The RNase R from Campylobacter jejuni Has Unique Features and Is Involved in the First Steps of Infection*

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Background: Members of the RNB family are involved in virulence; nothing is known about the Campylobacter jejuni homologue.

Results: Cj-RNase R is active in several conditions. It is also important for adhesion and invasion.

Conclusion: RNase R is important for C. jejuni biology and infection.

Significance: RNase R could be targeted to reduce infection by this pathogen.

Bacterial pathogens must adapt/respond rapidly to changing environmental conditions. Ribonucleases (RNases) can be crucial factors contributing to the fast adaptation of RNA levels to different environmental demands. It has been demonstrated that the exoribonuclease polynucleotide phosphorylase (PNPase) facilitates survival of Campylobacter jejuni in low temperatures and favors swimming, chick colonization, and cell adhesion/invasion. However, little is known about the mechanism of action of other ribonucleases in this microorganism. Members of the RNB family of enzymes have been shown to be involved in virulence of several pathogens. We have searched C. jejuni genome for homologues and found one candidate that displayed properties more similar to RNase R (Cj-RNR). We show here that Cj-RNR is important for the first steps of infection, the adhesion and invasion of C. jejuni to eukaryotic cells. Moreover, Cj-RNR proved to be active in a wide range of conditions. The results obtained lead us to conclude that Cj-RNR has an important role in the biology of this foodborne pathogen.

Campylobacter jejuni is a foodborne bacterial pathogen that is now considered the leading cause of human bacterial gastrointestinal disease worldwide, with ~400 million cases of campylobacteriosis diagnosed each year (1). The symptoms of this disease include malaise, fever, severe abdominal pain, and diarrhea. Post-infectious sequelae can occur, including septicemia and neuropathies such as Guillain-Barré syndrome (2–4). The principal reservoir of C. jejuni is the gut of avian species, which may contain up to 109 cfu/g of feces (5). The fecal contamination of poultry meat during food processing is the main cause of C. jejuni infections (6). Despite its specific microaerobic growth requirements, C. jejuni is ubiquitous in the aerobic environment, and it is capable of withstanding different stresses caused by growth or survival on a suboptimal carbon source, temperature changes, exposure to atmospheric oxygen, hypo- and hyper-osmotic stress, and desiccation. Moreover, during infection, C. jejuni has to withstand other stresses, including changes in pH and the host innate immune response (7, 8).

Cellular levels of RNA are determined by their rate of synthesis and their rate of degradation. As such, ribonucleases are important in the adaptation of organisms to new environments. In the case of C. jejuni, little is known about the RNA processing pathways and associated ribonucleases. However, the exoribonuclease polynucleotide phosphorylase has been shown to be important for cell survival at low temperature and has an important role in swimming, cell adhesion/invasion ability, and chick colonization (9, 10). Moreover, the endoribonuclease RNase III was recently characterized and was shown to be active in an unexpectedly large range of conditions (11). This endoribonuclease may have an important role under a Mn2+-rich environment, considering that Mn2+ is its preferred cofactor (11). Similarly to what was observed in other organisms, RNase III from C. jejuni is involved in the processing of 30 S rRNA (11, 12) and participates in the maturation of CRISPR RNAs, which are key elements in the CRISPR (clustered regularly interspaced short palindromic repeats) system of bacterial adaptive immunity (12).

RNase R is a 3’ to 5’ hydrolytic enzyme that belongs to the RNB family of enzymes. This family of exoribonucleases is widely distributed in all domains of life and plays important functions in the cell. The E. coli genome codes for two proteins of this family, RNase II and RNase R. They present a similar mechanism of action; however, they behave differently regarding product released and the ability to degrade structured RNA.

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TABLE 1
Strains used in this study

| Strain | Antibiotic resistance | Reference | Genetic |
|--------|------------------------|-----------|---------|
| E. coli DH5α | Invitrogen | recA1 endA1 |         |
| BL21 Star™(DE3) | Invitrogen | rnel131 |         |
| C. jejuni 81–176 | Korlath et al. (51) |         |         |
| Δrnr::aphA3 | Kanamycin | This study | C. jejuni Δrnr |

(13). The crystal structure of RNase II showed that the protein is formed by a central RNB domain, which is responsible for the catalytic activity. This domain is flanked by two N-terminal cold shock domains and a C-terminal 51 domain, important for RNA binding (14, 15). All the other members of this family have the same domain organization, but may have extra domains at the N-terminal region. For example, RNase R has a helix-turn-helix motif, whereas in eukaryotes there is an extra PINc domain, which has endonucleolytic activity (16, 17). In the catalytic region, there are several highly conserved residues that are important for the activity of the protein. The role of these residues seems to be conserved both in eukaryotes and in prokaryotes (18–21).

