonas campestris pv. campestris in the
856   interaction with the susceptible and the resistant cultivars of Brassica oleracea. *FEMS*

857   *Microbiology Letters* 298:260–266.

858 Wang Y., Kim SG., Wu J., Huh HH., Lee SJ., Rakwal R., Kumar Agrawal G., Park ZY., Young
859   Kang K., Kim ST. 2013. Secretome analysis of the rice bacterium Xanthomonas oryzae
860   (Xoo) using in vitro and in planta systems. *Proteomics* 13:1901–1912.

861 Wang L., Rong W., He C. 2008. Two Xanthomonas extracellular polygalacturonases, PghAxc
862   and PghBxc, are regulated by type III secretion regulators HrpX and HrpG and are required
863   for virulence. *Molecular plant-microbe interactions : MPMI* 21:555–563.

864 Weaver LM., Herrmann KM. 1997. Dynamics of the shikimate pathway in plants. *Trends in
865   Plant Science* 2:346–351.

866 Wengelnik K, Van den Ackerveken G and BU. 1996. HrpG, a Key hrp Regulator Protein of
867   Xanthomonas campestris pv. vesicatoria is Homologous to Two-Component Response
868   Regulators. :704–712.

869 Wessel AK., Liew J., Kwon T., Marcotte EM., Whiteley M. 2013. Role of Pseudomonas
870   aeruginosa peptidoglycan-associated outer membrane proteins in vesicle formation. *Journal
871   of Bacteriology* 195:213–219.

872 Wylie JL., Worobec E a. 1995. The OprB porin plays a central role in carbohydrate uptake in
873   Pseudomonas aeruginosa. *Journal of Bacteriology* 177:3021–3026.

874 Xu S., Luo J., Pan X., Liang X., Wu J., Zheng W., Chen C., Hou Y., Ma H., Zhou M. 2013.
875   Proteome analysis of the plant-pathogenic bacterium Xanthomonas oryzae pv. oryzae.
876   *Biochimica et biophysica acta* 1834:1660–70.

877 Yamazaki A., Hirata H., Tsuyumu S. 2008. HrpG regulates type II secretory proteins in
878   Xanthomonas axonopodis pv. citri. *Journal of General Plant Pathology* 74:138–150.

879 Zang N., Tang D-J., Wei M-L., He Y-Q., Chen B., Feng J-X., Xu J., Gan Y-Q., Jiang B-L., Tang
880   J-L. 2007. Requirement of a mip-like gene for virulence in the phytopathogenic bacterium
881   Xanthomonas campestris pv. campestris. *Molecular plant-microbe interactions : MPMI*
882   20:21–30.

883 Zijnge V., Kieselbach T., Oscarsson J. 2012. Proteomics of protein secretion by aggregatibacter
884   actinomycetemcomitans. *PLoS ONE* 7.

885 Zimaro T., Thomas L., Marondedze C., Garavaglia BS., Gehring C., Ottado J., Gottig N. 2013.
886   Insights into xanthomonas axonopodis pv. citri biofilm through proteomics. *BMC
887   microbiology* 13:186.

888 Zipfel C., Kunze G., Chinchilla D., Caniard A., Jones JDG., Boller T., Felix G. 2006. Perception
889   of the Bacterial PAMP EF-Tu by the Receptor EFR Restricts Agrobacterium-Mediated
890   Transformation. *Cell* 125:749–760.

891

892

893 **Figure Legends**

894

895 **Figure 1: (A)** Cluster of *hrp* genes in the *Xac* genome, highlighting the mutation of the *hrpB4*

896 gene induced by insertion of the transposition cassette EZ-Tn5 <Kan>. The insertion was made

897 at position 147 in the gene. **(B)** Structural composition of the corresponding apparatus to the type

898 III secretion system encoded by *hrp* genes. The HrpB4 protein is highlighted in red. (C)

899 Phenotype virulence alteration of the mutant *ΔhrpB4* (complete absence of symptoms) compared

900 to the wild-type strain (WT) after 12 days of infection in *Citrus sinensis* plants.

901

902 **Figure 2: Comparative analysis of *Xac* secretome. (A)** Comparison highlighting 55 total

903 detected proteins, correlating culture mediums (XAM1 × NB – A1) and strains (Xac and *ΔhrpB4*

904 – A2) analysed. The two circles represents the total proteins detected. **(B)** Categorization of

905 annotated protein functions, adapted from da Silva and colleagues (da Silva et al., 2002): I -

906 Intermediary metabolism II - Biosynthesis of small molecules, III – Metabolism of

907 macromolecules, IV - Cell structure, V - Cellular processes, VI - Mobile genetic elements, VII -

908 Pathogenicity, virulence, and adaptation, VIII - Hypothetical/Conserved hypothetical genes, XI -

909 Without a defined function **(C)** Venn Diagram highlighting each of the proteins detected under

910 the four different conditions tested. Proteins annotated as hypothetical are highlighted in red. The

911 same Venn diagram is shown on a smaller scale but with only the numbers of the proteins

912 detected in each condition.

