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1 **Pancreatic Amylase is an Environmental Signal for Regulation of Biofilm**
2 **Formation and Host Interaction in *Campylobacter jejuni***

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14

15 Running head: *C. jejuni* response to pancreatic amylase

16

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20

21 **Abstract**

22 *Campylobacter jejuni* is a commensal bacterium in the intestines of animals and
23 birds and a major cause of foodborne gastroenteritis in humans worldwide. Here we
24 show that exposure to pancreatic amylase leads to secretion of an α -dextran by *C.*
25 *jejuni* and that a secreted protease, Cj0511, is required. Exposure of *C. jejuni* to
26 pancreatic amylase promotes biofilm formation *in vitro*, increases interaction with
27 human epithelial cell lines, increases virulence in the *Galleria mellonella* infection
28 model and promotes colonisation of the chicken ileum. We also show that exposure
29 to pancreatic amylase protects *C. jejuni* from stress conditions *in vitro* suggesting
30 that the induced α -dextran may be important during transmission between hosts.
31 This is the first evidence that pancreatic amylase functions as an inter-kingdom
32 signal in an enteric micro-organism.

33

34 **Introduction**

35 *Campylobacter* infection is a major cause of foodborne gastroenteritis in humans
36 worldwide (1). Disease ranges from mild, non-inflammatory, self-limiting diarrhea to
37 prolonged, inflammatory diarrhea and occasional serious complications such as
38 Guillain-Barré syndrome and reactive arthritis (1). Ninety percent of human disease
39 is attributed to *C. jejuni*, while *Campylobacter coli* accounts for the remainder (2).

40 In contrast to human infection, *C. jejuni* establishes a largely asymptomatic
41 but persistent infection in animals and birds, and the major risk of human infection is
42 from handling and consumption of poultry meat (3, 4, 5). Campylobacters primarily
43 inhabit the lower gastrointestinal (GI) tract of poultry and contamination of the flesh

44 of the bird occurs at slaughter. *Campylobacter* spp. are present in the luminal crypts
45 of the chicken gut as densely-packed communities surrounded by mucus (6), an
46 arrangement suggestive of a biofilm, a structured community of bacteria enclosed in
47 a self-produced exopolymeric matrix (EPM) composed primarily of polysaccharides
48 (7). Biofilms have also been described on experimentally-infected human ileum *ex*
49 *vivo* suggesting that this mode of growth is also important during human infection (8,
50 9).

51 Despite several studies of *C. jejuni* biofilms (10-15), the EPM has not been
52 characterised. Polysaccharide is a major component of most bacterial EPMs and
53 provides the structural framework (7, 16, 17). *C. jejuni* produces several surface-
54 associated carbohydrate structures including lipooligosaccharide (LOS), capsular
55 polysaccharide (CPS) and both *N*- and *O*-linked glycoproteins (18), but there is no
56 evidence that these glycans contribute to biofilm formation (11, 19).

57 Enteric bacteria use environmental signals present in host environments to
58 regulate the expression of components required for interaction with the host and
59 signals include bile salts, mucus, low oxygen, bicarbonate and neuroendocrine
60 stress hormones (20-22). *C. jejuni* shows increased interaction with intestinal
61 epithelial cells (IECs) in response to low oxygen, bile salts and noradrenaline (23-
62 25). In addition, bile salts have recently been reported to enhance *C. jejuni* biofilm
63 formation by causing release of extracellular DNA (26).

64 Here we show that *C. jejuni* responds to the presence of pancreatic amylase
65 by secreting an α -dextran that is a component of the biofilm EPM. This is the first
66 definitive characterisation of the polysaccharide component of *C. jejuni* biofilms.
67 Importantly, pre-exposure to pancreatic amylase results in significant changes in the

68 interactions of *C. jejuni* with the host in both *in-vitro* and *in-vivo* infection models.
69 Pre-exposure to pancreatic amylase also promotes stress tolerance *in vitro*
70 suggesting that the α -dextran may be important during transmission. This is the first
71 evidence that pancreatic amylase is used by an enteric micro-organism as a signal
72 to regulate interaction with the host.

73

74 **MATERIALS AND METHODS**

75 **Bacterial strains and growth conditions.** Bacterial strains are listed in Table 1. *C.*
76 *jejuni* was stored at -70°C in Brucella broth (BB; Oxoid) containing 15% glycerol and
77 was grown on Mueller Hinton agar (MHA; Oxoid), Brucella agar (BA; Oxoid),
78 Columbia blood agar (CBA; Oxoid) containing 7% defibrinated horse blood (E & O
79 Laboratories) in a microaerobic atmosphere generated using Campypaks (Oxoid) in
80 gas jars at 37°C. Charcoal (0.4%) was added to BA to improve contrast for
81 photography. For broth cultures, BB or Eagle's Minimal Essential Medium- α (MEM-
82 α ; Sigma-Aldrich, M0894) were used and incubated with shaking (50 rpm) at 37°C in
83 5% CO₂. Kanamycin (Km; 40 μ g/ml) and chloramphenicol (Cm; 20 μ g/ml) were used
84 for selection.

85

86 **Sources of pancreatic amylase.** Hog pancreatic α -amylase (HPA) was purchased
87 from Sigma Aldrich. Polyhistidine-tagged human pancreatic amylase expressed and
88 purified from human cells was obtained from Sino Biologicals. For chicken pancreas
89 extract, 20 organs were homogenized in 0.05 M Tris, 0.9% NaCl, 0.05 M CaCl₂, pH

90 8.0 (27). After centrifugation (1,000 x g, 20 min), amylase activity in the supernatant
91 was quantified by enzymatic assay (28).

92

93 **EPM purification and characterisation.** Growth (48 h) removed from MHA with or
94 without 100 nM HPA was suspended in phosphate buffered saline (PBS) and the
95 extracellular material was recovered using modifications of a method previously used
96 to isolate exo-polysaccharide from *Vibrio parahaemolyticus* (29). Briefly, the bacterial
97 cell suspensions in PBS were washed on a rotary platform at 200 rpm for 1.5 h at
98 30°C, vortexed for 15 min and washed again at 200 rpm for 1.5 h at 20°C. The
99 supernatant was recovered (1,800 x g, 15 min) and precipitated overnight with 4
100 volumes of cold acetone at 4°C. The precipitate was recovered (450 x g, 5 min),
101 washed in distilled water (dH₂O), dried (Speedvac SPD1010, Thermo Scientific),
102 dissolved in dH₂O and stored at -70°C. Total carbohydrate was measured by phenol-
103 sulphuric acid assay (30) with glucose as standard. To determine the statistical
104 significance, a one-way ANOVA was used to compare the amount of secreted
105 carbohydrate in response to the seven different doses of pancreatic amylase. Within
106 the one-way ANOVA we constructed two contrasts, the first compared the joint effect
107 of the three lowest doses with the joint effect of the four highest doses, the second
108 compared the effect of a dose of 50 nM pancreatic amylase with 100 nM. As
109 independent samples of a bacterial culture were exposed to the different doses of
110 amylase in a closed system, we assumed independence between the seven
111 experiments. To analyse the amount of carbohydrate secreted by the wild-type,
112 *Cj0511* mutant and complemented strain, a two-way ANOVA was performed
113 comparing the amount of secreted carbohydrate in response to the four different

114 doses of pancreatic amylase in the three different strains. Contrasts of the 15 nM
115 joint with 50 nM concentrations, versus 100 nM joint with 500 nM concentrations
116 were constructed for *C. jejuni* 11168H, *Cj0511* mutant, and complemented strains.
117 Differences between these three contrasts were tested for statistical significance.
118 The contrasts and comparisons were planned *a priori* and thus no multiple testing
119 adjustments were required.

120

121 Further characterisation of the EPM carbohydrate was performed after dialysis (14
122 kDa MWCO) for 72 h at 4°C against three changes of dH₂O per day. The EPM was
123 freeze dried and characterized using NMR spectroscopy (31), monomer analysis
124 (32) and linkage analysis (33) in comparison with a commercial α -dextran standard
125 (Sigma-Aldrich).

126

127 **Genetic complementation of a *cj0511* mutant.** A *cj0511* mutant obtained from a
128 collection at the London School of Hygiene and Tropical Medicine (Table 1) was
129 used to construct a complemented strain by insertion of *cj0511* under the control of
130 the iron-inducible *fdxA* promoter into a pseudogene (*cj0046*) in the *cj0511* mutant as
131 previously described (34). The oligonucleotides used are listed in Table 2.

132

133 **Production of recombinant Cj0511 and proteolysis assay**

134 Codon optimized *cj0511* was synthesized by Celtek, USA and obtained in pGH. The
135 gene was cut by HindIII (New England Biolabs, UK) and the released DNA fragment
136 was cloned in pET-28a(+)(Novagen, UK) to generate pMAR31. Expression of *cj0511*

137 was achieved in *E.coli* BL21 (DE3) (New England Biolabs, UK). To localize the
138 recombinant protein in the periplasm of *E.coli*, recombinant Cj0511 was modified to
139 have a signal peptide from the *E.coli* DsbA protein fused to the N-terminal, while the
140 C-terminus was fused to a 6 x-His affinity tag. Recombinant Cj0511 was purified
141 using nickel affinity column (Qiagen) and eluted in 500 mM imidazole, 300 mM
142 sodium phosphate and 20 mM sodium chloride buffer. The eluted fractions were
143 combined and concentrated using an Amicon ultrafiltration column with a 100 kDa
144 cut off (Merck Millipore, UK) then resuspended in 10 mM sodium phosphate buffer.
145 One microgram of purified r-Cj0511 was mixed with 2 µg polyhistidine-tagged human
146 pancreatic amylase (Sino Biologicals) or casein (Sigma Aldrich) in 20 µL of 50 mM
147 sodium phosphate buffer (pH 7.5) and incubated at 37°C for a total of 2 h. The
148 reactions were stopped using 6 x Laemmli buffer and the reaction mixtures were
149 boiled for 10 min before being separated by SDS-PAGE (16% polyacrylamide) and
150 stained with Coomassie R-250.