In eukaryotes, the member of the RNB family of enzymes is called Dis3 and exists in three isoforms (Dis3/Rrp44, Dis3L1, and Dis3L2). These proteins locate in the cell differently. Dis3L2 prefers poly(U) RNAs and was recently shown to be involved in a new eukaryotic RNA degradation mechanism independent of the exosome (22). Dis3 and Dis3L2 were shown to be involved in important human diseases (23). In some pathogenic organisms, proteins from this family were shown to be crucial for the establishment of virulence (24).

Considering that C. jejuni is a foodborne bacterial pathogen with impact on human health, it is important to understand the mechanisms behind the infection process. In the genome of C. jejuni, we found a homologue of the RNB family of enzymes, which, based on sequence alignment, seems to be more similar to RNase R (from here on designated by Cj-RNR).6 Taking into account the involvement of RNase R-like proteins in the establishment of virulence in other pathogens, Cj-RNR seems to be an excellent candidate for studies in C. jejuni.

The aim of this work was to access the role of RNase R in growth and virulence of C. jejuni. We have also cloned, expressed, purified, and characterized the activity of this protein at different conditions. The importance of RNase R in C. jejuni virulence and in its adaptation to different environments is discussed.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—The strains used in this study are listed in Table 1. C. jejuni 81–176 and Δrnr mutant strains were routinely cultured on Karmali agar plates (Oxoid, Basingstoke Hampshire, UK) or in brain heart infusion broth (Merck), and kanamycin at 50 μg/ml was added for the culture of Δrnr mutant strain. Culture on plates and broth were incubated at 42 °C for 48 and 24 h, respectively, under a microaerophilic atmosphere in jars flushed with a gas mixture of 10% CO2, 5% O2, and 85% N2. E. coli strains were cultivated overnight at 37 °C in Luria-Bertani medium (Sigma, Steinheim, Germany). When necessary, 100 μg/ml ampicillin was added to the growth medium.

Construction of the C. jejuni Δrnr Mutant Strain—The protocol to obtain C. jejuni 81–176 rnr mutant strain was adapted from Ref. 25. Briefly, a DNA sequence coding a putative RNase R (CjR8176G659) was identified in the genome of C. jejuni 81–176 using National Center for Biotechnology Information (NCBI) BLAST features and the EMBOSS alignment (1935 bp). The C. jejuni 81–176 Δrnr deletion mutant (Δrnr::aphA3) was generated by homologous recombination using a PCR-amplified nonpolar cassette carrying the aphA3 kanamycin resistance gene flank ed by ~300-bp regions that flank either side of the rnr gene. Overlapping extension PCR protocol was used to amplify the deletion cassette. To prepare the three PCR products used as templates in overlapping extension PCR reaction, PCR was performed with Phusion high-fidelity DNA polymerase (Finnzymes, Thermo Fisher Scientific, Illkirch, France) and with three different primer sets: MO576 (217–199 upstream of rnr coding sequence) and MO577 (nucleotides 38–56 of rnr coding sequence) were used for the upstream region (273 bp), MO582 and MO583 were used to amplify the kanamycin resistance cassette (1388 bp), and MO578 (1892–1911 of rnr coding sequence) and MO579 (347–366 downstream of rnr coding sequence) were used for the downstream region (410 bp). The sequences of all primers used in this study are presented (see Table 3). The rnr fragments were amplified using genomic DNA isolated from C. jejuni 81–176 by using the DNeasy blood and tissue kit (Qiagen, Courtaboeuf, France), while the kanamycin resistance cassette was amplified from pBF14 (26). The reverse primer for the upstream fragment (MO577) and the forward primer for the downstream fragment (MO578) included 22 bp of DNA sequences (see Table 3, underlined) at the 5’ end that are complementary to MO582 and MO583, respectively, to allow joining of the kanamycin resistance cassette to the upstream and downstream fragments. These three fragments were purified from agarose gels (with QIAquick gel extraction kit, Qiagen) and then used in overlapping extension PCR (using MO576/579) to obtain the deletion cassette, which consisted of the upstream (273 bp) and downstream (410 bp) sequences of the target gene rnr with a kanamycin resistance cassette (1388 bp) inserted between them. This deletion cassette of 2071 bp was sequenced and then cloned into pGEM-T vector (Promega), which is a suicide vector for C. jejuni, leading to the recombinant plasmid pNH07. 5 μl of pNH07 was used to transform C. jejuni 81–176 competent cells. Transformants were selected by incubation in Columbia plates containing 5% sheep blood and 50 μg/ml kanamycin. The insertion of the cassette and deletion of the rnr gene were confirmed by PCR using primer set MO587/588.

