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Long-Term Survival of *Campylobacter jejuni* at Low Temperatures Is Dependent on Polynucleotide Phosphorylase Activity▽

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***Campylobacter jejuni* is a leading cause of bacterial gastroenteritis worldwide. Infection generally occurs after ingestion of contaminated poultry products, usually conserved at low temperatures. The mechanisms promoting survival of *C. jejuni* in the cold remain poorly understood despite several investigations. The present study provides insight into the survival mechanism by establishing the involvement of polynucleotide phosphorylase (PNPase), a 3′-5′ exoribonuclease with multiple biological functions in cold survival. The role of PNPase was demonstrated genetically using strains with altered *pnp* genes (which encode PNPase) created in *C. jejuni* F38011 and *C. jejuni* 81-76 backgrounds. Survival assays carried out at low temperatures (4 and 10°C) revealed a difference of 3 log CFU/ml between the wild-type and the *pnp* deletion (Δ*pnp*) strains. This did not result from a general requirement for PNPase because survival rates of the strains were similar at higher growth temperatures (37 or 42°C). *trans*-Complementation with plasmid pNH04 carrying the *pnp* gene under the control of its natural promoter restored the cold survival phenotype to the *pnp* deletion strains (at 4 and 10°C) but not to the same level as the wild type. In this study we demonstrate the role of PNPase in low-temperature survival of *C. jejuni* and therefore attribute a novel biological function to PNPase directly related to human health.**

Campylobacter jejuni is presently considered a leading cause of human gastroenteritis worldwide (1, 5, 17). In the United States, 2.5 million cases of *Campylobacter* infection occur yearly, and most of these infections (about 80%) are the result of food-borne transmission (28). Campylobacteriosis is characterized by several common clinical symptoms including watery, bloody diarrhea; abdominal pain; fever; headache; and nausea. Occasionally, extraintestinal infection or postinfection complications, such as reactive arthritis or neurological disorders, occur. Importantly, *C. jejuni* is the most recognized antecedent cause of Guillain-Barré syndrome, a polio-like form of paralysis that can result in respiratory and severe neurological dysfunction and even death (30).

Although the optimal growth temperature of *C. jejuni* is between 37 and 42°C (26), *C. jejuni* has the potential for remarkable survival under nonpermissive temperature conditions (7). It was determined that *C. jejuni* survived for only a few days when incubated at 20°C in surface water and microcosm water (sterilized water), but survival was prolonged to several weeks at 4°C (4, 41). In a viable but nonculturable state, *C. jejuni* survived for about 4 months at 4°C (37), and survival of nonculturable cells continued for up to 7 months based on signs of cellular integrity, respiratory activity, and intact DNA content (25).

Previous studies have shown that *C. jejuni* can survive on raw and cooked poultry samples during refrigeration at 4°C (8, 9, 26, 38). At 4°C, various biological activities including protein synthesis, oxygen consumption, catalase activity, ATP generation, and motility are still occurring in this bacterium (20, 25). The ability of *C. jejuni* to survive refrigeration is of major interest to food safety and public health because refrigeration is an intervention typically used in the control of bacterial growth in foods. The survival of this food-borne pathogen in the cold remains poorly understood, as is the ability of *C. jejuni* to adapt and regulate gene expression in response to cold temperature. Global transcriptional analyses have led to identification of a set of genes related to energy metabolism (39) but not revealed the control mechanisms.

Polynucleotide phosphorylase (PNPase) degrades RNA from 3′ to 5′, is a major component of *Escherichia coli* degradosomes (6, 36), and in other bacteria has shown multiple biological functions including adaptation to low temperatures (6, 10, 18, 27, 36, 39). In some species including *E. coli*, *Bacillus subtilis*, and *Yersinia enterocolitica* (18, 27, 45), PNPase is dispensable for growth at moderate temperatures but has an essential role at low temperatures, while in *Salmonella enterica* (10) PNPase is not required. It was reported that in *E. coli*, PNPase plays an essential role in stress adaptation by selectively degrading mRNAs for stress-response proteins to prevent overproduction of these proteins, which is deleterious to cells (45). To analyze the role of PNPase in *C. jejuni* survival at low temperatures, we inactivated the *pnp* gene to create a deletion-derivative strain and compared its growth characteristics to those of the wild-type strain at different temperatures.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or plasmid property	Resistance[a]	Source or reference
C. jejuni strains			
F38011	Wild-type strain		23
81-176	Wild-type strain		24
F38PNP	Isogenic *pnp* mutant of F38011	Kan	This study
81PNP	Isogenic *pnp* mutant of strain 81-176	Kan	Provided by C. M. Burns
F38PNPc1	F38PNP complemented with *C. jejuni* 81-176 *pnp* gene	Kan Cm	This study
E. coli strains			
DH5α	Wild-type strain		Laboratory collection
DH5PNP	Isogenic *pnp* mutant of DH5α	Cm	This study
DH5PNPc	DH5PNP complemented with *C. jejuni* 81-176 *pnp* gene	Cm Amp	This study
Plasmids			
pGEM-T	*E. coli* cloning vector	Amp	Promega
pNH01	*C. jejuni* 81-176 *pnp* cloned in pGEM-T	Amp	This study
pBF14	*E. coli* and *Campylobacter* cloning vector	Kan	44
pNH02	Linear pNH01 vector doubly digested with BclI/EcoRI		
pNH03	*pnp* in pGEM-T with *aphA3* insertion	Kan Amp	This study
pRY111	*Campylobacter* cloning vector	Cm	46
pNH04	Entire *C. jejuni* 81-176 *pnp* gene cloned in pRY111 vector	Cm	This study

[a] Kan, kanamycin; Cm, chloramphenicol; Amp, ampicillin.

