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Transcriptome analysis of *Campylobacter jejuni* polyphosphate kinase (*ppk1* and *ppk2*) mutants

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Campylobacter jejuni is a leading cause of bacterial foodborne diarrhea worldwide.[1] It is also associated with debilitating sequelae such as the Guillain-Barre and Miller Fisher Syndromes.[2] *C. jejuni* is primarily contracted via the consumption of contaminated poultry products; however, this pathogen is prevalent in a variety of sources, including other food animals, the farm environment, and water.[3] Given these diverse environments, *C. jejuni* must possess versatile mechanisms that allow it to survive and/or persist in a variety of niches. This environmental flexibility is notable, because *C. jejuni* has a relatively small genome, lacks many of the classical stress response factors associated with other enteric pathogens, and is generally considered fastidious under laboratory conditions. Consequently, analysis of potentially multipurpose mechanisms that can facilitate *C. jejuni*'s adaptation to disparate environments will result in a better understanding of the pathogenesis and ecology of this pathogen.

Recently, we have demonstrated that the metabolism of inorganic polyphosphate (poly-P), a molecule associated with survival and virulence phenotypes in many bacterial pathogens, contributes to the pathobiology of *C. jejuni*.[4-8] A key role for poly-P in a fastidious organism like *C. jejuni* is consistent with the ability of poly-P to efficiently store energy and phosphate,[9-10] which can in turn fuel essential cellular functions.[11] The poly-P metabolism in *C. jejuni* is mediated primarily by polyphosphate kinase 1 (PPK1) and polyphosphate kinase 2 (PPK2). Polyphosphate kinase 1 functions to mediate the synthesis of poly-P,[11-13] while PPK2 preferentially facilitates the generation of GTP from poly-P.[14-15] Both PPK1 and PPK2 (PPKs) contribute to survival, adaptation, and virulence in many bacterial pathogens.[11] For example, we have shown that the impairment of the *ppks (ppk1* and *ppk2*) can affect a wide range of phenotypes in *C. jejuni*,[4-6] including biofilm formation, and the resistance to osmotic, nutrient, and antimicrobial stresses. In addition, mutants show decreased ability to colonize chickens and infect human intestine epithelial cells in vitro. Despite these phenotypic observations, the mechanism(s) by which the PPKs impact these phenotypes in *C. jejuni* remain largely uncharacterized.

Previous studies have revealed differential expression of genes involved in stress response and virulence in both *ppk1* and *ppk2* mutants of disparate bacterial pathogens.[4,5,16-17] For example, 250 genes were up-regulated and more than 450 genes were down-regulated in a *ppk1* mutant of *Pseudomonas aeruginosa*.[11] Another study showed that 81 genes were differentially expressed in a poly-P-deficient mutant of *Pseudomonas sp*. B4.[18] Additionally, poly-P was shown to bind to the principal sigma factor of RNA polymerase in *Helicobacter pylori* during starvation.[19] Taken together, these observations suggest that poly-P and the PPKs might affect the expression of many genes and contribute to the regulation of various cellular mechanisms. If the PPKs impact gene expression and play similar role(s) in *C. jejuni*, this might allow us to associate genotype-phenotype relationships that underlie the pleiotropic effects associated with *ppks* impairment in *C. jejuni*. Here, we conducted RNA-Seq analysis on *C. jejuni* 81–176 (parental strain) and its *ppk1* and *ppk2* mutants.[4-5] We found that both *ppk1* and *ppk2* affected the expression of multiple genes which can be linked to these pleiotropic phenotypes. Our results further highlight the importance of poly-P metabolism in the survival and adaptation of *C. jejuni*.