C. jejuni Growth Experiments—Growth experiments were performed at optimal temperature (37 °C) and close to the minimal temperature of growth (32 °C) of C. jejuni to compare the behavior of the parental and rnr mutant strains. C. jejuni 81–176 parental 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
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**TABLE 2**

| Plasmid         | Antibiotic resistance | Reference        | Comments                      |
|-----------------|-----------------------|------------------|-------------------------------|
| pGEM-T          | Ampicillin            | Promega          | Commercial vector             |
| pET19b          | Ampicillin            | Novagen          | Commercial expression vector  |
| pHN07           | Ampicillin; kanamycin | This study       | pGEM-T vector containing the *rnr* deletion cassette |
| pGEMT-*rnr*     | Ampicillin            | This study       | Encodes *Cj-RNR*              |
| pETmrn-His      | Ampicillin            | This study       | Encodes His-Cj-RNR            |

**TABLE 3**

| Primers used in this study | Sequence (5′–3′) | Comments                                |
|----------------------------|------------------|-----------------------------------------|
| MO625                      | GTAAGGCGTGTCTCTTGAG | Primers used to amplify the beginning of *C. jejuni* *rnr* gene (fragment of 273 bp); the underlined region of MO677 is complementary to MO578 |
| MO591                      | CATATGAAAGAATTTTTAAA | Primers used to amplify the end of *C. jejuni* *rnr* gene (fragment of 410 bp); the underlined region of MO78 is complementary to MO83 |
| MO578                      | GTCTTTGGGTGATTTTAGCA | Primers used to amplify kanamycin resistance cassette from pBF14(26) |
| MO576                      | ATGAGCGTGTCCTTGAGAG | Primers used to check *C. jejuni* Δ*rnr* mutant strain |

to 20 ml of brain heart infusion medium and incubated with shaking for 24 h under microaerophilic conditions to obtain a starter culture. 100 ml of brain heart infusion medium was then inoculated with a 1/100 dilution of starter culture and incubated microaerobically at 32 and 37°C with agitation (110 rpm). Viable counts were measured by serial dilution and plating on Karmali agar at regular time intervals to estimate the total number of cfu/ml. The growth rate was calculated using the formula \( \ln \left( \frac{N_t}{N_0} \right) / t \), where \( N_t \) corresponds to the viable count in cfu/ml at time 0, \( N_0 \) corresponds to the viable count in cfu/ml at time \( t \), and \( t \) corresponds to the interval time between each points. Each experiment was performed four times independently, and for each experiment, the individual samples were plated in triplicate on Karmali agar plates.

Adhesion and Invasion Assays Using Ht-29 Epithelial Cells—Bacterial adhesion and invasion into Ht-29 cells were studied using the gentamicin protection assay, as described previously (27). Briefly, microplate wells were seeded with 2 × 10⁵ Ht-29 cells and incubated for 5 days at 37°C in a humidified, 5% CO₂ incubator. After washing, the Ht-29 monolayers were infected with a suspension of ~2–5 × 10⁵ cfu. To measure cell adhesion, the infected monolayers incubated for 1 h at 37°C in a humidified 5% CO₂ incubator were washed, and then Ht-29 adherent *Campylobacter* were enumerated on Karmali agar. To evaluate bacterial invasion, the infected monolayers were incubated during 3 h, gentamicin (250 μg/ml) was added, and the monolayers were incubated for 2 more hours. After washes and Ht-29 cell lysies, intracellular *Campylobacter* was enumerated on Karmali agar. Each experiment was done in duplicate and performed at least three times independently.

