



Polynucleotide phosphorylase has an impact on cell biology of *Campylobacter jejuni*

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Polynucleotide phosphorylase (PNPase), encoded by the *pnp* gene, is known to degrade mRNA, mediating post-transcriptional regulation and may affect cellular functions. The role of PNPase is pleiotropic. As orthologs of the two major ribonucleases (RNase E and RNase II) of *Escherichia coli* are missing in the *Campylobacter jejuni* genome, in the current study the focus has been on the *C. jejuni* ortholog of PNPase. The effect of PNPase mutation on *C. jejuni* phenotypes and proteome was investigated. The inactivation of the *pnp* gene reduced significantly the ability of *C. jejuni* to adhere and to invade Ht-29 cells. Moreover, the *pnp* mutant strain exhibited a decrease in *C. jejuni* swimming ability and chick colonization. To explain effects of PNPase on *C. jejuni* 81-176 phenotype, the proteome of the *pnp* mutant and parental strains were compared. Overall, little variation in protein production was observed. Despite the predicted role of PNPase in mRNA regulation, the *pnp* mutation did not induce profound proteomic changes suggesting that other ribonucleases in *C. jejuni* might ensure this biological function in the absence of PNPase. Nevertheless, synthesis

were modified in the *pnp* mutant strain suggesting a more specific role of PNPase in *C. jejuni*. In conclusion, PNPase deficiency induces limited but important consequences on *C. jejuni* biology that could explain swimming limitation, chick colonization delay, and the decrease of cell adhesion/invasion ability.

Keywords: *Campylobacter jejuni*, polynucleotide phosphorylase, *in vitro* virulence tests, chick colonization, 2D-electrophoresis

INTRODUCTION

Campylobacter jejuni is considered as the leading cause of human bacterial gastroenteritis in the world (Skirrow, 1994; Altekruze et al., 1999). The main reservoir of *C. jejuni* is the guts of avian species with up to 10⁹ CFU/g of feces (Newell and Fearnley, 2003). Although gut colonization is asymptomatic in mammals and birds, this bacterium causes diarrhea, fever, and abdominal pain in humans. Moreover, in rare but significant cases, *C. jejuni* triggers autoimmune disease such as Guillain-Barré syndrome (Nachamkin et al., 1998). Consumption and handling poultry meat products are the major sources of campylobacteriosis in developed countries (Cohn et al., 2007).

To analyze gene regulation in *C. jejuni* and its impact on cellular functions, several regulators have been identified and characterized. They are involved in oxidative stress (PerR; Palyada et al., 2009), in iron homeostasis (Fur; Holmes et al., 2005), in host cells interaction (Andersen et al., 2005; Fields and Thompson, 2008), in colonization (Guo et al., 2008; Palyada et al., 2009), and other have pleiotropic functions such as HspR (Andersen et al., 2005). Besides these regulators, RNA degradation mechanisms

have shown to be determinant for the post-transcriptional control of gene expression and are also known to be involved in cellular functions. RNases mediate the processing, decay and quality control of RNA, and could be endo- or exoribonucleases (Arraiano et al., 2010). In bacteria, many times mRNAs are first degraded into smaller fragments by endonucleases including RNase E, RNase G, RNase III, and RNase P (Mackie, 1998; Linton et al., 2000; Deutscher, 2006) and subsequently they are degraded in the 3′–5′ direction by exonucleases such as RNase II, polynucleotide phosphorylase (PNPase), and RNase R (Donovan and Kushner, 1986; Deutscher, 2006; Andrade et al., 2009). Complexes of RNases have been also described as RNA-degrading machines namely degradosomes, involved in RNA degradation control of bacteria including *Escherichia coli* (Carpousis, 2007; Arraiano et al., 2010). Such a protein complex has not been yet described in *C. jejuni* and little is known about post-transcriptional regulation mediated by ribonucleases. In *C. jejuni*, RNase E and RNase II responsible for the major RNA degradation in Gram-negative bacteria are absent, however a *pnp* gene has been identified in all sequenced genomes of the species. PNPase encoded by *pnp* gene is a 3′–5′ exoribonuclease

identified in a large number of both eukaryotic and prokaryotic organisms. It has both 3′–5′ exoribonuclease activity and 3′-terminal oligonucleotide polymerase activity (Mohanty and Kushner, 2000) and plays important roles in mRNA degradation, tRNA processing, and small RNA (sRNA) turnover. In most of bacterial sequenced genomes, orthologous genes to *pnp* have been identified (Leszczyniecka et al., 2004).

Polynucleotide phosphorylase does not seem to be essential at optimal temperature in *E. coli*, unless other exoribonucleases (RNase II or RNase R) are also missing. PNPase is however essential for *E. coli* growth at low temperatures (Luttinger et al., 1996; Zangrossi et al., 2000): It is required to grow after a cold shock in *E. coli* (García-Mena et al., 1999; Beran and Simons, 2001; Briani et al., 2007; Matus-Ortega et al., 2007) and it is involved in cold adaptation of *Bacillus subtilis* (Wang and Bechhofer, 1996), *Salmonella enterica* (Clements et al., 2002), and the psychrotrophic bacterium *Yersinia enterocolitica* (Goverde et al., 1998). *C. jejuni* can not grow below 30°C, but can survive several weeks at 4°C. In this context, we have previously shown that PNPase was also involved in the survival at refrigerated temperatures (under 10°C; Haddad et al., 2009). The role of PNPase in the other cellular functions in diverse bacterial species has been also investigated. It was reported to be implicated in antibiotic resistance and competence of *B. subtilis* (Luttinger et al., 1996; Bechhofer and Stasinopoulos, 1998; Bechhofer and Wang, 1998), and in resistance to oxidative stress in *E. coli* (Wu et al., 2009). This exoribonuclease enzyme also regulates virulence determinants such as host cell invasion, type III secretion systems, and infection in mouse in *S. enterica*, *Y. pestis*, and *Y. pseudotuberculosis* pathogens (Clements et al., 2002; Rosenzweig et al., 2005, 2007; Ygberg et al., 2006).

Considering the wide potential cellular functions of PNPase in the bacterial world and the absence of gene encoding the major exoribonuclease RNase II in *C. jejuni*, the aim of this study was to characterize the phenotypic effects of a *pnp* mutation in *C. jejuni* strain on the proteome, cell motility, *in vitro* cell adhesion/invasion and chick *in vivo* colonization.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Campylobacter jejuni 81-176 and F38011 strains and its derivative mutants defective in PNPase (*C. jejuni* 81Δ*pnp* and F38Δ*pnp*) were described in our previous paper (Haddad et al., 2009).

The *pnp* mutant was obtained by double crossover homologous recombination. *C. jejuni* was routinely cultured on Karmali agar plates (Oxoid, Basingstoke, Hampshire, England), or in brain and heart infusion (BHI) broth (Merck, Darmstadt, Germany) under 110 rpm shaking. *C. jejuni* was grown under microaerobic conditions (10% carbon dioxide, 5% oxygen, and 85% nitrogen) at 37° or 42°C. *C. jejuni* enumeration was performed on Karmali agar plates incubated 48 h at 42°C under microaerobic conditions. Kanamycin (Km) 50 μg/ml was used for *C. jejuni* 81Δ*pnp* strain growth. Strains and plasmids used in this study are listed in **Table 1**.

To determine the doubling time of the parental and the mutant strains, we first determined the growth rate in exponential growth phase. *C. jejuni* cultures were grown in BHI and incubated at different temperatures (32, 34, 37, and 42°C). Experiments were repeated three times independently.

COMPLEMENTATION

As described in Haddad et al. (2009), *trans*-complementation of the derivative strains was done using the entire *C. jejuni pnp* gene and its natural promoter cloned into pRY111 vector. The recombinant vector was introduced in the *C. jejuni* derivative strain (Δ*pnp*) by electroporation, and transformants resistant to both kanamycin at 50 μg/ml and chloramphenicol at 15 μg/ml were isolated.