The wildtype *C. jejuni* 81–176 (WT) and the previously described Δ*ppk1* and Δ*ppk2* mutants were used in this study.[4-5] The strains were grown in Mueller-Hinton broth (MH, Fisher Scientific, Pennsylvania, USA) under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) at 42°C with shaking (200 rpm) and harvested at both log (8 h) and stationary (18 h) phases of growth. RNA was isolated from both phases of growth for each strain as described previously.[20] Briefly, the bacterial cells were treated with RNAProtect Bacteria Reagent as described in the manufacturer's instructions (Qiagen Inc.., California, USA). The cells were then lysed by the addition of Proteinase K (1 mg/ml; Epicenter) followed by incubation at room temperature for 10 min. The total RNA was isolated and purified using RNeasy Mini Kit (Qiagen), and the samples were treated to remove any residual DNA by using the RNase-free DNase Set (Qiagen). Total RNA was then eluted in RNase-free water and an aliquot was subjected to an additional step of DNase treatment using TURBO DNA-*free*™ (Ambion, Life Technologies, New York, USA). The absence of DNA was confirmed by PCR amplification using *Campylobacter*-specific 16S rRNA primers,[21] and the

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quantity and purity of the RNA was determined by using the NanoDrop® ND-1000 spectrophotometer (ThermoFisher Scientific, New York, USA). Total RNA samples (~5 μg) were subjected to depletion of rRNA using Ribo-Zero™ rRNA removal kit for Gram-negative bacteria (Epicentre, Wisconsin, USA) and assessed using denaturing formaldehyde gel electrophoresis. The concentration and purity of enriched mRNA was determined by using the NanoDrop® ND-1000 spectrophotometer and the Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA).

RNA-Seq libraries were prepared from the enriched mRNA using the mRNA-Seq Sample Preparation Kit (Illumina, Wisconsin, USA) as described by the manufacturer. Briefly, the mRNA-Seq library construction consisted of the following steps, i) fragmentation, ii) first strand cDNA synthesis, iii) second strand cDNA synthesis, iv) end repair, v) A-tailing, vi) adapter ligation, vii) size selection of cDNA fragments in the 200 bp range and, viii) PCR enrichment. Libraries were generated for 6 bacterial samples [3 bacterial strains (WT, *Δppk1* and *Δppk2*) × 2 growth conditions (log phase + stationary phase)], which was biologically replicated in a separate experiment. Barcoded RNA-Seq libraries were analyzed on the Agilent Technologies 2100 Bioanalyzer to assess size, purity and concentration. The RNA-Seq libraries were sequenced on the Illumina Genome Analyzer IIX using Multiplexing Sequencing Primers, PhiX Control v3 and standard sequencing reagents as described by the manufacturer (Illumina). Reads were then aligned to the reference genome of *C. jejuni* and RNA-Seq analysis was carried out using the Partek Genomics Suite (Partek Inc.., Missouri, USA). Transcripts with a $\log_2$ fold change >1.5 or <1.5 and a p value ≤ 0.05 were considered to be significantly differentially expressed. To confirm the sequencing results, qRT-PCR was performed on a subset of genes (**Table S1**). For this purpose, RNA was re-isolated from the strains at log and stationary phases as described above and cDNA was synthesized with the Super-Script III First-Strand Synthesis System (Life Technologies). The SYBR® Green RT-PCR kit (Invitrogen, New York, USA) was used to perform the qRT-PCR analysis. Samples were tested in triplicates and the analysis was replicated at least 3 times. Relative gene expression was determined by the $2^{(-\Delta\Delta C(T))}$ method.[22]

The RNA-Seq analysis during the exponential growth phase revealed 15 differentially expressed (3 upregulated and 12 downregulated) genes in the Δ*ppk1* (**Table 1**). It should be noted that to focus the study, the genes that encoded hypothetical proteins were reported in **Table S2**. The downregulation of genes encoding heat shock proteins, such as *clpB, grpE* and *dnaK*, was prominently observed in the Δ*ppk1* (**Table 1**). This is important, because of the well-characterized roles of heat shock proteins in mediating the general stress response and for their role as chaperones in binding/refolding and degrading misfolded and aggregated proteins.[23-25] Despite the absence of heat stress in this study, both heat shock proteins and chaperones (DnaK and ClpB) are known to be important for protein homeostasis in this bacterium under non-stress conditions.[26] Furthermore, downregulation of these genes is consistent with the increased sensitivity observed for the Δ*ppk1* to a variety of stresses and with a previous study in *Mycobacterium* spp showing that PPK1 was associated with general stress tolerance and the response to heat stress.[4,27] Therefore, the differential expression of the heat shock proteins and chaperones was not surprising.