The derivative strain (*C. jejuni* Δ*pnp*) exhibited a difference of up to 3 log CFU/ml compared to the wild type, clearly demonstrating the involvement of PNPase in the long-term survival of *C. jejuni* at common refrigeration temperatures (below 10°C).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Experiments were performed with *C. jejuni* F38011 and *C. jejuni* 81-76 and their derivative strains. *C. jejuni* was routinely cultured on Karmali agar plates (Oxoid, Basingstoke Hampshire, England) or Columbia agar plates supplemented with 5% horse blood (Merck, Darmstadt, Germany; BioMérieux, Marcy l'Etoile, France). Growth/survival curves were determined in brain heart infusion (BHI) broth (Merck). All enumerations were done by plating appropriate 10-fold serial dilutions on Karmali agar plates and incubating cultures at 42°C for 48 h under a microaerophilic atmosphere in jars flushed with a gas mixture of 10% carbon dioxide, 5% oxygen, and 85% nitrogen. *E. coli* strains were cultivated overnight at 37°C in Luria-Bertani medium (Sigma, Steinheim, Germany), which corresponds to Miller's Luria-Bertani broth base, containing 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 1 liter of distilled water (pH 7). When necessary, appropriate antibiotics were added to the growth medium as follows: μg/ml kanamycin 50 and/or 15 μg/ml chloramphenicol for *C. jejuni* derivative strains and 25 μg/ml chloramphenicol and/or 100 μg/ml ampicillin for *E. coli* derivative strains. All strains and plasmids used in this study are listed in Table 1. Columbia plates were used to isolate mutant strains during construction of *C. jejuni* derivative strains.

Construction of *C. jejuni* and *E. coli* derivative strains (Δ*pnp*). A DNA sequence coding a putative PNPase was identified in the genomes of *C. jejuni* 81-76 and *C. jejuni* 11168 by using the BLAST features NCBI and the EMBOSS alignment. The DNA sequence encoding the putative PNPase ortholog showed 37% identity and 56% similarity to that of *E. coli*. This gene was PCR amplified from *C. jejuni* 81-76 chromosomal DNA obtained by a Wizard Genomic DNA Purification Kit (Promega, Charbonnieres, France) and primers MO001/MO002 (Table 2; see also Fig. 2A). The PCR program was 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min 30 s, with a final step at 72°C for 7 min. The amplified DNA product of 2,492 bp was cloned into pGEM-T vector (Promega), which is a suicide vector for *C. jejuni*, leading to the recombinant plasmid pNH01 (Fig. 1A). Plasmid pNH01 was doubly digested with BclI and EcoRI restriction enzymes in order to delete the central part of the *pnp* gene (about 1,083 bp), yielding a linear vector called pNH02 (Fig. 1B). A kanamycin cassette (*aphA3*) was amplified from plasmid pBF14 (42) (Table 1) using primers MO003/MO004, which contain BclI and EcoRI restriction sites, respectively (Table 2; see also Fig. 2A). The kanamycin cassette of a 1,408-bp fragment was purified, doubly digested with BclI and EcoRI restriction enzymes, and ligated to linear vector pNH02, leading to recombinant plasmid pNH03 (Fig.

TABLE 2. Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Target	Restriction site
MO001	AGATCTATTACAATTTATTTGGTGGAATTTGGTTTGATACTC	*pnp* gene and its promoter	
MO002	TCACTCGAGCTCACACAAATCCACAGAAATTTTTCCG	*pnp* gene and its promoter	
MO003	GCTGATCAACCATTTGAGGTGATAGGTAAG	*aphA3*	BclI
MO004	GGGAATTCGGTACTAAAACAATTCATCCAG	*aphA3*	EcoRI
MO005	CCTCTAACCGTCCAATACATC	*pnp* ORF	
MO006	TCACTCACACAAATCCACAG	*pnp* ORF	
MO007	CACTAAAGACTGGAGCGAAG	Part of *pnp* ORF	
MO008	GGAGAAGTTGAGGGTAGGG	CJJ81176_1270	
MO009	CATCCTTTTGAGCCTTTTT	CJJ81176_1270	
MO010	TAGGGTTGTCATTAGTCGC	*E. coli pnp* gene with insertion of Cm^r cassette	
MO011	CAAAGTCAACGATACGGGT	*E. coli pnp* gene with insertion of Cm^r cassette	

[a] Primers were based on the genome sequence from *C. jejuni* 81-176. Restriction sites are underlined.