151

152 **Biofilm assays.** A 16 h culture of *C. jejuni* in BB was diluted to OD₆₀₀ of 0.1 in BB
153 with or without 100 nM HPA, 100 nM recombinant human pancreatic amylase or an
154 equivalent activity of chicken pancreas extract, added to a borosilicate glass tube
155 (VWR International) and incubated without shaking at 37°C in 5% CO₂. After 48 h
156 the culture was decanted, the biofilms were washed with dH₂O, dried and stained for
157 5 min with 0.1% (w/v) crystal violet (CV; Sigma-Aldrich), washed with dH₂O and
158 dried. Bound CV was solubilised in 80% ethanol-20% acetone and quantified by
159 measuring OD₆₀₀. Statistical significance was assessed with a two-sample t-test
160 assuming unequal variance.

161

162 **Confocal laser scanning microscopy of biofilm.** A 16 h culture of *C. jejuni* was
163 diluted to OD₆₀₀ of 0.1 in BB with or without 100 nM HPA and placed in a 6-well
164 tissue culture plate (Sarstedt). Two glass coverslips were placed upright in each well
165 and incubated for 48 h at 37°C in 5% CO₂. The coverslips were washed in PBS,
166 stained with Live/Dead BacLight stain (Invitrogen) according to the manufacturer's
167 instructions and visualised with a BioRad Radiance 2100 confocal scanning system
168 attached to an Olympus BX51 upright microscope. Digital images were produced
169 using Image J software (National Institutes of Health).

170

171 **Adhesion and invasion of Caco-2 cells.** Human colon cancer cells (Caco-2) were
172 cultured as previously described (25) and 10⁸ bacteria, grown on MHA with or
173 without 100 nM HPA, were added at a multiplicity of infection (MOI) of 100:1 and
174 incubated at 37°C in 5% CO₂. Interacting and invading bacteria were enumerated at
175 3, 6 and 24 h as previously described (25) and statistical significance was assessed
176 using a two-sample t-test assuming unequal variance.

177

178 **Interaction with T84 human colonic epithelial cells.** T84 cells were grown in
179 complete Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM
180 (Invitrogen) in rat collagen pre-coated transwell dishes until the transepithelial
181 electrical resistance (TEER) measurement (35) was >800 Ω confirming formation of
182 tight-junctions in the monolayer. Co-culture studies were performed at a MOI of 10:1
183 in DMEM/F12 HAM medium containing 10% foetal bovine serum for 6 or 24 h.

184 Bacteria were grown on MHA with or without 100 nM HPA and viable counts were
185 performed. Statistical significance was assessed using a Wilcoxon matched paired
186 test. IL-8 levels were determined by ELISA (eBioscience) from the supernatant of the
187 apical surface at 24 h post-infection. Statistical significance was assessed using a
188 one-way ANOVA.

189

190 ***Galleria mellonella* infection model.** *G. mellonella* larvae were obtained
191 commercially (Cornish Crispa Co) and stored in containers with wood chips at 12°C.
192 *C. jejuni* strains were grown on MHA with or without 100 nM HPA for 48 h, harvested
193 in PBS and 10^6 cfu injected into the right foreleg of 10 *Galleria* larvae as previously
194 described (36). Statistical significance was assessed using a two-sample t-test,
195 assuming unequal variance.

196

197 **Infection of chickens.** Day-old Ross broiler chickens were obtained from a
198 commercial supplier and housed in biosecure housing. The study was performed
199 under a project licence issued by the UK Home Office under the Animals (Scientific
200 Procedures) Act 1986 (PPL 30/2599) and protocols and euthanasia techniques were
201 approved by the University of Bristol Animal Welfare Ethical Review Board. Birds
202 were fed commercial starter and grower diets (BOCM Pauls Ltd, Ipswich), had
203 access to food and water *ad libitum* and were exposed to a 12 h light/darkness cycle.
204 At 21 days old, groups of 30 birds were infected by oral gavage with 10^5 of *C. jejuni*
205 11168H or the *cj0511* mutant grown on MHA with or without 100 μ M HPA for 48 h
206 and resuspended in MH broth. Fifteen birds were euthanized by cervical dislocation

207 at 4 and 7 days post infection and ileum and liver samples removed for culture. Ileum
208 samples were serially diluted and plated on modified charcoal cefoperazone
209 deoxycholate agar (mCCDA; Oxoid) incubated at 37°C for 48 h in microaerobic
210 conditions. Liver samples were homogenised and enriched in modified Exeter broth
211 (37), incubated with minimal headspace at 37°C for 48 h and plated on mCCDA.
212 Differences in the number of birds colonised were assessed using a Chi-square test.
213 The distributions of numbers of bacteria found in samples from the birds were
214 checked for normality using a D'Agostino and Pearson omnibus normality test; as
215 data were not shown to follow a Gaussian distribution, differences in the number of
216 bacteria found at each site were analysed using a Kruskal-Wallis test with Dunn's
217 multiple comparison test, using a Benjamini and Hochberg correction to adjust for
218 multiple pairwise comparisons, as a post-hoc test.

219

220 **Resistance to environmental stress.** The growth from 48 h MHA plates with and
221 without 100 nM HPA was recovered into PBS (4°C) or MH broth (20°C), washed and
222 resuspended to an OD₆₀₀ of 2.0. The cultures were incubated with shaking (50 rpm)
223 at 37°C in 5% CO₂ and samples were removed at intervals for viable counting. The
224 statistical significance of the difference in viable counts in the presence and absence
225 of HPA was assessed using two sample t-tests, assuming unequal variance.

226

227 **RESULTS**

228 ***C. jejuni* responds to the presence of pancreatic amylase.** Physiological
229 concentrations of mammalian pancreatic α -amylase are estimated to be nanomolar
230 (38). When hog pancreatic α -amylase (HPA) was incorporated into agar at various

231 concentrations, we observed the formation of large mucoid colonies of *C. jejuni*
232 11168H at a minimum concentration of 100 μ M (Fig. 1A). It is important to note that
233 mucoid colonies were apparent on MHA and also on BA containing 100 μ M HPA;
234 since BA does not contain starch, this indicates that the response is independent of
235 amylase starch degrading activity. Whilst 100 μ M pancreatic amylase seem high
236 compared to the estimated *in vivo* concentrations, it is noteworthy that the number of
237 molecules of amylase that can interact with the bacteria growing on an agar plate is
238 likely to be low because of spatial confinement, in contrast to physiological
239 conditions where bacteria will be bathed in litres of pancreatic juice per day. Mucoid
240 colonies were also produced by other *C. jejuni* strains (81-176, 81116, G1, X) in
241 response to 100 μ M HPA but not by the periodontal pathogen, *Campylobacter rectus*
242 NCTC 11489 (data not shown).

243 **Pancreatic amylase promotes *C. jejuni* growth.** As the colonies on amylase were
244 larger in addition to being mucoid, the number of bacteria per colony was determined
245 for *C. jejuni* 11168H from HPA-containing (100 μ M) and amylase-free MHA. In three
246 experiments, colonies contained a mean of $5.3 (+/-3.2) \times 10^7$ cfu in the presence of
247 amylase, compared to $3.3 (+/-2.3) \times 10^6$ cfu in the absence of amylase, representing
248 a 16-fold increase. In MEM- α , supplementation with physiological concentrations
249 (100 nM) of HPA resulted in an increased growth rate (mean generation time of 180
250 (+/-0) minutes in the presence of pancreatic amylase compared to 280 (+/-35)
251 minutes in its absence). The difference in growth rate was statistically significant
252 ($p < 0.001$) at both mid-exponential phase (10 h) and stationary phase (15 h) (Fig.
253 1B).

254

255 **Mucoid colonies secrete increased amounts of carbohydrate.** As mucoid
256 colonies are suggestive of exo-polysaccharide production, we attempted to purify the
257 extracellular material from colonies of strain 11168H grown in the presence of
258 increasing concentrations of HPA by washing in PBS. Measurement of the
259 carbohydrate content of this preparation by phenol sulphuric acid assay showed that
260 exposure to physiological concentrations of HPA (100 nM) resulted in a 100%
261 increase in the soluble carbohydrate secreted by *C. jejuni* 11168H compared to
262 growth without HPA (Fig. 2A). A 11168H *kpsM* mutant, unable to export CPS (39),
263 and 11168H *waaF* and *waaC* mutants lacking the outer and entire core
264 oligosaccharide of LOS, respectively (40, 41), also showed increased carbohydrate
265 secretion of comparable magnitude to the wild-type (WT) strain in the presence of
266 HPA, indicating that the secreted carbohydrate is independent of both CPS and LOS
267 (Fig. 2B). The amount of carbohydrate secreted by the 11168H *kpsM*, *waaC* and
268 *waaF* mutants did not differ significantly from the wild-type strain in either the
269 presence or absence of pancreatic amylase.