Flagella-associated genes (CJJ81176_RS06440, CJJ81176_RS02650 and CJJ81176_RS02655) and flagella glycosylation-associated gene (*pglI*; CJJ81176_RS05480) were downregulated in the Δ*ppk1* in log phase (**Table 1**). A proteome analysis of a poly-P deficient *P. aeruginosa* revealed that genes encoding flagellar proteins were underrepresented, which supports our findings.[28] The Δ*ppk1* did not display significant motility defects,[4] which might indicate that the degree of downregulation of the flagella genes might not have been enough to impair motility. This observation can also be reconciled with previous studies that showed that flagellar glycosylation mutants were still motile but defective in autoagglutination.[29] Furthermore, a previous study reported the isolation of 4 mutants (Cj1318, Cj1333, Cj1340c, and Cj1062) that expressed wild-type levels of FlaA and were deficient in autoagglutination.[30] This is important, because impairment of autoagglutination and flagella can impede intestinal epithelial cells invasion and chicken colonization, respectively, which might partially explain the reduced ability of the Δ*ppk1* to colonize the chicken host.[4,30-31] However, it should be noted that autoagglutination phenotype of the Δ*ppk1* mutant of *C. jejuni* has not been characterized yet. Although further investigation is required to discern the importance of the downregulation of these genes, our results clearly highlight the contributions of *ppk1* to various phenotypes in *C. jejuni*.

We previously reported an upregulation of genes involved in phosphate uptake (*pstC, pstS, phosR*) and the stringent response (*spoT*) in the Δ*ppk1*.[4] However, this was not observed in the RNA-Seq analysis of the Δ*ppk1* during the log phase. It should be noted that in the former study, we were attempting to test gene expression under acid stress and only incubated the acid-treated and control cultures for 30 min before RNA isolation and subsequent qRT-PCR analysis.[4] Therefore, it is possible that upregulation of the genes involved in phosphate uptake and the stringent response might not have been detected in the curret study, because the bacterial cultures were incubated for a longer time to achieve log phase growth before the RNA-Seq analysis.

In stationary phase, 40 genes were downregulated and 13 were upregulated in the Δ*ppk1* (**Table 1**, **Table S2**). Similar to above, many of the downregulated genes were associated with the flagella, including genes encoding the flagellar hook protein (*flgE*), the flagellar L-ring protein (*flgH*), the flagellar hook-associated protein (*flgL*), the flagellar basal body rod protein (*flgG*), and flagellin modification protein (*pseA*). Other genes with decreased expression included the gene encoding the HrcA family transcriptional regulator (**Table 1**). The downregulation of *hrcA* is accompanied by upregulation of the stress response genes *groEL/S*.[32] However, the upregulation of these stress response genes (*groEL/S*) in the stationary phase were not detected in the Δ*ppk1* strain. Regardless, the differential expression of the genes encoding the HrcA

Table 1. Differentially expressed genes (DEG) in the Δ*ppk1* compared to the WT in the log and stationary phases. Genes with $\log_2$ fold change >1 .5 or <1 .5 with a *p*-value ≤0 .05 were considered significant. Genes of unknown functions and those that encode hypothetical proteins were not included in this table.