Construction of the Plasmid Expressing RNase R from *C. jejuni*—The *C. jejuni* 81–176 RNase R gene (*rnr*) (GenBank™ accession number YP_001000332) was amplified from genomic DNA using the primers MO625 and MO591 (see Table 3) (Ndel and BamHI sites, respectively, are underlined in the table). For *rnr* amplification by PCR, genomic DNA, 200 μM of each primer, and 1 unit of Phusion high-fidelity DNA polymerase (Finnzymes) were used. The resulting product (1944 bp) was purified (QIAquick PCR purification kit, Qiagen) and cloned into the pGEM-T plasmid (Promega) to generate pGEMT-*rnr*. To overexpress the *Cj-RNR* protein, the *rnr* gene double-digested with Ndel and BamHI was subcloned from pGEMT-*rnr* into the pET19b expression vector previously digested with the same enzymes (Novagen, Merck). Plasmids and primers used are presented in Tables 2 and 3, respectively. The resultant pETmrn-His was confirmed by DNA sequencing (Beckman Coulter Genomics).

Overexpression and Purification of Recombinant RNase R from *C. jejuni*—The plasmid harboring the histidine-tagged *Cj-RNR* protein (pETmrn-His) was introduced by transformation into *E. coli* BL21(DE3) Star to allow the production of the recombinant protein. Cells were grown at 37°C in 100 ml LB medium supplemented with 100 μg/ml ampicillin to an A₉₀₀ of 0.5, induced by the addition of 0.5 mM isopropyl-thio-β-D-galactopyranoside, and grown at 32°C for 4 h. Cells were pelleted by centrifugation and stored at −80°C. Purification was performed by immobilized metal affinity chromatography using HiTrap chelating HP columns (GE Healthcare) and ÄKTA FPLC system (GE Healthcare) following a protocol described previously (19). The purity of the protein was verified by SDS-PAGE using an 8% gel and visualized by Coomassie Blue staining. Proteins were quantified using the Bradford method (28), and 50% (w/v) glycerol was added to the final fractions prior to storage at −20°C.

In Vitro Transcription—Templates for tRNA<sup>Ser</sup> and 5 S rRNA were generated by PCR using the primers Cjej3 and Cjej4 and Cjej7 and Cjej8, respectively (Table 3). The phage T7 RNA polymerase promoter sequence was included in the forward
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primer sequences and is underlined. In vitro transcription was carried out using the purified amplicons as template in the presence of an excess of [α-32P]UTP over unlabeled UTP with the Riboprobe in vitro transcription system (Promega) and T7 RNA polymerase. The reaction mixture was incubated 2 h at 37 °C. To remove all trace of DNA, 1 unit of DNase (Promega) was added and incubated for 30 min at 37 °C. The unincorporated radioactive nucleotides were removed using a G50 column as instructed (GE Healthcare). The transcripts were purified by electrophoresis on an 8 m urea, 10% polyacrylamide gel. The gel slice was crushed, and the RNA was eluted overnight at room temperature into elution buffer (0.5 m ammonium acetate, pH 5.2, 1 mM EDTA, 2.5% (v/v) phenol, pH 4.3). The RNA was precipitated using ethanol and resuspended in RNase-free water. The yield of the labeled substrates (cpm/μl) was determined by scintillation counting.

**RNase R Activity Assays**—The activity assays were performed using different substrates: a poly(A) oligomer of 35 nt, a 16-mer oligoribonucleotide, a 30-mer oligoribonucleotide, a 24-mer DNA substrate, tRNA^ser^, and 5 S rRNA (transcribed in vitro as described in the above section) (Table 3). To obtain the double-stranded substrates 16–30(ds) and 16–16(ds), the 30-mer and the 16-mer oligoribonucleotides, respectively, were hybridized to the complementary unlabeled 16-mer oligoribonucleotide (5′-AGTGGTTGGTGTCGGG-3′) (Table 3). The hybridization was performed in a 1:1 (mol:mol) ratio by incubation for 5 min at 100 °C followed by 45 min at 37 °C. All the synthetic RNA molecules were labeled at its 5′ end with [γ-32P]ATP using T4 polynucleotide kinase. The RNA oligomers were then purified using a G25 column (GE Healthcare) to remove the unincorporated nucleotides. After optimization of the buffer, the activity assays were performed in a final volume of 20 μl with an activity buffer composed of 10 mM Tris-HCl, pH 7.5, 5 mM KCl, 0.5 mM MgCl2, and 0.1 mM DTT and 10,000 cpm of substrate. The temperature into elution buffer (0.5 M ammonium acetate, pH 5.2, 1 mM EDTA, 2.5% (v/v) phenol, pH 4.3). The RNA was precipitated using ethanol and resuspended in RNase-free water. The yield of the labeled substrates (cpm/μl) was determined by scintillation counting.

