Phosphate transporter PstSCAB of *Campylobacter jejuni* is essential for lactate-dependent growth and colonization in chickens

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Abstract

Campylobacter jejuni causes acute gastroenteritis world-wide and is transmitted primarily through poultry, in which it is often a commensal member of the intestinal microbiota. Previous RNASeq experiments showed that transcripts from an operon encoding a high affinity phosphate transporter (PstSCAB) of *C. jejuni* were among the most abundant when grown in chickens. Elevated levels of the *pstSCAB* mRNA were also identified in an RNASeq experiment from human infection studies. In this study, we explore the role of PstSCAB in the biology and colonization potential of *C. jejuni.* Our experimental results demonstrate that cells lacking PstSCAB survive poorly in stationary phase, nutrient-limiting media, and under osmotic conditions reflective of those in the chicken. Polyphosphate levels in the mutant cells were elevated at stationary phase, consistent with alterations in expression of polyphosphate metabolism genes. *C. jejuni* were highly attenuated in colonization of newly hatched chicks, recovered at levels several orders of magnitude below wild type. Mutant and wild type grew similarly in complex media but the *pstSCAB* mutant exhibited a significant growth defect in minimal media supplemented with L-lactate, postulated as a carbon source *in vivo*. Poor growth in lactate correlated with diminished expression of acetogenesis pathway genes previously demonstrated as important for colonizing chickens. The phosphate transport system is thus essential for diverse aspects of *C. jejuni* physiology and *in vivo* fitness and survival.

Importance:

C. jejuni causes millions of gastrointestinal infections annually worldwide. Poultry and poultry products are major sources of *C. jejuni* infection to human as the microbe is a commensal colonizer of the chicken gastrointestinal tract. Due to the emergence of multi-drug resistance in *C. jejuni*, there is need to identify alternative ways to control this pathogen. Genes encoding the high-affinity phosphate transporter PstSCAB were highly expressed during colonization of *C. jejuni* in chicken and human. In this study, we address the role this high-affinity phosphate transporter PstSCAB of *C. jejuni* on chicken colonization and for its general physiology. PstSCAB is required for colonization in chicken, metabolism and survival under different stress responses and during growth on lactate, a potential substrate for growth of *C. jejuni* in chickens. Our study highlights that PstSCAB may be an effective target to develop mechanisms to control the bacterial burden in both chicken and human.

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109 **Introduction**
110
111 *Campylobacter jejuni* is a microaerophilic, Gram-negative bacterium that causes acute
112 gastroenteritis worldwide (1). Common campylobacteriosis symptoms are self-limiting diarrhea
113 (which may be watery to bloody), with nausea, fever and abdominal cramping in human. As *C.*
114 *jejuni* is a common member of the chicken gastrointestinal microbiota, poultry meat is a major
115 source of human campylobacteriosis, either through direct consumption of poultry or through
116 cross-contamination of other food (2, 3). As consumption of poultry meat worldwide over the
117 last 20 years has approximately doubled, (4) the threat of campylobacteriosis remains high.
118 To explore mechanisms by which *Campylobacter* colonizes the chicken gastrointestinal
119 tract, we used RNASeq to determine the transcriptome of *C. jejuni* strain 81-176 harvested from
120 ceca of seven day-old chickens after experimental oro-gastric inoculation on day-of-hatch (5).
121 Among the more highly abundant transcripts in this population were those encoding a predicted
122 phosphate transporter (*pstSCAB*), suggesting that phosphate transport is important for the *in vivo*
123 fitness of *C. jejuni*, and perhaps that inorganic phosphate plays a key role of chicken colonization
124 (5).
125 Phosphorus, or inorganic phosphate (Pi), is one of the most important macronutrients for
126 microbial growth. It is required for biosynthesis of cellular components such as nucleic acid,
127 phospholipids and proteins, and is critical for maintaining a cellular energy source in ATP. It is
128 also involved in regulating carbon and nitrogen metabolism (6). Uptake and storage of inorganic
129 phosphate, a growth-limiting nutrient, is heavily regulated in bacteria. Generally, in Gram-
130 negative bacteria, a two-component sensor-kinase/response regulator system controls phosphate
131 transport in response to levels of available inorganic phosphate. In *C. jejuni*, a two component

132 regulatory system, PhosSR, controls the high affinity phosphate transporter operon (*pstSCAB*),
133 as well as other genes, in phosphate limiting condition (7). PstSCAB comprises an ATP binding
134 cassette (ABC) transporter system in the inner membrane of many Gram-negative bacteria, with
135 PstS the periplasmic phosphate binding protein, PstAC comprising transmembrane protein
136 permeases and PstB an ATP binding and hydrolyzing subunit. PstS bound to Pi is stimulated to
137 associate with PstA/C transmembrane proteins. This triggers ATP hydrolysis in the PstB dimer
138 which induces the conformational changes that facilitate release of Pi into the cytosol (8). In
139 many bacteria a PstSCAB-associated protein, PhoU, acts to reduce expression of the Pho regulon
140 under conditions of phosphate sufficiency (6), but a PhoU homologue has not been reported in *C.*
141 *jejuni*.

142 Phosphate transporters contribute to virulence in other microbes, although the molecular
143 mechanisms by which that occurs varies across species and in any event are incompletely
144 understood (9). For example, inoculation into chickens of an avian pathogenic *E.coli* mutant
145 strain lacking *pst* led to fewer extra-intestinal lesions than did inoculation of wild type, and the
146 mutant also showed higher sensitivity to rabbit serum (though not to chicken serum), polymyxin
147 B, and acid shock (10). A *pstS* mutant of virulent EHEC and EPEC showed significant adherence
148 and colonization defects in intestinal epithelial cells as well as in animal models, resulting from
149 altering expression of adhesin proteins (9, 11). *Mycobacterium tuberculosis* contains two
150 different phosphate transporters which regulate replication in macrophages and provide
151 protection from antimicrobial stress response (12, 13).

152 Given the high abundance of transporter mRNA during *in vivo* growth of *C. jejuni*, it
153 seems likely that the transporter is a determinant of colonization. We analyzed a mutant lacking
154 *pstSCAB* for several growth phenotypes as well as chicken colonization. We demonstrate a

155 severe colonization defect in mutants lacking the *pstSCAB* operon, and we also uncover
156 metabolic deficiencies in the mutant strain – particularly its inability to thrive on L-lactate at
157 concentrations approximating those in the chicken cecum - that likely contribute to loss of *in*
158 *vivo* fitness.

159 **Results**
160
161 **Mutagenesis of and characterization of the *C. jejuni pstSCAB* operon.** We constructed an
162 insertion allele using a cassette encoding resistance to kanamycin, inserted into the *pstS* gene of
163 *C. jejuni* strain 81-176 **(Fig.S1).** The resulting kanamycin resistant strain was used in qRT-PCR
164 experiments designed to analyze transcription of the *pstSCAB* locus. Consistent with the earlier
165 RNASeq study (5), qRT-PCR of the wild type locus using primers specific for the junctions
166 between open reading frames confirmed that the four genes are co-transcribed, a conclusion
167 further supported by the observation that insertion in the *pstS* gene, the most upstream gene of
168 the locus, resulted in loss of detectable downstream transcription (**Fig. 1**).