Δ*ppk1* DEG (Log phase)	$\log_2$ fold	Proposed Function (Abbreviation)
CJJ81176_RS06440	−2.58223	Flagellin A (FlaA)
CJJ81176_RS02475	−2.21966	Molecular chaperone (ClpB)
CJJ81176_RS05980	−2.06765	Cobalamin ABC transporter permease
CJJ81176_RS03630	−2.03358	Molecular chaperone (DnaK)
CJJ81176_RS02650	−1.89773	Flagellar protein (FlaG)
CJJ81176_RS02655	−1.71348	Flagellar cap protein (FliD)
CJJ81176_RS05935	−1.62294	Molecular chaperone (GroEL)
CJJ81176_RS03625	−1.52779	Co-chaperone protein (GrpE)
CJJ81176_RS05905	−4.55049	Membrane protein
CJJ81176_RS05480	−2.839	Glucosyltransferase (PglI)
CJJ81176_RS06615	−3.62449	Toluene tolerance protein
CJJ81176_RS05825	−2.58223	2-oxobutyrate oxidase
CJJ81176_RS00540	1.526291	Chromosome partitioning protein (ParB)
CJJ81176_RS03800	1.718938	2-oxoglutarate:acceptor oxidoreductase
CJJ81176_RS05940	3.909622	Sensor histidine kinase
DEG (Stationary phase)		
CJJ81176_RS02690	−2.89733	C4-dicarboxylate ABC transporter
CJJ81176_RS03575	−2.35115	Adhesin
CJJ81176_RS03825	−2.13977	Signal transduction histidine kinase
CJJ81176_RS04095	−2.02534	Membrane protein
CJJ81176_RS03320	−1.9993	Flagellar L-ring protein (FlgH)
CJJ81176_RS03620	−1.95134	HrcA family transcriptional regulator
CJJ81176_RS03800	−1.85067	2-oxoglutarate:acceptor oxidoreductase
CJJ81176_RS04090	−1.8288	Major Facilitator Superfamily (MFS) transporter
CJJ81176_RS04235	−1.82852	Flagellar hook-associated protein (FlgL)
CJJ81176_RS03370	−1.77805	Flagellar basal body rod protein (FlgG)
CJJ81176_RS01310	−1.74863	Tautomerase
CJJ81176_RS06535	−1.69151	Membrane protein
CJJ81176_RS03375	−1.69103	Flagellar basal body rod protein (FlgG)
CJJ81176_RS06110	−1.67824	Isomerase
CJJ81176_RS08145	−1.64488	50S ribosomal protein L18 (RplR)
CJJ81176_RS06410	−1.62838	Flagellin modification protein (PseA)
CJJ81176_RS07355	−1.59133	ABC transporter permease
CJJ81176_RS08350	−1.56166	Flagellar hook protein (FlgE)
CJJ81176_RS04755	−1.55111	Major Facilitator Superfamily (MFS) transporter major
CJJ81176_RS03630	−1.5464	Molecular chaperone (DnaK)
CJJ81176_RS04260	−1.52623	Chemotaxis protein (CheY)
CJJ81176_RS03290	−1.51055	ABC transporter
CJJ81176_RS06305	−1.50693	UDP-N-acetylglucosamine 4,6-dehydratase
CJJ81176_RS01785	−4.68234	Colicin
CJJ81176_RS03100	1.573296	peptide methionine sulfoxide reductase (MsrA)
CJJ81176_RS05130	1.577051	Carbon-nitrogenhydrolase
CJJ81176_RS04670	1.632199	Membrane protein insertion efficiency factor
CJJ81176_RS06600	1.636997	Guanine permease
CJJ81176_RS05135	1.658186	Exodeoxyribonuclease VII, small subunit
CJJ81176_RS01830	1.70258	Sulfoxide reductase catalytic subunit (YedY)
CJJ81176_RS08155	1.795694	30S ribosomal protein S8
CJJ81176_RS08295	1.866083	3-isopropylmalate dehydratase small subunit (LeuD)
CJJ81176_RS01905	1.911169	Tram-like protein
CJJ81176_RS02320	2.158265	Preprotein translocase subunit (SecE)
CJJ81176_RS02990	2.66359	Phosphate ABC transporter substrate-binding protein (PstS)
CJJ81176_RS07070	3.117623	Transformation system protein

family transcriptional regulator and the DnaK chaperone suggest that the *ppk1* might contribute to the thermotolerant properties of *C. jejuni*.

Among the 13 upregulated genes in the Δ*ppk1* in the stationary phase were genes that encode a phosphate ABC transporter substrate-binding protein (CJJ81176_RS02990), a preprotein translocase subunit (*secE*; CJJ81176_RS02320), a permease (CJJ81176_RS06600) and a carbon-nitrogen hydrolase (CJJ81176_RS05130) (**Table 1**).