MOTILITY ASSAY

The parental strains (*C. jejuni* 81-176 and F38011), the mutant strains (*C. jejuni* 81Δ*pnp* and F38Δ*pnp*) and the F38Δ*pnp* complemented strain were grown overnight on Karmali plates at 42°C under microaerobic conditions. They were harvested using BHI broth to obtain a cell suspension (OD₆₀₀ = 0.1). One microliter of the cell suspension was deposited in the center of BHI soft agar plates (0.25 or 0.4% agar). After 48 h incubation in microaerobic conditions at 37 or 42°C, the ability of the strain to move in the soft agar was evaluated by measuring the diameter of the spread colonies. The assays were done four times independently.

Ht-29 CELL INFECTION

Bacterial adhesion and invasion into Ht-29 cells was studied using the gentamicin protection assay as described previously (Haddad et al., 2010). Briefly, microplate wells were seeded with 2 × 10⁵ Ht-29 cells then incubated for 5 days at 37°C in a

Table 1 | Strains and plasmids used in this study.

Strain or plasmid	Genotype and plasmid property	Resistance	Source or reference
<i>C. jejuni</i> 81-176	Wild-type strain		Korlath et al. (1985)
<i>C. jejuni</i> 81Δ <i>pnp</i>	Isogenic <i>pnp</i> mutant of 81-176	Km	Provided by C. M. Burns
pBluescript	<i>E. coli</i> cloning vector	Amp	Stratagene
pUC4K	<i>E. coli</i> cloning vector	Amp, Km	
pGA28	pBluescript containing <i>C. jejuni</i> 81-176 <i>pnp</i> gene	Amp	Provided by C. M. Burns
pGA29	pBluescript containing <i>C. jejuni</i> 81-176 <i>pnp</i> gene disrupted with <i>aphA3</i> gene	Amp, Km	Provided by C. M. Burns

Km, kanamycin; Amp, ampicillin.

humidified, 5% CO₂ incubator. After washing, the Ht-29 monolayers were infected with a suspension of approximately 2 to 5 × 10⁷ CFU *Campylobacter*. To measure cell adhesion, the infected monolayers incubated for 1 h at 37°C in a humidified 5% CO₂ incubator were washed, then Ht-29 adherent *Campylobacter* were enumerated on Karmali agar. To evaluate bacterial invasion, the infected monolayers were incubated during 3 h and consecutively 2 h with gentamicin (250 μg/ml). After washes and Ht-29 cell lyses, intracellular *Campylobacter* was enumerated on Karmali agar. Each experiment was done in duplicate and performed at least three times independently. The significance of differences in adhesion and invasion efficiency was determined using Student's *t*-test. A *P*-value <0.05 was defined as significant.

CHICK COLONIZATION

The chick model was used to determine colonization potential of *C. jejuni*. Specific pathogen free (SPF) 2 days old chickens were obtained from the high-security livestock at the French Agency of Food, Environmental and Occupational Health Safety (ANSES). Chicks were housed and treated in accordance with the regulation of the local veterinary office (Direction des Services Vétérinaires des Côtes d'Armor, France). All the animals were reared in isolators with controlled airflow. Sixty SPF chickens were distributed into three groups of 20 animals and maintained in separate isolators with unlimited food and water. One group of 20 animals was kept as negative controls and placed in a separate isolator. At 2 days old, chicks were each dosed orally, by gavage, with 10⁷ CFU of *C. jejuni* in 0.1 ml of 0.1 M PBS, pH 7.2. During 4 weeks, every 7 days, chicks were sacrificed by cervical dislocation. Ceca were aseptically collected then *C. jejuni* enumerated on Karmali agar plate. The non-parametric Mann–Whitney test was used to assess the statistical significance of differences in colonization levels (*P* < 0.01).

A second independent experiment was performed using the same protocol but with 5 days old chicks.

BACTERIAL RNA ISOLATION AND PURIFICATION

To study expression of *pnp* gene versus growth phase and temperature incubation, *C. jejuni* 81-176 was grown in BHI broth at 37 or 42°C until the mid-log phase (5 h) or the stationary phase (16 h). *C. jejuni* cells from 10 ml of mid-log phase and

from 1 ml of stationary phase cultures were harvested (3,300 g, 6 min, 4°C) then resuspended in 1 ml of Extract All (Eurobio, Courtaboeuf, France) and 0.2 volumes of chloroform. After centrifugation at 12,000 g for 15 min at 4°C, the aqueous phase was removed. Total RNA was precipitated in isopropanol, rinsed in cold ethanol 75% then solubilized in 50 μl of sterile water. Isolation of total RNA was performed in triplicate from three independent cultures. DNA was degraded by treatment with DNase I Amplification Grade (Sigma-Aldrich, Saint Quentin Fallavier, France) then DNA removal was checked by PCR using MO001 primers (Table 2). Quality and quantity of RNA were checked using a NanoDrop 2000 (2000c Spectrophotometer, Thermo Scientific). The integrity of RNA samples was checked on a 2% agarose gel; electrophoresis was carried out in Tris–acetate–EDTA buffer for 45 min at 100 V. RNA concentrations were then standardized to 20 ng/μl for each sample prior to reverse transcription.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION PCR

cDNA synthesis was performed using M-MLV (Moloney murine leukemia virus) Reverse Transcriptase (Promega, Charbonnières, France) with random hexamer primers according to the manufacturer's instructions. Quantitative real-time PCR assay was performed using an Applied Biosystems 7300 Real-time PCR System and MO002 to MO007 primers (Table 2). The composition of the PCR mix was as follows: 5 μl of sample, reverse primer (1 μM), forward primer (1 μM), and qsp 25 μl of SYBR Green I Master Mix (Applied Biosystems, Foster City, CA, USA). Amplification program included an initial denaturing step at 95°C (10 min), followed by 40 cycles of 95°C (15 s) and 60°C (1 min). A negative control (without cDNA) was included to each run. A melting curve was obtained from a first step starting from 60 to 95°C, to control specificities of quantitative PCR reaction for each primer pair. Efficiency of amplifications was determined by running a standard curve with serial dilutions of cDNA. The efficiency (*E*) was calculated with the formula $E = [10^{(1/s)} - 1] \times 100$, where "*s*" is the slope of the standard curve. The C_T (critical threshold) stood for the first PCR cycle during which the fluorescence value was significantly distinct from the background and corresponded to a signal. Results were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method in which the amount of target RNA was adjusted to a reference [internal target RNA,

Table 2 | Primers used in this study.

Primer	Sequences		Gene amplified	Amplicon size (bp)
	Forward (5'–3')	Reverse (5'–3')		
MO001	GGATTCGTATTAACACAAATCGTGC	CTGTAGTAATCTTAAAACATTTTG	<i>flaB</i>	1703
MO002	CCAGGTTTTTCAGTAGGCGA	CCACGCTTGATAAAGTGCT	<i>pnp</i>	100
MO003	CGAGCTTGCTTTGATGATGAGTG	AGTCCCACAGGAAAACCTA	<i>rpoA</i>	109
MO004	TAATTGGAACACCTGATGAG	TCCGCATTGGTAGATATTAAG	<i>luxS</i>	116
MO005	CCTAATAGTGGAAGTGCAAGA	GTTCTATGTCAGGATTGCTTT	<i>peb3</i>	98
MO006	CAAACAGCTATGATAATAGCC	GGAGCATATCTTTGTGCTACG	<i>katA</i>	87
MO007	CCATAGCAAAAAGCGGTACTAA	CTAGCCACCATAAAAAGCAGAA	<i>hsp90</i>	118

rpoA (51)]. Relative expression level was obtained by the following formula: $2^{\Delta\Delta C_T}$ (37). RT-qPCR was performed in triplicate with, at least, three templates of RNA extracted from independent cultures.