913

914 **Figure 3: Model highlighting proteins related to virulence characterized in comparative**

915 **proteomics.** Three of these proteins (Ax21, FliC, and Ef-Tu) are characterized as PAMPs

916 capable of inducing a PTI response, which can consequently lead to the increased synthesis of

917 chorismate. Chorismate is a precursor in the synthesis of IAA, Trp, and SA, which are involved

918 in the ROS response and induction of plant defence. However, the secretion of PheA may reduce

919 plant defence responses by shifting the metabolism of chorismate to prephenate synthesis. Also

920 highlighted is the Peh-1 protein, which is an endopolygalacturonase regulated by HrpX and

921 cellulase (Egl), and endo-1,4-beta-glucanase (EngXca), which is regulated by RpfF.

922   Endopolygalacturonase and cellulase degrade pectin polymers and cellulose in the plant cell

923   wall, respectively. Phe - phenylalanine; Tyr - tyrosine; Trp - tryptophan; IAA - indole-3-acetic

924   acid; SA - salicylic acid; PTI - (PAMP) -triggered immunity; PAMPs - pathogen-associated

925   molecular pattern; ROS - reactive oxygen species; EF-Tu - elongation factor Tu; EFR - EF-Tu

926   receiver; Ax21 - Ax21-triggered immunity; FliC - flagellin; FLS2 - flagellin sensitive receiver 2;

927   PheA – chorismate mutase; Peh-1 - endopolygalacturonase; Egl and EngXca - cellulase.

928   HrpG/HrpX and RpfC/RpfF - two component systems (sensory and regulatory proteins,

929   respectively); Black - detected in *Xac* and *ΔhrpB4* grown in XAM1; Red - detected exclusively

930   in *Xac* grown in XAM1; Orange - detected in *Xac* grown in BOTH media (NB and XAM1); Blue

931   - Detected exclusively in *Xac* grown in NB. Dotted arrows indicate regulation.

932

933   **Figure 4: Model highlighting proteins related to adaptation in comparative proteomics.**

934   These proteins were grouped into five groups: lipoproteins, porins, outer membrane proteins,

935   secreted proteins, and ribosomal proteins. HrpG/HrpX, PhoQ/PhoP and PhoR/PhoB—two

936   component systems (sensory and regulatory proteins, respectively); Black—detected in *Xac* and

937   *ΔhrpB4* grown in XAM1; Red – detected exclusively in *Xac* grown in XAM1; Orange—detected

938   in *Xac* grown in both media (NB and XAM1); Blue - detected exclusively in *Xac* grown in NB.

939   Dotted arrows indicate regulation; Pi/PolyPi – inorganic phosphate and inorganic polyphosphate.

940

941

942

943

944   **TABLES**

945   **Table 1:** Proteins expressed/detected in wild type and Δ*hrpB4* strains only in infectious conditions

Gene ID	Gene Name	Product	Cat.	NCBI ID	Strains (culture media)				SP	PIP
					Δ*hrpB4* (NB)	*Xac* (NB)	Δ*hrpB4* (XAM1)	*Xac* (XAM1)		
XAC0223		Hypothetical protein	VIII	1154294	-	-	+	+	Y	N
XAC1466	*pcp*	Peptidoglycan-associated outer membrane lipoprotein	IV	1155537	-	-	+	+	Y	N
XAC2853		Cysteine protease	III	1156924	-	-	+	+	Y	N
XAC2504	*rpfN*	Regulator of pathogenicity factors	VII	1156575	-	-	+	+	N*	N
XAC1113	*slp*	Outer membrane protein Slp	IV	1155184	-	-	+	+	N*	N
XAC2562		Hypothetical protein	VIII	1156633	-	-	+	+	Y	N
XAC0677		Hypothetical protein	VIII	1154748	-	-	+	+	Y	N
XAC3141	*ompP6*	Outer membrane protein P6	IV	1157212	-	-	+	+	N*	N
XAC0552		Proteinase	III	1154623	-	-	+	+	Y	N
XAC0661	*peh-1*	Endopolygalacturonase	VII	1154732	-	-	+	+	Y	Y
XAC3647	*pheA*	Chorismate mutase	II	1157718	-	-	+	+	Y	N
XAC0232		Hypothetical protein	VIII	1154303	-	-	+	+	N*	N
XAC1585		peptidyl-prolyl cis-trans isomerase	III	1155656	-	-	+	+	Y	N