270

271 **The secreted carbohydrate is an α -dextran.** The soluble extracellular material
272 recovered from *C. jejuni* 11168H and the *kpsM* mutant provided ^1H and ^{13}C -NMR
273 spectra identical to an α -dextran standard (Fig. 2C) and distinct from starch (Fig.
274 S1). A series of 2D-NMR (COSY, HSQC & HMBC) were also recorded (data not
275 shown) to confirm the identity of the exo-polysaccharide as an α -dextran. Monomer
276 analysis was undertaken using high performance anion exchange chromatography in
277 combination with pulsed amperometric detection. After acid hydrolysis, the
278 extracellular material recovered from both *C. jejuni* 11168H and the *kpsM* mutant

279 gave chromatographs containing a single peak which co-eluted with a glucose
280 reference standard (Fig. S2). Monomer analysis thus confirmed that glucose was the
281 only monosaccharide present and linkage analysis showed exclusively 1,6-glycosidic
282 links (Fig. S3).

283

284 **Cj0511 is essential for the response to pancreatic amylase but does not**
285 **degrade it *in vitro*.** Since HPA improves the growth of *C. jejuni*, we reasoned that it
286 could be degraded by the bacterium. As Cj0511 is a protease (42) that is known to
287 be secreted in outer membrane vesicles (OMVs) (43) and is abundant in a *C. jejuni*
288 strain that colonises chickens efficiently (44), we hypothesized that it is involved in
289 the response to pancreatic amylase. Determination of the amount of secreted
290 carbohydrate showed that the increased secretion seen in the WT strain in response
291 to stimulation with HPA was absent in a *cj0511* mutant (Fig. 2D). Restoration of the
292 response to HPA in the complemented strain (Fig. 2D), shows that the defect in the
293 *cj0511* mutant is the result of inactivation of *cj0511* and not due to a polar effect or
294 spontaneous mutation elsewhere in the genome. To determine if Cj0511 degrades
295 pancreatic amylase, recombinant Cj0511 (r-Cj0511) was incubated with recombinant
296 human pancreatic amylase or with casein (Fig. 3). Partial and complete degradation
297 of casein was apparent at 30 min and 2 h, respectively. In contrast, there was no
298 apparent degradation of pancreatic amylase after 2 h.

299

300 **Exposure to pancreatic amylase promotes biofilm formation.** To determine if
301 exposure to pancreatic amylase contributed to biofilm formation in *C. jejuni*, biofilms

302 formed at the air/liquid interface in glass tubes in the presence or absence of
303 physiological concentrations of pancreatic amylase (100 nM) were measured by
304 crystal violet staining. With the exception of the *cj0511* mutant, all the strains showed
305 significant increases in biofilm formation in the presence of HPA (Fig. 4A). The
306 magnitude of the increase in biofilm in response to HPA in the *waaC*, *waaF* and
307 *kpsM* mutants was similar to the WT (2.1-2.5-fold) demonstrating that it is
308 independent of LOS and CPS. Although we observed increased biofilm formation by
309 the *cj0511* mutant compared to the WT in the absence of HPA ($p=0.003$), there was
310 no significant increase in the presence of HPA in this mutant (in contrast with the
311 complemented strain), demonstrating that a functional Cj0511 protease is required
312 for the response (Fig. 4A). To confirm that the increased biofilm was specifically due
313 to pancreatic amylase, the assay was repeated in the presence of 100 nM
314 recombinant human pancreatic amylase and an increase in biofilm density of similar
315 magnitude (2.2-fold; $p=0.001$) to that obtained with the preparation of HPA was
316 observed (Fig. 4B). Addition of a volume of chicken pancreas extract containing
317 equivalent amylase activity also resulted in an increase in biofilm density (2.2-fold;
318 $p<0.001$) (Fig. 4C).

319 Confocal laser scanning microscopy (CLSM) was used to visualise biofilms of
320 *C. jejuni* 11168H grown in the presence or absence of HPA. In the presence of HPA
321 there was typical biofilm structure at 48 h with adherent microcolonies of live bacteria
322 separated by dark channels, whereas there were few adherent bacteria in the
323 absence of HPA (Fig. 4D). Examination in the x-z plane showed typical three-
324 dimensional biofilm structure (of maximum height 160 μm) only in the presence of
325 HPA.

326

327 **Pre-exposure to pancreatic amylase promotes interaction with human IECs.** To

328 determine if pre-exposure of *C. jejuni* to HPA affects the interaction with IECs,
329 strains grown with or without 100 nM HPA were co-cultured with Caco-2 cells. *C.*
330 *jejuni* 11168H grown in the presence of HPA showed significant increases in the
331 number of interacting bacteria after 3, 6 and 24 h of co-culture (Fig. 5A). Growth of
332 *C. jejuni* 81-176 in the presence of HPA also resulted in significant increases in
333 interaction with Caco-2 cells (data not shown). In contrast, exposure of the *C. jejuni*
334 11168H *cj0511* mutant to HPA did not result in increased interaction, whereas the
335 complemented strain did show significant increases (Fig. 5A). Pre-exposure to HPA
336 also resulted in increased invasion by strains 11168H (Fig. 5B) and 81-176 (data not
337 shown). There was no increase in invasion by the *cj0511* mutant with pre-exposure
338 to HPA whereas the complemented strain showed a significant increase (Fig. 5B).

339 To explore the effect of pre-exposure to HPA on IEC cytokine responses, we
340 measured interleukin-8 (IL-8) secretion from T84 human colon cancer cells in
341 response to bacteria grown with or without HPA. At 24 h, we observed a significant
342 increase in IL-8 secretion in response to infection but growth on HPA had no effect
343 (Fig. 5C). A similar lack of cytokine response to HPA pre-treatment was observed in
344 THP-1 macrophages (data not shown). The effect of infection on transepithelial
345 electrical resistance (TEER) was also investigated. No disruption in tight-junction
346 integrity in response to *C. jejuni* grown on HPA was noted 6 h post-infection (data
347 not shown), but there was a significant increase in translocation of the bacteria pre-
348 exposed to HPA compared to those grown without HPA (Fig. 5D), a difference no
349 longer apparent at 24 h (data not shown).

350

351 **Pre-exposure to pancreatic amylase results in increased killing of *Galleria***
352 ***mellonella* larvae.** *C. jejuni* 11168H, 81-176 and the *cj0511* and *kpsM* mutants were
353 grown with or without HPA, injected into ten *G. mellonella* larvae and the number of
354 dead larvae determined at 24 h (Fig. 6). For both WT strains, a mean of 1 - 2 larvae
355 were killed when the bacteria were grown without HPA compared to means of ~9
356 when grown with HPA. The increased virulence in response to HPA was lost in the
357 *cj0511* mutant but restored in the complemented strain (Fig. 6), confirming the
358 essential role of Cj0511 in signal detection.

359

360 **Pre-exposure to pancreatic amylase promotes colonisation of broiler chickens.**

361 To determine whether pre-exposure to pancreatic amylase affects the ability of *C.*
362 *jejuni* to colonise chickens, strain 11168H and the *cj0511* mutant grown with or
363 without HPA were used to infect broiler chickens of 21 days old. At day 4, the WT
364 strain had colonised better than the *cj0511* mutant, but no difference was apparent
365 between treatments (Fig. 7A). By day 7, the number of birds colonised by the WT
366 strain exposed to HPA prior to infection was significantly greater than the number of
367 birds colonised by this strain not exposed to HPA ($p=0.005$) (Fig. 7B). Differences in
368 the numbers of bacteria present between infection groups were seen at day 4
369 ($p=0.0006$; Kruskal-Wallis test) and day 7 ($p=0.0026$). At day 4 in chickens infected
370 with strains grown in the presence of HPA the WT strain was present in significantly
371 higher numbers in the ileum (13/15 birds infected, median 3.0×10^4 cfu/g, range 0-
372 1.5×10^5 cfu/g) compared to the *cj0511* mutant (5/15 birds infected, median 0 cfu/g,
373 range 0- 1.5×10^4 cfu/g) (Fig. 7C). Growth on HPA also significantly increased the
374 numbers of the WT strain in the ileum at day 7 (11/15 birds infected, median 2.0×10^4

375 cfu/g, range 0-3x10⁴ cfu/g) compared to the unexposed WT (5/15 birds infected,
376 median 0 cfu/g, range 0-3x10⁴ cfu/g), whereas there was no such increase
377 detectable in the *cj0511* mutant (Fig. 7D).

378

379 **Pre-exposure to pancreatic amylase promotes survival in stress environments.**

380 To assess the role of the α -dextran in survival outside the host, we cultured *C. jejuni*
381 11168H in the presence or absence of 100 nM HPA, recovered the bacteria into
382 either broth or PBS without HPA and determined by viable counting the numbers of
383 surviving bacteria at room temperature and at 4°C in air. In both stress conditions,
384 pre-exposure to HPA prolonged survival suggesting that the α -dextran has a
385 protective effect (Fig. 8).