PROTEIN EXTRACTION

To avoid possible misinterpretation of results, proteins were extracted from *C. jejuni* 81-176 or 81 Δ *pnp* cultures at the late exponential phase showing similar optical density (OD) at 600 nm and cell counts on agar. Thus, the two strains were assumed to be in the same physiological state. Prior to each experiment, one colony grown on Karmali agar for 48 h at 42°C was resuspended in 50 ml of BHI broth then incubated at 42°C for 24 h in microaerobic conditions with shaking at 110 rpm. Two milliliters of the resulting culture were used to inoculate 200 ml of BHI broth incubated for 7 h in the same conditions (until OD₆₀₀ reached 0.2). Cells were harvested by centrifugation at 7,000 g for 20 min, washed consecutively with 200 mM glycine solution (Sigma-Aldrich) and 100 mM Tris-HCl pH 7.0 solution (Sigma-Aldrich) then resuspended in 10 ml of a 10 mM Tris-HCl pH 7.0 buffer. Cells were disrupted by series of 6 × 30 s sonications with 6 min intervals on ice (Vibracell 72434, Bioblock Scientific, Illkirch, France). To eliminate cell debris, samples were centrifuged twice at 10,000 g for 20 min at 4°C. Then, cytoplasmic proteins were separated from membrane fractions by ultracentrifugation at 188,000 g for 1 h at 4°C. The cytoplasmic protein fraction in the supernatant was treated with protease inhibitor cocktail tablets Complete™ (Roche Diagnostics, Mannheim, Germany) and nuclease solution containing RNase and DNase (Sigma-Aldrich). Protein samples were dialyzed using cellulose membrane tubing with a cut-off at 12,000 Da (Sigma-Aldrich) against Milli-Q water at 4°C in shaking conditions during 3 days, refreshing the dialyze bath each day. Total protein concentration was determined using the Micro BCA™ Protein Assay Kit (Perbio-Science, Brebieres, France).

TWO-DIMENSIONAL GEL ELECTROPHORESIS OF BACTERIAL PROTEINS

A quantity of 100 μg of protein was concentrated using Concentrator 5301 (Eppendorf, Le Pecq, France), at room temperature. Samples were diluted with 275 μl of a solution of 6 M urea, 2 M thiourea, 4% CHAPS, 0.4% DTT, bromophenol blue (BB; Sigma-Aldrich) and 2% Biolyte 3/10 (Bio-Rad, Marnes-la-Coquette, France). Proteins in the rehydration solution were absorbed overnight by 17 cm pH 4–7 or 6–11 IPG strips (Bio-Rad, GE Healthcare, Orsay, France). Then, the iso-electro-focalization (IEF) was performed using the Bio-Rad IEF program as follows: from 50 to 250 V for 3 h, from 250 to 6,000 V for 3 h, and at 6,000 V until reaching 54,000 Vh. Finally, each strip was soaked for 20 min in equilibration buffer [1.5 ml of a 6-M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8), 30% glycerol and BB] supplemented with 2% DTT and subsequently for 20 min in equilibration buffer with 4% iodoacetamide (Bio-Rad). The second dimension was performed in 12% acrylamide gels covered with 1% low-melting point agarose (Bio-Rad) and run at 40 mA/gel at 14°C using a Protean II xi cell (Bio-Rad) until the migration reached the base of the gels. Proteins in gels were finally silver stained and scanned with a GS-800 densitometer (Bio-Rad) operated with the Quantity One® software (Bio-Rad) at the resolution of 42.3 μm.

IMAGE, STATISTICAL ANALYSIS, AND PROTEIN IDENTIFICATIONS

The image analysis was performed using the Progenesis Samespots® software (NonLinear Dynamics, Newcastle upon Tyne, UK). The statistical analysis of the results was performed with two technical replicates of three independent experiments resulting in six gels for each strain. Differences between the parental and mutant strain proteome (2 × 6 gels) were validated by principal component analysis (PCA) and differences among matched spot intensities were statistically validated by ANOVA (at a 5% significance level) and by using a Power superior to 0.8. The PCA analysis ensured the reproducibility of the experiments and has shown a significant effect of the *pnp* disruption on *C. jejuni* proteome. Over- and down-expressed proteins were taken into account only if the mean difference in spot intensities passed the threshold of 1.8. When spots of interest were located, 2D-electrophoresis gels were performed again using 700 μg of proteins and were stained with BioSafe colloidal Coomassie blue (Bio-Rad). Spots of interest were excised from preparative gels and protein identification was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using PAPPSo platform facilities¹. Gel plugs were first washed twice with 50% (v/v) acetonitrile, 25 mM ammonium carbonate in water, and then dried in a vacuum speed concentrator. Aliquot (10 μl) of a trypsin solution (Promega, 12.5 ng/μl in 50 mM NH₄HCO₃) was added to each sample and digestion was performed for 6 h at 37°C. A 1-μl aliquot of each supernatant was spotted directly onto the MALDI plate then dried at room temperature before adding 1 μl of the matrix solution [α -cyano-4-hydroxycinnamic acid, 3 mg/ml in 50% (v/v) acetonitrile 0.1% (v/v) trifluoroacetic acid]. Mass spectra were acquired on a Voyager-DE-STR (Applied Biosystems, Framingham, MA, USA), equipped with a nitrogen laser (Laser Science, Franklin, MA, USA). Tryptic autodigestion ion peaks, (M + H)⁺ = 2211.104 and 842.509 Da, were used for internal calibration of spectra. The peptide mass lists were used for protein identification in a *C. jejuni*, strain 81-176, amino acid sequence database (UniProtKB, 09.09.2010), using the Protein Prospector v 3.2.1 software set with the following parameters: mass tolerance <20 ppm, four peptides required to match, one missed cleavage, oxidized methionine and carbamidomethylated cysteine respectively as possible and fixed modifications.

POLY(A) DEGRADING ACTIVITY IN *C. jejuni* CELL LYSATES

Campylobacter jejuni was grown in Mueller-Hinton medium to late log phase, lysates prepared using EasyLyse (Epicenter), and assayed for ribonuclease activity as described (Deutscher and Reuven, 1991), except that the reactions were done in a buffer optimized for measurement of RNase R (Cheng et al., 1998). Degradation of [³H]poly(A) (Amersham Product TRK.480) was quantified by scintillation counting [³H]nucleotide released into acid soluble form. Dependence of the reaction on lysates of *C. jejuni* parental and *pnp* mutant strains was measured in the presence and absence of 10 mM KPO₄ to distinguish hydrolytic from phosphorolytic activities. Less than 10% of substrate was consumed in the reactions.

¹<http://pappso.inra.fr>

IN SILICO ANALYSIS

DNA and protein homology searches were performed by BLAST analysis². Oligonucleotide primers were designed using Primer 3 program³.

RESULTS

BACTERIAL GROWTH CURVES AND *pnp* GENE EXPRESSION

IN *C. jejuni* 81-176

To determine the expression profile of *pnp* gene, quantitative RT-PCR experiments were assayed in four different growth conditions. *C. jejuni* 81-176 was grown in BHI broth at 37 or 42°C until the mid-log phase (5 h) or the stationary phase (16 h). C_T values were determined in four replicates from cDNA synthesized from RNA extracted from three independent cultures for each growth condition. The specificity of each primer pair (MO002 to MO007) was controlled by analyzing melting curves. PCR efficiencies of the primer pair used were homogenous and all over 90%. The *rpoA* gene was used as an internal control (Ritz et al., 2009) and the expressions at 37°C in mid-log phase were used as calibrators to calculate relative expression levels. No significant difference ($P < 0.05$) in expression of *pnp* gene was observed for the four growth conditions tested (Figure 1).

There is a difference between the growth of the parental and the mutant strain, however this difference is only significant below 35°C as shown in Figure 2.