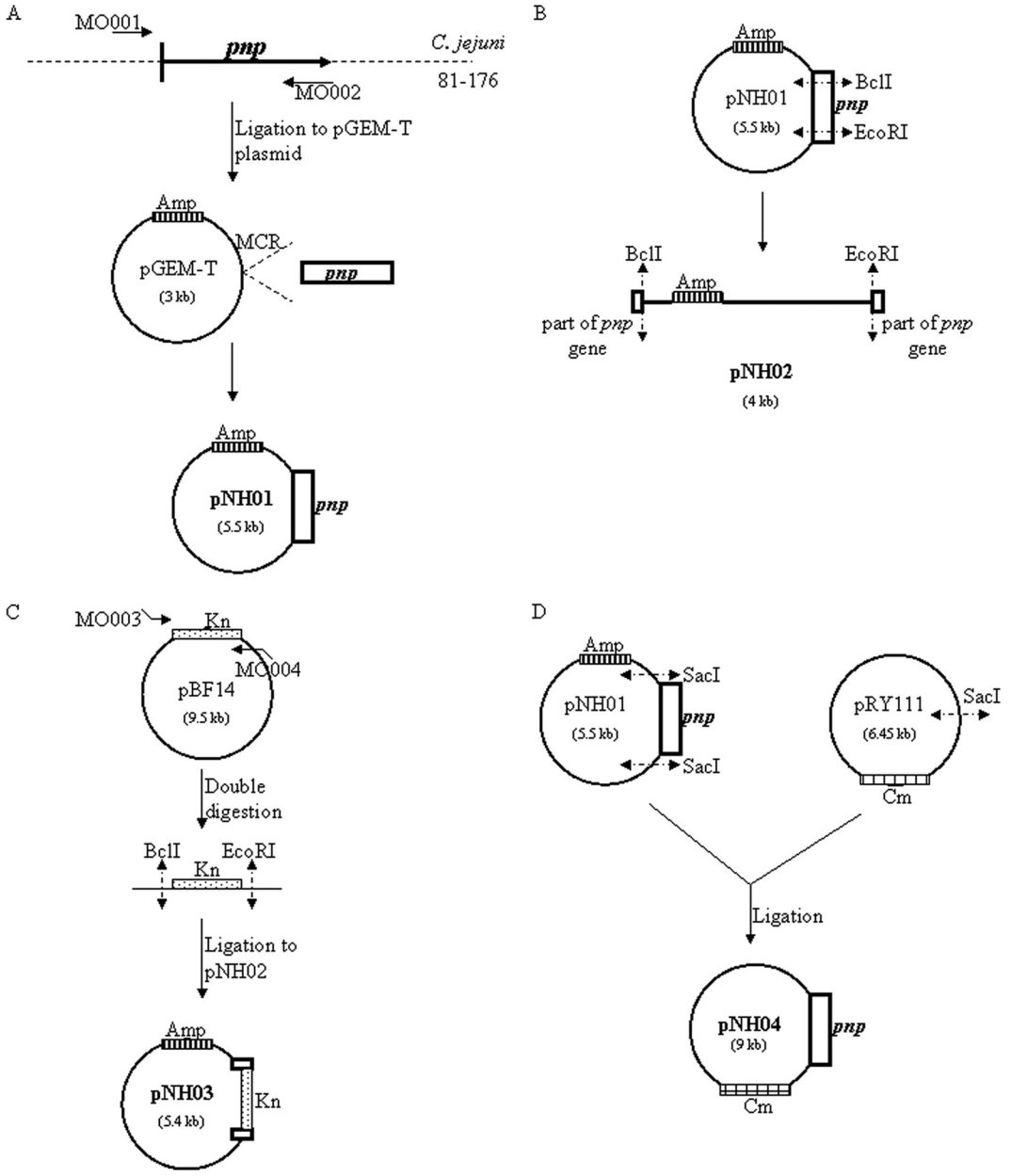

FIG. 1. Construction of different vectors used in this study and listed in Table 1: pNH01 (A), pNH02 (B), pNH03 (C), and pNH04 (D).

1C). The PCR program used to synthesize the kanamycin cassette was 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min 30 s, with a final step at 72°C for 7 min.

Insertion of the kanamycin cassette into plasmid pNH03 was confirmed by DNA sequencing (Genome Express, Meylan, France). Recombinant plasmids pNH01 and pNH03 conferred resistance to kanamycin and ampicillin antibiotics. It should be noted that only kanamycin resistance was used as a marker because of the natural resistance of *Campylobacter* strains to ampicillin. The recombinant plasmid pNH03 used to create the doubly homologous recombination was introduced into *C. jejuni* F38011 by electroporation with a Bio-Rad Gene Pulser with a pulse controller in 0.2-mm gap cuvettes (Eurogentec, Angers, France) at 2 kV, 25 μF, and 200 Ω. Cells were recovered in 200 μl of 2× yeast extract-tryptone medium and poured onto 5% horse blood (Columbia blood agar) plates. After 12 h of regeneration at 37°C in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 gas, cells were harvested and plated onto Columbia agar plates supplemented with 5% horse blood and kanamycin at 50 μg/ml.

C. jejuni strains exhibiting resistance to kanamycin were selected and checked for disruption of their *pnp* locus by the *aphA3* cassette by PCR analysis using different combinations of primers (i.e., primers MO001/MO002 and MO003/MO002) (see Fig. 2A). In the case of primers MO001/MO002, the PCR program was identical to that cited above (amplification of *pnp* gene), whereas the PCR program using primers MO003/MO002 contains slight modifications (melting temperature of 55°C and elongation time of 1 min). The disruption of the *pnp* gene was confirmed by DNA sequencing.

A derivative strain of *E. coli* DH5α altered in the *pnp* gene was constructed in this study using a previously described method (13). *E. coli* derivative strains were selected on agar plates supplemented with 25 μg/ml chloramphenicol and inspected for disruption of the *pnp* gene by PCR technology using the primers MO010/MO011 and the following program: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min, with a final step at 72°C for 7 min. The *E. coli* derivative strain was named DH5PNP (Table 1).

Complementation. For *trans*-complementation of the derivative strains, we produced the recombinant plasmid pNH04 (Fig. 1D), in which the *C. jejuni* 81-76 *pnp* gene and its natural promoter were cloned into the shuttle vector pRY111.

Briefly, plasmid pNH01 was digested with a SacI restriction enzyme to yield two distinct DNA fragments. The DNA fragment containing the *pnp* gene (2,500 bp) was purified from the agarose gel and ligated to plasmid pRY111, itself digested with a SacI restriction enzyme (Fig. 1). Plasmid pNH04 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, yielding *C. jejuni* F38PNPc1 (Table 1).

Cross-complementation. Complementation of the *E. coli* derivative strain DH5PNP was performed with plasmid pNH01 (Table 1), leading to the recombinant strain DH5PNPc. Growth of the wild-type, derivative, and complemented strains was performed at 37 and 20°C. Viable counts were measured by serial dilution and plating on agar plates containing appropriate antibiotics and inspected daily in order to determine the total CFU/ml.

RNA extraction and reverse transcription-PCR (RT-PCR) assays. Total RNA was extracted from *C. jejuni* 81-76 using a NucleoSpin RNA II Kit (Macherey-Nagel, Hoerdt, France) according to the supplier's instructions. The quantity of total RNA was determined by measuring the absorbance at 260 nm (A_{260}). The integrity of RNA samples was checked on a 0.8% agarose gel; electrophoresis was carried out in Tris-acetate-EDTA buffer for 1 h at 100 V. The gels were stained with ethidium bromide and visualized by UV light.

cDNA synthesis was performed using M-MLV (Moloney murine leukemia virus) Reverse Transcriptase, RNase H Minus, Point Mutant (Promega). Two micrograms of total RNA was mixed with random hexamer primers at 250 ng in a volume of 14 μl, heated at 70°C for 5 min, and immediately chilled on ice for 5 min. The reaction mixture was prepared in a total volume of 25 μl, which consisted of 1× RT buffer, 2 μg of heated RNA, 250 ng of random hexamer primer, 200 U of reverse transcriptase, and a 500 μM concentration of each deoxynucleoside triphosphate. Reverse transcriptase was replaced by RNase-free water for the negative control. The reaction mixture was incubated at room temperature for the initial 10 min and at 48°C for 50 min. The reaction was inactivated by heating at 70°C for 15 min. Using the cDNA obtained, several PCRs were performed with the primer pairs MO005/MO006, MO007/MO006, MO005/MO008, MO007/MO008, and MO009/MO008 (Table 2; see also Fig. 2A) and *Taq* polymerase (BioLab, Saint Quentin Yvelines, France), according to the supplier's instructions. All primers used in this study are described in Table 2. The gene ruler used was a 1-kb DNA Ladder (BioLab).