**RESULTS AND DISCUSSION**

**Analysis of C. jejuni RNase R**—RNase R-like proteins are very heterogeneous in their size and composition. In some organisms, they present the organization described for *E. coli* protein, with a helix-turn-helix domain at the N-terminal region followed by two cold shock domains, a central RNB domain, a C-terminal S1 domain, and a lysine-rich region (Fig. 1A). However, the N- and C-terminal regions are very variable. For instance, in *C. jejuni* there is no helix-turn-helix or Lys-rich region, and in archaea the existence of an RNase R that only has the RNB domain was described (33). Despite these differences, the RNB domain (characteristic from this family of enzymes) is very conserved, with all the residues involved in catalysis found in all members of the family (supplemental Fig. 1). We have constructed a phylogenetic tree based on the RNase R sequence of *C. jejuni*, which is also an Epsilonproteobacteria. Moreover, it is possible to see a clear separation in two groups: Gram-positive and Gram-negative bacteria (Fig. 1B).

**Cj-RNR Activity at Different Conditions**—To characterize the activity of Cj-RNR protein, we have first determined the optimal conditions for the catalysis. For this purpose, we have analyzed the activity of this protein at different conditions. We have cloned the *rnr* gene from *C. jejuni* in an expression vector. We tested several conditions for protein expression in different expression strains, and finally, we expressed Cj-RNR at 32 °C in a BL21 Star™ (DE3) (Invitrogen). We then purified the protein by histidine affinity chromatography as described under “Experimental Procedures.”

We started by analyzing the activity of the protein using two different types of salts, KCl and NaCl, using six different concentrations, 5, 10, 25, 50, 100, and 200 mM. The results show that Cj-RNR is active with both KCl and NaCl and prefers low con-
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centrations of monovalent cations (Fig. 2A). We have quantified the activity of the protein in these conditions. In fact, Cj-RNR has a maximum activity when lower concentrations are used and prefers KCl to NaCl (Fig. 2B). In contrast, it was described that E. coli RNase II and RNase R are more active with higher KCl concentrations (50–500 mM) (34).

We have also determined the effect of pH on the activity of Cj-RNR. With this purpose, we have performed activity assays at different pH values between 5.4 and 9.0. The E. coli proteins, RNase II and RNase R, present an optimal activity at a pH range between 7.5 and 9.5 (34). Cj-RNR activity is higher at a pH of 7.5, although it is active in a wide range of pH from 6.5 to 9.0 (Fig. 3). Based on these results, we used a buffer containing 5 mM KCl and with a pH of 7.5 in subsequent experiments.

Enzymes from the RNB family require a divalent ion for catalysis, normally Mg²⁺. However, they are also active in the presence of other ions. For that reason, we tested Cj-RNR activity in the presence of Mg²⁺, Mn²⁺, Ca²⁺, Zn²⁺, Ni²⁺, Cu²⁺, and Co²⁺. As it is possible to observe, Cj-RNR is active in the presence of Mg²⁺, Mn²⁺, Ca²⁺, and Ni²⁺ (Fig. 4A). For the first two ions, the enzyme seems to have the same activity, whereas for Ca²⁺ and Ni²⁺, there is a reduction in the activity, especially with regard to the degradation of the smaller fragments (Fig. 4A). We then tested different Mg²⁺ and Mn²⁺ concentrations. The results show that Cj-RNR prefers lower concentration of divalent ions (from 0.1 to 1 mM) (Fig. 4B and C). We then determined the activity of Cj-RNR in those conditions. We confirmed that it is more active in the presence of lower concentrations of Mg²⁺ and Mn²⁺. However, in contrast to what was described for other proteins of this family, Cj-RNR prefers Mn²⁺ and not Mg²⁺ (Fig. 4D). Manganese has emerged as a very important metal in virulence. Moreover, macrophages have poor magnesium and acidic and low oxygen environment (35). Campylobacter can survive within macrophages for a period of 24–30 h, and it is within the macrophages that manganese is thought to be important for C. jejuni (36). Manganese and magnesium contents in C. jejuni are not yet known. However, they were determined in E. coli, where they were shown to change accordingly to growth phase; their levels decline as growth progresses (37). One of the major challenges that bacteria have when growing in oxygenated environments is to efficiently resist or repair damages caused by reactive oxygen species. One strategy to reduce oxidative damage involves limiting intracellular iron content. In these situations, bacteria appear to have an absolute requirement for Mn²⁺ (38). The differences in the activity of Cj-RNR in the presence of both Mg²⁺ and Mn²⁺