169 In the earlier RNASeq study abundance of the *pstS* mRNA was significantly higher than
170 distal genes in the operon (5). Quantitative RT-PCR experiments confirmed that mRNA
171 encoding *pstS* is more abundant than that of the *pstCAB* genes **(Fig. S2A)**. Differential stability
172 within a polycistronic mRNA can be controlled through a conserved internal RNA hairpin that
173 protects the transcript from RNA decay (14). The protective RNA structure is often present in the
174 open reading frame of the downstream gene to protect the essential upstream gene from decay
175 (14). Consistent with such a mechanism acting on the *pstSCAB* operon, we identified a
176 thermodynamically stable hairpin loop structure (ΔG=-27.70) within the protein coding region of
177 *pstC* gene **(Fig. S2B and C**). We hypothesize this RNA structure in *pstC* protects *pstS* mRNA
178 from decay, leading to its elevated abundance relative to the other genes in the operon. Because

179 of polarity from the *pstS::kan* insertion, for further experiments we used a complementing
180 plasmid encoding the entire *pstSCAB* operon. In describing work with mutant and complemented
181 strains, the strain is generally designated by its genotype (*pstS::kan*), and the strain with the
182 mutation and carrying the complementing plasmid is designated *pstS::kan/C.*

183 **Phosphate storage and metabolism gene products are altered in the *pstSCAB* mutant.**
184 Survival of *C. jejuni* in stationary phase and nutrient limiting condition is dependent on
185 polyphosphate (poly-P) metabolism (15). *C. jejuni ppk1* and *ppk2* encode poly-P kinases, with
186 the former responsible for poly-P synthesis, using ATP generated from inorganic phosphate (16),
187 and the latter generating GTP from poly-P (17). In some microbes Ppk2 can produce poly-P
188 from GTP (17), but this activity has not been reported in *C. jejuni*. *C. jejuni* also encodes two
189 exo-polyphosphatases, Ppx1 and Ppx2, which degrade poly-P into inorganic phosphate and have
190 guanosine pentaphosphate hydrolase activity to generate guanosine tetraphosphate (ppGpp) (18).
191 With the regulatory gene product SpoT, ppGpp regulates the stringent response of *C.jejuni,*
192 contributing to survival in stationary phase (19). Another key gene of phosphate metabolism is
193 *ppa*, which encodes an inorganic pyrophosphatase that hydrolyses pyrophosphate to inorganic
194 phosphate (PPi→Pi)(19).

195 We investigated the effect of a *pstSCAB* mutation on these phosphate storage and stress
196 response mechanisms, examining expression in wild type, mutant, and complemented mutant
197 strains after 24 and 48 hours of growth in MHB. The gene largely responsible for poly-P
198 synthesis, *ppk1*, was unchanged in its level of expression in the mutant versus the wild type at
199 either time point. Expression of *ppk2,* which converts GDP to GTP using poly-P as a substrate -
200 and is reported in some microbes (although not in *C. jejuni*) to produce poly-P from GTP - was
201 elevated up to 10-fold in the mutant strain at 24 hours, returning to wild type expression levels
202 by 48 hours **(Fig. 2A)**. At 48 hours, expression levels of an exo-polyphosphatase gene (*ppx2*),

203 the inorganic pyrophosphatase gene *ppa,* and the stringent regulatory gene *spoT* were
204 significantly reduced in the mutant compared to wild type (**Fig. 2B**). As *ppx2* and *spoT* of
205 *C.jejuni* regulate synthesis of ppGpp, important for survival in stationary phase, reduced
206 expression of these two genes would likely result in decrease in the level of ppGpp in the
207 *pstS::kan* mutant strain.

208 At 24 hours, poly-P levels in the mutant were roughly half of those observed in the wild
209 type. At 48 hours, by which time the mutant had diminished in viability by several orders of
210 magnitude (see below, Fig. 3B), poly-P was elevated to about three times the levels observed in
211 wild type (**Fig. 2C and D)**. With elevated *ppk2* expression at 24 hours in the *pstS::kan* mutant,
212 decreased intracellular poly-P might be expected, as the Ppk2 enzyme consumes poly-P to
213 convert GDP to GTP. The higher levels of poly-P observed in the *pstS::kan* mutant compared to
214 wild type at 48 hours is readily explained by decreased expression of the exopolyphosphatase
215 gene *ppx2* in the mutant background, and is consistent with a similar report of a *pstS::kan* mutant
216 of *Pseudomonas aeruginosa* (20).

217 ***pstS* is essential for stationary phase survival and osmotic stress response.** Expression of *pstS*
218 is elevated approximately four-hundred-fold in 0.08 mM inorganic phosphate (Pi) compared with
219 1.6 mM Pi, through activation of the PhoRS system at the more limiting phosphate concentration
220 (7). The PhoRS-regulated alkaline phosphatase gene of *C. jejuni* (*phoX*) is also induced at lower
221 phosphate concentration, providing Pi for transport into the cell by the PstSCAB system (21).
222 We hypothesized that the *pstS::kan* mutant strain would signal phosphate limitation to the cell,
223 even at a concentration (1.6 mM) that does not typically result in *phoX* expression. We measured
224 alkaline phosphatase activity in wild type and *pstS::kan* mutant cells grown in 0.08 mM and 1.6
225 mM phosphate. Supporting our hypothesis, the mutant expressed significantly elevated alkaline
226 phosphatase activity at both the lower (0.08 mM) and higher (1.6 mM) concentrations, whereas
227 wild type (and the complemented mutant) expressed high levels of alkaline phosphatase activity

228 only at the lower phosphate concentration (**Fig. 3A**). We conclude that, even at elevated
229 phosphate concentrations, the *pstS::kan* mutant strain senses phosphate limitation.

230 To assess the effect of loss of *pstSCAB* on growth of *C. jejuni*, we cultured wild type,
231 mutant, and complemented mutant strains in Mueller-Hinton broth (MHB), a complex medium
232 of protein hydrolysate and infusion. All grew to similar levels for 24 hours, peaking at 10^{10}
233 cfu/ml **(Fig. 3B).** By 48 hours, the wild type and complemented mutant strains had dropped
234 about two orders of magnitude in plating efficiency, while the mutant strain had dropped by over
235 five orders of magnitude in plating efficiency, suggesting a severe defect in survival in stationary
236 phase and consistent with the diminished levels of poly-P we detected in the cells at that time-
237 point. Assessing survivability in media depleted of nutrients provided a more direct examination
238 of the challenge posed by loss of *pstSCAB*. In this experiment the wild type strain was grown for
239 48h in MH broth to reach stationary phase, after which the growth media was collected by filter
240 sterilization. We inoculated mid-log phase wild type, *pstS::kan* and the complemented mutant in
241 the sterilized depleted media, with initial inoculum (5×10^8 CFU/ml), and assessed viable cells at
242 24 hours. In contrast to wild type and complemented mutant, which were still recoverable at high
243 numbers in depleted media, the mutant was completely unrecoverable **(Fig. 3C)** confirming that
244 loss of the phosphate uptake system causes a significant defect in growth physiology. Likely
245 contributing to this loss of fitness under limiting nutrient conditions **(Fig. 3D)** is the decreased
246 expression demonstrated **(Fig. 2B)** in of *ppx2* and *spoT,* regulators of ppGpp synthesis.

247 In addition to stationary phase survival, poly-P metabolism contributes to survival under
248 osmotic stress, based on analysis of strains carrying mutations in the genes described above (16).
249 To assess whether phosphate transport *per se* contributes to osmotic survival, we analyzed wild

250 type and *pstS::kan* mutant growth and survival at 1% and 1.5% NaCl, which approximate
251 concentrations within the chicken gastrointestinal tract (22). The *pstS::kan* mutant strain
252 demonstrated a significant growth defect (**Fig. 3E)** and lower plating efficiency than wild type
253 on 1% and 1.5% NaCl **(Fig. 3F and G).** The mutant strain was restored to wild type levels of
254 plating by complementing with *pstSCAB* in trans, confirming that loss of viability at elevated
255 osmolarity is due to loss of phosphate transport. Thus, similar to mutant strains lacking *ppx1* and
256 *ppx2* (exo-polyphosphatases) (18), which are unable to degrade polyphosphate, the phosphate
257 transporter mutant survives poorly under osmotic stress conditions that are similar to those found
258 in the chicken gastrointestinal tract.

259 **Loss of PstSCAB compromises *C. jejuni* chicken colonization and fitness** We investigated
260 whether elevated abundance of *pstS* mRNA in *C. jejuni* harvested from chick ceca, compared to
261 *C. jejuni* grown *in vitro* (5), correlates to actual requirement for *pstSCAB* during growth in the
262 chicken. We orally inoculated day-of-hatch chickens with two different doses (10^3 or 10^6 CFU)
263 of wild type *C. jejuni* 81-176, the *pstS::kan* mutant derivative, and the complemented mutant,
264 and enumerated *C. jejuni* loads in cecal contents on days three and seven after inoculation.
265 Irrespective of the inoculum dose, the mutant strain exhibited a large colonization defect at both
266 days three and seven compared with wild type **(Fig 4A,B,C and D)**, being recovered
267 approximately five orders of magnitude less than wild type when introduced at the lower
268 inoculum and by roughly two orders of magnitude when introduced at the higher inoculum. In all
269 cases the complemented strain colonized at levels similar to wild type.