To check that phenotypic changes in the *pnp* mutant strain were specifically due to PNPase absence, we attempted genetic complementation of *pnp* in *C. jejuni* 81-176. Despite several attempts, we never obtained such a complemented strain in *C. jejuni* 81-176, but were able to complement the *pnp* mutation in *C. jejuni* strain F38011 (Haddad et al., 2009).

EFFECT OF *pnp* MUTATION ON *C. jejuni* 81-176 MOTILITY

Motility is a common bacterial virulence factor involved in colonization and invasion of host cells by pathogens. Swimming abilities of *C. jejuni* 81-176 parental and *C. jejuni* 81Δ*pnp* mutant strains are presented Figure 3 on soft agar plate 0.4%. Results indicated that inactivation of *pnp* gene caused a 5.3-fold reduction in motility on agar plates compared to the parental strain, at both 37 and 42°C. Because motility is phase variable in *C. jejuni*, measurements were achieved using independent cultures on different days. The results of four experiments were essentially identical, suggesting that reduced motility of the mutant strain is unlikely due to phase variation. To test if the motility defect observed is specifically linked to the inactivation of the *pnp* gene, we also tested the effect of genetic complementation of the *pnp* mutant with the wild-type *pnp* gene. As we were unable to complement the *pnp* mutation in *C. jejuni* 81-176, we used the complemented mutant of strain F38011 (Haddad et al., 2009). As shown in Figure 3C, complementation of the *pnp* mutation in trans restored partially wild-type motility, confirming that absence of PNPase results in reduced motility and associated virulence phenotypes.

Moreover, impaired mutant strain motility was also observed on soft agar plate 0.25% (data not shown).

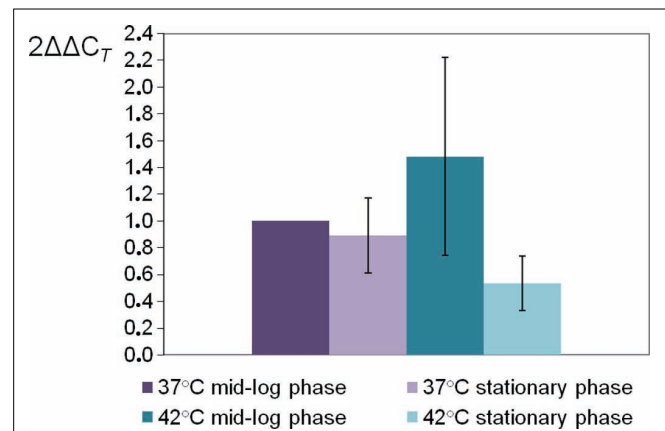


FIGURE 1 | Relative expression levels of *C. jejuni* 81-176 *pnp* gene at different conditions. Gene expression has been estimated using RT-qPCR and the comparative critical threshold ($\Delta\Delta C_T$) method. The *rpoA* gene was used as the internal control, and the expression at 37°C in mid-log phase as the calibrator. Error bars represent the standard deviations from the mean of three independent experiments.

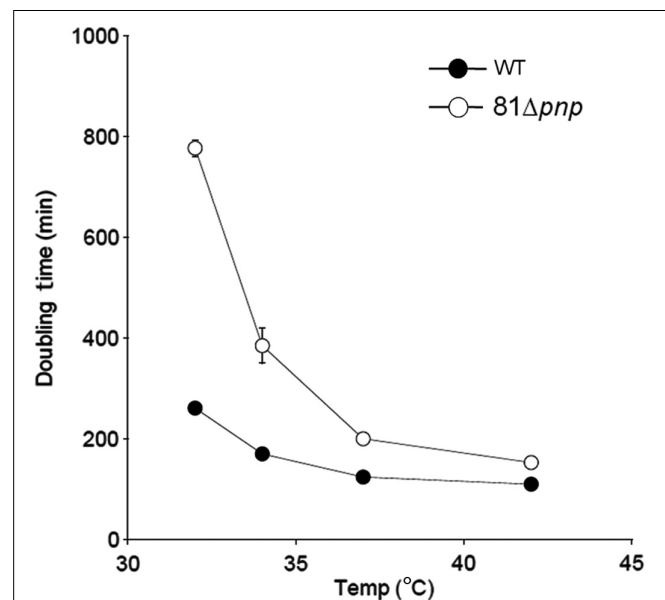


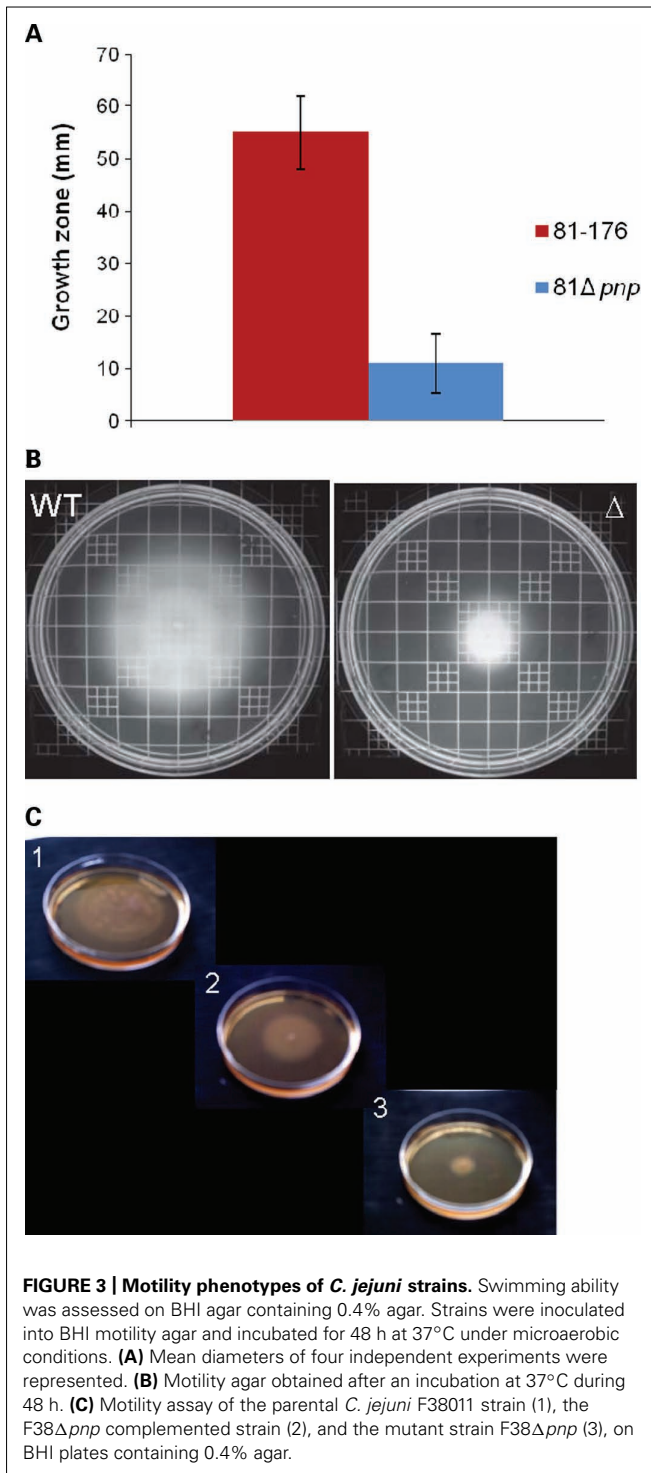
FIGURE 2 | Mean generation time of the parental strain (solid circles) versus the mutant strain (open circles). *C. jejuni* was cultivated in BHI broth and incubated in microaerobic condition at 32, 34, 37, and 42°C. Experiments were performed three times independently.