946   Gene_ID and Cat (primary category): according to da Silva (da Silva et al., 2002); Product: according to Kegg (Ogata et al., 1999); SP – Signal

947   Peptide; Y – Yes; N – No; N* - Secreted by non-classical pathways.

948 **Table 2:** Proteins expressed/detected only in wild type strain during infectious conditions

Gene ID	Gene Name	Product	Cat.	NCBI ID	Strains (culture media)				SP	PIP
					ΔhrpB4 (NB)	Xac (NB)	ΔhrpB4 (XAM1)	Xac (XAM1)		
XAC3472	oprO	Polyphosphate-selective porin O	IV	1157543	-	-	-	+	Y	N
XAC0678		Hypothetical protein	VIII	1154749	-	-	-	+	Y	Y
XAC3605	uptE	Hypothetical protein	IV	1157676	-	-	-	+	Y	N
XAC0435	virK	VirK protein	VII	1154506	-	-	-	+	Y	N
XAC0974	rplW	50S ribosomal protein L23	III	1155045	-	-	-	+	N*	N
XAC1497		Hypothetical protein	VIII	1155568	-	-	-	+	Y	N
XAC0029	egl	Cellulase	VII	1154100	-	-	-	+	Y	N
XAC1081	hupB	Histone-like protein	III	1155152	-	-	-	+	N*	N
XAC0989	rpsE	30S ribosomal protein S5	III	1155060	-	-	-	+	N	N
XAC3380		Hypothetical protein	VIII	1157451	-	-	-	+	Y	N
XAC0028	egl	Cellulase	VII	1154100	-	-	-	+	Y	N
XAC2682		Hypothetical protein	VIII	1156753	-	-	-	+	Y	N
XAC0612	engXCA	Cellulase	VII	1154683	-	-	-	+	Y	N
XAC0988	rplR	50S ribosomal protein L18	III	1155059	-	-	-	+	N	N

949 Gene_ID and Cat (primary category): according to da Silva (da Silva et al., 2002); Product: according to Kegg (Ogata et al., 1999); SP – Signal

950 Peptide; Y – Yes; N – No; N* - Secreted by non-classical pathways.

951 **Table 3:** Proteins expressed/detected only in ΔhrpB4 strain during infectious conditions

| Gene ID | Gene Name | Product | Cat. | NCBI ID | Strains (culture media) | | | | SP | PIP |
					ΔhrpB4 (NB)	Xac (NB)	ΔhrpB4 (XAM1)	Xac (XAM1)		
XACb0007	*mlt*	Lytic murein transglycosylase	IV	1158494	-	-	+	-	Y	N
XAC4344	*vacJ*	Lipoprotein	III	1158415	-	-	+	-	Y	N
XAC3664	*ompW*	Outer membrane protein	IV	1157735	-	-	+	-	Y	N
XAC1761		Hypothetical protein	VIII	1155832	-	-	+	-	Y	N
XAC2781	*leuS*	Leucyl-tRNA synthetase	III	1156852	-	-	+	-	N	N
XAC0541	*groES*	Co-chaperonin GroES	III	1154612	-	-	+	-	N*	N
XAC1479		OmpA family protein	IV	1155550	-	-	+	-	Y	N
XAC4342	*yrbC*	Toluene tolerance protein	VII	1158413	-	-	+	-	Y	N
XAC2328	*cycK*	C-type cytochrome biogenesis membrane protein	I	1156399	-	-	+	-	Y	N
XAC0645	*pepN*	Aminopeptidase	III	1154716	-	-	+	-	N*	N

952 Gene_ID and Cat (primary category): according to da Silva (da Silva et al., 2002); Product: according to Kegg (Ogata et al., 1999); SP – Signal

953 Peptide; Y – Yes; N – No; N* - Secreted by non-classical pathways.

954

955 **Table 4:** Proteins expressed/detected only in wild type strain during non-infectious conditions

Gene ID	Gene Name	Product	Cat.	NCBI ID	Strains (culture media)				SP	PIP
					ΔhrpB4 (NB)	Xac (NB)	ΔhrpB4 (XAM1)	Xac (XAM1)		
XAC2665	*pilY1*	Type IV pilus assembly protein PilY1	IV	1156736	-	+	-	-	Y	N
XAC1975	*fliC*	Flagellin	V	1156046	-	+	-	-	N*	N
XAC3314		Hypothetical protein	VIII	1157385	-	+	-	-	N*	N
XAC1974	*fliD*	Flagellar hook-associated protein 2	V	1156045	-	+	-	-	N*	N