386

387 **DISCUSSION**

388 Given that *C. jejuni* has been described as a “sugary bug” (45) it is somewhat
389 surprising that until the work described herein, no definitive identification of a
390 polysaccharide involved in biofilm formation had been made. Indeed it has been
391 suggested that DNA is a major component of the *C. jejuni* biofilm matrix (26).
392 However, when *C. jejuni* is freshly isolated from animal or human stools it is highly
393 mucoid (Allan, unpublished observation), suggesting that a polysaccharide matrix is
394 being produced. This led us to hypothesize that a host factor was responsible for
395 inducing the polysaccharide EPM. Such interkingdom signalling between eukaryotes
396 and prokaryotes was first identified over a century ago (46). Over the last decade or
397 so it has been established that interkingdom signalling between bacteria and hosts,
398 via “hormones” is widespread (47). One of the first reports of this was the effect of

399 adrenaline and noradrenaline on *E. coli* (48). There are a limited number of reports
400 on host cell molecules affecting biofilm formation by bacteria. For example, bile salts
401 enhance biofilm formation by *C. jejuni* (26), *Listeria monocytogenes* (49) and *Vibrio*
402 *cholerae* (50). In the case of *Streptococcus pneumoniae*, a number of host factors
403 affect biofilm formation and structure including norepinephrine (51), and
404 extracytoplasmic ATP (52). Our work shows for the first time that avian and
405 mammalian pancreatic amylase can act as an interkingdom signalling molecule and
406 enhance *C. jejuni* growth and biofilm formation. It is important to emphasise that a
407 mucoid colony is produced on *Brucella* agar in response to pancreatic amylase. This
408 medium does not contain starch, indicating that the response is directly to the
409 presence of amylase protein and is not dependent on amylase starch degrading
410 activity. This was confirmed in the biofilm assay which was also carried out in
411 medium lacking starch. To our knowledge this is only the second example of a
412 proteinaceous host component that can enhance bacterial biofilm formation, the first
413 being the cytokine interleukin 1 β which enhances biofilm formation by
414 *Staphylococcus aureus* (53). Interestingly, *C. rectus* did not appear to detect
415 pancreatic amylase suggesting that the response may be a specific adaptation to
416 host environments. It will be interesting to investigate in future work whether the
417 enteric and oral *Campylobacter* spp. are able to detect the presence of salivary
418 amylase, given its similarity in sequence and structure to pancreatic amylase.

419

420 The polysaccharide secreted by *C. jejuni* was definitively identified as α -dextran and
421 the ease with which it was removed from the bacterial cell surface, by simply
422 washing in saline, indicates that it is a loosely-associated 'slime polysaccharide' as
423 oppose to the capsular polysaccharides that are covalently attached to the cell wall

424 (16, 54). Given that *C. jejuni* is capable of producing complex glycan structures (18),
425 the production of a simple α -dextran is intriguing. Dextrans are produced by oral
426 streptococci and *Leuconostoc* spp. from sucrose using dextransucrases, cell wall-
427 anchored enzymes of the glycoside hydrolase superfamily (56). Searching the
428 translated *C. jejuni* genome sequences with known dextransucrase sequences did
429 not identify homologues, suggesting that dextran synthesis in *C. jejuni* occurs by a
430 novel mechanism. The source of the glucose monomer, whether it is taken up from
431 the environment or synthesized from amino acids, awaits experimental investigation.

432

433 The fact that HPA enhances the growth of *C. jejuni* suggested that it may be used as
434 a nutrient source and Cj0511, predicted as a serine protease of the C-terminal
435 protease (CTP) family and known to be secreted in OMVs (43), seemed a likely
436 candidate for degrading pancreatic amylase. Our data shows that a *cj0511* mutant
437 did not display the response to HPA, a phenotype that was restored in a
438 complemented strain, confirming the role of Cj0511 specifically. However, there was
439 no apparent change in migration of recombinant human amylase on an SDS-PAGE
440 gel following incubation with Cj0511 even though partial casein degradation was
441 apparent in 30 min and complete digestion in 2 h. The recombinant amylase is
442 tagged with 6 histidine residues at the C-terminus and C-terminal proteolysis of even
443 a single amino acid would result in a reduction of ~1 kDa in the molecular weight of
444 the protein which would be apparent on a 16% polyacrylamide gel. In this context, it
445 is important to emphasise that the polyhistidine-tagged human recombinant amylase
446 preparation was active, since we showed it induced biofilm formation in the wild-type
447 strain. Thus, interference by the polyhistidine tag cannot explain why we fail to see

448 cleavage by r-Cj0511 *in vitro*. Since Cj0511 is N-glycosylated in *C. jejuni* (Young et
449 al., 2002), an absence of glycosylation in r-Cj0511 is another plausible explanation.
450 However, we have shown that a *pglB* mutant (i.e. defective for N-glycosylation; 55)
451 still responds to pancreatic amylase (Jowiya and Allan, unpublished), which
452 discounts this explanation also. Thus we conclude that Cj0511 does not digest
453 pancreatic amylase, at least *in vitro*. Bacterial CTPs are involved in a range of
454 physiological processes. The *Escherichia coli* tail-specific protease processes
455 penicillin binding proteins and regulates cell morphology (57). CTPs with roles in
456 virulence have also been described: for example, CtpA of *Brucella suis* is required
457 for virulence in a mouse infection model (58). A recent study by Karlyshev and co-
458 workers showed that a *cj0511* mutant was severely attenuated in its ability to
459 colonise chickens (42). Since Cj0511 does not appear to degrade pancreatic
460 amylase, we conclude that it is involved at a later stage in the molecular mechanism;
461 elucidation of its precise role requires further research. Previous studies have shown
462 that Cj0511 is expressed in the absence of pancreatic amylase (43) suggesting that
463 its expression is constitutive. It will be interesting in future work to see if Cj0511
464 levels are increased in the presence of pancreatic amylase.

465

466 Having established that pancreatic amylase induces secretion of an α -dextran, we
467 next addressed the biological consequence. Growth on physiological concentrations
468 of HPA resulted in increased biofilm formation in two different WT strains. Moreover,
469 the increase in biofilm formation induced by HPA was dependent on Cj0511. Since
470 the purity of the commercial HPA preparation is not guaranteed, we tested purified
471 recombinant human pancreatic amylase in the biofilm assay and observed an

472 increase in biofilm formation of similar magnitude to that induced by HPA, confirming
473 that the response is to pancreatic amylase specifically. Biofilm formation was also
474 increased in response to an extract of chicken pancreas suggesting that the α -
475 dextran promotes biofilm formation in the intestines of both mammalian and avian
476 hosts.

477 The study next investigated the effect of HPA exposure on the interaction of
478 *C. jejuni* with IECs. Growth on HPA resulted in a significant increase in adhesion and
479 invasion of *C. jejuni* in Caco-2 cells. As the neutrophil chemoattractant IL-8 has a
480 role in the response to *C. jejuni* (59), we studied production by T84 cells as they are
481 more potent cytokine responders than Caco-2 cells (60). Surprisingly, no difference
482 in IL-8 secretion in response to bacteria grown with or without HPA was observed.
483 Pre-exposure to HPA led to a significant increase in bacterial translocation 6 h post-
484 infection, however, without loss of tight junction integrity. Collectively, these data
485 suggest that the α -dextran modulates early host-pathogen interactions by promoting
486 rapid intracellular transfer while avoiding overt host inflammatory responses. It is
487 intriguing to speculate that an α -1,6-dextran evolved as a colonisation factor since it
488 exhibits sufficient similarity to dietary starch to be tolerated by the immune system
489 yet is resistant to the α -1,4-glucan glucanohydrolase activity of pancreatic amylase.

490 In the *G. mellonella* infection model, growth of *C. jejuni* on pancreatic amylase
491 resulted in increased virulence and this is again dependent on Cj0511. *G. mellonella*
492 larvae have been used as a model to study infection by *C. jejuni* (36) and other
493 enteric pathogens as they possess both humoral and cellular immune systems (61).
494 The increased insecticidal activity of *C. jejuni* grown on HPA suggests that biofilm
495 formation may contribute to resistance to these immune mechanisms. It is notable

496 that the insecticidal activity observed for *C. jejuni* 11168H and 81-176 in the absence
497 of pancreatic amylase was lower than that reported by Champion *et al.* (36) for these
498 strains. We assume that the decreased virulence observed in our study is a
499 reflection of culture on Mueller Hinton agar as oppose to Columbia blood agar which
500 was used by Champion *et al.*

501

502 In the laboratory, *C. jejuni* is sensitive to environmental stresses such as
503 drying, chilling, and exposure to atmospheric oxygen yet the bacterium exhibits a
504 remarkable ability to persist in food processing facilities and natural environments
505 (62). We have shown that pre-exposure of *C. jejuni* to pancreatic amylase prolongs
506 survival in atmospheric conditions at ambient and refrigeration temperatures. This
507 demonstrates that the α -dextran, which will be expressed by *C. jejuni* expelled into
508 the environment from a host organism, is stable and may be important for the
509 environmental resilience of the bacterium.

510 In conclusion, we propose a new model in which *C. jejuni* uses host
511 pancreatic amylase, encountered as it passes the pancreatic duct, as a signal for
512 secretion of an α -dextran which promotes biofilm formation and contributes to
513 colonisation of the mammalian and avian intestine and increases resistance to host
514 immune functions. We have shown that Cj0511, a secreted serine protease, is
515 required for the response to pancreatic amylase although its precise function in the
516 response is currently unknown. The demonstration of a secreted dextran that leads
517 to biofilm formation is an important finding that is expected to enable the design of
518 intervention strategies to reduce the burden of *C. jejuni* in the food chain and
519 ultimately to reduce the incidence of human enteric disease.