Protein extraction and Western blotting analysis. *C. jejuni* 81-76 was grown on Karmali agar for 48 h at 42°C. After this period of incubation, one colony was randomly chosen and resuspended in 50 ml of BHI medium and then incubated at 42°C for 24 h. One milliliter of the resulting culture was used to inoculate 100 ml of BHI medium, which was incubated for 12 h at 42°C under microaerophilic conditions with shaking at 110 rpm. Cells were retrieved by centrifugation at 7,000 × *g* for 20 min and washed consecutively with 200 mM glycine solution (Sigma-Aldrich, Saint-Quentin Fallavier, France) and 100 mM Tris-HCl, pH 7.0, solution (Sigma-Aldrich). Pellets were resuspended in 10 ml of a 10 mM Tris-HCl, pH 7.0, solution, and cells were disrupted by a series of six 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. Protein concentration was determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL). Five micrograms of total protein was mixed with denaturing buffer and separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide (10%) gels. One gel was stained with Coomassie blue R-250 (Sigma). The second gel was fixed in a transfer buffer containing 25 mM Tris, 190 mM glycine, and 20% absolute ethanol for 10 min. Transfer to a nitrocellulose membrane (Bio-Rad, Marnes-la-Coquette, France) was carried out for 1 h at 250 mA in transfer buffer. The membrane was immersed overnight in a blocking buffer containing 10 mM Na_2HPO_4, 1.4 mM K_2HPO_4, 52 mM NaCl, and 2.7 mM KCl, to which 5% dried skim milk was added. After three washes for 10 min in the blocking buffer containing 0.05% Tween, the membrane was incubated with diluted *E. coli* PNPase antibodies (kindly provided by A. J. Carpousis) in blocking buffer for 1 h. The membrane was washed three times with phosphate-buffered saline-Tween and incubated for 1 h in phosphate-buffered saline to which rabbit anti-immunoglobulin G diluted 1/100,000 was added. After three washes for 5 min in the blocking buffer containing 0.05% Tween, proteins were visualized with an NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate) liquid substrate system (Sigma).

Determination of survival. *C. jejuni* F38011 and *C. jejuni* 81-76 wild-type and derivative strains were obtained from −80°C stocks and cultured for 48 h at 42°C under microaerophilic conditions on Karmali agar plates. Cells were transferred to 50 ml of BHI medium and incubated with shaking for 24 h under microaerophilic conditions in order to obtain a starter culture. Fifty milliliters of BHI medium was inoculated with 1/100 of working culture and incubated at 37°C, 10°C, and 4°C under microaerophilic conditions or ambient atmosphere. Under microaerophilic growth conditions, survival of *C. jejuni* was tested at 42°C instead of 37°C. Viable counts were measured by serial dilution and plating on Karmali agar every 2 days to estimate the total number of CFU/ml. To calculate survival, the following formula was used: $\log_{10} N_t - \log_{10} N_0$, where N_0 corresponds to the viable count in CFU/ml at time zero, and N_t corresponds to the viable count in CFU/ml at time t (the end of the experiment). Experiments to evaluate each survival condition were repeated three times. For each experiment, the individual samples were plated in triplicate on Karmali agar medium. The data points in each graph are the means of the survival values derived from three replicate experiments under each condition, with standard deviations shown.

In silico analysis. DNA and protein homology searches were performed by BLAST analysis (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using the EMBOSS alignment program (http://www.ebi.ac.uk/Tools/emboss/align/index.html). The transcriptional start site was predicted using the Softberry BPROM program (http://linux1.softberry.com/cgi-bin/programs/gfindb/bprom.pl).

RESULTS

Identification of putative PNPase in *C. jejuni*. Computer-assisted analysis of the genomic sequences of *C. jejuni* 81-76 and *C. jejuni* NCTC 11168 revealed genes predicted to encode PNPase orthologs, designated PNPase CJJ81176_1269 and PNPase Cj1253, respectively. Putative *pnp* genes were also found in the genomes of *Campylobacter coli*, *Campylobacter upsaliensis*, *Campylobacter lari*, and *Campylobacter fetus*. The putative PNPase of *C. jejuni* is composed of 719 amino acids and has a calculated molecular mass of 71.9 kDa.

A BLASTP search showed that the PNPase of *C. jejuni* strains exhibited 38% identity and 58% similarity to *E. coli* PNPase. *C. jejuni* 81-76 PNPase showed high amino acid sequence identity (>98.5%) to predicted PNPase enzymes in other sequenced *C. jejuni* strains. However, the identity decreases when the sequence is compared to PNPases of other *Campylobacter* species (69 to 87% for *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus*) and to PNPase-like enzymes of bacteria belonging to the epsilon group of the *Proteobacteria*, such as *Helicobacter*, and *Wolinella succinogenes* (49 to 58%). Alignment of the amino sequence of *C. jejuni* PNPase with sequences of the databases showed the presence of the five motifs highly conserved in the PNPase enzyme family. The PNPase_KH motif is an RNA-binding domain. The RNase_PH and RNase_PH_C motifs are the domains 1 and 2 of the 3′ exoribonuclease family, respectively. The PNPase motif is responsible for the catalytic activity of polynucleotide nucleotidyltransferase and an RNA-binding domain. Finally, the S1-like motif is involved in RNA binding.

Characterization of *pnp* locus and transcriptional analysis. In *C. jejuni* 81-76, the *pnp* gene is located in a cluster containing nine genes starting from open reading frame (ORF) CJJ81176_1263 and ending in ORF CJJ81176_1271. There are six ORFs upstream and two ORFs downstream of *pnp*; among genes flanking *pnp*, only *guaA* (CJJ81176_1264), *purD* (CJJ81176_1266), and CJJ81176_1267 are annotated, respectively, encoding a bifunctional synthase/amidotransferase protein, a ligase, and an organic solvent tolerance protein.