956 Gene_ID and Cat (primary category): according to da Silva (da Silva et al., 2002); Product: according to Kegg(Ogata et al., 1999); SP – Signal

957 Peptide; Y – Yes; N – No; N* - Secreted by non-classical pathways.

958

959   **Table 5: Xac annotated proteins expressed and secreted compared to other studies of**
960   **Xanthomonadaceae family organisms.**

Function / Product	Mesophyllic			Vascular			
	Xac[a]	Xac[b]	Xac[c]	Xoo[d]	Xoo[e]	Xcc[f]	Xf[g]
Hydroliticenzymes							
Alpha-amylases	X						
Aminopeptidases	X						
Cellulases	X					X	
Endopolygalacturonases	X	X					
Extracellular proteases	X						
Proteinases	X	X					
Cysteine protease	X	X			X		
Serine Proteases	X						
Motility/chemotaxis							
Flagellar associated protein	X						
Flagellin#	X			X	X	X	
Type IV pilus assembly protein PilY1#	X	X		X			
Twiching motility					X		X
Adaptation/Virulence							
Lytic murein transglycosylase#	X						
RpfN#	X		X				
T3SSe				X		X	
ROS				X	X		
Elongation factor Tu#	X		X	X	X	X	X
Membraneassociatedproteins							
TBDRs			X	X		X	
Iron/siderophores receptors				X	X	X	
ABC transporters				X			
Lipoproteins	X						
Outer membrane proteins	X		X	X	X	X	X
Polyphosphate-selective porin O#	X				X		
Toluene tolerance proteins	X						
Others							

	a	b	c	d	e	f	g
Chorismate mutase#	X						
Co-chaperonin GroES#	X		X	X			
C-type cytochrome biogenesis membrane protein#	X						
Histone-like protein#	X						
Leucyl-tRNA synthetase#	X						
VirK protein#	X	X		X	X		
Polyvinyl alcohol dehydrogenase#	X						
Molecular chaperone GroEL#	X		X	X	X		X
Peptidyl-prolyl cis-trans isomerase#	X			X	X		X
Ribosomal proteins	X		X	X	X		
ATP synthase			X		X		X

961   [#] unique proteins

962   [a] – This work (reference data), [b] – Yamazaki et al. (2008), [c] – Zimaro et al. (2013), [d] – Wang et al. (2013),

963   [e] – González et al. (2012), [f] – Sidhu et al. (2008) and [g] – Silva et al. (2011).