EFFECT OF *pnp* MUTATION ON ADHESION TO AND INVASION IN Ht-29 CELLS

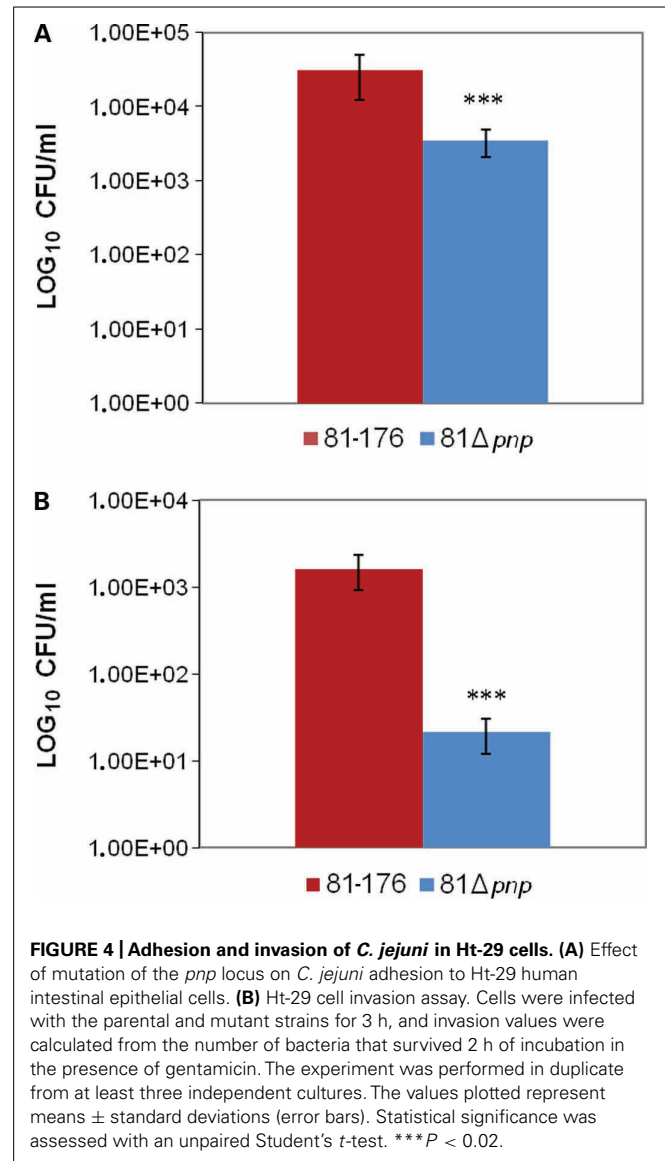
In *C. jejuni*, motility is critical both for invasion of gastrointestinal epithelial cells and for colonization of the mucus lining of the gastrointestinal tract (Yao et al., 1994). To examine the role of the *pnp* gene in *C. jejuni* infection, the *in vitro* adhesion and invasion abilities of the *pnp* mutant and parental *C. jejuni* 81-176 strains were compared. The *C. jejuni* 81Δ*pnp* mutant exhibited a significant ($P \leq 0.02$) decrease in adhesion ability compared to the parental strain (Figure 4A), and a 3 log-fold decrease in invasion

²<http://www.ncbi.nlm.nih.gov>

³<http://frodo.wi.mit.edu/primer3/>



of Ht-29 cells ($P < 0.02$; **Figure 4B**). No difference in susceptibility to gentamicin for both strains was observed, indicating that the invasion phenotypes of the strains were not due to differences in gentamicin susceptibilities (data not shown). Results of invasion assays are consistent with the lowered adherence of the mutant strain and indicate that inactivation of the *pnp* locus indirectly affects the interaction of *C. jejuni* with epithelial cells,



most likely via the reduced motility resulting from the absence of PNPase.

EFFECT OF PNPase DEFICIENCY ON COLONIZATION OF CHICK GUT BY *C. jejuni*

According to the motility and adhesion results, colonization experiments were also conducted. To determine the relative importance of PNPase in chicken colonization, we inoculated 2 days old chicks with the parental and PNPase mutant strains. Three groups of five birds were infected with 10^7 CFU of parental or mutant strains. On day 7, viable bacteria were found in all five chickens fed with the parental strain, whereas viable mutant bacteria were recovered from only two. In addition, the mean colonization concentration of the chicken inoculated with the parental strain was 1.5×10^7 CFU/g on day 7 and was significantly lower (2.4×10^5 CFU/g; $P < 0.01$) in the chickens inoculated with the mutant strain. On days 14, 21, and 28,

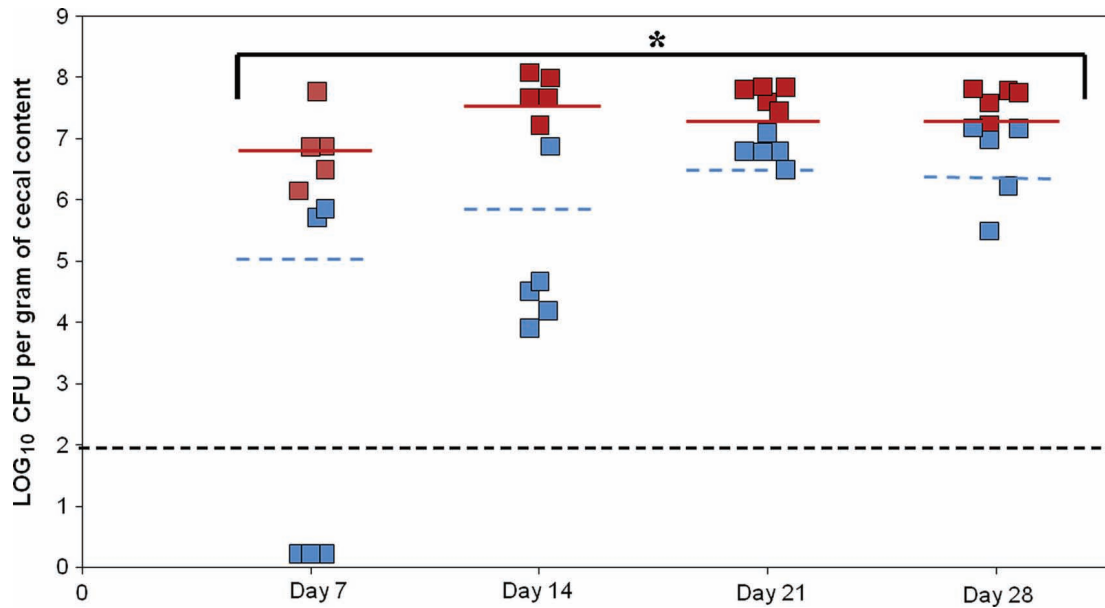


FIGURE 5 | Effect of the PNPase mutation on chick gut colonization by *C. jejuni*. The experiment included three groups of birds. Two days old chicks were orally gavaged with 1.4 to 1.5×10^7 CFU of *C. jejuni* 81-176 (red squares) or 81-176 derivative strain lacking production of PNPase (blue squares). Colonization levels were measured by enumeration of bacteria present in ceca on days 7, 14, 21, and 28 post-inoculation (LOG₁₀ CFU/g of

ceca). Each dot represents the load of *C. jejuni* in the cecum of an individual chick. The geometric mean of the bacterial loads from each set of chicks is denoted by the horizontal bar. The line designates the detection limit of 2×10^2 CFU/g of ceca. Statistical analysis was performed using the Mann-Whitney test (mean and *P*-value <0.01). * Significant difference between the parental and *pnp* mutant strains.

viable mutant bacteria were detected in all the chickens. However, throughout the experiment, the mean colonization level of the chicken inoculated with the mutant strain was significantly reduced compared to those inoculated with the parental *C. jejuni* (Figure 5; $P < 0.01$). The defect in colonization of the *pnp* mutant strain was confirmed by a second experiment using SPF 5 days old chicken in which, the mutant strain was unable to colonize chicken gut, while the parental strain reached a colonization level of 9.3×10^7 CFU/g at 29 days (data not shown).