270 In addition to individual colonization assays with wild type and *pstSCAB* mutant strains,
271 we carried out a competition assay between the wild type strain 81-176 and the *pstSCAB*

272 derivative, mixing the strains at 1:1 concentration in the inoculum and introducing the
273 combination at 10^6 CFU/ml by oral gavage to day-of-hatch chickens. We harvested cecal
274 contents on day seven post-infection and determined the ratios of the mutant and wild type
275 strains by measuring relative numbers of kanamycin resistant (*pstSCAB* mutant) and sensitive
276 (wild type) *C. jejuni* in the output **(Fig 4E)**. The wild type strain exhibited greater fitness in this
277 experiment than the mutant by 100-fold. The wild type strain also out-competed the mutant when
278 grown together in nutrient-rich media or in minimal media supplemented with 80 uM phosphate
279 **(Fig. S4A and B)**. These findings indicate the essential nature of *pstSCAB* for chick colonization
280 and extend the earlier observation of high-level *in vivo* expression of this operon in the
281 transcriptome study (5).

282 **The phosphate transporter is essential for lactate-dependent growth.** Lactate is present in
283 both D- and L- isomers at varying concentrations in the chicken gastrointestinal tract, generally
284 decreasing from the upper to the lower intestine and ceca (23). Although at high concentrations
285 lactate inhibits growth of *C. jejuni* (24) and reduces expression of some colonization traits (23),
286 many strains - including 81-176 - have pathways for uptake of and growth in lactate, including a
287 transporter for L-lactate encoded by *lctP* (CJJ81176_0113) and two NAD-independent
288 membrane-associated L-lactate dehydrogenase complexes (CJJ81176_0112-0110) and
289 (CJJ81176_1182), which convert L-lactate to pyruvate (25). Mechanisms for uptake and
290 assimilation of D-lactate are less understood (25).

291 Given the reduced fitness of the *pstSCAB* mutant in the cecum, we investigated the
292 growth of the mutant on this potentially relevant carbon source in that environment. We cultured
293 wild type, mutant, and complemented mutant strains on minimal media containing D- + L-lactate
294 (10 mM total lactate) in both low (80 micromolar) and high (1.6 millimolar) phosphate

295 concentrations. In both phosphate concentrations, the mutant grew to levels 100-1000 times
296 lower than wild type at 24 and 48 hours of growth (**Fig. 5A and B**). This contrasts to cells
297 cultured in media with pyruvate as the carbon source, where mutant and wild type grew to
298 similar levels in high phosphate media (1.6 mM) and differed by only an order of magnitude at
299 the lower phosphate concentration (80 μM; **Fig. S5A-B)**. When cultured on L-lactate alone, the
300 *pstS::kan* mutant strain was significantly limited compared to wild type, irrespective of
301 phosphate concentration, while on D-lactate alone, the mutant was diminished for growth during
302 initial exponential growth phase (irrespective of phosphate levels) but ultimately attained the
303 same level of growth as the wild type and the complemented mutant (**Fig. 6A-D).** Collectively
304 these data suggest that the phosphate transport mutant is challenged for growth on L-lactate in
305 particular; even when both D- and L- lactate are available *in vitro* the mutant is at a significant
306 disadvantage compared to wild type (**Fig. 5)**. Given that D- and L-lactate may serve as growth
307 substrates for *C. jejuni* growth in the chick cecum (25), these data also contribute to explaining
308 the fitness disadvantage of the mutant *in vivo*.

309 Lactate-derived pyruvate is converted to acetate in *C. jejuni* via phosphotransacetylase
310 (Pta) and acetate kinase (AckA); this acetogenesis pathway is critical for establishing chicken
311 colonization (23). Genes controlled by this acetogenesis pathway may contribute to fitness in the
312 chick ceca including those encoding γ-glutamyl transferase (Ggt), a component of an amino acid
313 transporter complex (Peb1c), and a putative di/tri-peptide transporter (Cjj81176_0683) (23). The
314 higher L-lactate levels in the upper intestine, compared to those of the lower intestine and ceca,
315 lead to reduced expression of these genes through an undefined regulatory mechanism that
316 correlates to relatively lower levels of *C. jejuni* in the upper intestine (23). We hypothesized that
317 the poor growth on lactate of the *pstSCAB* mutant might also correlate with diminished

318 expression of acetogenesis-regulated colonization traits. To test this, we carried out quantitative
319 RT-PCR to measure transcript levels of *ggt*, *peb1c* and *Cjj81176_0683*. Expression of each was
320 reduced by two-to-four-fold in the mutant compared to wild type **(Fig 7).** This reduction in
321 expression is similar in magnitude to their reduced expression in the upper intestine where *C.*
322 *jejuni* colonizes poorly (23). Thus, we conclude that in addition to poor growth on L-lactate by
323 the mutant lacking PstSCAB, the strain also expresses potentially key colonization determinants
324 at levels apparently insufficient for cecal colonization.

325 **Metabolomic analysis of wild type and *pstSCAB* mutant *C. jejuni.*** To explore further the
326 growth physiology of wild type and *pstSCAB* mutant on lactate, we measured intracellular
327 metabolites using mass spectrometry. We observed no significant difference in intracellular
328 lactate or pyruvate between wild type and the *pstS::kan* mutant strain **(Fig S6A)** indicating that
329 the phosphate transport system has no effect on lactate uptake or its conversion to pyruvate. Of
330 metabolites we measured, adenine - and its deamination product hypoxanthine - were
331 significantly lower in the mutant compared with the wild type (**Fig. 8 B and C**), and this was
332 reflected in diminished ATP levels in the mutant as well. (**Fig. 8 A).** The low intracellular Pi
333 concentration in the *pstS::kan* mutant (as reflected in elevated alkaline phosphatase activity; **Fig.**
334 **3A)**, correlated to decreased intracellular ATP levels and might account in part for its poor
335 fitness. This was confirmed with ATP assays on extracts of cells cultured for 24 hours in
336 minimal-L-lactate-media demonstrating intracellular ATP concentrations in the *pstS::kan* mutant
337 significantly below those of wild type, irrespective of phosphate concentration **(Fig 8A)**. *C.*
338 *jejuni* does not encode a purine/pyrimidine or nucleotide/nucleoside transporter (26), and indeed
339 adenine supplementation (1uM or 10uM) of L-lactate media did not restore growth or ATP levels
340 in the mutant (**Fig. S7).**

341 **Discussion**

342 The *pstSCAB* phosphate transporter locus of *C. jejuni* is among the more significantly
343 expressed transcripts identified by RNASeq from microbes harvested from the chick cecum (5)
344 and during infection of humans (27). A transcriptomic study of *C. jejuni* colonizing chicks using
345 microarray analyses (28) did not report high level expression of *pstSCAB*, although there are
346 significant differences in the way the RNASeq and microarray studies were carried out with
347 regards to chicken inoculation and colonization. The RNASeq study (5) used an inoculum of 10^3
348 CFU of *C. jejuni*, and harvested cecal contents for RNA preparation after seven days of
349 colonization. In contrast the microarray study used a much higher inoculum of 10^9 CFU and
350 harvested cecal contents after only 12 hours of colonization (28). It is conceivable that these
351 differences in inocula and time of colonization would result in different physiological outcomes
352 in the *C. jejuni* recovered from the cecal contents.

353 Generally, elevated level of expression of the high-affinity phosphate transporter in
354 bacteria is observed in a low-phosphate environment. For example, transcription of *pstS* in
355 *C.jejun*i is significantly increased at 0.08 mM concentration compared to 1.6 mM (7). We
356 determined phosphate concentrations within the chicken cecum as relatively high, 0.5- 0.8
357 mM/100mg of cecal content **(Fig. S3)**. Further, previous metabolite studies estimated that the Pi
358 concentration in intestinal lumen of healthy adults are varied between 15-30 mM on a Western
359 diet (29). At these *in vivo* levels of inorganic phosphate, we would not expect elevated
360 expression of *pstSCAB* during colonization in chicks and humans, so it remains unclear what
361 drives this outcome during host colonization. We hypothesize that, although present at high
362 detectable levels, phosphate is nevertheless unavailable to *C. jejuni*, perhaps due to competition
363 from other microbes. We previously demonstrated *in vivo* competition for zinc, resulting in poor

364 colonization of a *C. jejuni* mutant lacking the *znuA* high-affinity zinc transporter. In that case, the
365 colonization defect of the *znuA* mutant was mitigated in chicks reared in germ-free conditions
366 harboring a limited microbiota (30).