FIGURE 2. Salt dependence of Cj-RNR. A, 1 nm recombinant protein was incubated with 10 nm poly(A) at 37 °C for 5 min in a reaction buffer with different salt concentrations as indicated. Samples were taken during the reaction at the time points indicated. B, determination of the activity of Cj-RNR. Error bars indicate mean ± S.D.

FIGURE 3. pH dependence of Cj-RNR. A, 1 nm recombinant protein were incubated with 10 nm poly(A) at 37 °C for 5 min in a reaction buffer with different pH, ranging from 5.4 to 9.0. Samples were taken during the reaction at the time points indicated. Ctrl, control. B, determination of the activity of Cj-RNR. Error bars indicate mean ± S.D.
may allow *C. jejuni* to manipulate the enzymatic activity of RNase R according to the environment, thus altering gene expression. This feature can be an important advantage for *C. jejuni* to adapt and survive within macrophages. Additionally, it was shown that the endoribonuclease RNase III from *C. jejuni* may also have an important role under a Mn$^{2+}$/H$^{11001}$-rich environment (11). The optimal temperature of growth for *C. jejuni* is between 37 and 42 °C, the human and avian body temperatures, respectively (39). Although not able to grow below 30 °C, *C. jejuni* is able to survive at refrigerated temperatures (40). It was already shown that the exoribonuclease polynucleotide phosphorylase was important for the survival at refrigerated temperatures (9). Recently, it was also demonstrated *in vitro* that the endoribonuclease RNase III is active in an unexpectedly large range of temperatures from 4 to 42 °C (11). Taking this into account, we decided to analyze the activity of Cj-RNR at various temperatures. The activity assays were performed at four distinct temperatures: 4, 30, 37, and 42 °C. The results demonstrate that, in these conditions, this protein is active in all the temperatures tested, even at 4 °C (Fig. 5), where we would expect a drastic decrease in its activity.

**RNA Cleavage by Cj-RNR**—Taking into account that Cj-RNR is active in a broad range of conditions, for the following experiments we have used a buffer with pH 7.5, 5 mM KCl, 0.1 mM MgCl$_2$, and all the assays were performed at 37 °C. The activity of Cj-RNR was evaluated using three different single-stranded substrates (poly(A), 30ss and 16ss) and two different double-stranded RNAs (16–30ds and 16–16ds). Regarding the degradation of ssRNA, it is possible to see that the substrate is progressively degraded until a 2-nt fragment is released, similarly to what was described for RNase R-like proteins (Fig. 6A). It is also possible to observe the presence of an intermediate product with 4 nt of length (Fig. 6). A mixture of two different degradation products with 2 and 4 nt of length was also observed in *E. coli* RNase R protein. Because there is no crystal structure available for any RNase R protein, it was postulated that, similarly to what occurs in RNase II, some 4-nt fragments are released, although they are still partially “clamped” in the active site. Others are degraded to a 2-nt fragment (21). We tested the
activity of Cj-RNR protein using two structured substrates: 16–16ds and 16–30ds (see “Experimental Procedures” for a description of these substrates). When the substrate tested was the 16–30ds, we were able to see that, once again, the protein behaved like RNase R, being able to overcome the double-stranded structures (Fig. 6B). RNase R from E. coli requires a 3’ single-stranded region to cleave structured substrates (41). In contrast, Cj-RNR does not have such a strict requirement. In the conditions tested (10 nM RNA and 25 nM Cj-RNR), it is able to digest the perfect double-stranded 16–16ds substrate (Fig. 6B); however, the degradation of this substrate is less efficient when compared with the degradation of 16–30ds (Fig. 6B). We have tested the E. coli RNase R (Ec-RNR) in the same conditions and observed that it is not able to degrade the 16–16ds (data not show). E. coli RNase R has at the C terminus a region rich in lysines (Fig. 1A), which was shown to be involved in the degradation of double-stranded substrates, probably by helping to unwind the two strands (42). This region is absent in Cj-RNR. Together with the evidence that Cj-RNR is able to degrade the 16–16ds RNA, although with less efficiency, it seems that the mechanism by which the dsRNA is degraded by RNase R in C. jejuni is different from the one in E. coli; however, these differences seem to be minimal. In E. coli, one of the existing degradation pathways implies that the RNA molecules to be