964

965 **FIGURES**

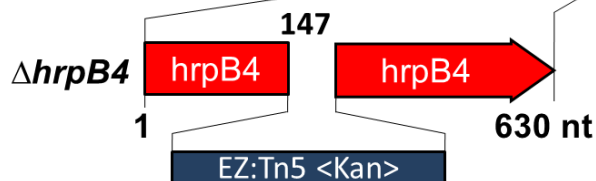
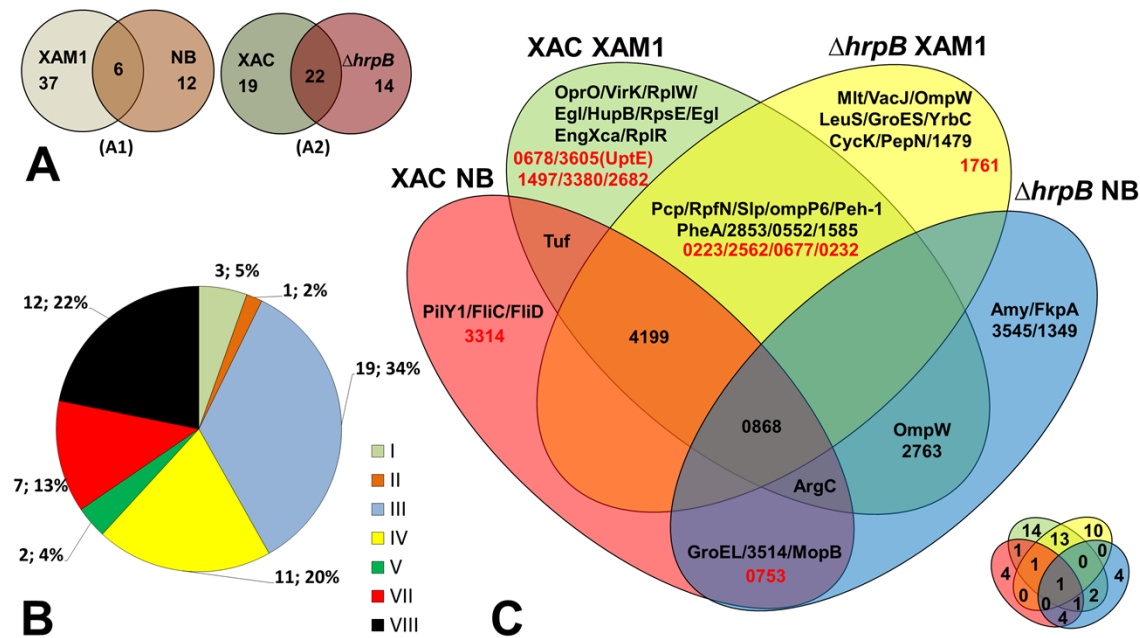
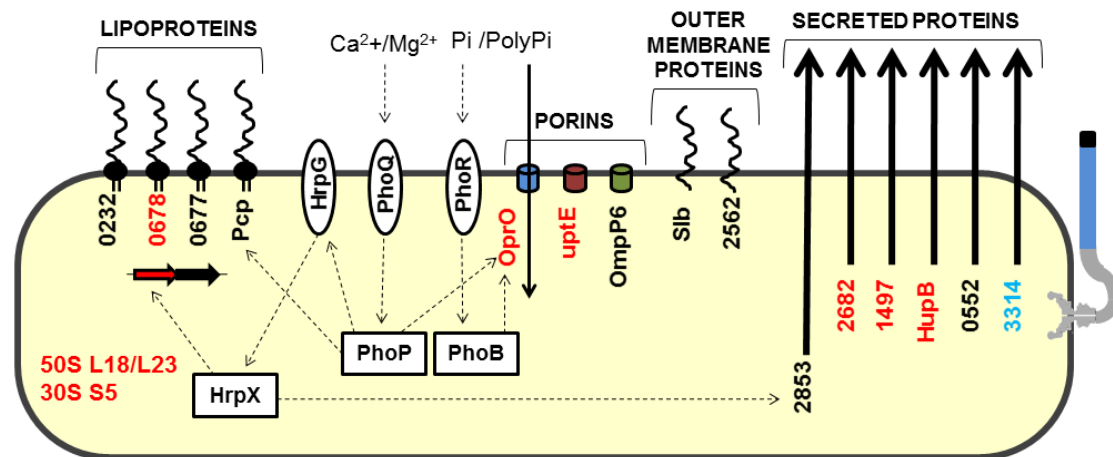
966
967 **Figure 1.**
968

969

970   **Figure 2.**

971

972
973   **Figure 3.**
974

975
976 **Figure 4.**
977

978 **SUPPLEMENTAL MATERIAL**

979 **Supplemental table 1**: Other proteins detected

Gene ID	Gene Name	Product	Cat.	NCBI ID	Strains (culture media)				SP	PIP
					ΔhrpB4 (NB)	*Xac* (NB)	*ΔhrpB4* (XAM1)	*Xac* (XAM1)		
XAC0753		Hypothetical protein	VIII	1154824	+	+	-	-	Y	N
XAC0542	*groEL*	Molecular chaperone GroEL	III	1154613	+	+	-	-	N	N
XAC3514		Serine Protease	III	1157585	+	+	-	-	Y	N
XAC1012	*mopB*	OmpA-OmpF porin, OOP family	IV	1155083	+	+	-	-	Y	N
XAC4199		Polyvinyl alcohol dehydrogenase	I	1158270	+	-	+	+	N*	N
XAC0868		Hypothetical protein	VIII	1154939	+	+	+	+	Y	N
XAC2763		Extracellular protease	III	1156834	+	-	-	+	Y	N
XAC3354	*ompW*	Outer membrane protein W	IV	1157425	+	-	-	+	Y	N
XAC0957	*tuf*	Elongation factor Tu	III	1155028	-	+	-	+	N	N
XAC2992	*argC*	Endoproteinase ArgC	III	1157063	+	+	-	+	Y	N
XAC0798	*amy*	Alpha-amylase	I	1154869	+	-	-	-	Y	N
XAC3545		Serine protease	III	1157616	+	-	-	-	Y	N
XAC1349		Serine protease	III	1155420	+	-	-	-	Y	N
XAC1550	*fkpA*	FKBP-type peptidyl-prolyl cis-trans isomerase	III	1155621	+	-	-	-	N*	N

980 Gene_ID and Cat (primary category): according to da Silva (da Silva et al., 2002); Product: according to Kegg(Ogata et al., 1999); SP – Signal

981 Peptide; Y – Yes; N – No; N* - Secreted by non-classical pathways.

982