520

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525

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751

Table 1. Bacterial strains and plasmids

Bacterial strain	Characteristics	Reference
<i>C. jejuni</i> 11168H	Hyper-motile variant of NCTC 11168; good coloniser of chickens	(63)
<i>C. jejuni</i> 81-176	Clinical isolate from human diarrhoea sample	(64)
<i>C. jejuni</i> 81116	Waterborne human outbreak strain	(65)
<i>C. jejuni</i> X	Clinical isolate from patient with enteritis	(66)
<i>C. jejuni</i> G1	Clinical isolate from patient with Guillain-Barré syndrome	(67)
<i>C. jejuni</i> 11168H <i>kpsM::aphA3</i>	Mutant in gene encoding ABC transporter involved in capsule assembly; no CPS expression	(68)
<i>C. jejuni</i> 11168H <i>waaC::aphA3</i>	Mutant in gene encoding heptosyltransferase I; expresses truncated LOS	<i>Campylobacter</i> Resource Facility (http://crf.lshtm.ac.uk/index.htm)
<i>C. jejuni</i> 11168H <i>waaF::aphA3</i>	Mutant in gene encoding heptosyltransferase II: expresses truncated core oligosaccharide	<i>Campylobacter</i> Resource Facility (http://crf.lshtm.ac.uk/index.htm)
<i>C. jejuni</i> 11168H <i>cj0511::aphA3</i>	Mutant in gene encoding secreted carboxyl-terminal protease.	<i>Campylobacter</i> Resource Facility (http://crf.lshtm.ac.uk/index.htm)
11168H <i>cj0511::aphA3</i> pWJ4	Complemented strain containing <i>cj0511</i> gene and Cam ^r inserted into <i>cj0046</i>	This study
<i>C. rectus</i> NCTC 11489	Type strain, from human periodontal pocket	Public Health England culture collections

<i>E. coli</i> JM109	Cloning host	Promega
<i>E. coli</i> BL21 (DE3)	Expression host	New England Biolabs
Plasmid		
pJMK30	pUC19 carrying <i>C. coli</i> Kan ^r gene <i>aphA-3</i>	(69)
pGEM-T-Easy	Cloning vector enables TA cloning	Promega
pC46fdxA	Complementation vector containing <i>fdxA</i> promoter, Cam ^r selection marker.	(34)

754

755

756 Table 2. Oligonucleotides

757

Name	Sequence (5'-3')	Use
WJ10F	GTC <u>CGTCTCACATG</u> TTGAAAACAAAACG	Amplification of <i>cj0511</i> for cloning in pC46fdxA (BsmBI site underlined)
WJ10R	GTC <u>CGTCTCACATG</u> TATTATGTCCTTGTTG	Amplification of <i>cj0511</i> for cloning in pC46fdxA (BsmBI site underlined)
WJ4	GTAAATTTTTGATTATCAAATTTACATTATTTAAG	Orientation of <i>cj0511</i> with respect to P_{fdxA} in pC46fdxA; anneals in P_{fdxA}
WJ11	GGAACACAGCAGAGCACTTG	Confirmation of <i>cj0511</i> complementing strain; anneals in <i>cjs03</i> RNA coding sequence located 5' to <i>cj0046</i>
WJ12	CCTGGAGAAGTATTAGATAGTAGCG	Confirmation of <i>cj0511</i> complementing strain; anneals in <i>cj0053c</i> located 3' to <i>cj0046</i>

758

759

760 **FIG. 1.** Exposure to pancreatic amylase promotes growth and results in a large,
761 mucoid colony. A. *C. jejuni* 11168H grown at 37°C for 72 h on BA containing 0.4%
762 charcoal without (left) or with (right) 100 μM HPA. The ruler marks 5 mm intervals. B.
763 *C. jejuni* 11168H growth with shaking (50 rpm) in 5% CO₂ at 37°C in MEM-α with
764 (squares) or without (diamonds) 100 nM HPA. Data reported as means and standard
765 deviations (SD) from three independent experiments. The statistical significance of
766 the difference in growth in the presence and absence of HPA was assessed at two
767 time points (10 and 15 h) using a two-sample t-test, assuming unequal variance;
768 ***p<0.001.

769

770 **FIG. 2. Physiological concentrations of pancreatic amylase induces α-dextran**
771 **secretion in *C. jejuni* and the secreted protease, Cj0511 is required.** (A) Total
772 extracellular carbohydrate in *C. jejuni* 11168H grown in the presence of increasing
773 concentrations of HPA. Data reported as mean and standard deviation from three
774 experiments (p<0.0001 for the contrast between the three lowest doses and the four
775 highest doses of pancreatic amylase; p<0.0001 for the contrast between 50 mM and
776 100 nM pancreatic amylase within one-way ANOVA). (B) Total extracellular
777 carbohydrate in different *C. jejuni* strains grown in the presence (black bars) or
778 absence (white bars) of 100 nM HPA. Data reported as mean and standard deviation
779 from three experiments. The *kpsM*, *waaC* and *waaF* mutants were created in strain
780 11168H. Asterisks indicate statistically significant increases in the presence of HPA
781 determined by a two-sample t test; ***p<0.001, **p<0.01, *p<0.05. (C) ¹H-NMR
782 spectra of extracellular material from *C. jejuni* 11168H, a *kpsM* mutant and a
783 commercial α-dextran recorded in D₂O at 7°C using acetone as an internal standard

784 on a Bruker Avance 400 MHz spectrometer. (D) Total extracellular carbohydrate in
785 *C. jejuni* 11168H (black bars), a *Cj0511* mutant (white bars) and a *Cj0511*
786 complemented strain (grey bars) grown on MHA with increasing concentrations of
787 HPA. A two-way ANOVA showed that the interaction between the bacterial strain
788 and the dose of pancreatic amylase was highly significant ($p < 0.0001$). The contrasts
789 comparing the pattern of the response between the wild-type strain and the *Cj0511*
790 mutant and between the mutant and the complemented strain were both statistically
791 significant ($p < 0.0001$).

792

793 **Fig. 3. Proteolysis of casein and pancreatic amylase by r-Cj0511.** Coomassie-
794 stained SDS-polyacrylamide gel showing no detectable proteolysis of polyhistidine
795 tagged human pancreatic amylase. Casein served as a positive control.

796

797 **Fig. 4. Exposure to pancreatic amylase promotes biofilm formation.** (A) Biofilm
798 formation at 48 h by *C. jejuni* strains on glass tubes in the presence (black bars) or
799 absence (white bars) of 100 nM HPA. Data reported as means and SD from three
800 independent experiments. Asterisks indicate statistically significant increases in the
801 presence of HPA; $***p < 0.0001$. (B) Biofilm formation at 48 h by *C. jejuni* 11168H on
802 glass tubes in the presence (black bars) or absence (white bars) of 100 nM
803 recombinant human amylase. Data reported as mean \pm SD from three independent
804 experiments. Asterisks indicate statistically significant increases in the presence of
805 pancreatic amylase; $***p < 0.001$. (C) Biofilm formation at 48 h by *C. jejuni* 11168H on
806 glass tubes in the presence (black bars) or absence (white bars) of chicken pancreas
807 extract. Data reported as mean \pm SD from three independent experiments.

808 Asterisks indicate statistically significant increases in the presence of pancreatic
809 amylase; *** $p < 0.001$. (D) CLSM of Live/Dead stained *C. jejuni* 11168H biofilm at 48
810 h. Top panels, confocal images of bacteria in x-y plane, bottom panels, digital
811 images in x-z plane.

812

813 **FIG. 5. Pre-exposure to pancreatic amylase promotes interaction with human**

814 **intestinal epithelial cells.** (A) Interaction with Caco-2 cells by *C. jejuni* 11168H
815 grown in the presence (black bars) or absence (white bars) of 100 nM HPA. Bacteria
816 were co-cultured with Caco-2 cells for 3, 6 and 24 h. (B) Invasion of Caco-2 cells by
817 *C. jejuni* 11168H grown in the presence (black bars) or absence (white bars) of 100
818 nM HPA. (C) Apical IL-8 levels in T84 cells at 24 h in response to *C. jejuni* 11168H,
819 the *Cj0511* mutant and the complemented strain grown in the presence (black bars)
820 and absence (white bars) of 100 nM HPA. (D) Translocation of *C. jejuni* in T84 cells
821 at 6 h. *C. jejuni* 11168H, a *Cj0511* mutant and a complemented strain were grown in
822 the presence (black bars) and absence (white bars) of 100 nM HPA. For (A) to (C),
823 data reported as mean \pm SD from three independent experiments. For (C), analysis
824 by one-way ANOVA showed that there was a significant increase in IL-8 secretion in
825 response to infection ($p < 0.05$); the construction of contrasts within ANOVA showed
826 that there was no significant increase in the levels of IL-8 in response to any of the
827 bacterial strains grown with pancreatic amylase compared to the same bacteria
828 grown in its absence ($p > 0.05$). For (D), data reported as mean \pm SD from three
829 independent experiments for *C. jejuni* 11168H and the *cj0511* mutant. For the *cj0511*
830 complemented strain, the mean \pm SD from two independent experiments is
831 reported as a result of contamination in the third experiment, thus no statistical
832 analysis is presented. *** $p < 0.0001$, * $p < 0.05$.