COMPARATIVE PROTEOMIC ANALYSIS

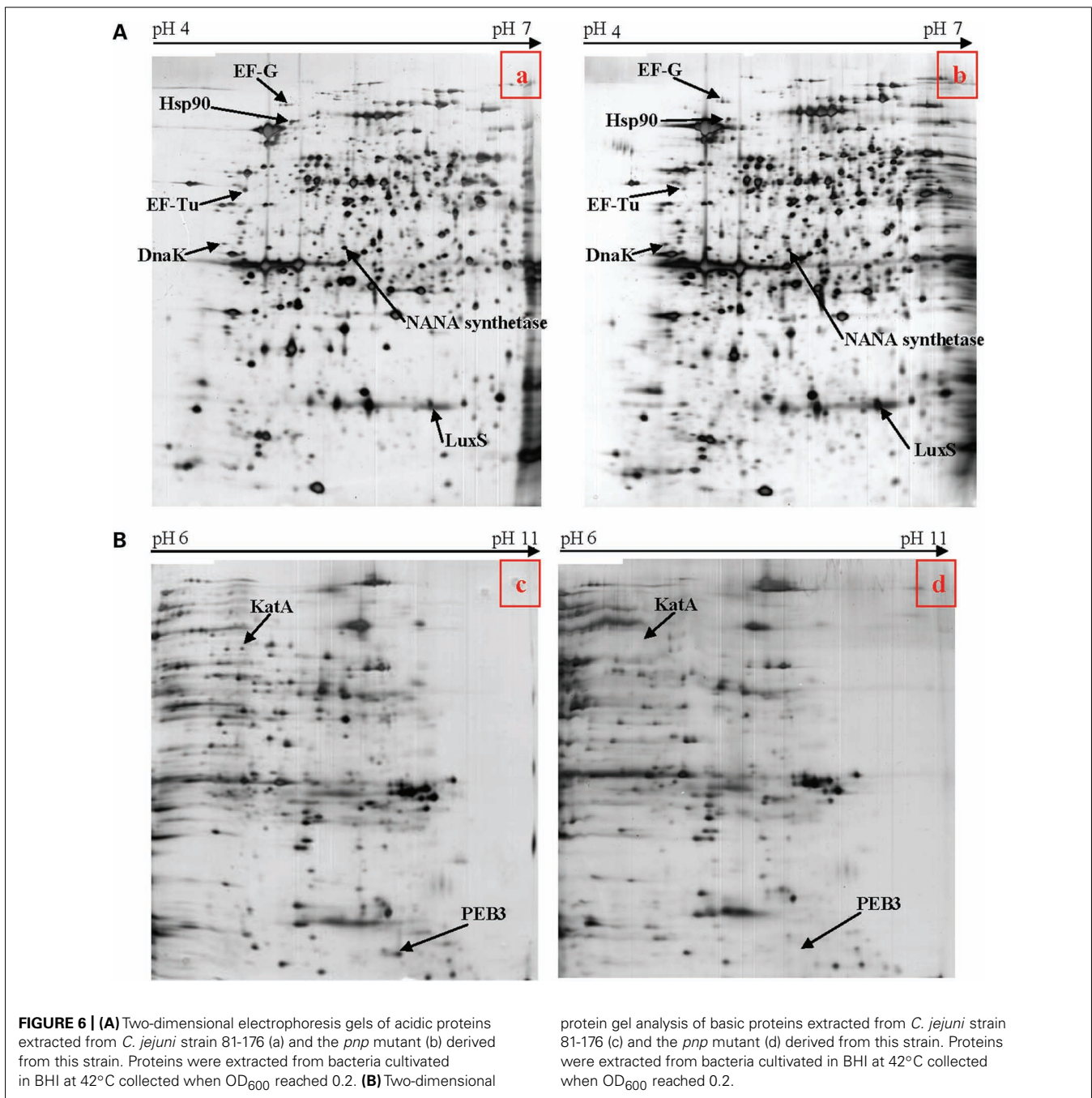
In order to pinpoint the effect of *pnp* mutation at a protein level, proteomic analyses were performed. Proteomes of the *pnp* mutant and its parental strains were compared using Progenesis SameSpots® software. Examples of digital images from 2-DE gels achieved in the 4–7 and 6–11 pI ranges are given in Figures 6A,B. This comparative analysis resulted in nine preponderant proteins showing a significant difference in expression, amongst which three were over-expressed and six were repressed in the *pnp* mutant strain compared to the parental strain (Table 3). As expected, spot occurrences corresponding to PNPase were absent in the mutant strain (Table 3). Among the down-expressed proteins, the heat-shock protein Hsp90, catalase KatA, the elongation factor EF-Tu, the *N*-acetylneuraminic acid (NANA) synthetase and, an antigenic peptide, PEB3 were identified. Among the over-expressed proteins, the elongation factor EF-G, the chaperone DnaK and S-ribosylhomocysteinase, LuxS were found (Table 3).

EFFECT OF *pnp* MUTATION ON *luxS*, *peb3*, *kata*, AND *hsp90* GENES EXPRESSION

To analyze if our proteomic data are correlated to gene expression, a quantitative RT-PCR analysis was performed on four genes known to be involved in adhesion, invasion, or colonization ability of *C. jejuni*. Therefore, gene expression of *luxS*, *peb3*, *kata*, and *hsp90* was measured (Figure 7). Levels of *peb3* and *kata* mRNA was significantly decreased in the *pnp* mutant strain as compared to the parental strain, showing the same trend of protein expression, while gene expression of *luxS* and *hsp90* remained unaffected by *pnp* mutant, but not protein expression.

RNA DECAY IN *C. jejuni*

An established method to assess the relative contributions of phosphorolytic and hydrolytic degradation in bacterial lysates was employed to characterize behavior of *C. jejuni*. Reactions were done to confirm measurements were made in the linear range of the assay. Figure 8 shows that poly(A) can be degraded in the parental *C. jejuni* cell lysates by a hydrolytic mechanism, revealing the existence of hydrolytic ribonucleases. However, phosphorolytic RNA degradation predominates as phosphate addition greatly increased activity. Moreover, poly(A) degradation by lysates of the *pnp* mutant strain was almost totally non-responsive to phosphate addition (Figure 8), indicating that the majority of phosphorolytic activity, and therefore the majority of poly(A) degrading activity in the parental cells, depends on the *pnp* gene. Comparison of hydrolytic poly(A) degrading activity in parental and mutant strains lysates revealed that *pnp* deletion increases this activity by almost fivefold.