In this study, 14 (9 downregulated and 5 upregulated) genes were differentially regulated in the Δ*ppk2* during the log phase (**Table 2**). The gene encoding catalase (CJJ81176_RS06675) and CJJ81176_RS01080 which encodes a serine protease

Table 2. Differentially expressed genes (DEG) in the Δ*ppk2* compared to the WT in the log and stationary phases. Genes with $\log_2$ fold change >1 .5 or <1 .5 with a *p*-value ≤0 .05 were considered significant. Genes of unknown and after functions those that encode hypothetical proteins were not included in this table.

Δ*ppk2* DEG (Log phase)	$\log_2$ fold	Proposed Function (Abbreviation)
CJJ81176_RS02990	−1.73	Phosphate ABC transporter substrate-binding protein
CJJ81176_RS06675	−1.72	Catalase (KatA)
CJJ81176_RS03985	−1.70	CoA-binding protein
CJJ81176_RS00395	−4.18	Cation-binding protein
CJJ81176_RS01080	−2.42	Serine protease
CJJ81176_RS02935	1.53	Sulfurase
CJJ81176_RS03255	1.60	C4-dicarboxylate ABC transporter (DcuB)
CJJ81176_RS04170	1.70	Arylsulfate sulfotransferase
CJJ81176_RS03005	1.96	Phosphate import ATP-binding protein (PstB)
DEG (Stationary phase)		
CJJ81176_RS04335	-1.56444	Alanine racemase
CJJ81176_RS04100	1.528314	Membrane protein
CJJ81176_RS02320	1.575105	Preprotein translocase subunit (SecE)
CJJ81176_RS08175	1.593406	50S ribosomal protein L14 (RplN)
CJJ81176_RS05955	1.867396	CopG family transcriptional regulator
CJJ81176_RS07160	1.886767	Membrane protein
CJJ81176_RS05845	1.997799	Membrane protein
CJJ81176_RS03005	2.149801	Phosphate import ATP-binding protein (PstB)

were among those downregulated in the Δ*ppk2* strain in the log phase. Serine proteases are known to have significant roles in prokaryotes, contributing to diverse functions such as metabolism, cell signaling, adaptation to the extracellular environment, cellular defenses, host invasion, and virulence.[33]

Interestingly, the survival of the Δ*ppk2* and the WT after exposure to hydrogen peroxide and paraquat (oxidative stress) was similar. However, the Δ*ppk2* showed decreased survival under aerobic stress, and the KatA has been shown to contribute to survival under aerobic stress.[5,34] Furthermore, the expression of the gene encoding DcuB (CJJ81176_RS03255), a protein that mediates aspartate transport under oxygen limitation and fumarate: succinate exchange during fumarate respiration,[35] was upregulated in the Δ*ppk2* during log phase (**Table 2**). It has been previously observed that *dcuB* was upregulated under acid shock conditions, and the authors speculated that *dcuB* might contribute to resisting cytoplasm acidification in *C. jejuni*.[36]

In the stationary phase, it was interesting to observe that the gene that encode alanine racemase, an enzyme involved in peptidoglycan biosynthesis, and *secE* which contributes to protein export were differentially expressed in the Δ*ppk2*. Similar to *ppk1*, these observations confirm that *ppk2* is an important contributor to the pathobiology of *C. jejuni*. However, unlike *ppk1*, the effect of *ppk2* is less pronounced in terms of the number of affected genes in the stationary phase.

To validate the RNA-Seq data, we performed qRT-PCR analysis on a subset of the differentially expressed genes in the *ppk* mutants (**Table S1**). Our results showed a high correlation between qRT-PCR and the RNA-Seq analysis of the gene expression in the Δ*ppk1* ($r = 0.8$) and the Δ*ppk2* ($r = 0.9$), respectively. In conclusion, this study showed that deletion of *ppk1* and *ppk2* impact the expression of many genes that are involved in important functions in *C. jejuni*, corroborating the pleiotropic effects associated with the *ppk1* and *ppk2* deletion mutants. Collectively, we also propose that the polyphosphate kinases play an important regulatory role in *C. jejuni*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Material

Supplemental data for this article can be accessed on the publisher's website.

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