833

834 **FIG. 6. Pre-exposure to pancreatic amylase promotes infection of *Galleria***
835 ***mellonella* larvae.** Killing of *G. mellonella* larvae by *C. jejuni* grown in the presence
836 (black bars) or absence (white bars) of 100 nM HPA. 10 larvae were infected with
837 each strain or with PBS alone. Injection with PBS did not kill any larvae. The *kpsM*
838 mutant and the *cj0511* mutant and complemented strain were derived from *C. jejuni*
839 11168H. Data reported as mean and SD from three independent experiments.
840 Asterisks indicate statistically significant increases in the presence of HPA;
841 **p=0.001.

842

843 **FIG. 7. Pre-exposure to pancreatic amylase promotes colonisation of broiler**
844 **chickens.** (A) and (B) number of birds from which *Campylobacter* was isolated from
845 the ileum at 4 d and 7 d post infection, respectively. *C. jejuni* was grown with (black
846 bars) or without HPA (white bars). n=15 birds per group. By day 7, exposure of the
847 WT strain to HPA prior to infection resulted in infection in a greater number of
848 chickens (p=0.005; indicated by bar). (C) and (D) cfu of *Campylobacter* per gram of
849 ileal contents in birds 4 d and 7 d post infection, respectively. Open symbols show
850 strains grown without HPA, closed symbols show strains grown with HPA. Significant
851 differences were seen between groups on days 4 (C; P=0.0006) and 7 (D;
852 P=0.0026) by Kruskal-Wallis test. Bars indicate groups between which differences
853 occur assessed by Dunn's multiple comparison test. n=15 birds per group.

854

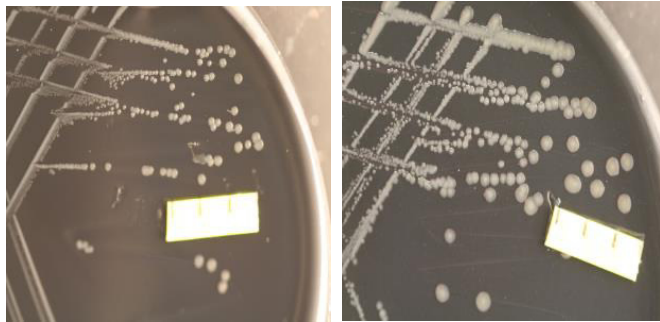
855 **FIG. 8. Pre-exposure to pancreatic amylase promotes survival in stress**
856 **environments.** (A). Survival at 4°C of *C. jejuni* 11168H grown with (squares) or

857 without (diamonds) HPA. (B). Survival at 20°C in air of *C. jejuni* 11168H grown with
858 (squares) or without (diamonds) HPA. Data reported as means +/- SD from three
859 independent experiments. Statistical significance between the counts in the
860 presence and absence of HPA was assessed using a two-sample t-test, assuming
861 unequal variance; **p<0.01, ***p<0.001.