![Exoribonucleolytic activity of Cj-RNR at different temperatures](image1)

![Exoribonucleolytic activity of Cj-RNR using ssRNA (A) or dsRNA (B) molecules](image2)
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degraded are tagged with poly(A) tails synthesized by poly(A) polymerase I (PAP I). These extra nucleotides serve as platform for the binding of RNases such as RNase R because these proteins require a single-stranded tail to proceed with the degradation of structured substrates. In C. jejuni, until date, there is no data on the poly(A) polymerase I protein, although a putative gene that could code for a poly(A) polymerase is found in its genome. Considering that Cj-RNR, to some extent, degrades perfect double-stranded RNAs, we can speculate that perhaps polyadenylation may play a different role in this organism.

We also determined the activity of Cj-RNR with different substrates and at various temperatures. To do it, we adjusted the reaction conditions, as described under “Experimental Procedures,” to ensure that less than 25% of the substrate was being degraded. The results show that there is no preference for a specific substrate, except at 42 °C, where the enzyme seems to prefer poly(A) (Fig. 7). Moreover, this experiment allowed us to determine that, in fact, there are some differences in the activity of the protein at different temperatures; at 4 and 30 °C, the activity is half that determined at 37 and 42 °C (Fig. 7). These differences were not visible by eye in the polyacrylamide gels presented before due to the conditions used (Fig. 5).

Cj-RNR Is Able to Cleave DNA Substrates—It is known that RNase II, the prototype of this family of enzymes, is able to bind to DNA molecules; however, it is not able to cleave them because there are specific requirements for a ribose in the second or fourth nucleotides counting from the 3′-end (43, 44). When RNase R was initially characterized, it was shown that is able to cleave DNA, albeit with a reduced efficiency and using higher protein concentrations (34). We decided to test the activity of Cj-RNR also with a DNA substrate and compare it with the E. coli counterparts. As found previously, E. coli RNase II is not able to cleave DNA, whereas E. coli RNase R can degrade a small percentage of the substrate (Fig. 8). When we tested Cj-RNR activity, we were able to see that it can cleave DNA in a distributive way. Moreover, it is more efficient in degrading DNA substrates when compared with the E. coli counterpart (Fig. 8). These results confirm that Cj-RNR behaves like an RNase R-like protein. Interestingly, the exoribonuclease polynucleotide phosphorylase from B. subtilis, in the presence of Mn²⁺ and low level of inorganic phosphate (Pᵢ), is able to degrade ssDNA. This activity was postulated to be important for DNA repair pathways (45). Why RNase R-like proteins are able to cleave DNA is still unknown, and structural studies need to be performed to understand the mechanism involved.

tRNAs Are Substrates for Cj-RNR—It was described that RNase R is able to degrade defective tRNAs or rRNA and mRNAs containing repetitive extragenic palindromic (REP) sequences (13). M. genitalium RNase R was shown to mature tRNAs (46). E. coli RNase R is able to efficiently cleave 23 S and 16 S rRNAs but acts poorly on 5 S rRNA and tRNA (34). We have tested the activity of Cj-RNR using 5 S rRNA and a tRNA molecule (we chose tRNASer). We transcribed both RNAs and performed the degradation assays as described under “Experimental Procedures.” The results obtained showed that, similarly to what was shown in E. coli, Cj-RNR acts poorly over 5 S rRNA; however, it is able to degrade tRNA molecules (Fig. 9). tRNA molecules contain very small 3′ single-stranded overhangs, which explains why E. coli RNase R is not able to efficiently cleave this substrate (34). As we discussed previously, Cj-RNR can cleave structured substrates in the absence of a 3′ single-stranded region, although with less efficiency (Fig. 6B). This characteristic may be useful to confer to this protein the ability to degrade tRNAs in C. jejuni.