367 The *pstSCAB* mutant of *C. jejuni* showed a significant colonization defect on chicken at
368 both inocula we tested (10^3 and 10^6 CFU), with the most significant defect at day three after
369 infection. From these data, we hypothesize that *pstSCAB* enables *C. jejuni* to survive initial
370 conditions after reaching the chicken gut. We also observed a dose dependent colonization defect
371 of the mutant at day seven. A previous study reported similar colonization levels between wild
372 type *C. jejuni* and a mutant lacking *phoRS*, which encodes a regulator of *pstSCAB* expression (6).
373 That study used a different strain of *C. jejuni*, and the animal data were not shown, so we do not
374 know what infection dose was used or what time points were assessed post-inoculation. Another
375 study demonstrated that mutants lacking the alkaline phosphatase PhoX have diminished
376 capacity for colonizing chickens compared to wild type (21) and that this is a dose-dependent
377 outcome with somewhat greater colonization defect of the mutant observed after a low dose (10^3)
378 inoculum by comparison to a higher dose inoculum (10^5).

379 As a proxy for analyzing fitness of wild type and *pstSCAB* mutant in humans, we
380 measured growth of WT and *pstSCAB* mutant strain *in vitro* in minimal media with 10% human
381 fecal extract, in which the phosphate concentration was approximately 500 μM **(Fig. S8A)**. The
382 mutant strain grew in this medium after 24 hours, but nevertheless grew to levels 10x lower than
383 wild type and the complemented mutant (**Fig. S8B**). Taken together with previous human
384 transcriptomic data demonstrating elevated levels of *pst* messages during human infection (27),
385 we conclude that phosphate transporter of *C. jejuni* is required for fitness in both chickens and
386 humans.

387 The *pstSCAB* operon is transcribed as a full-length polycistronic mRNA, demonstrated
388 both in the earlier RNASeq study and in our qRT-PCR presented in this work. Trancripts
389 encoded by the first gene in this operon, *pstS,* are present at much higher levels than those of the
390 three downstream genes (5). We postulate that a potential stem-loop structure encoded just
391 downstream of the *pstS* gene, which we postulate serves to stabilize *pstS* mRNA from
392 degradation, resulting in higher steady state levels compared to the other genes of the operon. A
393 similar mechanism is proposed for the *pstS2* operon of *Vibrio cholerae* (31).

394 In complex media, PstSCAB is dispensable until late in growth, after which it is required
395 for cells to remain viable in the nutrient-depleted environment (**Fig.3**). As transcription of the
396 locus is elevated during mid-logarithmic growth (compared with stationary phase growth (5)) we
397 conclude that assembly of the system and Pi uptake occurs early during growth and enables
398 survival later when the locus is far less transcriptionally active. Accumulation of poly-P in the
399 *pstSCAB* mutant late in growth may be the result of decreased expression of the exo-
400 polyphosphatase genes *ppx1* and *ppx2*, which encode enzymes that metabolize poly-P.
401 Consistent with a need to balance poly-P production and metabolism for wild type fitness in *C.*
402 *jejuni* others, have demonstrated that *phoX* and *ppk1/ppk2* mutants also have survival defects in
403 nutrient limiting condition (18). Increased poly-P was also observed in phosphate transport
404 mutants of *M. tuberculosis* (32).

405 The failure of the *pstSCAB* mutant to thrive - and its loss of viability - in 10 mM lactate,
406 on which wild type grows to high levels, is worth further analysis. The mutant strain exhibits
407 reduced expression of the *pta-ackA* genes in the acetogenesis pathway, which could be partly
408 responsible for the decreased ATP levels in the mutant compared to the wild type. Lactate at
409 high concentrations causes membrane damage to *C. jejuni* and is the basis for the suggestion that

410 *Lactobacillus* strains that produce high lactic acid levels might be useful probiotics to reduce *C.*
411 *jejuni* loads in poultry (24). Concentrations required for killing *C. jejuni* are much higher than 10
412 mM, but in the context of loss of *pstSCAB* perhaps the cell is sensitized to lactate toxicity. A
413 mutant of avian pathogenic *E. coli* lacking *pst* was demonstrated to be more sensitive to
414 membrane-targeting polymyxin B, but we did not observe that with *pstSCAB* mutant *C. jejuni*.
415 How *pstSCAB* contributes to lactate-dependent growth will be the subject of future research.
416

417 **Methods and Materials**

418 **Bacterial Strains and Media** All strains and plasmids used in the study are listed in Table 1.
419 The wild type strain used in all experiments is DRH212, a streptomycin resistant derivative of *C.*
420 *jejuni* 81-176 (33). All *C. jejuni* strains were routinely grown under microaerobic conditions
421 (85% N2,10%CO2, 5% O2) on Muller-Hilton (MH) agar or in broth under necessary antibiotics
422 at the following concentrations: chloramphenicol, 15 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; TMP, 10
423 μg ml^{-1}; streptomycin, 2 mg ml^{-1}. *C. jejuni* strains were stored in MH broth with 20% glycerol at
424 −80°C.

425 **Construction of a *pstS::kan* insertion mutant and complementation** *C. jejuni pstS*::*kan*
426 mutant was constructed by insertion of kanamycin resistance cassette into the gene as previously
427 described (34). Briefly, the 500bp of both upstream and downstream regions of *pstS* locus were
428 amplified by PCR with flanking region containing SmaI restriction site. The amplified upstream
429 and downstream region were joined by Soeing PCR. The 1000bp amplified PCR products was
430 cloned into pGEM-T easy. The *Campylobacter* kanamycin resistance cassette was excised from
431 PILL600 using SmaI and ligated between upstream and downstream cloned region of pGEM-T
432 construct. The plasmid was electroporated into DRH212 strain and grown on MH agar overnight

433 at 37°C under microaerobic conditions. Then cells were plated and grown on MH agar
434 containing kanamycin for 2-3 days, and successful integration of *pstS::kan* into the chromosome
435 was confirmed by PCR. As we demonstrate in Results, this insertion is polar on the *pst* genes
436 downstream of *pstS*. Therefore, for complementation, the coding sequences of whole *pstSCAB*
437 operon - from the second codon of *pstS* to the stop codon of *pstB* - were amplified by PCR and
438 cloned into the BamHI site of pECO102 plasmid (35), which was introduced into *E.*
439 *coli*DH5α/pRK212.1 strain (35). Conjugation to *C. jejuni* was performed as previously described
440 by Guerry et al (36).

441 **Growth Curve analysis in MH broth and Minimal media** *C. jejuni* strains WT, *pstS::kan*,
442 *pstS::kan/C* were grown from freezer stocks on MH agar containing desired antibiotics for 24h
443 under microaerobic condition. The strains again re-streaked and grown for 16h. The bacteria
444 were suspended in MH broth and inoculated in 25ml of MH broth containing desired antibiotics.
445 The initial O.D was approximately 0.05 and bacterial numbers were measured at different time
446 intervals up to 48h. To determine growth of *C. jejuni* strains in different concentrations of
447 inorganic phosphate, strains were grown in MCLMAN minimal media containing different
448 carbon sources including sodium L-pyruvate, D-lactate and L-lactate (37) as noted. Strains were
449 washed twice with minimal media and inoculated with 25ml of minimal containing different
450 concentration of Pi. Then, the growth was measured via absorbance at 600nm (OD_{600}) up to 48h
451 with the initial O.D was 0.05.

452 **ATP measurements** ATP levels in strains were determined by BacTitre-Glo™ Assay Kit
453 (Promega) as previous described. Briefly, strains were grown for 24h in minimal media (L-
454 lactate) with high (1.6mM) and lower concentration (0.08mM) of Pi and normalized OD at 0.3.