In derivative strain *C. jejuni* F38PNP, the *aphA3* cassette conferring resistance to kanamycin was inserted 570 bp downstream of the start codon of the *pnp* gene (Fig. 2A). Therefore, a putative transcriptional start site was identified 100 bp from the start codon by the Softberry-BPROM program (Fig. 2A); a −10 box (TATAAT) and a −35 box (TTTACA) were detected upstream of the transcriptional start site. An inverted repeat

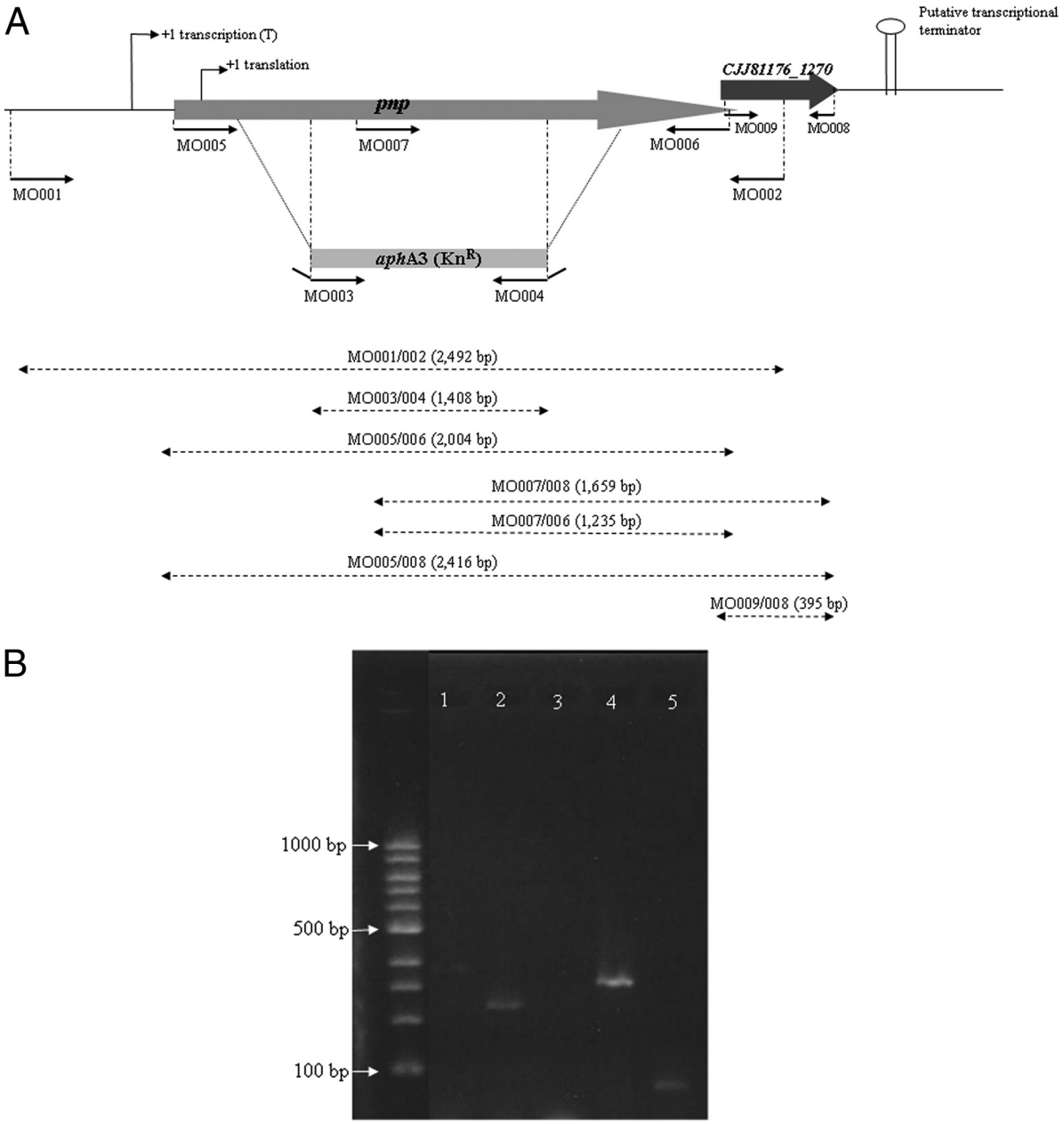

FIG. 2. (A) Genetic organization of the *pnp* CJJ81176_1270 cluster showing the putative transcriptional start site, localization of the insertion of *aphA3* within the *pnp* gene, putative transcriptional terminator site, and position of the different primers used in this study. Kn^R, kanamycin resistance. (B) RT-PCR assays performed using *C. jejuni* 81-76 mRNA. RT experiments were performed with random hexamer primers, and PCR was done with the primer pairs MO005/MO006 (lane 1), MO007/MO006 (lane 2), MO005/MO008 (lane 3), MO007/MO008 (lane 4), and MO008/MO009 (lane 5). A 1-kb DNA ladder (BioLab) is shown on the left.

with a $\Delta G°$ of −53.98 kcal/mol that could correspond to a transcriptional terminator was located downstream of ORF CJJ81176_1270, suggesting that these two genes could constitute an operon (Fig. 2A). Another transcriptional start site was predicted with a high score downstream of ORF CJJ81176_1270. This hypothesis that the *pnp* gene and ORF CJJ81176_1270 form an operon was definitely confirmed by RT-PCR analysis since these two genes were shown to be cotranscribed (Fig. 2B). PCR performed with primers MO005/MO006 allowed amplification of a DNA product of 2,004 bp corresponding to *pnp* gene, and that obtained with primers MO007/MO006 produced a DNA product of 1,235 bp corresponding to part of the *pnp* gene (Fig. 2A). PCR performed with primers MO007/MO008 allowed amplification of a 1,659-bp DNA product corresponding to part of the *pnp* gene and the entire ORF CJJ81176_1270 (Fig. 2A). Finally, PCR with primers MO005/MO008 permitted amplification of the entire *pnp* gene and ORF CJJ81176_1270, leading to a DNA

fragment of 2,416 bp, and primers MO009/MO008 amplified the whole ORF CJJ81176_1270 with a size of 395 bp (Fig. 2A). An RT-PCR experiment using primers that span the two genes *pnp* and ORF CJJ81176_1270 revealed the detection of the RNA product. These data indicated that these two genes are cotranscribed in *C. jejuni* 81-76.