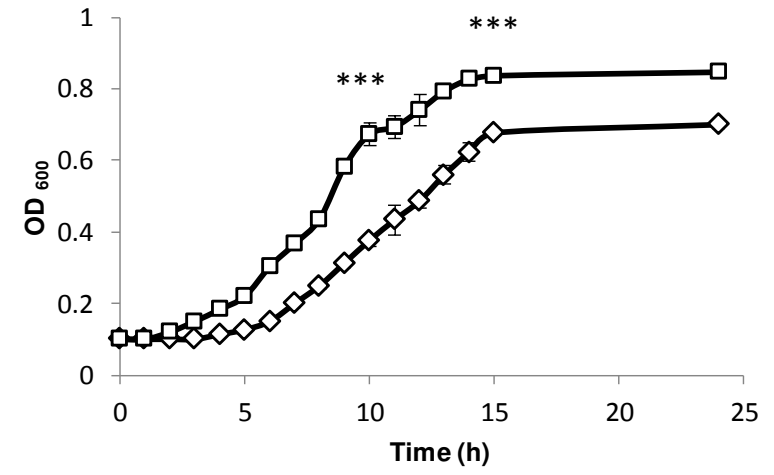
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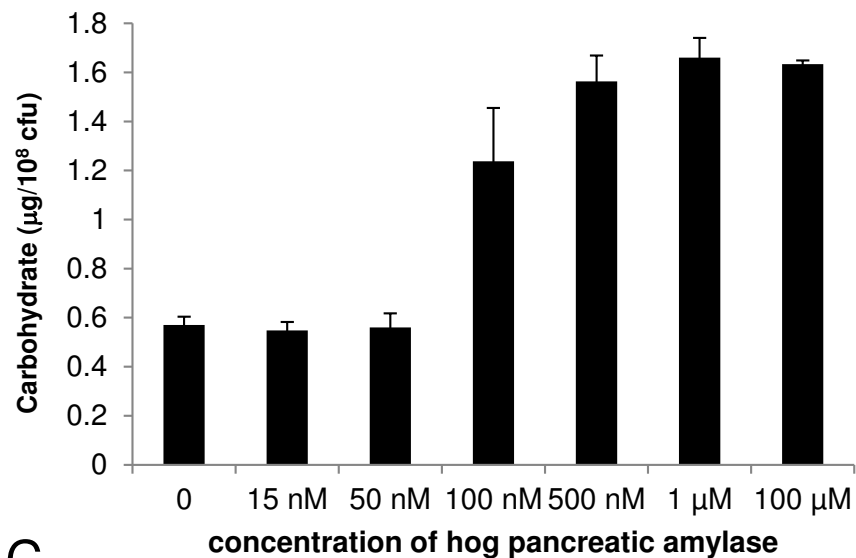
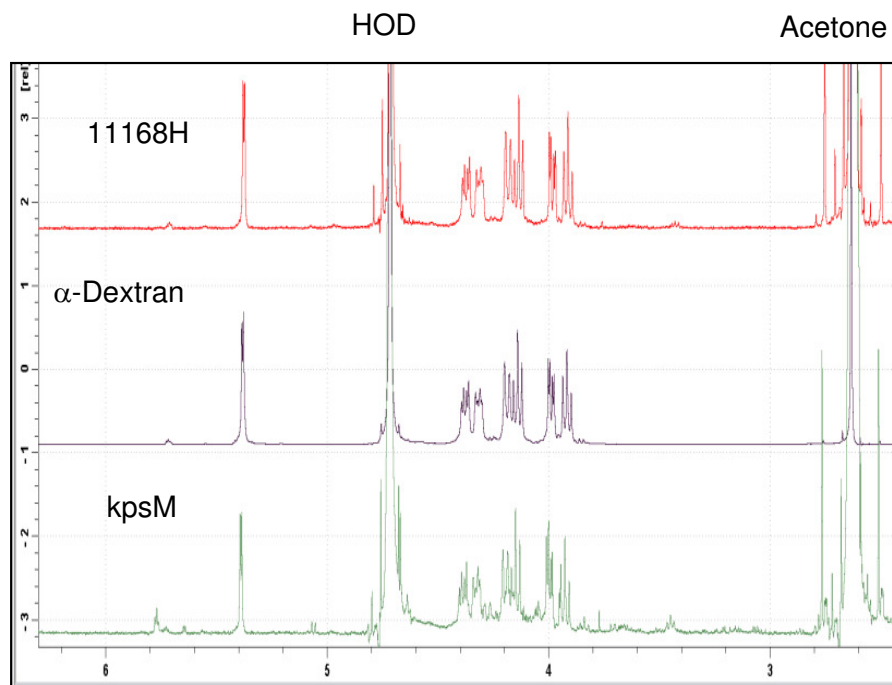
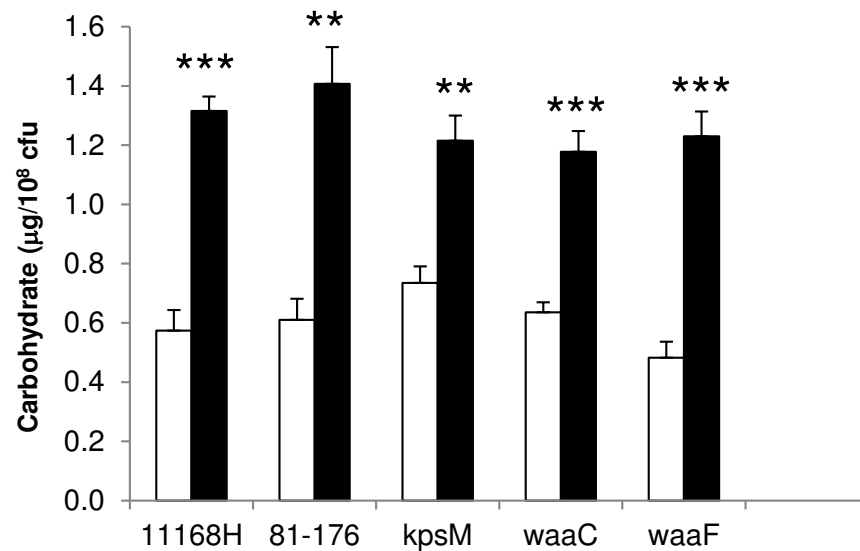
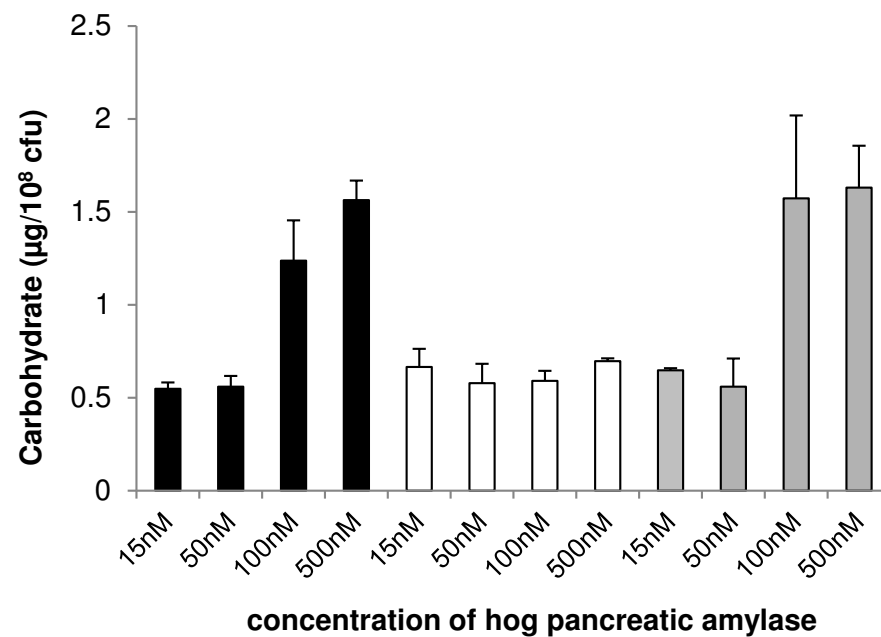
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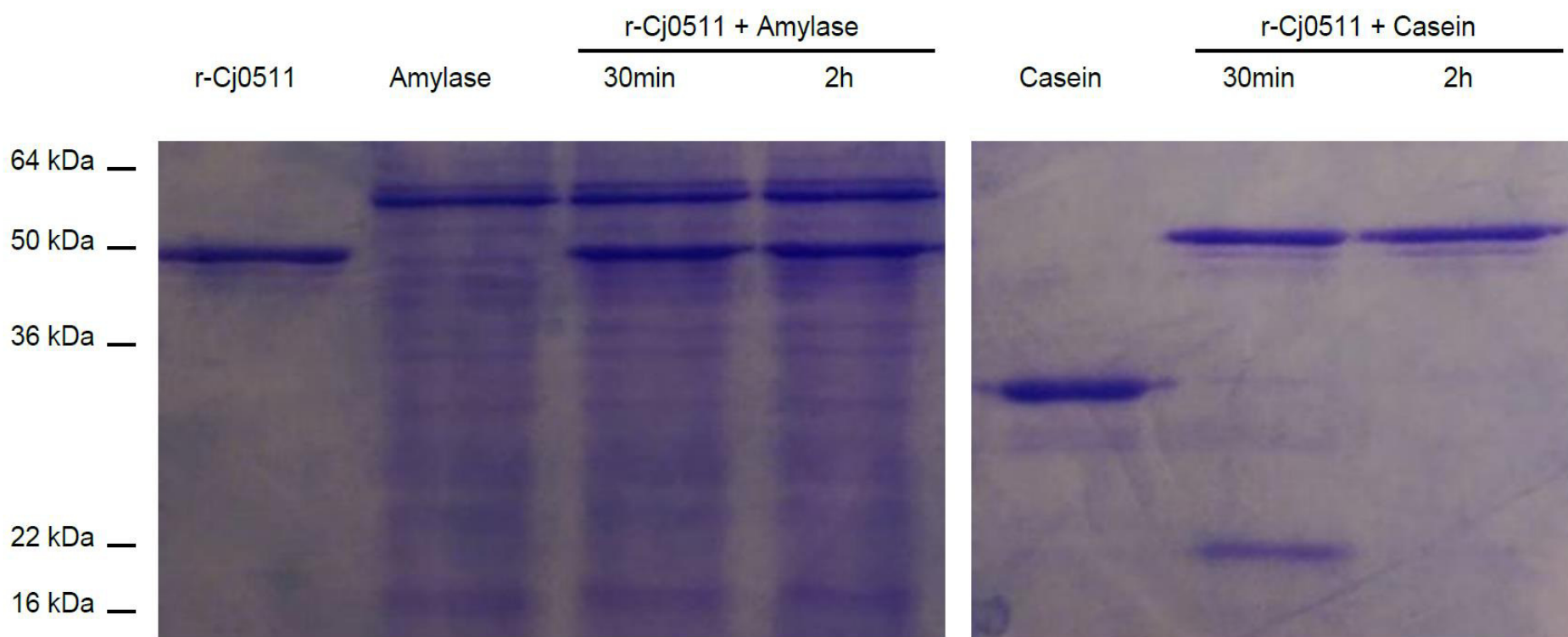
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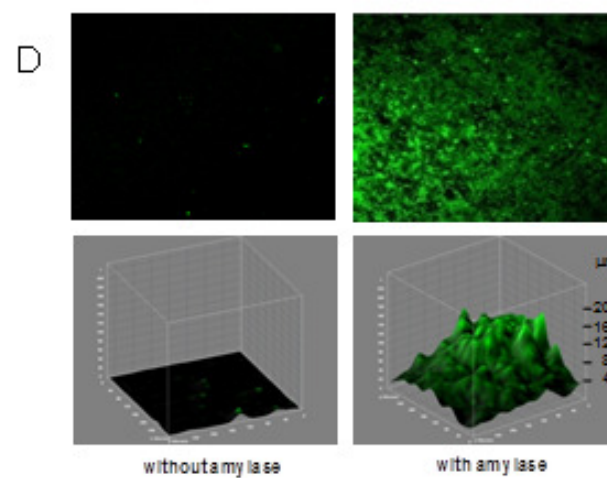
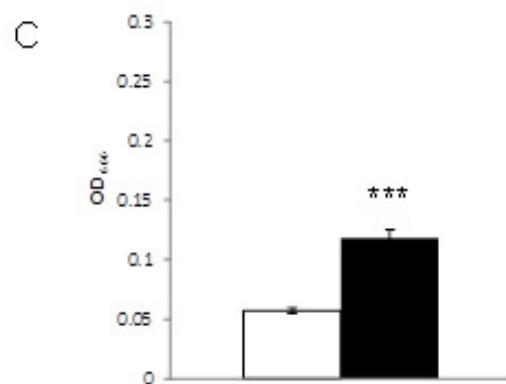
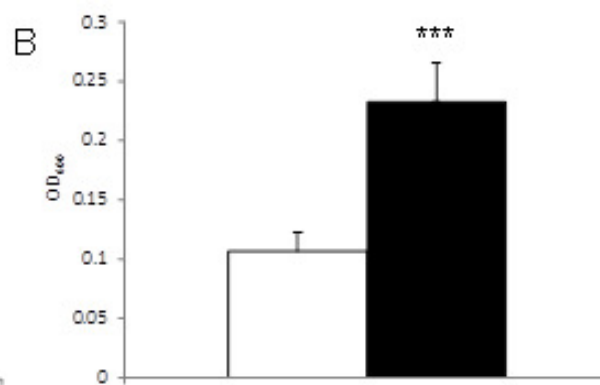
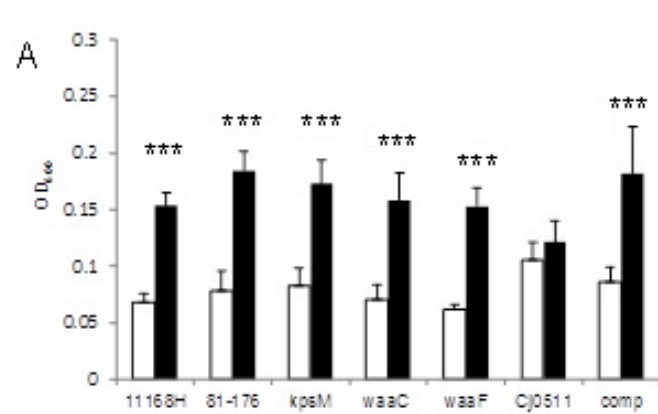