455 Then ATP level were measured according to the manufacture's protocol and ATP concentrations
456 were measure by standard curve (26).
457 **Measurement of intracellular metabolites** Wild type strain DRH212, *pstS::kan* were grown in
458 presence of L-lactate with higher and lower concentration of phosphate for 18h. The cells were
459 collected by centrifugation and washed twice with normal saline. Then all samples were
460 normalized approximately with OD. 1. Then the samples were extracted for determination of
461 metabolites. Samples on dry ice were combined with 800 microliters of ice-cold
462 acetonitrile:methanol:acetone (8:1:1, v:v:v) and suitable stable isotope-labeled or synthetic
463 internal standards including D4-glycochenodeoxycholic acid and myristoyl phosphatidylcholine
464 for estimation of metabolite recovery and for relative quantitation across experimental groups.
465 Samples were homogenized with zirconium oxide beads for 2 minutes in a Bullet Blender (Next
466 Advance) at 4 degrees Celsius, incubated for 30 minutes at 4 degrees Celsius, then centrifuged
467 for ten minutes at 10,000g at 4 degrees Celsius. Supernatants were filtered through 0.2 micron
468 syringe filters (Fisher Scientific), and evaporated to dryness in a SpeedVac. Samples were
469 resuspended in 95% acetonitrile for use in LC-MS analysis.

470 **Liquid chromatography-mass spectrometry** Untargeted metabolite identification utilized a
471 Thermo model LTQ-Orbitrap Velos mass spectrometer operating in full scan negative ionization
472 MS mode at 60,000 resolution with data-dependent HCD-MS/MS over a mass range of 50-1000
473 m/z. The mass spectrometer was coupled to a Shimadzu Prominence HPLC system through an
474 electrospray ionization source. Ten microliters of each metabolite extract was injected and
475 separated on a Phenomenex HILIC LC column, 2.0 mm x 100 mm, 3.0 micron particle size, 100
476 Angstrom pore size, using a gradient of (A) water containing 50 mM ammonium formate, and

477 (B) acetonitrile from 95% to 50% B over 14 minutes as previously descrived. (38). A guard
478 cartridge of matching chemistry was fitted to the analytical column.

479 **Data analysis** Compound identifications, isotope correction, chromatographic peak alignment,
480 and peak area quantification were performed with MAVEN software (39). Due to the need to
481 simultaneously analyze numerous disparate classes of metabolites in untargeted experiments,
482 only relative quantitation of analytes against a selected internal standard were performed for
483 comparison of values across experimental treatment groups. Multivariate statistical analysis was
484 performed using MetaboAnalyst version 4.0 software (https://www.metaboanalyst.ca/).

485 **Nutrient downshift survival** *C. jejuni* were grown overnight at 37°C in MH broth under
486 microaerobic condition. Then strains were collected by centrifugation at 6,000 rpm for 10 min
487 and washed twice with MCLMAN minimum media where no carbon and no phosphate present.
488 Strains were diluted to an OD600 of 0.05. After different time intervals, CFU were measured by
489 plating on MH agar plates.

490 **Osmotic stress survival** *C. jejuni* strains were grown overnight at 37°C in MH broth under
491 microaerobic condition. Then strains were centrifuged and resuspended in MH media containing
492 1% and 1.5% NaCl. The initial OD of the culture was 0.05 and survival was measured by CFU
493 count at different time intervals (22).

494 **Real Time PCR.** Wild type strain DRH212, *pstS::kan* strains were grown in different condition
495 *in vitro* and total RNA was isolated by the Trizol method and RNeasy Mini Kit (Qiagen). After
496 digestion with TURBO Dnase treatment, cDNA was made by SuperScript III First-Strand

497 Synthesis Supermix (Invitrogen). Then real time PCR was performed by Syber Green method.
498 The gene expression of different targeted genes was measured by $2^{\text{delta deltaCT}}$ method.

499 **Measurement of *in vivo* poly-P levels.** Intracellular poly-P levels were measured as described
500 (40) Briefly, strains were grown in MH broth for 24h and 48h. Strains were collected in each
501 time point by centrifugation and dissolved in 250 µL of GITC lysis buffer (4 M guanidine
502 isothiocyanate, 50 mM Tris-HCl, pH 7) and lysed by incubating at 95 °C for 10 min. Lysates
503 were stored at -80 °C. The protein concentration was determined by Bradford assay (Bio-Rad) of
504 a 5-µl aliquot. Poly-P was isolated using EconoSpin silica spin columns (Epoch Life Science)
505 and digested with 1 µg of PPX1 exopolyphosphatase from *Saccharomyces cerevisiae*. The
506 resulting free phosphate was measured using a malachite green colorimetric assay and
507 normalized to the total protein (40).

508 **Chicken colonization assay and *in vivo* phosphate determination** Day-of-hatch white
509 Leghorn chickens were inoculated orally with two doses (approximately $1x10^3$ and $1x10^6$
510 CFU/ml) of indicated *C. jejuni* strains (wild type strain 81-176, *pstS::kan* and *pstS::kan/C*) (8).
511 Birds were euthanized at day 3 and day 7 after infection, and cecal contents were collected as
512 described (41). Cecal contents were homogenized in sterile PBS and serial dilutions were plated
513 on Mueller-Hinton (MH) agar containing 10% sheep's blood, cefoperazone (40 µg/ml),
514 cycloheximide (100 µg/ml), trimethoprim (10 µg/ml), and vancomycin (100 µg/ml), selective for
515 *Campylobacter*. Plates were grown for 48 h under microaerobic conditions and colonies were
516 enumerated. For the competition experiment, wild type and *pstS::kan* strain were mixed equally
517 (approximately $1x10^6$ CFU/ml of each strain) and inoculated the day-of-hatch chicks by oral
518 gavage. At Day 7, cecal contents were collected and after serial dilution plated on

519 Campylobacter selective media with and without kanamycin (50 µg/ml). Competitive index
520 values were calculated as previously described (42).
521 Inorganic free phosphate was measured in chicken cecal content (100mg) at Day 3 and
522 Day 7 by inorganic free phosphate measurement ELISA Kit (Bioassay) as per manufacturer
523 protocol. All chicken experiments and protocols were approved by the Institutional Animal Care
524 and Use Committee in the Animal Care Program at Michigan State University.
525

526 **Acknowledgements**

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528 State University for providing human stool samples used in this study. This work was supported
529 in part by NIH award AI 111192 (to V.J.D.) and by the Rudolph Hugh Endowment of Michigan
530 State University.
531

532 **Table 1:**
533

Strain or plasmid	**Relevant characteristics**	**Source of Reference**
C. jejuni DRH212	Streptomycin-resistant wild type *C. jejuni* 81-176 (Sm^r)	34
DRH212 *pstS::kan*	*pstS* insertion mutant of *C. jejuni* DRH212 (Kn^r Sm^r)	In this study
DRH212 *pstS::kan/C*	*pstS* insertion mutant of *C. jejuni* DRH212 content *pstSCAB* cloned PECO212 ((Kn^r Sm^r Cm^r)	In this study
E.coli DH5a	*E. coli* strain used for cloning	
E.coli DH5a/pRK212.1	Contains conjugative plasmid for the conjugation of plasmid DNA into *Campylobacter*	36
Plasmids		
pGEMT	Subcloning vector (Ap^r)	
pECO102	pRY112 derivative with *cat* promoter in XhoI-BamHI	36
pILL600	contains *Campylobacter* kanamycin cassette	36

534

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660 **Fig 1:**

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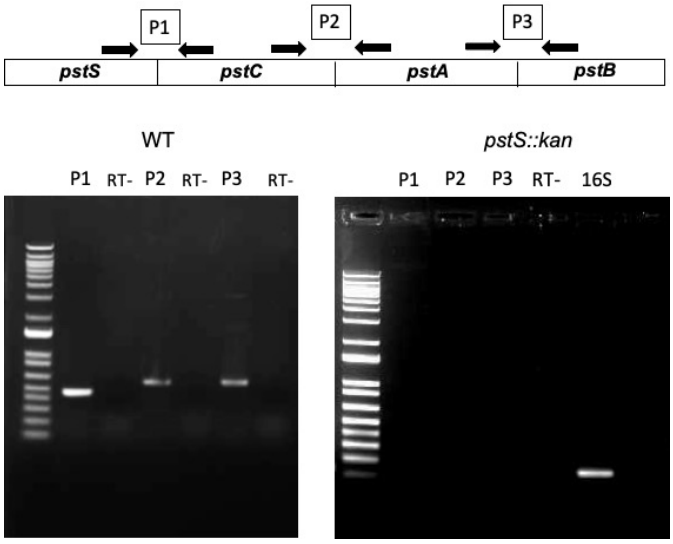

662

663 **Fig 1: Characterization of *pstSCAB* operon: A)** RT-PCR was performed to determine whether
664 the four genes (*pstS*, *pstC*, *pstA* and *pstB*) are cotranscribed. Three intergenic primer sets (P1, P2
665 and P3) were designed to amplify transcripts crossing the gene boundaries. The product sizes in
666 bp were correct as predicted in all cases. RT PCR with total RNAs were extracted from both
667 DRH212 (left panel) and *pstS::kan* mutant (right panel) using the designed three primer sets. RT
668 - negative control; 16S - as a positive control.