DISCUSSION

Ribonucleases are enzymes involved in post-transcriptional regulation of genes by mediating RNA degradation. In some Gram-negative bacteria, the endoribonuclease RNase E is essential for cellular growth and inactivation of *rne* gene (encoding RNase E) impedes processing and prolongs the lifetime of bulk mRNA (Cam et al., 1996; Ow and Kushner, 2002). For the exoribonuclease activity, RNase II is responsible for the majority of RNA phosphodiester bond cleavage in *E. coli* as most RNA (90%) is degraded via a hydrolytic mechanism (Deutscher and Reuven, 1991). Genes encoding RNase E and RNase II enzymes are absent

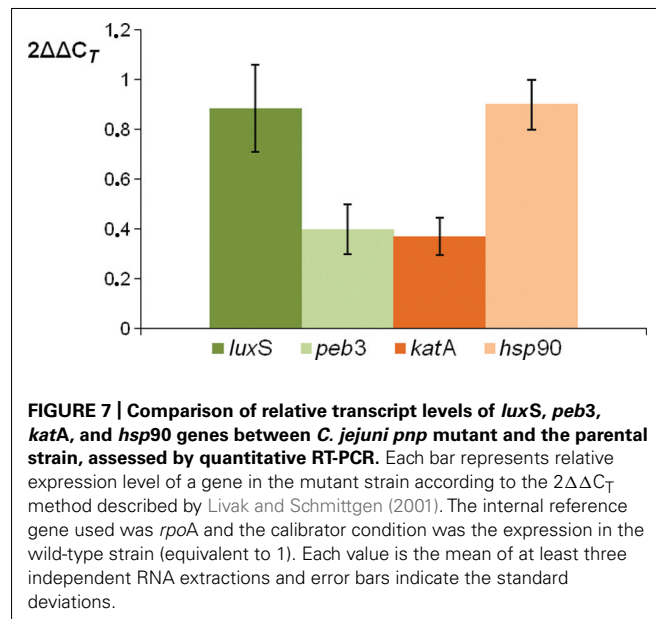
in the *C. jejuni* genome, suggesting a major role is played by other ribonucleases like PNPase. Because of the pleiotropic functions of PNPase described in various bacteria (Luttinger et al., 1996; Wang and Bechhofer, 1996; Bechhofer and Stasinopoulos, 1998; Goverde et al., 1998; Yamanaka et al., 1999; Clements et al., 2002; Rosenzweig et al., 2005, 2007; Ygberg et al., 2006; Wu et al., 2009), the present study broadly analyzed the role of PNPase in *C. jejuni*, beyond its role in long-term survival to cold (Haddad et al., 2009).

In the current study, we have examined the role of the putative post-transcriptional regulator PNPase and its consequences on *C. jejuni* proteome, which may then affect some biological

Table 3 | *Campylobacter jejuni* over- and down-expressed proteins in the *pnp* mutant as compared to the parental strain.

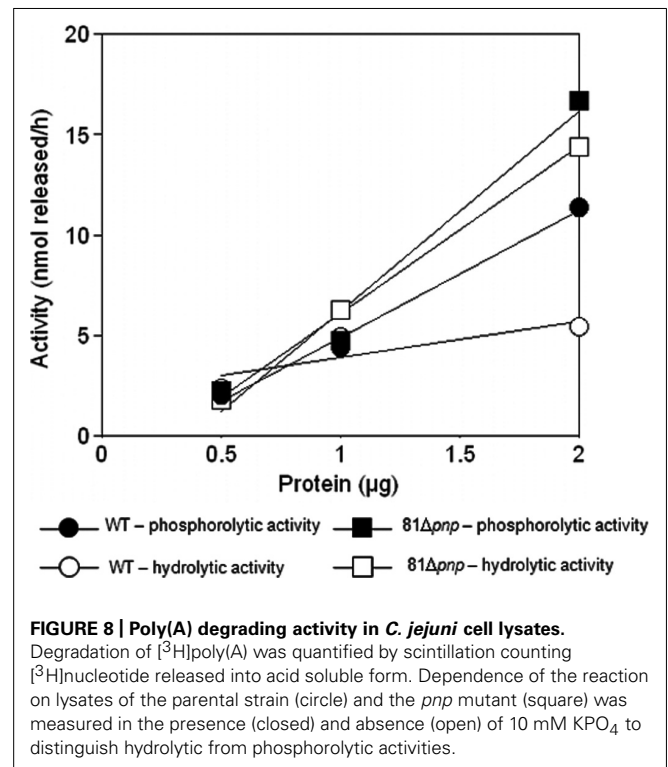
Protein identification	<i>C. jejuni</i> no.	Accession number	MW (kDa)/pI	MOWSE score	No. peptides (% masses matched)	Fold in <i>pnp</i> mutant
Heat-shock protein 90 (Hsp90)	CJJ81176_0546	YP_001000221	69.6/5.1	144	6/43 (13%)	-2.4
<i>N</i> -acetylneuraminic acid synthetase	CJJ81176_1334	YP_001000992.1	38.7/5.4	276	4/23 (17%)	-1.8
Polynucleotide phosphorylase (PNPase)	CJJ81176_1269	YP_001000929.1	79.1/5.3	893	9/64 (14%)	-3.5
Truncated PNPase	CJJ81176_1269	YP_001000929.1	79.1/5.3	153	6/56 (10%)	-2.2
Truncated PNPase	CJJ81176_1269	YP_001000929.1	79.1/5.3	479	4/47 (8%)	-2.2
Elongation factor EF-Tu	CJJ81176_0499	YP_001000177.1	46.6/5.1	4.8E + 07	15/65 (23%)	-2
Major antigenic peptide PEB 3	CJJ81176_0315	YP_001000003.1	27.5/9.4	33	6/29 (20%)	-3.1
Catalase (KatA)	CJJ81176_1387	YP_001001043.1	54.3/7.3	108	4/27 (14%)	-2.5
S-ribosylhomocysteinase (LuxS)	CJJ81176_1213	YP_001000873.1	18.3/5.9	557	6/30 (20%)	2.4
Elongation factor EF-G	CJJ81176_0513	YP_001000189.1	76.7/5.1	307	5/28 (17%)	2.3
Molecular chaperone DnaK	CJJ81176_0775	YP_001000443.1	67.4/5	2.3E + 04	7/64 (10%)	1.8

MW, molecular weight; pI, isoelectric point; No. peptides, number of peptides identified.



functions of the pathogen. Growth curve data show that, inactivation of the *pnp* gene has no significant effect on *C. jejuni* 81-176 growth rate at optimal temperature. Generation time increases with the decrease of the temperature of incubation, a finding that confirms the hypothesis that PNPase is involved in *C. jejuni* survival at temperatures below the optimal temperature of growth.

Transcript analyses revealed that expression of *C. jejuni* 81-176 *pnp* gene is not influenced by growth phase or by optimal temperatures of growth. In order to define the role of PNPase in the cellular biology of *C. jejuni*, proteomic profiles of parental and PNPase mutant strains were compared. The *pnp* gene mutation affects synthesis of proteins in *C. jejuni* 81-176; however, only few proteins vary significantly between the parental and the *pnp*-deleted strains. Although comparison of proteome of these



two strains verified the non-essential role of *pnp* gene in *C. jejuni* 81-176 strain, the majority of phosphorolytic ribonuclease activity seems to depend on PNPase. Moreover, deletion of *pnp* gene increases hydrolytic ribonuclease activity. These results suggest that PNPase either down regulates, or antagonizes function, of a hydrolytic ribonuclease in *C. jejuni*. The enzyme responsible for hydrolytic poly(A) degrading activity is unknown, but comparative sequence analysis predicts a single hydrolytic mRNA degrading exoribonuclease encoded by the *C. jejuni* genome, RNase R.

The swarming ability of the *C. jejuni pnp* mutant strain is reduced. As motility is directly linked to *C. jejuni* pathogenic features, we confirmed that the motility defect observed with the *pnp* mutant strain is due to the *pnp* mutation using the F38011 complemented strain. The motility of *C. jejuni* is conferred by polar flagella, and approximately 40 genes are predicted to be involved in flagella biosynthesis or function (Yao et al., 1994; Parkhill et al., 2000). Flagella-mediated motility is responsible for colonization of the mucous lining of the mammalian and avian gastrointestinal tracts as well as invasion of intestinal epithelial cells (Wassenaar et al., 1991; Grant et al., 1993; Rosenzweig et al., 2007). In proteomic analysis, the abundance of protein related to flagella was not affected by the mutation however a decrease in NANA synthetase production was observed in the *pnp* mutant strain, a factor supposed to be involved in *C. jejuni* motility. NANA synthetase, encoded by the *neuB* gene, catalyzes formation of NANA, the main component of *C. jejuni* lipo-oligosaccharide (LOS). Linton et al. (2000) hypothesized that *neuB3* gene is involved in the post translational modification of the flagellin subunit and in motility, as inactivation of *neuB3* leads to non-motile and aflagellate cells (Linton et al., 2000). This information is particularly interesting in that for the *C. jejuni* 81-176 genome, *neuB* is located in a gene locus encoding proteins involved in the formation of flagella or mobility. Because motility is a prerequisite to eukaryotic cells invasion, we expected that the *pnp* mutant would be less invasive than its parental strain. Studies using Ht-29 cells confirmed that mutation of *pnp* decreased the invasiveness of *C. jejuni* and also a decrease in adhesion ability. The putative adhesion PEB3 was 3.1-fold decreased in the *pnp* mutant and quantitative RT-PCR also revealed a diminution in *peb3* mRNA synthesis. PEB3 protein shares 51.8% sequence identity to Paa, an *E. coli* adhesion (Batisson et al., 2003) and 49% identity to AcfC, an accessory colonization factor from *Vibrio cholerae* (Peterson and Mekalanos, 1988). PEB3 of *C. jejuni* is considered as an adhesion based on the work performed with Hep-2 cells by Wren et al. (Drs. B. W. Wren and R. Langdon, personal communication; Rangarajan et al., 2007).