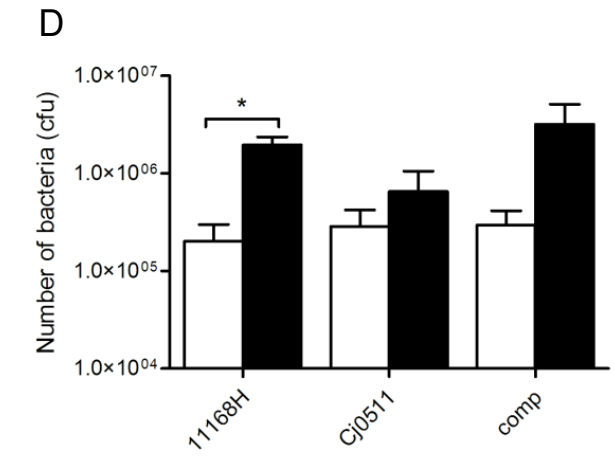
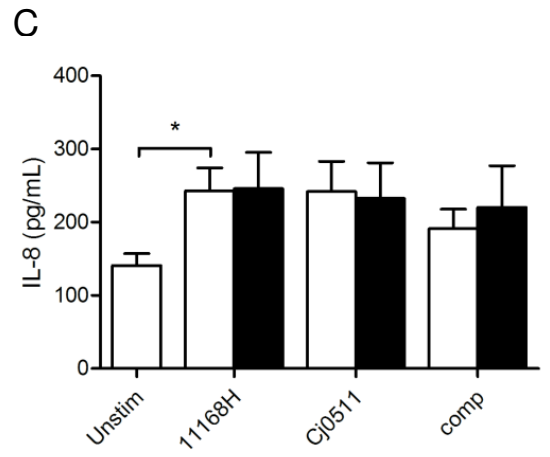
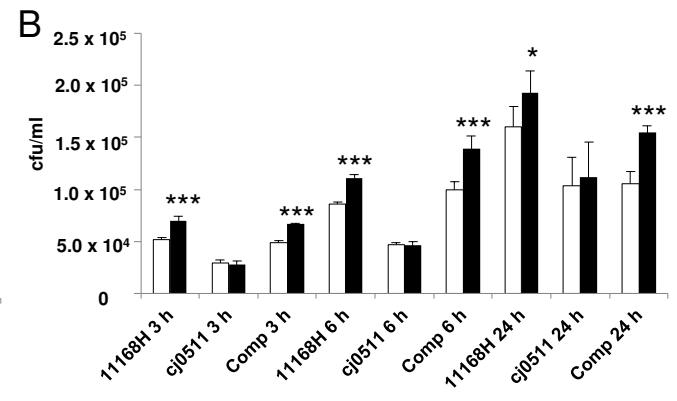
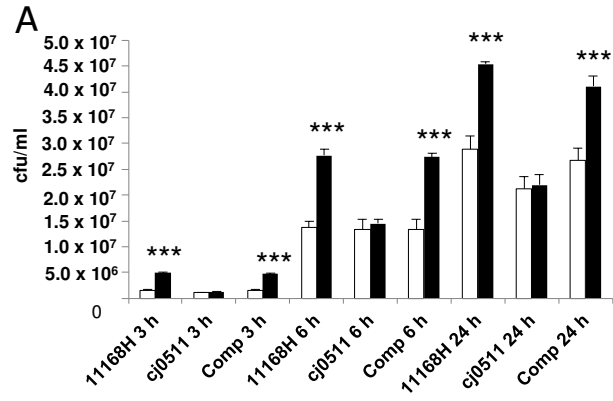
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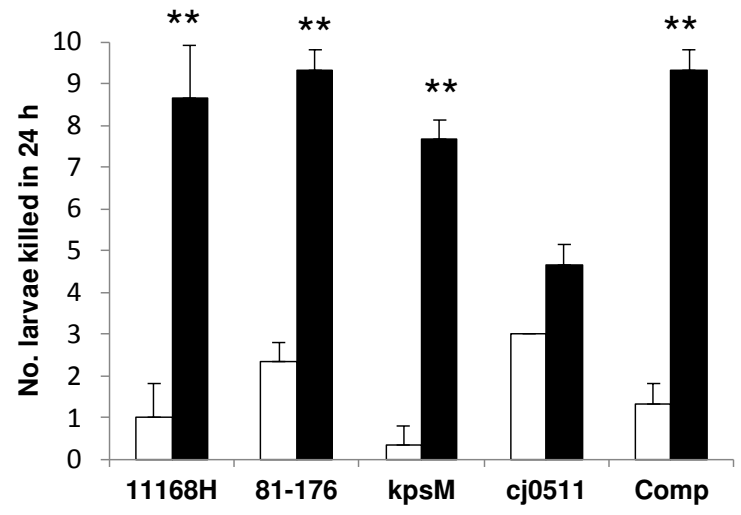


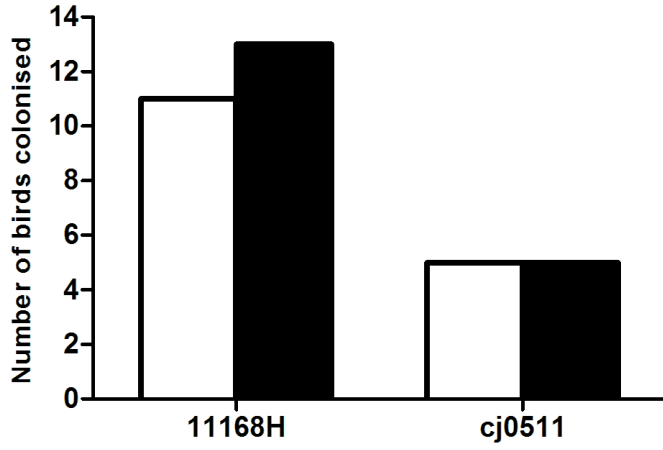
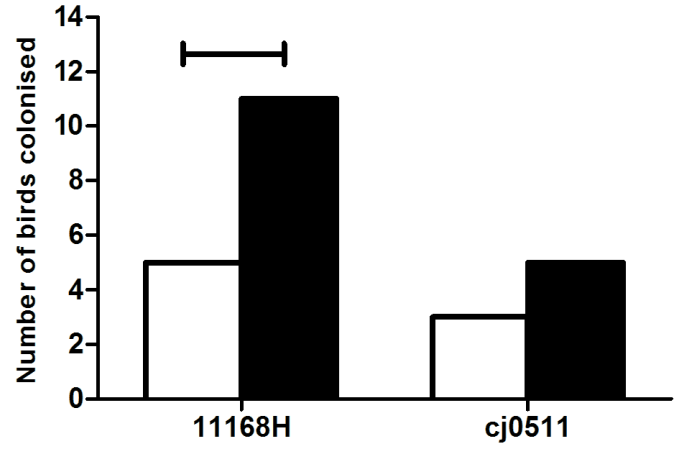
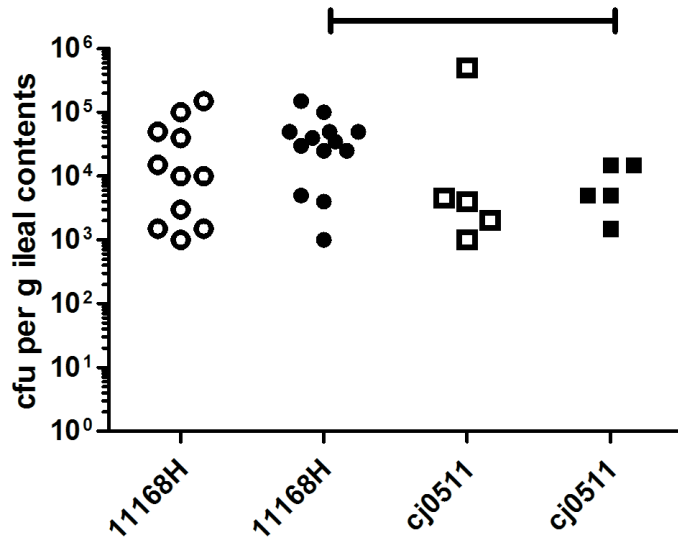
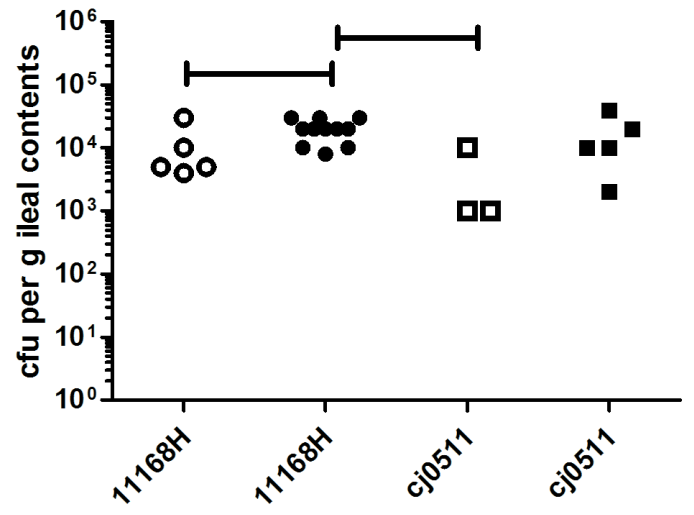
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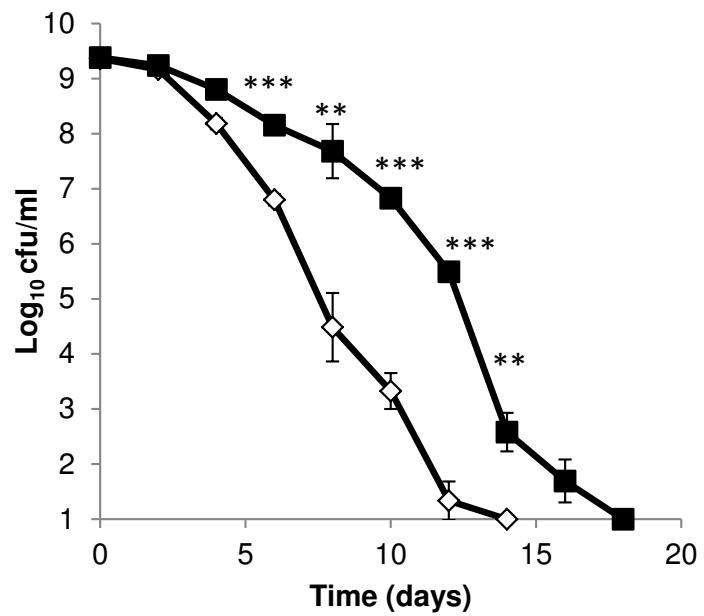








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