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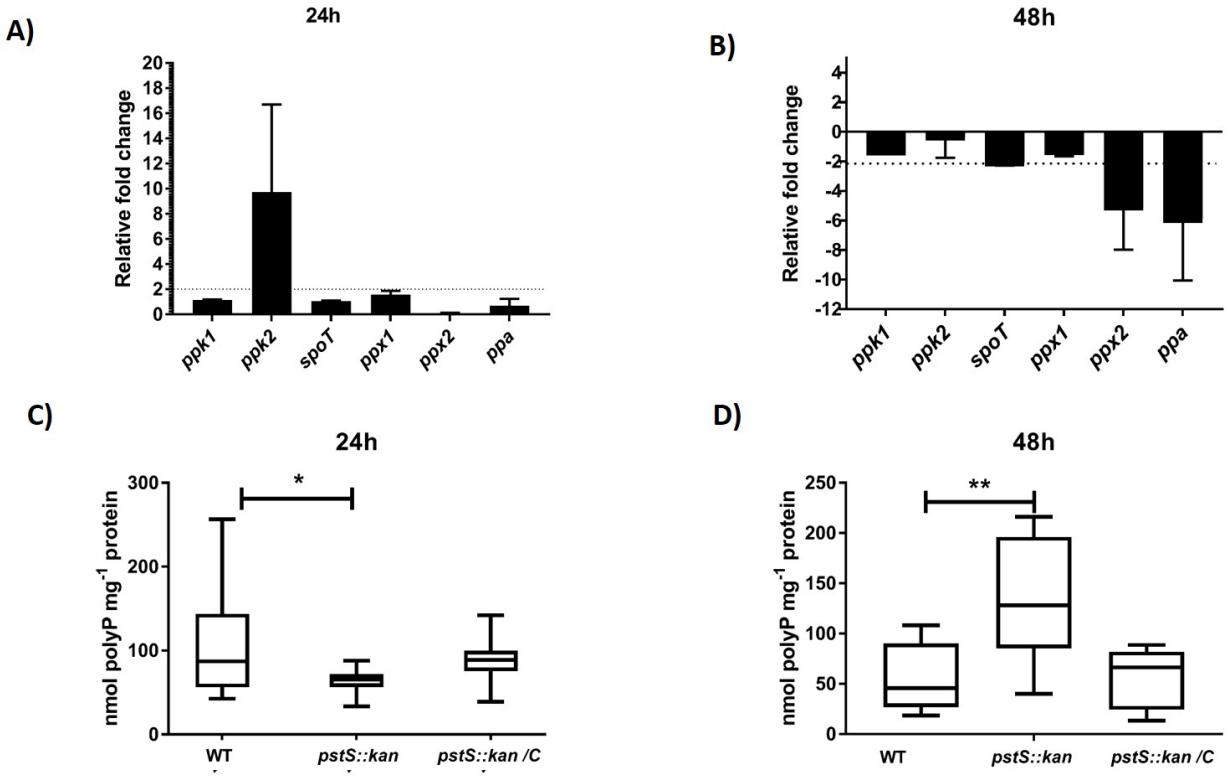
676 **Fig 2:**



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679 **Fig 2: Comparative analysis of Poly-P metabolism regulatory genes and Poly-P level**
680 **between DRH212 and *pstS::kan* mutant: A)** and **B)** Transcription analysis of different stress
681 regulatory genes *ppk1, ppk2, spoT* and *ppA* at 24h and 48h after growth of DRH212 and
682 *pstS::kan* by Quantitative RT PCR. Changes of gene expression in the *pstS::kan* mutant
683 compared with wild type DRH212 were determined by the $2^{-\Delta\Delta CT}$ method. Data are represented
684 as mean value of three independent experiments with standard deviation (±). **C)** and **D)** Levels of
685 Intracellular polyphosphate (Poly-P) of three strains (DRH212, *pstS::kan, pstS::kan/C*) were
686 measured at two different time intervals 24h and 48h. Statistical analysis were done using
687 Unpaired t-test and One way ANOVA with Holm-Sideak's multiple test; (n=8,±SD), $*$
688 ,P<0.05; $**, P < 0.005$.

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692 **Fig 3:**

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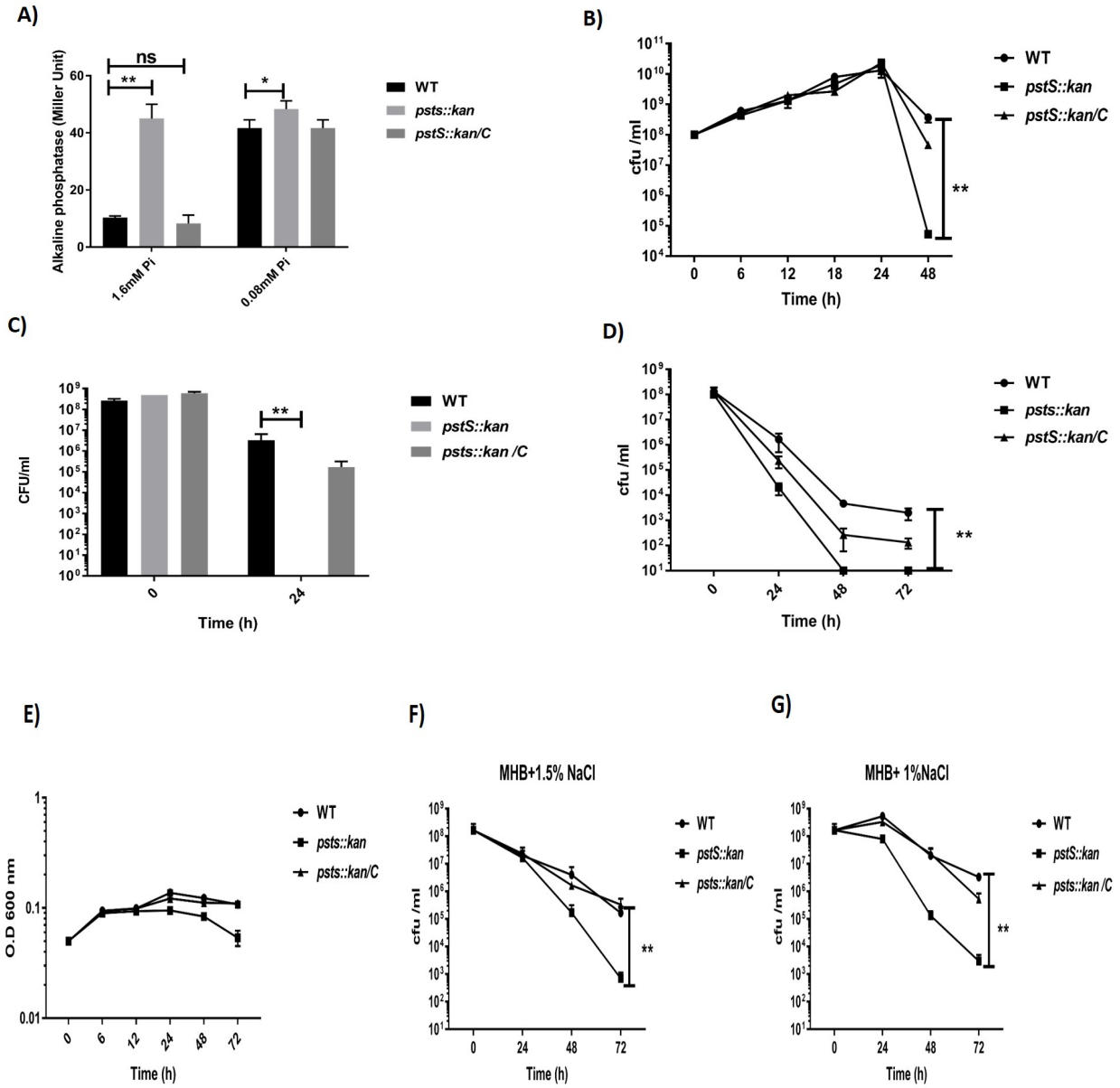