Mutant strains with altered chromosomal *pnp* genes were created in *C. jejuni* F38011 and *C. jejuni* 81-76 by double homologous recombination between *pnp* and a kanamycin cassette. Mutant strains were selected on agar medium supplemented with kanamycin, and all *pnp* loci (wild-type and mutant strains) were amplified by PCR and sequenced to confirm the disruption events. We observed that mutant strains were able to grow without PNPase, indicating that the *pnp* gene is not essential in *C. jejuni*.

Survival assays at different temperatures. The role of PNPase in promoting survival of *C. jejuni* was tested at three temperatures using both microaerophilic (4, 10, and 42°C) and aerobic (4, 10, and 37°C) growth conditions. Culturable cells were quantified as a function of storage time for both wild-type and *pnp* derivative strains to determine the influence of *pnp* on survival. Each experiment was done using three independent cultures of the wild-type or mutant strains, and the mean and standard deviation values were calculated.

The viable cell counts observed under a microaerophilic atmosphere over a 23-day period are shown in Fig. 3. At a normal growth temperature of 42°C, the reduction in viable counts over 19 days of *C. jejuni* (F38011 and 81-76) was small, with similar values for wild-type and derivative strains (1.8 to 2.1 [± 0.21] $\log_{10}$ CFU/ml), while *C. jejuni* 81-76 was even more stable, with a reduction of only about 0.6 (± 0.11) $\log_{10}$ CFU/ml (Fig. 3A).

The results at lower temperatures were very different. After a period of 21 days at 10°C, the survival levels of the wild-type strains *C. jejuni* F38011 and *C. jejuni* 81-76 were reduced 5.81 (± 0.29) and 4.11 (± 0.12) $\log_{10}$ CFU/ml, respectively (Fig. 3B); the derivative strains *C. jejuni* F38PNP and *C. jejuni* 81PNP were substantially more reduced, i.e., by 7.7 (± 0.17) and 7.1 (± 0.16) $\log_{10}$ CFU/ml, respectively (Fig. 3B). After 23 days at 4°C cell survival was dependent on the strain used: *C. jejuni* F38011 was reduced 4.8 (± 0.27) $\log_{10}$ CFU/ml while *C. jejuni* 81-76 was reduced only 1.3 (± 0.27) $\log_{10}$ CFU/ml (Fig. 3C). However, survival was highly sensitive to mutation of *pnp* because the derivative strains were reduced by 7.6 (± 0.22) and 3.4 (± 0.37) $\log_{10}$ CFU/ml, respectively (Fig. 3C). The differences observed between the wild-type and derivative strains at 10 and 4°C were significant.

The second part of the survival study was performed at 4°C, 10°C, and 37°C under ambient atmosphere (Fig. 4). After a period of 17 days at 37°C, the reduction in viable counts of *C. jejuni* (F38011 and 81-176) and derivative strains was between 1.4 and 1.9 (±0.12) $\log_{10}$ CFU/ml (Fig. 4A), and this difference was not significant. After 17 days at 10°C survival rates of the wild-type strains *C. jejuni* F38011 and *C. jejuni* 81-76 were reduced by 6.3 (± 0.14) and 4.5 (± 0.3) $\log_{10}$ CFU/ml, respectively (Fig. 4B), while the derivative strains F38PNP and 81PNP both declined 9.1 (± 0.15) $\log_{10}$ CFU/ml (Fig. 4B). The decreases in survival after 17 days at 4°C were 3.4 (± 0.23) and 2.9 (± 0.01) $\log_{10}$ CFU/ml for *C. jejuni* F38011 and *C. jejuni* 81-76 strains and 5.9 (± 0.01) and 6.0 (± 0.02) $\log_{10}$ CFU/ml

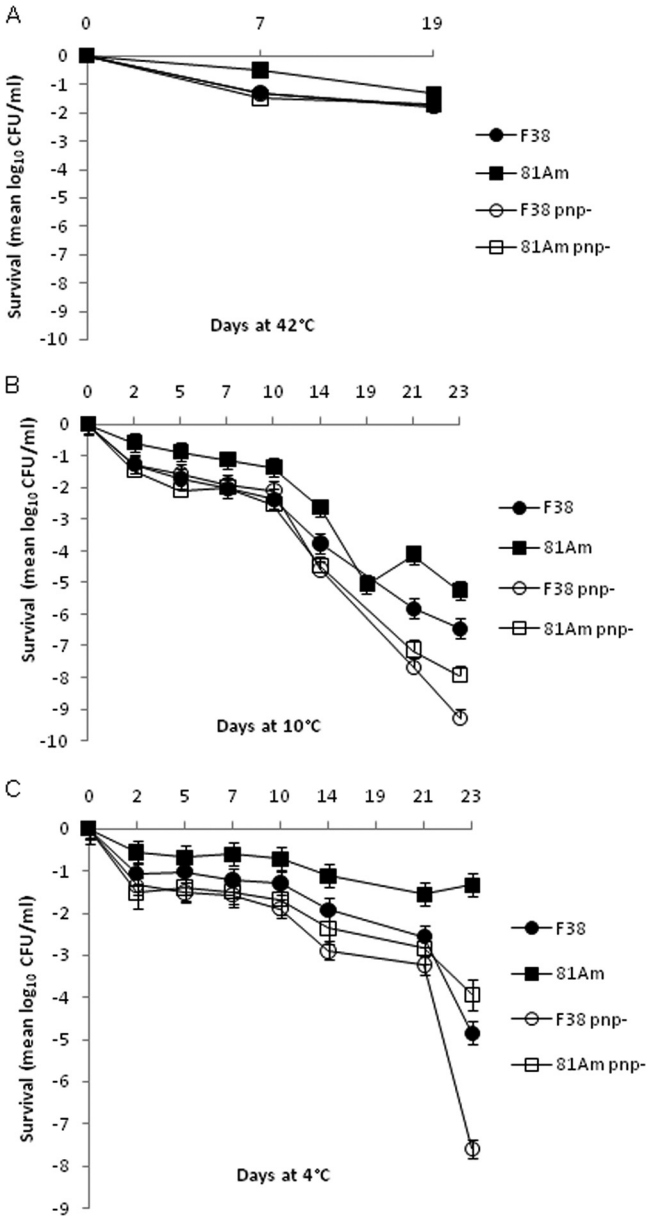

FIG. 3. Survival of *C. jejuni* strains F38011, 81-76, and their *pnp* mutant derivatives F38PNP and 81PNP at 42°C (A), 10°C (B), and 4°C (C) under microaerophilic conditions. The values plotted are means ± standard deviations (error bars).