Interestingly, PNPase was described as a factor affecting *S. enterica* invasion and establishment of chronic infection in a mouse model (Clements et al., 2002). In contrast with results obtained in *C. jejuni*, *S. enterica* pathogenicity islands are over-expressed in PNPase mutants, resulting in increased invasion into epithelial MDCK cells (Clements et al., 2002). However, the cell line used in Clements et al. (2002) study has been isolated from canine kidney and is largely different from those utilized in the current study. Moreover, studies on *S. enterica* PNPase use the typhoid strain of the bacteria while the current study showed interaction of *C. jejuni* with intestinal cells. Other ribonucleases have been shown to be involved in bacterial virulence. RNase R of *Shigella flexneri* has been reported to be required for the expression of the invasion factors IpaB, IpaC, and IpaD (Cheng et al., 1998).

As chickens are the principal origin of campylobacteriosis in humans, the potential colonization of the parental and mutant strains were also investigated in the avian intestine. Initial colonization was markedly different between the variants, indicating that PNPase is required to establish colonization. Hanel et al.

(2004) have used a chick model to study the colonization ability of different strains of *C. jejuni* (Hanel et al., 2004). Three groups of strains have been determined. The first group corresponds to strains which could not be re-isolated after chicken inoculation; the second one contains weak or delayed colonizers; the last group includes strong colonizers that could be re-isolated from all animals 7 days after inoculation. In the current study, the wild-type strain *C. jejuni* 81-176 could be considered as a “strong colonizer” because this strain was present in all animals up to 7 days after inoculation. However, *pnp* disruption results in a delayed colonization of the *C. jejuni* mutant strain, which can be linked to the group 2 of Hanel et al. (2004). This defect in colonization could be due to the defect in motility and adhesion abilities of the mutant strain. Moreover, the observed lower expression of *katA* gene and KatA protein in the *pnp* mutant strain could be linked to the deficiency in colonization. *C. jejuni* expresses a single heme-containing catalase, KatA, which is part of a defense system against oxidative stress (Haas and Goebel, 1992). In addition, KatA contributes to intramacrophage survival of *C. jejuni* and chick gut colonization (Day et al., 2000; Palyada et al., 2009). A $\Delta katA$ mutant is unable to colonize the chick ceca after 4 days of incubation (Palyada et al., 2009).

Proteomic experiments showed that LuxS, another protein involved in colonization, is 2.4-fold overproduced in the PNPase mutant strain, but no variation was observed in *luxS* mRNA level. Being the last enzyme in the AutoInducer-2 (AI-2) biosynthesis pathway, LuxS controls production of AI-2. While Holmes et al. (2009) have shown that AI-2 does not function as a quorum sensing molecule in *C. jejuni* during exponential growth *in vitro* (Holmes et al., 2009), LuxS seems to be involved in *C. jejuni* motility by modifying transcription rate of flagellin *flaA* mRNA without change the level of total flagellin protein in the cells (Jeon et al., 2003). Quiñones et al. (2009) have also demonstrated the implication of LuxS in chick gastrointestinal tract colonization and adherence to avian cells LMH, which could be correlated with phenotypic results obtained in this work (Jeon et al., 2005; Quiñones et al., 2009).

Proteomic results indicated that synthesis of two heat-shock proteins were modified by inactivation of the *pnp* gene suggesting a probable stress-like response in the mutant strain. Hsp90 was down-expressed, whereas DnaK chaperone was up-regulated in the *pnp* mutant. In the *C. jejuni* mutant strain, Hsp90 was 2.4-fold less abundant than in parental strain; however, transcript analysis did not reveal such variation. Transcript *hsp90* could be more stable in the absence of PNPase enzyme and accumulation of this mRNA would lead to a greater production of the protein. In *E. coli*, Hsp90 participates in *de novo* protein folding during a heat-shock condition by facilitating the ability of the DnaK–DnaJ–GrpE complex to interact with newly synthesized polypeptides (Thomas and Baneyx, 2000); however, Homuth et al. (2000) suggested that the probable role of this protein is in response to acid or oxidative stress, because Hsp90 is not over-expressed in *Helicobacter pylori* following a heat-shock (Homuth et al., 2000). Moreover, Hsp90 has been implicated in *Salmonella* pathogenesis by contributing to bacterial survival within host cells (Osman et al., 2009).

In the case of DnaK, this protein is known to be involved in DNA replication, protein folding, and in stress-response. DnaK

was up-regulated in the *C. jejuni pnp* mutant compared to the parental strain, suggesting accumulation of denatured proteins in the mutant cells. Interestingly in *E. coli*, DnaK is preferentially associated to PNPase in the degradosome, a multiprotein complex involved in RNA degradation (Regonesi et al., 2006); however, no degradosome has been yet described in *C. jejuni* particularly because the major component of this complex, RNase E, is not detected in the genome of *C. jejuni*. Production of two elongation factors (EF-Tu and EF-G) was also modified in the mutant strain, suggesting a role of PNPase in the translation mechanism or a rearrangement of protein production in absence of this exoribonuclease.

In conclusion, deficiency in PNPase production in *C. jejuni* results in a defect in its ability to swim, to adhere and invade intestinal cells, and to colonize chick gut. All these phenotypic features could be linked to the motility or export function of the flagella. Further analyses should focus on a possible link existing between PNPase and *C. jejuni* flagella. Moreover, mutation of the *pnp* gene induces a stress-like state of the bacterial cell, observed with overproduction of proteins such as Hsp90, DnaK, KatA, suggesting that PNPase could be involved in *C. jejuni* resistance to stress. Proteomic experiments showed that PNPase inactivation causes little variation in *C. jejuni* protein production, but, specific proteins such as PEB3 and KatA are nonetheless affected. These data suggest that in *C. jejuni*, PNPase is involved in gene expression in

multiple ways, in addition to directly degrading the primary transcript; however, PNPase is responsible for adding nucleotides to an existing poly(A) tail of mRNA through a poly(A) polymerase activity (Li and Deutscher, 1994; Mohanty and Kushner, 2000; Oussenko et al., 2005; Viegas et al., 2007). The heteropolymeric tails synthesized by PNPase may further enhance the degradation of mRNA intermediates targets. Further molecular studies using mRNA stability assays will be conducted to verify if the regulation of the genes identified in this study are potential targets of PNPase, either directly or through an alternative mechanism. Indeed, RNase R can be substituting PNPase since the *pnp* mutant strain has the hydrolytic activity increased. This could explain the little variation observed in proteomic results. In *E. coli*, RNase II antagonizes PNPase action on polyadenylated transcripts. In *C. jejuni*, PNPase may antagonize RNase R action. However, direct measurement of hydrolytic ribonuclease gene expression or combinatorial *in vitro* RNA degradation assays may also be required.

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