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696 **Fig 3: Characterization of *C. jejuni pstS::kan* strain in different *in vitro* conditions:**

697 **A)** Alkaline phosphatase activity was determined in pyruvate-minimal media with 1.6 mM or
698 0.08 mM Pi (n=3). Statistical analysis was done using two way ANOVA with Sidak's multiple
699 comparisons test. $*$ **,P<0.05;** $**$, $\boldsymbol{P < 0.005}$**, B)** *C. jejuni* strains were grown in Mueller-Hilton
700 (MH) broth in microaerobic condition for 48 h and after different time intervals CFU/ml were
701 determined**. C)** *C. jejuni* DRH212 was grown in MH broth for 48h. After 48h, sterile spent media

were collected by filter sterilization. Mid-log phase cultures of DRH212, *pstS::kan, pstS::kan/C* strains were inoculated (10^8 CFU/ml) in spent media and survival was measured at different time intervals by CFU count. **D)** In nutrient down shift assay, mid-log phase of DRH212, *pstS::kan, pstS::kan/C* strains were inoculated in minimal media where no carbon and phosphate source were present. The survival assay was measured by CFU count at different time intervals. **E)** Osmotic stress tolerance: strains were inoculated in MH broth containing 1% NaCl (**F**) or 1.5% NaCl (**G**) and growth was determined by measuring O.D at 600nm and cfu counts**.** Each experiment represents mean value of three independent experiments. Statistical significant was done student t test. $*$,P<0.05; $**, P < 0.005$.

Fig.4:

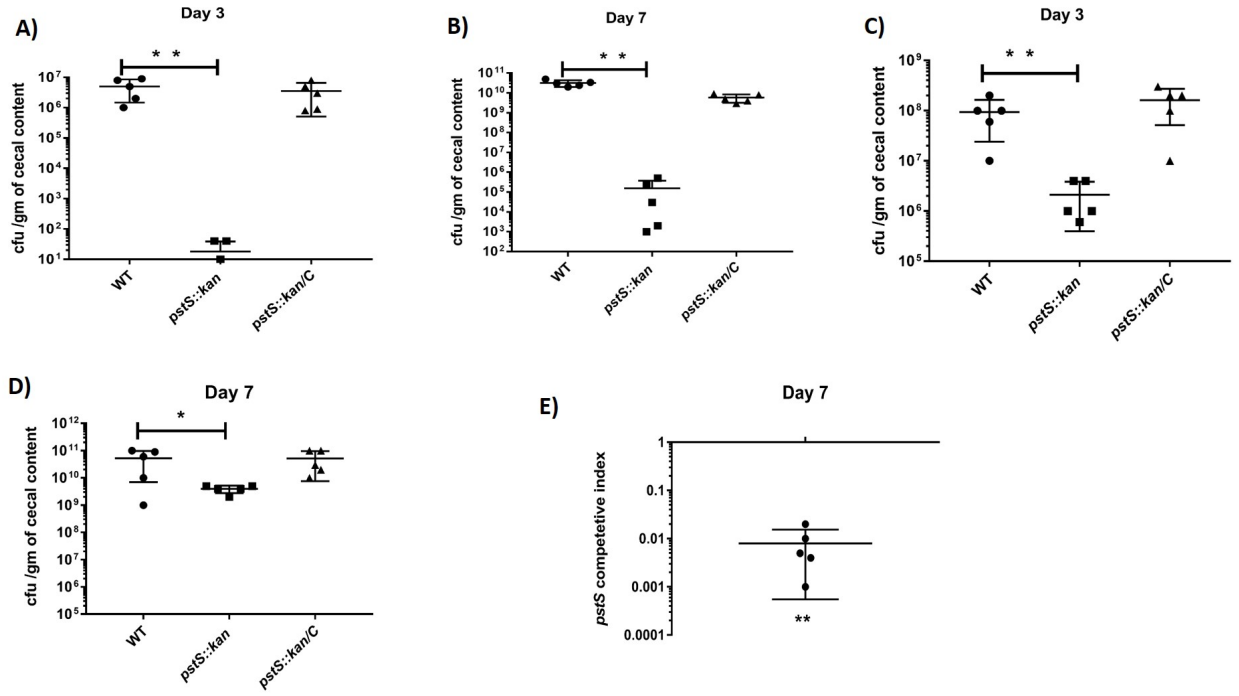

Fig 4: Colonization ability of *pstS::kan* mutant in chickens: Day-of-hatch white Leghorn chicks were infected with either DRH212, *pstS::kan* or *pstS::kan/C* strains of *C. jejuni* (two different doses 10^3 and 10^6 CFU/ml) and cecal loads were enumerated at day three and day seven. Comparative colonization ability between DRH212 and *pstS::kan* strains at day three and day seven after oral inoculation with 10^3 (**A, B)** and 10^6 CFU/ml **(C, D).** Statistical analysis was one way ANOVA with Tukey's multiple comparison test (n=5,±SD). **E)** In competition analysis,

719 DRH212 versus *pstS::kan* mutant, day-of-hatch chicks were infected with equal ratio of
720 DRH212 and *pstS::kan* mutant. At day seven the ratio of *pstS::kan* mutant to DRH212 was
721 determined and is presented as a competitive index. Statistical analysis was performed using a
722 one-simple t test against a hypothetical value of 1. (n=5 of each group). $*$,P<0.05; $**, P <$
723 0.005.

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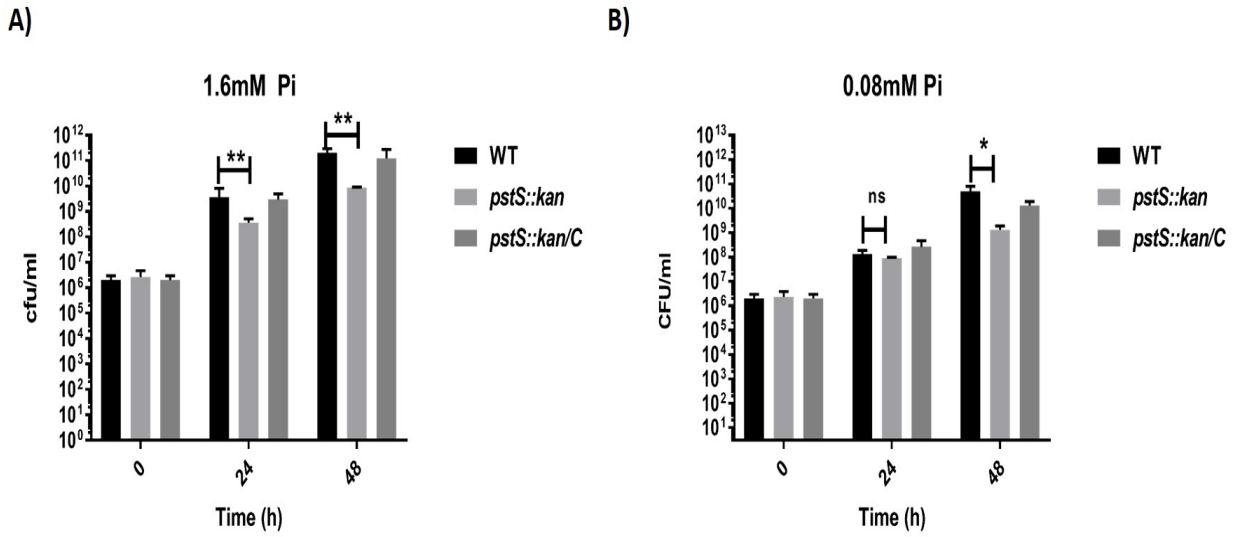
726 **Fig 5.**