for the respective derivative strains (Fig. 4C). The differences observed between wild-type and derivative strains at 4 and 10°C were significant.

trans-Complementation of *C. jejuni* derivative strains with plasmid pNH04 (Fig. 1D) containing the *pnp* gene partially restored survival at refrigerated temperatures, confirming that the activity depends on PNPase and not on the cotranscribed downstream ORF (survival curves not shown).

Complementation of *E. coli* (Δ*pnp*) with the *C. jejuni pnp* gene. The ability of the *C. jejuni pnp* gene to complement an *E. coli pnp* mutant was tested using the *E. coli* derivative strain DH5PNP and plasmid pNH01 carrying the *pnp* gene of *C.*

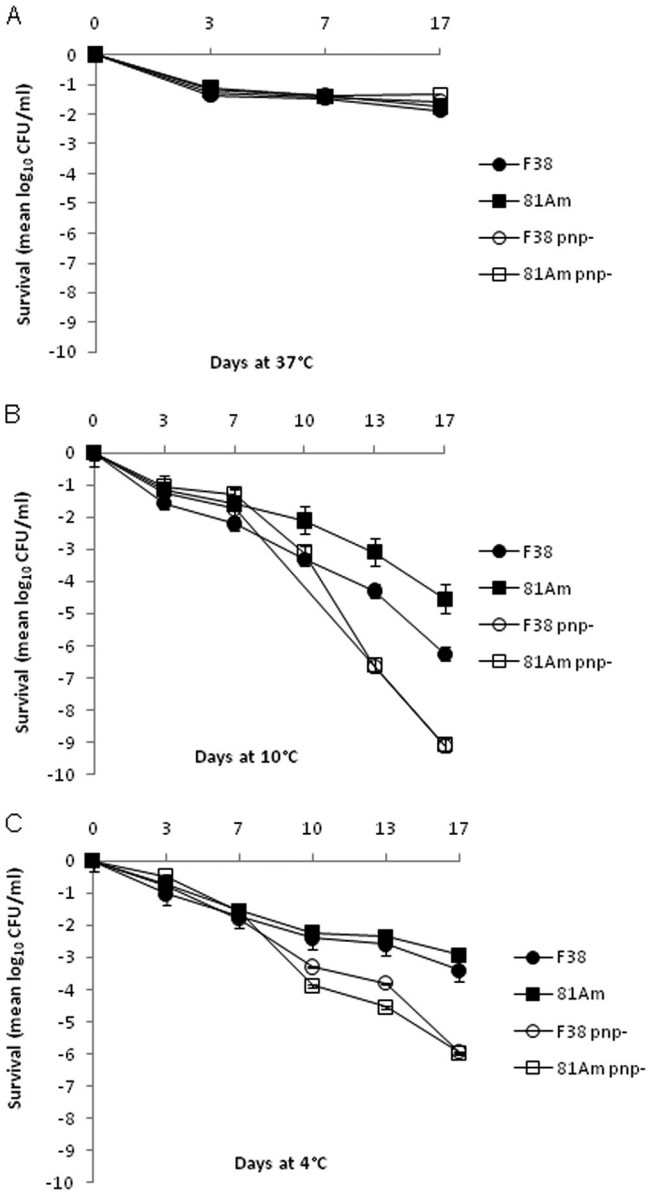

FIG. 4. Survival of *C. jejuni* strains F38011, 81-76, and their *pnp* mutant derivatives F38PNP and 81PNP at 37°C (A), 10°C (B), and 4°C (C) in the ambient atmosphere. The values plotted are means ± standard deviations (error bars).

jejuni 81-76 (Table 1). The colony-forming abilities of the *E. coli* wild-type, mutant, and complemented strains were compared at 37°C and 20°C according to the method of Yamanaka and Inouye (45). Unlike the wild-type strain, the mutant and complemented strains were unable to form colonies at 20°C, whereas at 37°C all of the strains could grow (data not shown). Despite the high degree of sequence similarity, polyclonal antibodies raised against *E. coli* PNPase did not show a cross-reaction with crude extracts of *C. jejuni* 81-76 (data not shown). These data argue that although they have overlapping cellular functions, the structure and cellular properties of the *E. coli* and *C. jejuni* PNPases are unique.

DISCUSSION

In most industrialized countries raw poultry products are commonly exposed to refrigeration for variable lengths of time before they reach the consumer. Since raw or undercooked chicken is considered to be an important risk factor for human campylobacteriosis (7, 32, 40), one would expect that this foodborne pathogen must have the ability to tolerate refrigeration. Many studies demonstrated the remarkable survival of *C. jejuni* under temperature conditions nonpermissive for growth (3, 7, 9, 11, 20), but the mechanisms *C. jejuni* uses to adapt and control gene expression in response to cold shock are poorly understood.

To investigate the ability of this pathogen to survive prolonged exposure to low temperatures, we focused on the *pnp* gene, which encodes PNPase, a 3′-5′ exoribonuclease with multiple cellular functions. We expected that *C. jejuni* deficient in PNPase might not be viable because the gene encoding RNase II (another 3′-5′ exoribonuclease) was not detected in the *C. jejuni* genome, and *E. coli* cells lacking both PNPase and RNase II are not viable (15). However, the *pnp* gene is not essential in *B. subtilis* or *Pseudomonas putida*, which also appears to lack RNase II (14, 16), and our data show that *C. jejuni* behaves similarly. Why some bacteria require *pnp* in the absence of RNase II and others do not remains unknown.