Growth and Viability of Parental and Δrrn Strains—In some bacteria, RNase R-like proteins are essential (13). However, as reported previously for H. pylori, it was possible to construct a C. jejuni strain deficient in RNase R, which shows that, in this organism, RNase R is not essential (47). This meant we were able to analyze the effect of rrn deletion in growth and viability. We tested this at two different temperatures, at 37 °C (the optimal temperature of growth is between 37 and 42 °C), and at 32 °C, which is considered as the minimal temperature of growth of C. jejuni (48).
If we compare the growth of the two strains at optimal temperature, 37 °C, we observe that the lag phase of the wild-type strain is more prolonged when compared with the mutant strain (Fig. 10A). Moreover, when we calculated the maximum rate growth, we were able to see that the mutant strain grows faster when compared with the wild type (0.49 and 0.3 h⁻¹, respectively).

At minimal temperature of growth, 32 °C, it is possible to observe the “Phoenix” effect described by Kelly et al. (49). This phenomenon is characterized by a decrease in viability after inoculation followed by an increase in survival to levels similar or higher to the initial ones. Both strains show an exponential phase of 78 h (Fig. 10B). In this phase, wild-type cells grow better than the mutant ones, although the viability was shown to be similar. This phase is followed by a decline phase, which reflects the high mortality due to stress induced by temperature. The bacterial cells that survived then start to regrow, and we can see that the Δrnr strain recovers viability sooner than the wild-type strain.

In conclusion, Cj-RNR is not an essential protein, and its deletion does not cause significant changes in growth and viability when compared with the wild-type strain. However, at lower temperatures (32 °C), the mutant strain recovers better than the wild type.

Involvement of RNase R in C. jejuni Adhesion and Invasion of Eukaryotic Cells—RNase R-like proteins have been involved in the establishment of virulence in several pathogenic organisms (24). In C. jejuni, it was already shown that polynucleotide phosphorylase, another exoribonuclease, has an important role...
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| Ratio WT/Δmr | Adhesion | Invasion |
|--------------|---------|---------|
| 0            |         |         |
| 1            |         |         |
| 2            |         |         |
| 3            |         |         |
| 4            |         |         |
| 5            |         |         |
| 6            |         |         |
| 7            |         |         |
| 8            |         |         |
| 9            |         |         |
| 10           |         |         |
| 11           |         |         |

**FIGURE 11.** Adhesion and invasion ability of *C. jejuni* 81–176 wild-type and Δmr strains. The results are expressed as the ratio between wild-type and Δmr strains. Error bars indicate mean ± S.D.

in adhesion and invasion (10). Moreover, we showed that *Cj*-RNR is active in a large range of conditions, which may be important for the adaptation of *C. jejuni* to different environments during the infection process. For that reason, we decided to address the role of *Cj*-RNR in adhesion and invasion. This was done using wild-type and Δmr strains of *C. jejuni* 81–176 and a eukaryotic cell line of intestinal origin, Ht-29. Several bacterial concentrations were tested, while the concentration of eukaryotic cells was set as $2 \times 10^5$ cells/well (multiplicity of infection of 100). Experiments were done three times, and the results correspond to the mean value calculated from the three experiments.

The results show that the wild-type strain is three times more adherent and six times more invasive than the mutant strain (Fig. 11). The results are in agreement with what has been reported for *Shigella flexneri* and *E. coli* EIEC, in which the Δmr mutant strains were shown to be less invasive (50). These results indicate that RNase R, similarly to what was shown for polynucleotide phosphorylase (10), is an important protein for the first steps of infection of *C. jejuni*.

**Conclusions**—In this study, we have undertaken a functional and biochemical analysis of *Cj*-RNR. We demonstrated that *Cj*-RNR is active in a wide range of conditions and determined the optimal conditions for its activity. We also demonstrated that *Cj*-RNR behaves like an RNase R-like protein regarding its ability to degrade structured RNAs. However, the mechanism of action seems to be different because it is able to degrade perfect double-stranded structures, although with less efficiency. Our results also showed that *Cj*-RNR is capable of acting on a variety of RNA molecules, namely highly structured RNAs such as tRNAs. Finally, we saw that, although not essential, *Cj*-RNR is important for *C. jejuni* adhesion and invasion ability. This demonstrates that RNase R plays an important role in the first steps of *C. jejuni* invasion, probably by regulating virulence factors.
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