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728 **Fig 5: PstSCAB transporter of *C. jejuni* is essential for growth in lactate:** *C. jejuni* strains
729 were grown in minimal media containing both D+L lactate (each 5mM) with two concentrations
730 of inorganic phosphate. Growth of DRH212 and *pstS::kan* was measured by CFU count in
731 minimal media (D+L lactate) with **A)** 1.6mM Pi and **B)** 0.08mM Pi at 24 and 48h. N=3; error bar
732 indicated SD. Statistical analysis was done by two-way analysis of variation (ANOVAs) with
733 Sidak's multiple comparisions test. $*$,P<0.05; $**, P < 0.005$**.**

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737 **Fig 6:**

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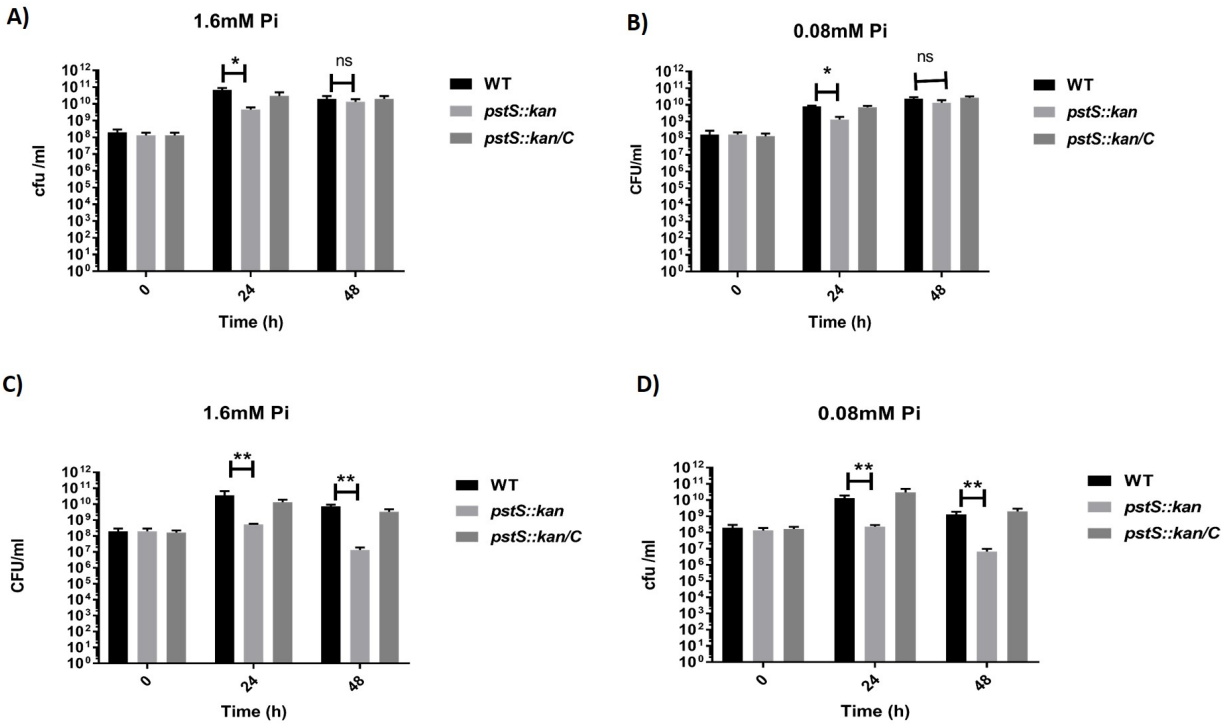

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740 **Fig 6: Comparative growth analysis of *C. jejuni* strains in minimal media with D- or L-**
741 **lactate**. *C. jejuni* strains were grown in minimal media with D-lactate or L-lactate as a carbon
742 source and high or low concentration of inorganic phosphate. Growth of *C. jejuni* strains was
743 measured in D-lactate minimal medium with **A)** 1.6mM and **B)** 0.08mM phosphate by CFU
744 counts at 24h and 48h. Growth of these three strains was also determined in L-lactate minimal
745 medium with 1.6mM **C)** and 0.08mM **D)** phosphate. N=3; error bar indicated SD. Statistical
746 analysis was done by two-way analysis of variation (ANOVAs) with Sidak's multiple
747 comparisons test. $*$,P<0.05; $**, P < 0.005$.

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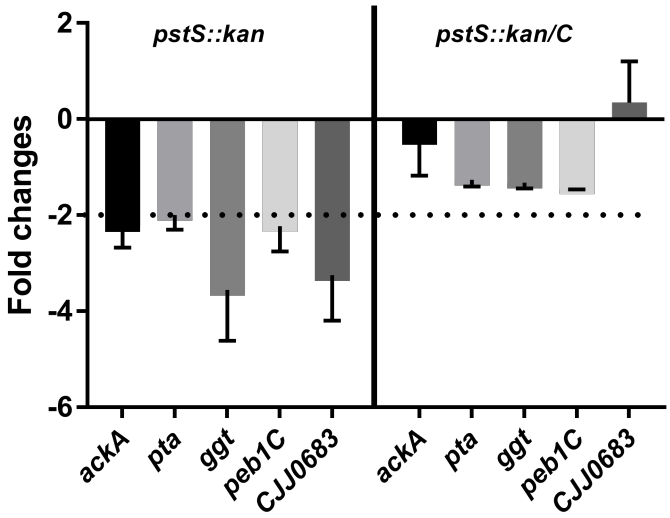
751 **Fig:7**



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755 **Fig 7: Transcript analysis of acetogenesis regulatory and acetogenesis dependent gene**

756 **expression in wild type, *pstS::kan*, and complemented strains**: Bacterial strains were grown in

757 minimal media containing L-lactate with 1.6mM Pi up to 18h, after which cells were harvested

758 for RNA isolation. Acetogenesis regulatory genes (*ackA* and *pta*) and acetogenesis dependent

759 genes (*ggt,peb1C* and *CJJ0683*) were measured in *pstS::kan* mutant and *pstS::kan/C* strains

760 relative to DRH212 by real time PCR. N=3; error bar indicated SD.

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767 **Fig 8:**

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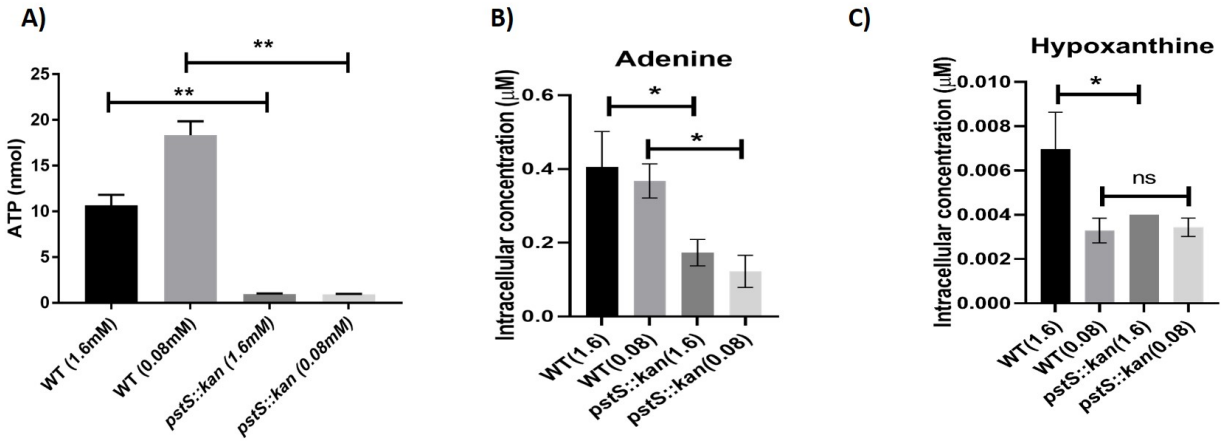

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771 **Fig 8: Intracellular ATP and metabolites in DRH212 and *pstS::kan* mutant grown in L-**

772 **lactate minimal media.** DRH212 and *pstS::kan* strains were grown in L-lactate minimal media

773 with 1.6mM and 0.08mM Pi at 37^0 C. After 18h, **A)** ATP levels in DRH212 , *pstS::kan* were

774 detetermined. N=3; error bar indicated SD. The intracellular concentration of **B)** adenine and **C)**

775 hypoxanthine were measured by liquid chromatography mass spectroscopy analysis. Data

776 represent mean value of two independent experiments. The statistical analysis was done by one

777 way ANOVA. $*$,P<0.05; $**, P < 0.005$.

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