In the current study, the survival of *C. jejuni* strains was observed at refrigerated temperatures (4 and 10°C) in a nutrition-rich medium (BHI medium) and found to be less than at the optimal growth temperature (37°C). It was reported that in surface waters and microcosms (sterilized water), survival of *C. jejuni* was limited to a few days at ambient temperature, but at 4°C *C. jejuni* could survive for several weeks (4, 41). The importance of this finding for food safety was confirmed by the observation that *C. jejuni* was able to persist on chicken skin fragments at 4°C (26).

C. jejuni survival at low temperatures involves active transcriptional machinery resulting in protein synthesis, motility, and oxygen consumption (20). Previous analysis of global transcription profiles showed that the most notable group of genes with reduced transcript abundance following cold shock encode proteins that are involved in chemotaxis, flagellin biosynthesis, and flagellar motility (39), while genes with increased transcript abundance are predominantly involved in energy metabolism (29). PNPase might be involved in motility control as this important virulence characteristic was completely lost in derivative *C. jejuni* 81-76 and *C. jejuni* NCTC 11168 strains with deletions of *pnp* (J. Li, P. Lin, and C. M. Burns, unpublished data).

Close to the maximum growth temperature, the growth rate suddenly decreases; interestingly, the same observation was also noted near the minimum growth temperature (20). This suggests that *C. jejuni* does elicit a cold-shock response that regulates gene expression at low temperatures. The ability of *C. jejuni* to adapt and regulate gene expression in response to cold shock or cold survival is not fully understood, which is why we focused on the putative PNPase found in *C. jejuni*.

The results obtained in this study revealed that a clinical isolate (*C. jejuni* 81-76) was more resistant to low temperatures than a poultry isolate (*C. jejuni* F38011). After 23 days of storage at 4°C under a microaerophilic atmosphere, the titer of

C. jejuni F38011 was reduced approximately 4.8 (±0.27) $\log_{10}$ CFU/ml, while *C. jejuni* 81-76 was reduced by only 1.3 (±0.27) $\log_{10}$ CFU/ml. These findings are in agreement with results reported by Chan et al. (7). It is of note that the influence of strain origin on survival rate was less pronounced when ambient air was used instead of a microaerophilic atmosphere.

PNPase is involved in the adaptation of *E. coli*, *B. subtilis*, *S. enterica*, and *Y. enterocolitica* to low temperatures (10, 18, 27, 45). In *Y. enterocolitica*, *E. coli*, and *B. subtilis*, *pnp* mutation caused severe restriction of cell growth at cold temperatures (18, 27, 43, 45), while in *S. enterica*, the growth rate was reduced about twofold in a *pnp* mutant strain compared to wild type (10). Therefore, it was previously established that PNPase was involved in growth at low temperatures; our results implicate PNPase in cold survival of *C. jejuni*. Either in ambient or microaerophilic atmosphere, *pnp* gene mutation decreases survival of *C. jejuni* 2 to 4 $\log_{10}$ CFU/ml at 4 and 10°C compared to the wild type.

The molecular mechanism by which PNPase promotes cold survival of *C. jejuni* remains unknown. In *E. coli*, PNPase is considered a cold shock protein (CSP) (22) and seems to be required for adaptive growth resumption of cold-shock-treated cells (2). Upon temperature downshift, *E. coli* increases the synthesis rate of 14 CSPs while synthesis of most other proteins appears to be repressed (19, 34, 35). This repression is responsible for the inhibition of bacteria growth called the acclimation phase. At the end of the acclimation phase at 15°C, PNPase selectively degrades CSP mRNA but not non-CSP mRNA (45). Moreover, in the *pnp* mutant strain, mRNA half-lives of CSP are prolonged upon cold shock, and this strain is unable to form colonies below 25°C (45). Thus, at the end of the acclimation phase, CSP expression is reduced or degraded, with resumption of synthesis of non-CSP proteins facilitating reinitiation of growth (42). Major CSPs such as CspA (33) have not been detected in sequenced *C. jejuni* genomes, yet the control of mRNA stability and translatability seems to play a major role in the cold temperature adaptive response (35). Like *E. coli*, a PNPase-deficient mutant of *Y. enterocolitica* is unable to degrade *cspA1* and *cspA2* mRNAs encoding the major CSP after a cold shock (31). In addition, PNPase seems to interact with the low temperature-induced CsdA RNA helicase to selectively degrade these CSP transcripts at the end of the acclimation phase (45). As we have found PNPase to be involved in cold survival of *C. jejuni*, it will be of interest to learn what mechanism is used in the absence of CsdA.

The genetic organization of *pnp* in *C. jejuni* is different from that of *E. coli* and *S. enterica*. In these two bacteria, the *pnp* gene is preceded by *rpsO*, which encodes the ribosomal protein S15. Transcription of the *pnp* gene can be mediated by two promoters, one located upstream of *rpsO* and the other located downstream. Both transcripts are endonucleolytically cleaved by RNase III in the 5′ untranslated region of *pnp* (12, 21). Expression of PNPase is posttranscriptionally autoregulated at the level of both translation and stability of the RNase III-processed mRNA (12, 21). In contrast, in the *C. jejuni* genome, no *rpsO*-like gene was found upstream of *pnp*. Instead, *pnp* is followed by a small ORF encoding an unknown protein. RT-PCR demonstrated that these two genes are cotranscribed (Fig. 2B). *trans*-Complementation of *C. jejuni* derivative strains with a plasmid (pNH04) carrying the *pnp* gene partially restored the adaptation to survival at low temperatures of *C. jejuni* F38011, confirming that this phenotype was due to lack of *pnp*, not the downstream gene. Although the phenotype was only partially restored, *trans*-complementation of *B. subtilis* deficient in PNPase activity (Δ*pnp*) was also only partially effective in restoring resistance to tetracycline (43).

The inactivation of the *pnp* gene in *C. jejuni* allowed us to gain insights into the survival capabilities and mechanisms of this strain at refrigeration temperatures. The approach developed in this work is expected to be pursued in order to further understand how to control growth of this food-borne pathogen and reduce its impact on human health.

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